

## **Klebsiella serotyping by counter-current immunoelectrophoresis**

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### SUMMARY

The development of a *Klebsiella* serotyping method by counter-current immunoelectrophoresis (CIE) is described.

Antisera were prepared against the capsular antigens of 72 type strains and tested for the specificity and strength of their precipitin reactions with antigens from homologous and heterologous serotypes. All antisera produced strong reactions with their homologous antigen: when diluted to titre 63 were highly specific, 3 cross-reacted strongly and 6 weakly with one other antigen. Pools of antisera for screening purposes were constructed on the basis of common cross-reactions: component serotypes of each pool could be detected strongly and specifically.

The technique is simple to perform, fairly rapid, and economical in the use of antisera. Results can be read easily and quickly and the intensity of cross-reactions compared directly. The technique appears to be more specific and is less time consuming than the Quellung method, but further assessment of its efficacy in typing routine clinical cultures is necessary.

### INTRODUCTION

In recent years multi-drug resistant klebsiellas have been implicated as the cause of many hospital-acquired infections. Treatment of patients with broad spectrum antibiotics and intestinal colonization may predispose towards auto-genous infection; resistant organisms present in the hospital environment may be transferred to susceptible patients by staff or equipment, and in either event serious infections such as septicaemia, meningitis and bronchopneumonia may ensue. Incidents of *Klebsiella* infection in hospitals have been described by Price & Sleight (1970), Selden *et al.* (1971), Montgomerie *et al.* (1970) and Noriega *et al.* (1975). The epidemiology of *Klebsiella* infections has been discussed by Martin, Yu & Washington (1971) and Casewell *et al.* (1977).

A rapid, reliable and reproducible typing method for *Klebsiella* strains is desirable for monitoring the distribution of 'hospital' strains, to study suspected incidents of cross-infection and to distinguish them from autogenous infections, thereby improving the understanding of the epidemiology, prevention and control of *Klebsiella* infections.

Bacteriocine and phage typing have not yet proved as effective in distinguishing

strains as serotyping which defines more than 72 capsular types. The Quellung reaction has traditionally been used for serotyping and an assessment of this technique using commercial antisera was made by Casewell (1975). The technique has been used successfully in epidemiological studies by several workers: Martin *et al.* (1971), Rennie & Duncan (1974), Richard (1973), and Selden *et al.* (1971). The Quellung test has certain disadvantages, namely, difficulty in distinguishing the many cross-reactions which occur within the *Klebsiella* group, difficulty in detecting weak specific reactions and those of poorly encapsulated strains, subjectivity, and tedium when a large number of strains are to be examined. Serotyping by indirect immunofluorescence (Riser, Noone & Bonnet, 1976*a*), has eliminated many of these disadvantages, but still involves long periods of microscopical examination and a subjective assessment of degrees of fluorescence. CIE is a rapid and effective technique with wide applications in microbiology. (Moody, 1976). Immunodiffusion experiments with *Klebsiella* capsular antigens and antisera (Eriksen & Henriksen, 1963; Eriksen, 1965), and work on the detection of *Klebsiella* precipitins in patients' sera (Burns, 1968) indicated that CIE could be easily adapted for *Klebsiella* serotyping.

#### *Preliminary investigations*

Preliminary investigations showed that when undiluted capsular antisera were electrophoresed in agarose gel against their homologous antigens (whole cell suspensions at an arbitrarily chosen concentration), strong precipitation lines appeared. Consistent results for all serotypes were obtained using a Tris-EDTA-NaCl buffer at pH 8.6. Most lines were detectable within an hour. A brief study was made of capsular polysaccharide extracts, but as the results were similar to those obtained with whole cell suspensions, the additional time taken to extract, ultracentrifuge and purify the polysaccharides was not considered worth while.

Many of the antisera had been made originally for a typing programme using the Quellung method. Pools of antisera based on their Quellung cross-reactions detected most of the 72 serotypes by CIE, but there were some non-specific reactions. Specific CIE typing with the component antisera of the pools was complicated by the same cross-reactions as had occurred with the Quellung method, but further dilution of the antisera, not possible with the latter technique, eliminated many of these.

A detailed assessment of the feasibility of *Klebsiella* serotyping by CIE was then made using the information gained from the preliminary investigations.

