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The development and assessment of a bacteriocin typing method for *Klebsiella*

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SUMMARY

Klebsiellas are generally typed by the method of capsular serotyping but, although this is a reliable method, it is time consuming, requires the production of a large number of antisera and is not generally available. For this reason another method for typing klebsiellas was sought.

A bacteriocin typing method involving mitomycin C induction was developed and the cultural conditions giving optimum klebecin production and the best methods of testing the sensitivity of the organisms to klebecins were determined.

Of 190 klebsiella strains screened for bacteriocinogeny, only 68 (35·8%) produced klebecin and after calculation of similarity values by computer analysis, a typing set of 15 producers was selected. This typing set allowed over 96% of klebsiella strains to be typed and tests of the reproducibility of the method and the variability of typing patterns in natural populations of klebsiella indicated that results of acceptable accuracy could be obtained, while retaining good discrimination if two or more differences were required between patterns before they were regarded as distinct.

A complete set of capsular antisera were prepared, enabling the results obtained from klebecin typing to be compared with those from serotyping. There was generally close agreement between the results from the two typing methods and greater discrimination was obtained between similar strains when the two methods were combined. Klebecin typing and serotyping revealed relationships between strains from five outbreaks of infection, and strains of the same serotype from different hospitals could frequently be distinguished by their klebecin typing patterns.

INTRODUCTION

Over the past two decades the incidence of hospital-acquired infections caused by klebsiellas has greatly increased (Steinhauer *et al.* 1966; Dans *et al.* 1970; Lancet, 1971), and multiply antibiotic resistant strains of *K. aerogenes* have caused serious outbreaks of infection which have often been extremely difficult to control (Price & Sleigh, 1970; Hable *et al.* 1972; Hill, Hunt & Matsen, 1974; Curie *et al.* 1978).

Epidemiological studies of nosocomial klebsiella infections have been hindered by the lack of readily available, standardized typing methods. The most common method of typing klebsiellas is capsular serotyping either by the quellung reaction,

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which has been used for many years (Kauffmann, 1949; Ørskov, 1955; Casewell, 1975), or more recently by fluorescent antibody techniques (Riser, Noone & Bonnet, 1976; Riser, Noone & Poulton, 1976). Although capsular serotyping has provided valuable epidemiological information, its use has been largely restricted to a few specialized laboratories due mainly to the need for 77 capsular antisera.

Other methods of typing klebsiellas have been described, such as biotyping (Ørskov, 1957; Duncan & Rennie, 1974), bacteriophage typing (Slopek *et al.* 1967) and sensitivity to bacteriocins (Slopek & Maresz-Babczyszyn, 1967; Hall, 1971; Buffenmeyer, Rycheck & Yee, 1976) but none of these methods has come into widespread use. This has led to the sources and modes of spread of klebsiellas in the hospital environment being little studied.

The main aim of this investigation was to develop a reliable and discriminatory typing method for klebsiellas which could be more widely used than capsular serotyping. Bacteriocin typing, by either the production of, or sensitivity to, klebsiella bacteriocins (klebecins) was considered to be of the greatest potential for an additional typing method. Klebsiella strains have also been serotyped to enable a comparison to be made with the results from klebecin typing, and serotyping and klebecin typing were combined to determine whether this would provide greater discrimination between klebsiella strains than either typing system alone.

MATERIALS AND METHODS

Sources of strains

The klebsiella strains used in the development of the bacteriocin typing method and in the screening for klebecin producers comprised (*a*) 6 bacteriocin producing strains of *K. aerogenes* provided by Dr A. A. B. Mitchell, Law Hospital, Carluke, who had obtained four of them from Dr F. A. Hall, Summerfield Hospital, Birmingham, (*b*) 141 strains of *K. aerogenes* from a variety of clinical specimens, mainly urine, sputum and wound swabs, (*c*) 30 strains of *K. aerogenes* from the faeces of patients, (*d*) 6 capsular type strains of *K. aerogenes* representing serotypes 23, 24, 29, 70, 71 and 72 obtained from Dr I. Ørskov, Statens Serum-institut, Copenhagen, (*e*) two cultures of *K. ozaenae*, two cultures of *K. pneumoniae*, and one culture each of *K. atlantae*, *K. edwardsii* and *K. rhinoscleromatis* obtained from the National Collection of Type Cultures.

In tests of the discrimination and reproducibility of the typing method, the following strains were used: (*a*) 200 strains of *K. aerogenes* from clinical specimens at Leeds General Infirmary, (*b*) the 77 capsular type strains of klebsiella, (*c*) 34 isolates of *K. aerogenes* from the faeces of five patients over a period of 2–4 weeks, (*d*) 45 isolates of *K. aerogenes* from the site of infection of patients with a klebsiella infection, and 45 isolates of *K. aerogenes* from the faeces of the same patients, (*e*) 296 isolates of *K. aerogenes* from outbreaks of infection in five hospitals.

