Real-time PCR detection of VRE

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On-demand, non-batched testing of Vancomycin-resistant Enterococci (VRE) with the Spartan DX™.

Introduction

VRE is currently the third-leading cause of nosocomial bloodstream infections in intensive care units in the United States (Ref 1). It is responsible for increased patient morbidity and mortality, and longer hospital stays. Van A and van B are the most clinically relevant Vancomycin-resistance genes. The main reservoir for these genes are *E. faecium* and *E. faecalis* (Ref 2,3,4). The van C gene is less significant because it only confers low-level resistance. Van C may be found in *E. gallinarum*, *E. casseliflavus,* and *E. flavencens* (Ref 5,6,7).

Infection control for VRE relies on rapid and sensitive testing. However, standard culture methods are labor-intensive and require 48-72 h to give a result. Furthermore, van B is challenging to identify because it has a broad Minimum Inhibitory Concentration (MIC) range of 8-512 mg/L. In contrast with culture, DNA-based methods such as real-time PCR provide faster and more accurate results by detecting van A, van B, and van C genes (Ref 8).

The purpose of this study was to develop two real-time PCR assays for VRE detection. The first identifies van A, van B, and van C, and the second, more rapid program, identifies only van A and van B. These assay were designed for use on the Spartan DX, a low-throughput DNA analyzer for on-demand, non-batched testing.

Materials and Methods

Characterization of clinical isolates

Bacterial isolates were recovered from rectal and stool specimens. Specimens were screened on bile esculin azide agar (BEAA-V6) plates. Positive colonies were sub-cultured onto blood agar plates, and identified as *Enterococci* based on Gram stain, and catalase and L-pyrrolidonyl-β-naphthylamide (PYR) tests.

Isolates identified as *Enterococci* were inoculated in Methyl-alpha-D-glucopyranoside broth to screen for Vancomycin resistance. *Enterrococci* identified as VRE were tested for MIC to vancomycin. Isolates were further speciated by sugar testing.

DNA extraction

Crude DNA was extracted using boiling lysis from 70 VRE isolates, 20 Vancomycin-sensitive *Enterococci* (VSE) isolates, and 10 non-*Enterococci* isolates. In brief, a sterile transfer device was used to pick 4 bacterial colonies from a culture plate. The colonies were resuspended in 200 µl of sterile water and boiled for 10 min. Extracted DNA was stored at -20°C.

Real-time PCR

Oligonucleotide primers were designed against van A, van B1, van B2/3, van C1, van C2, and van C3 genes that code for Vancomycin resistance. The primers were designed with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). One set of primers was designed for each gene. For example, the van B primers recognize both the van B1 and the van B2/3 subtypes, and similarly for the van C primers (Table 1). Primers were optimized to increase their melting temperatures using online software (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer).

Primer/Probe	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)
van A primers	tat gat ggc cgc tgc agg ta	cgg tga aat tat ccc aag tgg c	163
van A probe	6-FAM-tgc act tcc cga act g-BHQ*plus*		
van B primers	gcc atg caa aac cgg gaa ag	caa gcg att tcg ggc tgt ga	192
van B probe	6-FAM-tga gcc acg gta tct tc-BHQ*plus*		
van C primers	ggg aag atg gca gta tcc aag g	gct tga tgc agc agc cat tt	109
van C probe 1	6-FAM-cct tat gtt ggt tgc ca-BHQ1*plus*		
van C probe 2	6-FAM-tgc ctt atg tag gct gc-BHQ1*plus*		

6-FAM = 6-carboxy-fluorescein, BHQ*plus* = Black Hole Quencher *plus*

Table 1. Primer/probe sequences and amplicon sizes.

Oligonucleotide BHQplus probes were designed against van A, van B1, van B2/3, van C1, van C2, and van C3 genes using Biosearch Technologies' RealTimeDesign software (http://www.biosearchtech.com/products/probe_design.asp). One probe each was designed for the van A, van B1, and van B2/3 genes, whereas two probes were designed for the identification of the van C1, van C2, and van C3 subtypes.

