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Multiplex Real-Time PCR Assay for Rapid Detection of Methicillin-Resistant Staphylococci Directly from Positive Blood Cultures

Hye-young Wang,[a] Sunghyun Kim,[b,c] Jungho Kim,[b] Soon-Deok Park,[b,d] Young Uh,[d] Hyeyoung Lee[b]

M&D, Inc., Wonju, Republic of Korea[a]; Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University, Wonju, Republic of Korea[b]; Institute for Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea[c]; Department of Laboratory Medicine, Wonju College of Medicine, Yonsei University, Wonju, Republic of Korea[d]

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most prevalent cause of bloodstream infections (BSIs) and is recognized as a major nosocomial pathogen. This study aimed to evaluate a newly designed multiplex real-time PCR assay capable of the simultaneous detection of *mecA*, *S. aureus*, and coagulase-negative staphylococci (CoNS) in blood culture specimens. The Real-MRSA and Real-MRCoNS multiplex real-time PCR assays (M&D, Republic of Korea) use the TaqMan probes 16S rRNA for *Staphylococcus* spp., the *nuc* gene for *S. aureus*, and the *mecA* gene for methicillin resistance. The detection limit of the multiplex real-time PCR assay was 10^3 CFU/ml per PCR for each gene target. The multiplex real-time PCR assay was evaluated using 118 clinical isolates from various specimen types and a total of 350 positive blood cultures from a continuous monitoring blood culture system. The results obtained with the multiplex real-time PCR assay for the three targets were in agreement with those of conventional identification and susceptibility testing methods except for one organism. Of 350 positive bottle cultures, the sensitivities of the multiplex real-time PCR kit were 100% (166/166 cultures), 97.2% (35/36 cultures), and 99.2% (117/118 cultures) for the 16S rRNA, *nuc*, and *mecA* genes, respectively, and the specificities for all three targets were 100%. The Real-MRSA and Real-MRCoNS multiplex real-time PCR assays are very useful for the rapid accurate diagnosis of staphylococcal BSIs. In addition, the Real-MRSA and Real-MRCoNS multiplex real-time PCR assays could have an important impact on the choice of appropriate antimicrobial therapy, based on detection of the *mecA* gene.

The rapid diagnosis and treatment of bacterial sepsis are requisite to decrease mortality and morbidity rates, because it is a rapidly progressing, life-threatening condition that can cause shock and organ failure (1). Staphylococci are the most commonly isolated organisms, accounting for almost 30% of all hospital-acquired infections and 50% of bloodstream infections (BSIs) (2). *Staphylococcus aureus* is a leading cause of health care-associated infections ranging from mild conditions such as skin and soft-tissue infections to life-threatening sepsis. Methicillin-resistant *S. aureus* (MRSA) is recognized as a major nosocomial pathogen associated with intrahospital and interhospital transmission (3). Staphylococcal infections have resulted in significant morbidity, deaths, and longer hospital stays if not treated early with effective antibiotics. The prevalence of MRSA infections exceeds 49% in U.S. hospitals and continues to increase (4). Coagulase-negative staphylococci (CoNS) are also known to be the most common isolates from blood cultures, although many are contaminants.

Fast accurate discrimination of MRSA from methicillin-susceptible staphylococci directly from patient blood samples provides data for proper medical decisions regarding antimicrobial therapy, which plays an important role in the reduction of deaths resulting from sepsis. Currently, bacterial culture is required as a standard method for diagnosis of the presence of bacterial pathogens in clinical samples. However, this technique has some disadvantages with regard to desired detection speed and sensitivity (5). Generally, blood culture samples are incubated for 5 days until they show positive signals in continuous monitoring blood culture systems (CMBCSs). Moreover, blood cultures may lead to false-negative results when fastidious or slowly growing bacteria are involved or when samples are obtained after antimicrobial therapy has been started (6, 7). The early diagnosis and adequate treatment of bacterial infections have great impacts on the outcomes for patients with systemic infections.

Real-time PCR is significantly faster than conventional PCR and other detection methods. The combination of excellent sensitivity and specificity, low contamination risk, ease of performance, and speed has made real-time PCR technology appealing to clinical microbiology laboratories (8). In this study, a multiplex real-time PCR assay using probes specific for *S. aureus* and a methicillin resistance gene was tested for the rapid detection and identification of MRSA, methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant CoNS (MRCoNS), and methicillin-susceptible CoNS (MSCoNS) directly from positive blood cultures.

MATERIALS AND METHODS

Bacterial and fungal strains. A total of 67 bacterial and 28 fungal reference strains and 118 clinical isolates from various specimen types were used to determine the specificity of the multiplex real-time PCR assay (see Tables 2 and 3). A total of 350 positive blood culture samples, including 166 *Staphylococcus* spp. and 184 nonstaphylococcal strains, were collected to evaluate the diagnostic performance of the multiplex real-time PCR assay. All clinical isolates and positive blood culture samples were col-

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Address correspondence to Young Uh, u931018@yonsei.ac.kr, or Hyeyoung Lee, hyelee@yonsei.ac.kr.
H.-Y.W. and S.K. contributed equally to this article.
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TABLE 1 Primers and probes used in this study

Target and primer/probe name	Nucleotide sequence (5′ to 3′)	Modifications[a]
16S rRNA		
16S-472F	AGTGATGAAGGTCTTCGGATCGTAAA	
16S-575R	CGTGGCTTTCTGATTAGGTACCGTC	
16S-513P	GGAAGAACAWAYGTGTAAGTAACTRTGCACRT	Cy5 and BHQ2
nuc		
nuc263-F	AAAGCGATTGATGGTGATACGGTT	
nuc355-R	TGCTTTGTTTCAGGTGTATCAACCA	
nuc294-P	ATGTACAAAGGTCAACCAATGACATTYAGA	HEX and BHQ1
mecA		
mecA-1501F	GCTCAAATTTCAAACAAAAATTTAGATAATG	
mecA-1598R	TGAAAGGATCTGTACTGGGTTAATCAGT	
mecA-1542P	AGCTGATTCAGGTTACGGACAAGGTGA	FAM and BHQ1

[a] Cy5, cyanine fluorescein; BHQ, black hole quencher; HEX, 4,4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein; FAM, 6-carboxyfluorescein.

lected between January 2013 and May 2013 at Yonsei University Wonju Severance Christian Hospital (Wonju, Republic of Korea). The identification of organisms was conducted with the MicroScan system (Siemens Healthcare Diagnostics, Sacramento, CA) and the Vitek 2 system (bioMérieux, Durham, NC).

