

Preparation of Urine Samples for Use in Commercial Latex Agglutination Tests for Bacterial Antigens

GEOFFREY A. WEINBERG AND GREGORY A. STORCH*

Department of Pediatrics, Washington University School of Medicine, and Division of Infectious Disease, St. Louis Children's Hospital, St. Louis, Missouri 63110

Received 26 November 1984/Accepted 20 February 1985

The use of latex agglutination (LA) tests for bacterial antigen detection in urine specimens is hindered by troublesome reactions such as nonspecific agglutination. Therefore, procedures such as boiling or membrane filtration of urine specimens are often used before LA testing. We discovered that the composition of the membrane filter used in filtration has a marked effect on the performance of an LA test used for detection of *Haemophilus influenzae* type b antigen. False-positive LA reactivity was common in urine specimens from pediatric patients that were processed by membrane filtration through certain filters; furthermore, such reactivity also occurred in LA tests for antigens other than those of *H. influenzae*. A protein present in urine at low concentrations appeared to be responsible for these phenomena.

The rapid detection of bacterial antigens in body fluids by immunological tests such as latex agglutination (LA), counter-current immunoelectrophoresis (CIE), and staphylococcal coagglutination has been a valuable addition to clinical medicine (2, 3, 7). Although CIE was the first of these techniques to be widely used, LA is increasingly replacing CIE because of its simplicity, greater sensitivity, and ability to detect antigen earlier in the course of infection with organisms such as *Haemophilus influenzae* type b (2, 5, 6). Because bacterial antigens may be excreted in the urine, antigen detection tests are frequently performed on urine specimens, particularly those from patients with infections other than meningitis (1, 3).

Although detection of antigenuria by LA has theoretical appeal, troublesome reactions such as nonspecific agglutination of latex particles have limited the utility of the technique. Procedures used to improve the quality of urine as a specimen for testing include centrifugation, boiling, and membrane filtration of the urine. We recently discovered that false-positive reactivity was common in an LA test used to detect *H. influenzae* type b antigen in urine when the urine was prepared by filtration through certain membrane filters. Other membrane filter materials successfully eliminated the false-positive reactivity. The urinary substance responsible for the false-positive reactivity appeared to be a protein.

(This study was presented in part previously [G. A. Weinberg and G. A. Storch, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 24th, Washington, D.C., abstr. no. 114, 1984].)

MATERIALS AND METHODS

Prevalence survey. Consecutive urine samples submitted to the St. Louis Children's Hospital Microbiology laboratory for bacterial antigen testing were tested for *H. influenzae* type b antigen by using a commercial LA test kit (Bactigen; Wampole Laboratories, Cranbury, N.J.). All specimens were preserved by addition of sodium azide to a final concentration of 0.1% (wt/vol) and were stored at 4°C (maximum storage before testing, 72 h). Before testing, samples were split; one aliquot was processed by the standard laboratory protocol (boiling) and the other by the modified protocol

(membrane filtration) as described below. None of the patients from whom specimens were taken had blood or cerebrospinal fluid cultures positive for *H. influenzae* type b or *Neisseria meningitidis*; one patient had blood cultures positive for *Streptococcus pneumoniae*, although CIE of this urine was negative.

Sample processing. In the standard protocol, urine was prepared for LA testing by heating in a boiling water bath for 5 min. In the modified protocol, urine was passed through a 0.45- μ m-pore-size acrylic copolymer-nylon support membrane filter unit (Gelman Acrodisc 4218, Gelman Sciences, Inc., Ann Arbor, Mich.) attached to a 12-cc syringe. The heated or filtered urine was then centrifuged at 12,000 \times g for 60 s, and the supernatant was used for testing.

LA testing: Bactigen *H. influenzae* type b, *S. pneumoniae*, and *N. meningitidis* groups A, B, C, and Y. After the sample processing, urine specimens were tested according to instructions provided in the package insert. For each antigen tested, 50 μ l of urine was mixed on a serological slide with 10 μ l of a suspension of latex particles sensitized with antibody directed against the test antigen. The same quantity of urine was also mixed with 10 μ l of a suspension of latex particles coated with nonspecific antibody from the same animal species that was the source of the test latex suspension (negative control latex). In the *S. pneumoniae* and *N. meningitidis* tests, 10 μ l of specimen buffer supplied in the kit was preincubated with the urine sample according to the instructions of the manufacturer. In the *N. meningitidis* test, the procedure also included testing of processed urine diluted 1:11 with a diluent supplied in the kit.

