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Comparison of High-Level Gentamicin-Resistant *Enterococcus faecium* Isolates from Different Continents

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Eight clinical isolates of *Enterococcus faecium* highly resistant to gentamicin (MIC, >1,000 mg/liter) from patients in six hospitals on three continents were investigated for evidence of spread of either a clone of high-level gentamicin-resistant (HLGR) *E. faecium* or wide dissemination of a gentamicin resistance (Gm^r) plasmid. A combination of ribotypes, plasmid profiles, and extended antimicrobial susceptibilities enabled us to distinguish all but two of the isolates and did not suggest clonal dissemination of a single strain. Two isolates from hospitals situated close together appeared identical by these methods. All of the isolates carried Gm^r plasmids which appeared to be closely related following digestion with restriction endonucleases. Cross-hybridization studies confirmed extensive DNA homology between these plasmids. The restriction fragments of these plasmids which hybridized with a probe specific for the *aac6'aph2"* resistance gene did not resemble those seen in the Gm^r transposon Tn*5281*, which was characterized previously in *E. faecalis* HH22. This study suggests that there has been widespread dissemination of a single Gm^r plasmid and its derivatives amongst isolates of HLGR *E. faecium*, although a Gm^r plasmid from an HLGR *E. faecium* isolated in the United States showed little homology with the other Gm^r plasmids studied.

High-level gentamicin-resistant (HLGR; MIC, >1,000 mg/liter) strains of *Enterococcus faecalis* were first reported in France in 1979 (9) and have subsequently become disseminated worldwide (12). In *E. faecium*, HLGR appeared in the United States in 1986 (4), although the emergence of such strains had been predicted previously from in vitro transfers of gentamicin resistance (Gm^r) plasmids from *E. faecalis* to *Enterococcus faecium* (2). There have been a number of recent reports of the isolation of HLGR *E. faecium* in several countries, including the United Kingdom, Ireland, Singapore, and Australia (1, 5, 10, 17, 20, 21).

In both *E. faecium* and *E. faecalis*, HLGR is often plasmid mediated and is due to the production of the bifunctional aminoglycoside-modifying enzyme AAC(6')-APH(2") (4, 6, 21). Detailed analysis of pBEM10, a Gm^r plasmid which also encodes β-lactamase production in *E. faecalis* HH22, showed that the *aac6'aph2"* gene was part of a transposon, designated Tn*5281* (8). This transposon is highly related to Gm^r transposons found in *Staphylococcus aureus* and *Staphylococcus epidermidis* (8, 11).

Patterson and colleagues found that Gm^r plasmids from geographically diverse strains of *E. faecalis* were heterogeneous with respect to size, resistance markers carried, and restriction digestion patterns (14). We have reported that gentamicin resistance in isolates of *E. faecium* from diverse geographical areas was mediated by plasmids approximately 75 kb in size which also encoded resistance to trimethoprim (19). In the present study, we further investigated eight isolates of HLGR *E. faecium* from three continents for evidence of dissemination of a clone, and we used restriction endonuclease analysis to assess the relatedness of the Gm^r plasmids in these isolates.

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MATERIALS AND METHODS

Bacterial isolates. Eight HLGR *E. faecium* isolates (A through H), each isolated from clinical specimens and sent to the Antibiotic Reference Laboratory, were included in the present study. The isolates were from six hospitals on three continents (Table 1).

E. faecium 87-T24 was used as a control strain to compare transconjugants with. This strain is *E. faecium* GE-1 containing a 51-MDa Gm^r plasmid from a strain of HLGR *E. faecium* isolated in the United States (4).

Antimicrobial susceptibility testing and determination of plasmid content. Susceptibilities of the isolates to a range of antimicrobial agents were determined by an agar incorporation method (16), and their plasmid contents were determined by an alkaline lysis technique as described previously (16).

