

Bacterial Antigen Detection in Body Fluids: Methods for Rapid Antigen Concentration and Reduction of Nonspecific Reactions

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We sought procedures which would allow a rapid concentration in high yield of bacterial antigens from tissue fluids of patients and which could be applied also to protein-rich fluids like serum. Ethanol precipitation at a subzero temperature with albumin added as an antigen coprecipitant made it possible to achieve a more than 20-fold concentration of antigen in 15 min and a 200-fold concentration in 45 min. Heat-stable antigens could be concentrated from protein-rich fluids (like serum) after the sample had been deproteinized by boiling. Such heating (100°C, 3 min) also liberated bacterial polysaccharides from antibody complexes and eliminated the nonspecific interference of serum in enzyme-linked immunosorbent assay.

The sensitivity of bacterial antigen detection in tissue fluids can be increased by concentrating the antigen before analysis. The described procedures (1, 6, 9, 20) for such concentration cannot be readily applied to protein-rich fluids like serum and are either time consuming (6) or lead to loss of antigen (9, 20).

We will describe a modified ethanol precipitation method which allows the concentration in high yield of bacterial antigens from samples of low protein content (spinal fluid, some urines) in 15 min. The method could also be used for protein-rich samples, e.g., serum, provided they had been deproteinized.

Among the methods for deproteinization, boiling (3 min, 100°C) was preferred since, in addition to deproteinization, it caused liberation of antigen complexed with antibody and reduced the nonspecific interference caused by serum in enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Specimens. Samples of cerebrospinal fluid (CSF), serum, and urine were submitted by hospitals in Norway. Immediately upon receipt of the samples, portions were taken for culture, direct immunofluorescent antibody microscopy (for CSF only), and counterimmunoelectrophoresis (CIE).

Bacterial antigens. Purified polysaccharide from *Escherichia coli* strain K1 (D 699), antigenically identical to the group polysaccharide of *Neisseria meningitidis* group B, was a gift from John B. Robbins (Division of Bacterial Products, Bureau of Biologics, Food & Drug Administration, Bethesda, Md.). Purified polyribose ribitol phosphate from *Haemophilus influenzae* type b was provided by Tov Omland (Norwegian Defence Microbiological Laboratory). Bacterial antigens from *N. meningitidis* groups A, B, and C,

Streptococcus pneumoniae, *H. influenzae* type b, and *Legionella pneumophila* were the crude supernatants (centrifuged 10,000 × *g* for 10 min) of suspensions of bacteria from agar plates, harvested in phosphate-buffered saline (PBS). *N. meningitidis* and *S. pneumoniae* were grown on blood agar, *H. influenzae* was grown on chocolate agar with supplement B (Difco Laboratories), and the *L. pneumophila* strains were grown on CYE agar (8). The *Legionella* strains were a gift from Robert Weaver, Special Bacteriology Branch, Center for Disease Control, Atlanta, Ga.

Sera. Rabbit antiserum against *H. influenzae* type b was produced using biweekly intravenous injections of live bacteria (17). Burro anti-*H. influenzae* was supplied by John Robbins. Rabbit antisera against *N. meningitidis* groups A, B, and C were provided by Eirik Holten, Rikshospitalet, Oslo. Rabbit and goat antisera against *L. pneumophila* were supplied by John C. Feeley, Bacterial Immunology Branch, Center for Disease Control, Atlanta, Ga. Anti-*S. pneumoniae* omniserum and antiserum against *S. pneumoniae* type 3 were purchased from Statens Seruminstitut, Copenhagen, Denmark.

Countercurrent immunoelectrophoresis. Essentially we followed a modification (14) of the original procedure of Culliford (7). Microscope glass slides were covered with 2 ml of 1% agarose (Behringwerke, Marburg-Lahn, Germany). Antigen and antibody wells were of 3-mm diameter (7- μ l capacity), cut 5 mm apart. A 25 mM sodium barbital buffer (pH 8.6) and an electrical field of 6 V per centimeter of agarose were used. The gels were inspected after 15 min and 30 min of electrophoresis time. The untreated body fluid specimens were tested for antigen with anti-*N. meningitidis* groups A, B, and C; anti-*H. influenzae* type b monosera; and anti-*S. pneumoniae* pool serum. If negative, the tests were repeated with an ethanol concentrate (see below) of the sample. In the case of a positive reaction, the titer (highest dilution giving a clearly visible precipitation line) was determined.

