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Rapid Identification of Bacterial Pathogens in Positive Blood Culture Bottles by Use of a Broad-Based PCR Assay Coupled with High-Resolution Melt Analysis▽

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We evaluated a broad-based PCR assay coupled with high-resolution melt analysis for rapid bacterial identification in patients with bacterial sepsis. With a reference library of 60 clinically relevant bacterial species, 52 positive blood culture samples were tested. Our assay identified 46/52 samples at the species level, with 100% concordance to culture findings.

Bacterial sepsis is a significant cause of morbidity and mortality, with an estimated 20 million cases worldwide each year and up to 215,000 registered deaths per year in the United States alone (2, 8, 11). Early diagnosis of the pathogens causing bacteremia and sepsis is particularly challenging in clinical settings due to the lack of rapid and specific diagnostic assays. As a consequence, this often leads to the conservative approach of prescribing broad-spectrum empirical antibiotics. Previous studies reported lower mortality rates for patients given appropriate targeted antibiotics versus those treated with a broad-spectrum therapy (9). Thus, for patients with suspected bacterial sepsis, especially the immunocompromised, early identification of the causative bacterial agent is crucial for directing appropriate antimicrobial therapy and improving patient care and associated outcomes (4).

Detection of the etiologic agent is currently accomplished by a multistep, time-intensive process involving procurement of blood cultures and incubation of the bottles, followed by Gram staining and subculturing of the bottles when positive, and finally detailed biochemical analyses of the recovered organisms. The complete process typically requires 48 h (and up to 72 h for fastidious organisms), during which time clinicians receive incremental information regarding culture positivity, microscopic description, speciation, and finally antimicrobial susceptibility results (7). Further, false-negative results based on conventional methods may occur in up to 30% of sepsis cases, especially for fastidious organisms or in samples obtained after antimicrobial therapy has been started (1, 3, 12). Molecular techniques to detect microbial nucleic acids have been adapted to identify a wide range of bacterial pathogens found in sterile body fluids in efforts to circumvent the technical shortcomings of current culture-based methods. Unlike culture methods, such molecular assays do not require bacterial growth for detection and identification. While methods such as peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) and real-time nucleic acid amplification methods are currently available for rapid identification of organisms directly from a positive blood culture bottle, they are pathogen-specific assays and are costly to perform.

Nucleic acid amplification methods that target the bacteria-specific 16S rRNA gene can offer many advantages; the highly conserved sequences of the gene allow broad-based detection of almost any bacterial species, while the hypervariable sequences can be exploited for species-level identification (12). We have previously combined 16S rRNA PCR with high-resolution melt analysis (HRMA) for broad-range species identification. HRMA is not only simple, fast, and easy to integrate with PCR, but also it has the capacity to discriminate single-nucleotide differences between closely related species (10). Our objective was to evaluate the performance of a broad-based PCR-HRMA assay in identifying common bacterial pathogens in positive blood culture bottles.

A mixture of 60 clinical and reference strains of bacterial species representing a broad range of microbial pathogens, including the 18 most frequent causes of bloodstream infections among our patient population, were used as reference organisms for analysis (Table 1) (5). All organisms were subcultured onto Trypticase soy agar with 5% sheep blood and incubated at 37°C for 24 h. Ten to 15 colonies of each bacterial organism were inoculated into 200 μl of molecular-grade water prior to DNA extraction. Negative blood culture bottles were used as standard negative controls.

The Clinical Microbiology laboratory at the Johns Hopkins Hospital (JHH) uses the BacT/Alert 3D blood culture system (bioMerieux, Durham, NC). The bottles in use contain the standard aerobic and standard anaerobic media. None of the bottles tested contained charcoal. A convenience sample consisting of 52 positive blood culture bottles (28 aerobic and 24 anaerobic) collected from 36 unique patients presenting to the JHH from July 2009 to August 2009 with suspected blood-

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TABLE 1. Melt library of common infectious pathogens

