کارگاه‌های آموزشی مرکز اطلاعات علمی جهاد دانشگاهی

کارگاه آنلاین کاربرد نرم‌افزار SPSS در پژوهش
کارگاه آنلاین اصول تنظیم قرارداد‌ها
کارگاه آنلاین پروپوزال نویسی
Evaluation of a PCR Assay to Detect Enterococcus faecalis in Blood and Determine Glycopeptides Resistance Genes: Van A and Van B

Hamidreza Honarmand1, Mahsome Falah Ghavidel2, Iraj Nikokar3, Morteza Rahbar Taromsari4

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.

Please cite this article as: Honarmand H, Falah Ghavidel M, Nikokar I, Rahbar Taromsari M. Evaluation of a PCR Assay to Detect Enterococcus faecalis in Blood and Determine Glycopeptides Resistance Genes: Van A and Van B. Iran J Med Sci. 2012;37(3):194-199.

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

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 tycoplanan, and strains with Van B are resistant to vancomycin but sensitive to tycoplanan.10,11 Resistant enterococcal infections are usually
treated by synergistic action of a glycopeptide and an aminoglycoside.\textsuperscript{1,5} Vancomycin-resistant enterococci strains are usually transferred via the hands of healthcare workers, who are fecal carriers 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.\textsuperscript{12-14} Culture is the most-used method for detecting enterococcus in the blood,\textsuperscript{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 take about five days.\textsuperscript{14-17} Some rapid methods such as E test for MIC, API 20 and API 32 for characterization, and selective-differential specific media and chromogenic agars with spectrophotometer. Then, diluted solutions with some single colonies, which were grown on TSA 108 cfu/ml was made in normal saline by adding certain amount of defibrinated sheep blood, some blood samples with different bacterial content (104 cfu/ml, 103 cfu/ml, 102 cfu/ml, 101 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 37°C for 24 hours, passage to TSA and incubation in 37°C 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 \textit{E. faecalis} from \textit{E. faecium} was done by three tests including ability to use pyruvate, fermentation of sorbitol, and reduction of tellurite.\textsuperscript{15,24} For screening VRE, we used BEA including 6 µg/ml vancomycin.\textsuperscript{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 H2O and incubation in 37°C for 30 minutes, adding 500 µl red cell lysis buffer (\textit{NaHCO}$_3$ 10 mM, \textit{NH}$_4$CL 0.155M, pH=7) and incubation at 37°C 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: MR7732) and incubation at 37°C for one hour, adding 4 µl proteinase K (900 u/ml, Fermentaze, Lot: 00022411) and incubation at 65°C 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$_2$O 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 94°C for 7 minutes, 34 cycle at 94°C for 40 seconds, 46°C for 40 seconds, 72°C for 50

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

\textsuperscript{1}The gene of each primer; \textsuperscript{2}Name of primers; \textsuperscript{3}The reference for the primer design
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.

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.

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. Identification by API 20 and API 32 was associated with different sensitivity and specificity. The most-studied selective-differential medias are EVA, CAN-VGA, and BEAA with 60 µg/ml vancomycin. 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 to detect a number of standard strains and clinical isolates. Patel studied 100 clinical isolates (34 *E. faecalis*) using four primer pairs. The multiplex-PCR assay that with 4 primer pairs that Stake, 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%. Ke, use primers designed from tuf enterococcal gene to diagnose 14 of 20 enterococcal species. Angelleti, 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 H2O for lysis blood cell, proteinase K for eliminating PCR inhibitors, phenol-chloroform 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.

**Acknowledgment**

We thank Mr. Mojtaha Hoseinpour for his kind help.

**Conflict of Interest:** None declared.

**References**

1. Facklam RR, Sham DF. Enterococcus. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of clinical microbiology. 6th ed. Washington DC: American Society for Microbiology; 1995. p. 308-14.
2. Reimer LG, Wilson ML, Weinstein MP. Update on clinical detection of bacteremia and fungemia. Clin Microbiol Rev. 1997;10:444-65. PubMed PMID: 9227861; PubMed Central PMCID: PMC172929.
3. Doern GV, Vautour R, Gaudet M, Levy B. Clinical impact of rapid in vitro susceptibility testing and bacterial identification. J Clin Microbiol. 1994;32:1757-62. PubMed PMID: 7929770; PubMed Central PMCID: PMC263786.
4. Klouche M, Schröder U. Rapid methods for diagnosis of bloodstream infections.
Clin Chem Lab Med. 2008;46:888-908. doi: 10.1515/CCLM.2008.157. PubMed PMID: 18624614.

5 Vincent JL, Abraham E. The last 100 years of sepsis. Am J Respir Crit Care Med. 2006;173:256-63. doi: 10.1164/rcrm.200510-1604OE. PubMed PMID: 16239619.

6 Dutka-Malen S, Courvalin P. Update on glycopeptide resistance in enterococci. Antimicrob Newsletter. 1990;7:81-6. doi: 10.1016/0738-1751(90)90027-A.

