

Screening of Stool Samples for Identification of Vancomycin-Resistant *Enterococcus* Isolates Should Include the Methyl- α -D-Glucopyranoside Test To Differentiate Nonmotile *Enterococcus gallinarum* from *E. faecium*

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Received 6 February 1998/Returned for modification 24 March 1998/Accepted 12 May 1998

The methyl- α -D-glucopyranoside (MDG) test has been shown to be superior to motility testing in differentiating *Enterococcus faecium* from *E. gallinarum*. In the present study, 33 vancomycin-resistant enterococcus (VRE) isolates collected as part of a stool surveillance study were compared by using motility and MDG. Motility testing identified all 33 isolates as *E. faecium*, whereas MDG identified 11 of the 33 isolates as nonmotile *E. gallinarum*. The MDG results were confirmed by sequencing the 16S rDNA V6-to-V8 region. We conclude that the MDG test is a necessary component of routine VRE screening.

Enterococci have been shown to be the second most common cause of nosocomial infection in the United States (15). *Enterococcus faecalis* is responsible for the majority of enterococcal infections, whereas *E. faecium*, while responsible for significantly fewer infections, is more commonly associated with resistance to beta-lactams, fluoroquinolones, and glycopeptides (9, 10) and is associated with greater morbidity and mortality (7). As the prevalence of infections caused by vancomycin-resistant enterococci (VRE) is presently low, the screening of stool for identification of colonizers in high-risk patients is the focus of several epidemiological studies (10, 13). It is critical in such screening studies to differentiate *E. faecalis* and *E. faecium* from other enterococcal species, such as *E. gallinarum* and *E. casseliflavus*, which do not normally cause human disease but commonly demonstrate intrinsic low-level glycopeptide resistance (1). Recent reports suggest that motility alone as the criterion for differentiating between *E. gallinarum* and *E. faecium* (3) is inadequate and may lead to the misidentification of isolates of nonmotile *E. gallinarum* as *E. faecium* (5).

Recently, Devriese and coworkers have shown the methyl- α -D-glucopyranoside (MDG) test to be reliable and accurate in differentiating *E. casseliflavus* and *E. gallinarum* from *E. faecalis* and *E. faecium* (4). We were interested in determining the impact of the MDG test using a collection of vancomycin-resistant *E. faecium* isolates taken from a recent stool surveillance project (10) in which 1,500 enterococcal isolates had been identified to species level with a conventional identification algorithm, not containing MDG (8). The susceptibilities to glycopeptides vancomycin and teicoplanin and the glycopeptide resistance genotypes were also determined (6, 11).

Frozen stock cultures of 33 vancomycin-resistant *E. faecium* isolates (MIC, 4 μ g/ml) obtained in the VRE prevalence study were subcultured onto Trypticase soy-5% sheep blood agar and incubated at 37°C for 24 h for MDG testing and PCR ly-

sate preparation. MDG testing of all VRE isolates was performed as previously described by Devriese et al. (4).

Enterococcal species identification of each isolate was confirmed by sequencing the V6-to-V8 region (12) of the 16S rDNA which corresponds to bp 929 to 1369 of the *Escherichia coli* 16S rRNA sequence (2). The following ATCC strains were chosen for sequence determination: *E. faecalis* ATCC 29212, *E. faecium* ATCC 35667, *E. gallinarum* ATCC 35038, and *E. casseliflavus* ATCC 12755. Thirty-two enterococcal isolates from the stool surveillance project had also been sequenced to confirm the specificity and consistency of all sequences: 9 *E. faecium* isolates, 10 *E. faecalis* isolates, 5 *E. casseliflavus* isolates, and 8 *E. gallinarum* isolates. Subsequently, sequencing of the V6-V8 region of the 16S rDNAs of all 33 VRE isolates tested for MDG was performed. DNA extracts were prepared by using one or two colonies from the 24-h subcultures. DNA was isolated by using the QIAamp tissue kit (Qiagen, Santa Clarita, Calif.) in accordance with the manufacturer's protocol. PCR amplification was performed by using universal 16S rDNA gene primers 91E(G) (5'TCAAAGGAATTGACGGGGC) and 13B (5'AGGCCCGGGAACGTATTCAC) (14). A 50- μ l PCR mixture contained 5 μ l of DNA template; 5 μ l of 25 mM MgCl₂-10 \times PCR buffer; 1.25 mM each dCTP, dGTP, dATP, and dUTP · dTTP in an 8:1 ratio; 0.5 μ l of 100 mM each

TABLE 1. Comparison of MDG testing and species identification using sequencing with conventional testing of 33 VRE (*E. faecium*) isolates

No. of isolates	MIC (μ g/ml)		Genotyping result	Sequencing result	Conventional test ^a result	MDG result
	Vancomycin	Teicoplanin				
11	4-8	0.25-0.5	<i>vanC₁</i>	<i>E. cass/E. gall</i> ^b	<i>E. faecium</i> ^c	+
10	4-8	0.25-0.5	<i>vanB</i>	<i>E. faecium</i>	<i>E. faecium</i>	-
12	256-512	4-32	<i>vanA</i>	<i>E. faecium</i>	<i>E. faecium</i>	-

^a Includes biochemical reactions, motility, and pigment.

