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Comparison of a New, Rapid Enzyme-Linked Immunosorbent Assay with Latex Particle Agglutination for the Detection of *Haemophilus influenzae* Type b Infections

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A new, rapid enzyme-linked immunosorbent assay (ELISA) for the detection of polyribosylribitol phosphate of *Haemophilus influenzae* type b was compared with a commercially available latex particle agglutination (LPA) system (Bactigen; Wampole Laboratories, Cranbury, N.J.). By adding specimens and the anti-polyribosylribitol phosphate immunoglobulin–enzyme conjugate to the solid phase in a single step, it was possible to complete the ELISA procedure in 30 min. The ELISA was capable of detecting 0.3 ng of polyribosylribitol phosphate per ml in cerebrospinal fluid, 0.6 ng/ml in urine, and 1.2 ng/ml in serum; the in vitro sensitivity of LPA in these body fluids was 0.6, 0.3, and 0.3 ng/ml, respectively. Both procedures detected polyribosylribitol phosphate in specimens from 25 patients with bacteriologically confirmed *H. influenzae* type b infections. The specificity of ELISA appeared to be superior to that of LPA. ELISA was positive in only one of seven patients who had a positive LPA test and a clinical illness that was not compatible with haemophilus infection. Moreover, five patients with bacteriologically confirmed infections due to other pathogens (*Streptococcus pneumoniae* type 14 [two patients], *Neisseria meningitidis* group C, *Escherichia coli* K100, and *Staphylococcus aureus*) had false-positive LPA tests; only two (*E. coli* and *S. aureus*) were positive by ELISA. A total of 108 samples from 61 patients who had no evidence of haemophilus infections were negative by both procedures. The ELISA is a rapid, sensitive, and specific alternative to LPA for the detection of haemophilus polyribosylribitol phosphate.

Haemophilus influenzae type b causes a variety of serious infections in infants and young children, including bacteremia, meningitis, cellulitis, pericarditis, septic arthritis, pneumonia, empyema, and epiglottitis. Early treatment is critical, and antibiotics are often prescribed empirically because the physician cannot wait a day or longer for definitive culture results. In addition, cultures may not be helpful because many pediatric patients are already taking oral antibiotics when invasive haemophilus disease is first suspected. Moreover, in some infections, such as pneumonia in infants, culture material is difficult to obtain. Therefore, there has been considerable interest in developing rapid diagnostic tests that can detect the presence of haemophilus infection even when bacteria are no longer viable or when appropriate specimens for culture are unavailable.

Most of the tests introduced to date have relied on detection of polyribosylribitol phosphate (PRP) in body fluids such as cerebrospinal fluid (CSF), serum, and urine. Emphasis has been placed on the sensitivity of these tests, since failure to treat haemophilus infection promptly could have disastrous consequences. Thus, tests such as counterimmunoelectrophoresis, which cannot detect the low concentrations of PRP present in some invasive *H. influenzae* type b infections (1, 5, 9–11), have largely been abandoned in favor of more sensitive tests, such as latex particle agglutination (LPA). LPA can detect 1 ng or less of PRP per ml in clinical specimens and has been found to identify nearly all culture-documented haemophilus infections (3, 8–12). In addition, it is relatively inexpensive, technically straightforward, and extremely rapid.

Although sensitivity, speed, and simplicity are very important considerations in evaluating a diagnostic test, specificity is also critical. In our experience, pediatricians are very reluctant to disregard a positive test, even when observation of the patient diminishes the likelihood of haemophilus infection and cultures remain negative. A false-positive test can lead to needless hospitalization for a week or more, as well as intravenous therapy with potentially toxic agents, such as chloramphenicol.

