

# Rapid Real-Time Nucleic Acid Sequence-Based Amplification–Molecular Beacon Platform To Detect Fungal and Bacterial Bloodstream Infections<sup>∇</sup>

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**Bloodstream infections (BSIs) are a significant cause of morbidity and mortality. Successful patient outcomes are diminished by a failure to rapidly diagnose these infections and initiate appropriate therapy. A rapid and reliable diagnostic platform of high sensitivity is needed for the management of patients with BSIs. The combination of an RNA-dependent nucleic acid sequence-based amplification and molecular beacon (NASBA-MB) detection system in multiplex format was developed to rapidly detect medically important BSI organisms. Probes and primers representing pan-gram-negative, pan-gram-positive, pan-fungal, pan-*Candida*, and pan-*Aspergillus* organisms were established utilizing 16S and 28S rRNA targets for bacteria and fungi, respectively. Two multiplex panels were developed to rapidly discriminate bacterial or fungal infections at the subkingdom/genus level with a sensitivity of 1 to 50 genomes. A clinical study was performed to evaluate the accuracy of this platform by evaluating 570 clinical samples from a tertiary-care hospital group using blood bottle samples. The sensitivity, specificity, and Youden's index values for pan-gram-positive detection and pan-gram-negative detection were 99.7%, 100%, 0.997 and 98.6%, 95.9%, 0.945, respectively. The positive predictive values (PPV) and the negative predictive values (NPV) for these two probes were 100, 90.7, and 99.4, 99.4, respectively. Pan-fungal and pan-*Candida* probes showed 100% sensitivity, specificity, PPV, and NPV, and the pan-*Aspergillus* probe showed 100% NPV. Robust signals were observed for all probes in the multiplex panels, with signal detection in <15 min. The multiplex real-time NASBA-MB assay provides a valuable platform for the rapid and specific diagnosis of bloodstream pathogens, and reliable pathogen identification and characterization can be obtained in under 3 h.**

Bloodstream infections (BSIs) are a significant cause of morbidity and mortality in the United States. Approximately 250,000 nosocomial BSIs occur annually, with a crude mortality rate of 20 to 50% (2, 17, 38). Based on data from death certificates, BSIs are the 10th leading cause of death in the United States (33), and the age-adjusted death rate has risen by 78% during the past two decades (10). The mortality rates of BSI patients are increased with measurable delays in the institution of effective, appropriate antimicrobial therapy (19, 35). Inadequate therapy was an independent determinant of mortality for individual organisms such as *Candida* species (21). Yu et al., in a U.S. multicenter study, found that failure to start antimicrobial therapy within 24 h of illness onset was strongly correlated with a higher probability of a 28-day mortality (49). A rapid and reliable diagnosis may be the optimal method for avoiding such delays in the treatment of BSIs.

A variety of microorganisms can cause BSIs. A nationwide surveillance study showed that gram-positive bacteria, gram-negative bacteria, and fungi (including yeasts and molds) caused 65, 25, and 9.5% of monomicrobial BSIs, respectively.

The most common organisms associated with BSIs are the coagulase-negative staphylococci (CoNS), *Staphylococcus aureus*, *Enterococcus* spp., and *Candida* spp. and the gram-negative bacilli *Escherichia coli* and *Klebsiella* spp. (47). The conventional diagnostic test for BSI is blood culture, which usually is specific but can have limited sensitivity and is inherently slow in providing complete diagnostic information (14, 37). Even after the detection of growth in cultured blood (usually not before 6 to 12 h of incubation), conventional blood cultures require at least another 24 to 48 h for the definitive identification of the pathogen and the assessment of resistance to antibiotics (4, 37). In recent years, real-time PCR assays have been developed for bacterial and fungal detection from clinical samples (23, 32, 48). The limit of detection was observed as 40, 50, or 2,000 CFU/ml for *E. coli*, group B *Streptococcus* spp., and *Listeria monocytogenes*, and it was 18 CFU/ml for *Candida* species. The nucleic acid extraction and the PCR amplification could be done in about 4 h. Compared to current culture-based and biochemical methods, nucleic acid sequence-based amplification (NASBA) assays may be the most promising diagnostic approach, because they offer the ultrasensitive detection of pathogens from blood. Despite this progress, current PCR-based diagnostics have some inherent limitations. A variety of pathogen species-specific PCR-based assays are available; however, the probes developed are species specific, which restricts the range of pathogens that can be detected in a single

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assay. Therefore, the assays are best applied when a specific infection is suspected (37), which is rarely the case during the early stages of BSIs. In addition, a positive signal does not necessarily reflect viable microorganisms (25), as the amplification of DNA from dead cells can confuse clinical management, especially following therapy. Importantly, some amplified detection methods generally require a growth enrichment step, i.e., inoculation and growth in blood culture bottles, to achieve the sensitivity necessary for pathogen detection and identification.

The universal detection of bacteria or fungi in blood using broad-range PCR assays has been described (1, 16, 22, 42), but interpretations can be difficult, as assay contaminants can cause false-positive results (37). The early detection of BSIs requires reliable sensitivity, around 10 CFU/ml of blood for bacteria and fungi, which is below the level of most existing diagnostic platforms. As an alternative to PCR, NASBA provides the ultrasensitive detection of nucleic acids. It is an isothermal process that selectively amplifies RNA even in the presence of genomic DNA (44). The reaction is highly robust, resulting in  $10^{12}$  amplified copies of template in a 30-min assay (12), which is comparable to a nested PCR in terms of sensitivity. When used to target rRNA genes, which are present in multiple copies in the genomes and expressed at a high level (thousands of copies per cell) in all known human bacterial and fungal pathogens, a NASBA-based platform is capable of high-level detection. It detects both live and dead cells. The amplified single-stranded RNA can be detected easily in real time by molecular beacon (MB) probes, which are small, self-reporting, single-stranded nucleic acid hairpin probes that brightly fluoresce when bound to their targets (45). MB probes offer a superior detection modality to perform real-time NASBA assays, which are rapid, highly specific, and sensitive.

To address the special considerations of BSIs, we have developed a real-time NASBA-MB platform that rapidly identifies medically important bacterial and fungal pathogens for BSI. A cascade pathogen detection and identification approach was developed that recognizes increasing complexity ranging from kingdom-specific (e.g., pan-fungal) targets to genus-specific (e.g., pan-*Candida* and pan-*Aspergillus*) and species-specific targets to drug resistance targets. The bacterial primers and probes target 16S rRNA genes, while the fungal assays target the 28S rRNA genes. This is the first report of a comprehensive NASBA-based platform for the rapid detection at the genus level of the major bacteria and fungi that are responsible for BSIs. This platform is suitable for use in simplex or multiplex assays incorporating a range of probes and primer sets to meet a variety of clinical laboratory detection needs.

This initial proof-of-concept study was designed to assess the technical feasibility of this method to detect a large variety of bloodstream pathogens. Although the final goal of a rapid diagnostic is detection from directly drawn blood samples, these studies are essential to evaluate the potential of the amplification system, primers, and probes. Technical parameters evaluated included assay sensitivity and specificity. To evaluate the overall accuracy of this platform, a clinical study was performed from a tertiary-care hospital group using blood bottle samples.

