JOURNAL OF CLINICAL MICROBIOLOGY, June 1978, p. 519-523
0095-1137/78/0007-0519$02.00/0
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Vol. 7, No. 6
*Printed in U.S.A.*

# Biochemical Characteristics of *Haemophilus influenzae* in Relationship to Source of Isolation and Antibiotic Resistance

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Received for publication 13 February 1978

Based on a limited number of biochemical properties, a system for biotyping *Haemophilus influenzae* (M. Kilian, Acta Pathol. Microbiol. Scand. Sect. B**82**:835-842, 1976) was used to analyze the relationship of biotype to source of infection and antibiotic resistance for 600 clinical strains. The distribution of biotypes from bacteremic patients was significantly different ($P < 0.001$) from the distribution of biotypes from nonbacteremic patients. Although there appeared to be a correlation between biotype and source of isolation, no single biotype correlated with a specific clinical syndrome in bacteremic patients. The frequency of resistance to antibiotics (ampicillin, tetracycline, chloramphenicol, and kanamycin), which was known to be at least in part plasmid mediated, was determined. Of the 600 isolates, 43 were resistant to at least one antibiotic (30 were ampicillin resistant, 11 were tetracycline resistant, 1 was ampicillin-tetracycline resistant, and 1 was tetracycline-chloramphenicol resistant). Of these 43 resistant isolates, 42 were either biotype I or II. This distribution of biotypes among antibiotic-resistant isolates was significantly different from the overall distribution of biotypes ($P < 0.001$).

Kilian (12) recently described a system for biotyping *Haemophilus influenzae* isolates based on a limited number of biochemical properties and found an apparent correlation between biotype and source of isolation. This observation has been confirmed in a separate study (1), although both studies were notably deficient in blood isolates. The relationship of encapsulated type *b* organisms to invasive disease has been stressed by many investigators (15, 22, 25), but the association of serotypes other than *b* (2, 9) and nontypable strains (7, 10, 11) with invasive disease remains to be explained.

Approximately 500 clinical isolates of *H. influenzae* are reported yearly from the clinical Microbiology Laboratory of the Health Sciences Centre, University of Manitoba, Winnipeg. Between 1975 and 1977, 98 blood isolates from the Health Sciences Centre and 2 blood isolates from patients in affiliated hospitals were typed and further characterized. This study reports the results of biotyping, serotyping, and antibiotic susceptibility testing for these blood isolates and a large number of nonblood isolates.

## MATERIALS AND METHODS

**Bacterial strains.** Six hundred strains of *H. influenzae* were used. All but two were isolated in the Clinical Microbiology Laboratory of the Health Sciences Centre. Ninety-eight blood isolates were from consecutive bacteremic patients admitted to the Health Sceinces Centre. The two additional blood isolates were referred to the Clinical Microbiology laboratory of the Health Sciences Centre for further identification and were isolated from patients admitted to affiliated hospitals during the period of study. One hundred isolates from consecutive nonbacteremic patients on whom demographic data were available were used for determining the distribution of biotypes by source of isolation. The remaining 400 strains represented the majority of clinical isolates from nonbacteremic patients during 1976 and, in a limited number, may represent several isolates from the same patient. Demographic data on patients were available from the microbiology requisition sheets and a review of the hospital chart at the Health Sciences Centre.

**Culture methods.** Initial isolation was made on chocolatized blood agar incubated at 37°C in a 5% $CO_2$ atmosphere or in commerical B-D peptone broth. Organisms were identified as *H. influenzae* if they had typical colony morphology, were small, pleomorphic, gram-negative rods on Gram stain, and required both X and V factors for growth on Trypticase soy agar. Stock cultures were maintained at −70°C in defibrinated rabbit blood.

