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Novel Method of Biotyping *Haemophilus influenzae* That Uses API 20E

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One hundred *Haemophilus influenzae* isolates from various body sites were biotyped by conventional methods and by the API 20E system (Analytab Products, Plainview, N.Y.). By using a hemin- and a nicotinamide adenine dinucleotide-enriched saline solution as the inoculating fluid for the API 20E, a 100% correlation of results was obtained between the two methods. Ninety percent of the blood and cerebrospinal fluid isolates were biotype I. Biotype II was the predominant biotype encountered overall. No correlation was observed between beta-lactamase production and biotype. The API 20E is a reliable method and should prove useful for routine biotyping of *H. influenzae* in the clinical laboratory.

Traditionally, the requirement for hemin ("X factor") and nicotinamide adenine dinucleotide (NAD; "V factor"), in conjunction with typical colonial morphology, and tinctoral and morphological characteristics on Gram stain have been utilized in the identification of *Haemophilus* species. Kilian (5) characterized *H. influenzae* biochemically with conventional tube media and established five biotypes. More recent studies have reported that the Minitek System (BBL Microbiology Systems, Cockeysville, Md.) (2, 8, 10) and the Micro-ID (General Diagnostics, Morris Plains, N.J.) method (3) can be utilized to determine the biotype of *Haemophilus influenzae*.

This study was undertaken to develop another method for biotyping *H. influenzae*, which would be simple to apply to routine clinical usage and still be reliable. The API 20E (Analytab Products, Plainview, N.Y.), which is intended for the identification of members of *Enterobacteriaceae*, was utilized in this study.

A total of 100 consecutive clinical strains of *H. influenzae* were isolated in our laboratory. Isolates were identified by the procedures recommended by Kilian (6). All of the isolates exhibited typical colonial morphology and growth characteristics on microbiological media and typical tinctoral and morphological characteristics on Gram stain. The requirement for X and V factors was demonstrated by utilizing X-, V-, and XV-factor strips (BBL) on brain heart infusion agar (BBL). As a confirmatory test for the requirement for X factor, the strains were tested for their inability to synthesize porphyrins from delta-aminolevulinic acid (4). Isolates were also nonhemolytic when 24-h growth was examined on 5% (vol/vol) defibrinated horse blood agar (BBL) (5).

A saline solution containing X and V factors was prepared by placing two Taxo XV-factor strips (BBL) into a test tube containing 5 ml of sterile physiological saline. This test tube was blended on a Vortex mixer for 30 to 45 s to extract the X and V factors. The XV factor-saline solution was filter sterilized by using a 10-ml syringe and an Acrodisc with a 0.45-μm filter (Gelman Instrument Co., Ann Arbor, Mich.). *H. influenzae* isolates were suspended in 1 ml of XV factor-saline solution to a turbidity of not less than a 0.5 on a MacFarland turbidity standard.

The concentration of X factor in the XV strip is 3.5 mg per strip, and the concentration of V factor is 20 μg per strip. It is anticipated that the elution process is less than 100% efficient. It is also anticipated that during filter sterilization, some of the X and V factors may bind to the filter membrane. The final concentration of the X and V factors can be expected to be less than 1.4 mg/ml and 4 μg/ml, respectively.

The correlation of API 20E biochemical tests with conventional biochemical reactions was determined by performing taxonomic biotyping of each isolate and by utilizing modifications of the methods described by Kilian (5). Six to 8 drops of the XV factor-saline solution containing the *H. influenzae* isolate were added to 2.5 ml of 2% tryptone and 2.5 ml of Moeller ornithine decarboxylase (GIBCO Laboratories, Fairfield, N.J.), and 3 to 4 drops were added to a Christensen urea agar slant (GIBCO). The ornithine decarboxylase medium was overlaid with sterile mineral oil. All of the tests were incubated in

TABLE 1. Abbreviated biochemical characteristics of *H. influenzae* biotypes

H. influenzae biotype	Biochemical tests		
	Indole production	Urease activity	Ornithine decarboxylase activity
I	+	+	+
II	+	+	−
III	−	+	−
IV	−	+	+
V	+	−	+

a non-CO_2 incubator for 18 to 24 h. Biotypes were determined and are shown in Table 1.

Not all of the biochemical tests contained in the API 20E are useful for the taxonomic biotyping of *H. influenzae*. The biochemical tests utilized were indole, urea, and ornithine decarboxylase. Inoculation, utilizing a suspension of the isolate in XV factor-saline solution, and processing of the API 20E were as described by the manufacturer. The test strips were incubated at 35°C in a non-CO_2 incubator for 18 to 24 h.

All of the *H. influenzae* strains were tested for their possession of group a, b, c, d, e, or f antigens. The presence of antigens was determined with polyvalent antisera and individual group antisera (Difco Laboratories, Detroit, Mich.).

All ampicillin-resistant isolates were tested for the production of beta-lactamase. Detection of beta-lactamase was performed by the chromogenic cephalosporin method (9).