^b *E. cass*, *E. casseliflavus*; *E. gall*, *E. gallinarum*.

^c Misidentification of nonmotile *E. gallinarum*.

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Efa TCATTTAGTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGAGGAGGGTGGGGATGACGTCAAATCATCATGCCCCCTTATGACCTGGGCTACACAG
Efe .....C.....A.....
Ega .....T.....G.....
Eca .....T.....G.....

Efa TGCTACAATGGGAAGTACAACGAGTCGCTANACCGGAGGTCATGCAAATCTCTTAAAGCTTCTCTCAGTTTCGGATTGCAAGGCTGCAACTCGCCTGCATG
Efe .....T..G.AGT.....CT.A..T.....C.....G.....
Ega .....T..G.AGT.....CT.A..T.....T.....A.....
Eca .....T..G.AGT.....CT.A..T.....T.....A.....

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FIG. 1. Sequence variability in the V7-V8 region of the 16S rDNA gene (bp 1130 to 1330) among *E. faecalis* (Efa), *E. faecium* (Efe), *E. gallinarum* (Ega), and *E. casseliflavus* (Eca).

primer; 0.5 U of uracil DNA glycosylase (GibcoBRL, Burlington, Canada); 2.5 U of *Taq* DNA polymerase (Pharmacia Biotech, Baie d'Urfé, Canada); and 30 μ l of sterile distilled H₂O. The PCR was performed with a Perkin-Elmer GeneAmp PCR System 9600 with cycles of 37°C for 10 min and 95°C for 10 min and 30 cycles of 95, 55, and 72°C for 1 min each and incubated at 72°C for 10 min for final extension.

Sequencing reactions were performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, Calif.). The primer used for the sequencing reaction was 91E(G) or 13B diluted 1:100 in Tris-EDTA buffer. Cycle sequencing on the GeneAmp PCR System 9600 was performed, and sequencing analysis was performed with the PE-ABI 373 DNA sequencing system and Software 373 in accordance with the manufacturers' instructions.

The results obtained by MDG testing for all 33 VRE isolates demonstrated that 22 isolates were MDG negative, showing concordance with prior motility-based *E. faecium* identification. However, 11 VRE isolates tested MDG positive, indicating that they were in fact not *E. faecium* as originally reported but, instead, nonmotile *E. gallinarum* (Table 1). These 11 strains all possessed low-level glycopeptide resistance, with vancomycin and teicoplanin MICs of 4 to 8 μ g/ml and 0.25 to 0.5 μ g/ml, respectively.

The MDG results were confirmed by sequencing the 16S rDNA V6-to-V8 regions. *E. faecalis* and *E. faecium* each showed specific and consistent sequence variability. *E. gallinarum* and *E. casseliflavus* showed a 2-bp difference from *E. faecium* and a 10-bp difference from *E. faecalis* but could not be differentiated from each other by using this area of the 16S rDNA gene (Fig. 1). Therefore, sequencing served to differentiate *E. faecium* from *E. gallinarum* and *E. casseliflavus*. Sequencing of the 33 VRE isolates which had previously all been identified as *E. faecium* confirmed that 22 isolates were *E. faecium* and 11 were nonmotile *E. gallinarum* (Table 1). *E. gallinarum* was differentiated from *E. casseliflavus* by its lack of pigment. There have been reported cases of nonpigmented *E. casseliflavus* (16); however, from a clinical point of view, this is not significant.

The clinical significance of this work is apparent, as misidentification of vancomycin-resistant *E. gallinarum* as vancomycin-resistant *E. faecium* causes great concern. The prevalence of nonmotile *E. gallinarum* may be even higher due to the fact that vancomycin-sensitive nonmotile *E. gallinarum* can also be

misidentified as vancomycin-sensitive *E. faecium*, although this is not a great concern.

Sequencing, which provided us with definitive identification of *Enterococcus* species, is not a realistic approach for routine VRE screening. Yet, it can serve as a tool for definitive identification of important pathogens or of strains of questionable identity. We conclude that the MDG test is a reliable, rapid, and cost-effective method for identification of clinically relevant *Enterococcus* species and that it is a necessary component for routine VRE screening.

Nucleotide sequence accession numbers. The determined sequences comprising the V6-to-V8 regions of the 16S rDNA gene for each *Enterococcus* species used in this study have been submitted to GenBank with the following accession numbers: *E. faecalis*, AF023101; *E. faecium*, AF023102; *E. gallinarum*, AF023103; *E. casseliflavus*, AF023104.

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