Enzyme-Linked Immunosorbent Assay for Detection of
Streptococcus pneumoniae Antigen

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An enzyme-linked immunosorbent assay (ELISA) was developed for the detection
of Streptococcus pneumoniae polysaccharide antigen in cerebrospinal fluid and serum. Sensitivity and specificity were determined for purified antigen
preparations. Specificity was also evaluated in the rabbit meningitis model, and
the sensitivity was compared to counterimmunoelectrophoresis, using the infected
rabbits’ cerebrospinal fluid and serum. The ELISA was a specific technique for
detecting S. pneumoniae antigens. ELISA was 25 times more sensitive than
counterimmunoelectrophoresis for purified antigen and resulted in an increased
positivity of the cerebrospinal fluid and serum from infected rabbits. ELISA
should prove very useful in the diagnosis of pneumococcal infections.

Detection of Streptococcus pneumoniae polysaccharide antigen in spinal fluid, serum, urine, and other body fluids has been proven to be
highly specific for pneumococcal infection (1, 4–6, 8, 16). When applied to sputum, there is
conflicting data as to specificity for significant infection, with some authors finding false
positives in patients with upper respiratory infections (12) and others reporting positives only in
patients with pneumonia or chronic bronchitis (10). The techniques used include immunodif-
fusion, counterimmunoelectrophoresis (CIE), and latex agglutination. However, these tech-
niques are positive only in 25 to 50% of infected patient sera (4, 6, 15), making them insensitive in
pneumococcal infections other than when applied to cerebrospinal fluid (CSF) in meningitis
or pleural fluid in pulmonary infections.

The enzyme-linked immunosorbent assay (ELISA) described by Engvall and Perlman (3)
is a simple and very sensitive assay that has now been applied to the detection of a wide variety
of antibodies and antigens. In this report we describe a double-antibody sandwich ELISA for
the detection of pneumococcal antigen. This technique was evaluated for sensitivity and spec-
ficity and compared to CIE on the spinal fluid and serum of rabbits with pneumococcal men-
ingitis.

MATERIALS AND METHODS

Bacteria. The strains of S. pneumoniae and Hae-
mophilus influenzae used in these studies were de-
scribed previously (13, 14). The Klebsiella pneumo-
niae type I strain was obtained from R. Royds, Hoff-
mann-La Roche, and was originally isolated from a
neonate with meningitis. This strain has demonstrated
virulence for experimental animals, including guinea
pigs, mice, and rabbits (W. M. Scheld, D. D. Fletcher,
E. N. Fink, and M. A. Sande, submitted for publica-
tion). Overnight cultures of S. pneumoniae and H.
influenzae were grown and washed as previously de-
described (13, 14). The K. pneumoniae isolate was grown for 18 h in tryptic soy broth (Difco), centrifuged,
and washed three times in phosphate-buffered saline
(PBS). The inocula were used 5.0, 6.0 to 8.0, and 9.9
log10 colony-forming units (CFU)/ml for the K. pneu-
moniae, S. pneumoniae, and H. influenzae strains,
respectively.

Rabbit model. Two-kilogram New Zealand white
rabbits were prepared according to the method of
Dacey and Sande (2). A dental acrylic helmet was
attached to the animal’s skull to facilitate immobi-
lization in a stereotaxic frame. A Quincke spinal needle
(25 gauge by 9 cm) was inserted percutaneously by a
grounded electrode introducer into the cisterna magna,
and 0.3 ml of CSF was withdrawn. The bacterial
inoculum, in a volume of 0.25 ml, was then inoculated
intracisternally. The animals were then returned to
their cages. All animals developed meningitis as char-
acterized by fever (>40°C), neurological signs, a CSF
pleocytosis (0.8 × 103 to >25 × 103 cells/mm3, with greater
than 95% polymorphonuclear leukocytes), and CSF
bacterial titers ranging from >105 to less than 106 CFU/
ml. Simultaneous nontraumatic CSF and blood samples
were then obtained at 6 h (H. influenzae) or 16 h
(S. pneumoniae, K. pneumoniae) postinoculation. Re-
peat samples were also obtained 24 h later in those
animals that survived this interval. All animals died
within 48 h. The CSF and blood were quantitatively
cultured. The CSF and serum samples were then
tested by ELISA and CIE techniques for pneumococ-
cal polysaccharide antigen.

