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Counterimmunoelectrophoresis of Pneumococcal Antigens: Improved Sensitivity for the Detection of Types VII and XIV

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Rapid identification of *Streptococcus pneumoniae* has been reported using counterimmunoelectrophoresis for the detection of specific capsular antigens in serum, cerebrospinal fluid, and urine. Previous clinical studies have failed to detect type VII or XIV pneumococcal antigen. These two types, however, account for a significant portion of pneumococcal disease. The incorporation of a sulfonated derivative of phenylboronic acid in the buffer system provides a method for the sensitive detection of these types in artificial mixtures without greatly reducing the sensitivity for the detection of other pneumococcal types. A problem with false positives encountered using human serum and barbital buffer was reduced by the use of buffer containing sulfonated phenylboronic acid.

The detection of bacterial antigen in clinical specimens is gaining widespread acceptance as a sensitive method for the rapid diagnosis of infection. Antigen of *Streptococcus pneumoniae* has been detected by counterimmunoelectrophoresis (CIE) in a significant percentage of infections due to this organism (1, 4, 5, 9, 14). In the combined reports of Coonrod and Rytel (4) and Tugwell and Greenwood (14), representing 128 cases of pneumococcal pneumonia, antigen was detected and the serotype was determined by CIE in 77 cases. Not a single case of infection due to serotype VII or XIV was reported (10). This finding is consistent with the earlier report by Kenny et al. (9) that these serotypes were not detectable by the methods employed. They are, however, among the most common serotypes responsible for pneumococcal disease (8, 11, 12), and we believe that development of a CIE system capable of detecting these serotypes could increase the sensitivity of the test for the detection of pneumococcal infection.

The type-specific pneumococcal capsular antigens are polysaccharides. The chemical structures of types VII and XIV (17) indicate that they are neutral and would probably not show adequate anodal migration under the conditions used for CIE. The observed cathodal mobility of types VII and XIV (2, 9) is consistent with the neutral nature of these polysaccharides and could result from electroendosmosis. Neutral carbohydrates can be made to migrate anodally during electrophoresis in alkaline borate buffers owing to anionic complexes formed with the borate ions (6). Complex formation is dependent on the stereochemistry of the carbohydrate and the pH of the medium. Utilization of derivatives of boric acid can modify the specificity for the reaction and provide for efficient complex formation at lower pH (7, 16).

Our studies were designed to evaluate the effect of borate buffers on improving the sensitivity for detection of pneumococcal capsular types VII and XIV by CIE.

MATERIALS AND METHODS

Infrared spectra were determined on a Beckman model IR18 spectrophotometer and calibrated against polystyrene. Melting points were taken with a Fisher-Johns hot-stage apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories (Knoxville, Tenn.). CIE results were observed with a 10× hand lens and recorded photographically using dark-field illumination and Kodak high-contrast copy film.

Chemical reagents. Phenylboronic acid (benzene boronic acid) was purchased from Aldrich Chemical Co. (Milwaukee, Wis.) and Columbia Chemical Co. (Columbia, S.C.). Sodium barbital and barbital were from Sigma Chemical Co. (St. Louis, Mo.). All other reagents were of reagent grade, except acetone used for crystallization, which was National Formulary grade.

Preparation of SPB. Sulfonated phenylboronic acid (SPB) was prepared by the method of Garegg and Lindberg (7) from 49.2 g (0.40 mol) of phenylboronic acid. Difficulties were encountered during the crystallization step, and it was found that the addition of an equal volume or greater of acetone to the ethanol-water solution increased yield. The yield of SPB was 33.2 g (0.15 mol, 38%). Physical data have not been reported for this compound. We therefore report our data: the compound did not melt below

300 C; infrared spectra (KBr) were 620, 1040, and 1190 cm^{-1}. Data calculated for $C_6H_6BO_5SNa$ were: C, 32.19; H, 2.70; B, 4.82. Data actually found were: C, 32.42; H, 2.65; B, 4.70.

