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Comparison of SDS–PAGE protein patterns with other typing methods for investigating the epidemiology of '*Klebsiella aerogenes*'

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SUMMARY

Twenty-four cultures comprising 20 clinical isolates of '*Klebsiella aerogenes*' from two hospitals, a reference strain of '*K. aerogenes*' and the type strains of three other *Klebsiella* species, were characterized by one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell proteins. The protein patterns were highly reproducible and were used as the basis of a numerical analysis which divided the clinical isolates into 12 protein types. Comparison with established typing methods indicated that the level of discrimination of SDS–PAGE was similar to that achieved with conventional typing methods but the strains were grouped differently. Protein typing subdivided five serotype K3 isolates that could also be distinguished by phage typing. Conversely, three strains of protein type 11 were clearly distinguishable by both serotyping and phage typing. We conclude that high-resolution SDS–PAGE of proteins provides an effective adjunct to other methods for typing isolates of '*K. aerogenes*'.

INTRODUCTION

Eight species of *Klebsiella* currently have standing in nomenclature. Of these, *K. ozaenae*, *K. pneumoniae* and *K. rhinoscleromatis* show such high levels of DNA–DNA relatedness that they are considered biovars or subspecies of the single species, *K. pneumoniae* [1, 2]. In the UK, clinical microbiologists continue to recognize '*K. aerogenes*' (or *K. pneumoniae* ssp. *aerogenes* [3]) as a species separate from *K. pneumoniae* on clinical and biochemical grounds, although it has no standing in nomenclature; elsewhere strains of '*K. aerogenes*' are included within a more broadly defined *K. pneumoniae*.

'*K. aerogenes*' is clinically important as it causes widespread colonization of hospital patients, particularly of the respiratory tract. Clinical sepsis may develop in surgical wounds and in the urinary tract, whilst bacteraemic infections may prove fatal. Furthermore, '*K. aerogenes*' strains are naturally resistant to ampicillin but strains in hospitals are often also resistant to gentamicin and various cephalosporins. '*K. aerogenes*' is commonly carried in the bowel and hence it is important to distinguish self-infection from cross-infection. This has stimulated the development of effective typing schemes for the investigation of their epidemiology.

Since members of the *K. pneumoniae* complex are the most prevalent klebsiellas in clinical material, typing schemes have been developed primarily for this species. The most extensive scheme is based on serotyping by the capsular (K) antigens. The present total exceeds 80 and currently in the UK three types predominate, accounting for 30% of all isolates [4]. However, the prevalence of these particular types and the fact that 15% of strains may prove untypable [5], limit the usefulness of capsular serology as an epidemiological tool. Although somatic (O) antigens are recognized, making it possible to divide klebsiella strains into O groups and then further subdivide into capsular types, this is not routinely performed as the number of O groups is very small and they are technically difficult to determine. Bacteriocin typing has been described but typability may be as low as 58·5% [6] and even where higher, problems of adequate discrimination (37% of isolates fell into a single type [7]) or reproducibility (67% [8]) may be encountered. A phage typing scheme has been developed primarily as a secondary typing method for subdivision of the capsular serotypes prevalent in the UK but is also useful in typing strains of most serotypes; however, the poor reproducibility (71%) after storage is a cause for concern in retrospective studies of outbreaks [4]. Biotyping may be useful using standardized media but even where a commercial system was used, two-thirds of 259 isolates belonged to a single biotype [9].

High-resolution polyacrylamide gel electrophoresis (PAGE) of bacterial proteins has been used for identification at the species, sub-species and infra sub-specific levels [10, 11]. The technique using either conventionally stained or radiolabelled proteins, has been applied increasingly to the typing of a variety of clinically important species that have included *Acinetobacter calcoaceticus* [12], *Campylobacter pylori* [13], *Clostridium difficile* [14, 15], *Enterobacter cloacae* [16], methicillin-resistant *Staphylococcus aureus* [17, 18] and *Providencia alcalifaciens* [19].

Except for the more recently described *K. ornithinolytica* [20], all the other *Klebsiella* species have been studied by SDS–PAGE to determine their taxonomic relationships [21]. The data obtained confirmed the division into species and subspecies as determined by DNA relatedness studies [1, 21]. Although subdivision of the *K. pneumoniae* complex, on the basis of protein pattern heterogeneity could be made, it was considered that the strains represented a continuum. Detailed discrimination below the species level (i.e. typing) was not reported. The aim of the present study was therefore to compare the high-resolution SDS–PAGE whole cell protein patterns of a number of isolates of '*K. aerogenes*' from two well-defined hospital outbreaks. A computerized analysis of protein patterns was used to gain an objective evaluation of the technique as a typing tool and compare its efficacy with serotyping and phage typing.

