

## Differentiation of *Haemophilus* spp. in Respiratory Isolate Cultures by an Indole Spot Test

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Indole spot tests using isolated, nonhemolytic colonies of *Haemophilus* species were positive for 90 of 151 (60%) respiratory isolates of *Haemophilus influenzae*, whereas 67 of 72 (93%) isolates of *H. influenzae* from cerebrospinal fluid and blood specimens were indole positive. Only 4 of 117 (3%) *Haemophilus parainfluenzae* isolates were positive for indole spot tests. Thus, indole-positive, nonhemolytic *Haemophilus* isolates in respiratory cultures can be presumptively identified as *H. influenzae*.

Differentiation of *Haemophilus influenzae* from other *Haemophilus* species is commonly done by performing the satellite test to determine X (hemin) and V (NAD) growth requirements. Satellite testing usually requires 24 h beyond the time of initial isolation on primary culture plates. An alternative and more rapid confirmatory test is the determination of the utilization of delta-aminolevulinic acid for porphyrin synthesis (7). Lund and Blazevic (12) evaluated this method as a 4-h test and found it to be practical for use in the clinical laboratory.

Kilian (7) showed that biotyping may also be performed for the classification of *Haemophilus* species, and Edberg et al. (2) recently demonstrated that Micro-ID was also useful for the rapid (4-h) biochemical characterization of *Haemophilus* species. A disadvantage of the porphyrin production test and the use of Micro-ID is that relatively large inocula are required. Since primary culture plates of respiratory specimens usually contain a mixture of organisms in various numbers, a simple test for presumptive differentiation of *Haemophilus* species would be desirable. The production of indole is a consistent feature of some biotypes of *H. influenzae* and is not present with *H. parainfluenzae* (8, 13). We evaluated the production of indole in *Haemophilus* cultures by using a rapid spot test (11, 16) as a means of identifying *H. influenzae* isolated from respiratory specimens. Results were compared with those for organisms isolated in a pediatric hospital and in a general hospital and demonstrated that the indole spot test is a useful aid in the rapid identification of *H. influenzae* in respiratory tract cultures.

### MATERIALS AND METHODS

Routine bacteriological cultures of respiratory specimens were included in the study. Most respiratory

specimens, except those submitted to the two laboratories for beta-hemolytic *Streptococcus* culture only, were cultured for *Haemophilus*. Sputum specimens received at one hospital (University of Utah) were microscopically screened as previously described (19), and only those specimens fulfilling criteria for acceptability on the basis of enumeration of leukocytes and squamous epithelial cells were cultured. Specimens received on swabs (e.g., nasopharyngeal) were directly inoculated onto primary plating media and then streaked for colony isolation. Aspirated or expectorated specimens were selectively sampled by using a sterile loop or swab and inoculated as described above. Primary plating media included 5% sheep blood agar, 5% selectively enriched horse blood agar (SHBA) (10), MacConkey agar, and phenylethyl alcohol agar with 5% sheep blood. Regular sheep blood agar and SHBA plates were incubated at 35 to 37°C in 6% CO<sub>2</sub>. Other media were incubated at 35 to 37°C in ambient air. Plates were examined after 24 to 48 h of incubation.

*H. influenzae* isolated from blood or cerebrospinal fluid (CSF) cultures were studied for comparison with respiratory isolates. Isolates obtained from pediatric patients at Oklahoma Children's Memorial Hospital were tested either upon initial isolation or after storage in 1 ml of whole sheep blood at -70°C. Other isolates originally isolated from CSF or blood were obtained as lyophilized cultures through the courtesy of Melvin Marks, Division of Pediatric Infectious Diseases, Oklahoma University Health Sciences Center.

Definitive identification of *H. influenzae* and *H. parainfluenzae* was performed either with paper disks containing X or V factor or with media supplemented with X or V or both X and V factors to determine growth requirements. Growth of *H. influenzae* or *H. parainfluenzae* on selective horse blood agar plates was identified as small, moist, greyish colonies, frequently surrounded by a greenish but not hemolyzed blood medium. Individual colonies of each morphological type were picked with a straight needle and streaked on a brain heart infusion agar plate. X and V disks (Difco Laboratories, Detroit, Mich.) were placed in the center of the inoculated area and spaced approx-

imately 10 mm apart. These plates were incubated at 35 to 37°C in a CO<sub>2</sub> environment for 24 h. If growth occurred only between the two disks, isolates were identified as *H. influenzae*. If growth was located around the V disk only, isolates were identified as *H. parainfluenzae*. Alternatively, growth requirements were determined by using factor-supplemented media; tests were performed according to the manufacturer's (Regional Media Laboratories, Lenexa, Kans.) directions.

