A new multiplex PCR assay for the simultaneous detection of vancomycin-resistant enterococci from rectal swabs

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Summary Objectives: This study describes the diagnostic performance of a recently available multiplex PCR-based kit for the simultaneous detection and identification of Enterococcus faecium, Enterococcus faecalis, vanA, vanB, vanC1 and vanC2/C3 genes, directly from rectal swabs constituting the most complete existing molecular assay currently available.

Methods: The diagnostic performance of this assay was evaluated by a multicenter study involving three independent public hospitals and consisted in the analysis of 187 rectal swabs from patients at high risk for vancomycin-resistant enterococci colonization.

Results: When bacteria culture was used as the gold standard, the sensitivity, specificity, positive and negative predicted values for the assay were 96.8%, 76.0%, 67.7% and 97.9%, respectively. When a composite reference standard consisting of culture and DNA sequencing of PCR products was used as the gold standard, the sensitivity, specificity, positive and negative predicted values for the PCR-based assay were 97.8%, 96.9%, 96.7% and 97.9%, respectively.

Conclusions: Based on these results, we conclude that this assay is considerably more sensitive than traditional microbiological methods for detecting vancomycin-resistant enterococci from rectal swabs. It is also much faster than culture. We believe that the implementation of this assay in routine clinical laboratories could help to reduce hospital-acquired vancomycin-resistant enterococci infections.

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Introduction

Vancomycin-resistant enterococci (VRE) constitute one of the major causes of nosocomial infections and are associated with high mortality worldwide. At least three different phenotypes associated with the gene cassettes vanA, vanB, and vanC are responsible for vancomycin resistance in enterococci. During 2004, VRE caused about one third of infections in intensive care units, according to the U.S. Centers for Disease Control and Prevention (CDC).

The reservoirs for VRE are colonized and infected patients. CDC and the Society of Healthcare Epidemiology of America recognize the importance of active VRE surveillance to reduce or eliminate hospital-acquired infections. Any established surveillance program will benefit from the fast identification of VRE carriers by allowing the rapid isolation of those patients, thus minimizing the spread. Rapid identification become even more important in developing countries where isolation rooms are scarce.

Although bacteria culture is currently the method of choice for VRE screening, this technique has a long turnaround time (48–72 h) and limited detection sensitivity. Therefore, the availability of an assay capable of detecting VRE in few hours coupled to an appropriate infection control program could reduce hospital-acquired VRE infections, thus leading to a significant reduction of patient mortality by this particular agent.

Several nucleic acid amplification tests have been developed and evaluated for the detection of VRE. Limitations of these tests include the following: (i) the requirement of complex extraction and detection procedures or the requirement of a culture step when testing is done from a selective enrichment broth or isolates are recovered from solid media; (ii) high cost per sample and expensive equipment; and (iii) the most widely used molecular approach (e.g. detection of vanA-vanB by real time PCR) deliver less information than the culture test (e.g. species identification and detection of vanC genotypes), which limits the full integration of molecular approaches for VRE detection.

Recently, a new conventional multiplex PCR-based assay for the simultaneous detection of Enterococcus faecium, Enterococcus faecalis, vanA, vanB, vanC1 and vanC2/C3 genes became commercially available. This assay uses a simple processing method and the post-PCR step is performed on a microfluidics-based platform that allows the automated separation and detection of PCR products. Additionally, this platform comes with integrated software for automated interpretation and report generation. The results of the assay are available in less than 3.5 h from specimen extraction to report generation.

In this study we report the results obtained with this new multiplex PCR-based assay for epidemiologically relevant VRE (vancomycin-resistant E. faecium and E. faecalis) detection in a random sample of 187 rectal swabs from three public hospitals in Chile. The performance in VRE detection of this molecular biology test was evaluated by using two different criteria: 1) setting bacteria culture results as the gold standard and 2) using a composite reference standard that consisted of bacteria culture and PCR followed by DNA sequencing of PCR products.

Materials and methods

Patients and specimens

This multicenter study was conducted in the following Chilean public hospitals: Roberto del Río Hospital (200 beds), Dipreca Hospital (400 beds) and Barros Luco Trudeau Hospital (600 beds). The study was approved by each of the corresponding institutional review boards. For the clinical evaluation, rectal swabs were collected from November 2007 to April 2008 from 187 patients at high risk for VRE colonization. Eligible participants included patients with five or more days of hospitalization in intensive care or hematology-oncology units. A single rectal rayon swab (Copan Diagnostics, USA) per patient was obtained by nursing staff using the liquid Stuart’s BBL CultureSwab collection and transport system (BD diagnostics, USA).

