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Serum Antibody Response to *Moraxella catarrhalis* Proteins in Stringently Defined Otitis Prone Children

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Abstract

Background—*Moraxella catarrhalis* (*Mcat*) is a frequent pathogen of acute otitis media (AOM) in young children. Here we prospectively assessed naturally-induced serum antibodies to four *Mcat* vaccine candidate proteins in stringently defined otitis prone (sOP) and non-otitis prone (NOP) children age 6 to 36 months old following nasopharyngeal (NP) colonization, at onset of AOM and convalescence from AOM.

Methods—Serum IgG and IgM antibody against recombinant *Mcat* proteins, oligopeptide permease A (OppA), outer membrane protein (OMP) CD, hemagglutinin (Hag), and PilA clade 2 (PilA2), were quantitated by ELISA.

Results—During NP colonization by *Mcat* all four antigens were immunogenic in both sOP and NOP children. However, sOP children had lower antibody responses than NOP children across age 6-36 months, similar to our findings for protein vaccine candidates of *Streptococcus pneumoniae* (*Spn*) and Nontypeable *Haemophilus influenzae* (NTHi). sOP children displayed a later and lower

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peak of antibody rise than NOP children for all four antigens during NP colonization of *Mcat*. The age-dependent increase of antibody ranked as OppA > Hag5-9 > OMP CD > PilA2 in both sOP and NOP children. Lower serum antibody levels to the *Mcat* antigens were measured in sOP compared to NOP children at the onset of AOM. We did not find a consistent significant increase of antibody at the convalescence phase after an AOM event.

Conclusions—sOP children is a highly vulnerable population that mount lower serum antibody responses to *Mcat* candidate vaccine proteins compared to NOP children during asymptomatic NP carriage and at onset of AOM.

Keywords

Otitis prone; Nasopharyngeal colonization; Acute otitis media; Immunogenicity; Recombinant proteins; Carriage

1. Introduction

Acute otitis media (AOM) is the most common infectious disease to cause parents to seek medical care for their child and receive antibiotics. The primary otopathogens causing AOM are *Streptococcus pneumoniae* (*Spn*), nontypeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis* (*Mcat*).[1] *Mcat* is also a common cause of acute sinusitis in children and adults and acute exacerbations of chronic bronchitis in adults.[2] Our group has been studying the immune response to protein vaccine candidates of the three major otopathogens in two populations of children: (1) a group that has poor antibody and cellular immune responses to otopathogens and very frequent AOM episodes (termed stringently defined otitis prone (sOP) children) and (2) a group who show strong immune responses and experience few or no AOM episodes (termed non-otitis prone (NOP) children).[3-14] However, among the *Spn* and NTHi candidate vaccines we have studied thus far some antigens showed significantly greater immunogenicity than others.[3,4,15,16] Moreover, since sOP children would benefit the greatest from a vaccine to prevent AOM and their frequent illnesses cause greater morbidity and consume a disproportionately high amount of health care costs for otitis media care, study of this highly vulnerable population has merit.

Currently, there is no licensed vaccine available for *Mcat*. A number of vaccine targets have been identified.[17] Here, we studied four highly conserved, surface exposed *Mcat* proteins: OMP CD, OppA, Hag, and PilA2 in sOP compared to NOP children. OMP CD is a porin and an adhesin.[18] Oligopeptide permease protein A (OppA) is an oligopeptide binding protein of the oligopeptide permease ABC transport system, which mediates uptake of peptides and fitness of *Mcat* in the respiratory tract.[19,20] Hemagglutinin (Hag), also named Moraxella IgD binding protein (MID), is an adhesin and mediates adherence to primary cultures of human middle ear epithelial cells.[21] PilA clade 2 (PilA2) is a pilin, which is the major protein subunit of type IV pili and is essential for *Mcat* natural genetic transformation and can also enhance biofilm formation.[22]

Naturally-induced antibodies comprise a fundamental component of humoral immunity against infectious agents. We have previously shown that healthy children develop serum antibodies to OMP CD, OppA, Hag, and PilA2 following *Mcat* nasopharyngeal (NP)

colonization and AOM.[23] In this study, we analyzed serum antibody responses to the same four *Mcat* proteins in sOP children and compared them to NOP children. We sought to identify *Mcat* proteins capable of eliciting robust antibody immune responses in sOP children equivalent to those in NOP children, since natural priming in infancy and post vaccination boosting in the second year of life to those antigens among sOP children would be a desirable feature.

2. Materials and Methods

2.1. Subjects and sampling

The samples collected and analyzed were obtained during a prospective study supported by the National Institute of Deafness and Communication Disorders, as previously described.[15,16] Healthy children without previous episodes of AOM were enrolled at 6 mos. of age from a middle class, suburban socio-demographic pediatric practice in Rochester, NY during June, 2008 to March, 2014. Serum samples and NP and oropharyngeal cultures (hereafter termed NP samples) were obtained 7 times during the study period at 6, 9, 12, 15, 18, 24, and 30-36 mos. of age. During the study period whenever children experienced an AOM episode a confirmatory tympanocentesis was performed and middle ear fluid (MEF) samples were microbiologically assessed (an essential component to the definition of "stringently-defined" AOM) since virtually all prior studies have relied on only a clinical diagnosis that is known to be variably accurate). Serum and NP cultures were collected at time of the clinical diagnosis of AOM and, 3 weeks following an AOM (convalescent stage). The study was approved by the Rochester General Hospital Research Subjects Review Boards and written informed consent was obtained for participation and all procedures.

