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Haemophilus influenzae vaccine candidate outer membrane protein P6 is not conserved in all strains

Arthur Chang,[1] Ravinder Kaur,[1] Lea Vacca Michel,[2] Janet R. Casey[3] and Michael Pichichero[1,3,*]

[1]Rochester General Hospital; Rochester General Research Institute; Center for Infectious Diseases and Immunology; [2]Rochester Institute of Technology; [3]Legacy Pediatrics; Rochester, NY USA

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Abbreviations: NP, nasopharyngeal; OP, oropharyngeal; *NTHi*, nontypeable *Haemophilus influenzae*; AOM, acute otitis media

An outer membrane protein (OMP) of nontypeable *Haemophilus influenzae* (*NTHi*), P6, is a vaccine candidate because it has been characterized as conserved among all *H. influenzae* strains. Among 151 isolates from children, age 6 to 30 months, evaluating *NTHi* nasopharyngeal (NP) and oropharyngeal (OP) colonization and tympanocentesis confirmed acute otitis media we identified 14 strains (9.3%) that had variant protein sequences of P6. One atypical *omp* P6 isolate had sequence mutations in the binding site of a proposed major antigenic epitope of *omp* P6 identified by monoclonal antibody 7F3. Eight strains (5.3%) had non-homologous variations in amino acids that could result in significant changes to the protein structure of P6, and 5 other strains had amino acid substitutions at four previously described key residue sites. These results show that *NTHi omp* P6 is not invariant in its structure among respiratory isolates from children.

Introduction

Pioneering work by Murphy et al. characterized an outer membrane protein (OMP) of nontypeable *Haemophilus influenzae* (*NTHi*), called P6, as a vaccine candidate.[1-3] One attribute of P6 that has made it a particularly attractive vaccine candidate is its reported conservation among all *H. influenzae* strains. Indeed, previous studies have described outer membrane protein P6 (*omp* P6) as 100% homologous among all tested *NTHi* strains.[4]

Our group is conducting a multi-year, prospective study to evaluate the immune response to *NTHi* OMP vaccine candidates when children experience nasopharyngeal (NP) colonization and acute otitis media (AOM) caused by *NTHi*.[5,6] Here we describe that during the course of comparing the amino acid sequences of *omp* P6 we found that *omp* P6 was not invariant in its protein structure sequence among *NTHi* isolates.

Results

Microbiologic and PCR culture analysis identified a total of 151 isolates of *NTHi* from our pediatric samples. In total, out of 151 *NTHi* isolates, 14 (9.3%) had variations in their translated *omp* P6 sequences compared to the previously described *omp* P6 sequence which has been proposed to be conserved among all *NTHi* strains.

Table 1 shows *omp* P6 amino acid sequence variations of 14 *NTHi* isolates with variations throughout the *omp* P6 sequence and the sequence of a previously described urogenital strain.[7] For these 14 isolates, the amino acid substitutions could impact protein structure because the substitutions are non-conservative. The OP was the source of isolation of 11 of these 14 isolates, others were isolated from the NP (n = 2) or MEF (n = 1). The RapID NH system, confirmed the strains were classified as *NTHi* with >99% confidence. Partial 16S sequencing results were not able to distinguish these 14 *NTHi* isolates from *H. haemolyticus,* but the fucolose kinase gene was amplified for all of these isolates which indicated that these isolates are *NTHi*. One of the six isolates (HH13) had variations at amino acid positions 59 and 61. **Figure 1** shows the position of residues 59 and 61 on the 3 dimensional NMR structure of *omp* P6; the binding of monoclonal antibody 7F3 depends on the conformation and/or chemical properties of these two residues.[8] We therefore tested this isolate for binding to mAb 7F3 by whole-cell ELISA and showed mAb 7F3 did not bind to the P6 produced by this isolate. As a control we showed that the mAb 7F3 was capable of binding to *NTHi* strain 86-028NP. We show that a *H. influenzae* isolate (HH1) that does not have any mutations in the P6 protein, and *NTHi* (HH22) that has a mutation in amino acid position 33 which has been shown to be conserved in all previous *NTHi* strains also bound to mAb 7F3. HH1 was not statistically different from the

