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Evaluation of PCR-Reverse Blot Hybridization Assay, REBA Sepsis-ID Test, for Simultaneous Identification of Bacterial Pathogens and *mec*A and *van* Genes from Blood Culture Bottles

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Background: The aim of this study was to evaluate a newly developed PCR-based reverse blot hybridization assay (PCR-REBA), REBA Sepsis-ID (M&D, Wonju, Korea), to rapidly detect the presence of bacteremia and antimicrobial resistance gene in blood culture samples.

Methods: One thousand four hundred consecutive blood culture samples from patients with a delta neutrophil index greater than 2.7% were selected from March to July in 2013. Three hundred positive and 1,100 negative for bacterial growth in blood culture bottles samples were tested by conventional and real-time PCR-REBA, respectively.

Results: The overall agreement between the conventional identification test and the REBA Sepsis-ID test was 95.3% (286/300). Agreement for gram-positive bacteria, gram-negative bacteria, fungi, and polymicrobials was 94.5% (190/201), 97.3% (71/73), 100% (14/14), and 91.7% (11/12), respectively. The detection rate of the *mec*A gene from methicillin-resistant *Staphylococcus* isolates was 97.8% (90/92). The *van*A gene was detected in one blood culture sample from which vancomycin-resistant *Enterococcus* was isolated. When the cycle threshold for real-time PCR was defined as 30.0, 2.4% (26/1,100) of negative blood culture samples tested positive by real-time PCR.

Conclusions: The REBA Sepsis-ID test is capable of simultaneously and quickly detecting both causative agents and antimicrobial resistance genes, such as *mec*A and *van*, in blood culture positive samples.

Key Words: Real-time PCR, Blot, Hybridization, Bacteremia, *mec*A, Vancomycin resistance, Blood Culture

INTRODUCTION

Blood stream infections (BSIs) are associated with high rates of morbidity and mortality ranging from 20% to 70% worldwide [1-4]. BSIs are the 10th leading cause of death in the United States, accounting for 6% of all deaths [5]. An estimated 135,000 patients die each year of sepsis-associated complications in Europe [6]. Blood culture systems, which detect viable microorganisms in blood, are the current gold standard for BSI diagnosis. Patients with sepsis, defined as a clinical infection re-

sulting in a systemic inflammatory response, account for only about one third of the total positive cultures [7]. Although blood cultures are currently performed with continuous-monitoring blood culture systems (CMBCSs), several factors such as poor timing of collection, insufficient blood volumes, and the presence of antibiotics in the samples reduce the sensitivity of blood cultures [7, 8]. The main limitation of utilizing blood culture method is the vast amount of time required, as treating patients with empirical therapies before the blood culture analysis is complete may not serve to cure the illness. Kumar *et al*. [9] reported that the mean survival rate decreased by 7.6% every hour that effective antibiotic therapy was delayed following the onset of sepsis-related hypotension.

Recently, several PCR-based commercial assays that target a panel of clinically relevant bacterial and fungal bloodstream pathogens have been developed. Two types of commercial PCR-based assays are most common. The first type is designed for culture-positive samples. Examples of this type of assay are the peptide nucleic acid fluorescence *in situ* hybridization-based assay (AdvanDx, Woburn, MA, USA) [10], Hyplex Blood Screen (BAG, Lich, Germany) [11], and Prove-it Sepsis (Mobidiag, Helsinki, Finland) [12]. The second type is designed for direct blood samples. Examples of this type of assay are SepsiTest (Molzym, Bremen, Germany) [13] and Vyoo (SIRS-Lab, Jena, Germany) [14]. The PCR-based reverse blot hybridization assay (PCR-REBA, REBA Sepsis-ID; M&D, Wonju, Korea) was developed to rapidly detect bacterial and fungal pathogens and antimicrobial resistance genes in blood culture samples [15]. It uses pan-probes to distinguish gram-positive bacteria (GPB), gram-negative bacteria (GNB), and fungi. In addition, it uses probes for antibiotic resistance genes (i.e., the *mec*A gene of methicillin-resistant *Staphylococcus* spp. and the *van*A and *van*B genes of vancomycin-resistant enterococci).