2.2. Enzyme-linked immunosorbent assay

Recombinant *Mcat* proteins, OppA, OMP CD, Hag5-9 (truncated Hag protein), and PilA2 were expressed and purified as previously described.[18,20-22] All the proteins are conserved among *Mcat* strains [17] and have been demonstrated to display native epitopes on the surface of *Mcat* by a flow cytometry assay which showed binding of mouse immune sera against all four recombinant proteins displayed on the surface of multiple *Mcat* strains (data not shown). Protein-specific antibody concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using purified recombinant proteins. Ninety six-well Nunc MaxiSorp plates were coated with 1 μg/mL of individual proteins (100 μL/well) in phosphate-buffered saline (PBS, pH 7.4) and incubated at 37°C for 1 h. After five washes, the plates were blocked with 10% fetal bovine serum (FBS) in PBS (pH 7.4) at room temperature for 1 h (200 μl per well). After washing, 100 μl of serum 2-fold serially diluted in PBS/0.5%BSA/0.005% tween at a starting dilution of 1:50 was added to each well. Human serum IgGs, Carimune (CSL Behring AG, Bern, Switzerland) and Gammagard (Baxter, Deerfield, IL) were used as references and in-house control sera with high and low titers were run on each plate. The plates were incubated at room temperature for 30 min followed by the addition of affinity purified goat anti-human IgG/IgM antibody conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, TX) as a secondary antibody. The reaction products were developed with TMB Microwell Peroxidase Substrate System

(KPL, Gaithersburg, MD), stopped by addition of 1.0 M phosphoric acid and read by a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale, CA) using a 450-nm filter.

To provide quantitative results on antibody concentrations, the level of specific antibody in the samples was determined by comparison to an internal reference serum (Carimune for OMP CD and Gammagard for OppA, Hag, and PilA2). The levels of IgG and IgM in the reference serum were quantitatively measured by using a human IgG or IgM ELISA quantitation kit (Bethyl laboratories). A four-parameter logistic-log function was used to form the reference and sample curves. Precision, Calibration of Standard curve, Robustness/ Stability and Linearity were done with an in-house reference serum according to International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guidance.[24] The assay lower limit of detection was 171 ng/mL for OppA, 69 ng/mL for OMP CD, 191 ng/mL for Hag5-9, and 47 ng/mL for PilA2 for serum IgG, and 44 ng/mL for OppA, 55 ng/mL for OMP CD, 63 ng/mL for Hag5-9, and 30 ng/mL for PilA2 for serum IgM. Less than 3% of the serum samples had IgG and IgM titers that were below the limit of detection. The inter-assay coefficient of variation was ≤30% for all antigens and secondary antibody combinations.[16,23]

2.3 Microbiology

All NP samples and MEF were tested for the presence of *Mcat*, *Spn* and NTHi using standard methods as previously described.[15]

2.4. Statistical analysis

To compare the antibody response to *Mcat* NP colonization between sOP and NOP children at healthy visits, a linear mixed effects model was used to model response $\log_2$ Antibody concentration vs. predictors $\log_{10}$ Age and colonization status (per visit) vs. otitis prone (OP) status (per individual).[25] A time varying OP effect was estimated separately for each visit type, using suitable likelihood ratio tests (see Appendix for details). The model was applied to each antigen-specific IgG separately, and *P*-values adjusted using the Benjamini-Hochberg procedure.[26] Dunn's multiple comparisons test was employed to compare the difference of antibody concentration among different age time points for sOP and NOP children when NP colonization status was assessed. An unpaired t test was used to compare the difference of antibody concentrations between sOP and NOP children for the data in Gaussian distribution and Mann-Whitney test for data not in Gaussian distribution. A paired t test was used to compare the difference of antibody concentrations between AOM acute and convalescence phase. P values of < 0.05 were considered significant.

3. Results

3.1. Serum IgG antibody levels in sOP and NOP children after NP colonization

Fifty-one sOP children and 131 NOP children who had current and/or prior documented culture positive *Mcat* NP colonization were studied and their serum antibody levels are displayed in Figure 1 and Figure 2. For OppA, sOP children had significantly lower levels of antibody compared to NOP children at age 6-36 mos. ($P = 0.015$, Fig 1A and Table 3). At age 18 mos. sOP children had antibody levels higher than those measured at age 6 mos. ($P <$

