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ORIGINAL ARTICLE

Development and evaluation of a Quadruplex Taq Man real-time PCR assay for simultaneous detection of clinical isolates of *Enterococcus faecalis*, *Enterococcus faecium* and their *vanA* and *vanB* genotypes

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ABSTRACT

Background and Objectives: We developed and evaluated the utility of a quadruplex Taqman real-time PCR assay that allows simultaneous identification of vancomycin-resistant genotypes and clinically relevant enterococci.

Materials and Methods: The specificity of the assay was tested using reference strains of vancomycin-resistant and susceptible enterococci. In total, 193 clinical isolates were identified and subsequently genotyped using a Quadruplex Taqman real-time PCR assay and melting curve analysis. Representative Quadruplex Taqman real-time PCR amplification curve were obtained for *Enterococcus faecium*, *Enterococcus faecalis*, vanA-containing *E. faecium*, vanB-containing *E. faecalis*.

Results: Phenotypic and genotypic analysis of the isolates gave same results for 82 enterococcal isolates, while in 5 isolates, they were inconsistent. We had three mixed strains, which were detected by the TaqMan real-time PCR assay and could not be identified correctly using phenotypic methods.

Conclusion: Vancomycin resistant enterococci (VRE) genotyping and identification of clinically relevant enterococci were rapidly and correctly performed using TaqMan real-time multiplex real-time PCR assay.

Key words: Enterococci, Vancomycin, Multiplex TaqMan real-time PCR

INTRODUCTION

Enterococci are one of the major causes of hospital-acquired infections although they can also cause human infections in the community (1). Hospital acquired infection is defined as an infection which develops 48 h after hospital admission not being the reason of the admission. Enterococci are individual, paired, or short-chain Gram-positive, catalase-negative cocci. This organism is mainly commensals in gastrointestinal tract of healthy individuals but may become opportunistic pathogens in immune-compromised hosts and in patients who have received broad-spectrum antimicrobial therapy or had a prolonged hospital stay (2).

Enterococci display both intrinsic and acquired resistance patterns to many antimicrobials, such as glycopeptides, β-lactams, fluoroquinolones and aminoglycosides which dramatically reduce the remaining therapeutic options among patients infected with these organisms (3). Along with *E. faecalis*, the genus *Enterococcus* includes *E. faecium* which found less frequently than *Enterococcus faecalis* in clinical isolates and are significantly more resistant to vancomycin than *E. faecalis*.

E. faecalis and *E. faecium* are the main causative agents for serious relevant nosocomial infection in humans, thereby it is necessary to discriminate between the low-level vancomycin resistant *E. faecalis* and *E. faecium* isolates with the other low-virulence motile enterococcal species (4, 5). Moreover, since vancomycin offers as last line of

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Table 1: Sequence of primers and probes used in this study

Primer/probe	Forward (5'-3')	Reverse (5'-3')		Amplicon size
van A primers	ATCAACCATGTTGATGTAGC	AAGGGATACCGGACAATTCA	This study	136bp
van A probe:	5-JOE-TCCATCTTCACCTGACTTGCCA-BHQ1-3		This study	
van B primers	ACCCTGTCTTTGTGAAG	GAAATCGCTTGCTCAAT	This study	121bp
van B probe:	Tex Red-TCCATCATATTGTCCTGCTGCTTCTAT-Tamra		This study	
E.faecium primers	CGTAGCATTCTATGATTATGAAGCC	CATCGTGTAAGCTAACTTCG	This study	124bp
E.faecium probe:	FAM- CAGATTCCAGCCGAAGTGCC- TAMRA		This study	
E.faecalis primers	GACAGGAAAGAAACTAGGAGGAC	AAACAGACACATCGTGCT	This study	84bp
E.faecalis probe:	CY5-CACTTCTGCCGCCATACAACAA-Tamra		This study	

intravenous antibiotic therapy, so resistance to this antibiotic becomes an important clinical concern among *Enterococci* infections (6).The important role of *E. faecalis* and *E. faecium* isolates in hospital and community acquired infections makes detection and identification of this organism a priority for state and local health departments as well as laboratories that deal with pathogenic bacteria (7).

The culture techniques are routinely used for detecting of *E. faecalis* and *E. faecium* but PCR-based methods have given researchers the ability to detect small amounts of DNA to increase the sensitivity in diagnostics (8-10). A significant issue to replace conventional PCR for qPCR in diagnostics is the problem of contamination, which is almost zero in qPCR. Moreover, in diagnostics with conventional PCR, the nature of the PCR product on agarose gel was always confirmed by probe detection after Southern blotting (10-13). Real-time quantitative PCR (qPCR) using a combination of specific primers and fluorescent probes overcomes these deficiencies (14). In real-time PCR, no post amplification detection is needed. Besides, examining more genes in one reaction not only reduce experiment time but also decrease cost of the experiments. Several real-time and standard PCR assays have been developed in multiplex formats that allow the detection of multiple targets within a single reaction tube (15-18).

