JOURNAL OF CLINICAL MICROBIOLOGY, June 2001, p. 2367–2368 Vol. 39, No. 6
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.6.2367–2368.2001

Improved Primer Design for Multiplex PCR Analysis of Vancomycin-Resistant *Enterococcus* spp.

Kariyama and colleagues (5) recently described a multiplex PCR method for the detection of the *vanA*, *vanB*, *vanC1*, and *vanC2/C3* genes, in addition to primers for the detection of the *ddl* genes of *Enterococcus faecalis* and *E. faecium* and of 16S rRNA (PCR control). However, the *vanB* primers used by these authors were derived from the sequence of the *vanB1* subtype (1) and are incapable of detecting *vanB2* or *vanB3* subtypes, as previously described (2, 4, 7) and recently demonstrated in our laboratories.

Calgary Laboratory Services (CLS) recently implemented the PCR scheme described by Kariyama et al. (5). Three enterococcal strains which displayed a vancomycin-resistant enterococci phenotype were negative for the *van* genes using this methodology even though the $ddl_{E.\ faecalis}$ and 16S rRNA genes were amplified. PCR using the primer set for *vanB* alone yielded an amplicon of 1.1 kb for all three strains, which was larger than the expected 433 bp. The isolates were subsequently referred to the National Microbiology Laboratory (NML), Winnipeg, Manitoba, for further analysis. The NML has implemented a multiplex assay which uses novel, unpublished primers for the detection of *vanB* genes, the *ddl* genes of *E. faecalis* and *E. faecium*, and a published set of primers for the *vanA* gene (3). Using this assay, all three strains produced the *E. faecalis*-specific product and the *vanB* product. In order to explain the discordant results, the NML sequenced the 1.1-kb product produced using the Kariyama et al. (5) primer set. Sequence analysis revealed that the reverse primer bound correctly. However, the forward primer did not appear to anneal at the site 5′ GTG ACA AAC CGG AGG CGA GGA 3′ since the corresponding sequence of the *vanB2* gene was 5′ GTG ACA AGC CGG AGG CGG GTG 3′. There are four nucleotide changes, with three of them at the 3-prime end, which likely results in no primer binding. This is predicted to also occur with the *vanB3* gene (5′ GTG ACA AGC CGG AGA CGG GTG 3′). Further sequence analysis revealed that the forward primer is binding to the upstream *vanH* gene at the following site: 5′ GGA TGT GTT GGA GGG CGA GGA 3′. Note that the last 8 bp of this region and the forward primer used by Kariyama et al. (5) are identical. This primer binding would result in an amplicon of 1,086 bp, which is consistent with the PCR results.

The NML currently uses in-house primers developed from consensus *vanB* sequences (forward, 5′ AAG CTA TGC AAG AAG CCA TG 3′, and reverse, 5′ CCG ACA ATC AAA TCA TCC TC 3′) capable of detecting all *vanB* subtypes, with an amplicon size of approximately 536 bp. The multiplex assay described by Kariyama and colleagues (5) was easy to perform and reliably detected *vanA*, *vanC1*, *vanC2/vanC3*, and species-specific genes for *E. faecalis* and *E. faecium* from other isolates tested at both CLS and NML. However, if one were to substitute the *vanB* primers used by Kariyama et al. (5) with those developed by NML, the multiplex assay would be capable of detecting all *vanB* subtypes. Other *vanB* consensus primers have been developed (2); however, these primers cannot be used in the multiplex assay described by Kariyama et al. (5) since the *vanC2* or *vanC3* amplicon sizes would be identical.

In summary, the presence of *vanB* subtypes necessitates the use of consensus PCR primers for rapid and reliable detection. While the work was in progress Kawalec et al. (6) described similar results in a *vanB2 E. faecium* strain and determined that mispriming within *vanH* was responsible, though they did not precisely determine the anomalous primer binding site.

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Authors' Reply

We thank Elsayed and colleagues for their implementation of our multiplex PCR scheme (2) and the primer design for consensus *vanB* sequences. We have one comment for their letter. The first sentence written by Elsayed and colleagues does not correctly describe our primers. The sentence "in addition to primers for the detection of the *ddl* genes of *E. faecalis* and *E. faecium*" should be changed to "in addition to primers for species-specific genes of *E. faecalis* and *E. faecium*." In our article (2), the primer set for *E. faecalis* amplifies the *ddl* gene, but the primer set for *E. faecium* does not amplify the *ddl* gene (1).

