Development of a PCR Assay for Rapid Detection of Enterococci

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Enterococci are becoming major nosocomial pathogens, and increasing resistance to vancomycin has been
well documented. Conventional identification methods, which are based on culturing, require 2 to 3 days to
provide results. PCR has provided a means for the culture-independent detection of enterococci in a variety of
clinical specimens and is capable of yielding results in just a few hours. However, all PCR-based assays
developed so far are species specific only for clinically important enterococci. We have developed a PCR-based
assay which allows the detection of enterococci at the genus level by targeting the *tuf* gene, which encodes
elongation factor EF-Tu. Initially, we compared the nucleotide sequences of the *tuf* gene from several bacterial
species (available in public databases) and designed degenerate PCR primers derived from conserved regions.
These primers were used to amplify a target region of 803 bp from four enterococcal species (*Enterococcus
avium*, *E. faecalis*, *E. faecium*, and *E. gallinarum*). Subsequently, the complete nucleotide sequences of these
amplicons were determined. The analysis of a multiple alignment of these sequences revealed regions con-
served among enterococci but distinct from those of other bacteria. PCR primers complementary to these
regions allowed amplification of genomic DNAs from 14 of 15 species of enterococci tested (*E. solitarius* DNA
could not be amplified). There was no amplification with a majority of 79 nonenterococcal bacterial species,
except for 2 *Abiotrophia* species and several *Listeria* species. Furthermore, this assay efficiently amplified all 159
clinical isolates of enterococci tested (61 *E. faecium*, 77 *E. faecalis*, 9 *E. gallinarum*, and 12 *E. casseliflavus*
isolates). Interestingly, the preliminary sequence comparison of the amplicons for four enterococcal species
demonstrated that there were some sequence variations which may be used to generate species-specific internal
probes. In conclusion, this rapid PCR-based assay is capable of detecting all clinically important enterococci
and has potential for use in clinical microbiology laboratories.

Enterococci are members of the normal flora of the gastro-
intestinal tract in humans and animals (24). The incidence of
enterococcal infections has increased in recent years because
of widespread multiresistant enterococcal strains and increas-
ing numbers of immunosuppressed patients and catheter-re-
lated infections. In fact, enterococci are now the second most
common nosocomial pathogens in the United States (4, 31).
There are two major pathogenic species in humans, *Enterococ-
cus faecalis* and *E. faecium*, with occasional infections being
caused by *E. durans*, *E. gallinarum*, *E. casseliflavus*, *E. avium*, *E.
hirae*, *E. mundtii*, and *E. raffinosus* (24, 38). Devriese et al. (8)
suggested that the extensive agricultural use of glycopeptides
has created an animal reservoir of resistant enterococci which
may lead to more enterococcal species resistant to glycopep-
tides in animal sources and complicate the control of such
infections. Enterococci resistant to glycopeptides have been
isolated with increasing frequency and have become a major
concern worldwide. Rapid identification of enterococci is im-
portant in reducing the spread of multiresistant enterococci (2,
16).

Identification of enterococci through conventional methods,
i.e., by determining phenotypic characters, is complicated and
often requires 24 to 48 h (7, 11, 28). The automated methods

currently used are unable to reliably identify enterococci other
than *E. faecalis* and *E. faecium* (29, 32, 34). Furthermore,
phenotypic identification of some enterococcal species may be
occasionally difficult or even impossible because these species
lack typical characteristics. More rapid and accurate methods
would be helpful for microbiology laboratories. Several DNA-
based methods for the specific detection of *E. faecalis* or *E.
faecium* have been reported (5, 10, 27, 30). Other molecular
methods, such as contour-clamped homogeneous electric field
electrophoresis patterns, amplified ribosomal DNA spacer
polymorphisms, and randomly amplified polymorphic DNA
analysis, have been used to identify enterococci at the species
level (3, 9, 23, 26, 34). However, it is difficult to adapt these
tests for use in clinical microbiology laboratories because of
their complexity. An *Enterococcus* sp. assay based on the hy-
bridization of rRNA genes (Gen-Probe, San Diego, Calif.) is
commercially available for culture confirmation (6). The sen-
sitivity of this assay is unsatisfactory for direct detection from
clinical specimens.