Biochemical identification of klebsiella

Organisms were identified as belonging to the genus *Klebsiella* by inoculation into a screening medium (Donovan, 1966) and into Christensen's urea medium.

Non-motile, hydrogen sulphide negative inositol fermenting and urea hydrolysing strains were further divided into different *Klebsiella* species by the methods of Cowan & Steel (1974).

Development of klebecin typing method

As a first step two methods of bacteriocin typing were compared, using the six klebecin producers obtained from Dr A. A. B. Mitchell. These were (i) the cross-streaking method for bacteriocin typing of klebsiella described by Hall (1971), and (ii) the growth-in-broth method involving mitomycin C induction devised by Farmer (1972*a*) for bacteriocin typing of *Serratia marcescens*.

Initial experiments indicated that the growth-in-broth method was the most suitable (see Results) and further tests were made to define the optimum cultural conditions for klebecin production. For these experiments the six klebecin producers were grown in 100 ml volumes of four different media, Proteose peptone No. 3 broth, nutrient broth, trypticase soy broth and Worfel Ferguson broth, contained in 250 ml conical flasks, incubated at three different temperatures (27, 32 and 37 °C) and treated with mitomycin C (Sigma London Chemical Company, Kingston upon Thames, Surrey) at various concentrations (0·5, 1·0, 2·0, 3·0 and 5·0 μg/ml). Samples were withdrawn at intervals up to 24 h after the addition of mitomycin C and the amount of klebecin in each sample was assayed by the critical dilution method as described by Mayr-Harting, Hedges & Berkeley (1972). The amount of bacteriocin present was expressed as the reciprocal of the highest dilution showing inhibition of the corresponding indicator strain as arbitrary units (AU) per ml.

The most satisfactory means of testing the sensitivity of klebsiella strains to klebecins was also determined. Different methods of obtaining lawns containing approximately constant numbers of organisms were compared, and four media (proteose peptone agar, brain heart infusion agar, MacConkey agar and nutrient agar) were tested to determine on which the clearest inhibition zones were formed.

From these investigations the following method of klebecin typing was developed.

Klebecin producers were grown in 3 ml volumes of proteose peptone No. 3 broth (PP3) for 18 h at 37 °C, then 1 ml was added to 8 ml of PP3 contained in screw-capped test tubes and incubated in a 32 °C water bath. After 1–1½ h, when the cultures were in the exponential phase of growth, 1 ml of a 10 μg/ml solution of mitomycin C was added, to give a final concentration of 1 μg/ml. The tubes were returned to the 32 °C water bath for a further 18 h, then 0·5 ml chloroform was added to each induced culture and the tubes were vigorously shaken by hand for 10 sec. The killed cultures were immediately centrifuged at 3000 rev./min for 20 min, then the tops of the tubes were loosened and the tubes allowed to stand at room temperature for 30 min to ensure the complete evaporation of traces of chloroform. The supernatants, containing klebecins, were transferred to sterile containers and stored at 4 °C until required.

Lawns of test strains were prepared by growing the test strains in 3 ml volumes of PP3 for 18 h at 37 °C, making a 1/10 dilution of each culture in phosphate

buffered saline, pH 7·3, and then adding 0·1 ml of each 1/10 suspension to 3 ml of 0·7 % agar which had been melted and cooled to 50 °C. The inoculated agar was poured over the surface of a well-dried proteose peptone agar (PPA) plate to give an even layer, and after this overlay had set, the lid of the plate was removed, and the plate left to dry at room temperature for 1½–2 h.

Mitomycin-C-induced lysates, plus a control, containing uninoculated PP3 and mitomycin C, were then spotted onto the lawns and the plates incubated for 18 h at 37 °C, then results were read against a black background.

Selection of a typing set of klebecin producers

Using the above method, 190 klebsiella strains were screened for klebecinogeny against the same 190 strains with the aid of a multiple inoculator of the Ridgway–Watt system (Denley Instruments, Bolney, Sussex). Lysates causing inhibition of test lawns were tested for the presence of bacteriocin or bacteriophage by spotting dilutions onto lawns of the appropriate indicator. Bacteriocin activity was recognized by a gradual overgrowth of the inhibition zone as drops of higher dilution were applied, and bacteriophage activity by the formation of discrete plaques as higher dilutions were tested.