The objective of this study was to develop a quadruplexed real-time PCR assay which can quickly and accurately identify *E. faecalis* and *E. faecium* and their *vanA* and *vanB* genotypes, simultaneously. In this assay, Taqman probes were used to detect *ddl E. faecalis* , *ddl E. faecium* ,Tn1546 (vanA genotype) and Tn1549 (vanB genotype) genes in *E. faecalis* and *E. faecium*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The method was developed by using standard strains of *E. faecium* BM4147, *E. faecalis* 33186, *E. faecalis* ATCC 29122 and *E. coli* ATCC 25922. To ascertain the effectiveness of the assay, 235 clinical enterococcal isolates including urine, blood, sputum, cerebrospinal fluid (CSF), pleura, and wound were obtained in this study. The bacterial isolation from clinical samples was performed using sheep blood agar as previously described (19) and were identified as *Enterococcus* spp. based on Gram staining, the catalase test, the hydrolysis of esculin in the presence of bile, and growth in brain-heart infusion broth containing 6.5% NaCl (20). All isolates were stored in BHI broth with 15% glycerol at -70°C until further analysis.

Evaluation of susceptibility to vancomycin was done by standard disk diffusion method and E-test (AB Biodisk, Solna) according to CLSI guideline. For E-test, the turbidity of bacterial inoculums were adjusted at 0.5 McFarland and before plating on Mueller-Hinton (MH) agar. E-test strips were then placed on MH agar and incubated at 35°C for a full 24 hours. Isolates with MIC≥32 and 4< MIC<32 were selected for molecular analysis.

Preparation of DNA. Total genomic DNA was extracted from each isolate by DNA extraction Kit (DNA Technology, Denmark) and the DNA concentration was estimated using a Nano Drop spectrophotometer (ABI, USA).

Primer and *probe design.* Real-time PCR assays using 5′-hydrolysis Taqman probes were designed for *ddl* gene of *E. faecalis*, *E. faecium* , *vanA* and

Table 2: Prevalence of *vanA* and *vanB* genes regarding the minimum inhibitory concentration to vancomycin in *E. faecalis* isolates. N=193

Genes	MIC≥32/N	MIC<32) /(4<N	MIC≤4/N
	18/*(9/33%)	27/(13/98%)	148/(76/68%)
vanA (5)	5/(27/77%)	0	0
vanB (5)	5/(27/77%)	0	0
van A & van B (6)	6/(33/33%)	0	0

* Three vancomycin resistant *E. faecalis* isolates without *vanA* or *vanB* genes

vanB genes (Table 1). The sequences of these genes were obtained from completed genomic sequences for *E. faecalis*, *E. faecium* (GenBank accession no. U00457, U39790, vanA M97297 and vanB U00456, respectively). The BLASTN program at the National Center for Biotechnology Information (NCBI) confirmed that all of the PCR target regions had 100% similarity to two sequenced isolates of *ddl* gene of *E. faecalis*, *E. faecium*, *vanA* and *vanB* genes as well. The primers and probes were designed by Beacon Designer version 7 software.

Optimization of q*PCR*. First, variable parameters such as cycle temperatures, the number of PCR cycles, and length of annealing and replicating steps were optimized. Designed primers were evaluated with SYBR Green to optimize cycle temperatures and times. Each 25 µl single reaction consisted of 12.5 µl master mix Takara (Takara, Japan), 500 nM forward primer, 500 nM reverse primer, 200 nM probe, 50 ng target DNA and HPLC-grade H_2O to 25 µl. During the cycling phase, the extension temperatures were varied from 48 to 56 °C in single-degree increments to maximize the reaction. The optimized protocol identified and used for all singleplex assays was an initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 15 s, 52 °C for 15 s and 72 °C for 60 s. Quadruplex reaction conditions were optimized as follows: 500 nM forward primer , 500 nM reverse primer, 400 nM probe (*ddl E. faecalis*, *vanA* and *vanB* genes), 200 nM probe (*ddl E. faecium*), 50 ng target DNA and HPLC-purified H_2O to 25 µl. Thermal cycling conditions were 5 min at 95 °C, followed by 40 cycles of 15s at 95 °C, 15s at 52 °C and 60s at 72 °C. A positive signal was determined by the crossing of a fluorescence threshold of 25 before cycle 35.

