Typing of *Enterococcus* Species by DNA Restriction Fragment Analysis

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Enterococci are a frequent cause of hospital-acquired infection, being associated with urinary tract infections, wound sepsis, bacteremia, and endocarditis. The source of infection is usually thought to be endogenous, but some evidence points to cross-infection between patients. A better understanding of the epidemiology of enterococci has been limited by the lack of a good discriminatory typing system. This report describes the application of two DNA-based typing methods to *Enterococcus faecalis* and *Enterococcus faecium*: comparison of restriction fragments from total DNA by conventional electrophoresis and comparison of restriction fragments hybridizing to an rRNA gene probe (ribotyping). Comparison of restriction fragments (from *Sst*I digestion) by conventional electrophoresis was simple and highly discriminatory. The results of analysis of blood culture isolates and of repeat isolates from individual patients are reported. Ribotyping (with *Bst*CI digestion) was more applicable at the level of species discrimination.

Enterococci are increasingly recognized as common and sometimes problematic causes of nosocomial infection. Urinary tract infections are the most frequent in occurrence, but bacteremia and endocarditis also occur and may be difficult to treat because of both inherent and acquired antibiotic resistance. Although infections have traditionally been thought to arise from the patient's own flora, some reports have suggested that cross-infection between patients may be important (15, 16). Current understanding of the epidemiology of enterococci and their role in infection has been thoroughly reviewed by Chenoweth and Schaberg (2), Murray (7), and Lewis and Zervos (6); these articles highlight the lack of a generally useful typing system for this genus.

Various DNA-based typing methods are now being used for numerous genera of bacteria (10). The aim of this study was to apply such methods to enterococci, to assess the level of discrimination between isolates, and to examine the relationships between isolates of epidemiological interest. The typing methods chosen were digestion of total DNA with a restriction enzyme followed by conventional electrophoresis and hybridization of digested DNA on a Southern blot with an rRNA probe (ribotyping [13]). Recently, Murray et al. (8, 9) have used a third method, pulsed-field electrophoresis of DNA fragments, for typing *Enterococcus faecalis*.

MATERIALS AND METHODS

Bacterial isolates and growth of organisms. Enterococci had been collected at the Royal London Hospital from January to September 1989 (3a). Isolates were from a variety of clinical specimens and were not restricted to those considered clinically significant. Organisms had been identified to species level by eight biochemical tests selected from the scheme of Waitkins et al. (14): growth on 6.5% sodium chloride agar, arginine hydrolysis, pyruvate fermentation, esculin hydrolysis, and fermentation of lactose, arabinose, sorbitol, and mannitol. The reactions of enterococcal species

are given in Table 1. Isolates not identified by this scheme were tested by the commercial API 20S system and subsequently, where necessary, by the API 50S system (both from Analytab Products, Basingstoke, United Kingdom).

From the collection, 12 consecutive *E. faecalis* and 6 consecutive *Enterococcus faecium* isolates (all from different patients) were taken for analysis. All blood culture isolates of *Enterococcus* species available and identified at the start of this study were analyzed (12 isolates from 10 patients). To compare multiple isolates from individuals, 42 isolates (from various specimens) from 12 patients were selected.

Type strains of *E. faecalis* (NCTC 775) and *E. faecium* (NCTC 7171) were also used.

Isolates were stored frozen on beads at -20°C in 50% glycerol broth. Frozen stocks were recovered by plating onto agar containing 5% horse blood. Broth cultures were made in Todd-Hewitt broth (Oxoid Ltd.).

For repeated subculture to assess the stability of patterns, a single colony from each of two isolates was streaked onto nutrient agar and allowed to grow overnight, and then a single colony from the first plate was streaked onto a second plate. This was repeated for 25 subcultures.

