Real-time PCR surveillance of *vanA* for vancomycin-resistant *Enterococcus faecium*

TAEK SOO KIM[1], HYE LIN KWON[2], SANG HOON SONG[1,2], KYOUNG-HO SONG[3], HONG BIN KIM[3], KYOUNG UN PARK[1,2], JUNGHAN SONG[1,2] and EUI-CHONG KIM[1]

[1]Department of Laboratory Medicine, Seoul National University College of Medicine, Seoul; Departments of [2]Laboratory Medicine and [3]Internal Medicine, Seoul National University Bundang Hospital, Seongnam, Republic of Korea

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Abstract. In Korea, the incidence of vancomycin-resistant enterococci (VRE) infection has recently been increasing, with a clinical isolation rate of up to 25%. We evaluated the clinical usefulness of real-time PCR for the detection of the *vanA* gene over conventional culture in patients confirmed as being VRE-colonized. During the evaluation stage, 100 consecutive clinical specimens were analyzed for the presence of the *vanA* gene via real-time PCR, for which 4 specimen preparations were used. During the application stage, 1,115 specimens from 82 patients were monitored for 20 months by *vanA* real-time PCR. For isolates prepared via incubation in broth for 24 h, the PCR results were concordant with those of the conventional method. The median value of the time spent in isolation and the number of test repeats from detection to release from isolation was 71 days and 5.5, respectively. The optimal negative test number for the release from isolation was ≥3. In conclusion, the real-time PCR method is more sensitive than, and is expected to replace, the conventional VRE surveillance method.

Introduction

Enterococci have been recognized as an important cause of nosocomial infection in the past few decades (1). Clinical infections caused by *Enterococcus* species include urinary tract infections, bacteremia, bacterial endocarditis and diverticulitis (2). Moreover, vancomycin-resistant isolates of *Enterococcus faecium* (*E. faecium*) and *Enterococcus faecalis* (*E. faecalis*) have been described (3). Three genetic mechanisms of enterococcal resistance against glycopeptides have been described (4). Overall, vancomycin resistance in enterococci has been associated with more frequent episodes of recurrent bacteremia, persistent isolation of enterococci from primary infection sites, increased frequency of endovascular infection, and increased mortality (5).

Due to the rapid increase in the incidence of vancomycin-resistant enterococci (VRE) infections, particularly of those acquired in hospitals, the Centers for Disease Control and Prevention published recommendations for preventing the spread of vancomycin resistance in 1995 (6). These recommendations included a policy for deciding the time of removal of patients from isolation; that is, VRE-negative results on at least 3 consecutive occasions (≥1 week apart) in all cultures from multiple body sites. This recommendation has generally been accepted, but the rationale of this policy seems to stem from an empirical basis.

The conventional phenotypic method has been the gold standard for VRE surveillance. However, a previous report suggested that the molecular identification of VRE is more sensitive and accurate than the conventional culture method (7). Thus, the molecular method can be used for a limited population, such as VRE-colonized patients (8).

In Korea, VRE infections have recently been increasing, with a clinical isolation rate of up to 25% (9). Most of the cases of VRE infections are caused by *E. faecium* (10,11). A previous study on *E. faecium* isolates from 10 teaching hospitals showed that all isolated VRE harboured the *vanA* gene (12).

The aim of the present study was to determine the clinical usefulness of real-time PCR for the detection of the *vanA* gene over the conventional culture method in patients confirmed as being VRE-colonized, and to re-evaluate the optimal number of negative tests before deciding a patient's removal from isolation.

Materials and methods

Evaluation stage. We compared the results of real-time PCR for the detection of the *vanA* gene, for which 4 preparations (direct specimen, isolate cultured in blood agar plate for 24 h, isolate cultured in broth for 24 h and isolate cultured in blood agar plate for 24 h, as well as in broth for the previous 24 h) of identical clinical specimens were used, with the results of the conventional culture method. A total of 100 consecutive routine clinical specimens were collected for 180 days at the Seoul National University Bundang Hospital. All specimens were simultaneously confirmed as VRE-positive or vanco-

Correspondence to: Professor Kyoung Un Park, Department of Laboratory Medicine, Seoul National University Bundang Hospital, 166 Gumi-Ro, Bundang-Gu, Seongnam, Gyeonggi 463-707, Republic of Korea
E-mail: m91w95pf@snu.ac.kr

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Table I. Results of real-time PCR for the detection of *vanA* in 4 preparations of specimens from VRE-infected or VRE-colonized patients.

