ORIGINAL ARTICLE

Twenty-three years of *Klebsiella* phage typing: a review of phage typing of 12 clusters of nosocomial infections, and a comparison of phage typing with K serotyping

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Objective To review phage typing of 12 clusters of nosocomial *Klebsiella* infections which occurred between 1974 and 1997, and to compare phage typing and K serotyping.

Materials and methods A total of 489 clinical and laboratory *Klebsiella* isolates were phage typed using 110 different phage preparations and K typed by counter current immunoelectrophoresis against 77 K antisera.

Results A total of 152 phage types (PT) and 82 K types were found. Thirty-six phage types and 14 K types were represented only by the reference type strains. Of the remaining 68 K types, 60 could be subdivided into from two to 10 phage types . Ten out of 12 clusters of nosocomial *Klebsiella* infections could be verified as outbreaks by phage typing, whereas two clusters were found to be accumulations of sporadic cases. K typing performed retrospectively confirmed these results. In addition, for a subset of 104 epidemiologically unrelated isolates, O typing and pulsed field gel electrophoresis typing data were available. Based on these results the discriminative power of phage typing was found to be comparable with that of K typing, but phage types were less stable and reproducible.

Conditions In an outbreak situation, phage typing was found to be very useful, although it seems less suitable for long-term surveillance purposes.

Keywords *Klebsiella*, phage typing, K typing

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INTRODUCTION

Klebsiella species are important causes of nosocomial infections [1,2]. A recent study published in Israel showed that *Klebsiella* was the cause of 39% of 322 episodes of Gram-negative bacteraemia and was the most common pathogen of nosocomial infections in infants [3]. In recent years, the appearance and spreading of multiple antibiotic resistant *Klebsiella* strains have increased the therapeutic problems encountered [4–6]. When several concomitant or successive cases of infection occur, typing is essential (i) to clarify if these are part of an outbreak or just coincidental, sporadic cases, and (ii) to identify the source of infection and the route of transmission. Various typing methods have been used in these epidemiological studies: K-typing [7–9], biotyping [10,11], bacteriocin-typing [12], phage typing [13–16], typing by pulsed field gel electrophoresis (PFGE) [17], ribotyping [18], and DNA homology analysis [19].

For the last 23 years, phage typing of *Klebsiella* has been used at the Central Laboratories, Jerusalem, where it has proved to be useful in many epidemiological investigations. In this paper, we present a summary of some of these investigations, and a comparison between phage typing and K serotyping.

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MATERIALS AND METHODS

Strains

One hundred and nineteen isolates from 11 clusters of nosocomial infections in Israel; 40 isolates from an outbreak in

a neonatal intensive care unit (NICU) in India (Sperling V, manuscript in preparation); 145 isolates (102 blood, 41 urine and two peritoneal dialysate) from non-outbreak situations, Hvidovre Hospital, Denmark; 77 K-serotype reference strains, Statens Serum Institut, Copenhagen, Denmark; 108 phage-typing reference strains from the Central Laboratories, Jerusalem, corresponding to phage types 1–112 (reference strains 30 and 55 were lost together with their corresponding phages; reference strains 58 and 84 were also lost but the corresponding phage preparations were still available). The epidemiology of the 102 blood isolates from Hvidovre Hospital is described elsewhere [20]. A total of 489 *Klebsiella* isolates were included (407 *Klebsiella pneumoniae* strains, 62 *Klebsiella oxytoca* strains, 10 *Klebsiella planticola* strains, one *Klebsiella terrigena* strain and 9 *Klebsiella* spp.).

Most isolates from outbreaks and most of the pairs of isolates obtained from blood or urine from a single patient were identical by K typing and phage typing and may therefore be considered as clonal. Thus, the number of different strains available for a comparison between phage typing and K typing was 353: 42 from outbreaks, 97 from blood, 29 from urine, 77 K-serotype reference strains and 108 phage-type reference strains.

