Comparison of Bacteriophage Typing, Serotyping, and Biotyping as Aids in Epidemiological Surveillance of *Klebsiella* Infections

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Bacteriophage typing was used to subdivide *Klebsiella* obtained from patients in a surgical intensive care unit during a 2-year period. The 15 phages employed to type the strains were propagated by a soft-agar layer technique. In all, 23 phage types were found among the 120 clinical strains. The phage types of repeat isolates were reproducible. Only 70% of the strains tested were phage typable, but when used in conjunction with capsular serotyping and biotyping, a much greater subdivision of the *Klebsiella* strains was achieved. The addition of phage typing to serobiotyping for epidemiological analysis suggested that the number of cross-infecting *Klebsiella* strains in the intensive care unit was few, but that these strains persisted in the unit for long periods of time and could infect different body sites.

The importance of subdividing strains of a bacterial species into as many types as possible for epidemiological surveillance and infection control has been recognized for many years. While some bacteria like *Staphylococcus aureus* can be subdivided adequately by a single typing method, this has not proved satisfactory for *Klebsiella*. For this organism, capsular serotyping (4, 5, 11, 12), bacteriocin typing (9, 15), biotyping (8, 13), and bacteriophage typing (16) have all been tried; but applied alone, each method has lacked sufficient precision for epidemiological needs. In an earlier study (13), it was found that a combination of serotyping and biotyping was more discriminating than either method alone, but apparently identical serobiotypes of *Klebsiella* have been observed in which no clear epidemiological relationship existed (14).

The present investigation was designed to test whether bacteriophage typing could be applied to the typing of *Klebsiella* either as a single technique or as a supplementary method together with serotyping and biotyping. A persistent problem of serious infections caused by *Klebsiella* in patients in the surgical intensive care unit (SICU) of a Stockholm hospital provided the opportunity to investigate the reliability of phage typing as an epidemiological tool. This report gives an account of its use both when employed alone and as an adjunct to serotyping and biotyping for the subdivision of nosocomial *Klebsiella*.

MATERIALS AND METHODS

Bacterial strains. A collection of 31 cultures of *Klebsiella* and 18 cultures of other members of the tribe *Klebsiellae* was obtained from the American Type Culture Collection (ATCC), the National Collection of Type Cultures (NCTC), and the National Bacteriological Laboratory (NBL) collection, Stockholm, Sweden, for use in testing the spectrum and sensitivity of the typing phages. These are listed in Table 1. Clinical isolates of *Klebsiella* were obtained during a 2-year period from patients admitted to the SICU of Danderyds Hospital, a university referral clinic of the Karolinska Institute, Stockholm, Sweden. Biochemical identification of all the clinical isolates of *Klebsiella* was done at 37°C with a range of 24 tests by the scheme of Edwards and Ewing (7); media were made conventionally according to the manufacturers' instructions.

Bacteriophage typing. Fifteen *Klebsiella* phages (16) and their propagating strains were obtained through the courtesy of S. Slopek, Ludwig Hirsztfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland. The propagating strains were subcultured to Trypticase soy agar (Baltimore Biological Laboratory) and were stored at 4°C. The phages, designated I through XV, were propagated by a soft-agar layer technique (1) with 1.5% Trypticase soy agar in the bottom layer and 0.6% Trypticase soy agar in the top layer. After overnight incubation at 30°C, the crude soft-agar lysate was centrifuged at $2,500 \times g$ for 20 min at 4°C. The supernatant was then filtered through a 0.22- μ m nitrocellulose filter (Millipore Corp., Gothenburg, Sweden). The phage titers ranged from 10^9 to 10^{11} plaque-forming units per ml. These stock preparations were stored at 4°C.

TABLE 1. *Species and source of culture collection of strains of Klebsiellae*

Bacterial species	Source	Strain reference no. ^a
<i>K. pneumoniae</i>	ATCC	4352, 8308(1), 8308(2), 13882, 13883(1), 13883(2), 13886, 13887, 25955
	NCTC	204, 243(1), 243(2), 5056, 5057, 9495, 9496
	NBL	17726, A5054
<i>K. ozaenae</i>	ATCC	11296, 11297, 25926
	NCTC	9545, 9659
<i>K. rhinoscleromatis</i>	NBL	B5055(1), B5055(2)
	ATCC	6908, 9436, 13884
<i>E. cloacae</i>	NCTC	5046, 779, 10273
	ATCC	222, 13047, 23355
<i>E. aerogenes</i>	NCTC	9345, 10005
	NBL	U4
	NCTC	10336
<i>H. alvei</i>	NBL	U23
	ATCC	11604, 13337(1), 13337(2)
<i>Serratia liquefaciens</i> ^b	NCTC	8105, 8106
	ATCC	11367, 14460(1), 14460(2), 14461
	NBL	2182

^a (1) and (2), Morphological variants of type strains found on subculture.

