Biotypes of Serologically Nontypable *Haemophilus influenzae* Isolated from the Middle Ears and Nasopharynges of Patients with Otitis Media with Effusion

THOMAS F. DEMARIA,^{1*} DAVID J. LIM,¹ JEAN BARNISHAN,² LEONA W. AYERS,² AND HERBERT G. BIRCK^{1,3}

Otological Research Laboratories, Department of Otolaryngology,¹ and Department of Pathology,² The Ohio State University College of Medicine, and Department of Otolaryngology, Children's Hospital,³ Columbus, Ohio 43210

Received 6 April 1984/Accepted 30 August 1984

A biotype analysis was performed on serologically nontypable *Haemophilus influenzae* isolates from the middle ear effusions and nasopharynges of 33 children with chronic otitis media with effusion (serous otitis media). Over 50% of the *H. influenzae* isolates from the middle ears belonged to biotype II. The incidence of beta-lactamase production was 17%. The results of this study indicate that future clinical or experimental investigations of chronic otitis media with effusion induced by serologically nontypable *H. influenzae* should focus on this clinically important biotype.

The factors responsible for chronic otitis media with effusion (OME) have not been elucidated. During episodes of OME, a middle ear effusion (MEE) forms without any symptoms of infection and is commonly thought to be sterile. Recent studies, however, indicate that up to 97% of OME cases are preceded by an episode of acute otitis media (5) and that up to 45% of the MEEs from OME cases contain culturable bacteria (13). An even greater number of MEEs, 84%, contain bacteria, as determined by a Gram stain (13). *Haemophilus influenzae* is the most common bacterial pathogen associated with chronic OME, accounting for 25% of the total bacterial isolates. Approximately 95% of the *H. influenzae* isolates from OME cases are non-encapsulated and not typable with antisera specific for the six recognized capsular serotypes (12). Because of the lack of a useful classification system, *H. influenzae* isolates from OME cases have not yet been investigated in depth either clinically or experimentally.

The purpose of this study was to define the biochemical behavior of serologically nontypable *H. influenzae* isolates from the MEEs and nasopharynges of patients with OME, in accordance with the established biotyping system for *H. influenzae* (7, 10). In addition, the incidence of beta-lactamase production was evaluated in the middle ear and nasopharynx *H. influenzae* isolates.

(This work was presented in part at the Third International Symposium on Recent Advances in Otitis Media with Effusion, Fort Lauderdale, Fla., 17-20 May 1983.)

MATERIALS AND METHODS

Specimens. MEEs and nasopharyngeal samples were obtained from patients undergoing routine myringotomy and tube insertion for chronic OME at the Children's Hospital, Columbus, Ohio. The ages of the patients ranged from 3 months to 11 years. A total of 30 MEE isolates of serologically nontypable *H. influenzae* and the paired nasopharyngeal isolates were analyzed at the Clinical Microbiology Laboratory at The Ohio State University Hospitals, Columbus. For this study, only specimens that exhibited growth of

H. influenzae in both the MEE and the nasopharynx were included. Three cases in which only the MEE was culture positive for *H. influenzae* were excluded from the analysis.

Culturing and isolation of bacteria. All specimens were cultured for bacteria within 1 hour after surgery on blood and chocolate agars by procedures previously described (11). Presumptive *H. influenzae* isolates were identified by their typical colony morphology on chocolate agar. Definitive identification was determined by their growth requirements for NAD and hemin on brain heart infusion agar by means of X, V, and XV factor disks (Difco Laboratories, Detroit, Mich.). Nontypable *H. influenzae* isolates were defined as organisms that failed to agglutinate with commercial type-specific rabbit antisera prepared against types a through f (Difco).

