JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1980, p. 22-26
0095-1137/80/01-0022/05$02.00/0

Vol. 11, No. 1

Rapid Biochemical Characterization of *Haemophilus* Species by Using the Micro-ID

STEPHEN C. EDBERG,* ERVIN MELTON, AND JACQUES M. SINGER

Division of Microbiology and Immunology, Department of Pathology, Montefiore Hospital and Medical Center, The Albert Einstein College of Medicine, New York, New York 10467

Biotyping of *Haemophilus influenzae* into five types and *H. parainfluenzae* into three types based on indole production, ornithine decarboxylase, and urease has been reported (M. Kilian, Acta Pathol. Microbiol. Scand. Sect. B **82**:835-842, 1976). A commercially available test system designed for the 4-h identification of *Enterobacteriaceae*, Micro-ID, proved efficacious for the rapid biotyping of these two *Haemophilus* species. The nitrate reductase, indole production, ornithine decarboxylase, urease, and *o*-nitrophenyl-β-D-galactopyranoside hydrolysis tests in Micro-ID correlated over 99% with conventional methodology. By utilizing the indole and *o*-nitrophenyl-β-D-galactopyranoside tests it was possible, with 261 of 272 (96.1%) isolates, to distinguish *H. influenzae* from *H. parainfluenzae*. Cerebrospinal fluid isolates were over 90% *H. influenzae* biotype I, and conjunctival isolates were approximately 70% biotype II. Type b *H. influenzae* were predominantly biotypes I and II; these type b isolates were also overwhelmingly indole producers. Although over 90% of biotypes I and II have been reported to produce β-lactamase, this was not confirmed by the small number of β-lactamase producers encountered here. The 4-h Micro-ID should prove a useful mechanism, amenable to the routine clinical laboratory, for the further exploration of the association of *Haemophilus* with the site of isolation, antigenicity, and antibiotic resistance.

The identification of species in the genus *Haemophilus* is commonly based upon growth characteristics on various microbiological media and their requirements for nicotinamide adenine dinucleotide (or commonly V factor), hemin (or commonly X factor), or both. Testing is most often performed by making a suspension of organisms from a chocolate sheep blood agar plate and swabbing the suspension on a medium containing neither of these factors. Paper disks containing X factor alone, V factor alone, and X and V factor together are pressed on the agar. After incubation in the presence of carbon dioxide, growth around the paper strip(s) is taken as evidence of a requirement for either growth factor alone or for both growth factors together (16). *H. influenzae* and *H. parainfluenzae*, the most commonly isolated haemophili, respectively require both X and V factors and V factor only. Because as many as 18% of haemophili can be misidentified by the disk test (10), tests for the identification of this genus not based on growth stimulation have been sought.

Kilian reported that species of *Haemophilus* that require hemin lack the enzymatic capability to convert delta-amino levulinic acid to protophorphyrin. A rapid chemical method of identifying those *Haemophilus* which require only V factor was developed based on this pathway (6). It is also known that some *Haemophilus* are capable of metabolic activities such as the production of indole, the reduction of nitrate, and the fermentation of sugars. Because haemophili from human clinical isolates are nutritionally fastidious, performance of these biochemical tests is burdensome and, unless performed with exactitude, may not be reliable (7-9). On the basis of selected biochemical test reactions, Kilian proposed the subdivision of *H. influenzae* into five biotypes and *H. parainfluenzae* into three biotypes (7). This biotyping system was used to study the distribution of isolates from clinical specimens, and an association of biotypes with the site of isolation has been reported (1, 5, 9, 10).

This study was undertaken to develop a method that would both rapidly biotype clinical laboratory isolates and assist in the epidemiological study of *H. influenzae* and *H. parainfluenzae* and also would be amenable to routine clinical usage. The Micro-ID, a system intended for the 4-h identification of *Enterobacteriaceae*, was employed. Micro-ID does not depend upon growth of the organism, but detects the presence of constitutive enzymes in a bacterial suspension.

MATERIALS AND METHODS

Bacterial strains. *Haemophilus* species were fresh isolates from the General Bacteriology Laboratory of the Microbiology and Immunology Division, Department of Pathology, Montefiore Hospital and Medical Center, The Albert Einstein College of Medicine. They were identified by the procedures recommended by the *Manual of Clinical Microbiology* (16), which includes possession of typical colonial morphology and growth characteristics on microbiological media, requirement for X and V factors, ability to synthesize porphyrins from delta-amino levulinic acid (6, 12) and typical microscopic appearance by Gram strain. Chocolate sheep blood agar plates were used; the hemolytic nature of the isolates was not studied. Based on these criteria, 172 *H. influenzae* and 90 *H. parainfluenzae* were studied.

