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Increasing Resistance to β-Lactam Antibiotics among Clinical Isolates of *Enterococcus faecium*: a 22-Year Review at One Institution

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To identify any change in the antibiotic resistance of *Enterococcus faecium*, we examined the antibiotic susceptibilities of clinical strains ($n = 84$) isolated at one institution during the 22 years since 1968. A significant increase in resistance to penicillin was observed during the study period: the MICs of penicillin for 50 and 90% of isolates tested were 16 and 64 μg/ml, respectively, from 1969 to 1988 ($n = 48$; geometric mean MIC, 14 μg/ml), whereas they were 256 and 512 μg/ml, respectively, from 1989 to 1990 ($n = 36$; geometric mean MIC, 123 μg/ml) ($P < 0.001$). A comparable increase in resistance to ampicillin was also noted ($P < 0.001$). No strains produced detectable β-lactamase. In contrast, susceptibilities to vancomycin, teicoplanin, and ciprofloxacin remained stable. High-level resistance to gentamicin was observed in none of 48 isolates from 1969 to 1988, but was present in 22 of 36 strains (61%) from 1989 to 1990 ($P < 0.001$) and was significantly associated with resistance (MIC, ≥128 μg/ml) to penicillin ($P < 0.001$). To assess the potential evolution of antibiotic resistance in this species, clinical isolates ($n = 24$) were compared with strains isolated in 1968 from a human population in the Solomon Islands that was never exposed to antibiotics. Solomon Island isolates were significantly more susceptible than all clinical strains to penicillin, ampicillin, and vancomycin ($P < 0.001$ for each), but they exhibited no differences in susceptibility to teicoplanin or ciprofloxacin. The penicillin-binding affinity of penicillin-binding protein 5 (PBP 5) in penicillin-resistant clinical strains (MIC, 512 μg/ml) was notably lower than that in strains with more typical susceptibilities, suggesting an alteration in this PBP as a possible mechanism for increased penicillin resistance. Solomon Island strains most susceptible to penicillin demonstrated a prominent PBP 5* and the absence of PBP 5. These changes in the antibiotic resistance of *E. faecium* emphasize the importance of identifying this species in patients with serious enterococcal infections and the necessity of assessing its susceptibility to both beta-lactams and aminoglycosides if effective therapy is to be identified.

Clinical enterococcal isolates that are relatively resistant to beta-lactam antibiotics have been reported increasingly during recent years (1, 9–11). Whether these observations identify a trend toward increasing resistance to beta-lactams among this species or represent a greater awareness among investigators of antibiotic resistance in gram-positive organisms remains unclear. *Enterococcus faecium* and *Enterococcus raffinosus*, which frequently possess intrinsic relative resistance to beta-lactams (3, 7), have been notable in some reports (1, 9, 10). To identify any trend toward increasing antibiotic resistance among clinical isolates of *E. faecium*, we reviewed the susceptibilities of *E. faecium* strains that were isolated at one institution (Massachusetts General Hospital [MGH], Boston) and that were collected without bias to their antibiotic resistance during 1969 to 1990. Furthermore, to examine the potential evolution of antibiotic resistance in this species, we also studied strains isolated in 1968 from a human population in the Solomon Islands that was never exposed to antibiotics (6).

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MATERIALS AND METHODS

Strains and identification. Unduplicated clinical isolates of *E. faecium* obtained at MGH between 1969 and 1990 were collected and described by year of original isolation. Strains collected between 1969 and 1988 had been held in frozen storage and were retrieved. Isolates collected in 1989 and 1990 consisted of all isolates from blood obtained during those years and all consecutive isolates collected prospectively during one 3-month period in each year. *E. faecium* strains collected in the Solomon Islands in 1968 ($n = 24$) were obtained from fecal cultures taken from a human population that had never been exposed to antibiotics (6).

The identification of all strains was confirmed by using the API Rapid Strep Identification System (Analytab Products, Plainview, N.Y.) and in accordance with the criteria recommended by Facklam and Collins (4).

