EPIDEMIOLOGICAL TYPING

The use of bacteriophages to differentiate serologically cross-reactive isolates of *Klebsiella pneumoniae*

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Summary. In serological typing of *Klebsiella pneumoniae* strains from human, equine and environmental sources, the capsular identity of many isolates could not be determined because of serological cross-reactivity. A panel of 91 bacteriophages able to lyse each of the 77 capsular serotypes of *K. pneumoniae* was isolated and tested for the ability to distinguish between strains in a collection of 17 clinical isolates of *K. pneumoniae* which exhibited cross-reactivity with two or more capsular type sera. Most isolates could be assigned a capsular type by performing a simple streak test with bacteriophage, although some required the application of an efficiency of plating analysis to discern capsular type. Bacteriophage typing was found to be an effective, inexpensive and clinically practical adjunct to serotyping in distinguishing serologically cross-reactive *K. pneumoniae* isolates, irrespective of their origin.

Introduction

Klebsiella pneumoniae is an opportunist pathogen that is a major cause of nosocomial infections.^{1–4} In many hospitals, *Klebsiella* spp. are second only to *Escherichia coli* as the major cause of gram-negative bacteraemia.^{5,6} As an increasing proportion of *K. pneumoniae* isolates are resistant to commonly used antibiotics,^{1,7} it is not surprising that reported mortality rates for *K. pneumoniae* bacteraemia and pneumonia range from c. 25–50%.^{2,8} *Klebsiella* spp. are also important in veterinary medicine, as a cause of equine metritis and bovine mastitis.^{9,10} Consequently, in both human and veterinary medicine, it is important to monitor and control the presence of *K. pneumoniae*.

Klebsiella spp. have been typed by various techniques, including bacteriocin typing,¹¹ biotyping,¹² serotyping,^{13,14} bacteriophage typing,^{15–17} antibiotic-susceptibility testing,¹⁸ protein profiling,¹⁹ multilocus enzyme analysis,^{8,20} and several molecular techniques.^{8,18,21,22} Each typing method has drawbacks and recent reviews have detailed their limitations.^{23–25} Because of the limitations of employing a single approach to typing, some investigators have used two or more typing methods for improved discrimination between isolates.^{18,26–28}

Although the current trend is toward the use of

molecular typing techniques, serological and bacteriophage typing methods are attractive to diagnostic laboratories because they are simple and can be easily and rapidly applied to many isolates.^{16,29} Initially, we monitored *K. pneumoniae* outbreaks by serotyping. However, clinical isolates exhibiting serological cross-reactivity between two or more capsular serotypes were found regularly. As Przondo-Hessek,¹⁷ Sutherland,³⁰ and Rieger-Hug and Stirm³¹ have shown that it is possible to obtain bacteriophages specific to a particular capsular serotype, or groups of serotypes, of *Klebsiella* spp., bacteriophage typing was evaluated as a supplement to serotyping to differentiate cross-reacting *Klebsiella* serotypes. It was reasoned that bacteriophage typing would be an inexpensive, easy to perform method for differentiating *K. pneumoniae* isolates and would obviate the need for more complicated molecular typing methods. This study is the first report of the use of bacteriophages to differentiate between serologically cross-reacting capsular types of *K. pneumoniae* obtained from various sources.

Materials and methods

Klebsiella strains

Reference strains of *Klebsiella* serotypes K1–72, K74 and K79–82 were obtained from Dr I. Ørskov, Collaborative Centre for Reference Research on *Escherichia* and *Klebsiella*, World Health Organization, Statens Seruminstitut, Copenhagen, Den-

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mark.³² Isolates of *K. pneumoniae* from human, veterinary and environmental sources were obtained over a 5-year period through submission to one of us (R.P.R.) for epidemiological serotyping. Isolates 7-1406 and 41-50538 were from human infections; isolates Fibar-2, Fibar-3, UG-971, UG-972, UG-50888, CR-14974, CR-15831, CR-15857, CR-16131, CR-16452, CR-16661, CR-16941 and CR-17124 were from equine infections; CR-15881 was isolated from the floor of an equine breeding area; and isolate SCW-5489 was obtained from city water. Isolates were identified as *K. pneumoniae* by the AMS-Vitek Identification System (Hazelwood, MO, USA).

