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Original Article

Simple and Rapid Detection of Vancomycin-Resistance Gene from Enterococci by Loop-Mediated Isothermal Amplification

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We developed a simple and rapid method for detecting vancomycin resistance genes, such as *vanA* and *vanB*, using loop-mediated isothermal amplification (LAMP). To identify not only vancomycin resistance genes, but also the genus *Enterococcus*, primers were designed for *vanA*, *vanB*, and 16S rRNA. Screening for vancomycin susceptibility in *Enterococcus* was performed using Etest (bioMérieux Inc). The results of the LAMP assay were compared to those of real-time RT-PCR. The optimal conditions for the LAMP assay were 65℃ for 60 min. The detection limits of the LAMP assay for *vanA*, and *vanB* were 2×10^2 copies/reaction. Compared to RT-PCR, the sensitivities and specificities of LAMP for 16S rRNA, *vanA*, and *vanB* were 100/100%, 100/100%, and 100/100%, respectively. The *vanA* genotype-vanB phenotype accounted for 57.5% (46/80) of the vancomycin-resistant Enterococci samples collected from 2016 to 2019. In conclusion, the LAMP assay developed in this study showed high sensitivity and specificity for vancomycin-resistant genes. Moreover, due to the simplicity and rapidity of the LAMP assay, its use can be very useful in clinical microbiology laboratories.

Key Words: Vancomycin-resistant Enterococci (VRE), Loop-mediated isothermal amplification, *vanA*, *vanB*, 16S rRNA

INTRODUCTION

Vancomycin-resistant enterococci (VRE) has rapidly spread and emerged as a major nosocomial problem worldwide, since first being isolated in 1986 (Uttley et al., 1988; Bonten et al., 2001). VRE can cause a variety of invasive infections including intraabdominal infection, bacteremia, and endocarditis. Invasive VRE infections are difficult to treat and are associated with high mortality (Chiang et al., 2017). Moreover, the transferability of the *vanA* gene from Enterococci to *Staphylococcus aureus* can cause serious problems (Niederhäusern et al., 2011).

Rapid and accurate detection of VRE is required for timely antimicrobial treatment and infection control. Culture-based methods to detect VRE are time-consuming, taking several days to complete (2~5 days). Various PCR-based methods have been used for rapid detection of VRE in many hospitals. PCR-based methods are highly sensitive and specific for *vanA*-type VRE; however, these methods require special

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machines and experienced technicians. Moreover, many false-positive results are reported for *vanB* VRE, mainly due to the non-enterococcal *vanB* gene, which can be found in anaerobic bacteria in the gut (Ballard et al., 2005; Graham et al., 2008).

Recently, loop-mediated isothermal amplification (LAMP) has been used for the detection of various infections (Hara-Kudo et al., 2005; Misawa et al., 2007; Yamazaki et al., 2008). Compared to PCR, LAMP has the advantage of simplicity, rapidity of detection (by the naked eye) and a short amplification time under isothermal conditions. Moreover, LAMP can amplify DNA with high sensitivity and efficacy; it relies on autocycling strand displacement DNA synthesis performed using the Bst DNA polymerase large fragment. LAMP shows high specificity when a set of four specifically designed inner and outer primers are used (Notomi et al., 2000; Li et al., 2017).

This study aimed to develop and evaluate a LAMP assay designed for simple and rapid detection of the *vanA* and *vanB* genes.

MATERIALS AND METHODS

Bacterial strains

A total of 128 strains of Enterococci, including 88 strains of VRE and 40 strains of vancomycin-susceptible Enterococci (VSE), were included in this study. Among 88 VRE, 2 reference strain with *vanB* genotype and 2 clinical strains with *vanC* genotype were included. The remaining 84 VRE were clinical strains: 78 *vanA E. faecium*, 4 *vanA E. faecalis*, 1 *E. avium* and 1 *E. raffinosus* (Table 1). To evaluate the specificity of the LAMP assay for 16S rRNA, 12 reference strains, including 6 gram positive cocci (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pneumoniae* ATCC 49619, *Streptococccus agalactiae* ATCC 12386, *Streptococcus bovis* ATCC 49147), 5 gram negative rods (*Escherichia coli* ATCC 25922, *Klebsiella penumoniae* ATCC 70063, *Enterobacter cloacae* ATCC 700323, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606) and 2 yeasts (*Candia albicans* TIMM 3316 and *Candida parapsilosis*) were used.