TABLE 1. Bacterial and fungal strains used in this study

Species and organism type	No. of strains	Reference strain	Source
<b>Gram-positive bacteria</b>			
<i>Staphylococcus aureus</i>	8	MRSA-9897	Clinical strain
<i>Staphylococcus epidermidis</i>	8	10683	Clinical strain
<i>Enterococcus faecium</i>	8	11936	Clinical strain
<i>Enterococcus faecalis</i>	8	9199	Clinical strain
<b>Gram-negative bacteria</b>			
<i>Escherichia coli</i>	8	8781	Clinical strain
<i>Enterobacter cloacae</i>	8	10623	Clinical strain
<i>Pseudomonas aeruginosa</i>	8	10470	Clinical strain
<i>Acinetobacter baumannii</i>	8	10437	Clinical strain
<i>Klebsiella pneumoniae</i>	8	20856	Clinical strain
<b><i>Candida</i> spp.</b>			
<i>Candida albicans</i>	8	90028	ATCC strain
<i>Candida glabrata</i>	8	90030	ATCC strain
<i>Candida krusei</i>	8	6258	ATCC strain
<i>Candida tropicalis</i>	8	750	ATCC strain
<i>Candida parapsilosis</i>	8	22019	ATCC strain
<i>Candida guilliermondii</i>	8	6260	ATCC strain
<i>Candida lusitanae</i>	4	200950	ATCC strain
<i>Candida dubliniensis</i>	8	3949	NCPF strain
<b><i>Aspergillus</i> spp.</b>			
<i>Aspergillus fumigatus</i>	8	293	ATCC strain
<i>Aspergillus flavus</i>	8	204304	ATCC strain
<i>Aspergillus terreus</i>	8	9151	Merck strain
<i>Aspergillus niger</i>	8	9508	ATCC strain
<b>Other fungi</b>			
<i>Cryptococcus neoformans</i>	1	208821	ATCC strain
<i>Saccharomyces cerevisiae</i>	1	Y55	Laboratory strain

## MATERIALS AND METHODS

**Bacterial and fungal strains.** Bacterial strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Acinetobacter* spp. and fungal strains of *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Candida parapsilosis*, *Candida dubliniensis*, *Candida guilliermondii*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus niger* were obtained from the historical stock of the Public Health Research Institute (PHRI), which is collected from many sites across North America and Europe (Table 1).

**Bacterial and fungal cultures.** All reference bacterial strains were cultured on Luria-Bertani (LB) agar at 37°C, except for *S. aureus* strains that were grown on mannitol salt agar (MSA) at 37°C. *Candida* spp. reference strains were grown and maintained on yeast extract-peptone-dextrose (YPD) agar at 30°C, and *Aspergillus* spp. reference strains were cultured on potato dextrose agar (PDA) at 37°C. Brain heart infusion (BHI) medium, LB broth, or YPD broth was used to prepare overnight cultures for the nucleic acid extraction from bacteria or *Candida* reference strains, respectively.

**Primers and MB design.** 16S and 28S rRNA were chosen to be our detection targets for bacteria and fungi, respectively. The 16S rRNA gene sequences from 12 bacterial species were obtained from online databases (Table 2). The sequences were aligned using the online program ClustalW from the European Bioinformatics Institute (<http://www.ebi.ac.uk/Tools/clustalw/index.html>). Similarly, 28S rRNA gene sequences for 15 different fungal species were collected and aligned (Table 2). For bacteria, one region highly conserved for gram-positive bacteria but variable for gram-negative bacteria was selected as the diagnostic target for the pan-gram-positive probe. On the contrary, a region conserved for all of the gram-negative bacteria but variable for other microbes was chosen as the diagnostic target for the pan-gram-negative probe. Based on this principle, pan-fungal, pan-*Candida*, and pan-*Aspergillus* targets were selected accordingly. MB probes were designed by adding two artificial arm sequences to

TABLE 2. Strains and sequences used for primer and MB design

Species and organism type	Strain	Accession no.	Region
<b>Gram-positive bacteria</b>			
<i>Staphylococcus aureus</i>	MRSA252	NC_002952	16S rRNA
<i>Staphylococcus epidermidis</i>	RP62A	NC_002976	16S rRNA
<i>Streptococcus pneumoniae</i>	TIGR4	AAGY02000123	16S rRNA
<i>Enterococcus faecium</i>	DO ctg565	AAAK03000115	16S rRNA
<i>Enterococcus faecalis</i>	V583	NC_004668	16S rRNA
<b>Gram-negative bacteria</b>			
<i>Escherichia coli</i>	O157:H7 EDL933	NC_002655	16S rRNA
<i>Enterobacter cloacae</i>	CP1	DQ089673	16S rRNA
<i>Klebsiella pneumoniae</i>	MGH 78578	NC_009648	16S rRNA
<i>Pseudomonas aeruginosa</i>	PAO1	NC_002516	16S rRNA
<i>Acinetobacter baumannii</i>	ADP1	NC_005966	16S rRNA
<i>Proteus mirabilis</i>	HI4320	AM942759	16S rRNA
<i>Haemophilus influenzae</i>	86-028NP	NC_007146	16S rRNA
<b>Candida spp.</b>			
<i>Candida albicans</i>	SC5314	AACQ01000295	28S rRNA
<i>Candida glabrata</i>	CBS138	AY198398	28S rRNA
<i>Candida krusei</i>	NRRL Y-5396	EF550222	28S rRNA
<i>Candida tropicalis</i>	MYA-3404	AAFN01000124	28S rRNA
<i>Candida parapsilosis</i>	317	Sanger Institute <sup>a</sup>	28S rRNA
<i>Candida guilliermondii</i>	ATCC 6260	AAFM01000051	28S rRNA
<i>Candida lusitanae</i>	ATCC 42720	AAFT01000066	28S rRNA
<i>Candida dubliniensis</i>	CD36	Sanger Institute <sup>b</sup>	28S rRNA
<b>Aspergillus spp.</b>			
<i>Aspergillus fumigatus</i>	Af293	AAHF01000017	28S rRNA
<i>Aspergillus flavus</i>	NRRL3357	AAIH01002203	28S rRNA
<i>Aspergillus terreus</i>	NIH 2624	Broad Institute <sup>c</sup>	28S rRNA
<i>Aspergillus niger</i>	CBS 513.88	NT_166520	28S rRNA
<b>Other fungal species</b>			
<i>Cryptococcus neoformans</i>	B-3501A	AAEY01000011	28S rRNA
<i>Saccharomyces cerevisiae</i>	RM11-1a	AAEG01000105	28S rRNA
<i>Neosartorya fischeri</i>	NRRL 181	AAKE03000097	28S rRNA

<sup>a</sup> Genome sequence data used in this study were produced by the Pathogen Genomics group at the Wellcome Trust Sanger Institute and are available from <http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/>.

<sup>b</sup> Genome sequence data used in this study were produced by the Pathogen Genomics group at the Wellcome Trust Sanger Institute and are available from <http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/>.

<sup>c</sup> Sequence data were produced by the *Aspergillus terreus* sequencing project at the Broad Institute of Harvard and MIT (<http://www.broad.mit.edu>).

both ends of the target sequence. The secondary structure of the MB was evaluated by the software OligoAnalyzer 3.0 (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>). The MBs were labeled with the fluorophores 5-carboxyfluorescein (FAM), 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein (HEX), CAL fluor red (CR610), and Quasar 670 (Q670) at the 5' end and with benzoic acid succinimidyl ester (dabcyl) or black hole quencher at the 3' end. The MBs were purchased from Biosearch Technologies, Inc., Novato, CA. The pan-gram-negative MB has a perfect target sequence for most bacterial species tested in this study, except for *Acinetobacter* spp. and *Pseudomonas* spp., which both have two mismatches with the pan-gram-negative beacon. Therefore, two individual beacons targeting *Acinetobacter* spp. and *Pseudomonas* spp. were designed in the same region covered by the pan-gram-negative primers, and they were mixed with the pan-gram-negative MB for the following experiments. Similarly, three patch probes for pan-gram-positive MB (16S GP v1.21 to 16S GP v1.23) (Table 3) were designed, because three gram-positive species (*Corynebacterium* sp., *Propionibacterium* sp., and *Micrococcus* sp.) from the clinical samples were missed by the pan-gram-positive probe. NASBA primers also were designed based on the sequence alignments. A T7 promoter sequence was added at the 5' end of each P1 primer. All MBs and NASBA primers used in this study are listed in Table 3.

