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Biotyping of *Enterobacter cloacae*

DAVID C OLD

From the Department of Bacteriology, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY

SUMMARY A biotyping scheme for *Enterobacter cloacae* is proposed. Tests with seven substrates that gave reliable, reproducible results with 110 isolates of *E cloacae* formed the basis of the biotyping scheme which would allow recognition of 128 potential biotypes.

Among members of the tribe *Klebsielleae,* klebsiellae and serratiae are generally considered to be more commonly implicated in hospital-acquired infections than are enterobacters.[1] Accordingly, for the former groups, well-established methods for type discrimination within the species are available.[2–5] The occurrence of an outbreak due to *Enterobacter cloacae* revealed the lack of a suitable system for type discrimination within that species. Our previous experience with other enterobacteria[6,7] suggested that biotyping might provide an easy, reproducible system for that purpose; the basis of such a scheme is reported in this paper.

Material and methods

ORGANISMS: ISOLATION AND IDENTIFICATION

Most (85%) of the 105 clinical isolates of *E cloacae* examined were recovered from specimens originating from one maternity ward in a Dundee hospital during the three months from September to November 1978; the remainder (16 cultures) were stock cultures of *E cloacae* from that or other wards isolated before or after the "epidemic" period. Most of the cultures (42%) were isolated from vaginal or placental swabs; the remainder were from diverse clinical specimens including blood, expressed breast milk, faeces, urine, and pus from wounds. Five reference strains of *E cloacae* from the National Collection of Type Cultures, Colindale, London, were included: NCTC strains 9529, 9711, 9785, 9842 and 10005.

Presumptive coliform organisms present on diagnostic platings of clinical specimens on MacConkey Agar (Oxoid Ltd) were picked and identified as *E cloacae* by standard techniques.[8] The reactions of isolates judged to be *E cloacae* were uniform in the following tests: motile, indole-negative, urease-negative, Simmons's citrate-positive, arginine dihydrolase-positive, lysine decarboxylase-negative and ornithine decarboxylase-positive.

BIOTYPING MEDIA

The ability of isolates to ferment sugars or sugar alcohols was tested in peptone water (Oxoid CM9) medium, pH 6·8, with 0·002% (wt/vol) bromocresol purple as indicator, dispensed in 5 ml amounts in screw-capped, quarter-ounce (7 g) bottles. The substrates tested, at a final concentration of 1% (wt/vol) were: adonitol, dulcitol, inositol, α-methylglucoside, rhamnose and salicin. The preparation of aesculin agar[7] and malonate broth[8] have been described elsewhere.

INOCULATION OF MEDIA

A well-isolated colony from a pure culture of the test organism was spread on nutrient agar and after incubation for 24 h at 37°C, suspended (to 10^9 bacteria/ml) in saline (NaCl, 7·5 g/l). Biotyping media were inoculated with 0·02 ml of that suspension.

INTERPRETATION OF TESTS

All cultures were incubated at 37°C. Fermentation tests were examined daily for up to seven days for the production of acid (indicator change to yellow) in cultures of the test organism. As a result of these observations it became clear that tests for the fermentation of each carbohydrate were best evaluated at 24 h, the definitive time giving optimal separation of strains into genotypically fermenting and non-fermenting types. Utilisation of malonate was indicated by a colour change of indicator to deep blue after incubation for 24 and 48 h. Hydrolysis of aesculin in plate tests was indicated by the presence of black precipitate around areas of growth. In each typing test, results were recorded as positive (+) or negative (—) at the definitive time of reading. The biotype was expressed as a profile of positive and negative results (Table).

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Reactions of 110 isolates of Enterobacter cloacae in seven biotyping tests

Test	Biotype profile 1	2	3	4	5	6	7	8	9	10	11	12	13
Fermentation of:													
Adonitol	+	+	−	−	−	−	−	−	−	−	−	−	−
Dulcitol	−	−	+	+	+	+	−	−	−	−	−	−	−
Inositol	+	−	+	−	−	−	−	−	−	−	−	−	−
α-methylglucoside	+	+	+	+	−	−	+	+	−	−	−	−	−
Rhamnose	+	+	+	−	+	+	+	+	+	+	+	+	+
Utilisation of:													
Malonate	+	+	+	−	+	−	+	−	+	+	−	−	+
Aesculin*	+	−	+	+	+	−	−	+	+	−	+	−	+
No of isolates	1	6	1	86	1	1	2	1	2	5	1	2	1

*Or salicin.

Results

DISCRIMINATION

Biotyping of 110 isolates of *E cloacae* by their reactions in tests with eight substrates (Table) distinguished 13 biotypes, the most common being that represented by 86 replicate isolates of the "epidemic" strain (type 4, Table).

Differentiation of the isolates into fermenting and non-fermenting types in tests with the three sugar alcohols and the methyl sugars, α-methylglucoside and rhamnose, was best achieved with a definitive time of reading of 24 h. In order that the interpretation of the results should not be biased by consideration of such a large number of isolates (86) of the same strain, only one isolate of the epidemic strain will be considered in assessing the degree of discrimination achieved by the different tests. Thus, with reference to 25 strains (rather than 110 isolates) it was clear that different degrees of differentiation were obtained as follows: with α-methylglucoside, discrimination was excellent (48% of isolates fermenting (positive); 52% of isolates non-fermenting (negative)); with adonitol (28% positive; 72% negative) and dulcitol (16% positive; 84% negative) it was moderate; and, with inositol and rhamnose, each of which discriminated only 8% of the minority type (respectively, positive and negative), it was poor. On occasion, positive results were obtained between two and seven days of incubation in some tests with cultures that were negative at 24 h, a finding that indicated the origin of mutant fermenting types in the course of extended incubation in selective medium. Accordingly, with reference to the definitive time of reading, these irregular "late-positive" results were scored as negative.