Assessment of antibiotic susceptibility. Antibiotic susceptibility was determined by the standard agar dilution technique of the National Committee for Clinical Laboratory Standards (8) by using Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.), with antibiotic concentrations tested in twofold increments. The following antibiotics were tested: benzylpenicillin (Squibb-Marsam Inc.,

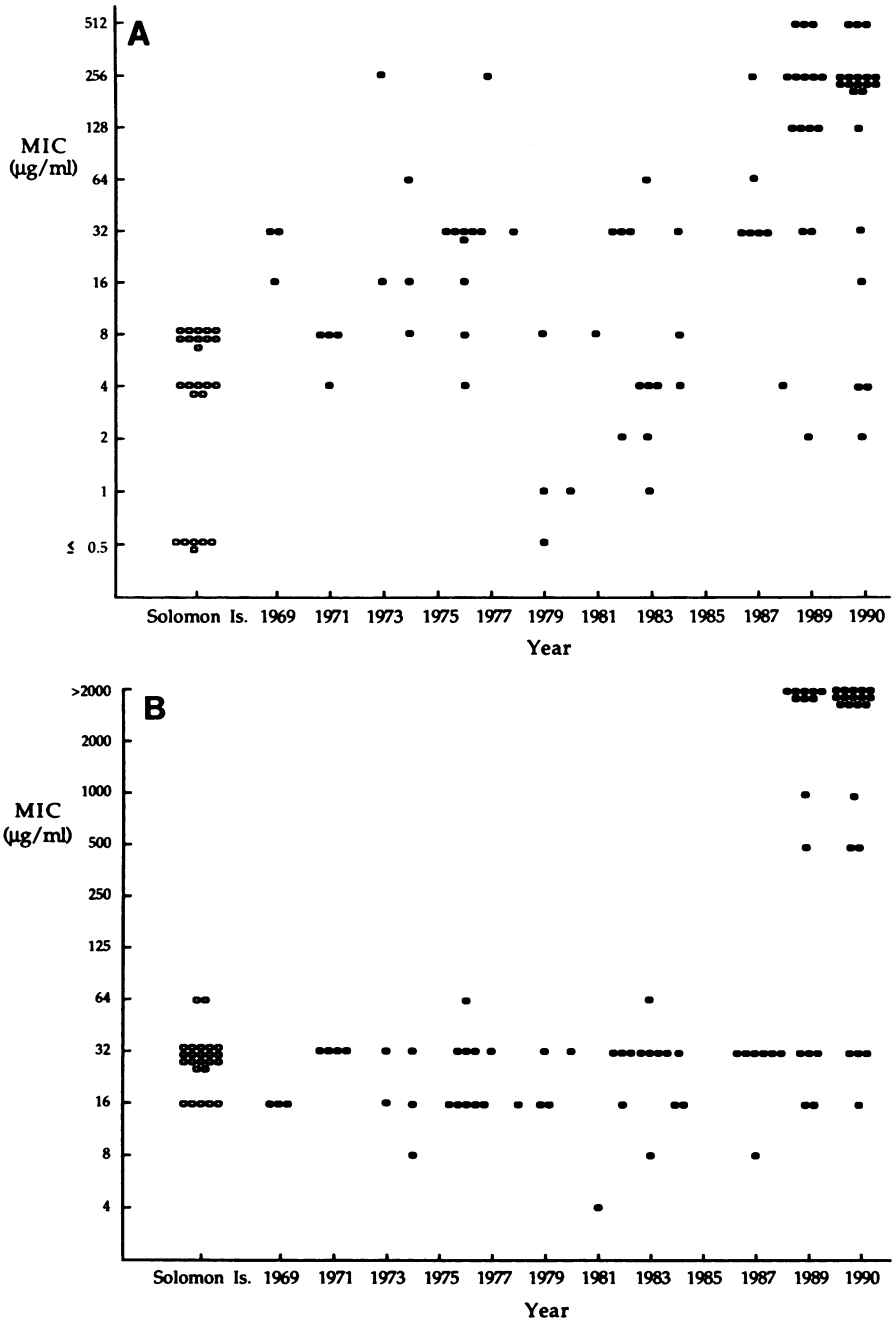

FIG. 1. Susceptibilities of *E. faecium* strains to penicillin (A) and gentamicin (B) by year of isolation. □, Solomon Island isolates; ■, clinical isolates.

