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Detection of Glycopeptide Resistance Genotypes and Identification to the Species Level of Clinically Relevant Enterococci by PCR

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A PCR assay that allows simultaneous detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci (*Enterococcus faecium*, *E. faecalis*, *E. gallinarum*, and *E. casseliflavus*) was developed. This assay was based on specific amplification of internal fragments of genes encoding D-alanine:D-alanine ligases and related glycopeptide resistance proteins. The specificity of the assay was tested on 5 well-characterized glycopeptide-resistant strains and on 15 susceptible enterococcal type strains. Clinical isolates of enterococci that could not be identified to the species level by conventional methods were identified by the PCR test. This assay offers a specific and rapid alternative to antibiotic susceptibility tests, in particular for detection of low-level vancomycin resistance.

Enterococci are increasingly responsible for nosocomial infections such as endocarditis, bacteremia, urinary tract infections, or neonatal sepsis (28). In a recent study these microorganisms represented the third most common cause of hospital-acquired bacteremia (28). Of the 20 enterococcal species described to date (4), *Enterococcus faecalis* and *E. faecium* represent ca. 90% of clinical isolates belonging to this genus (13, 19). *E. gallinarum* and *E. casseliflavus* are less represented, but the incidence of these species is underestimated because of frequent misidentification (33). Proper identification of enterococci to the species level is important for appropriate therapy of certain infections, in particular endocarditis, because of species-specific differences in the susceptibility to β-lactams and glycopeptides (19).

Glycopeptide resistance in enterococci is now a cause of clinical concern. Three glycopeptide resistance phenotypes can be distinguished on the basis of the level and inducibility of resistance to vancomycin and teicoplanin (1, 7). The VanA-type is characterized by acquired inducible resistance to both vancomycin and teicoplanin (17, 32). Strains of the VanB type have acquired inducible resistance to various levels of vancomycin but not to teicoplanin (24). Constitutive low-level resistance to vancomycin (VanC type) is an intrinsic property of motile enterococci, *E. gallinarum* (18, 33), *E. casseliflavus* (20, 33), and *E. flavescens* (20). Fourteen percent of enterococci isolated in the United States are glycopeptide resistant (30). The actual prevalence might even be higher since phenotypic detection of low-level resistance is difficult (27, 31). Several recent outbreaks of vancomycin-resistant enterococci (30, 32) emphasize the need for laboratories to be able to detect the various types of resistance.

The PCR has been extensively applied in medical diagnosis (4). It has been used for species identification of infectious agents (12, 16, 21) and specific detection of antibiotic resistance genes (23). We have developed a PCR assay which allows identification of the four glycopeptide resistance genotypes described for enterococci (*vanA*, *vanB*, *vanC-1*, and *vanC-2*) together with identification to the species level of four clinically relevant species of enterococci (*E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus*).

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MATERIALS AND METHODS

Bacterial strains and culture conditions. Well-characterized glycopeptide-resistant enterococci belonging to phenotypes VanA, VanB, and VanC were studied. These were *E. faecium* BM4147 (VanA) (17), *E. faecalis* V583 (VanB) (26), *E. gallinarum* BM4174 (VanC) (10), *E. casseliflavus* ATCC 25788 (VanC) (20), and *E. flavescens* CCM 439 (VanC) (22). The following susceptible type strains were used as controls: *E. avium* NCDO 2369, *E. cecorum* NCDO 2674, *E. columbae* STR345, *E. dispar* NCIMB 1300, *E. durans* NCDO 596, *E. faecalis* NCDO 581, *E. faecium* NCDO 942, *E. hirae* NCDO 1258, *E. malodoratus* NCDO 846, *E. mundtii* NCDO 2375, *E. pseudoavium* NCDO 2138, *E. raffinosus* NCTC 12192, *E. saccharolyticus* NCDO 2594, *E. salivarius* ATCC 102503, *E. seriolicida* ATCC 49156, *E. solitarius* NCTC 12193, and *E. sulfureus* NCDO 2379. All cultures were performed at 37°C in brain heart infusion broth and agar (Difco Laboratories, Detroit, Mich.). Twenty-six clinical isolates of enterococci isolated in 1993 at the Hôpital A. de Villeneuve (Montpellier, France) were also included.

