

Biotyping of *Haemophilus* using API 10S—an epidemiological tool?

S MEHTAR, S AMINI AFSHAR

From the Department of Microbiology, North Middlesex Hospital, Edmonton, London N18 1QX

SUMMARY One hundred and ninety-nine strains of *Haemophilus* isolates were biotyped by Kilian's method¹ and a modified API 10S strip and the results compared. One hundred percent correlation was found between the two systems. The ONPG test proved of value in differentiating between *Haemophilus influenzae* and *Haemophilus parainfluenzae* when there was growth factor disc failure.

Haemophilus influenzae a well-recognised pathogen, has been implicated in paediatric outbreaks particularly in the USA.^{2,3} The biotyping method described by Kilian has revived interest in *Haemophilus* as an organism involved in cross infection in hospitals and families. Capsular typing, though an important marker of invasiveness, has proved of limited value in such situations because of the heterogeneity of the *Haemophilus* population and the unreliability of the available typing antisera.

Biotyping has been found to be more consistent and we feel has proved to be a useful epidemiological tool.

Preparation of sugars in the laboratory by the described conventional method was tedious and time-consuming. API 10S strip contained the relevant biochemical tests and the suspending medium was modified to support growth of haemophili without interfering with results.

Material and methods

ISOLATES

All clinical isolates of *Haemophilus* identified in the laboratory between December 1981 and February 1982 were examined. *Haemophilus* was identified by picking off suspicious colonies from a chocolate agar plate (containing 10 units of bacitracin) after 18 h incubation in CO₂ at 37°C. Gram stain, growth dependence on "X" and "V", purity plate and antibiotic disc sensitivity testing were performed on a single colony suspended in ordinary peptone water. The purity plates were then used for biotyping.

Growth dependence and antibiograms were repeated and biochemical profiles determined by both methods. Pittman serotyping using Wellcome's antisera a-f was performed on *H influenzae* strains.

BIOTYPING

Kilian's method was used to prepare biochemical tests in the laboratory as described.¹ Four biochemical tests, urea, ornithine, indole and β -galactosidase (ONPG) were selected which adequately covered the identification of both *H influenzae* and *H parainfluenzae*.

API 10S strips were found to contain the same selection of biochemical tests required. After a pilot study using glucose broth, and API water supplemented with 10% X and V, supplemented peptone water was found to be the most suitable and was prepared as follows:

(a) *Peptone water* (Southern Group) commercially available in 3 ml quantities.

(b) *NAD (BDH)* made up according to the manufacturer's instructions. Distilled water (5 ml) was added to each vial containing 25 mg of NAD; 0.3 ml of this was added to peptone water.

(c) *Haemin 1% (BDH)* when reconstituted, was sufficient for over 3000 tests; 0.3 ml was added to the peptone water.

One colony of *Haemophilus* was suspended in ordinary peptone water, and the NAD and Haemin added after growth requirements and antibiograms had been performed. The API 10S was then inoculated. The indole microtube in the strip was overlaid with sterile mineral oil along with the urea and ornithine. Saline containing one colony of *Haemophilus* was used as a suspending medium to inoculate the conventional sugars. Both sets of sugars

were incubated at 37°C for 18 h aerobically. The next morning oil from the indole microtube in the API 10S strip was removed with a pipette and Kovac's reagent was added. Kovac's reagent was also used for detection of indole in the conventional sugars.

Known NCTC strains of haemophili, *H influenzae* biotypes I-V and *H parainfluenzae* biotypes I-III were used as control strains.

Results

A total of 199 strains of *H influenzae* were tested for biochemical reactions and Kilian's method and API 10S compared. Using growth requirements, 105 strains were identified as *H influenzae* and 94 as *H parainfluenzae* in the routine laboratory. Biotyping of these strains identified 105 *H influenzae*, 72 *H parainfluenzae*, 20 *H para-aphrophilus* (ONPG-positive only) and two non-typable strains (indole and ONPG-positive). The biochemical identification for each biotype of *H influenzae* and *H parainfluenzae* is shown in Table 1.

There was a 100% correlation between the two methods used. *H influenzae* biotypes I to V were easily identified by their lack of β -galactosidase. In contrast, *H parainfluenzae* strains were ONPG-positive in all but three cases: one strain belonged to biotype I and there were two biotype III strains. Twenty strains gave no reactions to urea, indole or ornithine in either system but were ONPG-positive (Table 1). These strains were non-haemolytic and "V" dependent and therefore were biochemically classed as *H parainfluenzae*. Two non-typable strains were indole-positive as well as being ONPG-positive. We found no ONPG-positive "X & V" dependent strains. ONPG was a good differential test between *H influenzae* and *H parainfluenzae* where there was growth factor disc failure and gave a 98.4% positive identification for *H parainfluenzae* in both systems (Table 1).

