JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1995, p. 3019–3021 Vol. 33, No. 11
0095-1137/95/$04.00+0

NOTES

Molecular Characterization and Multilaboratory Evaluation of *Enterococcus faecalis* ATCC 51299 for Quality Control of Screening Tests for Vancomycin and High-Level Aminoglycoside Resistance in Enterococci

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Received 1 December 1994/Returned for modification 14 February 1995/Accepted 2 August 1995

Studies were conducted to validate the use of *Enterococcus faecalis* ATCC 51299 (which is vancomycin resistant and resistant to high levels of gentamicin and streptomycin) and *E. faecalis* ATCC 29212 (which is susceptible to vancomycin and against which gentamicin or streptomycin and cell wall-active agents have synergistic killing activity) as controls in an agar screening test for vancomycin resistance and high-level streptomycin and gentamicin resistance and a broth microdilution screening test for high-level streptomycin and gentamicin resistance. Both organisms performed as expected in these tests and will serve as appropriate controls. However, *E. faecalis* ATCC 29212 was occasionally noted to produce light growth on the vancomycin screening plate with certain lots of agar. Quality control ranges for disk diffusion tests with disks with large amounts of streptomycin (300 μg) and gentamicin (120 μg) were established for *E. faecalis* ATCC 29212; zone limits are 16 to 22 mm for gentamicin and 14 to 19 mm for streptomycin. No zones of inhibition were seen when *E. faecalis* ATCC 51299 was tested with these high-content disks.

The National Committee for Clinical Laboratory Standards (NCCLS) has recently included recommendations for screening tests for the detection of both vancomycin resistance and high-level gentamicin and streptomycin resistance in their standards for disk diffusion (4) and dilution (3) testing. As part of the development of the screening tests, an evaluation of *Enterococcus faecalis* ATCC 51299, a new quality control organism, and *E. faecalis* ATCC 29212, a strain already used for quality control in dilution tests (3) (as resistant and susceptible controls, respectively), was undertaken in two separate multilaboratory studies. The results of those studies are reported here.

The parameters for an agar dilution screening method for the detection of vancomycin resistance in enterococci were first proposed by Willey et al. (9) and were further developed by a working group of NCCLS (6). Similarly, the parameters for agar dilution and broth microdilution screening tests and a disk diffusion screening test with gentamicin and streptomycin high-content disks were also studied (7) and were adopted by NCCLS (3). In the study of the aminoglycosides, two organisms were used for quality control purposes: *E. faecalis* ATCC 49532, a strain showing high-level gentamicin resistance only, and *E. faecalis* ATCC 49533, a strain showing only high-level streptomycin resistance. Both strains were susceptible to vancomycin. Because of the work required to maintain three control organisms (one susceptible and two resistant organisms) for the screening tests, a single multiply-resistant enterococcus that could be used as a control for all three screening tests was sought. *E. faecalis* ATCC 51299 met these requirements and underwent further testing.

E. faecalis ATCC 51299 was received at the Centers for Disease Control and Prevention (CDC) in 1989 from Barnes Hospital in St. Louis, Mo., for confirmation of its vancomycin resistance. Recently, it was included in a study to evaluate methods for the detection of antimicrobial resistance in enterococci (8). In that study it was referred to as strain NJ-3.

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TABLE 1. Screening methods for detection of vancomycin resistance and high-level aminoglycoside resistance in enterococci

Drug or drug group and screening procedure	Medium	Inoculum	Incubation time (h)	Concn			Endpoint
				Vancomycin	Gentamicin	Streptomycin	
Vancomycin, agar dilution	BHI	1×10^5–1×10^6 CFU/spot	24	6 μg/ml			Any growth > 1 colony
Aminoglycoside							
Agar dilution	BHI	1×10^6 CFU/spot	24[a]		500 μg/ml	2,000 μg/ml	Any growth > 1 colony
Broth microdilution	BHI	5×10^5 CFU/ml	24[a]		500 μg/ml	1,000 μg/ml	Any growth
Disk diffusion	MHA[b]	0.5 McFarland standard[c]	18–24		120 μg/disk	300 μg/disk	6 mm = resistant; 7–9 mm = inconclusive[d]; ≥10 mm = susceptible

[a] If streptomycin is negative at 24 h, reincubate for an additional 24 h.
[b] MHA, Mueller-Hinton agar.
[c] NCCLS disk diffusion standard (3).
[d] If the zone is 7 to 9 mm, the test is inconclusive and an agar dilution or broth microdilution test should be performed to confirm susceptibility or resistance.

