Technical method

Countercurrent immunoelectrophoresis: improved detection of pneumococcal capsular antigens in sputum by incorporation of a carboxylated derivative of phenyl boronic acid

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Counter-current immunoelectrophoresis (CIEP) is now a well-recognised technique for the detection of pneumococcal antigens in body fluids. Much has been reported on its use in pneumococcal chest infections, but reports vary as to its ability to detect all pneumococcal types. Pneumococcal capsular types 7 and 14 could not be detected by Kenny *et al.* (1972) or Coonrod and Rytel (1973). El-Refaie and Dulake (1975) could not detect type 14. Other authors report no difficulties in detecting these types (Tugwell and Greenwood, 1975; Spencer and Savage, 1976). In these studies there was no agreement on the test pH, which varied between 6.6 and 8.6.

During CIEP antibody will migrate towards the cathode by electroendosmosis, and the negatively charged capsular antigen will migrate towards the anode by electrophoresis. The capsular polysaccharides of type 7 and type 14 are neutral or carry a slight positive charge and hence cannot be expected to move towards the anode by electrophoresis. Anhalt and Yu (1975) describe the use of phenyl boronic acid derivatives in the buffer system to improve the sensitivity of CIEP by binding to the polysaccharide and increasing the likelihood of anodal migration. In later studies, *m*-carboxyphenyl-boronic acid (*m*CPB) was found to give the best results (J. P. Anhalt, personal communication) and was incorporated in the agarose support at a pH of 8.6.

The present study was carried out to assess whether *m*CPB could be used to improve the detection of pneumococcal capsular antigens in sputum, particularly those of type 7 and type 14.

Material and methods

COUNTERCURRENT IMMUNOELECTROPHORESIS

CIEP was performed at room temperature for 45 minutes using a Shandon Vokam DC power pack giving a constant current of 30 mA. The gel-support was 1% agarose on glass slides (2.5 × 7.5 cm) poured to a depth of 1 mm (3 ml/slide). Paired wells, 3 mm in diameter and 2 mm apart, were cut using a template. Ten microlitres of Omni-serum (Statens Serum Institut, Copenhagen, Denmark) was placed in the well nearest the anode and 10 µl of the specimen in the cathodal well. Up to eight specimens could be electrophoresed on a single slide. Results were read 5 minutes after completion of the electrophoresis and again after refrigeration overnight at 4°C. Barbitol buffer with an ionic strength of 0.05 and at pH 8.6 was used in the electrophoretic tank throughout.

SPUTUM SPECIMENS AND PNEUMOCOCCAL TYPING

All sputum specimens tested were received in the routine laboratory of this hospital. All were from inpatients and were cultured on the day of arrival or after overnight refrigeration. The diagnoses were varied, a large proportion being postoperative chest infections. Other reasons for requests for culture included pneumonia and purulent sputum from bronchitis and patients with other underlying chest diseases.

To ensure the presence of pneumococci in significant numbers a semiquantitative culture technique was employed (Dixon and Miller, 1965). The sputum was digested with an equal volume of *N*-acetyl-L-cysteine and then further diluted 1 in 100 with Ringer's solution. A 5 mm loop was plated on to blood agar and incubated at 37°C in 10% carbon dioxide. Only sputa containing pneumococci in almost pure culture were used in this study.

The pneumococci isolated were identified by Optochin sensitivity. A single colony was then placed in Todd-Hewitt broth with 1% glucose, incubated overnight, and centrifuged. The pellet was resuspended in a small volume of the broth and used for typing by slide agglutination with antisera supplied by the Statens Serum Institut, Copenhagen. It was thought sufficient to identify only the pool A-I, to which the pneumococcus belonged, except for types 7 and 14 which were agglutinated with their

specific antisera after the pool to which they belonged had been identified. For electrophoresis the digested sputum was used without further dilution. Statens Serum Institut Omni-serum diluted 1 in 2 and containing antibodies to all 83 types was used as the antiserum.

ANTIGEN CONTROLS

A type 7 and a type 14 pneumococcus supplied by NCTC, Colindale were incubated overnight in Todd-Hewitt broth with 1% glucose; the resulting cultures were ultrasonicated and centrifuged. The supernatants were used as antigen controls.