Table 1. The species of Enterococci used for evaluation of LAMP assay in this study

Vancomycin susceptibility (n)	Species of Enterococci (n)	Genotype by RT-PCR	
		vanA	*vanB*
VRE (88)	*E. faecalis* (6)	4	2*
	E. faecium (78)	78	0
	E. avium (1)	1	0
	E. raffinosus (1)	1	0
	E. casseliflavus (1)†	0	0
	E. gallinarum (1)†	0	0
VSE (40)	*E. faecalis* (20)	0	0
	E. faecium (20)	0	0

Abbreviations: RT, real-time; VRE, vancomycin-resistant Enterococci; VSE, vancomycin-susceptible Enterococci
**E. faecalis* ATCC 700802 and ATCC 51299
†*E. casseliflavus* and *E. gallinarum* has inherent *vanC* gene

DNA extraction from bacterial isolates

A single colony was diluted with 200 μL sterile saline and boiled for 10 min at 100℃. After boiling, the bacteria-containing liquid was centrifuged for 30 sec at 12,000 rpm. The supernatant was used as the template for real-time RT-PCR and LAMP.

Identification of bacteria and antimicrobial susceptibility test

Identification of bacteria was performed using the VITEK 2 system (bioMérieux Inc., Durham, NC, USA). The minimum inhibitory concentration (MIC) of vancomycin and teicoplanin was determined by the Etest (bioMérieux Inc.) according to the manufacturer's instructions. The concentration of 0.5 MF (1.0×10^8 CFU/mL) was inoculated to Muller-Hinton agar and incubated for 24 h at 35℃ in a non-CO_2 incubator. The breakpoints were also described in the Clinical and Laboratory Safety Institute guidelines (CLSI, 2018).

Primer design and optimization of reaction conditions for LAMP and RT-PCR assay

Gene sequences of 16S rRNA, *vanA*, and *vanB* were searched for in the GenBank database and analyzed with CLC Genomics Workbench (Qiagen, Hilden, Germany) to

Table 2. The sequences of primers for LAMP assay used in this study

Target gene	Primer	Sequence (5' → 3')
16S rRNA	F3*	GCCGCGGTAATACGTAGG
	B3*	TCGCCACTGGTGTTCCTC
	FIP	CGGGGGCTTTCACATCAGACTT-GTCCGGATTTATTGGGCGTA
	BIP	CTCAACCGGGGAGGGTCATTG-TTTCACCGCTACACATGGAA
	LF	AAGAAACCGCCTGCGCTCG
	LB	GAAACTGGGAGACTTGAGTGC
vanA	F3*	GGATTACTTGTTAAAAAGAACCATG
	B3*	TCCCAGCATTTTTCGCAA
	FIP	CCTTGTATGGATCCATCTTCACC-CCATGTTGATGTAGCATTTTCAG
	BIP	TGTTTGAATTGTCCGGTATCCCTT-CGATGTATGTCAACGATTTGTC
	LF	TGACTTGCCATGCAAAG
	LB	TGCGATATTCAAAGCTCAGCAA
vanB	F3*	TACGGAATGGGAAGCCGA
	B3*	CAAGCTGCGGAGCTTTGA
	FIP	ACGCCGTGTTTCGTATTCGCTT-GTCTCCCCGCCATACTCTC
	BIP	CTTTCCCGGTTTTGCATGGCAA-CCCACATAGGGGATACCAGA
	LF	CCATGCGTTTTCCTATCCGG
	LB	ATGCGGGGAGGATGGTG

*Two outer primers F3 and B3 were also used as primers of real-time PCR for 16S rRNA, *vanA* and *vanB*
Abbreviations: loop-mediated isothermal amplification; FIP, forward inner primer; BIP, backward inner primer; LF, forward loop primer; LB, backward loop primer

identify highly conserved regions. LAMP primer sets were designed using Explorer V5 software (Eiken Chemical Co. Ltd., Tokyo, Japan). Primer sets included two external primers (forward outer primer F3 and backward outer primer B3), two internal primers (forward inner primer FIP and backward inner primer BIP), and two loop primers (forward loop primer LF and backward loop primer LB). All primers were synthesized by Bionics, Inc. (Seoul, Korea). Detailed information for the three primer sets used in this study is presented in Table 2 and Fig. 1.

