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High-Level Penicillin Resistance and Penicillin-Gentamicin Synergy in *Enterococcus faecium*

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Thirty-seven *Enterococcus faecium* strains with different levels of penicillin susceptibility were studied in time-kill experiments with a fixed concentration (5 μg/ml) of gentamicin combined with different penicillin concentrations (6 to 600 μg/ml). Synergy was defined as a relative decrease in counts of greater than 2 $\log_{10}$ CFU per milliliter after 24 h of incubation when the combination of the antibiotics was compared with its most active component alone. The minimal synergistic penicillin concentrations found were 6 μg/ml for 16 of 16 strains for which penicillin MICs were ≤25 μg/ml, 20 to 100 μg/ml for 14 of 17 strains for which penicillin MICs were 50 to 200 μg/ml, and 200 to 500 μg/ml for 4 of 4 strains for which MICs penicillin were >200 μg/ml. Penicillin-gentamicin synergy was observed even in high-level penicillin-resistant *E. faecium* strains at penicillin concentrations close to one-half the penicillin MIC. The possibility of treating infections caused by high-level penicillin-resistant *E. faecium* strains with penicillin-gentamicin combinations in particular cases may depend on the penicillin levels attainable in vivo.

Before 1989, most *Enterococcus faecium* clinical isolates showed relative susceptibility to penicillin (MICs, 16 to 32 μg/ml), and only occasionally did papers refer to isolates for which MICs were higher (32 to >128 μg/ml) (11, 20, 26). From 1989 onwards, the problem of high-level penicillin resistance in *E. faecium* isolates began to be documented (4, 6, 16, 22, 25). In a recent American series, about one-third of the isolates showed high-level penicillin resistance (5). In a comparative retrospective study carried out at Massachusetts General Hospital, respective penicillin MICs for 50 and 90% of isolates increased from 16 and 64 μg/ml in the period from 1968 to 1988 to 256 and 512 μg/ml in the period from 1989 to 1990; for ampicillin, the corresponding increases were from 8 and 32 μg/ml to 64 and 128 μg/ml (15). In the same study, high-level penicillin resistance in *E. faecium* isolates (MIC, ≥128 μg/ml) increased from 6 to 78% from the first to the second period.

Ampicillin resistance correlates very closely with penicillin resistance, ampicillin MICs being generally one-half those of penicillin (15). At present, ampicillin resistance rates in *E. faecium* strains (MIC, ≥16 μg/ml) range from 22 to 59% in the United States (13, 18), United Kingdom (14), Canada (3), and Spain (2). High-level penicillin-resistant *E. faecium* strains show a decreased penicillin-binding affinity of one or more of their penicillin-binding proteins (1, 11, 15, 17, 26, 27). β-Lactamase production has been shown only in a single *E. faecium* strain, and it was probably not responsible for the high-level penicillin resistance (7).

The spread of high-level penicillin (or ampicillin) resistance may represent an important problem for the treatment of serious enterococcal infections, particularly when it is associated with vancomycin resistance (16). In this study, experiments on penicillin-gentamicin synergy were undertaken on high-level penicillin-resistant *E. faecium* strains to investigate the possibility of using such combinations in the therapy of human infections.

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MATERIALS AND METHODS

Strains. Thirty-seven *E. faecium* strains were studied; 28 were from clinical sources (San Millán Hospital, Logroño, Spain), and 9 were isolated from sewage. The strains were first identified by the API 20 Strep identification system (BioMerieux, La Balme Les Grottes, France) and later confirmed on the basis of the criteria recommended by Facklam and Collins (10).

Susceptibility testing. The antibiotics used in this investigation included streptomycin (Sigma Chemical Co., St. Louis, Mo.), gentamicin (Schering-Plough Research, Bloomfield, N.J.), kanamycin (Bristol-Myers Squibb Co., Princeton, N.J.), penicillin and ampicillin (Pfizer, Groton, Conn.), and imipenem and vancomycin (Abbott Laboratories, North Chicago, Ill.). MICs were determined on Mueller-Hinton agar plates (Difco Laboratories, Detroit, Mich.) by the standard agar dilution method recommended by the National Committee for Clinical Laboratory Standards (21). The bacterial inoculum was prepared by making appropriate dilutions of overnight broth cultures of organisms in fresh Mueller-Hinton broth (Difco) and deposited onto antibiotic-containing plates with a Steers replicator device, yielding a final inoculum size of approximately 10^4 CFU per spot. Plates were examined for growth after 18 to 24 h of incubation at 35°C. *Staphylococcus aureus* ATCC 24213 and *Enterococcus faecalis* ATCC 29212 were used as control strains. Each *E. faecium* strain was tested for the production of β-lactamase by use of a heavily inoculated nitrocefin disk (Cefinase; BBL Microbiology Systems, Cockeysville, Md.). Strains for which streptomycin, kanamycin, or gentamicin MICs were ≥2,000 μg/ml were considered to be in the high-level aminoglycoside resistance category.

