JOURNAL OF CLINICAL MICROBIOLOGY, July 1995, p. 1931–1933 Vol. 33, No. 7
0095-1137/95/$04.00+0

Comparison of Pigment Production and Motility Tests with PCR for Reliable Identification of Intrinsically Vancomycin-Resistant Enterococci

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Received 10 February 1995/Returned for modification 28 March 1995/Accepted 18 April 1995

Forty-eight clinical isolates identified as either *Enterococcus faecium* or *Enterococcus faecalis* with the MicroScan system (Dade International, MicroScan Inc., West Sacramento, Calif.) were further characterized by two supplementary biochemical tests (pigment production and motility). Twenty isolates (42%), all initially identified as *E. faecium*, were motile. Of these 20, 8 isolates (17%) produced yellow pigment and were identified as *Enterococcus casseliflavus* and the remaining 12 (25%) were nonpigmented and were identified as *Enterococcus gallinarum*. Identical identification results were obtained when PCR amplification of regions of the *vanC* gene was used as a technique for differentiating these organisms. The results of this study indicate that motility and pigment production tests together with commercial test systems are sufficient for reliable identifications of *E. faecium*, *E. casseliflavus*, and *E. gallinarum*.

Reports of vancomycin-resistant enterococci first appeared in the late 1980s (7), and the increasing epidemiologic problem posed by these organisms, particularly in intensive care units, has led to greater demands on laboratories to identify and report isolates (2). *Enterococcus gallinarum* and *Enterococcus casseliflavus* are among the more recently described species of enterococci. They exhibit low-level intrinsic resistance to vancomycin (MICs of between 2 and 16 μg/ml for these organisms), with resistance being conferred by possession of the *vanC* gene (8, 9). Unfortunately, *E. casseliflavus* and *E. gallinarum* are difficult to differentiate from other enterococci, particularly *Enterococcus faecium*, with commercial biochemical test systems, which may not even include these organisms in their databases (6). If the epidemiologic impact of a laboratory report of *E. faecium* as vancomycin resistant is considered, it is clearly of some import that laboratories be able to rapidly and reliably differentiate *E. gallinarum* and *E. casseliflavus* from *E. faecium*. Two biochemical tests have been reported to have the greatest utility in differentiating these three organisms. They are tests for motility (*E. gallinarum* and *E. casseliflavus* are positive; *E. faecium* is negative) and yellow pigment production (*E. casseliflavus* is positive; *E. faecium* and *E. gallinarum* are negative) (5). Some investigators have questioned the reliability of these conventional tests (13) and have described elaborate molecular approaches for identifying these organisms (3, 4, 13), including PCR amplification of regions of the *vanC* gene (4).

Reports of failures of automated susceptibility test systems to detect low-level vancomycin resistance in enterococci (12) led to the development of media designed to screen for vancomycin-resistant organisms (11). Initial reports describing these media indicated that the majority of isolates of *E. gallinarum* and *E. casseliflavus* could grow on them, regardless of the MIC of vancomycin for the isolates (11), but that only isolates of *E. faecium* for which MICs were ≥4 μg/ml could be cultivated on the vancomycin-containing screening agars. Thus, it is conceivable that growth on vancomycin resistance screening agar could be used as an additional test for identification of pigmented and/or motile enterococci.

In this study, we examined the value of simple tests for motility and pigment production to aid in the identification of *E. casseliflavus* and *E. gallinarum* and compared this approach with PCR amplification of regions of the *vanC1* (specific for *E. gallinarum*) and *vanC2* (specific for *E. casseliflavus*) genes. The MICs of vancomycin for clinical isolates identified at a >95% confidence level as *E. faecium* or *Enterococcus faecalis* with the MicroScan system (this system does not currently contain *E. gallinarum* and *E. casseliflavus* in its database) were determined with a commercially available microdilution system (MicroScan Pos MIC Panel Type 6). Panels were incubated for 24 h at 35°C, and growth was assessed visually with a TouchScan reader to ensure accurate determination of MICs. Isolates for which the MICs were at least 1 μg/ml were chosen for further testing. Motility and pigment production for these isolates were determined as described previously (7), and the identification of all nonmotile, nonpigmented isolates was confirmed by conventional biochemical tests (7). In addition, the ability of isolates to grow on a vancomycin resistance screening agar, brain heart infusion agar with 6 μg of vancomycin per ml (BHIV; PML Microbiologicals, Tualatin, Oreg.), was determined as described by Swenson et al. (11).

DNA extracts for PCR were prepared from 24-h broth (brain heart infusion) cultures of isolates by a standard alkaline lysis procedure (1). A multiplex PCR was then performed with primer pairs C_1/C_2 (for *vanC1*, the product is 822 bp) and D_1/D_2 (for *vanC2*, the product is 439 bp) (4). The reaction mixture (final volume, 20 μl) contained 100 ng of DNA template, 10 pmol of each oligonucleotide primer, 1× PCR Buffer II (Perkin-Elmer), 1.5 mM $MgCl_2$, deoxynucleoside triphosphates (200 μM each), and 2 U of *Taq* DNA polymerase (Perkin-Elmer). The PCR, performed in a MiniCycler (MJ Research), consisted of an initial denaturation step for 10 min at 94°C followed by 30 cycles for 15 s each at 94, 58, and 72°C.

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TABLE 1. Comparison of tests for identification of *E. gallinarum* and *E. casseliflavus*

Species (no.) as identified with MicroScan system	No. of isolates by phenotype			No. of isolates by PCR amplification			No. of isolates growing on BHIV
	Nonmotile	Motile and nonpigmented	Motile and pigmented	Not VanC	VanC1	VanC2	
Enterococcus faecalis (9)	9	0	0	9	0	0	0
Enterococcus faecium (39)	19	12	8	19	12	8	20[a]

[a] Two of these isolates were vancomycin-resistant *E. faecium.*

Following amplification, PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining.

