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Electrophoretic typing of *Enterobacter cloacae* with a limited set of enzyme stains

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SUMMARY

Hospital isolates of *Enterobacter cloacae* were analysed by polyacrylamide gel electrophoresis for enzyme polymorphism and the results were compared with established serotyping, phage typing and biotyping techniques. Initially, the diversity of electromorphs of 13 enzymes was determined on a representative set of 62 distinct strains. Two broad clusters of strains were found in the species, and analysis by serotype suggested a limited diversity within the most frequent O serotypes. A subset of three enzymes, lactate dehydrogenase, 6-phosphogluconate dehydrogenase, glutamate dehydrogenase and an unidentified marker, were selected and used to type groups of hospital isolates. There was good general agreement between the two systems, although the enzyme method failed to distinguish between some strains with the same serotype. This method provided useful epidemiological information and, in the absence of established typing systems, it is a practical approach to subdividing the species.

INTRODUCTION

Enterobacter cloacae is an opportunistic pathogen that can cause outbreaks of infection in hospitalized patients (1). Clinical isolates are phenotypically diverse in the characteristics usually used to sub-speciate strains and a number of useful typing methods have been described (2, 3). However, the reagents necessary for conventional typing schemes are only available in a small number of reference laboratories.

Recently, several electrophoretic typing techniques have been described. These methods have the advantage that they are applicable to a wide range of microorganisms, allowing hospital laboratories without access to complex reference systems to carry out detailed epidemiological investigations. One method, multilocus enzyme electrophoresis, has been successfully applied to a number of species, particularly for the analysis of clonal diversity (4–6). Many applications of this technique have involved the characterization of 20 or more enzymes, and this work load seriously detracts from the usefulness of the technique as a typing method for most hospitals.

In 1986, Goullet and Picard (7) found that esterase diversity, in combination

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with acid phosphatase and several dehydrogenases, could separate strains of *E. cloacae* from strains of *E. sakazakii*. They proposed that variations in electrophoretic patterns may be useful as epidemiological markers in these species. In this study, we have selected a set of four easily characterized enzymes and compared their ability to discriminate between groups of hospital isolates of *E. cloacae* with established typing methods.

MATERIALS AND METHODS

Strains

All isolates were selected from cultures sent to our laboratory for epidemiological studies. Distinct strains were selected on the basis of sending laboratory, serotype and phage sensitivity. The electrophoretic diversity in the species was assessed on 62 strains; 17 O-serotype strains and 45 strains from British laboratories. This panel represented the broad range of serotypes and sources of isolates routinely sent to us for typing, and included blood, urine and faecal strains.

Epidemiological typing

O serology and phage typing was performed by the methods described previously (8,9) with 30 O antisera, 7 additional experimental antisera and a set of 17 phages. Most isolates were also biotyped using a scheme modified from Old (10) by the exclusion of α-methylglucoside.

Preparation of bacterial enzyme extracts

Bacteria were grown overnight at 37 °C on 9 cm nutrient agar plates. Growth was harvested into 3 ml of phosphate-buffered saline and disrupted by sonication for 2 min at 4 °C. Bacterial debris was removed by centrifugation and the crude enzyme extracts were either used immediately or stored at −70 °C for up to 6 weeks.

Electrophoresis

Electrophoresis of extracts was performed on a polyacrylamide slab gel system with a non-dissociating discontinuous buffer system modified from Laemmli (11) by the exclusion of sodium dodecyl sulphate. Stacking gels were 5% acrylamide pH 6·8 and resolving gels were 6·5% acrylamide pH 8·8. Electrophoresis was carried out at 8 °C with a constant current of 35 mA per gel. Gels were run for 4–5 h and stained immediately to visualize enzyme bands.

In determining the diversity of enzyme electromorphs within the species, extracts from each strain were run at least twice. First to determine the diversity of types, and again to characterize accurately the mobility of the enzyme for each strain. Enzyme mobilities were coded numerically (5) and the genetic diversity of 62 strains was displayed as a phenogram based on their percentage similarity, clustered by the unweighted average linkage method.

