

Rapid Identification of Clinically Relevant *Enterococcus* Species by Fluorescence In Situ Hybridization[∇]

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Received 24 April 2007/Returned for modification 29 May 2007/Accepted 26 July 2007

A fluorescence in situ hybridization assay for the rapid identification of clinically relevant enterococci (*Enterococcus faecalis*, *E. faecium*, *E. gallinarum*, the VanC-type resistance group) was developed and evaluated with 33 reference strains, 68 clinical isolates, and 58 positive blood cultures. All probes showed excellent sensitivities and specificities.

Enterococci are frequent causes of nosocomial infections, like urinary tract infections and sepsis (13). The rapid identification of enterococci to the species level is important for treatment decisions, because different species show different resistance patterns. While most strains of *Enterococcus faecalis* are susceptible to penicillins, first-line therapy for infections caused by most strains of *Enterococcus faecium* requires the use of a glycopeptide antibiotic. In turn, members of the VanC-type resistance group, such as *Enterococcus gallinarum* and *Enterococcus casseliflavus*, are intrinsically resistant to vancomycin but are mainly susceptible to penicillins. Species identification of enterococci by phenotypic methods is time-consuming (5–7, 14, 20, 21), however, and the misidentification of *E. faecium* as *E. gallinarum* or *E. casseliflavus* and vice versa is a frequent problem (4, 7, 12).

Fluorescence in situ hybridization (FISH) with fluorescently labeled oligonucleotide probes targeting the rRNA is a rapid and easy-to-perform method that has already been used for the detection of *E. faecalis* and *E. faecium* in blood cultures (10, 11, 16, 17, 22). The published *Enterococcus*-specific probes have not been thoroughly evaluated, however (10, 11, 16, 17, 22), and are partly not specific, according to a current GenBank database analysis (1, 10, 11). Others are implemented under unusual conditions, such as with formamide concentrations of either 10% (22) or 25% (2) or at a hybridization temperature of 50°C (8), and may therefore not be combined with other DNA probes for FISH under standard hybridization conditions.

We therefore designed new FISH probes, including species-specific probes for *E. faecium*, *E. faecalis*, and *E. gallinarum*; a group-specific probe for the VanC-type resistance group; and two genus-specific probes for all enterococci (Table 1). The probes were designed by using ARB software and were directly 5' labeled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester or the sulfoindocyanine dye Cy3 (Thermo, Ulm, Germany). All probes were used with a formamide concentration of 30%, as described previously (17). This allows the simultaneous implementation of all probes on multiple field slides.

In the first step, the probes were evaluated with 14 *Enterococcus* spp. and 19 nonenterococcal reference strains: *Enterococcus avium* (ATCC 14025), *E. casseliflavus* (ATCC 25788, ATCC 12755), *Enterococcus durans* (ATCC 20633), *E. faecalis* (ATCC 29212, ATCC 51299, ATCC 19433), *E. faecium* (ATCC 19434), *Enterococcus flavescens* (ATCC 7370), *E. gallinarum* (ATCC 700425, ATCC 35038), *Enterococcus hirae* (ATCC 8043), *Enterococcus mundtii* (ATCC 43186), *Enterococcus raffinosus* (ATCC 49427), *Streptococcus agalactiae* (ATCC 13813), *Streptococcus anginosus* (ATCC 12395), *Streptococcus bovis* (ATCC 33317), *Streptococcus constellatus* (ATCC 27823), *Streptococcus mitis* (ATCC 49456), *Streptococcus mutans* (ATCC 25175), *Streptococcus oralis* (ATCC 35037), *Streptococcus parasanguis* (ATCC 15912), *Streptococcus pneumoniae* (ATCC 6303, ATCC 49619, ATCC 20566, ATCC 11865), *Streptococcus pyogenes* (ATCC 12344), *Streptococcus salivarius* (ATCC 7073), *Streptococcus sanguis* (ATCC 10556), *Staphylococcus aureus* (ATCC 29213, ATCC 43300), *Staphylococcus epidermidis* (ATCC 12228), and *Staphylococcus saprophyticus* (ATCC 15305). All probes correctly detected the respective target organisms. Apart from probes ENC 176 and ENU 1470, which cross-reacted with *Staphylococcus saprophyticus* and one isolate of *Enterococcus durans*, respectively, all probes had an analytical specificity of 100%. Because of the observed cross-reaction, five well-characterized isolates of *E. durans* (15) were additionally included and did not show any cross-reaction.

The probes were further evaluated with 68 clinical isolates, including 49 enterococcal strains and 1 *Abiotrophia adiacens* strain from blood cultures and 18 strains of vancomycin-resistant enterococci (VRE). The VRE were grown on VRE screening agar (BD, Heidelberg, Germany) and included 3 *E. faecium vanA* strains, 1 *E. faecium vanB* strain, and 14 *E. gallinarum* strains. All isolates were investigated by FISH. The clinical isolates were identified by growth on bile-esculin agar with esculin hydrolysis, use of the API 20 Strep system (BioMérieux, Nuertingen, Germany), formation of methyl- α -D-glucopyranoside (according to Devriese et al. [3]), and pigmentation. Sequencing of the complete 16S rRNA gene was performed as described previously for isolates with ambiguous phenotypic identification results ($n = 5$) (1, 9). All FISH probes consistently reacted in accordance with the identification results (Table 2).