The aim of this study was to evaluate the REBA Sepsis-ID test for rapid and accurate detection of pathogens and antimicrobial resistance genes in blood.

METHODS

This study was approved by the Institutional Review Board (CR312055) of Yonsei University Severance Hospital.

1. Collection of blood culture bottles

Three-hundred positive blood culture (PBC) and 1,100 negative blood culture (NBC) samples from patients with a delta neutrophil index (DNI) greater than 2.7% [16] were consecutively collected at Wonju Severance Christian Hospital from March to July in 2013. To avoid the redundancy of enrolled samples, only one blood culture sample per patient was allowed. The enrolled blood culture samples were simultaneously tested with the PCR-REBA and conventional microbiological tests. The overall positive rate of blood culture in this study period was 7.46% (1,640/ 21,979).

The PBC samples were eligible for enrollment if they had been flagged positive by BACTEC FX (Becton Dickinson, Sparks, MD, USA) or BacT/ALERT 3D (bioMérieux, Durham, NC, USA) with a positive Gram stain. The PBC bottles were then removed from the CMBCS and a 1,000 μL aliquot of the culture-broth mixture was aseptically collected by using a syringe and needle. The aliquot was then divided into halves. The first half (500 μL) was used to perform a Gram stain and subcultured on sheep blood agar and MacConkey agar, which were incubated at 35°C for 24-48 hr in 5% CO_2, and the second half (500 μL) was kept at -20°C for subsequent DNA extraction.

NBC samples were used if culture results were negative for five days of incubation in CMBCSs. After the blood culture bottles were removed from the CMBCS, 500 μL of blood suspension was collected and kept at -20°C for subsequent DNA extraction. All the NBC bottles were incubated at 35°C until PCR results were obtained. For the NBC samples with a positive real-time PCR result, 1,000 μL of blood suspension was used to inoculate routine subculture media (sheep blood agar, chocolate agar, and Sabouraud dextrose agar), and incubated at 35°C under 5% CO_2 for five days. For slow-growing bacteria, another 1,000 μL of blood suspension was used to inoculate special subculture media (plating count agar media [Becton Dickinson] [17] and Reasoner's 2A agar media [Becton Dickinson] [18]), which was incubated at 20°C low temperature incubator. Additionally, the remaining blood suspension was used to inoculate Luria-Bertani [19] and brain heart infusion broths (Becton Dickinson), which were incubated at 37°C incubator. Colonies isolated from the special subculture media and NBC samples that were positive by real-time PCR were confirmed by bacterial 16S rRNA and fungal 18S to 5.8S internal transcribed sequence analysis. The amplicons were sequenced by Xenotech Company (Daejeon, Korea). The conventional identification test and antimicrobial susceptibility test were done by using the MicroScan system (Siemens Healthcare Diagnostics, Sacramento, CA, USA).

2. DNA preparation

To prepare DNA templates from the 300 PBC and 1,100 NBC samples, DNA was extracted by using the following procedure. A

200 μL aliquot of the blood was mixed with 1,000 μL of erythrocyte lysis buffer (ELB) (Sigma, St. Louis, MO, USA) at room temperature for 10 min to disrupt erythrocytes. The supernatant was then removed after centrifugation at 13,000 g for 5 min. The pellet was washed with 1,000 μL of ELB to completely remove the erythrocytes and centrifuged under the same conditions. One hundred microliters of ELB was added to the pellet, which was then frozen and thawed twice. One hundred microliters of DNA extraction solution (M&D) was added to the mixture, and it was boiled for 15 min. After centrifugation at 13,000 g for 10 min, the supernatant was used as a DNA template for PCR.

