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Indirect Sandwich Enzyme-Linked Immunosorbent Assay for Rapid Detection of *Haemophilus influenzae* Type b Infection

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We report the development and testing of an enzyme-linked immunosorbent assay with excellent sensitivity for the detection of *Haemophilus influenzae* type b (HI_b) antigen in clinical specimens from patients with HI_b meningitis. The assay, an indirect sandwich technique, uses polystyrene balls as a solid phase and an alkaline phosphatase-labeled goat anti-rabbit globulin conjugate. Specimens are incubated with polystyrene balls armed with burro anti-HI_b antiserum, and recognition antibody is visualized by addition of alkaline phosphatase-labeled anti-globulin, together with the enzyme substrate *p*-nitrophenyl phosphate. Concentrations of antigen are determined from standard curves prepared by using purified HI_b capsular antigen polyribophosphate. The assay reproducibly detects polyribophosphate at concentrations between 1 and 5 ng/ml. Cross-reactions have not as yet been encountered in simulated and authentic clinical specimens containing other species including *Escherichia coli*, *Klebsiella pneumoniae*, group B *Streptococcus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria meningitidis*, and *Listeria monocytogenes*. In preliminary tests with 11 spinal fluid specimens, 2 serum specimens, and 5 urine specimens from patients with culture-proved HI_b meningitis, antigen was detected in all specimens in concentrations ranging from 1 to 7,000 ng/ml. Antigen was not detected in any of 62 clinical specimens which were culture negative for HI_b, including 11 spinal fluid specimens from patients with bacterial meningitis caused by microorganisms other than HI_b. The enzyme-linked immunosorbent assay technique described here is considerably simpler than radioimmunoassay and, based on concurrent tests with 14 positive clinical specimens, may be more sensitive than counterimmunoelectrophoresis. It seems, therefore, to hold considerable promise for clinical use in rapid detection of systemic HI_b infections.

There has long been a need for reliable noncultural techniques for the rapid diagnosis of bacterial infections. A variety of different approaches have been investigated, but to date none has been both sufficiently sensitive and technically simple enough to receive wide acceptance in clinical laboratories.

Several immunologically based techniques have shown promise for detection of bacterial antigen, most prominently, counterimmunoelectrophoresis (CIE), radioimmunoassay, and latex particle agglutination. CIE has been used mainly to detect antigens of the three major agents of bacterial meningitis, *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* type b (HI_b) (6, 12). In the largest recent study with CIE, which included 3,674 spinal fluid specimens (5), only 57% of the culture-positive specimens were positive by CIE; moreover, since antisera for CIE must be used nearly undiluted, false-positive reactions of culture-negative specimens and cross-reactions with heterologous organisms have also posed problems.

Radioimmunoassay has been shown to be a highly sensitive technique for detection of a variety of antigens and antibodies. Käyhty et al. (10) were able to detect 0.5 ng of HI_b polysaccharide by radioimmunoassay. The technique appears sufficiently sensitive and rapid to be clinically applicable, but the substantial technical skill required, the costly equipment and reagents (which have relatively short half-lives), and the potential for radiation exposure have thus far limited its use in clinical microbiology.

Two techniques simpler than either CIE or radioimmunoassay, which use protein A-treated antisera or latex particles coated with antisera, have also been used to detect HI_b antigen in

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body fluids (14, 18). In a recent study, using latex particle agglutination for the detection of HI_b antigen (18), 18% of serum specimens from children without HI_b infection showed false-positive reactions.

The first applications of enzyme-linked immunosorbent assays (ELISA) were published independently by Engvall and Perlmann (9) and Van Weeman and Schuurs (15) in 1971. With this technique, enzyme labels are covalently coupled to either antigens or antibodies, most often by using glutaraldehyde as described by Avrameas (1). There have been a large number of reports of applications of ELISA, primarily for virological diagnosis (8, 13, 16). As yet, relatively little work has been done on using ELISA to detect bacterial antigens in clinical specimens.

HI_b is the single most important cause of bacterial meningitis in the young and is a major cause of otitis media, epiglottitis, cellulitis of infants, bronchitis, and pneumonia. We undertook this study to determine the suitability of ELISA for rapid and reliable detection of HI_b antigen, and we report the successful development of a new indirect sandwich technique which is simple and reliable and may be more sensitive than CIE.