Biotype analysis. A total of 25 different biochemical tests were performed on each isolate, as described by Kilian (10), by means of a microtube broth method developed at The Ohio State University Hospitals Clinical Microbiology Laboratory (2). The biochemical substrates were dispensed into 96-well microtube plates (Microtiter Trays; Dynatech Laboratories, Inc., Alexandria, Va.) by means of a Sandy Springs Quick Spence II semiautomatic dispenser (Bellco Glass, Inc., Vineland, N.J.). These plates were sealed in plastic bags and stored at -80°C.

The biochemical formulations were based on those of Dowell et al. (4) but included some modifications. Modified Lombard-Dowell broth was the base medium for fermentation tests of the following carbohydrates: glucose, salicin, raffinose, sucrose, mannose, fructose, trehalose, rhamnose, mannitol, maltose, xylose, sorbitol, lactose, arabinose, inulin, and glycerol. Urease activity was determined with Stuart broth (17). Acid production from pyruvate was determined with the medium described by Gross et al. (8). Phenol red (0.12%) served as the indicator for the above-described biochemical tests. Heart infusion broth (Difco) was the base for the nitrate medium. Esculin hydrolysis was determined with the medium described by Vaughn and Levine (18). Thioglycolate medium (BBL Microbiology Systems, Cockeysville, Md.) without dextrose or indicator was the base for the 20% bile medium. All the biochemicals were

* Corresponding author.

TABLE 1. Biochemical characteristics of *H. influenzae* strains isolated from patients with chronic OME

Test or substrate	% Positive test reaction ^a of <i>H. influenzae</i> biotype:				
	I	II	III	V	VI
Ornithine	100	0	0	100	100
Indole	100	100	0	100	0
Urease	100	100	100	0	0
Glucose	100	100	100	100	100
Nitrate	100	100	100	100	100
Pyruvate	100	100	100	100	100
Xylose	80	79	85	100	100
Bile (20%)	40	2	0	0	0
Phosphatase	80	96	100	75	100

^a All strains were tested and found negative for salicin, esculin, raffinose, sucrose, mannose, levulose, rhamnose, mannitol, maltose, sorbitol, lactose, trehalose, arabinose, arginine, inulin, and glycerol.

supplemented with 5% Fildes (BBL). Beta-lactamase production was determined for each isolate by the acidometric Beta Test (Malcom Laboratory Supply, Cleveland, Ohio) and by the chromogenic cephalosporin method (PADAC; Calbiochem-Behring, La Jolla, Calif.).

Cultures for inocula from each paired specimen were incubated on chocolate agar at 35°C for 18 h. The organisms were suspended in Trypticase soy broth (BBL) to a McFarland number 1 turbidity standard. The inocula were transferred to the wells of the microtiter plates with disposable replicators (Dynatech). The biochemicals, except for the 20% bile, were reinoculated with a heavy suspension of organisms (McFarland number 7 turbidity standard or greater) in distilled water with 0.02% Tween. A Pasteur pipette was used, resulting in a delivery of ca. 0.4 ml of the suspension to each biochemical well. The plates were incubated for 24 h at 35°C. The indole, ornithine, and urease tests were used to assign biotype designations, in accordance with the classification system recently proposed by Gratten (7) (Table 1).

RESULTS

All the paired isolates were dependent on both X and V factors for growth, and all the strains could be accommodated in the currently recognized taxa of seven biotypes (7) (Table 1). The biotype designations were based primarily on three biochemical tests: ornithine decarboxylation, indole production, and urea hydrolysis. No biotype IV (ornithine decarboxylase positive, indole negative, urease positive) or biotype VII (ornithine decarboxylase negative, indole positive, urease negative) isolates were observed in this study. Xylose fermentation was variable among the biotype I, II, and III isolates, as was phosphatase production by the biotype I, II, and V isolates. A total of 40% of the biotype I and 2% of the biotype II isolates grew in 20% bile medium.

A total of 53% of the middle ear isolates examined belonged to biotype II (Table 2). All the *H. influenzae* isolates cultured from the nasopharynxes of the patients were also biotype II. A total of 23% of the middle ear *H. influenzae* isolates were designated as biotype III. The remaining middle ear isolates were biotype I (10%), biotype V (7%), or biotype VI (7%) (Table 2). With one exception, the nasopharyngeal biotypes were identical to the corresponding middle ear biotypes.

