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Synergistic Killing of Vancomycin-Resistant Enterococci of Classes A, B, and C by Combinations of Vancomycin, Penicillin, and Gentamicin

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Using both high and low inocula for time-kill curves, we examined the antibiotic killing of clinical isolates of glycopeptide-resistant enterococci (*Enterococcus faecium*, *E. faecalis*, and *E. gallinarum*) belonging to phenotypic resistance classes A, B, and C. None were resistant to high levels (>500 mg/liter) of gentamicin. Vancomycin-penicillin-gentamicin resulted in 2 or more logs of killing above that of the most effective two-antibiotic combination for all strains except two of three *E. gallinarum* (VanC) strains and a constitutive mutant of a VanB strain. This strategy may be useful clinically.

Enterococci resistant to various glycopeptide antibiotics have been described recently (4). These include *Enterococcus faecalis*, *E. faecium*, *E. avium*, and *E. gallinarum* (4, 12). The resistant strains are readily divided into three classes, VanA, VanB, and VanC, on the basis of the level of resistance to vancomycin and teicoplanin and whether the resistance is inducible (9, 13). VanA strains have inducible, high-level resistance to all the glycopeptides we have tested (11), while VanB strains show inducible, moderate levels of resistance to vancomycin but remain susceptible to teicoplanin and certain other glycopeptides (14). VanC strains show constitutive resistance to vancomycin only (13). For the VanA and VanB classes, synergistic inhibition of growth by vancomycin and penicillin has been demonstrated (5, 14). This synergy is thought to be due to an interaction between the carboxypeptidase that is induced by vancomycin and the normal penicillin-binding proteins of the cell, which may also have carboxypeptidase activity (1). Clinically, these glycopeptide-resistant strains may pose a problem in that *E. faecium* is frequently resistant to penicillin as well and is more resistant than *E. faecalis* to antimicrobial synergism (6). The vancomycin resistance observed for *E. gallinarum* may be a property of the species (8, 12). We sought to examine whether one could take advantage of the synergistic inhibition of growth by penicillin-vancomycin combinations to increase killing by combining these agents with gentamicin. We chose to use very low concentrations of antibiotics to mimic conditions which might be found in vegetations occurring in patients with endocarditis. We examined both high and low inoculum levels for the same reason.

The bacterial strains used (Table 1) included *E. faecium* D359 (penicillin-resistant, vancomycin-susceptible control) (15), *E. faecium* D399 (VanA; highly resistant to vancomycin and resistant to penicillin) (10), *E. faecium* D366 (VanB; moderately resistant to vancomycin, susceptible to teicoplanin, and resistant to penicillin) (14), and a constitutive vancomycin- and teicoplanin-resistant mutant of D366 (T4) (this work); *E. faecalis* JH2-2 (penicillin- and vancomycin-susceptible control) (3) and A256 (VanA; highly resistant to vancomycin) (11); and three strains of *E. gallinarum* (UCLA I, UCLA II, and SC I), all included within the VanC phenotype (13). Potassium penicillin G and gentamicin were obtained from Sigma Chemical Co. (St. Louis, Mo.), vancomycin was obtained from Elli Lilly & Co. (Indianapolis, Ind.), and teicoplanin was obtained from Merrell Dow Inc. (Cincinnati, Ohio). MICs were determined with serial two-fold dilutions of antibiotics through brain heart infusion (BHI) agar. A spot containing about 10^4 cells of each strain was used for each antibiotic concentration. The strains, their glycopeptide antibiotic resistance classes, and MICs of various antibiotics against them are shown in Table 1. For the time-kill curves, cells were grown overnight at 37°C without shaking in BHI broth. They were diluted 1:100 in fresh BHI in the morning, incubated for 1 h at 37°C, and rediluted 1:100 in BHI supplemented with the appropriate antibiotic or antibiotic combination, resulting in an initial concentration of about 10^5. For the high-inoculum experiments, the second 1:100 dilution was omitted. Gentamicin was used at 1 μg/ml, vancomycin was used at 8 μg/ml, and penicillin was used at 2 μg/ml to approximate reasonable, clinically achievable levels. All antibiotic combinations resulting in 2 or more logs of killing at 24 h against the low inoculum were retested against the high inoculum. Selected strains were tested at the high inoculum with higher penicillin concentrations. To induce vancomycin resistance, we grew and diluted cells in BHI supplemented with 8 μg of vancomycin per ml. Samples for viable counts were obtained at 0, 6, and 24 h. Samples (100 μl) were either plated directly on BHI agar without antibiotic or diluted in water and plated. Colonies were counted after incubation at 37°C overnight. Thus, the lower limit of detectable CFU per milliliter was 10 ($\log_{10} = 1$). The BHI agar plates contained 20 ml of agar, resulting in a 200-fold dilution of antibiotic, even from the undiluted samples. Given the low antibiotic concentrations that we used, we thought that antibiotic carryover was not likely to have been a problem. To rule out carryover, we performed a disk test with diluted broth containing antibiotic. The broth was diluted in the same manner as for the kill curves and compensating for the

