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Original Article

Evaluation of a PCR Assay to Detect *Enterococcus faecalis* in Blood and Determine Glycopeptides Resistance Genes: Van A and Van B

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Abstract

Background: Bacteremia due to *Enterococcus faecalis* is usually caused by strains resistant to most antibiotics. Effective management of the disease is dependent on rapid detection and characterization of the bacteria, and determination its sensitivity pattern to antimicrobial drugs. The aim of this study was to investigate a more rapid and reliable assay for simultaneous diagnosis of enterococcal bacteremia and its sensitivity pattern to antimicrobial drugs.

Methods: Several bacterial suspensions with different content of two standard strains of *Enterococcus faecalis* resistant to vancomycin were used for inoculation to defibrinated sheep blood samples. PCR and routine assay was performed on all blood samples with different bacterial content.

Results: Routine assay and PCR for all inoculated blood samples with ≥5 cfu/ml was positive. Mean time for PCR and routine assays was 10 hours and 5 days, respectively.

Conclusion: PCR is a more rapid and sensitive assay for simultaneous detection and characterization for *Enterococcus faecalis*, and determination of its sensitivity pattern to vancomycin.

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Keywords ● *Enterococcus faecalis* ● multiplex-PCR ● Van A ● Van B

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Introduction

Enterococcus faecalis is the cause of 85-90% of enterococcal, and third cause of nosocomial infections, especially bacteremia, sepsis in children, endocarditis, urinary tract infection (UTI), and wound infections.[1,2] It plays a significant role in treatment of the disease,[3,4] Knowledge of bacterial resistance pattern to antimicrobial agents is important for the successful management of diseases.[5] Most hospital isolates are resistant to most usual antibiotics including vancomycin.[6-8] There are five resistance genes whose products are responsible for resistance to glycopeptides antibiotics in vancomycin-resistant enterococci strains (VRE). Two of such genes (Van A and Van B) are most common than others, especially in *E. faecalis* and *E. faecium*.[6,9] Strains with Van A gene are resistant to vancomycin and tycoplanin, and strains with Van B are resistant to vancomycin but sensitive to tycoplanin.[10,11] Resistant enterococcal infections are usually

treated by synergistic action of a glycopeptide and an aminoglycoside.[1,5] Vancomycin-resistant enterococci strains are usually transferred via the hands of health care workers, who are fecal carrier and are in close contact with patients. Those patients who have long-time hospital stay and long-time antibiotic therapy, as well as children and the elderly with a critical situation, such as those who are hospitalized in intensive care units (ICU), are more prone to take the disease.[12-14] Culture is the most-used way for detecting enterococci in the blood,[2] however, for effective treatment of enterococcal bacteremia, characterization of the bacteria and their pattern of resistance to antibiotics is necessary. This requires some diagnostic biological tests as well as determination of its antibiogram pattern and MIC, which usually takes about five days.[14-17] Some rapid methods such as E test for MIC, API 20 and API 32 for characterization, and selective-differential specific media and choromogenic agars for direct detection of VRE such as EVA, CAN-VGA, and BEAA with 6 µg/ml vancomycin have been introduced in recent years.[15,18] These methods have shown different sensitivity and specificity in different studies,[20-23] and need 2-3 days for final confirmation of their results.[15,18,19] Therefore, a more rapid and reliable test is needed. PCR is reported to be a suitable alternative.[2]

The aim of this study was to evaluate the feasibility of a PCR assay (four-pair primers) as an alternative for a routine method, which is time-consuming and expensive, to characterize *E. faecalis* in the blood and to reveal its resistance type to vancomycin.