Cherry Hill, N.J.), ampicillin (Pfizer, Groton, Conn.), vancomycin (Abbott Laboratories, North Chicago, Ill.), teicoplanin (Merrell Dow Pharmaceuticals Inc., Cincinnati, Ohio), ciprofloxacin (Miles Inc., West Haven, Conn.), kanamycin (LyphoMed Inc., Melrose Park, Ill.), streptomycin (Eli Lilly & Co., Indianapolis, Ind.), and gentamicin (Elkins-Sinn Inc., Cherry Hill, N.J.). Final inocula of 10^4 CFU per spot were applied to agar plates by using a multiprong inoculating device. Plates were inoculated at 35°C and were examined for evidence of growth at 18 h. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as controls. β-Lactamase production was tested by using nitrocefin disks (Cefinase; BBL).

Analysis of PBPs. Labeling of penicillin-binding proteins (PBPs) in whole cells was performed as described previously (14), with a final concentration of either 100 or 500 μg of [^{3}H]benzylpenicillin per ml incubated for 60 min (5, 12). [^{3}H]benzylpenicillin ethylpiperidinium salt (57.83 mCi/mg) was a generous gift from Merck Sharp & Dohme Research Laboratories, Rahway, N.J. PBPs were analyzed by slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography as described previously (14, 15). Separat-

TABLE 1. Antimicrobial susceptibility of *E. faecium*

Antibiotic	MIC_{50}/MIC_{90} (μg/ml)[a]		
	Solomon Island isolates, 1968 (n = 24)	MGH clinical isolates	
		1969–1988 (n = 48)	1989–1990 (n = 36)
Penicillin	4/8[b,c]	16/64[b,d]	256/512[c,d]
Ampicillin	2/2[e,f]	8/32[e,g]	64/128[f,g]
Vancomycin	≤0.5/≤0.5[h,i]	1/2[h]	1/2[i]
Teicoplanin	1/1	1/1	1/1
Ciprofloxacin	4/4	4/32	4/8

[a] MIC_{50}/MIC_{90}, MICs for 50 and 90% of isolates tested, respectively. Values with the same letter are significantly different (P <0.001).

TABLE 2. Prevalence of penicillin resistance and HLR to aminoglycosides in *E. faecium*[a]

Source	% Isolates with:			
	Penicillin resistance[c]	HLR to aminoglycosides[b]		
		Strepto-mycin	Kana-mycin	Genta-micin
Solomon Islands (n = 24)	0	0	0	0
MGH (1968–1988; n = 48)	6[d]	42[e]	29[f]	0[g]
MGH (1989–1990; n = 36)	78[d]	83[e]	89[f]	61[g]

[a] Values with the same letter are significantly different (P <0.001).
[b] MIC, ≥2,000 μg/ml.
[c] MIC, ≥128 μg/ml.

ing gels consisted of 7% acrylamide and 0.12% bisacrylamide. The exposure time for fluorographs was 3 to 7 days at −70°C.

Statistical analysis. Statistical analysis of changes in the susceptibilities of *E. faecium* isolates was done by either the Kruskal-Wallis test or the chi-square test.

RESULTS

Eighty-four strains of *E. faecium* were isolated and collected during the 22-year period from 1969 to 1990 (1969 to 1988, n = 48; 1989, n = 15; 1990, n = 21). All strains were collected without bias to their antibiotic resistance, except that 6 strains isolated in 1987 were known to be resistant to cefoperazone. The original sites of isolation and the number of isolates (1969 to 1988/1989 to 1990) were as follows: blood, 34 (30/4); urine, 25 (5/20); wound, 9 (7/2); biliary, 3 (0/3); abdominal fluid/drain, 6 (0/6); and unknown, 7 (6/1).

The susceptibilities of the isolates to penicillin and the year of their original isolation are given in Fig. 1A. The pattern of susceptibility to ampicillin was almost identical to that for penicillin, except that MICs were generally 1 dilution lower. Resistance to penicillin and ampicillin has increased significantly during recent years (P < 0.001), with the geometric mean MICs of penicillin and ampicillin for strains isolated between 1969 and 1988 being 14 and 5 μg/ml, respectively, versus 123 and 50 μg/ml, respectively, for strains isolated in 1989 and 1990. In comparison, there was no significant change in the susceptibilities of MGH *E. faecium* isolates to vancomycin, teicoplanin, or ciprofloxacin during the study period (Table 1), although an MIC of 32 μg of vancomycin per ml was noted for one strain isolated in 1990. All *E. faecium* isolates failed to produce detectable β-lactamase.