Time-kill synergy studies. To perform time-kill synergy studies with each *E. faecium* strain, the method described by Sahm and Torres was used (24). In brief, organisms were grown overnight in brain heart infusion (BHI) tubes (Difco) at 35°C, and the turbidity was adjusted to a 0.5 McFarland standard by adding fresh BHI. Cultures were again diluted in BHI to yield a final concentration of 10^7 CFU/ml. Prior to

TABLE 1. Antimicrobial susceptibility of 37 *E. faecium* strains at various penicillin MICs[a]

Antibiotic	Antimicrobial susceptibility of strains for which the penicillin MIC (μg/ml) was as follows:								
	≤25 (*n* = 16)			50–100 (*n* = 5)			≥200 (*n* = 16)		
	Mode	MIC range	% of strains resistant	Mode	MIC range	% of strains resistant	Mode	MIC range	% of strains resistant
Ampicillin	1	1–16	6	16	16–64	100	64	64–256	100
Imipenem	4	1–16	19	32	16–64	100	128	64–256	100
Vancomycin	1	0.5–2	0	1	1	0	1	0.5–>512	12
Streptomycin	16	16–>4,000	25	2,000	64–4,000	60	>4,000	64–>4,000	94
Kanamycin	64	32–>4,000	19	>4,000	64–>4,000	60	>4,000	128–>4,000	94
Gentamicin	8	8–16	0	8	8–16	0	16	8–16	0

[a] Breakpoints for resistance: ampicillin, ≥16 μg/ml; imipenem, ≥16 μg/ml; vancomycin, ≥32 μg/ml; streptomycin, kanamycin, and gentamicin, ≥2,000 μg/ml.

inoculation, each tube of fresh BHI was supplemented with penicillin (final concentrations, 6, 10, 20, 50, 100, 150, 200, 300, 400, 500, and 600 μg/ml, depending on the penicillin MIC for the strain tested), either alone or in combination with gentamicin (final concentration, 5 μg/ml). A positive growth tube without antibiotics was used as a control. Test tubes were incubated at 35°C, and the number of CFU per milliliter was determined after 0, 4, 24, and 48 h of incubation.

The relative decrease in counts (RDC) after 24 h of incubation between the combination of penicillin and gentamicin and its most active component alone was measured and expressed as $\log_{10}$ CFU per milliliter. Synergy was defined as an RDC value of >2. The bactericidal effect was defined as the reduction in bacterial growth expressed in $\log_{10}$ CFU per milliliter with the antibiotic combination after 24 h of incubation compared with the initial inoculum. A bactericidal effect of >2.5 was also required for synergy. With the method used, colony counts as low as 1.2 $\log_{10}$ CFU/ml could be detected. Each experiment was performed at least twice, and the results are expressed as the average. In addition, experiments were carried out to exclude a significant antibiotic carryover effect. In these experiments, a small number of bacteria (final concentration, 1 to 3 $\log_{10}$ CFU/ml) was inoculated into tubes containing every antibiotic concentration tested in synergy studies, either alone or in combination (23). After plating on antibiotic-free medium, viable counts obtained were compared with those of the initial inoculum. The differences were below 5% in all cases.

RESULTS

Correlation of antibiotic susceptibility and penicillin MICs. Table 1 shows the antimicrobial susceptibility of the 37 *E. faecium* strains, depending on the penicillin MICs. As expected, a small number of strains with a basal penicillin susceptibility level (MIC, ≤25 μg/ml) were resistant to ampicillin (6%; MIC, ≥16 μg/ml) and imipenem (19%; MIC, ≥16 μg/ml).

