Epidermiol. Infect. (1993), **111**, 81–88
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A miniaturized biotyping system for strain discrimination in *Escherichia coli*

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(*Accepted 12 February 1993*)

SUMMARY

A two-tier miniaturized scheme of eight tests was devised for biotyping strains of *Escherichia coli* in microwell plates. Primary biotypes were defined by positive and negative reactions in tests for fermentation of raffinose, sorbose, dulcitol and 2-deoxy-D-ribose and for decarboxylation of ornithine when read after specified periods of incubation; subtypes were identified within primary biotypes according to results in secondary tests for rhamnose fermentation, lysine decarboxylation and motility. The method gave reproducible results on different occasions of testing.

Among 100 *E. coli* strains from various sources, 26 of the 32 possible primary biotypes and 56 full biotypes, as defined by results in both primary and secondary tests, were identified, thus demonstrating a high index of strain discrimination ($D = 0{\cdot}98$).

The scheme is recommended as a simple, reliable, inexpensive and efficient method of differentiating strains of *E. coli*.

INTRODUCTION

The choice of typing methods for the differentiation of bacterial strains depends not only on the species involved but also on available laboratory resources.

For *Escherichia coli*, the two-tier biotyping scheme of Crichton and Old [1], comprising four primary and six secondary tests, can be applied in any laboratory to provide excellent subspecific discrimination of strains as 'biotypes'. Because types are stable even after storage on non-selective medium for many years [1], the scheme can be used for both prospective and retrospective analyses [2, 3] and its flexibility allows extension by addition of any new tests that become available.

Although the methodology is simple and requires no specialized equipment or technical expertise it is, as originally described, rather cumbersome. Reduction of substrate volumes and time taken to provide results would make the scheme even more valuable.

In this study, the biotyping system of Crichton and Old [1] was modified for use in microwell plates. Further substrates were also examined for possible inclusion in the scheme, with a view to increasing its already high index of discrimination [4].

https://doi.org/10.1017/S0950268800056703 Published online by Cambridge University Press

MATERIALS AND METHODS

Bacteria

Of the 100 strains of *E. coli* examined in this study, 76 had been biotyped previously [1]; the additional 24 strains were isolated in the Department of Medical Microbiology, Ninewells Hospital, Dundee, from faeces (11), urine (5), wounds (4) and body fluids (4) of different patients. Strains, identified as *E. coli* by established methods [5], were stored on Dorset's egg medium at room temperature (*c.* 20 °C) until they were plated on MacConkey Agar (Oxoid) for testing.

Biotyping media

The fermentation medium of Crichton and Old [1], containing Oxoid Peptone Water 15 g/L and, as pH indicator, bromocresol purple 0·02 g/L, was modified (a) by adding Difco-Bacto Agar 1·5% w/v and (b) by incorporating the carbohydrates D-raffinose, L-sorbose, L-rhamnose and dulcitol at one or more concentrations (0·5, 1·0 or 1·5% w/v). The preparation of semi-solid/motility medium, decarboxylase media and aesculin agar has been detailed before [1]; the motility of 50 strains was also assessed in duplicate in a commercially available Motility Test Agar (Mast Diagnostics Ltd, Bootle, Merseyside, England). Sterile media were dispensed in 200 μl amounts from a repetitive pipette (Boehringer Corp., Lewes, Sussex) into microwell plates, which were wrapped in clingfilm and stored in the dark at 4 °C for $\leqslant$ 28 days.

Fimbriation status and growth-factor requirements of strains were not assessed in this study because they were considered inappropriate for a rapid, miniaturized system.

Inoculation of biotyping tests

On each occasion of testing, half of a colony from MacConkey Agar was inoculated for confluent growth on Nutrient Agar (Oxoid) and incubated overnight at 37 °C. All biochemical tests received an inoculum (10 μl) of the resulting growth suspended at *c.* 10^{12} cfu/L in physiological saline (8·5 g/L NaCl); motility medium was 'stabbed' with a straight wire charged with material from the colony. Inoculated decarboxylase media were overlaid with one drop of sterile mineral oil from a pasteur pipette. Plates were sealed with sterile adhesive film and incubated at 37 °C for $\leqslant$ 72 h.

Interpretation of biotype test results

With a Titertek plate reader (ICN Flow, High Wycombe, Bucks., England), plates were examined at intervals for a colour change in carbohydrate fermentation tests from purple to yellow (positive); lysine and ornithine decarboxylation were recorded as positive when the test medium was purple whilst the basal medium without amino acid remained yellow; motility tests were examined for growth throughout the medium (positive) and aesculin hydrolysis tests were examined for the presence of a brownish-black precipitate (positive).