0.05; Fig 2A) vs. age 15 mos. for NOP children ($P < 0.05$; Fig 2B), consistent with a later age for measureable increases in total IgG antibody to OppA. The maximum level of antibody (ng/ml) was 3906 ± 629, measured at age 24 mos. for sOP and 5623 ± 1811 measured at age 24 mos. for NOP children (Fig 2A and 2B). For OMP CD, sOP children had lower levels of antibody compared to NOP children at age 6-36 mos. ($P = 0.0055$, Fig 1B and Table 3). sOP children did not have an increase of IgG across 6-36 mos. age ($P > 0.05$, Fig 2C). Differently, NOP children showed an earlier increase of IgG to OMP CD at 12 mos. of age ($P < 0.05$ compared to 6 mos. age, Fig 2D) and further increases at 18-36 mos. of age ($P < 0.001$ compared to 6 mos. age, Fig 2D). The maximum level of antibody (ng/ml) was 2487 ± 684, measured at age 18 mos. for sOP and 3537 ± 918 measured at age 18 mos. for NOP children (Fig 2C and 2D). For Hag5-9, sOP children had lower levels of antibody compared to NOP children at age 6-36 mos. old ($P = 0.02$, Fig 1C and Table 3). At age 18 mos. sOP children had antibody levels higher than those measured at age 6 mos. ($P < 0.05$; Fig 2E) vs. age 15 mos. for NOP children ($P < 0.05$; Fig 2F), consistent with a later age for measureable increases in total IgG antibody to Hag5-9. The maximum level of antibody (ng/ml) was 2407 ± 392 measured at age 24 mos. for sOP and 4174 ± 1422 measured at age 24 mos. for NOP children (Fig 2E and 2F). For PilA2, although no difference of antibody levels between sOP and NOP children was observed across age 6-36 mos. ($P = 0.32$, Fig 1D and Table 3), sOP children displayed a later rise and lower peak of antibody than NOP children (Fig 2G and 2H). sOP children did not have a significant increase of IgG across 6-36 mos. of age ($P > 0.05$, Fig 2G). In contrast, NOP children showed an increase of IgG at 30-36 mos. of age ($P < 0.05$ compared to 6 mos. age, Fig 2H). The maximum level of antibody (ng/ml) was 1929 ± 358, measured at age 30-36 mos. for sOP and 3563 ± 1362 measured at age 24 mos. for NOP children (Fig 2G and 2H).

In sum, the increase of antibody to the four *Mcat* antigens can be ranked as OppA > Hag5-9 > OMP CD > PilA2 in regard to earlier rises and higher peak levels of natural antibody in both sOP and NOP children based on overall.

3.2. Serum IgG and IgM antibody levels in sOP and NOP children at onset of AOM

Serum IgG and IgM were examined in sOP and NOP children at the time of clinical diagnosis of AOM confirmed to be caused by *Mcat* using tympanocentesis to obtain MEF for microbiologic culture. Because we found that age was a significant variable for both sOP and NOP children with regard to mounting an antibody response (Fig 1 and Fig 2), we confined this analysis of serum antibody response to AOM infections occurring in children at age 11 to 22 months old to minimize an age effect as a confounder for comparisons. For OppA, IgG levels at onset of AOM were significantly higher for NOP compared to sOP children ($P = 0.039$, Fig 3A), and IgM and total IgM + IgG levels trended higher for NOP children ($P = 0.1515$, Fig 3A). For Hag5-9, IgG levels at onset of AOM were significantly higher for NOP compared to sOP children ($P = 0.025$, Fig 3C). The immune response to Hag5-9 was remarkable for the significantly higher IgM compared to IgG levels in both sOP and NOP children ($P = 0.0035$ and 0.0012, respectively, Fig 3C). For OMP CD (Fig 3B) and PilA2 (Fig 3D), the quantity of IgM and IgG in serum at onset of AOM was not significantly different between sOP and NOP children, suggesting that there was approximately equal early primary responses and late primary or secondary responses to these antigens. There

were 8.3% of *Mcat*-caused AOM episodes in which *Mcat* was isolated with a second otopathogen (*Spn* or NTHi) at the same time from the middle ears of these children. We did not find a difference of the serum antibody responses to the *Mcat* proteins between colonization or infection of *Mcat* only from co-colonization or co-infection of *Mcat* with *Spn* and/or NTHi in the children.

3.3 Serum IgG and IgM antibody levels in sOP and NOP children at onset of AOM vs. convalescence

Fifteen to nineteen pairs of sera from sOP children and 20-22 pairs of sera from NOP children were examined to assess the difference of IgM and IgG levels at onset of AOM compared to after recovery from AOM caused by *Mcat* for the same child (Table 1). sOP children and NOP children showed 5%-16% and 0-5%, respectively, of > 2-fold increase of serum IgM to *Mcat* proteins at convalescence stage after an AOM episode (Table 1). There were 0-20% and 0-15% of > 2-fold increases of serum IgG to *Mcat* proteins in sOP children and NOP children, respectively, at convalescence stage vs. the acute AOM phase (Table 1). Antibody change of each individual child revealed that serum IgM and IgG exhibited a difference between acute and convalescence levels in all three directions: rising, dropping and unchanged among both sOP and NOP children (Table 2).