ELISA. A four-layer multiple-antibody sandwich

ELISA was set up for *H. influenzae* type b antigen and for *L. pneumophila* group I antigen (using reagents based upon the Philadelphia I strain). The procedure follows closely the ELISA for *Legionella* antigen described by Berdal et al. (1), except that whole serum (instead of purified immunoglobulin G [IgG]) was used for coating the plates. For the *H. influenzae* type b ELISA, burro anti-*H. influenzae* type b made up the first layer (coating), and rabbit anti-*H. influenzae* type b made up the third one. The ELISAs for both *H. influenzae* type b and *Legionella* (Philadelphia I) antigen were 10 times more sensitive than CIE in detecting the antigen in a given solution.

Coagglutination. Formalin-stabilized protein A-containing staphylococci (strain Cowan I) were made up by the method of Kronvall (12). The batch used was a gift from J. C. Siebke, Virus Department, Norwegian Institute of Public Health. Ten milliliters of a 1% staphylococcal cell suspension was incubated (1 h at 22°C) with 0.1 ml of rabbit anti-*H. influenzae* type b serum, washed twice in PBS, and suspended to a final concentration of 1% in PBS. This reagent was used for slide agglutination, following the principles of Olcén et al. (16). The co-agglutination method was 1.5 times more sensitive than CIE in detecting *H. influenzae* type b antigen in a given solution.

Fluorescent-antibody technique. Detection of bacteria in spinal fluid specimens by this method was as described by Føre and Gaustad (10).

Procedures for ethanol precipitation of antigen in body fluid specimens. Different procedures were used depending on the volume of the sample and its content of ethanol-insoluble material (protein). Procedure A₁ was used for samples of 3 ml or less which contained little protein, e.g., spinal fluid, urine. Procedure A₂ was used for samples (3 ml or less) of protein-rich fluids. The presence of ethylenediaminetetraacetic acid during boiling made it possible to sediment the denatured protein by centrifugation. In the absence of ethylenediaminetetraacetic acid, boiled serum formed a gel trapping the antigen. Dilution of the serum with 3 or more volumes of PBS before boiling also prevented the formation of a gel. Procedure B was used for samples of volume above 3 ml regardless of their protein content.

(i) **One-step precipitation.** Procedure A₁: 1 volume (0.5 to 3 ml) of sample fluid was mixed with 0.1 volume of a solution of 3 M sodium acetate with 1% (wt/vol) bovine serum albumin and 0.1% (wt/vol) sodium azide. Ethanol (96% vol/vol, stored at -20°C) was then added to give a final concentration of 70% (vol/vol), and the mixture was blended in a Vortex mixer. The ethanol precipitate was pelleted by centrifugation (10,000 × *g* for 10 min at 4°C), and the pellet was suspended in 0.1 ml of PBS. Procedure A₂: One volume (0.5 to 3 ml) of serum or other protein-rich fluid was mixed with 0.4 volume of 100 mM disodium ethylenediaminetetraacetic acid (adjusted to pH 7.4 by the addition of NaOH). The mixture was placed in a boiling water bath for 3 min and centrifuged as above. The supernatant was ethanol precipitated and further treated as described above A₁.

(ii) **Two-step precipitation.** The first ethanol precipitation step was as described for A₁. The pellet was thoroughly suspended in up to 3 ml of PBS and boiled

in a water bath for 3 min. The insoluble residue was removed by centrifugation for 5 min at 10,000 × *g*, and this supernatant was re-precipitated with ethanol as described above (A₁).

RESULTS

Ethanol precipitation of bacterial antigens. In preliminary experiments it was found that when bovine serum albumin (1 mg/ml) was included along with precooled (20°C) ethanol, an excellent recovery of the antigen resulted. This was the case even when the antigen was highly diluted and the centrifugation was started immediately after addition of the ethanol.

