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Evaluation of numerical analysis of SDS-PAGE of protein patterns for typing *Enterobacter cloacae*

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SUMMARY

Twenty cultures comprising 13 clinical isolates of *Enterobacter cloacae* from two hospitals, the type and another reference stain of *E. cloacae* and the type strains of four other *Enterobacter* sp. and of *Escherichia coli*, 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 nine clearly defined protein types. Comparison with established typing methods indicated that the discrimination of SDS-PAGE was similar to that achieved with conventional typing methods and all strain groups recognized by combined sero/phage typing were also found by SDS-PAGE. In addition, protein typing sub-divided a group of four serotype O3 isolates that were difficult to distinguish by phage typing. We conclude that high-resolution SDS-PAGE of proteins provides an effective method of typing isolates of *E. cloacae*.

INTRODUCTION

The genus *Enterobacter* is composed of Gram-negative motile bacteria that conform to the definition of the family Enterobacteriaceae. Currently, nine species of *Enterobacter* are recognised with *E. cloacae* and *E. aerogenes* being the species isolated most frequently from clinical material. Although not primary human pathogens, both species are capable of causing opportunistic infections usually in compromised or debilitated patients. *E. cloacae* and *E. aerogenes* show resistance to ampicillin and cephalosporins and are of increasing importance in nosocomial infections. A number of outbreaks of *E. cloacae* infection have been documented over recent years and this has increased the need for effective typing schemes for the investigation of the epidemiology of this species (1).

Extensive serotyping schemes, based on both O- and H-antigens have been described for *E. cloacae*. However, together with other typing systems, these suffer from a number of drawbacks. The O-serotyping scheme has problems of discrimination in that although 85% of isolates can be typed, approximately 43% of isolates were found to be of the three most frequently encountered types (2, 3). The H-serotypes gave a low typability of only 53% of all isolates. In addition, large sets of antisera, up to 30 O-types and 25 H-types, are needed and none are

available commercially. Although 13 biotypes have been identified using biochemical test reactions, the two most frequent account for up to 80% of all isolates (1, 4). A number of bacteriocin based schemes have been described with up to 23 types being defined (5–7). Both reproducibility and typability were high but again discrimination was low with a single reaction accounting for 20% of isolates. More recently a bacteriophage based typing scheme utilizing a set of 18 phages has been developed as a secondary typing method to sub-divide the common O serotypes (8, 9). Typability was high; 89% of isolates could be typed, but the reproducibility was reduced after prolonged storage of isolates and it was recommended that typing should be carried out on the same day to ensure comparability (1).

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 *Clostridium difficile* (12, 13), methicillin-resistant *Staphylococcus aureus* (14), *Providencia alcalifaciens* (15), *Campylobacter pylori* (16) and *Acinetobacter calcoaceticus* (17).

The aim of the present study was to compare the high-resolution SDS-PAGE whole cell protein patterns of a number of isolates of *E. cloacae* 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 biotyping, serotyping and phage typing.

MATERIAL AND METHODS

Bacterial cultures

The 20 cultures used in this study are listed in Table 1 together with their respective bio-, sero-, phage-type (where these are available) and PAGE types. Fifteen of the cultures were *E. cloacae* and included 13 hospital isolates: 7 from a London hospital (Hospital I; Ref. nos. 3–9) and 6 from a hospital in Portsmouth, England (Hospital II; Ref. nos. 10–15), together with 2 reference strains, one of which was the type strain NCTC 10005 and the other NCTC 9396. In addition 5 other strains, 4 type strains of different species of *Enterobacter*, and the type strain of *Escherichia coli* NCTC 9001 were also included as study references.

Strains were grown on nutrient agar containing: Nutrient Broth No. 2 (Oxoid: CM67), 25 g/l; Japanese agar (Davis), 15 g/l, for 24 h at 37 °C.

