Biotypes of *Haemophilus parainfluenzae* from the respiratory secretions in chronic bronchitis

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Summary. A total of 2401 isolates of *Haemophilus parainfluenzae* was isolated from respiratory secretions of 36 healthy adults and 128 patients with chronic bronchitis over a period of 1 year. The isolates were allocated to eight biotypes, by their production of indole, urease and ornithine decarboxylase. Biotypes I and II constituted most of the isolates of *H. parainfluenzae* from the oropharynx of controls (75%) and chronic bronchitics (c. 90%). Among the patients, there was no difference in the isolation rate between oropharyngeal swabs and sputum specimens. Biotypes III, IV, VI, VII and VIII were isolated less frequently, as was a new taxon defined here as biotype V which does not produce indole, urease or ornithine decarboxylase. Biotype III was isolated significantly less frequently from cases of chronic bronchitis than from controls, whereas biotype II was isolated somewhat more frequently from the patients, especially during acute episodes.

Introduction

In recent years *Haemophilus parainfluenzae* has been recognised as both a normal inhabitant and an opportunist pathogen of the upper respiratory tract (URT). It is thought that dental, oropharyngeal, pulmonary or middle ear infections frequently precede systemic infections caused by this organism.¹ The most commonly reported infection is endocarditis^{1,2} although cases of pneumonia,^{3,4} septic arthritis⁴ and bacteraemia⁵ have been reported. Frederiksen and Kilian⁶ have argued that reports of meningitis and epiglottitis caused by *H. parainfluenzae* represent misidentified strains of *H. influenzae*.⁷ *H. parainfluenzae* constitutes at least 74% of the isolates of *Haemophilus* spp. from the pharynx;⁸ it is commonly isolated from the URT and lower respiratory tract (LRT) secretions of chronic bronchitics but is generally considered to be non-pathogenic. However, there is accumulating evidence to suggest that this organism may cause disease of a similar spectrum to that of non-typable *H. influenzae* in the LRT. Rhind *et al.*⁹ presented evidence that *H. parainfluenzae* was a pathogen in respiratory tract infections, and other investigators^{10–12} have demonstrated a high isolation rate in sputum specimens. Studies have shown that *H. parainfluenzae* slows ciliary activity in the respiratory tract.¹³ This may

contribute to diminished mucociliary clearance of micro-organisms and, possibly, chronic bronchial sepsis.¹¹ With its high isolation rate in respiratory secretions and its increasing implication as an opportunist pathogen, emphasis has been placed on the importance of characterising isolates of *H. parainfluenzae* from the respiratory secretions in chronic bronchitis.

Over a 1-year period, we investigated the distribution of biotypes of *H. parainfluenzae* in the URT and LRT of chronic bronchitic patients, some apparently free of infection and others with acute exacerbations; and we compared these strains with URT isolates from age-matched healthy adults.

Materials and methods

Patients and healthy controls

We recruited 148 patients from a hospital outpatient clinic to participate in a prospective study of chronic bronchitis, defined according to American Thoracic Society criteria;¹⁴ median age 66, range 18–84 years. An age-matched control group of 36 subjects with no recognised lung disease were also studied; median age 66, range 48–75 years. Whereas 44% of the chronic bronchitic subjects smoked, only 11% of the controls smoked. Oropharyngeal swabs were taken from both groups monthly for 1 year. Samples of sputum were collected from the bronchitic subjects at the same time when possible. The sputum specimens were checked

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by Gram's stain and microscopy; by the criteria of Courcol *et al.*,¹⁵ those with greater than four squamous epithelial cells per low power field were rejected. Episodes of acute bronchitis were defined as "production of increased volume and purulence of sputum". Routine samples were taken from patients who had no episode of acute bronchitis for at least 21 days. Cultures of *H. parainfluenzae* were isolated from 128 of the patients and from all 36 control subjects.

Bacterial culture and identification

Specimens were incubated overnight at 37°C, in CO₂ 5% in air, on chocolate agar (defibrinated horse blood 5%) containing bacitracin (Sigma) 300 µg/ml. Up to four morphologically distinct colonies were selected from each sample. Small gram-negative pleomorphic coccobacilli from a single colony were subcultured to chocolate agar and incubated overnight. The V-factor requirement was assessed by the observation of satellite growth beside a streak of the Oxford strain of *Staphylococcus aureus* on blood agar, on which the presence or absence of haemolysis was noted also. The X-factor requirement was deduced from ability to metabolise δ -aminolaevulinic acid and produce porphyrins.¹⁶

Biochemical tests

Isolates that were V-dependent and X-independent were biotyped by their production of indole, urease and ornithine decarboxylase¹⁷ with the Minitek Differentiation System (BBL Microbiology Systems).^{18,19} Tests were read after incubation for 4 h at 37°C.

