Outer Membrane Protein Profiles of Paired Nasopharyngeal and Middle Ear Isolates of Nontypable *Haemophilus influenzae* from Mexican Children with Acute Otitis Media

Alberto Villaseñor-Sierra* and José Ignacio Santos²

We studied nontypable *Haemophilus influenzae* (NTHi) isolates from simultaneous cultures of nasopharyngeal exudates (NEs) and middle ear fluids (MEFs) obtained by tympanocentesis from 57 children with acute otitis media (AOM). Preparations of outer membrane proteins (OMPs) from 14 pairs of NTHi strains recovered from NEs and MEFs from 10 children with unilateral AOM and four with bilateral AOM were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The NTHi subtypes were determined by comparing the OMP profiles of the isolated strains with those of eight reference NTHi subtypes. Of the 14 pairs, 10 (71%) were identical, and one (8%) was different; three strains isolated from NEs (21%) did not correspond to any of the reference subtypes (nontypable). Subtypes 4, 6, 5, 3, and 8 were isolated in the present study, thereby showing that their distribution is similar to that of subtypes isolated from children with AOM in the United States and suggesting that common otogenic strains are widely distributed in North America.

Acute otitis media (AOM) is considered the third most common reason for pediatric consultation [1]. It occurs as a single episode in 82% of children, as a second event in 14.5%, and as a third episode in 3.5% [2]. The incidence is highest in the first 36 months of life and between 6 and 9 years of age. In countries with temperate climates, AOM is most frequent during the winter months, December through March. The clinical manifestations include local and systemic signs and symptoms. Pneumatic otoscopy reveals the classic features of opaqueness, rounding, and hypomobility of the tympanic membrane [3].

Various explanations for the high incidence of AOM in early childhood have been considered; these include the immunologic immaturity of the young infant and child; a shorter, straighter, and more patulous eustachian tube in the child; not having been breast-fed; and various factors related to bottle-feeding, including development of the facial muscles that affect the function of the eustachian tube, thus increasing the risk of retrograde aspiration of milk into the middle ear [4].

Capsulated *Haemophilus influenzae* strains are classified according to their agglutination with specific antiserum (a−f), and noncapsulated strains are designated as nontypable. Of the etiologic agents of AOM, nontypable *H. influenzae* (NTHi) is one of the most frequently isolated [5]. In a review of 4,675 cases of AOM in the United States, Switzerland, and Finland, *H. influenzae* was the second most common etiologic agent (21% of all isolates); most of these strains were nontypable [6]. Other studies [5, 7] including one in Mexico [8] found similar results.

Little is known about the pathogenesis of *H. influenzae* AOM indicating that the development of specific antibodies by the host and the marked difference in bacterial adherence, invasiveness, virulence, and resistance to the bactericidal effect of human serum may be related to ultrastructural and macromolecular markers of the organism, such as the outer membrane proteins (OMPs) [13, 14]. OMPs of NTHi strains have been characterized. In 1983, Murphy et al. [13] described the OMP profiles of 48 clinical isolates of NTHi from adults and children with use of SDS-PAGE. These investigators found that each strain had from 10 to 20 OMPs, including two major bands that stained more densely than the others; the molecular weights ranged from 32,000 to 42,000. Between the two major bands, eight intermediate bands with well-defined electrophoretic patterns were clearly identified; these bands were proposed as a subtyping scheme for identifying eight different subtypes.
The two major bands of OMPs of NTHi have been recently designated as protein P2 or b/c by Murphy and Bartos [15]. Restriction fragment length polymorphism analysis of the P2 of NTHi strains showed a marked heterogeneity in and around the ompP2 locus. This heterogeneity could be related to the antigenic heterogeneity of the P2 molecule [7].