*Correspondence to: Michael E. Pichichero; Email: Michael.pichichero@rochestergeneral.org
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Table 1. Amino acid sequence variation of all isolates that did not have 100% amino acid homology with either typical *NTHi* or *H. haemolyticus*

	Source	AA11	AA33	AA42	AA59[a]	AA61[a]	AA77	AA81	AA114	AA152
Typical *NTHi*	N/A	Ala	Ala	Ala	Asp	Thr	Ala	Ala	Ala	Ala
Invasive Urogenital[b]	N/A	Ala	Gly	Ser	Asn	Glu	Ala	Ala	Ala	Ser
HH13	OP	Ala	Gly	Ala	Asn	Glu	Ala	Ala	Ala	Ala
HH22	OP	Ala	Gly	Ala	Asp	Thr	Ala	Ala	Ala	Ala
HH39	OP	Ala	Gly	Ala	Asp	Thr	Ala	Ala	Ala	Ala
HH75	OP	Ala	Gly	Ala	Asp	Thr	Ala	Ala	Ala	Ala
HH77	OP	Ala	Gly	Ala	Asp	Thr	Ala	Ala	Ala	Ala
HH86	OP	Ala	Gly	Ala	Asp	Thr	Ala	Ala	Ala	Ala
HH2	NP	Ala	Ala	Ala	Asp	Thr	Ala	Thr	Ala	Ser
HH5	NP	Ala	Ala	Ala	Asp	Thr	Ala	Ala	Thr	N/D
HH40	MEF	Ala	Ala	Ala	Asp	Thr	Ala	Thr	Ala	N/D
HH67	OP	Ala	Ala	Ala	Asp	Thr	Ala	Ala	Thr	Ala
HH69	OP	Ala	Ala	Ala	Asp	Thr	Glu	Ala	Ala	Ala
HH70	OP	Ala	Ala	Ala	Asp	Thr	Glu	Ala	Ala	Ala
HH80	OP	Ala	Ala	Ala	Asp	Thr	Ala	Ala	Thr	Ala
HH88	OP	Thr	Ala	Ala	Asp	Thr	Ala	Ala	Ala	Ala

Source abbreviations are defined as OP is oropharyngeal; NP is nasopharyngeal; MEF is Middle Ear fluid; N/A is not applicable. The sequence reaction for HH5 and HH40 did not cover the region for amino acid 152 (N/O). Shaded cells indicate amino acid differences from typical *NTHi*. Note: [a]AA59 and AA61 of *omp* P6 is part of the epitope that binds mAB 7F3. [b]The invasive urogenital amino acid pattern is from the reported unique *NTHi* isolate by Murphy et al.[7]

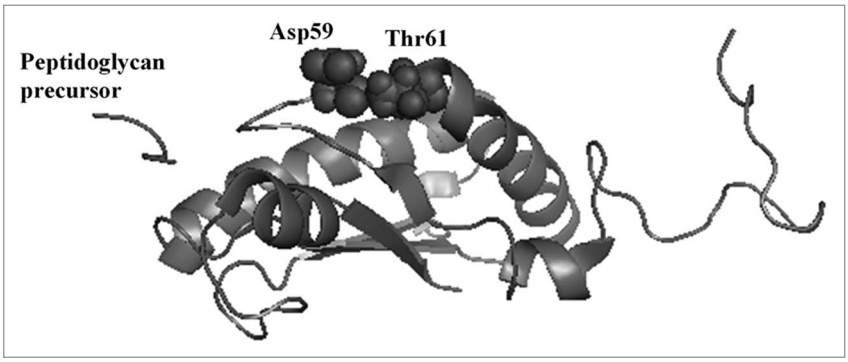

Figure 1. P6 NMR structure (Protein Data Bank ID 2AIZ) with residues 59 and 61 highlighted in ball-and-stick, prepared using PyMOL Molecular Viewer. Copyright 2009 DeLano Scientific LLC.

control while HH22 was. As a second control we tested an *H. haemolyticus* isolate that did not bind mAb 7F3 either. We also tested all of the remaining P6 variant *NTHi*; all showed binding to mAb 7F3 of which only 3 were statistically different from the control (data not shown).