Blood culture and collection of blood culture bottle samples. Two or three pairs of culture bottles for aerobes or anaerobes were incubated in a BacT/Alert 3D (bioMérieux), Bactec 9240 (Becton, Dickinson Diagnostic System, Spark, MD), or Bactec FX (Becton, Dickinson) blood culture system for 5 days after inoculation with blood drawn from the patient at the bedside. If bacterial growth was not detected within 5 days, then the blood culture result was considered negative. When bacterial growth was noted, the culture sample was inoculated onto both blood agar and MacConkey agar plates, which were then cultured overnight at 35°C in a 5% CO_2 incubator. Isolates were identified based on colony morphology, Gram staining, and biochemical test results and were further verified with a Vitek 2 (bioMérieux), MicroScan (Siemens), or Vitek 2 yeast identification card (bioMérieux) system. Antimicrobial susceptibility tests were performed with the Clinical and Laboratory Standards Institute (CLSI) recommended disk diffusion test, the Vitek 2 (bioMérieux) system, and the MicroScan (Siemens) system.

DNA preparation. To prepare DNA templates for the multiplex real-time PCR assay, one colony of each type strain and clinical isolate was suspended in 100 μl DNA extraction solution (M&D, Wonju, Republic of Korea). The suspended bacterial solution was boiled for 10 min. After centrifugation at 13,000 × *g* for 10 min, the supernatant was used as the DNA template. For preparation of DNA templates from positive blood culture bottle samples, 0.5 ml of blood suspension was taken directly from the blood culture bottle and 1.0 ml phosphate-buffered saline (PBS) (pH 8.0) was added. After centrifugation at 13,000 × *g* for 1 min, the supernatant was removed and the pellet was resuspended in 1.0 ml of sterile red blood cell (RBC) lysis buffer, for RBC lysis, and centrifuged at 13,000 × *g* for 1 min. This washing step was repeated twice, and the pellet was resuspended in DNA extraction solution as described above.

Multiplex real-time PCR TaqMan assay. The multiplex real-time PCR TaqMan assay was carried out with the Real-MRSA and Real-MRCoNS multiplex real-time PCR assay kits (M&D, Wonju, Republic of Korea) using the CFX-96 real-time PCR system (Bio-Rad, Hercules, CA), which was used for thermocycling and fluorescence detection. The real-time PCR amplification was performed in a total volume of 20 μl containing 10 μl of 2× Thunderbird probe quantitative PCR mixture (Toyobo, Osaka, Japan), 5.0 μl of primer and TaqMan probe mixture, and 5 μl of template DNA; distilled water (DW) was added for a final volume of 20 μl. The specific primers and probes used for the identification of *Staphylococcus* species and *S. aureus* and for detection of antibiotic resistance are listed in Table 1. The multiplex real-time PCR assay detected the 16S rRNA, *nuc*, and *mecA* genes simultaneously, in a single tube, by incorporating three target-specific TaqMan probes labeled with different fluorophores (Cy5, HEX, and FAM).

Positive and negative controls were included throughout the procedure. No-template controls with sterile DW instead of template DNA were incorporated in each run under the following conditions: 95°C for 3 min and 35 cycles of 95°C for 20 s and 60°C for 40 s in a single real-time PCR assay. The bacterial load was quantified by determining the cycle threshold (C_T), which is the number of PCR cycles required for the fluorescence to exceed a value significantly higher than the background fluorescence level.

Spiking experiment to determine the assay detection limit. To determine the detection limit of the multiplex real-time PCR assay, the cultured *S. aureus* (ATCC 25923) bacterial reference strain was suspended in 1× (PBS), and the density of the bacterial suspension was adjusted to 0.5 McFarland units (approximately 1.5×10^8 cells/ml). A series of 10-fold dilutions, which ranged from 10^8 to 10^0 CFU/ml, were prepared from the bacterial suspensions, and 100 μl from each dilution was spread on a blood agar plate. Plates were incubated at 37°C for 16 h to count CFU. Each dilution was spiked into 1.0 ml of a negative blood culture sample for 5 days, and each spiked sample was used for genomic DNA extraction with DNA extraction solution (M&D, Wonju, Republic of Korea). The eluted DNA (5.0 μl) was used as the template for the multiplex real-time PCR assay.

PCR-reverse blot hybridization assay. To confirm the results of the multiplex real-time PCR assay for the identification of *Staphylococcus* spp. and the determination of methicillin resistance with positive blood culture samples, the PCR-reverse blot hybridization assay (REBA), the REBA Sepsis-ID test (M&D), was performed according to the manufacturer's instructions. PCR was performed using a 50-μl reaction mixture (GeNet Bio, Daejeon, Republic of Korea) containing 25 μl of 2× master mix, 10 μl of 1× primer mixture, 5.0 μl of sample DNA, and sterile DW for a final volume of 50 μl. The first 10 PCR cycles consisted of denaturation at 95°C for 30 s followed by annealing and extension at 60°C for 30 s. These 10 cycles were followed by 40 cycles of denaturation at 95°C for 30 s followed by annealing and extension at 54°C for 30 s. After the final cycle, samples were maintained at 72°C for 10 min to complete the synthesis of all strands. The amplified target was visualized as a single band corresponding to a length of 100 to 200 bp, using the ChemiDoc system (Vilber Lourmat, Germany).