**Biochemical tests.** Biochemical tests were performed as follows. (i) Hemin requirement was confirmed by the lack of ability to synthesize porphyrins from δ-aminolevulinic acid (13, 17). (ii) Biotyping was by the method of Kilian (12), using the biochemical tests included in the study of *H. influenzae* by Bruun and Friis-Møller (1; see Table 1). (iii) Serotyping was by counterimmunoelectrophoresis of overnight broth

filtrates, using commercially available (Difco Laboratories) polyvalent and monovalent (capsular types *a* to *f*) *H. influenzae* typing sera. (20). (iv) Preliminary screening for antibiotic resistance to ampicillin (Ap), tetracycline (Tc), chloramphenicol (Cm), and kanamycin (Km) was determined by disk sensitivity testing on chocolate agar by using an inoculum of $10^8$ colony-forming units per ml from a log-phase culture in Eugon broth supplemented with 1% Fildes enrichment. Resistance was confirmed by determination of the minimal inhibitory concentration in microtiter plates in Eugon broth supplemented with 1% Fildes enrichment by using an inoculum of $10^5$ colony-forming units per ml. End points were read as no visible growth after 18 h of incubation at 37°C in a 5% $CO_2$ atmosphere (modified from 23). (v) Ap-resistant isolates were screened for the production of $\beta$-lactamase with a chromogenic cephalosporin (21).

**Statistical tests.** Probability values for differences in frequency distributions were determined by chi-square analysis using an on-line computer statistics program.

## RESULTS

On the basis of the biochemical characteristics shown in Table 1, 591 isolates could be assigned to biotypes I to V. The remaining nine isolates corresponded to the strains tentatively described by Kilian as a sixth biotype (12).

The age distribution and clinical syndromes associated with *H. influenzae* bacteremia in 100 patients are shown in Fig. 1 and Table 2. Of the bacteremic patients, 43% were under 1 year of age and 11% were over the age of 20. Clinical syndromes in adults associated with bacteremia included epiglottitis (1), pneumonia (3), pharyngitis (1), and genitourinary tract infections (5). Ninety-three percent of the blood isolates could be assigned to either biotype I or II. No single biotype correlated with a specific clinical syndrome.

The distribution of biotypes by source of isolation in 100 nonbacteremic patients is given in Table 3. Of the isolates from nonblood sources, 44% were from patients over the age of 20. There was no significant difference, however, in the distribution of biotypes for isolates from patients over the age of 20 when compared with isolates from patients under the age of 20. The overall distribution of biotypes among isolates from bacteremic patients was significantly different ($P < 0.001$) from the distribution of biotypes among isolates from nonbacteremic patients. Biotypes II and III were associated with 23 of 27 conjunctival isolates and, among bacteremic patients, were associated with infections arising from the genitourinary tract. The distribution of serotypable organisms by biotype was significantly different ($P < 0.001$) from the distribution of nontypable organisms (Table 4). Only two of the nonblood isolates were typable serologically and both were capsular type *b* organisms. Seven blood isolates were nontypable. The capsular type *e* organism was typable by agglutination as well as by counterimmunoelectrophoresis, using monovalent Difco type *e* antiserum. The two

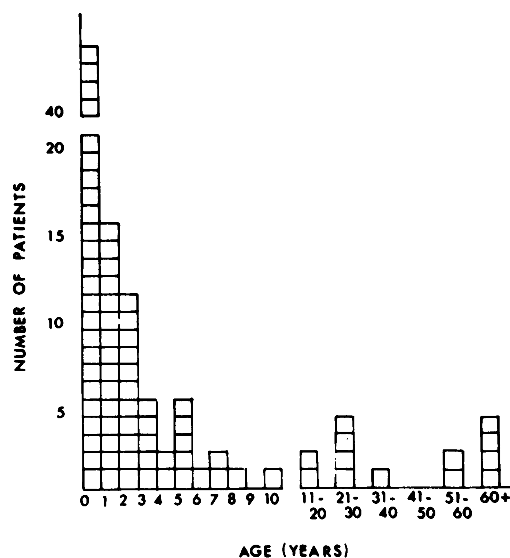


FIG. 1. *Age distribution of patients with H. influenzae bacteremia.*

TABLE 1. *Biochemical characteristics of H. influenzae*[a]

