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Direct Screening of Blood by PCR and Pyrosequencing for a 16S rRNA Gene Target from Emergency Department and Intensive Care Unit Patients Being Evaluated for Bloodstream Infection

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Here we compared the results of PCR/pyrosequencing to those of culture for detecting bacteria directly from blood. DNA was extracted from 1,130 blood samples from 913 patients suspected of bacteremia (enrollment criteria were physician-ordered blood culture and complete blood count [CBC]), and 102 controls (healthy blood donors). Real-time PCR assays for beta-globin and Universal 16S rRNA gene targets were performed on all 1,232 extracts. Specimens identified by Universal 16S rRNA gene PCR/pyrosequencing as containing staphylococci, streptococci, or enteric Gram-negative rods had target-specific PCR/pyrosequencing performed. Amplifiable beta-globin (melting temperature [T_m], 87.2°C ± 0.2°C) occurred in 99.1% (1,120/1,130) of patient extracts and 100% (102/102) of controls. Concordance between PCR/pyrosequencing and culture was 96.9% (1,085/1,120) for Universal 16S rRNA gene targets, with positivity rates of 9.4% (105/1,120) and 11.3% (126/1,120), respectively. Bacteria cultured included staphylococci (59/126, 46.8%), Gram-negative rods (34/126, 27%), streptococci (32/126, 25.4%), and a Gram-positive rod (1/126, 0.8%). All controls screened negative by PCR/pyrosequencing. Clinical performance characteristics (95% confidence interval [CI]) for Universal 16S rRNA gene PCR/pyrosequencing included sensitivity of 77.8% (69.5 to 84.7), specificity of 99.3% (98.6 to 99.7), positive predictive value (PPV) of 93.3% (86.8 to 97.3), and negative predictive value (NPV) of 97.2% (96.0 to 98.2). Bacteria were accurately identified in 77.8% (98/126) of culture-confirmed sepsis samples with Universal 16S PCR/pyrosequencing and in 76.4% (96/126) with follow-up target-specific PCR/pyrosequencing. The initial PCR/pyrosequencing took ~5.5 h to complete or ~7.5 h when including target-specific PCR/pyrosequencing compared to 27.9 ± 13.6 h for Gram stain or 81.6 ± 24.0 h for phenotypic identification. In summary, this molecular approach detected the causative bacteria in over three-quarters of all culture-confirmed cases of bacteremia directly from blood in significantly less time than standard culture but cannot be used to rule out infection.

For decades, automated blood culturing with constant monitoring for growth of microorganisms has served as the gold standard, albeit imperfect, in the clinical microbiology laboratory for diagnosing bloodstream infections (BSI). Factors impacting blood culture's sensitivity include the number of blood culture sets drawn and the volume of blood collected per bottle (1). More recently, several molecular approaches, including both nucleic acid amplification tests and non-amplification-based tests like fluorescence *in situ* hybridization using peptide nucleic acid probes, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF), and microarrays have been implemented to more rapidly identify microorganism(s) (2). Some of these newer approaches, like the Verigene Gram-positive and Gram-negative tests (Nanosphere Inc., Northbrook, IL) and the BioFire Film Array assays (BioFire Diagnostics LLC, Salt Lake City, UT), also incorporate screening for antimicrobial resistance genes, e.g., *mecA*, *vanA*, and *vanB* for Gram-positive bacteria and several *bla* gene targets associated with Gram-negative bacteria (3–5).

However, all of the approaches mentioned above have, as their starting sample, fluid from positive broth cultures, so the amount of time saved in detecting and identifying the microorganisms compared to culture is relevant only to the time after which the instrument detects growth. In contrast, in this study we describe using real-time PCR/pyrosequencing with blood directly, reducing the time needed to detect and identify bacterial pathogens relative to the time of collection.

Currently, the literature describing screening of blood directly for microorganisms is limited. The reason is that this approach is much more demanding, requiring lower limits of detection and the use of molecular-grade reagents to minimize contamination when amplifying highly conserved targets like 16S rRNA genes. The SeptiFast assay from Roche Molecular Diagnostics (Branchburg, NJ) is the most common platform evaluated to date; this real-time multiplex assay specifically screens for 19 bacterial and 6 fungal pathogens. In a recent meta-analysis of 43 studies, the sensitivity and specificity of the SeptiFast assay compared to culture

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are 0.68 (95% confidence interval [CI], 0.63 to 0.73) and 0.86 (95% CI, 0.84 to 0.89), respectively (6). The authors conclude that firm recommendations cannot be made about the clinical utility of the SeptiFast assay in the setting of sepsis. In a second meta-analysis of 34 studies enrolling 6,012 participants, the overall sensitivity and specificity of the SeptiFast assay are 0.75 (95% CI, 0.65 to 0.83) and 0.92 (95% CI, 0.90 to 0.95), respectively (7). Here the authors conclude that the SeptiFast assay does provide valuable information for early detection of sepsis.