^b See reference 7, p. 309.

The lytic spectrum of the phage preparations against the 15 propagating strains was determined as for staphylococci (3). The reactions were recorded as: +++, confluent or almost confluent lysis; ++, more than 30 isolated plaques; +, 15 to 30 isolated plaques; ±, 5 to 15 plaques. The phage dilution giving a ++ reaction on heterologous propagating strains was compared to the dilution giving the same reaction on the homologous strain.

For the determination of the routine test dilution, each phage suspension was titrated against its propagating strain. Overnight broth cultures of the organisms were diluted 100-fold in fresh Trypticase soy broth and were incubated with agitation at 37°C for 2 h. Trypticase soy agar plates (0.6% agar) were flooded with the broth cultures, drained, and dried well. Ten-fold dilutions of the phage suspensions were then applied, and the plates were incubated at 30°C for 4 h and then stored overnight at 4°C. The highest dilution of phage showing almost confluent lysis was chosen as the routine test dilution. For clinical isolates of *Klebsiella*, the plates were flooded with the strains and the phages were applied at the routine test dilution and 100 × the routine test dilution. Phage types of these strains were defined by +++ or ++ reactions.

Capsular serotyping and biotyping. The capsular serotypes of the *Klebsiella* strains were determined by the Quellung reaction (4) with the 72 antisera of the international set. These were prepared in rabbits by one of us (R.P.R.) and were absorbed to remove cross-reacting antibodies (7). All the *Klebsiella* strains were subdivided by a numerical biotyping scheme (13). Eleven tests—indole and Voges-Proskauer (acetyl-methyl carbinol production) and citrate utilization; lactose and sucrose fermentation and malonate and D-gluconate utilization; dulcitol fermenta-

tion, lysine and ornithine decarboxylation and urease production—were used. The results of the 11 tests were converted to a three-digit code as given in Tables 2, 3, and 4 of reference 13. The results of all three typing methods were then combined as the sero-biophage type of the strain.

RESULTS

Phage typing. The lytic spectrum of the 15 *Klebsiella* bacteriophages against their propagating strains was found to be stable in our hands and, as identified in Table 2, their spectrum was similar to that observed previously by Slopek et al. (16).

Only, 19 (61%) of the 31 culture collection strains of *Klebsiella* were lysed by phages in the existing set (Table 3). The *K. pneumoniae* strains were of a variety of phage types. However, all six strains of *K. rhinoscleromatis* were lysed by phage VI only, and none of the seven strains of *K. ozaenae* were lysed. If this latter group of seven strains was removed, the percentage of typable strains increased to 77% for the culture collection strains of *Klebsiella*. Over 35% of the type cultures of *Enterobacter* species cross-reacted with the *Klebsiella* phages, but these reactions were in part species related; none of the *E. cloacae* and *Hafnia alvei* were lysed by any phages, but the two strains of *E. aerogenes* were typable. These organisms were easily distinguished from *Klebsiella* by their biochemical reactions.

During the 2-year survey period in which phage typing was done, 120 strains of *Klebsiella* were isolated from 93 patients in the SICU of Danderyds Hospital by using the initial selection criteria of different phage types to identify a strain. Of the 120 strains, one strain of *K. ozaenae* was seen and the rest were all *K. pneumoniae*. Repeat isolates lysed by the same phages were saved to test the reproducibility of the typing technique, but were not included among the 120 strains. Of the final series of 120 strains, 57 were obtained from respiratory tract specimens, 19 from the urinary tract, and 44 from blood cultures or from surgical wound sites other than tracheostomies. Almost all the *Klebsiella* from the respiratory tract were isolated from tracheal suction specimens.

