

Comparison of Methods for Serotyping Isolates of *Haemophilus influenzae*

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This study compared four methods for serotyping isolates of *Haemophilus influenzae*. Slide agglutination with commercial antisera (Difco Laboratories and Wellcome Diagnostics), coagglutination (Phadebact *Haemophilus* Test [Pharmacia Fine Chemicals]), latex agglutination with affinity-purified anticapsular antibody, and counterimmunoelectrophoresis with multiple antisera were used to serotype 80 isolates of *H. influenzae*. Coagglutination and counterimmunoelectrophoresis correctly identified all 80 isolates as either type b or not type b. Slide agglutination and latex agglutination each successfully identified 76 of the 80 isolates; however, each of these two methods failed to type four isolates because of agglutination of controls. We recommend slide agglutination or coagglutination as the serotyping methods of choice in most laboratories because they are simple, accurate, and rapid. Slide agglutination with Difco antiserum can be performed at the lowest cost.

Serotyping of isolates of *Haemophilus influenzae* is important for both clinical and epidemiological purposes. Because it is now recommended that certain contacts of patients with invasive disease caused by type b strains of *H. influenzae* receive rifampin prophylaxis (3), clinical laboratories require a rapid and reliable method to identify type b strains. The most frequently used serotyping method is slide agglutination, using commercially available antisera. Several published reports (10, 11), as well as our own past experience, have suggested that this method may not be consistently reliable. The most common error is the misidentification of nontypable strains as type b. In an effort to assess the accuracy of the slide agglutination procedure in differentiating type b from non-type b strains, we compared it with three other serotyping methods which are suitable for use in a clinical laboratory.

MATERIALS AND METHODS

Isolates. Serotyping methods were compared by using 80 isolates of *H. influenzae*. Of these, 49 were fresh clinical isolates cultured from specimens of blood, cerebrospinal fluid, eye drainage, abscess, or respiratory tract secretions submitted to our laboratory from May to September 1983. Isolates were identified as *H. influenzae* by their typical colonial morphology on chocolate agar and by their requirement for X and V factors (BBL Microbiology Systems, Cockeysville, Md.) on Trypticase soy agar plates (BBL) (4). Serotyping was performed either on growth taken directly from the original media or after one subculture to chocolate agar. Thirty-one additional isolates (freezer isolates) were kindly supplied by Dan Granoff. These isolates had been recovered from various body sites and had been sent to the laboratory of D. Granoff for further analyses. Included among these 31 were isolates representing capsular types a to f, as well as nontypable strains which had caused difficulty in serotyping. In the present study, serotyping of these isolates was performed on initial subculture from frozen stock or after one subculture.

Slide agglutination. Slide agglutination testing was performed with two commercially available antisera to *H.*

influenzae type b (Difco Laboratories, Detroit, Mich., and Wellcome Diagnostics, Research Triangle Park, N.C.). One drop of a turbid suspension of the test organism in normal saline was mixed separately with 1 drop of antiserum and 1 drop of normal saline (autoagglutination control) on a glass slide. Rapid (<1 min) agglutination of organisms by the antiserum with clearing of the background and no agglutination of organisms in the normal saline control were interpreted as a positive test (6).

Coagglutination. Staphylococcal coagglutination was performed with the Phadebact *Haemophilus* Test (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) according to the manufacturer's instructions. A very small amount of growth was removed from a pure subculture plate, using a sterile swab. This inoculum was thoroughly rubbed onto two circles of the cardboard slide supplied with the test kit. A drop of type b reagent was applied to one circle, and a drop of types a plus c to f reagent was applied to the second circle. Reagent and inoculum were thoroughly mixed in each circle, using a wooden applicator stick. The slide was rocked for 30 to 60 s while observing for agglutination. Visible agglutination was interpreted as a positive test. "Stringiness" without true agglutination was interpreted as a negative test.

