Combined Biochemical and Serological Typing of Clinical Isolates of *Klebsiella*

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In a series of 640 strains of *Klebsiella* isolated from clinical specimens over a 7-month period, there were sufficient biochemical differences between strains to allow a biochemical typing system to be established. Biochemical tests were done in solid media inoculated with a modified Steers inocula replicator. Biotypes were designated by a numerical coding system; 29 distinct biotypes were found among the 640 strains of *Klebsiella*. Serotyping of 270 of the strains was done by the Quellung reaction, and 40 capsular types were identified. Numerical biotypes and serotypes of strains appeared to vary independently. When used in conjunction, the two methods subdivided the strains into many more distinct types than either used alone. With the combined method over 100 types of *Klebsiella* were distinguished among the 270 isolates.

The importance of members of the genus *Klebsiella* as a cause of nosocomial infections has been stressed in a number of reports (5, 10, 13, 20). In the C.D.C. cooperative study of nosocomial infections in 69 U.S. hospitals, the overall rate of nosocomial infection in 1972 was 3 to 4%, and 9% of the infections were due to *Klebsiella* (3). The urinary tract is the most common site of nosocomial infection by *Klebsiella* (3, 10, 20), but major infections such as bacteraemia (20), meningitis (18), and pneumonia (16) are also encountered.

In clinical bacteriology laboratories these bacteria are identified only to the generic level or at most are subdivided into either three or six species (4, 11). This degree of subdivision is of little assistance in hospital epidemiological investigations, although differentiation of isolates into six species has been used in studies of the relative pathogenicity of different species in respiratory infections (6, 12, 14). For epidemiological studies the most useful method has been capsular serotyping (1, 7, 9, 15, 17). We have found that our own clinical isolates of *Klebsiella* show considerable differences in biochemical reactions. This has enabled us to devise a system of biochemical typing which divides our stains into sufficiently different types to be potentially useful for epidemiological investigations either alone or in conjunction with capsular serotyping. This report describes the typing system and its reproducibility as assessed by typing serial isolates from a number of patients.

**MATERIALS AND METHODS**

*Klebsiella* isolates. All of the 640 strains of *Klebsiella* examined were isolated during a 7-month period from specimens submitted to the diagnostic microbiology laboratory at Sunnybrook Medical Centre. For clinical purposes they were identified and reported only as a species of *Klebsiella* on the basis of Gram staining, motility, lactose and inositol fermentation, H₂S and indole production, utilization of citrate and gluconate, and decarboxylation of lysine and ornithine. Strains were then stored for further investigation at −70°C in a solution containing 40% (vol/vol) glycerol and 5% (wt/vol) tri-sodium citrate.

**Control strains of bacteria.** Isolates of *Enterobacter hafniae*, *Escherichia coli*, *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Citrobacter diversus* were obtained from the diagnostic laboratory for use as control organisms in the biochemical tests. These were maintained by monthly subculture on brain heart infusion agar (BBL), and were included in all sets of biochemical tests.

**Biochemical tests.** Frozen stored strains of *Klebsiella* and the control bacteria were cultured on brain heart infusion agar. After overnight incubation at 37°C single colonies were picked and inoculated into a minimal salts medium with 0.5% (wt/vol) glucose. The basic medium consisted of NaCl, 1 g; MgSO₄, 0.1 g; (NH₄)₂SO₄, 1 g; K₂HPO₄, 0.5 g; KH₂PO₄, 1 g; distilled water, 1,000 ml. After 4 h of incubation at 37°C portions of the suspensions were pipetted into the wells of a sterile stainless steel block of a Steers inocula replicating apparatus (19). An inoculating head which consisted of 36 sharp-pointed stainless steel needles instead of the flat-ended rods of the original apparatus was used. This made stab inoculations into solid media contained in square
intergrid petri dishes (Fisher Scientific, Toronto, Canada).

The biochemical tests and media are described in detail in Table 1. Commercial media were prepared according to the manufacturers' instructions. Additional medium constituents and all reagents were made up in wt/vol concentrations. The medium for gluconate utilization was the same minimal salts medium as that used for preincubation of test strains but with 1% potassium gluconate substituted for 0.5% glucose. The agar concentration of all media was made up to a final concentration of 3% (wt/vol). For most tests 40 ml of medium per plate was employed. For the VP test 25 ml was used so as to allow adequate absorption of the reagents added when the test was read after incubation. With glucose agar 60 ml was used because the larger bubbles of gas produced in the greater depth of medium were more readily observed.

Results of biochemical tests were recorded after 18 to 20 h of incubation at 37 C. The indole, MR, and VP tests were performed by placing drops of the appropriate reagents onto individual growth spots. For indole, Kovacs' reagent was used, and for methyl red an alcohol solution of 0.04% methyl red was employed; these tests were read immediately. In the VP test a drop of 10% KOH was added followed by a drop of a 5% solution of α-naphthol in alcohol and the test read 30 min later. Utilization of gluconate was measured by the presence or absence of growth. Reactions of the other tests were recorded by changes in the color of indicators incorporated in the media.