MATERIALS AND METHODS

Bacterial cultures

The 24 cultures used in this study are listed in Table 1 together with their respective capsular serotype, phage sensitivity pattern and PAGE protein type. Twenty-one of the cultures were '*K. aerogenes*' and included 20 hospital isolates: 8 from a Liverpool hospital (Hospital I; Ref. nos. 2–9) and 12 from a hospital in Great Yarmouth (Hospital II; ref. nos. 10–21), together with one reference strain,

NCTC 8172. In addition, the type strains of three other *Klebsiella* species were included as study references.

Strains were grown in Nutrient Broth No. 2 (Oxoid: CM67, 25 g/1), with shaking for 3 h at 37 °C.

Serotyping and phage typing

Capsular (K) serotyping and phage typing were all performed using methods described previously [4, 22]. Isolates were divided initially on the basis of capsular serotype and then were further sub-divided by phage typing. Minor variations in phage susceptibility pattern were not considered significant.

Preparation of protein samples and electrophoresis

For each protein sample, approximately 0·02–0·04 g wet weight of the bacteria were harvested after centrifugation (2500 g) from the nutrient broth and suspended in about 60 μl of double strength lysis buffer (20% v/v glycerol, 1·5% v/v 2-mercaptoethanol, 4% w/v sodium dodecyl sulphate [SDS] and 70% v/v stacking gel buffer). The protein samples were extracted as described previously [23].

Samples were run on discontinuous SDS–polyacrylamide gels which were cast to allow for a 10 mm stacking gel. The final polyacrylamide concentrations were 10% w/v for the separation gel and 5% w/v for the stacking gel. Full details of the methods used in gel preparation and electrophoresis were described previously [24].

Scanning of gels and computations

The stained protein patterns in the dried gels were scanned using a LKB Ultroscan XL laser densitometer (Pharmacia-LKB Biotechnology, Sweden). Absorbance was recorded at 160 μm intervals along the gel yielding 625 values per 10 cm gel. The absorbance range was set from 0·15 to 0·6 absorbance units (full scale). A rectangular line beam (800 μm × 50 μm) was used to scan each track three times (with no overlap in scan positions) resulting in a multiple track scan of 2·4 mm width. Multiple scanning was carried out in order to reduce the effect of inconsistencies which may be encountered across a track. The mean absorbance of the area scanned was recorded, *via* an RS232C interface, as raw data on the magnetic disk of a computer.

The initial (stacking gel/separation gel interface) and final (bromophenol blue marker) bands were deleted and protein patterns corrected for gel-to-gel variation using a reference bacterial standard ('*K. aerogenes*' 1240: Ref. no. 14). A replicate of the reference bacterial standard on the subsequent (second) gel was used to calibrate patterns against the reference on the first gel. Segmented linear correction was performed using a total of 18 discernible marker positions (usually peaks) on the reference pattern and by marking the same positions on the calibration pattern replicate. Linear correction (expansion or compression) to the reference distances was carried out within each of the 17 defined segments for each track by three-point quadratic interpolation [25]. The length-corrected traces on the reference gel were composed of 587 absorbance values after removal of the initial and final bands. A general background trend (0·3: fraction of absorbance)

in each trace was removed to increase discrimination between patterns. Similarity between all possible pairs of traces was expressed as the Pearson product-moment correlation coefficient, which was converted for convenience to a percent value. The best fit between each pair of traces was obtained by laterally shifting one corrected trace with respect to the other in single point steps of 160 μm up to three points on either side of the initial alignment. Strains were then clustered by the method of unweighted pair group average linkage (UPGMA). Computations were carried out on a Compaq 386 microcomputer using a program package written in Turbo Pascal [17, 25].

RESULTS

Capsular serotyping and phage typing

The results for capsular serotyping and phage typing of the outbreak and reference strains are shown in Table 1.

Both K-serotyping and phage typing proved to be effective methods for characterizing the isolates. Six isolates, all from an outbreak of gentamicin resistant '*K. aerogenes*' from an orthopaedic ward, were of serotype K15, the most frequent type in this study. Five strains were serotype K3, the next most frequent type and two strains were serotype K11 (one of which also reacted with K26 antiserum). The remaining strains were of eight different K-serotypes or were non-typable (three strains) using the capsular antisera available.