For REBA Sepsis-ID, the hybridization and washing processes were performed according to the manufacturer's instructions. In brief, biotinylated PCR products were denatured at 25°C for 5 min in denaturation solution and diluted in 970 μl hybridization solution on the REBA membrane strip, in the provided blotting tray. Denatured single-stranded PCR products were used to hybridize with the probes on the strip at 55°C for 30

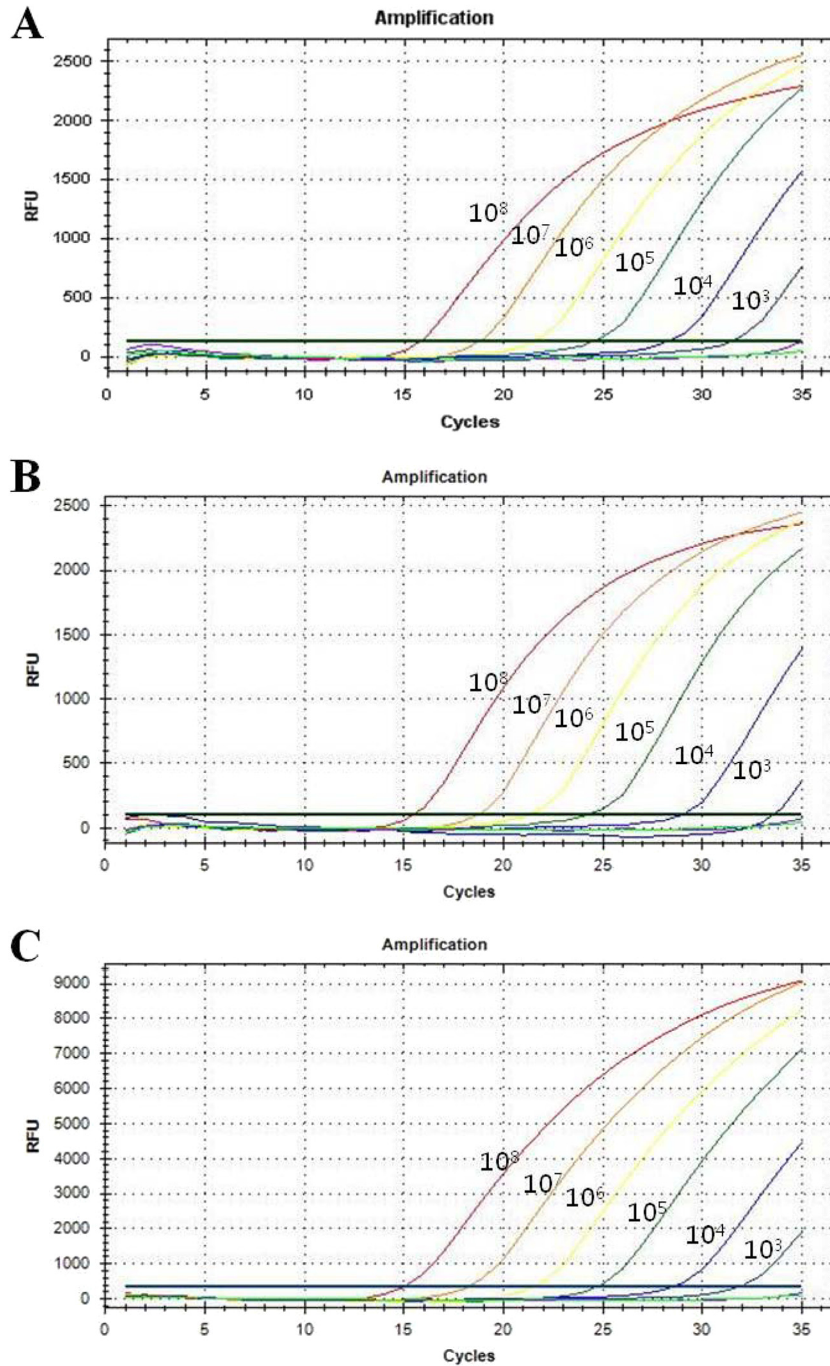

FIG 1 Detection limits for the three target probes from 10-fold serial dilutions of spiked samples. Serially diluted *S. aureus* DNA ranging from 10^8 to 10^0 CFU/ml was used to determine the detection limit of the multiplex real-time PCR assay. (A) Amplification curve for the 16S rRNA probe for detecting *Staphylococcus* spp. (B) Amplification curve for the *nuc* probe for detecting *S. aureus*. (C) Amplification curve for the *mecA* probe for detecting methicillin resistance. The overall detection limits of this assay for the 16S rRNA, *nuc*, and *mecA* genes were approximately 10^3 CFU/ml. RFU, relative fluorescence units.

min. The strips were then washed twice in 1.0 ml washing solution at 55°C for 10 min with gentle shaking, incubated at 25°C for 30 min with streptavidin-alkaline phosphatase conjugate (Roche Diagnostics, Mannheim, Germany) diluted 1:2,000 in conjugate diluent solution (CDS), and washed twice with 1.0 ml CDS at room temperature for 1 min. Colorimetric hybridization signals were visualized with the addition of alkaline phosphatase-mediated staining solution, i.e., nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) (Roche Diagnostics), diluted 1:50, and strips were incubated until the color was detected. Finally, the band patterns were read and interpreted.