4. Discussion

Stringently defined otitis prone (sOP) children should be a primary target for novel vaccines against AOM infections caused by *Mcat* because these children would particularly benefit from vaccination due to their exceptional susceptibility to recurrent middle ear infections. Our previous publication (Ren et al, Vaccine 2015) analyzed the serum antibody responses of the combined sOP and NOP child populations [23], while the current study compared the difference of serum antibody responses between sOP and NOP children. This is the first report of antibody responses to *Mcat* antigens in sOP children. We have previously shown that sOP children have significantly lower serum antibody responses to *Spn* and NTHi NP colonization and AOM infections caused by those respiratory pathogens.[3,4] In the current work we similarly found that sOP children displayed impaired serum antibody responses to *Mcat* candidate protein vaccine antigens compared to age-matched NOP children after asymptomatic *Mcat* NP colonization and *Mcat*-caused AOM. The immune deficits among sOP children have been characterized by our group and shown to include immature innate and adaptive immune responses to otopathogens.[5,7,11]

The current results are relevant to consideration of vaccine trials involving young children in hopes of preventing AOM caused by *Mcat*, as indicated by our previous observations that 23% of sOP children often exhibit absent or significantly reduced antibody responses to many routine pediatric vaccines given in the first year of life.[8] Among the four *Mcat* candidate protein vaccines we found that OppA was most immunogenic followed by Hag5-9, OMP CD, and then PilA2. The immunogenicity of a protein antigen is mainly determined by the binding strength of epitopes of the protein to B- or T-cell human major histocompatibility complex (MHC) or human leukocyte antigen (HLA) molecules and the density of MHC/HLA binding motifs on each epitope.[27] Differed immunogenicity of these

Mcat proteins may be attributed to differing strength of binding to B- and T-cells. However, we note that all four antigens were immunogenic in both sOP and NOP children in the age range of 6 to 36 months old. There are two clades of pilin proteins, PilA1 comprising subclades of PilA1a (32%) and PilA1b (10%), and PilA2 (58%) among *Mcat* strains.[22] Although there is high conservation among the pilins [22], each protein may elicit serum antibodies without cross reaction with the other pilins upon *Mcat* NP colonization and middle ear infection because each *Mcat* strain expresses only one subclade of pilin. In addition, PilA2 is a smaller 16-kD protein [22] and may present fewer reactive epitopes than other larger protein antigens on the surface of *Mcat*, such as OMP CD, OppA, and Hag. All these factors may be responsible for the lower serum antibody levels detected in young children in this study.

The current results of a more gradual rise in serum antibodies to *Mcat* antigens in sOP compared to NOP children in response to NP colonization and AOM infections are consistent with our prior observations on the antibody responses to *Spn* and NTHi.[3,4] In the current work we quantitated antibody by weight whereas in our prior work results were reported in ELISA units [3,4] so direct comparisons in antibody quantity from the current study with prior work by our group is not possible. However, the age of sOP and NOP children when measurable rises to specific candidate protein antigens were identified can be compared. In that regard we observed that natural exposure to *Mcat* resulted in serum antibody rises to all 4 of the proteins we studied at a later age than we observed for either *Spn* or NTHi. Specifically for *Spn* proteins PhtD, LytB, PcpA, PhtE and Ply and NTHi proteins Protein D, P6 and OMP26, significant rises in serum antibody were measured at age 9-12 months.[3,4,15,16] In comparison for *Mcat* proteins OppA, OMP CD, Hag, and PilA2, significant rises in serum antibody were measured at age 12-36 months. The relevance of these findings relates to potential of natural priming by natural exposure to *Mcat* prior to and concurrent with the likely age of vaccination of young infants in the first year of life. That is, our data suggest that a natural priming immune response from *Mcat* exposures may be more delayed than what might occur for *Spn* and NTHi, suggesting that immune responses to *Mcat* requires a more mature immune system in infants. We previously found that *Spn* predominates over *Mcat* to cause AOM when both organisms co-colonized the NP of young children.[28] Pettigrew et al. showed that *Spn* colonization is negatively associated with colonization by *Haemophilus influenzae* on 968 swabs collected from 212 American children.[29] We hypothesize that colonization with *Spn* and/or NTHi may suppress the colonization and proliferation of *Mcat* in the NP of children, which may account for the delayed natural priming from *Mcat* and development of antibodies to *Mcat* antigens. Future studies on the microbial interactions in the NP among these otopathogens may shed light on the mechanisms of this observation. We did not observe a significant difference of *Mcat* protein-specific antibodies in the sera of sOP and NOP children when samples were taken during a current vs. a previous *Mcat* NP colonization. The level of serum *Mcat*-specific antibody was more associated with the age of the children than the time of the colonization and the gradual effect of repeated exposures to the organism.