**Extraction of TNA and RNA.** A 200- $\mu$ l aliquot of test culture was mixed with 800  $\mu$ l NucliSENS lysis buffer (bioMérieux, Durham, NC) in lysing matrix tubes (MP, Biomedicals, Inc., Solon, OH) and vigorously vortexed on a FastPrep instrument (MP, Biomedicals, Inc., Solon, OH) for 45 s, followed by a 30-min incubation at 37°C. Cell lysates were centrifuged at 16,000  $\times$  g for 10 min, and then 800  $\mu$ l of supernatant was transferred and mixed with 2 ml NucliSENS lysis

buffer in the NucliSENS easyMAG (bioMérieux) sample cartridge for the total nucleic acid (TNA) extraction. Magnetic silica solution was added, and the TNA extraction was performed according to the easyMAG user manual. The final elution volume of the TNA for each dilution was 110  $\mu$ l. For *Aspergillus* spp., conidia were collected from an *Aspergillus fumigatus* culture grown on a PDA plate at 37°C for 3 days, using sterile saline with 0.01% Tween 20. The conidial suspension was counted under a microscope using a disposable cell counting chamber, and 200  $\mu$ l of 10<sup>7</sup> CFU/ml conidial suspension was extracted for total nucleic acids. Tenfold serial dilutions were made from the extracts. In some experiments, pure RNA was isolated using the RNeasy kit according to the procedure of the manufacturer (Qiagen, Valencia, CA). The RNA concentration was determined by spectrophotometry using a Nanodrop DN1000 device (Nanodrop Technologies, DE), and the 260-nm wavelength/280-nm wavelength and 260-nm wavelength/230-nm wavelength ratios were calculated.

**Real-time NASBA.** The NASBA reactions were performed with the NucliSENS basic kit version 2 (bioMérieux). Reactions were carried out in a total volume of 20  $\mu$ l containing reagent mix, 0.2  $\mu$ M each primer, 0.1  $\mu$ M MB, 5  $\mu$ l of RNA or TNA template, and 5  $\mu$ l of NASBA enzyme mix (nucleic acid-free enzyme; provided by bioMérieux, France). The enzyme mixture contained T7 RNA polymerase, avian myeloblastosis virus reverse transcriptase, RNase H, and bovine serum albumin, and the mixture was added to the reaction mixture after a two-step incubation of 2 min at 65°C and 2 min at 41°C. For simplex NASBA assays, amplification and real-time detection were performed on the NucliSENS EasyQ analyzer (bioMérieux) at 41°C for 90 min. The fluorescence signal was measured with an interval time of 30 s for each independent reaction at two wavelengths using the accompanying NucliSENS EasyQ Director software (ver-

TABLE 3. Primers and MBs for each assay

Oligonucleotide or primer and organism type	Type	Sequence (5'-3') <sup>h</sup>	Position
<b>Gram-positive bacteria</b>			
PLK1	Forward primer	TACGGGAGGCAGCAGT	351–366 <sup>a</sup>
PLK2N v1.4	Reverse primer	<i>AATTCTAATACGACTACTATAGGGGCTGCTGGCACGTA</i> <i>GTTAGCCGTGGCTTTC</i>	533–504 <sup>a</sup>
16S GrP MB	MB	<u>CGAGCTAGCAACGCCGCGTGAGTGAAGCTCG</u>	401–419 <sup>a</sup>
16S GP v1.21	MB	<u>CGAGCTAGCAACGCCGCGTGGGTGAAGCTCG</u>	401–419 <sup>a</sup>
16S GP v1.22	MB	<u>CGAGCTAGCAACGCCGCGTGAGGGAAGCTCG</u>	401–419 <sup>a</sup>
16S GP v1.23	MB	<u>CGAGCTAGCGACGCCGCGTGGGGGAAGCTCG</u>	401–419 <sup>a</sup>
<b>Gram-negative bacteria</b>			
Grm neg 16S P2	Forward primer	CCTGATGCAGCCATGCCGCGTG	385–406 <sup>b</sup>
Grm neg 16S P1	Reverse primer	<i>AATTCTAATACGACTACTATAGGGCACGGAGTTAGCC</i> <i>GGTGCTT</i>	517–498 <sup>b</sup>
Grm neg 16S MB	MB	<u>CGAGCTTGAAGAAGGCCCTTCGGGTTGTAAAGAGCTCG</u>	409–433 <sup>b</sup>
Grm neg16S-Acin	MB	<u>CGAGCTTGAAGAAGGCCCTTATGGTTGTAAAGAGCTCG</u>	397–421 <sup>c</sup>
Grm neg16S-Pseu	MB	<u>CGAGCTAGCACTTTAAGTTGGGAGGAAGCTCG</u>	427–446 <sup>d</sup>
<b>Bacteria</b>			
GP/GN P1 <sup>g</sup>	Reverse primer	<i>AATTCTAATACGACTACTATAGGGGTATTACCGCGGCT</i> <i>GCTGGCAC</i>	544–523 <sup>a</sup>
<b>Fungi</b>			
28SPF P2	Forward primer	CGGCTCTTCCTATCATAACCG	2850–2869 <sup>e</sup>
28SPF P1	Reverse primer	<i>AATTCTAATACGACTACTATAGGGCTAAACCCAGCTCA</i> <i>CGTTCC</i>	2931–2912 <sup>e</sup>
28SPF MB	MB	<u>CGCGATATTCGGTAAGCGTTGGATTGATCGCG</u>	2877–2896 <sup>e</sup>
<b>Candida species</b>			
28SPC P2	Forward primer	GGAATCCGCTAAGGAGTGTG	1246–1265 <sup>e</sup>
28SPC P1	Reverse primer	<i>AATTCTAATACGACTACTATAGGGCCATCCATTTTCAG</i> <i>GGCTAGT</i>	1309–1289 <sup>e</sup>
28SPC MB	MB	<u>CGCGATTAACAACCTACCGGCCGAATATCGCG</u>	1266–1285 <sup>e</sup>
<b>Aspergillus species</b>			
28SPAS	Forward primer	CAGCAGTTGGACATGGGTTA	1503–1522 <sup>f</sup>
28SPAASN	Reverse primer	<i>AATTCTAATACGACTACTATAGGGGAGAATCCACATCC</i> <i>AGGTGC</i>	1625–1606 <sup>f</sup>
28SPAMB	MB	<u>CGACCGCATAGGGAAGTTCCGTTTGGTGC</u>	1534–1553 <sup>f</sup>

<sup>a</sup> The position is based on *S. aureus* MRSA252 NC\_002952.

<sup>b</sup> The position is based on *E. coli* O157:H7 EDL933 NC\_002655.

<sup>c</sup> The position is based on *A. baumannii* ADP1 NC\_005966.

<sup>d</sup> The position is based on *P. aeruginosa* PAO1 NC\_002516.

<sup>e</sup> The position is based on *C. albicans* SC5314 AACQ01000295.

<sup>f</sup> The position is based on *A. fumigatus* Af293 AAHF01000017.

<sup>g</sup> The primer was used for bacterial multiplex detection.

<sup>h</sup> T7 promoter sequences are labeled in italics, and beacon arm sequences are underlined.

sion 2). For multiplex NASBA assays, reactions were carried out on a Stratagene Mx3005P real-time PCR system (Stratagene, La Jolla, CA) with the setting of 180 cycles at 41°C with 30 s for each cycle. The fluorescence signal was measured once for each cycle. The cutoff threshold for the positive signal was set as 10% higher than the end point signal from the no-template control, which used 5 µl of nuclease-free water instead of nucleic acids in the reaction mixture. The time to positive was defined as the time point when the positive signal crossed the threshold. Two multiplex real-time NASBA assays (pan-gram positive/pan-gram negative and pan-fungal/pan-*Candida*/pan-*Aspergillus*) were developed for bacterial and fungal detection, respectively.