RESULTS

Detection of *E. faecium* and *E. faecalis* and vancomycin susceptibility rate. In this study, of 235 clinical specimens, 226 specimens were positive for *E. faecium* and *E. faecalis* in which, 193 isolates (82.2%) were *E. faecalis* and 33 isolates (14.04%) were *E. faecium*.

Antimicrobial susceptibility by phenotypic test showed 166 (73.45%) isolates were susceptible to vancomycin which 28 isolates had intermediate susceptibility to vancomycin and 32 isolates were fully resistant. The E-test and disk diffusion method were 100% concordant for the determination of the vancomycin susceptibility of the isolates (Table 2,3).

TaqMan real-time PCR. Singelplex TaqMan real-time PCR was first performed on DNAs extracted from four reference strains as positive controls for the four possible genes (Fig. 1).

TaqMan real time PCR on DNA extracts of Enteroccoci and *E. coli* control strains produced

Table 3: Prevalence of *vanA* and *vanB* genes regarding the minimum inhibitory concentration to vancomycin in *E. faecium* isolates. N=19

Genes	MIC≥32/N	MIC<32) /(4<N	MIC≤4/N
	14/*(42/42%)	1/(3/03%)	18/(54/55%)
van A (7)	7/(50%)	0	0
van B (1)	1/(7/14%)	0	0
van A & van B (3)	3/(21/43%)	0	0