The classification of NTHi into eight different subtypes has been used as an epidemiological tool to identify the relative prevalence of these strains. In 1987, Murphy et al. [16] compared the subtypes of 19 pairs of NTHi strains isolated simultaneously from middle ear fluids (MEFs) and nasopharyngeal exudates (NEs) from children with AOM and recurrent AOM. Of 19 pairs, 16 (84%) were identical. Subtypes 5, 7, 6, 3, 4, and 2 were isolated in decreasing order of frequency.

Reports from other countries regarding NTHi subtypes isolated from MEFs, as well as from other clinical sources, have shown regional variability [7, 17, 18]. The findings from these few studies could be related to nonrepresentative samples, a distinctive host genetic susceptibility to infection by certain subtypes, or a genetic variability of the NTHi P2. Identification of NTHi subtypes that is based on OMP profiles has been of great value for the classification of this heterogeneous group for the following reasons: the technique for obtaining OMPs is reproducible and they remain stable under experimental manipulation; their identification is not dependent on the bacterial growth phase; they do not change while undergoing culture or animal experimentation; the results are useful for the purposes of epidemiological surveillance; and their homogeneity makes them potential vaccine candidates [13, 14, 19].

In Mexico, reported information regarding the epidemiology and etiology of AOM is limited, and there is no information on the prevalence of NTHi subtypes, data that are of paramount importance for a global perspective on the development of a vaccine specifically targeted to prevent otitis media by this organism.

In the present study, OMP profiles were used to determine and correlate the subtypes of NTHi isolated from cultures of paired MEFs and NEs from children with AOM or recurrent AOM.

Materials and Methods

Study subjects. Children from 1 month to 13 years of age were recruited for the study from the outpatient clinic and the Ear, Nose, and Throat Service at Hospital Infantil de México “Federico Gómez” (Mexico City) from May 1991 to January 1993.

Inclusion criteria were a clinical diagnosis of AOM or recurrent otitis media and isolation of NTHi simultaneously from the middle ear and nasopharynx. The presence of local and systemic signs and symptoms as well as the triad of signs in the tympanic membrane including redness, bulging, and decreased mobility was required for the diagnosis of AOM [20].

Exclusion criteria were chronic otitis media, a perforated tympanic membrane, antibiotic therapy within 72 hours before study entry, and immunodeiciencies.

Case definition. Our case definition was based on the simultaneous isolation of NTHi from the MEF of one or both ears and from the nasopharynx of a single child.

Tympanocentesis was performed by an otolaryngologist and by one of the investigators (A.V.-S.); the patient was previously sedated by a pediatric anesthesiologist. The external auditory canal was sterilized with 70% ethyl alcohol for 3 minutes, and a culture specimen was obtained. The tympanic membrane was punctured in the posterior quadrant by using a surgical head otoscope and a sterile 20-gauge lumbar puncture needle specially adapted for the procedure [21, 22].

The MEF was cultured on sheep blood agar and chocolate agar; the culture was incubated at 35°C in a 5% CO₂ environment [23]. Simultaneously, an exudate sample from the nasopharynx was obtained with a sterile cotton swab moistened in Stuart medium (Difco Laboratories, Detroit), and the sample also was cultured on sheep blood agar and chocolate agar.

Classification of H. influenzae. Each isolated strain was identified by its colonial morphology, microscopic visualization, and the requirement of X (hemin) and V (nicotinamide-adenine dinucleotide) factors for growth. The strains were classified as nontypable when there was no agglutination with the specific capsular antiserum (Phadebact KARO-BIO, Huddinge, Sweden).

Maintenance of NTHi strains and obtaining OMPs. From the initial cultures of MEF and NE, one to six colonies were taken and subcultured separately on chocolate agar; the subcultures were incubated at 35°C for 18 to 20 hours in a 5% CO₂ atmosphere [14]. Each strain was then inoculated into 5 mL of brain-heart infusion broth (Difco Laboratories) enriched with 10 μg of hemin/mL (Sigma, St. Louis) and 10 μg of nicotinamide-adenine dinucleotide/mL (Sigma).