A variety of conserved genes, including rRNA genes (3, 19,
22), the heat shock protein 60 (HSP60 or CPN60) gene (13,
14), the major cold shock protein gene (12), and the *sod* gene
(39), have been exploited for the detection of bacteria. The *tuf*
gene, encoding elongation factor EF-Tu, is involved in peptide
chain formation and is an essential constituent of the bacterial
genome (15). These characteristics make it a target of choice
for diagnostic purposes. PCR-based assays in which the *tuf*
gene serves as the target sequence have been developed for
Mycoplasma fermentans (1) and *M. pneumoniae* (20). We re-

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TABLE 1. Sources of the 159 enterococcal clinical isolates used in this study

Species	No. of strains from the following source[a]:								
	A	B	C	D	E	F	G	H	Total
E. casseliflavus			12						12
E. faecalis	47	2		2	12	9	1	4	77
E. faecium	7			1	38	10	1	4	61
E. gallinarum	1				7		1		9
Total	55	2	12	3	57	19	3	8	159

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port here the development of a PCR-based assay that targets the *tuf* gene, that can detect most enterococcal species with excellent sensitivity and acceptable specificity, and that has potential for the development of species-specific internal probes.

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MATERIALS AND METHODS

Bacterial strains. Twenty enterococcal strains obtained from the American Type Culture Collection (ATCC), Manassas, Va., were used in this study. These strains represent the following species: *E. avium* (ATCC 14025), *E. casseliflavus* (ATCC 25788), *E. cecorum* (ATCC 43198), *E. dispar* (ATCC 51266), *E. durans* (ATCC 19432), *E. faecalis* (ATCC 19433, ATCC 29212, ATCC 33186, ATCC 49533, and ATCC 51299), *E. faecium* (ATCC 19434 and ATCC 51559), *E. flavescens* (ATCC 49996), *E. gallinarum* (ATCC 49573), *E. hirae* (ATCC 8043), *E. mundtii* (ATCC 43186), *E. pseudoavium* (ATCC 49372), *E. raffinosus* (ATCC 49427), *E. saccharolyticus* (ATCC 43076), and *E. solitarius* (ATCC 49428). An additional 159 clinical isolates of enterococci obtained from various sources were also used in this study (Table 1).

The specificity of the PCR-based assay was verified by use of a battery of ATCC reference strains consisting of 44 gram-negative and 50 gram-positive bacterial species (Table 2). The 159 clinical isolates of enterococci (61 *E. faecium*, 77 *E. faecalis*, 9 *E. gallinarum*, and 12 *E. casseliflavus*) from various origins (Table 1) were also tested to further validate the *Enterococcus*-specific PCR-based assay. The reference strains as well as the clinical isolates were all identified by conventional methods or with an automated MicroScan Autoscan-4 system equipped with a Positive BP Combo Panel Type 6 (Dade Diagnostics, Mississauga, Ontario, Canada). Bacterial strains were grown from frozen stocks kept at $-80°C$ in brain heart infusion medium containing 10% glycerol and were cultured on sheep blood agar or in brain heart infusion broth.

PCR primers. The *tuf* gene sequences available from public databases were analyzed with GCG programs (version 8.0) (Genetics Computer Group, Madison, Wis.). Based on multiple sequence alignments, regions of the *tuf* gene highly conserved among eubacteria were chosen, and PCR primers were derived from these regions with Oligo primer analysis software (version 5.0) (National Biosciences, Plymouth, Minn.). When required, the primers contained inosines or degeneracies at one or more variable positions. Oligonucleotide primers were synthesized with a model 391 DNA synthesizer (Perkin-Elmer Corp., Applied Biosystems Division, Mississauga, Ontario, Canada). PCR primers used in this study are listed in Table 3.

DNA sequencing. An 803-bp portion of the *tuf* gene was sequenced for *E. avium*, *E. faecalis*, *E. faecium*, and *E. gallinarum*. Amplification was performed with 1 ng of genomic DNA prepared by use of a G NOME DNA kit (Bio 101, Vista, Calif.). The 20-μl PCR mixtures used to generate PCR products for sequencing contained 1.0 μM each universal primer (U1 and U2; Table 3), 200 μM each deoxyribonucleoside triphosphate (Pharmacia Biotech Inc., Baie d'Urfé, Québec, Canada), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 0.5 U of *Taq* polymerase (Promega Corp., Madison, Wis.), and TaqStart antibody (Clontech Laboratories Inc., Palo Alto, Calif.). The TaqStart antibody, which is a neutralizing monoclonal antibody for *Taq* DNA polymerase, was added to all PCR mixtures to enhance the efficiency of the amplifications (18).

The PCR mixtures were subjected to thermal cycling (3 min at 95°C and then

35 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, with a 7-min final extension at 72°C) with a PTC-200 DNA Engine thermocycler (MJ Research Inc., Watertown, Mass.). The amplified PCR mixtures were resolved by electrophoresis through 1.5% agarose gels at 4 V/cm for 90 min; the gels were then stained with ethidium bromide and visualized under 312-nm UV light. Subsequently, PCR products having the predicted sizes were recovered from the gels with a QIAquick gel extraction kit (QIAGEN Inc., Mississauga, Ontario, Canada).