All except three isolates reacted with one or more of the typing phages. The isolates which were non-typable using phages belonged to serotypes K3 and K20, with one strain (1224; ref. no. 2) being non-typable using both capsular serotyping and phage typing.

Hospital I: eight isolates from six patients, on a single ward, were typed. There was clear evidence to indicate that cross-infection had occurred in five of these patients. Six isolates from rectal swabs or a catheter urine specimen of patients B to F were indistinguishable using both serotyping (type K15) and phage typing (type 6). Only one of the two isolates from patient A was sero-typable (type K20) and neither reacted with any of the phages employed.

Hospital II: a total of 12 cultures, each from different patients, was typed. The cultures were isolated from a variety of sites and included six from blood cultures and two each from sputum and throat swabs. Three isolates were serotype K3 and two were of serotype K11. The latter strains were nonetheless distinguishable using serotyping, as one also reacted with type K26 antiserum, and also using phages, as each gave a different pattern of sensitivities. Two of the serotype K3 strains were typable using phages and although each gave a different pattern of sensitivities there were a number of common features in pattern between these isolates. The remaining serotype K3 strain was non-typable using phages. Two strains (1237 and 1241; ref. nos. 11 & 15) had identical phage sensitivity patterns but were of different capsular serotype. The heterogeneity in both serotype and phage pattern amongst this set of strains indicated that there did not appear to be any evidence for cross-infection.

Table 1. *Strains analysed by whole cell SDS–PAGE protein patterns*

Ref. no. in dendrogram	Strain no.	Patient: Source	Capsular serotype	Phage sensitivity pattern	Protein type
Klebsiella aerogenes'					
1	NCTC 8172	—	64	$7^{w}/13$	8
		Hospital I, Orthopaedic ward: Liverpool, England			
2	1224	A: Rectal swab	NT	NT	8
3	1225	A: Catheter specimen urine	20	NT	7
4	1226	B: Rectal swab	15	6	13
5	1227	C: Rectal swab	15	6	13
6	1228	C: Catheter specimen urine	15	6	13
7	1229	D: Rectal swab	15	6	13
8	1230	E: Rectal swab	15	6	13
9	1231	F: Rectal swab	15	6	13
		Hospital II: Great Yarmouth, England			
10	1236	G: Umbilical swab	3	$2/4/6/7^{w}/8^{w}/15$	9
11	1237	H: Throat swab	5	$2/4/5^{w}/7^{w}$	11
12	1238	I: Blood culture	NT	3/4	11
13	1239	J: Throat swab	3	NT	14
14	1240	K: Sputum	11/26	$5^{w}/6^{w}/8^{w}/13$	7
15	1241	L: Blood culture	3	$2/4/5^{w}/7$	1
16	1242	M: Rectal swab	35	2/13	11
17	1243	N: Sputum	16	$6/10^{w}$	4
18	1244	O: Blood culture	9	$3/4/5^{w}$	3
19	1245	P: Blood culture	11	$4^{w}/12$	10
20	1248	Q: Blood culture	80	2	12
21	1249	R: Blood culture	NT	4^{w}	2
bsiella pneumoniae					
22	NCTC 9633*	—	3	$2/3/4/5^{w}/8^{w}$	7
bsiella rhinoscleromatis					
23	NCTC 5046*	—	3	$4/9^{w}$	5
bsiella ozaenae					
24	NCTC 5050*	—	4	2	6

* Type strain; w, weak (+ or ±); NT, non-typable.

General features of PAGE protein patterns

One-dimensional SDS–PAGE of whole cell protein extracts of the 24 cultures included in this study produced patterns containing approximately 40 discrete bands with molecular weights of 18–100 kDa. Proteins of less than 18 kDa were not resolved under the electrophoretic conditions used in this study. PAGE protein patterns are illustrated in Fig. 1. The protein patterns of the klebsiella isolates were in general very similar to each other. Qualitative differences between isolates were evident principally in the protein bands with molecular weights in the range 30–45 kDa.

