

In Vitro Susceptibility Studies of Vancomycin-Resistant *Enterococcus faecalis*

DANIEL F. SAHM,^{1,2*} JESSICA KISSINGER,¹ MICHAEL S. GILMORE,³ PATRICK R. MURRAY,^{4,5}
ROSS MULDER,⁶ JOANNE SOLLIDAY,⁶ AND BARBARA CLARKE⁶

Clinical Microbiology Laboratories¹ and Department of Pathology,² The University of Chicago, Chicago, Illinois 60637-1463; Department of Microbiology and Immunology, Oklahoma University Health Sciences Center, Oklahoma City, Oklahoma 73190³; Vitek Systems Inc., Hazelwood, Missouri 63042-2395⁶; and Clinical Microbiology Laboratory, Barnes Hospital,⁴ and Washington University School of Medicine,⁵ St. Louis, Missouri 63110

Received 30 January 1989/Accepted 13 June 1989

Vancomycin resistance exhibited by *Enterococcus faecalis* isolates V583, V586, and V587 is described. The vancomycin MICs ranged from 32 to 64 µg/ml. Although resistant to vancomycin, the isolates were susceptible to teicoplanin (MIC, ≤0.5 µg/ml). Such a glycopeptide susceptibility profile has not been previously described for *E. faecalis*. Time kill studies showed that vancomycin resistance adversely affected the synergistic activity that vancomycin and aminoglycoside combinations usually demonstrate against enterococci. However, the ability to detect vancomycin resistance varied with the susceptibility testing method used. Whereas broth microdilution, broth macrodilution, and agar dilution methods detected resistance, disk-agar diffusion and the AutoMicrobic system Gram-Positive GPS-A susceptibility card (Vitek Systems Inc., Hazelwood, Mo.) did not. To detect vancomycin resistance reliably and establish the incidence of such *E. faecalis* isolates, adjustments in some susceptibility testing methods may be necessary.

Vancomycin combined with an aminoglycoside provides effective alternative therapy for serious enterococcal infections that occur in patients who cannot tolerate the penicillin-class antibiotics usually used in combination with aminoglycosides (8, 25, 26). Although vancomycin resistance has rarely been described (19, 22), recent reports from Europe (10, 11, 20, 23; F. W. Goldstein, A. Y. Buu-Hoi, R. Williamson, and J. F. Acar, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1022, 1987) indicate that such resistance among enterococci is an emerging problem.

While determining the antibiotic susceptibility profiles of several *Enterococcus faecalis* isolates, we encountered three isolates that, by broth microdilution, exhibited vancomycin resistance. We established the drug susceptibility profiles of these isolates and studied the abilities of various in vitro susceptibility testing methods to detect this vancomycin resistance. We also investigated the effect of vancomycin resistance on the synergistic activity usually achieved by vancomycin-aminoglycoside combinations against *E. faecalis* (8, 25, 26). To our knowledge, this report is the first detailed description of vancomycin-resistant, teicoplanin-susceptible *E. faecalis* strains encountered in the United States.

MATERIALS AND METHODS

Organisms. The vancomycin resistance of three *E. faecalis* isolates, designated V583, V586, and V587, was discovered while antimicrobial susceptibility profiles for 170 different *E. faecalis* isolates were being determined. Retrospective investigation of the sources of the isolates revealed that all three originated from Barnes Hospital, St. Louis, Mo. Two isolates (V583 and V586) were recovered from blood cultures collected from the index patient on 12 and 23 February 1987. Additional cultures with these organisms included blood collected on 4 March 1987 and multiple urine

and stool cultures collected from 1 March to 1 May. This patient had received vancomycin for 12 days prior to the first positive culture. The isolate from the second patient (V587) was recovered in a urine specimen collected on 26 February 1987 and two blood cultures collected on 9 March 1987. This patient was in the same intensive care unit as the first patient but had not received vancomycin before the first positive culture.

Identification of each isolate was confirmed by colony morphology, Gram stain, and recommended conventional biochemical characteristics, including hydrolysis of esculin in the presence of bile, growth in the presence of 6.5% NaCl, fermentation of sorbitol but not arabinose or raffinose, pyrrolidonylarylamidase (Remel, Lenexa, Kans.) activity, and reactivity with Burroughs Wellcome group D streptococcal antisera (3-5). Additionally, motility tests performed at 30°C were done to further differentiate the isolates from *Enterococcus gallinarum*, a motile enterococcus previously described as being vancomycin resistant (9). Along with these conventional methods, AutoMicrobic system Gram-Positive (AMS; Vitek Systems Inc., Hazelwood, Mo.) identification cards were used, according to the manufacturer's directions and specifications, to confirm the identification of the isolates.