Enzyme stains

The following enzymes were assayed: malate dehydrogenase (MDH), leucine dehydrogenase (LED), lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH),

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isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6P), glutamate dehydrogenase (GD2), 6-phosphogluconate dehydrogenase (6PG), fumarase (FUM), phosphoglucose isomerase (PGI), aconitase (ACO) and leucine aminopeptidase (LAP). With the exception of ACO, enzymes bands were visualized by the staining protocols given by Selander and colleagues (5) except tetranitro blue tetrazolium was used in place of dimethylthiazol tetrazolium. Typically, gels were placed in 50 ml of substrate/coenzyme buffer immediately following electrophoresis and incubated at 37 °C in the dark until enzyme bands were visible. ACO was demonstrated by direct application of the stain to the gel, rather than as an agar overlay. Gels stained for LAP required pH adjustment before the addition of the substrate. Pre-incubation of the gel with phosphate buffer (13·6 g/l KH_2PO_4, pH 4·4) for 20 min produced clear well-defined bands. A negatively staining band was visualized on gels incubated with NAD-containing buffer. For the purposes of this analysis, it was considered to be an enzyme marker and was recorded as an unidentified enzyme band (UIE).

RESULTS

Detection of enzyme electromorphs

Polyacrylamide gel electrophoresis of the cell lysates generally gave good resolution of individual enzyme bands. The sensitivity of the method varied considerably between stains and overloading the lane for the more sensitive stains had a deleterious effect on the resolution of the major enzyme band. Optimal sample loading ranged from 15 μl for G6P, to 75 μl for LAP.

The electromorphs of most enzymes were detected in the middle third of each gel, LAP was most mobile, and bands were developed 1–2 cm from the dye front. Several enzyme stains produces additional bands on prolonged incubation. These were strongest with gels stained for ACO, where they showed a high degree of polymorphism between strains. Three sets of enzyme data, for LDH, ADH and LED, were substantially identical, which may indicate that the same, or very similar, oxidoreductases were detected by the three stains.

The differences in mobility of the various electromorphs of a few enzymes were very small (1–2 mm) and simple measurements of their mobility in relation to the dye front was insufficient for consistent identification of electromorphs. As a consequence of this, all extracts were run two or three times, with control strains interspersed between the test strains, before a mobility type was assigned. Table 1 shows the electromorphs found in 17 O-serotype strains.

Electrophoretic diversity

The percentage similarities of 62 distinct strains were computed from the electrophoretic mobilities of 12 enzymes. The relationships are given as a phenogram in Fig. 1. ACO was not included in the comparison because of the difficulty in comparing the complex patterns of bands that developed with most extracts.

There was considerable enzyme polymorphism, and with exception of 10 strains belonging to serotypes of O 3 and O 8, strains with the same serotype could be separated by enzyme mobilities. Two broad clusters were found and a subgroup

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Table 1. *Electromorph profiles of strains representing the most frequent O serotypes in* Enterobacter cloacae

Serotype strain	Electromorph for enzyme												
	MDH	LDH	ADH	IDH	LED	G6P	GD2	FUM	LAP	PGI	UIE	6PG	ACO*
1	1	2	1	1	1	1	1	1	2	1	1	1	2
2	1	1	2	1	2	1	4	0	2	1	1	1	1
3	1	1	2	1	2	1	1	2	3	1	1	1	2
4	1	1	2	2	2	1	1	2	3	1	3	2	2
7	2	1	2	3	2	1	3	2	2	2	1	0	1
8	1	2	1	1	1	1	1	1	2	1	1	1	2
9	1	1	2	1	2	1	1	2	2	1	1	0	1
10	1	1	2	1	2	1	1	0	1	1	1	0	2
11	1	0	0	1	0	1	1	1	2	1	1	0	2
12	1	1	2	1	2	1	1	2	3	1	1	3	5
13	1	1	2	3	2	1	1	2	2	1	1	1	3
16	1	1	2	2	2	2	1	4	1	3	2	3	4
19	1	2	1	1	1	1	1	1	2	4	1	3	2
20	1	2	1	1	1	1	1	1	2	1	1	1	2
24	1	2	1	5	1	1	1	1	2	1	1	1	3
25	1	2	1	1	1	1	4	1	2	1	1	1	2
27	1	2	1	4	1	2	3	4	2	2	2	4	6
Number† of types	2	2	2	5	2	4	4	5	4	5	5	4	—

* General mobility types only; the number of minor bands made reproducible characterization difficult.

† Number of mobility types found in all 62 distinct strains tested.

within each cluster was predominated by serotype O 8 or O 3 strains. These are the two most frequent serotypes in the species. Both serotypes can be readily subdivided by phage typing, and O 3 can also be subdivided on the basis of H-antigens.

Investigation of hospital isolates

Three enzymes, LDH, 6PG and GD2, were selected for the ease by which electromorphs could be recognized. This set was augmented with the UIE marker, which was read from the LDH gel. Isolates from nine incidents of infection in eight hospitals were studied, and the enzyme profiles were compared with the results of conventional epidemiological typing methods.