To optimize the reaction conditions of LAMP, various reaction temperatures and times were used. LAMP was carried with a master mix solution containing 5 μL of WarmStart® colorimetric LAMP master mix (New England Biolabs Inc., Ipswich, MA, USA), 1 μL of F3 and B3 primer, 1 μL of FIP and BIP primer, and 1 μL of LF and LB primer for each reaction. A volume of 2 μL DNA template extracted from the bacterial isolate was added to the master mix and incubated at 65℃ for 60 min. The mixture was then heated at 80℃ for 10 min for enzyme inactivation. A color change of phenol red pH indicator from pink to yellow, due to a decrease in pH in the presence of extensive amplified DNA, indicated a positive LAMP reaction. LAMP results were also confirmed by 2% agarose gel electrophoresis and the "ladder-like" amplified DNA products indicated a positive LAMP reaction. To confirm the LAMP results, RT-PCR (CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was also performed using SYBR Green (Bio-Rad) with 10 pmol of outer primers (F3 and B3) and 2 μL DNA template. The PCR conditions were as follows: initial denaturation at 95℃ for 5 min, 35 cycles of denaturation at 95℃ for 30s, annealing at 60℃ for 30s, elongation at 72℃ for 30s, and a final elongation step of 72℃ for 5 min.

Detection limits of LAMP assay for the 16S rRNA, *vanA* and *vanB* genes

To determine the detection limits of the LAMP assay for

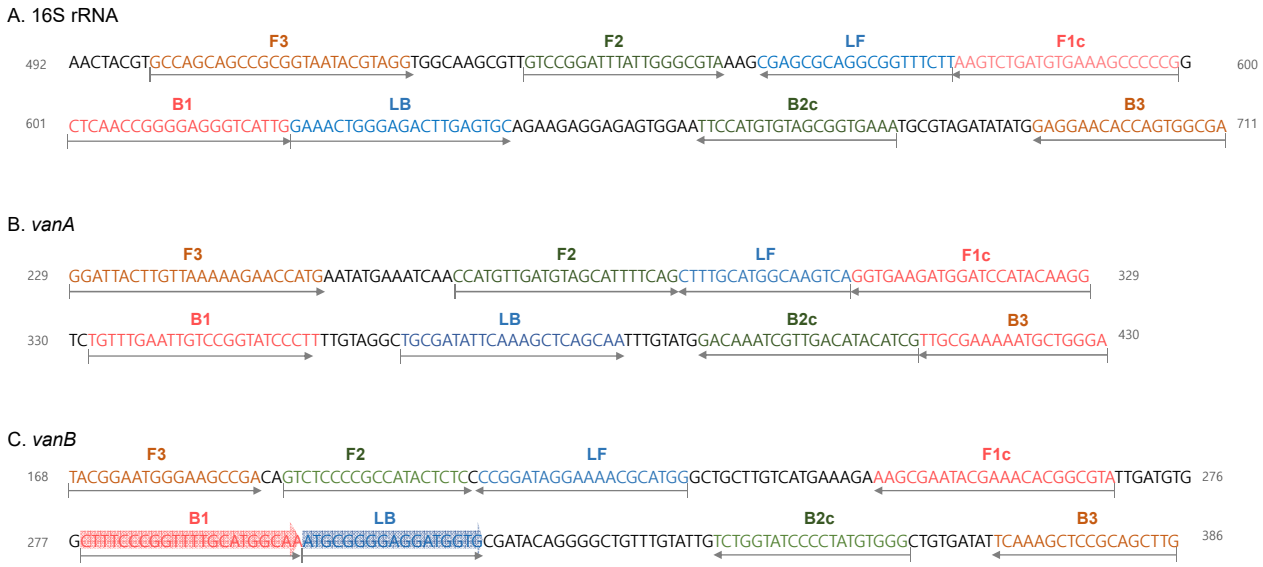

Fig. 1. Primer designed for 16S rRNA, *vanA*, *vanB* loop-mediated isothermal amplification (LAMP) assays. Nucleotide sequences of 16S rRNA (A), *vanA* (B), *vanB* (C) and the location of LAMP primers. The forward and backward inner primers are F1c-F2 and B1c-B2 sequences, respectively. The forward and backward outer primers are F3 and B3, respectively.