Pathogen	Grouping code of analysis subset[b]			Signature code[c]	Pathogen	Grouping code of analysis subset[b]			Signature code[c]
	V1	V3	V6			V1	V3	V6	
Acinetobacter sp. ATCC 5459	e	a	a	eaa	*Mycobacterium fortuitum*	m	l	e	mle
Aerococcus viridians	h	e	n	hen	*Mycoplasma pneumoniae*[a]	b	m	i	bmi
Bacteroides fragilis[a]	g	f	c	gfc	*Mycoplasma hominis*[a]	j	c	k	jck
Bordetella pertussis	j	d	f	jdf	*Neisseria meningitidis* ATCC 6250	a	d	l	adl
Bordetella parapertussis	l	i	c	lic	*Neisseria gonorrhoeae*[a]	g	i	l	gil
Campylobacter jejunii	c	j	l	cjl	*Oligella urethralis*	i	h	l	ihl
Cornyebacterium sp.[a]	a	j	a	aja	*Pasteurella multocida*	a	e	l	ael
Chlamydia pneumoniae[a]	g	a	b	gab	*Pseudomonas aeruginosa* ATCC 10145	f	c	l	fcl
Chlamydia trachomatis[a]	m	n	n	mnn	*Propionibacterium acnes*	f	j	l	fjl
Citrobacter freundii[a]	a	b	m	abm	*Proteus mirabilis*[a]	a	b	c	abc
Clostridium difficile	h	d	a	hda	*Proteus vulgaris*[a]	j	g	m	jgm
Clostridium perfringens	d	b	b	dbb	*Salmonella* sp. ATCC 31194	c	g	m	cgm
Enterobacter aerogenes	c	b	e	cbe	*Serratia marscecens* ATCC 8101	a	d	a	ada
Enterobacter cloacae[a]	e	d	j	edj	*Staphylococcus aureus* ATCC 25923	h	e	g	heg
Enterococcus casseliflavus[a]	e	c	c	ecc	*Staphylococcus epidermidis* ATCC 12228	n	e	a	nea
Enterococcus faecium	j	c	d	jcd	*Staphylococcus caprae*[a]	d	e	a	dea
Enterococcus faecalis ATCC 29212	c	h	a	cha	*Staphylococcus haemolyticus*[a]	b	e	b	beb
Enterococcus gallinarum	j	k	l	jkl	*Staphylococcus hominis*[a]	b	a	b	bab
Enterococcus raffinosus[a]	a	c	d	acd	*Staphylococcus lugdunensis*	d	a	e	dae
Escherichia coli ATCC 25927	a	g	d	agd	*Staphylococcus sapropyticus*[a]	b	b	b	bbb
Helicobacter pylori[a]	g	b	f	gbf	*Streptococcus pneumoniae* ATCC 49619	b	e	a	bea
Haemophilus influenzae ATCC 49247	a	e	b	aeb	*Streptococcus pyogenes*[a]	m	a	d	mad
Klebsiella pneumoniae[a]	e	i	d	eid	*Streptococcus agalactiae* ATCC 13813	m	c	c	mcc
Legionella pneumophila ATCC 33495	c	d	a	cda	*Streptococcus mitis* ATCC 49456	d	a	c	dac
Listeria monocytogenes ATCC 7648	g	c	f	gcf	*Streptococcus mutans* ATCC 25175	d	b	i	dbi
Micrococcus luteus[a]	g	i	h	gih	*Streptococcus salivarius* ATCC 13419	g	d	b	gdb
Morganella morganii[a]	j	h	j	jhj	*Streptococcus sanguis* ATCC 10556	g	e	i	gei
Moraxella catarrhalis	d	h	a	dha	*Streptococcus oralis* ATCC 10557	d	b	c	dbc
Mycobacterium kansasii	g	j	h	gjh	*Streptococcus gordonii* ATCC 10558	k	b	c	kbc
Mycobacterium gordonae	k	b	b	kbb	*Treponema pallidum*[a]	c	d	l	cdl

[a] Clinical isolate.
[b] Difference plots generated for each organism were grouped based on curve similarity within each analysis subset (V1, V3, and V6), and the corresponding unique letter code was assigned to each group with a distinct curve shape.
[c] Combined grouping code letters of the three analysis subsets.

stream infections were obtained from the hospital microbiology laboratory after standard blood culture testing was completed. Samples were deidentified for research purposes. The study was approved by The Johns Hopkins University Institutional Review Board.

The extraction of DNA from culture colonies was performed using a Roche MagNA Pure instrument (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. For the isolation of bacterial DNA from clinical blood culture bottles, a 500-μl aliquot drawn from the blood culture bottle was treated with 50 μl of lysis buffer (MagNA Pure LC Kit-I; Roche Diagnostics) and incubated at room temperature for 20 min. The specimen was centrifuged at 4,000 × *g* for 10 min, and the cell pellet was resuspended in 100 μl molecular-grade water. The specimen was then treated with 130 μl of lysis buffer (MagNA Pure LC Kit-III; Roche Diagnostics), 20 μl of lysing enzyme mixture (10 μl of 0.8 μg/μl lysozyme and 10 μl of 1.25 μg/μl lysostaphin; Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 20 min. The specimen was then treated with 20 μl of 1× proteinase K (MagNA Pure LC Kit-I; Roche Diagnostics) and incubated at 65°C for 10 min. Subsequently, the specimen was subjected to a brief freeze-thaw at −80°C for 10 min and 95°C for 5 min and then sonicated for 10 min (T9000; Brasonic, Shelton, CT). The processed samples then underwent a final DNA extraction using the Roche MagNA Pure instrument (Roche Diagnostics) in accordance with the manufacturer's instructions.