Reproducibility

The protein patterns of the isolates examined were highly reproducible both within and between gels. Duplicate protein samples of '*K. aerogenes*' 1240 (ref. no. 14), run on the two gels used in this study, gave similarity values of 97% ($96{\cdot}2 \pm 0{\cdot}7$% when an additional set of gels was used). Molecular weight protein

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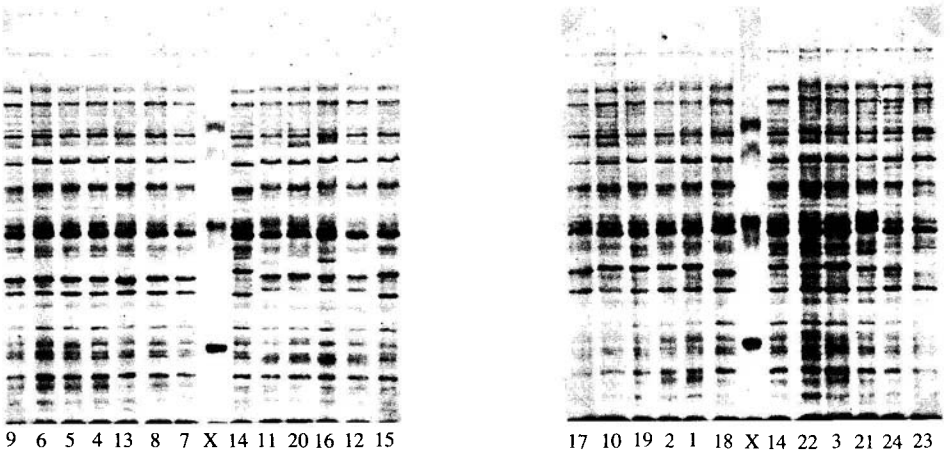

Fig. 1. Electrophoretic protein patterns of '*Klebsiella aerogenes*' and reference strains of other species of the *Klebsiella pneumoniae* complex. The numbers refer to those used in Table 1 and Fig. 2. Molecular weight markers (track labelled X) are (from top to bottom): ovotransferrin, 76–78 kDa; albumin, 66·25 kDa; ovalbumin, 42·7 kDa; carbonic anhydrase, 30 kDa; myoglobin, 17·2 kDa.

standards were also included on each gel and in this case estimates of their similarity were 99% although they provided a less objective measure of reproducibility as they were based on only four bands. The level of reproducibility achieved in this study was higher than that quoted as acceptable by Jackman [10] and was well above the minimum acceptable value [26]. Previous studies using similar methods have reported levels of at least 93% similarity between duplicate samples in separate electrophoretic runs [13, 19, 23, 24]. The dendrogram (Fig. 2) and protein types recognized in the analysis proved to be extremely robust when the computations were repeated using different levels of trace alignment, background subtraction and duplicate gels.

Numerical analysis

Numerical analysis of PAGE total protein profiles based on the determination of the Pearson product-moment correlation coefficient and UPGMA clustering revealed, at the 92% (S) similarity level, a total of 14 distinct protein types for the 24 klebsiella strains (12 protein types for the 20 hospital isolates). The majority of the types defined could be separated visually on the basis of the banding pattern in the 30–45 kDa range. Protein types 8, 9 and 10 were more difficult to separate from each other, as were types 13 and 14, because the molecular weights of the bands in this region were very similar. The type strains of *K. ozaenae* (NCTC 5050) and *K. rhinoscleromatis* (NCTC 5046) had protein patterns unique to themselves and distinct from the patterns of the hospital isolates. However, the clusters representing these strains were closely associated with, and were not separated from, those representing the '*K. aerogenes*' isolates. The type strain of *K. pneumoniae* (NCTC 9633) and the reference strain of '*K. aerogenes*' (NCTC 8172) were clustered separately with other hospital isolates from which they were indistinguishable on protein pattern.

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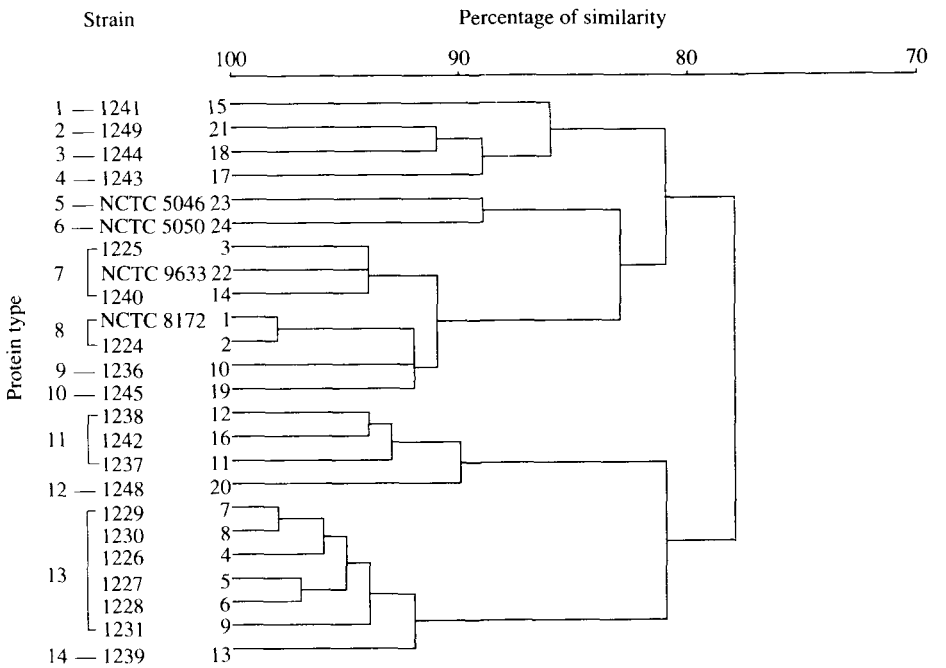