Table 1 Biochemical profile of 199 isolates of *Haemophilus*

	API/CS Urea	API/CS Ornithine	API/CS Indole	API/CS ONPG
<i>H influenzae</i>	Total 105			
Biotype I	19/19	19/19	19/19	0/0
II	57/57	0/0	57/57	0/0
III	21/21	0/0	0/0	0/0
IV	6/6	6/6	0/0	0/0
V	0/0	2/2	2/2	0/0
<i>H parainfluenzae</i>	Total 72			
Biotype I	0/0	29/29	0/0	28/28
II	28/28	28/28	0/0	28/28
III	15/15	0/0	0/0	13/13
<i>H aphrophilus</i>	Total 20			
20	0/0	0/0	0/0	20/20
Non-typable	2	0/0	2/2	2/2

CS = Kilian's method. API = API 10S strip.

DISTRIBUTION OF BIOTYPES

Amongst *H influenzae*, biotype II was the commonest. Of the 150 strains biotyped, biotype I was 18.2%, biotype II 54.8%, biotype III 20.1%, biotype IV 5.7% and biotype V 1.9%. The 72 *H parainfluenzae* strains were 40.2%, 38.8% and 20% respectively in biotypes I, II, III. *H para-aphrophilus* made up of 10% of the total number of haemophili isolated; there were only two non-typable strains. Thirty-four (32.3%) capsulated strains of *H influenzae* were found. These were mainly amongst biotypes I and II: the commonest being Pittman type b (Table 2).

Twenty-one of the 34 strains belonged to biotype II (36.8%) and to biotype I (42%). We feel the number of capsulated strains would have been higher but the agglutination method is limited by the available antisera and the selection of single colonies from the original plates for purity.

CORRELATION WITH ISOLATION SITES

Haemophilus influenzae was isolated from sputum, CSF, blood, conjunctiva, nose, throat, antral washouts (Table 3). In contrast, *H parainfluenzae* strains were restricted to the respiratory tract and were repeatedly isolated from purulent and mucopurulent sputum from patients with obvious chest infections where no other pathogens were found. Some strains of *H para-aphrophilus* were found mixed with *H influenzae* strains and were regarded as commensals. One hundred and eighty-five of 199 strains were isolated from the respiratory tract (92%), 2.5% from conjunctiva and 1.5% from CSF. *H influenzae* strains found in sputum belonged mainly to biotypes I and II. CSF isolates belonged to biotype I but only one of them was capsulated. This strain was isolated from the blood as well. The *H influenzae* biotype II was isolated from the CSF of a 55-year-old woman and was non-capsulated. Isolates from the conjunctiva belonged only to biotypes II and III of *H influenzae*. The association of various biotypes of *H influenzae* and *H parainfluenzae* associated with antibiotic resistance will be published separately.

Table 2 Capsular type distribution amongst the various biotypes of *H influenzae*

Biotype	Pittman type					
	a	b	c	d	e	f
I (19)	1	6	0	1	0	0
II (57)	9	7	4	1	0	0
III (21)	0	2	3	0	0	0
IV (6)	0	0	0	0	0	0
V (2)	0	0	0	0	0	0
Total (34)	10	15	7	2	0	0

Table 3 *Distribution of various biotypes from clinical specimens*

Organism	Sputum	Eye	CSF	Blood	T/s	Nose	Antrum
<i>H influenzae</i>							
Biotype I	17	—	2	1*	—	—	—
II	50	2	1	—	2	1	1
III	17	3	—	—	1	—	—
IV	6	—	—	—	—	—	—
V	2	—	—	—	—	—	—
<i>H parainfluenzae</i>							
Biotype I	29	—	—	—	—	—	—
II	28	—	—	—	—	—	—
III	15	—	—	—	—	—	—
Non-typable	1	—	—	—	1	—	—
<i>H para-aphrophilus</i>	20	—	—	—	—	—	—

* Isolated from CSF as well.

Table 4 *Distribution of capsular and biotypes of H influenzae showing a bimodal distribution of age*

Age (yr)	0-5			6-15			16-40			≥41			Total		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Capsular type a		2	1							1	8		1	10	1
b	5	3					2		1	4			11	9	1
c							1				4	4	1	4	4
d										3	1	1	3	1	1
e										1			1		
f										1			1		
Total	5	5	1				3		1	10	21	5	18	24	7

The age distribution in relation to various biotypes and serotypes is given in Table 4. *H influenzae* showed a biphasic distribution between the ages of 0-5 yr and > 40yr. The isolates from patients below 5 yr of age were isolated from CSF, blood and conjunctiva whereas those above the age of 40 yr were mainly from the respiratory tract with one from CSF, another from an antral wash out and one from the eye. Below the age of five, biotypes I and II were equally distributed and capsular type b was commonest. After the age of 40 yr, most of the isolates were from the respiratory tract. Even though biotype II was predominant amongst *H influenzae*, capsular serotypes "a" and "b" were evenly distributed with types "c" and "d" being the next commonest. The other two biotypes of *H influenzae*—that is, IV and V, were found to be non-capsulated.