Table 1 shows primer sequences and expected amplicon sizes. Components of the PCR amplification mixture are listed in Table 2, and cycling parameters are listed in Tables 3 and 4. Reactions were performed using the Spartan DX instrument and 0.2 ml thin-wall flat cap PCR tubes (VWR, Cat. No. 53550-106). Reactions were overlaid with 15 µl of PCR-grade mineral oil (Biotools, Cat. No. 20.032) to prevent evaporation. Negative controls consisted of reactions without DNA template.

Reactions were preformed as per Table 3 and table 4. VRE Program 1 total run time was 70 min Program 2 total run time was 30 min.

Assay reproducibility was determined by testing three isolates: van A, van B1, and van B2/3. Each isolate was tested 20 times using the same assay, with Program 1 (Table 6).

In parallel, samples were tested for van A and van B using the LightCycler® VRE Detection Kit (Roche, Cat. No. 03 334 996 001) on the Roche LightCycler 2.0 instrument.

DNA analysis

Fluorescence results from the Spartan DX were analyzed using Spartan Analyzer software. Real-time PCR results were confirmed by gel electrophoresis.

Reaction Component	Final Amount
10X PCR Reaction Buffer (No $MgCl_2$) (Invitrogen)	1X
$MgCl_2$ (Invitrogen)	2.5 µM
dNTP mix (Invitrogen)	0.125 mM
Platimun Taq DNA polymerase (Invitrogen)	1 U
Primers (Integrated DNA Technologies)	1 µM each
BHQplus probes (Biosearch Technologies)	10 nM (van A & B)
BHQplus probes (Biosearch Technologies)	20 nM each (van C)
Template DNA	2 µl
Sterile water	
Total reaction volume	**20 µl**

Table 2. Components of PCR amplification mixture.

Results

For the 70 VRE samples, all were identified as either van A, van B, or van C, with the exception of one culture-positive sample that tested negative using both the Spartan and Roche assays (Table 5). Cycle threshold (Ct) values were reproducible within 1.5 standard deviations (Table 6). All of the non-VRE samples were found to be negative for van A, van B, and van C. Results were 100% concordant between the Spartan and the Roche assays. Gel electrophoresis confirmed the expected amplicon sizes for all of the real-time PCR results (Figure 1).

Discussion and Conclusions

Two real-time PCR assays were successfully developed to identify the van A, van B, and van C genes for Vancomycin resistance in VRE. Both assays were found to be specific and reproducible when tested against 100 previously-identified clinical isolates, including 70 VREs, 20 VSEs, and 10 non-*Enterococci* strains. One culture-positive VRE sample tested negative by both the Spartan and Roche assays. It is possible that this sample contained a less common Vancomycin resistance gene.

One of the assays allows the user to test for van A, van B, and van C with a slower program. The second assay tests for only van A and van B with a faster program. This provides flexibility for urgent testing, when faster results are required.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	10 s	1
Denaturation	95°C	30 s	50
Annealing/extension	50°C	50 s	50

Table 3. Cycling parameters for Program 1 that identifies van A, van B, and van C.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	24 s	1
Denaturation	95°C	16 s	50
Annealing/extension	50°C	16 s	50

Table 4. Cycling parameters for Program 2 that identifies van A and van B.

Bacterial Type† (n=100)	Spartan DX		LightCycler
	Program 1	Program 2	VRE Assay
VRE - van A	24	24	24
VRE - van B	34	34	34
VRE - van C	11	---	---
VRE - Unidentified	0	0	0
VSE (20)	0	0	---‡
Non-*enterococci* (10)	0	0	---‡

† Isolates genotyped using standard culture methods
‡ VSE and non-*Enterococci* samples were not tested on the LightCycler

Table 5. Results with Spartan DX and Roche LightCycler instruments & kits.

Sample	Isolate 1	Isolate 2	Isolate 3
Genotype‡	van A	van B1	van B2/3
N	20	20	20
Average CT	27.52	29.19	32.75
Std Dev	0.51	1.36	1.29

‡ As determined by the Spartan VRE Assay and the Roche LightCycler VRE assay

Table 6. VRE reproducibility results.

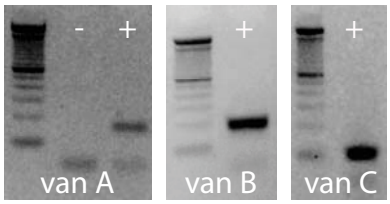

Figure 1. Gel electrophoresis results for van A, van B, and van C.

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