Other organisms, used as controls for the susceptibility tests, included *E. faecalis* ATCC 29212 (14) and *E. faecalis* UC73, a clinical isolate from the University of Chicago Clinical Microbiology Laboratories that is known to exhibit high-level resistance (MIC, >2,000 µg/ml) to gentamicin and streptomycin.

Antimicrobial agents. The drugs used for this study were supplied as follows: vancomycin and daptomycin (LY146032), Eli Lilly & Co., Indianapolis, Ind.; teicoplanin, Merrell Dow Pharmaceuticals Inc., Cincinnati, Ohio; streptomycin, Sigma Chemical Co., St. Louis, Mo.; and gentamicin, Schering Corp., Bloomfield, N.J.

In vitro susceptibility tests. Methods used to investigate antimicrobial susceptibility included broth microdilution

* Corresponding author.

TABLE 1. Vancomycin susceptibility as determined by various methods^a

<i>E. faecalis</i> isolate	Zone size (mm)/NCCLS category by disk-agar diffusion	MIC (μg/ml)/NCCLS category			
		AMS Vitek	Broth micro-dilution	Broth macro-dilution	Agar dilution
ATCC 29212	18/S	2/S	2/S	4/S	2/S
UC73	19/S	≤0.5/S	≤1/S	2/S	1/S
V583	15/S	≤0.5/S	>16/R	64/R	64/R
V586	16/S	≤0.5/S	>16/R	32/R	64/R
V587	14/S	≤0.5/S	>16/R	32/R	32/R

^a S, Susceptible; R, resistant. NCCLS interpretive categories are explained in references 13 and 14.

(Pasco Gram-Positive MIC Panels; Difco Laboratories, Detroit, Mich.), the AMS GPS-A susceptibility card (Vitek Systems Inc., Hazelwood, Mo.), broth macrodilution, agar dilution, and disk-agar diffusion tests, performed by procedures outlined by the National Committee for Clinical Laboratory Standards (NCCLS) (13, 14) and the manufacturer's recommendations. After MICs were determined by broth macrodilution, MBCs were ascertained according to NCCLS guidelines (15). By this method, drug concentrations that yielded ≤10 CFU on subculture were taken as the MBC.

Additional tests performed with the *E. faecalis* isolates included the Cefinase disk for β-lactamase production (BBL Microbiology Systems, Cockeysville, Md.) and high-level aminoglycoside resistance screens performed by using broth microdilution (Pasco Gram-Positive MIC panels) and disk-agar diffusion with high-content streptomycin (300-μg) and gentamicin (120-μg) disks (18).

Time kill studies. Methods outlined in a previous study were used to perform time kill tests with each enterococcal isolate (17). The drug concentrations selected for testing, based on clinically achievable levels in blood were as follows: gentamicin, 5 μg/ml; streptomycin, 20 μg/ml; vancomycin, 10 μg/ml; teicoplanin, 5 μg/ml; and daptomycin, 10 μg/ml. Inoculated broths were incubated in an ambient atmosphere at 35°C. At 0-, 4-, and 24-h intervals after inoculation, a 0.5-ml portion was removed from each of the tubes and subjected to serial 10-fold dilutions. From each diluent tube, 0.1-ml samples were taken and plated over the entire surface of Trypticase soy blood agar plates (BBL Microbiology Systems, Cockeysville, Md.). By using the viable counts determined at each time interval, a 24-h time kill curve was established for each *E. faecalis* strain. Synergy was defined as a ≥100-fold increase in killing by a drug combination over the killing accomplished by the most active of the two drugs in the combination when tested separately (12).

RESULTS

Both conventional testing and the AMS Gram-Positive identification cards confirmed the identities of V583, V586, and V587 as *E. faecalis*. All three isolates exhibited pyrrolidonylarylamidase activity, for which other vancomycin-resistant gram-positive bacteria such as *Pediococcus* spp., *Leuconostoc* spp., and most *Lactobacillus* spp. are negative (4, 5). Additionally, the AMS Vitek identified the isolates as *E. faecalis* with a 99% probability. In our experience, the AMS does not identify *Leuconostoc* spp., lactobacilli, and pediococci as *E. faecalis* (unpublished observations). Because none of these isolates exhibited motility at 30°C, the possibility that they were strains of *E. gallinarum* was ruled out.