Shortly after we began to offer LPA testing for PRP (Bactigen; Wampole Laboratories, Cranbury, N.J.) on a routine basis at Children's Hospital in 1918, pediatricians complained that some patients with positive serum or urine tests (or both) did not have clinical evidence of invasive *H. influenzae* type b disease. Careful review of our test procedures did not reveal any technical errors. In reviewing the literature, we found that most investigators believe the test to have acceptable specificity (8), but others have documented false-positivity (3). The availability of a newly developed, rapid enzyme-linked immunosorbent assay (ELISA; Seragen Diagnostics, Indianapolis, Ind.) provided us with an opportunity to confirm our LPA results with an independent test system. This comparative study also permitted us to evaluate the sensitivity of the Seragen ELISA.

MATERIALS AND METHODS

Reagents. Rabbit anti-PRP antibody was prepared at Seragen Diagnostics. Immunoglobulin G was isolated from immune rabbit serum by ammonium sulfate precipitation. Horseradish peroxidase (Worthington Diagnostics, Freehold, N.J.) was conjugated to anti-PRP immunoglobulin G by the method of Nakane and Kawaoi (6). Purified PRP was obtained from Porter Anderson, Rochester, N.Y. *ortho*-Phenylenediamine substrate tablets and diluent (phos-

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phate-citrate buffer solution) were obtained from Abbott Laboratories, North Chicago, Ill. Serum buffer (0.1 M glycine, 0.1% sodium azide, 0.05% sodium polyanethole sulfonate, 0.35% normal burro serum, 0.02 M dithiothreitol), pH 6.5, was supplied by George Siber, Boston, Mass.

Clinical specimens. CSF, serum, urine, and joint fluid were obtained from patients at Children's Hospital, Boston. Specimens from 25 patients with bacteriologically confirmed *H. influenzae* type b infections were tested by both LPA and ELISA. Samples from 61 patients who had cultures (including at least one blood culture) than did not grow *H. influenzae* were also tested by both methods. All samples for ELISA and LPA testing from these culture-negative patients were obtained within 24 h of the negative cultures. Specimens from an additional 26 patients were tested because the Bactigen LPA test was positive on at least one sample. Specimens were prepared as follows. Whole blood was allowed to clot at room temperature. Serum was separated from the cells by centrifugation. If the total volume of the serum specimen was less than 100 μl, it was routinely inactivated at 56°C for 30 min. If the volume was greater than 100 μl, heat inactivation was performed only on samples that were positive or inconclusive by LPA. Heated samples were also diluted 1:5. For the LPA test, serum buffer (10 μl) was added to a 50-μl sample of serum and preincubated at room temperature on a Yankee rotator (Clay Adams, Parsippany, N.J.) at 175 rpm for 5 min. Urine was routinely boiled for 10 min and clarified by centrifugation. Urine samples were also concentrated 10-fold with a Minicon Concentrator (Amicon Corp., Lexington, Mass.). CSF was clarified by centrifugation when necessary.

Assay procedures. The Bactigen LPA test was performed according to the manufacturer's instructions. A 10-μl sample of latex beads sensitized with equine anti-PRP antiserum was added to 50-μl samples of specimens and controls on a glass slide, and 10 μl of unsensitized beads was run in parallel as a control. The glass slide was placed on a rotator, covered, and rotated for 10 min at approximately 175 rpm. The slide was removed from the rotator, and the agglutination was read immediately while gently rocking the slide over oblique light with a black background. For the ELISA procedure, 12- by 75-mm polystyrene tubes (Clinical Plastics, Inc., Leominster, Mass.) were coated with 2.5 μg of anti-PRP immunoglobulin G in 200 μl of 0.5 M phosphate buffer (pH 8.6). This was the optimal dilution of anti-PRP antibody as determined by checkerboard titration. The tubes were allowed to stand covered at room temperature overnight and were then washed five times with 2 ml of 0.01 M Tris-gelatin buffer (pH 7.6) for 1 h at room temperature. The contents of the tubes were aspirated, and the tubes were dried and stored with dessicant in plastic bags at 4°C. Substrate solution was prepared by dissolving a tablet of *ortho*-phenylenediamine in 5 ml of phosphate-citrate buffer diluent.