Biotyping, serotyping and phage typing

Biotyping, O-serotyping and phage typing were all performed using methods described previously (2, 4, 8). Isolates were divided initially on the basis of O-serotype and those with the same O-serotype were 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 0·05–0·1 g wet weight of the bacteria were harvested directly from the nutrient agar plates 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 (18). 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 (19).

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 0·15–0·8 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 disc 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 (*Escherichia coli* NCTC 9001: Ref. no. 20). 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 16 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 15 defined segments for each track by three-point quadratic interpolation (20). The length-corrected traces on the reference gel were composed of 588 absorbance values after removal of the initial and final bands. A general background trend (0·4: 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 two 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. (16, 19).

RESULTS

Biotyping, serotyping and phage typing

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

Biotyping was of limited use in discriminating between strains. Twelve of the 13

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

Ref. no. in dendrogram	Strain no.	Source	Biotype	Serotype	Phage type	Protein type
Enterobacter cloacae						
1	NCTC 10005*	Cerebrospinal fluid, USA, 1958	—	—	—	9
2	NCTC 9396	Water, Uruguay, 1955	—	—	—	8
Hospital I: London, England						
3	2324	Patient A blood	OO	(1249)	D/K	11
4	2326	Patient B blood	22	8	A/C/E/F/I/M/N	6
5	2327	Patient B sputum	22	8	A/C/E/F/I/M/N	6
6	2328	Patient C blood	22	3	G^w/R	5
7	2329	Patient C wound swab	22	13	A/B/C/D	3
8	2330	Patient C blood	22	3	R	5
9	2331	Patient C blood	22	29	NT	10
Hospital II: Portsmouth, England						
10	2349	Vaseline pot	22	16	A/C/D/K/L	7
11	2350	Sheepskin (clean) surface	22	3	A/F/G^w/H/I/K/L	2
12	2351	Sheepskin (dirty) surface	22	3	A/F/H/I/K/L	1
13	2352	Patient D blood	22	3	K/L	4
14	2353	Patient E blood	22	3	A/F/G/H/I/K/L	2
15	2354	Patient E endo-tracheal tube	22	3	A/F/G^w/H/I/K/L	2
Enterobacter asburiae						
16	NCTC 12123*	Lochia exudate, USA				
Enterobacter amnigenus						
17	NCTC 12124*	Soil				
Enterobacter intermedium						
18	NCTC 12125*	Surface water				
Enterobacter taylorae						
19	NCTC 12126*	Human arm wound				
Escherichia coli						
20	NCTC 9001*	Urine, cystitis, Denmark, 1982				

*, type strain; ( ), serum prepared against strain 1249; w, weak (±); NT, not typable against phages A–R

isolates were characterized by biotype 22, the most frequent type in clinical isolates. The remaining isolate, biotype 00, represented negative reactions in all six biochemical tests.

Both serotyping and phage typing proved to be effective methods for characterizing the isolates. Seven isolates belonged to serotype O3, the most frequent type, and two belonged to serotype O8, the next most frequent type. All except one isolate reacted with one or more of the typing phages. The isolate which was non-typable using phages belonged to serotype O29 which is rare in isolates from clinical specimens.

Hospital I: Seven isolates were typed from three patients. There was no typing evidence to indicate that cross-infection had occurred. Two isolates from the blood and sputum of patient B were identical. In contrast four isolates from patient C represented three serotypes, O3, O29 and 1249 (an unclassified experimental type). The two O3 isolates were very similar by phage typing.

Hospital II: Three isolates were typed from two neonates, together with three isolates from the patient associated environment. The patient isolates were all serotype O3; the two isolates from patient E were indistinguishable by phage typing but were distinct from the isolate from patient D. Both of the sheepskin isolates gave either the same, or very similar phage sensitivity patterns, to those of patient E. The isolate from the vaseline pot represented a distinct strain.

