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New Bacteriophage Typing Scheme for Subdivision of the Frequent Capsular Serotypes of *Klebsiella* spp.

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A bacteriophage typing scheme for hospital isolates of *Klebsiella* spp. was developed. The scheme was designed specifically as a secondary typing method to discriminate between strains of serotypes K2, K3, and K21 but proved to be an efficient general typing method for strains of most serotypes. The set of 15 phages gave 87.3% typeability on 236 strains of more than 70 different serotypes. Typeability within the K2, K3, and K21 strains was 93, 89, and 91%, respectively. There was a mean of 3.2 reactions strain^{-1} for all phage-typeable strains. Of the serologically nontypeable strains, 76.7% were susceptible to one or more phages. The most common pattern accounted for only 7% of the strains. The lytic patterns were reproducible if strains were typed on the same day, but differences were observed if strains were stored for 1 week or more before retyping. A total of 96.5% of the strains were typeable by a combination of capsular serology and phage typing.

Klebsiella pneumoniae and *Klebsiella oxytoca* are important causes of nosocomial infections (5, 20). Epidemics caused by single strains that acquire multiple antibiotic resistance can be accompanied by high mortality and have proved difficult to control (6). Epidemiological studies of outbreaks have been facilitated by serotyping of capsular (K) antigens. There are 77 K serotypes which can be determined by a variety of methods (12, 13, 19), but because of the difficulty of producing reliable antisera to the large number of antigens, serotyping is usually restricted to reference centers. A number of workers have recognized the practical limitations of capsular typing and have developed alternative, simpler, typing schemes. These include biotyping (17, 18, 21), bacteriophage typing (11, 14–16), and bacteriocin typing (2, 4, 7–9, 22).

We have produced antisera to the K serotype strains and used them to type *Klebsiella* isolates from a number of British hospitals. Three serotypes have predominated: K2 (11.0%), K3 (8.8%), and K21 (11.0%). The frequency of these types severely limited the usefulness of K serology as an epidemiological marker, and a further method of distinguishing between strains of the common K types was required. The aim of this study was to develop a phage typing scheme for *Klebsiella* spp. to be used specifically as a secondary typing method for the subdivision of the K serotypes common among clinical isolates.

MATERIALS AND METHODS

Bacterial strains and capsular serotyping. All cultures of *K. pneumoniae* and *K. oxytoca* were isolated from clinical specimens from British hospitals between 1984 and 1986. For the purposes of this study, *K. pneumoniae* and *K. oxytoca* were considered together as *Klebsiella* spp. Isolates were typed according to their capsular polysaccharide antigens by countercurrent immunoelectrophoresis and Quellung reaction (12, 13). Antisera were prepared as described by Palfreyman (13).

Media. Bacterial cultures were grown in tryptone soya broth (Oxoid Ltd.) or on MacConkey agar (Oxoid). Double-strength nutrient broth (double-strength NB; Oxoid base) containing 400 μg of $CaCl_2$ ml^{-1} was used for phage enrichment of sewage. Phages were propagated in NB with 400 μg of $CaCl_2$ ml^{-1}. Phage typing agar consisted of 2% (wt/vol) NB powder (Oxoid), 0.5% (wt/vol) NaCl, and 0.7% (wt/vol) agar (Oxoid); 0.24% (wt/vol) $CaCl_2$ was added to the molten phage typing agar at 50°C before it was poured into petri dishes. All phage typing tests were incubated at 32°C for 18 h.

Isolation of phages. Eleven *Klebsiella* phages were taken from the typing set of Ślopek et al. (23). All other phages were isolated from samples of untreated sewage settled for 24 h, which had been obtained from three geographically unrelated sewage treatment works in the United Kingdom.

A set of 57 distinct strains that included at least one representative of each of the common K serotypes was selected from recent clinical isolates. Common serotypes were defined as those for which we had received seven or more isolates for serotyping between 1984 and 1986 (1,257 isolates were received in this period). This phage isolation set included a number of K2, K3, and K21 strains. Enrichment experiments were performed to isolate phages active on each strain of the phage isolation set as follows: 5 ml of sewage was mixed with 5 ml of double-strength NB and 0.1 ml of a 6-h NB culture of the enrichment strain. After overnight incubation, 2 ml of chloroform was added and the broth was mixed vigorously for 30 s. The bacterial cells were removed by centrifugation (4,000 × *g* for 20 min), and 20-μl portions of 10-fold dilutions of the supernatant were plated on phage typing agar seeded with a 4-h NB culture of the test strain. After incubation, the bacterial lawn was examined for phage activity. A representative of each morphological type of plaque was selected, purified, and propagated by standard methods (1).