**Preliminary in vitro specificity and sensitivity. (i) Quantifying target template.** An artificial rRNA fragment for each pathogen was synthesized by in vitro transcription from a PCR product containing the corresponding genomic DNA using a specific primer set (Table 4), of which the forward primer was linked with a T7 promoter sequence. The specific PCR products either were purified by paramagnetic beads (Agencourt AMPure, Beverly, MA) or extracted from agarose gels (Montage; Millipore, Billerica, MA). After purification, the PCR product was transcribed in vitro for 1 h at 37°C using an RNAMaxx High Yield Transcription kit (Stratagene) and then treated by RNase-free DNase I (Ambion, Austin, TX) for 30 min at 37°C. RNA products were purified using the

RNeasy kit according to the RNA cleanup protocol (Qiagen). Purified RNA targets were quantified by a Quant-iT RiboGreen RNA quantification kit (Molecular Probes, Invitrogen, Carlsbad, CA). For TNA quantification, overnight cultures were subcultured in the same broth and grown for 3 to 4 h until log phase and then were harvested by centrifugation at 4,000 rpm for 5 min at 4°C. The cells were washed twice with sterile phosphate-buffered saline (PBS). The numbers of structurally intact cells were determined microscopically using a disposable cell counting chamber. Cultures were serially diluted from 10<sup>7</sup> to 10<sup>0</sup> CFU/ml accordingly. TNA was extracted from each dilution as described above. The accurate CFU was enumerated by plating 100 µl of each dilution onto LB agar for bacteria (MSA for *S. aureus*) or YPD agar for *Candida* and incubating it at 37°C for 24 h.

**(ii) Sensitivity and specificity testing.** The sensitivities of the real-time NASBA assays were assessed by amplifying both in vitro RNA transcripts and TNAs isolated from serial dilutions of the target organism cultures. As described above, the in vitro RNA transcripts were synthesized, purified, and quantified, and the number of RNA copies was calculated according to the concentration and the size of the RNA transcript. Tenfold serial dilutions were prepared covering a range of 8-log (10<sup>7</sup> to 10<sup>0</sup>) copies of RNA molecules per NASBA reaction. These dilutions of RNA were added directly into the NASBA mix. For



TABLE 4. Primers used for in vitro transcription

Primer	Sequence (5'-3')	Position
SA16S F	AATTCTAATACGACTCACTATAGGGTAACGGCTTACCAAGGCAAC	269-288
SA/Enter16S R	ACCTTCCGATACGGCTACCT	1533-1514 <sup>a</sup>
Enter16S F	AATTCTAATACGACTCACTATAGGGCACATTGGGACTGAGACACG	303-322
SE16S F	AATTCTAATACGACTCACTATAGGGCAAGTCGAGCGAACAGACGA	59-78
SE16S R	TGTCACCGGCAGTCAACTTA	1181-1162
Ecoli/Kleb16S F	AATTCTAATACGACTCACTATAGGGGATCATGGCTCAGATTGAACG	15-35 <sup>b</sup>
Ecoli/Kleb16S R	GCTGGCAACAAAGGATAAGG	1132-1113 <sup>b</sup>
28SPF F	AATTCTAATACGACTCACTATAGGGGAGGCTAGAGGTGCCAGAAA	2756-2775 <sup>c</sup>
28SPF R	TCTGACTTAGAGGCGTTCAGC	3101-3081 <sup>c</sup>
Calb28S F	AATTCTAATACGACTCACTATAGGGCGATACTGCCAGCCTAGACC	566-585
Calb28S R	CGCTCTCCAGCCATAAGAC	1751-1732
Afum28S-F	AATTCTAATACGACTCACTATAGGGGTGAAGCTCCTTCGACGAGT	250-269
Afum28S-R	TCTCCGCGTTCAGTTACGTT	1651-1632

<sup>a</sup> The position is based on the *S. aureus* 16S sequence from NC\_002952.

<sup>b</sup> The position is based on the *E. coli* 16S sequence from NC\_002655.

<sup>c</sup> The position is based on the *C. albicans* 28S sequence from AACQ01000295.

the TNA isolated from the bacterial or fungal culture serial dilutions, the NASBA assay was performed on each of these extracts. To mimic the clinical environment, blood culture bottles containing 8 ml of whole blood were spiked with 10-fold serially diluted bacterial cultures of *Enterobacter cloacae* and yeast cultures of *Candida glabrata* and were subjected to TNA extraction. A 200- $\mu$ l aliquot of the spiked blood bottle culture was used for TNA extraction at a concentration ranging from  $4 \times 10^6$  to  $4 \times 10^0$  CFU/ml. Blood culture bottles lacking the spiking pathogen were subjected to extraction in parallel as the negative control. All of the real-time NASBA assays described above were repeated at least three times. Preliminary probe specificity was evaluated by testing the nucleic acids from the target organisms, as well as the nucleic acids from other related microbes. For instance, the pan-gram-positive probe was tested against nucleic acids from four gram-positive bacterial species as well as a panel of five gram-negative species, eight *Candida* spp., *C. neoformans*, *S. cerevisiae*, and four *Aspergillus* spp., as listed in Table 1. The no-template control also was tested in parallel, and at least eight TNA samples from different clinical isolates of the same species were tested for each probe.

**Clinical evaluation: sample collection, preparation, and NASBA detection.** To assess the clinical performance of the NASBA assays, aerobic and anaerobic blood culture bottles submitted for routine clinical testing were processed according to the manufacturer's instructions in either the BacT/Alert 3D (bioMérieux) or the BacTec 9240 (Becton Dickinson, Sparks, MD) automated blood culture system. Five-milliliter aliquots from 520 randomly selected positive blood culture bottles (BacTec Aerobic Plus,  $n = 250$ ; BacTec Anaerobic Plus,  $n = 232$ ; BacTec Anaerobic Lytic,  $n = 6$ ; BacTec Peds Plus,  $n = 12$ ; and BacT/Alert Aerobic,  $n = 20$ ) were collected at the time blood bottles were flagged as positive by the automated blood culture systems and processed for routine microbiologic testing. The identification of the organism(s) present in the positive blood culture bottles was not known at the time of selection. They were selected in this manner so as not to bias results and to mimic the pattern of clinical isolates routinely encountered in our laboratory. The blood culture aliquots were deidentified by the North Shore-Long Island Jewish Health System Laboratories and stored at 4°C until shipment to PHRI on a weekly basis. Gram stain and culture on solid media were performed according to standard laboratory procedures for microbe identification and the determination of sensitivity to antibiotics. Microbe identification and the methicillin susceptibility profiles for *S. aureus* isolates were not provided to PHRI until the completion of the study. In addition, 5-ml aliquots from 50 negative blood culture bottles, collected on day 5 at the completion of routine culture, also were deidentified. The blood aliquots were stored at 4°C and transported within 7 days to PHRI for real-time NASBA identification. Briefly, 200  $\mu$ l of the blood culture was mixed with 800  $\mu$ l of a mixture of lysis buffer and proteinase K (25  $\mu$ g/ml). FastPrep for 45 s, as described above, was followed by a 1-h incubation at 55°C and then a hard spin of the lysates at 16,000  $\times g$ . The supernatants were processed as described for TNA extraction from laboratory cultures. The final volume of each TNA sample eluate was 110  $\mu$ l, 5  $\mu$ l of which was used for each real-time NASBA assay. Blood bottle samples were tested by multiplex real-time NASBA assays. Studies were performed under an Institutional Review Board-approved protocol.

