

# An improved test for *Haemophilus influenzae* precipitins in the serum of patients with chronic respiratory disease

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**SYNOPSIS** The detection of antibacterial precipitins in the serum of patients with chronic lower respiratory infections has been shown to be of great value in the assessment of pathogenicity of the corresponding bacterial species in the sputum. The test used hitherto, however, is time-consuming and cumbersome owing to the need to distinguish, by immunoelectrophoresis, between antibodies specific to the organism in question and those which react with antigens common to various Gram-negative species. A method has now been devised for the preparation of an extract of *Haemophilus influenzae* containing species-specific antigens only. Use of this extract obviates the need for immunoelectrophoresis and improves the sensitivity of the test.

The value of the detection of serum precipitins as an index of chronic *Haemophilus influenzae* infection of the lower respiratory tract was discussed by Burns and May (1967), and extension of the principle to the diagnosis of chronic infection by *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and enterobacteria was subsequently reported (Burns and May, 1968; Burns, 1968a and b). The findings obtained in these investigations in London were later confirmed by Burns (1972) in subjects with chronic bronchitis, asthma, or pulmonary suppuration studied in Australia; and May, Herrick, and Thompson (1972) used the precipitin test to define patterns of bacterial infection in patients with cystic fibrosis. The test was also used by Jenne, MacDonald, Lapinski, Bratberg, and Hall (1970) to monitor the response of patients with chronic haemophilus infections to chemotherapy, while May, Peto, Tinker, and Fletcher (1973) used it in a study of the relationship between smoking and haemophilus infection.

In this laboratory in the last five years some 5000 precipitin tests have been carried out on the serum of subjects with various respiratory disorders, and experience gained from these studies, together with those in the published reports, leaves us in no doubt of the value of the test in the assessment of the pathogenic significance of different bacterial species in chronic respiratory disease. This experience,

together with details of the technique used hitherto, has been summarized by May (1972).

The test, however, has its limitations, and prominent among these is the cumbersome and time-consuming nature of the technical procedure. This arises from the fact that crude antigen extracts, prepared by ultrasonic disruption of bacterial suspensions, of the three Gram-negative species against which patients' serum is routinely tested (*H. influenzae*, *P. aeruginosa*, *K. pneumoniae*) contain some antigens common to all three species in addition to those specific to the species in question. If serum is tested against such extracts by double diffusion in agar gel it is often not possible to distinguish precipitates arising from reactions between antibodies and specific antigens from those between 'non-specific' components. In order to make this distinction, serum shown by the double-diffusion test to contain antibody is retested in agar against electrophoresed antigen extract. Burns and May showed, by neutralization tests, that the specific antibodies found most commonly in patients' sera reacted with two antigens, one of which migrated towards the cathode during electrophoresis in agar at pH 8.2 while the other remained at the starting point. The designations H<sub>1</sub> and H<sub>2</sub> respectively were used for these antigens in tests of *H. influenzae* extracts; P<sub>1</sub> and P<sub>2</sub> in tests of pseudomonas extracts; and E<sub>1</sub> and E<sub>2</sub> (E for enterobacteria) in tests of klebsiella extracts. Antigens which migrated towards the anode were shown in the main to be common to each of the Gram-negative species.

<sup>1</sup>Professor Robert May died on 23 February 1974.

Thus the practical test involves an initial screening of the patient's serum by the double-diffusion technique followed, if positive, by immunoelectrophoresis to determine whether or not antibody to specific antigens is present. This procedure, apart from its cumbersome nature, has the additional disadvantage that immunoelectrophoresis is less sensitive than the double-diffusion test, with the result that some sera giving weak positive reactions in the latter may fail to give a precipitate when tested by immunoelectrophoresis. 'False negative' readings may therefore occur. Further, the use of an antigen extract containing a mixture of specific and non-specific components precludes the adaptation of the test to obtain a quantitative estimate of the amount of specific antibody present.