3. PCR amplification

Conventional PCR amplification was performed according to the manufacturer's instructions to evaluate the PBC samples. Taq-Man real-time PCR assays were carried out by using Real-GP (gram-positive), -GN (gram-negative), and -CAN (*Candida*) real-time PCR kits (M&D) according to the following procedure to evaluate the NBC samples. The reaction mixture contained 10 μL of real-time PCR mixture, 5 μL of primer and probe mixture, 0.04 μL of 50× ROX reference dye, 5 μL of sample DNA, and sterile distilled water to give a final volume of 20 μL. The thermal cycling conditions were: 10 min at 94°C, followed by 40 cycles of 30 sec at 94°C and 30 sec at 60°C. Each TaqMan real-time PCR assay included a positive control and an internal control, which was used to control for the effect of PCR inhibitors in the reaction. The cycle threshold (C_T) values resulting from reactions in the master mix with and without specimen were compared. The bacterial load was quantified by determining the C_T, the number of PCR cycles required for the fluorescence to exceed a value significantly higher than the background fluorescence. All reactions were performed by using an ABI 7500 FAST instrument (Applied Biosystems, Foster City, CA, USA). The C_T value was analyzed by using 7500 Software version 2.0.4 (Applied Biosystems). Fifteen PBC samples were evaluated by using real-time PCR TaqMan assay as a pilot study before evaluating the NBC samples. The C_T values resulting from this pilot study ranged from 11 to 20.94 cycles. The real-time PCR assay was defined as positive if the C_T value was below 30.0.

4. PCR–reverse blot hybridization assay

The REBA Sepsis-ID test was performed according to the standard protocol provided by the manufacturer [15]. The membrane used in the REBA Sepsis-ID test contained DNA probes for GPB, including *Staphylococcus aureus*, *Staphylococcus* spp., *Streptococcus pneumoniae*, *Streptococcus* spp., *Entero-*

coccus spp., and *Mycobacterium* spp.; DNA probes for GNB including *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Salmonella* spp., *Shigella* spp., *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*; DNA probes for *Candida* species including *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. krusei*; and DNA probes for antimicrobial resistance genes including *mec*A, *van*A, and *van*B. Indicator lines on the REBA strips were evaluated by using a template provided with the kit. A universal control band was used to evaluate the intensity of faint bands (only the bands with color intensity equal to or greater than that of the control band were considered positive). The results indicated by band patterns on the developed strips specific for each species and resistance gene were compared to the conventional microbiologic results.

5. 16S rRNA sequence analysis

All the isolates and PCR amplicons with discrepant results between the REBA Sepsis-ID test and conventional methods were subjected to 16S rRNA sequence analysis. The REBA Sepsis-ID test, bacterial 16S rRNA, and fungal 18S to 5.8S internal transcribed sequence analysis were performed on these samples. The results were compared with those of blood cultures.

6. Statistical analysis

All PBC and NBC data (1,400 samples) were analyzed by using IBM SPSS Statistics version 20 (SPSS Inc., Chicago, IL, USA). The degree of agreement between conventional culture method and REBA-Sepsis ID test was determined by kappa coefficient and its corresponding *P* value.

RESULTS

1. Positive blood culture samples –monomicrobial bacteremia

Two hundred eighty-eight of 300 PBC samples contained a single organism as determined by the culture method. Of the monomicrobial PBC samples, 69.8% (201/288) contained GPB including *S. aureus*, coagulase-negative *Staphylococcus* (CoNS), *S. pneumoniae*, *Streptococcus* spp., *Enterococcus* spp., and anaerobic GPB. An additional 25.3% (73/288) contained GNB including *E. coli*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *C. freundii*, and anaerobic GNB. A total of 4.9% (14/288) contained *Candida* spp. including *C. albicans*, *C. parapsilosis*, *C. glabrata*, and *C. tropicalis* (Table 1). Two of 27 *S. aureus* and three of 103 CoNS isolates gave discrepant results between the

Table 1. The spectrum of isolates and comparison of results between conventional culture method and REBA Sepsis ID test