	Method				
	Culture	Real-time PCR			
Specimen type	Broth and BAP	Direct	BAP	Broth	Broth and BAP
Other[a]	3	2	1	3	3
Fluid	4	4	3	4	4
Gastrointestinal	31	16	18	31	26
Genitourinary	9	4	5	9	9
Respiratory	5	4	4	5	5
Total	52	30	31	52	47

[a]Includes specimens from wounds, sores, etc. BAP, blood agar plate; VRE, vancomycin-resistant enterococci.

mycin-susceptible enterococci (VSE)-positive through the conventional method. Screening and identification of isolates were performed by plating in brain heart infusion agar with 6 μg vancomycin and via MicroScan WalkAway (Siemens Healthcare Diagnostics, West Sacramento, CA, USA), respectively. Additionally, antimicrobial susceptibility test profiles and minimum inhibitory concentraion (MIC) levels were determined by the disc diffusion method and E-test (bioMérieux, Marcy l'Etoile, France). Specimens used in the present study were extracted from the gastrointestinal tract (56 isolates), genitourinary tract (16 isolates), respiratory tract (8 isolates) and fluid (4 isolates); 16 isolates were from other sources.

Application stage. Between May 2008 and December 2009, a total of 1,115 clinical consecutive specimens from 82 previously established VRE-infected or VRE-colonized patients at the Seoul National University Bundang Hospital were evaluated for VRE surveillance. Specimens were obtained from 21 blood (8 patients), 16 fluid (5 patients), 636 gastrointestinal tract (79 patients), 320 genitourinary tract (48 patients), 98 other (16 patients) and 24 respiratory tract (7 patients) samples. All specimens were incubated in broth for 24 h.

For both the evaluation and the application, nucleic acid extractions were performed on each pre-treated specimen. In the direct specimen culture, a few colonies grown on blood agar plate were transferred to phosphate-buffered saline (PBS) via a cotton swab. The amount of 1 ml of mixture or broth was centrifuged at 15,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 1 ml PBS through centrifugation. After mixing the pellet with 20 μl of Chelex solution, the tube was heated at 100˚C for 20 min and cooled subsequently. After centrifugation at 15,000 rpm for 3 min, 100 μl of the supernatant was obtained for real-time PCR.

Real-time PCR for the detection of the *vanA* gene was performed with 3 μl of template DNA, 0.6 μl of each primer (*vanA* 3F, 5'-CTG TGA GGT CGG TTG TGC G-3'; *vanA* 3R, 5'-TTT GGT CCA CCT CGC CA-3'), 2.4 μl of $MgCl_2$, and 2 μl of LightCycler FastStart DNA Master HybProbe (*vanA* 3P, FAM-5'-CAA CTA ACG CGG CAC TGT TTC CCA AT-3'-TAMRA; Roche Diagnostics GmbH, Germany). The final volume was adjusted to 20 μl with distilled water. PCR was performed under the following conditions: 95˚C for 3 min; 40 cycles of 95˚C for 10 sec, 58˚C for 20 sec and 72˚C for 20 sec; followed by a final extension at 40˚C for 30 sec. As an internal control, real-time PCR for 16S ribosomal RNA (rRNA) was performed under the same conditions.