Capsular serotyping

The capsular serotypes of clinical isolates were determined by the Quellung test as described previously.³³ A light suspension of bacteria was prepared by touching several individual colonies from MacConkey agar plates and mixing in 1 ml of formalin 1% in saline. A 10- μ l sample of this suspension was mixed with an equal volume of pooled *K. pneumoniae* antiserum, placed on a slide and viewed by phase contrast microscopy. Each pool of antisera comprised three to six individual sera.³⁴ When a positive Quellung reaction was obtained, individual capsular antisera were used to determine which capsular type had produced the positive reaction. Capsular cross-reactivity between isolates was also screened in this manner.

Capsular type antisera were prepared by immunising New Zealand White rabbits with each strain from the Danish set of 77 *Klebsiella* serotypes as described by Edwards and Ewing.³⁴ All rabbits were pre-screened to ensure that they were pasteurised-free and to ascertain any background titre to *klebsiella* capsular antigens. Antibody end-point titres were determined by a Quellung reaction with two-fold dilutions of serum in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 8 mM Mg₂SO₄ (SM buffer). The highest serum dilution that gave a positive Quellung reaction was taken as the titre. The titre of serologically cross-reacting sera was determined similarly. All antisera were stored at -70°C after adding sodium azide 0.1% w/v.

Bacteriophage isolation

Bacteriophages able to replicate in each of the 77 *Klebsiella* serotypes were isolated from raw sewage. Fifty ml of double strength Luria Broth (LB) were mixed with 50 ml of sewage and 10 ml of an overnight culture of each of the serotypes. After incubation for 18 h at 37°C with shaking (100 rpm) in a water bath, chloroform 1% v/v was added. The sample was then incubated at room temperature for 30 min to lyse the bacteria. After centrifugation at 10000 *g* for 10 min to remove cellular debris, the supernate was removed and filtered through a 0.45- μ m Nalgene filter, Type S (Nalgene Company, Rochester, NY, USA). To con-

firm the presence of bacteriophage in the filtrate, a drop was streaked on to a plate containing an agar overlay of the *Klebsiella* serotype used to seed the raw sewage. The plate was examined for lytic activity after incubation at 37°C. All bacteriophages were plaque-purified five times, and the final cell lysate was assayed to titre and stored over chloroform 1% v/v at 4°C. Most of the bacteriophage suspensions had a titre in the range 10⁸-10¹⁰ plaque forming units/ml. In this report, each bacteriophage is identified by the capsular serotype number of its initial host bacterium. Two or more distinct bacteriophages isolated from the same *Klebsiella* serotype (based upon plaque morphology) are designated by lower case letters.

Determination of bacteriophage cross-infectivity

The routine test dilution (RTD) was taken to be the dilution of bacteriophage that just failed to give confluent lysis of the *Klebsiella* host strain on which it was first isolated. RTDs are used routinely in phage typing because phages have different burst and particle sizes, both of which can influence the concentration at which a particular phage will give semi-confluent lysis of its bacterial host. For phage typing, a 0.45-ml sample of an overnight culture of the *Klebsiella* isolate to be typed was diluted in 5 ml of LB-soft agar (0.75% agar), poured over an LB agar plate (9 cm diameter), and allowed to dry for 30 min. A 25- μ l sample of each bacteriophage suspension at its RTD was placed as a drop on to the agar overlay of the test organism and dragged as a streak across the overlay with a small strip (8 $\times$ 60 mm) of sterile Whatman No. 1 filter paper. This filter streaking technique resulted in a concentration gradient of phage across the plate and eliminated the possibility of obtaining non-infectious lysis of the test bacteria as can occur with too high a concentration of phage. After overnight incubation at 37°C, the plate was scored for the presence of lytic activity as indicated by the presence of 20 or more plaques, or a clear or turbid lytic area, along the streak line. Cultures to be typed were each tested a minimum of three times with the appropriate bacteriophages. Identical results were obtained each time.