General features of PAGE protein patterns

One-dimensional SDS-PAGE of whole cell protein extracts of the 20 cultures included in this study produced patterns containing approximately 40 discrete bands with molecular weights of 18–100 kDa. Proteins of < 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 enterobacter isolates were in general very similar to each other. Qualitative differences between isolates was evident principally in the protein bands with molecular weights in the range 30–40 kDa. Additional heavy bands which appear to be strain specific were evident at > 78 kDa in a number of the patterns (Ref. nos. 4, 5, 6, 8 and 10: Table 1). The *Escherichia coli* protein pattern did not differ markedly from those of representative *Enterobacter* species, reflecting the close phylogenetic relationships between genera within the Enterobacteriaceae (21).

Reproducibility

The protein patterns of the isolates examined were highly reproducible both within and between gels. Duplicate protein samples of *E. cloacae* 2351 (Ref. no. 12) and *Escherichia coli* NCTC 9001 (Ref. no. 20) run on different gels, and separate gel runs, gave similarity values of 94% and 96% respectively. Molecular weight protein standards were also included on each gel and in this case estimates of their similarity were 96% 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 similar to that quoted by Jackman (10) and was well above the minimum acceptable value of 80% (22). Previous studies using similar methods have reported levels of at least 93% similarity between duplicate samples in separate electrophoretic runs (15, 16, 18, 19). The dendrogram and

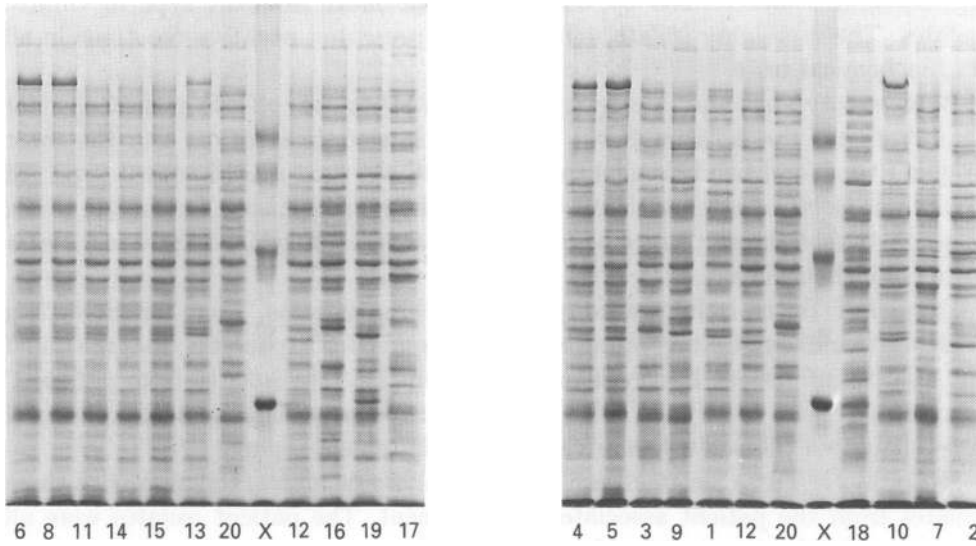

Fig. 1. Electrophoretic protein patterns of *Enterobacter cloacae* and reference strains of other *Enterobacter* species. The numbers refer to those used in Table 1 and Fig. 2. Molecular weight markers (track labelled X) are (from top to bottom): ovotransferrin, 76–78 kDa; albumin 66·25 kDa; ovalbumin,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

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 90% (S) similarity level, a total of 11 distinct protein types for the 15 *E. cloacae* (9 protein types for the 13 hospital isolates). The two *E. cloacae* reference strains had patterns unique to themselves, distinct from the patterns of the hospital isolates, but more similar to these than to the other species examined. In addition, all five reference strains representing different species gave distinct patterns as reflected in the dendrogram in Fig. 2.

