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Detection of capsulated *Haemophilus influenzae* in chest infections by counter current immunoelectrophoresis

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SUMMARY The application of counter current immunoelectrophoresis to the detection of *Haemophilus influenzae* capsular antigen in sputum is described. The method, technically simple, provided results within 30 minutes. *H. influenzae* capsular antigen was detected in 12% of patients and in 54·8% of the *H. influenzae* strains isolated. The test was not impaired by prior antibiotic therapy.

The isolation of *Haemophilus influenzae* from patients with respiratory tract infection, continues to present clinicians and clinical bacteriologists with a problem of interpretation since the species can form part of the normal upper respiratory tract flora. Moreover, if antibiotic therapy is started before culture of the sputum it may prevent isolation of the causative organism.

Immunological methods are at present being used to detect bacterial antigens in sputum (Tugwell and Greenwood, 1975; Coonrod and Rytel, 1972; El Refaie and Dulake, 1975) and have proved specific and of diagnostic and prognostic value.

In this study, counter immunoelectrophoresis (CIE) has been adapted to the detection of *H. influenzae* capsular antigen in sputum.

Material and methods

PATIENTS

Consecutive sputa from 300 patients aged 28 to 90 years with suspected chest infection were tested over a two-month period (May-July 1976). Over 70% of the patients had received antibiotic therapy before bacteriological investigation.

For the purpose of this study the results of more than one specimen received from any patient in a week were treated as one specimen.

SPUTUM CHARACTERISTICS AND CULTURE

On receipt the sputa were recorded, according to their macroscopic appearance, as either salivary, mucoid, or purulent. Salivary specimens were not examined. Of 300 sputa tested, 61% were mucoid and 39% purulent. For culture, the sputa were diluted 1 in 2 with Sputolysin[1] and thoroughly mixed on a Vortex mixer to speed homogenisation. One standard loopful, containing 10 μl of the homogenate, was mixed in 10 ml of sterile peptone water. From this, one standard loopful was inoculated onto chocolate agar and incubated in air plus 5% CO_2. A second loopful was inoculated onto blood agar and incubated anaerobically, and a third one onto MacConkey agar and incubated aerobically. All cultures were incubated at 37°C for 18 hours.

Gram-stained films of homogenate were examined for the presence of neutrophils and scored according to their number per high-power field (HPF), using 100× oil immersion lens, as less than 5, 5-10, 10-20, and over 20. Direct sensitivity tests were done if a predominating organism was present in the Gram-stained film. On chocolate agar five colonies of *H. influenzae* represent 10^6 organisms per ml of original sputum and were taken as significant. Identity was confirmed by X and V factor dependence.

SALIVA CONTROLS

Thirty healthy volunteers provided salivary specimens for an investigation into the carriage rate of capsulated types of *H. influenzae* in the oropharynx. These were treated in the same way as the sputa.

COUNTER CURRENT IMMUNOELECTROPHORESIS

This was performed according to the method of El Refaie and Dulake (1975) with only minor modi-

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[1]Sputolysin is a product of Calbiochem, California, USA.

fications. The pH of the stock buffer was 8·6 and that of the agarose 6·6. Glass plates 8 × 8 cm were covered with 8 ml of 1% agarose to give an approximate depth of 0·6 mm. The well pattern was made with a template to give three parallel columns of nine paired wells, 3 mm in diameter, with a 2 mm inter-well space in each column. The antisera were obtained from Hyland (Costa Mesa, California, USA) as individual sera, some of which were pooled and used as polyvalent serum for screening procedures. Any sputum giving a precipitin line with polyvalent serum was typed using the individual sera. Two microlitres of the sputum diluted 1 in 2 with Sputolysin was placed in the well nearest the anode, and 2 μl of the antiserum in the well nearest the cathode. Electrophoresis was carried out at room temperature using a constant voltage of 200 volts for 20 minutes. The plates were then examined with an oblique light for the presence of precipitin lines which occurred midway between the two wells. All plates were held at 4°C and re-examined after two and 18 hours.

To test for the specificity of the antisera and to check for cross reactions with other organisms isolated with *H. influenzae*, suspensions of *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus viridans*, *Klebsiella aerogenes*, *Neisseria catarrhalis*, *Citrobacter* sp, and *Candida* sp. were prepared in saline, Sputolysin, and 5% serum broth and tested by CIE.

Cultures of *H. influenzae* types a, b, c, d, e, and f were obtained from the National Collection of Type Culture (NCTC) and used in broth suspensions as positive controls.