Latex agglutination. Latex agglutination was performed with reagents prepared in our laboratory. The affinity-purified *H. influenzae* type b anticapsular antibody preparation used to sensitize latex particles was prepared as previously described (9). In brief, type b capsular polysaccharide purified by the method of Kuo et al. (7) was activated with cyanogen bromide and then bound to ω -aminohexyl-Sepharose (Sigma Chemical Co., St. Louis, Mo.) (8). Antiserum raised in burros to *H. influenzae* type b (burro 132 antiserum kindly provided by John Robbins) was incubated with the affinity matrix for 2 h at room temperature. After the column was washed with phosphate-buffered saline-albumin, anticapsular antibodies were eluted with 3.5 M MgCl₂ and immediately diluted with 1 volume of phosphate-buffered saline-albumin followed by dialysis against phosphate-buffered saline at 4°C. This affinity-purified anticapsular antibody preparation was used to sensitize 0.81- μ m-diameter polystyrene latex particles (Dow Chemical Co.,

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TABLE 1. Isolates of *H. influenzae* not successfully typed by all methods

Source	Isolate no.	Reaction by ^a :						
		Slide agglutination		Coagglutination	Latex agglutination	CIE		
		With Difco antiserum	With Wellcome antiserum			With Wellcome antiserum	With burro antiserum	With affinity-purified burro antiserum
Serum	1	A ^b	A	—	—	—	—	—
Blood	2	A	A	—	NS	—	—	—
Tracheal aspirate	3	A	A	—	NS	—	—	—
Freezer	4	A	A	—	—	—	—	—
Sputum	5	—	—	—	NS	—	—	—
Freezer	6	—	—	—	NS	—	—	—

^a —, Not type b. A, Autoagglutination, fine clumping without clearing of the background, seen when the isolate was mixed with saline. NS, Nonspecific reaction, agglutination of the isolate with both the *H. influenzae* type b and control latex preparations.

^b This isolate showed slight autoagglutination in saline and strong agglutination with the Difco antiserum.

Midland, Mich.) (12). A control latex preparation was similarly prepared with a nonimmune burro antiserum.

The latex agglutination test was performed by suspending the test organism in normal saline at a turbidity adjusted to equal a 0.5 McFarland standard. Samples (50 μ l) of the suspension were mixed on a glass slide with 10 μ l of the *H. influenzae* type b-specific preparation and with 10 μ l of control latex preparation. The reaction was read by eye after rotation for 1 min. Agglutination with the *H. influenzae* type b-specific latex and not with the control latex was interpreted as a positive test.

CIE. Counterimmunoelectrophoresis (CIE) was performed with three different antisera: the Wellcome Diagnostics antiserum, which was also used for slide agglutination; the burro affinity-purified anticapsular antibody, which was also used for latex agglutination; and the original burro antiserum (burro 132), from which the affinity-purified anticapsular antibody was made. The antigen preparation used was a suspension of the test organism in normal saline with turbidity adjusted to equal that of a 0.5 McFarland standard. Antigen and antibody preparations were placed in 5-mm wells spaced 3 mm apart on a 1% agarose-coated glass slide. The slides were electrophoresed in a barbital buffer (pH 8.6) at 6 V/cm for 30 min. The presence of a sharp precipitin band was interpreted as positive. Plates showing broad precipitin bands were soaked in normal saline overnight and observed again the following day (1).

Analysis. The study coordinator (C.A.H.) reviewed the results of all testing methods. When discrepancies among methods were noted, the test methods yielding discrepant results were repeated to distinguish reproducible from technical discrepancies. The true serotype of each isolate was considered to be that reflecting the consensus of the test methods.

RESULTS

Of the 80 test isolates, 39 (49%) were type b, including 28 of the 49 isolates from the clinical laboratory and 11 of the 31 "freezer isolates." As expected, most (95%) of the 29 isolates from blood and cerebrospinal fluid were type b, and most (95%) of the 28 isolates from other sites (respiratory, eye, and abscess) were not type b.

Coagglutination correctly identified all 80 isolates. CIE also correctly identified all 80, although two isolates were incorrectly identified on first testing. One type b strain was initially typed as non-type b by CIE. When the discordance

of this result with the other test methods was recognized, CIE was repeated and correctly identified the strain as type b. A human error seems likely as the explanation for this discrepancy. One other strain that was nontypable was initially identified as type b by CIE, using the burro antiserum. This error occurred because of the presence of a broad nonspecific band that did not completely soak out and was read as positive. On repeat testing, the band did disappear after overnight soaking. A similar broad band was seen with eight other isolates on CIE testing with the burro antiserum; in these instances the band disappeared after overnight soaking and was easily recognized as a nonspecific finding.