**Coding of biochemical test results.** For recording purposes the biochemical tests were divided into three groups, A, B, and C, which are detailed in Tables 2–4. The various combinations of reactions in each group of tests were allocated code numbers. The numerical biotype of a strain was expressed by combining the code numbers for each group of tests. Thus, a strain with the reactions (−−+) in group A, (++++) in group B, and (+−−+) in group C was given a numerical biotype of 1/1/1.

**Capsular serotyping.** A complete set of 72 capsular type strains of Klebsiella was kindly provided by Ida Ørskov of the Statens Serum Institut, Copenhagen. Antisera to these type strains were purchased.

| TABLE 1. Biochemical tests for identification and biotyping of Klebsiella |
|-----------------------------|-----------------------------|
| Test                        | Medium constituents and pH |
| Indole                      | Tryptone, 1% (Difco): pH 7.2 |
| Methyl red                  | MR—VP medium (Difco): pH 6.9 |
| Voges-Proskauer             | Peptone, 0.25%; yeast extract, 0.1%; sodium pyruvate, 2%; glucose, 1%; pH 6.7 |
| Citrate                     | Simmons citrate agar (Difco): pH 6.8 |
| Lactose                     | Phenol red broth base (BBL); lactose, 10%; pH 7.4 |
| Sucrose                     | Phenol red broth base (BBL); sucrose, 0.5%; pH 7.3 |
| Malonate                    | Phenylalanine malonate broth (Difco); bromothymol blue, 0.004% final conc; pH 6.3 |
| Gluconate                   | Minimal salts medium; potassium gluconate, 1%; pH 7.0 |
| Dulcitol                    | Phenol red broth base; dulcitol, 0.5%; pH 7.3 |
| Lysine                      | Peptone, 0.5%; yeast extract, 0.3%; glucose, 0.35%; lysine-hydrochloride, 0.5%; bromocresol purple, 0.002%; pH 6.8 |
| Ornithine                   | Yeast extract, 0.5%; glucose, 0.4%; ornithine-hydrochloride, 0.5%; bromocresol purple, 0.002%; pH 6.7 |
| Urease                      | Urea agar base concentrate (Difco): pH 6.8 |
| Glucose (acid/gas)           | Phenol red broth base; glucose, 1%; pH 7.2 |

| TABLE 2. Coding system for numerical biotyping of Klebsiella group A tests |
|-------------------------------|-----------------------------|
| Biochemical reactions         | Code |
| Indole                        | VP | Citrate |
| −                            | +  | +       | 1    |
| −                            | −  | +       | 2    |
| −                            | +  | −       | 3    |
| +                            | −  | +       | 4    |
| +                            | −  | −       | 5    |
| +                            | +  | −       | 6    |
| +                            | −  | −       | 7    |
| +                            | −  | −       | 8    |

| TABLE 3. Coding system for numerical biotyping of Klebsiella group B tests |
|-------------------------------|-----------------------------|
| Biochemical reactions         | Code |
| Lactose                       | Sucrose | Malonate | Gluconate |
| +                            | +       | +       | +         | 1    |
| +                            | +       | +       | −         | 2    |
| +                            | +       | +       | −         | 3    |
| +                            | −       | −       | +         | 4    |
| −                            | −       | −       | +         | 5    |
| −                            | −       | +       | −         | 6    |

| TABLE 4. Coding system for numerical biotyping of Klebsiella group C tests |
|-------------------------------|-----------------------------|
| Biochemical reactions         | Code |
| Dulcitol                      | Lysine | Ornithine | Urea |
| +                            | +       | −       | +     | 1    |
| −                            | +       | −       | +     | 2    |
| −                            | +       | −       | −     | 3    |
| −                            | +       | −       | −     | 4    |
| −                            | −       | −       | +     | 5    |
| −                            | −       | −       | −     | 6    |
commercially (Difco). The antisera available consist of 18 pools and 72 specific sera. Single colonies of Klebsiella were subcultured from brain heart infusion agar to Worfel-Ferguson agar (Difco). After overnight incubation at 37 °C Quellung reactions were done by the method of Casewell (2).