The vancomycin MIC for this strain is usually 16 to 32 μg/ml after 24 h of incubation, but it can be as high as 128 μg/ml after 48 h of incubation. The gentamicin and streptomycin MICs for the strain with cation-adjusted Mueller-Hinton broth are >2,000 μg/ml.

E. faecalis ATCC 51299 is essentially identical in its vancomycin and gentamicin resistance characteristics to *E. faecalis* V583, also isolated at Barnes Hospital, which carries the *vanB* gene (2, 5). DNA hybridization studies (1) with a probe specific for the *vanB* gene (2) and a PCR assay specific for the presence of the bifunctional gentamicin resistance gene *aac(6′)+aph(2″)* (7) were used to confirm the presence of these genes in *E. faecalis* ATCC 51299. In addition, a PCR assay specific for the *ant(6)-I* gene, which mediates high-level streptomycin resistance (7), was used to confirm that this gene had been acquired by *E. faecalis* ATCC 51299, a trait that differentiated it from *E. faecalis* V583 (5). The PCR assay demonstrated positive results with *E. faecalis* ATCC 51299 and negative results with *E. faecalis* ATCC 29212. Additional positive and negative controls, including *E. faecalis* JH1, *E. faecalis* ATCC 49532, and *E. faecalis* ATCC 49533, produced appropriate reactions (data not shown). This confirmed that *E. faecalis* ATCC 51299 contained the *vanB*, *ant(6)-I*, and *aac(6′)+aph(2″)* resistance genes, which correlated with its vancomycin and high-level gentamicin and streptomycin resistance phenotype.

The screening procedures recommended by NCCLS are summarized in Table 1. Evaluation of the performances of strains ATCC 51299 and ATCC 29212 for the vancomycin agar dilution screening test was done during the study to establish the best parameters for that test (6). Evaluation of their performances in tests to detect aminoglycoside resistance was undertaken in a separate multilaboratory study (7). Inoculum suspensions for all of the studies were prepared in either Mueller-Hinton broth or 0.85% saline, as recommended by NCCLS (3, 4).

For both the agar and broth dilution tests studied, media from the following five manufacturers of brain heart infusion (BHI) medium were included: Acumedia Manufacturers Inc., Baltimore, Md.; Adams Scientific Inc., West Warwick, R.I.; BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.; Difco Laboratories, Detroit, Mich.; and Oxoid, Unipath Co., Ogdensburg, N.Y. Two inoculum concentrations (10^5 and 10^6 CFU per spot) were used in the vancomycin agar dilution screen; only one (10^6 CFU per spot) was used in the aminoglycoside agar test. The larger inoculum (10^6 CFU) was spotted with a 10-μl micropipet; the smaller inoculum (10^5 CFU) was spotted with either a 1-μl calibrated loop or a 1-μl micropipet. In studies of the agar dilution screening procedures with both vancomycin and the aminoglycosides, all laboratories tested the organisms on a common lot of plates prepared at CDC and on unique lots of plates prepared in each of the participating laboratories. For the evaluation of the broth microdilution test for aminoglycoside resistance, plates were prepared at CDC with two lots each of BHI broth obtained from five manufacturers, and the plates were distributed to the study participants.

Zone diameter ranges for the aminoglycoside tests for *E. faecalis* ATCC 29212 were determined with a common lot of Mueller-Hinton agar (Remel, Lenexa, Kans.) and unique lots provided by each laboratory (Difco, BBL, or Oxoid). High-content gentamicin (120 μg) and streptomycin (300 μg) disks were prepared commercially by three manufacturers: Becton Dickinson Microbiology Systems, Remel, and Difco. At least 20 separate results were generated in each laboratory for each test by performing replicate tests on 5 separate days.

Results of the performances of the control strains on the vancomycin agar dilution screening plates are given in Table 2 as the number of times that growth was recorded for both of the strains for the two inocula (10^6 and 10^5 cfu per spot) read at 24 and 48 h. All media except that from Oxoid gave ≥95% correct performance. The susceptible control strain (ATCC 29212) grew on the Oxoid medium with the higher inoculum or

TABLE 2. Performance of common and unique lots of BHI agar with 6 μg of vancomycin per ml and two control strains, *E. faecalis* ATCC 51299 and *E. faecalis* ATCC 29212

Source of BHI medium	No. of isolates[a]	No. of times growth was recorded[b]							
		E. faecalis ATCC 51299				*E. faecalis* ATCC 29212			
		10^5 CFU		10^6 CFU		10^5 CFU		10^6 CFU	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Acumedia[c]	120	120	120	120	120	0	1	0	1
Adams	21	21	21	21	21	0	0	0	0
BBL	42	42	42	42	42	0	0	1	1
Difco	84	83	84	83	84	0	0	0	0
Oxoid	21	21	21	21	21	2	21	19	21

[a] Common lots of plates were tested 20 times (5 times on 4 different days) in each of six laboratories. Unique lots were tested 21 times in each laboratory (5 times on 4 days and 1 time on an additional day). Two laboratories used BBL medium and four laboratories used Difco medium.
[b] Correct value for *E. faecalis* ATCC 51299 is any growth and that for *E. faecalis* ATCC 29212 is no growth.
[c] Common lot of medium.