Efficiency of plating (EOP) assays were also performed to quantitate the ability of bacteriophages to cross-infect other *Klebsiella* serotypes. The EOP value is a ratio indicating how well a bacteriophage plates on a heterologous *Klebsiella* serotype compared to the *Klebsiella* serotype first used to isolate the bacteriophage.³⁵ An EOP of < 100% indicates that some form of host restriction is occurring. Samples of serial 10-fold dilutions (in SM buffer) of the respective phage suspensions were incubated for 15 min with the isolating host and with a heterologous host. Each mixture was diluted into 5 ml of LB-soft agar and layered on to an LB agar plate. The plates were incubated overnight at 37°C and scored for the presence of plaques. In this laboratory, EOP values for the various bacteriophages have remained stable for > 3 years.

Results

Capsular serotyping of *K. pneumoniae*

Most of the rabbit antisera generated against the 77 *Klebsiella* capsular serotypes had titres ≥ 16 and a few had titres as high as 128 (partial listing in table I). However, for several capsular types, the antiserum titre could not be raised above 8 and, in two cases, the titre could not be raised above 4. The majority of the 77 rabbit *klebsiella* capsular antisera reacted with more than one capsular serotype (partial listing in table I). Initial attempts were made to adsorb the antisera with cross-reacting capsular serotypes, but in several cases, this eliminated the Quellung titre for the capsular serotype used for immunisation. Because the titre against a cross-reacting capsular type was frequently $< 25\%$ of the titre against the capsular type used for immunisation, attempts were made to reduce the interference posed by cross-reactivity by diluting the antisera when performing Quellung reactions. Because of the subjectivity inherent in interpreting the Quellung reaction, this approach to discriminating serotypes was difficult to reproduce and could not be done at all when the titres to the immunising and cross-reacting capsular types were very similar.

Isolation of *klebsiella* capsular type-specific bacteriophages

A total of 91 bacteriophages was isolated which lysed the 77 *Klebsiella* capsular serotypes. For several of the capsular types, including K22, K46 and K64,

Table I. Serologically cross-reactive strains of *Klebsiella**

Capsular type (Quellung titre)	Cross-reacting capsular type (Quellung titre)
K2 (16)	K13 (2)
K7 (16)	K10 (8); K61 (2)
K8 (8)	K25 (2)
K10 (64)	K7 (2); K61 (32)
K11 (64)	K21 (2)
K12 (16)	K29 (4); K42 (8)
K13 (32)	K30 (8)
K14 (16)	K64 (16)
K18 (32)	K44 (8)
K21 (64)	K11 (16)
K22 (64)	K37 (32)
K24 (32)	K43 (4)
K25 (8)	K8 (2)
K27 (32)	K46 (16)
K29 (16)	K12 (2); K42 (2)
K30 (16)	K13 (4)
K37 (64)	K22 (32)
K42 (32)	K29 (2)
K43 (4)	K24 (4)
K44 (32)	K18 (8)
K46 (64)	K27 (16)
K61 (8)	K7 (2); K10 (4)
K64 (16)	K14 (2)

* The serological cross-reactivities shown relate to the cross-reactive serotypes found for the 17 *Klebsiella* isolates listed in table III.

Table II. Efficiency of plating (EOP) of bacteriophages on their natural and heterologous cross-reacting *Klebsiella* serotype hosts

Host capsular type	Bacteriophage plated	EOP (%)
K2	2	100
	13	0
	30	0
K7	7	100
	10	7.3
	61	0
K8	8	100
	25	0
K10	10	100
	7	0
K11	61	0
	11	100
	21	0
K12	12	100
	29	2.5×10^{-4}
	42	0
K13	13	100
	2	1.4×10^{-4}
	30	0
K14	14	100
	64a	2.0×10^{-2}
	64b	0
K18	18	100
	44	0
K21	21	100
	11	85
K22	22a	100
	22b	100
K24	37	0
	24	100
	43	0
K25	25	100
	8	0
K27	27	100
	46a	100
	46b	0
K29	29	100
	12	13
	42	0
	30	100
K30	2	0
	13	0
	37	100
K37	22a	1.4
	22b	56
K42	42	100
	12	0
K43	29	7.2×10^{-6}
	43	100
K44	24	0
	44	100
K46	18	0
	46a	100
	46b	100
K61	27	0
	61	100
	7	0
K64	10	2.0×10^{-3}
	64a	100
	64b	100
	14	4.0×10^{-2}

more than one bacteriophage was isolated. An EOP analysis was used to determine how effectively each bacteriophage was able to replicate in a cross-reacting heterologous capsular serotype compared to the *Klebsiella* capsular serotype used for isolation. Because