RESULTS

Numerical biotyping. In our series of 640 clinical isolates of Klebsiella 29 numerical biotypes were distinguished (Table 5). The six numerical biotypes defined as typical had the same biochemical reactions as the corresponding species of the two recognized systems of classification. The remaining biotypes were allocated to each species according to the majority of their biochemical reactions. As shown in Table 6, the five most common indole-negative numerical biotypes all had the biochemical reactions in groups A and B which are considered typical for the genus Klebsiella. Only reactions in group C tests showed variations. In all, 16 indole-negative numerical biotypes were identified which accounted for 84% of the 640 isolates of Klebsiella tested. A breakdown of indole-positive strains, which accounted for 16% of the 640 Klebsiella isolates, is also given in Table 6. It can be seen that 66 of the 99 indole-positive strains were either numerical biotype 5/1/1 or 5/1/2. These biochemical patterns were identical in all other tests to the two most common numerical biotypes.

Capsular serotyping. All of the pooled and monospecific commercial sera were tested by Quellung reaction against the 72 type strains of Klebsiella provided by Ørskov to check their homologous and heterologous titers. Approximately 50% of the 72 specific antisera gave positive Quellung reactions with more than one type strain of Klebsiella. In these cases, titrations were required to identify the specific capsular type. No problems were encountered with most Quellung cross-reactions because the heterologous titers were clearly lower than the homologous titer. However, the cross-reactions between types 3 and 68, types 10 and 61, types 14 and 64, and types 22 and 37 showed only a twofold difference in titer in both directions. In addition, four type strains, 19, 40, 43 and 55, did not react with any of the 18 antisera pools. The serological identity of these strains was determined only after testing with specific antisera. Despite these difficulties, it was possible to serologically differentiate all 72 type strains and over 90% of the clinical isolates of Klebsiella examined with the commercial antisera.

Reproducibility of the numerical biotyping method. Repeat isolates of Klebsiella were obtained on two or more occasions from 37 patients. The repeat isolates from 24 of these patients, in whom cross-infection did not seem clinically probable, were found consistently to have either the same or a closely related numerical biotype and the same capsular type, indicating persistence of the same strain. Minor changes noted in the numerical biotype of Klebsiella isolated on repeated occasions from 7 of these 24 patients were due to variable urease production. In repeat isolates from 8 of the 37 patients, major changes in numerical biotype were observed. The new biotype in every case was of a different capsular type from the original strain. The later isolates from five patients were of different capsular types from the original but had the same numerical biotype. They all belonged to one of our three commonest biotypes, each of which is associated with many different serotypes. All but 1 of the 13 patients showing a change of biotype or serotype had in-dwelling bladder catheters, tracheostomies,

### Table 5. Comparison of numerical biotyping with the common species classifications of Klebsiella

| Species nomenclature | Numerical biotypes |
|-----------------------|-------------------|
| Ewing and Edwards (11) | Cowan et al. (4) |
| *K. aerogenes* | 1/1/1 3 |
| *K. pneumoniae* | 2/1/1 6 |
| *K. edwardsii var. edwardsii* | 1/2/2 6 |
| *K. rhinoscleromatis* | 4/3/3 5 |
| *K. ozaenae* | 4/6/6 3 |

### Table 6. Distribution of 640 Klebsiella isolates by numerical biotype

| Biotype | Strains |
|---------|---------|
| Indole negative | No. | % |
| 1/1/2 | 141 | 22 |
| 1/1/1 | 134 | 21 |
| 1/1/4 | 122 | 19 |
| 1/1/3 | 124 | 8 |
| 1/1/5 | 19 | 3 |
| Others (11) | 71 | 11 |
| Indole positive | | |
| 5/1/1 | 56 | 9 |
| 5/1/2 | 10 | 2 |
| Others (11) | 33 | 5 |
| Total | 640 | 100 |
Comparison of biotyping and serotyping.

Of the 640 strains of *Klebsiella* which were biotyped, 270 strains isolated from 125 patients were serotyped. Twenty-seven of the 29 different numerical biotypes found in the total series were represented among the serotyped strains. The results of serotyping these 270 strains are shown in Table 7. As there were repeat isolates in the series this gives only a general indication of the serotypes more prevalent in the hospital. In all, 40 different capsular types were identified. Of the strains tested, 93% were typable and only four strains did not produce enough capsular material for typing even after two subcultures on Worfel-Ferguson agar.

Analysis of the results of serotyping and biotyping showed that strains which were all apparently the same by one typing method could regularly be subdivided further by the other method of typing. For example, nine different numerical biotypes were found among the 36 strains of capsular type 24, the most prevalent serotype. By comparison, numerical biotype 1/1/2, the most common biochemical type of *Klebsiella* isolated, was associated with 21 different capsular types. When each group of strains of the same capsular type was further subdivided on the basis of numerical biotypes, 102 different types of *Klebsiella* could be recognized. By comparison, species subdivision of strains by the scheme of Ewing and Edwards (11) in conjunction with capsular serotyping recognized 45 distinct types.