RESULTS

Multiplex real-time PCR assay sensitivity and specificity with reference strains. Data from a spiking experiment showed that

TABLE 2 Specificity of the multiplex real-time PCR assay for detecting the 16S rRNA, *nuc*, and *mecA* genes in 95 bacterial and fungal reference strains

Organism type and genus and species or serovar	Standard strain[a]	C_T for:		
		16S rRNA	*nuc*	*mecA*
Gram-positive bacteria				
Staphylococcus				
S. aureus	ATCC 29213	28.33	29.94	UD[b]
	ATCC 25923	13.39	17.13	UD
	ATCC 13565	14.09	14.26	UD
	ATCC 33586	16.07	15.86	UD
	ATCC BAA-2312	22	15.76	15.24
S. xylosus	ATCC 29971	22.30	UD	UD
S. epidermidis	ATCC 12228	12.32	UD	UD
Enterococcus				
E. hirae	ATCC 9790	UD	UD	UD
E. raffinosus	ATCC 49427	UD	UD	UD
E. sulfureus	ATCC 49903	UD	UD	UD
E. durans	ATCC 19432	UD	UD	UD
E. casseliflavus	ATCC 700327	UD	UD	UD
	ATCC 49997	UD	UD	UD
E. faecium	ATCC 19434	UD	UD	UD
E. faecalis	ATCC 29212	UD	UD	UD
E. mundtii	ATCC 43186	UD	UD	UD
E. cecorum	ATCC 43198	UD	UD	UD
E. gallinarum	ATCC 49573	UD	UD	UD
E. faecalis	ATCC 51299	UD	UD	UD
E. solitarius	ATCC 49428	UD	UD	UD
E. faecium	ATCC 35667	UD	UD	UD
E. malodoratus	ATCC 43197	UD	UD	UD
E. saccharolyticus	ATCC 43076	UD	UD	UD
E. casseliflavus	ATCC 25788	UD	UD	UD
Streptococcus				
S. pneumoniae	ATCC 49619	UD	UD	UD
S. agalactiae	ATCC 13813	UD	UD	UD
Micrococcus luteus	ATCC 49732	UD	UD	UD
Mycobacterium				
M. avium	ATCC 25291	UD	UD	UD
M. chelonae	ATCC 35749	UD	UD	UD
M. gastri	ATCC 15754	UD	UD	UD
M. kansasii	ATCC 12478	UD	UD	UD
M. nonchromogenicum	ATCC 19530	UD	UD	UD
M. phlei	ATCC 11758	UD	UD	UD
M. smegmatis	ATCC 19420	UD	UD	UD
M. triviale	ATCC 23292	UD	UD	UD
M. aurum	ATCC 23366	UD	UD	UD
M. farcinogenes	ATCC 35753	UD	UD	UD
M. gilvum	ATCC 43909	UD	UD	UD
M. neoaurum	ATCC 25795	UD	UD	UD
M. parafortuitum	ATCC 19686	UD	UD	UD
M. peregrinum	ATCC 14467	UD	UD	UD
M. septicum	ATCC 700731	UD	UD	UD
M. abscessus	ATCC 19977	UD	UD	UD
Corynebacterium diphtheriae	ATCC 11913	UD	UD	UD
Gram-negative bacteria				
Escherichia				
E. coli	ATCC 25922	UD	UD	UD
	ATCC 35218	UD	UD	UD
Enterobacter aerogenes	ATCC 1304	UD	UD	UD
Citrobacter freundii	ATCC 6750	UD	UD	UD

(Continued on following page)

TABLE 2 (Continued)

Organism type and genus and species or serovar	Standard strain[a]	C_T for:		
		16S rRNA	*nuc*	*mecA*
Shigella				
S. boydii	DML 399	UD	UD	UD
S. dysenteriae	DML 400	UD	UD	UD
S. flexneri	ATCC 9199	UD	UD	UD
Serratia liquefaciens	ATCC 27952	UD	UD	UD
Salmonella				
S. Typhi	ATCC 19430	UD	UD	UD
S. Enteritidis	ATCC 13076	UD	UD	UD
S. Paratyphi	ATCC 11511	UD	UD	UD
S. Typhimurium	ATCC 13311	UD	UD	UD
S. Newport	ATCC 6962	UD	UD	UD
Klebsiella				
K. pneumoniae	ATCC 13883	UD	UD	UD
K. oxytoca	ATCC 700324	UD	UD	UD
Proteus				
P. alcalifaciens	ATCC 51902	UD	UD	UD
P. vulgaris	ATCC 49132	UD	UD	UD
P. mirabilis	ATCC 49132	UD	UD	UD
Pseudomonas				
P. cepacia	ATCC 25608	UD	UD	UD
P. aeruginosa	ATCC 27853	UD	UD	UD
Haemophilus influenzae	ATCC 49247	UD	UD	UD
Leclercia adecarboxylata	ATCC 23216	UD	UD	UD
Bordetella bronchiseptica	ATCC 10580	UD	UD	UD
Fungi				
Candida				
C. albicans	ATCC 36802	UD	UD	UD
	ATCC 10231	UD	UD	UD
C. tropicalis	ATCC 14506	UD	UD	UD
C. glabrata	ATCC 38326	UD	UD	UD
C. parapsilosis	ATCC 7330	UD	UD	UD
C. lusitaniae	ATCC 34449	UD	UD	UD
C. guilliermondii	ATCC 56822	UD	UD	UD
C. krusei	ATCC 2159	UD	UD	UD
Penicillium				
P. camemberti	ATCC 58608	UD	UD	UD
P. paneum	KACC 44823	UD	UD	UD
Aspergillus				
A. oryzae var. *oryzae*	KACC 44847	UD	UD	UD
A. oryzae var. *effusus*	ATCC 1010	UD	UD	UD
A. clavatus	ATCC 66443	UD	UD	UD
A. sydowii	KACC 41869	UD	UD	UD
A. fumigatus	KCMF 10773	UD	UD	UD
A. flavus	KCMF 10777	UD	UD	UD
A. tamari	ATCC 20054	UD	UD	UD
Fusarium acuminatum	ATCC 10466	UD	UD	UD
Aureobasidium pullulans	KACC 41291	UD	UD	UD
Bipolaris sorokiniana	KACC 44841	UD	UD	UD
Cryptococcus neoformans	KCMF 20047	UD	UD	UD
Kodamaea ohmeri	KCMF 20430	UD	UD	UD
Saccharomyces cerevisiae	KCMF 50427	UD	UD	UD
Trichophyton				
T. rubrum	KCMF 10444	UD	UD	UD
T. mentagrophytes	KCMF 10515	UD	UD	UD
Microsporum canis	KCMF 10531	UD	UD	UD
Epidermophyton floccosum	ATCC 52063	UD	UD	UD
Malassezia furfur	KCMF 20409	UD	UD	UD