Phage typing

Based on previous experience with phage typing of *Salmonella* [21,22], this method was adapted to *Klebsiella* in 1974 at the Central Laboratories, Jerusalem, as a tool to study a hospital outbreak in an infant ward. *Klebsiella* cultures with well-developed capsules were selected, and phages that were active on them were isolated from crude sewage [23]. The phages obtained were purified by three times single plaque isolation, and were tested in ten-fold dilutions by the spot technique [23]. Since that time, whenever a culture was found to be resistant to the previous phage preparations, a new phage was isolated, plaque purified, and tested on all the previous isolates. During the years 1974–90, a phage-typing scheme was established based on sensitivity to 110 phage preparations (numbered 1–112, as phages 30 and 55 were lost) [24]. Testing was performed in two stages, first with pools and then with the respective monovalent phages at 10 times routine test dilution (RTD), i.e. if the RTD is 10^{-4} the testing is performed with the dilution 10^{-3}. This concentration was preferred in order to avoid weak and unclear reactions. Overnight broth cultures were used for testing, but suspensions of one colony in 1 mL of broth can also be used. A provisional result may be read after only 6 h of incubation at 36 °C, so the final result may be read in the evening of the same day or after overnight incubation. The phage type (PT) of a strain is noted with the number of the phage that is active on it. If a strain is resistant to all the phages at 10 RTD, it is tested with undiluted phages and in this case the type is noted with the number in brackets. A strain that is sensitive to two or three phages is noted as, for example PT 35/42/107. Two strains reacting to the same phage, one at 10 RTD and the other only undiluted, or two strains reacting, one to a single phage and the other to the same phage and to one or two additional phages, are considered closely related. A strain resistant to all undiluted phages was recorded as nontypable (PTNT), and a strain sensitive to more than three phages was considered polysensitive (PS).

K typing was carried out at Statens Serum Institut by countercurrent immuno-electrophoresis (CCIE) using a modification of the method described by Palfreyman [25]. An extract was used as antigen instead of a whole cell suspension [26]. The extract was modified in that it was only heated once for 1 h at 100 °C before centrifugation. All strains with negative or doubtful reactions in CCIE were tested by the classical Quellung reaction [27].

For a subset of 104 isolates (102 blood isolates and two isolates from peritoneal dialysate, Hvidovre Hospital) O typing and PFGE typing were available. O typing recognizing the O serogroups O1, O2, O2ac, O3, O4, O5, O7, O8 and O12 was carried out by an inhibition enzyme-linked immunosorbent assay method as previously described [28]. PFGE typing was performed at Statens Serum Institut by incubation of whole cell DNA in agar plugs overnight with *Xba*I [17]. Electrophoresis was carried out on a CHIEF-DR™ II (Bio-Rad Laboratories, Richmond, CA, USA). Interpretation of the PFGE patterns was carried out according to Tenover et al. allowing up to two genetic events resulting in up to six bands difference between two lanes for identical/closely related strains [29].

RESULTS

Results of Phage, K, O and PFGE typing

Phage typing of the 489 isolates identified 152 phage types, whereas 27 isolates were PS and two isolates were PTNT. One hundred and ten types were sensitive to one phage preparation, and 42 types were sensitive to two or three phages. Thirty-six phage types were only found among the phage-type reference strains. Most of the reference phage-type strains and the isolates from the Israeli outbreaks, which were phage typed close to the date of isolation, reacted with only one phage, whereas among cultures tested after a longer period of preservation in agar stabs or frozen at −70 °C in 10% glycerol broth 27% were PS. The highest percentage of PS cultures (68%) was found among the urine isolates. No correlation between *Klebsiella* species and phage type was found.

K typing of the 489 isolates identified 82 different K types: 77 K prototypes, four complex types related to more than one K type (K10,61, K25,59, K33,35, and K39,46) and one provisional type (K″i28–94″). Seventeen isolates were nontypable (KNT). Fourteen K types were represented only by the

K type reference strains: K4, K13, K22, K36, K40, K49, K50, K51, K59, K65, K66, K70, K72 and K82.

Among the subset of 104 isolates, 83 isolates were found to belong to five O groups (O1, O2, O3, O4, and O5), whereas 21 strains were non-typable (ONT) or rough. PFGE typing of the 104 isolates resulted in 88 different types whereas five isolates were non-typable (PFGENT) as they repeatedly gave a smear on the gels.

Sub-division of K types by phage typing

Disregarding the 14 K types represented only by their prototype strain, only seven K types corresponded each to a single phage type: K5, K33,35, K42, K52, K58, K69, and K71 corresponded to PT23, PT77, PT19, PT67, PT99, PT14, and PT16, respectively. Inversely, however, only four of these phage types (PT23, PT19, PT67, PT16) corresponded only to a single K type.

The remaining 61 K types could each be subdivided into from two to 10 phage types; some examples of the subdivision of K types by phage typing are shown in Table 1. For 42 K types two or more strains were found to belong to a given phage type (e.g. for K type K27, three strains belonged to phage type 105, whereas two other strains were PT61 and PT63, respectively).

Phage typing compared with O:K serotyping and PFGE typing

By combining O and K serotyping, PFGE typing, and clinical information we found that the subset of 104 isolates from Hvidovre Hospital consisted of 81 single isolates, seven pairs of identical isolates, two triplets of identical isolates, and finally a group of three isolates giving unclear results. Two of these last three isolates could theoretically be identical, as PFGE type NT is not regarded as an exclusive type (all three isolates were *K. pneumoniae* O3:K31, but had PFGE type 14, 39 and NT, respectively). All pairs/triplets of the same PFGE type belonged to the same O:K serotype (data not shown). Using these data as references the phage-typing results were evaluated.