Ribotyping. Restriction fragment length polymorphisms (RFLPs) of the rRNA genes in the HLGR *E. faecium* were determined as described previously (22). Briefly, genomic DNA was digested with *Bam*HI (GIBCO BRL, Uxbridge, United Kingdom), separated on 0.8% agarose gels, and transferred to nylon membranes (Hybond N; Amersham, Aylesbury, United Kingdom). The DNA was investigated for hybridization with a biotinylated cDNA probe derived from 16S and 23S rRNAs of the type strain of *E. faecalis* (strain NCTC 775). Ribotypes were differentiated by using a one-band difference rule (22).

Conjugation and analysis of transconjugants. Transfer of gentamicin resistance in vitro from HLGR *E. faecium* isolated from clinical specimens to a recipient strain of *E. faecium*, GE-1 (4), was investigated by using a cross-streak mating method (7). Gentamicin-resistant transconjugants were selected and screened for plasmid content as described previously (19, 21). Eight transconjugants (designated A' through H') corresponding to the eight donor isolates were studied.

Preparation of plasmid DNA for digestion. Gm^r plasmids were prepared from the transconjugants by using a miniprep-

TABLE 1. Characteristics of HLGR transconjugants and Gm^r plasmids

Transconjugant	Source of donor (hospital no. and country)	Reference	Markers transferred[a]	Size[b] of plasmid (kb)	Size(s) (kb) of restriction fragment(s) hybridizing with *aac6'aph2"* probe	
					*Cla*I	*Hae*III
A'	1. Ireland	20	Gm, Tr	53	3.0	2.8
B'	2. United Kingdom	20	Gm, Tr	65	3.0	2.8
C'	3. United Kingdom	17	Gm, Tr	65	3.0	2.8
D'	4. Singapore	10	Gm, Tr	65	3.0	2.8
E'	5. United Kingdom	1	Gm, Tr	65	3.0	2.8
F'	5. United Kingdom	1	Gm, Tr	70	3.0	2.8
G'	6. Australia	5	Gm, Tr	67	3.0	2.8
H'	4. Singapore	10	Gm, Sm, Tr, Ery	77	1.6, 3.0, 3.2, 4.0	2.8, 3.1, 3.6, 4.4, 9.0
87-T24	7. United States	4	Gm, Sm, Ery	80[c]	2.5	8.0

[a] Gm, gentamicin; Tr, trimethoprim; Sm, streptomycin; Ery, erythromycin.
[b] Mean of two estimates calculated from *Cla*I restriction fragments.
[c] Converted from published size of 51 MDa.

aration method which yielded enterococcal plasmid DNA in a form suitable for direct digestion with restriction endonucleases. This method consisted of a modification of the alkaline lysis technique used previously (16). Enterococci were grown overnight on Columbia blood agar (Unipath, Basingstoke, United Kingdom). A rice grain-sized pellet of bacterial growth was suspended in 100 μl of suspending buffer (25% sucrose in TE buffer [10 mM Tris, 1 mM EDTA, pH 8.0] plus 10 mg of lysozyme per ml) and incubated at 37°C for 30 min. The cells were lysed by the addition of 200 μl of alkaline sodium dodecyl sulfate (0.2 M NaOH, 1% sodium dodecyl sulfate), and the samples were incubated at 56°C for 1 h. After addition of 150 μl of 3 M potassium acetate (pH 4.8), the samples were left on ice for 20 min. The samples were centrifuged for 5 min in a microcentrifuge, and the precipitated debris was removed by decanting the supernatant into a fresh tube through a double thickness of medical gauze (Boots plc, Nottingham, United Kingdom). An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and samples were again spun for 5 min in a microcentrifuge. The DNA from 200 μl of the supernatant was precipitated with 2 volumes of cold ethanol at room temperature for 2 min and harvested by centrifugation for 5 min.

The DNA pellets were dried at 37°C for 1 to 2 h and suspended in distilled water containing 1 μg of RNase (Sigma, Poole, United Kingdom). Plasmid DNA was digested overnight with 40 U of the restriction endonuclease (GIBCO BRL) in the presence of the recommended incubation buffer. The samples were electrophoresed through 1% agarose gels at 90 V for 2 to 3 h. The electrophoresis buffer was 0.5× TBE (1× TBE is 89 mM Tris, 89 mM borate, and 2 mM EDTA [pH 8.0]) (15).