The incidence of high-level resistance (HLR) to aminoglycosides (MIC, ≥2,000 μg/ml) was significantly higher in isolates obtained in 1989 and 1990 than it was in strains obtained earlier (P < 0.001; Table 2). HLR to gentamicin was present in 61% of strains isolated in 1989 and 1990, with no such resistance being noted prior to this period (P < 0.001; Fig. 1B). Furthermore, HLR to gentamicin was significantly associated with resistance to penicillin (MIC, ≥128 μg/ml), with all 22 isolates with HLR to gentamicin being resistant to penicillin (P < 0.001). A correlation between antibiotic susceptibility and the site of original isolation failed to reveal any significant associations. In particular, three of four isolates obtained from blood in 1989 and 1990, while constituting a smaller proportion of strains collected during this period (11%) than during 1969 to 1988 (63%), demonstrated penicillin resistance and HLR to gentamicin; this rate is similar to that for isolates obtained from other sites during this time period.

To assess the possibility that the high rate of penicillin and aminoglycoside resistance among *E. faecium* isolates during recent years may have simply represented a localized nosocomial outbreak, we examined all *E. faecium* isolates obtained from blood at another hospital (New England Deaconess Hospital, Boston, Mass.) during 1990. Similar results were noted, whereby an MIC of ≥128 μg of penicillin per ml was identified for six of seven isolates, and four of seven isolates demonstrated HLR to gentamicin. To be certain that increased resistance to penicillin at that institution was not limited to blood culture isolates, we also examined 17 strains of *E. faecium* recovered from cultures of urine submitted in 1990. MICs of penicillin for all isolates were 256 to 512 μg/ml.

In comparison, *E. faecium* isolates from the Solomon Islands were significantly more susceptible to penicillin (P < 0.001), ampicillin (P < 0.001), and vancomycin (P < 0.001) than were MGH strains obtained either from 1969 to 1988 or during 1989 and 1990, but their susceptibilities to teicoplanin and ciprofloxacin were similar to those of MGH isolates. No HLR to aminoglycosides was noted in these strains.

PBPs from 10 strains were examined to assess any features that could account for differences in penicillin susceptibility. The typical PBP pattern for *E. faecium* was identified in all strains (Fig. 2), including isolates with both PBP 5 (79.5 kDa) and PBP 5* (81 kDa) (e.g., Fig. 2, lanes 3, 5, and 6) and isolates with hyperproduction of PBP 5 without PBP 5* (Fig. 2, lane 4), as described previously by Williamson et

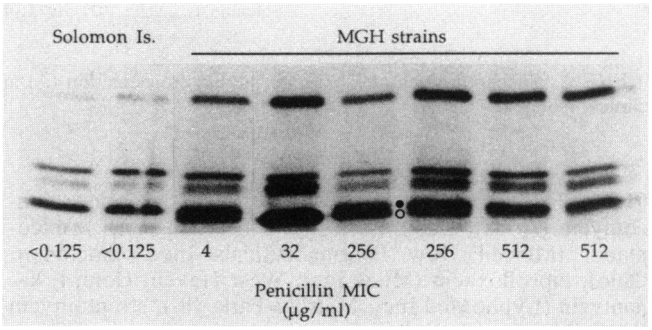

FIG. 2. PBPs of eight *E. faecium* strains incubated with 100 μg of [^{3}H]benzylpenicillin per ml. Solomon Island strains that were hypersusceptible to penicillin (MIC, ≤0.125 μg/ml) demonstrated PBP 5* (●) without PBP 5 (○). Clinical isolates demonstrated the PBP pattern(s) reported previously (13), except that PBP 5 was less clearly visualized in resistant isolates (see Fig. 3).