Reproducibility of biotype test results

To assess reproducibility of results with each substrate, tests were set up on 12 individual colonies from cultures of 4 representative strains. All strains were then

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tested in duplicate in medium containing the substrate concentration considered optimal for inter-assay reproducibility.

Additional substrates tested

Each of 24 randomly selected *E. coli* strains was also tested for fermentation of D-arabitol, 2-deoxy-D-ribose, fucose, 5-keto-D-gluconic acid, melibionic acid and D-tagatose at different concentrations at 37 °C for $\leqslant 72$ h. Inter-assay reproducibility was investigated as above.

Biotype reproducibility

All 100 strains were biotyped on 2 occasions, 4 months apart and thereafter at weekly intervals for 4 weeks, with the complete set of selected tests (see below). The results for 76 of the strains were compared with those obtained by the standard method originally described, i.e. tests performed in 4 ml volumes of liquid medium in bijou bottles [1].

Numerical index of discrimination

The discrimination indices of primary and full biotyping (i.e. of primary tests with and without secondary tests) [1] were calculated for the 100 strains of *E. coli* examined. That index (D) [6] measures the probability that two unrelated strains chosen at random will be of different types and is derived by the formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1),$$

where N is the total number of strains tested, s is the total number of different types identified and n_j is the number of strains of the jth type,

RESULTS

Reproducibility of biotype test results

Tests of inter-assay reproducibility, in which 12 individual colonies of each of 4 strains were examined, indicated clearly that most of the tests of the standard biotyping scheme of Crichton and Old [1] were readily adaptable to the miniaturized system. Thus, results of tests for decarboxylation of ornithine and lysine were reproducible after incubation for between 6 and 24 h; those for fermentation of sorbose and rhamnose fermentation were reproducible when read at 24 h. In the rhamnose-fermentation test, it was important that only a bright yellow colour was interpreted as positive to distinguish strong-fermenting (+) from weak- and non-fermenting (−) strains. Similarly, reliable results were obtained for fermentation of raffinose and dulcitol after 48 h incubation. Although motility results were reproducible when read after 24 h, interpretation of tests made in the standard semi-solid agar proved difficult because of condensate; assessment of motility was simplified by the use of a commercial semi-solid medium in which the red reduction product (formazan) of triphenyltetrazolium choloride indicates bacterial growth. In contrast to those described above, results of aesculin hydrolysis were not reproducible and were not studied further.

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Table 1. *Reactions of 100* E. coli *strains in biotyping tests*

Percentage of *E. coli* strains giving a positive result in test* for								
raf	sor	mot	orn	dul	rha	lys	ara	2DR
52	62	62	68	74	79	88	3	40

* Fermentation of raf, raffinose; sor, sorbose; dul, dulcitol; rha, rhamnose; ara, arabitol; 2DR, 2-deoxy-D-ribose; decarboxylation of orn, ornithine; lys, lysine; mot, motility.

Of the new substrates examined, arabitol and 2-deoxy-D-ribose gave reproducible results in tests read at 24 h. On the other hand, results of tests with fucose, 5-keto-D-gluconic acid, melibionic acid and tagatose read at intervals $\leqslant$ 72 h were not reproducible. They were thus unlikely to have any value for *E. coli* strain discrimination when tested by this miniaturized system and were not examined further.

In summary, strains of *E. coli* gave identical biotype results in tests with the following substrates incorporated in the medium at the concentrations indicated, when read at the definitive time: raffinose (0·5% w/v, read at 48 h); sorbose (0·5, 24 h); motility (24 h); ornithine (0·5, $\leqslant$ 24 h); dulcitol (1·0, 48 h); rhamnose (0·5, 24 h); lysine (0·5, $\leqslant$ 24 h); arabitol (0·5, 24 h); and 2-deoxy-D-ribose (1·0, 24 h) (Table 1).

Reproducibility of biotype

When they were biotyped by the miniaturized system, all 100 strains gave the same results in tests made 4 months apart on medium that had been stored at 4 °C for $\leqslant$ 8 weeks and with plates that had been stored at 4 °C for $\leqslant$ 28 days. Biotype results for 76 of the strains were the same as those generated by the traditional method 10–12 years previously [1].