Measurement of IgM and IgG antibody levels at the time of clinical diagnosis of AOM allows additional comparisons among vaccine candidate proteins. However, the results do not inform regarding protective levels of antibody. We pursued these studies primarily to

understand the relative frequency of early primary serum responses (predominant IgM) to the studied vaccine candidates versus late primary (equivalent IgM and IgG) or secondary (predominant IgG) responses. In our past study of natural antibody levels to NTHi protein antigens D, P6 and OMP26, IgG levels to the individual proteins were higher than the corresponding IgM levels in both sOP and NOP children.[3] In our current *Mcat* study, we observed similar levels of IgG and IgM to OppA, OMP CD and PilA2 in both sOP and NOP children. However, IgM levels to Hag5-9 were much higher than IgG levels in both sOP and NOP children. We interpret the findings to suggest that earlier exposures to Hag5-9 following NP colonization and AOM infections resulted in less frequent primary immune responses than the other 3 proteins studied because the responses were IgM predominant (Fig 3). The implication might be that natural priming for Hag5-9 responses occurs less frequently in young infants compared to the other vaccine candidates at a younger age.

We found that the *Mcat* antigens elicited varying IgM and IgG antibody responses in both sOP and NOP children when acute to convalescent measurements were made surrounding an AOM infection. These results are consistent with our earlier studies of *Spn* and NTHi IgG responses.[3,4] It supports our observation that acute to convalescent changes in antibody to various candidate protein antigens of all three otopathogens surrounding an AOM event reflect an ongoing immune response initiated by NP colonization and not a specific response to the AOM infection. Similar observations of this variability in acute to convalescent antibody levels surrounding an AOM event have been made by another group regarding *Mcat* protein responses [30].

More than 90% of AOM are preceded by a viral upper respiratory infection (URI).[31] For some children NP colonization may have occurred weeks before an intercurrent viral URI triggered pathogenesis of AOM.[32-34] But for other children acquisition of the potential otopathogen may have occurred only a few days before an intercurrent viral URI, thus accounting for our observing IgM and IgG antibody levels rising, remaining unchanged or falling during the 3 week time span between collection of acute and convalescent sera. We do not consider it likely that the timing of the collection of the acute serum sample in relation to the onset of AOM clinical infection varied widely among the study children because prior work has shown that nearly all AOM infections occur 3 to 5 days after onset of viral URI.[34-36]

Overall this study adds further evidence that sOP children are immunologically different from NOP children. We have previously reported that sOP children suffer from delayed immune maturation and we have proposed the term “prolonged neonatal-like immune profile (PNIP) as applicable to these children because our studies of their B cells and T cells are consistent with a neonatal-like profile.[5,7,8,11,37] The underlying immune immaturity likely accounts for the impaired antibody responses to *Mcat* infection during asymptomatic colonization and AOM. Although they have not studied *Mcat* proteins, we note that studies by the group led by Thornton and Richmond involving Australian children who experience recurrent AOM have not observed lower antibody responses to *Spn* proteins or capsular polysaccharides or to NTHi proteins.[38-40] However there are important differences in the two study populations. We study stringently-defined otitis prone (sOP) children who have every clinical diagnosis confirmed by tympanocentesis and every otopathogen identified by

culture, while the Australian study population is diagnosed clinically by health care providers not directly involved in the research and in some cases middle ear fluid obtained from ruptured eardrums.[38-40] Also only 26% of our sOP children require ventilation tubes (unpublished data) while all of the Australian OP children studied for antibody responses to protein antigens were recruited at time of ventilation tube surgery.[38-40] Moreover, they found that Australian aboriginal (possibly similar to OP) children with otitis media displayed lower serum IgG to NTHi but not *Spn* proteins than non-aboriginal (possibly similar to NOP) or healthy children.[41] Consistent with our findings, these observations also suggest that OP children in different geographic areas may have similar defects in producing serum antibodies to certain otopathogens.

In conclusion, vaccine candidate proteins OppA, OMP CD, Hag5-9 and PilA2 of *Mcat* are naturally immunogenic in young children who experience *Mcat* NP colonization and AOM. sOP children produce lower naturally-induced serum antibody against these *Mcat* protein antigens compared to NOP children, which supports previous work in identifying the immune defects in this population. Purified proteins, particularly when adjuvanted, and administered by inoculation may elicit a different pattern and robustness of antibody response than we measured following natural exposure to *Mcat* by NP colonization and AOM infection. Further studies of functionality of the serum antibodies and analysis of mucosal antibodies would provide insights to their potential as vaccines and such studies are currently underway in our laboratory.

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Appendix

Statistical models for OP (otitis prone) effects on antibody response to NP colonization of *Mcat*

There are n subjects, from which data from multiple visits are observed. Index pair (i, j) refers to the jth of n_i visits from subject i. The variables used are:

A_{ij} = Age of subject i at jth visit (in months),

COL_{ij} = 1 if subject i is colonized at visit j; = 0 if subject otherwise has colonization history,

OP_i = 1 if subject i is sOP,

Y_{ij} = Antibody titer of subject i at jth visit. Four antibodies were modeled (OppA, OMP CD, Hag5-9, and PilA2).