Antisera and antigens. Rabbit antisera were ob-
tained from the Statens Seruminstitut in Copenhagen,
Denmark, as “omniserum” which contains antibodies
against all 83 capsular types and has been precipitated with Na₂SO₄, dialyzed, lyophilized, and concentrated (9). Pneumococcal vaccine was obtained from Merck, Sharp and Dohme, West Point, Pa., as a source of polysaccharide antigen. The vaccine is formulated to contain 50 μg of polysaccharide per 0.5-ml volume. Purified type-specific pneumococcal polysaccharide antigens (types 4, 5, 7, 9, 12, 14, 18, 19, and 23) were provided by R. Baker, Eli Lilly and Co., Indianapolis, Ind. Neisseria meningitidis group A vaccine was obtained from Connaught Laboratories, Swiftwater, Pa. H. influenzae type B vaccine was provided by J. Colabico, University of Rochester, Rochester, N.Y. All purified antigens were diluted in PBS to the desired concentrations.

CIE for pneumococcal antigen. CIE was performed using the Austigen II CIE system (Hyland Laboratories). The wells on the anodal side were filled with 25 μl of omission. The wells on the cathodal side were filled with undiluted spinal fluid or serum. The system was controlled by including pneumococcal polysaccharide at a concentration of 1 μg/ml in one specimen well of each plate. Precut agar plates containing 22 pairs of wells, 0.01 M barbital buffer, pH 8.6, and disposable sponge wicks are provided with the system. Electrophoresis was run at 40 mA for 60 min. The plates were flooded with 0.85% saline for 30 min before reading for precipitin arcs using indirect light.

ELISA reagents. Horseradish peroxidase type VI was conjugated to omission by using the method described by Nakane and Kawai (11). A tenfold determination was used for 1 ml of borohydride and separation of conjugate from free enzyme by ammonium sulfate precipitation at 0.5 saturation were the only modifications to the conjugation method (7). Working dilutions of unlabeled coating antibody and horseradish peroxidase-labeled antibody were determined by checkerboarding with a serum specimen containing antigen and a negative serum. Dilutions were chosen that showed maximum reactivity of the positive and minimum reactivity of the negative specimen. Some lots of omission are clearly superior to others in their ability to adsorb to polyvinyl chloride plates used as the solid phase, and this variable had to be optimized before the assay was used.

ELISA for pneumococcal antigen. The double-antibody sandwich ELISA method used is outlined schematically in Fig. 1. Polyvinyl chloride microtiter plates (Cooke Laboratory Products, Alexandria, Va.) were coated with 200 μl of diluted omission containing 5 μg of immunoglobulin G in 0.05 M carbonate buffer (pH 9.5). The plates were incubated for 3 h at 37°C and washed three times with PBS containing 0.05% Tween 20. The plates were used immediately or stored at 4°C in a moist chamber. Two hundred microliters of sample to be tested was added to each well. Spinal fluid was tested undiluted. Serum was heat inactivated for 30 min at 56°C and diluted 1:2 with PBS-Tween before testing to remove a heat-labile inhibitor present in rabbit sera. Samples were incubated at 37°C for 3 h and then washed three times with PBS-Tween. Two hundred microliters of diluted horseradish peroxidase-labeled omission with an approximate concentration of immunoglobulin G of 1 μg/ml was added and incubated for 3 h at 37°C or over-night at 4°C. Plates were washed three times with PBS-Tween, and 200 μl of freshly prepared 5-amino-salicylic acid with 0.05% H₂O₂ (9:1) was added and incubated at 25°C for 1 h. The enzyme-substrate reaction was stopped by adding 50 μl of 3 N NaOH. A positive reaction could be read visually as a dark brown color or spectrophotometrically at 450 nm. A positive reaction was defined as an absorbance value of greater than or equal to twice that of the average of three negative controls. The extinction value at 450 nm (E₄₅₀) of negative controls averaged 0.3 to 0.4 and most positive reactions reached a maximum E₄₅₀ of 2.0 at the end of the substrate incubation. However, any specimen with an E₄₅₀ of 0.8 or greater would be considered positive. Even weak reactions could be accurately read visually.

RESULTS

Sensitivity and specificity. The ELISA was able to detect 1 to 3 ng of pneumococcal polysaccharide antigen per ml in PBS. The limit of detection for the same antigen by CIE was 25 to 50 ng/ml. No cross-reactivity for the purified antigens of H. influenzae type B and N. meningitidis group A was seen when concentrations up to 1 μg/ml were tested.