Strain differentiation afforded by biotyping

The results of raffinose fermentation by 100 strains of *E. coli* tested in microwell plates showed clearly that that substrate gave excellent type discrimination, dividing strains almost optimally (52% positive: 48% negative); results of tests for fermentation of sorbose and dulcitol, and for decarboxylation of ornithine also provided good type discrimination. Because rhamnose and lysine tests were able to discriminate only 21 and 12%, respectively, of the minority, negative types (Table 1), they were designated as secondary tests; as was motility which, though providing good type differentiation (62% positive: 38% negative), is found difficult to interpret by some workers [1].

Of the new substrates, 2-deoxy-D-ribose gave good discrimination of strains (40% positive: 60% negative) (Table 1) whereas arabitol provided such poor discrimination (only 3% of the minority, positive type identified) (Table 1) that it was not studied further.

The 100 strains included examples of each of the 16 (2^4) 'old' primary *E. coli* biotypes defined by positive and negative results in tests with raffinose, sorbose, ornithine and dulcitol (Table 2). When 2-deoxy-D-ribose was included as a fifth primary test, 26 of the 32 (2^5) possible types (Table 2) were identified, i.e. all types

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Table 2. *Reactions of primary biotypes of* Escherichia coli

Old biotype no. with four primary tests	No. of strains of that biotype	Result of test with raf*	sor	orn	dul	2DR	New biotype no. with five primary tests	No. of strains of that biotype
1	21	+	+	+	+	+	1	13
						−	2	8
2	6	+	+	+	−	+	3	2
						−	4	4
3	5	+	+	−	+	+	5	0
						−	6	5
4	2	+	+	−	−	+	7	0
						−	8	2
5	9	+	−	+	+	+	9	2
						−	10	7
6	4	+	−	+	−	+	11	1
						−	12	3
7	2	+	−	−	+	+	13	0
						−	14	2
8	3	+	−	−	−	+	15	1
						−	16	2
9	20	−	+	+	+	+	17	14
						−	18	6
10	1	−	+	+	−	+	19	1
						−	20	0
11	3	−	+	−	+	+	21	1
						−	22	2
12	4	−	+	−	−	+	23	0
						−	24	4
13	5	−	−	+	+	+	25	2
						−	26	3
14	2	−	−	+	−	+	27	0
						−	28	2
15	9	−	−	−	+	+	29	2
						−	30	7
16	4	−	−	−	−	+	31	1
						−	32	3

* Raf, raffinose; sor, sorbose; orn, ornithine; dul, dulcitol; 2DR, 2-deoxy-D-ribose.

except 5, 7, 13, 20, 23 and 27. The commonest of the 16 'old' primary types 1 and 9 (21 and 20 strains respectively) were subdivided by their 2-deoxy-D-ribose results into the 'new' primary types 1 and 2 (13 and 8 strains) and 17 and 18 (14 and 6 strains), illustrating the refinement provided by this additional test (Table 2).

Inclusion of 2-deoxy-D-ribose as a primary test also improved the biotype discrimination among 28 cultures of common urinary serogroups O 6 and O 75 in this study (Table 3).

Discriminatory index of the miniaturized biotyping system

Biotyping tests performed in microwells with 4 primary substrates (raffinose, sorbose, ornithine and dulcitol) and 3 secondary substrates (rhamnose, lysine and motility) identified 16 primary and 45 full biotypes, respectively, among the 100 strains studied; the corresponding discrimination indices were 0·89 and 0·96

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Table 3. *Discrimination by biotyping of 28* E. coli *strains of serogroups O 6 and O 75*

E. coli O group and no. of strains of that biotype	Old* primary biotype no.	New† primary biotype no.	Secondary biotype characters‡ (and no. of strains of that type)
O6			
4	1	1	b(1), e(3)
8	9	17	a(6), b(1), e(1)
1	9	18	a(1)
O75			
7	1	1	a(4), b(2), c(1)
2	1	2	e(2)
1	2	3	b(1)
2	2	4	e(2)
1	4	8	e(1)
1	9	18	a(1)
1	16	32	e(1)

* Old primary biotypes are defined by reactions in tests with raffinose, sorbose, ornithine and dulcitol.

† New primary biotypes are defined by reactions in tests with raffinose, sorbose, ornithine, dulcitol and 2-deoxy-D-ribose.

‡ Secondary characters are: negative results in tests for: b, rhamnose fermentation; c, lysine decarboxylation; and e, motility; a, positive results in all secondary tests.

