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Detection of *Haemophilus influenzae* Type b Antigens in Body Fluids, Using Specific Antibody-Coated Staphylococci

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Protein A-rich staphylococci coated with *Haemophilus influenzae* type b antiserum agglutinate specifically with homologous bacterial cells or with cell-free supernatant fluids of cultures of the organism. Antibody-coated staphylococci were used to detect soluble antigens in body fluids of patients infected with *H. influenzae* type b. Cerebrospinal fluid from 36 cases of meningitis caused by this organism showed positive coagglutination tests in 86% of patients prior to initiation of therapy. Antigens could be detected in 46% of sterile cerebrospinal fluid specimens obtained from the same cases 1 to 10 days after therapy. Soluble antigens were also detectable in sera (58%) and urine specimens (67%) of patients with *H. influenzae* type b septicemia, when such specimens were tested within 10 days of onset of illness. No antigen could be detected in body fluids beyond 10 days. The coagglutination test was positive in 57% of all body fluids examined; contercurrent immunoelectrophoresis (CCIE) was positive in only 27%. All specimens positive by CCIE were also positive by coagglutination. No false-positive reactions were noted by either test in body fluids from controls. The coagglutination test is simple, specific, and more sensitive than the CCIE method and could be a valuable tool for detecting antigens in body fluids of patients with various infections.

The encapsulated form of *Haemophilus influenzae*, primarily type b, is a common cause of serious pyogenic infections in a pediatric population. Identification of this organism in a diagnostic microbiology laboratory rests primarily on the demonstration of capsular swelling (quellung test). Although the test is simple and rapid, it requires the presence of whole visible cells.

In recent years, detection of the soluble polyribophosphate (PRP) capsular antigen of *H. influenzae* type b in certain body fluids has been achieved. The countercurrent immunoelectrophoresis (CCIE) test (4, 7, 9, 14, 15) and the latex agglutination method (13) have been employed for this purpose. These tests have aided in establishing a rapid diagnosis of *H. influenzae* type b infections in some cases.

Strains of *Staphylococcus aureus* containing protein A on their outer surface bind immunoglobulin G (8). Binding occurs at the Fc fraction of immunoglobulin G (12), thus leaving the Fab fraction free. The addition of homologous antigen to the antibody-coated staphylococci results in an agglutination reaction. This coagglutination test has been utilized by some investigators in the identification of certain bacterial isolates (3, 5, 6, 11).

The present report describes the usefulness of the coagglutination test in the identification of *H. influenzae* type b and in the detection of soluble antigens of this bacterium in various body fluids.

MATERIALS AND METHODS

Clinical specimens. Bacterial isolates tested were ones recovered in the diagnostic microbiology laboratory at the Children's Hospital of Michigan, Detroit. In addition, six American Type Culture Collection strains of *H. influenzae* types a through f were used.

Cerebrospinal fluid (CSF) specimens, sera, and unconcentrated urine samples were obtained from patients admitted to the hospital with clinical illnesses. Specimens that could not be tested immediately were frozen at −20°C until used.

Materials. Antisera against *H. influenzae* types a through f were obtained from a commercial source (Hyland, Div. of Travenol Laboratories, Inc., Costa Mesa, Calif.). Purified PRP was kindly supplied by John B. Robbins. The protein A-rich staphylococcus used was the Cowan 1 strain (ATCC 12598).

Procedure. A stabilized suspension of *S. aureus* was prepared by the method of Kronvall (11). The Cowan 1 strain of *S. aureus* was grown in tryptic soy broth for 18 h, and the harvested cells were washed three times in phosphate-buffered saline (PBS; 0.12 M NaCl-0.03 M Na_2HPO_4 [pH 7.3]). The bacteria

were then suspended in 0.5% formaldehyde in PBS and kept at 25°C for 3 h. The cells were washed three times in PBS and finally adjusted to a concentration of 10% (vol/vol). The cell suspension was heated at 80°C for 1 h. After cooling this suspension, 0.1 ml of *H. influenzae* antiserum was added to 1 ml of the stabilized cell suspension, and the mixture was kept at 25°C for 1 h with occasional shaking. To the mixture 8.9 ml of PBS was added. This final suspension was used as the antibody-coated staphylococcal reagent and was stable at 4°C for at least 4 months. Controls of stabilized staphylococcal cell suspensions not mixed with antiserum or mixed with normal rabbit serum were also prepared.

