

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

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Based on a limited number of biochemical properties, a system for biotyping *Haemophilus influenzae* (M. Kilian, Acta Pathol. Microbiol. Scand. Sect. B82:835-842, 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 Sciences 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 chocolateized blood agar incubated at 37°C in a 5% CO₂ atmosphere or in commercial 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% CO₂ atmosphere (modified from 23). (v) Ap-resistant isolates were screened for the production of β -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

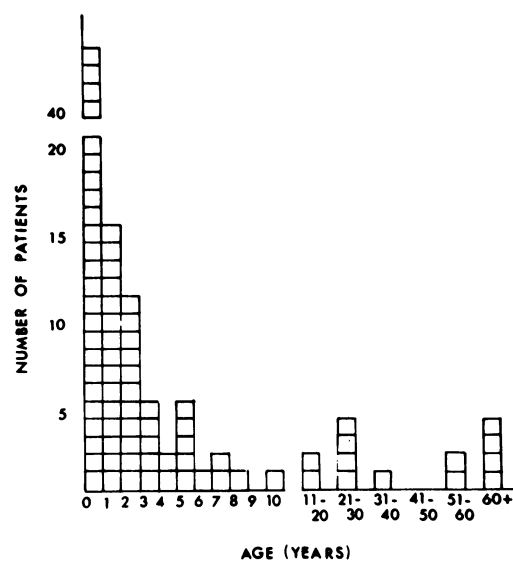


FIG. 1. Age distribution of patients with *H. influenzae* bacteremia.

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 <i>b</i> ; 1 <i>e</i>
Epiglottitis	10	5	0	0	0	0	15 <i>b</i>
Pneumonia	4	5	2	0	0	0	7 <i>b</i> ; 2 <i>f</i> ; 2 NT ^a
Septic arthritis	5	0	0	0	0	0	5 <i>b</i>
Cellulitis	8	3	0	0	0	0	11 <i>b</i>
Miscellaneous							
OPSI ^b	0	1	0	0	0	0	1 <i>b</i>
Pericarditis	1	0	0	0	0	0	1 <i>b</i>
Pharyngitis	1	0	0	0	0	0	1 <i>b</i>
G-U ^c	0	2	2	1	0	0	1 <i>b</i> ; 4 NT
Unknown	2	0	0	1	0	0	2 <i>b</i> ; 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 <i>b</i>
Sputum	21	20	10	1	3	0	1 <i>b</i>
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
<i>b</i>	65	25	2	0	0	0
<i>f</i>	2	0	0	0	0	0
<i>e</i>	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 β -lactamase when assayed by the chromogenic cephalosporin method. Although all 18 resistant isolates could be assigned to biotype 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 β -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 TnA 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.

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