Only biotype II and V isolates produced beta-lactamase. The overall incidence of beta-lactamase production was 17%, and the results for the middle ear isolates matched those for the paired nasopharyngeal isolates. All the biotype V isolates produced beta-lactamase and accounted for more

than half of the ampicillin-resistant isolates in the present study.

DISCUSSION

Non-encapsulated *H. influenzae* are antigenically diverse, and a serological classification system for these organisms is not available at this time (1). Current data suggest that the outer membrane profile of this organism also appears to be too variable and that the lipopolysaccharide is too biochemically homogeneous to provide the framework for a classification system (1, 9). At present, biotyping is the only available framework for the classification of these organisms.

The predominance of biotypes II and III in the middle ears of patients with OME observed in our study differs, except for a report by Barenkamp et al. (1), from the results of other reports for patients with acute otitis media. Long et al. (15) observed that 67% of the nontypable *H. influenzae* strains from patients with acute otitis media were biotype I, and Kilian (10) reported an approximately equal distribution of biotypes I, II, and V. It may be, as discussed in a report by Granato et al. (6), that certain biotypes are endemic to a particular geographical area, which may account for the variability observed in the biotypes isolated from patients with acute otitis media. Additional biotype analyses of isolates from patients with chronic OME are needed to determine whether there is an association between a particular biotype and this disease.

The presence of concordant biotypes in samples from the nasopharynxes and middle ears of children with acute bacterial otitis media has been previously reported by Long et al. (14). The results from the present study indicate that, with only one exception, concordant biotypes also exist in the middle ears and nasopharynxes of patients with chronic OME. This finding supports one current theory that the natural entry for bacteria into the middle ear is most likely the nasopharynx via the eustachian tube during periods of disrupted eustachian tube ventilatory function (16).

The reason that biotype II organisms were predominant in the MEEs of patients with chronic OME is not known. Biotypes II, III, IV, and V are regarded as upper respiratory tract commensals in 14 to 35% of healthy children (15). However, recent reports indicate that non-encapsulated biotypes II, III, and V can also be important causes of serious invasive diseases in adults (19). In contrast to their role in acute otitis media, the role of bacteria in the pathogenesis of chronic OME has not been conclusively established. Therefore, it cannot be ascertained whether the present data simply reflect an extension of the predominant normal flora biotypes into the middle ear or whether a specific predilection and pathogenic role can be ascribed to the nontypable biotype II isolates.

In the chinchilla model with *H. influenzae* biotype II,

TABLE 2. Biotypes of nontypable *H. influenzae* from paired MEEs and nasopharyngeal isolates

Biotype	No. of paired isolates	% of isolates of each biotype
I	3	10
II	16	53
III	7	23
IV	0	0
V	2	7
VI	2	7
VII	0	0

experimental OME that appears to mimic certain types of clinical OME in children has been successfully induced (3). This finding suggests that the presence of this organism in MEEs may have significant clinical relevance and may represent an active bacterial pathogenic role in OME rather than a mere entrapment of normal respiratory flora in the middle ear. Further study is needed to better understand the clinical course of *H. influenzae* otitis media. According to our data, future experimental studies of OME should focus on this clinically important biotype.

ACKNOWLEDGMENTS

This work was supported in part by grants from The Deafness Research Foundation and the National Institute of Neurological and Communicative Disorders and Stroke (Public Health Service grant NS 08854).

We thank Valerie Slusarz, Shakuntala Lamgaday, and Katherine Adamson for their assistance.