After an 18-hour incubation, the 5-mL culture was placed in 50 mL of brain-heart infusion broth enriched with X and V factors in a 100-mL flask, and the flask was incubated at 35°C with agitation for 18–24 hours. The bacteria were collected by ultracentrifugation at 40,000g for 20 minutes at 4°C and frozen at −20°C in sterile cryogenic polypropylene vials for subsequent use.

Each isolate was resuspended in 10 mL of 0.01 M HEPES buffer (pH 7.4) (Sigma) and disrupted by sonication (Branson Sonifier 250, Branson, Danbury, CT) at 100 W during five 20-second periods in plastic tubes submerged in ice. The remaining intact bacteria and debris were removed by centrifugation at 1,700g for 30 minutes, and the bacterial membranes were collected by centrifugation at 10,000g for 60 minutes at 4°C.

The resulting pellet was resuspended in 1 mL of 0.01 M HEPES buffer and 1 mL of 2% N-lauroylsarcosine sodium salt (Sigma) in vol/vol 0.01 M HEPES buffer, and the pellet was
homogenized by agitation for 30 minutes at room temperature to dissolve the inner membrane proteins.

The detergent insoluble fraction (rich in outer membranes) was recollected by centrifugation at 100,000g for 60 minutes at 4°C, and the pellet was resuspended in 100 µL of distilled water. The protein concentration was determined by the method of Lowry et al. [24].

Electrophoresis in polyacrylamide gels (SDS-PAGE). The membrane preparations were adjusted in a 1:1 dilution with SDS/sample buffer (5 mL of 0.5 M Tris-HCl [pH 6.8], 0.5 mL of 10% SDS, 0.5 mL of glycerol, 10 mg of bromophenol blue, and 9 mL of distilled water) to obtain a concentration of ~15 µg of protein. The membrane protein was heated at 100°C for 5 minutes and later abruptly chilled in ice water to facilitate protein denaturation.

Electrophoresis was performed in a minichamber (Mighty Small II, model SE 250; Hoefer Scientific Instruments, San Francisco). Aliquots of ~15 µg of each sample protein were applied to each of the gel slots. The stacking gel contained 3% acrylamide (Sigma) and 0.08% N,N-methylene-bis-acrylamide (Sigma), and the separating gel contained 11% acrylamide and 0.3% N,N-methylene-bis-acrylamide. Aliquots of 1.5 µL of standardized molecular weight markers (low range, 14,400–97,400; Bio-Rad, Hercules, CA) were applied in the first lane of each gel.

Electrophoresis was performed at 4°C and 30 V until the sample reached the separation gel and then at 100 V until the color indicator migrated to within 1 mm of the outer lower gel bud.

Once each run was completed, the gel was placed in a concentrated staining solution (10% acetic acid, 25% methanol, 0.25% Coomassie blue R) for 1 hour, and then the gel was placed in destaining solution (10% acetic acid, 25% methanol). Identification of NTHi subtypes according to OMP patterns was done in two phases. First, gels with OMP samples from NTHi strains isolated from middle ear and nasopharyngeal samples as well as from eight reference strains (kindly donated by Dr. Timothy F. Murphy, Infectious Diseases Unit, University of Buffalo, Buffalo) were run in parallel (figure 1). A preliminary comparison of the OMP profiles of clinical isolates with those of the reference strains was made visually. Second, another gel was run with the OMP samples from paired MEF and NE isolates from each patient, followed by a gel with an OMP from the most likely corresponding reference subtype in an adjacent lane. This procedure was repeated until the bands between 32 and 42 kD for OMPs from each of the clinical samples were matched visually with those for OMPs from any of the eight reference subtypes.