7 Morris JG Jr, Shay DK, Hebben JN, McCarter RJ Jr, Perdue BE, Jarvis W, et al. Enterococci resistant to multiple antimicrobial agents, including vancomycin. Establishment of endemicity in a university medical center. Ann Intern Med. 1995;123:250-9. PubMed PMID: 7611590.

8 Uttley AH, George RC, Naidoo J, Woodford N, Johnson AP, Collins CH, et al. High-level vancomycin-resistant enterococci causing hospital infections. Epidemiol Infect.1989;103:173-81. doi: 10.1017/S0950268800030478. PubMed PMID: 2506070; PubMed Central PMCID: PMC2249484.

9 Leclercq R, Derlot E, Duval J, Courvalin P. Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. N Eng J Med. 1988;319:157-61. doi: 10.1056/NEJM198807213190307. PubMed PMID: 2968517.

10 Cetinkaya Y, Falk P, Mayhall CG. Vancomycin-resistant enterococci. Clin Microbiol Rev. 2000;13:668-707. doi: 10.1128/CMR.13.4.668-707.2000. PubMed PMID: 11023964; PubMed Central PMCID: PMC88957.

11 Dutka-Malen S, Molinas C, Arthur M, Courvalin P. The VANA glycopeptide resistance protein is related to D-alanyl-D-alanine ligase cell wall biosynthesis enzymes. Mol Gen Genet. 1990;224:364-72. doi: 10.1007/BF00262430. PubMed PMID: 2266943.

12 Devriese LA, Pot B, Collins MD. Phenotypic identification of the genus Enterococcus and differentiation of phylogenetically distinct enterococcal species and species groups. J Appl Bacteriol. 1993;75:399-408. doi: 10.1111/j.1365-2672.1993.tb02794.x. PubMed PMID: 8300442.

13 Kanchana MV, Deneer H, Blondeau J. Cost-effective algorithm for detection and identification of vancomycin-resistant enterococci in surveillance cultures. Eur J Clin Microbiol Infect Dis. 2000;19:366-9. doi: 10.1007/s100960050496. PubMed PMID: 10898139.

14 Sahm DF, Free L, Smith C, Eveland M, Mundy LM. Rapid characterization schemes for surveillance isolates of vancomycin-resistant enterococci. J Clin Microbiol. 1997;35:2026-30. PubMed PMID: 9230375; PubMed Central PMCID: PMC229896.

15 Facklam RR, Collins MD. Identification of Enterococcus species isolated from human infections by a conventional test scheme. J Clin Microbiol. 1989;27:731-4. PubMed PMID: 2656745; PubMed Central PMCID: PMC267406.

16 Hanson KL, Cartwright CP. Comparison of simple and rapid methods for identifying enterococci intrinsically resistant to vancomycin. J Clin Microbiol. 1999;37:815-7. PubMed PMID: 9986863; PubMed Central PMCID: PMC84568.

17 Sahm DF, Olsen L. In vitro detection of enterococcal vancomycin resistance. Antimicrob Agents Chemother. 1990;34:1846-8. doi: 10.1128/AAC.34.9.1846. PubMed PMID: 2126696; PubMed Central PMCID: PMC171946.

18 Satake S, Clark N, Rimland D, Nolte FS, Tenover FC. Detection of vancomycin-resistant enterococci in fecal samples by PCR. J Clin Microbiol. 1997;35:2325-30. PubMed PMID: 9276411; PubMed Central PMCID: PMC229963.

19 Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995;33:24-7. PubMed PMID: 7699051; PubMed Central PMCID: PMC227872.

20 Swenson JM, Hill BC, Thornberry C. Problems with the disk diffusion test for detection of vancomycin resistance in enterococci. J Clin Microbiol. 1989;27:2140-2. PubMed PMID: 2778080; PubMed Central PMCID: PMC267762.

21 Hamilton-Miller JM, Shah S. Identification of clinically isolated vancomycin-resistant enterococci: comparison of API and BBL Crystal systems. J Med Microbiol. 1999;48:695-6. doi: 10.1099/00222615-48-7-695. PubMed PMID: 10403421.

22 Sader HS, Biedenbach D, Jones RN. Evaluation of Vitek and API 20S for species identification of enterococci. Diagn Microbiol Infect Dis. 1995;22:315-9. PubMed PMID: 8582135.

23 Landman D, Quale JM, Oydna E, Willey B, Ditore V, Zaman M, et al. Comparison of five selective media for identifying fecal carriage
of vancomycin-resistant enterococci. J Clin Microbiol. 1996;34:751-2. PubMed PMID: 8904453; PubMed Central PMCID: PMC228885.

24 van Horn KG, Gedris CA, Rodney KM. Selective isolation of vancomycin-resistant enterococci. J Clin Microbiol. 1996;34:924-7. PubMed PMID: 8815109; PubMed Central PMCID: PMC228918.

