Published in final edited form as:

Vaccine. 2018 February 21; 36(9): 1133–1135. doi:10.1016/j.vaccine.2018.01.021.

***Haemophilus influenzae*-Protein D Specific Antibody Correlate with Protection against Acute Otitis Media in Young Children**

Anthony Almudevar, PhD¹ and Michael E Pichichero, MD^{2,*}

¹Department of Biostatistics and Computational Biology, University of Rochester Medical Center, Rochester NY

²Center for Infectious Disease and Immunology, Rochester General Hospital Research Institute, Rochester, NY

Abstract

Background: *Haemophilus influenzae* (*Hi*) is a common bacterial pathogen of respiratory infections and pathogenesis of this microbe begins in the human nasopharynx (NP). The objective of this study was to assess the correlation of NP colonization-induced serum antibody levels to *Hi* protein D with risk of acute otitis media (AOM) in children <2yr.

Methods: 455 sera from 213 children (age 6-24 months old) were collected when they were colonized with *Hi* and when the child developed AOM. Presence of *Hi* during AOM was confirmed by culture of middle ear fluid. Quantitative serum IgG against protein D antigen was measured by ELISA.

Results: Asymptomatic *Hi* NP colonization reduced the risk of future AOM infections. Higher serum IgG titers against *Hi* protein D correlated with reduced future AOM risk.

Conclusion: Colonization by *Hi* reduces future AOM risk. Higher antibody levels against protein D correlates with lower risk of AOM caused by *Hi*.

Keywords

Haemophilus influenzae; correlate of protection; antibody response

Introduction

Respiratory tract infections are the most common pediatric disease associated with significant morbidity and socioeconomic cost [1-3]. *Haemophilus influenzae* (*Hi*) is a common bacterial pathogen that causes pneumonia, acute exacerbations of bronchitis, acute

*Correspondence: Michael E. Pichichero, Rochester General Hospital Research Institute, Center for Infectious Diseases and Immunology, 1425 Portland Avenue, Rochester, NY 14621, U.S.A., michael.pichichero@rochesterregional.org.

Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

sinusitis, and acute otitis media (AOM) [1]. The first step of respiratory bacterial infection is nasopharyngeal (NP) colonization [4, 5], and NP colonization usually precedes upper and lower respiratory bacterial infections [3, 6]. We have studied natural immune responses of children to three highly conserved surface-exposed proteins among *Hi* strains that have shown significant potential as vaccine candidates: protein D, P6 and OMP26 [7, 8]. In this study, serum IgG concentration against *Hi* surface protein D was compared among cohorts of children during *Hi* NP colonization and AOM to determine if elevated protein D specific antibody levels correlate with protection against AOM in young children.

Materials and Methods

Subjects and study design

This study was part of a 10-year prospective, longitudinal evaluation of human child immunity to *Streptococcus pneumoniae* (*Spn*) and *Hi* supported by the National Institute of Deafness and Communication Disorders as described previously [9, 10]. Data here are from healthy children without known, medically attended previous episodes of pneumonia, sinusitis, or AOM. They enrolled at age 6 months from a middle class, suburban socio-demographic pediatric practice in Rochester, New York. NP, oropharyngeal (OP), hereafter referred to collectively as NP samples, and serum samples were collected from healthy children at 6, 9, 12, 15, 18 and 24 months of age and data here involve children who had not received antibiotics for at least 3 weeks prior to sampling. The NP samples were obtained for determining NP colonization of *Spn* and *Hi* by standard culture as described previously [11]. The serum samples were used for determining antibody response by quantitative ELISA. All of the children received standard vaccinations including PCV7 (Prevnar, Wyeth Pharmaceuticals, Collegeville, PA) as appropriate for age. The study was approved by the Institutional Review Board (IRB) of Rochester General Hospital. Written informed consent was obtained from parents or guardians of all child subjects prior to enrollment in the study.

Quantitative ELISA for antigen-specific antibody

Serum IgG levels against *Hi* outer membrane protein D were measured using quantitative ELISA in the GLP lab of Rochester General Hospital Research Institute. A sera pool from three adult donors with high endpoint ELISA antibody titers of IgG against protein D was used as reference serum for *Hi* antigen-specific ELISA. Total IgG in the reference serum was quantified using Human IgG ELISA Quantitation Kits (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's protocol with some modification. The wells of a 96-well microtiter plate for generating a standard curve were coated with affinity purified human IgG capture antibodies, while the wells for measuring specific antibodies were coated with 100 ng of recombinant protein D (provided as a gift from GSK as vaccine grade) in 100 µl of coating buffer (carbonate-bicarbonate, pH 9.6) [8]. Antigen specific antibody was calculated using the standard curve generated with SoftMax Pro version 5.2 (Molecular Devices Corp., Sunnyvale, CA) using a commercial sera pool containing known amounts of total IgG and total IgA (Bethyl RS10-101). Antibody concentrations were quantified using a four-parameter logistic-log standard curve generated from reference serum with SoftMax Pro version 5.2 (Molecular Devices Corp., Sunnyvale, CA). The lower limit of detection was calculated from the lowest concentration in the standard curve whose

OD was at least two standard deviations above the mean OD of blank controls. The lower detection limits for anti-protein D IgG was 3.5 ng/ml.

