Methods for Serotyping Nasopharyngeal Isolates of *Haemophilus influenzae*: Slide Agglutination, Quellung Reaction, Countercurrent Immunelectrophoresis, Latex Agglutination, and Antiserum Agar

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Nasopharyngeal isolates of *H. influenzae* were typed by the slide agglutination test, the Quellung reaction, the latex agglutination test, countercurrent immunoelectrophoresis, and the antiserum agar test. These tests gave essentially comparable results, with countercurrent immunoelectrophoresis and latex agglutination being slightly more sensitive. Cross-reactive problems encountered with latex agglutination and the expense of performing countercurrent immunoelectrophoresis or the antiserum agar test made these tests less practical than the slide agglutination test to identify single strains that were already isolated. The Quellung reaction and slide agglutination were the most rapid tests used to type an organism. For mass screening of multiple samples, countercurrent immunoelectrophoresis was the simplest technique. The antiserum agar test was slow but was the best technique to screen nasopharyngeal swab cultures to identify the presence of any encapsulated strains in the mixed flora. Whether any of the above techniques were as sensitive as the immunofluorescence test was not evaluated in this study.

In 1931, Pittman used specific antisera in the slide agglutination test and the precipitin test to divide encapsulated *Haemophilus influenzae* into six different antigenically distinct serotypes (14). The Quellung reaction (capsular swelling) was used later as a simple typing method (1, 2, 12). Recently, four other techniques, countercurrent immunoelectrophoresis (CIE) (3, 4), the latex agglutination test (LA) (10, 11), antiserum agar (culture plates containing specific antibody) (8), and immunofluorescence (4, 6, 13, 15) have been employed as alternative methods for typing *H. influenzae*. This study was designed to determine whether the slide agglutination test, Quellung reaction, CIE, LA, and antiserum agar gave comparable results when used to type nasopharyngeal isolates of *H. influenzae* and to examine the advantages and disadvantages of using each of these tests for this purpose.

MATERIALS AND METHODS

Isolation and identification of strains of *H. influenzae*. Between September 1970 and June 1977, strains of *H. influenzae* were obtained by a phosphate-buffered saline nasopharyngeal wash from normal children in day care at the Frank Porter Graham Child Development Center in Chapel Hill, N.C. These children were cultured biweekly when well and with each

illness. The nasopharyngeal washing was plated on chocolate agar (Granite Diagnostics, Burlington, N.C.) and incubated overnight at 37°C without CO₂. *H. influenzae* strains were tentatively identified by odor and by colonial morphology. One colony was transferred to a second chocolate agar plate and incubated overnight at 37°C, and a piece of colonized agar was transferred to 2 ml of sterile skim milk and frozen at -70°C. For typing, a loopful of the thawed skim milk was transferred to 1 ml of Leventhal's broth and incubated for 20 h at 37°C; a Gram stain was then performed, and a portion of the culture was plated on chocolate agar and incubated overnight at 37°C to examine for contaminants, and on rabbit blood agar to determine hemolytic activity. X and V factor dependence was determined by using tubes containing 1% neopeptone broth (pH 7.5) with one of the following: (i) factor X (hemoglobin; Difco Laboratories, Detroit, Mich.) plus brain heart infusion broth (BHI) (Baltimore Biological Laboratory, Cockeysville, Md.); (ii) factor V (B nicotinamide adenine dinucleotide) (Sigma Chemical Co., St. Louis, Mo.); or (iii) both factors (Leventhal's broth plus BHI in a ratio of 12:30). These tubes were inoculated from the chocolate agar subculture by using an inoculating loop to carefully touch the top of an isolated colony and transfer it to the tube. These tubes were examined visually for turbidity after 6 h of incubation at 35°C.

Antibody preparation. Antibody specificity was tested by the five typing methods using *H. influenzae* antibodies a to f (Hyland Labs, Costa Mesa, Calif.)

and 18-h Leventhal's broth cultures of *H. influenzae* types a to f (American Type Culture Collection, Rockville, Md.). American Type Culture Collection typable *H. influenzae* lot numbers were: type a, 9006; type b, 9795; type c, 9007; type d, 9008; type e, 8142; and type f, 9833. The only cross-reaction noted was by CIE, slide agglutination, and the Quellung reaction, in which *H. influenzae* type d antibody cross-reacted with the broth culture of *H. influenzae* type f. After incubation for 18 h in CO₂ at 35°C, *H. influenzae* type f bacteria were sedimented by centrifugation, washed three times in saline plus 1% formaldehyde, added to the *H. influenzae* type d antibody, incubated at 35°C for 30 min, and centrifuged. The adsorbed *H. influenzae* d antibody was then used in all five typing methods once it was determined by all of these tests that it no longer contained cross-reacting antibody.