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MATERIALS AND METHODS

Solid phase for assay. One-quarter-inch (ca. 0.6-cm) polystyrene balls with specular finish were purchased from the Precision Plastic Ball Co., Chicago, Ill.

Sera. Commercial antisera used in these studies included rabbit anti-HI_b antiserum (Hyland Laboratories, Costa Mesa, Calif.) and goat anti-rabbit antiserum (Miles Laboratories, Elkhart, Ind.). Goat anti-rabbit immunoglobulin G was obtained from the latter antiserum by diethylaminoethyl-cellulose chromatography. Total protein was determined spectrophotometrically at 280 nm. Burro anti-HI_b antiserum was kindly provided by John Robbins (National Institutes of Health, Bethesda, Md.). Normal burro serum was obtained from the Department of Veterinary Medicine, University of Wisconsin, Madison, Wis. Normal rabbit serum and fetal calf serum (FCS) were purchased from Gibco Diagnostics, Grand Island, N.Y.

Antigens. Purified HI_b polyribophosphate (PRP) was also provided by John Robbins. An unrefined mixture of soluble antigens of HI_b was prepared by passing intact organisms through a French pressure cell at approximately 10,000 lb/in^2, centrifuging them at 5,500 × g for 10 min, and retaining the supernatant. This material had a total protein content of 3.1 mg/ml as determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) which is based on a dye binding method originally described by Bradford (2), and had a total carbohydrate content of 0.1 mg/ml as determined with Dreywood anthrone reagent (11). HI_b antigen could be detected at a 1:10,000 dilution, using the indirect sandwich assay.

Enzyme-linked conjugate. Goat anti-rabbit immunoglobulin G (2 mg/ml) was conjugated with alkaline phosphatase (2 mg/ml) (Sigma Chemical Co., St. Louis, Mo.), using glutaraldehyde as described by Avrameas (1). *p*-Nitrophenyl phosphate (5-mg tablets, Sigma) was used as the enzyme substrate.

Organisms. HI_b, type 3 *S. pneumoniae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and group B *Streptococcus* were obtained from the stock culture collection of the diagnostic microbiology laboratory of Madison General Hospital, Madison, Wis. *E. coli* K-100 (Easter) was obtained from John Robbins.

Reagents. Buffers used were those described by Voller et al. (17) with the exception that phosphate-buffered saline (PBS)-Tween contained 1 ml of Tween-20 per liter. The substrate, *p*-nitrophenyl phosphate, was dissolved in diethanolamine buffer, pH 9.8, to a final concentration of 1 mg/ml.

Bacteriologically complex specimens. Test organisms were added to a pooled sputum base in concentrations of between 10^6 and 10^7 colony-forming units per ml. It is noteworthy that the pooled sputum base already contained large numbers of viable organisms, including *P. aeruginosa*, various *Enterobacteriaceae*, *S. aureus*, and beta-hemolytic streptococci. Test organisms which were added to the pool included type 3 *S. pneumoniae*, *K. pneumoniae*, *P. aeruginosa*, *E. coli*, *S. aureus*, group B *Streptococcus*, and *E. coli* K-100 (Easter). Viable HI_b organisms were also added to aliquots of the pooled sputum base as a positive control.

Simulated clinical specimens. Four simulated clinical specimens were prepared from pooled human urine, serum, sputum, or spinal fluid specimens. Each pool contained materials from three patients except the spinal fluid pool, which was comprised of eight individuals' specimens. None of these patients was known to have an infectious disease, and each sputum specimen had yielded uncharacterized "oropharyngeal flora" only. Dilutions of the purified HI_b capsular antigen (PRP) were made in these pools.

True clinical specimens. Samples of spinal fluid, serum, and urine which had been submitted to the clinical laboratories of seven hospitals in Madison and Milwaukee, Wis., were studied. All specimens were processed by conventional bacteriological techniques, and all isolates were identified through species; *Haemophilus influenzae* strains were serotyped with commercial antiserum (Difco Laboratories, Detroit, Mich., or Hyland Laboratories, Costa Mesa, Calif.).

CIE. Fourteen of 18 clinical specimens from patients with HI_b meningitis and most of the spinal fluid specimens negative for HI_b in culture were also tested by CIE at the State Laboratory of Hygiene, Madison, Wis., using a standardized technique (12).

Indirect sandwich ELISA technique. (i) Preliminary processing of tested specimens. Before

testing, all clinical specimens were heated in a water bath at 56°C for 20 min to inactivate any intrinsic alkaline phosphatase which might be present (this step was found not to reduce detectably the sensitivity of the assay). Spinal fluid and serum received no further treatment. Urine specimens were first adjusted to pH 8 with 0.1 N NaOH.