25 Ledeboer NA, Das K, Eveland M, Roger-Dalbert C, Mailler S, Chatellier S, et al. Evaluation of a novel chromogenic agar medium for isolation and differentiation of vancomycin-resistant Enterococcus faecium and Enterococcus faecalis isolates. J Clin Microbiol. 2007;45:1556-60. doi: 10.1128/JCM.02116-06. PubMed PMID: 17329453; PubMed Central PMCID: PMC1865861.

26 Patel R, Uhl JR, Kohner P, Hopkins MK, Cockerill FR 3rd. Multiplex PCR detection of vanA, vanB, vanC-1, and vanC-2/3 genes in enterococci. J Clin Microbiol. 1997;35:703-7. PubMed PMID: 9041416; PubMed Central PMCID: PMC229654.

27 Jayaratne P, Rutherford C. Detection of clinically relevant genotypes of vancomycin-resistant enterococci in nosocomial surveillance specimens by PCR. J Clin Microbiol. 1999;37:2090-2. PubMed PMID: 10325390; PubMed Central PMCID: PMC85045.

28 Ke D, Picard FJ, Martineau F, Ménard C, Roy PH, Ouellette M, et al. Development of a PCR assay for rapid detection of enterococci. J Clin Microbiol. 1999;37:3497-503. PubMed PMID: 10523541; PubMed Central PMCID: PMC85677.

29 Angeletti S, Lorino G, Gherardi G, Battistoni F, De Cesaris M, Dicuonzo G. Routine molecular identification of enterococci by gene-specific PCR and 16S ribosomal DNA sequencing. J Clin Microbiol. 2001;39:794-7. doi: 10.1128/JCM.39.2.794-797.2001. PubMed PMID: 11158155; PubMed Central PMCID: PMC87824.

30 Paule SM, Trick WE, Tenover FC, Lankford M, Cunningham S, Stosor V, et al. Comparison of PCR assay to culture for surveillance detection of vancomycin-resistant enterococci. J Clin Microbiol. 2003;41:4805-7. doi: 10.1128/JCM.41.10.4805-4807.2003. PubMed PMID: 14532226; PubMed Central PMCID: PMC254367.

31 Kariyama R, Mitsuhashi R, Chow JW, Clewell DB, Kumon H. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. J Clin Microbiol. 2000;38:3092-5. PubMed PMID: 10921985; PubMed Central PMCID: PMC87194.

32 Kane TD, Alexander JW, Johannigman JA. The detection of microbial DNA in the blood: a sensitive method for diagnosing bacteremia and/or bacterial translocation in surgical patients. Ann Surg. 1998;227:1-9. PubMed PMID: 9445103; PubMed Central PMCID: PMC1191165.

33 Zhang Y, Isaacman DJ, Wadowsky RM, Rydquist-White J, Post JC, Ehrlich GD. Detection of Streptococcus pneumoniae in whole blood by PCR. J Clin Microbiol. 1995;33:596-601. PubMed PMID: 7751363; PubMed Central PMCID: PMC227996.

34 Newcombe J, Cartwright K, Palmer WH, McFadden J. PCR of peripheral blood for diagnosis of meningococcal disease. J Clin Microbiol. 1996;34:1637-40. PubMed PMID: 8784560; PubMed Central PMCID: PMC229085.

35 Klauserger A, Hell M, Berger A, Zinober K, Baier S, Jones N, et al. Gram type-specific broad-range PCR amplification for rapid detection of 62 pathogenic bacteria. J Clin Microbiol. 1999;37:464-6. PubMed PMID: 9889245; PubMed Central PMCID: PMC84344.

36 Anthony RM, Brown TJ, French GL. Rapid diagnosis of bacteremia by universal amplification of 23S ribosomal DNA followed by hybridization to an oligonucleotide array. J Clin Microbiol. 2000;38:781-8. PubMed PMID: 10655385; PubMed Central PMCID: PMC86203.

37 Rothman RE, Majmudar MD, Kelen GD, Madico G, Gaydos CA, Walker T, et al. Detection of bacteremia in emergency department patients at risk for infective endocarditis using universal 16S rRNA primers in a decontaminated polymerase chain reaction assay. J Infect Dis. 2002;186:1677-81. doi: 10.1086/345367. PubMed PMID: 12447747.

38 Sahm DF, Kissinger J, Gilmore MS, Murray PR, Mulder R, Sollliday J, et al. In vitro susceptibility studies of vancomycin-resistant Enterococcus faecalis. Antimicrob Agents Chemother. 1989;33:1588-91. doi: 10.1128/AAC.33.9.1588. PubMed PMID: 2554802; PubMed Central PMCID: PMC172707.
کارگاه‌های آموزشی مرکز اطلاعات علمی جهاد دانشگاهی

کاربرد نرم افزار SPSS در بیوشیمی

کارگاه آنلاین اصول تنظیم قراردادها

کارگاه آنلاین پرورش نویسی