Evaluation of phages. Phages were evaluated for their potential usefulness in a typing scheme by testing them at the routine test dilution on a primary evaluation set of 105 selected strains representing 41 serotypes (E1 set). The routine test dilution was defined as the dilution of phage that just failed to give confluent lysis of its propagating (isolating) strain. Cultures to be typed were inoculated into 5 ml of tryptone soya broth and incubated overnight at 37°C. Broth

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cultures were diluted approximately 1 in 200 in sterile broth, and the surface of a phage typing agar plate (diameter, 9 cm) was flooded with approximately 2 ml of the diluted culture. The excess fluid was removed, and the plates were dried for 30 min at room temperature. Phages were applied with a multiloop applicator that dispensed approximately 0.01-ml aliquots of each phage (10). The plates were incubated for 18 h, and the lytic reactions were recorded.

Numerical taxonomy. Computerized taxonomic analysis was performed at the University of Leicester, Leicester, United Kingdom, with the TAXPAK suite of programs by M. Sackin. The phage isolates were compared on the basis of their ability to lyse each of the test strains in the E1 set (3). For the purposes of the numerical analysis, only lytic reactions involving 20 or more plaques were considered positive.

The Jaccard coefficient (S_j), calculated as follows, was used as the basis for the comparisons: $S_j = a/(a + b + c)$, where a was the number of positive matches and b and c were the numbers of dissimilar test results (24). The unweighted average linkage algorithm was used to cluster the phages on the basis of the similarity data; the relationships are shown as a dendrogram (Fig. 1).

Selection of the typing set. Phages active on the K2, K3, and K21 strains in the E1 set were tested on a larger collection of isolates for each of the serotypes. The results of this analysis were used initially to select a set of phages that produced maximum discrimination of the K2 strains. This partial set was extended by additional phages necessary to discriminate between K3 strains. Phages necessary to discriminate between K21 strains were then added. The provisional typing set was completed by the addition of phages that gave distinct lytic patterns or contributed to the discrimination of serotypes other than K2, K3, and K21 or both. The provisional typing set was then evaluated on a large collection of distinct strains (E2 set).

RESULTS

A total of 74 *Klebsiella* phages were propagated, 11 from the set of Slopek et al. (23) and 63 from sewage. No differences were observed in the frequency of *Klebsiella* phages in the three sewage samples.

Similarities between the candidate phages. The 74 phages were tested at the routine test dilution on 105 *Klebsiella* strains (E1 set). The similarity of these phages as determined by S_j and the unweighted average linkage clustering method is shown in Fig. 1. The phages did not form distinct groups, but some loose associations were observed and the lytic reactions of seven subsets (S1 to S7 [Fig. 1]) were selected for further examination (Table 1).

In five of the seven phage subsets, some serotype associations were apparent but none was uniquely associated with a K serotype. Phages in the S1 group reacted with strains of serotypes K18, K20, K21, K22, and K27. S2, S3, and S6 phages were specific mainly for K38, K30, and K2 isolates, respectively. S5 phages reacted with strains belonging to serotype K18 or K21. Conversely, phages in groups S4 and S7 were highly reactive, and each phage lysed an average of 35.4 and 35.3 of 105 strains, respectively. No serotype associations were found in these groups.

Selection of provisional typing set. (i) Phages active on K2 isolates. Of the 74 phages, 18 lysed one or more of the six K2 strains present in the E1 set, and nine distinct patterns of lytic reaction were evident. The 18 phages were then tested on 48 K2 isolates from nine hospitals. Three phages gave identical reactions, and eleven of the remaining phages were at least 50% similar to two or more other phages (as assessed by S_j). Analysis of the lytic spectra showed that seven phages was the minimum number necessary to give the best available discrimination among the 48 isolates, and this number of phages was selected for the provisional typing set.