Strains for which penicillin MICs were ≥50 μg/ml were always resistant to ampicillin and imipenem. Nevertheless, for penicillin-resistant strains for which penicillin MICs were relatively low (50 ≤ MIC ≤ 100 μg/ml), ampicillin and imipenem MICs were also lower (16 to 64 μg/ml) than those for the more resistant strains (penicillin MICs, ≥200 μg/ml), for which the ampicillin and imipenem MIC range was 64 to 256 μg/ml. β-Lactamase detection was consistently negative in all penicillin-resistant strains.

High-level streptomycin and kanamycin resistance (MIC, ≥2,000 μg/ml) was relatively infrequent among penicillin-susceptible strains (25 and 19%, respectively), but this frequency increased among resistant strains for which penicillin MICs were 50 to 100 μg/ml (60% for both aminoglycosides). Most resistant strains for which penicillin MICs were ≥200 μg/ml were resistant to both streptomycin and kanamycin (94%). No strains exhibiting high-level gentamicin resistance were found in our series (gentamicin MIC, <32 μg/ml). Two strains with high-level vancomycin resistance (MIC, >512 μg/ml) were also highly resistant to penicillin (MIC, 400 μg/ml).

Correlation of penicillin MICs and synergistic effect of penicillin and gentamicin. Time-kill synergy studies were performed on all 37 *E. faecium* strains to determine the minimal penicillin concentration required to obtain a synergistic effect (MSPC) with a fixed gentamicin concentration (5 μg/ml). The MSPCs obtained were closely proportional to the corresponding penicillin MICs (Table 2).

Sixteen *E. faecium* strains for which penicillin MICs were ≤25 μg/ml were susceptible to penicillin-gentamicin synergy at an in vivo attainable concentration of penicillin (6 μg/ml), with an RDC value of 3.2 ± 0.6 (mean ± standard deviation). The bactericidal effect on these strains was 3.7 ± 0.7 (mean ± standard deviation).

For five strains for which penicillin MICs were 50 or 100 μg/ml, MSPCs were 20 to 100 μg/ml. Three of these strains, for which the penicillin MIC was 50 μg/ml, were susceptible to synergy at a minimal penicillin concentration of 20 μg/ml (RDC value, 2.9 ± 0.2; bactericidal effect, 3.1 ± 0.6). For the

TABLE 2. Correlation between penicillin MICs and MSPCs with gentamicin at 5 μg/ml for 37 *E. faecium* strains[a]

Penicillin MIC (μg/ml)	No. of strains	MSPC (μg/ml)	RDC[b] (mean ± SD)	Bactericidal effect[b] (mean ± SD)
≤25	16	6	3.2 ± 0.6	3.7 ± 0.7
50	3	20	2.9 ± 0.2	3.1 ± 0.6
100	1	50	2.1	4.2
	1	100	2.6	3.7
200[c]	9	100	3.1 ± 0.4	3.2 ± 0.5
	1	150	3.5	4.7
400	3	200	3.4 ± 1.0	3.5 ± 0.7
800	1	500	2.8	2.6

[a] None of the strains showed high-level gentamicin resistance.
[b] Evaluated at the MSPC.
[c] Two additional strains for which the penicillin MIC was as shown were not susceptible to synergy, even when a 600-μg/ml penicillin concentration was used.