Antigens and antisera. Purified pneumococcal polysaccharides for types III, VII-L (American type 51), and XIV were kindly provided by D. W. McCoy of Lederle Laboratories (Pearl River, N.Y.). Lot analyses provided with these polysaccharides showed approximately 5% protein and less than 2% nucleic acid impurities. Stock solutions containing 50 μg of each antigen per ml were prepared in 0.001 M phosphate-buffered 0.85% saline, pH 7.4 (PBS). All solutions of antigen contained sodium azide (0.05%) as preservative and were stored at 5 C. Normal human serum (NHS) was obtained by venipuncture from a healthy laboratory worker. Cerebrospinal fluid (CSF) was obtained from a patient incidental to myelography. Serial twofold dilutions of each antigen were prepared in PBS and NHS from the 50 μg/ml antigen stock solutions. Appropriate concentrations of antigen in CSF were prepared by the addition of 50 μg/ml antigen stock solutions. Serial twofold dilutions in CSF were not prepared because of the small amount of CSF available.

Antiserum active against the 83 pneumococcal types (omniserum) and type-specific antisera were purchased from the Statens Seruminstitut (Copenhagen, Denmark). These antisera were stored at 5 C and used undiluted.

Concentration of pneumococcal antigens added to urine. Midstream urine was collected from a healthy laboratory worker. Sodium azide (0.05%) was added as a preservative, and the solution was filtered through a membrane filter (pore size, 0.45 μm). The stock solutions of antigen were added to this urine to the appropriate concentrations. Urine containing added antigen and blank urine were concentrated by the method of Coonrod and Rytel (3); however, PBS was used for resuspension of the precipitated material, and undissolved material was not removed prior to use in CIE.

Preparation of buffers. Barbital buffer (0.05 M, pH 8.6) was prepared from 6.2 g (33.7 mmol) of 5,5'-diethyl-barbituric acid (barbital), 34.2 g (166 mmol) of sodium barbital, 1.6 g (5.1 mmol) of calcium lactate pentahydrate, and 4 g (30 mmol) of NaN_3 dissolved to 4 liters in distilled water. SPB (0.024 M)-barbital (0.05 M) buffer (pH 8.6) was prepared from 5.44 g (24.3 mmol) of SPB, 10.3 g (50 mmol) of sodium barbital, and 0.4 g (1.2 mmol) of calcium lactate pentahydrate dissolved to 1.0 liter. Adjustment of pH was usually not necessary. The composition of other buffers employed is summarized in Table 1. In general, buffers that contained barbital and derivatives of boric acid were prepared by the addition of a triple-strength solution of the boronic acid to a triple-strength solution of sodium barbital containing calcium lactate (1.2 g/liter); pH was adjusted using 1 N NaOH, followed by dilution to the appropriate concentration with distilled water. Borate buffers were prepared by titration of a concentrated solution of sodium borate with boric acid to the proper pH, followed by dilution to the appropriate concentration. Alternatively, for borate buffers of low pH, a concentrated boric acid solution was titrated with 1 N NaOH to the proper pH and diluted. Buffers containing only SPB or phenylboronic acid (PBA) were prepared by titration of a triple-strength solution of the boronic acid containing calcium lactate (1.2 g/liter) with 1 N NaOH and diluted.

CIE. The apparatus used for electrophoresis was constructed from a solid plastic block. Each buffer reservoir contained 80 ml of buffer. Power supplies from Lambda Electronics (Melville, N.Y.) (model LL905) and Schlumberger Products (St. Joseph, Mich.) (Heathkit model IP17) were used interchangeably. Agarose from L'Industrie Biologique Française (IBF) was purchased through Fisher Scientific Co. (Chicago, Ill.) (lot 744181). SeaKem agarose (lot 10645) was purchased from Marine Colloids (Rockland, Me.). A premarketing sample of agarose isolated from *Gracilaria* agar (RE7257) was a gift from Marine Colloids. A melted solution of the appropriate agarose and buffer (6 ml) was poured onto a glass slide (50 by 75 mm) on a leveled surface. After the agarose had gelled, the slide was stored in a moist chamber at 5 C and used within 48 h. A plastic template was used for punching antigen and antibody wells in the agarose. Five pairs of wells, 3 mm in diameter, were spaced equally across the width of the slide. Antigen and antibody wells were separated 2 mm edge to edge. The wells were filled to the brim using disposable capillary micropipettes.