The CCIE test was performed by a previously described procedure (2), using the same antisera as above. The agar was examined for precipitin lines within 0.5 h after termination of the electrophoresis and was reexamined 16 to 18 h later.

RESULTS

When a suspension of *H. influenzae* type b was mixed with type b antibody-coated staphylococci on a glass slide, agglutination occurred instantaneously (Fig. 1A). This coagglutination occurred when a single colony from a solid medium or a loopful of a broth culture was used. No agglutination was detected when *H. influenzae* isolates other than type b were substituted (Fig. 1B). Also, no agglutination occurred when *H. influenzae* type b was mixed with PBS (Fig. 1C).

The specificity of the coagglutination test in identifying *H. influenzae* type b isolates is shown in Table 1. No false-negative or false-positive reactions were noted. An antibody-free staphylococcal cell suspension and a suspension coated with normal rabbit serum were used as controls. No agglutination with any bacterial isolate occurred with these controls.

Antibody-coated staphylococcal suspensions were also prepared by using rabbit antiserum against each of the other *H. influenzae* types (types a, c, d, e, and f). In all instances agglutination occurred only with the homologous strain.

Undiluted cell-free supernatant fluids from broth cultures of 40 *H. influenzae* type b isolates were also tested with type b antiserum-coated staphylococci. Strong and immediate agglutinations were observed in every instance. This observation prompted the comparison of the sensitivities of the coagglutination and CCIE methods in detecting soluble antigens. Twofold dilutions of cell-free supernatant fluids from five *H. influenzae* type b cultures were made in PBS, and detection of soluble antigens was attempted in the various dilutions. The

TABLE 1. *Specificity of coagglutination in the identification of clinical isolates, using type b rabbit antiserum*

Organism	No. tested	% Positive by:	
		Quellung	Coagglutination
H. influenzae type b	76	100	100
H. influenzae, not type b	93	0	0
Haemophilus spp.	21	0	0
Other[a]	30	0	0

[a] A total of 13 *E. coli*, 11 *S. pneumoniae*, 2 β-hemolytic streptococci, and 1 each of *N. meningitidis*, *K. pneumoniae*, *Proteus* sp., and *Pseudomonas* sp.

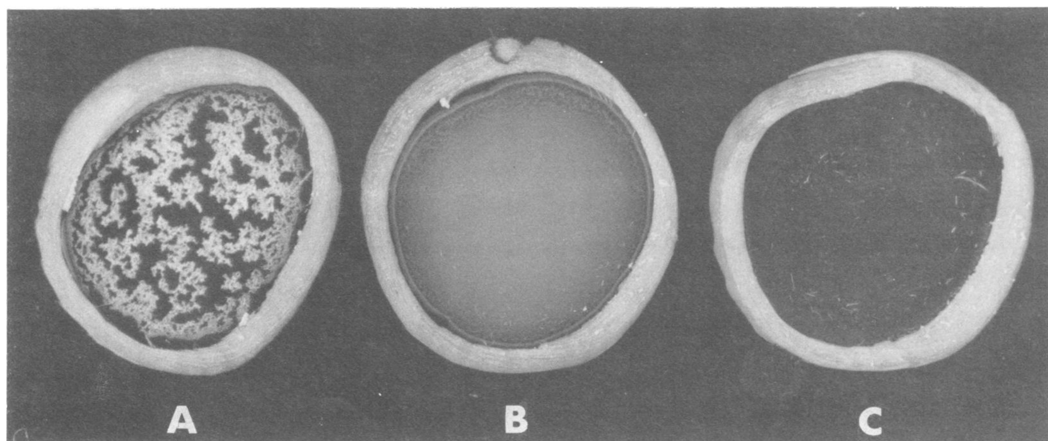

FIG. 1. *Coagglutination of H. influenzae type b, using a staphylococcal suspension coated with type b rabbit antiserum (A). The same suspension did not agglutinate with H. influenzae types a, c, d, e, and f or with nontypable H. influenzae (B). H. influenzae type b cells mixed with PBS as a control (C).*

results are shown in Table 2. The superiority of the coagglutination test over the CCIE in detecting antigens at higher dilutions is obvious.

