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Evaluation of a New Tube Latex Agglutination Test for Detection of Type-Specific Pneumococcal Antigens in Urine

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A modified tube agglutination test using type-specific latex reagents for detection of pneumococcal capsular polysaccharide antigens in alkalinized, unconcentrated urine samples was evaluated in reconstituted urine samples and in groups consisting of 26 children with clinical and roentgenographic evidence of acute lower respiratory tract infection, six patients with blood culture-proven infection of nonpneumococcal etiology, and 30 healthy individuals. The sensitivity of the tube latex agglutination method for pneumococcal polysaccharides was 2 to 10 times higher than that of the slide agglutination method. Positive antigen findings were obtained for 42% of urine samples from patients with acute lower respiratory tract infection but in neither patients with nonpneumococcal septicemia nor healthy controls. Fifty-five percent of the antigen-positive patients also showed evidence of pneumococcal involvement by pneumococcal antibody assay or antigen detection in acute-phase serum.

Etiologic diagnosis of bacterial acute lower respiratory tract infection (ALRI) is difficult to establish if only isolation of infecting organisms from blood or pleural fluid is used. Blood cultures are positive in only 10 to 30% of pneumonia cases in adults (4, 8) and about 5% of those in children (2, 11, 18). Lung aspiration by needle, which has been used successfully in a number of studies in research settings, has not been adopted in routine clinical practice because of the risk of serious complications due to the invasiveness of the method. Pneumococci are considered the most common bacterial pathogen of pneumonia in both adults and children (4, 17, 18). Therefore, serological methods, including detection of pneumococcal antigens in urine and acute-phase serum (2, 11, 13–15), as well as measurement of antibody responses to pneumococcal polysaccharides or to a protein exotoxin, pneumolysin (1, 3, 4), have been developed for etiologic diagnosis of invasive pneumococcal infection.

Latex agglutination (LA) is a rapid test most commonly used for detection of bacterial polysaccharide antigens in biological fluids in both routine clinical diagnostics and research settings. The low sensitivity of the LA method in detection of pneumococcal polysaccharides in both urine and serum obtained from patients with ALRI, however, has been reported in several previous studies (4, 11, 14, 15). Various modifications of the LA test have therefore been devised to improve its sensitivity, i.e., concentration of urine samples from 10- to 100-fold (11, 13, 15), use of type-specific latex reagents together with concentrated urine samples (13), and use of a tube modification of the LA test with special instrumentation for reading (7).

The aim of our study was to develop a rapid, inexpensive, and sensitive LA test for detection of pneumococcal polysaccharide antigen in urine which could be used in large patient materials for etiologic diagnosis of ALRI, especially in less industrialized countries. We developed a tube modification of the LA test (TLA) based on the use of unconcentrated urine and studied its sensitivity and specificity with reconstituted urine samples with both known amounts of added polysaccharides and with clinical samples obtained from patients with ALRI and from controls.

MATERIALS AND METHODS

Reconstitution samples. Type-specific pneumococcal polysaccharides (Merck Sharp & Dohme, West Point, Pa.) were added to phosphate-buffered saline (PBS; pH 8.2) and pooled urine from healthy laboratory personnel. Dilutions ranging in concentration from 10 μg/ml to 1 ng/ml were prepared.

Patients and controls. The patients studied consisted of two groups: 26 children with clinical and roentgenographic findings of severe but nonbacteremic ALRI enrolled in a separate study regardless of etiologic diagnosis made with both serological and microbiological methods (16) and 6 adults with blood culture-proven nonpneumococcal infections (*Escherichia coli* and *Staphylococcus aureus*). Controls consisted of 30 healthy children and adults with or without nasopharyngeal carriage of pneumococci.

Sample processing. Voided urine samples from the three study groups were collected and kept at −20°C until tested. Blood was cultured by using standard techniques (Orion Diagnostics, Espoo, Finland). Throat swab specimens were taken from healthy controls by using Dacron-tipped swabs (Hardwood Products Co., Quilford, Maine) and cultured on crystal violet-nalidixic acid-gentamicin agar plates to isolate *Streptococcus pneumoniae* (12). The plates were incubated in a 5% CO_2 atmosphere at +36°C for 24 h. Alpha-hemolytic colonies resembling pneumococci were tested for optochin sensitivity.

Latex particle reagents. Latex particles (diameter, 0.81 μm; Difco Laboratories, Detroit, Mich.) were coated with monospecific antisera (Statens Serum Institute, Copenhagen, Denmark) to pneumococcal types and groups 1, 4, 6 to 9, 11, 14, 15, 19, and 23, which commonly cause invasive pneumococcal disease in Finnish children (unpublished data), prepared as described previously (5). The type-specific latex reagents detected a minimum concentration of purified pneumococcal polysaccharide in PBS from 1 to 25 ng/ml, depending on the polysaccharide (see Table 1).