The purified DNA fragments were cloned into pCR2.1 vector (Invitrogen Corp., Carlsbad, Calif.). Plasmids were isolated from transformed *Escherichia coli* with a QIAGEN plasmid mini-kit. The presence of DNA inserts in the recombinant plasmids was confirmed by digesting purified plasmid DNA with *Eco*RI (New England Biolabs, Ltd., Mississauga, Ontario, Canada), which allowed excision of the inserted fragments. Both strands of the DNA inserts for each of the selected recombinant plasmids were sequenced with a PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit and an Applied Biosystems 373A sequencer (Perkin-Elmer). In order to exclude the possibility of sequencing errors attributable to misincorporations by *Taq* polymerase, each strand of the insert was sequenced from three different clones.

PCR amplification. For all bacterial species, amplification was performed from purified genomic DNA or from a bacterial suspension whose turbidity was adjusted to that of a 0.5 McFarland standard, which corresponds to approximately 1.5×10^8 bacteria per ml. One nanogram of genomic DNA or 1 μl of standardized bacterial suspension was transferred directly to a 19-μl PCR mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM MgCl₂, 0.2 μM each *Enterococcus*-specific primer (Ent1 and Ent2; Table 3), 200 μM each deoxynucleoside triphosphate (Pharmacia Biotech), 3.3 μg of bovine serum albumin (BSA) (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) per μl, 0.5 U of *Taq* polymerase (Promega), and TaqStart antibody (Clontech). PCR amplification and agarose gel analysis of the amplified products were performed as previously described (21).

The Superlinker phagemid pSL1180 (Pharmacia Biotech) linearized by digestion with *Eco*RI (New England Biolabs) and the primers (IC1 and IC2; Table 3) derived from the multiple cloning sites of this plasmid were used to provide an internal control for all *Enterococcus*-specific PCR-based assays. These primers can amplify a 252-bp product. The internal control was integrated into the PCR-based assays to verify the efficiency of the amplifications and to ensure that significant PCR inhibition was absent. Four thousand copies of the linearized plasmid were added to each PCR. The concentrations of the internal control primers were adjusted to ensure that there was no detrimental effect on the *Enterococcus*-specific amplification. We found that concentrations of 0.1 and 0.04 μM were optimal for 30- and 40-cycle PCRs, respectively.

For determination of the sensitivities of the PCR-based assays, twofold dilutions of purified genomic DNA were used to determine the minimal number of genomes which can be detected.

Nucleotide sequence accession numbers. GenBank accession numbers for the 751-bp partial sequence of the *tuf* gene (excluding the sequences of the two universal amplification primers) are as follows: AF124220 for *E. avium*, AF124221 for *E. faecalis*, AF124222 for *E. faecium*, AF124223 for *E. gallinarum*, AF124224 for *Abiotrophia adiacens*, and AF124225 for *A. defectiva*.

RESULTS

Sequencing of a portion of the *tuf* gene from four enterococcal species. The *tuf* sequences from a number of selected bacterial species, including *E. coli* (J01690), *M. genitalium* (U39732), *Haemophilus influenzae* (U32746), *Neisseria gonorrhoeae* (L36380), *Salmonella typhimurium* (X55116), and *Micrococcus luteus* (M17788), were aligned and compared. Two highly conserved regions were identified, and a pair of primers (U1 and U2) amplifying a region of 803 bp was designed (Table 3). Several degeneracies and inosines were incorporated into these two primers because some positions are variable among eubacteria. These primers allowed the amplification of *tuf* sequences from a wide variety of bacteria, including 14 enterococcal species. By using these primers, we were able to amplify the 803-bp portion of *tuf* for four enterococcal species: *E. avium*, *E. faecalis*, *E. faecium*, and *E. gallinarum*. After purification from agarose gels, the 803-bp PCR product was cloned into a TA cloning vector. Subsequently, the sequence of the inserted DNA fragment was determined by sequencing of three randomly selected clones for each enterococcal species to ensure that no sequencing errors were attributable to misincorporation by the *Taq* polymerase. In order to facilitate the selection of *Enterococcus*-specific primers, we conducted a multiple sequence analysis using the sequences

TABLE 2. Specificity test performed with the *Enterococcus*-specific 40-cycle PCR assay and DNA from a variety of gram-positive and gram-negative bacterial species