The ELISA test was performed by adding 50 μl of specimen or control and 200 μl of the anti-PRP immunoglobulin G–enzyme conjugate to the coated tubes in a single step. Tubes were incubated at room temperature for 10 min. After incubation, the contents of the tubes were aspirated, and the tubes were washed five times with 2 to 3 ml of deionized water containing 0.5% Tween. After the final wash, the tubes were drained, and 200 μl of *ortho*-phenylenediamine solution was added to each tube and a blank tube. All tubes were incubated in the dark for 15 min at room temperature. The reaction was stopped by adding 1 ml of 1 M HCl, and the tubes were read at 492 nm on a Gilford Straser III spectrophotometer. A sample was considered positive if the absorbance was 5 standard deviations greater than the mean absorbance of the negative controls.

In vitro sensitivity of ELISA and LPA. To compare the in vitro sensitivity of LPA and ELISA, purified PRP (5 ng/ml) was serially diluted in pooled normal serum, urine, and CSF. Assays were performed in duplicate.

Clinical assessment. Patients with positive LPA tests were grouped into three categories on the basis of a review of medical records and bacteriology reports conducted by a pediatric infectious diseases specialist who was not involved in this study. Patients who had *H. influenzae* type b isolated from blood, CSF, or other normally sterile body sites were classified as definite cases. Children who had a febrile illness that was compatible clinically with *H. influenzae* type b infection were considered probable cases. Children who had a febrile illness, but had a clinical presentation that was not consistent with invasive *H. influenzae* infections, were classified as doubtful cases. A positive LPA test in a patient from whom another etiological agent was recovered was considered false-positive.

RESULTS

Comparison of LPA and ELISA using pooled normal CSF, serum, and urine spiked with PRP revealed only slight differences in in vitro sensitivity. ELISA detected 0.3 ng of PRP per ml in CSF, and LPA detected 0.6 ng/ml. In urine, ELISA detected 0.6 versus 0.3 ng/ml for LPA. LPA detected less PRP than did the ELISA in serum (0.3 versus 1.2 ng/ml, respectively).

There were 25 definite, culture-documented cases of invasive *H. influenzae* type b disease, including 10 cases of meningitis, 9 cases of epiglottitis, 3 cases of cellulitis, 2 cases of pneumonia, and 1 case of septic arthritis (Table 1). In all cases, ELISA confirmed the positive results obtained with LPA. Forty-seven samples of CSF, serum, urine, and joint fluid from these 25 patients were tested by both methods. There was complete concordance of results, except for one serum sample that was positive by LPA and negative by ELISA; an unconcentrated urine sample from this patient gave positive results with both techniques. Thus, when specimens from multiple sites were included, the sensitivity of both methods was 100% in this study.

Fourteen patients were classified as probable cases of *H. influenzae* type b invasive disease; three of these patients had received partial antibiotic therapy (Table 2). In five specimens (three urines and two sera) from four patients (one with pneumonia, two with otitis, and one with sepsis), ELISA was negative, whereas LPA was positive.

There were seven patients who were categorized as doubtful cases (one case each of encephalopathy, upper respiratory tract infection, "viral syndrome," "fever of unknown origin," otitis, cellulitis, and right middle lobe pneumonia) (Table 3). In only one case did ELISA confirm the positive results obtained with LPA testing. This was a patient with cellulitis who had positive LPA and ELISA tests on both unconcentrated and concentrated urine specimens. In two cases (the viral syndrome and fever of unknown origin), both serum and urine were positive by LPA, but negative by ELISA.

The five patients with false-positive LPA tests are listed in Table 4. Two of these cases (the *Staphylococcus aureus* wound infection and the *Escherichia coli* urinary tract infection) also had a positive ELISA on at least one specimen. After phenol extraction, the *E. coli* strain agglutinated the Bactigen haemophilus latex, suggesting that the strain was

E. coli K100. All specimens from the other three patients were negative by ELISA. LPA and ELISA were both negative in 108 specimens (14 CSFs, 54 sera, and 40 urines) from 61 patients who did not have *H. influenzae* type b infections. Nine of the patients had blood cultures that grew other bacterial pathogens, including pneumococcus types 14 (two cases), 12, 33, 19, and nontypable, group A streptococcus, *S. aureus*, and nontypable *H. influenzae*. Thus, the specificity of LPA was 92.4%, and the specificity of ELISA was 97% (or 83.6 and 95.9%, respectively, when doubtful cases are included).