Identification of VRE by Enterococcosel plate culture

Each swab was used to inoculate a plate of Enterococcosel agar (BD diagnostics, USA) containing vancomycin at 6 μg/ml and then stored at –20 °C until DNA extraction for the PCR-based testing was performed. The cultures were then incubated aerobically at 35 °C for 3 days and suspected positive colonies were analyzed using a GPI card in a VITEK equipment (Biomerieux, France). Enterococcus positive isolates were then studied for vancomycin resistance using E-test (AB Biodisk) and phenotype recognition (vanA or vanB) with teicoplanin discs (Oxoid). The clinical laboratory and standards institute (CSLI) breakpoints of 2008 were used for susceptibility interpretation.

Identification of VRE by PCR-based assay

DNA was extracted from the swabs already used to inoculate plates, with the DNAExtract Swab Kit (TAAG Diagnostics, Chile) according to manufacturer instructions. The extracted DNA was kept at 4 °C until the PCR reaction was performed. PCR was carried out using the QuickTAAg® VRE Kit (TAAG Diagnostics, Chile) according to the manufacturer protocol. This test is a multiplex PCR assay that simultaneously amplifies specific target sequences of E. faecium and E. faecalis species in addition to vanA, vanB, vanC1 and vanC2/C3 genes, and includes an internal positive control (plasmid DNA containing an Arabidopsis thaliana promoter sequence). All PCR primers in this kit were specifically designed to work in a single tube multiplex amplification reaction and have not been previously described in the literature. PCR products were then automatically analyzed with the TAAg® MultiDetection System, that consists of a microfluidics based equipment and software (TAAG Diagnostics, Chile). VRE positive results were considered as those samples where E. faecium and/or E. faecalis plus vanA and/or vanB target was found. Otherwise the result was informed as negative.

Since a culture step is not required, molecular testing of 32 samples can be carried out in approximately four hours: one hour is needed for specimen processing (extraction of
nucleic acid from the sample) and three hours for PCR, analysis and reporting of results. PCR-negative controls for each bundle of samples processed at the same time were also included, and consisted of sterile water added to the last sample tube.

Quality control

To ensure the correct work of culture media, susceptibility testing, biochemical reagents and PCR assay, in each sample batch tested, a positive control of either a vanA-resistant E. faecium strain (ATCC 51559) or a vanB-resistant E. faecalis strain (ATCC 51299) was included.

Detection limits

To obtain an estimate of the detection limit of the multiplex-PCR based assay, several dilutions with a known number of CFUs of E. faecium sensitive strain (ATCC 35667) and E. faecalis vanB-resistant strain (ATCC 51229) were tested. The detection limits obtained were 50 CFUs/assay for E. faecium strain and 12 CFUs/assay for E. faecalis strain.

Specificity panel evaluation of PCR-based assay

The specificity of the PCR-based assay for Enterococcus targets was tested with DNA extracted from clinical isolates of the following bacteria: Enterococcus durans (ATCC 11576), Enterococcus hirae (ATCC 10541), vancomycin sensitive Enterococcus gallinarum (ATCC 49573), vancomycin sensitive Enterococcus casseliflavus (ATCC 25788), Escherichia coli (ATCC 51446), Klebsiella pneumoniae (ATCC 13883), Lactococcus plantarum (ATCC 8014), Listeria innocua (ATCC 33090), Listeria monocytogenes (ATCC 19115), Proteus mirabilis (ATCC 7002), Providencia alcalifaciens (ATCC 51902), Salmonella enterica serovar typhimurium (ATCC 14028), Salmonella enterica serovar enteritidis (ATCC 13076) and Staphylococcus aureus (ATCC 29213).

Data analysis

The results obtained with the PCR-based assay were first compared to the those obtained with Enterococcus plate culture. Therefore, in this initial analysis, the Enterococcus plate culture was considered as the gold standard. Following a recommended practice in the assessment of new diagnostic tests, and because of the known low sensitivity of the Enterococcus plate culture test, in a second round of analysis the gold standard was switched to a composite reference standard that consisted of Enterococcus plate culture and PCR, followed by DNA sequencing of PCR products. In this composite reference standard analysis, positive VRE specimens were defined as those that tested positive either by one of the methods that constitute the composite scheme, otherwise the specimen was defined as negative (i.e. a sample was defined as positive when culture test resulted positive or, if culture test resulted negative but PCR resulted positive, it was required that the PCR product confirmed that the sample had at least two positive targets: vanA/vanB and E. faecium/E. faecalis). Confidence intervals for sensitivity, specificity, and positive and negative predictive values were calculated based on exact binomial probabilities. Fisher’s exact test was used to measure association and to determine statistical significance.