These difficulties could be overcome if an extract containing only specific antigens could be prepared. Immunoelectrophoresis would then not be required and full advantage of the greater sensitivity of the double-diffusion test could be taken. It should also be relatively simple to devise a quantitative procedure for the determination of antibody titre. In this paper we describe a method for the preparation of the specific antigens of *H. influenzae* (H<sub>1</sub> and H<sub>2</sub>).

## Material and Methods

### MICHAELIS' BUFFER

#### Solution A

1/7M sodium acetate + 1/7M sodium barbitone (19.43 g sodium acetate + 29.43 g sodium barbitone in 1 litre of de-ionized water).

#### Solution B

0.1N hydrochloric acid.

For pH 8.0, 400 ml A + 208 ml B + de-ionized water to 2 litres.

For pH 5.0, 400 ml A + 704 ml B + de-ionized water to 2 litres.

### PRELIMINARY OBSERVATIONS ON FRACTIONATION OF THE ANTIGEN EXTRACT

Knowing that at pH 8.2 H<sub>1</sub> and H<sub>2</sub> antigens could be separated electrophoretically from non-specific antigens (H<sub>3</sub>, H<sub>4</sub>, and H<sub>5</sub>) it seemed probable that bulk separation could be achieved by ion-exchange chromatography. The choice of ion-exchanger was made by a pilot experiment using available materials—one anionic exchanger, DEAE-Sephadex A-25, and one cationic exchanger, CM-Sephadex C-50.

The exchangers were swollen in Michaelis' barbital sodium acetate buffer at pH 8.0 for the A-25 and pH 5.0 for the C-50. After equilibration equal quantities of crude antigen dissolved in the same

buffers were mixed with each Sephadex 'slurry'. The Sephadex was allowed to sediment and the supernatant fluid was then removed, dialysed, and freeze-dried. The resultant powders were re-dissolved in de-ionized water and their antigenic composition was determined in immunoelectrophoresis tests using human serum known to contain the full range of antibodies to *H. influenzae*.

### Result

The supernatant fluid from the A-25 exchanger contained H<sub>1</sub> and H<sub>2</sub> antigens only, while that from the C-50 exchanger contained the full range of haemophilus antigens.

Thus at pH 8.0 DEAE-Sephadex can absorb out the non-specific antigens from a solution of crude *H. influenzae* extract, and it should be possible to achieve the practical preparation of H<sub>1</sub> and H<sub>2</sub> antigens in bulk simply by adding A-25 exchanger at pH 8.0 at the appropriate stage in the preparation of the extract.

### THE PRACTICAL PROCEDURE

1 Ten g DEAE-Sephadex A-25 is mixed with 250 ml buffer, pH 8.0. Complete swelling takes place with three changes of buffer in 24 hours at room temperature.

2 Four hundred and fifty ml of a suspension of *H. influenzae* is prepared as described by May (1972), using buffer at pH 8.0 as the suspending fluid instead of distilled water.

3 The suspension is divided into 25 ml aliquots for ultrasonic disintegration (May, 1972), the aliquots being subsequently pooled and centrifuged at 10000 rpm for 30 minutes at 10°C.

4 The supernatant is mixed with 40 ml DEAE-Sephadex slurry and allowed to stand at room temperature for 24 hours with frequent stirring.

5 The supernatant is removed and cleared of all traces of Sephadex by filtration through a Seitz clarifying filter. It is then dialysed against 0.02M NH<sub>4</sub> HCO<sub>3</sub> for seven days and freeze-dried.

6 For use the antigen is dissolved in phosphate-buffered saline containing sodium azide 1 g per 100 ml. Two concentrations are used in the double-diffusion test—10 mg/ml and 1 mg/ml. Inclusion of the weak solution reduces the risk that some sera containing low antibody concentrations may present as 'false negatives' owing to failure of the precipitate to form in the presence of excess antigen.