Sputum was liquefied by adding an equal volume of either 2% *N*-acetyl cysteine or Sputolysin (Calbiochem, La Jolla, Calif.), blending the mixture vigorously in a Vortex mixer, and centrifuging it for 10 min at 250 × *g*; the supernatant was used for testing.

Dilutions of clinical specimens, simulated clinical specimens, purified antigens (standards), and controls were made in PBS (pH 7.4).

(ii) Polystyrene ball preparation. The sequence of steps in the assay is illustrated schematically in Fig. 1. The solid-phase polystyrene balls were first armed with burro anti-HI_b antibodies (step 1) by immersing them in the antiserum diluted 1:500 in coating buffer (pH 9.6), using screw-capped tubes (12 by 125 mm), and incubating them for 3 to 4 h at room temperature on a tissue culture roller drum. Control balls were prepared in the same manner, except that normal burro serum was used. Supernatant fluids were suctioned off with a Pasteur pipette attached to a vacuum source, after which the balls were washed three times in PBS-Tween. (This wash was done routinely after each step of the assay.) Step 2 consisted of immersing the washed balls in 10% fetal calf serum (FCS) in PBS (pH 7.4) with 0.2% sodium azide for 1 h at room temperature. (We have found this step is necessary to block unfilled protein binding sites on the balls.) The FCS was then suctioned off, and the balls were placed on gauze in a petri dish at room temperature for rapid drying. Armed balls were stored at 4°C in capped polystyrene tubes (12 by 75 mm).

(iii) Assay steps. Burro anti-HI_b-armed polystyrene balls were placed in disposable glass tubes (16 by 100 mm) to which 300 μl of the test specimen (standard, control, simulated clinical specimen, or clinical specimen) was added for antigen capture (Fig. 1, step 3). After incubation in a 40°C water bath for 45 to 60 min, the balls were again washed three times in PBS-Tween. Antigen captured was recognized in step 4 by the addition of 300 μl of rabbit anti-HI_b antiserum and incubation for 45 to 60 min at 40°C. After a further washing, identifying rabbit antibody was developed by the addition of 300 μl of alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G and incubated for