Statistical Analysis

Serum IgG titers were expressed in ng/mL. For the purpose of statistical analysis, antibody titers below the lower limit of detection was assigned a value equal to half the lower limit of detection for the assay. $P < 0.05$ was considered to indicate statistical significance. The strategy used here was to estimate the association of immunological outcomes (colonization rate, AOM risk and humoral antibody concentration) with immunological history. Odds ratios for risk were estimated using logistic modeling. Subject level correlation was modeled using generalized estimating equation methods. Details are given in the Methodological Supplement.

Results

In our longitudinal clinical study of 3755 visits by 762 children, we observed NP colonization by *Hi* in 336 children (44.1%), and *Hi* AOM in 149 children (19.6%). This study analysis involved 455 NP and serum samples from 494 visits of 213 children collected when they were healthy at 6, 9, 12, 15, 18 and 24 months of age. The characteristics of the children are shown in Table 1. Fourteen percent of the child population experienced their first AOM before age 6 months old, 50% between age 6 months and 1 year, 24% between age 1 year and 1.5 years and the balance beyond age 1.5 years.

NP colonization by *Hi* is protective for future *Hi* AOM infection

Asymptomatic *Hi* colonization events most likely occurred in many of the children prior to age 6 months old, because at first sampling at age 6 months, antibody to *Hi* protein D was measureable, confirming our previous reports.[7, 8] However, at 6 months of age maternal IgG may still be present and thus anti-protein D IgG measured might not exclusively reflect previous *Hi* exposure. In addition, asymptomatic *Hi* colonization events presumably occurred between the periodic 3-month sampling intervals since antibody levels rose between samplings. Nevertheless, comparisons of antibody levels among those who experienced an AOM and those children who did not is feasible since the detected exposure to *Hi* would allow the opportunity for the child to immunologically respond.

We modeled the dependence of AOM risk on prior detected asymptomatic *Hi* colonization history. We calculated the odds ratio (OR) for the change in AOM risk associated with detected *Hi* colonization history among children with no prior AOM. A history of prior detected *Hi* colonization significantly reduced future AOM risk, with OR of 0.54 (95% CI = [0.34, 0.87]; $P = 0.012$, Table 2), consistent with the notion that *Hi* colonization elicits an immune response that can be protective against AOM. Prior AOM infections would occur in the context of NP colonization and prior AOM infections with *Hi* correlated with protection against future AOMs, with an OR of 0.64 (95% CI = [0.45, 0.92]; $P = 0.017$).

Protection conferred by immunity to *Hi* protein D

We modeled antibody responses to the *Hi* antigen protein D according to subject age, colonization history, the presence of detected colonization at a given visit, and interactions with colonization history. Among patients with no observed AOM infection at any time, only age was a significant predictor of antibody response. This model was then fit using all visits for which antibody response was observed. An indicator predictor was added identifying visits at which subjects had no current or previous AOM infection, but had a future AOM infection. The antibody concentration to protein D for visits prior to an AOM infection caused by *Hi* were significantly lower by a fold-change of 0.66 (95% CI = (0.44, 0.97), $p = 0.0299$), across the age span of the children studied (Figure 1).

Discussion

We analyzed *Hi* colonization rates, *Hi* AOM, and naturally induced specific antibody responses to *Hi* protein D in a cohort of children studied prospectively. We found that specific antibody responses to *Hi* protein D, most often elicited by NP colonization, were associated with reduced risk of subsequent AOM.

Vaccines that prevent the risk of AOM would be highly desirable as a means to reduce the morbidity, cost and usage of antibiotic treatment for these infections. Our observation that higher serum antibody titers against protein D are associated with reduced risk of future AOM supports clinical studies where the pneumococcal conjugate vaccine, PHiD-CV (Synflorix™, GSK Vaccines) that contains protein D showed a trend of protective efficacy against AOM caused by *Hi*. [12] Development of a vaccine targeting *Hi* is actively being pursued because a vaccine to reduce AOM caused by *Hi* could also have efficacy in reducing other diseases caused by *Hi* such as sinusitis, non-bacteremic pneumonia and exacerbations of chronic obstructive pulmonary disease.