Material and Methods

We used a standard strain VRE (PTCC 1447, and PTCC 1237) prepared by the division of Bacteria and Fungi Collections, Iranian Institute of Industrial and Scientific Researches, Tehran, Iran). A suspension 10^8 cfu/ml was made in normal saline by adding some single colonies, which were grown on TSA by adjusting its optical density to half McFarland solution and checking their absorbance in 700 nm with spectrophotometer. Then, diluted solutions with different bacterial contents (10^6 cfu/ml, 10^4 cfu/ml and 10^2 cfu/ml) were made by diluting it in normal saline. They are used for inoculating to blood. By adding certain amount of each bacterial solution to certain amount of defibrinated sheep blood, some blood samples with different bacterial content (10^4 cfu/ml, 10^3 cfu/ml, 10^2 cfu/ml, 10^1 cfu/ml, 5 cfu/ml and zero as control) were prepared. Ten-milliliter-samples of each dilution were prepared to be used in ten experiments of each of the PCR and routine assays. For routine assay, we used initial enrichment procedure for each specimen by inoculating to TSB and incubation at 37ºC for 24 hours, passage to TSA and incubation in 37ºC for more 24 hours, identifying by catalase test, PYR test, growth on TSA with 6.5% Nacl, and hydrolysis esculin in the presence of bile on BEA. Differentiation of *E. faecalis* from *E. faecium* was done by three tests including ability to use pyruvate, fermentation of sorbitol, and reduction of tellurite.[15,24] For screening VRE, we used BEA including 6 µg/ml vancomycin.[10,15,24]

The extraction of DNA was achieved using the following procedure. Transferring 100 µl of each blood sample to a 2 ml ependorf vial contain 400 µl sterile double distilled H_2O and incubation in 37ºC for 30 minutes, adding 500 µl red cell lysis buffer ($NAHCO_3$ 10 mM, NH_4CL 0.155M, pH=7) and incubation at 37ºC for one hour, centrifugation at 10,000 rpm for 15 minutes, discarding the supernatant, adding 200 µl lysis buffer for bacteria (Tris 10 mM, sucrose 0.3 M, $MgCl_2$ 5 mM) to the pellet with 10 µl lysozyme (0.1 mg/ml, Sinagen, Lot: MR7735) and incubation at 37ºC for one hour, adding 4 µl proteinase K (900 u/ml, Fermentaze, Lot: 00022411) and incubation at 65ºC for one hour, extraction of DNA by standard phenol-chloroform method and precipitation of DNA by cold isopropanol. PCR mix was prepared as 3 µl of 10x PCR buffer, 2 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTP 10 mM, 100 pM of each primer,0.2 µl DNA pol (5 u/µl), 2 µl DNA, and double distilled H_20 to final volume of 25 µl. Special features of primers that were used in this study are shown in table 1.

PCR programs was adjusted as one cycle at 94ºC for 7 minutes, 34 cycle at 94ºC for 40 seconds, 46ºC for 40 seconds, 72ºC for 50

Table 1: Special features of the used primers

Gene[1]	Name[2]	Sequence	Size of PCR product (bp)	Reference[3]
rrs (16S rRNA)	C1 C2	5-GGATTAGATACCCTGGTAGTCC-3 5-TCGTTGCGGGACTTAACCCAAC-3	320	25
ddl E. faecalis	D1 D2	5-ATC AAG TAC AGT TAG TCT TTA G-3 5-ACG ATT CAA AGC TAA CTG AAT CAG T-3	941	19
Van A	A1 A2	5-GGG AAA ACG ACA ATT GC-3 5-GTA CAA TGT GGC CGT TA-3	732	19
Van B	B1 B2	5-ATG GGA AGC CGA TAG TC-3 5-GAT TTC GTT CCT CGA CC-3	635	19

[1]The gene of each primer; [2]Name of primers; [3]The reference for the primer design

seconds, and final extension at 72ºC for 10 minutes. Electrophoresis of PCR product was done on 1.5% agarose gel including Etidium bromide by 100 volts for one hour.

Results

All blood samples with different bacterial content of 5 cfu/ml were positive in the routine assay and PCR (figure 1). So, the sensitivity of PCR was the same as that of routine test.

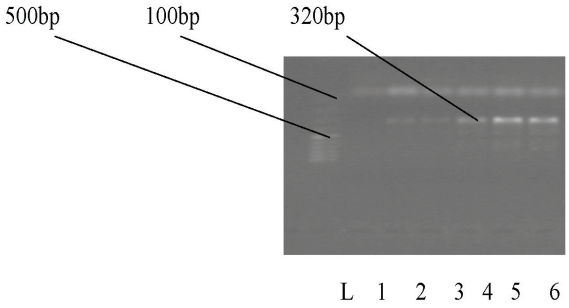

Figure 1: Gel electrophoresis of PCR with genus specific primers C1 and C2) to Enterococcus on blood samples with different contents of *Enterococcus faecalis* (ptcc 1447). L: ladder 100 bp; 1: negative control (blood without bacteria); 2: blood with 5 cfu/ml; 3: blood with 10 cfu/ml; 4: blood with 10^2 cfu/ml; 5: blood with 10^3 cfu/ml; 6: blood with 10^4 cfu/ml; Size of all bands is 320 bp.