**Statistical evaluation.** After breaking the code of the blinded blood bottle samples, the accuracy of the real-time multiplex NASBA assay was assessed by

calculating the sensitivity, specificity, Youden's index (6), and predictive values for each probe.

## RESULTS

### Specificity of the broad-range real-time NASBA platform.

The specificity of the assay was defined as the ability to exclusively detect the target of interest. Like PCR-based methods, broad-range NASBA assays also are more vulnerable to trace amounts of nucleic acid contamination than the species-specific assays, which can lead to false-positive results. To overcome this potential problem, a nucleic acid-free enzyme mix (R&D Group, bioMérieux, France) was used for amplification in the broad-range bacterial and fungal pathogen detection system. All probes showed 100% specificity in initial tests using reference strains. Specific signal was observed for all target species but not for any other nontarget organism. Pan-gram-positive and pan-gram-negative probes were able to detect four (*S. aureus*, *S. epidermidis*, *E. faecium*, and *E. faecalis*) and five (*E. coli*, *P. aeruginosa*, *A. baumannii*, *E. cloacae*, and *K. pneumoniae*) medically important species, respectively, and no cross-reactivity was observed between each group or with fungi. Pan-fungal, pan-*Candida*, and pan-*Aspergillus* probes also demonstrated 100% specificity. Eight *Candida* species (*C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. lusitaniae*, and *C. dubliniensis*), four *Aspergillus* species (*A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*), *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* (Table 1) were detected easily by the pan-fungal probe without any cross-reactivity with the bacterial probes. Pan-*Candida* and pan-*Aspergillus* probes selectively recognized all of the *Candida* or *Aspergillus* species among all of the fungal species listed in Table 1. All probes were validated with at least eight clinical isolates.

**Sensitivity of real-time NASBA assays: simplex assays.** *C. albicans* and *A. fumigatus* were selected as representative species for the pan-fungal, pan-*Candida*, and pan-*Aspergillus* sensitivity evaluations. As shown in Table 5, the analytical sensitivity of the assay was  $10^4$  copies of target RNA transcripts for pan-fungal and pan-*Candida* assay and  $10^3$  copies of target RNA for pan-*Aspergillus* detection. To assess the effect and the

TABLE 5. Sensitivity of real-time NASBA

Probe and species	Sensitivity in simplex assay		Sensitivity in multiplex assay	
	Copy no.	CFU/reaction	Copy no.	CFU/reaction
<b>GrP16S</b>				
<i>E. faecium</i>	10 <sup>3</sup> ~10 <sup>4</sup>	0.2	10 <sup>5</sup> ~10 <sup>6</sup>	2
<i>E. faecalis</i>	10 <sup>3</sup> ~10 <sup>4</sup>	0.56	10 <sup>5</sup>	5.6
<i>S. epidermidis</i>	10 <sup>4</sup>	4.5	10 <sup>6</sup>	45
<i>S. aureus</i>	10 <sup>4</sup>	1.3	10 <sup>5</sup> ~10 <sup>6</sup>	13
<b>GrN16S</b>				
<i>E. coli</i>	10 <sup>4</sup>	5.5	10 <sup>6</sup>	55
<i>A. baumannii</i>	10 <sup>4</sup>	1.1	10 <sup>5</sup> ~10 <sup>6</sup>	11
<i>K. pneumoniae</i>	10 <sup>4</sup>	1.1	10 <sup>5</sup> ~10 <sup>6</sup>	11
<i>E. cloacae</i>	10 <sup>4</sup>	1.7	10 <sup>6</sup>	17~170
<i>P. aeruginosa</i>	10 <sup>4</sup>	1.1	10 <sup>5</sup> ~10 <sup>6</sup>	11
<b>28SPF</b>				
<i>C. albicans</i>	10 <sup>3</sup> ~10 <sup>4</sup>	1.8	10 <sup>4</sup>	1.8
<i>A. fumigatus</i>	10 <sup>3</sup> ~10 <sup>4</sup>	0.14	10 <sup>4</sup>	1.4
<b>28SPC</b>				
<i>C. albicans</i>	10 <sup>4</sup>	1.8	10 <sup>5</sup>	1.8
<b>28SPA</b>				
<i>A. fumigatus</i>	10 <sup>3</sup>	0.14	10 <sup>4</sup>	1.4

efficiency of the extraction process, the TNAs isolated from serially diluted fungal cultures were used to evaluate the limit of detection of the assay. All fungal probes were able to detect fewer than 2 CFU of target organisms. Similar sensitivities were observed for both pan-gram-positive and pan-gram-negative probes, with a detection limit ranging from 10<sup>3</sup> to 10<sup>4</sup> RNA copies and around 1 to 5 CFU of target organisms (Table 5). To investigate the applicability of our pan-NASBA assay, specificity and sensitivity tests were performed using TNAs extracted from blood culture bottles containing whole blood that had been spiked with serially diluted *C. glabrata* or *E. cloacae* cultures. Robust signals were detected at as low as 0.28 CFU of *C. glabrata* by the pan-fungal and the pan-*Candida* probes, and the pan-gram-negative probe could detect 3.1 CFU of *E. cloacae* in a single NASBA reaction.

**Multiplex assays.** Compared to the ultrasensitive simplex NASBA reactions, multiplex NASBA assays were less sensitive (10- to 100-fold) for most of the probes. Interactions among different MBs and primer sets were expected to occur in these multiplex detections, which accounts for the diminished sensitivity. To improve the sensitivity, one common P1 (GP/GN P1) was designed and used for the bacterial panel. The common P1 demonstrated that approximately 10<sup>5</sup> to 10<sup>6</sup> copies of target RNA molecules or 5 to 50 CFU of organisms (Table 5, Fig. 1) could be detected by the pan-gram-positive/pan-gram-negative assays. Additional patch probes for gram-positive bacteria did not change the sensitivity of the multiplex assay. In contrast, less competition was seen in the fungal panel, of which the detection limit ranged from 10<sup>4</sup> to 10<sup>5</sup> copies of *C. albicans* or *A. fumigatus* RNA or around 1 CFU of target organism (Table 5, Fig. 2).

**Clinical evaluation.** A total of 520 randomly selected samples from positive blood bottles were identified to the subkingdom or genus level by multiplex NASBA, and the results

were compared to those obtained by conventional culture systems. Based on the culture identification, 362 samples contained gram-positive bacteria, 149 samples contained gram-negative bacteria, and 10 samples contained *Candida* spp. A wide BSI pathogen spectrum was seen in these samples, consisting of eight gram-positive bacterial genera and 21 species (*Corynebacterium* spp. [including *C. jeikeium*], *Enterococcus* spp. [*E. casseliflavus*, *E. faecalis*, *E. faecium*, and *E. gallinarum*], *Lactobacillus casei*, *Micrococcus* sp., *Propionibacterium acnes*, *Staphylococcus aureus* [methicillin sensitive and resistant], CoNS [including *S. lugdunensis*], and *Streptococcus* spp. [*S. agalactiae*, *S. bovis*, *S. milleri* group, *S. mitis* group, *S. pneumoniae*, *S. pyogenes*, *S. sanguis*, and *S. salivarius*]), 17 gram-negative bacterial genera and 20 species (*Acinetobacter baumannii*, *Alcaligenes* sp., *Bacteroides* sp., *Burkholderia cepacia*, *Citrobacter* spp. [*C. freundii* and *C. koseri*], *Enterobacter* spp. [*E. aerogenes* and *E. cloacae*], *Escherichia coli*, *Fusobacterium nucleatum*, *Haemophilus influenzae*, *Klebsiella* spp. [*K. oxytoca* and *K. pneumoniae*], *Morganella morganii*, *Prevotella loescheii*, *Proteus mirabilis*, *Providencia stuartii*, *Pseudomonas aeruginosa*, *Salmonella* sp. group B, and *Serratia marcescens*), and 3 *Candida* species (*C. albicans*, *C. parapsilosis*, and *C. tropicalis*). High-level specificity was observed for all probes in the multiplex NASBA detections (Table 6). In a set of controlled experiments, 50 culture-negative blood bottle samples were evaluated and failed to elicit any detectable fluorescent signals. In addition, full sensitivity as described above was unaltered with blood bottle media used as the background matrix.