Among the 104 isolates, 63 different phage types and 45 K types were found. Eleven isolates could not be assigned to any phage type (two PTNT and nine PS) and six strains were KNT.

The 81 single strains belonged to 53 phage types (nine isolates were PS), and to 43 K types (six isolates were KNT). Out of the seven pairs of identical strains, four pairs had the same phage type: PT28, PT40, PT105 and PTNT; one pair had partially identical phage types: PT37 versus PT (37,112); and two pairs differed in phage type: PT41 versus PT(112) and PT(38) versus PT(69,105). Among both triplets, two of the strains were of same phage type whereas the third strain either had a partially identical phage type or a different phage type: PT104,45, PT104,45, PT104; and PT(73), PT(73), PT83. For the triplet of possible identical strains, the phage types were identical or very similar: PT35, PT35, PT35,42. Thus, in seven out of these nine pairs/triplets, phage typing correctly indicated an epidemiological relation between strains. However, three pairs of strains identical in O:K and PFGE type had different PTs: O1:K39 PFGE type 98, but PT41/PT(112); O1:K81 PFGE type 113, but PT(38)/PT(69 105); and O1:K3 PFGE type 61, but PT83/PT(73).

Among the pairs (and triplets) that were identical by all four typing systems were three pairs of isolates from three patients each with two bacteremia episodes. The three pairs of isolates were obtained 3, 8 and 31 weeks apart, respectively.

Phage-typing results from 12 nosocomial outbreaks

Table 2 summarizes the typing results of the 12 clusters of nosocomial infections.

Table 1 Examples of subdivision of K types by phage typing

K type	Phage types	K type	Phage types
1	37, 93	25	35, 42, 107, (35, 42, 107)
2	40, 93	27	105, 61, 63
3	28, 43, 56, 73, 83, 100	28	7, 25, 64
6	28, 73, 99	31	21, 35, 37, 54, 87, 101
7	22, 79	38	52, 60, 70
8	102, 2, 106	39	41, 90
9	46, 45	45	25, 108, 110, 111
12	38, 39, 89	47	24, 38, 47, 52
14	57, 51, 77, 96, 109	54	9, 29, (9, 83)
15	32, 39, 106	55	42, 35, 45
16	12, 27, 65, 83 (12, 27)	60	87, 88, (1, 87)
17	36, 15, 84, (15, 36), (36, 84)	62	53, 86, 20
18	33, 78, 96, 13, 91, (13, 33)	74	69, 14, 56, 87

Phage type underlined: phage type found more than once in the same K type.
Phage type in brackets: Strain sensitive to two or three phage preparations.

Table 2 Phage and K types of strains from 12 clusters of nosocomial *Klebsiella* infections

	Period	Hospital	Phage type, K type (no. of cases; source)
1	Aug.–Sept. 1974	Tz	PT57, K14 (9; milk powder)
2	June 1978	Af	PT22, K80 (1); PT23, K5 (1); PT24, K47 (1); PT25, K45 (1)
3	Feb.–March 1979	Af	PT8, K54 (14); PT26, K46 (1); PT28, K6 (1); PT29, K54 (1)
4	April–June 1980	SZ	PT34, K21 (10); PT57, K14 (9); PT71, K61 (1); PT72, K″i28–94″[a] (1); PT73, K6 (1); PT74, K35 (1); PT76, KNT (1); PT78, K18 (1)
5	Sept. 1980	HI	PT34, K21 (5); PT40, K2 (3; hands of nurse and balance for infants)
6	Nov. 1980	HI	PT17, K20 (1); PT42, K25 (1); PT98, K24 (1); PT100, K3 (1); PT102, K8 (1)
7	March 1985	W	PT102, K8 (7)
8	June 1986	TH	PT106, K35 (11); PT40K2 (10)
9	Aug. 1987	HI	PT14, K69 (9); PT37, K1 (1); PT52, KNT (1)
10	Feb.–March 1994	NICU[b]	PT3, K″i28–94″[a] (28; blood-neonates: 2; nasal and skin swab – staff: 1; water for feed preparation); PT2, KNT (1; tap water); PT40, K2 (4; throat swab – staff); PT53, K62 (2; throat and skin swab – staff); PT84, K17 (1; throat swab – staff); PT49, K23 (1; milk after feeding)
11	Dec. 1996	TH	PT14,28, K3 (4)
12	Feb. 1997	W	PT35, K25 (3 infants and 1 adult)

[a] Provisional K type. [b] Neonatal intensive care unit in India.
Remarks: In cluster 1, 5, and 10 a source was identified. Cluster 4, 5, and 8 was caused by two different phage types. Cluster 4, 9 and, 10 included also sporadic cases. Cluster 2 and 6 only sporadic cases.