With either the Wellcome or the Difco antisera, the slide agglutination test correctly identified 76 of the isolates. The remaining four (isolates 1 to 4) could not be tested by slide agglutination because of autoagglutination (Table 1). One of these four isolates (isolate 1) showed strong agglutination with clearing of the background when tested with the Difco antiserum, but also showed weak agglutination in saline (autoagglutination). This result was potentially confusing because the antiserum agglutination was much stronger than the observed autoagglutination. A similar result was not observed with the Wellcome antiserum. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the outer membrane proteins of this isolate yielded a pattern not previously observed among type b strains and suggestive of a nontypable strain (2). One additional isolate that was type b initially was reported as non-type b with the Difco antiserum. On repeat testing, the Difco antiserum produced a positive test. The most likely explanation for the initial incorrect result was that the slide was not observed for a long enough period to observe the agglutination.

The latex agglutination test also correctly identified 76 of the 80 isolates. Four isolates (isolates 2, 3, 5, and 6) produced results that were uninterpretable because of agglutination of the negative control latex as well as the *H. influenzae* type b latex preparation (Table 1).

DISCUSSION

This study, comparing four immunological methods for serotyping strains of *H. influenzae*, was stimulated by reports (10, 11) that the slide agglutination technique frequently yielded erroneous results, especially the misidentification of nontypable strains as type b. In contrast to these

studies, we found good agreement among all methods tested. This finding is consistent with the study reported by Ingram et al. (5) in which nearly complete correlation was observed among the results of slide agglutination, Quellung reaction, CIE, and latex agglutination. However, in that study, serotyping results for nontypable strains were not presented.

Although we found the slide agglutination technique to be accurate, the method does contain potential pitfalls. It is mandatory to include a saline control to detect autoagglutination. Four strains could not be typed by slide agglutination because of autoagglutination. All four were non-type b strains that might have been identified as type b if autoagglutination had not been recognized. Even with the autoagglutination control, one of these strains could easily have been interpreted as type b when tested with the Difco antisera because the agglutination produced by the Difco antiserum was much stronger than that observed in saline. Another potential pitfall of slide agglutination is that some nontypable strains can produce slight clumping with the type b antiserum, thought to result from cross-reactivity of somatic antigens (6). Accordingly, it is important to consider only strong agglutination with clearing of the background as a positive test. We did not observe such reactions in this study. Finally, the time of reading of the slide agglutination test is important. In this study, several type b strains yielded strong agglutination with the Wellcome antiserum only after more than 30 s. In addition, fine autoagglutination may not be observed until more than 30 s have elapsed. Thus, it is important to observe both the antiserum and the saline control for 30 to 60 s before determining the result.

Drawing on our experience in this study, we have several recommendations for clinical laboratories performing serotyping of *H. influenzae*. Both slide agglutination and the commercial coagglutination test are simple, rapid, and accurate methods for identifying type b strains of *H. influenzae*. The cost of materials is least for slide agglutination, using the Difco antiserum. The per-test costs in our laboratory were as follows: slide agglutination (Difco), \$0.94; slide agglutination (Wellcome), \$1.65; coagglutination, \$1.60. If laboratories choose to use the slide agglutination test, it is important that personnel performing the test be familiar with the characteristic reactions. In addition, results of the slide agglutination test should be compared with colony morphology. We were uniformly able to differentiate encapsulated from nonencapsulated strains on chocolate agar, by virtue of the larger, more mucoid colony morphology of the encapsulated strains. If the slide agglutination test is positive on a strain that has the appearance of a nonencapsulated strain, the test should be repeated and verified with another method. For laboratories using slide agglutination, we recommend either CIE or coagglutination as alternative or backup procedures. Finally, it is important to remember that the quality of the

antiserum used is a major determinant of the accuracy of any immunological serotyping method.

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