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Haemophilus influenzae Is Frequently Detected with Monoclonal Antibody 8BD9 in Sputum Samples from Patients with Cystic Fibrosis

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To determine the frequency of *Haemophilus influenzae* in sputum from patients with cystic fibrosis (CF), 477 sputum samples obtained from 86 CF patients were analyzed by standard culture and by the in situ immunoperoxidase staining technique with monoclonal antibody 8BD9. *H. influenzae* was isolated from 109 sputum samples (23%) from 45 patients (52%) and detected by immunoperoxidase staining in 175 sputum samples (37%) obtained from 63 patients (73%). The results of this study demonstrate the frequent presence of *H. influenzae* in sputum samples from CF patients.

Nonencapsulated *Haemophilus influenzae* is an important pulmonary pathogen in patients with cystic fibrosis (CF) (3, 7), and antibiotic treatment should be considered, irrespective of how few are isolated (14, 15). Recovery of *H. influenzae* by standard culture techniques from sputum samples from CF patients is difficult. The growth of *H. influenzae* is suppressed in the presence of mucoid *Pseudomonas aeruginosa* (16, 17) and by overgrowth of other microorganisms such as swarming *Proteus* spp. (11). Furthermore, during antibiotic treatment, growth of *H. influenzae* is inhibited and the colonizing microbial load of *H. influenzae* may be reduced (2, 11). The use of immunoperoxidase (IP) staining can avoid problems encountered with culturing *H. influenzae*, as previously shown by Groeneveld and coworkers (4, 5) for patients with chronic obstructive pulmonary disease. In this study, we compared the frequency of *H. influenzae* in sputum samples from CF patients as determined by IP staining and standard culturing.

In total, 86 CF patients (42 females and 44 males) visiting the Academic Medical Center in Amsterdam and the Leyenburg Hospital in The Hague from 1 February 1990 to 1 January 1991 were enrolled in the study. All consecutive CF patients from whom sputum samples of the lower respiratory tract were obtained for routine culture during the study period were included. The age distribution of the patients ranged from 2 to 51 years (median age, 24 years).

Sputa were collected in sterile vials and stored at 4°C, generally within 45 min of collection. A sputum specimen was washed twice in phosphate-buffered saline (pH 7.2) (12) and examined microscopically after Gram staining. Sputum samples obviously contaminated with oropharyngeal flora were excluded from further examination (6, 13). A purulent portion of the washed sputum specimen was cultured for *H. influenzae* on chocolate agar medium with an antimicrobial disk of bacitracin (40 U; Rosco Diagnostica, Taastrup, Denmark) applied to the surface of the medium at the beginning of the second set of streaks. To suppress the growth of *Pseudomonas* spp., plates were incubated for 48 h under strict anaerobic conditions at 37°C (17). *Haemophilus*-like colonies were isolated and identified by the method of Kilian (10), using growth requirement of isolates for hemin and NAD in a disk test with X and V factors. For the isolation of *P. aeruginosa* and *Staphylococcus aureus*, cultures were done on such aerobically incubated media as cystine-lactose-electrolyte-deficient medium, horse blood agar, and Columbia agar with 5% (vol/vol) horse blood supplemented with nalidixic acid (10 mg/liter) incubated in air enriched with 5% CO_2. The isolates were identified by usual methods (8).

For IP staining, a smear of washed sputum was dried on a glass slide and fixed with methanol. A smear from a pure culture of *H. influenzae* was always included as a positive control. IP staining was performed as previously described with monoclonal antibody 8BD9 directed against outer membrane protein P6 of *H. influenzae* (5). The slides were counterstained with 0.5% (wt/vol) methylene blue in distilled water. The number of stained bacteria was determined semiquantitatively by microscopic examination at ×1,000 magnification with oil immersion. At least 30 separate microscope fields were examined. A sputum specimen was considered positive by IP staining if more than 10 morphologically typical IP-stained bacteria were observed in at least one field. If less than 10 IP-stained bacteria per field were observed in all fields studied, the sputum specimen was considered negative because *H. influenzae* is commonly present in the commensal throat flora.

The McNemar test was used to compare isolation and detection rates of *H. influenzae*. P values of <0.05 were considered significant.