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TABLE 1. MICs of various antibiotics for vancomycin-resistant enterococci

Strain[a]	Species	Phenotypic class[b]	MIC (μg/ml)		
			Penicillin	Vancomycin	Gentamicin
D359	*E. faecium*	S	32	1	16
JH2-2	*E. faecalis*	S	1	1	16
D399	*E. faecium*	A	64	>1,000	16
D366	*E. faecium*	B	16	32	16
D366 IND	*E. faecium*	B	0.50	32	16
D366 T4	*E. faecium*	B	0.50	64	16
A256	*E. faecalis*	A	0.50	256	16
A256 IND	*E. faecalis*	A	0.50	256	16
UCLA I	*E. gallinarum*	C	4	16	16
UCLA II	*E. gallinarum*	C	1	16	16
SC I	*E. gallinarum*	C	1	16	16

[a] IND, Induced with 8 μg of vancomycin per ml.
[b] S, Susceptible to vancomycin.

smaller amounts spotted on the disks. Thirty microliters was spotted on a sterile disk. A susceptible strain, JH2-2, was planted in a lawn by the Kirby-Bauer method, the disk was placed on the lawn, and zone sizes were measured after overnight incubation at 37°C. No carryover was detected by this method.

Time-kill curves for several strains with penicillin, vancomycin, and gentamicin in various combinations are shown in Fig. 1, and the results for all strains are shown in Table 2. For our purposes, bactericidal synergy was defined as the killing of at least 2 $\log_{10}$ CFU above that of the most effective comparative agent or most effective comparative combination of agents (7). In Fig. 1, the log of 0 CFU per milliliter was <1. As noted in Table 1, no strains were resistant to high levels of gentamicin. The penicillin-resistant, vancomycin-susceptible control strain of *E. faecium* and the susceptible control strain of *E. faecalis* were only killed by the vancomycin-gentamicin combination, and penicillin added nothing to that level of killing. When we retested *E. faecalis* JH2-2 at the high inoculum with 10 μg of penicillin per ml, most of the killing was caused by penicillin alone (3 logs), and gentamicin added little to that level of killing (Table 2). When we retested *E. faecalis* A256 with 4 instead of 2 μg of penicillin plus 1 μg of gentamicin per ml, the regrowth seen after 24 h with the lower concentration of penicillin (Table 2 and Fig. 1) was abolished (data not shown). Thus, at the low inoculum, all vancomycin-resistant *E. faecium* and *E. faecalis* strains (including uninduced VanA and VanB strains) and one of three *E. gallinarum* strains (VanC) were resistant to penicillin-gentamicin synergy (Table 2). For the VanA and VanB strains, no difference between the high and low inocula was noted (data not shown). The two *E. gallinarum* strains killed by penicillin-gentamicin at the low inoculum were not killed at the high inoculum (data not shown), and the addition of vancomycin had no discernible effect on either inoculum. On the other hand, among the vancomycin-resistant enterococci, *E. faecalis* A256, two *E. faecium* strains, and one *E. gallinarum* strain resistant to penicillin-gentamicin synergistic killing were killed by vancomycin-penicillin-gentamicin (Fig. 1 and Table 2), even at the high inoculum (data not shown). We repeated the kill curve determinations for D366 and SC I with 10 instead of 2 μg of penicillin per ml and the high inoculum. No change in the results was noted (data not shown). For A256, our claim of an increased level of killing by the triple combination was based on the regrowth seen in the presence of penicillin-