Overall, there was good agreement between the two systems (Table 2). The main area of discrepancy was the inability of the limited enzyme set to separate some strains belonging to the same serotype. In four incidents, there was minor variation in the mobility of LDH between isolates that represented the same sero/phage/bio-type.

Incident 1. Six isolates from five patients in an intensive care unit and which demonstrated inducible resistance to cephalosporins. All isolates represented distinct strains and gave a unique enzyme profile including two isolates from different sites on the same patient.

Incident 2. Sixteen cultures from an obstetric unit, isolated during a trial of a

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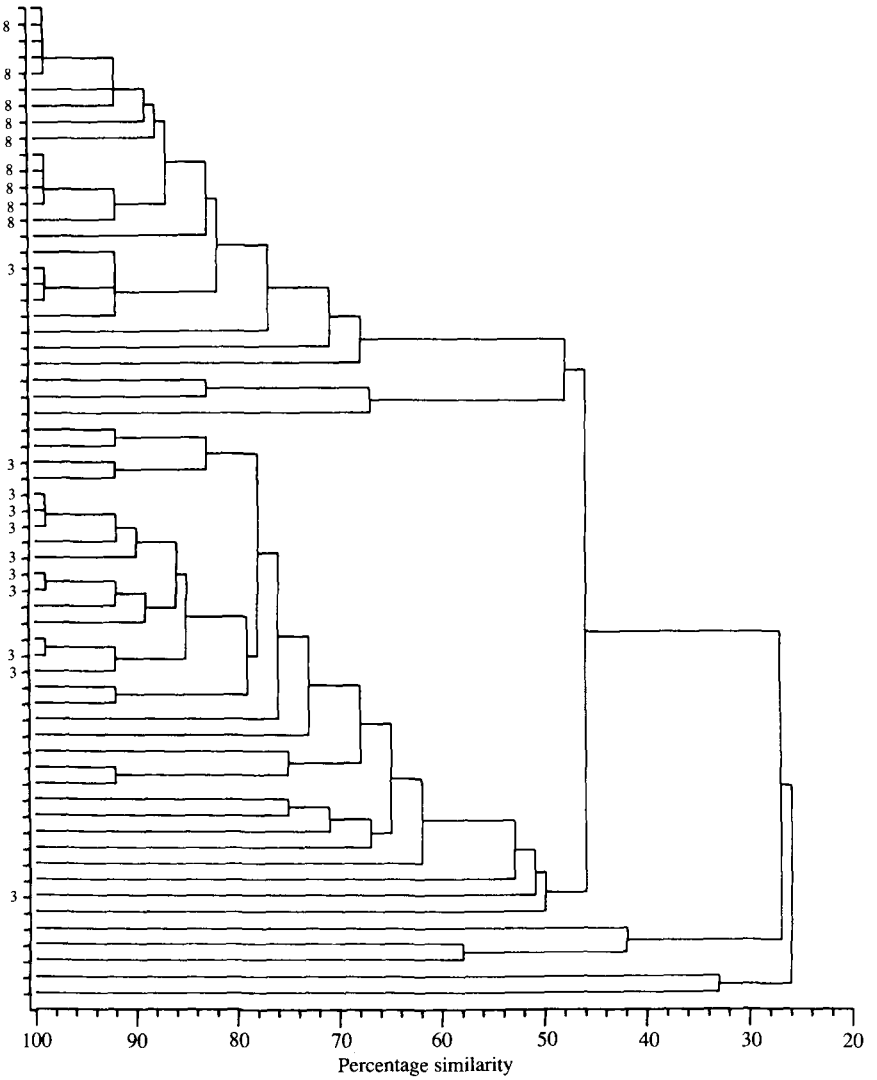

Fig. 1. Relationships among 62 strains of *Enterobacter cloacae* based on enzyme mobility of 12 enzymes in polyacrylamide gels. Numbers on the vertical axis represent strains belonging to serotypes O 3 and O 8.

broad-spectrum cephalosporin. The eight isolates not belonging to serotype O 8 were correctly characterized by their enzyme profiles. Phage typing identified three strains among the eight serotype O 8 isolates, but seven gave the same enzyme profile. The remaining strain gave a reaction with LDH, whereas no reaction was seen in extracts of the other isolates. The extra reaction may have been the result of a variation in the efficiency of the extraction process. Pairs of isolates from two patients, and from different body sites, differed in both sero/phagetype and enzyme profile.

