Evaluation of PCR-based screening for vancomycin-resistant enterococci compared with a chromogenic agar-based culture method

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Rapid detection of vancomycin-resistant enterococci (VRE) infection is very important for control and prevention of nosocomial spread of these bacteria. A multiplex PCR method for rapid screening of VRE has recently been developed. We performed a prospective study of VRE screening tests to compare the performance of PCR to that of a chromogenic agar-based culture method. From January to December 2009, a total of 8815 rectal swab specimens were tested simultaneously for VRE by VRE selective culture and by PCR. The specimens were inoculated onto ChromID VRE agar containing 8 μg vancomycin ml⁻¹ and examined after 24 and 48 h of incubation. Identification and antibiotic susceptibility tests were performed using the automated VITEK-2 system and a supplementary E-test and disk diffusion test. Detection of the vanA and vanB genes was performed with the Seeplex VRE detection kit. Specimens were inoculated in enterococcosel broth for 16–24 h before PCR for enrichment of VRE. VRE were isolated from 741 of the 8815 specimens by chromogenic agar-based culture (8.4 %). vanA and vanB genotypes were detected in 758 (8.6 %) and 3 (0.03 %) specimens, respectively, by multiplex PCR. Sensitivity, specificity, positive predictive value and negative predictive value of PCR for detection of VRE were 98.2 %, 99.6 %, 95.7 %, and 99.8 %. No VRE were isolated from vanB-positive specimens. The overall performance of PCR is comparable to that of a chromogenic agar-based culture method for screening of VRE, so PCR could be an alternative or supportive method for effective control of nosocomial VRE infection.

INTRODUCTION

Emergence of vancomycin-resistant enterococci (VRE) has become a serious issue for nosocomial infection control worldwide. The US Centers for Disease Control and Prevention reported that the incidence of colonization with VRE has rapidly increased since 1989 (CDC, 1995). In Korea, the prevalence of VRE has increased with the use of oral vancomycin since 1998 (Shin et al., 2003). VRE are resistant to most major classes of antibiotics and can transfer vancomycin resistance to highly virulent meticillin-resistant Staphylococcus aureus (Weigel et al., 2003). In addition to high morbidity and mortality associated with VRE, substantial increase of patient care cost has also been reported (Carmeli et al., 2002; DiazGranados et al., 2005). Therefore, rapid detection of VRE is very important for control and prevention of nosocomial spread of VRE.

Current techniques used for detection of VRE include selective culture or combination of culture with molecular detection of genes responsible for vancomycin resistance. Although culture is the method of choice for confirmation of VRE, it is time-consuming and may delay isolation of patients carrying VRE (Petrich et al., 1999; Roger et al., 1999). On the other hand, molecular detection of resistance-related genes requires a relatively short period of time. A multiplex PCR kit (Seeplex VRE detection, Seegene, Seoul, Korea) for rapid screening of VRE has recently been developed. The assay detects vanA and vanB genes by use of a dual-priming oligonucleotide system. The purpose of this study was to compare the performance of multiplex PCR with that of a chromogenic agar-based culture method for screening of VRE.

Abbreviations: NPV, negative predictive value; PPV, positive predictive value; PYR, pyrrolidonyl arylamidase; VRE, vancomycin-resistant enterococci.
METHODS

Patients and specimens. This study was conducted at a tertiary care hospital in Seoul, Korea, and approved by the institutional review board. From January to December 2009, we examined a total of 8815 rectal swab specimens from 7377 patients for whom VRE rectal examination was requested for the purpose of VRE surveillance or follow-up of proven VRE infection. Among them, 8427 specimens were collected from inpatients who were being admitted to general wards or intensive-care units, 370 from the emergency room, and the remaining 18 from outpatients during the study period. Rectal swabs were directly inoculated onto a chromogenic agar plate (ChromID VRE agar, bioMérieux, France) containing 8 μg vancomycin ml⁻¹ and incubated at 35 °C aerobically. The agar plates were screened for growth of presumptive colonies after 24 and 48 h of incubation. For colonies resembling enterococci, Gram stain and pyrrolidonyl arylamidase (PYR) test were performed, and PYR-positive, Gram-positive cocci were screened for vancomycin resistance. Colonies were inoculated onto blood agar plates containing a 30 μg vancomycin disk. Following an incubation period of 24 h, colonies that demonstrated vancomycin resistance were submitted to identification and antimicrobial susceptibility testing.