The coagglutination and CCIE tests were also compared as to their sensitivity in detecting purified PRP of *H. influenzae* type b. By using the same antiserum, 4.0 μg of PRP per ml could be detected by the CCIE test, and 2.0 μg/ml could be detected by the coagglutination test. It should be emphasized, however, that the amount of antiserum employed in the CCIE test is 100-fold that used in the coagglutination test.

When it was evident that agglutination occurred with soluble antigens of *H. influenzae* type b and not only with the whole organism, attempts were made to detect such antigens in body fluids of patients infected with this bacterium. SCF samples, sera, and unconcentrated urine specimens were collected from such patients at various stages of their infections, and the CCIE and coagglutination methods were used to detect antigens. The findings are summarized in Table 3.

The CSF samples were obtained from cases of meningitis, and in every instance *H. influenzae* type b was recovered prior to initiation of therapy (zero time). All CSF samples were sterile at subsequent taps (day ≥ 1). The coagglutination test was more sensitive than the CCIE test at all observation times. All specimens positive by CCIE were also positive by coagglutination. Only 5 of 36 specimens obtained prior to initiation of therapy were not diagnosed immediately by the coagglutination test (false negatives). Of 37 CSF specimens examined 1 to 10 days after initiation of therapy (at a time when cultures were sterile), 17 (46%) were positive by the coagglutination test, whereas only 6 (16%) were positive by the CCIE.

Examinations of 21 sterile CSF specimens from patients not suffering from meningitis and 11 CSF specimens yielding bacteria other than *H. influenzae* (six *Escherichia coli*, two *Streptococcus pneumoniae*, and one each of *Neisseria meningitidis*, *Pseudomonas aeruginosa*, and group B beta-hemolytic streptococcus) were performed as controls. No agglutinations were detected in any of these, and none was positive by the CCIE test.

Sera were obtained from patients with *H. influenzae* type b septicemia (with or without meningitis) and tested by both methods (Table 3). The coagglutination test was again more sensitive than the CCIE. All but one of the positive sera were specimens obtained within 48 h of initiation of therapy. In testing sera by the coagglutination test, it was essential to include a control of a stabilized staphylococcal suspension not containing the *H. influenzae* type b antiserum. Agglutination with antibody-coated staphylococci but not with the control was interpreted as positive (Table 3). Agglutination with both antibody-coated and antibody-free staphylococci was noted in two sera (not included in Table 3). Adsorption of these two sera with the Cowan 1 strain of *S. aureus* eliminated the agglutination with both test and control suspensions.

Three control sera were also tested by the CCIE and coagglutination tests. These sera were from patients with septicemia due to *S. aureus*, *Klebsiella pneumoniae*, or enterococci. All were negative by the CCIE test with *H. influenzae* type b antiserum. The serum from the patient with staphylococcal septicemia gave

TABLE 2. *Comparison of coagglutination and CCIE in detecting H. influenzae type b antigens in supernatant fluids*

Fluid concn	No. of positive/no. tested	
	Coagglutination	CCIE
≤1:16	5/5	5/5
1:32	5/5	3/5
1:64	5/5	2/5
1:128	5/5	0/5
1:256	2/5	0/5
1:512	1/5	0/5
1:1,204	0/5	0/5

TABLE 3. *Temporal detection of H. influenzae type b soluble antigen in body fluids*[a]

Time (days)	No. of positive CSF samples/no. tested		No. of positive sera/no. tested		No. of positive urine samples/no. tested	
	CCIE	Coagglutination	CCIE	Coagglutination	CCIE	Coagglutination
0	26/36	31/36			1/3	3/3
1	2/9	4/9	6/11	7/11	5/13	7/13
2	3/11	8/11	3/3	3/3	3/13	10/13
3–5	0/7	2/7	0/4	1/4	5/35	27/35
6–10	1/10	3/10	0/1	0/1	3/32	17/32
>10	0/18	0/18	0/5	0/5	0/5	0/5

[a] A total of 15 of 32 CSF specimens and 8 of 17 urine specimens positive by CCIE were detected only when read at 16 to 18 h. Sera positive by CCIE were detected within 30 min.

a positive agglutination test with antibody-coated and antibody-free staphylococci. These agglutinations were eliminated after adsorbing the sera with the Cowan 1 strain. The sera from the other two patients did not agglutinate.