DISCUSSION

LPA tests for the detection of *H. influenzae* type b PRP have achieved considerable commercial success because of their sensitivity, rapidity, reagent stability, and reasonable cost. LPA testing is also relatively simple to perform, although the technologist's workload has been increased slightly by the buffering and heat inactivation or filtration steps required to reduce the rate of inconclusive results with serum and urine. The only significant problem that has been encountered with LPA is an apparent lack of specificity in serum and urine testing. Daum et al. (3) identified five persons with positive Bactigen LPA tests among 170 patients in whom *H. influenzae* type b disease was considered doubtful. In addition, two definite false-positive tests were encountered in patients with *E. coli* K100 and meningococcal infections. It has also been the impression of the pediatricians at Children's Hospital that the Bactigen LPA test occasionally yields positive results in patients who are unlikely to have *H. influenzae* type b infection. This clinical assessment is supported by the results of our study. Five patients who had infections caused by pathogens other than *H. influenzae* had positive LPA tests for PRP. In three cases, the positive results obtained with the Bactigen LPA could not be confirmed with the Seragen ELISA. These three infections were caused by *Streptococcus pneumoniae* type 14 (two cases) and *Neisseria meningitidis* group C; neither of these bacteria is known to express antigens that are serologically related to PRP. In two infections, clinical specimens were positive by both LPA and ELISA. One of these infections was caused by *E. coli* K100, a capsular serotype known to cross-react with PRP. The other was caused by *S. aureus*. Some staphylococci, such as *S. aureus* variant

TABLE 1. Results of LPA and ELISA in 25 definite cases of *H. influenzae* type b infection[a]

Diagnosis (no. of cases)	LPA				ELISA			
	CSF	Serum	Urine[b]		CSF	Serum	Urine	
			1×	10×			1×	10×
Meningitis (10)	+	+	+, +	+, +	+	+	+, +	+, +
	+	+		NT	+	+	+	NT
		+	+	NT		+	+	NT
	+				+			
	+	+	+	NT	+	+	+	NT
		+	+	NT		+	+	NT
	+	+			+	+		
			+	NT			+	NT
	+	+	+	NT	+	+	+	NT
	+				+			
Epiglottitis (9)		+	+	+		+	+	+
		+				+		
		+	+	+		+	+	+
		+	+	+		+	+	+
		+	+	NT		+	+	NT
			+	NT			+	NT
		+	+	NT		+	+	NT
			+	NT			+	NT
		+	+	NT		+	+	NT
Cellulitis (3)			+	NT			+	NT
		+	+	NT		+	+	NT
		+				+		
Septic arthritis (1)		+[c]	+	NT		+[c]	+	NT
Pneumonia (2)		+	+	NT		+	+	NT
		+	+	NT		−	+	NT

[a] Each + or − indicates the results on testing a single clinical sample; two urine specimens were sent in one case. NT, Not tested.
[b] For urine testing 1× and 10× indicate unconcentrated and 10-fold concentrated samples, respectively.
[c] Joint fluid sample.

TABLE 2. Results of LPA and ELISA in 14 probable cases of *H. influenzae* type b infection[a]

Diagnosis (no. of cases)	LPA				ELISA			
	CSF	Serum	Urine		CSF	Serum	Urine	
			1×	10×			1×	10×
Meningitis (4)			+	+			+	+
		+				+		
		+				+		
			+	+			+	+
Partially treated meningitis (1)			−	+			NT	+
Cellulitis (2)			−	+			NT	+
			+	+			+	+
Pneumonia and otitis (1)[b]		+, +				+, +		
Pneumonia (2)			+	NT			+	NT
			+	NT			−	NT
Bronchitis and otitis (1)			+	NT			+	NT
Otitis (2)		+				−		
			+	NT			−	NT
Sepsis (1)		+	+	NT		−	−	NT

[a] See footnotes *a* and *b* of Table 1.
[b] Two serum samples were sent.