One blood culture, flagged as positive by the automated blood culture instrument, was culture negative, and the NASBA assays identified a gram-negative organism. A second blood culture collected from the same patient several hours later grew *Pseudomonas aeruginosa*. A blood culture from another patient had very poor growth that did not initially allow for the identification of the two pathogens present in the sample. The NASBA assays were positive for both a gram-positive and a gram-negative organism. Conventional culture took more than 1 week to finally confirm the presence of a gram-positive rod in the same bottle. A gram-negative rod was not recovered from this bottle, but another blood culture drawn on the same day contained *E. coli*.

It was observed that the uncommon organisms *Corynebacterium* spp., *Propionibacterium* sp., and *Micrococcus* sp. were falsely identified as gram-negative bacteria. To address this issue, three patch probes were designed for pan-gram-positive detection (Table 3). The use of a mixture of four pan-gram-positive MBs increased the specificity of pan-gram-positive detection to >99%. A total of 13 out of 14 missed gram-positive samples subsequently were picked up by the new pan-gram-positive detection, and no cross-reaction was seen from gram-negative bacteria and fungi. In addition, the pan-gram-negative probe failed to recognize the anaerobe *Prevotella loescheii* in the clinical blood culture, and no detectable signal was seen from other probes as well. In control experiments, no organisms were detected in the 50 negative blood cultures submitted for testing.

The overall performance of multiplex NASBA assays was measured by sensitivity, specificity, Youden's index, and predictive values for each primer/probe set (Table 6). High sensitivity (96.1 and 98.6%), specificity (100 and 95.9%), and