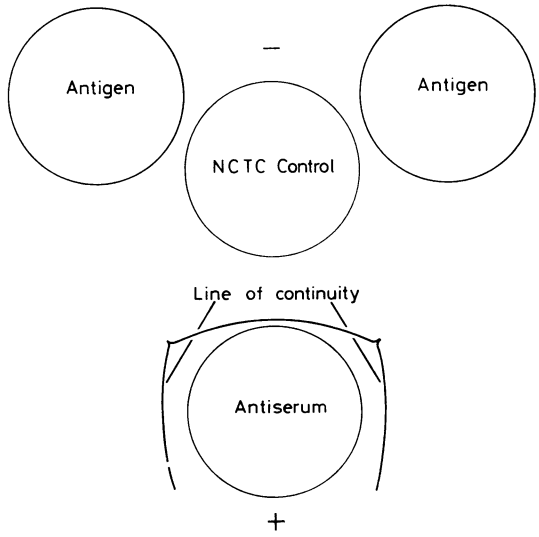

Figure *Well pattern to demonstrate lines of continuity.*

The accuracy of typing by CIE was confirmed by parallel testing with counter current immunoelectrophoresis and the Quellung reaction. Furthermore, to ensure that all capsular antigens detected by CIE had been correctly identified, tests for lines of continuity were made as indicated in the Figure: two wells contained the antigen under test, the third had the corresponding NCTC strain, and the fourth the appropriate antiserum. A continuous pattern was obtained when the test strain was identical with the control.

In order to determine the least amount of capsular antigen detectable by CIE, a simple dilution procedure was used; Levinthal broth cultures of *H. influenzae* types a to f were prepared, and, from these, 10-fold dilutions from 10^{-1} to 10^{-6} were made in sterile peptone water. Miles and Misra surface plate counts and CIE were performed on each neat and diluted suspension.

The use of CIE for the detection of pneumococcal antigen in sputum is an established procedure in this laboratory and so the frequency of pneumococcal isolation and antigen detection was also investigated in the same specimens.

Results

The relationship between purulence, *H. influenzae* isolation, and capsular antigen detection is summarised in Table 1.

Table 2 shows that *H. influenzae* was cultured from 46 of 300 sputum specimens. Quellung tests performed on the 46 *H. influenzae* strains isolated showed that 16 were capsulated; the same capsular antigens were detected in the corresponding 16

Table 1 *Relationship between purulence*, H. influenzae *isolation, and capsular antigen detection*

H. influenzae	*Neutrophils per HPF*	
	5 or <5	*>5*
Isolated	30*	16†
Capsular antigen detected	0	36

*Noncapsulated
†Capsulated

Table 2 *Relationship between culture and CIE*

CIE	*Culture*	
	Positive	*Negative*
Positive	16*	20†
Negative	30‡	234

* 2 on antibiotics
†17 on antibiotics
‡ 6 on antibiotics

sputa. Antibiotics had been taken by two of the 16 patients.

H. influenzae capsular antigen was also detected in 20 specimens from which *H. influenzae* had not been isolated and these patients had recently completed a course of antibiotics. The remaining 30 samples grew *H. influenzae* but all attempts at typing the isolated strains by Quellung and the sputa by CIE failed. These were believed to be non-capsulated strains of *H. influenzae*.

Table 3 analyses the distribution of *H. influenzae* cultured, their serotypes, and CIE results in patients with chest infections according to their clinical condition.

Table 3 *Culture, serotypes, and CIE in relation to clinical condition*

Patient's condition	*Culture*		*CIE*		*Total number*	*Serotypes*
	+	−	+	−		
Category I						
Acute bronchitis	7	0	7	0	7	2a, 3b, 2d
Exacerbation of chronic bronchitis	6	14	20	0	20	12b, 1a, 4e, 2f, 1c
Carcinoma of bronchus	1	1	2	0	2	2c
Postoperative chest infection	2	2	4	0	4	2f, 2d
Category II						
Congestive cardiac	0	1	1	0	1	1e
Pulmonary tuberculosis	0	2	2	0	2	1a, 1e
Category III						
Chronic bronchitis	24	0	0	24	24	Non-capsulated
Urinary tract infection	4	0	0	4	4	
Peptic ulcer	2	0	0	2	2	
Total	46	20	36	30	66	4a, 15b, 3c, 4d, 6e, 4f
Percentage	69·6	30·3	54·8	45·4	100	

Three categories were defined:

Category I Acute infections where CIE was positive either alone or in conjunction with culture. Type b was prevalent (15 out of 33).

Category II Severe underlying disease with negative cultures and positive CIE. This small group contained two type e and one type a.

Category III Chronic conditions and unrelated disease. CIE was negative and cultures yielded non-capsulated types of *H. influenzae*.

Two cases were of particular interest.