Results

The study included a total of 57 infants and children with AOM; 38 (67%) of the patients were male, and the greatest proportion of patients were between 2 and 4 years of age (table 1). Tympanocentesis was performed unilaterally in 47 cases (25 right ears and 22 left ears) and bilaterally in 10 cases. There were no complications during or after the procedures. In all cases, the material obtained from the middle ear was cultured, the isolated bacteria were identified, and antimicrobial susceptibility was determined by the Kirby-Bauer method.

Microbiology of the middle ear. Bacterial cultures of MEFs from 40 (70%) of 57 children were positive. Briefly, of the positive cultures, 21 (52%) yielded NTHi, and 19 (48%) yielded other bacteria, including Staphylococcus aureus, H. influenzae type b, Neisseria sicca, Neisseria subflava biovar flav a, and Streptococcus pyogenes. Of the 21 children with MEF cultures positive for NTHi, the majority, 14 (16%), also had NE cultures positive for this organism. However, of 19 children in whom other bacteria were isolated from the MEF, only three (16%) had concomitant NE cultures positive for NTHi. Finally, of 17 children with negative MEF cultures, three (18%) had NE cultures positive for NTHi or Hib.

Simultaneous isolation of NTHi from MEFs and NEs. NTHi was simultaneously isolated from MEFs and NEs from 14 (25%) of 57 children (10 with unilateral AOM and four with bilateral AOM).

Paired subtypes isolated from MEFs and NEs. All NTHi strains isolated from the middle ear of the 14 children with unilateral or bilateral AOM were subtyped according to the OMP patterns of the eight reference strains. For all of these strains, the major bands (between 32 and 42 kD) were identical to those for the corresponding reference strains.

Correspondence of subtypes isolated from MEFs and NEs. For 10 (71%) of 14 children, the subtype isolated from the middle ear was identical to the one isolated simultaneously from the nasopharynx (figure 2). For three (21%) of 14 patients, the strain isolated from the middle ear was subtypable, whereas the strain isolated simultaneously from the nasopharynx was
Table 1. Distribution of 57 children with acute otitis media by age and gender.

<table>
<thead>
<tr>
<th>Age range (mo)</th>
<th>No. (%) of children</th>
<th>No. of males</th>
<th>No. of females</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–23</td>
<td>11 (19)</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>24–47</td>
<td>19 (33)</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>48–71</td>
<td>8 (14)</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>72–95</td>
<td>8 (14)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>96–119</td>
<td>8 (14)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>120–143</td>
<td>2 (4)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>144–167</td>
<td>1 (2)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>57 (100)</td>
<td>38</td>
<td>19</td>
</tr>
</tbody>
</table>

Figure 2. Representative nontypable *Haemophilus influenzae* (*NTHi*) isolates of identical subtypes in cultures of middle ear fluid and a nasopharyngeal exudate from a child with acute otitis media. *Lane S*, molecular weight standards; *lanes A* and *B*, the outer membrane protein (OMP) profiles of the *NTHi* strains isolated simultaneously from the middle ear fluid and nasopharyngeal exudate, respectively; *lane C*, the OMP profile corresponding to that of the reference subtype 4.

Figure 3. Different nontypable *Haemophilus influenzae* (*NTHi*) subtypes isolated simultaneously in cultures of middle ear fluid and a nasopharyngeal exudate from a child with acute otitis media. *Lane S*, molecular weight standards; *lanes A* and *B*, the outer membrane protein (OMP) profile of the *NTHi* strain isolated from the middle ear fluid and nasopharyngeal exudate, respectively; *lanes C* and *C’*, the OMP profiles of the reference subtypes 6 and 3, respectively.

Figure 4. Nonsubtypable strains of *Haemophilus influenzae* isolated from nasopharyngeal exudates from children with acute otitis media. *Lane S*, molecular weight standards; *lanes A, B, C, and D*, the outer membrane protein (OMP) profiles of nonsubtypable strains of *H. influenzae*. To avoid possible misclassification because of apparent similarity in the OMP profile between strains, the OMP profile of reference subtype 1 (lane ST-1) is shown after that of a nonsubtypable strain (lane B).

not subtypable. Finally, for one child with AOM, the subtype isolated simultaneously from the middle ear and the nasopharynx was different (figure 3).