| Biotype | Hemin requirement | NAD[b] requirement | Hemolysis | Indole production | Urease activity | Ornithine decarboxylase activity | Glucose fermentation | Sucrose fermentation | Lactose fermentation | Xylose fermentation | Mannitol fermentation |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I | + | + | − | + | + | + | + | − | − | + | − |
| II | + | + | − | + | + | − | + | − | − | d+ | − |
| III | + | + | − | − | + | − | + | − | − | d+ | − |
| IV | + | + | − | − | + | + | + | − | − | + | − |
| V | + | + | − | + | − | + | + | − | − | + | − |

[a] +, 95 to 100% positive; d+, 75 to 95% positive; −, 0 to 5% positive.
[b] NAD, Nicotinamide adenine dinucleotide.

TABLE 2. *Distribution of biotypes by disease entity in bacteremic patients*

| Disease entity | No. of patients with biotype: | | | | | | Serotype(s) |
|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | Non-I-V | |
| Meningitis | 34 | 12 | 0 | 1 | 0 | 0 | 46 *b*; 1 *e* |
| Epiglottitis | 10 | 5 | 0 | 0 | 0 | 0 | 15 *b* |
| Pneumonia | 4 | 5 | 2 | 0 | 0 | 0 | 7 *b*; 2 *f*; 2 NT[a] |
| Septic arthritis | 5 | 0 | 0 | 0 | 0 | 0 | 5 *b* |
| Cellulitis | 8 | 3 | 0 | 0 | 0 | 0 | 11 *b* |
| Miscellaneous | | | | | | | |
| OPSI[b] | 0 | 1 | 0 | 0 | 0 | 0 | 1 *b* |
| Pericarditis | 1 | 0 | 0 | 0 | 0 | 0 | 1 *b* |
| Pharyngitis | 1 | 0 | 0 | 0 | 0 | 0 | 1 *b* |
| G-U[c] | 0 | 2 | 2 | 1 | 0 | 0 | 1 *b*; 4 NT |
| Unknown | 2 | 0 | 0 | 1 | 0 | 0 | 2 *b*; 1 NT |

[a] NT, Nontypable.
[b] OPSI, Overwhelming postsplenectomy infection.
[c] G-U, Genitourinary tract infection.

TABLE 3. *Distribution of biotypes by source of isolation in nonbacteremic patients*

| Source | No. of patients with biotype: | | | | | | Serotype |
|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | Non-I-V | |
| Eye | 1 | 19 | 4 | 2 | 0 | 0 | 1 *b* |
| Sputum | 21 | 20 | 10 | 1 | 3 | 0 | 1 *b* |
| Lung | 1 | 6 | 2 | 0 | 0 | 0 | |
| Pharynx | 1 | 2 | 0 | 0 | 0 | 0 | |
| Ear | 1 | 1 | 1 | 0 | 0 | 1 | |
| Miscellaneous | | | | | | | |
| Thyroglossal duct cyst | 1 | 0 | 0 | 0 | 0 | 0 | |
| G-U tract[a] | 0 | 0 | 0 | 1 | 0 | 0 | |
| Gastric aspirate | 0 | 1 | 0 | 0 | 0 | 0 | |

[a] G-U tract, Genitourinary tract.