Only three of the protein types contained two or more isolates, the remainder being unique for each isolate. Protein type 2 was represented by three isolates; two were from different sites in the same patient (E; Ref. nos. 14 & 15) and the other was from an environmental site (Ref. no. 11) from the same ward as this patient. All three isolates had the same serotype and phage type. A fourth isolate with a similar serotype and phage type was characterized as protein type 1, and clustered with protein type 2 at the 88% similarity level. Protein type 5 included two isolates from differing blood samples of the same patient (C; Ref. nos. 6 & 8). The other two samples from this patient (Ref. nos. 7 & 9) gave protein pattern types 3 and 10 respectively and were also found to give different serotypes and phage types. The two isolates included in protein type 6 were also from a single patient (B; Ref. nos. 4 & 5) but from differing samples (blood and sputum). The patients

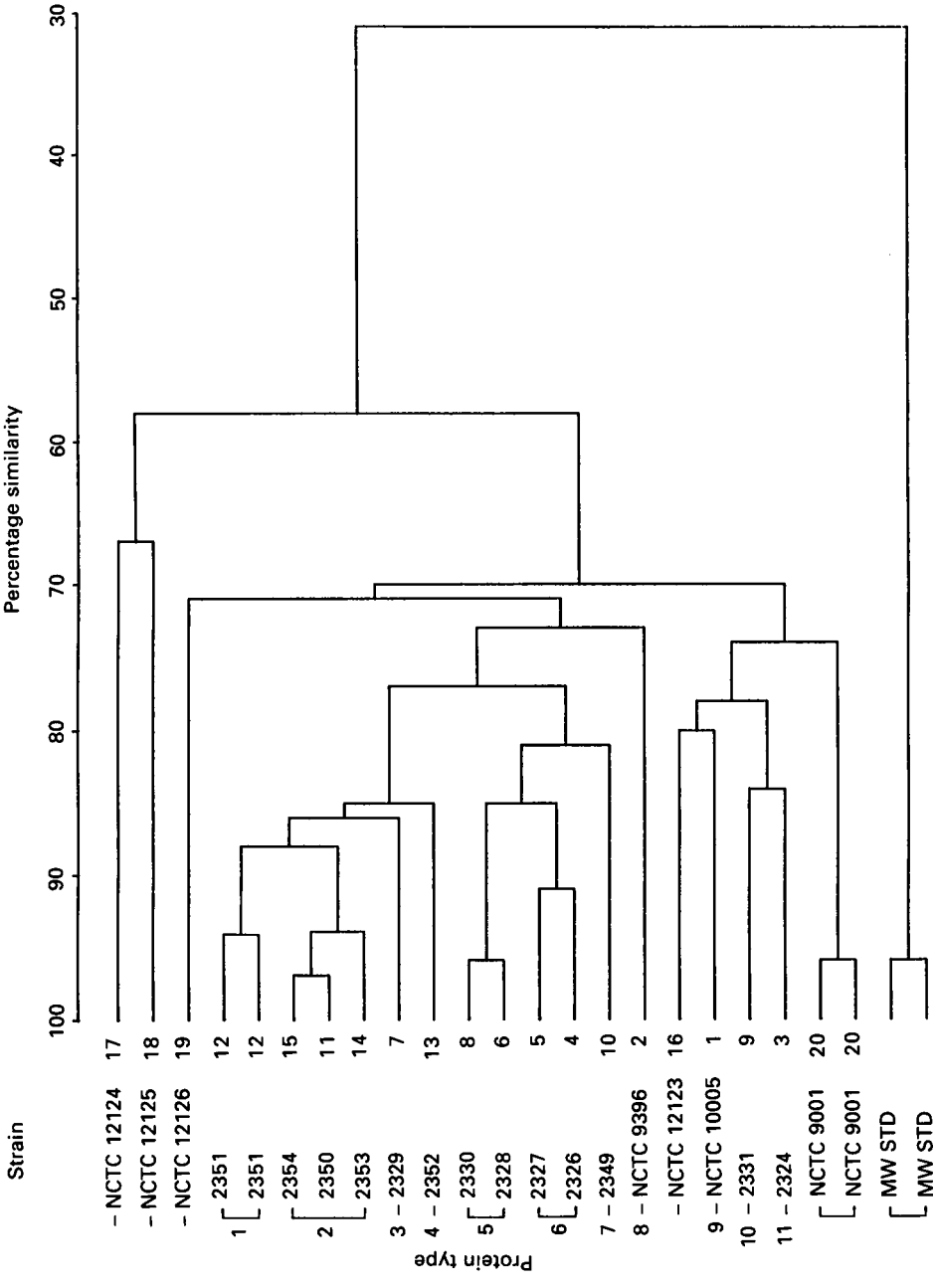

Fig. 2. Dendrogram of the cluster analysis based on total protein content of strains (vertical axis) listed in Table 1. The numbers on the horizontal axis indicate the percentage similarities as determined by the Pearson product moment correlation coefficient and UPGMA clustering. Protein types were formed at the 90% similarity level.

which were represented by isolates with protein types 5 and 6 were from the same ward and were also characterized by a heavy protein band at > 78 kDa. They had, however, quite distinct serotypes (O3 and O8 respectively) and phage types (see Table 1).

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 (12, 15, 16, 19). Previously only limited data was available to evaluate the sensitivity and reliability of SDS-PAGE with respect to existing typing methods (13, 17). In this study we have compared the effectiveness of this technique against a panel of other methods for typing a number of isolates of *Enterobacter cloacae*.

The 13 hospital isolates included in this study were divided on the basis of SDS-PAGE of protein patterns into nine clearly defined types. This level of discrimination was similar to that obtained using the combination of serotyping and phage typing, and there was an excellent correlation between strains defined by sero/phage typing and electrophoretic typing. Indeed, each of the sero/phage types defined in this study was represented by a separate and unique electrophoretic protein pattern, herein referred to as an electropherotype. However, the SDS-PAGE technique enabled an electropherotype to be given to *E. cloacae* strain 2331 (Ref. no. 9) which was non-typable using the phages available.