The full linear model is

$$log_2(Y_{ij}) = log_{10}(A_{ij}) \times COL_{ij} \times OP_i + u_i + e_{ij}, i = 1, \ldots, n, j = 1, \ldots, n_i, \quad (1)$$

where $log_{10}(A_{ij}) \times COL_{ij} \times OP_i$ includes all main effects and interactions, u_i is a subject level random effect and e_{ij} is the remaining error term. It is assumed that the random effects and error terms are entirely independent, with $u_i \sim N(0, \sigma_u^2)$, $e_{ij} \sim N(0, \sigma^2)$.

Model (1) can be interpreted as 2 × 2 factorial design with age covariate, with design points = $(COL_{ij}, OP_i) \in [(0,0), (0,1), (1,0), (1,1)]$, alternatively denoted $t \in [ćó, ćo, cć, co]$ respectively. In the full model a separate simple linear regression fit is associated with each design point t:

$$log_2(Y_{ij}) = \alpha_t + \beta_t\, log_{10}(A_{ij}) + u_i + e_{ij}, i, j \text{ for which } (COL_{ij}, OP_i) = t, \quad (1)$$

with pooled estimates of σ_u^2 and σ^2 . In this way an OP effect is assessed separately for the colonization +ve (colonization positive at current visit) and -ve (colonization negative at current visit but positive at prior visit (s)) visits, by testing hypotheses:

$$H_{0:c}: \alpha_{có} = \alpha_{co} \text{ and } \beta_{có} = \beta_{co} \text{ vs } H_{1:c}: \alpha_{có} \neq \alpha_{co} \text{ or } \beta_{có} \neq \beta_{co} \quad [col + ve]$$
$$H_{0:ć}: \alpha_{ćó} = \alpha_{ćo} \text{ and } \beta_{ćó} = \beta_{ćo} \text{ vs } H_{1:ć}: \alpha_{ćó} \neq \alpha_{ćo} \text{ or } \beta_{ćó} \neq \beta_{ćo} \quad [col - ve].$$

Hypotheses $H_{1:c}$ and $H_{1:ć}$ are equivalent in the sense that they both represent the full model (1), with 8 model degrees of freedom. Hypotheses $H_{0:c}$ and $H_{0:ć}$ each introduce two constraints to the full model, and are reduced submodels of (1) with 6 degrees of freedom. These represent no OP effect for colonization +ve and -ve visits. The hypotheses can therefore be tested using the likelihood-ratio test statistic $X^2 = 2log(l_{FULL}/l_{RED})$, where l_{FULL}, l_{RED} are the maximum likelihood values of the appropriate full and reduced model. Under the null hypothesis X^2 possesses an approximate χ^2 distribution with d degrees of freedom, equaling the difference in model degrees of freedom.

The models were fit using function **lme** from the **R** package **nlme**, using the maximum likelihood method (the restricted log-likelihood (REML) method is also available).[25] OP effect on antibody response to each antigen for colonization +ve and -ve visits is shown in Table 3.

Distributional assumptions were assessed by examining subject and population level residuals, and the fitted values for random effect u_i. No apparent deviation from normality

was observed for the random effect. A moderate amount of right-skewness was observed for some of the residuals. To assess the effect of this on estimates of significant levels, the log transform $log_2(Y)$ for antibodies was replaced by the Box-Cox transform $Y_\lambda = (Y^\lambda - 1)/\lambda$ for values of $\lambda \in [-0.5,0.5]$.[42] This transformation converges to the log transformation as λ approaches 0, and the parameter λ otherwise controls skewness. By adjusting λ, skewness in the residual distributions was reduced without significantly changing the observed significance levels. We therefore conclude that skewness does not affect the inference.

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Abbreviations

Mcat	*Moraxella catarrhalis*
AOM	acute otitis media
sOP	stringently defined otitis prone
NOP	non-otitis prone
OP	otitis prone
NP	nasopharyngeal
OMP	outer membrane protein

Spn	*Streptococcus pneumoniae*
NTHi	nontypeable *Haemophilus influenzae*
OppA	oligopeptide permease A
MID	Moraxella IgD-binding protein
Hag	hemagglutinin
PilA2	PilA clade 2
mos	months
MEF	middle ear fluid
Escherichia coli	*E. coli*
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
ICH	International Conference on Harmonisation
ANOVA	analysis of variance
SEM	standard error of the mean
URI	upper respiratory infection
PNIP	prolonged neonatal-like immune profile