The results of the activity of each klebecin producer on the 190 test strains were subjected to computer analysis using a computer program which had been developed for a numerical taxonomy study (Hayes, 1975). The necessary modifications to this program were made by Mr R. R. Hughes, Centre for Computer Studies, Leeds University. A dendrogram was constructed from the similarity matrix produced by the computer using the single linkage method of Sneath (1957), and a typing set of 15 producers were selected from the relationships exposed in the dendrogram.

Recording of results

Results were scored as + if there was a clear inhibition zone, ± if there was some resistant growth but still a discernible suppression of growth compared with the control, and − if there was no discernible inhibition. When comparing the typing patterns given by different strains, or by the same strain on different occasions, a difference in strength of reaction (between − and ±, or ± and +) was ignored, and only the difference between − and + reaction was termed a strong reaction difference.

When recording large numbers of typing patterns it was convenient to use the code devised by Farmer (1970) in which the reactions of the bacteriocin producers are divided into triplets, with each triplet denoted by a number from 1 to 8. Since 15 producers were used in this typing scheme, each bacteriocin type was coded as a five-digit number. For the purposes of coding, reactions recorded as ± or + were considered positive.

Serological typing

Klebsiella strains were serotyped by the quellung reaction as described by Kauffmann (1949) with 77 capsular antisera produced in this laboratory using an

immunization schedule provided by Dr P. Bradstreet, Standards Laboratory for Serological Reagents, London. The antisera were grouped into 19 pools and a set of specific antisera at working dilution was also prepared. Wild strains of klebsiella were tested against the pooled antisera, and then against working dilutions of the component antisera of the pool or pools giving a positive quellung reaction.

RESULTS

Development of the klebecin typing method

In the preliminary comparison of the cross-streaking method and the growth-in-broth method, 100 unrelated klebsiella strains from clinical specimens were tested by each method, using the six klebecin producers obtained from Dr A. A. B. Mitchell, Law Hospital, Carluke. More strains were typable by the growth-in-broth method (85 typable) than by the cross-streaking method (73 typable). In addition there were certain difficulties with the cross-streaking method for the mucoid nature of the primary streak of the klebecin producers often led to problems in removing the growth from the agar surface, and frequently there were considerable differences in the size of the inhibition zones on test strains when duplicate plates of the same producer were compared. For these reasons the growth-in-broth method was developed further.

The addition of mitomycin C to cultures at a concentration of 1 μg/ml increased the amount of klebecin produced by 100–1000-fold compared with uninduced cultures, but no further significant increase in klebecin production was obtained when the concentration of mitomycin C was raised above 1 μg/ml. Greater amounts of klebecin were obtained by growing producers in PP3 at 32 °C with an induction period of 18 h, than under any other combination of cultural conditions. The most discrete inhibition zones were formed on lawns prepared by the agar overlay method using 1/10 dilutions of overnight cultures, and proteose peptone agar was the most suitable medium for sensitivity testing since on brain heart infusion agar, nutrient agar, and particularly MacConkey agar, inhibition zones were often obscured by the mucoid nature of the growth, making results difficult to read.

Selection of a typing set of klebecin producers

Of the 190 strains screened for bacteriocinogeny, only 68 (35·8 %) were klebecin producers. Each of the seven strains of *K. atlantae*, *K. edwardsii*, *K. ozaenae*, *K. pneumoniae* and *K. rhinoscleromatis* produced klebecin, but only 33 % of the strains of *K. aerogenes* were klebecinogenic. This low incidence of bacteriocinogeny indicated that it would be impracticable to base a typing scheme for klebsiellas on klebecin production, and therefore in the computer analysis the klebecin producers were treated as operational taxonomic units and inhibition of growth of any test strain as a positive character for binary coding. As a result of the analysis a preliminary set of 19 producers was selected and used to type 277 unrelated klebsiella strains, 200 of which had been isolated from clinical specimens, and the 77 standard capsular type strains. The wild klebsiella strains were chosen