The susceptibility profiles of the three isolates, as determined by broth microdilution, were the same; vancomycin, >16 μg/ml; penicillin, 1 μg/ml; ampicillin, 0.25 μg/ml; erythromycin, >4 μg/ml; tetracycline, ≤0.5 μg/ml; chloramphenicol, 8 μg/ml; ciprofloxacin, 0.5 μg/ml; rifampin, ≤0.5 μg/ml; streptomycin, <2,000 μg/ml; and gentamicin, >2,000 μg/ml. High-level resistance to gentamicin, but not streptomycin, was also demonstrated by the disk test. All three isolates were negative for β-lactamase production.

Vancomycin susceptibility results obtained with various testing methods are given in Table 1. Resistance was detected by broth microdilution, broth macrodilution, and agar dilution but not by the automated system (AMS Vitek) or disk-agar diffusion. When the incubation period of the disk-agar diffusion plates was extended from 24 to 48 h, a fine haze of minute colonies could be observed growing up to the vancomycin disk. When this 48-h growth was used to inoculate another disk-agar diffusion plate, the organisms still appeared to be susceptible at 24 h, with barely detectable growth up to the disk occurring only after 48 h of incubation. Another approach to aid the detection of vancomycin resistance by disk-agar diffusion was to test disks of various potencies, including 5, 10, 15, 20, 25, and 30 μg. Even with the lowest-potency disk used (5 μg), the smallest inhibition zone obtained (11 mm) was greater than the 9-mm interpretive zone size recommended for determining vancomycin resistance (1, 13).

The comparative activities of vancomycin, teicoplanin, and daptomycin are given in Table 2. Vancomycin and teicoplanin MICs obtained by broth macrodilution correlated well with those obtained by agar dilution. Daptomycin MICs were higher by agar dilution. Both teicoplanin and daptomycin demonstrated notably greater activity than vancomycin against V583, V586, and V587. The MBCs were generally considerably higher than the MICs.

Results of the time kill studies showed that after 24 h of incubation, isolate V583, which was highly resistant to gentamicin but not streptomycin, actively grew in the presence of vancomycin alone and in the presence of vancomy-

TABLE 2. Comparative in vitro activities of vancomycin, teicoplanin and daptomycin against *E. faecalis*

Isolate	MIC/MBC (μg/ml) by broth macrodilution			MIC (μg/ml) by agar dilution		
	Vancomycin	Teicoplanin	Daptomycin	Vancomycin	Teicoplanin	Daptomycin
ATCC 29212	4/128	≤0.5/32	≤0.5/0.5	2	≤0.5	4
UC73	2/32	≤0.5/64	≤0.5/8	1	≤0.5	2
V583	64/256	≤0.5/16	≤0.5/8	64	≤0.5	2
V586	32/128	≤0.5/32	≤0.5/2	64	≤0.5	2
V587	32/256	≤0.5/4	≤0.5/≤0.5	32	≤0.5	2

cin combined with gentamicin. The combination of vancomycin and streptomycin showed substantial bactericidal activity after 4 h of incubation, but by 24 h considerable regrowth had occurred in the presence of these two drugs. Teicoplanin alone was only minimally bactericidal against V583. Streptomycin, but not gentamicin, demonstrated synergy with teicoplanin. When tested alone, the killing activity of daptomycin was so effective that after 24 h of incubation, any synergistic effect with an aminoglycoside could not be ascertained at the concentrations tested. Time kill results obtained with V586 and V587 were comparable to those obtained with V583.

DISCUSSION

The *E. faecalis* isolates investigated demonstrated resistance to vancomycin (MICs, 32 to 64 $\mu\text{g/ml}$) but not to teicoplanin (MIC, ≤ 0.5 $\mu\text{g/ml}$), a glycopeptide antibiotic similar to vancomycin in spectrum of activity and mode of action (16). This susceptibility profile substantiates the biochemical results that differentiated these *E. faecalis* isolates from other vancomycin-resistant gram-positive bacteria. *Leuconostoc* spp., *Pediococcus* spp., and *Lactobacillus* spp. exhibit high-level vancomycin resistance (MIC, ≥ 256 $\mu\text{g/ml}$), heavy growth up to a 30- μg vancomycin disk, and high resistance to teicoplanin (5, 9; J. Swenson, R. Facklam, and C. Thornsberry, 28th ICAAC, abstr. no. 1321, 1988).