Biotyping did not prove useful in the differentiation of these hospital isolates as only two biotypes were represented in this study. The O-serotyping scheme when used alone proved to be more discriminating than biotyping with six different O-types being found. The distribution of O-types decreased the effectiveness of this technique as seven of the isolates gave the same serotype, O3, which has been previously reported as being of common occurrence (20% of all isolates; 2, 3). It can thus be seen that electrophoretic typing is extremely effective as a typing method for *E. cloacae* and gives discrimination comparable to the best of the other methods currently available.

The three London patients (A–C) infected with *E. cloacae* did not have strains which were related to each other. Indeed, patient C appeared to have a multiple infection with three clearly different strains of *E. cloacae*. The various typing techniques employed were in complete agreement in terms of defining the status of types found in each patient. The infections reported in these patients appeared to be quite independent and unrelated to each other. The Portsmouth patients (D and E) were also shown to be infected with different strains of *E. cloacae* when using phage and protein typing but not on serotyping alone. It is interesting to note that one of the isolates from the sheepskin (2350: Ref. no. 11) gave exactly the same electropherotype and phage typing data as patient E and could therefore have been the source of infection for this patient.

With *E. cloacae* the main advantage of SDS-PAGE protein typing over 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 (23).

The higher discrimination shown here for SDS-PAGE protein patterns *versus* serotyping has also been described by Delmée and colleagues (24) and Mulligan and colleagues (13) for *Clostridium difficile* where both showed superior discrimination for protein typing over serotyping. In the former study ten types could be defined using serogrouping, a number easily exceeded by the 21 electropherotypes differentiated for the same set of strains (24). Walia and co-workers, (23) in their comparison of these two typing methods for *Pseudomonas aeruginosa* again noted the superior discrimination achieved using electrophoretic typing over serotyping but also described an additional advantage of being able to differentiate strains that are replaced during therapy by a different strain with the same serotype.

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.

At lower levels of similarity the clusters associated with the *E. cloacae* strains do not appear to be exclusive to this species but include other reference species of *Enterobacter* (Fig. 2). It is evident therefore that preliminary characterization of strains is required before typing using this technique.

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