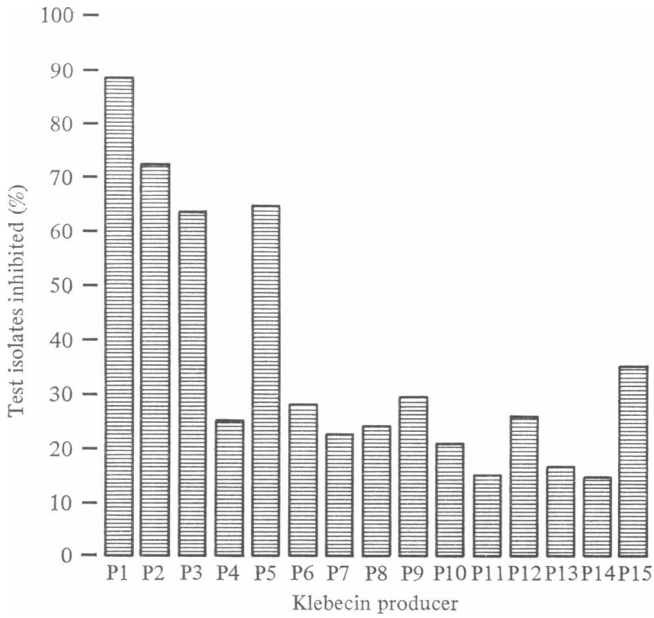

Fig. 1. Histogram showing percentage of 277 klebsiella isolates inhibited by each of the 15 klebecin producers of the typing set.

to include as wide a range of serotypes as possible. A preliminary examination of results indicated that two producers could be omitted as they inhibited a very similar range of strains to another producer, and another two were excluded as they frequently gave inhibition reactions of variable intensity which were difficult to read. This left a typing set of 15 producers, of which 12 were *K. aerogenes*, one was *K. atlantae*, one *K. edwardsii*, and one *K. rhinoscleromatis*. Further examination of the sensitivity of the 277 test strains to these 15 producers showed that all but 9 were typable, and 130 different typing patterns were represented, when patterns differing by at least one strong reaction were regarded as distinct. The percentage of test strains inhibited by each of the 15 producers is shown in Fig. 1, and indicates that many different patterns are possible. The omission of any producer led to a decrease in both the number of typable strains and the number of different patterns.

Although a large number of different typing patterns were found it was necessary to determine the reproducibility of the method before an accurate assessment of its discriminatory power could be obtained.

Tests of the reproducibility of the klebecin typing method

Two aspects of reproducibility were examined: firstly, a laboratory test of the reproducibility of the method was made by typing a set of unrelated klebsiella strains on three consecutive weeks, and secondly, the variability of typing patterns in natural populations of klebsiella was tested using the following four sets of cultures: (*a*) multiple colonies picked from one primary culture date, (*b*) sequential cultures from the faeces of a single patient, (*c*) cultures from different sites on

Table 1. *Variations in typing patterns of* 98 *klebsiella isolates typed twice at an interval of one week using* (*a*) *freshly prepared klebecins and* (*b*) *klebecin preparations stored at* 4 °*C*

(Percentage of isolates with corresponding number of strong-reaction differences between their two typing patterns.)

	No. of strong-reaction differences between the pair of typing patterns for each isolate				
	0	1	2	3	> 3
Fresh klebecin preparations	67%	22%	3%	3%	3%
Stored klebecin preparations	46%	33%	8%	7%	4%

the same patients, and (*d*) isolates from outbreaks of infection in five different hospitals.

Laboratory test of reproducibility

A hundred klebsiella strains obtained from clinical specimens were typed on 3 consecutive weeks using two sets of mitomycin-C-induced lysates. One set of lysates was freshly prepared each week and the other set was prepared the first week and then stored at 4 °C. An indicator strain was chosen for each producer and each lysate was assayed on the appropriate indicator before being used for typing. The variations in the pair of patterns obtained for each strain after the first 2 weeks of tests are given in Table 1 in terms of strong-reaction differences. Two strains were non-typable and have been omitted from the table. The typing patterns obtained on the third week using freshly made lysates showed a similar amount of variation when compared with the patterns obtained on the first 2 weeks to that found between the patterns of the first 2 weeks. The patterns of strains typed on the third week with lysates which had been stored at 4 °C showed greater variation when compared with the patterns obtained on the first week than the variations between the patterns of the first 2 weeks. Only 38 strains showed no changes in pattern over the 3 weeks when typed with stored lysates. The major cause of the greater variability of patterns using stored lysates was a rapid loss of potency of lysates from four producers, which contained 64 AU of klebecin per ml when freshly made. This resulted in strains which were inhibited by these lysates on the first week becoming insensitive to their action on subsequent weeks. Lysates from the other 11 producers, which contained 10^2–10^4 AU/ml, were less frequently involved in the variations in patterns and maintained a high level of activity when stored at 4 °C for up to 4 months.