Fig. 2. Dendrogram of the cluster analysis based on total protein content of strains (vertical axis) listed in Table 1. The numbers on the horizontal axis indicate the percentage similarities as determined by the Pearson product-moment correlation coefficient and UPGMA clustering. Protein types were formed at the 92% similarity level.

Only four of the protein types contained two or more isolates, the remainder being unique for each isolate. Protein type 7 was represented by three isolates; two were from patients in different hospitals (ref. nos. 3 & 14) and the other was the type strain of *K. pneumoniae*. All three isolates had a different K-serotype and phage type (one was non-typable using phages). Protein type 8 included two isolates, one of which was a reference strain. The three isolates (ref. nos. 11, 12 & 16) included in protein type 11 were all from different patients in hospital II. Each had a unique phage pattern and the two which could be typed using capsular serotypes were different. The six isolates (ref. nos. 4–9) which were included in protein type 13 all shared the same K-serotype and phage type. There appeared to be no correlation between the site of isolation and the protein type but five of the six isolates of protein type 13 were from rectal swabs, the remaining strain was a catheter urine specimen from a patient (C) who also had an identical strain isolated from a rectal swab. With the exception of protein type 13, strains with the same K-serotype (type K3; ref. nos. 10, 13, 15, 22 & 23) were not included within the same protein type, and similarly, strains with the same phage type (ref. nos. 11 & 15) were also located in separate protein types.

Strains were assigned to a protein electropherotype on the basis of overall similarity ($\geqslant 92\%$) in protein pattern but since types can be ultimately defined by 'absolute identity' it is possible that further types can be recognized within these electropherotypes by a visual assessment of differences in banding pattern. This

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is clearly evidenced in electropherotypes 7 and 11 where the strains included in each show clear differences in banding pattern (Fig. 1) but are nonetheless clustered loosely together at 94% S and 93% S respectively. The heterogeneity in protein pattern within these electropherotypes correlates well with the heterogeneity in serological and phage typing results for the strains.

DISCUSSION

The analysis of bacterial SDS–PAGE protein patterns is an effective means of differentiating medically important bacteria at the species and infra sub-specific levels and can provide a novel method of typing, especially where there are no other typing methods available [13, 14, 19, 24]. Previously only limited data were available to evaluate the sensitivity and reliability of SDS–PAGE with respect to existing typing methods [12, 15]. In this study we have compared the effectiveness of this technique against other methods for typing isolates of the *Klebsiella pneumoniae* complex.

The 20 hospital isolates included in this study were divided on the basis of SDS–PAGE of protein patterns into 12 clearly defined protein electropherotypes (although we refer to protein types throughout we do not here propose that they should be considered as definitive types). This level of discrimination was not quite as great as that obtained using the combination of serotyping and phage typing. However, the SDS–PAGE technique enabled an electropherotype to be given to '*K. aerogenes*' strain 1224 (ref. no. 2) which was non-typable by both capsular serotyping and phage typing, as well as to strains 1225 (ref. no. 3), 1238 (ref. no. 12), 1239 (ref. no. 13) and 1249 (ref. no. 21) which were typable by only one of these two methods. In some cases there was an excellent correlation between strains defined by sero/phage typing and electrophoretic typing, as in the case of the six strains of serotype 15. Typing on the basis of SDS–PAGE of protein patterns generally achieved a higher level of discrimination than could be achieved by serotyping alone, as with the five strains of serotype K3 each of which belonged to a different electropherotype. Since the latter five strains also each belonged to a different phage type there is some indication of a similarity in degree of discrimination between phage typing and typing by SDS–PAGE. However, phage typing can differentiate strains of a single electropherotype as in the case of the three strains of electropherotype 11. In some instances, serotyping will also differentiate strains of the same electropherotype as in the case of the three strains of electropherotype 7. Nevertheless, the strains in both electropherotypes could be differentiated by visual inspection of their protein patterns (Fig. 1). This raises a number of fundamental questions regarding how SDS–PAGE protein patterns are interpreted for epidemiological studies. In the first instance it is extremely difficult to define objectively the term 'significant difference' as applied to protein patterns. Therefore a delineation of types by 'absolute identity' becomes questionable as the assessment of differences in pattern may vary between observers. The approach used in this study, i.e. that based on overall similarity, removes the element of subjectivity as it clusters strains together even though they may show minor differences in pattern. This inevitably places a conservative estimate on the number of types defined but may be more reproducible when