Incident 3. Five cultures, from a renal unit, with decreased sensitivity to gentamicin. Three indistinguishable O 13 isolates gave the same enzyme profile

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Table 2. *Comparison of conventional typing methods with electrophoretic mobility of four enzymes*

	Patient	O serotype	Strain code*	Enzyme† LDH	6PG	UTE	GD2
Incident 1	Zi	7	A	1	1	1	1
	Ma	3	B	2	2	1	2
	Ha	AA	C	0	2	2	0
	Ha	12	D	0	2	2	3
	Bu	3	E	3	2	1	4
	Ho	NT	F	3	2	1	5
Incident 2	An	8	A	0	0	1	1
	Cj	8	A	0	0	1	1
	Aw	8	A	0	0	1	1
	Ca	8	A	0	0	1	1
	Wo	8	A	0	0	1	1
	Ma	8	B	0	0	1	1
	Ma	8	B	2	0	1	1
	Fa	8	C	0	0	1	1
	An	MA	D	0	1	1	1
	Wa	3	E	0	3	1	2
	Ne	3	E	0	3	1	2
	Ne	NT	F	0	0	2	3
	Ba	NT	F	0	0	2	3
	De	NT	F	0	0	2	3
	Gr	NT	F	0	0	2	3
	Ny	NT	F	0	0	2	3
Incident 3	Sa	1	A	1	1	1	1
	Ca	1	B	1	1	1	1
	Le	13	C	2	1	1	1
	Pe	13	C	2	1	1	1
	Li	13	C	2	1	1	1
Incident 6	Th	16	A	1	1	1	1
	Ha	13	B	1	2	1	2
	Pe	3	C	1	2	1	3
	Ja	8	D	2	2	1	4
	Ja	8	D	(3)	2	1	4
Incident 7	Sw	MA	A	1	1	1	1
	Sw	MA	A	1	1	1	1
	Po	MA	B1	1	1	1	1
	Sw	MA	B2	1	1	1	1
	Sw	MA	B3	1	1	1	1
	Mc	MA	B4	1	0	1	1
	Mc	MA	C	1	3	1	1
	Mc	MA	D	1	4	1	1
	Mc	MA	E	1	4	1	1
	Sw	MA	B1/F	3	1	1	1
	Ob	8	G	3	0	1	3
	Mc	8	H	1	4	1	3
	Fa	3	I	4	0	1	4
	Ha	27	J	2	2	2	2
Incident 8	Ey	8	A	2	1	1	1
	Wn	8	A	2	1	1	1
	Ch	8	A	2	1	1	1
	Ta	8	A	2	1	1	1
	We	8	A	2	1	1	1
	Pe	3	B	1	1	1	2
	Ke	3	B	(3)	1	1	2
	Sk	3	B	1	1	1	2
	Tr	AA	C	0	2	0	0

Incident 4	Kn	8	A	1	1	1	1	Incident 9	Rw	8	A	1	1	1	1
	Pu	8	A	1	1	1	1		Rw	8	A	1	1	1	1
	Po	8	A	1	1	1	1		—	8	A	1	1	1	1
	Po	8	A	1	1	1	1		—	27	B	0	2	2	2
	Co	8	A	(2)	1	1	1		Env	3	C	1	2	1	3
	Sm	8	A	1	1	1	1		An	NT	D	1	2	1	2
	Sm	8	A	1	1	1	1		Env	NT	E	2	3	1	1
	Ja	8	A	1	1	1	1		An	1	F	0	1	3	1
	Ab	8	A	1	1	1	1		Env	NT	G	1	2	1	1
	Ab	8	A	(2)	1	1	1		Lj	8	H	1	1	1	3
	La	NT	B	3	2	2	2		Co	MA	I	3	2	1	3
									Hl	3	J	1	2	0	3
Incident 5	Ch	13	A	1	1	1	1		Om	3	K	3	1	1	1
	Ch	13	A	1	1	1	1		At	3	K	3	1	1	1
	Sq	3	B	2	0	1	2		—	NT	L	1	3	2	4
	Bi	24	C	1	1	1	1								
	Ga	8	D	1	1	1	3								
	Sm	8	E	1	0	1	3								
	Sp	E1233	F	0	2	1	1								
	Ma	3	G	2	2	2	1								

NT, non-typable; MA, multi-agglutinable; AA, auto-agglutinable; Env, environmental isolate.
* Strain identification based on serotype, phage susceptibility, and biotype (where available).
† Enzyme codes only applicable within each incident.
Codes in parentheses indicate a minor variation in the mobility of LDH when compared to other isolates of the same strain.