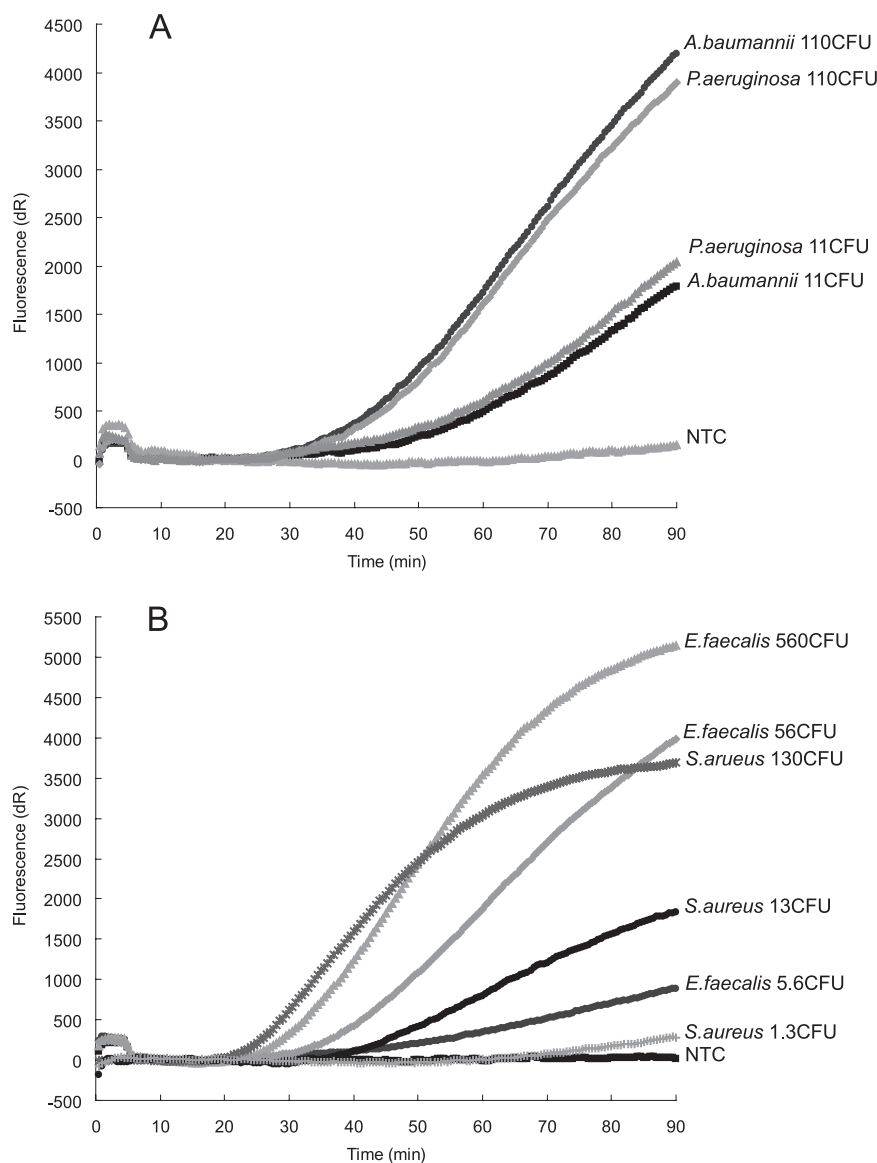


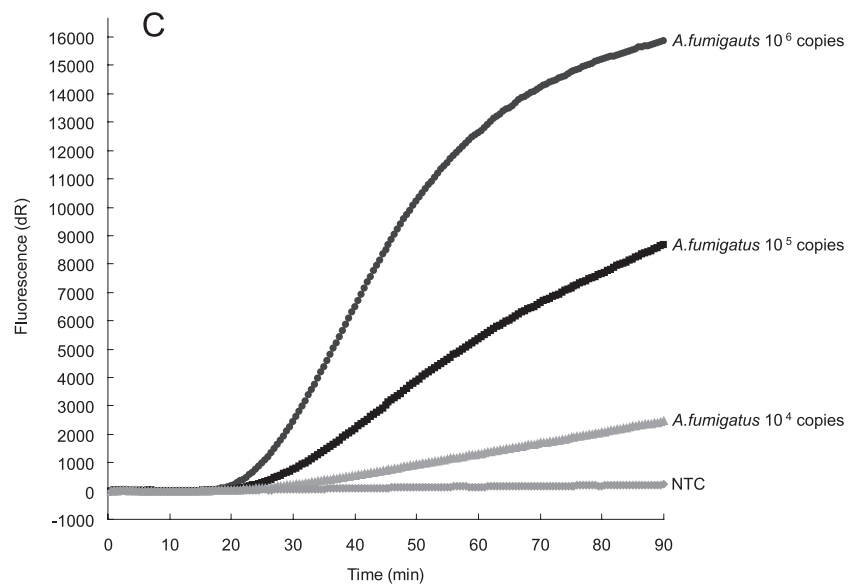
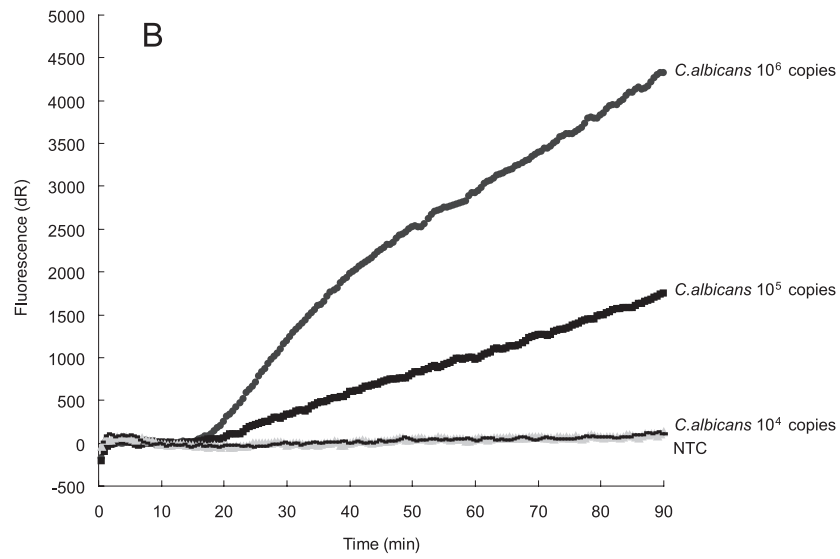
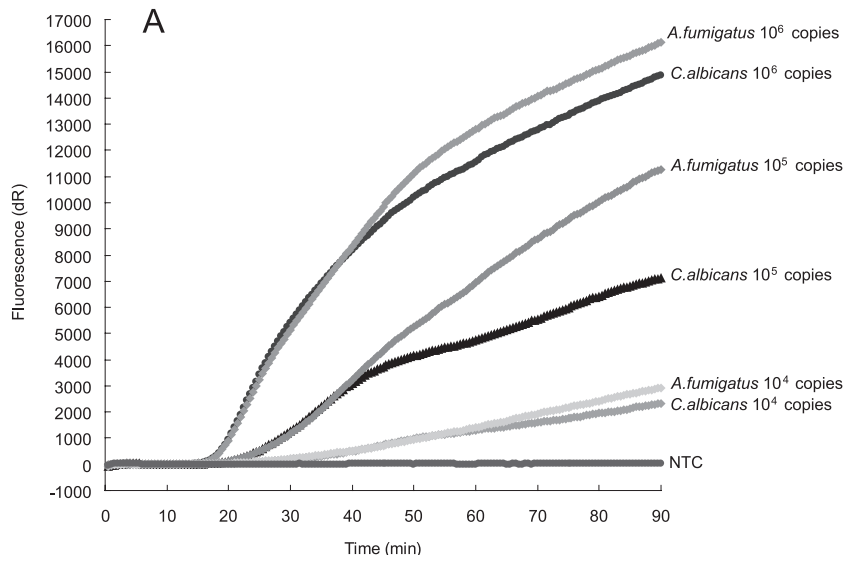
FIG. 1. Sensitivity of the multiplex real-time NASBA assay for bacteria. Sensitivity of pan-gram negative (FAM) (A) and pan-gram positive (HEX) (B) probes in multiplex format using a 10-fold dilution series of TNA isolated from cells of *A. baumannii* and *P. aeruginosa* (A) and *S. aureus* and *E. faecalis* (B). Oligonucleotides 16S GrP MB and Grm neg 16S MB and the primer set of PLK1, primer Grm neg 16S P2, and primer GP/GN P1 were used in this multiplex assay.

Youden's indices for pan-gram-positive and pan-gram-negative detection demonstrate the great accuracy of this multiplex assay. The positive predictive values (PPV) for pan-gram-positive and pan-gram-negative probes were 100 and 90.7%, respectively, and the corresponding negative predictive values (NPV) for these two probes were 91.8 and 99.4% (Table 6). The relatively low NPV for pan-gram-positive probe and low PPV for pan-gram-negative probe were due largely to the false identifications for three gram-positive organisms, *Corynebacterium* spp., *Propionibacterium acnes*, and *Micrococcus* sp., which counted for 13/14 samples falsely identified as gram-negative pathogens. However, with the addition of the patch probes, the NPV for the pan-gram positive assay was increased to 99.4%. Meanwhile, the fungal panel was observed to be highly reli-

able. Both pan-fungal and pan-*Candida* probes demonstrated 100% sensitivity, specificity, Youden's index, PPV, and NPV, and the pan-*Aspergillus* probe also showed a 100% negative predictability (Table 6). It should be noted that the high PPV needs to be viewed in context with the limited number of positive fungal cultures obtained in this study. Robust signals were observed for all probes in the multiplex panels, with signal detection in less than 15 min.

## DISCUSSION

Conventional diagnostic methods for BSIs require several days to complete, and both false-positive (15, 26, 34) and false-negative results (24, 40, 41) have been reported. Delayed





diagnosis correlates strongly with the high mortality of BSIs (19, 35, 49). Thus, there is an urgent need for a rapid and reliable diagnostic method that will allow clinicians to make faster and better-informed treatment choices for BSI patients. In this study, we describe a rapid, highly specific, and sensitive diagnostic platform for BSI, using NASBA and MB technology to perform real-time assays. Five broad-range MBs and the corresponding primer sets were developed and evaluated in this study. The primer/probe sets were able to rapidly and accurately identify most medically important BSI pathogens to the genus level. Furthermore, the simultaneous detection and characterization of the BSI pathogen at the subkingdom or genus level could be done by the two multiplex real-time NASBA detection panels presented in this study. Reliable pathogen identification and characterization can be obtained in under 3 h. This approach holds promise for the rapid diagnosis of BSIs.

As a powerful diagnostic tool, real-time NASBA has been used widely for detecting RNA viruses such as enterovirus and human immunodeficiency virus (27, 31, 39), certain microbial pathogens including *Legionella* species, *Vibrio cholerae*, etc. (18, 30), and pathogens from food and environmental samples (11, 36). However, only a few studies have reported using conventional NASBA to detect *Candida* spp. and *Aspergillus* spp. (7, 29, 46), and no real-time NASBA methods had been reported for BSIs. Conversely, during the past decade, many PCR-based assays were developed and dominated the methods used for the identification of microorganisms and the diagnosis of infections. Compared to PCR, a major advantage of NASBA is the fast amplification kinetics. It is an isothermal process, and the single-stranded RNA amplicons produced can be used directly for the subsequent rounds of amplification or probe detection without the need for denaturation or strand separation. When using the multicopy rRNA as the target, NASBA results in high sensitivity and allows for a faster identification of the pathogens.

The universal PCR-based detection of bacteria or fungi has been attempted because of its powerful potential to rapidly detect and identify a broad range of pathogens, but the clinical value of this testing still is debatable (8). The most significant limitation to date is the potential for DNA or RNA contamination from a variety of uncertain sources, including enzymes, reagents, nucleic acid extraction kits, columns, water, the environment, and so on. Attempts to decontaminate PCR materials (9, 13) are not entirely effective or reproducible. Some real-time PCR assays were not able to detect pathogens presenting at low levels without reporting false positives caused by low-level endogenous contamination. In our preliminary experiments, the availability of nucleic acid-free enzyme preparations and high-quality nucleic acid-free reagents has helped to eliminate the contamination problem. In this context, five broad-range real-time NASBA assays were developed to detect a wide variety of BSI pathogens. Several medically impor-

tant pathogens, including 9 bacterial species and 12 fungal species, were used to evaluate these real-time NASBA detections. All of these assays have great selectivity for their target organisms, and no cross-reaction was observed in tested species. The great diversity of bacterial species represented in the clinical study was extremely helpful in highlighting false-negative results for three gram-positive bacterial organisms, *Corynebacterium* sp., *Propionibacterium* sp., and *Micrococcus* sp., when tested using the original pan-gram-positive probe. With the exception of immune-compromised persons or persons with indwelling devices, these bacterial species generally do not cause invasive disease, can be found on human skin, and usually are present in blood cultures as a result of contamination during sample collection. However, since there are circumstances in which these organisms are clinically relevant, several new pan-gram-positive probes were designed and tested. The mixture of these new probes and the old pan-gram-positive probe then could easily detect all species. Similarly, the pan-gram-negative probe failed to identify *Prevotella* spp. from the blood cultures, although a patch probe was not designed against this genus.