Gram-positive bacteria (*n* = 50)	PCR	Gram-negative bacteria (*n* = 44)	PCR
Abiotrophia adiacens ATCC 49175	+	*Acinetobacter baumannii* ATCC 19606	−
Abiotrophia defectiva ATCC 49176	+	*Acinetobacter haemolyticus* ATCC 17906	−
Corynebacterium aquaticus ATCC 14665	−	*Bordetella pertussis* ATCC 9797	−
Enterococcus avium ATCC 14025	+	*Bulkholderia cepacia* ATCC 25416	−
Enterococcus casseliflavus ATCC 25788	+	*Citrobacter diversus* ATCC 27028	−
Enterococcus cecorum ATCC 43198	+	*Citrobacter freundii* ATCC 8090	−
Enterococcus dispar ATCC 51266	+	*Enterobacter aerogenes* ATCC 13048	−
Enterococcus durans ATCC 19432	+	*Enterobacter agglomerans* ATCC 27155	−
Enterococcus faecalis ATCC 19433	+	*Enterobacter cloacae* ATCC 13047	−
Enterococcus faecium ATCC 19434	+	*Escherichia coli* ATCC 25922	−
Enterococcus flavescens ATCC 49996	+	*Haemophilus ducreyi* ATCC 33940	−
Enterococcus gallinarum ATCC 49573	+	*Haemophilus haemolyticus* ATCC 33390	−
Enterococcus hirae ATCC 8043	+	*Haemophilus influenzae* ATCC 9007	−
Enterococcus mundtii ATCC 43186	+	*Haemophilus parahaemolyticus* ATCC 10014	−
Enterococcus pseudoavium ATCC 49372	+	*Haemophilus parainfluenzae* ATCC 7901	−
Enterococcus raffinosus ATCC 49427	+	*Hafnia alvei* ATCC 13337	−
Enterococcus saccharolyticus ATCC 43076	+	*Kingella indologenes* ATCC 25869	−
Enterococcus solitarius ATCC 49428	−	*Klebsiella oxytoca* ATCC 13182	−
Gemella haemolysans ATCC 10379	−	*Klebsiella pneumoniae* ATCC 13883	−
Lactobacillus acidophilus ATCC 4356	−	*Moraxella atlantae* ATCC 29525	−
Listeria grayi ATCC 19120	−	*Moraxella catarrhalis* ATCC 25240	−
Listeria innocua ATCC 33090	+	*Moraxella osloensis* ATCC 19976	−
Listeria ivanovii ATCC 19119	+	*Morganella morganii* ATCC 25830	−
Listeria monocytogenes ATCC 15313	+	*Neisseria caviae* ATCC 14659	−
Listeria murrayi ATCC 25401	−	*Neisseria elangata* ATCC 25295	−
Listeria seeligeri ATCC 35967	+	*Neisseria gonorrhoeae* ATCC 35201	−
Micrococcus luteus ATCC 9341	−	*Neisseria meningitidis* ATCC 13077	−
Staphylococcus aureus ATCC 25923	−	*Neisseria mucosa* ATCC 19696	−
Staphylococcus capitis ATCC 27840	−	*Pasteurella aerogenes* ATCC 27883	−
Staphylococcus epidermidis ATCC 14990	−	*Proteus mirabilis* ATCC 25933	−
Staphylococcus haemolyticus ATCC 29970	−	*Proteus vulgaris* ATCC 13315	−
Staphylococcus hominis ATCC 27844	−	*Providencia alcalifaciens* ATCC 9886	−
Staphylococcus lugdunensis ATCC 43809	−	*Providencia rettgeri* ATCC 9250	−
Staphylococcus saprophyticus ATCC 15305	−	*Providencia rustigianii* ATCC 33673	−
Staphylococcus simulans ATCC 27848	−	*Providencia stuartii* ATCC 29914	−
Staphylococcus warneri ATCC 27836	−	*Pseudomonas aeruginosa* ATCC 27853	−
Streptococcus agalactiae ATCC 13813	−	*Pseudomonas fluorescens* ATCC 13525	−
Streptococcus anginosus ATCC 33397	−	*Pseudomonas stutzeri* ATCC 17588	−
Streptococcus bovis ATCC 33317	−	*Salmonella typhimurium* ATCC 14028	−
Streptococcus constellatus ATCC 27823	−	*Serratia marcescens* ATCC 8100	−
Streptococcus crista ATCC 51100	−	*Shigella flexneri* ATCC 12022	−
Streptococcus intermedius ATCC 27335	−	*Shigella sonnei* ATCC 29930	−
Streptococcus mitis ATCC 33399	−	*Stenotrophomonas maltophilia* ATCC 13843	−
Streptococcus mutans ATCC 25175	−	*Yersinia enterocolitica* ATCC 9610	−
Streptococcus parasanguis ATCC 15912	−		
Streptococcus pneumoniae ATCC 6303	−		
Streptococcus pyogenes ATCC 19615	−		
Streptococcus salivarius ATCC 7073	−		
Streptococcus sanguis ATCC 10556	−		
Streptococcus suis ATCC 43765	−		