Table III. Bacteriophage differentiation of *K. pneumoniae* isolates showing serological cross-reactivity

Isolate no.	Cross-reactive serotypes*	Bacteriophage streaked†	Lytic activity	Phage-determined type‡
7-1406	K22/37	22a 22b 37	+ — —	K22
41-50538	K22/37	22a 22b 37	— — +	K37
Fibar-2	K18/44	18 44	— +	K44
Fibar-3	K27/46	27 46a 46b	+ — —	K27
SCW-5489	K14/64	14 64a 64b	— + —	K64
UG-971	K12/29/42	12 29 42	+ + —	K29§
UG-972	K24/43	24 43	— +	K43
UG-50888	K8/25	8 25	— +	K25
CR-14974	K22/37	22a 22b 37	— + —	K22
CR-15831	K7/10/61	7 10 61	— + +	K61
CR-15857	K7/10/61	7 10 61	+ + —	K7
CR-15881	K11/21	11 21	— +	K21
CR-16131	K14/64	14 64a 64b	+ + +	K64
CR-16452	K7/10/61	7 10 61	+ + —	K7
CR-16661	K2/13/30	2 13 30	— — +	K30
CR-16941	K14/64	14 64a 64b	+ + —	K14§
CR-17124	K2/13/30	2 13 30	+ + —	K13

* Serotype of the clinical isolate determined by the Quellung reaction.

† Bacteriophages were used at a concentration of 1 RTD, except as follows: bacteriophages 37, 22a, 22b were used at 16 RTD; bacteriophage 11 was used at 1024 RTD; and bacteriophage 21 was used at 512 RTD.

‡ The type assigned to the isolate on the basis of the lytic pattern obtained with the indicated bacteriophage.

§ Phage typing assignment required a complete EOP analysis (see table IV).

the intent was to validate the use of bacteriophages to distinguish between serologically cross-reactive *Klebsiella* isolates, the EOP values were determined only for those combinations of phage and host serotypes relevant to the clinical isolates examined later (see below). The EOP results (table II) revealed that while most bacteriophages were specific for the host capsular type on which they were isolated initially, some, such as 11, 22b and 46a, replicated almost equally well on other *Klebsiella* capsular types as they did on the capsular types in which they were first isolated.

Bacteriophage typing of serologically cross-reactive K. pneumoniae

Over a 5-year period, 17 isolates of *K. pneumoniae* which were serologically untypable because of cross-reactivity with two or more capsular antisera were collected from various sources. Bacteriophages showing specificity for each of the cross-reacting capsular serotypes (table II) were tested for their ability to produce plaques on these isolates. As shown in table III, 15 of 17 isolates could be directly assigned to a

Table IV. Differentiation of cross-reactive serotypes of *K. pneumoniae* by bacteriophage EOP analysis

Isolate no.	Cross-reactive serotypes	Bacteriophage plated	EOP (%)*
UG-971	K12/29/42	12	5
		29	42
		42	0
CR-16941	K14/64	14	2.0×10^{-2}
		64a	1.8×10^{-3}
		64b	0

* Plating of the indicated bacteriophage on the *Klebsiella* capsular type used for the initial isolation of the phage was taken as 100%.

particular capsular type from their sensitivity to type-specific bacteriophage. For example, Fibar-2, UG-972 and UG-50888 were found to be capsular types K44, K43 and K25, respectively, because the bacteriophages isolated originally in these capsular serotypes were able to replicate on the three clinical isolates. These capsular assignments are validated by the EOP values in table II which show that, within this particular group of serotypes, bacteriophages 44, 43, and 25 show complete specificity.

