Indirect Sandwich Enzyme-Linked Immunosorbent Assay for Rapid Detection of *Streptococcus pneumoniae* Type 3 Antigen

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An indirect sandwich enzyme-linked immunosorbent assay for the detection of the polysaccharide antigen of type 3 pneumococcus (SSS-III) is reported. Polystyrene balls with attached anti-SSS-III antibody serve as the solid phase, and antigen is detected using an alkaline phosphatase-labeled antiglobulin conjugate. The reaction is quantitated by spectrophotometry. Concentrations of antigen are estimated by comparison with standard curves prepared with purified SSS-III. For this assay, the practical lower limit of detection of SSS-III is approximately 2 to 3 ng/ml, thus making the test sensitivity about 25 times that reported for counterimmunoelectrophoresis. In preliminary tests with simulated human clinical specimens, none of the body fluids tested (spinal fluid, serum, urine, and sputum) interfered with detection of antigen, nor did they produce false-positive or false-negative results. *Streptococcus pneumoniae* type 3 whole organisms were readily detected in simulated clinically positive sputum specimens. Cross-reactions were not observed with *Haemophilius influenzae* type b, group B *Streptococcus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.

There are an estimated 200,000 to 1,000,000 cases of pneumococcal pneumonia in the United States annually, resulting in 13,200 to 66,000 deaths (1). Diagnosis depends upon the use of Gram-stained smears, which is rapid, or the more time-consuming culture of sputum; unfortunately, the results of either test are often not adequate for diagnosis. Bartlett (3) estimates that more than one-third of the sputum specimens submitted to clinical laboratories are unsuitable for diagnostic purposes because of contamination by oropharyngeal microflora. Development of a rapid and reliable immunological technique for detecting pneumococcal antigen could help obviate the shortcomings of conventional methods in early diagnosis of pneumococcal pneumonia. A rapid indirect sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of antigen in clinical specimens taken from patients with serious infections caused by *Haemophilius influenzae* type b has recently been reported from our laboratory (7). The detection of antibody to pneumococcus by an ELISA technique has been reported (5), but there are no reports of ELISA tests designed to detect pneumococcal antigen in clinical specimens of hospitalized patients with possible serious pneumococcal infections. Such a system would permit diagnosis before antibody is evident. We report here the development of an indirect sandwich ELISA assay that is capable of detecting low levels of the polysaccharide antigen of *Streptococcus pneumoniae* type 3 in clinical samples of human spinal fluid, urine, serum, and sputum. This assay appears promising for a rapid and accurate method of diagnosing serious pneumococcal infections.

**MATERIALS AND METHODS**

**Solid phase for assay.** Polystyrene balls (0.25 in., ca. 0.6 cm) with specular finish were purchased from the Precision Plastic Ball Co., Chicago, Ill.

**Sera.** Commercial antisera and normal sera used in these studies were as follows (source): rabbit anti-pneumococcus serum pool B (Difco Laboratories, Detroit, Mich.); normal rabbit serum (GIBCO Diagnostics, Grand Island, N.Y.); goat anti-rabbit globulin (Miles Laboratories, Elkhart, Ind.); normal horse serum and fetal calf serum (GIBCO). Horse anti-type 3 pneumococcus serum was kindly provided by Philip Baker (National Institutes of Health, Bethesda, Md.).

**Antigen.** Purified pneumococcus type 3 polysaccharide (SSS-III) was also provided by Philip Baker.

**Enzyme-linked globulin conjugate.** The immunoglobulin G fraction of goat anti-rabbit globulin (2 mg/ml), obtained by diethylaminomethyl-cellulose chromatography, was conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.), 2 mg/ml, using glutaraldehyde as described by Avrameas (2). *para*-Nitrophenyl phosphate (5-mg tablets, Sigma) was employed as the enzyme substrate.

**Reagents.** Buffers used were essentially those described by Volier et al. (11). The phosphate-buffered saline-Tween was modified by addition of 1 ml of
phenyl phosphate, 0.1 ml of 1 mg/ml of alkaline phosphatase activity (step 6). The sputum was dissolved in diethanolamine buffer (pH 9.8) to a final concentration of 1 mg/ml. The sputum-liquifying agent (Sputolysin) was obtained from Calbiochem, La Jolla, Calif.

**Performance of the indirect sandwich ELISA.** The steps in the assay, similar to those described previously (7), are illustrated schematically in Fig. 1. The polystyrene balls (step 1) were armed with horse anti-type 3 pneumococcus antiserum by immersion in a 1:500 dilution of the antiserum and incubation for 3 to 4 h at room temperature on a tissue culture roller drum. Blockage of unfilled binding sites on balls (step 2) was accomplished by immersion of the washed balls in 10% fetal calf serum. Antigen capture (step 3) was achieved by exposure of the armed, blocked balls to 300 μl of a given specimen (standard, control, or clinical test) in glass test tubes, followed by incubation for 45 min at 40°C. Captured antigen was recognized (step 4) by addition of 300 μl of rabbit anti-type 3 pneumococcus serum and incubation for 45 min at 40°C. Development of the recognition antibody (step 5) was accomplished by addition of 300 μl of alkaline phosphatase-labeled goat anti-rabbit globulin and incubation for 45 min at 40°C. After a final washing, the balls were transferred to clean cuvettes (10 by 75 mm) for visualization of alkaline phosphatase activity (step 6). In this final step, 1 ml of substrate (p-nitrophenyl phosphate in diethanolamine buffer) was added, followed by incubation for 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.