Bacterial identification and antimicrobial susceptibility test. The species of presumptive isolates were identified by the automated VITEK-2 system (bioMérieux). We performed the methyl d-glucopyranoside (MGP) test in order to distinguish Enterococcus gallinarum or E. casseliflavus from E. faecium. Antimicrobial susceptibility and MICs of vancomycin and teicoplanin were determined by the same instrument. For isolates determined to be either susceptible or having intermediate resistance to vancomycin by VITEK-2 system and E-test, the isolate was proven resistant by bacterial identification and antimicrobial susceptibility tests were designated VRE. Enterococci with natural resistance to vancomycin were referred to as VRE only when they were proven to carry the vanA gene. Sensitivity, specificity, positive predictive value and negative predictive value of multiplex PCR were evaluated in comparison with the results of chromogenic agar-based culture.

Chromogenic agar-based culture. Rectal swabs were directly inoculated onto a chromogenic agar plate (ChromID VRE agar, bioMérieux, France) containing 8 μg vancomycin ml⁻¹ and incubated at 35 °C aerobically. The agar plates were screened for growth of presumptive colonies after 24 and 48 h of incubation. For colonies resembling enterococci, Gram stain and pyrrolidonyl arylamidase (PYR) test were performed, and PYR-positive, Gram-positive cocci were screened for vancomycin resistance. Colonies were inoculated onto blood agar plates containing a 30 μg vancomycin disk. Following an incubation period of 24 h, colonies that demonstrated vancomycin resistance were submitted to identification and antimicrobial susceptibility testing.

Multiplex PCR for vanA and vanB gene detection. For enrichment of VRE prior to PCR, one cotton swab was inoculated into enterococcosel broth containing 6 μg vancomycin ml⁻¹ and incubated for 16–24 h at 35 °C, after which 50 μl culture broth was boiled at 100 °C for 10 min and centrifuged at 12000 r.p.m. for 5 min for DNA extraction. Then 3 μl supernatant was added to 17 μl PCR Mastermix [consisting of 4 μl 5 × VRE primer, 3 μl 8-methoxypsoralen and 10 μl 2 × Multiplex Master Mix (Seegene)] to make a final volume of 20 μl. PCR amplification was performed with a GeneAmp PCR System 9700 (Applied Biosystems) using the following conditions: initial denaturation at 94 °C for 15 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, extension at 72 °C for 1 min; final extension at 72 °C for 10 min. vanA- and vanB-positive enterococci isolated from clinical specimens including Enterococcus faecalis were included as external positive controls. The Screen Tape System (Lab901) was used for analysis of PCR products for the vanA and vanB genes.

Data analysis. In this study, the chromogenic agar-based culture method was considered as the reference method for VRE screening. Enterococcus species that were demonstrated to be vancomycin resistant by bacterial identification and antimicrobial susceptibility tests were designated VRE. Enterococci with natural resistance to vancomycin were referred to as VRE only when they were proven to carry the vanA gene. Sensitivity, specificity, positive predictive value and negative predictive value of multiplex PCR were evaluated in comparison with the results of chromogenic agar-based culture.

RESULTS

Chromogenic agar-based culture

A total of 741 VRE were isolated from 574 patients, and overall culture positivity rate was 8.4% (741/8815). All of the VRE recovered were resistant to vancomycin (MIC ≥ 32 μg ml⁻¹) by the VITEK-2 system and E-test. Among the isolates, E. faecium was the predominant Enterococcus species (n=729, 98.4%), followed by E. faecalis (n=5, 0.7%), E. avium (n=4, 0.5%), E. hirae (n=2, 0.3%) and E. gallinarum (n=2, 0.3%). E. gallinarum was isolated from two separate specimens from the same patient and demonstrated to have the VanA phenotype by the VITEK-2 system and E-test. Also, the isolate was proven to have the vanA gene by multiplex PCR. A considerable number of VRE carrying the vanA gene were determined to have intermediate resistance or be susceptible to teicoplanin by the VITEK-2 instrument. According to our VRE testing manual, we did not carry out an E-test for

Fig. 1. Flowchart showing the shortest course to report positive results of VRE screening. For the chromogenic agar-based culture, a preliminary report was provided after 24 h of incubation whether the presumptive organism grew or not. EB, enterococcosel broth; BAP, blood agar plate; AST, antimicrobial susceptibility test.
teicoplanin susceptibility for vancomycin-resistant isolates. The mean time required from specimen submission to final report was 4.9 days (range 2.4 to 11.7 days). However, a presumptive VRE-positive report was provided as early as the day after submission for the Gram-positive cocci that grew on ChromID VRE agar and were positive for the PYR test.