Bacterial pathogen or resistance gene by conventional methods (N)	Identification with the REBA Sepsis-ID test	
	Consistent results (N)	Discrepant results (N)
Gram positive		
Staphylococcus aureus (27)	*Staphylococcus aureus* (25)	No result (1)*, *Staphylococcus* spp. (1)[†]
Staphylococcus epidermidis (46)	*Staphylococcus* spp. (45)	Gram positive (1)[†]
Staphylococcus hominis (27)	*Staphylococcus* spp. (27)	
Staphylococcus capitis (21)	*Staphylococcus* spp. (21)	
Staphylococcus haemolyticus (9)	*Staphylococcus* spp. (9)	
Staphylococcus saprophyticus (2)	*Staphylococcus* spp. (1)	pan bacteria (1)[†]
Staphylococcus intermedius (1)	*Staphylococcus* spp. (1)	
Staphylococcus schleiferi (1)	*Staphylococcus* spp. (0)	pan bacteria (1)[†]
Streptococcus pneumoniae (5)	*Streptococcus pneumoniae* (5)	
Streptococcus mitis (6)	*Streptococcus* spp. (5)	*Streptococcus* spp. & *S. aureus* (1)[‡]
Streptococcus agalatiae (5)	*Streptococcus* spp. (5)	
Streptococcus salivarius (3)	*Streptococcus* spp. (3)	
Streptococcus anginosus (2)	*Streptococcus* spp. (2)	
Streptococcus pyogenes (2)	*Streptococcus* spp. (2)	
Streptococcus sanguinis (2)	*Streptococcus* spp. (1)	pan bacteria (1)[†]
Streptococcus bovis (1)	*Streptococcus* spp. (1)	
Streptococcus dysgalactiae (1)	*Streptococcus* spp. (0)	*Streptococcus* spp. & *S. aureus*[‡]
Enterococcus faecium (6)	*Enterococcus* spp. (6)	
Enterococcus faecalis (5)	*Enterococcus* spp. (5)	
Enterococcus avium (1)	*Enterococcus* spp. (0)	*Enterococcus* spp. & *C. freundii* (1)[‡]
Enterococcus gallinarum (1)	*Enterococcus* spp. (1)	
Corynebacterium spp. (10)	Gram positive (10)	
Bacillus spp. (9)	Gram positive (7)	Gram positive & *Streptococcus* spp. (2)
Micrococcus spp. (8)	Gram positive (8)	
Anaerobe bacteria (4)	Gram positive (4)	
Gram negative		
Escherichia coli (35)	*Escherichia coli* (34)	Gram negative (1)[§]
Klebsiella pneumoniae (11)	*Klebsiella pneumonia* (11)	
Acinetobacter baumannii (6)	*Acinetobacter baumannii* (6)	
Pseudomonas aeruginosa (5)	*Pseudomonas aeruginosae* (5)	
Aeromonas hydrophila (3)	Gram negative (3)	
Morganella morganii (2)	Gram negative (2)	
Enterobacter cloacae (2)	Gram negative (2)	
Enterobacter aerogenes (1)	Gram negative (1)	*E. aerogenes* & *A. baumannii* (1)[‡]
Citrobacter freundii (1)	*Citrobacter freundii* (1)	
Klebsiella oxytoca (1)	Gram negative (1)	
Serratia marcescens (1)	Gram negative (1)	
Chryseobacterium indologenes (1)	Gram negative (1)	
Delftia acidovorans (1)	Gram negative (1)	

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Table 1. Continued

Bacterial pathogen or resistance gene by conventional methods (N)	Identification with the REBA Sepsis-ID test	
	Consistent results (N)	Discrepant results (N)
Moraxella catarhalis (1)	Gram negative (1)	
Ochrobactrum anthropi (1)	Gram negative (1)	
Anaerobe bacteria (1)	Gram negative (1)	
Candida		
Candida albicans (7)	*Candida albicans* (7)	
Candida parapsilosis (4)	*Candida parapsilosis* (4)	
Candida glabrata (2)	*Candida glabrata* (2)	
Candida tropicalis (1)	*Candida tropicalis* (1)	
Antimicrobial resistance		
Methicillin resistance (92)	*mec*A (90)	No detection (2)[II]
Vancomycin resistance (1)	*van*A (1)	