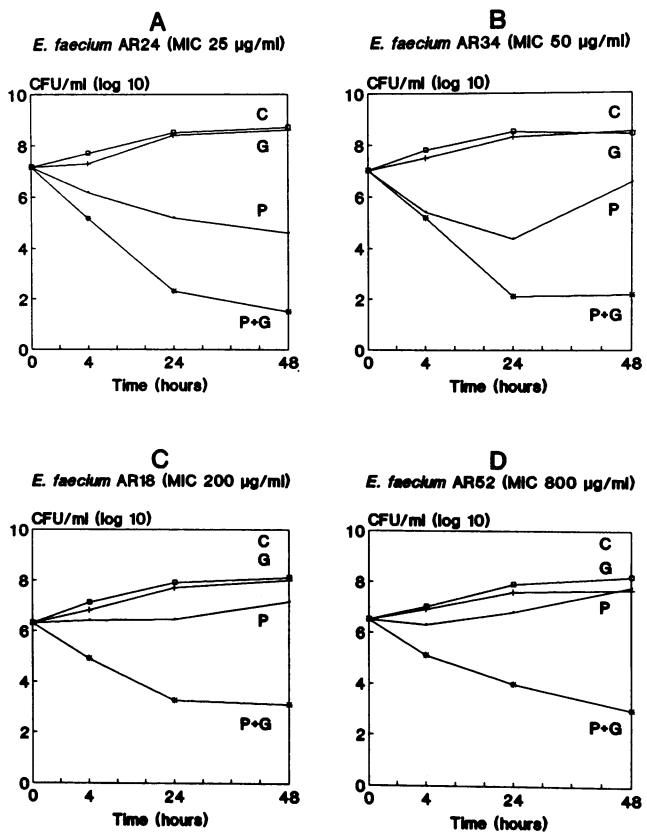

FIG. 1. Time-kill curves obtained with penicillin (P), gentamicin (G), and the combination (P+G) and without antibiotics (C) for four *E. faecium* strains with different levels of penicillin resistance. The bactericidal activity of a fixed gentamicin concentration (5 μg/ml) alone or in combination with the MSPCs (6, 20, 100, and 500 μg/ml in A, B, C, and D, respectively) is represented.

other two *E. faecium* strains, for which the penicillin MIC was 100 μg/ml, the MSPCs were 50 and 100 μg/ml.

For 12 strains the penicillin MIC was 200 μg/ml. For nine of them, synergy was found at a minimal penicillin concentration of 100 μg/ml, with an RDC value of 3.1 ± 0.4 and a bactericidal effect of 3.2 ± 0.5. For one *E. faecium* strain, the MSPC was 150 μg/ml (RDC value, 3.5; bactericidal effect, 4.7). The two remaining strains for which the penicillin MIC was 200 μg/ml were not susceptible to penicillin-gentamicin synergy, even with 600 μg of penicillin per ml. All three strains for which the penicillin MIC was 400 μg/ml were susceptible to synergy at a minimal penicillin concentration of 200 μg/ml, with an RDC value of 3.4 ± 1.0 and a bactericidal effect of 3.5 ± 0.7. *E. faecium* AR52, with the highest penicillin resistance (MIC, 800 μg/ml), was susceptible to penicillin-gentamicin synergy (RDC value, 2.8; bactericidal effect, 2.6) only when the penicillin concentration reached 500 μg/ml.

All penicillin-resistant *E. faecium* strains for which penicillin-gentamicin synergy was obtained at noninhibitory penicillin concentrations were also susceptible to synergy at inhibitory concentrations.

Typical time-kill curves obtained for four *E. faecium* strains with different levels of penicillin resistance are shown in Fig. 1. In this figure, the represented penicillin concentrations are those corresponding to the minimal concentrations required in all cases to obtain synergy with gentamicin. *E. faecium* AR24 (penicillin MIC, 25 μg/ml) had an RDC value of 2.9 after 24 h of incubation with the combination of penicillin (6 μg/ml)-gentamicin in comparison with penicillin alone. The bactericidal effect was 4.8. For *E. faecium* AR34 (penicillin MIC, 50 μg/ml), the penicillin (20 μg/ml)-gentamicin RDC value was 2.2, and the bactericidal effect was 4.9. For *E. faecium* AR18 (penicillin MIC, 200 μg/ml), the penicillin (100 μg/ml)-gentamicin RDC value was 3.2, and the bactericidal effect was 3.0. Finally, for *E. faecium* AR52 (penicillin MIC, 800 μg/ml), the penicillin (500 μg/ml)-gentamicin RDC value was 2.8, and the bactericidal effect was 2.6. After 48 h, the decreases in CFU per milliliter were even higher for the penicillin-gentamicin combinations than for the most active antibiotics or the initial inoculum.

DISCUSSION

In this study, a close correlation between the MICs of penicillin and ampicillin or imipenem was found (Table 1). These results were expected, considering the mechanism of resistance presumptively involved in such strains (penicillin-binding protein modifications) (11, 15, 17, 26, 27), and are in agreement with previously published data (13, 20). In our study, as in most of these studies, ampicillin MICs were generally one dilution below the corresponding penicillin MICs. Imipenem MICs were also increased with penicillin resistance. In fact, *E. faecium* is more resistant to imipenem than *E. faecalis* (25), and the local appearance of high-level penicillin resistance in this species has been associated with imipenem overconsumption (4).