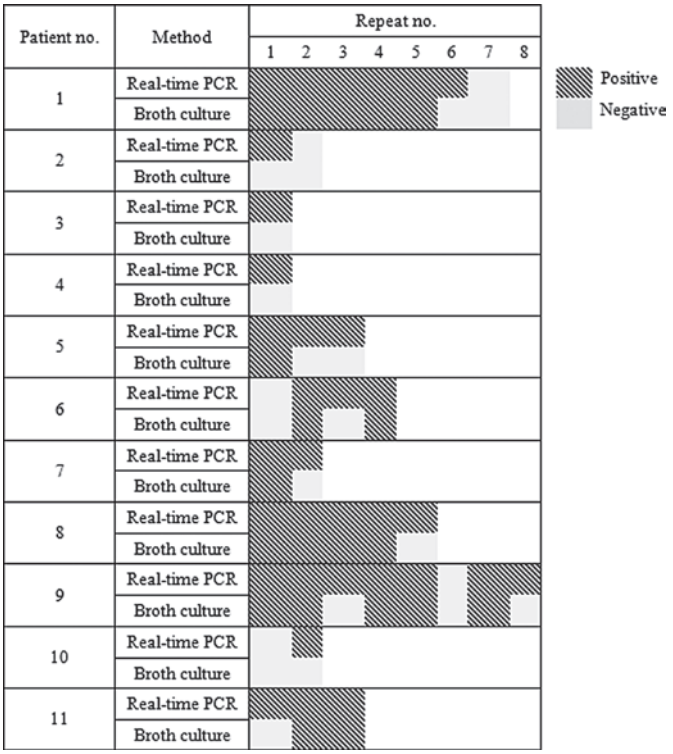

Figure 1. Discrepancies between the results of *vanA* real-time PCR and conventional broth culture for 11 of 19 patients.

Results

Evaluation stage. Between March 2007 and September 2007, a total of 100 clinical isolates of *E. faecium* were obtained consecutively from 19 patients at the Seoul National University

Table II. Comparison of real-time PCR for the detection of *vanA* and conventional culture for total and gastrointestinal specimens.

	Total no. of specimens		Gastrointestinal specimens	
real-time PCR for *vanA*	VRE detected by conventional culture n (%)	No VRE detected by conventional culture n (%)	VRE detected by conventional culture n (%)	No VRE detected by conventional culture n (%)
Positive	52 (52.0)	20 (20.0)	30 (53.6)	13 (23.2)
Negative	0	28 (28.0)	0	13 (23.2)

VRE, vancomycin-resistant enterococci.

Table III. Days and number of tests performed before release from isolation and specimen types in 23 patients.

Patient no.	Days	Repeats	Last specimen type	Initial specimen type
1	1	6	Other	Other
2	277	6	Gastrointestinal	Gastrointestinal
3	51	10	Gastrointestinal	Genitourinary
4	52	9	Gastrointestinal	Genitourinary
5	163	25	Other	Other
6	63	10	Gastrointestinal	Genitourinary
7	44	8	Gastrointestinal	Genitourinary
8	472	12	Gastrointestinal	Genitourinary
9	21	4	Genitourinary	Genitourinary
10	47	8	Gastrointestinal	Gastrointestinal
11	19	3	Gastrointestinal	N/A
12	55	9	Gastrointestinal	Genitourinary
13	21	3	Gastrointestinal/Genitourinary	Genitourinary
14	417	15	Gastrointestinal	N/A
15	102	16	Gastrointestinal	Other
16	13	3	Gastrointestinal/Other	Other
17	14	3	Gastrointestinal/Genitourinary	Blood
18	16	4	Gastrointestinal/Genitourinary	Other
19	236	10	Gastrointestinal	Genitourinary
20	19	4	Gastrointestinal	Genitourinary
21	17	3	Gastrointestinal	Other
22	142	22	Gastrointestinal	Other
23	33	6	Genitourinary	Genitourinary
Median	71	5.5		

N/A, no information available.

Bundang Hospital. All 100 isolates were identified as *E. faecium*. A total of 52 of these isolates were resistant and 48 were susceptible to vancomycin. Clinical specimens with VRE included fluid (n=4), gastrointestinal tract (n=30), genitourinary tract (n=9), respiratory tract (n=6) and others (n=3).

The results of real-time PCR for the detection of *vanA* in 52 isolates, for which 4 preparation methods were used, are shown in Table I. For isolates prepared via incubation in broth for 24 h, the PCR results were concordant with those of the conventional method. The results of real-time PCR for the detection of *vanA* in isolates prepared by the other 3 preparation methods showed relatively low sensitivity.