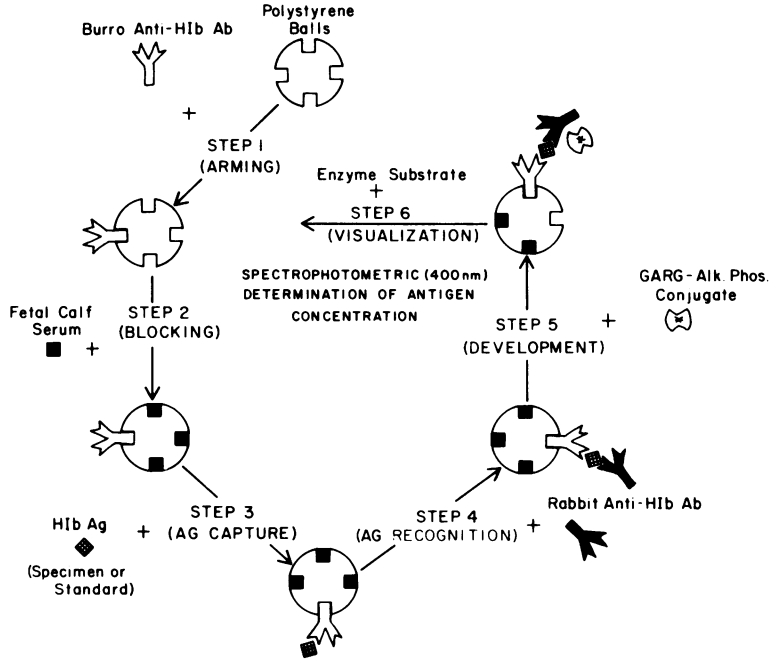

FIG. 1. *Schematic representation of ELISA indirect sandwich assay. (Step 1) Polystyrene balls are armed with burro anti-HI_b antibodies (anti-HIb Ab) by incubating both in coating buffer for 3 to 4 h at room temperature. (Step 2) Unfilled protein binding sites on the balls are blocked with 10% FCS for 1 h at room temperature. (Step 3) HI_b antigen (HIb Ag) in test specimens is captured by burro anti-HI_b antibodies adsorbed to the balls during 45 to 60 min of incubation at 40°C. (Step 4) Captured antigen is recognized by rabbit anti-HI_b antibodies during incubation for 45 to 60 min at 40°C. (Step 5) Recognition antibody is developed by the addition of goat anti-rabbit antibodies conjugated with alkaline phosphatase (GARG-Alk. Phos. conjugate). (Step 6) Alkaline phosphatase substrate (p-nitrophenyl phosphate) is added, and after 45 min incubation at 40°C, enzyme activity (which is proportional to the concentration of bound antigen) is quantitated spectrophotometrically at 400 nm.*

45 min at 40°C (step 5). After washing for a final time, the balls were transferred to clean cuvettes (10 by 75 mm) for determination of alkaline phosphatase activity (step 6). One milliliter of substrate, *p*-nitrophenyl phosphate in diethanolamine buffer (final concentration, 1 mg/ml), was added. After incubation for another 45 min at 40°C, color development was stopped by the addition of 0.1 ml of 3 N NaOH. The absorbance for each tube was read on a spectrophotometer at 400 nm; all determinations were run in duplicate. Control tests included polystyrene balls coated with normal burro serum, use of normal rabbit serum instead of anti-HI_b rabbit serum, and clinical specimens known to be positive or negative for HI_b antigen.

(iv) Interpretation. Concentrations of HI_b capsular antigen in test specimens were determined from standard curves prepared with each set of experiments. Concentrations of purified PRP of 1, 5, 10, 100, and 1,000 ng/ml were used to construct standard curves.

RESULTS

Selection of serum dilutions. A 1:500 dilution of burro anti-HI_b antiserum was used routinely for arming the polystyrene balls, based on results of earlier experiments with dilutions of antiserum ranging from 1:50 to 1:2,000 under the following trial conditions: (i) purified antigen concentrations ranging from 1 to 1,000 ng/ml; (ii) a 1:500 dilution of rabbit anti-HI_b antiserum; and (iii) a conjugate dilution of 1:200, which in turn was based on the results of titrations performed on each new lot of conjugate prepared (described below).

The 1:500 dilution of rabbit anti-HI_b antiserum was selected by a similar experimental protocol, but with the dilutions of burro antiserum held constant at 1:500. The optimum dilution of each new lot of conjugate was determined by testing dilutions ranging from 1:100 to 1:500 with purified PRP at concentrations from 1 to 1,000 ng/ml and 1:500 dilutions of both rabbit and burro anti-HI_b antisera. The conjugate dilution which showed the greatest sensitivity under the test conditions was chosen and used routinely for that particular lot of conjugate.

Selection of incubation times and temperatures. The use of room temperature (25°C) for arming polystyrene balls was based on preliminary experiments which showed no enhanced binding of antibody at higher (37°C) or lower (5°C) temperatures. The selection of the 3- to 4-h incubation period for arming the balls with antibody was based on trial results showing little differences with incubation times ranging from 3 to 4 h to overnight. With periods less than 3 to 4 h, we observed some loss of sensitivity. Moreover, Engvall and Perlmann (9) found that 20 to 30% of adsorbed protein material is leached off of polystyrene after 16 h or more. A temperature of 40°C was chosen for incubation in the remainder of the steps in the assay, based on the protocol accompanying a commercial ELISA kit for the detection of hepatitis B surface antigen (Cordis Laboratories, Inc., Miami, Fla.), which also used alkaline phosphatase-labeled antibodies, and the reported effects of temperature on the activity of alkaline phosphatase (4). Forty-five-minute incubation times at each step of the assay gave results equivalent to using 1 h. Earlier experiments showed that incubation periods significantly less than 45 min may result in some loss of sensitivity, but the optimal period of incubation requires further study.

Importance of using FCS to prevent nonspecific binding. Our early studies were complicated by nonspecific binding of conjugate to polystyrene balls after step 1, presumably at unfilled binding sites. We subsequently found that incubating balls in FCS after initially arming them effectively eliminated the problem. Table 1 shows the results of experiments done to demonstrate the efficacy of FCS as compared with bovine serum albumin and controls. Whole serum was chosen over bovine serum albumin because of its greater blocking effect. The actual component(s) responsible for blocking remain unidentified.