[a] ATCC, American Type Culture Collection; DML, Diagnostic Microbiology Laboratory, Biomedical Laboratory Science, Yonsei University; KACC, Korean Agricultural Culture Collection; KCMF, Korea Culture Collection Medical Fungi.
[b] UD, undetermined.

TABLE 3 Results of the multiplex real-time PCR assay for the discrimination of MRSA, MSSA, MRCoNS, and MSCoNS among 118 clinical isolates

Culture identification	Total no. of samples	No. of isolates positive for:			C_T values	
		16S rRNA	*nuc*	*mecA*	Range	Average
Staphylococcus aureus	12	12	12	9	21.12–30.85	24.98
Staphylococcus epidermidis	4	4	0	3	24.18–31.03	27.98
Staphylococcus haemolyticus	3	3	0	3	23.55–25.34	24.29
Staphylococcus capitis	1	1	0	1	31.66	31.66
Streptococcus spp.	5	0	0	0	UD[a]	UD
Enterococcus faecalis	4	0	0	0	UD	UD
Enterococcus faecium	10	0	0	0	UD	UD
Enterococcus mundtii	1	0	0	0	UD	UD
Corynebacterium spp.	1	0	0	0	UD	UD
Escherichia coli	16	0	0	0	UD	UD
Klebsiella pneumoniae	13	0	0	0	UD	UD
Pseudomonas aeruginosa	13	0	0	0	UD	UD
Acinetobacter baumannii	11	0	0	0	UD	UD
Enterobacter asburiae	1	0	0	0	UD	UD
Enterobacter cloacae	1	0	0	0	UD	UD
Enterobacter asburiae	1	0	0	0	UD	UD
Moraxella catarrhalis	1	0	0	0	UD	UD
Serratia marcescens	1	0	0	0	UD	UD
Providencia rettgeri	1	0	0	0	UD	UD
Morganella morganii	1	0	0	0	UD	UD
Proteus mirabilis	1	0	0	0	UD	UD
Aeromonas spp.	1	0	0	0	UD	UD
Citrobacter freundii	2	0	0	0	UD	UD
Candida albicans	5	0	0	0	UD	UD
Candida glabrata	1	0	0	0	UD	UD
Candida parapsilosis	3	0	0	0	UD	UD
Candida tropicalis	2	0	0	0	UD	UD
Saccharomyces cerevisiae	2	0	0	0	UD	UD
Total	118					

[a] UD, undetermined.

the detection limit of the multiplex real-time PCR assay for the 16S rRNA, *nuc*, and *mecA* genes was 10^3 CFU/ml (Fig. 1). The C_T values for the 16S rRNA, *nuc*, and *mecA* genes with each cell concentrate (10^8 to 10^3 CFU/ml) ranged from 15 to 31.77, from 15.76 to 31.36, and from 15.24 to 31.15, respectively.

To determine the assay specificity, primers and probes for detecting the 16S rRNA, *nuc*, and *mecA* genes were tested with 88 nonstaphylococcal reference strains, including 37 Gram-positive and 23 Gram-negative bacteria and 28 fungi. The multiplex real-time PCR assay for detecting the 16S rRNA, *nuc*, and *mecA* genes yielded negative results with all nonstaphylococcal reference strains; thus, no cross-reactivity was detected (Table 2).

All seven staphylococcal reference strains, including five *S. aureus* strains, one *Staphylococcus xylosus* strain, and one *Staphylococcus epidermidis* strain, showed positive fluorescence signals for 16S rRNA in the multiplex real-time PCR assay. Five *S. aureus* reference strains showed positive results and two non-*S. aureus* reference strains were negative for the *S. aureus*-specific *nuc* gene in the multiplex real-time PCR assay (Table 2). Therefore, the specificity of the multiplex real-time PCR assay for detecting 16S rRNA, *nuc*, and *mecA* genes in reference strains was 100%.

Results of the multiplex real-time PCR assay with clinical isolates. The results of conventional methods and the real-time PCR assay with 118 clinical isolates, including 41 Gram-positive and 64 Gram-negative bacteria and 13 fungi, were completely concordant (100%). All 20 staphylococcal clinical isolates, including 12 *S. aureus* strains, four *S. epidermidis* strains, three *Staphylococcus haemolyticus* strains, and one *Staphylococcus capitis* strain, showed positive fluorescence signals for detecting 16S rRNA with the multiplex real-time PCR assay. All of the *S. aureus* and CoNS isolates showed positive and negative signals for the *nuc* gene, respectively, in the multiplex real-time PCR assay (Table 3).

Antibiotic susceptibility test results revealed that nine of 12 *S. aureus* (75%) and seven of eight CoNS (87.5%) clinical isolates with known oxacillin resistance ($\geq$4.0 μg/ml) were all positive for the *mecA* gene, based on results of the multiplex real-time PCR assay (Table 3). These data reveal that the sensitivity and specificity of the multiplex real-time PCR assay for detecting the 16S rRNA, *nuc*, and *mecA* genes were 100% with clinical isolates.