mentioned above (available in public databases) as well as partial *tuf* sequences from staphylococci, streptococci, enterococci, *Listeria* spp., and *Abiotrophia* spp. (all obtained from our laboratory; unpublished data). By using this approach, we were able to identify regions conserved in the four enterococcal species but variable in the other bacterial species. The *Enterococcus*-specific PCR primers were derived from these regions. A similarity comparison of the *tuf* sequences for the enterococcal species *E. avium*, *E. faecalis*, *E. faecium*, and *E. gallinarum* as well as for two *Abiotrophia* species is given in Table 4. The sequence similarities for the 803-bp fragment in these species ranged from 85 to 91%.

The selected primers revealed no more than two mismatches within the *tuf* sequences of the enterococcal species, except for

E. faecium and two *Abiotrophia* species, in which three mismatches were present at the 5′ end of the 5′ primer. Importantly, more than five mismatches were found in the corresponding regions of the other bacterial species mentioned above. Since the mismatches in the other bacteria were clustered at the 3′ end of the primers, a position critical for discriminatory PCR amplification, the amplification of bacterial species other than enterococci could be efficiently prevented.

Amplifications with the *Enterococcus*-specific PCR assay. The specificity of the assay was assessed by performing 30-cycle and 40-cycle PCR amplifications with the panel of gram-positive (50 species from 9 genera) and gram-negative (44 species from 21 genera) bacterial species listed in Table 2. The PCR assay was able to detect 14 of 15 enterococcal species tested in

TABLE 3. PCR primers used in this study

Primer	Sequence	Nucleotide positions[a]	Product length (bp)
Universal amplification			803
U1	5′-AAYATGATIACIGGIGCIGCICARATGGA-3′	271–300	
U2	5′-AYRTTITCICCIGGCATIACCAT-3′	1051–1073	
Enterococcus specific			112
Ent1	5′-TACTGACAAACCATTCATGATG-3′	618–639	
Ent2	5′-AACTTCGTCACCAACGCGAAC-3′	708–729	
Internal control			252
IC1	5′-TCTCGAGCTCTGTACATGTCC-3′		
IC2	5′-GTTCTAGAGGTACCGGTTGTT-3′		

[a] The nucleotide positions given are for the *E. coli tuf* sequence (GenBank accession no. J01690).

both 30-cycle and 40-cycle regimens. *E. solitarius* was the only enterococcal species tested that was not amplified by the *Enterococcus*-specific assay (Fig. 1). For 30-cycle PCR, all bacterial species tested other than enterococci were negative, except for two *Abiotrophia* species, *A. adiacens* and *A. defectiva*. For 40-cycle PCR, four *Listeria* species, *Listeria innocua*, *L. ivanovii*, *L. monocytogenes*, and *L. seeligeri*, were also positive in addition to the enterococci and *Abiotrophia* species. The other species tested remained negative. The internal control was always efficiently amplified when no target DNA was present, thereby showing the absence of PCR inhibitors. On the contrary, the internal control was not amplified when target DNA was present in a sample (Fig. 1). This result is explained by the fact that the concentrations of the internal control primers were limited in order to favor the amplification of the target DNA. Tests of a collection of clinical isolates comprising *E. faecalis* (n = 77), *E. faecium* (n = 61), *E. gallinarum* (n = 9), and *E. casseliflavus* (n = 12) showed a uniform amplification signal with both the 30-cycle and the 40-cycle PCR assays and a perfect relationship between the genotype and classical means of identification.

The sensitivity of our *Enterococcus*-specific assay with 30-cycle and 40-cycle PCR protocols was determined by using purified genomic DNA from 14 enterococcal species. For PCR with 30 cycles, we found a detection limit ranging from 330 to 1,700 copies of genomic DNA, depending on the enterococcal species tested (Table 5). In order to enhance the sensitivity of the assay, we increased the number of cycles. For PCR with 40

cycles, the detection limit was lowered to two to eight genome copies (Table 5).