Standard curve for detection of PRP capsular antigen of HI_b. Figure 2 shows a typical curve obtained when PRP was diluted in PBS to final concentrations ranging from 1 to 1,000 ng/ml. With the sandwich assay, it was possible to detect reproducibly between 1 and 5 ng of PRP equivalent per ml. Concentrations of PRP in test specimens were estimated from the parallel straight-line portions of the standard curve.

Tests for cross-reactions with bacterio-

TABLE 1. *Prevention of nonspecific conjugate binding by treatment with FCS or bovine serum albumin*

Reagent added to balls[a]	Absorbance (400 nm)[b]
10% FCS	0.009–0.012
2% Bovine serum albumin	0.046–0.089
PBS-Tween	0.125–0.420
Coating buffer only (control)	0.256–0.538

[a] Polystyrene balls were exposed to coating buffer (pH 9.6) for 1 h at room temperature, after which they were exposed for an additional 1 h to either 10% FCS, 2% bovine serum albumin, or PBS-Tween or received no further exposure (control); they were then processed through steps 5 and 6 (Fig. 1) of the indirect sandwich assay.

[b] The absorbances shown are the ranges for three individual determinations. Results shown are representative of those obtained in a number of similar experiments.

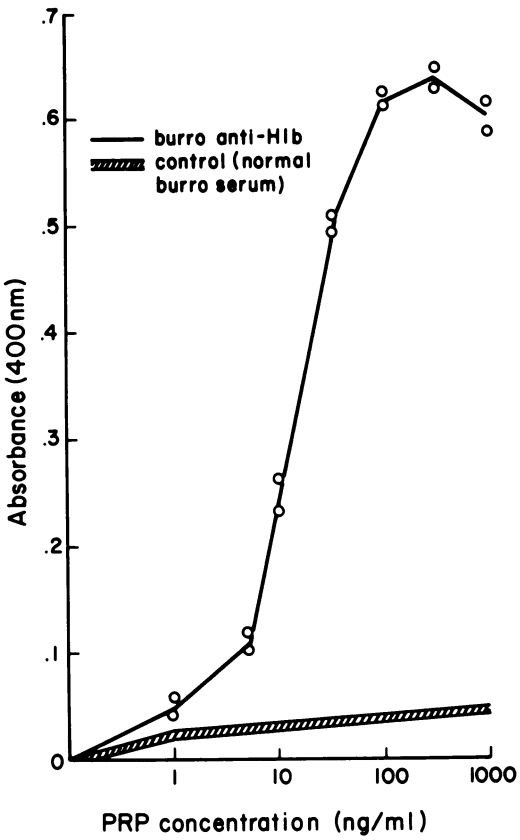

FIG. 2. *Typical standard curve prepared in PBS used for the detection of PRP in clinical specimens by the indirect sandwich ELISA assay. The standard curve was prepared by using burro anti-HI_b-armed polystyrene balls; the control was normal burro serum-armed balls.*

logically complex specimens. To determine if species commonly found in clinical specimens, some of which might possess cross-reacting antigens, would interfere with the assay, type 3 *S. pneumoniae*, *K. pneumoniae*, *P. aeruginosa*, *E. coli*, *S. aureus*, group B *Streptococcus*, and *E. coli* K-100 (Easter) were tested in a pooled sputum base. In seven complex specimens containing these species, no ELISA HI_b reactivity was detected with any of the species other than HI_b (Table 2). Absorbances of the specimens containing organisms other than HI_b in the sputum pools ranged from 0.013 to 0.053, compared with 0.705 with viable HI_b organisms and 0.693 for PRP in PBS (100 ng/ml). Assays of these organisms in pure broth culture also showed minimal reactivity (absorbances, 0.029 to 0.068).

The presence of cross-reacting antigens on *E. coli* K-100 (Easter) was confirmed by testing it along with HI_b in antiserum agar, using the method described by Counts and Turck (7).

TABLE 2. *Demonstration of specificity of the indirect sandwich ELISA assay by testing with HI_b organisms and others inoculated into a complex sputum pool*

Species or antigen added to sputum pool[a]	CFU/ml[b] or antigen concn	Absorbance (400 nm)
HI_b (positive control)	22×10^6	0.705
PRP (positive control)	100 ng/ml	0.693
S. pneumoniae type 3	10×10^6	0.053
K. pneumoniae	30×10^6	0.045
P. aeruginosa	50×10^6	0.048
E. coli	40×10^6	0.049
S. aureus	50×10^6	0.057
S. group B	30×10^6	0.039
E. coli K-100 (Easter)	22×10^7	0.013

[a] Test organisms grown overnight in liquid media were diluted to a final concentration of approximately 10^7 colony-forming units (CFU) per ml; a 0.1-ml aliquot was added to 1 ml of pooled sputum which was then used as a test specimen in the indirect sandwich ELISA assay. The sputum pool was made from three pooled sputum specimens, one of which contained large numbers of *E. coli* and *Klebsiella*, the second of which contained *P. aeruginosa*, and the third of which contained *S. aureus* and beta-hemolytic streptococci.