### METHODS AND MATERIALS

#### *Preparation of antisera*

Capsular serotypes of *Klebsiella* were obtained from the National Collection of Type Cultures and from the Statens Seruminstitut, Copenhagen. Formolized overnight cultures in Worfel-Ferguson broth (Difco) were checked for adequate capsular production by Indian-ink wet film. Satisfactory suspensions were used to inoculate New Zealand white rabbits, via the marginal ear vein, in a programme

of 6 injections over a two week period. Five days after the last injection a blood sample was obtained and the serum tested by the Quellung method: if titres were satisfactory, that is, if a strong reaction was observed with serum diluted 1/8, blood was drawn from the heart. Further injections were sometimes necessary to increase titres, and in a few cases the titre could not be raised above 4. An average of 100 ml of serum was obtained from each rabbit, preserved with 0.08 % sodium azide, and stored at  $-20^{\circ}\text{C}$ .

#### *CIE method*

##### *Antigen preparations*

Stock capsular serotypes of *Klebsiella* were subcultured to fresh Worfel-Ferguson agar plates and allowed to grow at room temperature for 48 h. Using a sterile Q-tip the growth was emulsified in normal saline and homogenized for a few seconds on a vortex mixer. The concentration of antigen, as whole organisms, was adjusted by comparison with an opacity standard corresponding to  $3 \times 10^4$  organisms/ml, the concentration previously used for slide agglutination tests.

##### *Plate preparation and electrophoresis*

Glass plates 100 mm square were coated with a 1 mm thickness of 1 % agarose in Tris-EDTA-NaCl buffer, pH 8.6. Parallel rows of wells, 3 mm apart, were cut with a 2.5 mm diameter suction cutter (Hoechst Pharmaceuticals Limited) using a perspex template. Each plate had 5 pairs of rows with up to 15 wells in a row. The edges of the plate were avoided, as inconsistent results had been obtained when wells were less than 15 mm from the edge. The wells were filled to the brim with antigen and antiserum using a very fine pipette. The electrophoresis tank contained 500 ml of buffer connected to the agarose by strips of Whatman chromatography paper. Constant current electrophoresis was carried out at 20 mA per slide for 90 min. Plates were then examined for precipitation lines in a strong light, against a dark background.

##### *Titration of antisera with homologous serotypes*

The first step in the development of the serotyping system was to establish optimum titres of the antisera and an appropriate antigen concentration for use when typing unknown strains. Doubling dilutions of serum, from 1/2 to 1/128, were electrophoresed against doubling dilutions, from undiluted to 1/64, of homologous antigen, in chess-board fashion. All dilutions were made in normal saline.

##### *Testing antisera for specificity*

The 72 capsular antisera were tested for specificity at titre by electrophoresis against all 72 capsular antigens. The cross-reactions detected are listed in Table 1.

##### *Composition of pools*

The contents of pools of antisera were based on the strong cross-reactions found in the early trials using the Quellung technique. Although many of these cross-reactions were resolved by CIE, it was thought possible that the system might be

Table 1. *Cross-reactions of antisera at titre*

Antiserum	Cross-reacting antigen
10	61 (weak)
22	37 (weak)
24	40 (weak)
29	12 (weak)
37	22
38	41
42	29 (weak)
53	47
64	14 (weak)

Table 2. *Pool composition*

Pool no.	Constituent antisera	Related serotypes
I	1 2 3 4 5 68	1, 4; 3, 68
II	7 8 9 11 21 26	11, 21, 26
III	6 27 46 12 29 42	6, 27, 46; 12, 29, 42
IV	13 14 15 16 64 65	14, 64, 65
V	17 18 20 22 23 37	22, 23, 37
VI	24 25 28 30 40 69	24, 40; 25, 69
VII	31 32 33 34 36 43	
VIII	38 41 44 45 47 48	38, 41
IX	49 50 51 52 54 55	
X	56 57 58 59 60 61	
XI	62 66 67 70 71 72	
XII	10 35 39 53 63	

Antiserum 19 was used alone because its optimum titre was only 2.  
Pool XII consisted of low-titre antisera mixed undiluted.

more sensitive for freshly isolated strains, and that known strong cross-reactions could persist. The final arrangement is shown in Table 2. The component antisera of each pool were diluted in sterile saline containing 0.08% sodium azide, mixed to titre, divided into volumes of 0.5 ml and stored at  $-20^{\circ}\text{C}$ .

The ability of each pool to detect its corresponding capsular antigens was checked. Subsequently the pools were tested for cross-reactions with all 72 capsular serotypes.

## RESULTS

### *Titration of antisera with homologous serotypes*

The titre was taken as the highest dilution of antiserum which gave a satisfactory precipitation line with the highest dilution of antigen. A well-defined endpoint was obtained with most antisera: titres ranged from 4 to 128 with the majority at 8 and 16. The antigens, however, reacted equally over a range of dilutions against their antisera at optimum titre: to accommodate differences in these ranges of reactivity, an intermediate dilution of the antigen preparation of 1/6 was chosen for the future screening of freshly-isolated strains. This dilution was used for the specificity and pool tests and was found to be satisfactory for all serotypes.