Results of the multiplex real-time PCR assay with positive blood culture samples. To evaluate the performance of the multiplex real-time PCR assay with positive blood culture samples, a total of 350 positive blood culture bottle samples were used. Among 350 positive blood culture samples, 166 cases were identified as *Staphylococcus* spp. by the standard culture method. They were all positive and 184 non-*Staphylococcus* cases were negative for 16S rRNA in the multiplex real-time PCR assay (Table 4). These results show that the results of the multiplex real-time PCR assay in targeting 16S rRNA to differentiate *Staphylococcus* spp. from non-*Staphylococcus* spp. in positive blood culture samples were totally concordant with standard culture results.

Among 166 *Staphylococcus* cases, 36 were identified as *S. aureus*

TABLE 4 Comparison of the multiplex real-time PCR assay, conventional culture identification, and antimicrobial susceptibility test with 166 *Staphylococcus*-positive blood culture samples

		Multiplex real-time PCR assay results (*n* [%]) with[b]:							
		Staphylococcus-specific probe ($n = 166$)		*S. aureus*-specific probe ($n = 166$)		Methicillin-resistant staphylococci probe ($n = 118$)			
						MRSA ($n = 23$)		MRCoNS ($n = 95$)	
Culture identification(s)	No. of samples ($n = 166$)	16S rRNA$^+$	16S rRNA$^-$	*nuc*$^+$	*nuc*$^-$	*mecA*$^+$	*mecA*$^-$	*mecA*$^+$	*mecA*$^-$
Staphylococcus									
S. aureus	36	36 (100)	0 (0)	35 (97.2)	1 (2.8)	22 (95.7)	1 (4.3)		
CoNS									
S. epidermidis	63	63 (100)	0 (0)	0 (0)	63 (100)			48 (100)	0 (0)
S. hominis	26	26 (100)	0 (0)	0 (0)	26 (100)			18 (100)	0 (0)
S. capitis	23	23 (100)	0 (0)	0 (0)	23 (100)			15 (100)	0 (0)
S. haemolyticus	12	12 (100)	0 (0)	0 (0)	12 (100)			10 (100)	0 (0)
S. saprophyticus	2	2 (100)	0 (0)	0 (0)	2 (100)			2 (100)	0 (0)
S. xylosus	1	1 (100)	0 (0)	0 (0)	1 (100)			1 (100)	0 (0)
S. warneri	1	1 (100)	0 (0)	0 (0)	1 (100)			0 (0)	0 (0)
S. intermedius	1	1 (100)	0 (0)	0 (0)	1 (100)			1 (100)	0 (0)
S. epidermidis, *S. warneri*[a]	1	1 (100)	0 (0)	0 (0)	1 (100)			0 (0)	0 (0)
Total	166	166 (100)	0 (0)	35 (21.1)	131 (78.9)	22 (95.7)	1 (4.3)	95 (100)	0 (0)

[a] Polymicrobial infection.

[a] Superscript plus symbols indicate the presence of the gene, and superscript minus symbols indicate its absence.

by bacterial culture methods, and all except one case were positive for the *S. aureus*-specific *nuc* gene in the multiplex real-time PCR assay. A total of 130 cases, including 63 *S. epidermidis* cases (48.5%), 26 *Staphylococcus hominis* cases (20%), 23 *S. capitis* cases (17.7%), 12 *S. haemolyticus* cases (9.2%), two *Staphylococcus saprophyticus* cases (1.5%), one *S. xylosus* case (0.8%), one *Staphylococcus warneri* case (0.8%), one *Staphylococcus intermedius* case (0.8%), and one polymicrobial infection (*S. epidermidis* and *S. warneri*) case (0.8%), were identified as CoNS by bacterial culture and all were negative for the *nuc* gene by multiplex real-time PCR assay testing (Table 4). These results show that, except for one case, the results of the multiplex real-time PCR assay in targeting the *nuc* gene to differentiate *S. aureus* from CoNS in positive blood culture samples were highly concordant with standard culture results.

Among 36 *S. aureus* cases, 23 were resistant to oxacillin and 22 were positive for the methicillin resistance *mecA* gene (Table 4). Among the 130 CoNS cases, 95 cases, including 48 *S. epidermidis* cases, 18 *S. capitis* cases, 15 *S. hominis* cases, 10 *S. haemolyticus* cases, two *S. saprophyticus* cases, and one case each of *S. xylosus* and *S. intermedius*, were resistant to oxacillin and all were positive for the *mecA* gene in the multiplex real-time PCR assay (Table 4). These results show that, except for one case, the results of the multiplex real-time PCR assay in targeting the *mecA* gene to detect methicillin resistance in *S. aureus* and CoNS in positive blood culture samples were highly concordant with the results of standard antimicrobial susceptibility tests.

Comparison of bacterial identification and methicillin susceptibility test results with the multiplex real-time PCR assay and the PCR-REBA. Another molecular assay, the PCR-REBA, was performed with the same DNA samples from positive blood cultures in order to confirm false positivity of the multiplex real-time PCR assay. The results were compared with standard culture method results (Fig. 2). All 166 staphylococcal positive samples from the multiplex real-time PCR assay showed positive signals with Gram-positive strain- and *Staphylococcus* genus-specific probes in the REBA Sepsis-ID test. Results from the multiplex real-time PCR assay and the PCR-REBA for detecting methicillin resistance were all concordant except for two cases. One case was identified as MRSA by the standard culture method and the PCR-REBA (positive for *S. aureus* and the *mecA* gene); however, the multiplex real-time PCR assay results were discordant with the results of those two assays (negative with the *nuc* and *mecA* gene-specific probes) (Table 5). The other sample was identified as MRCoNS by the standard culture method and the multiplex real-time PCR assay (positive for the *nuc* and *mecA* genes), but the PCR-REBA results were discordant with the results of those two assays (negative with the *mecA* gene-specific probe) (Table 5).