Discussion

This is the first study to identify variations of *omp* P6 among *NTHi* respiratory isolates in humans. Our findings have implications for selecting *omp* P6 as a vaccine component against *NTHi*. Until now, previous studies have shown that *NTHi omp* P6 had 100% homology among human respiratory isolates from adults and children.[9,10] Those studies used a variety of methods to determine *omp* P6 conservation including restriction fragment length polymorphism, direct DNA sequencing, and a variety of immunoassays.[1,3,9,10] Also *omp* P6 has been characterized as a surface exposed antigen that induces high bactericidal antibody titers, which makes *omp* P6 a vaccine candidate.[1,3,11,12] Our discovery that ~10% of *NTHi* strains in a pediatric population have variations in P6 protein structure does not eliminate this antigen as a potential vaccine ingredient since conservation of the protein among 90% of *NTHi* strains is of considerable value and still may allow *omp* P6 to be included in a multi-component vaccine.

The atypical *omp* P6 isolates of *NTHi* in our study population that did not bind to mAb 7F3 differs from a previously described invasive urogenital *NTHi* isolate which was also incapable of binding to mAb 7F3.[7] A previous study by McCrea et al. found that 3 (3.4%) of 88 *NTHi* strains they studied did not react with the mAb 7F3; these results may have been due to amino acid substitutions at key 7F3 antibody residues, similar to what we have seen in this study.[13] However, McCrea et al. did not comment or pursue this finding further.

We observed differential binding of mAb 7F3 to the different variant isolates. This could be due to the expression differences of P6 in different isolates or the amino acid variations may be altering the ability of the mAb to access the 7F3 epitope.

It is important to recognize the limitations of our study. The amino acid substitutions altered the chemical nature of P6, but it is unknown how or to what extent these changes altered the structure of P6. We also did not evaluate the impact of the variations in *omp* P6 regarding its reactivity with antibody generated

during *NTHi* NP colonization or mucosal infection in children or adult hosts. Studies are now underway by our group to specifically evaluate the impact of structural variations in *omp* P6 induced by the amino acid substitutions we have identified.

Materials and Methods

Study design and population. The samples evaluated in this report were collected as part of a prospective study that commenced in June 2006 and is ongoing. The children were enrolled at 6 months of age and followed prospectively. NP and oropharyngeal (OP) samples were obtained at 6, 9, 12, 15, 18, 24 and 30 months of age for microbial culture analysis. Middle ear fluid (MEF), OP and NP samples were also obtained to determine the etiology of AOM when a child developed their first and any subsequent (AOM) episodes and 3 weeks after an AOM. A second group of enrolled children were defined as otitis prone, with recurrent AOM (3 AOM episodes in 6 months or 4 episodes in 12 months) and younger than 36 months. In that group, MEF, OP and NP samples were obtained at the time of diagnosis of AOM and 3 weeks after an AOM episode. The study was approved by the University of Rochester and subsequently by the Rochester General Hospital IRB and written informed consent was obtained.

Definition of AOM. AOM was diagnosed by pneumatic otoscopy by two of the authors (JC, MEP), who are both validated otoscopists, when children with acute onset of otalgia have tympanic membranes (TMs) that were: (1) bulging or full; and (2) a cloudy or purulent effusion was observed, or the TM was completely opacified; and (3) TM mobility was reduced or absent.

MEF sampling. MEF for cultures were obtained by puncture of the inferior portion of an intact TM with a 20-gauge spinal needle attached to a 3 mL syringe using a hand-held operating otoscope. If a small sample of MEF was obtained on aspiration, any MEF was aspirated through the spinal needle, inoculated onto agar plates and into broth (as described below), and then after 24 hours of growth, aliquoted for storage at -80°C.

Nasopharyngeal (NP) and oropharyngeal (OP) sampling. At each sampling visit, a cotton-tipped wire swab was inserted into each nares for NP samples and an OP swab was obtained by rubbing both tonsils and the posterior pharynx.