TABLE 3. Results of LPA and ELISA in seven doubtful cases of *H. influenzae* type b infection[a]

Diagnosis	LPA			ELISA		
	Serum	Urine		Serum	Urine	
		1×	10×		1×	10×
Encephalopathy		+	+		−	−
Upper respiratory infection		+	NT		−	NT
Viral syndrome	+	+	+	−	−	−
Fever of unknown origin	+	+	NT	−	−	NT
Otitis		+	NT		−	NT
Cellulitis		+	+		+	+
Pneumonia		+	NT		−	NT

[a] See footnotes *a* and *b* of Table 1.

TABLE 4. False-positive results with LPA and ELISA in five patients[a]

Infection (no. of cases)	LPA			ELISA		
	Serum	Urine		Serum	Urine	
		1×	10×		1×	10×
S. pneumoniae type 14 bacteremia (2)	+, +	+	NT	–, –	–	NT
	+			–		
N. meningitidis group C bacteremia (1)	+			–		
S. aureus wound infection (1)		+	+		+	+
E. coli urinary tract infection (1)	+	+, +	+, NT	–	+, +	+, NT

[a] See footnotes *a* and *b* of Table 1. Two serum samples were sent for one patient, and two urine samples were sent for another.

Copenhagen, have a cell wall component that cross-reacts with PRP, but the strain recovered from the Children's Hospital patient was unavailable for further testing.

Examination of the results of LPA and ELISA testing in the seven doubtful cases in our study raises additional concerns about the specificity of the Bactigen test. In six of these patients, urine or serum specimens (or both) were positive by LPA, but negative by ELISA. It is possible that these patients had unrecognized haemophilus infections. Alternatively, PRP may have reached the circulation as a result of undetected *H. influenzae* type b colonization of the nasopharynx. Neither of these possibilities seems likely. The in vitro sensitivity of ELISA was only very slightly less than that of LPA in serum and urine spiked with known concentrations of PRP. However, ELISA detected all 25 confirmed cases of *H. influenzae* type b infection. Although four probable cases of haemophilus infection (two cases of otitis, one case of pneumonia, and one case of sepsis) were missed by ELISA, the classification of these patients was somewhat arbitrary in the absence of cultures growing *H. influenzae*. In retrospect, the data from these so-called probable cases may be a further reflection of the non-specificity of LPA, rather than an isolated indication of ELISA insensitivity.

After this study was completed, the manufacturer made several changes in the Bactigen procedure, including a new specimen buffer and instructions to filter unconcentrated urine samples. Our experience with these modifications is insufficient to determine whether they have improved the specificity of the test.

Previous investigators have noted the potential sensitivity and specificity of ELISA systems for the detection of haemophilus PRP (2, 4, 7, 9, 13). In addition, ELISA has theoretical advantages over LPA, including nonsubjective endpoints. However, the ELISA technology has not been embraced by clinical bacteriology laboratories because most previously described procedures take at least several hours to perform and involve multiple washings and incubations.

In an effort to improve the performance characteristics of ELISA PRP detection systems, Yolken and Leister (14) studied the effect of reducing the incubation time to 10 min. Although sensitivity was reduced 10-fold, the ELISA could still detect approximately 1 ng of PRP per ml, which is comparable to the sensitivity of LPA. Yolken and Leister also found that there was not a further loss of sensitivity when the specimen containing PRP and the anti-PRP immunoglobulin G–enzyme conjugate were added to the sensitized solid phase in single step. These principles have been modified for incorporation into the Seragen ELISA PRP detection system, which yields an objective endpoint in only 30 min. If the Seragen ELISA can be adapted to a microtiter system or another solid-phase system suitable for testing multiple specimens at one time, it should provide a rapid, sensitive, and specific alternative to LPA.

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