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Development of a Novel Immunochromatographic Assay for Rapid Detection of VanA Ligase-Producing Vancomycin-Resistant Enterococci

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We developed a novel immunochromatographic assay (ICA) (EZ-Step VanA rapid kit; Dinona, Korea) for the detection of VanA ligase from vancomycin-resistant enterococci (VRE). Of eight monoclonal antibodies screened by ELISAs, the VanA ligase ICA constructed with 1H9 plus 3G11 showed the greatest reactivity. The detection limit of the kit was 6.3×10^6 CFU per test. Of 127 vancomycin-resistant microorganisms, 100 *vanA* VRE were positive in the VanA ligase ICA, and 27 non-*vanA* vancomycin-resistant isolates were negative. These results were consistent with those of the PCR analyses. Thus, our ICA is a reliable and easy-to-use immunological assay for detecting VanA-producing VRE in clinical laboratories.

Keywords: Vancomycin resistance, VanA, immunochromatographic assay, rapid detection, *Enterococcus*

Since vancomycin-resistant enterococci (VRE) first emerged in the United Kingdom in 1986 [13], the occurrence of VRE has spread worldwide [8, 6, 15]. Among the nine gene clusters (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*) that are known to mediate glycopeptide resistance, the two genotypes of greatest clinical importance are *vanA* and *vanB* [7, 16]. The *vanA* genotype, which is the most commonly isolated VRE genotype, is associated with the transfer of high-level vancomycin resistance from enterococci to *Staphylococcus aureus* [5].

Real-time PCR can be used to detect vancomycin-resistant genes (*vanA, B, C*) rapidly and with great sensitivity; thus, it is commonly used to detect VRE in rectal swabs or in stool [2, 3]. However, the assay requires trained personnel and specialized equipment that may not be readily available in small laboratories and it may be less cost-effective than culturing. The development of a simpler method than PCR for detecting VRE (*e.g.*, immunologic methods) may be useful to overcome the limitation of PCR or to confirm colonies selected from the screening media for VRE [9].

In this study, we designed an immunochromatographic assay (EZ-Step VanA rapid kit; Dinona, Iksan, Korea) using novel monoclonal antibodies (mAbs) specific for VanA ligase and evaluated the performance of the rapid kit using VRE.

To express recombinant VanA ligase, the *vanA* gene was PCR-amplified from vancomycin-resistant *E. faecium* isolated from a clinical specimen using specific primers (5′-caccatgaatagaataaaagttgcaa-3′ and 5′-ccccttttaacgctaatacgatca-3′). The amplified *vanA* gene of 1,768 bp, corresponding to the VanA ligase, was ligated directly into the pET101/D-TOPO cloning and expression vector (Invitrogen, Carlsbad, CA, USA) by the heat-shock method [1]. The ligation products were transformed into competent *Escherichia coli* BL21 Star (DE3) cells (Invitrogen), and the transformants were selected on LB agar containing 50 μg/mL ampicillin. Anti-VanA ligase mAbs were prepared as described previously [12]. Purified His-tagged recombinant VanA ligase was used for immunization and mAbs were screened from hybridomas by indirect ELISAs as described below. A microplate coated with rVanA ligase was incubated with

hybridoma supernatant for 1 h, followed by HRP-conjugated goat anti-mouse IgG (Dinona). After 1 h, tetramethyl benzidine substrate solution (Invitrogen) was added and the reaction was stopped using 1.0 N sulfuric acid. To determine which mAb were suitable for use as the capture antibody in the ICA from among the previously selected mAbs, a sandwich ELISA was performed as follows: a microplate coated with anti-VanA antibody (1.0 μg/ml) was incubated for 1 h with rVanA solution, and then HRP-conjugated anti-VanA mAb (the detector antibody) were added. In addition, to prepare the test and control lines of ICA, 1.0 mg of mouse mAb specific for VanA ligase (as the capture antibody) and 0.3 mg of anti-mouse IgG (Dinona) per test were coated onto a nitrocellulose membrane (Millipore, Billerica, MA, USA) at 30 and 35 mm distal from the sample application area, respectively. Colloidal gold particle conjugated with anti-VanA monoclonal IgG (as the detector antibody) was sprayed onto a conjugated pad (Millipore) placed on an adjacent sample pad and lyophilized overnight.