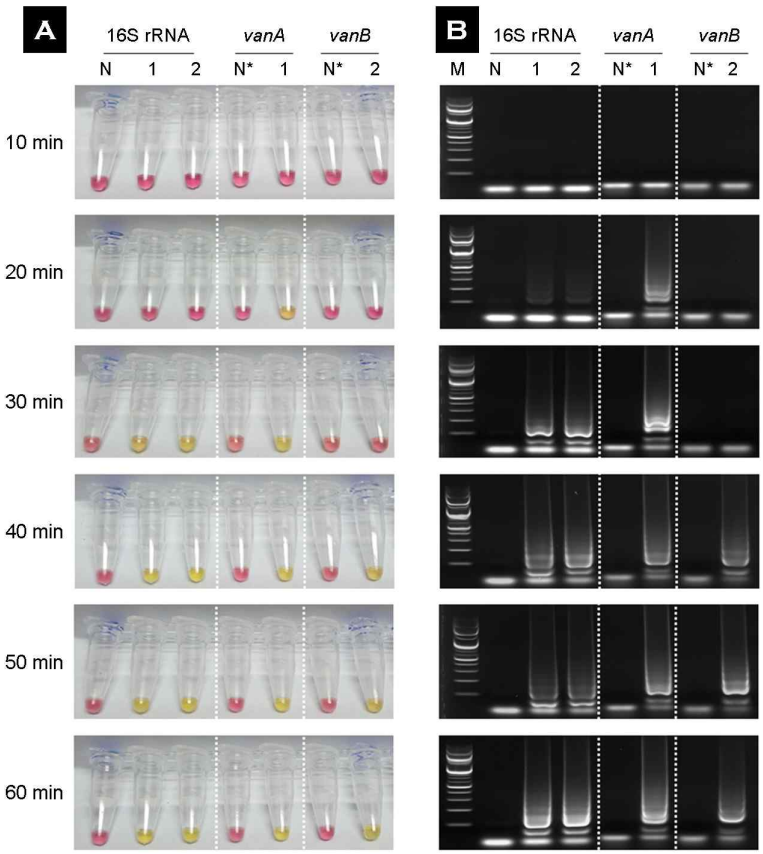

Fig. 2. Visual (A) and agarose gel (B) images of the 16S rRNA, *vanA*, *vanB* loop-mediated isothermal amplification (LAMP) product of vancomycin-resistant Enterococci (VRE) on various reaction times. The yellow color change of pH indicator was interpreted positive for amplification of DNA (A). The electrophoresis was performed at 2% agarose gel and amplified products typically showed the ladder like shape (B). The positive reaction of LAMP assay for *vanA*, 16S rRNA and *vanB* was observed at 20 min, 30 min, and 40 min, respectively and the best reaction of the three genes was obtained at 65℃ and 60 min. Abbreviations: N, negative (non-*Enterococcus*, *Staphylococcus aureus* ATCC 25923); N* (vancomycin susceptible *Enterococcus*, *E. faecalis* ATCC 29212).

the 16S rRNA, *vanA*, and *vanB* genes, the inoculum with VRE was continuously subjected to 10-fold step dilution from 10^8 to 10^2 CFU/mL. The experiment was repeated three times.

Specificity of the LAMP assay for 16S rRNA in the genus *Enterococcus*

The specificity of the LAMP assay for 16S rRNA was evaluated using 12 reference strains (see Bacterial strains section in Material and Methods).

Performance of the LAMP assay using clinical strains

To evaluate the performance of the LAMP assay for detection of the *vanA* and *vanB* genes, the LAMP assay and RT-PCR were performed simultaneously on 128 strains of Enterococci with resistance (88) or susceptibility (40) to vancomycin. RT-PCR served as the reference method.

RESULTS

In the reference strains of VRE (*vanA* and *vanB* type) and VSE, LAMP to the *vanA*, 16S rRNA, and *vanB* genes was observed at 20, 30, and 40 min after amplification, respectively. The optimal conditions for the LAMP assay were 65℃ for 60 min (Fig. 2). Therefore, subsequent tests were performed under those conditions.

Detection limit of LAMP

The detection limits of LAMP were 20 copies/reaction (10^4 CFU/mL) for 16S rRNA and 200 copies/reaction (10^5