[b] As determined by standard plate counts, using serial dilutions of each pool after the organisms were added.

Definite halos were observed around colonies of both species. Halos were not observed around the negative controls, *S. aureus* and *P. aeruginosa*.

Testing for PRP and mixed soluble antigens in simulated clinical specimens. The four body fluid pools described in Materials and Methods were each used as diluents for purified HI_b capsular antigen, PRP. Standard curves were derived and compared with standard curves prepared with PRP dissolved in PBS. In each case, the standard curve prepared in the body fluid pool was almost superimposable on the one derived simultaneously with PBS used as the liquid phase (Fig. 3). The mixture of soluble antigens of HI_b prepared in a French pressure cell was detected as well in the sputum pool as in PBS and was calculated to contain the equivalent of 108,000 ng of PRP per ml.

Tests with authentic clinical specimens. (i) Sterile specimens. Twenty spinal fluids which were bacterial culture negative produced test absorbances in the indirect ELISA technique ranging from 0.009 to 0.028; 11 of these specimens were from patients with spinal fluid pleocytosis and, by presumption, aseptic meningitis. Fifteen urines and 15 sera submitted for routine tests gave absorbances ranging between 0.009 and 0.119. All of these specimens were tested undiluted.

(ii) Sputa. Twenty sputum specimens, among

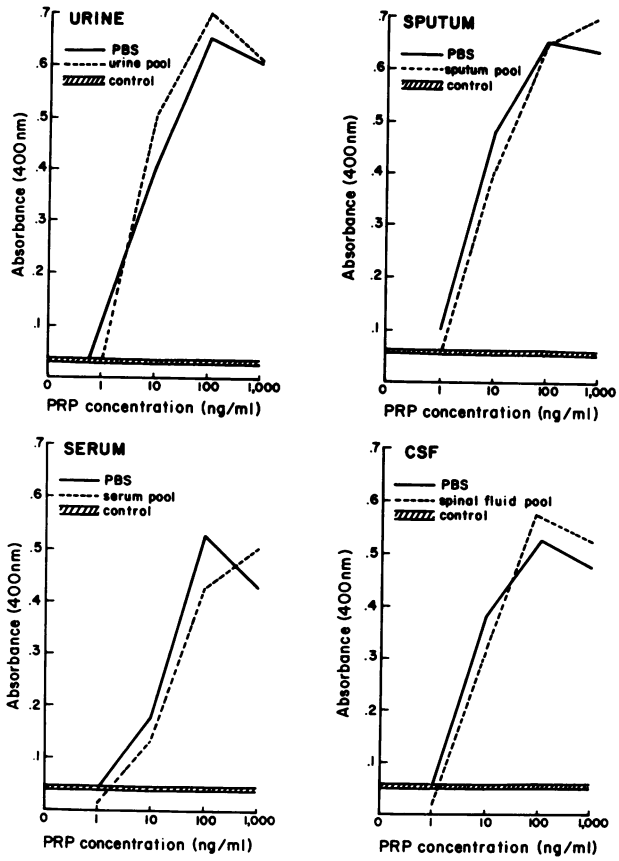

FIG. 3. *Comparison of standard curves for the indirect sandwich ELISA assay prepared with PRP dissolved in PBS and in various body fluid pools: urine, sputum, serum, and spinal fluid. Control serum results in each experiment were obtained by using normal burro serum-armed balls.*

which 2 contained *S. aureus*, 2 contained *S. pneumoniae*, 1 contained beta-hemolytic streptococci, 5 contained nontypable *H. influenzae*, and 14 contained normal oropharyngeal flora, were tested. No specimen had an absorbancy above 0.075 in a 1:2 dilution.