The slide was rotated at 160 rpm within a humidified chamber at ambient temperature and examined for agglutination after 10 min. Agglutination was graded 4+ when the agglutination mixture cleared and large clumps of latex particles were observed, 3+ when the mixture was slightly cloudy but large clumps were observed, 2+ when moderate clumps were readily observed against a cloudy background, 1+ when small but definite clumps were observed after closer inspection, \pm when a fine granular background was seen, and negative when the mixture remained cloudy without agglutination. When there was agglutination in the negative control latex, the test was interpreted as showing nonspecific agglutination. Specimens showing a 1+ or greater

* Corresponding author.

TABLE 1. Results of LA testing for multiple bacterial antigens performed on urine specimens from subjects without evidence of systemic bacterial infection^a

Specimen no.	<i>H. influenzae</i> type b by:		<i>S. pneumoniae</i> by:		<i>N. meningitidis</i> serogroups by:					
	Bactigen	Directigen	Bactigen	Directigen	Bactigen				Directigen	
					A	B	C	Y	A	C
1	2+	-	-	-	1+	1+	-	-	-	-
2 ^b	3+	-	-	-	-	-	-	-	-	-
3	3+	-	-	1+	-	-	-	-	-	-
4	2+	-	2+ to 3+	-	±	1+ to 2+	-	-	-	-
5	2+	-	2+ to 3+	±	±	2+	-	-	-	-

^a All urine specimens were filtered through 0.45- μ m-pore-size acrylic copolymer filters before LA testing. All specimens were negative for all antigens when tested after boiling.

^b Patient from whom specimen 2 was obtained had blood cultures positive for *S. pneumoniae*; CIE of his urine for *S. pneumoniae* was negative.

degree of agglutination of the test latex, but not the negative control latex, were considered positive. Positive and negative antigen control specimens supplied in the kit were run daily.

A false-positive reaction was defined as one in which the urine prepared by filtration (modified protocol) but not that prepared by boiling (standard protocol) produced agglutination of the specific latex suspension but not the negative control latex suspension.

LA testing. Directigen Meningitis kit. Selected urine specimens were also tested for *H. influenzae* type b, *N. meningitidis* groups A and C, and *S. pneumoniae* antigen in urine using the Directigen Meningitis kit (Hynson, Westcott, and Dunning, Baltimore, Md.), according to instructions provided in the package insert. For each antigen tested, 50 μ l of urine was mixed on a serological slide with 15 μ l of a suspension of latex particles coated with antibody directed against the test antigen. The slide was rotated as for the Bactigen test except that the speed of rotation was 100 rpm. The Directigen kit did not include a negative control latex suspension to assess nonspecific agglutination.

Filter survey. Five urine specimens that yielded false-positive reactions in the Bactigen *H. influenzae* test were selected for further study. Samples of each of these urine specimens were processed by membrane filtration through different membrane filters. The filters used were nitrocellulose and hydrophilic polyvinylidene difluoride (Millipore Corp., Bedford, Mass.) and mixed cellulose esters, cellulose acetate, acrylic copolymer, and polysulfone (Gelman Sciences, Inc.). All filters were 25-mm-diameter disks and had a pore size of 0.45 μ m. A glass chimney and support system (Millipore) attached to a suction pump was used for filtration.

Trypsin treatment. After filtration through the acrylic copolymer filter as described above, three of the above five urine samples were treated with bovine pancreas trypsin (L-1-tosylamide-2-phenylethyl chloromethylketone treated; Sigma Chemical Co., St. Louis, Mo.) by addition of a stock solution made in 0.001 normal hydrochloric acid to a final trypsin concentration of 10 μ g/ml. The reactions were allowed to proceed for 30 min at room temperature and were stopped with an equal volume of soybean trypsin inhibitor (Sigma Chemical Co.) prepared in phosphate-buffered saline (pH 7.5).

Dialysis. One of the above five urine samples was dialyzed against phosphate-buffered saline (pH 7.5), using Spectrapor tubing (Spectrum Medical Industries Inc., Los Angeles, Calif.) with an exclusion size of 12,000 to 14,000 daltons. Dialysis was carried out over 40 h with three buffer changes, using a 1:100 ratio of specimen to buffer.