Conventional biochemical tests. To determine the correlation of Micro-ID biochemical tests with conventional biochemical reactions, 50 randomly selected strains of *H. influenzae* and 30 randomly selected strains of *H. parainfluenzae* were compared. Not all of the biochemical tests contained in the Micro-ID are useful for the classification of *Haemophilus*, and only those showing taxonomic utility were performed by conventional methods. Conventional tests and test formulations employed were those recommended by Kilian (7–9). Reduction of nitrate to nitrite was determined by the method of Cowan and Steel (4) after 96 h of incubation in Leventhal broth containing 0.1 and 0.01% KNO_3. Indole production was determined by the method of Clarke and Cowan (3) after 48 h of incubation in Leventhal broth. Decarboxylation of ornithine and lysine was determined by the method of Moeller (13) with hemin (Eastman Kodak, Rochester, N.Y.) and nicotinamide adenine dinucleotide grade III (Sigma Chemical Co., St. Louis, Mo.), each incorporated at a final concentration of 10 μg/ml (7). Urease production was determined by the method of Lautrop (11). *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis was detected by the production of a yellow color (7).

Micro-ID. The Micro-ID consists of 15 biochemical reactions on paper impregnated disks, each in its own compartment in a hard plastic tray. The biochemical reactions include the Voges-Proskauer (production of acetoin), nitrate reductase, phenylalanine deaminase, production of hydrogen sulfide, production of indole, ornithine decarboxylase, lysine decarboxylase, malonate utilization, urease, esculinase, hydrolysis of ONPG, and the fermentation of the sugars arabinose, adonitol, inositol, and sorbitol. Inoculation and processing were as recommended by the manufacturer for *Enterobacteriaceae*. *Haemophilus* was suspended in 3.5 ml of saline to equal a 0.5 MacFarland turbidity standard (approximately three to five colonies from chocolate sheep blood agar). Each compartment of the Micro-ID was inoculated with 0.2 ml of bacterial suspension. After 4 h of incubation in air at 35°C, 2 drops of 20% KOH were added to the Voges-Proskauer well. The Micro-ID was tilted to wet the detection disks in the first five compartments, and the reactions, either positive or negative, were recorded as per instructions from the manufacturer for *Enterobacteriaceae*. Incubation for 15 min at room temperature was permitted for development of the Voges-Proskauer test.

Serological methods. The same 50 strains of *H. influenzae* examined by conventional biochemical tests were assayed for their possession of group b antigen. Fresh isolates were incubated for 48 h in Leventhal broth with the addition of 0.5% glucose. The presence of group b antigen was determined by counterimmunoelectrophoresis (Millipore Corp., Bedford, Mass.), using rabbit anti-*Haemophilus* b antiserum (Hyland-Travenol Laboratories, Costa Mesa, Calif.) (14).

Beta-lactamase. Detection of beta-lactamase was performed by the method of Thornsberry and Kirven (15).

RESULTS

There was excellent agreement between Micro-ID and conventional biochemical test results (Table 1). Of the biochemical tests useful for the classification and biotyping of *Haemophilus*—nitrate reductase, indole production, ornithine decarboxylase, urease production, and ONPG hydrolysis—Micro-ID and conventional procedures agreed in over 99%. Two of 50 *H. influenzae* and 1 of 30 *H. parainfluenzae* were falsely ornithine negative by Micro-ID. One strain of *H. influenzae* yielded a falsely negative indole reaction. All other biochemicals agreed completely. To determine whether biochemical reactivity was reproducible, three strains of *H. influenzae* and two strains of *H. parainfluenzae* were tested 10 times each by conventional and Micro-ID procedures. Test results did not vary.