DNA extraction. DNA extraction was based on the method of Pitcher et al. (11). Isolates were incubated overnight in Todd-Hewitt broth, and then 3 ml of culture was pelleted and resuspended in 0.1 ml of TE (10 mM Tris HCl, 1 mM EDTA [pH 8.0]). Lysozyme was added to 5 mg ml^{-1} , and mixtures were incubated at 37°C for 30 min. Cells were lysed by adding 0.5 ml of GES (5 M guanidium thiocyanate, 0.1 M EDTA [pH 8.0], 0.5% *N*-laurylsarcosine) and incubating at room temperature for at least 10 min until lysis was observed. Cold 7.5 M ammonium acetate (0.25 ml) was added, and the mixture was placed on ice for 10 min. Proteins were extracted by mixing with 0.5 ml of chloroform-isoamylalcohol (24:1) and then centrifuged for 10 min in a microcentrifuge, and the upper phase was recovered. Cold isopropanol (0.54 volume) was added, and the precipitated DNA was recovered by spinning for 5 min in a microcentrifuge. DNA was resuspended in 0.2 ml of TE, reprecipitated with 0.5 ml

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TABLE 1. Identification scheme for *Enterococcus* species

Test	Reaction of ^a :				
	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. casseliflavus</i>	<i>E. avium</i>	<i>E. durans</i>
Lactose	+	+	+	+	+
Mannitol	+	+	+	+	-
Arabinose	-	+	+	+	-
Sorbitol	+	-	V	+	-
Growth on 6.5% NaCl	+	+	+	+	+
Esculin	+	+	+	+	+
Arginine	+	+	+	-	-
Pyruvate	+	-	-	-	-

^a Reactions of the species, taken from reference 14: +, positive; -, negative; V, variable (i.e., some strains positive, some strains negative).

of cold ethanol, and finally resuspended in 0.1 ml of TE or enough to dissolve the DNA.

DNA restriction and electrophoresis. Restriction digestion of 10- μ l samples of DNA was performed by overnight incubation with buffers and conditions supplied by the manufacturers. DNA fragments were separated by electrophoresis at 120 V in 0.9% agarose in TBE buffer (0.9 M Tris-borate, 0.004 M EDTA) by standard methods as de-

FIG. 1. DNA restriction patterns of *E. faecalis*. Total DNA extracted from 12 consecutive isolates of *E. faecalis* (lanes 1 through 12) was digested with *Sst*I and separated on an agarose gel. NCTC 775, *E. faecalis* type strain, is also shown (lane A). Molecular size markers (lanes m) are 1.6 kb and 2 to 12 kb (in 1-kb increments) (1-kb ladder from Life Technologies, Paisley, United Kingdom). Diagram below (taken from original Polaroid negative) shows all bands in the 1.6-to-8-kb size range.

FIG. 2. DNA restriction patterns of *E. faecium*. Total DNA extracted from six consecutive isolates of *E. faecium* (lanes 13 through 18) was digested with *Sst*I and separated on agarose gels. NCTC 775, *E. faecalis* type strain, and NCTC 7171, *E. faecium* type strain, are also shown (lanes A and B, respectively). (Isolate 14 was subsequently identified as *E. avium*.) Molecular size markers (lanes m) are 1.6 kb and 2 to 12 kb (in 1-kb increments) (1-kb ladder from Life Technologies, Paisley, United Kingdom). Diagram below (taken from original Polaroid negatives) shows all bands in 1.6-to-8-kb size range.

scribed by Sambrook et al. (12). Gels were stained with ethidium bromide after electrophoresis. When two patterns were thought to be identical, this was confirmed by running the samples in adjacent lanes on a gel.

Ribotyping. Southern transfers of gels with *Bsc*II-digested DNA were made by capillary blotting to nylon membranes (Hybond-N; Amersham International) (12).