Results

Specificity panel evaluation of the PCR-based assay

Isolates of 11 bacteria that can be found in the gastrointestinal tract were used to test the specificity of this assay. These bacteria included many enterococci as well as other gram-positive/negative bacteria (see Methods for the complete list). All these bacteria tested negative with this assay.

Evaluation of PCR-based assay versus culture

A total of 187 rectal swabs were evaluated. Some examples of typical results obtained with this multiplex-PCR based assay are shown in Fig. 1. Using bacteria culture analysis, 125/187 (66.8%) samples were negative for any VRE and 62/187 (33.2%) samples were VRE positive. Using the PCR-based assay, 97/187 (51.9%) were VRE negative and 90/187 (48.1%) were VRE positive. Assuming bacteria culture method as the gold standard, the sensitivity and specificity values for the PCR-based assay were 96.8% (60/62) and 76.0% (95/125), respectively. The positive and negative predictive values in these samples were 67.7% and 97.9%, respectively. The diagnosis results of the PCR-based assay compared to culture are shown in Table 1.

Evaluation of PCR-based assay versus the composite reference standard

When the composite reference standard scheme was adopted as the gold standard in the analysis, 89 specimens were VRE positive and 98 VRE negative with the PCR-based assay. The sensitivity and specificity values were 97.8% (87/89) and 96.9% (95/98), respectively. The positive and negative predictive values in these samples were 96.7% and 97.9%, respectively. In this setting, with the composite reference standard as the gold standard, the sensitivity and specificity values of the Enterococcus plate culture test were 69.7% and 100.0%, respectively. The positive and negative predictive values of the culture method in these samples were 85.5% and 100.0%, respectively. The diagnosis results of the culture and PCR-based assay compared to the composite gold standard are shown in Table 2. The difference in sensitivity between the molecular assay and culture was statistically significant (P < 0.001). Performances of culture and the PCR-based assay using the composite gold standard are shown in Table 3.

Discussion

The laboratory diagnosis of VRE is challenging, because the conventional gold standard (i.e. bacteria culture) has a low sensitivity. Therefore, when culture is used as the gold
A standard for assessing a new test, the sensitivity estimate of the new test will be unbiased but the specificity estimate will be biased in the direction of lower estimates. This bias, known as the imperfect gold standard bias, occurs because many new test positive results from truly colonized or infected patients will be assigned as false positives.

To reduce imperfect gold standard bias, in this work we have additionally used a composite reference standard approach. This approach is a better alternative to using culture results as the gold standard in diagnostic accuracy studies.

To our knowledge this is the first time that a composite reference standard approach for a VRE assay has been reported in the literature. However, similar approaches have been successfully used in the performance assessment of tests for Bordetella pertussis, Streptococcus pneumoniae and Trichomonas vaginalis, among others.

Figure 1  Example results of the multiplex-PCR based assay. (A–F) Electroferogram view of results obtained with some real clinical samples. Expected amplicon sizes of the multiplex-PCR reaction are the following: E. faecium 260 bp (base pairs), E. faecalis 460 bp, vanA 380 bp, vanB 140 bp, vanC1 90 bp, vanC2/C3 680 bp and the internal positive control 120 bp. Though E. gallinarum and E. casseliflavus species are not targeted by the multiplex-PCR reaction, their presence in a sample is indirectly inferred by detecting vanC1 and vanC2/C3 genes, respectively. The signals at 15 bp and 1,500 bp correspond to the internal molecules used by the system to adjust the minimum and maximum values that define the allowed size range. (A) E. faecalis sensitive strain. (B) E. faecalis vanB strain. (C) E. faecalis sensitive strain and E. gallinarum vanC1 strain. (D) E. faecium and E. faecalis with vanA and vanB genes. (E) E. casseliflavus with vanC2/C3 gene. (F) E. gallinarum with vanC1 gene.
The results of the present study indicate that the new PCR-based assay is considerably more sensitive than culture for detecting VRE from rectal swabs. The sensitivity improvement rate obtained in this study was similar to other studies using real-time PCR-based assays for the direct detection of vanA/vanB genes from rectal swabs.  