The glycopeptide susceptibility profiles exhibited by V583, V586, and V587 have been reported previously for *E. faecium* and *E. gallinarum* but not for *E. faecalis* (9, 23; Goldstein et al., 27th ICAAC). For these *E. faecium* and *E. gallinarum* strains, vancomycin MICs were 32 and 16 $\mu\text{g/ml}$, respectively, and teicoplanin MICs were 0.25 and 1 $\mu\text{g/ml}$, respectively. In contrast, the *E. faecalis* isolates reported by Uttley et al. (23) were resistant to both vancomycin and teicoplanin, and the vancomycin MICs were >64 $\mu\text{g/ml}$. Similarly, the vancomycin- and teicoplanin-resistant *E. faecium* strains reported by Leclercq et al. (10, 11), also resistant to both vancomycin and teicoplanin, had vancomycin MICs that ranged from 512 to 1,024 $\mu\text{g/ml}$. The *E. faecalis* strain reported by Shlaes et al. (20) had vancomycin and teicoplanin MICs of 256 and 16 $\mu\text{g/ml}$, respectively. These variations in enterococcal susceptibility to vancomycin and teicoplanin indicate that differences may exist both in the modes of action of these two glycopeptides and in the mechanisms of enterococcal resistance to them. Further studies designed to investigate the physiological basis for these differences in resistance are under way.

Daptomycin MICs for these vancomycin-resistant strains were comparable to those for vancomycin-susceptible and -resistant isolates published in previous reports (7–10). The differences between daptomycin MICs obtained by broth microdilution and those obtained by agar dilution were reproducible and may be due to possible differences in the calcium contents of the agar and broth MH media used in this study (6). Daptomycin activity, as measured by both methods, was comparable to that reported by other investigators (2, 7, 9–11, 21, 23, 24).

Certain evidence strongly suggests that the isolates described in the present study are the same strain. All three have the same antibiotic susceptibilities, including resistance to vancomycin but not teicoplanin. Two isolates were from the same patient (V583 and V586), and the third isolate (V587) was from a second patient whose time of stay in an intensive care unit overlapped that of the first patient. Nosocomial dissemination of enterococci has been docu-

mented (27) and may explain the isolation of these *E. faecalis* strains with unique glycopeptide resistance profiles from different patients. Plasmid analysis of V583 and V586 showed that, although both isolates were from the same patient, their plasmid profiles differed slightly. Of the three extrachromosomal bands observed in V583, only two were seen in V586. The plasmid profile of V587 was the same as that of V583.

The incidence of such vancomycin resistance among *E. faecalis* is probably quite low, but the failure of disk-agar diffusion and the AMS Vitek System, two commonly used susceptibility-testing procedures, to detect this resistance may be why such *E. faecalis* isolates have not been encountered previously. However, specific suggestions for altering currently recommended disk-agar diffusion testing methods (13) to facilitate detection of vancomycin resistance must await investigations that include a greater variety of vancomycin-resistant enterococcal strains. Also needed are evaluations to determine how well various commercial systems, both automated (e.g., AMS Vitek) and nonautomated, detect enterococcal vancomycin resistance.

Finally, because of the resistance that enterococci already exhibit to a variety of antimicrobial agents, the emergence of vancomycin resistance is troublesome. The incidence of this resistance and its impact on the therapeutic management of patients can only be better understood after more in vitro and in vivo studies. Further investigations that enhance our knowledge and understanding of the mechanisms and genetic transferability of this resistance are also needed.

LITERATURE CITED

1. Barry, A. L., C. Thornsberry, and R. N. Jones. 1986. Evaluation of teichoplanan and vancomycin disk susceptibility tests. *J. Clin. Microbiol.* 23:100–103.
2. Eliopoulos, G. M., C. Thauvin, B. Gerson, and R. C. Moellering, Jr. 1985. In vitro activity and mechanism of action of A219738C₁, a novel cyclic lipopeptide antibiotic. *Antimicrob. Agents Chemother.* 27:357–362.
3. Facklam, R. R., and R. B. Carey. 1985. Streptococci and aerococci, p. 154–175. In E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), *Manual of clinical microbiology*, 4th ed. American Society for Microbiology, Washington, D.C.
4. Facklam, R. R., and M. D. Collins. 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. *J. Clin. Microbiol.* 27:731–734.
5. Facklam, R. R., D. Hollis, and M. D. Collins. 1989. Identification of gram-positive coccid and coccobacillary vancomycin-resistant bacteria. *J. Clin. Microbiol.* 27:724–730.
6. Faruki, H., A. C. Niles, R. L. Heeren, and P. R. Murray. 1987. Effect of calcium on in vitro activity of LY146032 against *Clostridium difficile*. *Antimicrob. Agents Chemother.* 31:461–462.
7. Fass, R. J., and V. L. Helsel. 1986. In vitro activity of LY146032 against staphylococci, streptococci, and enterococci. *Antimicrob. Agents Chemother.* 30:781–784.
8. Harwick, H. J., G. M. Kalmanson, and L. B. Guze. 1973. In vitro activity of ampicillin or vancomycin combined with gentamicin or streptomycin against enterococci. *Antimicrob. Agents Chemother.* 4:383–387.
9. Kaplan, A. H., P. H. Gilligan, and R. R. Facklam. 1988. Recovery of resistant enterococci during vancomycin prophylaxis. *J. Clin. Microbiol.* 26:1216–1218.
10. Leclercq, R., E. Derlot, J. Duval, and P. Courvalin. 1988. Plasmid-mediated resistance to vancomycin and teichoplanin in *Enterococcus faecium*. *N. Engl. J. Med.* 319:157–161.
11. Leclercq, R., E. Derlot, M. Weber, J. Duval, and P. Courvalin. 1989. Transferable vancomycin and teichoplanin resistance in *Enterococcus faecium*. *Antimicrob. Agents Chemother.* 33:10–15.