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and were distinct from two O 1 isolates. However, two O 1 isolates which were distinguishable by phage typing could not be separated by their enzyme profiles.

Incident 4. Eleven multi-resistant cultures from several wards. Minor variation occurred in the mobility of LDH in two isolates, otherwise the enzyme profiles corresponded with the results of conventional typing methods.

Incident 5. Eight blood isolates from a haematology unit. There were six enzyme profiles, two indistinguishable O 13 isolates gave the same profile as an O 24 isolate.

Incident 6. Five cultures from four neonates, each with a distinct strain. There was minor variation in the mobility of LDH between two O 8 isolates from the same patient, otherwise the results corresponded with the established typing methods.

Incident 7. Fourteen cultures from a leukaemia unit. We had considerable difficulty in interpreting the results of conventional typing methods for these isolates. Many belonged to a group of experimental serotypes and showed extensive cross-reactions, and in the absence of absorbed antisera they could not be assigned to a specific type, i.e. they were classified as multi-agglutinable. Phage and biotyping were also inconclusive. Four distinct isolates with clearly defined serotypes/phagetypes were also distinct by enzyme profile. Five of the 10 cross-reactive isolates had the same enzyme profile but showed four phage susceptibility patterns, and a further pair of isolates with identical profiles also showed different phage susceptibility. Overall, the enzyme method was as effective as the other methods in establishing useful types in this group of isolates.

Incident 8. Nine isolates from a neonatal unit that represented three distinct strains. With the exception of one isolate that showed minor variation in the mobility of LDH, all the representatives of the same strain gave identical enzyme profiles.

Incident 9. Fifteen cultures from patients associated with a surgical unit. Isolates that were indistinguishable by conventional methods had the same enzyme profile.

DISCUSSION

In our experience, the majority of hospital isolates of *E. cloacae* represent distinct strains. Most clusters of isolates are polymorphic and common source/cross-infection outbreaks such as incident 4 are rare. Given the small set of enzymes and the limited diversity within each locus, it is not surprising that many of the distinctions between strains were made on the basis of one enzyme, often on a null reaction. Nevertheless, there was a good correlation between the strains defined by conventional typing methods and the discrimination achieved with the enzyme markers. This method therefore provided useful epidemiological information, and in the absence of established systems, it appears to be a practical approach to typing hospital isolates.

Iso-enzyme analysis has a number of potential advantages over other electrophoretic typing techniques, such as the comparison of whole-cell protein profiles (WCPPs). Both techniques may be used to study small numbers of isolates, particularly if extracts of all the strains can be run together on the same

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gel. With larger numbers of isolates, it is important to achieve a consistent interpretation between different gels. Enzyme electromorphs are more readily classified into simple numerical codes than the complex patterns of WCPPs. Data from large studies should therefore be easier to analyse as electromorph codes. Phenograms showing the interrelationships of large groups of strains (such as Fig. 1) can be constructed from WCPPs and have been shown to correlate well with DNA–DNA reassociation studies (13). However, this analysis requires consistent high resolution electrophoresis, an expensive laser densitometer and complex computer software. WCPPs are therefore unlikely to be a suitable typing method for most hospital laboratories which have a requirement to investigate large outbreaks of nosocomial infection or have a continuous surveillance program. The potential disadvantages of iso-enzyme analysis for hospital laboratories are the high costs of the co-enzymes used in many stains and the necessity to run a number of acrylamide gels for each strain.

An ideal iso-enzyme typing method would involve a single electrophoretic run and would produce only one major discriminatory band per strain. Goullet and Picard have reported a high degree of diversity in the esterases produced in several species and this system may be candidate for a simplified enzyme typing method (7, 12, 14, 15). In a study on *Serratia marcescens*, we found that both esterase and alkaline phosphatase gave a very high degree of strain definition (unpublished data), close to that achieved with phage typing. In contrast, we were unable to achieve satisfactorily consistent results with these enzymes in preliminary studies on *E. cloacae*, and they could not be used for typing in our system. Of the enzymes tested, the diversity of electromorphs was greatest for ACO but most of the distinctions were made on the basis of minor bands. We found that the ACO patterns were difficult to reproduce and therefore were not a sufficiently robust system for routine use. Nevertheless, the diversity of bands seen in the ACO stain may warrant further investigation.

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