DISCUSSION

Until recently, initial treatment with antimicrobials for bacteremia often followed the identification of bacterial species by Gram staining of positive blood cultures. However, this process requires 48 to 72 h (2 to 3 days) for the identification of pathogens such as MRSA, MSSA, MRCoNS, and MSCoNS, in addition to the accurate determination of antimicrobial sensitivities for prompt optimal patient therapy and infection-control initiatives (9). Molecular diagnostic methods such as real-time PCR are now becoming established in clinical laboratories, and coupling this technology with traditional methods has provided rapid, specific, sensitive detection of microbial pathogens from blood cultures within 2 to 4 h (10, 11).

In this study, a multiplex real-time PCR assay that targets bacterial 16S rRNA to distinguish *Staphylococcus* spp., the *nuc* gene to distinguish *S. aureus* from other *Staphylococcus* spp., and the *mecA* gene for the detection of methicillin resistance was evaluated with reference strains and clinical samples. Results of the present study showed that the multiplex real-time PCR assay was rapid, with a

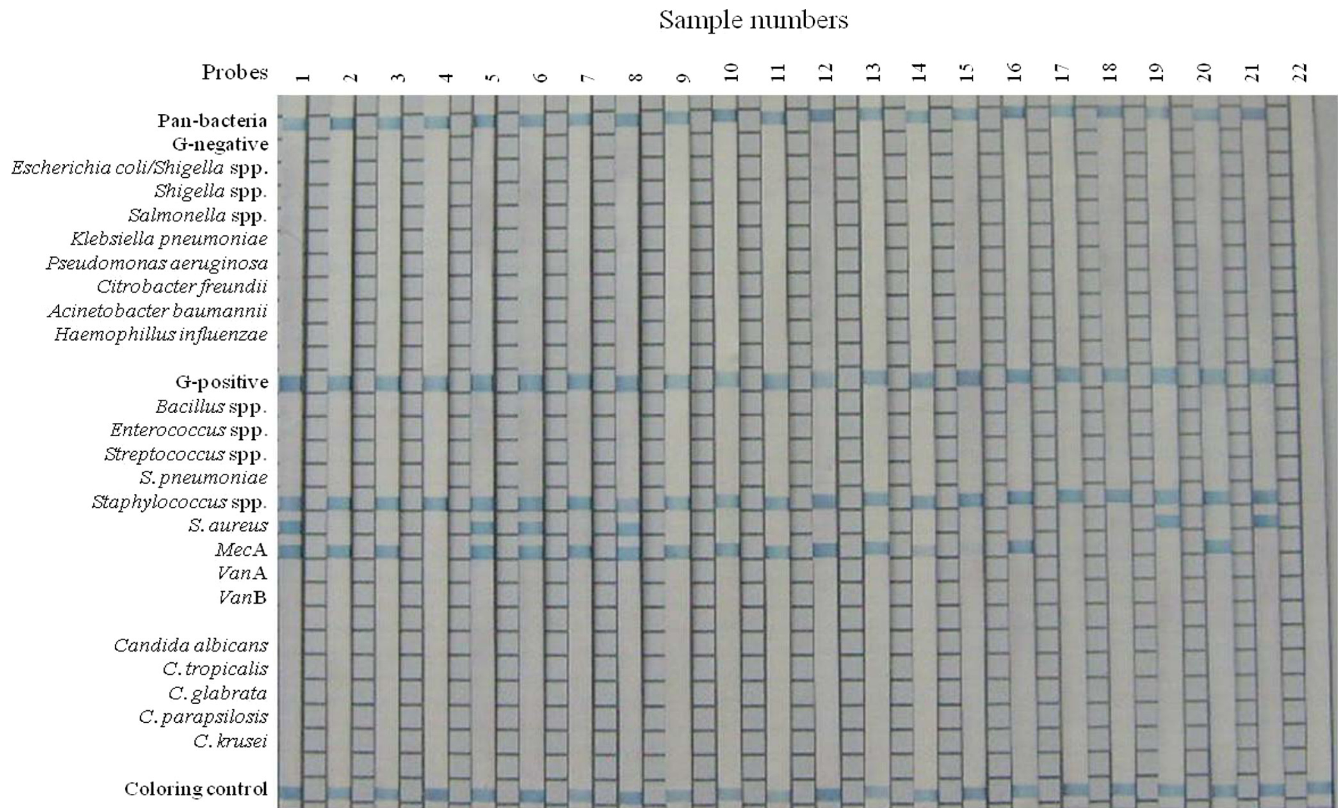

FIG 2 Typical results of the PCR-REBA with blood culture bottle samples positive for *Staphylococcus* spp., as a confirmative assay for the multiplex real-time PCR assay. Lanes 1, 5, 6, and 8, MRSA; lanes 2, 3, 7, 9, 10, 11, 12, 13, 14, 15, 16, and 20, MRCoNS; lanes 4, 17, and 18, MSCoNS; lanes 19 and 21, MSSA; lane 22, negative control. G, Gram.

turnaround time of usually no more than 4 h, which included 1 h for DNA preparation and 1.5 h for target DNA amplification. It allowed for the rapid identification of *S. aureus* and CoNS and their methicillin resistance status without a post-PCR process (e.g., agarose gel electrophoresis), and amplicon recognition was achieved by monitoring the accumulation of specific products during each cycle, in comparison with other PCR assays. Another advantage of this assay over standard PCR assays is that the entire process from amplification to analysis is performed in the same tube. This differs from standard PCR assays, in which the PCR product is moved and manipulated into other formats. As a result, there is a decreased possibility of contamination of the product with real-time PCR methods. Furthermore, the assay was very specific and sensitive, because the results were highly correlated with standard bacterial identification and antimicrobial susceptibility test results (Table 6). A possible limitation of the assay is the occurrence of false-negative results due to the presence of PCR inhibitors or polymicrobial infections in direct blood samples.