The results of this study demonstrate that the addition of pigment production and motility test results to those produced with the biochemical panel of the MicroScan system enables efficient, rapid, and simple identification of *E. casseliflavus* and *E. gallinarum* (Table 1) and gives results identical to those found by PCR amplification of genes specific to these two organisms. The identification of *E. faecalis* with the MicroScan system was completely reliable (at the >95% confidence level used in our laboratory), conventional biochemical testing confirmed the identities of all nine isolates, and MICs of vancomycin for all isolates were 1 μg/ml. Of the *E. faecium* isolates examined, 20 of 39 (51%) were motile and 8 of 39 (21%) produced yellow pigment. These relatively high frequencies of motile and pigmented organisms identified as *E. faecium* reflect the fact that an MIC of vancomycin of 1 μg/ml was used as a selective criterion in identifying isolates to be examined in this study. Since *E. gallinarum* and *E. casseliflavus* are intrinsically resistant to vancomycin, the MICs of vancomycin for them are typically greater than those for the majority of vancomycin-susceptible isolates of other enterococcal species. Thus, the use of the MIC of vancomycin as a screen enabled us to enrich the population of isolates tested with *E. casseliflavus* and *E. gallinarum*. Indeed, the MICs of vancomycin for only two isolates of *E. faecium* were >1 μg/ml (32 μg/ml and >256 μg/ml). MICs for *E. casseliflavus* and *E. gallinarum* varied between 2 μg/ml and 16 μg/ml, with MICs for *E. gallinarum* isolates being, on average, considerably higher than those for *E. casseliflavus*, a phenomenon noted by previous investigators (11, 13). Significantly, of the 20 isolates of *E. casseliflavus* and *E. gallinarum* studied, only 4 (all isolates of *E. gallinarum*) would have been reported as vancomycin resistant (MIC of >4 μg/ml) after being tested with the MicroScan Pos Breakpoint Combo Panel Type 6 used by us for routine susceptibility testing and identification of enterococci. Given the epidemiologic importance of a report of the isolation of a vancomycin-resistant enterococcus, these four isolates would have been subjected to additional biochemical testing in our laboratory to confirm their identities as *E. faecium*, and they would consequently have been identified as *E. gallinarum*. The remaining 16 isolates of *E. casseliflavus* and *E. gallinarum* would, however, have been reported as vancomycin-susceptible isolates of *E. faecium.*

In addition, we screened our isolates for their ability to grow on a vancomycin resistance screening agar (BHIV) and found that only 18 of 20 (90%) isolates of *E. casseliflavus* and *E. gallinarum* were cultivatable on this medium (Table 1). The two organisms that failed to grow were both *E. casseliflavus* isolates for which the MICs of vancomycin were 2 μg/ml; interestingly, several isolates of this organism for which the MICs were comparable could grow on BHIV, and all isolates of *E. casseliflavus* possessed the *vanC2* gene as determined by PCR. Similar results were reported by Swenson et al. (11), and it seems, therefore, that growth on BHIV has little utility as a test for the identification of intrinsically vancomycin-resistant enterococci. It remains to be seen if the selective growth pattern observed with BHIV is an indication that only certain isolates of *E. casseliflavus* and *E. gallinarum* express the VanC phenotype or if it is simply a reflection of the lack of sensitivity of this screening test.

A previous study by Vincent et al. (13) indicated that motility and pigment production tests were unreliable for the identification of *E. casseliflavus* and *E. gallinarum*. These authors reported that 12 isolates of *E. casseliflavus* identified by DNA homology comparison with known *E. casseliflavus* isolates were tested and that 2 were nonmotile and 2 were nonpigmented. This report led to a number of studies exploring the use of elaborate molecular methods for the identification of these organisms, including the PCR approach used in this paper. Our results indicate that motility and pigment production tests are extremely reliable for the identification of these organisms in a clinical laboratory, since no isolate was misclassified when these tests were used in conjunction with the MicroScan system. This discrepancy between our results and those of Vincent and colleagues probably represents a difference in the population of organisms studied. The previous work used American Type Culture Collection strains and isolates obtained from the culture collections of other laboratories, while our study was conducted prospectively on organisms recently isolated from clinical specimens. Repeated passage of organisms in vitro is likely to affect such labile phenotypic markers as pigment production and motility, and this is a possible explanation for the poor results obtained by Vincent et al.

Recent reports have indicated that a third intrinsically vancomycin-resistant motile enterococcus, *Enterococcus flavescens*, can occasionally be found in clinical specimens (10), although no isolates of this organism were found in the present study. This organism appears to be very closely related to *E. casseliflavus* since it possesses the *vanC2* gene (4) and differs from *E. casseliflavus* in only one or two biochemical reactions (10). This complication notwithstanding, differentiation of the intrinsically vancomycin-resistant group of enterococci from *E. faecium* should remain the primary goal of the clinical laboratory, and this can readily be attained by screening isolates identified with the MicroScan system as *E. faecium* for motility and pigment production. Further identification can then be performed, depending on the clinical significance of the isolate. It is hoped that improving the accuracy with which isolation of *E. casseliflavus*, *E. gallinarum*, and possibly *E. flavescens* is reported will make possible a better assessment of the clinical importance, or lack thereof, of these organisms and, furthermore, will provide data on the epidemiologic significance of the VanC phenotype.

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