All outbreaks occurred among infants or neonates. The *Klebsiella* isolates were cultured from patients and the environment during periods varying from 7 to 43 days. Isolates were primarily phage typed, whereas K typing was performed later.

In outbreaks 1, 7, 11, and 12 all isolates belonged to same phage and K type, whereas in outbreaks 4, 5, and 8 isolates belonged to two phage and K types. In addition to the outbreak strain (s) in outbreaks 4, 9, and 10 strains also belonging to various other phage types were isolated from patients or staff members (colonization/sporadic infections). Finally, a common source of infection was ruled out in the two clusters 2 and 6, since all isolates were of different phage types (and K types).

DISCUSSION AND CONCLUSIONS

Phage typing of 12 clusters of nosocomial infections confirmed 10 of them as outbreaks, whereas two clusters were shown to consist of sporadic infections. In addition, the source of infection or the route of transmission could be demonstrated in several cases. One cluster was shown to be made up of two consecutive outbreaks. These phage-typing results were later found to be in total agreement with results based on K typing.

The use of typing in epidemiological studies relies on the supposition that the type of the outbreak strain remains constant. Phage typing is based on the presence of a phenotypic element, a specific receptor at the surface of the bacterial cell, and a genotypic element, the recognition of the phage-DNA by the enzymes of the strain [30]. The phage type of a strain can be modified by loss, gain or alteration of the surface receptor, by lysogenization or by incorporation of a plasmid in the cell [31]. These events may reduce the sensitivity of a strain to a specific phage, or even render it completely resistant to one or more phage(s). The reverse, however, could also take place, the strain could become sensitive towards more phages.

The appearance of sensitivity to one or more additional phages was described many years ago by Anderson and Felix as 'the process of degradation' which 'may take place in a series of progressive stages' [32]. In the most advanced stages, degradation results in sensitivity to many phages (polysensitivity). The stability of the phage types found in the three patients with bacteremia episodes 3, 8 and 31 weeks apart illustrates that it is not in the living organism, but after repeated passages on artificial media or in cultures preserved for long periods that the phenomenon of degradation is mainly observed. We found that, in several cases when phage types were different in otherwise identical strains, this difference could be interpreted as a result of the beginning of degradation of one of the isolates.

Our experience, as illustrated by the study of the 12 clusters of nosocomial infections presented here, for which phage typing was performed shortly after the isolation of the cultures, is that modification of phage types does rarely occur during outbreaks of limited time. However, as Pieroni et al. have shown [14], it is important that any conclusion regarding epidemiological significance that is based on the identity of phage types should always be supported by the epidemiological evidence.

Although some phages are used for identification purposes as, for example, of species [33], of serogroup [34], or of serotype [35], the main use of phages is for typing. As reported by others [14,16], we found that most *Klebsiella* K types could be subdivided by phage typing. This makes it possible to achieve

a more discriminating typing by using phage typing in a hierarchical system. But, as indicated above, not all phage types and K types are independent, as the specific receptor for some phages is the capsular polysaccharide (K antigen). Using such phages for 'serotyping' is a well-known approach in *E. coli* where normal serotyping, for example the K1 and K5 antigens, is made difficult by their poor immunogenicity.

Evaluation of phage typing in a non-outbreak situation was possible by comparing phage typing with O:K serotyping and PFGE typing for a subset of 104 clinical isolates. We found that the discriminative power of phage typing is comparable with that of K typing. When looking at number of types and number of strains per type, phage typing even seems to perform better (more types), but phage typing is less stable and reproducible than K typing. This probably reflects the fact that the K antigen is expressed with more stability than most of the factors involved in phage typing. The implication of this is that although phage typing in an outbreak situation has been found very useful, it seems less suitable for long-time surveillance purposes.

One advantage of phage typing is the speed. As soon as the bacterium has grown, it can be phage typed using a suspension of a single colony in broth. Provisional results can be read after 6 h, and the final results can be obtained in the evening of the same day.

At present, phage typing is only available at a few specialized centres. However, in a setting where nosocomial *Klebsiella* infections are common and economic resources are insufficient for DNA technologies, phage typing is still an attractive typing method. Phage typing can in principle be performed in any laboratory willing to invest the necessary time and effort, and it is cheap as no special investment for equipment or reagents is needed.

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