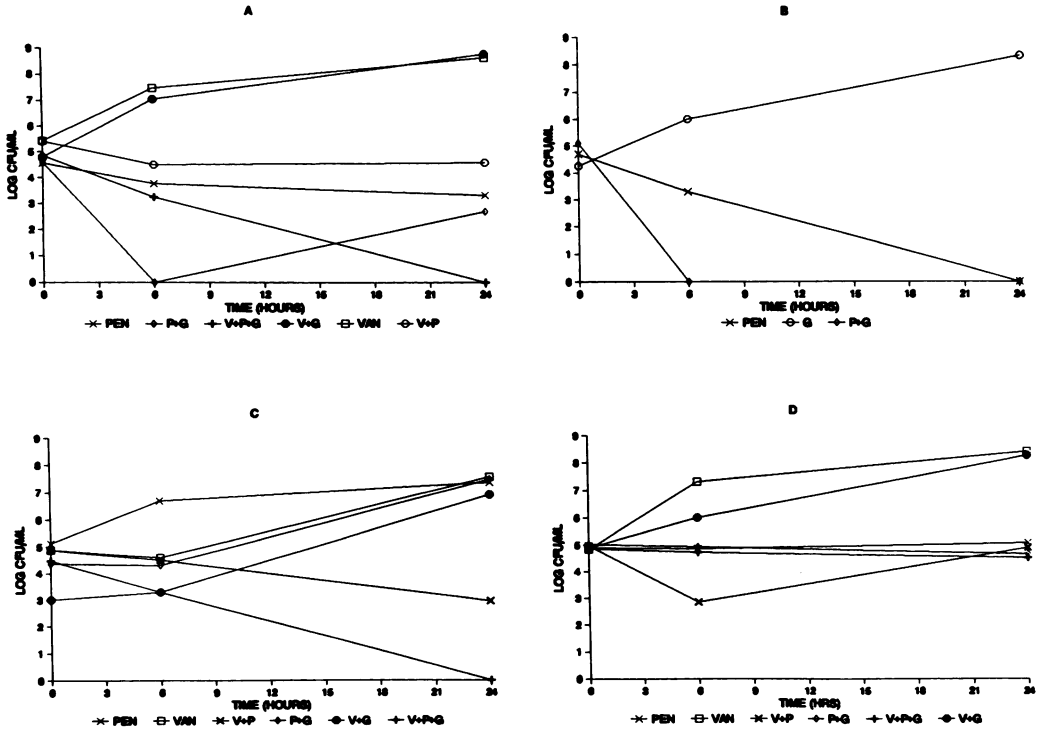

FIG. 1. Time-kill curves for vancomycin-resistant enterococci. (A) *E. faecalis* A256 (VanA). (B) A256, induced. (C) *E. faecium* D366 (VanB). (D) D366 T4. PEN and P, Penicillin; VAN and V, vancomycin; G, gentamicin.

TABLE 2. Killing of enterococci by vancomycin, penicillin, and gentamicin in combination

Organism	Time (h)	Change in $\log_{10}$ CFU with[a]:						
		Penicillin (2 μg/ml)	Vancomycin (8 μg/ml)	Gentamicin	Vancomycin-penicillin	Penicillin-gentamicin	Vancomycin-gentamicin	Vancomycin-penicillin-gentamicin
E. facecalis JH2-2								
Low inoculum (penicillin at 2 μg/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	6.00	−0.05	0.02		−0.27	−0.08	−4.19	−2.14
	24.00	1.69	2.16		−0.39	−0.44	−2.29	−2.54
High inoculum (penicillin at 10 μg/ml)	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	6.00	−1.13	0.02		−0.40	−2.02	−4.19	1.77
	24.00	−2.86	2.16		−0.52	−4.09	−2.29	−2.42
E. faecium D359	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	6.00	0.76	−0.08		−0.13	0.39	−2.27	−2.16
	24.00	3.31	−0.26		−0.68	3.29	−4.27	−2.38
E. faecium D399 (VanA)	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	6.00	1.73	0.73		−0.60	2.38	1.79	−4.60
	24.00	2.62	2.51		−1.94	3.20	4.12	−4.60
E. faecalis A256 (VanA)	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	6.00	−0.78	2.05		−0.90	−4.59	2.26	−1.60
	24.00	−1.24	3.23		−0.81	−1.90	4.02	−4.86
E. faecalis A256 (VanA; induced [8 μg of vancomycin per ml always present])	0.00	0.00		0.00		0.00		
	6.00	−1.40		1.74		−5.10		
	24.00	−4.70		4.04		−5.10		
E. faecium D366 (VanB)	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	6.00	1.59	−0.28		−0.36	−0.06	0.30	−1.18
	24.00	2.25	2.68		−1.92	3.11	3.93	−4.48
E. faecium D366 (VanB; induced [8 μg of vancomycin per ml always present])	0.00	0.00		0.00		0.00		
	6.00	−0.48		1.92		−0.34		
	24.00	−2.78		3.58		−4.70		
E. faecium D366 T4 (VanB)								
Low inoculum (penicillin at 2 μg/ml)	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	6.00	−0.03	2.46		−2.12	−0.10	1.10	−0.13
	24.00	0.18	3.55		−0.08	−0.37	3.34	−0.35
High inoculum (penicillin at 10 μg/ml)	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	6.00	−1.15	2.46		−0.55	−0.27	1.10	−0.08
	24.00	−1.15	3.55		−1.26	−2.17	3.34	−2.38
E. gallinarum UCLA I (VanC)	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	6.00	0.42	0.06		−0.43	−0.55	−0.08	−0.73
	24.00	2.45	3.15		−1.38	−0.50	3.26	−0.70
E. gallinarum UCLA II (VanC)	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	6.00	−0.45	−0.93		−0.54	−1.75	−0.41	−3.29
	24.00	−1.54	−1.47		−1.08	−4.75	−0.57	−4.79
E. gallinarum SC I (VanC)	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	6.00	−0.34	0.35		−0.70	−1.71	0.49	−0.83
	24.00	−1.40	1.77		−1.05	−2.76	2.20	−1.18