The results from this laboratory test of reproducibility indicated that even when freshly prepared lysates were used, only 67% reproducibility could be obtained if patterns differing by one strong reaction were regarded as distinct, and to improve reproducibility a difference rule would have to be applied. The application of a two-reaction difference rule, that is regarding as distinct only those patterns differing by two or more strong reactions, gave 89% reproducibility, and 92% reproducibility was obtained with a three-reaction difference rule. Although

Table 2. *Constancy of klebecin type in sequential isolates of klebsiella from the faeces of five patients*

Patient	No. of isolates	No. of isolates of same serotype and klebecin type	Combined serotype/ klebecin type
A	5	5	66/27888
B	7	7	31/12688
C	7	7	35/11288
D	9	9	27/16888
E	6	6	63/14588

these difference rules increased reproducibility they caused a reduction in discriminatory power, for 60 patterns differing by at least one strong reaction were found among the 98 typable strains while if two or more strong-reaction differences were required there were 53 distinct patterns, and if three or more strong-reaction differences were required there were 42 distinct patterns. Another indication of the discriminatory power of a typing method is the number of isolates of the most common type. The most common single typing pattern amongst the 98 strains represented 6·2% of the strains, but the percentage of strains represented by patterns differing from the 'modal' typing pattern, i.e. the pattern which varied least from the greatest number of other patterns, by less than two strong reactions, was 18·4%, and by less than three strong reactions, 26·7%. In comparison, 82 of the 100 strains were typable serologically, and 42 different serotypes were represented, with the most common, type 62, representing 8·5% of the typable strains.

Variability of typing patterns in natural populations of klebsiella

Multiple colonies from one primary culture plate

Ten klebsiella colonies were picked from each of eight plates on which faecal swabs had been cultured. All ten colonies in each set were of the same serotype. There were no strong-reaction differences between the klebecin typing patterns of the ten colonies within each set, although variations in the intensity of some inhibition reactions were evident.

Sequential cultures from the faeces of a single patient

A total of 34 klebsiella isolates were obtained from the faeces of five different patients over a period of 2–4 weeks. The results from serotyping and klebecin typing these isolates are given in Table 2. The isolates in each set were of the same serotype and klebecin type, although again there were variations in the degree of inhibition caused by certain producers on isolates from the same set.

Cultures from different sites on the same patient

Fifty-five specimens of faeces were obtained from patients with klebsiella infections and 45 (81·8%) contained klebsiella. The same serotype was present in the faeces and in the infection in 21 (38·2%) patients, and 19 specimens of faeces contained a different klebsiella from that in the infection. In five patients,

Table 3. *Variability of klebecin typing patterns of pairs of isolates obtained from the faeces and site of infection of patients with a klebsiella infection*

	No. of strong-reaction differences between the typing patterns of each pair of isolates			
	0	1	2	> 2
No. of pairs of isolates of same serotype with typing patterns differing by corresponding number of strong reactions	16	3	0	2
No. of pairs of isolates of different serotypes with typing patterns differing by corresponding number of strong reactions	3	1	1	14

faeces and specimen from the infection contained non-typable klebsiella. Only one of the 45 pairs of isolates was non-typable by klebecin typing and out of the remaining 44 pairs, 20 had identical typing patterns, 4 pairs had patterns differing by one strong reaction, 1 pair had patterns differing by two strong reactions and 19 pairs had patterns differing by more than two strong reactions. The variability of patterns of pairs of isolates of the same, and different serotypes, is given in Table 3. Five pairs of isolates have been omitted since they were non-typable by one or other of the typing methods. There was much less variability in the typing patterns of isolates of the same serotype than in pairs of isolates of different serotypes. Only 2 of the 21 pairs of the same serotype were distinguishable by their typing patterns when either a two- or three-reaction difference rule was applied, and 4 pairs of isolates had patterns differing by less than two strong reactions, and 5 pairs of isolates had patterns differing by less than three strong reactions, although they were of the same serotype.

Isolates from outbreaks of infection

Isolates from outbreaks of infection in five different hospitals (designated A–E) were obtained for study, and in certain of these outbreaks there was good epidemiologic evidence that single strains of klebsiella had caused the infections, while in others the epidemiology was more obscure, especially at Hospital E, where all previous attempts to type the isolates had revealed no clear relationships between them.

The outbreak of infection at Hospital A involved ten patients who had undergone surgery on the urinary tract and who subsequently developed urinary-tract infections caused by antibiotic-resistant *K. aerogenes* when they were all in the same ward. Isolates from nine of the patients had identical antibiotic sensitivity patterns and the tenth differed in only one respect in that it was sensitive to kanamycin whereas the others were resistant. The nine isolates with identical

antibiotic sensitivity patterns were all of serotype 9 and also possessed identical klebecin typing patterns. The tenth isolate was clearly a different strain, for it was of serotype 2 and had a typing pattern which differed from that of the other nine isolates by eight strong reactions.