*One was not amplified and gave no result by the REBA Sepsis-ID test; †The 16S rRNA sequence analysis reported as uncultured bacterium; ‡REBA Sepsis-ID test results agreed with 16S rRNA sequence analysis; §The PCR product weakly hybridized with the *E. coli* probe; IIThe *mec*A gene was not identified in two *S. saprophyticus* isolates by the REBA Sepsis-ID test.
Abbreviation: REBA Sepsis-ID, PCR – based reverse blot hybridization assay.

culture method and the REBA Sepsis-ID test. One *S. aureus* case was not identified by PCR amplification, and another *S. aureus* case was not identified as the species level. Three CoNS cases were identified at the pan-GP or pan-bacteria level by the REBA Sepsis-ID test. All five *S. pneumoniae* isolates were correctly identified. Three of 22 *Streptococcus* spp. showed discrepant results between culture and the REBA Sepsis-ID test. The presence of both *Streptococcus* spp. and *S. aureus* was indicated in two of these discrepant samples by the REBA Sepsis-ID test. Subsequent 16S rRNA sequence analysis confirmed the presence of two isolates in each sample. Twelve of 13 *Enterococcus* spp. were concordant. One sample resulted in two bands corresponding to *Enterococcus* spp. and *C. freundii* by the REBA Sepsis-ID test despite only *Enterococcus avium* being isolated. Subsequent 16S rRNA sequence analyses confirmed the presence of *E. avium* and *C. freundii* in the blood culture sample. The other 27 GPB including 10 *Corynebacterium* spp., 9 *Bacillus* spp., 8 *Micrococcus* spp., and 4 anaerobic GPB were not included in the REBA probes. PCR products from all but two samples hybridized with the pan-GP probes. The agreement rate between the culture method and the REBA Sepsis-ID test for GPB and GNB was 94.5% (190/201) and 97.3% (71/73), respectively. Three GPB monomicrobial samples were revealed to have more than one isolate by the REBA Sepsis-ID test. *E. coli* was isolated from one GNB monomicrobial sample, but the PCR product hybridized only with the pan-GN probe. In the only other GNB monomicrobial sample, *Enterobacter aerogenes* was isolated,

and the PCR product hybridized with both of the pan-GN and *A. baumannii* probes. This sample was confirmed to contain both of *E. aerogenes* and *A. baumannii* by 16S rRNA sequence analysis. All 14 *Candida* species were identified by the REBA Sepsis-ID test.

2. Positive blood culture samples –polymicrobial bacteremia
Of the 12 polymicrobial PBC samples, the agreement rate between the culture method and the REBA Sepsis-ID test was 91.7% (11/12). *E. coli* plus *Streptococcus anginosus* isolated by the culture method in one case was identified to be only *E. coli* by the REBA Sepsis-ID test (Table 2).

3. Negative blood culture samples
Among the NBC samples, 97.6% (1,074/1,100) had C_T values above 30.0. The remaining 2.4% (26/1,100) NBC samples had C_T values below 30.0, including four with values less than 25.0, one between 26.0 and 27.0, and 21 between 28.0 and 30.0. Of these samples, 69.2% (18/26) were Real-GN positive, 19.2% (5/26) were Real-GP positive, and 11.5% (3/26) were Real-CAN positive by TaqMan real-time PCR assay (Table 3). Most Real-GP, and Real-GN positive cases produced PCR products that weakly hybridized to the pan-bacteria probe. Isolates from two Real-CAN positive cases were identified as containing *C. tropicalis* and one was identified as containing *C. tropicalis* plus *C. parapsilosis* by the REBA Sepsis-ID test. Thirteen of 26 NBC samples with positive real-time PCR results were not amplified

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Table 2. Comparison of results from conventional culture methods and the REBA Sepsis-ID test for 12 polymicrobial blood culture samples