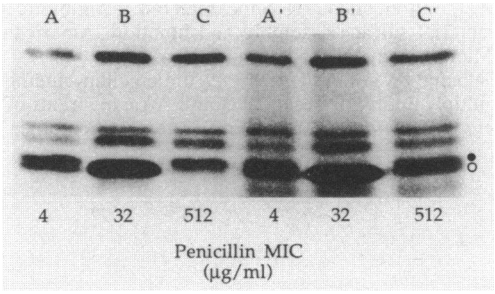

FIG. 3. PBPs of three *E. faecium* strains incubated with 100 μg (lanes A, B, and C) and 500 μg (lanes A′, B′, C′) of [^{3}H]benzylpenicillin per ml. ●, PBP 5*; ○, PBP 5. Unlike the strains in lanes A (MIC, 4 μg/ml) and B (MIC, 32 μg/ml), PBP 5 in strain C (MIC, 512 μg/ml) was better visualised when the strain was incubated with 500 μg of [^{3}H]benzylpenicillin per ml, suggesting low penicillin-binding affinity.

al. (13). However, two additional features were noted. In MGH strains for which penicillin MICs were 512 μg/ml, both PBP 5 and PBP 5* were present, however, PBP 5 demonstrated a very low penicillin-binding affinity, requiring incubation with a higher concentration of [^{3}H]benzylpenicillin (500 μg/ml) to adequately demonstrate this PBP than was required for isolates that were more susceptible to penicillin (Fig. 3). Furthermore, unlike previous descriptions in which PBP 5 was a constant feature of this species (12, 13), Solomon Island strains which were hypersusceptible to penicillin (MIC, ≤0.125 μg/ml) demonstrated PBP 5* alone, without PBP 5 (Fig. 2).

DISCUSSION

Recent reports of resistance to penicillin and ampicillin among clinical enterococcal isolates have relied on previously published data regarding the expected susceptibility pattern of this species. Bush et al. (1) prospectively examined clinical enterococcal isolates for penicillin resistance during a 6-month period in 1988 and noted that 10 of the 30 *E. faecium* strains that they collected were highly resistant (MIC, ≥200 μg/ml); however, no assessment for such resistance in isolates from earlier years was undertaken. Our study of a relatively large *E. faecium* collection is notable since isolates were collected at one institution over a 22-year period, from a variety of sites, without bias to their antibiotic susceptibilities. A significant increase in penicillin and ampicillin resistance to clinically unachievable concentrations was noted among isolates obtained during 1989 and 1990 in comparison with that noted among isolates from earlier years ($P < 0.001$). Furthermore, HLR to gentamicin increased dramatically during this same period and was significantly associated with this resistance to penicillin ($P < 0.001$). Importantly, susceptibility to vancomycin and teicoplanin did not change during the 22-year study period, and remained at clinically achievable levels.

PBP analyses support the proposed role of PBP 5 in the relative resistance of this species to penicillin (5, 13). Among strains with marked penicillin resistance (e.g., MIC, 512 μg/ml), the penicillin-binding affinity of PBP 5 was notably lower than that observed for *E. faecium* strains with more typical susceptibility patterns. Furthermore, the association between PBP 5* and penicillin susceptibility (13) was supported by the prominence of this PBP (and absence of PBP 5) in hypersusceptible isolates from a human population in the Solomon Islands that was never exposed to antibiotics. To our knowledge, this is the first demonstration of this phenomenon in naturally occurring isolates.

MGH isolates were significantly more resistant to penicillin, ampicillin, and vancomycin than Solomon Island isolates were ($P < 0.001$), yet they were no different in their susceptibility to teicoplanin or ciprofloxacin. This pattern of flux in glycopeptide resistance among MGH strains compared with that among Solomon Island strains, namely, increased resistance to vancomycin but stable teicoplanin susceptibility, is similar to that recently identified in enterococcal isolates with acquired low-level vancomycin resistance (2).

Results of this study suggest that there has been a dramatic increase in the incidence and level of beta-lactam and aminoglycoside resistance among *E. faecium* isolates in recent years and that this resistance may be widespread. Furthermore, these findings highlight the importance of beta-lactam and aminoglycoside susceptibility testing in patients with serious enterococcal infections if effective antibiotic therapy is to be identified.

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