Use of the substrate delta-aminolevulinic acid was determined by the method of Kilian (8) as subsequently described by Lund and Blazevic (12). Results were read after 4 h of incubation by using the fluorescent method (12), with a Wood lamp in a dark room. A positive test (lack of requirement for X factor) was indicated by red fluorescence.

Indole production by *Haemophilus* colonies was detected by using a tube test to which Kovacs reagent was added to a tryptophan broth suspension of organisms (16). Spot methods (4, 11, 16, 18) were also adapted to identify indole-positive colonies of *Haemophilus*. Isolated colonies on SHBA or chocolate agar were picked and touched to a filter paper circle moistened with the indole test reagent. Reagents employed were 1 and 5% (wt/vol) *p*-dimethylaminobenzaldehyde (DABA) and 1% *p*-dimethylaminocinnamaldehyde (DACA) prepared in 10% (vol/vol) concentrated hydrochloric acid. Positive reactions were interpreted as color development (pink, DABA; blue, DACA) within 10 s. Inocula for growth factor requirements and for indole testing were taken from primary culture plates when the amount of growth permitted.

To further confirm the identity of some strains and to determine whether a correlation between indole production and serotype existed, we performed slide agglutination tests with *H. influenzae* antiserum (Difco).

## RESULTS

Colonies of *Haemophilus* were usually evident after 24 h on SHBA. *H. influenzae* and *H. parainfluenzae* were small, moist, colorless to greyish, and nonhemolytic. *H. influenzae* commonly caused greening of the surrounding medium. Hemolytic colonies on SHBA were considered to be *H. haemolyticus* or *H. parahaemolyticus* and were not further identified. Hemolytic and nonhemolytic *Streptococcus* species, *Staphylococcus* species, *Neisseria* species, and diphtheroids do not grow on the medium owing to the incorporation of bacitracin as the selective agent (10). Yeasts or other gram-negative organisms were not inhibited but usually were easily differentiated by colonial morphology or the Gram stain. Occasional specimens, such as those from some cystic fibrosis patients, yielded heavy growth of *Pseudomonas aeruginosa* or members of *Enterobacteriaceae* and had to be excluded from the study. Of 84 consecutively isolated nonhemolytic *Haemophilus* isolates from pediatric patients, 82 were *H. influenzae* and 2 were *H. parainfluenzae* (ratio of 41:1).

Specimens from the mixed-age groups, similarly studied, yielded 69 *H. influenzae* and 115 *H. parainfluenzae* isolates (ratio of 0.6:1).

The results of indole spot tests on *Haemophilus* species from respiratory or blood and spinal fluid sources are shown in Table 1. Since the results of respiratory isolate testing depended on the accuracy of species identification (as *H. influenzae* or *H. parainfluenzae*), species verification was performed on selected isolates by determination of delta-aminolevulinic acid utilization. Lack of utilization as determined by the rapid porphyrin production test correlated with X and V requirement testing on 37 of 37 isolates identified as *H. influenzae*. A positive porphyrin test correlated with demonstration of the V requirement for 12 of 12 isolates identified as *H. parainfluenzae*. All isolates designated as hemolytic *Haemophilus* species produced distinct beta-hemolysis on primary SHBA plates.

Variations of the spot indole test and correlation of spot testing with the tube test were investigated. Of 37 isolates identified as *H. influenzae* by growth factor requirements and confirmed by the porphyrin production test, 20 (54%) were positive by the tube method, 19 (51%) were positive by the spot test with 1% DABA, and 27 (73%) were positive by the spot test with 1% DACA. To determine whether the greater rate of positivity with 1% DACA represented false-positive results or greater sensitivity, we tested 1% DACA compared with the other methods and with 5% DABA by using *H. influenzae* and *H. parainfluenzae* isolates in pure culture. Of 52 strains of *H. parainfluenzae*, 4 were weakly positive by the 1% DACA method. All 52 strains were negative by the other indole testing methods, including the 5% DABA method. Of 48 *H. influenzae* strains tested by the four methods, 8 were positive by the 1% DACA or 5% DABA methods, only. However, those which were positive by at least three of the four methods tended to give stronger reactions with 5% DABA than with 1% DABA or the tube test. In view of the absence of indole-positive reactions of *H. parainfluenzae* with 5% DABA