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Concentration of urine samples. A portion of urine samples was concentrated 25-fold in miniconcentrators (Minicon, Amicon Corp., Danvers, Mass.).

TLA. Samples were boiled in a water bath for 5 min and centrifuged at 4,000 × *g* for 5 min. The supernatant was placed in a 10-ml test tube, and the pH was checked with an indicator (Neutralit; E. Merck, Darmstadt, Germany) before addition of 2 N NaOH to attain a pH of 8.0. All control urines were alkalinized. Urine samples from the 26 patients with ALRI were run both at the original pH and alkalinized. Five hundred microliters of supernatant was pipetted into cryotubes (1.8 ml; NUNC, Roskilde, Denmark). Forty microliters of type-specific latex reagent was then added, and the mixture was incubated on a mechanical rotator (CM-9; Sarstedt, Nümbrecht, Germany) at 500 rpm for 5 min at room temperature. The 5-min incubation time was chosen after a time series test (5, 10, 15, 20, and 30 min) showed similar agglutination reactions with all of the types and groups after all of the time intervals. After incubation, the mixture was centrifuged at 4,000 × *g* for 5 min. Most of the supernatant was discarded, leaving approximately 150 μl with the sediment in the tube. The contents were mixed by in-and-out pipetting and transferred onto a black plastic plate (Bactplate; Laboratories Design AB, Lidingö, Sweden). The plate was manually rocked for 2 min, after which the results were read visually. Agglutination was defined and graded as large clumps against a clear background (3+), large clumps against a slightly cloudy background (2+), small but definite clumps against a cloudy background (1+), or negative (−) in the absence of agglutination.

Positive and negative controls were tested in each run. The positive control was urine without antigen from a healthy person mixed with the respective purified pneumococcal polysaccharide at a concentration of 10 ng/ml. PBS and urine without added polysaccharide served as negative controls.

SLA. The slide agglutination test (SLA) was performed by placing 30 μl of a boiled and concentrated urine sample and 30 μl of a sensitized latex reagent suspension onto a black plastic plate and then manually rocking it for 2 min. The results were read and graded similarly as in the TLA method.

Enzyme immunoassays. Immunoglobulin G class antibodies to pneumococcal pneumolysin were measured by enzyme immunoassay in paired sera of 26 children with ALRI. An at least twofold increase in antibody titer was considered indicative of pneumococcal involvement in acute infection, on the basis of studies done on infected and healthy adults (3) and 158 healthy children in different age groups with paired sera (6). Pneumococcal antigen in acute-phase serum was detected by using a commercial latex particle agglutination test (Bactigen *S. pneumoniae*; Wampole Laboratories, Division of Carter-Wallace Inc., Cranbury, N.J.).

RESULTS

Results of detection of pneumococcal capsular polysaccharide in the reconstituted samples by both the SLA and TLA methods are shown in Table 1. Samples containing known amounts of purified pneumococcal polysaccharide were tested by using both SLA and TLA. Both concentrated and unconcentrated samples were tested simultaneously. With the TLA method, both concentrated and unconcentrated urine samples showed 1+ agglutination at 1 ng/ml with all types and groups, except for type 23, which gave a 3+ reaction. At a concentration of 100 μg/ml, all types and groups gave negative reactions. With the SLA method, the lowest concentration at which the capsular polysaccharide was detected in PBS and concentrated pooled urine varied. Unconcentrated urine was not tested with the SLA method, since inadequate amounts of samples were available. The detection threshold with the TLA method was two to three times lower than with the SLA method.

TABLE 1. Comparison of the TLA and SLA methods for detection of pneumococcal capsular polysaccharide in reconstituted samples

Medium	Method	Detection limit of capsular polysaccharide (ng/ml)[a] of type or group:						
		1	4	8	19	6, 7, 9, 11	14	23
PBS	SLA	50	25	25	25	10	1	1
	TLA	25	10	10	1	1	1	1
Concentrated urine	SLA	10	10	25	10	10	1	1
	TLA	1	1	1	1	1	1	1
Unconcentrated urine[b]	TLA	1	1	1	1	1	1	1

[a] Concentrations tested: 10 μg/ml and 100, 50, 25, 10, and 1 ng/ml.
[b] Unconcentrated urine was not tested with the SLA method because of insufficient sample volume.