TABLE 3. Distribution of zones of inhibition against *E. faecalis* ATCC 29212 with 120-μg gentamicin disks[a]

Laboratory	No. of times the following zone diam (mm) was recorded										Zone diam range (mm)
	15	16	17	18	19	20	21	22	23	24	
A				6	19	22	21	13	7	1	7
B	5	13	28	11	13	14	3	1			8
C	3	4	45	33	5						5
D						3	46	39	2		4
E						13	44	33			3
F		1	2	16	28	30	13				6
All[b]	8	18	75	66	65	82	127	86	9	1	

[a] Each laboratory recorded 75 zone diameters on their unique lots of Mueller-Hinton agar with three different lots of disks and an additional 15 zone diameters on a common lot of Mueller-Hinton agar. All readings are combined in this table.
[b] A total of 97% of values is included in the range from 16 to 22 mm.

with an increased incubation time. The overall performance of the agar screening test for the determination of vancomycin resistance with all five media was ≥99% with an inoculum of 10^5 CFU per spot and incubation for only 24 h. The performances of the control organisms by the agar screening test and the broth microdilution screening test for high-level gentamicin and streptomycin resistance (data not shown) were ≥99% for both tests on all media.

For the disk diffusion test with aminoglycoside high-content disks, only *E. faecalis* ATCC 29212 was evaluated to define the range of acceptable zone diameters, since *E. faecalis* ATCC 51299 was known not to produce zones of inhibition around these disks. The distributions of all zone diameters determined (both on the common lot and unique lots) are given in Table 3 for gentamicin and in Table 4 for streptomycin. Zone diameter limits that encompass at least 95% of the values were determined to be 16 to 22 mm for gentamicin and 14 to 19 mm for streptomycin.

TABLE 4. Distribution of zones of inhibition against *E. faecalis* ATCC 29212[a] with 300-μg streptomycin disks

Laboratory	No. of times the following zone diameter was recorded:									Zone diam range (mm)
	13	14	15	16	17	18	19	20	21	
A		4	4	9	28	30	11	2	2	8
B	5	29	23	15	15	3				6
C		5	23	46	16					4
D					1	46	40	3		4
E					18	51	20			3
F	3	18	15	13	25	8	8			7
All	8	56	65	83	103	138	79	5	2	

[a] Each laboratory recorded 75 zone diameters on their unique lots of Mueller-Hinton agar with three different lots of disks and an additional 15 zone diameters on a common lot of Mueller-Hinton agar. All readings are combined in this table.
[b] A total of 97% of values is included in the range of 14 to 19 mm.

Both *E. faecalis* ATCC 51299 and *E. faecalis* ATCC 29212 performed acceptably as control organisms for screening procedures for the determination of vancomycin resistance and high-level aminoglycoside resistance in enterococci. Because *E. faecalis* ATCC 51299 displays low-level vancomycin resistance, it sometimes grows only weakly on the vancomycin screening plates. *E. faecalis* ATCC 29212 is susceptible to vancomycin. However, the vancomycin MIC for *E. faecalis* ATCC 29212 is in the range of 1 to 4 μg/ml. This strain grew on one of the manufacturer's lots of BHI agar, especially at the higher inoculum and with 48 h of incubation. The reason for this is not known. However, the performance of the screening plates with clinical isolates that were susceptible to vancomycin was not a problem (6). Before use in these tests, candidate lots of media should be evaluated for correct performance by using both *E. faecalis* ATCC 51299 and *E. faecalis* ATCC 29212.

We thank the following people for excellent and invaluable technical efforts: Laurie Free, Washington University School of Medicine; Jean Spargo, Massachusetts General Hospital; Angela Brueggemann, University of Massachusetts Medical Center; Louise Maher and Leticia McElmeel, University of Texas Health Science Center at San Atonio; G. Morthland, C. Grant, and J. Rhine-Chalberg, Oregon Health Sciences University; Jackie Thorpe, Duke University; Karen Jones and Judy Rothberg, Robert Wood Johnson Medical School; and Anthony R. DiNuzzo, University of Texas Medical Branch at Galveston.

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