Bacterial pathogen by conventional methods (N)	Identification with the REBA Sepsis-ID test (N)
Staphylococcus aureus and *Enterococcus faecalis (2)*	*Staphylococcus aureus* and *Enterococcus* spp. (2)
Staphylococcus epidermidis and *Staphylococcus warneri* (1)	*Staphylococcus* spp. (1)
Staphylococcus haemolyticus and *Candida utilis* (1)	*Staphylococcus* spp. and fungus (1)
Enterococcus durans and *Staphylococcus epidermidis* (1)	*Enterococcus* spp. and *Staphylococcus* spp. (1)
Enterococcus faecium and *Candida albicans* (1)	*Enterococcus* spp. and *Candida albicans* (1)
Enterococcus avium and *Staphylococcus epidermidis* (1)	*Enterococcus* spp. and *Staphylococcus* spp. (1)
Escherichia coli and *Enterococcus gallinarum* (1)	*Escherichia coli* and *Enterococcus* spp. (1)
Escherichia coli and *Streptococcus anginosus* (1)	*Escherichia coli* (1)
Klebsiella pneumoniae and *Enterococcus casseliflavus* (1)	*Klebsiella pneumoniae* and *Enterococcus* spp. (1)
Klebsiella pneumoniae and *Enterobacter cloacae* (1)	*Klebsiella pneumoniae* and Gram negative (1)
Proteus mirabilis and *Enterococcus faecalis* (1)	Gram negative and *Enterococcus* spp. (1)

Abbreviation: REBA Sepsis-ID, PCR-based reverse blot hybridization assay.

by sequencing reactions. Eight NBC samples produced colonies that grew on subculture media (Table 3). A significant agreement was found between conventional culture method and REBA-Sepsis ID test (kappa coefficient=0.916, $P<0.001$).

4. Identification of *mec*A and *van*A genes

Ninety of 92 (97.8%) blood culture samples with methicillin-resistant *Staphylococcus* isolates were *mec*A positive. *Mec*A was not detected in the two methicillin-resistant *Staphylococcus saprophyticus* isolates. No *mec*A genes were identified by PCR-REBA in the 44 methicillin-susceptible *Staphylococcus* spp. The *van*A gene was detected in one blood culture sample, from which vancomycin-resistant *Enterococcus* was isolated (Table 1).

DISCUSSION

The clinical treatment of bacterial infections with antibiotics depends on the bacterial species, and especially differs among GPB, GNB, and fungal infections. Antibiotic therapies are usually selected empirically until antimicrobial susceptibility test results are completed. Therefore, rapidly identifying pathogens and their resistance genes is important for treating septic patients. Diagnostic methods that can reduce the time to identify a BSI pathogen, and its antimicrobial susceptibility have great potential to improve patient care. Recently, a molecular diagnostic approach was proposed to be advantageous [20, 21]. The advantage of molecular approaches is notable when the infectious agent is fastidious or fungal, when blood culture fails to identify the causative agent, or when a quick diagnosis is needed.

The agreement rates between the conventional culture method

and the REBA Sepsis-ID test in identifying GPB, GNB, fungi, and the *mec*A gene in 300 PBC samples were 94.5%, 97.3%, 100%, and 97.8%, respectively. Steindor *et al.* [22] reported correct identification of 96.1% of GPB, 89.9% of GNB, and 92.9% of *mec*A using the PCR-based DNA strip assay, GenoType BC. Results from our PBC samples demonstrated that the overall agreement rate between the culture method and the REBA Sepsis-ID test was high at 95.3%, which was similar to that indicated in other reports [23, 24].

Two monomicrobial PBC samples with discrepant results between culturing and the REBA Sepsis-ID test were proven to have additional *S. aureus* isolates. In contrast, five samples containing *Staphylococcus* spp., including *S. aureus* isolates, were not correctly identified the by the REBA Sepsis-ID test. It is difficult to accurately identify more than one organism in a sample by using the culture method, as additional blind subculturing of PBC samples with positive flagging is not a common practice in clinical laboratories. PCR inhibitors have been an obstacle to obtaining accurate results. Achieving accurate PCR results from blood culture samples is difficult owing to PCR inhibitors in the blood such as sodium polyanetholsulfonate, heme, hematin, hemoglobin, lactoferrin, and IgG [25-29]. An optimal PCR sample preparation procedure should efficiently lyse resistant bacterial cell walls, including those in GPB, without being too harsh on the DNA released from the cells [30]. Differentiating *S. pneumoniae* from mitis group streptococci is difficult because of their close genetic relationship [31]. However, the REBA Sepsis-ID test discriminated between *S. pneumoniae* and other streptococci, including viridians group streptococci.