Isolates that gave negative results in these three biochemical tests were further characterised, with the Minitek system, by their production of acid from glucose, sucrose, lactose, xylose, mannose, mannitol and galactose.¹⁷ In triplicate tests on each strain, no discrepancies were found.

To test for catalase production, a small amount of the culture was removed with a clean sterile glass rod

and emulsified in a drop of H₂O₂ solution on a glass slide; the drop was observed for effervescence.

To test for oxidase production by Kovacs' method,²⁰ a portion of a colony was removed with a sterile glass rod and rubbed on a strip of filter paper impregnated with a freshly prepared solution of tetramethyl-*p*-phenylenediamine dihydrochloride 1%; oxidase-positive colonies gave a deep purple colour within 10 s.

Results

By their production of indole, urease and ornithine decarboxylase, we differentiated 2401 isolates of *H. parainfluenzae* into eight biotypes (table I). The distribution of these biotypes in controls and chronic bronchitic patients is shown in table II. The isolates were predominantly of biotypes I and II. From the oropharynx, biotype I was isolated at a similar frequency from the control group (43%) and the chronic bronchitic group (47%). Biotype II constituted 32% and 42% of the oropharyngeal isolates, respectively. Biotype III was isolated less frequently—16% and 7%, respectively. Biotypes I, II and III had similar

Table I. Differentiation of biotypes of *H. parainfluenzae* by tests for three metabolic products

Biotype*	Result of test for production of		
	indole from tryptophan	urease	ornithine decarboxylase
I	—	—	+
II	—	+	+
III	—	+	—
IV	+	+	+
V	—	—	—
VI	+	—	+
VII	+	+	—
VIII	+	—	—

* Biotype V is defined in the present study. The other seven have been described previously: biotypes I–III;¹⁷ biotype IV;⁵ biotypes VI and VII;²⁶ biotype VIII.²⁵

Table II. Biotypes of *H. parainfluenzae* isolated from chronic bronchitic patients and from healthy controls

Subject	Specimen	Number of isolates of <i>H. parainfluenzae</i>	Number (%) of isolates of biotype							
			I	II	III	IV	V	VI	VII	VIII
Healthy control	Oropharyngeal swab	231	100 (43)	75 (32)	36 (16)	9 (4)	10 (4)	0	0	1 (0.4)
Chronic bronchitic patient	Oropharyngeal swab									
	Routine*	1257	589 (47)	530 (42)	87 (7)	28 (2)	20 (2)	2 (0.2)	0	1 (0.1)
	Acute episode†	246	115 (47)	112 (46)	12 (5)	4 (2)	2 (1)	0	1 (0.4)	0
	Sputum									
	Routine*	533	251 (47)	237 (44)	27 (5)	0	18 (3)	0	0	0
	Acute episode†	134	60 (45)	66 (49)	8 (6)	0	0	0	0	0

* Routine sample taken in absence of acute exacerbation.

† Sample taken during acute exacerbation.

isolation rates from oropharyngeal and sputum specimens in the chronic bronchitic population. Little difference was observed in the frequencies of isolation in the presence or absence of acute bronchitis. Biotypes IV–VIII were isolated at a much lower frequency.

We report 50 V-dependent isolates which did not produce indole, urease or ornithine decarboxylase. Acid was produced from glucose, sucrose, mannose and galactose but not from lactose, xylose or mannitol. The isolates were oxidase positive, and the catalase reaction was variable; no haemolysis was observed. We propose that these 50 isolates be classified as *H. parainfluenzae* biotype V. This group of bacteria accounted for c. 2% of oropharyngeal isolates and 3% of sputum isolates.

Discussion

In 1976, in a comprehensive study which included 125 strains of *H. parainfluenzae*, Kilian¹⁷ proposed a classification of the genus *Haemophilus* in which strains of *H. parainfluenzae* were assigned to one of three biotypes (I–III) by differences in their production of indole, urease and ornithine decarboxylase. These isolates were mainly from the oral cavities and throats of healthy persons. In 1979, Oberhofer and Back¹⁰ described two groups of isolates that could not be classified by the Kilian system. They tentatively assigned to a biotype IV seven strains of *H. parainfluenzae* that did not produce indole, urease or ornithine decarboxylase, and they suggested a fifth biotype for three isolates that gave positive results in all three tests. However, in their ensuing discussion, they disclosed that the tentative fifth biotype was actually a mixture of *H. parainfluenzae* and *H. influenzae*.