We conclude that children are naturally immunized when exposed to *Hi* and antibodies against *Hi* protein D display behavior expected of a vaccine correlate of protection, where heightened antibody titers were associated with a reduced likelihood of developing AOM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by NIH, NIDCD R01-DC008671. The authors thank Dr. Janet Casey and other staff of Legacy Pediatrics for sample collection.

Abbreviations:

AOM	Acute otitis media
<i>Hi</i>	Haemophilus influenza
NP	Nasopharynx

OR	odds ratio
<i>Spn</i>	<i>Streptococcus pneumoniae</i>

References

- [1]. Cappelletty D. Microbiology of bacterial respiratory infections. *Pediatr Infect Dis J.* 1998;17:S55–61. [PubMed: 9727651]
- [2]. Bogaert D, Keijsers B, Huse S, Rossen J, Veenhoven R, van Gils E, et al. Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. *PLoS One.* 2011;6:e17035. [PubMed: 21386965]
- [3]. Murphy TF, Bakaletz LO, Smeesters PR. Microbial interactions in the respiratory tract. *Pediatr Infect Dis J.* 2009;28:S121–6. [PubMed: 19918134]
- [4]. Margolis E, Yates A, Levin BR. The ecology of nasal colonization of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*: the role of competition and interactions with host's immune response. *BMC Microbiol.* 2010;10:59. [PubMed: 20178591]
- [5]. Bogaert D, De Groot R, Hermans PW. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis.* 2004;4:144–54. [PubMed: 14998500]
- [6]. Garcia-Rodriguez JA, Fresnadillo Martinez MJ. Dynamics of nasopharyngeal colonization by potential respiratory pathogens. *J Antimicrob Chemother.* 2002;50 Suppl S2:59–73.
- [7]. Kaur R, Casey JR, Pichichero ME. Serum antibody response to three non-typeable *Haemophilus influenzae* outer membrane proteins during acute otitis media and nasopharyngeal colonization in otitis prone and non-otitis prone children. *Vaccine.* 2011;29:1023–8. [PubMed: 21129398]
- [8]. Pichichero ME, Kaur R, Casey JR, Sabirov A, Khan MN, Almudevar A. Antibody response to *Haemophilus influenzae* outer membrane protein D, P6, and OMP26 after nasopharyngeal colonization and acute otitis media in children. *Vaccine.* 2010;28:7184–92. [PubMed: 20800701]
- [9]. Pichichero ME. Ten-Year Study of Acute Otitis Media in Rochester, NY. *Pediatr Infect Dis J.* 2016;35:1027–32. [PubMed: 27182898]
- [10]. Pichichero ME. Ten-Year Study of the Stringently Defined Otitis-prone Child in Rochester, NY. *Pediatr Infect Dis J.* 2016;35:1033–9. [PubMed: 27273691]
- [11]. Casey JR, Adlowitz DG, Pichichero ME. New patterns in the otopathogens causing acute otitis media six to eight years after introduction of pneumococcal conjugate vaccine. *Pediatr Infect Dis J.* 2010;29:304–9. [PubMed: 19935445]
- [12]. Clarke C, Bakaletz LO, Ruiz-Guinazu J, Borys D, Mrkvan T. Impact of protein D-containing pneumococcal conjugate vaccines on non-typeable *Haemophilus influenzae* acute otitis media and carriage. *Expert Rev Vaccines.* 2017;16:1–14.

FIGURE 1:
Protein D antibody response model. No AOM History (Hx)/Post-AOM antibody titers are significantly higher ($p=0.0299$) compared to subjects just prior to an AOM who developed AOM caused by *Hi*. P-value represents evidence of association between antibody concentration and *Pre-AOM* visit status.

Table 1

Characteristics of children (n=213)

	Total number (%)
Gender	
Female	107 (50.2%)
Male	106 (49.8%)
Race (Caucasian)	
Yes (Caucasian)	149 (70%)
No (other)	63 (30%)
Breast Feeding	
Yes (> 3 months)	128 (60.1%)
No	83 (39.9%)
Smoking Exposure	
Yes	29 (14%)
No	184 (87%)
Daycare	
Yes	72 (33.8%)
No	141 (66.2%)
PCV-7 vaccination Up-To-Date	
Yes	211 (99%)
No	2 (1%)

Table 2.

Predictive models for AOM rates based on colonization history and AOM history

Pathogen	Outcome	Groups	OR (95% CI)	P
<i>Hi</i>	AOM	Prior <i>Hi</i> Colonization history	0.54 (0.34-0.87)	0.012
	AOM	Prior AOM caused by <i>Hi</i>	0.64 (0.45-0.92)	0.017