12. Moellering, R. C., Jr., C. Wennersten, and A. N. Weinberg. 1971. Synergy of penicillin and gentamicin against enterococci. *J. Infect. Dis.* **124**(Suppl.):S207-S209.
13. National Committee for Clinical Laboratory Standards. 1984. Performance standards for antimicrobial disk susceptibility tests. M2-A3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
14. National Committee for Clinical Laboratory Standards. 1985. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. M7-A. National Committee for Clinical Laboratory Standards, Villanova, Pa.
15. National Committee for Clinical Laboratory Standards. 1987. Methods for determining bactericidal activity of antimicrobial agents. M26-P. National Committee for Clinical Laboratory Standards, Villanova, Pa.
16. Parenti, F., G. Beretta, M. Berti, and V. Ariotti. 1978. Teichomycins, new antibiotics from *Actinoplanes teichomyceticus* nov. sp. I. Description of the producer strain, fermentation studies and biological properties. *J. Antibiot.* **31**:276-283.
17. Sahm, D. F., and C. Torres. 1988. Effects of medium and inoculum variations on screening for high-level aminoglycoside resistance in *Enterococcus faecalis*. *J. Clin. Microbiol.* **26**: 250-256.
18. Sahm, D. F., and C. Torres. 1988. High-content aminoglycoside disks for determining aminoglycoside-penicillin synergy against *Enterococcus faecalis*. *J. Clin. Microbiol.* **26**:257-260.
19. Schneierson, S. S., and D. Amsterdam. 1957. In vitro sensitivity of erythromycin-resistant strains of staphylococci and enterococci to vancomycin and novobiocin. *Antibiot. Chemother.* **7**:251-254.
20. Shlaes, D. M., A. Bouvet, C. Devine, J. H. Shlaes, S. Al-Obeid, and R. Williamson. 1989. Inducible, transferable resistance to vancomycin in *Enterococcus faecalis* A256. *Antimicrob. Agents Chemother.* **33**:198-203.
21. Stratton, C. W., C. Liu, H. B. Ratner, and L. S. Weeks. 1987. Bactericidal activity of daptomycin (LY146032) compared with those of ciprofloxacin, vancomycin, and ampicillin against enterococci as determined by time-kill studies. *Antimicrob. Agents Chemother.* **31**:1014-1016.
22. Toala, P., A. McDonald, C. Wilcox, and M. Finland. 1969. Susceptibility of group D streptococcus (enterococcus) to 21 antibiotics in vitro, with special reference to species difference. *Am. J. Med. Sci.* **258**:416-430.
23. Uttley, A. H. C., C. H. Collins, J. Naidou, and R. C. George. 1988. Vancomycin-resistant enterococci. *Lancet* **i**:57-58.
24. Wanger, A. R., and B. E. Murray. 1987. Activity of LY146032 against enterococci with and without high-level aminoglycoside resistance, including two penicillinase-producing strains. *Antimicrob. Agents Chemother.* **31**:1779-1781.
25. Watanakunakorn, C., and C. Bakie. 1973. Synergism of vancomycin-gentamicin and vancomycin-streptomycin against enterococci. *Antimicrob. Agents Chemother.* **4**:120-124.
26. Westenfelder, G. O., P. Y. Paterson, B. E. Reisberg, and G. M. Carlson. 1973. Vancomycin-streptomycin synergism in enterococcal endocarditis. *J. Am. Med. Assoc.* **223**:37-40.
27. Zervos, M. J., C. A. Kaufman, P. M. Therasse, A. G. Bergman, T. S. Mikesell, and D. R. Schaberg. 1987. Nosocomial infection by gentamicin-resistant *Streptococcus faecalis*. An epidemiologic study. *Ann. Intern. Med.* **106**:687-691.