**Quantitation of antigen.** Standard curves were generated utilizing purified SSS-III at concentrations of 0, 1, 5, 10, 100, and 1,000 ng/ml. Concentrations of SSS-III in test specimens were determined by comparison with the standard curve.

**Organisms.** *S. pneumoniae* type 3, *H. influenzae* type b, group B *Streptococcus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were obtained from the stock culture collection of the diagnostic microbiology laboratory of Madison General Hospital, Madison, Wis.

**Simulated clinical specimens.** The four types of simulated human clinical specimens tested were urine, sputum, and spinal fluid. Each type of specimen was composed of materials from three patients, except for the spinal fluid pool, which was comprised of specimens from eight individuals. None of the patients contributing specimens was known to have any infectious disease, and each individual sputum specimen test yielded "oropharyngeal flora" only. The sputum pool was liquified by addition of an equal quantity of

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**Fig. 1. Schematic representation of ELISA indirect sandwich assay.** Step 1: Polystyrene balls are armed with horse anti-*S. pneumoniae* type 3 antibodies (Ab). Step 2: Unfilled protein-binding sites on the balls are blocked with 10% fetal calf serum. Step 3: *S. pneumoniae* type 3 antigen (Ag) in test specimens is captured by horse anti-*S. pneumoniae* type 3 antibodies adsorbed to the balls. Step 4: Captured antigen is recognized by rabbit anti-SSS-III antibodies. Step 5: Recognition antibody is developed by the addition of goat anti-rabbit antibodies (GARG) conjugated with alkaline phosphatase. Step 6: Alkaline phosphatase substrate (p-nitrophenyl phosphate) generates color reaction, which is quantitated spectrophotometrically at 400 nm.
Sputolysin. Purified SSS-III was added to those pools at various concentrations for testing in the assay.

Selection of serum dilutions. A 1:500 dilution of horse anti-SSS-III serum was used routinely, based on preliminary experiments using dilutions of antisera ranging from 1:50 to 1:2,000. The remaining conditions of the test were: (i) purified antigen concentrations ranging from 1 to 1,000 ng/ml; (ii) a 1:500 dilution of rabbit anti-SSS-III serum, based on a titration in which horse anti-SSS-III was held constant at 1:500, and (iii) a conjugate dilution of 1:200, which in turn was based on results of titrations performed with dilutions of both horse and rabbit antisera held constant at 1:500.

Selection of incubation times and temperatures. Room temperature (25°C) for arming the polystyrene balls was chosen on the basis of preliminary experiments which showed no enhanced binding of the antibody at higher (37°C) or lower (5°C) temperatures. Selection of the 3- to 4-h incubation period for arming the balls with antibody was based on results showing no real improvement with longer incubation times (to 18 h) but some loss of sensitivity with periods of less than 3 h. Incubation at 40°C after substrate addition was patterned after the protocol using alkaline phosphatase in a commercial ELISA kit for detection of hepatitis B surface antigen (Cordis Laboratories, Miami, Fla.). Incubation times of 45 min for all remaining steps of the assay results were chosen because results were equivalent to those of 1 h or longer; some loss of sensitivity was observed with shorter periods.

Standard curve for detection of purified SSS-III. Figure 2 shows four typical curves (top lines, Fig.

![Fig. 2. Comparison of standard curves of the indirect sandwich ELISA prepared with SSS-III in phosphate-buffered saline with those prepared in various body fluids: (a) urine, (b) sputum, (c) serum, and (d) spinal fluid. Control in each experiment was obtained using normal horse serum-coated balls.](http://jcm.asm.org/)
2A to D) obtained with SSS-III diluted in phosphate-buffered saline to final concentrations of 1, 10, 100, and 1,000 ng/ml. Values for the assay performed in the complete absence of antigen but with balls treated with horse anti-SSS-III serum were essentially identical to those obtained at all levels of antigen but with balls treated with normal horse serum (base line). Results obtained at an SSS-III concentration of 1 ng/ml were in all cases above the base-line (control) level, but only marginally so. To avoid equivocal readings, estimation of SSS-III concentrations in test specimens should be restricted to that portion of the curve which is clearly above the base line. Thus the absolute lower limit of test sensitivity appears to be 1 ng/ml, but with the above restriction, the practical lower limit of detection is approximately 2 to 3 ng/ml.

RESULTS

Effect of body fluids on the detection of SSS-III. Comparisons of standard curves for purified SSS-III diluted in clinical materials (spinal fluid, urine, serum, and sputum) with curves prepared in phosphate-buffered saline are also shown in Fig. 2. All of the standard curves were essentially similar, although those for the clinical materials showed slightly reduced sensitivity, especially in the case of sputum.