Microbiology. MEF, NP and OP samples are inoculated onto trypticase soy broth, trypticase soy agar with 5% sheep blood plates, and chocolate agar plates. All samples are incubated at 37°C with 5% carbon dioxide. Bacterial isolates were identified by standard Clinical Microbiology Procedures Handbook methods. An isolate was identified as *NTHi* based on colony morphology, porphyrin reactivity, and growth requirement for hemin and nicotinamide adenine dinucleotide using Haemophilus ID Quad plates.[9]

PCR amplification of omp P6. The PCR reactions were performed on genomic DNA isolated from *NTHi* strains targeting the P6 gene using oligonucleotide primers described by Murphy et al.[9] Amplification of omp*P6* was conducted using PCR Sprint (Hybaid) as followed: 94°C for 5 minutes followed by 30 cycles (94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds) and finished with 10 minutes at 72°C.

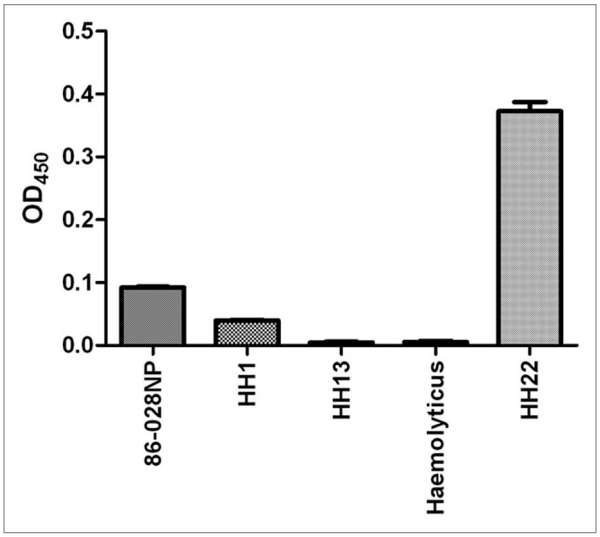

Figure 2. ELISA results showing the binding of monoclonal antibody 7F3 with standard deviation bars. *NTHi* HH13 and *H. haemolyticus* did not show any reactivity with mAb 7F3. HH22 and HH1 are representative of all of the variant P6 isolates tested.

Additional identification tests. The RapID NH system (IDS) was used to confirm the identity of *Haemophilus influenzae* following the manufacturer's protocol.[14] Partial 16s rRNA sequencing, fucolose kinase gene amplification (a marker to distinguish *NTHi* from *H. haemolyticus*), and 7F3 monoclonal binding (an attribute associated primarily with *H. influenzae*) were used to further confirm the identity of isolates.[10,13]

Whole cell ELISA. Isolated *NTHi* was cultured overnight at 37°C on Chocolate II agar with IsoVitalax (BD) and then suspended into 10 mL of Dulbecco's PBS, and the OD was read at 600 nm. Optical density of the samples was adjusted to 0.4. Adjusted OD samples were then sonicated 3x for 10 seconds. 100 μl of sample was placed into each well of an Apogent medium binding plate (Nunc) and incubated at room temperature overnight. Plates were washed 5 times with PBS with 0.1% TWEEN-20. The plate was then blocked with 200 μl of PBS with 0.1% Gelatin for 1 hour at 37°C. Plates were again washed 5 times with PBS with 0.1% Tween-20. The monoclinal antibody (mAb) 7F3 (provided by Dr. Timothy F. Murphy, University of Buffalo) was diluted in PBS with 0.05% Tween-20 and 0.2% Gelatin. 100 μl of mAb was used in each well and was allowed to incubate at room temperature for an hour. Plates were then washed 5 times. Goat anti-mouse IgG with HRP was used as the secondary antibody with a dilution 1:10,000. 100 μl of secondary antibody was placed in each well and allowed to incubate at room temperature for one hour. After washing, 100 μl of TMB substrate (KPL) was placed in each well and allowed to develop for one hour before being stopped by 100 μl of 1 M phosphoric acid. The plates were read using an automated ELISA reader at 450 nm.

Acknowledgements

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