The results of real-time PCR for the detection of *vanA* in isolates prepared by 24-h incubation in broth were compared with the results of the conventional culture method. The results obtained from the total isolates and from those from the most dominant isolation site are summarized in Table II. Both specimen groups had 100% sensitivity. On the other hand, the specificity was 71.4% in the total number of specimens and 50% in the gastrointestinal specimens.

Disagreements between the results of real-time PCR for the detection of *vanA* and the conventional method were observed in 11 of 19 patients (Fig. 1). More than 1 discordant result was detected in 2 patients (patient nos. 5 and 9).

Application stage. For the preparation, we applied the 24-h broth incubation method to a total of 1,117 clinical follow-up isolates from 82 VRE-infected or VRE-colonized patients between June 2008 and December 2009. Of the 1,117 isolates, 372 were resistant to vancomycin as detected via real-time PCR for the detection of *vanA*. The most prevalent collection site was the gastrointestinal tract (including stool), with up to 636 isolates (56.9%).

Three consecutive negative results from real-time PCR for the detection of *vanA* were found in 23 patients; the median period until removal from isolation was 71 days, and the median number of tests was 5.5 (Table III). In these 23 patients, there were only 2 (8.3%) initial specimens from the gastrointestinal tract, whereas 20 (83.3%) gastrointestinal specimens were submitted for release from isolation (Table III). Two out of the 23 patients showed 2 consecutive negative real-time PCR results. After 1 negative result, the test results were once again positive in 5 patients. In 7 patients, only one type of specimen was submitted, whereas in 16, more than 2 types of specimen were submitted consecutively. Gastrointestinal specimens were submitted in the case of all patients, except in 1 specimen labelled 'other'. A total of 5 of 16 patients with 2 specimen types showed negative results simultaneously.

Discussion

Vancomycin resistance is an independent predictor of mortality among patients with enterococcal bloodstream infection (13). Considering the clinical significance of VRE colonization, infection and spread, we evaluated the usefulness of the real-time PCR method in comparison to the conventional phenotypic method. It is widely acknowledged that real-time PCR shows more rapid, sensitive and reproducible results than conventional PCR. Moreover, it can minimize the carryover contamination rate (14). Particularly in the clinical laboratory, contamination can lead to wrong diagnosis and treatment.

As previously reported (15), enterococcal broth culture is more effective for VRE detection than enterococcal agar culture. Similarly, the most efficient preparation method for real-time PCR is 24-h incubation in enterococcal broth, which produced the same results as conventional PCR in a previous study (16).

On the other hand, real-time PCR was found to have a much higher positive rate of VRE detection in all types of samples than the conventional method (data not shown). This may be due to the administration of antibiotics or the difference in the limit of detection. In the clinical laboratory, real-time PCR of isolates prepared by 24-h broth incubation may be more adequate and easier to perform, given that broth medium is more convenient and less time-consuming to use than agar medium.

There are 3 consecutive negative results in VRE surveillance accepted as the standard period until removal from isolation (6). As shown in Fig. 1 (particularly in the cases of patient nos. 5 and 10), real-time PCR showed a higher detection rate than the conventional method. However, to be released from isolation, 3 or more consecutive negative results of real-time PCR maybe optimal, which remains the current recommendation in the conventional method. This is supported by the fact that in the application stage, 2 of the 23 patients removed from isolation tested positive for VRE after 2 consecutive negative results of real-time PCR for the detection of *vanA*. Moreover, as shown in Fig. 1, real-time PCR was much more sensitive than conventional culture.

Three consecutive negative results of *vanA* real-time PCR were shown in 23 patients, with a median period for removal from precautionary isolation of 71 days, which is shorter than that of the previous report (246 days) (16). The median number of tests was 5.5. This may be due to the outlier in the evaluation of the mean and median values.

A previous study reported urine to be the most frequent site from which VRE was isolated (17) and the number of cultures counted was up to 88 out of 240 isolates (36.7%). Similarly, 11 of 19 'cleared' patients were primarily identified with a positive urine culture.

We believe that our study proves the usefulness of real-time PCR for surveillance purposes. Considering the geographical difference, real-time PCR may replace the conventional culture method in VRE surveillance.

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