DISCUSSION

Enterococci can be encountered throughout the environment, from human, animal, and food sources. The emergence and dissemination of vancomycin-resistant enterococci (VRE) underscore the importance of the rapid detection of these organisms (16). Conventional identification of some enterococci is difficult because no phenotypic criteria are available to unequivocally separate the genus *Enterococcus* from the other genera of gram-positive cocci. A number of tests, such as Lancefield group D antigen, the ability to grow in 6.5% NaCl broth, the pyrolidonylarylamidase test, the leucinearylamidase test, and the bile esculin test, are all valuable for the identification of enterococci. Unfortunately, none of these tests alone or in combinations provides a phenotype unique to the enterococci (7). Furthermore, such tests require 24 to 48 h to provide results after pure cultures are obtained. Even with automated systems, incubation for up to 24 h is routinely required, and some additional tests may have to be carried out for species differentiation (29, 32). Since the vancomycin resistance genes are transferable among different enterococcal species or even

TABLE 4. Sequence homology for an 803-bp portion of the *tuf* gene and *Enterococcus*-specific PCR products for four enterococcal species and two *Abiotrophia* species

Species	% of sequence similarity[a] for:					
	A. adiacens	*A. defectiva*	*E. avium*	*E. faecalis*	*E. faecium*	*E. gallinarum*
A. adiacens		85	89	89	89	87
A. defectiva	89.8		87	85	86	85
E. avium	92.7	97.1		91	90	90
E. faecalis	92.7	91.3	92.7		89	90
E. faecium	95.7	89.8	91.3	91.3		90
E. gallinarum	94.2	95.7	95.7	95.7	94.2	

[a] The numbers in the upper right triangle are the percentage of similarity for the sequenced 803-bp fragment, and those in the lower left triangle represent the percentage of similarity for the 69-bp sequences flanked by the two *Enterococcus*-specific PCR primers. The similarity scores were obtained by use of the PILEUP program of the GCG software package.
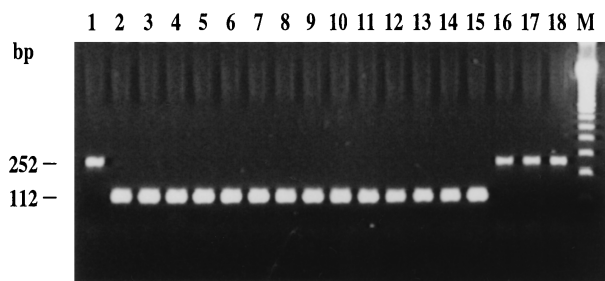

FIG. 1. Example of multiplex PCR amplifications with the *Enterococcus*-specific PCR assay and the internal control. PCR assays (40 cycles) were performed with 1 μl of genomic DNA (1 ng/μl) from various bacteria and 4,000 copies of linearized pSL1180 as an internal control. The internal control was not amplified when target DNA was present due to competitive inhibition by amplification of *Enterococcus* DNA. Lanes: 2, *E. avium* ATCC 14025; 3, *E. casseliflavus* ATCC 25788; 4, *E. cecorum* ATCC 43198; 5, *E. durans* ATCC 19432; 6, *E. dispar* ATCC 51266; 7, *E. faecalis* ATCC 19433; 8, *E. faecium* ATCC 19434; 9, *E. flavescens* ATCC 49996; 10, *E. gallinarum* ATCC 49573; 11, *E. hirae* ATCC 8043; 12, *E. mundtii* ATCC 43186; 13, *E. pseudoavium* ATCC 49372; 14, *E. raffinosus* ATCC 49427; 15, *E. saccharolyticus* ATCC 43076; 16, *E. solitarius* ATCC 49428; 17, *E. coli* ATCC 25922; 1 and 18, controls to which no DNA was added; M, 100-bp molecular size standard ladder.

TABLE 5. Detection limits of the *Enterococcus*-specific PCR assays for 14 enterococcal species

Species	Detection limit (no. of genomes) for PCR with the following no. of cycles[a]:	
	30	40
E. avium	1,700	8
E. cecorum	1,700	2
E. casseliflavus[b]	670	4
E. dispar	ND	8
E. durans	330	4
E. faecalis	670	2
E. faecium	670	8
E. flavescens[b]	ND	8
E. gallinarum	330	8
E. hirae	330	4
E. mundtii	1,700	8
E. pseudoavium	330	2
E. raffinosus	1,700	8
E. saccharolyticus	330	2

[a] The genome size for all enterococcal species was considered the same as that of *E. faecalis* (3.0 Mb). Serial twofold dilutions of genomic DNA from each enterococcal species were tested to determine the detection limit of the assay for each species. ND, not determined.

[b] *E. casseliflavus* and *E. flavescens* are now considered the same species (33).

among different genera (38), the inability to detect enterococci promptly may cause delays in reporting VRE; this situation may lead to complex and costly containment efforts to eliminate VRE colonization and infection.