### Results

The table shows the results obtained in double-diffusion tests of serum samples of 20 patients. Also shown are the results obtained by double-diffusion

Serum No.	Occurrence of Precipitation when Serum Tested against				
	Crude Extract			Purified Extract	
	20 mg/ml	10 mg/ml	Immuno-electrophoresis	10 mg/ml	1 mg/ml
556	+	+	H <sub>1,2,3,4,5</sub>	+	+
872	+	+	H <sub>1,2</sub>	+	-
3870	+	+	H <sub>2,3,5</sub>	+	+
3938	+	+	H <sub>1,2,3</sub>	+	+
3960	+	+	H <sub>1,2,3</sub>	+	+
4014	+	+	H <sub>1,2,3,4</sub>	+	+
901	+	+	H <sub>3</sub>	-	-
3566	+	+	H <sub>5</sub>	-	-
3910	+	+	H <sub>3,4</sub>	-	-
3977	+	+	H <sub>3,5</sub>	-	-
3978	+	+	H <sub>4</sub>	-	-
2757	-	-	-	-	-
3195	-	-	-	-	-
3812	-	-	-	-	-
3915	-	-	-	-	-
3933	-	-	-	-	-
3963	-	-	-	-	-
2333	+	+	H <sub>3,5</sub>	+	+
3939	+	+	H <sub>3,4</sub>	+	-
796	-	+	-	-	+

Table Comparison of results of precipitin tests on the sera of 20 patients using crude and purified *H. influenzae* extracts

and immunoelectrophoresis testing using the original crude extract at 20 mg/ml and 10 mg/ml. The results have been grouped into five categories:

1 SAMPLES 556, 872, 3870, 3938, 3960, 4014 These were shown by immunoelectrophoresis to contain both specific (H<sub>1</sub> or H<sub>2</sub>) and non-specific (H<sub>3</sub>, H<sub>4</sub>, or H<sub>5</sub>) antibodies and they also reacted with the specific extract at both concentrations tested. Both tests were in agreement.

2 SAMPLES 901, 3566, 3910, 3977, 3978 Immunoelectrophoresis indicated that these contained only non-specific antibody and they failed to react with specific extract. Both tests were in agreement.

3 SAMPLES 2757, 3195, 3812, 3915, 3933, 3963 These were shown by both tests to contain no antibody. Both tests were in agreement.

4 SAMPLES 2333, 3939 Immunoelectrophoresis using crude extract indicated that these contained only non-specific antibody. However, they gave precipitates in double-diffusion tests with purified specific antigen. These sera contained specific antibodies but they were too weak to be detected by immunoelectro-

phoresis. The original test, therefore, gave a false negative result.

#### 5 SAMPLE 796

This sample reacted with crude antigen at 10 mg/ml but not at 20 mg/ml. The antibody was too weak to be detectable by immunoelectrophoresis but did react in a double-diffusion test with purified specific antigen at 1 mg/ml.

The original test gave a false negative result.

#### Discussion

These findings confirm that the precipitin test as previously described (Burns and May, 1967) inevitably gives some false negative results, and that the number of these can be reduced by using the purified specific antigen extract, described here, in a double-diffusion test. This modification not only allows full advantage to be taken of the high sensitivity of the double-diffusion test but also, by obviating the need for immunoelectrophoresis, greatly reduces the time required for the test.

It is perhaps worth pointing out that, apart from the addition of DEAE-Sephadex in the preparation of the antigen, another change has been made to the procedure originally described. This relates to the dialysis against NH<sub>4</sub> HCO<sub>3</sub> (stage 5 above) instead of tap water as used previously. It has been found that the resultant powder after dialysis against NH<sub>4</sub> HCO<sub>3</sub> is completely water-soluble at the working strengths whereas that prepared after dialysis against water was only partly so (Burns and May, 1967).

Since antigens specific to *P. aeruginosa* and enterobacteria have similar electrophoretic mobilities to those of *H. influenzae* it should be possible to make purified preparations of them by a procedure similar to that described above. This has been confirmed in principle by preliminary attempts to purify pseudomonas extract, although incomplete absorption of P<sub>3</sub> has been observed in some batches. Experiments are now proceeding to determine the technical modifications necessary to overcome this difficulty.

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