TABLE 1. *Buffer composition*

Buffer[a]	Concentration range (mM)[b]				pH range
	BAR	BOR	PBA	SPB	
BAR	50				8.2–9.0
BOR		50–300			8.2–9.2
PBA			20–90		8.6–9.0
SPB				20–50	8.2–9.0
BAR/BOR	30–50	10–70			8.2–9.0
BAR/PBA	30–70		10–50		8.0–9.0
BAR/SPB	30–50			5–50	8.0–9.0

[a] BAR, Barbital; BOR, boric acid.

[b] Concentrations are expressed as the total acid plus salt, and for buffers containing derivatives of boron they represent equivalents of boron. In buffer solutions that resulted in high electroendosmosis, NaCl (10 to 25 mM) was added to decrease electroendosmosis and deformation of the agarose gel.

Approximately 15 μl of solution was required to fill each well. Antigen was placed in wells nearest the cathode. Strips of Telfa pad (Kendall Co., Chicago, Ill.) were used as connecting wicks to the buffer compartments. Depending on the buffer system employed, electrophoretic conditions varied slightly. In general, electrophoresis was performed for 1 h using a current of 12 to 15 mA for each slide. With 0.024 M SPB-0.05 M barbital (pH 8.6), a current of 14 mA for each slide resulted in a gradient of about 6 V/cm measured in the gel (60 V at the source). After electrophoresis, the slides were observed immediately, after 1 to 4 h at 5 C in a moist chamber, and after overnight washing with 0.85% saline.

Determination of optimal buffer composition. More than 50 buffer solutions covering the range of compositions shown in Table 1 were evaluated for detection of type VII pneumococcal polysaccharide. Agarose gels (1% wt/vol; IBF) were prepared with each buffer. For electrophoresis done at pH 9.0 or 9.2, the electrolyte compartments contained the same buffer as used in the agarose gel. Otherwise, electrophoresis was performed with 0.05 M barbital of the same pH as used for the agarose gel in the electrolyte compartments. Serial twofold dilutions of antigen in PBS were run, using each buffer to determine sensitivity. The optimal compositions were determined by independently varying the borate or boronate concentration and pH using sodium barbital or NaCl to maintain a sodium ion concentration of 50 to 75 mM. Sensitivity for pneumococcal polysaccharide types III and XIV was determined in the same manner for selected buffers.

Comparison of agarose from different sources. Three lots of agarose were evaluated: (i) IBF agarose obtained from a *Gracilaria* agar; (ii) SeaKem agarose (Marine Colloids) isolated from a *Gelidium* agar; and (iii) a premarketing sample of agarose (RE7257) obtained from Marine Colloids isolated from *Gracilaria* agar. Agarose gels containing 1% (wt/vol) IBF agarose or 0.75% (wt/vol) SeaKem or RE7257 agarose were prepared using barbital (0.05 M, pH 8.6) and 0.024 M SPB-0.05 M-barbital (pH 8.6) buffers. Sensitivity for detection of pneumococcal polysaccharides dissolved in PBS and NHS was determined using each agarose.

False positives obtained with NHS. The human serum used in this report for preparing solutions of pneumococcal polysaccharides gave a precipitate with pneumococcal antisera in the absence of added antigen. This serum was obtained from a male subject with type O, Rh_0-negative blood. A partial phenotype revealed that he was Le^a and Le^b negative and a secretor. Serum from this subject was tested against omniserum and types VII and XIV monovalent antisera using IBF agarose (1% wt/vol) and barbital (0.05 M, pH 8.6) buffer.