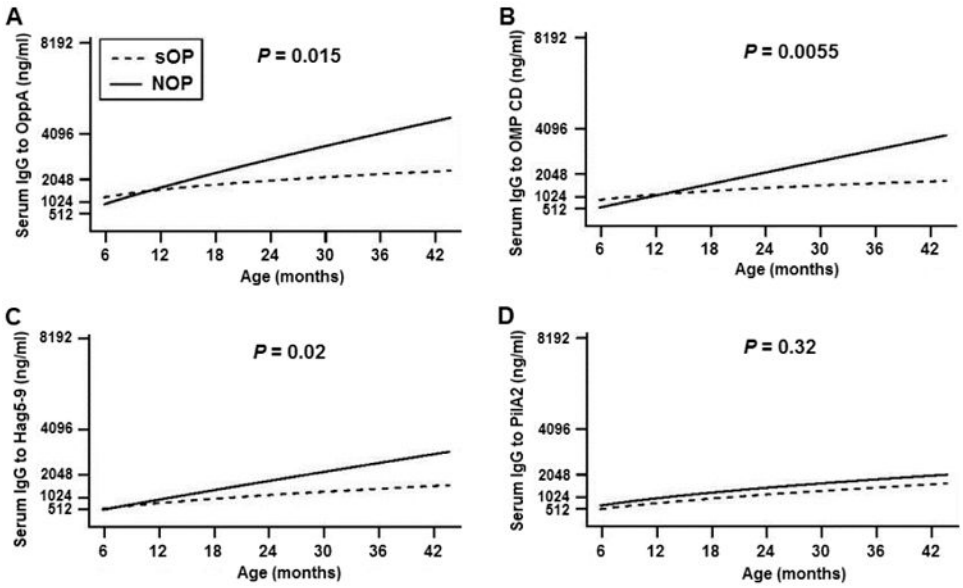

Figure 1.

Modeled comparison of serum IgG antibody level against *Mcat* proteins of OppA (A), OMP CD (B), Hag5-9 (C), and PilA2 (D) between the sOP (dash line) and NOP (solid line) children at their healthy visits. Serum was obtained from the children who had current and/or prior NP colonization of *Mcat* at each visit confirmed by culture. Serum anti-*Mcat* protein specific IgG antibody concentrations (ng/ml) were determined with a quantitative ELISA for the sOP children (n = 51) and NOP children (n = 131) at age of 6-36 mos., modeled to 42 mos. Fitted curves are plotted for all antibody values against age of children. *P*-value is for OP effect among colonization positive visits.

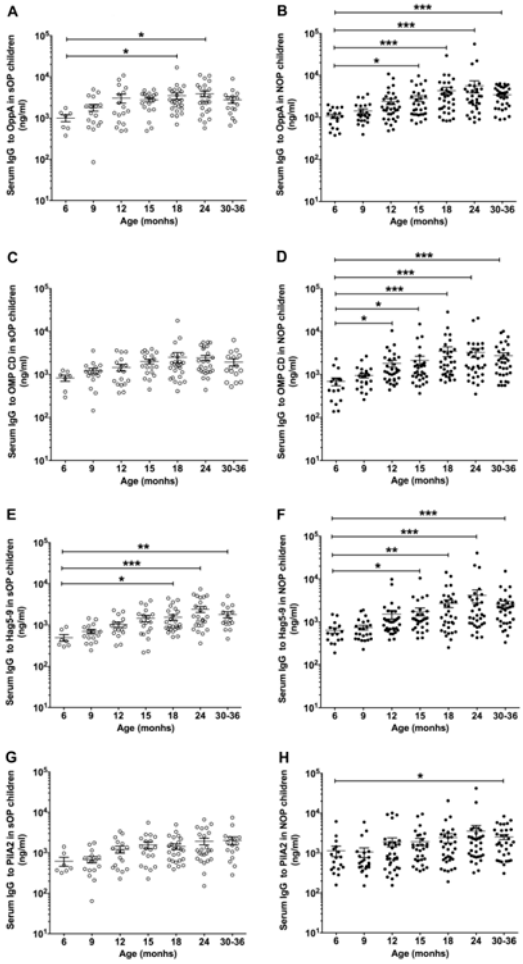

Figure 2.

Comparison of age-dependent increase of serum IgG antibody against *Mcat* proteins of OppA (A and B), OMP CD (C and D), Hag5-9 (E and F), and PilA2 (G and H) between the sOP and NOP children at their healthy visits. Serum was obtained from the children who had NP *Mcat* colonization prior to and/or at each visit confirmed by culture. Serum anti-*Mcat* protein specific IgG antibody concentrations (ng/ml) were determined with a quantitative ELISA for the sOP children (n = 51) and NOP children (n = 131) at age of 6-36 mos. Data are represented by mean ± standard error of the mean (SEM). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