Protein assay. The protein content of urine was measured by using a modification of the method of Lowry et al. (4) after cold precipitation with 10% trichloroacetic acid. Urine protein was also estimated with urine dipsticks (Bili Labstix, Ames Co, Elkhart, Ind.).

RESULTS

Pilot studies. Pilot studies were performed to ensure that minor variations from the instructions of the manufacturer did not affect test results. These included: (i) testing nonfiltered versus acrylic copolymer-filtered urine to ensure that filtration itself did not induce reactivity, (ii) testing urine before and after addition of sodium azide, and (iii) testing urine specimens processed by centrifugation at 12,000 \times g for 60 s versus those processed at 1,000 \times g for 10 min. No difference in degree of reactivity was seen in any of these experiments (data not shown).

Frequency of false-positive tests. A total of 37 consecutively submitted urine specimens that yielded negative results when tested by the Bactigen *H. influenzae* type b test after being boiled were tested by the same test after 0.45- μ m-pore-size acrylic copolymer membrane filtration. A total of 15 (41%) were positive, with scoring as follows: 1 (3%) was 1+, 10 (27%) were 2+, 4 (11%) were 3+. A total of 19 (51%) were negative, and 3 specimens (8%) were \pm .

Other antigen detection tests. Five urine specimens that yielded false-positive results when tested with the Bactigen *H. influenzae* type b kit were also tested with other antigen detection kits for *H. influenzae* type b, *S. pneumoniae*, and *N. meningitidis* groups A, B, C, and Y antigens. Portions of each urine specimen were processed by the standard and the modified protocols before testing for each antigen (Table 1). None of the urine specimens were positive when tested with

TABLE 2. Effect of different membrane filter materials on the results of LA testing for *H. influenzae* type b

Filter material ^a	Specimen no.			
	1	2	3	6
Nitrocellulose	-	-	-	-
Mixed cellulose esters	-	-	-	-
Cellulose acetate	-	±	-	-
Acrylic copolymer	1+ to 2+	2+	2+	±
Polysulfone	2+	2+	2+	1+ to 2+
Hydrophilic polyvinylidene difluoride	1+ to 2+	2+	2+	1+ to 2+

^a Filters are arranged in rough order of nonspecific protein binding, from most (nitrocellulose) to least (hydrophilic polyvinylidene difluoride) according to information provided by the manufacturer.

any kit after being boiled. However, one or more specimens that had been processed by acrylic copolymer membrane filtration were positive when tested with the Bactigen *S. pneumoniae* and *N. meningitidis* kits as well as with the Directigen *S. pneumoniae* test. The appropriate negative control latex test was negative in all of the Bactigen tests.

Filter survey. Six different membrane filters were used to prepare four urine specimens known to yield false-positive reactions in the Bactigen *H. influenzae* type b test after acrylic copolymer filtration. False-positive reactions occurred only when the urine was filtered through filters with low-protein-binding characteristics (Table 2).

Nature of urinary substance producing false-positive reactions. The protein concentration of five urine specimens that produced false-positive reactions was estimated with urinary dipsticks; all gave negative dipstick reactions (test sensitivity; 100 to 250 mg/liter). The protein concentration of one of these urine specimens measured by the modified method of Lowry was 40 mg/liter. Dialysis of this urine specimen increased the reactivity that occurred when the specimen was tested with the Bactigen *H. influenzae* type b and *N. meningitidis* groups A and B test kits. In addition, the dialyzed specimen produced a positive reaction in the Directigen *S. pneumoniae* test that was not observed when the specimen was tested before dialysis. The same urine sample was subjected to trypsin treatment; the results of Bactigen *H. influenzae* type b testing before and after treatment were 2+ and ±, respectively. (Trypsin treatment did not alter the reactivity of positive and negative controls.) The results of trypsin treatment of the other two urine specimens were a decrease in reactivity from 2+ to ± and from 2+ to 1+.