Of the eight mAbs screened by indirect ELISA, 3G11 showed the highest reactivity against the *vanA* VRE extract; this mAb was selected as the detector antibody (Fig. 1A). Four mAbs (1B7, 1C11, 1G10, and 1H9) were found to be suitable for use as capture mAbs in sandwich ELISA when 3G11 was used as the detector mAb (Fig. 1B). To develop the ICA, 3G11 was labeled with colloidal gold particles as the detector of VanA ligase, whereas 1B7, 1C11, 1G10, and 1H9 were immobilized on a nitrocellulose membrane.

Of the ICA kits constructed (four combinations), the kit that included 3G11 plus 1H9 showed the greatest reactivity against the *vanA* VRE extract; therefore, these two mAbs were used to prepare the VanA ligase ICA. The detection limit for bacteria was 6.3×10^6 CFU (10.3 ng) per test or 7.8×10^7 CFU per test on 6 μg/ml vancomycin-containing agar or Mueller Hinton (MH) broth (BD, Sparks, MD, USA) with 4 μg/ml vancomycin, respectively.

To prepare specimens for ICA, bacterial colonies incubated on Enterococcosel agar (BD) with 6 μg/ml vancomycin were suspended in phosphate-buffered saline (PBS), and the cells were collected by centrifugation at 5,000 ×*g* for 1 min. The supernatants were discarded and 30 μl of lysis solution (8.0 M urea in 20 mM phosphate buffer with pH 7.4) was added. The bacterial cells were further lysed by vortexing with glass beads for 1 min. Finally, 120 μl of dilution buffer was added, and 0.1 ml of the bacterial lysate was added to the test plate. The tests were scored visually within 20 min as per the manufacturer's instructions. In addition, pellets from MH broth with vancomycin were also suspended in PBS and were processed in the same way, as described previously. Of 127 vancomycin-resistant microorganisms, 100 *vanA* genotype VRE were positive by the VanA ligase ICA in both media, and 27 non-*vanA* vancomycin-resistant isolates were negative. The results were fully consistent with those of PCR analyses (Table 1 and Fig. 2).

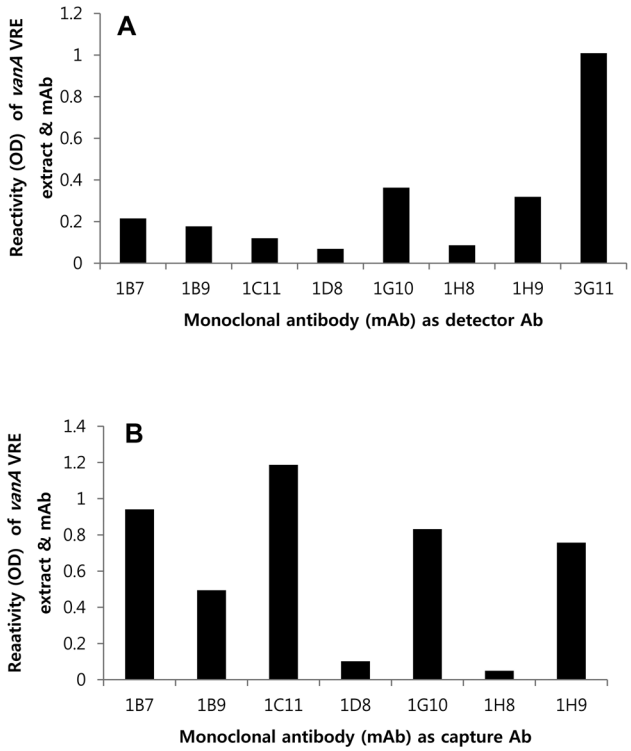

Fig. 1. Selection of monoclonal antibody pair to detect VanA ligase by indirect and sandwich ELISAs.
(**A**) 3G11 shows the highest reactivity against 2.0 μg of an extract of *vanA* vancomycin-resistant enterococci. (**B**) Four antibodies (1B7, 1C11, 1G10, and 1H9) were suitable for use as a capture antibody in sandwich ELISA when 3G11 was used as the detector antibody.

After VRE have been cultured and identified, molecular methods are necessary to determine their genotype; such methods are labor-intensive and costly, yet indispensable, and their use other than in epidemiological investigations is controversial [16]. The immunological assay is useful for confirming the genotype of VRE in this situation, which is encountered daily in microbiological laboratories worldwide. Moreover, this VanA ligase ICA can incorporate multiple test lines and will facilitate control of nosocomial infection by VRE together with ICAs for VanB ligase. The analytical sensitivity of the assay was 6.3×10^6 CFU (10.2 ng) per test in agar containing 6 μg/ml vancomycin. This detection limit is sufficient because the samples used were found to

Table 1. Diagnostic performance of the immunochromatographic assay for VanA ligase in clinical isolates cultured from Enterococcosel agar or Mueller-Hinton broth containing vancomycin compared with PCR detection of vancomycin-resistant enterococci.