Escherichia coli rRNA (Sigma) was extracted with phenol, ethanol precipitated, and then labelled with digoxigenin by reverse transcription by a method adapted from the Non-radioactive DNA Labelling Kit of Boehringer Mannheim. RNA (1 μ g in 13 μ l of H₂O) was heated to 65°C for 5 min and then cooled on ice. Two microliters of RT buffer (0.5 M Tris HCl [pH 8.0], 0.4 M KCl, 0.1 M MgCl₂, 0.1 M dithiothreitol), 2 μ l of hexanucleotide primers from the kit, 2 μ l of deoxynucleoside triphosphates from the kit (including digoxigenin-dUTP), and 20 units of reverse transcriptase were added to the RNA, and the mixture was incubated at 37°C for 1 h. Labelled DNA was precipitated with ethanol in the presence of 0.4 M LiCl and redissolved in 60 μ l of TE.

Membranes were prehybridized and hybridized with digoxigenin-labelled probe at 55°C and washed in 0.2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate at 55°C, and hybridization was detected exactly as described for the Non-radioactive DNA Detection Kit (Boehringer Mannheim).

FIG. 3. Ribotyping of (a) *E. faecalis* and (b) *E. faecium*. Total DNA extracted from 12 consecutive isolates of *E. faecalis* (lanes 1 through 12) and from 6 consecutive isolates of *E. faecium* (lanes 13 through 18) was digested with *Bsc*I, separated on agarose gels, blotted to a filter, and hybridized with cDNA made from *E. coli* rRNA. NCTC 775, *E. faecalis* type strain, and NCTC 7171, *E. faecium* type strain, are also shown (lanes A and B, respectively). (Isolate 14 was subsequently identified as *E. avium*.) Bars mark the five typical bands for *E. faecalis*.

Hybridization was repeated on a second blot to confirm results for most isolates.

RESULTS

Selection of restriction enzymes for digestion of total DNA.

DNA isolated from a small number of samples was digested with four different restriction enzymes: *Bsc*I, *Eco*RI, *Sal*I, and *Sst*I. Digestion with *Sst*I produced a pattern of several distinct bands in the range of 1.6 to 8 kb, which could readily be compared between isolates. *Bsc*I digested the DNA at more sites, generating a complex pattern of bands but leaving few large unresolved fragments. The *Bsc*I enzyme was chosen for probing for rRNA genes.

Comparison of consecutive isolates. Total DNA was extracted from 12 consecutive *E. faecalis* isolates and 6 consecutive *E. faecium* isolates (all from different patients) obtained in the course of a survey at the Royal London Hospital. The DNA was digested with *Sst*I, and the fragments were separated by electrophoresis. The results are shown in Fig. 1 and 2. Secondly, the DNA was digested with *Bsc*I, separated by electrophoresis, transferred to a membrane, and probed for rRNA genes (ribotyping). The results are shown in Fig. 3. All of the isolates were analyzed twice by both methods (starting with digestion of DNA), giving the same results each time. Digestion with *Sst*I and staining of total DNA gave patterns by which all 12 *E. faecalis* isolates could be distinguished from one another, although isolates 8 and 10 differed only slightly (Fig. 1). Certain bands were held in common by several isolates, but no species-specific characteristics could be identified.

E. faecium isolates were more similar to one another by total DNA pattern than were *E. faecalis* isolates (Fig. 2). Five of the isolates and the type strain were identical in all but one or two bands; nevertheless, all could be distinguished. Isolate 14 was very different from the other isolates and was subsequently identified as *Enterococcus avium* by the API 50S system, API 20S, and the eight-test scheme used in the survey, had given an ambiguous result for this isolate.

Ribotyping with *Bsc*I digestion produced highly related patterns for the 12 *E. faecalis* isolates. Five major bands were common to most isolates, as indicated in Fig. 3a, with

all isolates containing at least four of these bands. Several isolates had additional strong or weak bands. Ribotyping could not distinguish all isolates; for example, isolates 8, 9, and 10 had the same pattern, as did isolates 4 and 5.

The *E. faecium* isolates were even more highly related by ribotyping but had patterns quite different from those of *E. faecalis*. Again the pattern of isolate 14, *E. avium* by API 50S, differed considerably from the common *E. faecium* pattern (Fig. 3b).