A near total recovery of purified capsular polysaccharide antigen was noted with this method, whether the antigen had been dissolved in PBS or body fluids (Table 1). Samples of CSF could be concentrated 5 to 20-fold in 15 to 20 min by this procedure. The degree of concentration depended upon the volume of CSF available (0.5 to 2.0 ml). In the case of body fluids with high contents of ethanol-insoluble material, e.g., serum and some urines, a thorough suspension of the ethanol precipitate was necessary to dis-

TABLE 1. Recovery of bacterial antigens from various solutions with and without ethanol precipitation^a

Bacterial antigens and solutions	Original solution		Concentrated solution	
	Vol (ml)	Titer	Vol (ml)	Titer
PRP ^b in PBS	1	2	0.1	16
PRP in PBS	1	0.2	0.1	2
PRP in human serum	1	2	0.2	8
PRP in human urine	60	0.15 ^c	0.2	32
CSF (<i>H. influenzae</i> type b was isolated from the sample)	1	^d	0.1	8
<i>N. meningitidis</i> group A supernatant in serum	1	2	0.2	8
<i>N. meningitidis</i> group B supernatant in serum	1	2	0.2	8
<i>S. pneumoniae</i> type 3 supernatant in serum	1	2	0.2	8
<i>L. pneumophila</i> group 1 (strain Philadelphia I) in PBS	2	4	0.1	64
<i>L. pneumophila</i> group 1 (strain Philadelphia II) in PBS	2	4	0.1	64
<i>L. pneumophila</i> group 1 (strain Knoxville I) in PBS	2	4	0.1	64

^a Some examples of the concentrations of bacterial antigens achieved with ethanol precipitation are given. The titers indicate the highest dilution (of a twofold dilution series) giving a clearly visible precipitation line in CIE.

^b PRP, Polyribosyl ribitol phosphate (*H. influenzae* type b capsular antigen).

^c Titer calculated from the amount of PRP with known titer added to the sample.

^d —, Unconcentrated CSF was negative in CIE.

solve trapped antigen. In such cases, excess protein was removed by heating (in a boiling water bath for 3 min), followed by centrifugation to remove insoluble material. Re-precipitation with ethanol gave further concentration. Samples of urine (50 ml) could be concentrated up to 200 times in less than 1 h by this two-step precipitation procedure.

Heat treatment of bacterial antigens. Purified bacterial group-specific polysaccharides (*H. influenzae* types a and b, *N. meningitidis* group B) as well as antigens from supernatants of suspensions of *H. influenzae* type b, *N. meningitidis* groups B and C, *S. pneumoniae* type 3, and *L. pneumophila* (strains Philadelphia I and II, Knoxville I, Togus I, Bloomington II, and Los Angeles I), were detected equally well by CIE before and after heating (100°C for 3 min). Likewise, heating had no effect on the titer of polyribosyl ribitol phosphate (purified or from crude *H. influenzae* type b supernatants) whether in ELISA or in staphylococcal co-agglutination tests.

Use of heating to recover bacterial antigens from immune complexes. The heat resistance of the bacterial antigens made it possible to liberate them from antigen-antibody complexes, as shown in Fig. 1.

Effect of heating of samples upon false-positive reactions in ELISA. ELISA setups for *Legionella* antigen, *H. influenzae* type b antigen, cholera enterotoxin, and staphylococcal

B enterotoxin were all subject to nonspecific reactions (false-positives) due to substances present in human and rabbit sera, human urine, and CSF. The degree of nonspecific reaction varied conspicuously from specimen to specimen, but was similar in the various ELISA setups for any particular specimen.

The false-positive reactions were not altered by freezing or thawing the sera, or storage for 2 weeks at 4°C. They were not eliminated by ethanol precipitation of the sera. (The resuspended precipitate was tested.) However, the heat treatment of sera (100°C, 3 min), urines, and CSF samples completely eliminated this non-specificity, leaving the antigens intact for detection.

Diagnosis of bacterial meningitis. Only a limited number of clinical specimens have been examined so far. Ethanol concentration has permitted an etiological diagnosis: 1 *N. meningitidis* group A, 2 *N. meningitidis* group B, 2 *H. influenzae* type b in five specimens of CSF and 1 *N. meningitidis* group B and 2 *H. influenzae* type b in three specimens of urine from patients with meningitis, where CIE with the unconcentrated samples was negative.

In one of the CSF specimens (*N. meningitidis* group B), gram-negative diplococci were evident in direct microscopy. In another (*H. influenzae* type b), both culture and direct immunofluorescent-antibody microscopy were positive. No sample with positive culture or direct immunofluorescent-antibody microscopy has been negative in CIE after concentration of the sample. These results, although for a small number of cases, suggest that CIE, when combined with antigen concentration, is more sensitive than culture or microscopy.