(iii) Spinal fluid specimens from cases of meningitis caused by bacteria other than HI_b. Eleven spinal fluid specimens positive on culture for species other than HI_b were tested by the indirect sandwich assay. The following species had been recovered from these fluids: *S. pneumoniae* from six, *E. coli* from one, group G *Streptococcus* from one, *N. meningitidis* from two, and *Listeria monocytogenes* from one. Absorbances ranged from 0.009 to 0.024, compared with 0.600 and 0.560 obtained from two spinal fluid specimens from which HI_b had been isolated run concurrently at the same 1:5 dilutions. (A 1:5 dilution was used because of the limited volumes of spinal fluid available for study with many of these specimens. However, all of the 18 specimens positive by ELISA for HI_b antigen undiluted were also positive in a 1:5 dilution).

Most of these specimens and the spinal fluid specimens from cases of aseptic meningitis were also tested for HI_b antigen by CIE at the Wisconsin State Laboratory of Hygiene; all were negative.

(iv) Spinal fluids, sera, and urines from patients with HI_b infections. A total of 18 clinical specimens (spinal fluid, 11; urine, 5; serum, 2) from 11 patients with culture-proved HI_b meningitis were obtained for testing by the indirect sandwich ELISA assay (Table 3). Fourteen of these 18 specimens were also tested by CIE; 4 could not be because of an insufficient volume of sample. Testing both the undiluted specimen and a 1:5 dilution, the sandwich assay detected HI_b antigen in all 18 specimens, even in the 1:5 dilutions, in concentrations ranging from 1 to 7,000 ng of PRP equivalent per ml. Twelve of the 14 tested specimens were also positive by CIE, but 3 were positive by CIE only after preliminary concentrations with a Minicon B-15 concentrator (Amicon Corp., Lexington, Mass).

TABLE 3. *Comparison of the indirect sandwich ELISA assay and CIE in detecting HI_b antigen in 18 clinical specimens from 11 patients with HI_b meningitis*

Specimen	Patient no.	HI_b antigen detected by:	
		ELISA (ng/ml)[a]	CIE
Spinal fluid	1	4,000	+
Serum	1	1,000	+
Urine	1	7	+[b]
Spinal fluid[c]	2	4	−
Spinal fluid[d]	2	5	−
Spinal fluid	3	3,000	+
Spinal fluid	4	4,000	+
Spinal fluid	5	7,000	+
Spinal fluid[e]	5	1	ND[f]
Urine	6	7	+[b]
Spinal fluid	7	3,000	+
Spinal fluid	8	4,000	+
Spinal fluid	9	200	+
Urine	9	30	ND
Serum	9	20	ND
Urine	10	150	+[b]
Urine[g]	10	6	ND
Spinal fluid	11	2,000	+

[a] Results are given as PRP-equivalent antigen; if more than one determination was done, the mean is given.
[b] Positive only after preliminary concentrations of the specimen.
[c] Spinal fluid obtained 2 days after the first specimen.
[d] Spinal fluid obtained 13 days after the first specimen.
[e] Spinal fluid obtained 12 days after the first specimen.
[f] ND, Not done; insufficient specimen available.
[g] Urine obtained 5 days after first specimen.

The overall results of the aforementioned tests on 80 clinical specimens for the presence of HI_b antigen by the indirect sandwich ELISA technique are shown in Table 4. With each type of specimen, the difference between the mean absorbance of specimens from patients with HI_b infection and that of patients without HI_b infection was statistically highly significant (Table 4).

Reproducibility of the assay. Aliquots of eight clinical specimens positive for HI_b antigen by ELISA were tested repeatedly on different days. The five spinal fluid specimens could only be tested twice because of limited volumes of specimen available. One positive serum specimen was tested four separate times, a culture-positive sputum was tested on two occasions, and a urine specimen was tested three different times. The results, given as equivalent quantities of PRP, showed the following: the five paired spinal fluid assays yielded 150 and 200, 2,000 and 2,500, 4,000 and 4,000, 2,000 and 2,000, and 1 and 7 ng/ml; the four tests of one serum specimen yielded 4,000 and 4,000, and 5,000 and 5,000 ng/ml; and the urine specimen yielded 30, 30 and 25 ng/ml.