Another outbreak of urinary-tract infection caused by multiply antibiotic-resistant *K. aerogenes* occurred at Hospital B, and 12 isolates were obtained for study: seven from the urine of different patients and five from various environmental sources in the ward. Five of the isolates from urine and four from the environment were of serotype 21 and seven of these isolates also possessed identical klebecin typing patterns. The remaining two serotype 21 isolates, one from urine and one from a nurse's hands, had patterns differing from the pattern of the other serotype 21 isolates by five and eight strong reactions respectively. These large differences suggested that these two isolates were distinct from one another and from the other seven isolates of serotype 21, although the fact that all nine isolates were resistant to a similar range of antibiotics and were isolated from the same ward over a short period of time tended to support the interpretation from serotyping that they were all the same strain. The three isolates from this outbreak which were not of serotype 21 were of distinct klebecin types and were of no epidemiological significance.

A larger outbreak occurred in the Intensive Care Unit and associated wards at Hospital C. Serotyping suggested that either cross-infection or infection from a common source had occurred since 19 of the 24 isolates were of serotype 21. Klebecin typing provided a different interpretation of the relationships between the isolates as 14 of the serotype 21 isolates had identical typing patterns, which were coded as 88866, while four other serotype 21 isolates had a different pattern, coded as 72116, which differed from the former pattern by eight strong reactions. This suggested that there were two distinct strains of serotype 21 in the hospital, and other evidence supported this conclusion. For instance, the four isolates of klebecin type 72116 were isolated from the same ward over a short period of time, while the isolates of klebecin type 88866 were isolated from six different wards over a longer period, and there were slight differences between the antibiotic-sensitivity patterns of the two strains. The remaining isolate of serotype 21 was obtained from the same ward and at the same time as the four isolates of klebecin type 72116, and since it had a pattern differing from this type by only three strong reactions, it may have been the same strain.

Six isolates of multiply antibiotic-resistant *K. aerogenes* were obtained from a small outbreak of urinary-tract infection which occurred in the urological and a few other wards at Hospital D. Five of these isolates were of serotype 9 and possessed the same klebecin typing pattern, which coded as 16888, and the other was of serotype 21, klebecin type 88824. Over the next 6 months another three isolates of serotype 9, klebecin type 16888, and two isolates of serotype 21, klebecin type 88824, were obtained from sporadic urinary-tract infections in the same wards. During the next 3 months there was a larger outbreak of urinary-tract infection in these wards, caused by *K. aerogenes* with similar antibiotic sensitivity patterns to previous isolates. Eleven of the 17 isolates from this out-

break were of combined serotype/klebecin type: 9/16888, and were apparently the same as one of the strains which had caused infections in previous months. The serotypes and klebecin types of the remaining isolates showed that they were of no epidemiological significance.

Therefore, in the outbreaks of infection at Hospitals A, B, C and D, there was good agreement between the results from serotyping and klebecin typing. Assuming isolates of the same serotype from the same hospital to be the same strain, except at Hospital C where there were two distinct strains of serotype 21, out of 51 isolates regarded as the same by serotyping, 48 were regarded as the same by klebecin typing whether a difference rule was applied or not. This corresponds to an agreement of 94% between the two typing methods.

These four outbreaks were all of limited duration and the strains were typed shortly after their isolation, but the epidemic which occurred in the neurosurgical unit at Hospital E was much more prolonged and the strains had been stored for 8 years before they were obtained for study. Previous attempts to type the strains had failed to establish any relationships between isolates, with many different types emerging, but they had never been serotyped (Price & Sleigh, 1970). Two hundred and fourteen isolates were obtained and serotyping was attempted first, but since only two of the first 20 isolates were typable serologically, the isolates were tested instead against the typing set of klebecin producers. One hundred and ninety-four isolates were typable and 83 had the same typing pattern; 25 had patterns differing from this pattern by one strong reaction and 29 differed by two strong reactions. The remaining 57 isolates had typing patterns differing from the common one by more than two strong reactions and were represented by 28 distinct patterns. One of the 83 isolates which had the common typing pattern, strain G23, was tested for quellung reactions with undiluted antisera produced against the 77 recognized capsular serotypes, but did not react with any antiserum. Antiserum was, therefore, raised against this strain and was tested for cross-reactions against suspensions of the 77 capsular type strains. Only one cross-reaction occurred, with type strain 57, but this was a strong reaction and was absorbed out with difficulty. Despite this strong reaction, the lack of a reaction between a suspension of strain G23 and type 57 antiserum suggests that the capsular antigen possessed by strain G23 may represent a new, distinct serotype. The 214 isolates were screened against this new antiserum, beginning with the 137 isolates with the same, or similar typing patterns, and isolates giving a positive reaction were recorded as serotype G23. Isolates which did not react with the new antiserum were tested against the 19 pools of antisera in the normal manner. One hundred and twenty-seven isolates were of serotype G23, 39 were non-typable and the remaining 48 were of 20 different serotypes. The variations in typing patterns of isolates of serotype G23, and those of other serotypes, is given in Table 4.