The distribution of phage types of the 120 strains is shown in Table 4. In all, 23 types were identified among the strains, but 29% were not typable by the criteria of ++ or +++ reactions used to define a lytic reaction. About 40% of the strains were lysed by phages I or II alone or both I and II together. The single strain of *K. ozaenae* seen in the clinical series, like the culture collection strains of this species, was not

TABLE 2. *Lytic spectrum of Klebsiella bacteriophages*

Propagating strain	Bacteriophage no. ^a														
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
K67/264-1	5														
K59/2212	4	5			5									5	
K14/1193	5		5												
K40/8581				5											
K68/265-1	5	4			5										
K3/L-174	4	4				5									
K30/7824							5							4	
K58/636								5							4
K2/B-5055		3		5					5						
K11/390	4	5								5			2		
K24/1680											5	2			
K42/1702												5			
K71/B	4	5		3	4		4						5		
K62/344B	4	5	5										3	5	
K9/97B															5

^a The numbers refer to plating efficiencies (++) reactions) of each bacteriophage on heterologous propagating strains at phage dilutions: 5, the same as on the homologous propagating strain; 4, 10 to 10² times reduced; 3, 10³ to 10⁴ times reduced; 2, 10⁵ to 10⁶ times reduced. Blanks indicate negative reactions at the most concentrated dilutions used.

TABLE 3. *Phage typing of culture collection strains of Klebsiellae*

Bacterial species	No. of strains	No. of typable strains
<i>K. pneumoniae</i>	18	13
<i>K. ozaenae</i>	7	0
<i>K. rhinoscleromatis</i>	6	6
<i>E. cloacae</i>	6	0
<i>E. aerogenes</i>	2	2
<i>H. alvei</i>	5	0
<i>Serratia liquefaciens</i>	5	3

TABLE 4. *Phage types of 120 clinical strains of Klebsiella*

Phage type	Strains	
	No.	%
I	10	9
II	17	14
I/II	24	20
II/VI	5	4
II/V	5	4
X	3	2
II/IV	2	2
III, VIII, XII ^a	6	5
13 others ^b	13	11
Nonlytic	35	29

^a Two each.

^b One each.

phage typable. No *K. rhinoscleromatis* were found.

Combined typing. The biotypes and capsular serotypes of the 120 strains were also determined. Twenty-four different numerical biotypes were seen, and 35% of the strains were

TABLE 5. *Biotypes among 120 strains of Klebsiella in the SICU*

Biotype ^a	Strains	
	No.	%
Indole negative		
1/1/2	29	24
1/1/1	18	15
1/1/5	9	8
2/1/2	7	6
Other (10 types)	15	12
Indole positive		
5/1/1	21	18
5/1/2	4	3
6/3/4	4	3
5/3/3	3	3
Other (6 types)	10	8

^a Numerical codes given in Tables 2, 3, and 4 of reference 13.

indole positive (Table 5). This frequency of indole-positive strains is greater than usually seen, but no relationship between indole production and serotype or phage type was found. The distribution of capsular serotypes is given in Table 6. The 37 serotypes encountered were not unusual, but 17% were not serotypable. This is a higher percentage of nontypable strains than has been reported before (13), but may well be due to sampling in only a single hospital area. Of the 21 strains that could not be serotyped, 16 were lysed by one or more phages in the collection.

The independent variation of serotyping and biotyping was used previously to increase the precision with which *Klebsiella* could be typed

TABLE 6. Subdivision of the capsular types of 120 *Klebsiella* strains by biotype and phage type

Capsular type	No. of strains	No. of types when capsular type was further subdivided by:		
		Biotype	Phage type	Both
55	15	4	6	9 ^a
68	11	6	6	11
61	8	6	4	8
32	6	1	1	1 ^a
21	5	3	3	5
28	5	3	2	5
60	5	2	3	5
7	3	3	3	3
19	3	3	3	3
22	3	2	2	3
39	3	1	3	3
16	2	2	2	2
18	2	2	2	2
24	2	2	2	2
38	2	2	2	2
43	2	2	2	2
69	2	1	2	2
Other (20 types)	20	20	20	20
Nontypable	21	10	14	16 ^a

^a More than one strain with the same combined type was found (Table 7).

(13). In the present study we observed that phage types were unrelated to serotypes or biotypes. Table 6 shows also the degree of subdivision obtained when capsular types were further subdivided by biotypes and phage types. Among the 120 strains, 75 distinct serobiotypes were identified. By combining serotyping and phage typing, we recognized 82 types, and the combination of all three methods identified 104 types among the 120 strains. Even though many strains were lysed by phage I or II or both, the triple-typing method was capable of separating these strains without the necessity of finding new phages. When repeat isolates were examined that had the same phage type and were isolated from the same infected site or from different sites of one patient, results showed that three-method typing was reproducible. For example, the *Klebsiella* isolated from a patient's tracheal suction specimens and from his lungs at autopsy were all of phage type III/IX. Each isolate also had the same biotype 1/1/1, and none of them could be serotyped. Where the phage types of repeat isolates were different, we found that the biotypes or serotypes were different also.