The development of rapid and sensitive DNA-based assays which are applicable for the direct detection of enterococci from clinical specimens may improve the rapidity and the accuracy of the diagnosis of enterococcal infections. The validity and versatility of PCR in applications for the detection of nucleic acids from a variety of infectious agents have been well reviewed by Whelen and Persing (36). In the present study, we have developed a rapid PCR-based assay to improve the diagnosis of enterococcal infections. Initially, PCR primers complementary to highly conserved regions of the *tuf* gene among eubacteria were designed and used to amplify an 803-bp portion of the *tuf* gene from 14 enterococcal species. By sequencing four enterococcal species, which represented four different enterococcal subgroups based on 16S rRNA gene analysis, we have obtained adequate sequence information to design a pair of *Enterococcus*-specific PCR primers. Subsequently, a PCR-based assay was set up and optimized to be simple and rapid. Among 50 species of gram-positive (representing 9 genera) and 44 species of gram-negative (representing 21 genera) clinically important bacteria tested, this PCR-based assay was able to detect all enterococcal species tested, except for *E. solitarius*, which in fact does not appear to be a member of the genus *Enterococcus* based on a phylogenetic analysis (25, 37). Two *Abiotrophia* species were also amplified efficiently under the same amplification conditions. Moreover, amplification of four *Listeria* species was also observed when the number of PCR cycles was increased from 30 to 40. It should be noted that DNA from *Listeria* species was amplified about 100 times less efficiently than enterococcal DNA. However, most clinically important bacteria can be easily differentiated from enterococci by this assay, indicating its usefulness for the detection of enterococci. The concomitant use of species-specific internal probes should allow exclusion of *Abiotrophia* and *Listeria* species by increasing the specificity of the *Enterococcus*-specific PCR-based assay.

To elucidate the fact that the developed PCR-based assay failed to detect *E. solitarius*, which rarely causes infections in humans, we amplified and sequenced an 890-bp portion of *tuf* encompassing the 803-bp region by using another pair of primers (unpublished data). The sequence similarity between *E. solitarius* and other enterococci ranged from 79 to 81%, while that between the other enterococci ranged from 89 to 91%. This finding supports 16S rRNA data reported by others (25, 37) suggesting that *E. solitarius* is not a member of the genus *Enterococcus* (93.0 to 94.8% homology) but is phylogenetically more closely related to the genus *Tetragenococcus* (97.8% homology). Sequence data revealed that there were six mismatches at the *Enterococcus*-specific 3′-primer binding site which led to a failure in the amplification of *E. solitarius* by the developed PCR-based assay.

The finding that *A. adiacens* and *A. defectiva*, formerly referred to as nutritionally variant streptococci (17), were also positive in our *Enterococcus*-specific PCR assay indicates a high level of similarity of the *tuf* genes at the primer binding sites in the genus *Abiotrophia* and the genus *Enterococcus*. Sequencing of the 803-bp region of *tuf* showed that the sequence similarities between the enterococcal and *Abiotrophia* spp. ranged from 85 to 89%, values which are quite high. For comparison, the *tuf* sequences among enterococci are 89 to 91% similar. Therefore, it is not surprising that PCR primers derived from the *tuf* sequence amplify DNA from *Abiotrophia* spp. as well. In fact, there are only two mismatches at the 5′ end of the *Enterococcus*-specific primers compared to the sequences of *A. defectiva* and *A. adiacens*. Consequently, the sensitivity level achieved for the two *Abiotrophia* species was similar to that obtained for the enterococcal species. In addition, several *Listeria* species were detectable when 40-cycle PCR was performed, even though three mismatches located near the 3′ end of the upper primer were found between the four *Listeria* species and enterococci. However, it should be noted that *Listeria* DNA was amplified approximately 100 times less efficiently than enterococcal DNA. The four *Listeria* species, *L. monocytogenes*, *L. innocua*, *L. ivanovii*, and *L. seelegeri*, showed sequence similarities to enterococci ranging from 82 to 83% (data not shown). Based on the level of sequence divergence for the *tuf* gene, it should be possible to develop enterococcal species-specific internal probes which allow discrimination of members of the genera *Abiotrophia* and *Listeria* from those of the genus *Enterococcus*.

Others have developed *E. faecalis*-specific and *E. faecium*-specific PCR-based assays by targeting various genes. The target genes include (i) *ddl* (coding for D-alanine-D-alanine ligase) for the species-specific detection of *E. faecalis* and *E. faecium* (10, 30) and (ii) *PBP5* (coding for penicillin binding protein) for the species-specific detection of *E. faecalis* (27). A PCR-based assay amplifying different *vanC* genes (coding for intrinsic vancomycin resistance in *E. gallinarum*, *E. casseliflavus*, and *E. flavescens*) has also been developed to specifically detect *E. gallinarum*, *E. casseliflavus*, and *E. flavescens* (10). A sequence of unknown coding potential, selected from an *E. faecium* genomic library, has been used to develop an *E. faecium*-specific PCR-based assay (5). Although *E. faecalis* and *E. faecium* account for a majority of enterococcal infections, other enterococci may be associated with infections. However, currently available systems based on culturing for the identification of gram-positive cocci are often unable to correctly identify these less frequently encountered enterococci (29, 32, 34).