LITERATURE CITED

1. Barenkamp, S. J., R. S. Munson, Jr., and D. M. Granoff. 1982. Outer membrane protein and biotype analysis of pathogenic nontypable *Haemophilus influenzae*. *Infect. Immun.* **36**:535-540.
2. Bushing, W., J. R. Svirbely, and L. W. Ayers. 1983. Evaluation of the Anaerobe-Tek system for identification of anaerobic bacteria. *J. Clin. Microbiol.* **17**:824-829.
3. DeMaria, T. F., B. R. Briggs, N. Okazaki, and D. J. Lim. 1984. Experimental otitis media with effusion following middle ear inoculation of nonviable *H. influenzae*. *Ann. Otol. Rhinol. Laryngol.* **93**:52-56.
4. Dowell, V. R., Jr., G. L. Lombard, F. S. Thompson, and A. Y. Armfield. 1977. Media for isolation, characterization, and identification of obligately anaerobic bacteria. Center for Disease Control, Atlanta, Ga.
5. Giebink, G. S., E. L. Mills, J. S. Huff, C. K. Edelman, M. L. Weber, S. K. Juhn, and P. G. Quie. 1979. The microbiology of serous and mucoid otitis media. *Pediatrics* **63**:915-919.
6. Granato, P. A., E. A. Jurek, and L. B. Weiner. 1983. Biotypes of *Haemophilus influenzae*: relationship to clinical source of isolation, serotype, and antibiotic susceptibility. *Am. J. Clin. Pathol.* **79**:73-77.
7. Gratten, M. 1983. *Haemophilus influenzae* biotype VII. *J. Clin. Microbiol.* **18**:1015-1016.
8. Gross, K. C., M. P. Houghton, and L. B. Senterfit. 1975. Presumptive speciation of *Streptococcus bovis* and other group D streptococci from human sources by using arginine and pyruvate tests. *J. Clin. Microbiol.* **1**:54-60.
9. Inzana, T. J. 1983. Electrophoretic heterogeneity and interstrain variation of the lipopolysaccharide of *Haemophilus influenzae*. *J. Infect. Dis.* **148**:492-499.
10. Kilian, M. 1976. A taxonomic study of the genus *Haemophilus*, with the proposal of a new species. *J. Gen. Microbiol.* **93**:9-62.
11. Kilian, M. 1982. *Haemophilus*, p. 330-336. In E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), *Manual of clinical microbiology*, 3rd ed. American Society for Microbiology, Washington, D.C.
12. Lim, D. J., and T. F. DeMaria. 1982. Panel discussion: pathogenesis of otitis media. *Bacteriology and immunology. Laryngoscope* **92**:278-286.
13. Liu, Y. S., D. J. Lim, R. W. Lang, and H. G. Birck. 1980. Chronic middle ear effusions: immunochemical and bacteriological investigation. *Arch. Otolaryngol.* **101**:278-286.
14. Long, S. S., F. M. Henretig, and M. J. Teter. 1983. Nasopharyngeal flora and acute otitis media. *Infect. Immun.* **41**:987-991.
15. Long, S. S., M. J. Teter, and P. H. Gilligan. 1983. Biotypes of *Haemophilus influenzae*: correlation with virulence and ampicillin resistance. *J. Infect. Dis.* **147**:800-806.
16. Sanyal, M. A., F. W. Henderson, E. C. Stempel, A. M. Collier, and F. W. Denny. 1980. Effect of upper respiratory tract infection on eustachian tube ventilatory function in the pre-school child. *J. Pediatr.* **97**:11-15.
17. Stuart, C. A., E. van Stratum, and R. Rustigian. 1945. Further studies on urease production by *Proteus* and related organisms. *J. Bacteriol.* **49**:437-444.
18. Vaughn, R. H., and M. Levine. 1970. Media and tests for differentiation of *Enterobacteriaceae*. Center for Disease Control, Atlanta, Ga.
19. Wallace, R. J., D. M. Musher, E. J. Septimus, J. E. McGowan, Jr., F. J. Quinones, K. Wiss, P. H. Vance, and P. A. Trier. 1981. *Haemophilus influenzae* infections in adults: characterization of strains by serotypes, biotypes, and β -lactamase production. *J. Infect. Dis.* **144**:101-106.