DNA hybridization. Intact or digested plasmid DNA separated on agarose gels was transferred to a nylon membrane (Hybond N) by using a VacuGene vacuum blotting apparatus (Pharmacia-LKB, Milton Keynes, United Kingdom). To assess the degree of homology between the Gm^r plasmids, a representative plasmid (from transconjugant B') was digested with *Cla*I, and the resulting fragments were labeled with digoxigenin by using a random primer labeling kit (Boehringer Mannheim UK, Lewes, United Kingdom). The plasmids were also examined for hybridization with a Gm^r probe, i.e., the digoxigenin-labeled 1.5-kb *Eco*RI-*Hin*dIII fragment of plasmid pSF815A specific for the *aac6'aph2"* gene used previously (19, 21). All hybridizations were carried out under stringent conditions in accordance with the manufacturer's instructions (Boehringer Mannheim UK).

RESULTS AND DISCUSSION

Characterization of HLGR *E. faecium*. The clinical isolates of HLGR *E. faecium* were investigated for evidence of dissemination of a clone. The RFLPs of rRNA genes allowed the eight isolates to be divided into six ribotypes (Fig. 1) when genomic DNA was digested with *Bam*HI and hybridized with a cDNA probe derived from rRNA from the type strain of *E. faecalis* (strain NCTC 775). The eight isolates showed seven distinct plasmid profiles (data not shown). Isolates B and C, which were from hospitals situated close to each other, were of the same ribotype and had identical plasmid profiles. Isolates A and F also belonged to a single ribotype, but this particular ribotype corresponded to that previously designated FmI and is common among epidemi-

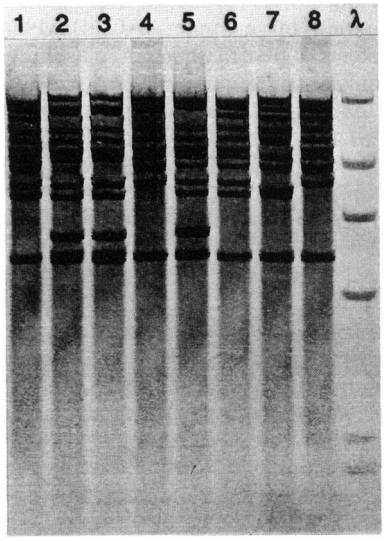

FIG. 1. Southern transfer of a 0.8% agarose gel showing the RFLPs of rRNA genes in HLGR *E. faecium* isolates A through H (lanes 1 through 8, respectively) after digestion with *Bam*HI. A *Hin*dIII digest of phage lambda is shown as a molecular weight standard.

ologically unrelated isolates of *E. faecium* (22). Isolates A and F had markedly different plasmid profiles. The antimicrobial susceptibilities of the isolates did not provide further discrimination (data not shown). All were resistant to multiple antimicrobial agents, although all were sensitive to vancomycin. Only isolate E did not show high-level resistance to streptomycin (MIC, >2,000 mg/liter).

Thus, a combination of ribotyping, plasmid profiles, and extended antimicrobial susceptibilities allowed most HLGR *E. faecium* from widespread areas to be distinguished. Indeed, even pairs of isolates from hospital 4 (isolates D and H) and hospital 5 (isolates E and F) differed markedly. This did not suggest that clonal dissemination of a single strain of HLGR *E. faecium* had occurred. Despite this, two isolates from hospitals situated close to each other (hospitals 2 and 3), between which patients are transferred, appeared identical by each of these three methods. This raises the possibility that interhospital spread of a strain occurred in this case. Similarly, a previous study found no evidence of intercontinental spread of β-lactamase-producing *E. faecalis*, although spread of a clone within the Middle Atlantic region of the United States was observed (13).

Preparation of transconjugants. Each HLGR *E. faecium* isolate was able to transfer gentamicin resistance readily to *E. faecium* GE-1. Those Gm^r transconjugants from each mating which had acquired a single plasmid were examined in greater detail in order to confirm that the acquired plasmid hybridized with the Gm^r probe. Further studies were undertaken by using one transconjugant which fulfilled these criteria (designated A′ through H′; Table 1) from each donor strain. In subsequent investigations, the characteristics of these eight transconjugants were compared with those of *E. faecium* 87-T24.