It seems reasonable to assume that all the isolates of serotype G23 were the same strain for it is unlikely that several different strains of the same new serotype would have been isolated from the same epidemic. This being the case, 40 of the 127 isolates regarded as the same strain by serotyping were distinguish-

Table 4. *Variation in klebecin typing patterns of isolates of serotype G23, and isolates of other serotypes*

	No. of strong-reaction differences from modal typing pattern			
	0	1	2	> 2
No. of isolates of serotype G23 with corresponding number of strong-reaction differences from modal pattern	78	9	18	22
No. of isolates of serotypes other than G23 with corresponding number of strong-reaction differences from modal pattern	1	15	4	28

Table 5. *Typing patterns of groups of isolates of the same serotype from different hospitals*

Hospital	No. of isolates of corresponding serotype	No. of isolates of same klebecin typing pattern	Klebecin producer no. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
			Typing patterns of groups of isolates														
	Serotype 9																
A	9	9	+	+	+	−	+	−	−	−	+	+	+	+	−	+	+
D	18	18	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−
	Serotype 21																
B	9	7	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−
C	19	14	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−
		4	−	−	+	+	+	−	+	+	+	+	+	+	−	+	−
D	5	3	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+

able by klebecin typing using a two-reaction difference rule, corresponding to 68·5% agreement between serotyping and klebecin typing, and 83·5% agreement between the two typing methods was obtained using a three-reaction difference rule. Thus isolates of the same serotype from Hospital E showed much greater variability in their typing patterns than isolates of the same serotype from other hospitals. A possible explanation for this discrepancy is that changes in cell wall structure, and hence bacteriocin receptor sites, may have occurred during the long period the strains were stored, resulting in increased variability in typing patterns. Additionally the epidemiology of this outbreak was extremely complex, as previous attempts to type the isolates had indicated, and it is unlikely that such complex epidemiology could be fully resolved after the long delay between the isolation and typing of the isolates. Nevertheless, klebecin typing was of

value as it provided an indication of the relationships between isolates and permitted serotyping to be more efficiently performed.

After consideration of the results from all the tests of reproducibility, it was decided that acceptable reproducibility could be obtained if two or more differences were required between patterns before they were regarded as distinct. This difference rule allowed good discrimination as demonstrated by the large number of distinct patterns possessed by unrelated strains, and by the fact that strains of the same serotype from different hospitals could often be readily distinguished by their typing patterns, as shown in Table 5. The ability to subdivide strains of the same serotype by klebecin typing was particularly useful at Hospital C, in determining that two outbreak strains of serotype 21 were present.

DISCUSSION

The two most common methods of bacteriocin typing are the cross-streaking method, based on the technique of Abbott & Shannon (1958), and the growth-in-broth method using some form of induction, usually mitomycin C. Several groups of Gram-negative bacteria have been typed by these methods, and each method has been preferred in turn by different workers. For example, Al-Jumaili (1975) considered that the growth-in-broth method was more suitable for bacteriocin typing of *Proteus*, than the cross-streaking method of Cradock-Watson (1965), but more recently Senior (1977) has preferred a scrape-and-streak method. Similarly, Traub, Raymond & Startsman (1971) and Farmer (1972*a*, *b*) used the growth-in-broth method for bacteriocin typing of *Serr. marcescens*, whereas Anderhub *et al.* (1977), after a comparative study, found that a cross-streaking method was slightly more discriminatory. The comparison of the two methods described in this study indicated that the growth-in-broth method was more suitable for klebsiella. The major disadvantages of the scrape-and-streak method were that it was extremely difficult to stimulate bacteriocin production by cultures grown on agar and that variation in inhibition reactions was common.

It was important to determine the cultural conditions leading to optimum bacteriocin production since factors such as the temperature of incubation and composition of media may have considerable effects on the amount of bacteriocin produced by different groups of bacteria (Farmer & Herman, 1969; George, 1974; Pau & Terry, 1976). The cultural conditions giving optimum klebecin production were similar to those for other Gram-negative bacteria, except that a longer period of induction was necessary. The standard methods determined for klebecin production and for testing the sensitivity of test strains to klebecins ensured that each strain was treated in the same way and reduced the possibility of variations in typing patterns caused by differences in the amounts of klebecin in preparations used for typing, and in the number of organisms comprising lawns of test strains.