PREPARATION OF *m*CPB

Carboxylated phenyl boronic acid was prepared in the Department of Chemistry, University College, University of London. A modification of the method of Bean and Johnson (1932) was used to manufacture *m*-tolylboronic acid, which was then used in the final preparation of *m*CPB using a modification of the method of Richter (1901). The infrared spectrum of the purified chemical agreed with those values reported by Anhalt (personal communication).

BUFFERS AND GEL-SUPPORT

Barbital buffer, pH 8.6, was used in the electrophoretic tank at all times (ionic strength 0.05 M: barbituric acid 0.92 g, sodium barbital 10.3 g, sodium acetate 11.3 g, and distilled water to 1 litre).

The gel-support was made with agarose for

electrophoresis supplied by BDH Laboratory Reagents. It was dissolved in either the barbital buffer or *m*CPB/barbital buffer (ionic strength 0.03 M *m*CPB and 0.05 M barbital: *m*CPB 0.99 g, sodium barbital 2.06 g, calcium lactate penta-hydrate 0.08 g, dissolved in 200 ml of distilled water).

To vary the pH of the test, the buffer in which the agarose was dissolved was adjusted with *m*-HCl or *m* NaOH. The gel-support was used at three pHs, 8.6, 7.1 (the lowest pH before *m*CPB precipitated), and 6.6 (barbital buffer only).

Results

The results of the electrophoresis of 80 sputa are given in Table 1. Refrigeration overnight increased the percentage of positives for all buffer/gel-support systems by between 12% and 17% except with the barbitone buffer at pH 8.6 when the increase was 34%. The *m*CPB/barbitone buffer at pH 7.1 detected the highest proportion of pneumococcal antigens (92%).

There were four sputa containing type 7 and four containing type 14 pneumococci. All buffer systems were able to detect antigen in some of these sputa. As a test of sensitivity, the control antigens were titrated in the different buffer systems (Table 2); *m*CPB/barbitone at pH 8.6 proved the most sensitive system for detecting these controls. This was also the only system to detect all the type 7 pneumococci in sputa.

Five specimens were negative for antigen with all

Table 1 Numbers of sputa in which pneumococcal antigen could be detected using the different buffer/gel-support systems, after refrigeration

	Buffer/gel-support				
	Barbitone pH 8.6	<i>m</i> CPB/barbitone pH 8.6	Barbitone* pH 7.1	<i>m</i> CPB/barbitone* pH 7.1	Barbitone pH 6.6
All sputa tested (total 80)	60 (75%)	69 (86%)	60 (75%)	72 (92%)	60 (75%)
Type 7 (total 4)	3	4	3	3	3
Type 14 (total 4)	2	2	2	3	3

*Only 78 sputa tested.

Figures in parentheses—percentages of total examined.

Table 2 Antigen controls titrated against Omni-serum using the different buffer/gel-support systems, after refrigeration

	Reciprocal maximum titre detectable				
	Barbitone pH 8.6	<i>m</i> CPB/barbitone pH 8.6	Barbitone pH 7.1	<i>m</i> CPB/barbitone pH 7.1	Barbitone pH 6.6
Type 7	8	16	4	4	2
Type 14	4	16	4	4	4

buffers, of which one contained type 14 pneumococci. The other negative specimens contained one type from pool A, two from pool D, and one from pool F. Two sputa gave a precipitation line only when a gel-support with *m*CPB was used (pH 8·6 and 7·1); one contained a type 7 pneumococcus, and the other, one from pool D.

Discussion

Boric acid will form anodal migrating complexes only at a high pH. However, its substituted derivatives are able to complex at a lower pH (Garegg and Lindberg, 1961), allowing their use over the pH range required for detecting pneumococcal capsular antigen.

Buffers containing *m*CPB were more effective, detecting antigen more frequently in the sputa than did the barbitone buffer. This was most marked at the more acid pH. No pH effect was detected with the barbitone buffers, all detecting antigen in 75% of the sputa after overnight refrigeration.

Both buffer systems seemed able to detect type 7 and type 14 capsular antigen in the sputa, but the *m*CPB/barbitone gel-support at pH 8·6 was most sensitive in detecting the control antigens. The greater sensitivity for the controls at the more alkaline pH can be explained by the higher binding potential of boric acid derivatives at these pHs.

Substituted phenyl boronic acid derivatives increase the sensitivity of CIEP for pneumococcal antigens in sputa, including the poorly charged or neutral capsular antigens. The use of these derivatives should be further explored in other CIEP detection systems.

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