Slide agglutination test. A 1-ml amount of an 18-h Leventhal's broth culture was centrifuged at 12,000 × *g* for 2 min. The supernatant was decanted and stored at -20°C. One drop on Loeffler's alkaline methylene blue was added to the sediment, and half of the mixture was diluted with equal volumes of 1% formalized saline. One drop of this dilution was mixed with one drop of monovalent *H. influenzae* antibody types a to f separately on a glass slide, rotated for 2 min, and examined for agglutination.

Quellung reaction. The Quellung reaction was performed, using the antibody manufacturer's recommendations (Hyland Labs, Costa Mesa, Calif.). A 0.25-ml amount of 2% peptone solution containing 1 g of NaCl plus 1 g of dehydrated peptone (Fisher Scientific Co., Pittsburgh, Pa.) in 50 ml of sterile water was added to the remaining stained sediment not used for the agglutination test. Then one loopful of the stained mixture was mixed on glass slides with one loopful of each one of the monovalent *H. influenzae* antibodies separately, covered with a cover slip, and after 5 to 10 min, examined under an oil immersion lens (×1,000) for capsular swelling and compared with a positive control.

CIE. CIE was performed on the 18-h broth culture supernatant fluid, using a Hyland Austigen II (Hyland Labs, Costa Mesa, Calif.) power pack, and plates were refilled with 16 ml of a 1:1 mixture of 1% Noble agar (Difco) and agarose (Fisher Scientific Co., Pittsburgh, Pa.) in barbital-hydrochloride buffer (0.05 M; pH 8.0) plus 1% sodium azide at 30 mA (2.9 V/cm) for 1 h at 4°C. The chamber sponges were soaked with 25 ml of barbital-hydrochloride buffer (0.5 M; pH 9.2). Pairs of 5-μl wells 5 mm apart were filled with 5 μl of broth supernatant on the cathodal side and 5 μl of a pool made of equal portions of monovalent *H. influenzae* a to f antibodies on the anodal side. Any visible precipitation line between the wells was read as positive. CIE was repeated, using monovalent *H. influenzae* antibodies a to f on broth supernatants typable by CIE using the pooled antibody, the slide agglutination test, the Quellung reaction, LA, or antiserum agar.

LA. LA was performed by a modification of the method of Newman et al. (11), using polystyrene latex particles (Dow Chemical Co., Indianapolis, Ind.) sensitized with either a pool of *H. influenzae* antibodies types a, b, c, and f mixed 1:2:1:1 and diluted 1:600 in 0.1 M, pH 8.2 glycine-buffered saline, a pool of *H.*

influenzae antibodies types d and e mixed 1:2 diluted 1:600 in GBS, or monovalent *H. influenzae* antibodies types a to f diluted 1:800 in glycine-buffered saline. By using a plastic tray with wells (Dispo Bact Cidal Assay Trays, Linbro Chemical Co., New Haven, Conn.), 25 μl of an 18-h broth supernatant was added to 25 μl of glycine-buffered saline plus 0.1% bovine serum albumin (Matrix, Chicago, Ill.). Then 25 μl of latex particles, sensitized with one of the two pools or with monovalent antibody, was added. The tray was covered and shaken for 2 h at 120 cpm at 25°C. Any agglutination was read as positive. All strains were initially typed by LA, using the two pools of antibody. All strains typable by the agglutination test, the Quellung reaction, CIE, or LA using the antibody pools, as well as 72 strains not typable by any of these methods, were subjected to typing by LA using monovalent antibodies.

Antiserum agar. Culture plates containing monovalent *H. influenzae* type-specific antibody were prepared by the method of Michaels et al. (8). All strains typable by the above methods were transferred to the plates from the unspun broth culture, streaked for isolation, and incubated for 72 h at 37°C. Any obvious precipitation forming a halo about a colony was read as indicating a typable strain.

RESULTS

Between 1970 and 1977, 955 nasopharyngeal isolates of *H. influenzae* were isolated. Of these, 45 were typable by at least 2 of the 5 tests examined. Of these 45 typable strains, 22 were type b, 2 were type d, 12 were type e, and 9 were type f. No *H. influenzae* types a or c were found. The results comparing the five different methods of typing are shown in Table 1.

The slide agglutination test detected 44 of the 45 typable strains and did not detect one type f strain. The agglutination reactions were easily read except those for type f, which were weak. The Quellung reaction detected 42 of 45 typable strains. The same type f strain determined to be nontypable by slide agglutination was also nontypable by the Quellung reaction. Two type d strains were not detected by the Quellung test when the *H. influenzae* type d antibody adsorbed with type f organisms was used. The Quellung reaction was the only procedure employed in which this adsorbed antibody could not detect the two nasopharyngeal strains of type d. To determine whether the inability of the Quellung reaction to detect nasopharyngeal type d strains was due to adsorbing the type d antibody with type f organisms, the test was repeated, using unadsorbed type d antibody which then did detect the two type d strains. The Quellung reactions were moderate to weak except for type b strains, which consistently gave obvious positive reactions. CIE detected all 45 typable strains. The precipitation lines were sharp and easily observed (Fig. 1). The antise-