In one case (serum from a patient with *N. meningitidis* group B meningitis), multiple precipitation lines against *N. meningitidis* groups A and B antisera were observed in CIE. After heat treatment of the specimen, only one precipitation line against *N. meningitidis* group B remained. Preincubation of the *N. meningitidis* group B antiserum with purified group-specific *N. meningitidis* group B polysaccharide abolished the precipitation line in the heat-treated sample, whereas precipitation lines were still present with the unheated specimen. Thus, it appears that the heat treatment enhanced the diagnostic specificity by destruction of heat-labile cross-reacting antigens, leaving the type-specific polysaccharide intact.

DISCUSSION

An etiological diagnosis of bacterial meningitis may be possible in less than 1 h by latex agglutination, co-agglutination, or CIE. These meth-

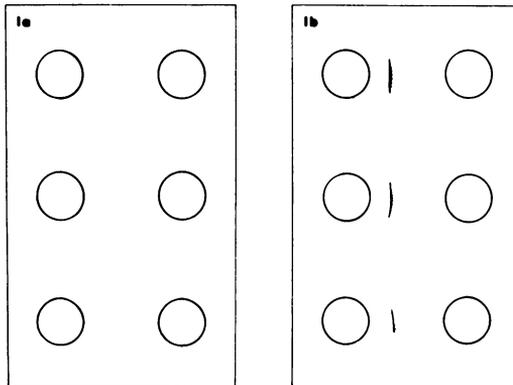


FIG. 1. Heat treatment as a means to recover bacterial polysaccharide complexed with antibody. Polyribosyl ribitol phosphate, the purified *H. influenzae* type b capsular antigen (0.01 ml; titer, 5,129) was incubated (for 2 h at 22°C) in 0.8 ml of rabbit anti-*H. influenzae* type b antiserum. The antigen-containing serum was then diluted 1:4 (upper wells), 1:16 (middle wells), or 1:64 (lower wells) in PBS and tested for antigen by CIE, either untreated (a) or after heating to 100°C for 3 min (b). The antigen-antiserum mixture was placed in the antigen wells, with anti-*H. influenzae* type b serum in the antibody wells.

ods have a sensitivity comparable to those of microscopy and culture (3, 5, 10, 11), but are considerably less sensitive than ELISA or radioimmunoassay (2, 19).

By the present quick procedure of precipitation with ethanol (using ethanol at -20°C and including serum albumin as coprecipitant), bacterial antigen was concentrated up to 20 times in less than 20 min, with a near complete recovery of antigen (Table 1). To achieve maximal concentration of antigen in large volumes of high protein content, a second ethanol precipitation followed a protein denaturation step (heating in a boiling bath for 3 min). In this way, antigen in urine was concentrated more than 200 times in less than 1 h. The usefulness of ethanol precipitation is not limited to capsular (group-specific) polysaccharides, as antigenic substances different from the capsular polysaccharides have been precipitated from the serum and CSF of a patient with meningitis due to *N. meningitidis* group B (unpublished observations). Likewise, antigen released into PBS from *L. pneumophila*, presumably protein lipopolysaccharide (23), could be concentrated by ethanol precipitation.

Ethanol precipitation has been used for decades to concentrate antigen from bacterial cultures (13, 21), but its use for concentration of bacterial antigens in body fluids has been limited to urine, where 20-fold concentration has been achieved (6). The reason for the limited use of ethanol precipitation until now may have been the delay (2 h) caused by the early procedures (6).

Membrane filtration has also proved useful for the concentration (from urines) of pneumococcal, meningococcal, and *H. influenzae* type b antigens (9) and the *Legionella* antigen (1). This method has given up to 20-fold concentration of the antigen in 1 h, but a considerable (more than 50%) loss of antigen occurs (9). A considerable loss of antigen also occurs when CSF is concentrated with dehydrated polyacrylamide gel absorption (20). The heat stability of capsular polysaccharides is the basis for the simple liberation of such antigens from complexes with antibody by heating (Fig. 1). The method is rapid (3 min) and is far simpler than pepsin digestion (18) or two-dimensional electrophoresis (4).

We have observed that normal sera, CSF specimens, and urines can give false-positive reactions in an ELISA sandwich setup. These observed reactions were considered to be nonspecific, since the same degree of interference was noted in several ELISA setups in which reagents of different origins were used. Heat treatment of samples before analysis by ELISA eliminated these false-positive reactions. Others, using latex agglutination or co-agglutination (with protein

A-coated staphylococci), have noted that rheumatoid factor (19, 22) and other unidentified heat-labile factors (15) may cause false-positive reactions.

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