Even with mitomycin C-induction, the incidence of bacteriocinogeny in klebsiella (35·8%) was much lower than in most other studied groups of Gram-negative bacteria. The high incidence of bacteriocinogeny in *E. coli*, *Ps. aeruginosa* and *Serr. marcescens* has enabled typing schemes based on both the production

of, and sensitivity to, bacteriocins to be introduced for these organisms, but the only practicable means of bacteriocin typing klebsiella appears to be by sensitivity to a set of klebecin-producing strains.

It was interesting to note that whereas only 33% of the 183 strains of *K. aerogenes* screened for klebecin production were klebecinogenic, each of the seven strains of *K. atlantae*, *K. edwardsii*, *K. ozaenae*, *K. pneumoniae* and *K. rhinoscleromatis* tested produced klebecin. This suggests that there may be some difference between these species with regard to bacteriocin production, although many more strains would have to be tested before a definite conclusion was reached.

A visual examination of the results from testing the sensitivity of the 190 test strains to the 68 klebecin producers revealed obvious relationships between certain producers, but computer analysis allowed a more accurate assessment of the relationships to be quickly made. There was only one cluster of related producers formed at the 85% similarity level and containing 14 producers, while the remaining producers were clearly separate from this cluster and from each other. The 15 producers selected for the typing set possessed varied activity spectra and the preliminary test of the typing method showed that the majority of strains were typable and many different patterns were obtained. However, it was necessary to assess the reproducibility of the method before using the scheme for epidemiological investigations since one of the major hazards in developing a typing scheme, particularly one based on sensitivity to bacteriocin and bacteriophage, is variability in typing patterns.

The importance of making extensive tests of the reproducibility of a typing method using groups of epidemiologically related strains was first noted by Williams & Rippon (1952), who found they could obtain acceptable reproducibility using their bacteriophage typing scheme for *S. aureus* only if two or more strong-reaction differences were required between patterns before they were regarded as distinct. Similar difference rules have been introduced for bacteriocin typing schemes of *Ps. aeruginosa* (Rampling, Whitby & Wildy, 1975) and *Serr. marcescens* (Anderhub *et al.* 1977), although in many typing schemes a difference of one reaction is considered sufficient for differentiating between patterns. The danger of not assessing the reproducibility of a typing scheme is that if no allowance is made for variability, isolates which are related may be regarded as distinct by their typing patterns, giving incorrect epidemiological information.

The tests of the reproducibility of this klebecin typing method, by typing 100 unrelated klebsiella strains on consecutive weeks, and by typing groups of related strains from natural populations of klebsiella showed that over 90% reproducibility could be obtained by the application of a two-reaction difference rule. This decreased the discriminatory power of the method, as 16·8% of the 100 unrelated strains had patterns differing from the 'modal' pattern by less than two strong reactions, but there were many distinct patterns among the remaining strains. Strains of no epidemiological significance from each of the studied outbreaks of infection were generally clearly differentiated from the outbreak strain, and from each other, by their klebecin types, and klebecin typing also allowed strains of

the same serotype from different hospitals to be discriminated. This was particularly useful with regard to strains of serotype 21, and at Hospital C two distinct strains of this serotype were identified by their typing patterns. Serotype 21 is one of the more commonly encountered serotypes and shows strong mutual cross-reactions in antisera produced against certain other types, especially types 11 and 33, which occasionally makes the determination of serotype 21 difficult. A means of distinguishing between strains of this type is therefore of practical value.

There was generally close agreement between the results from serotyping and klebecin typing, except for the isolates from Hospital E, where the epidemic was large and complex. A study was made of the extent of variation in the capsular antigens of wild strains of klebsiella by determining the serological reactions of sequential cultures obtained either from the faeces of patients or from the urine of patients with an infection of the urinary tract. These tests indicated that although changes in the cross-reactions of types occurred, larger antigenic variations which resulted in a change in the serotype of a strain were rare. Thus, when there was conflict between the results from serotyping and klebecin typing, isolates which were of different serotypes tended to be regarded as different strains whether their klebecin typing patterns were identical or distinct, whereas other epidemiological evidence was considered before making a decision as to the relationships between isolates of the same serotype, but distinct klebecin types.

This klebecin typing method is simpler to perform than serotyping, and its discriminatory power and reproducibility suggest that it will be of value, particularly in laboratories where capsular serotyping is unavailable, and will, it is hoped, help more workers to study klebsiella epidemiology. In laboratories where capsular antisera are available, the typing method is probably best used as an adjunct to serotyping with the two methods in combination providing greater discrimination between klebsiella isolates, allowing more detailed epidemiological information to be obtained.

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