TABLE 4. *Distribution of serotype strains by biotype*[a]

| Serotype | No. of strains of biotype: | | | | | |
|---|---|---|---|---|---|---|
| | I | II | III | IV | V | Non-I-V |
| *b* | 65 | 25 | 2 | 0 | 0 | 0 |
| *f* | 2 | 0 | 0 | 0 | 0 | 0 |
| *e* | 0 | 0 | 0 | 1 | 0 | 0 |
| Total | 67 | 25 | 2 | 1 | 0 | 0 |
| NT[b] | 26 | 50 | 19 | 6 | 3 | 1 |

[a] Chi-square = 47.36; $P < 0.001$.
[b] NT, Nontypable.

capsular type *f* organisms were typable only by counterimmunoelectrophoresis. The methodology used in serotyping *H. influenzae* isolates may produce variable results. The commercial typing sera are prepared against whole organisms and may produce agglutination or precipitation reactions with noncapsular antigens. The identification of the capsular type of organisms was confirmed by R. Schneerson, Bureau of Biologics, Food and Drug Administration, Bethesda, Md., using purified capsular polysaccharide.

The frequency of resistance to antibiotics (Ap, Km, Tc, and Cm), which is known to be at least in part plasmid mediated, was determined (Tables 5 and 6). The distribution of antibiotic resistance by biotype for 100 blood and 100 nonblood isolates is given in Table 5. All Ap-resistant isolates produced $\beta$-lactamase when assayed by the chromogenic cephalosporin method. Although all 18 resistant isolates could be assigned to biotpye I or II, the biotype distribution was not significantly different from the antibiotic-sensitive isolates for this small sample. The observation that antibiotic resistance was limited to biotypes I and II prompted a further survey of 400 nonblood isolates, and the results for the 500 nonblood isolates are shown in Table 6. Fermentation characteristics were not determined for the additional 400 isolates, and the assignment of biotype for these additional isolates was based on an abbreviated schema which included only indole production, urease activity, and ornithine decarboxylase ac-

TABLE 5. *Distribution of antibiotic resistance by biotype for 100 blood and 100 nonblood H. influenzae isolates*

| Source | Antibiotic sensitivity | No. of isolates of biotype: | | | | | |
|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | V | Non-I-V |
| Blood | Resistant[a] | 8 | 0 | 0 | 0 | 0 | 0 |
| | Sensitive | 57 | 28 | 4 | 3 | 0 | 0 |
| Nonblood | Resistant[b] | 2 | 8 | 0 | 0 | 0 | 0 |
| | Sensitive | 24 | 41 | 17 | 4 | 3 | 1 |

[a] Seven isolates were Ap resistant and one was Ap-Tc resistant.
[b] Seven isolates were Ap resistant and three were Tc resistant.

TABLE 6. *Distribution of antibiotic resistance by biotype for 500 nonblood H. influenzae isolates*[a]

| Antibiotic sensitivity | No. of isolates of biotype: | |
|---|---|---|
| | I/II | Non-I/II |
| Resistant[b] | 34 | 1 |
| Sensitive | 306 | 159 |

[a] Chi-square = 13.28; $P < 0.001$.
[b] Of 35 isolates, 23 were Ap resistant, 11 were Tc resistant, and 1 was Tc-Cm resistant.

tivity. Isolates were identified as biotype I/II if they produced indole and were positive for urease activity. Sensitive biotype I/II isolates were not further identified. All other organisms (non-I/II) were identified as to their specific biotype. The biotype distribution of antibiotic-resistant isolates from the larger sample was significantly different ($P < 0.001$) from the antibiotic-sensitive isolates, and this difference was maintained when the blood isolates were included. The single resistant nonbiotype I/II isolate was an Ap-resistant biotype III strain. A similar isolate has recently been reported by Marraro et al. (19).

## DISCUSSION

The age distribution and clinical syndromes associated with *H. influenzae* bacteremia in this study were similar to those reported in previous studies (18, 24). The 11% incidence of bacteremic patients over the age of 20 was in keeping with recent studies reporting an increase prevalence of *Haemophilus* bacteremia in older children and adults (5, 7, 16).