High-level streptomycin and kanamycin resistance (MIC, ≥2,000 μg/ml) was much more frequently observed among *E. faecium* strains with high-level penicillin resistance (14 of 16) than among penicillin-susceptible strains (4 and 3 of 16, respectively). This correlation has also been documented by other authors (4), and the reason remains obscure. High-level kanamycin and streptomycin resistance in *E. faecium* isolates is generally due to the presence of plasmid-mediated aminoglycoside-modifying enzymes produced in addition to a poorly expressed chromosomally-mediated AAC(6′) enzyme. The study of the ability of enterococci with altered penicillin-binding proteins to accept plasmids could provide an answer to this question. Another possible mechanism of resistance to streptomycin that should be considered involves mutations affecting the ribosome binding sites of the drug, as has been shown for *E. faecalis* (9).

The distribution of *E. faecium* strains according to penicillin MICs shows a bimodal shape (Table 2). The first distribution peak (16 strains) represents the more susceptible strains, for which MICs were ≤25 μg/ml. All these strains were susceptible to penicillin-gentamicin synergy at low penicillin concentrations (6 μg/ml). The second peak (12 strains) represents strains for which the penicillin MIC was 200 μg/ml. For most of them (nine strains), synergy was found, with an MSPC of 100 μg/ml. Between both peaks, strains for which penicillin MICs were 50 to 100 μg/ml were subject to synergy at 20 to 100 μg of penicillin per ml. Even the three strains for which the penicillin MIC was 400 μg/ml were susceptible to synergy at 200 μg of penicillin per ml. That result occurred despite the lack of bactericidal activity of penicillin alone at high concentrations on these highly resistant enterococcal strains in comparison with the more susceptible ones (Fig. 1).

Therefore, most of penicillin-resistant *E. faecium* strains studied, even those for which penicillin MICs were very

high, were susceptible to a synergistic effect when gentamicin (5 μg/ml) was associated with a penicillin concentration below the penicillin MIC (generally one-half). Preliminary experiments suggested that subinhibitory concentrations of penicillin could affect aminoglycoside uptake (data not shown). This observation was previously described with other cell wall-active agents (12). Two strains for which the penicillin MIC was 200 μg/ml were resistant to synergy, even at a penicillin concentration of 600 μg/ml. Alternate mechanisms of resistance to synergy could be implicated in these strains.

According to these results, the clinical categorization of *E. faecium* strains as high-level penicillin resistant may depend on the possibility of achieving relatively high penicillin concentrations in vivo. It has been shown that a 1-h intravenous infusion of 5,000,000 U of penicillin G can achieve a peak level in serum of 135 μg/ml (19). Synergy with other penicillins able to reach very high levels in serum with intravenous infusion, such as amoxicillin and piperacillin, can also be expected. Bush et al. (5) did not obtain synergy of penicillin and gentamicin in vivo (experimental endocarditis in rats) for an *E. faecium* strain for which the penicillin MIC was 200 μg/ml, despite a peak penicillin level of 120 μg/ml. This result can be explained by the high MSPC found for this strain (>200 μg/ml). Moreover, high penicillin levels are usually reached for a short time, and the extent of bactericidal activity may depend on the length of bacterial exposure to penicillin (8). Nevertheless, for strains for which the MSPC was 50 or 100 μg/ml, there may be a chance for high-dose penicillin treatment in some situations (for instance, in the case of seriously ill patients infected with vancomycin-resistant strains).

A more accurate classification of *E. faecium* isolates according to penicillin or ampicillin MICs could have clinical implications. *E. faecium* strains for which penicillin MICs are >200 μg/ml should undoubtedly be considered resistant to synergy with aminoglycosides, as the MSPC exceeds 150 μg/ml. Strains for which penicillin MICs are in the range of 50 to 200 μg/ml (in most cases, with MSPCs of between 20 and 100 μg/ml) could eventually be considered for treatment with high penicillin doses in patients without better therapeutic alternatives. Strains for which penicillin MICs are <25 μg/ml (or <32 μg/ml) should be considered susceptible to synergy with aminoglycosides (MSPC, 6 μg/ml). Experimental animal models with continuous-infusion penicillin therapy or penicillin therapy in association with probenecid-like agents retarding elimination, as well as observations on selected individual patients, would cast some light on the clinical relevance of these findings.

In any case, and considering the increasing frequency of isolation of *E. faecium* strains with penicillin-binding protein changes, it could be advisable to detect not only high-level aminoglycoside resistance but also high-level penicillin resistance in *E. faecium* clinical isolates.

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