In a similar manner, unconcentrated urine samples from patients with *H. influenzae* type b septicemia were tested (Table 3). Only 17 of 101 specimens gave a positive reaction by the CCIE test, whereas 64 were positive by coagglutination. Control urine specimens from 15 patients (12 sterile urine specimens, 2 urine specimens from patients with an *E. coli* urinary tract infection, and 1 urine specimen from a patient with a *K. pneumoniae* infection) were also tested. All were negative by the CCIE and coagglutination tests.

H. influenzae type b antigen could not be detected by either method in CSF, serum, or urine specimens beyond 10 days of onset of illness.

DISCUSSION

The data presented in this report indicate the usefulness of the staphylococcal coagglutination test in the identification of the various *H. influenzae* serotypes. Identification of pneumococci (11), gonococci (3), different groups of beta-hemolytic streptococci (6), and *Salmonella* and *Shigella* (5) has been achieved by other investigators using the staphylococcal coagglutination test. These findings have provided a new and needed dimension to diagnostic bacteriology.

The most significant finding in the present report is the detection of *H. influenzae* type b soluble antigens in body fluids of patients infected with this organism. Such detection establishes a rapid diagnosis of the etiological agent and obviates the possible erratic interpretation of Gram stain preparations. More importantly, however, soluble antigens can be detected in CSF samples of patients after treatment for their *H. influenzae* meningitis. This observation should provide a useful tool in the coagglutination test for establishing the etiological agent in cases of partially treated meningitis, where bacterial cultures may be negative. Both the CCIE test (4, 7, 9, 14, 15) and the latex particle agglutination method (13) have been used for the detection of *H. influenzae* soluble antigens in body fluids. Although the latex agglutination test has been shown to be more sensitive than the CCIE test (10), several false-positive reactions have been noted with the latex test (10, 13).

In our experience, the coagglutination test was consistently more sensitive than the CCIE test. In every instance when the CCIE test was positive, the coagglutination test was also positive. There were several specimens, however, in which the antigens were detectable only by the coagglutination test. Furthermore, the coagglutination test detected smaller quantities of soluble antigens in vitro. The nature of the antigens (other than PRP) detected by the coagglutination test was not determined in the present studies but is under investigation. It has been suggested that the CCIE test measures only the PRP antigen of *H. influenzae* type b, whereas the latex agglutination test detects other antigens in addition (10). It is possible that the coagglutination test is similar to the latex test in this regard. The superiority of the coagglutination test over the CCIE test in detecting *H. influenzae* type b antigens, but the comparable effectiveness of the two tests in measuring PRP, lends credence to this possibility.

Some false-positive reactions were observed in sera. These reactions can probably be attributed to the almost universal occurrence of antibodies to staphylococci in human sera. The inclusion of an antibody-free staphylococcal suspension as a control and the adsorption of sera with false-positive reactions would delineate most of false reactions, however.

We have not enountered false-positive reactions with the coagglutination test in any of the control CSF specimens or urine. Several of the controls contained organisms other than *H. influenzae* type b, and a variety of gram-positive and gram-negative bacteria were represented. There is an obvious need to examine a larger number of body fluid specimens containing various bacteria to assess the specificity of the coagglutination test. It is well recognized that several bacterial strains contain antigens that cross-react with the PRP of *H. influenzae* type b (1). These cross-reacting antigens are unlikely to be encountered in body fluids, however, since most of the responsible bacteria are nonpathogenic (1). Also, the cross-reacting pneumococcal strains belong to the higher serotypes (9, 10), and such strains are usually not encountered in human infections.

The data presented are very encouraging as to the value of the coagglutination test in the identification of *H. influenzae* serotypes and in the detection of soluble antigens in body fluids. The applicability of this test may well be much wider in scope, and concerted effort should be made to assess its usefulness in detecting and quantitating antigens of varied natures.

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