Frequently, authors fail to include sufficient characteristics to verify that their strains belong to the named species of the *Haemophilus* genus. In a recent definition of the genus,²¹ the main criterion for placing an aerobic or facultatively anaerobic gram-negative coccobacillus or rod in that genus is a requirement for one or both of the growth factors X and V. The taxonomically important character distinguishing *H. parainfluenzae* from *H. influenzae* is the capacity to synthesise porphyrin.¹⁶ The differentiation of *Haemophilus* spp. by X and V disks, as used by Oberhofer and Back, has been noted by several investigators to be misleading;^{22,23} at least 12% of strains of *H. influenzae* may be mis-identified as *H. parainfluenzae*.²⁴ Moreover, the biochemical methods employed do not allow differentiation of Oberhofer and Back's *H. parainfluenzae* biotype IV from other V-dependent species such as *H. segnis*, which also does not produce indole, urease or ornithine decarboxylase. Their seven isolates were oxidase negative but, according to Kilian,¹⁷ *H. parainfluenzae* is oxidase positive. This throws doubt on the interpretation of their data and on their biotype classification. In 1984, Brunn *et al.*⁵ described an isolate of *H. parainfluenzae* biotype IV,

which gave positive results in the indole, urease, and ornithine decarboxylase tests and was considered to be the aetiological agent in a patient with bacteraemia. More recently, *H. parainfluenzae* biotypes VI, VII and VIII have been reported^{25,26} among isolates from the genital and respiratory tracts.

In the present study, those isolates that were V-dependent and X-independent were biotyped. Among 2401 such isolates, 2351 were allocated to one of the seven *H. parainfluenzae* biotypes, other than biotype V, as listed in table I. Biotypes I, II and III in the sputum specimens of the bronchitics were also found with similar frequency in the oropharynx: biotypes I and II together constituted c. 90% of the *H. parainfluenzae* isolates while biotype III was isolated less frequently (c. 6%). These findings suggest that there may be free exchange of these three biotypes between the URT and LRT, in contrast with our findings for *H. influenzae*.²⁷ *H. parainfluenzae* biotypes I and II were isolated from the oropharynx of control subjects at a similar frequency to that of the chronic bronchitics, though the proportion of biotype II was somewhat greater in cases than in controls, especially during acute episodes. Although comparatively rare, biotype III was cultured approximately twice as often from the oropharynx of the controls as from patients ($p < 0.01$). The remainder of the 2351 isolates were distributed among biotypes IV, VI, VII and VIII, and these were isolated only from the oropharynx. Consistent with our findings, other investigators have reported that biotypes I–III predominated in both the URT and LRT, though the dominant biotype depended on the age and health of the group studied.^{10,25,26,28,29}

The 50 V-dependent isolates which were oxidase-positive but did not produce indole, urease or ornithine decarboxylase were classified as *H. parainfluenzae* biotype V (table I). This biotype was cultured from oropharyngeal swabs of the controls and bronchitics as well as the sputum specimens of the latter group. It was differentiated biochemically from other V-dependent strains of *Haemophilus* found in the respiratory tract which also do not produce indole, urease or ornithine decarboxylase—*H. segnis* and *H. paraphrophilus*. The species *H. parainfluenzae* ferments glucose, sucrose, mannose and galactose but not lactose, xylose or mannitol; and it is oxidase-positive. In contrast, *H. segnis* does not ferment mannose and is oxidase-negative; *H. paraphrophilus* does not ferment galactose but does produce acid from lactose. Kilian and Biberstein²¹ consider lactose fermentation to be a reliable criterion to differentiate *H. paraphrophilus* from *H. parainfluenzae*, regardless of CO₂ requirement. However, it has been reported that *H. paraphrophilus* is commonly misidentified as *H. parainfluenzae*,³⁰ and that at least 83% of strains submitted to the Centers for Disease Control were identified incorrectly.³¹

In conclusion, *H. parainfluenzae* seems to be part of the indigenous respiratory microflora and has little role in the pathogenesis of chronic bronchitis.

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