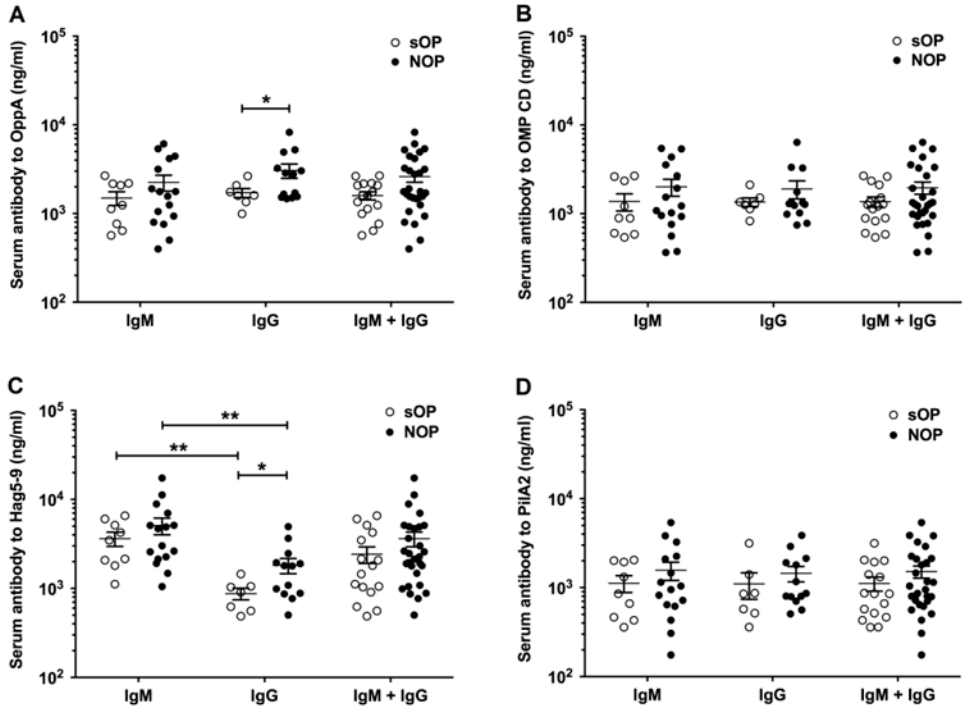

Figure 3.

Comparison of serum IgG and IgM antibody against *Mcat* proteins of OppA (A), OMP CD (B), Hag5-9 (C), and PilA2 (D) between the sOP and NOP children at their acute visit of AOM. Serum anti-*Mcat* protein specific IgG and IgM antibody concentrations (ng/ml) were determined with a quantitative ELISA for the sOP children (n = 7-9) and NOP children (n = 13-16) at age of 11-22 mos. Data are represented by mean ± SEM. **P* < 0.05 and ***P* < 0.01.

Table 1

Comparison of serum IgG and IgM antibody concentration against *Mcat* proteins between the sOP and NOP children at their AOM vs. convalescence stage[a]

Protein	Group of children[b]	N	IgM (ng/ml, mean±SEM)		IgG (ng/ml, mean±SEM)		Percentage of children with >2-fold increase in antibody at convalescence stage vs. AOM	
			Acute	Convalescence	Acute	Convalescence	IgM	IgG
OppA	sOP	15-19	1581±497	1352±368	2339±564	1688±214	**11%**	**0%**
	NOP	20-22	2019±335	1536±206	2405±420	2539±481	**5%**	**0%**
OMP CD	sOP	15-19	1471±413	1356±314	2593±730	2567±454	**16%**	**20%**
	NOP	20-22	1799±328*	1331±199	2324±455	2663±662	**5%**	**10%**
Hag5-9	sOP	15-19	3196±466	2831±337	1908±646	1109±180	**11%**	**0%**
	NOP	20-22	5205±903*	3943±603	1273±252	1697±559	**0%**	**15%**
PilA2	sOP	15-19	1237±387*	937±286	1888±509	1315±268	**5%**	**7%**
	NOP	20-22	1437±271*	1057±171	1073±200	1545±526	**5%**	**10%**

[a] children at age 6-36 mos. old were observed.

[b] sOP children have 3-8 AOM episodes each and NOP children have 1-2 AOM episodes each.

* $P < 0.05$ for acute versus convalescence serum.

Table 2

Change of serum IgG and IgM antibody level against *Mcat* proteins at convalescence stage vs. AOM in sOP and NOP children[a]

Protein	Group of children[b]	N	Percentage of children with nincrease in antibody at convalescence stage vs. AOM (%)		Percentage of children with no change in antibody at convalescence stage vs. AOM (%)		Percentage of children with decrease in antibody at convalescence stage vs. AOM (%)	
			IgM	IgG	IgM	IgG	IgM	IgG
OppA	sOP	15-19	16	33	58	40	26	27
	NOP	20-22	13	29	65	57	22	14
OMP CD	sOP	15-19	21	31	47	63	32	6
	NOP	20-22	9	24	52	71	39	5
Hag5-9	sOP	15-19	21	14	58	64	21	21
	NOP	20-22	9	20	57	65	35	15
PilA2	sOP	15-19	5	13	53	38	42	50
	NOP	20-22	9	50	43	11	48	39

[a] children at age 6-36 mos. old were observed.

[b] sOP children have 3-8 AOM episodes each and NOP children have 1-2 AOM episodes each.

Table 3

Tests for OP effect on antibody response to each antigen for colonization +ve and -ve visits

Antibody response	Colonization +ve[a]		Colonization -ve[a]	
	X^2	*P*-value	X^2	*P*-value
OppA	8.40	0.0150	0.53	0.7670
OMP CD	10.42	0.0055	1.49	0.4754
Hag5-9	7.82	0.0201	3.13	0.2095
PilA2	2.31	0.3154	1.65	0.4380

[a]The likelihood-ratio test statistic X^2 for each hypothesis are listed, along with the *P*-value for rejection of the null hypothesis of no group-specific OP effect. X^2 has an approximate χ^2 distribution with 2 degrees of freedom under the null hypothesis.