Antigen detection in rabbits with experimental pneumococcal meningitis. The ELISA technique was found to be more sensitive than CIE for pneumococcal antigen detection in both CSF and serum (Table 1). Sixteen hours after inoculation of S. pneumoniae into the cisterna magna, 20 of 20 (100%) CSF specimens gave positive results for antigen by ELISA, whereas antigen was detected in 16 of 20 (80%) by CIE. CSF bacterial titers ranged from 10⁴ to 10⁶ CFU/ml. Seven of seven CSF samples tested after 40 h (day 2) were positive by both techniques, and CSF bacterial titers ranged from 10⁶ to 10⁹ CFU/ml.

Similar results were found in serum. Eleven of 20 (55%) animals had antigen detected in serum by ELISA 16 h after infection, whereas 8 of 20 (40%) had antigen detected by CIE. At 40 h nine of nine (100%) surviving rabbits had antigen detected by ELISA, whereas only five of nine (55%) had antigen detected by CIE.

Twelve of the 20 day 1 blood cultures were positive, of which 8 were positive by ELISA (Table 2). Antigen was also detected by ELISA in three of the eight day 1 samples that were negative by culture. Eight of the nine day 2 blood cultures were positive, and antigen was detected in all. Blood bacterial titers ranged from 10 to 10⁷ CFU/ml.

Serum antigen detection by ELISA correlated with both CSF and blood bacterial titers (Fig. 2). Antigen was detected in serum when CSF contained more than 10⁶ CFU/ml of CSF and when blood contained more than 10 CFU/ml.
direct correlation between the numbers of organisms present in quantitative cultures of CSF or blood and the titer of antigen detected by ELISA. When blood cultures were negative the ELISA titer was 1:4 or less. However, some specimens with an ELISA titer of 1:2 or 1:4 had positive blood cultures. The higher ELISA titers, although associated with positive cultures, were not predictive of the quantitative number of organisms in the CSF or blood.

**DISCUSSION**

The detection of *S. pneumoniae* polysaccharide antigens in serum and body fluids has contributed greatly to the rapid accurate diagnosis of pneumococcal infections (1, 4–6, 8, 10). Antigen has been shown to be present even when Gram strains and cultures are negative (5, 8, 15). Methods for detecting antigen do not require the time delays inherent in culture detection and identification. The use of polyvalent antisera (omniserum) has made the detection of the many different capsular polysaccharide types possible with a single set of reagents (9). However, there appears to be significant lot-to-lot variability of omniserum for its use as a coating antibody preparation with an ELISA technique. This commercially available reagent is costly, and producing it in one’s own laboratory is difficult and also very costly. A single reagent for all capsular types of pneumococci is essential if this is to be a clinically useful, economically feasible test. Commercially available reagents that have been standardized and quality controlled for an ELISA application would be an ideal solution to this problem.

The ELISA technique, although more time-consuming than CIE, can still provide same-day results. In addition, ELISA requires no specialized equipment and could be performed in most
laboratories if the reagents were commercially available. The shelf life of enzyme-labeled reagents is long, making this technique more practical than radioimmunoassay while possessing similar sensitivity. Visual interpretation of a positive or negative reaction was accurate in all cases with the horseradish peroxidase enzyme and 5-aminosalicylic acid substrate combination.

Double-antibody sandwich ELISA is a sensitive and specific technique for demonstrating the presence of pneumococcal polysaccharide antigens in the CSF and sera of infected rabbits. This technique is 25 times more sensitive for purified antigen than CIE. This increased sensitivity resulted in 100% positivity by ELISA for infected CSF specimens and an overall positive rate on serum of 70%. By day 2 100% of serum specimens were positive by ELISA.

Antigen levels as reflected by ELISA titers did not correlate with the concentrations of viable bacteria present. This has also been the experience of Wheat et al. (16) when measuring circulating antigen levels in staphylococcal infections.

ELISA for pneumococcal antigens could be used as an initial diagnostic technique on serum and other sterile body fluids. It could also be used as a second procedure on CIE-negative specimens. The ELISA may prove particularly useful in cases of partially treated infection as the greatest difference in positivity rates compared to CIE occurred in the animals receiving the lower dose of S. pneumoniae with subsequently lower CSF bacterial titers. The increase in sensitivity would probably prevent its use on sputum as it is likely that sputum specimens from asymptomatic S. pneumoniae carriers would be positive.

In summary, this sensitive and specific technique for the detection of pneumococcal polysaccharide antigens in sera and sterile body fluids should prove very useful in the rapid and accurate diagnosis of pneumococcal infection.

LITERATURE CITED