PCR detection of *vanA* gene	Microorganism (No. of isolates)	Immunochromatographic assay for VanA ligase		Sensitivity (%)	Specificity (%)
		Positive	Negative		
Positive	*E. faecium* (92)	92	0		
	E. faecalis (7)	7	0		
	E. durans (1)	1	0		
	Total (100)	100	0	100	
Negative	*E. faecalis* or *faecium* (4)[a]	0	4		
	E. gallinarum (4)[b]	0	4		
	E. casseliflavus (6)[b]	0	6		
	Pediococcus spp. (1)[c]	0	1		
	Leuconostoc spp. (2)[c]	0	2		
	Candida albicans (2)[c]	0	2		
	GNR (8)[c,d]	0	8		
	Total (27)	0	27		100

[a]*vanB* type VRE, including *E. faecalis* ATCC700802 and *E. faecalis* ATCC 51299.

[b]*vanC* type VRE.

[c]Bacteria cultured in Mueller-Hinton broth with vancomycin were used for VanA ligase immunochromatographic assay owing to a lack of growth in Enterococcosel agar.

[d]GNR, gram-negative rod. Clinical isolates composed of *Escherichia coli* (2), *Klebsiella pneumoniae* (2), *Pseudomonas aeruginosa* (2), and *Acinetobacter baumannii* (2).

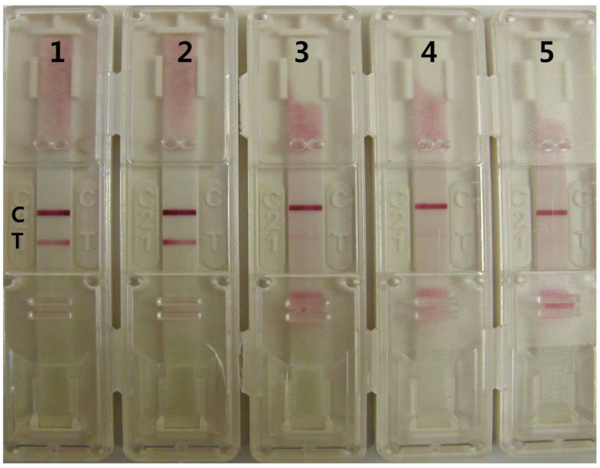

Fig. 2. Results of an immunochromatographic assay to detect VanA ligase from *vanA* and *vanB* vancomycin-resistant enterococci cultured on Enterococcosel agar with vancomycin.
Lanes 1 and 2 (*vanA* type) show distinct positive results, whereas lanes 3 and 4 (*vanB* type) show negative results. Lane 5 is the negative control (phosphate buffer).

contain 10^8 to 10^9 CFU. In addition, the VanA ligase concentration range as assessed by ELISAs in 20 clinical samples collected from Enterococcosel agar containing 6 μg/mL vancomycin was 109–1,100 ng/test (data not shown).

In this study, we included only a few isolates carrying the ligase gene encoding D-Ala:D-Lac ligase for the synthesis of peptidoglycan precursors with low affinity for glycopeptides except *vanA*, including *vanB*, *vanD*, and *vanM* (*vanB* type, four isolates). *vanB* VRE are the second most common type globally, but they have not been reported in Korea since 1999 [4, 10, 11, 14]. Moreover, the *vanD* and *vanM* type VRE are not isolated in Korea. Additional reagent to enable testing for these types, which show partial homology to VanA ligase, may become a necessary part of the VanA ligase kit in future.

Potential drawbacks of the VanA ligase kit are its requirements for broth enrichment with vancomycin or incubation in vancomycin-containing agar medium to induce VanA ligase. However, a 4 h incubation time was sufficient for induction of VanA ligase in broth containing 4 μg/ml vancomycin, and no further incubation was required for colonies cultured in vancomycin-containing screening agar (Enterococcosel agar containing 6 μg/ml vancomycin in this study), which is commonly used in clinical laboratories for VRE screening.

In conclusion, the novel ICA described here is a reliable,

easy-to-use, and rapid immunologic method for the detection of VanA ligase-producing VRE from conventionally cultured or screened bacterial colonies. The additional development of rapid immunological assays for VanB ligase will facilitate the control of VRE infection and epidemiological investigations.

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