In previous reports from other study groups, CoNS was reported to be the major causative microorganism in sepsis (12, 13). In this study, *S. epidermidis*, which is a CoNS member, was isolated most often from positive blood culture samples, with a total of 76 cases (58.5% of CoNS cases). This was identical to the results from other studies (14).

Among 166 *Staphylococcus* cases, 118 (71.1%) were resistant to methicillin by the conventional antimicrobial susceptibility tests, and their drug resistance was also tested by the multiplex real-time PCR assay. These results were confirmed with the PCR-REBA mo-

TABLE 5 Comparison of results between the multiplex real-time PCR assay and the PCR-REBA

Culture identification	No. (%) identified	Multiplex real-time PCR assay results (no. [%] of isolates)[a]				PCR-REBA results (no. [%] of isolates)[a]			
		16S rRNA$^+$, *nuc*$^+$, *mecA*$^+$	16S rRNA$^+$, *nuc*$^+$, *mecA*$^-$	16S rRNA$^+$, *nuc*$^-$, *mecA*$^+$	16S rRNA$^+$, *nuc*$^-$, *mecA*$^-$	16S rRNA$^+$, *nuc*$^+$, *mecA*$^+$	16S rRNA$^+$, *nuc*$^+$, *mecA*$^-$	16S rRNA$^+$, *nuc*$^-$, *mecA*$^+$	16S rRNA$^+$, *nuc*$^-$, *mecA*$^-$
MRSA	23 (100)	22 (95.7)	0 (0)	0 (0)	1 (4.3)	23 (100)	0 (0)	0 (0)	0 (0)
MSSA	13 (100)	0 (0)	13 (100)	0 (0)	0 (0)	0 (0)	13 (100)	0 (0)	0 (0)
MRCoNS	95 (100)	0 (0)	0 (0)	95 (100)	0 (0)	0 (0)	0 (0)	94 (98.9)	1 (1.1)
MSCoNS	35 (100)	0 (0)	0 (0)	0 (0)	35 (100)	0 (0)	0 (0)	0 (0)	35 (100)
Total	166	22	13	95	36	23	13	94	36

[a] Superscript plus symbols indicate the presence of the gene, and superscript minus symbols indicate its absence.

TABLE 6 Overall sensitivity and specificity of the multiplex real-time PCR assay in comparison with the standard blood culture method with positive blood culture samples

Multiplex real-time PCR assay result	No. (%)					Sensitivity (%)	Specificity (%)
	Staphylococcus spp. ($n = 166$)		Methicillin-resistant staphylococci ($n = 118$)				
	S. aureus ($n = 36$)	CoNS ($n = 130$)	MRSA ($n = 23$)	MRCoNS ($n = 95$)	Nonstaphylococcal strains ($n = 184$)		
16S rRNA							
Positive	36 (100)	130 (100)			0 (0)	100	100
Negative	0 (0)	0 (0)			184 (100)		
nuc							
Positive	35 (97.2)	0 (0)			0 (0)	97.2	100
Negative	1 (2.8)	0 (0)			184 (100)		
mecA							
Positive			22 (95.7)	95 (100)	0 (0)	99.2	100
Negative			1 (4.3)	0 (0)	184 (100)		

lecular diagnostic test. The results of the two different molecular diagnostic assays in detecting methicillin resistance among 118 *Staphylococcus* positive blood culture samples were highly concordant each other, except for two cases (Table 5); one of the cases was a polymicrobial infection case in which the patient was infected with both *Enterococcus faecalis* and *S. aureus* but the case was negative for both the *nuc* and *mecA* genes in the multiplex real-time PCR assay. The negative results were attributable to the fact that the signal strength for *E. faecalis* was much stronger than that for *S. aureus* in the REBA Sepsis-ID assay, because the *E. faecalis* genomic DNA concentration may have been much greater than that of *S. aureus* for target amplification, and the fluorescence signal for *E. faecalis* could not be detected in the multiplex real-time PCR assay because the 16S rRNA gene of the assay was specifically detected in *Staphylococcus* spp. The other case was identified as *S. saprophyticus* that was resistant to oxacillin. However, the multiplex real-time PCR assay results were concordant with the conventional assay results, while the PCR-REBA results were not concordant with the results of the two assays. Therefore, the overall concordance rates between the two molecular diagnostic assays for detecting the 16S rRNA, *nuc*, and *mecA* genes were 100%, 98.6%, and 99.5%, respectively.

Additionally, the multiplex real-time PCR assay results for detecting the three target genes were negative for all 184 nonstaphylococcal positive blood culture samples; thus, no cross-reactivity was demonstrated, and the overall specificity of this assay was 100% for each of the target genes. The overall sensitivities of the multiplex real-time PCR assay were 100% (166/166 positive blood culture samples), 97.2% (35/36 positive blood culture samples), and 99.2% (117/118 positive blood culture samples) for detecting the 16S rRNA, *nuc*, and *mecA* genes, respectively, in S*taphylococcus* positive blood culture samples (Table 6).

In conclusion, the use of the multiplex real-time PCR assay showed rapid, highly sensitive, specific results for detecting MRSA, MSSA, MSCoNS, and MRCoNS species directly from positive blood culture bottle samples. Although the use of molecular diagnostic technology is more expensive than conventional methods, the clinical and economic benefits of saving time in treatment remains to be elucidated. Therefore, the Real-MRSA and Real-MRCoNS multiplex real-time PCR assays may provide the essential information to accelerate therapeutic decisions for earlier and adequate antibiotic treatment in the acute phase of sepsis.

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