The biochemical test results obtained by Micro-ID were in close agreement with conventional biochemical tests reported in the literature (7). All *Haemophilus* produced nitrate reductase. All strains tested by the Micro-ID were unable to produce acetoin, phenylalanine de-

TABLE 1. *Correlation of conventional and Micro-ID biochemical reactivity*

Species	Test	No. positive		% Agreement
		Conventional	Micro-ID	
H. influenzae	Nitrate	50	50	100
	Indole	40	39	97.5
	Ornithine	26	24	92.3
	Urease	46	46	100
	ONPG	0	0	100
H. parainfluenzae	Nitrate	30	30	100
	Indole	0	0	100
	Ornithine	25	24	96
	Urease	17	17	100
	ONPG	25	25	100

aminase, H_2S, and esculinase, could not utilize malonate, and were not able to ferment arabinose, adonitol, inositol, and sorbitol. Five strains of *H. influenzae* (2.9%), but none of the strains of *H. parainfluenzae*, produced lysine decarboxylase. Because only *H. influenzae* produced indole (79.7%) and only *H. parainfluenzae* hydrolyzed ONPG (81.1%), one can use these characteristics to distinguish the two species in 96.1% of the encounters (Table 2). Only 3.9% of *H. influenzae* and *H. parainfluenzae* tested were both indole and ONPG negative. The identification of these isolates by Micro-ID was inconclusive, and data were not sufficient to provide a species name. Because *H. influenzae* biotypes III and IV are inherently indole negative, and *H. parainfluenzae* biotype III is inherently ONPG negative (7–9), only these biotypes can result in no identification. The percentage of isolates not identifiable to species will vary directly with the percentage of these biotypes isolated. By biochemical testing, some misidentification may result among indole-positive *H. influenzae* biotypes I, II, and IV and the ONPG-positive *H. parainfluenzae* biotypes I and II (biotype II is approximately 75% ONPG positive). The frequency of isolation of the biotypes (Tables 3 and 4) reported here agrees with that reported in the literature (1, 5, 7–9).

Based on the indole, ornithine, urea, and ONPG tests, it was possible to divide *H. influenzae* into the five biotypes and *H. parainfluenzae* into the three biotypes described by Kilian (7–9) (Tables 3 and 4). In concordance with the biotypes of isolates reported by other groups (1, 5, 7–9), it was found that a substantial majority (9 of 11) of *H. influenzae* isolates from the cerebrospinal fluid belonged to biotype I, whereas the majority (13 of 18) of *H. influenzae* isolates from the conjunctiva were biotype II (Table 3). The distribution of other biotypes of *H. influenzae* and *H. parainfluenzae* did not fall into a discernible pattern.

Fifty strains of *H. influenzae* were tested by counterimmunoelectrophoresis; 33 were type b. Of these 33, 30 (92%) produced indole as detected by both Micro-ID and conventional methods. Of the 17 cultures in which a type b capsule could not be demonstrated, 7 produced indole. All 11 *H. influenzae* isolated from the cerebrospinal fluid were type b; 10 produced indole. This observation correlated with the observation that types a, b, c, and f produce indole, whereas types

TABLE 3. *Biotypes of H. influenzae from body sites*

Site	No. from site	No. of biotype:					No. unclassified
		I	II	III	IV	V	
Cerebrospinal fluid	11	9	1	—[a]	—	1	
Epiglottitis	3	2	1	—	—	—	
Joint	1	1	—	—	—	—	
Skin (cellulitis)	1	1	—	—	—	—	
Eye	18	1	13	3	1		
Sputum	116	32	47	22	5	8	2
Ear	2	1	1	—	—	—	
Naso-pharynx	14	3	7	2	1	1	
Blood	6	4	2	—	—	—	
Total	172	54	72	27	7	10	2
% of total	100	21.3	41.9	15.7	4.1	5.8	1.2

[a] —, Biotype not encountered at site.

TABLE 4. *Biotypes of H. parainfluenzae from body sites*

Site	No. from site	No. of biotype:		
		I	II	III
Cerebrospinal fluid	2	1	1	—[a]
Epiglottitis	0	—	—	
Joint	0	—	—	—
Skin (cellulitis)	0	—	—	—
Eye	3	1	1	1
Sputum	65	31	19	15
Ear	0	—	—	—
Naso-pharynx	17	8	6	3
Blood	3	1	2	—
Total	90	42	29	19
% of total	100	46.7	32.2	21.1

[a] —, Biotype not encountered at site.

TABLE 2. *Micro-ID biochemical test results of 262 Haemophilus species*

Species	No. tested	% Positive with:[a]													
		VP	Nitrate	PD	H_2S	Indole	Ornithine	Lysine	Malonate	Urease	Esculin	ONPG	AR	AD	IN	SO
H. influenzae	172	0	100	0	0	79.7	42.4	2.9	0	93.6	0	0	0	0	0	0
H. parainfluenzae	90	0	100	0	0	0	78.9	0	0	53.3	0	81.1	0	0	0	0

[a] VP, Voges-Proskauer; PD, phenylalanine; H_2S, hydrogen sulfide; AR, arabinose; AD, adonitol, IN, inositol; SO, sorbitol.

d and e do not. Nontypable strains were indole variable (16).