PCR with species specific primers (D1 and D2) on two blood samples with different bacterial contents of *E. faecalis* (ptcc 1447) is shown in figure 2.

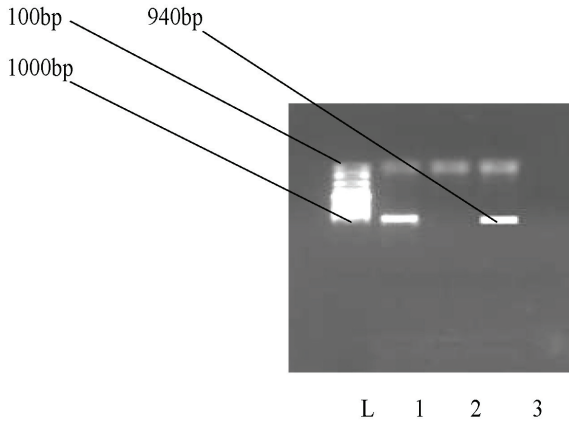

Figure 2: Gel electrophoresis of PCR with species specific primers (D1 and D2) on a blood samples. L: ladder 100 bp; 1: Blood with 10^3 cfu/ml *Enterococcus faecalis* (ptcc 1237); 2: negative control (blood without bacteria); 3: Blood with 10^3 cfu/ml *Enterococcus faecalis* (ptcc 1447); Size of both specific bands is 940 bp.

PCR with species specific primers Van A (A1, A2) and Van B (B1, B2) on two blood samples with the same number but different strains of *E. faecalis* (ptcc 1447) and *E. faecalis* (ptcc 1237) is shown in figure 3.

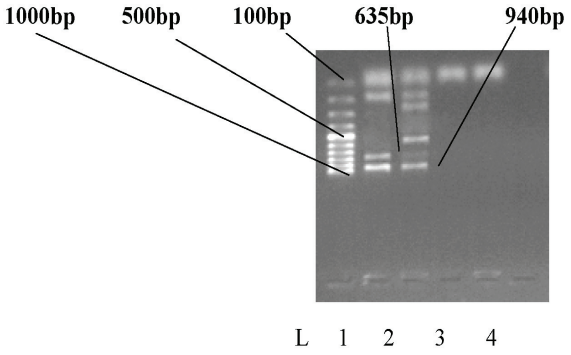

Figure 3: Gel electrophoresis of PCR with specific primers for Van A (A1, A2) and Van B (B1, B2) on two blood samples. L: ladder 100 bp; 1: Blood with 10^3 cfu/ml *Enterococcus faecalis* (ptcc 1447). Two specific bands are obvious: 940 bp for ddl gene and 732 bp for Van A; 2: Blood with 10^3 cfu/ml *Enterococcus faecalis* (ptcc 1237). Two specific bands are obvious: 940 bp for ddl gene and 635 bp for Van B; 3: negative control (blood without bacteria for *Enterococcus faecalis* ptcc 1447); 4: negative control (blood without bacteria for *Enterococcus faecalis* ptcc 1237)

The routine assay needed five days, but the PCR assay needed 10 hours. The sensitivity of the test was 95.4% and the specificity was 100%.