Stability and reproducibility of restriction pattern. Two clinical isolates were each replated 25 times in succession, and each time a single colony was picked and streaked out. DNA was extracted before and after the 25 subcultures and compared by digestion with *Sst*I. No difference was found after repeated subculture.

The type strains of *E. faecalis* and *E. faecium* had DNA extracted and restriction pattern and ribotyping performed on more than 15 occasions. The pattern of bands was always constant, although the overall intensity and degree of background staining varied somewhat. (Whenever the pattern for any isolate was not sufficiently clear, DNA extraction was repeated, but this was not often necessary.)

Blood culture isolates. Blood culture isolates from nine patients with *E. faecalis* and one patient with *Enterococcus casseliflavus* were examined. It is possible that systemic infection could be associated with one strain or subset of *E. faecalis*, perhaps one with particularly invasive properties. To determine whether the blood culture isolates were related, these were all typed by comparison of band patterns after digestion with *Sst*I. Figure 4 illustrates patterns obtained from the *E. faecalis* isolates of nine patients (two patients had duplicate isolates; see below). Overall, there is no strong similarity among the nine patterns which might indicate that a single strain was involved. There are three pairs of closely related patterns: a and c, d and h, and g and i (Fig. 4). The *E. casseliflavus* isolate had a completely different pattern (not shown).

Multiple isolates from individuals. Many patients had enterococci isolated on more than one occasion during the survey period, and 12 of these were investigated. For eight individuals, all isolates from each were identical by restriction enzyme digestion pattern (with *Sst*I). These eight in-

FIG. 4. DNA restriction patterns of nine *E. faecalis* blood culture isolates from different patients. DNA was digested with *Sst*I. Molecular size markers (lanes m) are 1.6 kb and 2 to 12 kb (in 1-kb increments) (1-kb ladder from Life Technologies, Paisley, United Kingdom). Diagram below (taken from original Polaroid negative) shows all bands in 1.6-to-8-kb size range.

cluded two individuals who had isolates from two different sites (Table 2). The isolates from four of these eight individuals were also compared by ribotyping, and again each individual had only one type.

Patient A had a second blood culture isolate after 3 months; this was identified as an asaccharolytic *E. faecalis* isolate by the eight-test scheme and as *Streptococcus lactis* by API 20S. This organism produced a DNA restriction pattern indistinguishable from that of the earlier *E. faecalis* isolate from the same individual, suggesting that it did originate from the earlier infection.

The other four patients had isolates which produced very different patterns. Patient G had two types of wound isolate, H had two types of urine isolate, and L had two types of fecal isolate, all of *E. faecalis*. For G, the different types could also be recognized by ribotyping, but for H they could not (L isolates were not checked by ribotyping). Patient K had five identical *E. faecium* isolates followed by four identical *E. faecalis* isolates.

DISCUSSION

DNA analysis for typing enterococci. There is no accepted conventional system for typing enterococci, and this has

TABLE 2. Repeat isolates from individual patients

Patient	No. (site) of isolates	Time span ^a	No. of pattern types ^b
A	2 (blood)	3 months	1 ^c
B	2 (blood)	3 weeks	1
C	3 (urine), 1 (feces)	3 days	1
D	1 (urine), 2 (wound)	1 day	1
E	3 (wound)	2 weeks	1
F	2 (urine)	5 days	1
G	3 (wound)	1 day	2
H	2 (urine), 2 (wound)	3 days	2
I	4 (urine)	10 days	1
J	2 (urine)	7 days	1
K	9 (urine)	3 months	2 ^d
L	2 (urine), 2 (feces)	3 weeks	2

^a Time span from first to last isolate.

^b Number of different patterns from each individual (all *E. faecalis* isolates unless specified).

^c Second isolate was biochemically different.

^d One pattern from *E. faecium* and one pattern from *E. faecalis* isolates.

impeded understanding of the epidemiology of enterococcal infections. We have previously used DNA analysis to confirm the identity of methicillin-resistant *Staphylococcus aureus* in this hospital (5), and the approach is being used for a rapidly increasing number of bacterial species (10).