Nine of 262 (3.4%) strains of *H. influenzae* and *H. parainfluenzae* produced β-lactamase. Three were *H. influenzae* biotype I, two were *H. influenzae* biotype II, two were *H. influenzae* biotype III, one was *H. parainfluenzae* biotype I, and one was *H. parainfluenzae* biotype II. Although the number of β-lactamase-producing strains was small, no biotypes could clearly be associated with the production of this enzyme.

Seven strains of *H. paraphrophilus* and three strains of *H. aphrophilus* were isolated during the course of this study. Although it appears that the Micro-ID accurately measured the constitutive enzymes of these organisms, the numbers of strains tested were too small for analysis.

DISCUSSION

Since the development of a scheme for biotyping *H. influenzae* and *H. parainfluenzae* by Kilian (7), the association of these biotypes with the anatomical sites of isolation, antigenic characteristics, and antibiotic resistance (1, 5, 7–9) have been noted. Micro-ID was efficacious for the rapid (4-h) classification and biotyping of *H. influenzae* and *H. parainfluenzae* based on the Kilian system. There was over 99% agreement between Micro-ID biochemical tests and conventional media. Conventional media have an inherent disadvantage for the routine clinical laboratory in being difficult to prepare and cumbersome to use. Commercially prepared kits can obviate the problem and make characterization of the genus accessible to the unspecialized laboratory (2). Since the Micro-ID agrees closely with conventional media, this system is applicable in place of tubed tests without the need for a change in the data base. By using the biochemical tests of indole, ornithine, ONPG, and urea, it was possible to divide *H. influenzae* into its five biotypes and *H. parainfluenzae* into its three biotypes in 4 h, and based on the indole and ONPG tests, to separate 96.1% of these two species. The Micro-ID should be especially useful as a rapid aid in identifying cerebrospinal fluid isolates, because over 90% are *H. influenzae* biotype I, which produces indole. *H. parainfluenzae* does not produce indole. Small numbers of other species of *Haemophilus* have been studied, but extrapolating from conventional biochemical tests reported in the literature, other species of human clinical isolates of *Haemophilus* should not be confused with either *H. influenzae* or *H. parainfluenzae*.

Unlike the situation which exists with respect to the *Enterobacteriaceae*, where large numbers of tests are required to biotype species and the biotypes generated may not be acceptably reproducible, a limited number of reproducible tests can differentiate *H. influenzae* and *H. parainfluenzae* biotypes. Unlike the *Enterobacteriaceae*, biotypes of *Haemophilus* are associated with the site of isolation. Albritton et al. (1) found that the distribution of *H. influenzae* biotypes from bacteremic patients were significantly different from the distribution of biotypes from nonbacteremic patients. In their series of 600 clinical isolates, 93% of *H. influenzae* from all sites from patients with bacteremia were type I or II, whereas 75% of *H. influenzae* isolates of all sources were types I and II from nonbacteremic patients. Of 47 isolates from the cerebrospinal fluid, 97.8% were biotypes I and II, 34 (72.3%) were biotype I, and 12 (25.5%) were biotype II. Kilian (10) also noted the preponderance of biotype I isolates from the cerebrospinal fluid, with this biotype accounting for 121 of 130 (93%) *H. influenzae* strains from Norway and Denmark. Cerebrospinal fluid isolates from the Mayo Clinic (5) and this hospital, with 16 of 18 (88.8%) and 9 of 11 (81.8%) biotype I isolates, respectively, indicate the increased frequency of this biotype from patients with meningitis. All reports have demonstrated an association of *H. influenzae* biotype II with its presence as a conjunctival pathogen. Studies by Kilian (43 of 100; 43% of isolates), Albritton et al. (19 of 26; 73% of isolates) and Goldberg and Washington (9 of 12; 75% of isolates), and in this study (13 of 18; 72% of isolates), all noted this association. Other than isolates from all sources from patients with *Haemophilus* bacteremia, cerebrospinal fluid, and conjunctival isolates, there does not appear to be an association of any biotype of *H. influenzae* with any site of isolation, although the numbers of strains from each of the variety of body sites described in the literature at this time are not many. There does appear to be a tendency of strains isolated from systemically ill patients (e.g., those with septic arthritis and cellulitis) to be biotype I. Relatively few strains of *H. parainfluenzae* biotypes have been reported (5, 7), but there does not appear to be an association between biotype and site of isolation.