[a] Experiments for the low inoculum only are shown unless otherwise indicated.

gentamicin at 24 h of incubation. This finding was a reproducible one, and our limits of detection, especially for the high-inoculum experiments, allowed us to show an increased level of killing by the triple combination equal to or greater than 2 $\log_{10}$ CFU above that by penicillin-gentamicin. However, as noted earlier, this regrowth was inhibited by increasing concentrations of penicillin, diminishing the increase in killing by the triple combination as compared with penicillin-gentamicin as well.

Vancomycin-penicillin resulted in about 2 logs of killing of our VanA and VanB strains of *E. faecium*. Again, the triple combination was reproducibly more bactericidal than was vancomycin-penicillin by about 2 logs at 24 h. Vancomycin-gentamicin was, as expected, not bactericidal for any vancomycin-resistant strain tested.

To explore further the role of antibiotic combinations in killing, we examined the killing of induced VanA and VanB strains and of a constitutive VanB mutant. When the VanB

protein was expressed in VanB strains, the MIC of penicillin decreased from 16 to 0.125–0.5 μg/ml. We used 2, 10, and 25 μg of penicillin per ml in our experiments (Fig. 1 and Table 2). The results suggested that, at least under our conditions, penicillin can be bactericidal for some induced vancomycin-resistant enterococci. On the other hand, no antibiotic, alone or in combination, was bactericidal against our constitutively resistant VanB mutant. Even with increasing concentrations of penicillin (the results obtained with 25 μg of penicillin per ml were similar to those obtained with 10 μg/ml and are not shown), penicillin alone accounted for 1 of the 2 logs of killing seen with penicillin-gentamicin, and the addition of vancomycin to this combination added nothing to that level of killing. These data suggested that there is more involved here than just the presence of the VanB protein.

E. faecalis strains, on the other hand, were frequently not killed, even by very high concentrations of penicillin. For our VanA strain (A256), the penicillin-vancomycin combination did not result in much killing. However, like our VanB strain (D366), when induced with vancomycin A256 was killed by 2 μg of penicillin per ml and by penicillin-gentamicin, even at a high inoculum.

Under our conditions of low concentrations of penicillin and gentamicin, the triple combination of vancomycin, penicillin, and gentamicin appeared to be more bactericidal for all of the VanA and VanB strains tested than was either vancomycin-penicillin or penicillin-gentamicin. In one outbreak, 13 of 15 vancomycin-resistant enterococcal isolates were resistant to high levels of gentamicin, precluding gentamicin synergy (2). For vancomycin-resistant *E. faecium* strains that are also resistant to gentamicin synergy, it is possible that a combination of vancomycin and penicillin may yield a clinically useful bactericidal effect. This possibility should be confirmed with such strains.

We cannot yet explain why our constitutive VanB mutant was not killed by any antibiotic or combination. Since such constitutive mutants are easy to select by growth of the VanB strain in the presence of teicoplanin in the laboratory, there is a danger that they may arise clinically during treatment with teicoplanin.

On the basis of our in vitro tests, we cannot recommend a regimen for the treatment of *E. gallinarum* infections, but it appears likely that standard synergy testing with higher concentrations of gentamicin and penicillin will yield positive results. In that case, strains highly resistant to gentamicin will not be amenable to synergistic bactericidal therapy.

Our results differ from those recently reported by Leclercq et al. (5), who found that, with VanA strains of *E. faecium* and *E. faecalis*, vancomycin-penicillin combinations were not more bactericidal than penicillin alone and that the addition of gentamicin added little killing. The differences between their results and ours may be explained by differences in antibiotic concentrations or by differences in media. Leclercq et al. (5) did not study vancomycin-resistant enterococci with lower levels of resistance (VanB and VanC classes).

We believe that our results may suggest clinically useful strategies for the treatment of infections caused by vancomycin-resistant enterococci belonging to phenotypic classes A and B, and we recommend that animal models be used to investigate these strategies further.

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