applied by different laboratories. As with strains of the same serotype, where further sub-division can be made using other techniques such as phage typing, so there is no reason why a visual interpretation of protein patterns cannot be made after a more objective computer generated assessment. Secondly, when further division is based on minor band differences undue weight is placed on these features as opposed to the remainder of the pattern. As a consequence of these shortcomings, computer-assisted numerical analysis has evolved in order objectively to define types which are clearly difficult to define visually.

From a practical view-point no single typing method gives a distinct epidemiological picture in the present study. In hospital II, the three strains of serotype K3 belonged to different phage types and electropherotypes, the two strains of phage sensitivity pattern 2/4/5/7 belonged to different serotypes and electropherotypes, whilst the three strains of electropherotype 11 belonged to different serotypes and differed in phage sensitivity pattern. Clearly, at least two of these techniques must be used in combination in detailed epidemiological studies. In assessing which methods to use one may have to take into consideration other features, such as typability, reproducibility, discrimination and ease of interpretation of results.

The distribution of serotypes (K2, K3, K21, K30, K38 and K39 account for over 40% of UK isolates [5]) was not reflected in the present study but there were five strains of serotype K3 each of which was clearly different by the other two typing methods.

An outbreak was clearly apparent in the orthopaedic ward of hospital I. Patient A was not involved in the outbreak and appeared to be harbouring two different strains (both points most effectively demonstrated by SDS–PAGE). Patients B to F were shown to be clearly part of an outbreak by all three typing methods. In hospital II an epidemiological relationship could not be demonstrated between any of the isolates so an outbreak was not in progress. The infections reported in these patients appeared to be quite independent and unrelated to each other.

With the *K. pneumoniae* complex the main advantage of SDS–PAGE protein typing over serotyping and phage typing is that it offers potentially 100% typability since a protein pattern can be produced for all strains. Another advantage is that the level of reproducibility reported for the SDS–PAGE technique is extremely high and this is not affected by long-term storage of strains [27].

Where there is a requirement merely to determine whether strains are identical or not, which is often the case in outbreaks of infection, a simple visual interpretation of patterns on gels can be successfully used to differentiate isolates. However, for definitive recognition of electrophoretic types using dendrogram analysis, a high-resolution scanner and numerical analysis by computer are required.

Since strains belonging to the *K. pneumoniae* complex are members of a single species, not only may it sometimes be difficult to ascribe a strain to a particular subspecies on biochemical tests, but the serotyping and phage typing schemes will also not distinguish between subspecies. In the present study, the respective type strains of *K. pneumoniae* ssp. *pneumoniae* and of *K. pneumoniae* ssp. *rhinoscleromatis* as well as three field strains of '*K. aerogenes*' all belonged to serotype

K3. Similarly, the type strain of *K. pneumoniae* ssp. *ozaenae* and a field strain of '*K. aerogenes*' both belonged to phage sensitivity pattern 2. Possibly serotyping and phage typing would not even distinguish between strains of the *K. pneumoniae* complex and other *Klebsiella* species not included in the present study. Although SDS–PAGE should more closely reveal the phenetic relationships between organisms and in a previous study the technique supported the division of the genus into species and subspecies as determined by DNA relatedness studies, it was nevertheless felt that the strains of the *K. pneumoniae* complex represented a continuum. Even in the present study the type strain of *K. pneumoniae* (ssp. *pneumoniae*) and a field strain of '*K. aerogenes*' both belonged to electropherotype 7. It is evident therefore that preliminary characterization of strains by biochemical tests will prove extremely useful before typing by any of the three techniques.

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