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Numerical index of the discriminatory ability of biotyping and resistotyping for strains of *Escherichia coli*

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SUMMARY

A discrimination index was applied to assess the value of biotyping and resistotyping, used alone or together, for the subspecific discrimination of *Escherichia coli*. The index was high when 599 strains from a wide variety of sources were examined by full biotyping with 10 tests. Although the discrimination achieved was lower when a sub-collection of 333 strains was examined, that finding probably indicated that many of the latter strains were of urinary origin and represented a limited number of uropathogenic clones. Combined biotyping and resistotyping provided a higher level of strain discrimination than either method used on its own. Results suggest that these typing methods may be used with confidence for the discrimination of strains of *E. coli*.

INTRODUCTION

A variety of techniques has been used for the differential typing of bacteria at the level of subspecies and both phenotypic and genotypic markers are available for strain discrimination [1, 2]. Those typing methods generally considered to have been of most value for tracing the spread or persistence of epidemic strains usually fulfil certain criteria, crucial among which is probably the requirement that typing results should be reproducible on different occasions of testing, so that members of an epidemic strain are readily recognized as belonging to the same type. Again, it is helpful if a typing method allows type designations to be assigned to all strains examined and an adequate number of types, though not so many as to be cumbersome, is recognized. Furthermore, the tests incorporated in a typing scheme should be those giving optimal differentiation among a large collection of randomly selected, epidemiologically unrelated strains. The choice of typing method, whether it be serotyping, phage typing, bacteriocin typing, plasmid-profiling or restriction fragment-length analysis, depends ultimately on the observed variation in the chosen markers in the bacterial population studied.

Biotyping of *Escherichia coli* strains by their reactions at definitive times in a carefully selected series of biochemical and physiological tests has proved helpful for analysing confined outbreaks of gastroenteritis in infants [3] and for assessing the history of urinary-tract and other infections in individual patients [4, 5]; biotyping proved effective even when used independently of complete serotyping,

a method generally considered to be the 'gold standard' for discrimination of strains of *E. coli*. With biotyping, all infecting strains could be assigned to 1 of 16 primary biotypes on the basis of results in 4 primary tests: 6 secondary tests provided further strain discrimination [6]. Biotypes proved to be stable on different occasions of testing the same strain, whether tests were made on cultures of strains that had been freshly isolated or stored for extended periods [6].

Because cultures from different sources that have been isolated over a period of time may be shown to belong to a common type or to have undergone genetic variation *in vivo* resulting in a change in their typing characters, we have always preferred to supplement the results of any typing scheme, however reliable, with information from one or more additional methods. Although our experience indicated that the combination of biotyping and resistotyping provided excellent discrimination of strains of *E. coli*, it was difficult to assess the discriminatory power of these and other typing methods because of the absence of a single numerical value of discrimination. The calculation of Simpson's index of diversity, based on the probability that any two randomly chosen strains will belong to different types [7], allowed us to make an objective assessment of the discrimination provided by our favoured *E. coli* typing methods.

MATERIALS AND METHODS

Bacteria

The 599 strains of *E. coli* biotyped included 177 O-serogroup strains from the National Collection of Type Cultures, Colindale, London, and another 422 strains isolated by the authors from diverse clinical sources. The latter group of 422 strains was represented by 1065 clinical isolates from 265 individual patients; from some of these patients, only 2 cultures were obtained from different clinical specimens taken on the same day whereas for others, multiple specimens yielded isolates over periods of up to 3 years [4,5,8,9]. All 599 strains were examined by more than one typing method, but only 333 strains (comprising 819 isolates) were biotyped and resistotyped [3–6,8–10]. For the purpose of analysis of this series of strains, cultures of the same type from the same patient have been scored as replicate isolates of one strain, regardless of the interval of time and the diversity of clinical specimens from which they were obtained.

All isolates were confirmed as *E. coli* by conventional methods [11] and were stored on Dorset's egg medium at ambient temperature (*c.* 20 °C).