*Two vancomycin resistant *E.fae*cium isolates without *van*A or *van*B genes

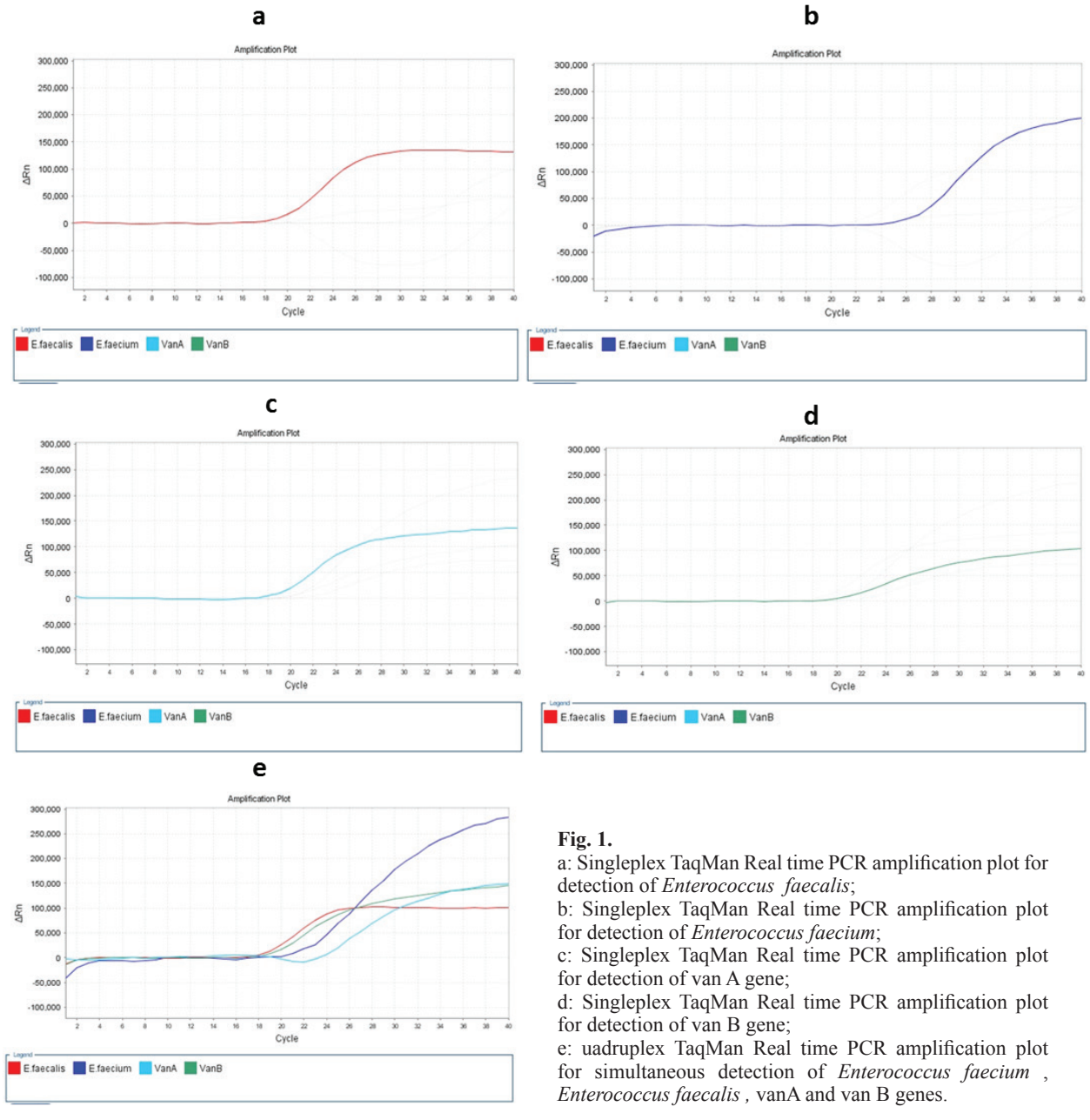

Fig. 1.
a: Singleplex TaqMan Real time PCR amplification plot for detection of *Enterococcus faecalis*;
b: Singleplex TaqMan Real time PCR amplification plot for detection of *Enterococcus faecium*;
c: Singleplex TaqMan Real time PCR amplification plot for detection of van A gene;
d: Singleplex TaqMan Real time PCR amplification plot for detection of van B gene;
e: uadruplex TaqMan Real time PCR amplification plot for simultaneous detection of *Enterococcus faecium* , *Enterococcus faecalis* , vanA and van B genes.

expected signals for *E. faecium* BM4147, *E. faecalis* 33186, *E. faecalis* ATCC 29122 and *E. coli* ATCC 25922 and cross-fluorescence was not observed during these experiments.

Using serial dilutions of DNA extracted from standardized inoculums adjusted at 10^7 CFU/ml of these strains, we determined the lower limit of detection. The lowest pure Enterococci DNA concentration always delivering a positive result corresponded to 2.8, 1.2, 2.8, and 1.65 cells per µl of extracted DNA for *E. faecium* BM4147, *E. faecalis* 33186, *E. faecalis* ATCC29122 and *E. coli* ATCC 25922, respectively. At the detection limit, C_T values varied between 19 and 25.

To confirm that the TaqMan real-time PCR could detect the simultaneous presence of different genotypes, the technique was carried out by using mixtures in various proportions of DNA from TaqMan real-time PCR strains (from 50 to 1%). For all three *E. faecium* BM4147, *E. faecalis* 33186 and *E. faecalis* ATCC 29122, the TaqMan real-time PCR was able to detect DNA until reaching concentration

as low as 10%. All 226 extracted were further tested by TaqMan real-time PCR. Vancomycin susceptible isolates detected by the phenotypic methods were found susceptible by TaqMan real-time PCR in all cases. Moreover, all phenotypic resistant isolates were also found resistant by TaqMan real-time PCR. Mixed populations were detected by TaqMan real-time PCR, whereas they were not detected by culture and E-test in nine cases. In these nine cases, the resistance genotype was detected by TaqMan real-time PCR but was missed by phenotypic methods. In total, TaqMan real-time PCR test showed higher sensitivity (100%) and specificity (94.86%)

DISCUSSION

Recently, real-time PCR assays have been widely used in different areas of medical microbiology including diagnosis and molecular epidemiology of bacterial (21-24) and viral infections (25, 26), evaluation of drug susceptibility as well as microbial involvement in other human diseases (27-28). In this study, we developed an easy-to-use real-time PCR technique based on the amplification of fragments of the *ddl* , *vanA* and *vanB* genes with TaqMan real-time PCR primers and probes. This specific method allows detecting four possible genes including *E. faecium* BM4147, *E. faecalis* ATCC 33186 and *E. faecalis* ATCC 29122. The four fluorescence results are readable on an ABI 7500 thermocycler.

The determination of the C_T cut off to distinguish positive from negative TaqMan real-time PCR assays was based on several results. The sensitivity and specificity of the TaqMan Real-time PCR are equivalent to those reported for real-time PCR assays (29, 30). The use of a culture result as a gold standard to evaluate performances of the TaqMan real-time PCR has certainly shortened the specificity of the test (31, 32). A quantitative PCR assays targeting 16S rRNA gene has been developed by Ryu *et al*. for identification of *Enterococcus* spp. in environmental samples (33). The results we obtained for several specimens that require 4 days to be processed by the laboratory with a positive TaqMan real-time PCR and negative culture highlight the advantage of PCR techniques that do not require viable bacteria.

Conclusion. Our TaqMan real-time PCR technique is rapid and simple. It can be performed about two hours for 40 cycles. As DNA extraction of isolates takes one hour, the simultaneous detection of isolates and their vancomycin susceptibility could be performed in less than three hours. Two hundred twenty-six specimens have been tested with perfect concordance with culture and E-test, except for minor discrepancies concerning mixed populations. The control using detection of the human ACTB gene did not detect any PCR inhibition.

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