The *Klebsiella* with the same sero-bio-phage types that were isolated from more than one patient in the SICU are identified in Table 7. Because two of these four types were not serotypable and their biotypes were similar, the dif-

TABLE 7. Isolation of four *Klebsiella* sero-bio-phage types causing more than a single infection in the SICU

Source and no. of isolates	Sero-bio-phage type ^a
Tracheal secretions, 2 Catheter urine, 2 Abdominal wound, 1 Blood culture, 1	K32:1/1/1:NL
Tracheal secretions, 3 Abdominal wound, 4	K55:5/1/1:I/II
Tracheal secretions, 2 Catheter urine, 1 Colonic fistula, 1	NT:2/1/2:II/V
Catheter urine, 2 Multiple sites (sputum, bile drain), 1	NT:1/1/2:X

^a NL, nonlytic; NT, not serotypable.

ferent phage types were important in distinguishing between them. Epidemiological data revealed that these *Klebsiella* had caused serious sporadic infections in the SICU during periods varying from a month to more than a year. No single type was restricted to any specific anatomical site. In all cases, these *Klebsiella* were isolated while the patient was in the SICU, but the period of stay in the unit of a patient newly infected with a particular type rarely overlapped that of a previous patient infected with a *Klebsiella* of the same sero-bio-phage type. The longest gap in time between isolations of strains of the same type was 5 months.

DISCUSSION

Only a slight increase in the percentage of phage-typable strains of *Klebsiella* over that found previously by Slopek et al. (16) was observed in the present study. The high proportion of strains lysed by phage I or II or both underscores the necessity of discovering new phages to subdivide these strains if phage typing is to be useful alone for typing *Klebsiella*. Whether new phages could be found to decrease the percentage of nonlytic strains is not clear. It is noteworthy that all the culture collection strains of *K. ozaenae* and our one clinical isolate of this species were not lysed by any phages. In Slopek's investigation (16), most of the *K. ozaenae* were also nonlytic. Strains of this species characteristically produce large amounts of watery, mucoid capsular substance, and this feature may affect their susceptibility to bacteriophage (10). It will be necessary to try to depress the production of capsular antigens on selective media to determine whether the susceptibility of strains of *K. ozaenae* or even other *Klebsiella* to bac-

terioophage particles can be altered. In this study it was observed that 16 of the 21 non-serotypable strains were susceptible to bacteriophages. It may be possible to use phage typing to differentiate these strains.

When used alone, phage typing had serious shortcomings, but when it was combined with capsular serotyping and biotyping, it proved of value in defining more clearly the extent of cross-infection in the SICU during the 2-year survey period. The four types of *Klebsiella* shown in Table 7 each caused sporadic infections separated by various periods of time. Each episode caused by these types could have been due to a separate introduction of the particular strain carried into the SICU by the patient concerned. Alternatively, the infections may have been derived from the intensive care environment, perhaps from contact with attending staff (6). In Toronto (14), strains of *Klebsiella* were observed to reemerge in different areas of the hospital after latent periods similar in duration to those seen in the SICU in the present study. In that investigation, the rarity of the types involved and their possession of specific antibiotic resistance markers strongly suggested that the same strain had reemerged to cause nosocomial infections. In this survey, the strains infecting several patients were no more resistant to antibiotics than strains isolated only once. Antibigrams were found to have no value in differentiating the sporadic from the more frequently isolated types of *Klebsiella*. Because the present study was restricted to strains of *Klebsiella* isolated from patients in the SICU, it cannot be concluded that the *Klebsiella* of each sero-biophage type shown in Table 7 were derived from the same original sources. Strains of an identical type can be different if no epidemiological relationship exists.

Our study supports in a practical way the suggestion of Barr (2) that multiple typing methods may be better able to identify strains of *Klebsiella* of varying pathogenic potential or perhaps strains that have a better capacity to survive and persist in hospital environments. Phage typing was shown to be of value in com-

bination with serotyping and biotyping in the separation of *Klebsiella* causing infections in the SICU into many different types. We consider that it has promise as an epidemiological aid for recognizing hospital strains of *Klebsiella*.

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