Poor accuracy in the identification of more than one organism

Table 3. Comparison of results from 16S rRNA sequence analysis, real-time PCR-REBA, and subculture

Real-time PCR (C_T value)*			REBA Sepsis-ID test	16S rRNA sequence analysis		Clinical sign[‡]
Real-GN	Real-GP	Real-CAN		Colonies from subculture[†]	Blood suspension after extended incubation	
16.83	ND[§]	ND	Pan	Uncultured *Sphingomonas*, Uncultured prokaryote	No amplification	Yes
28.49	ND	ND	Pan, GP	*Bacillus infantis*, *Bacillus neonatiensis*, Uncultured bacterium	*B. infantis*, *B. neonatiensis*, *Nocardia coeliaca*, *Rhodococcus* spp.	Yes
29.03	ND	ND	Pan (weak band)	*Pseudomonas putida*, *Actinobacterium* spp., *Cellulosimicrobium* spp., *Janthinobacterium* spp., *Lactobacillus* spp., *Microbacterium* spp., *Streptomyces* spp.	No amplification	Yes
29.09	ND	ND	Pan	*Agrococcus* spp., Uncultured bacterium	*Janthinobacterium* spp., Uncultured bacterium	No
29.67	ND	ND	Pan (weak band)	*Staphylococcus hominis*, Uncultured bacterium	*Rhodococcus erythropolis*, *Rhodococcus* spp., *Acrinomycetales bacterium*, *N. coeliaca*	Yes
29.82	ND	ND	Pan (weak band)	*Bacillus aryabhattai*, *Bacillus megaterium*	No amplification	Yes
29.85	ND	ND	Pan (weak band)	*Bacillus acidiceler*, *Bacillus luciferensis*, *Bacillus* spp.	No amplification	No
29.91	ND	ND	Pan (weak band)	No colony	*Janthinobacterium* spp., Uncultured bacterium	No
26.10	ND	ND	Pan (weak band)	No colony	No amplification	Yes
28.64	ND	ND	Pan (weak band)	No colony	No amplification	Yes
28.67	ND	ND	Pan (weak band)	No colony	*A. bacterium*, *R. erythropolis*, *Rhodococcus* spp.	Yes
28.78	ND	ND	Pan	No colony	No amplification	Yes
28.90	ND	ND	Pan	No colony	No amplification	Yes
29.06	ND	ND	Pan (weak band)	No colony	*Janthinobacterium* spp., Uncultured bacterium	Yes
29.12	ND	ND	Pan	No colony	No amplification	Yes
29.40	ND	ND	Pan (weak band)	No colony	*A. bacterium*, *N. coeliaca*, *R. erythropolis*	Yes

(Continued to the next page)

Table 3. Continued

Real-time PCR (C_T value)*			REBA Sepsis-ID test	16S rRNA sequence analysis		Clinical sign[‡]
Real-GN	Real-GP	Real-CAN		Colonies from subculture[†]	Blood suspension after extended incubation	
29.45	ND	ND	Pan (weak band)	No colony	No amplification	Yes
29.80	ND	ND	Pan (weak band)	No colony	No amplification	Yes
ND	17.08	ND	*Staphylococcus* spp.	*Staphylococcus epidermidis*	*S. epidermidis*	Yes
ND	24.26	ND	*Staphylococcus* spp.	No colony	*Staphylococcus warneri*	Yes
ND	26.01	ND	Pan (weak band)	No colony	*A. bacterium*	Yes
ND	28.91	ND	Pan (weak band)	No colony	No amplification	Yes
ND	23.83	ND	Pan	No colony	No amplification	Yes
ND	ND	29.17	*Candida tropicalis* (weak band)	No colony	*C. tropicalis*	Yes
ND	ND	29.21	*C. tropicalis* and *C. parapsilosis* (weak band)	No colony	Uncultured fungus	Yes
ND	ND	29.85	*C. tropicalis* (weak band)	No colony	*C. tropicalis*	Yes