Analysis of transconjugants. The antimicrobial susceptibilities of the nine HLGR transconjugants (including strain 87-T24) were determined in order to ascertain which antimicrobial resistance markers were cotransferred with HLGR. Seven of the transconjugants studied also acquired resistance to trimethoprim (Table 1), while one, transconjugant H′, acquired resistance to trimethoprim and erythromycin and high-level resistance to streptomycin in addition to HLGR. Strain 87-T24 had high-level resistance to both gentamicin and streptomycin and resistance to erythromycin (Table 1).

Comparison of Gm^r plasmids. The Gm^r plasmids from the nine HLGR transconjugants were compared further after digestion with restriction endonucleases. In this study, enterococcal plasmids were prepared for digestion by using a modification of an alkaline lysis technique. Most rapid methods are unsuitable for preparing enterococcal plasmids (13), and many laboratories continue to use cesium chloride-ethidium bromide density gradient centrifugation (see, for example, references 8 and 14). However, the method we used was quick, allowed multiple samples to be prepared simultaneously, and gave reproducible digestion patterns. The *Eco*RI digestion pattern obtained using this method for the Gm^r plasmid in strain 87-T24 was consistent with previous findings (3).

Digestion of the Gm^r plasmids with *Cla*I (Fig. 2) or *Hae*III (data not shown) yielded more than 10 fragments per plasmid and indicated a high degree of similarity between most of the Gm^r plasmids studied. With both enzymes, the plasmids of transconjugants B′, C′, D′, and E′ were indistinguishable, while those from A′, F′, and G′ showed relatively few differences. The plasmid of transconjugant H′ showed more differences, although several bands were shared with the other plasmids. The Gm^r plasmid of strain 87-T24 had unique *Cla*I and *Hae*III digestion patterns. Digestion with *Eco*RI allowed further differences between plasmids to be detected (data not shown), although this enzyme gave only four or five fragments for all plasmids except that of strain 87-T24. The *Eco*RI digestion patterns of plasmids from B′ and C′ (derived from two indistinguishable isolates) were slightly different (data not shown). The similarities between the Gm^r plasmids described here are in marked contrast to the earlier finding that Gm^r plasmids in *E. faecalis* from North America are heterogeneous with respect to the resistance markers carried and restriction digestion patterns (14).

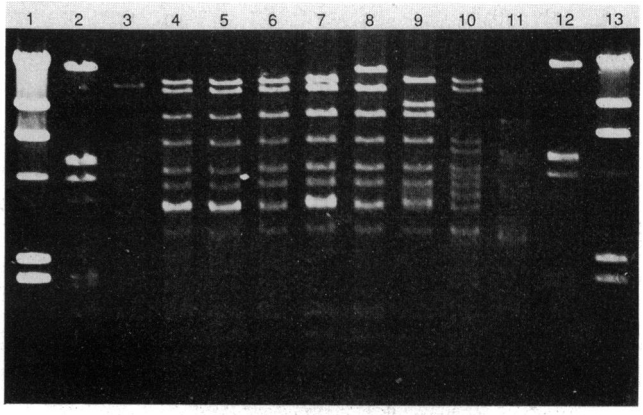

FIG. 2. Agarose gel (1%) electrophoresis of Gm^r plasmids digested with *Cla*I. Lanes shown are transconjugants A′ through H′ (lanes 3 through 10, respectively) and 87-T24 (lane 11). Molecular weight standards are lambda phage DNA digested with *Hin*dIII (lanes 1 and 13) and digoxigenin-labeled fragments from digestion of lambda DNA with *Eco*RI and *Hin*dIII (lanes 2 and 12; Boehringer Mannheim UK).

Cross-hybridization studies. When the Gm^r plasmid in transconjugant B′ was labeled and used in cross-hybridization studies, extensive homology between the Gm^r plasmids of transconjugants A′ through H′ was observed (Fig. 3), confirming the apparent similarities seen in digestion patterns. Only two fragments of the reference Gm^r plasmid in strain 87-T24 hybridized with this probe, indicating that there was little homology between this and the other plasmids.