Discussion

Rapid diagnosis is very critical in the treatment of bacteremia. Routine assay is time-consuming and expensive, and commercial automatic screening tests and disc diffusion agar are not efficient for highly resistant bacteria that make most of the hospital isolates.[20] Identification by API 20 and API 32 was associated with different sensitivity and specificity.[21-23] The most-studied selective-differential medias are EVA, CAN-VGA, and BEAA with 60 µg/ml vancomycin.[15,18] Although is more specific than CAN-VGA, EVA is slower (24 *vs* 48 hours). Several investigators have used multiplex-PCR with multiple pairs of specific primers. Dutka-Malen used six primer pairs todetect a number of standard strains and clinical isolates.[19] Patel studied 100 clinical isolates (34 *E. faecalis*) using four primer pairs.[26] The multiplex-PCR assay that with 4 primer pairs that Stake,[18] used for screening many clinical isolates, had a sensitivity of 85.0% and a specificity of 100% specificity, but the one that with three primers that Jayartne used for screening of 657 isolates had a sensitivity of 95.4% and a specificity of 99.8%.[27] Ke,[28] use primers designed from tuf enterococcal gene to diagnose 14 of 20 enterococcal species. Angelleti,[29] used four pair of primers to detecting 279 isolates, and found that it was more rapid than routine assay. It is in

agreement with our study. Paul,[30] used two pairs of specific primers for Van A and Van B to screen clinical isolates, and found it was more sensitive than culture methods. In our study, the sensitivity of PCR was the same as that of routine test. It seems that the quality and quantity of DNA, which is related to DNA extraction method, is critical to the higher sensitivity. Kariyama,[31] used Multiplex-PCR with seven pairs of primers to screen many clinical isolates and standard strains, and found that it was simpler and more efficient than the routine method. It is also compatible with our study.

False-positive cases is mainly the result of amplifying DNA of dead bacteria in the sample and amplifying resistance genes like Van A and Van B that are present in some other bacteria. This is critical for fecal samples that contain different bacteria, but not for blood ones.[25] Regulating the concentrations of several primers in PCR mix is a technical problem for multiplex-PCR. Kariyama found that inhibition of Van A primers can be neutralized by increasing their concentrations to two-folds.[31] Angeletti,[29] and Stake,[18] performed the multiplex-PCR in two steps, and in one step they only used Van A primers. We used one step and the same concentrations of primers, which seems to be the cause of weak view of bands (figure 3). The other cause of false-positivity is the specificity of primers. Primers designed by Ke,[28] from tuf gene could amplify two species of Abiotrophia and four species of Lysteria. However, they are not the usual causes of bacteremia and this problem is important for fecal screening. It has been recommended to use molecular typing methods such as RFLP on PCR product,[26] or very specific primers for *E. faecalis*,[19,27] for characterization. The present study used the latter method (figure 2). It has been recommended to use genus specific or universal primers as the internal control for detecting false-negative cases.[27] However, we used species specific primers to diagnose *E. Faecalis* and as an internal control (figure 2). One of the main technical problems in the diagnosis of bacteremia by PCR is the obtainment of high quality and quantity bacterial DNA from the whole blood. This may not be easily possible because of high contents of PCR inhibitors. Therefore, DNA extraction method is critical.[25,32] Zang,[33] used Quiagen kit, and detected five cfu/ml bacteria in the blood.[33] Newcomb,[34] used Boom method and Klausegger,[35] used DNA ZOl buffer for lysis bacteria in blood. However, Anthony,[36] used double distilled H_20 for lysis blood cells and boiling for extraction bacterial DNA. Rothman,[37] recommended initial enrichment process of blood sample in TSB before DNA extraction. We used sterile double distilled H_2O and red cell lysis buffer for lysis blood cell, proteinase K for eliminating PCR inhibitors, phenol-cholroform and alcohol precipitation to extract DNA of bacteria in blood, however, the quality of extracted DNAs was not very good. It seems that the poor quality of DNA was the main cause of weak bands of PCR products in our study (figure 1). We recommend a very efficient method or a commercial kit to increase the accuracy of PCR assays in such a case. We also recommend further studies to overcome other technical problems of PCR assay to make it more simple and reliable relative to new and very improved culture based methods such as VRE-BMX chromogenic commercial media.[38] Such kits has several advantages such as differentiation at the species level, inhibition of growth of sensitive enterococci to vancomycin, promoting growth of resistant strains, and shorter duration than the traditional methods (24 *vs* 48 hours).

Conclusion

PCR is a more rapid and a sensitive method for simultaneous detection and characterization of *E. Faecalis*, and determination of its antimicrobial pattern to vancomycin. It can be considered as an alternative assay, but a more efficient DNA extraction method to increase the sensitivity of the assay is suggested.

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Conflict of Interest: None declared.

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