Three conserved primers (V1-F, V1-R, V3-F, V3-R, V6-F, and V6-R) flanking three hypervariable regions (V1, V3, and V6) within the 16S rRNA gene were designed and used for PCR amplification as previously described (25). Extracted DNA from each bacterial organism or clinical sample was subjected to three PCR amplifications, each targeting the three hypervariable regions. The PCR setup and cycling conditions were as previously described (25).

Each post-PCR sample amplicon was subjected to HRMA on the LightScanner instrument (Idaho Technology). Melting temperatures ranged from 60°C to 95°C. Fluorescence data acquisition was obtained for every 0.1°C increase in temperature. Each PCR sample was examined in triplicate for HRMA and analyzed using the Light Scanner software version 2.0 (Idaho Technology). Melting curves generated from HRMA were analyzed as previously described (10). Briefly, derivative plots were generated to assess the number of melting peaks. Analysis subsets (V1, V3, and V6) were defined by the primer sets used. "Autogrouping" was performed on the difference plots to group all positive samples with similar curve shapes within the same analysis subset. A unique letter code was assigned for each group identified, starting with the letter a and progressing alphabetically. A combination of each letter from

TABLE 2. Identification of 52 blood culture bottle samples by PCR-HRMA and conventional blood culture methods

Conventional culture identification (no. of isolates)	Grouping code of analysis subsets			Signature code	PCR-HRMA identification (no. of isolates)	Analysis result for discordant samples (based on reculturing/ sequencing)
	V1	V3	V6			
Concordant samples						
S. aureus (10)	h	e	g	heg	*S. aureus* (10)	
CoNS (9)	n	e	a	nea	*S. epidermidis* (4)	
	b	e	b	beb	*S. haemolyticus* (2)	
	d	a	e	dae	*S. lugdunensis* (1)	
	b	a	b	bab	*S. hominis* (1)	
S. agalactiae (5)	m	c	c	mcc	*S. agalactiae* (5)	
MRSA[a] (3)	h	e	g	heg	*S. aureus* (3)	
P. aeruginosa (3)	f	c	l	fcl	*P. aeruginosa* (3)	
H. influenzae (3)	a	e	b	aeb	*H. influenzae* (3)	
E. coli (2)	a	g	d	agd	*E. coli* (2)	
K. pneumoniae (2)	e	i	d	eid	*K. pneumoniae* (2)	
M. morganii (2)	j	h	j	jhj	*M. morganii* (2)	
M. luteus (2)	g	i	h	gih	*M. luteus* (2)	
S. epidermidis (1)	n	e	a	nea	*S. epidermidis* (1)	
E. faecalis (1)	c	h	a	cha	*E. faecalis* (1)	
E. aerogenes (1)	c	b	e	cbe	*E. aerogenes* (1)	
Enterobacter sp. (1)	c	b	e	cbe	*E. aerogenes* (1)	
S. pyogenes (1)	m	a	d	mad	*S. pyogenes* (1)	
Discordant samples						
F. varium (1) (unidentified)[b]						*Staphylococcus xylosus*
CoNS (1) (unidentified)[b]						Mixed β *Streptococcus* species
S. pyogenes (2) (polymicrobial)[c]						α *Streptococcus* species, *Enterobacteriaceae* species
Viridans streptococci (2) (polymicrobial)[c]						α *Streptococcus* species, *Enterobacteriaceae* species

[a] MRSA, methicillin-resistant *S. aureus*.
[b] A single melt curve was generated for each analysis subset but was not associated with a pathogen within our reference organism library.
[c] Multiple melt curve peaks indicated polymicrobial infection.

each of the variable regions was accumulated to provide a signature code for each organism. A reference library based on the signature codes of 60 reference organisms was established accordingly for use in pathogen identification (Table 1).