RESULTS AND DISCUSSION

Buffer composition. The minimal detectable concentrations of type VII polysaccharide found using IBF agarose and different buffers are shown in Table 2. Sensitivity for detection of type VII polysaccharide generally increased in the pH range of 8.2 to 9.2; however, under the more alkaline conditions, sensitivity for detection of type III polysaccharide decreased. Optimization of buffer conditions, therefore, required a balance of the competing effects of borate-polysaccharide complex formation (6, 16) with inhibition or dissolution of antigen-antibody precipitates (13). Buffer systems incorporating SPB gave greater sensitivity than did systems containing PBA at the same pH and concentration of boronic acid. This observation is consistent with the assumption that an increased negative charge, resulting from ionization of the sulfonate group in complexes with SPB (7), would result in greater mobility of a neutral polysaccharide at lower ratios of boronate to polysaccharide.

A major disadvantage of buffer systems containing optimal concentrations of PBA was encountered using antisera from the Statens Seruminstitut. These antisera are normally supplied containing methylene blue as a coloring agent (E. Lund, Statens Seruminstitut, Copenhagen, Denmark; personal communication). With such antisera, a dark blue band

TABLE 2. *Optimal buffer compositions for detection of type VII polysaccharide in IBF agarose*

Buffer[a]	Composition (mM)[b]				pH	Minimal detectable concn of type VII polysaccharide (μg/ml)
	BAR	BOR	PBA	SPB		
BAR	50				8.2–8.8	ND[c]
BOR		100			9.2	3.2
PBA			75		8.8	0.8
SPB				37	8.6	1.6
BAR/BOR	38	50			9.0	1.6
BAR/PBA	40		37		8.8	0.8
BAR/SPB	50			24	8.6	0.8

[a] BAR, Barbital; BOR, boric acid.

[b] Concentrations are expressed as the total acid plus salt, and for buffers containing derivatives of boron they represent equivalents of boron.

[c] ND, Not detectable at up to 25 μg of antigen per ml using polyvalent or monospecific antisera.

formed midway between the antigen and antibody wells and obscured detection of a precipitate. The blue band could be removed by washing the agarose with saline or water after electrophoresis, but this required additional time. Antisera without methylene blue were not evaluated using optimal concentrations of PBA because PBA was found to be a potent lacrimator. SPB did not have this disadvantage, and in subsequent experiments the optimal 0.024 M SPB-0.05 M barbital buffer (pH 8.6) was used.

Comparison of agarose from different sources. Representative CIE results for the detection of type VII polysaccharide in PBS and NHS using SPB-barbital buffer and each of the agarose preparations are shown in Fig. 1. Comparison of the results obtained with PBS solutions of antigen showed only slight differences (one dilution or less) in sensitivity with the different agarose preparations. Greater differences in sensitivity were observed using serum solutions of antigen. In this case, RE7257 agarose gave superior sensitivity to the other agarose preparations. In addition, the precipitin lines observed using RE7257 agarose were more distinct and were located farther from the antigen well. In SeaKem agarose, isolated from *Gelidium* agar, a precipitin arc formed on the cathodal side of the antigen well containing NHS (Fig. 2). The anodal tailing of this arc interfered with the detection of weak antigen-antibody reactions located close to the anodal side of the antigen well. This phenomenon occurred to a lesser extent in IBF and RE7257 agarose isolated from *Gracilaria* agar. SeaKem and RE7257 agarose are purified by similar procedures (R. B. Cook, Marine Colloids, Rockland, Me.; personal communication). Therefore, the intensity of this cathodal arc may be related to the variety of agar used for isolation of the agarose.