We performed the multiplex PCR assay (2) by using a novel combination of primer sets which included a primer set for consensus *vanB* sequences (referred to here as *vanB* consensus primer set) designed by Elsayed and colleagues. We found that *E. faecalis*-specific amplification was inhibited by a novel primer combination with the *vanB* consensus primer set if we used the same primer concentrations as previously described

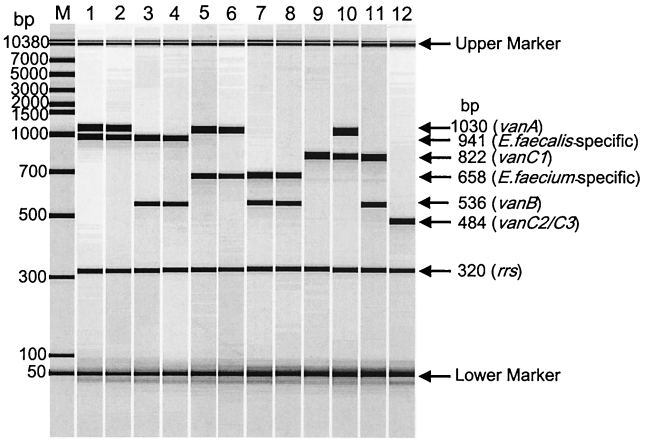

FIG. 1. Gel image generated by the Agilent 2100 bioanalyzer of amplified *vanA*, *vanB*, *vanC1*, *vanC2*/*C3*, *E. faecalis*-specific, *E. faecium*-specific, and *rrs* genes by the optimized multiplex PCR assay (2) containing a novel primer combination as described above. Lanes: M, DNA ladder; 1, an *E. faecalis vanA* isolate; 2, an *E. faecalis vanA* isolate; 3, an *E. faecalis vanB* isolate; 4, an *E. faecalis vanB* isolate; 5, an *E. faecium vanA* isolate; 6, an *E. faecium vanA* isolate; 7, an *E. faecium vanB* isolate; 8, an *E. faecium vanB* isolate; 9, an *E. gallinarum vanC1* isolate; 10, an *E. gallinarum vanC1* and *vanA* isolate; 11, an *E. gallinarum vanC1* and *vanB* isolate; 12, an *E. casseliflavus* or *E. flavescens vanC2* or *vanC3* isolate.

(2). Then, we optimized the *vanB* consensus primer concentration, which was found to be one-half of the concentration previously described (2). As shown in Fig. 1, the best result was observed when the novel primer combination was added to the reaction mixtures at 5 pmol each of *vanA*, *E. faecalis*-specific primers; 2.5 pmol each of the *vanC1*, *vanC2*/*C3*, *rrs* primers; and 1.25 pmol each of the *vanB*, *E. faecium*-specific primers.

Using the novel primer combination, vancomycin-resistant enterococci (low- to high-level resistance) from our collection were tested. For all isolates, PCR products with two or three intense bands on agarose gels were generated as shown previously (2). Of 139 isolates tested, 14 *E. faecalis* (*vanA*) isolates, 29 *E. faecalis* (*vanB*) isolates, 42 *E. faecium* (*vanA*) isolates, 9 *E. faecium* (*vanB*) isolates, 24 *E. gallinarum* (*vanC1*) isolates, 2 *E. gallinarum* (*vanC1* and *vanA*) isolates, 2 *E. gallinarum* (*vanC1* and *vanB*) isolates, and 17 *E. casseliflavus* or *E. flavescens* (*vanC2* or *vanC3*) isolates were clearly identified. The results indicated that we missed four isolates possessing a *vanB2* or *vanB3* gene previously.

Figure 1 shows a gel image of PCR products obtained by the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, Calif.) which utilizes chip-based nucleic acid separation technology (3). The LabChip7500 (Caliper Technologies, Mountain View, Calif.) was used to analyze PCR products. This instrument improves DNA analysis compared with agarose gel electrophoresis and is suitable for routine laboratories (3).

We appreciate Elsayed and colleagues, who designed the improved primer for consensus *vanB* sequences and gave us an opportunity to show new data obtained by the Agilent 2100 bioanalyzer. In summary, the novel primer combination using the optimized primer concentration described above is more reliable for the detection of vancomycin-resistant enterococci, since this combination detects all *vanB* subtypes.

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