A total of 477 sputum samples from 86 CF patients were analyzed by culture and IP staining. The mean number of sputum samples per patient was 5.5 (range, 1 to 25). From 68 patients, more than one sputum sample was obtained. The most frequently isolated bacterial species was *Pseudomonas* spp., which was obtained from 332 of 477 (70%) sputum samples. The majority of *Pseudomonas* spp. was mucoid *P. aeruginosa* cultured from 307 sputum samples (64%). From 128 sputum samples (27%), *S. aureus* was isolated. *S. aureus* together with *Pseudomonas* spp. were cultured from 59

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TABLE 1. Comparison of culture results and IP staining for detection of *H. influenzae* (477 sputum specimens from 86 CF patients)

Culture	IP staining	No. of specimens[a]	No. of patients
+	+	107 (31)	45
−	−	300 (46)	60
−	+	68 (8)	31
+	−	2 (1)	2

[a] The numbers in parentheses are the initial sputum specimens from individual patients tested.

sputum samples. *H. influenzae* was recovered from 109 sputum samples (23%) obtained from 45 patients (52%). Cultures positive for *H. influenzae* included 66 sputum samples from which also *Pseudomonas* spp. ($n = 26$), *S. aureus* ($n = 23$), or a combination of both microorganisms ($n = 17$) was isolated. In three of these sputum samples, *Proteus vulgaris* was also cultured. Eleven sputum samples from one patient yielded *Pseudomonas* spp. together with swarming *P. vulgaris*. Seven sputum samples harbored other microorganisms, and no bacterial pathogens were isolated from 43 sputum samples. IP staining was positive in 175 sputum samples (37%) obtained from 63 patients (73%). Less than 10 IP-stained bacteria per field were found in 23 sputum specimens obtained from 20 patients.

Table 1 summarizes the comparison of culture and IP staining results for detection of *H. influenzae*. *Pseudomonas* spp. were isolated from 47 of 68 IP-positive sputum samples and yielded 34 sputum samples with mucoid *P. aeruginosa*. Mucoid and nonmucoid *P. aeruginosa* were tested for cross-reactivity with monoclonal antibody 8BD9, and neither showed a reaction. Swarming *P. vulgaris* was isolated from 12 sputum samples, always together with *Pseudomonas* spp. The remaining 21 IP-positive sputum samples were obtained from patients receiving antibiotic treatment. Culture and IP staining results were significantly different for all sputum specimens ($P < 0.0001$). To eliminate the effect of multiple contributions per patient, the initial sputum specimen obtained from each patient was also analyzed ($P < 0.05$). To determine whether the discrepancies between culture and IP staining results were restricted to specimens from particular patients, we evaluated the number of discrepancies according to the number of sputum samples per patient tested. The discrepancies were randomly found in patients above 10 years of age. In all patients under 10 years of age, *H. influenzae* was recovered by culture and also detected by IP staining.

In the present study, *P. aeruginosa* was isolated from 64%, *S. aureus* from 27%, and *H. influenzae* from 23% of the sputum samples. In a 1986 survey of CF centers in the United States, *P. aeruginosa* was found in 60%, *S. aureus* in 27%, and *H. influenzae* in only 11% of the respiratory tract cultures (9). *H. influenzae* was recovered from 12% of the patients at a German CF center (1) and from 14% at a Danish CF center (7). The high recovery rate of *H. influenzae* in our study may primarily be ascribed to the use of anaerobically incubated chocolate agar medium (with a bacitracin disk [40 U]) (1, 17). However, enhanced recovery of *H. influenzae* by quantitative culturing has also been reported (18). By IP staining, *H. influenzae* was present in 68 additional sputum samples obtained from 31 patients. In total, *H. influenzae* was present in 175 sputum samples (37%) obtained from 63 patients (73%), which is 60% more frequently than recovered by culture and more than twice the frequency as previously reported. In this study, the sensitivity of the IP staining was 98.2%. The specificity is also high as shown previously. Monoclonal antibody 8BD9 cross-reacted only with the subspecies *H. aegyptius*, *H. haemolyticus*, and with 13% of the *H. parainfluenzae* strains (5). However, it is unlikely that *H. aegyptius* and *H. haemolyticus* are present in sputum since these species are rarely isolated from patients with respiratory tract infections (10). Strains of *H. parainfluenzae* that show cross-reactivity may belong to a special subgroup of *H. parainfluenzae* (5). In this study, *H. parainfluenzae* was cultured from only eight of the sputum samples, and a negative result was always obtained by IP staining.

The discrepancy between culture and IP staining in sputa with *Pseudomonas* spp. is probably due to the production of substances by *Pseudomonas* spp. which exert an inhibitory effect on the growth of *H. influenzae* (16). Overgrowth by swarming *Proteus* spp. also severely hampers the isolation of *H. influenzae* (11). In the remaining 21 sputum samples, the discrepancies could be related to an inhibitory effect associated with antibiotic treatment. All these patients were receiving antimicrobial treatment at the time the specimen was obtained. The low occurrence of sputum samples positive by culture and negative by IP staining for *H. influenzae* may be related to the unequal distribution or a low concentration of *H. influenzae* in the sputum specimen (11).

The high recovery rate of *H. influenzae* by IP staining stresses the need for improvement of culture techniques to perform antibiotic sensitivity testing of an organism apparently often present in the respiratory tract of CF patients. We conclude that IP staining is a valuable addition in laboratory detection and quantification of *H. influenzae* in sputum from patients with CF.

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