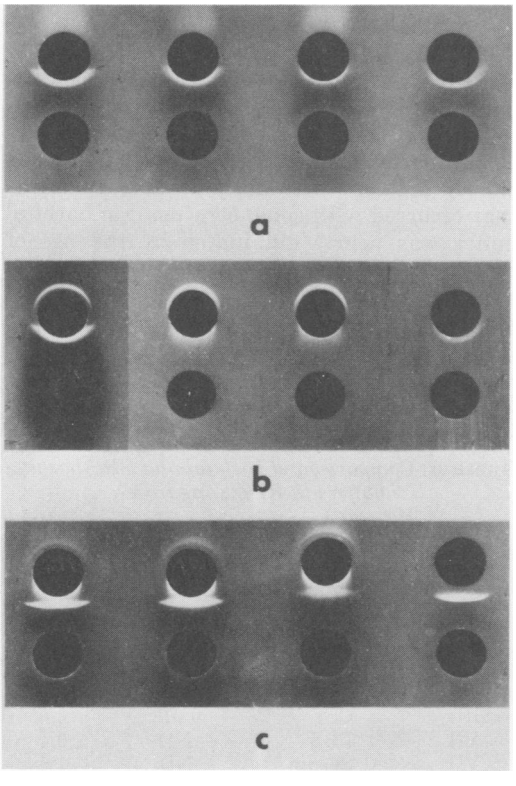

FIG. 1. *Comparison of agarose from different sources for detection of type VII polysaccharide in PBS and NHS. Antigen concentrations (left to right): 6.4 μg/ml in NHS; 3.2 μg/ml in NHS; 1.6 μg/ml in NHS; and 1.6 μg/ml in PBS. (a) One percent IBF agarose; (b) 0.75% SeaKem agarose; (c) 0.75% RE7257 agarose. Upper well of each pair contained antigen solution.*

False positives. The NHS in these experiments gave a precipitin line in the absence of added antigen between the antigen and antibody wells when barbital buffer was used (Fig. 3). This effect was independent of the source of the agarose; however, various degrees of precipitate formation were observed with sera from different individuals. Since soluble blood group substances had been reported to cross-react with pneumococcal type XIV antisera (15), we assumed that this effect might be related to blood group substances in serum. In this context, NHS reacted with omniserum and monovalent type XIV antiserum but not with monovalent type VII antiserum (Fig. 4). A large number of sera have not been tested; however, our preliminary results are consistent with the assumption that cross-reactivity with soluble

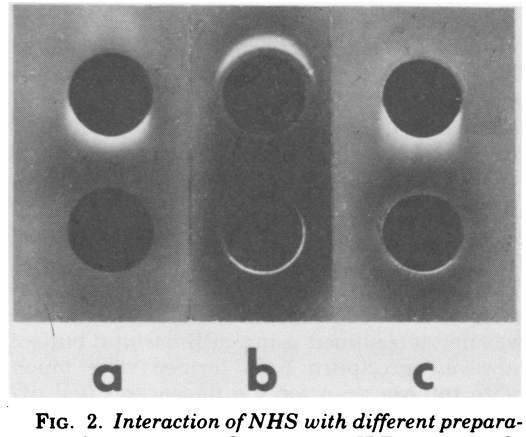

FIG. 2. *Interaction of NHS with different preparations of agarose. (a) One percent IBF agarose; (b) 0.75% SeaKem agarose; (c) 0.75% RE7257 agarose. Upper well of each pair contained NHS.*

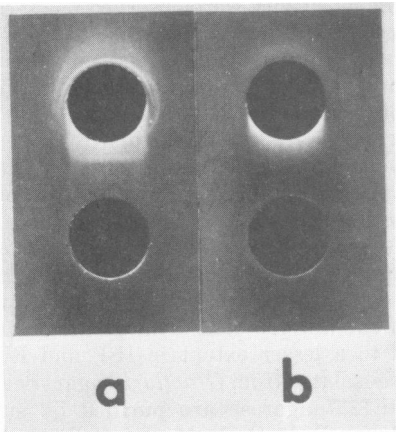

FIG. 3. *False-positive reactions observed with NHS. (a) Barbital buffer (0.05 M, pH 8.6); (b) SPB-barbital buffer (optimal composition). Upper well of each pair contained NHS.*