TABLE 1. *Haemophilus* spot indole results by species and source of isolation

Identification sources	No. of isolates spot indole positive/no. tested <sup>a</sup>
<i>H. influenzae</i> (respiratory)	90/151 (60) <sup>b</sup>
<i>H. parainfluenzae</i> (respiratory)	4/117 (3)
<i>H. influenzae</i> (blood-CSF)	67/72 (93)

<sup>a</sup> Spot testing for indole production with isolated colonies on primary SHBA plates (respiratory) or 24-h SHBA containing pure cultures (blood-CSF).

<sup>b</sup> Numbers in parentheses are percentages of positive isolates. Results are for tests done with the 5% DABA reagent.

TABLE 2. Indole testing results with serotype B *H. influenzae*

Source	No. serotyped	Total no. indole positive	No. indole positive type B/total no. type B
CSF	25	25	25/25
Respiratory	35	17	6/9

and the presence of stronger positive reactions of *H. influenzae* with 5% DABA as compared with 1% DABA, the 5% reagent was chosen for general use. Subsequently, four additional strains of *H. parainfluenzae* gave indole-positive reactions from primary media (Table 1). However, these false-positive results appeared to be due to adjacent indole-producing non-*Haemophilus* species.

The results of serotyping and indole testing on CSF and respiratory isolates of *H. influenzae* are presented in Table 2. Whereas all CSF isolates were both serotype B and indole positive, only 9 of 35 respiratory isolates were serotype B, and of those, only 6 were indole positive.

#### DISCUSSION

The clinical relevance of identification of *H. influenzae* in respiratory specimens is primarily based on the diagnosis of acute infection or for epidemiological surveillance in children. Respiratory sources of *H. influenzae* in adults may be associated with chronic or acute pulmonary disease. The traditional marker for determining the potential invasiveness of *H. influenzae* has been the presence of type b capsular antigen. Three recent case reports (3), however, reemphasize the virulence potential of non-type b organisms in adults, and the occurrence of otitis media in children caused by nontypable strains is well known (15).

Biotyping provides alternative markers for grouping *H. influenzae*. Of the six biotypes that have been proposed (13), three of them (I, II, and V) are characteristically indole positive. Kilian et al. (9) found that 127 of 130 meningitis isolates were biotype I or II. Albritton et al. (1) showed that *H. influenzae* isolated from bacteremic patients were, predominantly, biotype I or biotype II, and Golberg and Washington (5) showed that conjunctivitis isolates are usually biotype II.

In response to the renewed interest in *Haemophilus* biotyping, Edberg et al. (2) reported a rapid and reliable means of biotyping *Haemophilus* species by using a commercially available test system (Micro-ID). An indole screening test, as we describe, for presumptive biotyping may also prove useful for not only differentiating

*H. influenzae* from *H. parainfluenzae*, but also for predicting the potential relationship of a particular isolate to disease. Evidence for this latter correlation was actually shown many years ago. A partial correlation of indole production with pathogenicity was demonstrated experimentally by Rosher (14) in 1931. Further study needs to be undertaken in experimental systems to test the relationship between biotype, or indole production, and virulence.

The characterization of *Haemophilus* species by indole spot testing has been reported by Sutter and Finegold (17), but only in the context of case studies involving *H. aphrophilus* infections. The spot test has been applied more extensively in rapid identification schemes for lactose-fermenting members of *Enterobacteriaceae* (6) and in anaerobic bacteriology (16).

The results of spot tests with colonies from primary culture plates may occasionally be falsely positive because of adjacent indole-positive colonies of other species. This was encountered with four of our *H. parainfluenzae* isolates and has been observed by other investigators (6, 16, 18) using the indole spot test with other groups of bacteria. False-negative results were not proven, but are suggested by the occurrence of five CSF isolates, all of which had been lyophilized before testing, that were indole negative. All other CSF isolates of *H. influenzae*, fresh and after lyophilization, were indole positive.

In conclusion, the results of this study indicate that if a nonhemolytic *Haemophilus* species from the respiratory tract (isolated on selective horse blood agar) is positive for the indole spot test, it is *H. influenzae*.

An indole-negative isolate may require further diagnostic testing. Since the majority of invasive *H. influenzae* are indole positive, the indole spot test may also be used as a rapid screening procedure for potentially pathogenic *H. influenzae*.

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