Computer analysis of sequence data. Nucleotide sequences were aligned with the Genetics Computer Group software (5) by using the progressive alignment method (15). The GenBank database was screened to detect homology between the oligodeoxynucleotides used and known sequences.

PCR. Oligodeoxynucleotides were synthesized (Laboratoire de Chimie Organique, Institut Pasteur, Paris, France) by the methoxyphosphoramidite method on an Applied Biosystems (Foster City, Calif.) 380 DNA synthesizer. Total DNA was prepared by a rapid alkaline lysis method as described previously (8). PCR was performed on a DNA thermal cycler (model 480; Perkin-Elmer Cetus, Emeryville, Calif.) in a final volume of 100 μl containing 250 ng of DNA as template; 50 pmol of each oligodeoxynucleotide primer; 500 μM (each) dATP, dCTP, dGTP, and dTTP; 67 mM Tris-HCl (pH 8.8); 7 mM $MgCl_2$; 17 mM ammonium sulfate; 10 mM β-mercaptoethanol; and 2 U of *Taq* DNA polymerase. The cycles used were 94°C for 2 min for the first cycle; 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min for the next 30 cycles; and 72°C for 10 min for the last cycle. PCR products were resolved by electrophoresis on a 1.5% agarose–Tris–borate–EDTA gel containing 0.5 μg of ethidium bromide per ml.

Enzymes and reagents. Lysozyme was purchased from Sigma Chemical Co. (St. Louis, Mo.). *Taq* DNA polymerase was obtained from Amersham (Little Chalfont, United Kingdom). Dideoxynucleotides, dATP, dCTP, dGTP, and dTTP, were obtained from Pharmacia (Uppsala, Sweden).

RESULTS AND DISCUSSION

Design of oligodeoxynucleotides. The use of PCR for simultaneous identification of enterococcal species and glycopeptide resistance genotypes was based on specific detection of genes

TABLE 1. Oligodeoxynucleotide primers

Amplified gene	Size of PCR product (bp)	Oligodeoxynucleotide		Position	GC content (%)
		Pair	Sequence (5′ to 3′)[a]		
vanA	732	A_1	+GGGAAAACGACAATTGC	175–191	53
		A_2	−GTACAATGCGGCCGTTA	907–891	53
vanB	635	B_1	+ATGGGAAGCCGATAGTC	173–189	53
		B_2	−GATTTCGTTCCTCGACC	807–791	53
vanC-1	822	C_1	+GGTATCAAGGAAACCTC	246–272	53
		C_2	−CTTCCGCCATCATAGCT	1067–1051	50
vanC-2, *vanC-3*	439	D_1	+CTCCTACGATTCTCTTG	455–486	47
		D_2	−CGAGCAAGACCTTTAAG	885–869	47
$ddl_{E.\ faecalis}$	941	E_1	+ATCAAGTACAGTTAGTCT	98–116	38
		E_2	−ACGATTCAAAGCTAACTG	1038–1021	39
$ddl_{E.\ faecium}$	550	F_1	+TAGAGACATTGAATATGCC	NA[b]	37
		F_2	−TCGAATGTGCTACAATC	NA	39

[a] +, sense primer; −, antisense primer.
[b] NA, not applicable.

encoding D-alanine:D-alanine (D-Ala:D-Ala) ligases and related glycopeptide resistance enzymes. VanA- and VanB-type resistances are due to incorporation of D-alanyl-D-lactate (D-Ala-D-Lac) into peptidoglycan precursors that have reduced affinity for glycopeptides (3, 7). D-Ala-D-Lac is synthesized by D-Ala:D-Ala ligase-related proteins VanA (6) and VanB (11), respectively. In resistant strains, these enzymes are present in addition to the chromosomally encoded D-Ala:D-Ala ligase, Ddl in *E. faecium* (unpublished results) or Ddl in *E. faecalis* (11). Resistance in *E. gallinarum* is associated with peptidoglycan precursors terminating in D-alanyl-D-serine (D-Ala-D-Ser) (25) synthesized by VanC-1 (10). Similar ligases, VanC-2 and VanC-3, are present in *E. casseliflavus* and *E. flavescens*, respectively (20).