TABLE 1. Characteristics of clusters of *Klebsiella* phages with similar lytic patterns[a]

Group	No. of phages	Reactivity		Similarity (%)	Serotype(s)
		Range	Mean		
S1	7	11–17	12.9	55	K18, K20, K21, K22, K27
S2	4	2–4	3.5	62	K38
S3	3	5–7	6.0	77	K16, K30
S4	5	25–51	35.4	42	None
S5	4	2–4	3.3	46	K18, K21
S6	6	3–10	5.3	42	K2
S7	13	26–48	35.3	41	None

[a] Reactivity is expressed as the number of strains lysed phage^{-1}. Similarity is expressed as the mean similarity within the cluster, calculated by S_j. Only the predominant serotypes lysed by each group are shown.

(ii) Phages active on K3 isolates. Twenty-three phages lysed one or more of the six K3 strains present in the E1 set and gave 12 distinct lytic patterns. These phages were then tested on 30 K3 isolates from seven hospitals, and two pairs and one triplet of phages gave identical reactions. Overall, there were two clusters of phages active on K3 isolates: one cluster of 4 phages and another of 10 phages showed 73 and 37% or greater internal similarity, respectively. The discriminatory power of the seven typing phages in the partial set (those selected for K2 strains) was assessed on the 30 K3 isolates, and it was found that an additional five K3 phages were required for maximal discrimination. These phages were added to the partial set.

(iii) Phages active on K21 isolates. In our laboratory some clinical isolates of the K11 and K21 serotypes were difficult to distinguish without using absorbed antisera. We therefore considered these two groups to be similar, and K11 strains were grouped together with K21 strains for purposes of this analysis. Forty-one phages were active on one or more of the 10 K21 strains in the E1 set. They gave 21 distinct lytic patterns on the K21 strains.

The 41 phages were tested on 55 K21 isolates from eight hospitals. No pairs of identical reactions were found, but 20 phages formed a loose cluster with an internal similarity of 21 to 92%. The 12 phages in the partial typing set, i.e., those selected for the K2 and K3 strains, provided a high degree of discrimination between K21 isolates, and it was only necessary to add two of the 41 phages to this set to achieve a high degree of discrimination.

(iv) Other phages. An additional 11 phages were added to the 14 phages in the partial typing set. They were chosen from the remaining candidate phages by an exclusion procedure as follows: phages were excluded if they (i) had similar lytic patterns to those of other phages in the partial set; (ii) had poor lytic morphology, i.e., the reactions were difficult to read; or (iii) reacted with less than 5% of the strains.

Selection of the final typing set. The provisional set of 25 phages was tested on a collection of 236 strains (E2 set). The collection comprised 76 serotype strains and 160 clinical strains which represented 52 serotypes. The overall typeability with the 25 phages was 89.9%. The mean number of reactions strain^{-1} was 5.0, with a range of 0 to 13. There were 170 distinct lytic patterns, and only 3 patterns occurred