TABLE 1. Number of encapsulated strains of *H. influenzae* detected by five methods of typing

Typing method	No of encapsulated strains detected with <i>H. influenzae</i> antibody:							Total
	Pools ^a	a	b	c	d ^b	e	f	
Slide agglutination	—	0	22	0	2	12	8	44
Quellung reaction	—	0	22	0	0	12	8	42
CIE	a-f, 45	0	22	0	2	12	9	45
Antiserum agar	—	0	21	0	2	12	9	44
LA	abcf, 24	0	22	0	2	12	9	45
LA	de, 11							

^a Pools containing *H. influenzae* antibody of the types noted. —, No pool used.

^b Adsorbed with *H. influenzae* type f.

FIG. 1. CIE plate showing precipitation lines between two of the three pairs of wells. The two pairs of wells showing precipitation lines contain *H. influenzae* type b broth culture on the cathodal side and *H. influenzae* type b antiserum on the anodal side. The pair of wells showing no reaction contains *H. influenzae* type e in broth and *H. influenzae* type b antiserum.

rum agar method allowed the detection of all the typable strains except one type b organism that failed to produce a halo but was typable by the other four tests. The halos produced by the other typable strains were easily detected (Fig. 2).

FIG. 2. *H. influenzae* antiserum-agar medium showing halos of precipitation around typable colonies.

Using the LA and monovalent antibody all 45 typable strains were detected. By using pools of antibody, only 35 of 45 strains were detected. The LA reactions were often very weak, especially when testing type f strains. These weak reactions were difficult to interpret. The LA was the only test in which cross reactions were noted. Four of the 12 nasopharyngeal type e strains and 3 of the 9 type f strains cross-reacted with latex particles coated with *H. influenzae* type b antibody. American type culture collection strains of *H. influenzae* types a-f were properly identified with easily detectable reactions by all tests.

DISCUSSION

Encapsulated strains of *H. influenzae* are separated into types a through f, based on their production of one of six serologically distinct capsular polysaccharides (14). These capsular

polysaccharides are produced in various amounts, with mutant strains producing very little (4, 6), nasopharyngeal isolates producing more (7), and strains isolated from blood or cerebrospinal fluid often producing large amounts. The use of Leventhal's broth or agar may enhance production of capsular polysaccharides (2). Some investigators have noted that encapsulated strains may lose their ability to produce capsular polysaccharides after multiple passages in artificial media (2, 4, 5, 14).

To type nasopharyngeal isolates which produce only a moderate amount of capsular polysaccharides, each of the five tests enumerated has advantages and disadvantages. The slide agglutination test is the simplest test to perform and interpret. Although organisms may be tested directly from the growth on agar media, ideally an 18-hour broth culture is used. The test requires only 2 min. Also, the test uses very small amounts of antibody. The Quellung reaction is also rapid (5 min) and simple to perform, but perhaps the capsular swelling is more difficult to read than the slide agglutination test. When nasopharyngeal isolates were examined in this study, there was often minimal capsular swelling as compared with that of positive controls. The test uses small amounts of antibody. Its major advantage is that it may be used directly on organisms from a culture plate, although better results are obtained when at least a 6-h Leventhal's broth culture is used (12).

The LA requires at least an 18-h broth culture supernatant (10). If performed on a glass slide it only takes 2 min. If plastic trays are used, as was done in this study, the test requires additional incubation time, but multiple samples may be screened simultaneously. This test requires the least amount of antibody of the five procedures compared in this study. The problems associated with it are that weak agglutination is difficult to read, and that cross-reactions are seen with this procedure.

CIE requires more sophisticated equipment, is more expensive, and requires more time to perform than the previous tests, i.e., 1 h after an 18-h broth culture supernatant is available. It requires very small amounts of antibody. The advantages of this procedure are that the results are easy to interpret, multiple specimens may be screened at once, and cross-reactions do not occur.

The antiserum agar technique has the advantage that nasopharyngeal specimens may be plated directly onto it, and all the colonies containing encapsulated organisms can be typed without further passage and loss of encapsulation. The disadvantage associated with this test is that large amounts of antibody are required,

making it expensive, especially when typing multiple specimens. The test also requires a minimum of 2 days to perform.

Immunofluorescence can detect mutant strains and nasopharyngeal strains undetectable by slide agglutination or the Quellung reaction (4, 6, 7, 13, 15). This test requires expensive equipment. Whether it is more sensitive than CIE, LA, or the antiserum agar test was not evaluated in this study.

In this study, the slide agglutination test, the Quellung reaction, CIE, LA, and the antiserum agar test were essentially comparable in sensitivity when typing nasopharyngeal isolates, except that CIE and LA serotyped one more strain than did any of the other three tests. Although essentially equivalent in sensitivity to CIE, the LA test demonstrated less specificity.

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