Discussion

Biotyping of haemophili is a reproducible method of epidemiological tracing. Capsular typing poses problems of a heterogeneous population of *H influenzae* and is also limited by the agglutination results with the available antisera. The selection of single colonies of the various biotypes may account for the 30% of capsulated strains found in this study; had twenty or more colonies been tested, possibly a

higher incidence of capsulated strains would have been found.

Other rapid methods such as Micro ID⁴ and the Minitek⁵ have been used as a rapid biotyping method and compared with Kilian's method. The Micro ID gave 100% correlation except with ornithine which was 96%. The Minitek was less favourable with a 97.5% correlation in the indole test and 92.3% in the ornithine. However, using the API 10S strip a 100% correlation in all four tests was achieved when compared with Kilian.

The ONPG test proved helpful in differentiating *H influenzae* (ONPG negative) from *H parainfluenzae* particularly when there was diffusion of growth factors through the agar; 98.4% of *H parainfluenzae* were ONPG +ve; ONPG +ve "V" dependent strains that did not utilise substrates were classified as *H para-aphrophilus* by Kilian. However, Oberhoffer and Back⁶ reasonably suggest these be included in *H parainfluenzae* as biotype IV. β -galactosidase-positive strains which are also indole-positive but "V" dependent were considered non-typable.

Welch *et al*⁷ have reported a higher incidence of indole-producing *H influenzae* from CSF than the respiratory tract, thus pointing out the relevance of *H influenzae* biotype I and II in meningitis. Therefore an indole and ONPG positive strain is unlikely to be *H influenzae* and we feel should be classified separately.

The capsulated strains found under the age of 5 yr made up approximately half the total *H influenzae* isolates. It is therefore possible that serious infection can be caused by non-capsulated strains of *H influenzae* in the young.

After the age of 40 yr the incidence of *H influenzae* increased, but only a third of these strains was capsulated. This might reflect the lack of or loss of immunity in these patients. Long term colonisation of chronic bronchitis can cause the loss or gain of capsules and a mixed population of *Haemophilus*. The administration of antibiotics in acute exacerbation of chronic bronchitis could possibly induce resistant strains of other species to transfer plasmids to haemophili regardless of their capsules. Antibiotic resistance and the transfer of plasmids between similar or different biotypes of *H influenzae* and *H parainfluenzae* is becoming increasingly important. Capsular typing, a marker for virulence, may fall short when multiresistant non-capsulated strains of *H influenzae* produce severe systemic infection or where there may be cross infection.⁸ We feel that using the API 10S, biotyping is easy, reliable and rapid and can be a useful adjunct to the existing methods in the identification of haemophili.

References

- ¹ Kilian M. A taxonomic study of the genus *Haemophilus* with the proposal of a new species. *J Gen Microbiol* 1976;**93**:9–62.
- ² Glode MP, Schiffer MS, Robbins JB, *et al*. An outbreak of *Haemophilus influenzae* type B meningitis in an enclosed hospital population. *J Pediatr* 1976;**88**:36–40.
- ³ Ginsburg CM, McCracken GH, Roe S, Park JG. *Haemophilus influenzae* type B disease: incidence in a day centre. *JAMA* 1977;**238**:604–7.
- ⁴ Back AE, Oberhoffer TR. Use of the Minitex system for biotyping *Haemophilus* species. *J Clin Microbiol* 1978;**7**:312–3.
- ⁵ Edberg CS, Melton E, Singer JM. Rapid biochemical characterization of *Haemophilus* species by using Micro-ID. *J Clin Microbiol* 1980;**11**:22–6.
- ⁶ Oberhoffer TR, Back AE. Biotypes of *Haemophilus* encountered in clinical laboratories. *J Clin Microbiol* 1979;**10**:168–74.
- ⁷ Welch DF, Ahlia PA, Matsen JM. Differentiation of *Haemophilus* species in respiratory isolates cultures by an indole spot test. *J Clin Microbiol* 1982;**2**:216–9.
- ⁸ Mehtar S, Law C. Outbreak of β -lactamase producing *Haemophilus influenzae* (biotype III) in a geriatric ward. *J Hosp Infect* 1980;**1**:357–8.

Requests for reprints to: Dr S Mehtar, Department of Microbiology, North Middlesex Hospital, Edmonton, London N18 1QX, England.