Some variations between the clinical isolates and the reference strains were noted in the bands for the minor proteins and in the intensity of the major bands. For all four children with bilateral AOM, the subtype isolated from the right and left ears was identical, and in each case, the subtypes recovered from MEFs and NEs were identical. The subtypes isolated from MEFs, in increasing order of frequency, were 4, 6, 5, 3, and 8. *NTHi* strains isolated from NEs from 11 children were subtypable, whereas for three children, the OMP profile of the subtype isolated from the NE did not correspond to those of any of the reference subtypes (figure 4). There was a single case in which the subtypes isolated from the MEF and NE were identical and a nonsubtypable *H. influenzae* strain was also isolated from the NE. The subtypes isolated from the nasopharynx in decreasing order of frequency were 4, 5, 6, nonsubtypable, and 3.

*NTHi* recurrent AOM. There were two children with recurrent AOM. Recurrent AOM was defined as a history of three or more episodes of AOM within the preceding 6 months or four episodes in 1 year [25]. One patient had an episode in the left ear and another episode in the right ear; the interval between
episodes was 39 days. In each episode, the subtype isolated from the middle ear was different (subtypes 3 and 6, respectively). The second patient had one episode of otitis media in the left ear, a second episode 25 days later in both ears, and a third episode 15 days later in the left ear. A single subtype (subtype 4) was isolated from the middle ear in all three episodes of recurrent AOM.

Of all 28 NTHi subtypes isolated from both the middle ear and the nasopharynx, 25 (89%) belonged to five of the eight reference subtypes. In decreasing order of frequency, the isolates were subtypes 4, 6, 5, 3, nontypable, and 8 (table 2). Murphy et al. [16] found that 16 (84%) of 19 subtypes isolated from simultaneous MEFs and NEs were identical. There was no significant difference ($P = .639$) between the proportion of identical NTHi subtypes isolated simultaneously from MEFs and NEs in our study and the study by Murphy et al. [16].

In a study of typable *H. influenzae* and NTHi strains isolated from Pakistani children with respiratory infections [26], most isolates (82%) exhibited a clonal restriction to five subtypes, with a different pattern and frequency than were reported here or in the study by Murphy et al. [16].

The high proportion of identical NTHi subtypes isolated from both MEFs and NEs suggests that generally, at any given time, the nasopharynx is colonized by a single subtype. A recent study using PCR fingerprints to identify six NTHi strains isolated from each of five children colonized with NTHi [27] demonstrated that the children were colonized by a single strain at any one time. Finding identical subtypes in MEFs and NEs also supports that retrograde aspiration of nasopharyngeal secretions through the eustachian tube may play an important role in the inoculation of pathogenic bacteria from the nasopharynx into the middle ear space [28].

For one child, the isolated subtype from the middle ear and nasopharynx was different. In another child, two different NTHi strains were isolated from the NE: the first was identical to the one isolated simultaneously from the middle ear (subtype 5) and the second was a nontypable strain. One possible explanation is that occasionally the nasopharynx is colonized simultaneously by more than one NTHi subtype. The fact that subtypes 1, 2, and 7 were not found in our study could be due to selection bias, sample size, or perhaps a low frequency of these subtypes in children with AOM. The different OMP patterns of nontypable strains could be the consequence of the mixing of different subtypes in a single colony, the OMP pattern of capsule-deficient *H. influenzae* type b strains [29] not detected by the slide agglutination method used in this study, or the presence of subtypes not previously described.