The results obtained with clinical samples by the TLA method are shown in Table 2. Of the 26 urine samples from patients with ALRI, 2 (8%) were positive for pneumococcal antigen without alkalinization of urine with the TLA method. The positive yield increased to 11 (42%) when the samples were alkalinized, and they remained positive after reboiling for 5 min. Three nonalkalinized samples from the healthy group showed agglutination for several types or groups (three or more), but these reactions disappeared when the leftover urine was alkalinized, reboiled for an additional 5 min, and retested by TLA. Reactions for more than one serotype were not observed in the ALRI study group. *S. pneumoniae* was isolated in 1 of 30 throat samples of healthy controls, and the alkalinized urine sample from the same person gave a negative reaction.

In the 11 ALRI cases, the pneumococcal antigens detected were of type or group 6 (two patients), 14 (one patient), 15 (four patients), 19 (three patients), and 23 (one patient).

Six (55%) of the patients with antigenuria had other evidence of *S. pneumoniae* involvement. Three had a significant antibody response to pneumolysin by enzyme immunoassay in paired sera, while in the other three, pneumococ-

TABLE 2. Detection of pneumococcal antigen in urine by the modified TLA method

Study group	Urine alkalinized	No. tested	No. (%) positive
Children with ALRI	No	26	2 (8)
	Yes	26	11 (42)[a]
Patients with positive blood cultures[b]	Yes	6	0
Healthy children and adults	Yes	30	0[c]

[a] The positive reaction remained after reboiling.
[b] *E. coli*, $n = 5$; *S. aureus*, $n = 1$.
[c] Three samples were initially positive for several types or groups, but agglutination disappeared after reboiling.

cal antigen was detected in the acute-phase sera by a commercial latex particle agglutination test.

DISCUSSION

Several techniques for the diagnosis of pneumococcal infection have been described, including various methods for detection of pneumococcal polysaccharides in body fluids by latex agglutination, coagglutination, and counterimmunoelectrophoresis. The range of urinary antigen levels in infected individuals is extremely wide, depending on the time since the onset of infection, the severity of infection, and the amount of antigen-binding antibody the individual is able to produce. Excretion ranges of *Haemophilus influenzae* type b have been documented by Murphy et al. (10) in both infected individuals and healthy carriers, with an overlap in the range from <1 to 20 ng/liter. Healthy children carrying *S. pneumoniae*, however, seldom have antigenuria of >2.5 ng/liter, as shown by O'Neill et al. (13).

The TLA method we evaluated was sensitive in detecting pneumococcal capsular polysaccharide antigen in alkalinized, unconcentrated urine. An important finding was the improved sensitivity of the test when alkalinization of urine was used. This finding is in agreement with our earlier observations on the different factors of urine that affect both the sensitivity and the specificity of the SLA test, such as pH, leukocyte count, and bacterial growth. A pH above 7 provided an optimal milieu for the test (unpublished data). In our present study, most of the samples initially had a low pH. Adjusting the pH closer to that of the latex reagent (pH 8.0) possibly resulted in a more stable assay milieu. This, together with continuous mixing during incubation, may have enhanced the antigen-antibody binding reaction.

Urine samples from patients with sepsis of nonpneumococcal etiology gave no positive results. Agglutination in several types and groups was seen in 5% of the samples from healthy controls, but the reactions were not seen after testing following reboiling. Nonspecific agglutination seen simultaneously with several pneumococcal polysaccharide types and groups may have been caused by agglutinating substances in urine other than bacterial capsular polysaccharides.

The positive antigen findings obtained with other serological methods support our observation that the positive reactions obtained with the TLA method were valid findings. In the remaining five cases, several reasons may explain why no other supporting evidence for pneumococcal involvement was found. The initial concentration of antigen circulating in the serum or the amount of breakdown product in the urine may be below the detection limit. Possible sequestration of antibodies and antigen in circulating immune complexes may further lessen the amount of circulating antigen (9). In all positive cases, there was clinical and roentgenographic evidence of pulmonary infection.

Urine samples are usually concentrated to achieve better sensitivity of polysaccharide antigen detection. Commercial concentrators are disposable and expensive instruments. The procedure also increases the work load and the time needed to assay samples. In addition, a large volume of concentrated urine is required to test several types or groups if the type-specific SLA method is used. The TLA method avoids the costly concentration step. It requires only a small volume of urine for each serotype; with a 5-ml sample, 10 of the most common pneumococcal serotypes can be tested. With the TLA method, both unconcentrated and concentrated urine samples gave similar agglutination reactions.

Our findings are in line with a recent study of Gambian children which reported that the latex particle agglutination test was a sensitive method for identification of pneumococcal pneumonia when type-specific latex reagents were used (13).

A wide range of clinical specimens will be needed for the final evaluation of the reliability of the TLA method and its correlation with other immunological assays. In view of the small sample volume needed and the short time required for detection, this method seems useful for both clinical routine diagnostics and epidemiologic studies.

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