Albritton et al. (1) reported that *H. influenzae* biotypes I and II were 34 times more likely to be ampicillin resistant than biotypes III, IV, and V. Goldberg and Washington (5) found that of the 2 (of 78) *H. influenzae* isolates they studied which were β-lactamase producers, both were biotype II. This study, with a small number of strains, found β-lactamase producers in *H. influenzae* biotypes I, II, and III, and in *H. parainfluenzae* biotypes I and II.

The use of so-called "rapid" tests for the iden-

tification of nutritionally fastidious, slow-growing organisms is not unique to this paper. One should not confuse the complex nutritional requirements for an organism for growth with its ability to produce measurable enzymes during growth. The Micro-ID, as do all bacterial biochemical tests requiring 4 h or less, primarily measures constitutive rather than inducible enzymes. The enzyme mass need only be present in sufficient quantity in a bacterial population for a test to prove workable. For example, the niacin test for the identification of *M. tuberculosis* requires only minutes to perform, yet the bacterial culture is weeks old. To better understand and extend our knowledge of the relationship of *H. influenzae*, *H. parainfluenzae*, and possibly other hemophili with the site of isolation, disease syndrome, and antibiotic sensitivity pattern, a rapid, inexpensive, and facile system amenable to the routine clinical laboratory for the classification and biotyping of this genus would be of considerable benefit. The 4-h Micro-ID is such a vehicle.

LITERATURE CITED

1. **Albritton, W. L., S. Penner, L. Slaney, and J. Brunton.** 1978. Biochemical characteristics of *Haemophilus influenzae* in relationship to source of isolation and antibiotic resistance. J. Clin. Microbiol. **7**:519–523.
2. **Back, A. E., and T. R. Oberhofer.** 1978. Use of the Minitek system for biotyping *Haemophilus* species. J. Clin. Microbiol. **7**:312–313.
3. **Clarke, P. H., and S. T. Cowan.** 1952. Biochemical methods for bacteriology. J. Gen. Microbiol. **29**:187–197.
4. **Cowan, S. T., and K. J. Steel.** 1965. Manual for the identification of medical bacteria, p. 148. Cambridge University Press, Cambridge.
5. **Goldberg, R., and J. A. Washington.** 1978. The taxonomy and antimicrobial susceptibility of *Haemophilus* species in clinical specimens. Am. J. Clin. Pathol. **70**: 899–904.
6. **Kilian, M.** 1974. A rapid method for the differentiation of *Haemophilus* strains. The porphyrin test. Acta Pathol. Microbiol. Scand. Sect. B **82**:835–842.
7. **Kilian, M.** 1976. A taxonomic study of the genus *Haemophilus* with the proposal of a new species. J. Gen. Microbiol. **93**:9–62.
8. **Kilian, M., C. H. Modrhorst, C. R. Dawson, and H. Lautrop.** 1976. The taxonomy of haemophili isolated from conjunctivae. Acta Pathol. Microbiol. Scand. Sect. B **84**:132–138.
9. **Kilian, M., and C. R. Schiott.** 1975. Haemophili and related bacteria in the human oral cavity. Arch. Oral Biol. **20**:791–796.
10. **Kilian, M., I. Sørensen, and W. Frederiksen.** 1979. Biochemical characteristics of 130 recent isolates from *Haemophilus influenzae* meningitis. J. Clin. Microbiol. **9**:409–412.
11. **Lautrop, H.** 1960. Laboratory diagnosis of whooping-cough or *Bordetella* infections. Bull. W.H.O. **23**:15–31.
12. **Lund, M. E., and D. J. Blazevic.** 1977. Rapid speciation of *Haemophilus* with the porphyrin production test versus the satellite test for X. J. Clin. Microbiol. **5**:142–144.
13. **Moeller, V.** 1955. Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. Acta Pathol. Microbiol. Scand. **36**:158–172.
14. **Myhre, E. B.** 1974. Typing of *Haemophilus influenzae* by counterimmunoelectrophoresis. Acta Pathol. Microbiol. Scand. Sect. B **82**:164–166.
15. **Thornsberry, C., and L. A. Kirven.** 1974. Ampicillin resistance in *Haemophilus influenzae* as determined by a rapid test for beta-lactamase production. Antimicrob. Agents Chemother. **6**:653–654.
16. **Young, V. M.** 1974. *Haemophilus*, p. 302–307. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.