### DISCUSSION

Epidemiological investigation of nosocomial infections is greatly facilitated by the use of a precise typing system for the organism involved. This is particularly true when the organism is one, like *Klebsiella*, which is isolated with relative frequency. To provide additional precision to the commonly employed serological typing system, further subdivision of *Klebsiella* by a second method would be preferable to serotyping alone. The numerical biotyping system provides this. It allows a concise differentiation of each strain of *Klebsiella* without bias from over-emphasis on any one biochemical test. As shown in Table 5, our *Klebsiella* isolates were subdivided into many more biochemical types than the presently recognized species. This degree of subdivision would be of real value for epidemiological investigations.

To perform the biochemical tests on relatively large numbers of *Klebsiella*, use of solid media and the Steers inocula replicating apparatus was less costly than conventional tube biochemical tests and proved equally reliable. Even the alteration of tests for gluconate utilization to solid media read for presence or absence of growth did not affect the results so long as the test strains were preincubated in minimal salts medium. As shown in Table 8, this prevented residual growth on the solid medium of strains which did not utilize gluconate.

All tests except MR and gas production from glucose gave clear readings after 18 h of incubation. With MR and glucose, however, some

| Type | Strains |
|------|---------|
| No.  | %       |
| 24   | 36      | 13    |
| 55   | 24      | 9     |
| 43   | 20      | 7     |
| 7    | 19      | 7     |
| 27   | 15      | 6     |
| 61   | 15      | 6     |
| 18   | 10      | 4     |
| 21, 68 (9 each) | 18 | 7 |
| 2, 9, 20, 49, 69 (6 each) | 30 | 11 |
| 10, 25, 37, 64 (5 each) | 20 | 7 |
| 35, 42, 46, 60 (4 each) | 16 | 6 |
| 3, 30, 39 (3 each)  | 9  | 3     |
| 8, 38, 53, 54 (2 each) | 8 | 3 |
| 4, 12, 14, 16, 19, 23, 40, 44, 52, 63, 65 (1 each) | 11 | 4 |
| Insufficient capsule | 4 | 1 |
| Capsulated nontypable | 15 | 6 |
| Total | 270 | 100 |

### Table 8. Comparison of methods for testing the utilization of gluconate

| Test organism | Solid medium* inoculum pre-incubated in | Broth culture* |
|---------------|----------------------------------------|----------------|
|               | Peptone water | MSM* |                  |
| *Klebsiella* species | + | + | + |
| *Klebsiella* species | + | + | + |
| *Citrobacter diversus* | + | - | - |
| *Escherichia coli* | + | - | - |
| *Enterobacter aerogenes* | + | + | + |
| *Klebsiella* species | + | - | - |

*Positive (+) or negative (-) growth end point on solid medium.

*MSM, Minimal salts medium.

* Color change end point with Benedict's reagent.
strains gave strong reactions at 18 h, whereas others did not give any reaction until 2 or 3 days. Thus, if all were tested at 2 or 3 days the reactions of the strong early positives tended by that time to obscure the reactions of strains in adjacent locations on the plate. For these reasons these two tests were not used in our biotyping system and are not included in the numerical codes described in Tables 2-4. Nevertheless, it was observed that biochemically active strains, which in the coding system were given low code numbers, generally produced gas from glucose within 18 h. The methyl red test may prove useful as an additional marker for identifying less common numerical biotypes; this is being investigated further.

Indole-positive strains accounted for 16% of our isolates as compared with a figure of 6% given in the standard text by Edwards and Ewing (8). This was not an artefact due to our solid media technique because all these indole reactions were checked by the conventional tryptone water test. We are now investigating the part played by these strains in infections in our own hospital and comparing their frequency in other hospitals.

Reported investigations of hospital infections caused by Klebsiella have relied heavily on capsular serotyping for differentiation of Klebsiella strains. Our experience with the commercially available antisera has shown that they must be used with caution. According to the pamphlet supplied with each monospecific antiserum the Quellung reactions are specific. Our results do not substantiate this. Many cross-reactions were observed when the type strains of Klebsiella were tested against each commercial antiserum. The same cross-reactions were found when clinical isolates were examined. This was a serious technical disadvantage, because the titrations required to identify the homologous capsular type are tedious. Since the commercial antisera are supplied in 1-ml volumes, absorptions to remove heterologous antibodies were not practical. Moreover, we have found that few of the sera give adequate Quellung reactions if used at 1:8 to 1:16 dilutions as is stated to be possible in the accompanying leaflet. We found it necessary to use them undiluted. For these reasons we are now producing our own antisera in rabbits with acetone-dried whole bacterial antigens.

Our preliminary survey of repeat isolates of Klebsiella from various patients has demonstrated the reproducibility of numerical biotyping and its potential value in epidemiological studies when used in conjunction with capsular serotyping. Use of the two methods together allows the recognition of many more types than is possible with only one of the methods. We are now using the combined typing system in our continuing study on the epidemiology of nosocomial Klebsiella infections.

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