There was 100% agreement between the results of the 100 strains of *H. influenzae* tested by the API 20E procedure and the conventional biochemical test results. Preliminary studies utilizing only XV factor-saline solution to inoculate the appropriate biochemical wells of the API 20E revealed that all reactions were negative. When the API 20E was inoculated with saline suspensions of *H. influenzae* biotype I, the results of the ornithine decarboxylase test were difficult to interpret and were not reproducible. However, when the same strains were tested with a suspension prepared in XV factor-saline solution, reliable results were obtained. To determine whether the reactions obtained from the API 20E were reproducible, we tested two strains of *H. influenzae* biotype I by the API 20E system five times each. The results of these studies did not vary.

The distribution of biotypes according to body site is presented in Table 2. Ninety percent of the *H. influenzae* isolates obtained from blood and cerebrospinal fluid were categorized as biotype I. A substantial majority (44.0%) of *H. influenzae* isolates from respiratory tract specimens were categorized as biotype II. Similarly, biotype II was the predominant biotype encountered overall. The distribution of other biotypes of *H. influenzae* did not produce a distinguishable pattern.

Thirty-three percent of the isolates tested were encapsulated. Twenty-six (96.3%) of the *H. influenzae* type b isolates were biotype I. Of the serotype b, biotype I strains, 18 were recovered from respiratory tract specimens, 5 were recovered from cerebrospinal fluid, and 3 were recovered from blood specimens. One strain of an encapsulated serotype b, biotype II isolate was from a thyroglossal cyst of a 7-year-old patient. Three nonencapsulated biotype I strains were isolated in this study. Two of these strains were recovered from nasopharyngeal specimens, and one was recovered from a bronchial aspirate. Two encapsulated serotype f, biotype I strains were isolated from blood specimens. Two encapsulated biotype IV strains, serotypes d and e, were isolated from blood and respiratory specimens, respectively.

A total of 11 beta-lactamase-producing strains were isolated; 6 were biotype I, 2 were biotype II, and 1 each was biotype III, IV, and V.

Since Kilian (5) developed the scheme for biotyping *H. influenzae*, various commercial systems intended for the identification of *Enterobacteriaceae* have been modified and utilized in determining these biotypes. A study of 200 strains of *H. influenzae*, which utilized the Minitek biotyping procedure, showed a 97.7% correlation with the conventional tube method and noted discrepancies with the indole and

TABLE 2. Biotypes of 100 *H. influenzae* isolates according to source

Source (no.)	No. (%) of biotype:				
	I	II	III	IV	V
Blood (5)	4 (80.0)	—[a]	—	1 (20.0)	—
Cerebrospinal fluid (5)	5 (100)	—	—	—	—
Wound (2)	—	1 (50.0)	1 (50.0)	—	—
Cul de sac (1)	—	1 (100)	—	—	—
Eye (3)	—	1 (33.3)	1 (33.3)	—	1 (33.3)
Respiratory (84)	23 (27.4)	37 (44.0)	20 (23.8)	2 (2.4)	2 (2.4)

[a] —, Biotype not encountered at source.

ornithine decarboxylase tests (10). Similarly, in a study of the biotypes of 172 *H. influenzae* isolates, the Micro-ID method provided a 94% correlation, also with discrepancies with the indole and ornithine decarboxylase tests (3). Although in our study the number of strains tested was smaller, the API 20E procedure provided 100% correlation.

The distribution of biotypes according to the site of isolation is in agreement with previous reports (1, 5, 7, 8, 10). Kilian et al. (7) reported, in their study of 130 strains of *H. influenzae* isolated from patients with meningitis, that biotype I was the predominant biotype. Similarly, in our study, all five *H. influenzae* isolates from cerebrospinal fluid were biotype I. Respiratory isolates accounted for 92.5% of the biotype II isolates. Biotype II, not surprisingly, represented the predominant biotype isolated from respiratory tract specimens, which may indicate that this biotype represents commensal oral flora.

The predominance of encapsulated serotype b, biotype I strains isolated from cerebrospinal fluid and blood specimens is suggestive of possible additional virulence factors associated with biotype I strains. This observation has been reported previously (7, 10). The possibility that additional virulence factors are associated with biotype I strains of *H. influenzae* merits further investigation.

Of the 11 beta-lactamase strains of *H. influenzae*, 8 were either biotype I or II; however, in this study, this distribution was not significantly different from the sensitive isolates studied. Beta-lactamase production was detected in all five biotypes; thus, there was no apparent correlation between beta-lactamase production and biotype. These results are in agreement with those of other studies (3, 10).

Retrospective studies were performed to determine whether the concentrations of X and V factors eluted from one XV-factor strip were sufficient to obtain reliable results with the API 20E test system. Preliminary results of these studies revealed that reliable, reproducible results were obtained.

The results obtained in this study indicate that the API 20E provides excellent correlation with the conventional method of biotyping *H. influenzae*. The procedure described provides accurate, reliable results, is less cumbersome to perform than conventional methods, and is comparable in cost with the Micro-ID system. Small clinical laboratories which may not have the capabilities of biotyping *H. influenzae* by conventional, Micro-ID, or Minitek methods should find that this procedure is a highly acceptable procedure and lends itself to application in the clinical bacteriology laboratory.

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