DISCUSSION

In this study, 41% of urine specimens from hospitalized children produced false-positive reactions in a commercial LA kit for *H. influenzae* type b antigen when testing was carried out after membrane filtration of the urine through certain filter materials. The agglutination occurred only with the *H. influenzae* type b antiserum-sensitized latex and not with normal serum-sensitized latex; therefore, this reaction appeared to be a true-positive reaction rather than nonspecific agglutination, which would cause agglutination of both latex suspensions. However, we believe that the reactions were actually false-positive ones because they were eliminated by boiling the urine specimens before testing. The *H. influenzae* type b polysaccharide antigen that the reagent detects is heat stable (Bactigen product insert, April 1981); many proteins are not. Additionally, the children from whom the specimens were obtained did not have other evidence of disease caused by *H. influenzae* type b.

Testing of a small number of urine specimens revealed that the false-positive reactivity was not limited to tests for *H. influenzae* type b, but also occurred with *N. meningitidis* and *S. pneumoniae* LA kits from the same manufacturer (Wampole). One urine specimen from a patient with no evidence of *S. pneumoniae* disease reacted with the *S. pneumoniae* reagent from a different manufacturer, suggesting that the problem is not strictly manufacturer-related. Differences in test performance between kits from the two manufacturers could be explained by differences in one of several components of the kits. For example, differences in reactivities of the antisera used to coat the latex particles, reflecting differences in animal species, immunogen, or immunization schedule could explain the varying frequencies of false-positive test results.

The substance in urine samples that was responsible for the false-positive reactivity was heat labile, nondialyzable, and trypsin sensitive. Filtration of the urine specimens through membrane filters with high-protein-binding capacity eliminated the false-positive reactions; filtration with filters of low-binding capacity did not. These results suggest that the substance present in urine responsible for the false-positive agglutination was proteinaceous in nature. Since there was no evidence for pathological proteinuria in the urine samples, it is possible that a normal urinary protein present at low concentration is responsible for the false-positive reactivity, possibly as a result of immunological cross-reactivity with the bacterial antigens being sought. Alternatively, as the antisera used in the kits are polyclonal, this protein may actually be reacting with antibodies other than those directed against carbohydrate capsular antigens.

The practical implications of this work for clinical laboratories are clear. When membrane filtration is used to prepare urine specimens for LA testing, the choice of membrane filter is crucial. One or more proteins commonly found at a low concentration in human urine may react with the antisera used to prepare the latex agglutination reagents, resulting in false-positive tests that are not recognized as nonspecific agglutination because there is no agglutination of the negative control latex reagent. Proper processing of urine specimens before LA testing is essential to eliminate the false-positive reactivity. Since the role of membrane filtration appears to be removal of a protein that produces false-positive reactions, a membrane filter with high-protein-binding characteristics, such as nitrocellulose, should be used. Further studies are needed to define more completely the applicability of different membrane filter materials to LA testing for various bacterial antigens.

ACKNOWLEDGMENTS

We greatly appreciate Wampole Laboratories, Hynson, Westcott and Dunning, Millipore Corp., and Gelman Sciences, Inc., for donating materials. We also appreciate the assistance of personnel of the Serology Laboratory of St. Louis Children's Hospital.

LITERATURE CITED

1. Coonrod, J. D. 1983. Urine as an antigen reservoir for diagnosis of infectious diseases. *Am. J. Med.* 75(Suppl. 1B):85-92.
2. Daum, R. S., G. R. Siber, J. S. Kamon, and R. R. Russell. 1982. Evaluation of a commercial latex particle agglutination test for rapid diagnosis of *Haemophilus influenzae* type b infection. *Pediatrics* 69:466-471.
3. Feigin, R. D., M. Wong, P. G. Shackelford, B. W. Stechenberg, L. M. Dunkle, and S. Kaplan. 1976. Countercurrent immunoelectrophoresis of urine as well as CSF and blood for diagnosis of bacterial meningitis. *J. Pediatr.* 89:773-775.
4. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
5. Scheifele, D. W., R. S. Daum, V. P. H. Syriopoulou, G. R. Siber, and A. L. Smith. 1979. Comparison of two antigen detection techniques in a primate model of *Haemophilus influenzae* type b infection. *Infect. Immun.* 26:827-831.
6. Scheifele, D. W., J. I. Ward, and G. R. Siber. 1981. Advantage of latex agglutination over countercurrent immunoelectrophoresis in the detection of *Haemophilus influenzae* type b antigen in serum. *Pediatrics* 68:888-891.
7. Shackelford, P. G., J. Campbell, and R. D. Feigin. 1974. Countercurrent immunoelectrophoresis in the evaluation of childhood infections. *J. Pediatr.* 85:478-481.