Multiplex PCR

A comparison of the results of multiplex PCR and chromogenic agar-based culture is presented in Table 1. A total of 761 specimens from 592 patients were positive for vanA or vanB (758 vanA-positive and 3 vanB-positive). Mean turnaround time for PCR was 1.8 days. Compared to the culture method, the multiplex PCR assay had an overall sensitivity of 98.2% and a specificity of 99.6%. The positive predictive value (PPV) and negative predictive value (NPV) were 95.7% and 99.8%, respectively. Overall agreement of PCR with culture was 99.5%. PCR inhibition was not observed in our study.

Discrepant specimens

Forty-six specimens showed discrepant results between culture and PCR. Among them, 13 tested culture-positive and PCR-negative; 30 tested culture-negative and vanA PCR-positive; and 3 tested culture-negative and vanB PCR-positive. Of the 13 culture-positive and PCR-negative specimens, 12 were E. faecium and 1 was E. faecalis. An investigation of the PCR-positive but culture-negative results was undertaken by time-series analysis of VRE screening results tested around the screening date. Eight specimens that were positive by multiplex PCR with broth enrichment, but culture negative, were obtained from patients for whom at least one other specimen collected before and 1 week after the screening date was culture positive. Two specimens that were collected from one patient at 2 day intervals repeatedly showed positive PCR results, despite the fact that no VRE was isolated from the same specimens by the culture method. It is of note that E. faecium was isolated from the specimen collected 3 days before the test date.

Table 1. Comparison of chromogenic agar-based culture with PCR for the vanA and vanB genes

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<thead>
<tr>
<th>PCR</th>
<th>Chromogenic agar-based culture</th>
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<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>PCR positive</td>
<td></td>
</tr>
<tr>
<td>vanA</td>
<td>728</td>
</tr>
<tr>
<td>vanB</td>
<td>728</td>
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<tr>
<td>PCR negative</td>
<td>13</td>
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<td>Total</td>
<td>741 (8.4%)</td>
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DISCUSSION

Infections with VRE are significant issues for nosocomial infection control because they are associated with high patient care cost and an increased mortality rate. The impact of active surveillance on reduction of the VRE infection rate was demonstrated in one study which compared two hospitals, one with no routine VRE screening and the other with an active surveillance programme (Price et al., 2003). Also, Lee et al. (2005) reported that a VRE surveillance programme reduced the overall cost of care by nearly 200-fold. Thus, improvements in VRE diagnostics using reliable screening methods may improve the rate of early detection of VRE carriers and reduce the risk of VRE transmission in health-care settings.

A nucleic acid-based detection system provides a rapid and sensitive method for detection of the presence of resistance genes. Since the PCR method for detection of glycopeptide resistance was first described in 1995 (Dutka-Malen et al., 1995), use of PCR-based systems for detection of VRE has increased significantly in clinical microbiology laboratories. PCR has advantages over phenotypic methods by reducing the time required for detection of resistance and allowing more timely implementation of infection control interventions.

Among studies that have evaluated the performance of PCR for surveillance of VRE, results from this study were obtained from one of the largest collections of specimens. The results of our study demonstrated that the performance of multiplex PCR with enrichment broth is comparable to that of culture for screening of VRE using rectal swab specimens. The sensitivity and specificity of multiplex PCR in our study (98.2% and 99.6%, respectively) were higher than those of Lee et al. (2010) (92.5% sensitivity, 98.9% specificity), who evaluated the performance of the same PCR kit in comparison with chromogenic agar and blood agar plates with vancomycin disks with 180 rectal swab specimens. Previous studies that evaluated the performance of PCR for VRE screening have shown good results for detection of the vanA gene. However, low specificity and poor PPV for vanB-positive results have been a limitation of the PCR method (Mak et al., 2009; Stamper et al., 2007). The presence of vanB-containing transposons Tn5382 and Tn1549 in non-enterococcal anaerobic bacteria, such as Clostridium spp., Eggerthella lenta and Ruminococcus spp., may be a possible explanation for the high vanB false-positive rate (Ballard et al., 2005a, b; Domingo et al., 2005). However, only three specimens were positive for vanB PCR in our study and this had almost no effect on overall performance. In this study, no VRE was isolated from vanB PCR-positive specimens. One possible explanation is that some vanB-containing Enterococcus spp. with low-level resistance to vancomycin may have been inhibited by vancomycin at 8 or 6 μg ml⁻¹ (Cetinkaya et al., 2000; Grabsch et al., 2008a). ChromID VRE agar exhibits good performance for early detection and identification of clinically important VRE species, because it
confers characteristic colours for *E. faecium* (purple) and *E. faecalis* (blue or blue-green) that are different from other *Enterococcus* species and Gram-positive bacilli. However, ChromID VRE agar contains 8 μg vancomycin ml⁻¹, so *vanB*-containing VRE with lower vancomycin MICs may not be readily isolated on ChromID VRE agar compared to bile aesculin agar containing 6 μg vancomycin ml⁻¹.