TABLE 4. *Summary results with ELISA assay for HI_b antigen with 80 actual clinical specimens*

Patient group	Anatomic source (no.)	Absorbance (400 nm)[a]	
		Mean ± standard error	Range
Culture negative for HI_b (controls)	CSF—normal (10)	0.013 ± 0.001	0.009–0.015
	CSF—aseptic meningitis (9)	0.010 ± 0.001	0.006–0.017
	CSF—bacterial meningitis (11)[c]	0.025 ± 0.003	0.015–0.045
	Urine (15)	0.014 ± 0.002	0.002–0.045
	Serum (17)	0.035 ± 0.08	0.011–0.105
	Total (62)	0.022 ± 0.003	0.002–0.105
HI_b meningitis	CSF (11)	0.469 ± 0.046	0.139–0.640
	Urine (5)	0.303 ± 0.093	0.109–0.490
	Serum (2)	0.434 ± 0.169	0.159–0.606
	Total (18)	0.420 ± 0.042	0.109–0.640

[a] Comparison of each HI_b meningitis subgroup and total versus each corresponding HI_b culture-negative subgroup and total, significant at $P < 0.001$.
[b] CSF, Cerebrospinal fluid.
[c] *S. pneumoniae* (6), *N. meningitis* (2), *E. coli* (1), group B *Streptococcus* (1), *Listeria* (1).

DISCUSSION

Our results with this indirect sandwich ELISA test with clinical specimens indicate that the assay is specific and possesses sufficient sensitivity for the accurate diagnosis of HI_b meningitis and, possibly, other systemic life-threatening HI_b infections. At this time, we are using it as a qualitative test; with further refinement and testing, it promises to develop into a highly reproducible and sensitive quantitative assay. It appears that the majority of spinal fluids, serums, and urines from patients with HI_b meningitis contain between 10 and 4,000 ng of PRP-equivalent antigen per ml. Concentrations of PRP-equivalent HI_b antigen are calculated from the straight-line portions of standard curves and appear to be relatively accurate in this area.

The indirect sandwich ELISA may be more sensitive than CIE inasmuch as antigen was detected in two spinal fluid specimens from a patient with culture-proven HI_b meningitis which were negative by CIE. Antigen was also detected in five unconcentrated urine specimens from patients with meningitis, three of which were positive by CIE only after preliminary concentration. It should be possible to monitor a patient's clinical progress by following antigen levels in spinal fluid, urine, and serum. Although

we did not perform quantitative radioimmunoassay tests for comparison, the results of our ELISA test presented in Table 3 suggest that its sensitivity is comparable to that reported with radioimmunoassay (10).

Cross-reactions with other species such as *S. pneumoniae* or *N. meningitidis* thus far have not posed a problem, probably because the antisera are highly diluted. This finding is particularly significant in the case of *E. coli* K-100 (Easter), which we verified as having cross-reactive antigen(s) by the agar precipitin assay of Counts and Turck (7). This lack of cross-reactivity is in agreement with the findings of F. J. Crosson, Jr., J. A. Winkelstein, and E. R. Moxon (Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, C104, p. 294), who reported minimal cross-reactivity with similar species, using a different ELISA technique for the detection of HI_b antigen. It will be necessary, however, to test large numbers of clinical specimens containing strains known to have cross-reacting antigens with HI_b to firmly establish the assay's specificity.

One of the most attractive features of our ELISA method is that most of the reagents are available commercially and are stable for periods of 1 month or more when stored at ordinary refrigerator temperatures. We have found that antibody-coated polystyrene balls are fully active after storage for more than 1 month. Commercially available anti-HI_b antiserum prepared in rabbits against whole organisms is satisfactory without further treatment. Reagents are relatively inexpensive because they are used at high dilution. Moreover, the only equipment required is a spectrophotometer, which is available in almost all clinical laboratories.

It appears to be critically important with this indirect sandwich technique to add FCS after arming the polystyrene balls in order to prevent nonspecific binding, which otherwise substantially reduces the sensitivity and specificity of the assay (Table 1). We agree with Bullock and Walls (3) that PBS-Tween alone is sufficient for washing the solid phase between assay steps and that it is not necessary to include either FCS or bovine serum albumin in the wash solution.

The use of polystyrene balls as the solid phase may well be a key factor in our assay's specificity and sensitivity. Their diameters are extremely uniform, and the balls have contact with the bottom of the container at only one minute point. We have found the ease of manipulation of the balls together with their minimal space requirements for storage to be a distinct advantage over the use of microtiter plates.

Results with the indirect sandwich assay for the detection of HI_b antigen are available within 3.5 h. This time may be reduced even further with additional modifications of the test. The specificity, sensitivity, and technical simplicity of this technique for detection of HI_b antigen shows considerable promise for routine use in clinical laboratories.

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