The analytical sensitivity tests of the real-time NASBA assays were performed. All five probes were able to detect approximately  $10^3$  to  $10^4$  copies of artificial RNA targets in one NASBA reaction, as well as genomic RNA extracted from around 1 to 5 CFU of bacterial or fungal cultures, assuming 100% efficiency of the extraction. In comparison, the limit of broad-range bacterial detection was 10 to 100 CFU per reaction for *E. coli* and 100 to 1,000 CFU per reaction for *S. aureus* (20, 50) in regular PCR, and 1 to 10 CFU per reaction was observed in real-time PCR (20, 50). NASBA fungal assays showed limits of detection comparable to those of pan-fungal PCR assays (3, 43). The detection limits of pan-*Candida* and pan-*Aspergillus* assays are in agreement with the results reported by other studies that used traditional NASBA detection (electrochemiluminescence) for *Candida* spp. (28, 46) or *Aspergillus* spp. (29).

The clinical applicability of our real-time multiplex NASBA assays was evaluated by testing blinded blood cultures. To our knowledge, this is the first study to compare real-time NASBA assays with the capability to detect a wide range of potential pathogens to the classical cultural approach for the diagnosis of BSIs. Since this pilot study was conducted using samples derived from positive blood culture bottles, one might argue that there is little benefit to be obtained by using multiple molecular tests instead of a simple and inexpensive gram stain as the first diagnostic step. This is true for the majority of the samples tested in this pilot study. However, an example of a notable exception with a clear diagnostic benefit would be the identification of *Candida krusei*, since this yeast is intrinsically resistant to a first-line treatment drug, fluconazole. The ultimate goal in the development of such assays is to significantly reduce the time to initial pathogen detection, not to completely

FIG. 2. Sensitivity of the multiplex real-time NASBA assay for fungi. A 10-fold dilution series of in vitro 28S rRNA transcripts for *C. albicans* and *A. fumigatus* were used for the evaluation. Shown is the sensitivity, in multiplex format, of pan-fungal probe (CR610) (A), pan-*Candida* probe (FAM) (B), and pan-*Aspergillus* probe (Q670) (C). The same primer-MB sets for pan-fungal, pan-*Candida*, and pan-*Aspergillus* simplex detection (Table 3) were used in combination for this multiplex assay.

TABLE 6. Comparison of results from real-time multiplex NASBA detection and blood culture system

Probe type and NASBA ID <sup>c</sup>	No. with culture ID:						Sensitivity (%)	Specificity (%)	Youden's index <sup>a,t</sup>	PPV (%)	NPV (%)
	Gram <sup>+</sup>	Non-gram <sup>+</sup>	Gram <sup>-</sup>	Non-gram <sup>-</sup>	Fungal/ <i>Candida</i> spp.	Non-fungi/ non- <i>Candida</i> spp.					
Pan-gram positive (with patch pan-gram-positive MBs) <sup>d</sup>											
Gram <sup>+</sup>	348 (361)	0								100.00 (100.00)	
Non-gram <sup>+</sup>	14 (1)	156									91.76 (99.36)
Total	362	156					96.13 (99.72)	100.00 (100.00)	0.961 (0.997)		
Pan-gram negative											
Gram <sup>-</sup>			147	15							
Non-gram <sup>-</sup>			2	354						90.74	99.44
Total			149	369			98.66	95.93	0.946		
Pan-fungal/pan- <i>Candida</i> spp.											
Fungal/ <i>Candida</i> spp.					10	0				100.00	
Non-fungal/non- <i>Candida</i> spp.					0	260					100.00
Total					10	260	100.00	100.00	1		
Pan- <i>Aspergillus Aspergillus</i> spp.											
Non- <i>Aspergillus</i> spp.										NA <sup>b</sup>	100.00
Total					0	270	NA <sup>b</sup>	100.00	NA <sup>b</sup>		

<sup>a</sup> Youden's index is calculated as sensitivity plus specificity minus one.

<sup>b</sup> NA indicates that sensitivity, Youden's index, and PPV for the pan-*Aspergillus* spp. probe were not applicable because of no positive blood culture.

<sup>c</sup> ID, identification.

<sup>d</sup> Values obtained by using the mixture of the original pan-gram-positive probe and the patch probes are shown in parentheses when those values are different from those obtained by using the original pan-gram-positive probe only.

replace blood cultures. In the majority of cases, susceptibility studies still must be performed, and the detection of all pathogens must be guaranteed. With improved nucleic acid isolation and concentration methods, detection from directly drawn specimens may be possible. However, if this is shown not to be sufficiently sensitive, reducing the time to blood culture testing, i.e., sampling the bottle before it is flagged as positive, may be a viable alternative approach.

Although our testing was performed on positive blood cultures, in fact, our real-time NASBA assays demonstrated several advantages that could make it a valuable complement to traditional blood culture for the diagnosis of BSIs. First, the NASBA assays demonstrated a high predictability to rule out a BSI. The data also suggest that it can be used for accurate diagnosis when a pathogen is present. For example, one blood culture, reported as positive but from which no organism was grown, was positive by NASBA for a gram-negative organism and was confirmed by the isolation of *P. aeruginosa* from another blood culture bottle that became positive hours after the first blood bottle. The earlier detection of the gram-negative organism in the first bottle suggests that the increased sensitivity of the NASBA assay accurately identified a BSI in this patient much sooner than traditional culture methods. Second, the assays allow for a faster identification of the pathogen(s). This was demonstrated in particular by the blood culture that grew too poorly for timely identification using conventional test methods, yet the NASBA assays identified both a gram-positive and a gram-negative organism in the blood culture at the time the sample was flagged as positive. Third, in consideration of the high sensitivity of the assay, provided that pathogen nucleic acid extraction from the whole blood is sufficiently efficient, the assays could be used for detecting BSI pathogens from a direct blood draw without the need for a primary culture step. Studies to evaluate the potential of testing direct blood draws and blood culture bottles at various time points after inoculation but prior to instrument detection currently are in progress. Additionally, studies to include other blood culture bottle types and a more extensive testing of yeast and fungal isolates are indicated to comprehensively assess the performance of the assays. Finally, there is the possibility that amplified methods lead to an increase in false-positive results due to the detection of dead organisms or low-level contaminants. In such cases, as we currently must do with routine blood cultures, the information must be evaluated in the clinical context of the patient's symptoms, disease, and underlying comorbidities.

Wisplinghoff and colleagues reported that polymicrobial infection constituted 13% of all episodes of nosocomial BSIs in the United States (47), which is associated with higher mortality than monomicrobial BSI and tends to be more resistant to empirical therapy. The early diagnosis of polymicrobial infection is valuable to the patient's management, because the adjustment of empirical treatments often were required due to polymicrobial episodes (5). With the capability to simultaneously detect multiple targets, multiplex real-time NASBA could be not only more suitable to meet the need of rapidly recognizing polymicrobial infections but also more efficient and cost-effective than a simplex assay. In fact, the NASBA assays detected several gram-positive/gram-negative mixed infections, including CoNS with *Corynebacterium* spp., CoNS

with *E. coli* and *P. stuartii*, *E. faecalis* with *P. mirabilis*, CoNS with *E. cloacae* and *A. baumannii*, and CoNS with *Alcaligenes* sp. These data suggest that the multiplex assays are highly suitable for the primary screening of BSI pathogens.

In conclusion, this study demonstrates the feasibility of using an NASBA-MB approach as a broad-range rapid diagnostic method suitable for detecting BSIs.

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