Typing methods

Cultures were assigned to primary biotypes according to their positive or negative reactions after precise incubation periods [3, 12] in four primary tests chosen for their high discriminatory value and reliability: these were fermentation of raffinose, sorbose and dulcitol in peptone water and decarboxylation of ornithine (Table 1). Results in six secondary tests gave additional strain discrimination, though at a lower level than the primary tests; these were rhamnose fermentation, lysine decarboxylation, aesculin hydrolysis, motility, type-1 fimbriation and identification of growth-factor requirements. Thus, on the basis of 10 primary and secondary tests, strains were assigned to full biotypes [6].

The chemicals used for resistotyping were: A, sodium arsenate; B, phenylmercuric nitrate; C, 4:4′-diamidinodiphenylamine dihydrochloride; D, boric acid; E, acriflavine; F, 4-chlororesorcinol; G, cupric sulphate; and H, malachite green. Resistotyping, modified from the method of Elek and Higney [13], is based on the resistance or sensitivity of different strains of *E. coli* to these chemicals. For each chemical, strains were tested on a series of plates of buffered agar containing a narrow range of concentrations of the test chemical around the discriminating optimum. The simultaneous testing of five reference strains of known growth pattern in the presence of each chemical allowed test cultures to be assigned to a resistotype. For example, a strain of resistotype ACdef was fully resistant to chemicals A and C, partially resistant to chemicals D, E and F and (when the chemical code is absent from the resistotype profile) sensitive to chemicals B, G and H at the concentrations tested.

Full details of biotyping and resistotyping methodology, interpretation of results and type designation have been presented previously [4–6, 8–10, 12].

Index of discrimination

The ability of biotyping and resistotyping used alone or in combination to discriminate among *E. coli* strains can be given by a numerical value, the index of discrimination of Hunter and Gaston [7], which measures the probability that two unrelated strains chosen at random will belong to different types. The index of discrimination (D) is given by:

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

where N is the total number of cultures tested, s is the total number of different types identified and n_j is the number of isolates of the jth type. The application of this index has been fully explained elsewhere [7].

RESULTS

Biotyping

Among 599 strains examined by primary biotyping, each of the 16 (2^4) theoretically attainable biotypes identifiable on the basis of definitive positive and negative results in the four primary tests was recognised. Biotypes 1 (110 strains) and 9 (81 strains) were the most common; biotype 10 was rare, accounting for only 9 of the strains tested. Full details of the distribution of strains in the different primary types are given in Table 1.

Complete biotyping of the 599 strains according to definitive results obtained in both primary and secondary tests identified 213 full biotypes (Table 2) of which biotype Ide (29 strains) was the most common; of the other 212 biotypes, 93 contained more than one strain. The discrimination indices of primary biotyping and full biotyping in the examination of this series of 599 strains were 0·900 and 0·987, respectively (Table 2). Biotyping of the smaller series of 333 strains (that was also examined by resistotyping, see above) identified 16 primary and 134 full biotypes and gave discrimination indices of 0·886 and 0·984, respectively (Table 3).

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Table 1. *Reactions in primary biotyping tests of 599 strains of* Escherichia coli

Primary biotype designation	Result at definitive time in test for metabolism of				Number of strains of primary biotype
	Raffinose	Sorbose	Ornithine	Dulcitol	
1	+	+	+	+	110
2	+	+	+	−	42
3	+	+	−	+	49
4	+	+	−	−	12
5	+	−	+	+	49
6	+	−	+	−	21
7	+	−	−	+	19
8	+	−	−	−	11
9	−	+	+	+	81
10	−	+	+	−	9
11	−	+	−	+	10
12	−	+	−	−	13
13	−	−	+	+	21
14	−	−	+	−	18
15	−	−	−	+	65
16	−	−	−	−	69

Table 2. *Discrimination indices of primary and full biotyping in the examination of 599 strains of* Escherichia coli

Typing method	Number of types	Size (%) of largest type	Discrimination index
Primary biotyping	16	110 (18·4)	0·900
Full biotyping	213	29 (4·8)	0·987