We have shown that the $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ genes are specific for *E. faecalis* (7) and *E. faecium* (unpublished results), respectively, whereas the *vanA* and *vanB* genes are associated with VanA (8) and VanB types (24) of glycopeptide resistance. The *vanC-1*, *vanC-2*, and *vanC-3* genes are specific for *E. gallinarum* (18), *E. casseliflavus* (20), and *E. flavescens* (20), respectively. In the PCR assay described, species identification of *E. faecium*, *E. faecalis*, and *E. gallinarum* relies on specific amplification of fragments intragenic to $ddl_{E.\ faecium}$ $ddl_{E.\ faecalis}$, and *vanC-1*, respectively, whereas acquired glycopeptide resistance genotypes are identified by specific amplification of portions of the *vanA* and *vanB* genes. The *vanC-2* and *vanC-3* genes of *E. casseliflavus* and *E. flavescens* are almost identical (98% identity) (20). A primer pair intended to amplify fragments internal to both genes was chosen. Thus, these two species which are phenotypically very similar are not distinguished by our assay. The nucleotide sequence of the $ddl_{E.\ faecalis}$ *vanA*, *vanB*, *vanC-1*, and *vanC-2* genes and the partial sequence of the $ddl_{E.\ faecium}$ gene (unpublished results) were aligned (data not shown). Six pairs of oligodeoxynucleotides, each intended to prime amplification of a fragment internal to a gene, were selected in nonconserved regions. Table 1 shows positions and sequences of the oligodeoxynucleotides. Primers of similar size (17 to 19 bases) and GC content ranging from 37 to 53% were selected (Table 1) to avoid variations in annealing temperature and to allow simultaneous use of the six pairs in a single reaction. Each amplification product could be assigned to a gene on the basis of its size (Table 1).

Specificity of primer pairs. Five clinical isolates with glycopeptide resistance genotypes previously identified by DNA-DNA hybridization and nucleotide sequencing were studied: *E. faecium* BM4147 (VanA), *E. faecalis* V583 (VanB), *E. gallinarum* BM4174 (VanC), *E. casseliflavus* ATCC 25788 (VanC), and *E. flavescens* CCM 439 (VanC). First, pairs of oligodeoxynucleotide A to F (Table 1), were evaluated for their ability to amplify fragments intragenic to their targets. Each pair allowed specific amplification of a fragment of the predicted size from the corresponding DNA preparation only (data not shown). As an example, pair A yielded a band corresponding to 732 bp with DNA from *E. faecium* BM4147 (VanA) as a template but not with DNA from the other strains.

In a second step, PCR was performed with DNA from every strain as a template by using the six pairs of oligodeoxynucleotides simultaneously. One or two bands were generated, depending upon the template (Fig. 1). Two PCR products with sizes of ca. 550 and 732 bp resulting from amplification of portions of the $ddl_{E.\ faecium}$ and *vanA* genes (Table 1) were