For six of these 15 isolates (CR-15831, CR-15857, CR-16131, CR-16452, CR-16661 and CR-17124), it was necessary to use additional information provided by the EOP values from table II before a capsular identity could be determined. For example, the clinical isolate CR-15831 demonstrated K7/10/61 capsular cross-reactivity by serotyping and was lysed by bacteriophages 10 and 61, but not by bacteriophage 7 (table III). By comparing the EOP values of bacteriophages 7, 10 and 61 on *Klebsiella* capsular types K7, K10 and K61, and by comparing this to the ability of these three bacteriophages to grow on isolate CR-15831, it was determined that the isolate had capsular type K61. Similar reasoning applied to the other five isolates.

For the last two *Klebsiella* isolates included in this study, a simple streak test or a streak test combined with an EOP analysis as above, did not allow us to discern directly the capsular type. Isolate UG-971 was either K12 or K29 and isolate CR-16941 was either K14 or a K64 variant (table III). Therefore, the EOP for bacteriophages 12, 29 and 42 on isolate UG-971, and bacteriophages 14, 64a and 64b on isolate CR-16941 were determined. Results presented in table IV indicate that clinical isolates UG-971 and CR-16941 were of capsular types K29 and K14, respectively.

Of interest was the observation that phage typing of isolates 7-1406, 41-50538, Fibar-3, SCW-5489, CR-14974 and CR-15881 (table III) suggested that they were capsular type variants of *K. pneumoniae* serotypes K22, K37, K27, K64, K22 and K21, respectively. For example, isolate 7-1406 was a K22 capsular type variant because bacteriophage 22a was able to plaque on this isolate, while bacteriophage 22b was not, even though both bacteriophages show identical EOP values for growth in the original *K. pneumoniae* K22

host strain (table II). Similarly, isolate 41-50538 was typed as a capsular K37 variant because it was lysed by bacteriophage 37 but not bacteriophages 22a and 22b, despite the fact that these latter two phages could grow with a reduced EOP in the original *Klebsiella* capsular type K37 (table II).

Finally, it should be noted that bacteriophages were obtained which were also able to discriminate *Klebsiella* capsular types 1 and 6, 2 and 69, 3 and 68, 33 and 35, and 70 and 72. This was determined by using the Danish set of *Klebsiella* serotypes as host strains (data not shown). Clinical isolates have not yet been encountered which possess these capsular serotypes. Nevertheless, this bacteriophage panel allows the typing of a wider range of serologically cross-reacting *Klebsiella* capsular types than is shown in table III.

Discussion

Serotyping continues to be used in the epidemiological surveillance of *K. pneumoniae* outbreaks,^{16, 29} although it tends to be restricted to reference centres or large hospitals that can underwrite the cost of generating antisera to the 77 *Klebsiella* capsular types. However, in the serotyping of *K. pneumoniae*, two main drawbacks are encountered. The first is the weak immune response induced by *Klebsiella* capsular polysaccharide which results in sera of low titre.^{36, 37} The second is the occurrence of serological cross-reactivity between various capsular types (table I), because > 50% of capsular antisera show cross-reactivity with one or more capsular types.^{14, 36, 37} This serological cross-reactivity is likely to be due to the limited number of moieties comprising the capsular material³¹ and to the presence of shared lipopolysaccharide groups which may intercalate between the capsule and be accessible at the surface.³⁸ Therefore, it is not surprising that in attempting to obtain monospecific capsular antiserum by adsorbing with the cross-reacting capsular type, a significant reduction or total elimination of antibodies reacting with the immunising capsular type was observed. Moreover, other workers^{36, 37} and ourselves have found that antisera to the same capsular type can display variable capsular cross-reactivity.