Although the presence of the type *b* capsular polysaccharide would appear to be the major virulence determinant in *H. influenzae*, the biochemical heterogeneity of both encapsulated and non-encapsulated organisms was apparent, and the source of isolation as well as invasiveness appeared to be correlated with particular biochemical patterns. The results of the present study further confirm previous reports indicating a correlation between a particular biotype of *H. influenzae* and its source (1, 12). The predominance of biotypes II and III among conjunctival isolates has been previously described (14), but the association of these same biotypes with invasive disease originating from the genitourinary tract has not been previously noted. The association between genital tract infections and conjunctivitis caused by gonococci is well known, and it is tempting to speculate that the properties of *H. influenzae* biotypes II and III that allow establishment of infection in the conjunctiva are involved in the establishment of genital tract infections as well.

Sixty-seven of the 74 capsular type *b* organisms reported in the studies by Kilian (12) and Bruun and Friis-Møller (1) were biotype I organisms. The predominance of biotype I strains in invasive disease seen in our study may merely reflect a different frequency of encapsulation among the various biotypes (Table 4), but this could not be assessed independently because the number of encapsulated strains from nonblood sources was too small.

If the overall distribution of encapsulated strains, particularly type *b*, is independent of the biotype, it may be possible to identify additional virulence factors that are associated with biotype I and II organisms and responsible for invasive disease due to strains which are nontypable or to capsular types other than *b*.

The widespread occurrence in *H. influenzae* of Ap resistance associated with a plasmid carrying a translocatable fragment of DNA specifying a TEM-type $\beta$-lactamase has been followed by the demonstration of apparently plasmid-mediated resistance to Cm (26), Tc (3, 6), and Km (4). The nonrandom distribution of biotypes among these antibiotic (Ap, Tc, Cm, and Km)-resistant isolates (Table 6) may be due to: (i) a different frequency of carriage of cryptic plasmids capable of accepting the Tn*A* piece in the five biotypes; (ii) restricted transfer of antibiotic resistance determinants between biotypes; or (iii) more frequent association of biotype I and II organisms with other organisms capable of

transferring antibiotic resistance determinants to *H. influenzae,* as suggested by the nonrandom distribution of biotypes by source of isolation.

Future studies of the frequency of transfer of genetic determinants of capsule production and antibiotic resistance between the biotypes should provide some information on the molecular basis for the association of both antibiotic resistance and type *b* capsule production with biotype I and II organisms.


## ACKNOWLEDGMENTS

We express our appreciation to Evelyn Witwicki for her help in serotyping the strains and to R. Schneerson for typing three of the isolates and offering helpful comments on the manuscript.

This work was supported by funds from the Winnipeg Clinic Research Institute, the Children's Hospital Research Foundation, and the Medical Research Council of Canada (MA-6327).