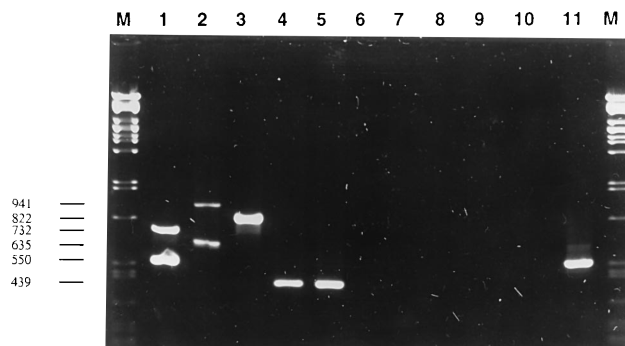

FIG. 1. PCR analysis of DNA from glycopeptide-resistant and -susceptible enterococci. Lanes: 1, *E. faecium* BM4147 (VanA); 2, *E. faecalis* V583 (VanB); 3, *E. gallinarum* BM4174 5 (VanC); 4, *E. casseliflavus* ATCC 24788 (VanC); 5, *E. flavescens* CCM439 (VanC); 6, *E. avium* NCDO 2369; 7, *E. cecorum* NCDO 2679; 8, *E. columbae* STR345; 9, *E. dispar* NCIMB 3000; 10, *E. durans* NCDO 596; 11, *E. faecium* NCDO 942; M, bacteriophage λ DNA (Pharmacia) digested with *Pst*I used as a size standard; the sizes (in base pairs) of the PCR products are indicated on the left.

observed with DNA from *E. faecium* BM4147 (VanA) (Fig. 1, lane 1). Similarly, two bands of 941 and 635 bp corresponding to the $ddl_{E.\ faecalis}$ and *vanB* genes, respectively, (Table 1) were obtained with DNA from *E. faecalis* V583 (VanB) (Fig. 1, lane 2). With *E. gallinarum* BM4174 (VanC), *E. casseliflavus* ATCC 25788 (VanC), and *E. flavescens* CCM 439 (VanC), a single PCR product with a size of 822 bp (Fig. 1, lane 3) or 439 bp (Fig. 1, lanes 4 and 5) corresponding to amplification of *vanC-1*, *vanC-2*, and *vanC-3*, respectively, was generated.

We investigated the specificity of the six primer pairs by testing reference strains of the 20 described enterococcal species. Amplification products with the expected sizes were obtained with DNA from *E. faecium* (Fig. 1, lane 11) and *E. faecalis* (data not shown). By contrast, the primer pairs did not amplify DNA from the other species (Fig. 1, lanes 7 to 10, and data not shown).

The PCR assay was then used on 26 clinical isolates of enterococci that could not be identified to the species level by API 50CH galleries (bioMérieux, Marcy l'Etoile, France). Species were identified for 23 strains including 13 *E. faecalis* strains, 7 *E. faecium* strains, and 3 *E. gallinarum* strains. No PCR product was observed with DNA from two strains, suggesting that they belong to enterococcal species other than those identified by our assay. PCR with DNA of the last strain generated a fragment with an unexpected size (ca. 590 bp) that is currently being sequenced. Genes *vanA* and *vanB* were not detected among the 26 clinical isolates.

Identification to the species level of enterococci is important, in particular for species that are intrinsically resistant to antibiotics (e.g., *E. faecium* to β-lactams or motile enterococci to vancomycin). Unfortunately, identification of *E. faecium*, *E. gallinarum*, and *E. casseliflavus* is based on physiological tests, such as motility at 30°C and yellow pigmentation (13), that are not totally reliable (33). In addition, it has been demonstrated in an animal model of endocarditis that low-level resistance of *Enterococcus* spp. to vancomycin results in therapeutic failure (14). Finally, recent reports have documented the inability of several automated susceptibility testing systems (27, 31, 34) and disk-agar diffusion (27, 29, 31) to detect low-level vancomycin resistance in enterococci (strains with a VanC phenotype and certain strains of the VanB type). These observations emphasize the need for accurate identification of enterococcal species and detection of acquired or intrinsic glycopeptide resistance. Our PCR assay provides a specific and rapid alternative to phenotypic or DNA-DNA hybridization methods for detection of glycopeptide resistance, in particular to low levels of vancomycin, and for identification of clinically relevant enterococci.

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