In this study, two methods were used. Total DNA was digested with a suitable restriction enzyme (*Sst*I), and the pattern of bands produced after electrophoresis and staining was used for comparison of isolates. Secondly, DNA digested with a different enzyme (*Bsc*I) was separated by electrophoresis and then transferred to a membrane and hybridized with an rRNA-derived probe (3) (ribotyping [13]). Both methods differentiated a number of types among 12 consecutive *E. faecalis* isolates, but whereas the *Sst*I restriction pattern distinguished all 12 isolates, ribotyping with *Bsc*I distinguished 9 types. Similarly, all *E. faecium* isolates could be distinguished by the *Sst*I pattern but not by ribotyping.

The results indicate that digestion of total DNA with *Sst*I is a good method for epidemiological studies on enterococci. The relatively simple pattern of bands in the range of 1.6 to 8 kb, well resolved by conventional electrophoresis, was easy to compare between isolates. Not only was this more discriminating than ribotyping, but results are available more quickly and it is easier and cheaper to perform. This method also yields results more quickly and is less technically demanding than pulsed-field electrophoresis, a technique used effectively by others (8, 9). An experienced worker can extract DNA from 12 to 24 isolates in 1 day and obtain a result from electrophoresis on the second day.

Ribotyping is more likely to have a role in species discrimination, particularly when biochemical tests yield an ambiguous identification. Less differentiation between isolates is to be expected with this method, as rRNA genes form probably the most conserved region of the genome.

A theoretical disadvantage of restriction analysis without probing compared with ribotyping is the effect of unstable elements in the genome. These would include plasmids, transposable elements, and lysogenic bacteriophages. The present study demonstrated that patterns remained constant after 25 rounds of subculture of two clinical isolates and upon more than 15 extractions of the type strains. Nevertheless, such elements are likely to contribute to minor

differences between strains. This, in fact, highlights one of the attractions of restriction fragment analysis, which is that it is able to demonstrate closely related as well as identical isolates.

Epidemiology of enterococci. As a result of the highly discriminatory typing system described here, it has been possible to address questions about isolates that had been collected at the Royal London Hospital. The first of these questions was whether the blood culture isolates could have a common origin, indicating that a distinct subset of the species might have the properties required to invade the bloodstream. The result was that different patients' isolates gave a range of different patterns. Although there were three cases in which a pair of isolates had similar patterns, further work (submitted for publication) has shown that patterns similar to those of these pairs are also common in urine and fecal isolates. It does not appear from this small sample that any particular strain is predominantly responsible for systemic infection by *E. faecalis*. A recently published article about a study performed in an American hospital found that gentamicin-resistant, hemolytic *E. faecalis* bacteremia was associated with an increased mortality over gentamicin-susceptible, nonhemolytic infections (4). We note that the two similar isolates a and c (Fig. 4) were also gentamicin resistant and hemolytic.

The second question concerned the relationship between multiple isolates from individuals. When isolates are obtained some time apart, or when there are isolates from different sorts of specimens, it would be informative to know whether the same or a different organism is involved. For 8 out of 12 patients, all isolates were identical (Table 2). For instance, two repeat blood culture isolates had retained identical patterns after 3 weeks and 3 months, although the isolate at 3 months had altered biochemical test results. Of the exceptions, patient K had one type of *E. faecium* repeatedly isolated from urine for 1 week and then a month later had one type of *E. faecalis* repeatedly isolated for 2 months. Patient L had identical urine and fecal isolates at one time but had had a different fecal isolate 3 weeks previously. Only patients G and H had two different isolate types at one time (Table 2). In these three cases, the difference in patterns was extensive and could not have resulted from simple changes in plasmid carriage or other mobile genetic elements.

The results discussed above represent a preliminary study of the epidemiology of enterococci with strains isolated in a survey at the Royal London Hospital. We believe that they demonstrate the potential of a highly discriminating but simple DNA-based typing system for understanding the spread of these (and other) organisms and their appearance as nosocomial pathogens.

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