On the other hand, the majority of discordant results between PCR and culture were observed in *vanA*-positive specimens (30 of 33, 91%). Non-enterococcal isolates harbouring *vanA* genes may contribute to false-positive results. Albeit in limited numbers, *Bacillus circulans*, *Arcanobacterium haemolyticum*, *Oerskovia turbata* and *S. aureus* have been reported to acquire *vanA* genes (Ligozzi *et al.*, 1998; Power *et al.*, 1995; Sievert *et al.*, 2008). However, the possibility of true VRE positivity of these specimens should not be discounted. Eight of these PCR-positive but culture-negative specimens were from patients for whom at least one other specimen collected before and 1 week after the screening date was culture positive. And two obtained from one patient at 2 day intervals repeatedly showed a positive PCR result despite the fact that no VRE was isolated from the same specimens by the culture method. The fact that *E. faecium* was isolated from this patient 3 days before the test date implies that *vanA*-containing enterococci were present in those specimens. Stool density of VRE also can be a factor influencing the sensitivity of the culture method (D’Agata *et al.*, 2002). A rectal swab specimen collected for one set of screening tests consists of two cotton swabs, and each swab may contain a different concentration of VRE. A low density of VRE in specimens used for culture may yield a false-negative result, whereas a resistance gene is detected by the PCR method. And nonviable or viable but nonculturable *Enterococcus* spp. may cause culture-negative but PCR-positive results (Lleo *et al.*, 2003). Specimens with culture-negative and PCR-positive results remain problematic in infection control strategies. This problem may be resolved partially by a broth enrichment step prior to culture. Two previous studies that evaluated the performance of ChromID VRE agar reported that an overnight enrichment in brain heart infusion broth containing 3 μg vancomycin ml⁻¹ prior to inoculation significantly increased the sensitivity of the medium (Grabsch *et al.*, 2008b; Kuch *et al.*, 2009).

Albeit in small numbers, PCR-negative but culture-positive results were observed in 13 specimens. A possible explanation for this discrepancy may be that the bacterial load in those specimens was near the detection limit of the PCR assay. Some mutations, particularly those within the primer binding sequence, may also yield false-negative results (Usacheva *et al.*, 2010).

Discrepancy between genotype and phenotype determined by the automated VITEK-2 system was frequently observed. A considerable number of isolates carrying the *vanA* gene were determined to have intermediate resistance or be susceptible to teicoplanin by the instrument. Hashimoto *et al.* (2000) demonstrated that the VanB phenotype in VRE carrying the *vanA* gene was attributable to point mutations in *vanS*. In Korea, the mechanism of the VanB phenotype in VRE carrying the *vanA* gene was an insertion of IS1216v into the *vanX–vanY* intergenic region (nt 8625–9051) and subsequent deletion of *vanY* and *vanZ* (Park *et al.*, 2006). However, isolates which seemed to have the VanB phenotype in our study were indeed demonstrated to have the VanA phenotype by disk diffusion test. Thus, we concluded that the discrepancy between genotype and phenotype arose from the incomplete ability of an automated instrument to determine the MIC of teicoplanin. Supplementary phenotypic methods, such as the disk diffusion test and E-test, may easily resolve this problem.

PPV and NPV of a diagnostic test in clinical practice depend on the prevalence of the targeted abnormality in the population being tested. Low prevalence of *vanB* (3 in 8815 specimens) may significantly compromise the PPV of PCR, compared to results for *vanA*, in which the prevalence was high (758 in 8815 specimens). Overall PPV and NPV of the PCR method nearly equalled those of *vanA* PCR in our study. Thus, implementation of infection control intervention can be undertaken immediately when *vanA* PCR is positive. On the other hand, intervention may be delayed when the assay is positive for *vanB* only.

In conclusion, multiplex PCR with enrichment broth showed good correlation with a chromogenic agar-based culture method for screening of VRE and offered results at least 2 days earlier than culture. Use of multiplex PCR would have important implications for effective control of nosocomial VRE infection.

REFERENCES