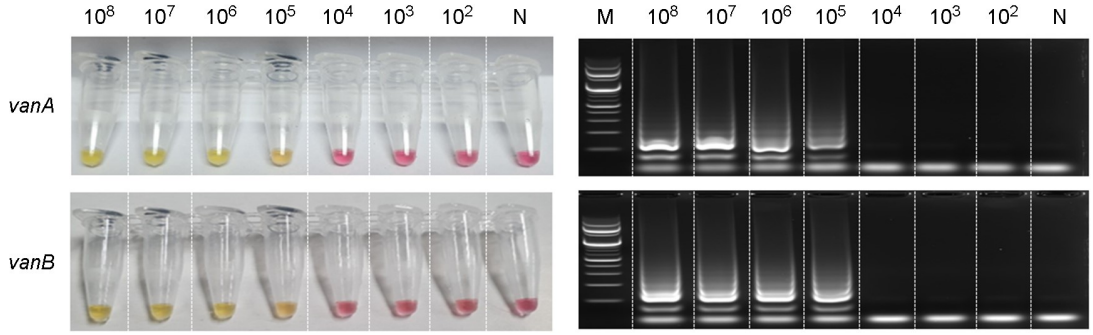

Fig. 3. Visual (A) and agarose gel (B) images of the *vanA* and *vanB* loop-mediated isothermal amplification (LAMP) product of VRE isolated at serial diluted concentration (10^2~10^8 CFU/mL). The amplified products typically showed the ladder like shape at 10^5 CFU/mL for *vanA* and *vanB*, which is 200 copies per reaction. Abbreviations: N, negative, M, molecular size ladder

Table 3. The results of loop-mediated isothermal amplification method for the detection of *vanA*, *vanB* and 16s rRNA gene from 140 strains

Phenotypic ID*	AST†	Gene‡	LAMP results for *vanA*, *vanB* and 16S rRNA			
			vanA+*vanB*-	*vanA*-*vanB*+	*vanA*-*vanB*-	16S rRNA
Enterococci (128)	VRE (88)	*vanA* (84)	84	0	0	84
		vanB (2)	0	2	0	2
		vanC (2)	0	0	2	2
	VSE (40)	ND	0	0	40	40
Non-Enterococci (12)§			0	0	12	0

Abbreviations: ID, identification; AST, antimicrobial susceptibility testing; VRE, vancomycin-resistant Enterococci; VSE, vancomycin susceptible Enterococci; ND, not detection; LAMP, loop mediated isothermal amplification.
*Identified by Vitek system and various biochemical reaction test (see text)
†Antimicrobial susceptibility testing for vancomycin and teicoplanin by E-test
‡Gene for vancomycin resistance detected by real-time PCR
§Included 5 GPC (*S. aureus*, *S. epidermidis*, *S. pneumoniae*, *S. bovis*, *S. agalactiae*), 5 GNR (*E. coli*, *K. pneumoniae*, *E. cloacae*, *P. aeruginosa*, *A. baumannii*) and 2 yeasts (*C. albicans*, *C. parapsilosis*)

CFU/mL) for *vanA* and *vanB* gene (Fig. 3).

Sensitivity and specificity of LAMP for 16S rRNA

Among 140 isolates, including 128 Enterococci and 12 non-Enterococci, the ability of RT-PCR and LAMP assay to detect the 16S rRNA gene of Enterococci was identical. The sensitivity and specificity of LAMP for 16S rRNA were both 100% (Tables 3, 4).

Evaluation of the performance of LAMP in detecting *vanA* and *vanB* from clinical strains

Among 86 VRE strains, with the exception of 2 intrinsic vancomycin-resistant isolates (*vanC*-type VRE including *E. casseliflavus* and *E. gallinarum*), 84 isolates were found to be *vanA* type and 2 were found to be *vanB* type by RT-PCR. For 86 VRE strains, the ability of LAMP to detect the *vanA* and *vanB* genes was the same as that of RT-PCR (Table 3, 4).

The MIC of vancomycin and teicoplanin in vancomycin-resistant *E. faecium* from 2016 to 2019

Among 80 isolates of *E. faecium* harboring the *vanA* gene collected from clinical specimens from 2016 to 2019, antimicrobial susceptibility testing revealed that 46 (57.5%) strains were of the vanB phenotype (Table 5).

Table 4. The performance of loop mediated isothermal amplification for real time PCR for detection of *vanA*, *vanB* and 16S rRNA gene from 140 strains

	Performance of loop-mediated isothermal amplification for three genes		
	vanA	*vanB*	16S rRNA
Sensitivity (%)	84/84 (100%)	2/2 (100%)	128/128 (100%)
Specificity (%)	56/56 (100%)	138/138 (100%)	12/12 (100%)

DISCUSSION

Various methods, including conventional antimicrobial susceptibility testing, use of chromogenic media (Delmas et al., 2007), and PCR (Palladino et al., 2003) have been used to detect vancomycin resistance from Enterococci. Recently, studies have been conducted to detect these genes using LAMP, which is faster and does not require special equipment (Kim et al., 2014; Huang et al., 2019). In this study, we developed a LAMP method that detects not only the *vanA* and *vanB* genes, but also the 16s rRNA gene in clinical strains of Enterococci.