Similarity of NTHi subtypes isolated from Mexican children and children from the United States was evident, with four of the six subtypes being concordant (subtypes 3, 4, 5, and 6). This similarity could suggest that racial factors, as well as regional differences, may be insufficient to explain the heterogeneity of NTHi subtypes that has been reported in other studies [17, 18] and that otogenic NTHi strains exist in a wide geographic distribution.

For the two children with recurrent AOM, the difference in isolated subtypes from MEFs appeared to be dependent on the time between episodes. In the first case, the persistence of the same subtype in three episodes can be interpreted as a consequence of a relapse, an inability to develop protective antibodies [30], or low serum levels of IgG subclass 2, which was previously described in otitis-prone children [4]. In the second case, the presence of a new subtype in the MEF 39 days after the initial episode undoubtedly represented a new

### Table 2. Nontypable *Haemophilus influenzae* subtypes isolated from MEFs and NEs from children with acute otitis media.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Ear affected</th>
<th>MEF</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bilateral</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Bilateral</td>
<td>5</td>
<td>5 and NT</td>
</tr>
<tr>
<td>3</td>
<td>Bilateral</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Left</td>
<td>8</td>
<td>NT</td>
</tr>
<tr>
<td>5</td>
<td>Left</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6*</td>
<td>Left</td>
<td>3</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>Right</td>
<td>3</td>
<td>NT</td>
</tr>
<tr>
<td>8</td>
<td>Left</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>Right</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>Left</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>11*</td>
<td>Right</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>12*</td>
<td>Bilateral</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>13</td>
<td>Right</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>14*</td>
<td>Left</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

**NOTE.** MEF = middle ear fluid; NE = nasopharyngeal exudate; NT = nontypable. * and † Episodes of recurrent acute otitis media in two different children.
event as evidenced by the concomitant identification of a different subtype in cultures of the MEF and NE. Similar observations have been described in previous reports [16, 31].

Finally, in the cases of isolation of an NTHi strain from the middle ear, simultaneous isolation from the nasopharynx (67%) was fourfold higher than in cases in which other bacteria were isolated from the middle ear (16%). These observations are in agreement with the findings of Faden et al. [32] and Long et al. [33] on the positive predictive value of isolating NTHi from NEs from children with AOM to predict the isolation of NTHi from MEFs.

In conclusion, all of the NTHi strains isolated from MEFs and most NTHI strains isolated from NEs were subtypable, thereby confirming the utility of the OMP subtyping system [13] for NTHi as an epidemiological tool. Finding identical OMP profiles of isolates from MEFs and NEs was the rule in most of the cases in the present study.

For the children with recurrent AOM, the NTHi subtype was identical to the one previously isolated if the time between episodes was ≤30 days and different if >30 days had elapsed between episodes. There was clonal restriction in the subtypes isolated from all cases and a high degree of correspondence between the NTHi subtypes isolated from Mexican and U.S. children with AOM [13]. We also demonstrated the presence of potentially new subtypes isolated from NEs from some children with AOM.

To define the prevalence of different subtypes and to identify possibly new subtypes, additional studies are required in developing as well as developed countries. Moreover, other techniques such as PCR fingerprinting, long PCR ribotyping, restriction fragment length polymorphism analysis, and pulsed-field gel electrophoresis [34–36] should be added to the OMP subtyping system to facilitate the strain characterization in future epidemiological studies.

Acknowledgments

The authors thank Dr. Adolfo García Sainz and colleagues (Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City) for technical support and counseling; Dr. Veronica Flores Sánchez (Department of Anesthesia, Hospital Infantil de México, Mexico City) and Dr. Carlos de la Torre (ENT Service, Hospital Infantil de México) for their support in performing tympanocentesis; Dr. Dennis L. Stevens (Veterans Affairs Medical Center, Boise, Idaho, USA) for the critical review of the manuscript; John J. Mangan for photographic work (Veterans Affairs Medical Center, Boise, Idaho, USA); and Michael R. Wyett (Veterans Affairs Medical Center, Boise, Idaho, USA) for layout work.

References