Table 3. *Discrimination indices of biotyping and resistotyping, used alone or in combination, in the examination of 333 strains of* Escherichia coli

Typing method	Number of types	Size (%) of largest type	Discrimination index
Primary biotyping	16	72 (21·6)	0·886
Full biotyping	134	18 (5·4)	0·984
Resistotyping	69	44 (13·2)	0·952
Primary biotyping with resistotyping	182	31 (9·3)	0·985
Full biotyping with resistotyping	280	11 (3·3)	0·998

Resistotyping

Although resistotypes assigned as described above are generally stable, the method is so sensitive that different isolates of some strains may show apparent change from partial to complete resistance, and vice versa, to one or two of the eight chemicals when tested at different times on different plates [10, 13]. Accordingly, in this analysis it seemed appropriate to consider only two states; thus, sensitivity to any chemical was scored as being distinct from resistance, whether that chemical resistance was full or partial. Among the 333 strains tested,

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69 of the theoretically attainable 256 (2^8) resistotypes were identified (Table 3). Forty-four strains belonged to the commonest resistotype CDEFH; of the other 68 resistotypes, 40 contained more than one strain. The index of discrimination of resistotyping in the examination of this series of 333 *E. coli* strains was 0·952 (Table 3).

Combined use of biotyping and resistotyping

Primary biotyping or full biotyping used together with resistotyping divided the 333 strains into 182 different primary biotype-resistotype combinations and 280 different full biotype-resistotype combinations. The discrimination indices of these combined methods were 0·985 and 0·998, respectively (Table 3). The numbers of different types identified and the number of strains of the commonest types are also shown (Table 3).

DISCUSSION

Many techniques, some of which may be suitable for use in reference laboratories only, are now available for tracing the spread of epidemic strains and clones. The choice of method depends not only on the bacterial species but also on the efficiency of the method and on the available resources.

Our previous studies have shown that biotyping of *E. coli* with a carefully selected spectrum of tests is helpful as a preliminary indicator of strain relatedness (and clonality when genetic relationships have been ascertained) among isolates from individual patients or from temporally or spatially confined outbreaks of infection. The method is easily performed without resort to expensive equipment and is preferable to strain characterization with commercial kits that are formulated for identification, rather than subspecific typing, of Enterobacteriaceae. All strains are typable and, even in inexperienced hands, the system has proved reliable and results were reproducible on different occasions of testing the same strain, whether freshly isolated from a patient whose infection had persisted for many years [9] or stored for extended periods on non-selective medium [6]. Thus, biotyping fulfilled most of the criteria considered essential in a good typing scheme [1]. The flexibility of our two-tier system allows for the inclusion at the secondary level of further tests, such as those for the metabolism of sucrose [3, 6], lactose [14], 5-ketogluconate [15, 16], deoxyribose and tagatose [14]; the latter, though relatively expensive substrates, seem to provide additional information useful for strain discrimination. Claims of unreliability for *E. coli* biotyping may be traced to various factors, such as inoculum size [17], but are due primarily to a failure to interpret results after the accurately defined incubation periods that clearly separate genotypically fermenting strains from non-fermenting ones; failure to do so yields late-positive results due to the occurrence of fermenting mutants. Unlike the more stringent biochemical fingerprinting of *E. coli* [18], in which the rate of substrate metabolism is measured, differences in biotype profiles of members of epidemic strains have not proved problematic; and when, rarely, minor changes in type were identified, these were frequently caused by mutation of daughter cells to auxotrophy, and a new growth-factor requirement was concomitantly revealed among secondary biotype test results.

Although it was obvious from our previous studies that biotyping of *E. coli* was clinically helpful, it was impossible until now to calculate its discriminating ability. We have corrected that deficiency by calculating Simpson's index of diversity [7], which provides an indication of the number of types in any collection and the distribution of strains among these types. Thus, discrimination is best and its index greatest when a large collection of randomly sampled strains is divided into many small clusters of different types. Complete biotyping as carried out on our collection of 599 strains from different sources gave a discrimination index ($> 0{\cdot}987$) so high as to indicate that results could be interpreted with confidence [7]; indeed, use of only the four primary tests provided a surprisingly high level (0·900) of strain discrimination. The finding that primary biotyping of the smaller series of 333 strains achieved poorer discrimination (0·886) was perhaps not surprising because of the high proportion of urinary isolates in that sub-collection and, thus, the less random nature of the strains examined.