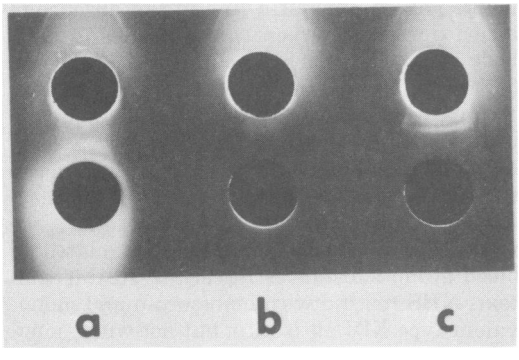

FIG. 4. *Reaction of NHS with different pneumococcal antisera. (a) Type XIV antiserum; (b) type VII antiserum; (c) omniserum. Upper well of each pair contained NHS.*

blood group substances in serum may lead to false-positive reactions for pneumococcal antigens. A precipitin line was not observed using SPB-barbital buffer, although a less distinct precipitate was sometimes present (Fig. 3).

Concentration of peumococcal antigens in urine. A 20-fold concentration of pneumococcal polysaccharide in urine using ethanol precipitation has been reported (3). Our results confirmed the earlier observation of increased sensitivity. The maximal sensitivity of the method was not determined using SPB-barbital buffer; however, precipitin lines formed were much more intense than for the unconcentrated urine. No problem with interfering substances in the concentrated urine was encountered.

Comparison of barbital and SPB-barbital buffers. Comparative results for sensitivity for the three antigen types tested using barbital and SPB-barbital buffers are summarized in Table 3. The highest concentration of antigen tested in each case was 25 μg/ml. Neither type VII nor XIV was detectable using barbital buffer and omniserum. Type XIV antigen, however, could be detected at 6.4 μg/ml in PBS using barbital buffer and monovalent antiserum. In this case, the precipitin line was faint and near the antibody well. Anodal mobility of type XIV antigen has been reported previously by Coonrod and Rytel (3); however, this is in conflict with other reports of cathodal mobility for this antigen (2, 9). Our results are consistent with the assumption that heterogenicity or decomposition in the antigen preparation could result in the presence of an antigenically similar material that has good anodal mobility and is detectable only with more potent monovalent antiserum.

Conclusions. CIE using barbital buffer was ineffective in detecting pneumococcal types VII and XIV polysaccharides. The incorporation of SPB in the buffer greatly increased the sensitivity of CIE for these two capsular types without greatly reducing the sensitivity for type III. In addition, the optimal buffer composition was 0.05 M barbital containing 0.024 M SPB at pH 8.6. A problem with false-positive reactions that occurred with some sera using a barbital buffer was nearly eliminated by the use of SPB-barbital buffer. An adverse interaction of serum with certain lots of agarose was observed and may be related to the source of the agar from which the agarose is extracted. Best results were obtained using an agarose isolated from *Gracilaria* agar and provided by Marine Colloids.

TABLE 3. *Comparison of barbital and SPB-barbital buffers in RE7257 agarose*

Antigen type	Solution	Minimal detectable antigen concn (μg/ml)	
		Barbital[a]	SPB-barbital[b]
III	PBS	0.012	0.05
III	Serum	0.05	0.05
III	CSF	0.012	0.05
VII	PBS	ND[c]	0.4
VII	Serum	ND	1.6
VII	CSF	ND	0.4
XIV	PBS	ND	0.8
XIV	Serum	ND	1.6
XIV	CSF	ND	0.4

[a] 0.05 M, pH 8.6.
[b] 0.024 M SPB, 0.05 M barbital, pH 8.6.
[c] ND, Not detectable at up to 25 μg of antigen per ml.

ADDENDUM

Shortly after the development of this buffer system, *S. pneumoniae* was isolated by our laboratory from the CSF of a patient with meningitis. The CSF obtained from this patient was negative by CIE using barbital buffer but was strongly positive using SPB-barbital buffer. Capsular typing by the quellung method showed that the organism was type VII.

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