Against this background of problems inherent in serotyping, the aim of this study was to determine if bacteriophages isolated by their ability to lyse specific *Klebsiella* capsular types could differentiate between isolates showing serological cross-reactivity. Bacteriophages able to replicate in each of the 77 *Klebsiella* capsular types were readily isolated from sewage. Evidence that these bacteriophages could be used to discriminate serologically cross-reactive *K. pneumoniae* capsular types came from EOP values for growth of the bacteriophages in those capsular type strains of *Klebsiella* that presented cross-reactivity problems. The EOP data showed that within a group of serotypes, bacteriophages could be identified which

were completely specific for one of the serotypes within that group. It must be noted that because a large-scale EOP analysis of all possible bacteriophage-host serotype combinations has not been done, it is possible that many of these phages will infect other serotypes of *Klebsiella*. This has been reported previously by Gaston *et al.*¹⁶ who identified groups of serotypes lysed by particular phages but not phages which were completely type-specific. Importantly, however, EOP analysis revealed that within a limited group of *Klebsiella* serotypes, specifically those which show serological cross-reactivity, it is possible to obtain phages that discriminate within that group. To demonstrate the practicality of this approach, a panel of bacteriophages was used in a simple streak test to assign directly a capsular type to 15 of 17 clinical isolates of *K. pneumoniae* which had previously been untypable due to serological cross-reactivity. For most of the isolates, a bacteriophage RTD of 1 was sufficient, although for isolates CR14974 and CR15881, higher RTDs were required. The reason for this is not known, although it is common to use higher bacteriophage RTD values than used here to type organisms.^{27,39}

For isolates UG-971 and CR-16941, the capsular type could not be discerned directly by a simple streak test. However, by using an EOP analysis for bacteriophage replication in these two isolates (table IV), a capsular type was assigned in both cases. Earlier, Przondo-Hessek¹⁷ showed that a bacteriophage able to infect several different *Klebsiella* capsular types lost this cross-infectivity if passaged at least 10 times on the capsular type used for initial isolation. Should this procedure be generally applicable for generation of capsular-specific bacteriophages, applying it to bacteriophages 12 and 29, for example, might render these two phages specific for capsular types K12 and K29. Such phages could then be used in a simple streak test and obviate the need to perform an EOP analysis to determine a capsular type.

We do not know whether the *Klebsiella* bacteriophages require a specific capsular type for infectivity or whether other factors such as a cellular bacteriophage receptor are also involved. Nonetheless, the data on the typing of serologically cross-reactive isolates suggests that at least some of the bacteriophages do not simply recognise the capsule of their *Klebsiella* host. This is illustrated by the disparate typing patterns of bacteriophages 22a and 22b with *K.*

pneumoniae isolates 7-1406 and CR14974, which showed serological cross-reactivity but were clinically distinct (table III). If the capsule was the only factor involved in infectivity, bacteriophages 22a and 22b should each have infected both isolates, because both phages replicate equally well on *Klebsiella* capsular type K22. The inability of a bacteriophage to replicate in a particular host could be due to a number of factors, including changes in a bacteriophage receptor,⁴⁰ host restriction mechanisms, or immunity resulting from lysogeny.⁴¹ Therefore, variability in bacteriophage susceptibility points to some degree of divergence among the bacteria themselves. As evidence of this, six isolates were encountered that were termed variants of their respective capsular type based upon the bacteriophage typing results. Presumably, typing with antisera lacks the sensitivity to detect and discriminate among such variants.

Other studies have used bacteriophage typing alone or in combination with other typing techniques to differentiate, classify, and monitor *K. pneumoniae* infections.^{15-18,26,27} Ayling-Smith and Pitt,²³ for example, considered phage typing to be an excellent secondary typing method when used in conjunction with serotyping. This study has shown for the first time that bacteriophage typing readily differentiates between serologically cross-reacting *K. pneumoniae* capsular types irrespective of their human, animal or environmental origins. Although molecular typing techniques can be used in epidemiological investigations, these methods are costly, labour intensive, and lack standardised interpretive criteria,^{24,28} which so far has limited their use to reference centres or research laboratories. In contrast, bacteriophage typing is inexpensive and can be used in clinical laboratories without great technical expertise, as the streak assay and even EOP assays are straight-forward to perform and give easily interpreted results. Bacteriophage typing proved to be a simple and cost-effective method which has the ability to distinguish serologically cross-reacting *K. pneumoniae* capsular types. As such, it should be of considerable benefit in epidemiological investigations.

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