## LITERATURE CITED


1. **Bruun, B., and A. Friis-Møller.** 1976. Ampicillin sensitivity and biotypes of recent Danish isolates of *Haemophilus influenzae.* Acta Pathol. Microbiol. Scand. Sect. B **84:**201-204.
2. **Buck, L., and G. W. Douglas.** 1976. Meningitis due to *Haemophilus influenzae* type e. J. Clin. Microbiol. **4:**381.
3. **Dang Van, A.** 1975. Resistance plasmidique a la tetracycline chez *H. influenzae.* C.R. Acad. Sci. **280:** 1321-1323.
4. **Dang Van, A., F. Goldstein, J. F. Acar, and D. H. Bouanchaud.** 1975. A transferable kanamycin resistance plasmid isolated from *Haemophilus influenzae.* Ann. Inst. Pasteur (Paris) **126A:**397-399.
5. **Drapkin, M. S., M. E. Wilson, S. M. Shrager, and R. H. Rubin.** 1977. Bacteremic *Haemophilus influenzae* type b cellulitis in the adult. Am. J. Med. **63:**449-452.
6. **Elwell, L. P., J. R. Saunders, M. H. Richmond, and S. Falkow.** 1977. Relationships among some R plasmids found in *Haemophilus influenzae.* J. Bacteriol. **131:**356-362.
7. **Everett, E. D., A. E. Rahm, R. Adaniya, D. L. Stevens, and T. R. McNitt.** 1977. *Haemophilus influenzae* pneumonia in adults. J. Am. Med. Assoc. **238:**319-321.
8. **Harkness, N. K., and M. L. Murray.** 1977. Occurrence of cryptic plasmids in *Haemophilus influenzae.* FEMS Microbiol. Lett. **1:**239-241.
9. **Harlow, M. S., S. M. K. Chung, and S. A. Plotkin.** 1975. *Haemophilus influenzae* septic arthritis in infants and children. J. Pediatr. **83:**215-219.
10. **Honig, P. J., P. S. Pasquariello, and S. E. Stool.** 1973. *H. influenzae* pneumonia in infants and children. J. Pediatr. **83:**215-219.
11. **Khuri-Bulos, N., and K. McIntosh.** 1975. Neonatal *Haemophilus influenzae* infection. Am. J. Dis. Child. **129:**57-62.
12. **Kilian, M.** 1976. A taxonomic study of the genus *Haemophilus,* with the proposal of a new species. J. Gen. Microbiol. **93:**9-62.
13. **Kilian, M.** 1974. A rapid method for the differentiation of *Haemophilus* strains: the prophyrin test. Acta. Pathol. Microbiol. Scand. Sect. B **82:**835-842.
14. **Kilian, M., C. H. Mordhorst, C. R. Dawson, and H. Lautrop.** 1976. The taxonomy of Haemophili isolated from conjunctivae. Acta. Pathol. Microbiol. Scand. Sect. B: **84:**132-138.
15. **Leidy, G., Hahn, E., Zamenhof, S., and H. E. Alexander.** 1960. Biochemical aspects of virulence of *Hemophilus influenzae.* Ann. N.Y. Acad. Sci. **88:**195-1202.
16. **Levin, D. C., M. I. Schwartz, R. A. Matthay and F. M. LeForce.** 1977. Bacteremic *Hemophilus influenzae* pneumonia in adults. Am. J. Med. **62:**219-224.
17. **Lund, M. E., and D. J. Blazevic.** 1977. Rapid speciation of *Haemophilus* with the prophyrin production test versus the satellite test for X. J. Clin. Microbiol. **5:**142-144.
18. **McGowan, J. E., J. O. Klein, L. Bratton, M. W. Barnes, and M. Finland.** 1974. Meningitis and bacteremia due to *Haemophilus influenzae*: occurrence and mortality at Boston City Hospital in 12 selected years, 1935-1972. J. Infect. Dis. **130:**119-124.
19. **Marraro, R. V., F. K. McCleskey, and J. L. Mitchell.** 1977. Pneumonia due to *Haemophilus influenzae* (*H. aegyptius*) biotype 3. J. Clin. Microbiol. **6:**172-173.
20. **Myhre, E. B.** 1974. Typing of *Haemophilus influenzae* by counterimmunoelectrophoresis. Acta Pathol. Microbiol. Scand. B **82:**164-166.
21. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of $\beta$-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1:**283-288.
22. **Sell, S. H. W., and D. T. Karzon (ed.).** 1973. *Haemophilus influenzae*: proceedings of a conference on antigen-antibody systems, epidemiology and immuno-prophylaxis. Vanderbilt University Press, Nashville, Tenn.
23. **Slaney, L.** 1977. *Haemophilus influenzae*: susceptibility to ampicillin. Can. J. Med. Technol. **39:**84-88.
24. **Todd, J. K., and F. W. Bruhn.** 1975. Severe *Haemophilus influenzae* infections: spectrum of disease. Am. J. Dis. Child. **129:**607-611.
25. **Turk, D. C., and J. R. May.** 1967. *Haemophilus influenzae*: its clinical importance. The English Universities Press Ltd., London.
26. **van Klingeren, B., J. D. A. van Embden, and M. Dessens-Kroon.** 1977. Plasmid-mediated chloramphenicol resistance in *Haemophilus influenzae.* Antimicrob. Agents Chemother. **11:**383-387.