Case 1, a 62-year-old patient with unresolved chest infection, was referred by his general practitioner. He had received a course of ampicillin and had shown no improvement. The first sample received was purulent, showing 20 neutrophils per HPF, and culture yielded a pure growth of *H. influenzae*. The CIE performed on the sputum was positive with polyvalent serum and, when tested against individual sera, was found to be type d. Quellung reaction and CIE on the isolated strain were also positive with type d serum. After receipt of the culture report the patient was given a second course of ampicillin. A week later a sputum, then mucopurulent, was tested. Culture did not yield *H. influenzae* but CIE on the sputum still showed the presence of type d capsular antigen. A third sample received five days later was mucoid with flecks of pus and showed five neutrophils per HPF. *H. influenzae* was not recovered but capsular antigen type d was still detectable in the sputum although the line had become much weaker in intensity. Subsequent specimens from this patient neither yielded *H. influenzae* nor gave a precipitin line by CIE.

Case 2, a 71-year-old patient, was admitted to Dulwich Hospital with left ventricular failure, atrial fibrillation, and suspected bronchopneumonia. The first sputum received after admission was mucopurulent and contained 10-20 neutrophils per HPF. A significant growth of *H. influenzae* type e was isolated and its antigen was detected in the sputum. The patient was treated with co-trimoxazole; three days later another specimen gave the same results. After six days of antibiotic therapy a culture failed to yield *H. influenzae* but type e capsular antigen was detectable in the sputum. On the 12th day after admission a request for further investigation was received. The clinical diagnosis was 'acute exacerbation—rise in temperature—green sputum'. The sputum was purulent, contained 20 neutrophils per HPF, and yielded a pure growth of *H. influenzae* type f. The CIE was again positive with a faint line with type e serum and a much stronger line with type f antiserum. *H. influenzae* type f and its antigen continued to be detectable for a further three days. The patient improved on a further course of co-trimoxazole and specimens tested 18 and 23 days after admission neither yielded *H. influenzae* nor gave a precipitin line by CIE.

The two cases described illustrate the association of pus, infection, and the detection of capsular antigen. Case 2 also showed that early re-infection with a different capsular type can occur and was associated with renewed purulence and rise in temperature.

Results obtained from the routine application of CIE to the detection of pneumococcal antigen in sputum were compared with those obtained with *H. influenzae*. Of 300 sputa, 105 showed evidence of pneumococcal infection: 20 yielded a pure growth

of *Strep. pneumoniae* and were also positive for pneumococcal antigen; nine yielded *H. influenzae* and *Strep. pneumoniae*, and in these both antigens were detected; and, finally, in 76 sputum specimens pneumococcal antigen alone was detected. Thus evidence of infection with *H. influenzae* or *Strep. pneumoniae* was detected in 141 out of 300 patients with nine double infections.

The investigation into the possible carriage of capsulated strains of *H. influenzae* in the saliva of healthy volunteers showed that none grew *H. influenzae* and none was positive by CIE. Three had over 10^6 organisms per ml of *H. parainfluenzae*. Table 4 shows the least number of bacteria giving a positive CIE when 10-fold dilutions of the six NCTC antigens were used. These were found to vary according to type. The highest was obtained with type a (380 organisms per 2 μl) and the lowest with type d (290 organisms per 2 μl).

Table 4 *Relationship between minimum number of organisms present and antigen detection*

		Positive CIE	
H. influenzae *type*	*No. of organisms per ml original broth*	*No. of organisms at lowest dilution*	*No. of organisms per 2 µl*
a	190×10^5	$1{\cdot}9 \times 10^5$	380
b	180×10^6	$1{\cdot}8 \times 10^5$	360
c	160×10^5	$1{\cdot}6 \times 10^5$	320
d	145×10^5	$1{\cdot}45 \times 10^5$	290
e	160×10^6	$1{\cdot}6 \times 10^5$	320
f	175×10^6	$1{\cdot}75 \times 10^5$	350

Tests for lines of continuity performed on all CIE positive sputa and Quellung positive cultures showed a continuous pattern, thereby proving that the strains detected contained the polyribophosphate capsular antigen of *H. influenzae*. Cross reactions were not observed in this study.

Discussion

It is generally believed that capsulated strains of *H. influenzae* from chest infection form a minority of the strains isolated. However, using CIE, it was possible to identify the polyribophosphate capsular antigen of *H. influenzae* in 12% (36 of 300) of the patients and in 54·8% of the *H. influenzae* strains isolated. These differ from the results obtained by Turk and Holdaway (1967); in their study of patients mainly between 5 and 17 years old, only 5% were capsulated; type e was found to be most common, followed by type f. In the categories defined in this study (Table 3), an overall predominance of type b, followed by type e, was noted. However, absence of capsular antigen does not mean that infection was not present, but noncapsulated types were more often found in patients with chronic and unrelated diseases (Table 3, category III), implying that their infection was less severe.

The diagnostic efficiency of CIE for the typing and detection of capsulated strains of *H. influenzae* has been demonstrated. The test was not impaired by prior antibiotic therapy and has the additional advantages of speed and early detection of changes in capsular types.

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