Strong utilisers of malonate invariably gave positive results on testing of different colonies from the same culture at the same time and testing of cultures of the same isolate at different times. Some cultures which at 24 h were malonate-negative gave positive results irregularly when readings were at 48 h. Thus, although the latter time is often recommended for tests with malonate, best differentiation with this series of *E cloacae* was achieved at 24 h, at which time discrimination was moderately good (76% positive; 24% negative).

A marked correlation between ability of *E cloacae* strains to ferment salicin in peptone water and to hydrolyse aesculin in agar was noted, when tests were read at 24 h. The single isolate which gave discrepant results fermented salicin, but did not hydrolyse aesculin, in 24 h.

REPRODUCIBILITY

When each of 110 isolates was tested on three different occasions over three years, discrepancies in results were not observed on any occasion for any culture for fermentation tests or malonate tests read at their definitive times. The least satisfactory test in terms of reproducibility was that for salicin hydrolysis in peptone water; thus, some non-hydrolysing isolates gave positive results on some occasions of testing. This test gave reproducible results, however, when several (up to six) colonies from each culture were tested and the results given by the majority of colonies taken. The irregular positive results obtained with some cultures of salicin non-hydrolysing isolates were probably due to the presence of hydrolysing mutants in the cultures. Similar anomalies have been noted with salicin testing of *Escherichia coli*.[7] On the other hand, discrepancies were not observed with the aesculin test which was chosen, therefore, as the test more likely to give reproducible results. On the basis of the results with aesculin hydrolysis, discrimination was good (36% positive; 64% negative).

Discussion

Because members of the *Klebsielleae* are often antibiotic resistant, their association with infections in

hospitalised patients presents considerable problems. The recognition of the importance of this particular group of organisms in nosocomial infections has stimulated the development of different kinds of typing methods for those members more usually implicated in hospital infections.[2-5] The species of *Enterobacter: aerogenes, cloacae* and *sakazakii*[9 10] have, however, received less attention.[1]

The commonly recommended method for typing *E cloacae,* for example, is serotyping which allows recognition of more than 70 serotypes.[11] That method, however, is unlikely to prove acceptable in most laboratories because commercial sera are not available and the establishment of the system *de novo* and the performance of the tests, is likely to prove burdensome and time-consuming. Biotyping of *E cloacae,* if feasible, would provide with simple reagents an inexpensive method for rapid typing, as it did for *E aerogenes*[1] and *E sakazakii*.[10]

In the establishment of a biotyping scheme for any bacterial species, tests suitable for identification of the species must be distinguished from those tests suitable for the discrimination of types within a species. Thus, discriminatory tests separate isolates into phenotypically positive or negative types. Though optimal separation (50% positive; 50% negative) is rarely achieved, tests may still be used for biotyping even when the separation they achieve departs considerably from the optimum. In the present study, each test afforded strain discrimination; some—for example, inositol and rhamnose, were less satisfactory than others—for example, α-methylglucoside. The strong correlation found between the ability to hyrolyse aesculin and salicin was not unexpected since the same β-glucosidase is known to hydrolyse these two structurally related compounds.[12] In view of the observed discrepancies with the salicin test, the aesculin test is to be preferred. At the definitive time of reading, only two phenotypes (+, −) were considered for each test and so the scheme, as presented, allowed recognition of 128 potential biotypes with the use of the seven substrates: adonitol, dulcitol, inositol, α-methylglucoside, rhamnose, malonate, and aesculin. This series of isolates, though primarily of local origin, was represented, nevertheless, by 13 biotypes; and each of the five NCTC reference strains (9529, 9711, 9785, 9842 and 10005) belonged to different biotypes (3, 5, 2, 13 and 7, respectively).

In typing for the discrimination of members of a bacterial species, it is important that: (i) most of the isolates are typeable, (ii) there is a high discriminating ability, (iii) reliability and reproducibility are achieved, and (iv) tests are simple to perform. Each point was fulfilled with the present system, and the phenotypic diversity demonstrated among this series of isolates indicates the potential value of biotyping for *E cloacae.*

It was not the purpose of the present paper on *E cloacae* to discuss the clinical or epidemiological aspects of the outbreak, the details of which will be presented elsewhere (D Parratt, R Traynor, personal communication 1981). Nevertheless, it should be noted that the epidemic strain (represented by 86 isolates) was unusual in being dulcitol-fermenting and rhamnose non-fermenting and, hence, was readily discriminated from other types of *E cloacae* which are generally dulcitol non-fermenting (87%) and rhamnose-fermenting (92%).[8] Though of a rare biotype, the strain readily identified as *E cloacae* by its reactions in tests with arginine, lysine and ornithine (+, −, +, respectively). Furthermore, by its lack of a yellow pigment, its fermentation of sorbitol and its resistance to ampicillin and cephaloridine (DC Old, unpublished results), it was clear that it was not an isolate of *E sakazakii* which has many biochemical properties in common with *E cloacae,*[10] and may, on occasion, be associated with conditions of clinical significance.[10 13]

Thus, biotyping would seem to have potential value for discriminating types of *E cloacae.* It allowed identification of the epidemic strain in this outbreak; its application in future epidemiological studies remains to be evaluated.

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Requests for reprints to: Dr DC Old, Department of Bacteriology, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY, Scotland.