Even with a biotyping scheme of proven value, such as this was, we recognized that relatedness among isolates was best assessed by a multiple-typing approach in which similarities and differences in the profiles achieved by one typing method were mirrored in the profiles of others [4]. For most purposes we found that problems of strain identity in *E. coli* were resolved by the combined use of biotyping and resistotyping. Indeed, in all of our multiple-typing studies, phenotypic methods were used because of difficulty at that time of routinely applying genotypic methods. However, with improvements in the ease of carrying out examination of chromosomal DNA and analysis of membrane proteins as means of establishing clonality, these techniques have rightly gained favour but remain limited by the difficulty of presenting results in a concise form. Nevertheless, the use of molecular methods is mandatory for detecting subtle evolutionary drift in otherwise identical isolates of epidemic strains that are known to be members of a clone – for example, verotoxin-producing strains of *E. coli* of serogroup O 157 [19, 20].

Early indications of the clonal nature of uropathogenic strains of *E. coli* stemmed from observations that isolates from patients with urinary-tract infections tended to show common characteristics including the possession of certain O, K and H antigens [21, 22] and were extended by later findings on fimbriation, haemolysin production and outer membrane-protein profiles [23]. The more recent application of multilocus enzyme electrophoresis to characterize chromosomal genotypes of *E. coli* further supports the concept of widespread distribution of a limited number of pathogenic clones which cause most cases of disease [24–26]. It is interesting, then, that 29·4% (98) of our collection of 333 strains, many of which had been isolated from urine, belonged to the common urinary serogroups O 2, O 4, O 6 and O 75 (data not shown) and that among these groups there was some evidence of biotype-resistotype clustering. For example, among a total of 32 isolates of serogroup O 75, 18 were of primary biotype 1 (metabolising all 4 primary substrates) and 7 of these were of resistotype BCDEFH; of 18 strains belonging to serogroup O 4, 14 were of primary biotype 9 (raffinose non-fermenting) and 7 of these were of resistotype CDEFH. Whilst strains of serogroups O 2, O 4 and O 75 were fairly heterogeneous with respect to biotype, strains of serogroup O 6 were restricted to biotypes 1 (8 strains) and 9

(21), an observation of interest in view of the restricted number of patterns found in serogroup O 6 strains by multilocus enzyme electrophoresis [27]. In addition, all 29 strains of serogroup O 6 were sensitive to chemical A (sodium arsenate) and 15 strains belonged to the same bio-resistotype, 9-CDEFH.

Other interesting, non-urinary clusters included four strains (11 isolates) of (sorbitol-fermenting) *E. coli* of serogroup O 157. The finding that all belonged to biotype 12 and that in three cases had been isolated from various sites on ill babies within a 4-month period suggested that these infections might have been nosocomially acquired. Thus, unusual typing profiles may help to pinpoint epidemic strains and clones.

The ability to calculate a numerical index for every published typing scheme, as envisaged by Hunter and Gaston [7], is of immense value because it allows objective comparisons of the discriminatory ability of different typing systems to be made. For that reason we first tested their method by assessing the discriminatory power of biotyping for salmonellae of different serotypes and showed that biotyping, used alone or together with phage typing, achieved excellent strain discrimination; that study indicated that our biotype information for salmonellae could be interpreted with confidence [28]. Similarly, we had been unable to include the index of discrimination in the original descriptions of the biotyping and resistotyping schemes that we used for *E. coli*, but have been greatly encouraged by the result of its application in this retrospective study and look forward to comparing the discrimination indices of biotyping and resistotyping with those of other typing systems used for strain discrimination of *E. coli* as they become available.

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