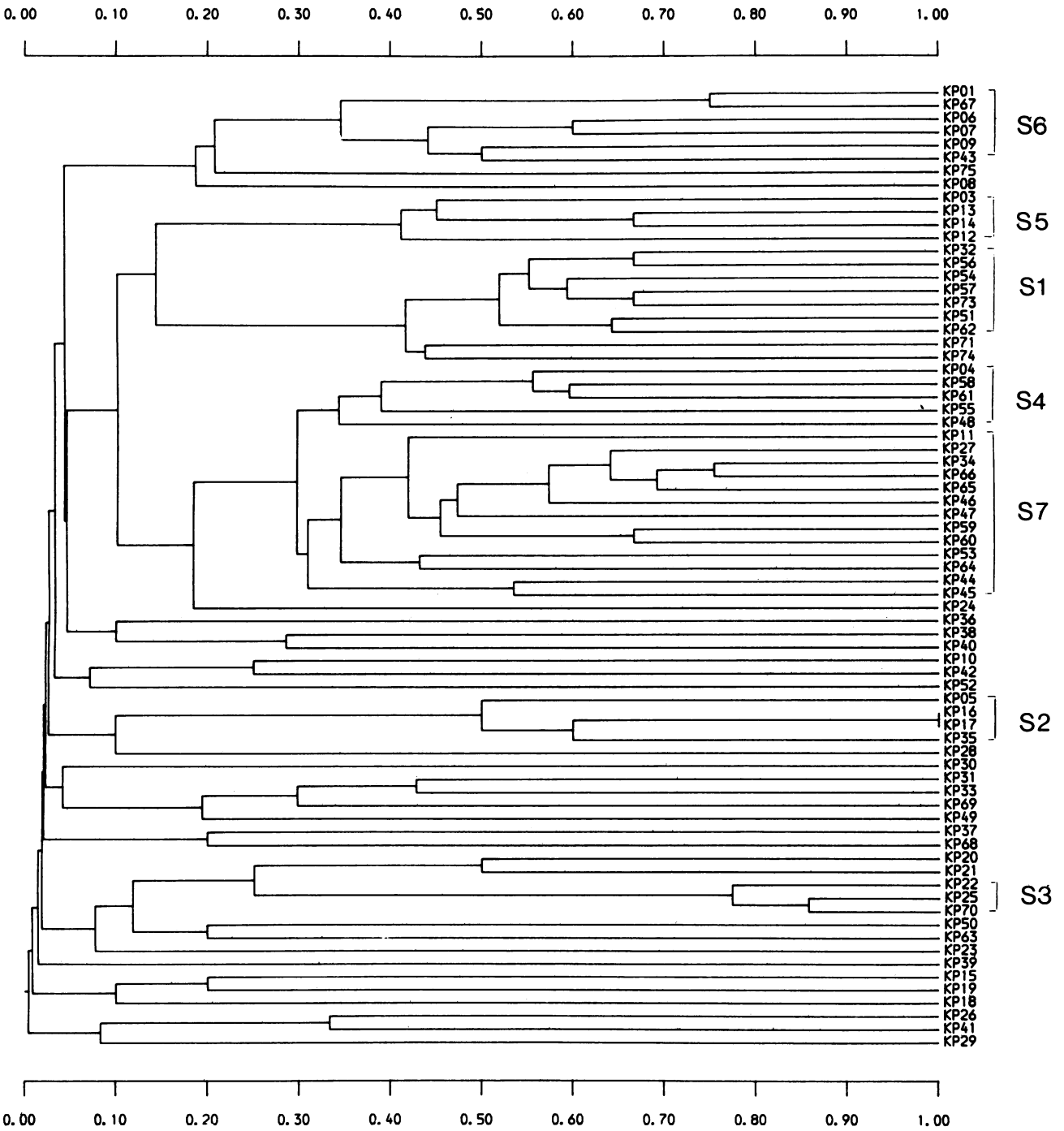

FIG. 1. Dendrogram of similarities among 74 *Klebsiella* phages based on S_j and unweighted average linkage. The horizontal axis represents the degree of similarity among *Klebsiella* phage (KP) isolates (1.00 = 100% similarity). S1 to S7 indicate clusters of similar phages.

five or more times. The number of strains lysed by each phage ranged from 1 to 144 (61%), with a mean of 46.8 (19.8%). Numerical analysis of the lytic patterns showed that nine pairs of phages had 50% or greater similarity.

Examination of the lytic patterns of five of the phages that reacted with five or fewer strains showed that they did not contribute significantly to the discrimination of the set, and they were discarded. One additional phage was discarded because it gave a variety of plaque types on some of the strains. Four phages, designated 34, 40, 60, and 61 (Fig. 1), showed more than 60% similarity to other phages in the set. This degree of similarity resulted in a high degree of redundancy in the information provided, and these phages were also discarded. The final typing set therefore consisted of 15 phages, all of which originated from sewage.