It was further assessed whether FISH may also be used for the rapid identification of enterococci directly from positive blood cultures. Samples from 58 positive blood cultures micro-

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[∇] Published ahead of print on 1 August 2007.

TABLE 1. Sequences of oligonucleotide probes used in this study

Probe	Target organism	Probe target	Sequence	Reference
EUB 338	All bacteria	16S rRNA	GCT GCC TCC CGT AGG AGT	2
ENC 176	<i>Enterococcus</i> spp. ^a	23S rRNA	CA GTT CTC TGC GTC TAC CTC	This study
ENC 221	<i>Enterococcus</i> spp. ^b	16S rRNA	CAC CGC GGG TCC ATC CAT CA	This study
EGAC 183	VanC group of enterococci ^c	16S rRNA	CAA CTT TCT TCC ATG CGG AAA AT	This study
ENF 191	<i>Enterococcus faecalis</i>	16S rRNA	GAA AGC GCC TTT CAC TCT TAT GC	This study
ENU 1470	<i>Enterococcus faecium</i>	23S rRNA	GAC TCC TTC AGA CTT ACT GCT TGG	This study
ENU 140	<i>Enterococcus faecium</i>	23S rRNA	TTACACAATCGTAAACATCCTA	This study
EGA 141	<i>Enterococcus gallinarum</i>	23S rRNA	ATCACAACTGTGTAACATCCTAT	This study

^a Also *Carnobacterium* spp.

^b Also *Granulicatella* spp. and *Abiotrophia* spp.

^c Comprising *Enterococcus gallinarum*, *E. flavescens*, and *E. casseliflavus*.

scopically showing gram-positive diplococci or cocci in chains suggestive of enterococci were included. One additional sample was investigated but was not included in the data analysis because the FISH slide contained no bacteria, most probably because of a mistake during sample preparation. For sample preparation, the blood culture medium was centrifuged at 130 × g for 10 min in order to remove the erythrocytes. The supernatant was again centrifuged at 1,800 × g for 5 min, and the bacterial pellet was suspended in 0.9% saline. Ten microliters of the suspension was applied to each field of an eight-well glass slide, air dried, and fixed in methanol for 10 min. Five of the 58 samples contained mixtures of different enterococci or enterococci and other bacteria. Including the mixtures and pure cultures, 32 *Enterococcus* spp. and 28 *Streptococcus* spp. and related species were cultured. All enterococci were correctly identified to the species level by FISH, including the cases in mixed cultures (Table 2). All 28 nonenterococcal isolates gave correct negative results with the probes (*Streptococcus mitis*, n = 6; *S. pneumoniae*, n = 5; *S. oralis*, n = 3; *S. pyogenes*, n = 3; *S. sanguis*, n = 2; *S. anginosus*, n = 2; *S. salivarius*, n = 1; *Gemella* spp., n = 3; *Lactococcus lactis*, n = 2; and *Abiotrophia adiacens*, n = 1).

Altogether, the newly designed FISH probe set proved highly sensitive and specific for the rapid identification of clinically relevant enterococcal species in the clinical microbiology laboratory. Because the assay contains multiple probes in a hierarchical manner (18), rare cases of malfunctioning of single probes will become apparent through contradictory binding patterns. A considerable advantage of the FISH assay is the

ability to discriminate reliably between *E. faecium* and enterococci of the VanC complex, which are often confused by commercial phenotypic methods (4–7, 12, 14, 20, 21). Identification of these strains is of major importance because of their typical resistance patterns. In addition, FISH was very useful during a VRE outbreak in our hospital, allowing the rapid differentiation of the *E. faecium* outbreak strain from VanC-harboring *E. gallinarum* isolates also growing on the VRE screening plates and showing ambiguous results with the API system. The black pigmentation of the colonies caused by esculin hydrolysis did not interfere with the FISH procedure. In addition to the identification of enterococci from pure cultures, FISH proved highly useful for direct application to positive blood cultures, even including blood cultures with polymicrobial growth. The FISH assay may thus significantly shorten the time to the retrieval of results in the clinical microbiology laboratory and to the initiation of pathogen-adapted antimicrobial therapy, especially for septic patients.

We thank Jan Peters (Federal Institute for Protection of Consumers and Veterinary Medicine, Berlin, Germany) for supplying us with strains of *E. durans*.

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TABLE 2. Identification of clinical isolates by FISH

Isolated species	No. of isolates identified/total no. tested (%):	
	Identification by FISH of clinical isolates from subculture	Direct identification by FISH of enterococci in microscopically positive blood cultures
<i>E. faecium</i>	23/23 (100)	18/18 (100)
<i>E. faecalis</i>	26/26 (100)	12/12 (100)
<i>E. avium</i>	3/3 (100) ^a	
<i>E. gallinarum</i>	15/15 (100)	2/2 (100)
Streptococci		27/27 ^b
<i>Abiotrophia adiacens</i>	1/1 ^b	1/1 ^b
Total	68/68 (100)	60/60 (100)

^a Correct identification as *Enterococcus* spp. other than *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. casseliflavus*, and *E. flavescens*.

^b Correct identification as non-*Enterococcus* spp.

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