LAMP showed high sensitivity and specificity for vancomycin resistance genes, and avoided the missed diagnoses associated with phenotypic detection of VRE. A response of LAMP to the *vanA*, 16S rRNA, and *vanB* genes was observed at 20, 30, and 40 min after amplification, respectively. The optimal conditions for the LAMP assay were 65℃ for 60 min (Fig. 2). Therefore, subsequent tests were performed under those conditions. The quick response of LAMP to *vanA* (20 min) would be very useful in many clinical situations where only that gene needs to be detected. The detection limits of LAMP for *vanA* and *vanB* were 200 copies per reaction respectively, which is sufficient to detect these genes in a VRE colony. However, in clinical specimens, the amount of VREs is less than in colonies, so it is necessary to improve the detection ability.

The ability of the LAMP assay to detect *vanA* and *vanB* genes in 88 VREs and 40 VSEs was consistent with that of RT-PCR. In Korea, *vanB* type VRE is rarely isolated, and VREs are mostly composed of *E. feacium*. In this study,

Table 5. Distribution of the MIC of vancomycin and teicoplanin in 80 VRE with *vanA* genotype isolated from clinical specimen during 2016-2019 year

Type		Range of MIC (μg/mL)		Number of isolates (%)
Genotype	Phenotype	Vancomycin	Teicoplanin	
vanA	vanA	≥256	≥32 ~ ≥256	34 (42.5%)
vanA	vanB	≥256	0.5 ~ 16	46 (57.5%)
Total		≥256	0.5 ~ ≥256	80 (100%)

Abbreviation: MIC, minimal inhibitory concentration

only two *vanB* type VREs and six vancomycin-resistant *E. feacalis* were included. Additional *vanB* genotypes and *Enterococcus* species should be investigated to validate the LAMP assay. The *vanC*-type VRE has an innately low level of resistance to vancomycin, and is not routinely detected in clinical laboratories (Cetinkaya et al., 2000). Therefore, the detection of *vanC* gene was not performed in this study.

It is useful to simultaneously detect 16S rRNA and vancomycin resistance genes, because the *vanB* gene can be isolated from bacteria other than *Enterococcus* (Ballard et al., 2005; Graham et al., 2008). To evaluate the 16S rRNA detection performance of the LAMP assay, it was performed on 128 strains, including 12 reference strains; accurate results were obtained for all strains (Table 3, 4).

The *vanA*-genotype-vanA phenotype accounts for the majority of cases, but several studies have shown that the prevalence of the *vanA*-genotype-vanB phenotype is increasing, both in Korea and worldwide (Lauderdale et al., 2002; Lee et al., 2004). According to Jung et al. (Jung et al., 2014), the *vanA* genotype-vanB phenotype comprised 70% of cases in tertiary hospitals in Korea from 2010 to 2011. Therefore, it is becoming more difficult to distinguish VRE by phenotype alone, and genetic tests for the *vanA* and *vanB* genes are important for the treatment of infected patients and infection control. In this study, 80 VREs isolated from 2016 to 2019 had the *vanA* genotype-vanB phenotype, representing 56.7% of cases (46/80) (Table 5). The molecular basis of the *vanA* genotype-vanB phenotype discrepancy has yet to be identified, but it has been suggested that impairment of accessory proteins VanY and VanZ, genetic rearrangement (including deletion of both *vanY* and *vanZ* following insertion of IS1216V), and mutations in the *vanS* regulatory gene may be responsible for the loss of teicoplanin resistance (Simonsen et al., 2000; Lauderdale et al., 2002; Lee et al., 2004; Jung et al., 2014).

A limitation of this study was that the VRE colony was directly used in the LAMP reaction; the inhibitory effect of LAMP on clinical samples, such as fecal samples, was not evaluated. In the future, it will be necessary to evaluate the applicability of this method to clinical samples.

In conclusion, the multiplex LAMP assay developed in this study showed excellent sensitivity and specificity for the *vanA* and *vanB* genes, and good agreement with the RT-PCR results. Due to the simplicity and rapidity of the LAMP assay, its use can be very useful in clinical microbiology laboratories.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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