Characteristics of the typing set. The similarity between the phages in the final typing set based on their reactions on 236 strains (E2 set) is shown in Table 2. Only seven pairs of phages showed 40% or more similarity. Six phages were active on less than 10% of the strains (Table 2). The lytic

TABLE 2. Similarity among the 15 phages of the *Klebsiella* typing set[a]

Phage	% Similarity with phage:															% of strains lysed
	3	4	48	44	55	59	49	50	24	25	7	1	8	16	31	
3																10.2
4	14															43.6
48	15	58														35.2
44	10	40	31													38.6
55	11	44	34	57												48.7
59	16	45	34	37	48											61.0
49	11	32	29	18	15	23										19.1
50	7	20	20	24	26	21	22									15.7
24	6	11	14	13	13	11	9	5								9.3
25	5	9	6	3	7	6	11	10	0							5.9
7	4	9	12	1	4	7	1	0	2	3						8.1
1	5	5	6	1	2	3	1	0	0	3	40					5.1
8	10	19	13	10	11	13	7	4	0	10	25	13				12.7
16	0	2	2	5	5	4	3	0	6	0	0	0	0			3.8
31	3	2	3	2	0	3	3	0	0	0	0	0	0	0		3.0

[a] The data are based on S_j. The reactivity of the phages is based on their reactions on 236 strains.

spectrum of the phages on the propagating strains is shown in Table 3. There were only 12 propagating strains since three strains were used to propagate two phages each. Five phage filtrates inhibited the growth of five propagating strains but did not produce visible plaques on the lawns of these strains, and these inhibition reactions were easily distinguished from phage lysis.

Typeability. The final typing set gave 87.3% typeability on the 236 strains tested. The number of reactions strain^{-1} ranged from 0 to 8, with a mean of 3.2. The typeability of strains belonging to the K2, K3, and K21 serotypes was 93.3, 88.9, and 90.9%, respectively. The corresponding mean number of reactions on strains belonging to these serotypes was 3.8, 2.9, and 6.0. Of 31 serologically nontypeable strains, 76.9% were lysed by one or more of the phages, with a mean number of reactions of 2.1 strain^{-1}.

The phages were active on both *K. pneumoniae* and *K. oxytoca*, although only 17 strains of *K. oxytoca* were tested. When used in conjunction with serological typing, a typeability rate of 96.5% was achieved. Only 1 of 47 strains of *Serratia marcescens* and 11 of 50 strains of *Enterobacter* spp. were lysed by the final typing set. The 11 *Enterobacter*

TABLE 3. Lytic spectrum of *Klebsiella* bacteriophages

Strain	Susceptibility[a] to phage:														
	3	4	48	44	55	59	49	50	24	25	7	1	8	16	31
K21	×	×	4		4	5	0								
768			×	5		0	4								
802				×	4	4									3
562		5	5		×		4		5						
293		5	5	5	4	×			4				5		
788		4	0	5		0	×	×							
394					3	5			×						
677		5				4				×			5		
804						0					×	4	×		
K2		3	5			5					5	×			
161					0	3								×	
977															×

[a] 0, Inhibition; 5, titer equal to titer on propagating strain; 4, 10- to 100-fold reduction in titer; 3, 1,000- to 10,000-fold reduction in titer; ×, propagating strain.

TABLE 4. Subdivision of K3 cultures from one hospital by *Klebsiella* phage typing

Strain[a]	Susceptibility[b] to phage:															Type[c]
	3	4	48	44	55	59	49	50	24	25	7	1	8	16	31	
781				S	C											A1
786				++	S											A1
905				++	S											A1
916				++	S											A1
917				C	C											A1
918				S	S											A1
984				++	S											A1
1058				++	C											A1
1059				++	S											A1
1107				++	S											A1
1235				++	C											A1
919					S											A2
1106					S											A2
1043		++														B
1060		S			++	C										C
1232											++					D
1233											++					D
1234											++					D

[a] All cultures were isolated from patients with urinary tract infections between June 1985 and June 1986.

[b] S, Semiconfluent lysis; C, confluent lysis; ++, 50 or more plaques.

[c] Isolates with patterns A1 and A2 may be related.

strains were all *E. aerogenes*, and most were sensitive to phage 44, 55, 49, or 25.

Discrimination. A total of 126 distinct lytic patterns were produced on the 236 strains (0.53 strain^{-1}), and four patterns occurred five or more times. The most common pattern, which occurred 16 times (6.8%), was a single reaction with phage 59. The set was able to distinguish between most distinct strains of serotypes K2, K3, and K21. The number of distinct patterns achieved strain^{-1} for these serotypes was 0.88, 0.78, and 0.81, respectively. The set was less effective on K-NT strains; only 0.62 distinct pattern strain^{-1} was found.