Fifty-two positive blood culture bottles were tested using our PCR-HRMA assay, and melting curve data generated from each of the samples were compared to our reference library for pathogen identification. Fifty of 52 (96.2%) positive blood culture samples contained a pathogen included in our library. Our assay results were concordant with original blood culture findings in 46/52 (88.5%) samples, with detailed species identification (Table 2). In 4/6 discordant samples, HRMA generated multiple melting peaks in their derivative plots, suggesting the presence of multiple pathogens. Further microbiologic analysis of these blood culture samples (reculturing and sequencing) confirmed polymicrobial growth in all four samples (Table 2). The other 2/6 discordant samples were not identifiable by our HRMA analysis. These samples were positive for *Fusobacterium varium* and coagulase-negative staphylococci (CoNS) by conventional blood culture methods. Both samples generated a single melting curve in HRMA but were not associated with any specific pathogens in our reference library. The unidentified CoNS sample was recultured and sequenced for definitive discrepant analysis, and the isolate was confirmed as *Staphylococcus xylosus*. Total PCR-HRMA assay time was approximately 3 h (2 h for DNA extraction, 1 h for PCR amplification, and 5 min for HRMA).

In this study, we developed a strategy combining traditional blood cultures with broad-based PCR and HRMA for rapid identification of bacterial pathogens. Discrete pathogen identification based on our reference panel of 60 bacterial organisms was obtained in 88.5% of the positive blood culture samples tested, but more importantly, 100% of these PCR-HRMA identifications were concordant with the culture results at the species level. Due to the ease of integrating HRMA with PCR, our approach offers a simple work flow with an assay time of 3 h without laborious post-PCR procedures (i.e., sequencing, microarray analysis, and mass spectrometry). However, the actual turnaround time to results when integrated into the workflow of a clinical laboratory may be longer. The use of sophisticated instruments (i.e., a SmartCycler for faster thermal cycling) for automated back-to-back testing may offer more real-time results than batched testing. Given that traditional identification methods based on biochemical analyses typically require a minimum of 26 h after initial culture positivity (6), our approach may provide results ~23 h earlier than the standard methods.

One of the unique features of our assay platform is the potential range of identifiable pathogens. We have included in our reference panel not only the top 20 (or 95% of) organisms

commonly recovered from blood cultures but also other clinically relevant organisms found in other body sites for future testing (5). The total number of organisms identifiable can be easily expanded (5). In our prior study, we demonstrated that identical signature codes were shared among various strains of the same species (10). Moreover, nonmatching signature codes generated from a positive amplification reaction can either represent the presence of polymicrobial infection if multiple peaks are observed in the melt curves or signify the presence of an uncommon, mutant, or emerging pathogen if a single dominant peak is seen. A similar strategy can also be adapted for fungal detection, and such work is under way. Of note, signature letter codes assigned to each organism may change if a different panel of reference organisms is selected (which explains the differences in the signature codes for the same organisms listed in Table 1 versus those listed in our previous publication [10]).

Our PCR-HRMA assay also demonstrated superior discriminating power over conventional methods for speciation. Among the 52 samples tested, conventional methods were only able to identify the organisms at the genus level in 11 samples (i.e., 10 CoNS and 1 *Enterobacter*). Our PCR-HRMA assay was able to further differentiate 9 of 10 CoNS isolates at the species level and 1 isolate of *Enterobacter* as *Enterobacter aerogenes* (Table 2). Although most CoNS are regarded as contaminants, the ability to discriminate CoNS species may help ascertain their clinical relevancy.

One of the current limitations of PCR-HRMA is its inability to resolve polymicrobial infections. Although the number of dominant peaks in the derivative melting curve may suggest the number of etiologic agents involved, the ability to identify the individual organisms is still lacking (10). Nonetheless, in our study, four discrepant samples with initial monomicrobial growth by culture were found to have multiple organisms by PCR-HRMA, which was confirmed by reculturing and sequencing. These findings suggest either subsequent contamination or a potential advantage of PCR-HRMA over conventional methods for determining the presence of polymicrobial infections.

Knowing the species of a pathogen at an earlier time point may offer significant clinical benefit. The information would be useful in de-escalating antibiotic coverage based on institutional susceptibility patterns to reduce emergence of drug-resistant pathogens, prompt an escalation in antibiotic use (for example, adding colistin for *Acinetobacter* or double-covering for *Pseudomonas*), or allow clinicians to disregard the report of a positive culture altogether in the case of identification of a common contaminant. Although PCR detection directly from whole blood without culturing would offer even more drastic time saving, the technical challenges in achieving adequate detection sensitivity still exist. Currently under way is an examination of an alternative approach to enrich the bacterial concentration in whole blood with brief culturing (2 to 3 h) prior to PCR-HRMA. Given that PCR-HRMA does not provide antibiotic susceptibility information, however, this method should not replace but rather offer an adjunctive role to standard culture methods.

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