Tests with human clinical specimens culture negative for S. pneumoniae. A series of tests was performed on clinical specimens that were culture negative for pneumococcus; all such tests gave results below the base-line level (generally, absorbances less than 0.07). Specifically, 15 spinal fluids that were bacterial culture negative produced absorbances ranging from 0.01 to 0.03, and 15 sera and 5 urines submitted for routine tests gave absorbances ranging between 0.01 and 0.06. Five individual sputum specimens that contained only a mixture of normal oropharyngeal flora produced absorbances between 0.01 and 0.07.

Detection of S. pneumoniae organisms. To determine whether S. pneumoniae could be detected in clinical specimens, viable organisms were added to normal sputum pools. Absorbances between 0.02 and 0.03 were obtained in tests of the unaltered sputum pools, whereas values between 0.60 and 0.70 were obtained routinely with specimens containing 10⁸ colony-forming units of S. pneumoniae type 3 per ml. Absorbances of 0.60 to 0.70 correspond to concentrations of approximately 100 ng of purified SSS-III polysaccharide per ml. This agrees with observations by Coonrod and Drennan (6), who detected pneumococcal antigen in human clinical specimens by using counterimmunoelectrophoresis. They reported detection of 50 to 100 ng of pneumococcal polysaccharide per ml by using 10⁸ pneumococci per ml.

Tests for cross-reactions by other organ-

isms. K. pneumoniae, E. coli, H. influenzae, P. aeruginosa, group B streptococci, and S. aureus have been shown to possess antigens cross-reactive with those of S. pneumoniae (4, 8, 10). Coonrod and Drennan (6) determined that 10⁶ colony-forming units per ml must be present in clinical specimens for detection by counterimmunoelectrophoresis. To determine whether these organisms would interfere with the species specificity of the assay, 2 × 10⁶ to 5 × 10⁶ colony-forming units per ml in phosphate-buffered saline were tested. Absorbances for all of the above organisms other than S. pneumoniae type 3 ranged from 0.03 to 0.05, whereas S. pneumoniae type 3 produced absorbance of 0.60 to 0.70.

DISCUSSION

Our results using human clinical specimens, with or without S. pneumoniae type 3 organisms or purified SSS-III antigen, indicate that this indirect sandwich ELISA for the detection of pneumococcal type 3 polysaccharide may possess sufficient sensitivity for the rapid and accurate diagnosis of serious pneumococcal infections. Broadly defined curves such as those presented in Fig. 2 allow qualitative determination of the presence of antigen in clinical materials and provide some estimate of the antigen level; strict quantitation of the antigen level would of course require more extensively defined standard curves. Harding et al. (9) have suggested that the extreme sensitivity of the ELISA might actually preclude its use in examining sputum samples for pneumococcal antigen because of the probability of positive results with asymptomatic S. pneumoniae carriers. Although such carriers might indeed be positive qualitatively, refinement of the assay to allow quantitative assessment might also permit establishment of minimal antigen levels reflecting numbers of organisms constituting a clinical concern. Specificity, at least at the species level, is supported by the finding that false-positive results are not produced by the presence of known cross-reactive organisms such as H. influenzae b, group B streptococci, E. coli, S. aureus, K. pneumoniae, or P. aeruginosa, even in numbers approximating or exceeding those found in clinical specimens.

Several features of the ELISA procedure make it highly attractive as a clinical test. The antisera are highly diluted, making the assay inexpensive. Ease of the assay to perform and availability of results in approximately 3 h are distinct advantages over cultural identification, which takes 18 to 24 h. Such rapid identification would make it possible to administer appropriate antibiotics promptly in lieu of interim ad-
ministration of broad-spectrum antibiotics with their potentially toxic side effects.

An ELISA test similar in principle to the one reported here but differing in several important aspects has been reported very recently (9). Both assays have the advantages of being about 25 times more sensitive than counterimmunoelectrophoresis, and of detecting pneumococcal antigen, thus permitting diagnosis before detectable antibody has developed. The assay described here, however, has several additional advantages. Results with polystyrene balls are available within 3 h, rather than 7 h as described for the use of microtiter plates (9). Moreover, this ELISA technique can readily be adapted to screening panels in which not only individual serotypes of pneumococcus but also other species of organisms involved in pneumonia or meningitis can be rapidly identified with a single enzyme-conjugated preparation. This advantage of using only one labeled antibody has been recognized and applied in radioimmunoassays and fluorescent-antibody assays.

Such adaptation, however, requires the use of several antisera not yet commercially available. The identification of pneumococcus at the species level, for example, requires two antigenically distinct polyvalent (species-specific) anti-pneumococcal antibodies; the first (i.e., capture) antibody can be prepared in any convenient species. The second antibody, which both recognizes captured antigen and serves as a target for the enzyme-conjugated anti-globulin reagent, should be prepared in a second species to avoid interaction of the conjugate with the first capture antibody. The second antibody may be either polyvalent (species specific) or monovalent (serotype specific), and as long as all second antibody preparations are of the same species, only one conjugate is required. At present, the species-paired polyvalent anti-pneumococcus antisera needed for this assay are not available commercially. The potential clinical usefulness of this assay, when the required antisera become available, is considerable.

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