The phages greatly improved the identification of strains in studies of both outbreaks and endemic infections. An example of the ability of the phage set to discriminate between *K. oxytoca* K3 isolates from one hospital is shown in Table 4. Each isolate originated from a separate patient with a urinary tract infection, and four distinct patterns were found. Cultures with phage susceptibility patterns A1 and A2 were considered to be similar and probably represented the same strain; the single variable reaction may have been due to experimental conditions. The phages were less useful when outbreaks were caused by uncommon serotypes, such as K57 and K19, since the index strains were often nontypeable.

Reproducibility. The long-term reproducibility of results with the phage set has yet to be assessed, but analysis of short-term reproducibility suggested that considerable attention to quality control will be necessary. Cultures typed twice on the same day showed no strong-reaction differences. Similarly, 20 distinct colonies of two strains and 10 colonies of an additional strain typed on the same day gave identical results.

However, of 55 strains which were retyped after 7 days, 14 (26%) showed one or more differences in phage susceptibility. On retyping after 5 weeks of storage, 17 of 59 strains showed one or more differences, giving 71% reproducibility. Most of these differences occurred with phages 4, 48, 44, 55, and 59. These phages gave very turbid lysis on some of the

strains, which was difficult to detect without careful observation in oblique lighting. Misreading of this reaction may contribute to errors during routine phage typing.

DISCUSSION

Numerical analysis was first applied to the selection of phages by Bergan in 1972 (3). In our laboratory we have recently used these methods in the development of a phage typing scheme for *Enterobacter cloacae* (M. A. Gaston, J. Med. Microbiol., in press). We have found numerical taxonomic procedures to be extremely useful in the analysis of the lytic reactions of the candidate phages for both the *Enterobacter* and *Klebsiella* typing sets. The advantages of this technique are that a large amount of data can be analyzed simultaneously and an objective evaluation of the lytic reactions is possible. S_j determinations (24) disregard conegative matches, and the similarity data obtained with this coefficient bear a close resemblance to a subjective analysis of test results. In this study, taxonomic analysis was used to identify identical or highly similar phages and allow multiple isolates of these phages to be discarded. It did not contribute directly to the selection of an efficient typing set, and an empirical positive selection procedure was used.

By dividing the genus into small groups, i.e., serotypes, and using numerical analysis to avoid redundancy, a useful typing set was readily selected from the large number of candidate phages. One clear advantage of this approach was that the 74 candidate phages were characterized by their lytic reactions on strains of known serotype. If the spectrum of serotypes prevalent among clinical isolates changes sufficiently to reduce the usefulness of the present scheme, we should be able to select additional or replacement phages suitable for distinguishing between strains of the new problem serotypes.

The ability to subdivide a bacterial species into a maximum number of valid types is an important factor in epidemiological surveillance. A number of established criteria must be met when choosing methods to identify individual strains, i.e., typeability, discrimination, and reproducibility. The 87.3% typeability rate achieved with the scheme compared well with other typing methods for *Klebsiella* spp. Przondo-Hessek and Ślopek (15, 16) compared the lytic activity and serological relations of the 12 phages of Milch and Deák (11) and the 35 phages of Przondo-Hessek (14). Of the 851 isolates tested, only 19.5% were typed by the set of Milch and Deák and only 67.7% were typed by the Przondo-Hessek set. Overall, 71.5% were typable with 47 phages. Although designed primarily for isolates from the United Kingdom, the set has been equally effective for typing K2 and K21 isolates from Australia, France, and North America.

The bacteriophage scheme described here was highly discriminatory: only 7% of the strains fell into the most common sensitivity pattern. Discrimination within the K2, K3, and K21 strains was also satisfactory. The scheme was simple to perform, and despite being developed primarily for K2, K3, and K21 strains, it proved to be very useful for the subdivision of strains of most serotypes. The poor reproducibility (71%) after storage may be a cause for concern in retrospective studies of *Klebsiella* outbreaks and indicates that it would not be suitable as a primary typing system for this genus.

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