Tyrrell et al. (35) reported using an internally transcribed spacer region PCR to identify enterococcal species based on characteristic amplicon profiles and the different patterns of *Sau*3A-digested PCR amplicons. Donabedian et al. (9) dem-

onstrated that contour-clamped homogeneous electric field electrophoresis patterns and DNA-DNA hybridization with biotin-labeled genomic DNAs from type strains of enterococci used as probes may be suitable for species differentiation of some enterococci. However, neither method is feasible for routine use in the clinical laboratory because of high complexity and must be used in conjunction with phenotypic tests to provide reliable results (9, 35). Recently, Quednau et al. (26) and Monstein et al. (23) identified enterococci to the species or species group level by using randomly amplified polymorphic DNA methods. A simple test for the detection of *Enterococcus* spp. has been developed by Gen-Probe for use as a culture confirmation assay; this test was 100% accurate in identifying enterococcal isolates from cultures by hybridization to rRNA (6). However, this assay does not offer potential for enterococcal species identification. Moreover, the sensitivity of the Gen-Probe assay is not sufficient for direct detection of enterococci from clinical specimens.

Besides identification of bacterial species by species-specific PCR-based assays, identification may also be performed by coupling the amplification of a highly conserved gene with hybridization to internal probes or DNA sequencing (1, 14, 20, 22). Many conserved genes have been selected as targets for this purpose. Among them, the 16S rRNA gene has been used to detect a wide variety of eubacteria because the presence of conserved regions and variable regions in this gene provides the possibility of developing PCR-based assays suitable for detecting and identifying bacteria at the species level or higher taxonomic levels (22). Other genes have been exploited for similar purposes. The *sod* gene, coding for superoxide dismutase, has been used as a target to amplify 28 species of mycobacteria and to differentiate one from another with probes recognizing species-specific regions (39). A similar approach has been used for the detection of staphylococci by targeting the chaperonin 60 (*cpn60*) gene (13, 14). These methods are specific, but their sensitivity remains unclear. The latter is critical when such tests are used to detect bacteria directly from clinical specimens. Berg et al. (1) have developed a PCR-based detection system for *M. fermentans* by targeting the *tuf* gene; the system has a high sensitivity. A similar assay has also been used for the identification of *M. pneumoniae* (20).

The *tuf* gene acts in translation to bring aminoacylated tRNA molecules to the ribosome. This gene represents an ideal candidate target for diagnostic purposes because it is highly conserved at the nucleotide level and ubiquitous in bacteria. By analyzing a rather small *tuf* sequence data set available in public databases, we were able to design PCR primers which could amplify an 803-bp portion of the *tuf* gene from a variety of bacteria, including members of the genera *Enterococcus*, *Streptococcus*, and *Staphylococcus*. We found that there were more nucleotide sequence variations in the *tuf* gene sequences than in the corresponding 16S rRNA gene sequences for four enterococcal species (i.e., *E. faecalis*, *E. faecium*, *E. avium*, and *E. gallinarum*). An analysis of the *tuf* sequences from gram-positive bacteria allowed the development of a PCR-based assay amplifying 14 of 15 enterococcal species tested. The PCR-based assay described here differentiates enterococci from most clinically relevant bacteria, indicating that the *tuf* gene is a target of choice for the molecular detection of enterococci. Furthermore, the amplicon sequence polymorphism should be sufficient to provide discriminatory internal probes specific for clinically important enterococcal species. The sensitivity of the 30-cycle PCR-based assay varies from 330 to 1,700 copies of enterococcal genomes for the 14 enterococcal species detected. This sensitivity level is sufficient

for culture confirmation assays. It is possible to efficiently increase the sensitivity of the assay. For example, in sensitivity assays performed with 40-cycle PCR, the detection limit was reduced to about two to eight copies of enterococcal genomes for the same 14 enterococcal species. This sensitivity level should be sufficient for the direct detection of enterococci in clinical specimens, such as fecal or urine samples. The use of this PCR-based assay coupled with species-specific internal probes specific for clinically important enterococci and other PCR assays targeting vancomycin resistance genes should provide a useful screening test for VRE from rectal swabs. Such an assay is currently under development in our laboratory.

In conclusion, we have developed a PCR-based diagnostic assay which is simple to conduct and reliable for the detection of enterococci. This assay offers an alternative to currently used methods and should allow the identification of clinically important enterococcal species if coupled with species-specific probes complementary to internal regions of the amplicons. This new diagnostic tool may lead to the early diagnosis of enterococcal infections, which is essential for the prevention and control of transmission of the infections.

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