Table 4. *Discrimination indices for 100 strains of* E. coli *by primary and full biotyping*

Biotyping tests	Number of types	Size of largest type	Discrimination index
Primary			
4* tests	16	21	0·89
5† tests	26	14	0·94
Full			
7‡ tests	45	14	0·96
8§ tests	56	10	0·98

* Tests with raffinose, sorbose, ornithine and dulcitol.

† Tests with raffinose, sorbose, ornithine, dulcitol and 2-deoxy-D-ribose.

‡ Four primary tests and three secondary tests (for rhamnose, lysine and motility).

§ Five primary tests and three secondary tests.

(Table 4). When 2-deoxy-D-ribose was included as a fifth primary substrate, the numbers of primary and full biotypes identified among these 100 strains were 26 and 56 and the discrimination indices were increased to 0·94 and 0·98, respectively (Table 4), illustrating the high index of strain discrimination attainable.

DISCUSSION

The epidemiological value of information provided by any bacterial typing system depends on identification of typing markers that are stable as well as highly discriminating at the subspecific level. Crichton and Old [1, 7] gave consideration to these criteria when developing a successful two-tier biotyping scheme of primary and secondary tests for the differentiation of *E. coli* strains. In

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the present study, tests for the metabolism of raffinose, sorbose, ornithine and dulcitol, Crichton and Old's primary tests, were adapted for use in microwell plates; the excellent correlation of results with those obtained by the standard method indicates that the discrimination provided by the two systems is equally high. Apart from including agar in the medium, which made for easier handling and increased colour intensity of the pH indicator, the only modification required was an increase in concentration of the sugar alcohol dulcitol to 1·0% w/v; that alteration was also necessary in a miniaturized biotyping system for differentiating strains of salmonella (D. C. Old, unpublished results). Although discriminating at a lower level, results of rhamnose fermentation, lysine decarboxylation and motility were also valuable strain markers. The test for aesculin hydrolysis, which assays the inducible enzyme β-glucosidase, proved unsatisfactory in microwells, a finding that was not unexpected in light of previous studies [1].

Other workers have identified 5-keto-D-gluconic acid, melibionic acid, tagatose and fucose as valuable test substrates for biotyping or biochemical fingerprinting of *E. coli* strains [8, 9]. It was disappointing that, in this study, none of these four substrates gave reliable results in replicate tests made on the same strain. It is obvious that biochemical fingerprinting, which relies on the rate of fermentation rather than on a positive or negative result, is very 'finely tuned' and not directly comparable with biotyping as done in this study. Our results, however, indicated that arabitol and 2-deoxy-D-ribose fermentation are reliable biotyping tests. The small minority (3%) of the arabitol-positive type identified here may be due to the preponderance of isolates from urinary-tract infections (55%), where a limited number of clones is implicated [10, 11]. Whilst arabitol, with its low discriminating ability, has not been included among our biotyping tests, the minority arabitol-fermenting phenotype, like the sorbitol non-fermenting phenotype of entero-haemorrhagic *E. coli* O 157, may be a useful marker of an epidemic clone. On the other hand, results of 2-deoxy-D-ribose fermentation gave excellent strain discrimination and were helpful for distinguishing different types within recognized urinary pathogenic O serogroups. It was interesting that, of 13 strains of serogroup O 6 (from different patients) included in this study, 8 belonged to biotype 17, a biotype that was rarely identified among strains of other O serogroups (data not shown). Although biotype may correlate well with genetic markers of clonal identity [12], it may also be an adaptation to environment [13] and genetic methods, such as multilocus enzyme electrophoresis [13, 14] would be required to assess the clonal relatedness among these strains of serogroup O 6. Nevertheless, biotype markers are of proven value in epidemiological studies involving strains of *E. coli* and other Enterobacteriaceae [3, 4, 15, 16].

Hunter and Gaston [6] suggested that, for confident interpretation of typing results, a discrimination index of $> 0{\cdot}9$ is desirable. Results with the five primary biotyping tests detailed here can achieve that desired level in *E. coli* (Table 4); strain discrimination is further enhanced by considering results of secondary tests for rhamnose fermentation, lysine decarboxylation and motility, which are designated by the characters b, c and e, respectively, placed after the primary biotype number (Table 3).

This miniaturized biotyping system is recommended as a reliable, simple and inexpensive method of discriminating among *E. coli* strains. Linkup to an

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automated plate reader would enable large numbers of strains to be analysed efficiently; but its ease of interpretation by relatively inexperienced personnel means that the system can be used as a epidemiological tool in the smallest laboratory.

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