The low sensitivity of culture compared to the PCR-based assay can be explained by several reasons, which include: (i) nonviable enterococci may have been present in some swabs; (ii) viable but nonculturable enterococci may have been present in some swabs; (iii) the growth of some VRE may have been inhibited by vancomycin at 6 μg/ml; and (iv) the load of VRE in some cases was below the threshold required to successfully culture them. Related to this last point, it is not clear how much is the risk of VRE transmission in those patients that carry low levels of VRE. Detection of low-level VRE carriers that have other risk factors could be epidemiologically important, because the use of antimicrobial agents in those patients is likely to increase the burden of VRE quickly.

The most widely-used molecular approach detects vanA/vanB directly from rectal swabs. Because the vanA/vanB genotypes can be found in non-enterococcal species, those assays that only detect vanA-vanB markers are expected to have a higher rate of false positive results than the PCR-based assay used in this study. This is because the procedure used here is based on the simultaneous detection of an enterococcal species marker and a vancomycin resistance marker. Additionally, species identification is not only valuable in epidemiological studies, but can also have a direct incidence in control programs. For example, the ratio of E. faecalis to E. faecium bacteremia for pediatrics and pediatric oncology patients has been reported to be 6.5 to 1. This has been suggested that differentiation between E. faecium and E. faecalis could define different infection control programs and specific patient care.

Because the new PCR-based assay used here allows the simultaneous identification of important species of Enterococcus and their vancomycin resistance genotypes (vanA, vanB, vanC1 and vanC2/C3), the assay has a broad applicability. For instance, this assay can also be used to identify VRE cultured on conventional agar plate media or in blood culture bottles, shortening the time of the VRE identification process by over 24 hours. Unlike other molecular kits, this new assay also detects vanC1 and vanC2/C3 genotypes (genes carried by resistant Enterococcus gallinarum and Enterococcus casseliflavus/flavescens, respectively). Although the clinical significance of these species is not yet fully established, because these organisms have been recovered infrequently from clinical specimens, they may also cause serious invasive infections.

Fast assays for detecting VRE would allow more efficient and cost-effective active VRE surveillance than using traditional methods. Several reports have demonstrated that active VRE surveillance produces significantly fewer laboratory studies ordered per patient, decreases the mortality rate due to bacteremias and it is cost saving.

In addition, early detection of VRE may also allow a rapid discharge of patients to long-term-care or rehabilitation facilities (which often require patient testing for VRE infection or colonization prior to transfer). A previous study has shown that the use of a PCR-based assay for VRE detection allowed to decrease the lengths of stay for patients discharged to long-term care by almost 2 days, saving to the hospital an estimate of USD$675,000 to $3,600,000 annually through prevention of VRE colonization and bacteremia. In addition, early detection of VRE may also allow a rapid discharge of patients to long-term-care or rehabilitation facilities (which often require patient testing for VRE infection or colonization prior to transfer). A previous study has shown that the use of a PCR-based assay for VRE detection allowed to decrease the lengths of stay for patients discharged to long-term care by almost 2 days, saving to the hospital an estimate of USD$675,000 to $3,600,000 annually through prevention of VRE colonization and bacteremia. In addition, early detection of VRE may also allow a rapid discharge of patients to long-term-care or rehabilitation facilities (which often require patient testing for VRE infection or colonization prior to transfer). A previous study has shown that the use of a PCR-based assay for VRE detection allowed to decrease the lengths of stay for patients discharged to long-term care by almost 2 days, saving to the hospital an estimate of USD$675,000 to $3,600,000 annually through prevention of VRE colonization and bacteremia.

In summary, the new PCR-based assay for VRE detection tested here allows a timely identification of patients colonized or infected with VRE which, along with an appropriate infection control program, may assist in the reduction or elimination of hospital-acquired VRE infections.

### Conflict of interest

The company TAAG Genetics declares that has funded all the reagents and costs required to carry out this investigation. Though RM and GL are employees of TAAG Genetics, they have not been imposed any condition nor received any benefit from TAAG Genetics depending on the results of the research reported here. DB, MSM, JA, LP and FM declare that they have not received any salary or monetary incentive from TAAG Genetics to carry out this research report. All authors also declare that no future incentive from TAAG Genetics upon the results of this research work have been negotiated and thus they have not any conflict of interest with the research results reported here.

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