Hybridization with the Gm^r probe. On *E. faecalis* plasmid pBEM10, the *aac6′aph2″* gene is part of transposon Tn*5281*. This transposon yields 2.5-kb *Cla*I and 3.9-kb *Hae*III fragments, which hybridize with the Gm^r probe (8). Therefore, in order to determine whether the *aac6′aph2″* gene in the HLGR *E. faecium* resided on a transposon similar to Tn*5281*, the Gm^r plasmids were examined to establish which restriction fragment hybridized with the Gm^r probe. Seven plasmids had common 3.0-kb *Cla*I and 2.8-kb *Hae*III fragments which hybridized with the probe (Table 1). This common pattern of hybridization suggests that in the *E. faecium* isolates studied, the *aac6′aph2″* gene is not part of an element identical to Tn*5281*. One plasmid examined (in transconjugant H′) had multiple fragments which hybridized with the probe, which is consistent with it carrying more than one copy of the *aac6′aph2″* gene. Although a 2.5-kb *Cla*I fragment of the reference Gm^r plasmid of strain 87-T24 hybridized with the probe, which is consistent with the presence of Tn*5281*, the hybridization pattern after digestion

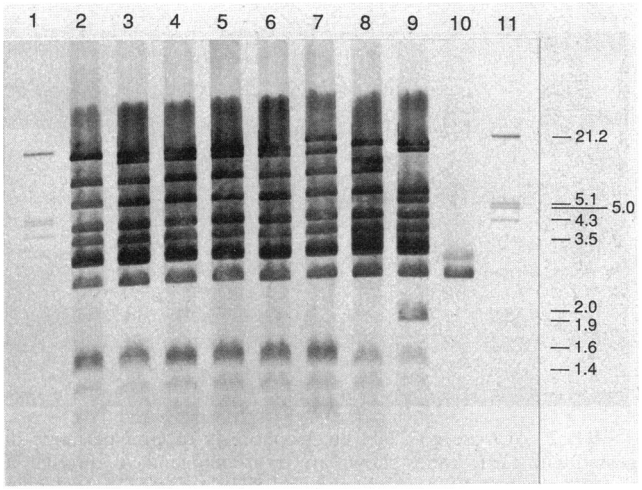

FIG. 3. Southern blot of *Cla*I-digested Gm^r plasmids cross-hybridized with digoxigenin-labeled Gm^r plasmid from transconjugant B′. Lanes shown are transconjugants A′ through H′ (lanes 2 through 9, respectively) and 87-T24 (lane 10). Molecular weight standards (in kilobases) are digoxigenin-labeled fragments from digestion of lambda DNA with *Eco*RI and *Hin*dIII (lanes 1 and 11; Boehringer Mannheim UK).

with *Hae*III was distinct (i.e., yielding a fragment of 8.0 not 3.9 kb; Table 1).

In conclusion, we have identified a group of highly related plasmids encoding HLGR and trimethoprim resistance in isolates of HLGR *E. faecium* from three continents. The reasons for such extensive homology between Gm^r plasmids in isolates from diverse geographical areas are unclear. Several of the isolates were from patients in liver transplant units (1, 5, 17), and although the possibility of transfer of patients and/or staff between units in the United Kingdom could not be excluded, this seems unlikely for units on different continents. Furthermore, the differences observed between the clinical isolates do not support direct spread of a single strain. As suggested previously (19), trimethoprim usage may have exerted selective pressure for the maintenance and dissemination of these highly related plasmids, which also encode trimethoprim resistance, within enterococcal populations. Trimethoprim is used extensively, alone or in combination with sulfamethoxazole, for the treatment of a variety of conditions including urinary tract infections, of which enterococci are a relatively common cause (12). The Gm^r plasmids reported here could be transferred readily between strains of *E. faecium* either directly or through an intermediate *E. faecalis* host (18). Such selective pressure would result in concomitant dissemination of HLGR, a genetically linked resistance marker of greater clinical significance.

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