*The cycle threshold value for real-time PCR was 30.0 in this study; [†]Samples were subcultured on sheep blood, MacConkey, chocolate, R2A, and plating agar; [‡]Clinical signs follow the systemic inflammatory response syndrome definition; [§]Undetermined result or cycle threshold value was over 30.0 by real-time PCR.
Abbreviations: REBA Sepsis-ID, PCR-based reverse blot hybridization assay; CT, cycle threshold; Real-GN, 16S rRNA gram-negative primers; Real-GP, 16S rRNA gram-positive primers; Real-CAN, 16S rRNA gram-positive primers; Pan, broad-range bacterial 16S rRNA probe.

in a sample is a well-known drawback of PCR-based tests. Molecular tests that detect a limited number of targets may report only one species, which can be misleading. Buchan *et al.* [32] reported that the agreement rate between a reference culture and a microarray-based nucleic acid test for polymicrobial cultures was only 72%. However, the REBA assay in the present study had an agreement rate of 91.7%. REBA may not detect multiple types of bacteria in a blood culture sample owing to technical limitations, such as interference between multiple probes and undetectable concentrations of minor bacterial constituents. The five PBC samples that had a single bacterial species by blood culture contained two isolates as determined by the REBA Sepsis-ID test, which was confirmed by 16S rRNA sequence analysis. The reason that the REBA Sepsis-ID test detected more isolates than the blood culture method is not clear. However, *C. freundii* was detected by the blood culture method on the day following its detection by using the REBA Sepsis-ID test. This result indicates that the REBA Sepsis-ID test may be able to detect nonviable bacteria or those at a low concentration that is not detectable by CMBCS. Therefore, the REBA Sepsis-ID test may have practical benefits in the clinical setting, particularly for patients on empirical antibiotic treatment before culture results are obtained.

The REBA Sepsis-ID test did not identify two (2.2%) methicillin-resistant *S. saprophyticus* isolates, which were identified by the conventional method. The two *mec*A-negative *S. saprophyti-*

cus isolates were likely methicillin-susceptible organisms. The Clinical and Laboratory Standards Institute interpretive criteria for detecting *mec*A-mediated resistance in *S. saprophyticus* may overestimate resistance [33].

Real-time PCR was used to evaluate NBC samples because it reduces the time required to obtain results, decreases contamination during the PCR procedure, and increases PCR sensitivity. Among the 26 NBC samples with real-time PCR positive results in this study, 13 cases were considered false positive results because they were not amplified by sequencing reactions. Matsuda *et al.* [34] reported that the positivity rate of PCR-hybridization using 500 µL of blood from NBC samples was 10.5% (11/105) and that the 11 culture negative, PCR-hybridization positive samples contained nine CoNS and two GPB species. Steindor *et al.* [22] evaluated a PCR-based DNA strip assay, GenoType BC, which detects bacteria from PBC samples and emphasized that the assay required abundant bacteria in the blood. Kocoglu *et al.* [35] reported that of 904 NBC samples, 2.6% were positive by reculture but not by PCR-based methods. They concluded that subculture was valuable in diagnosis using NBC samples, especially when only one set of blood cultures was taken. In this study, using a real-time PCR C_T of 30 resulted in a very low positivity rate in NBC samples. Colonies isolated from subculture media may also have been contaminated as the sequence analysis results were not concordant between the blood suspension and colonies. These results suggest

that it is not necessary for a clinical laboratory to perform supplemental subculture as a routine work after 5 days of incubation on CMBCS.

Although the REBA Sepsis-ID test will not absolutely replace the conventional culture method, it is likely to rapidly discriminate between PBC and NBC samples and provide clinical information relevant to patients by detecting important pathogens and antimicrobial resistance genes.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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