Precipitation lines were generally thin, sharp, and midway between the two wells. Rather blurred lines, close to the antigen well, were obtained with the few cultures that produced very tenacious capsular material. This did not affect their ease of recognition.

#### *Specificity of antisera*

The cross-reactions of the antisera when tested at their optimum titres against all 72 antigens are shown in Table 1. Three strong and 6 weak cross-reactions were detected: strong reactions were of equal or near equal intensity to the specific reaction, and weak reactions were much less intense. The remaining 63 sera were completely specific.

#### *Testing of pools*

All the pools gave strong reactions with the serotypes corresponding to their component antisera, with the exception of pools IX, X and XII, which reacted weakly with types 51, 60 and 63 respectively. 1 ml of each of the latter pools was supplemented with 0.1 ml of the appropriate undiluted antiserum. On retesting them satisfactory results were achieved.

The specificity tests showed that the pools reacted only with their component serotypes with the exception of pool XII, which also reacted with serotypes 47 and 61 due to previously determined cross-reactions with antisera 53 and 10.

### DISCUSSION

From our results CIE appears to have certain advantages over the Quellung method, the most important of these being greater specificity. With sera at their optimum titre, CIE detected 3 strong and 6 weak cross-reactions, whereas in previous tests the Quellung method had detected 11 strong and 15 weak cross-reactions. CIE completely resolved complex cross-reactions, for example, those between serotypes 11, 21 and 26, which had been almost impossible to distinguish by the Quellung technique despite the fact that the optimum titres of the antisera were identical for both methods. The cross-reactions that persisted (Table 2) presented few problems because serotypes could be identified by their pattern of reaction (Casewell, 1975; Riser, Noone & Poulton, 1976*b*); for example, a strain of serotype 47 could be identified by its positive reaction with antisera 47 and 53, whereas serotype 53 reacted only with its specific antiserum. With some badly cross-reacting antisera, several attempts had to be made to achieve the required specificity; previous experience had shown that antigens from different sources and different stocks of rabbits produced antisera of varying specificity. Nevertheless some cross-reactions appeared to represent inherent relationships and those between serotypes 11, 21 and 26; 12, 29 and 42; 22 and 37; 38 and 41; and 47 and 53 have also been described by other workers (Casewell, 1975; Edwards & Ewing, 1972). The CIE method was moderately economical in the use of antiserum, approximately 5  $\mu$ l being used for each test. The titres of the majority of antisera were in the range 8–16, so that 1 ml of undiluted serum would give 1600 or 3200

tests. Twenty-eight antisera had higher titres by CIE than Quellung, 24 antisera had the same titre, and 15 had lower titres by CIE. The variation in sensitivity is probably due to the differing rates of electrophoretic mobility of antigens towards the anode, which is in turn governed by the number of polar groups on the antigen, and thus its net negative charge at the working pH.

CIE proved to be less tedious and time consuming than Quellung for examining large numbers of cultures. Ten strains could be tested simultaneously with 12 pools, or 20 strains could be tested against specific components of pools. Setting up a run, including plate and antigen preparation, filling wells and reading results, took approximately one hour; on average 7 strains could be typed per hour of operator time. While electrophoresis was being carried out biochemical tests could be set up. The equivalent time necessary for the individual examination of 7 strains by the Quellung technique was much longer, particularly when cross-reactions occurred.

One outstanding advantage of CIE was the ease and speed with which results could be read, in contrast with the Quellung method, in which delineation of the capsule was often difficult to discern, especially when dilute antisera were used in an attempt to resolve cross-reactions. With CIE, precipitin lines were clear, sharp and strong enough to be seen without magnification or staining, and, except in the known instances of cross-reaction, were completely unequivocal. The reactions of strains with very small capsules were often difficult to determine by the Quellung method, as the bacilli were only slightly enlarged, rather than surrounded by a distinct line of precipitation. With CIE the size of the capsule had little effect, and two strains, serotypes 40 and 41, with particularly small capsules gave reactions as strong as well-encapsulated strains. Another useful advantage of CIE was the ability to compare reactions in parallel, thereby reducing subjectivity, rather than individually as in a microscopic technique.

The variation in reactivity of the antigens mentioned in the results possibly reflects differences in antigenic structure and optimum antigen/antibody proportions, but could also be due to the concentration of capsular antigen in a suspension being a variable factor dependent on the size and solubility of the capsules, or to the difficulty in accurate standardization by eye of the concentration of a suspension of whole organisms.

This work was initiated by Dr Evelyn Finnie, but sadly her death occurred before its development and completion.

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