The MagicPlex Sepsis test (MST) (Seegene Inc., Concord, CA) is another real-time multiplex PCR assay that analyzes blood samples directly. This test detects 73 Gram-positive bacteria along with *vanA*, *vanB*, and *mecA* resistance markers, 12 Gram-negative bacteria, and 6 fungi. Ljungström et al. (8) screened 382 suspected cases of sepsis and demonstrated moderate agreement (kappa, 0.50) between culture and the MST assay. The authors conclude that the strength of this approach is the timely detection of microorganisms, when results are positive, compared to culture. Likewise, Carrara et al. (9) assessed MST compared to culture among 267 patients in the intensive care unit (ICU), emergency department (ED), or hematology department and also found moderate agreement (kappa, 0.45) between the two methods, concluding that while specificity is high, improvements in design are needed to increase the sensitivity of the assay.

Another technology that screens blood directly for bloodstream infections is the Abbott IRIDCA technology, which uses PCR electrospray ionization mass spectrometry (PCR/ESI-MS). In a recent study, the PCR/ESI-MS demonstrated 83% sensitivity and 94% specificity compared to culture, with a total time to results of 5.9 h (10). Lastly, the Molzyme SepsiTest kit, which screens whole blood directly using broad-spectrum primers, provides results in 4 h. The only information on the performance of this kit is the manufacturer's stated sensitivity of 87% ($n = 187$). To date, none of these technologies that screen whole blood directly are currently available for use in the United States.

In this prospective, observational research study, we evaluated a real-time PCR/pyrosequencing approach to screen blood samples directly from patients being evaluated for BSI in the ICU or ED and compared to the results of standard automated blood culturing with phenotypic identification. Assay performance characteristics were calculated, and discordant testing was performed. Additionally, the theoretical times needed to generate these PCR/pyrosequencing results were compared to the actual time it took to report the Gram stain results and to enter the final phenotypic identification into the hospital's laboratory information system (LIS).

MATERIALS AND METHODS

Participant demographics. Of the total number of participants, 49% were recruited through an ED admission, and 51% through one of three adult ICUs. Fifty-six percent of the participants were male, and 58% of the total self-reported as black.

Enrollment. Enrollment occurred between July 2009 and September 2012, with 918 eligible and consented subjects participating in this observational study. A total of 1,130 blood samples were drawn from 918 subjects for testing; multiple blood samples drawn from the same participant represented different hospital admission dates. To be eligible, study participants needed to be ≥18 years and have physician-ordered blood cultures and complete blood count (CBC) drawn. Potential participants were ineligible if they declined to participate or if a blood sample could not be obtained.

The control group consisted of 102 healthy donors attending a blood center. One-milliliter volumes of EDTA-containing blood samples collected from these donors were shipped overnight at 4°C to the laboratory. The next day, DNA was extracted from 500 μl of each sample and screened for bacterial 16S rRNA gene target by real-time PCR/pyrosequencing. No blood culturing was performed on specimens from the control group. Because these specimens were discarded, deidentified blood samples, the research done with them was considered not to be research involving human subjects. The George Washington University (GWU) and Medical Center, Office of Human Research, approved this study.

Automated blood culturing and phenotypic identification. Blood was collected either by venous or by arterial draw or from an existing line, with 8 to 10 ml inoculated directly into each pair of physician-ordered pairs of blood culture bottles (aerobic bottle, catalog no. 442192; anaerobic bottle, catalog no. 442191; Becton Dickinson [BD], Franklin Lakes, NJ), which were placed into the Bactec 9240 instrument (BD) within 1 h from the time of receipt into the clinical laboratory. Bottles with detectable growth had fluid removed for Gram staining. Based on the Gram stain result, fluid from the positive blood culture bottle(s) was removed and subcultured onto Trypticase soy agar with 5% sheep blood, chocolate agar, Columbia CNA agar with 5% sheep blood, and/or MacConkey agar (BBL prepared media; BD). Phenotypic identification of cultured isolates was performed using the Vitek 2 instrument (bioMérieux, Durham, NC). Bottles without detectable growth were discarded after 5 days of incubation in the Bactec 9240 and reported out as having no growth. For the purposes of this evaluation, blood cultures positive for yeast or fungi were excluded.

Blood collection for direct molecular testing. One 8- to 10-ml K_2EDTA-containing Vacutainer tube (catalog no. 366643; BD) was drawn concurrently with that drawn for culturing and CBC testing. Blood samples were drawn either by venous or arterial draw or from an existing line. These research samples were transported to the clinical microbiology lab along with the physician-ordered blood culture bottles and stored at ambient temperature while awaiting pickup by research personnel. Research samples were processed either the day they were drawn or the following morning.

DNA extraction from blood samples. DNA was extracted from blood using a bead-based lysis technique. Five hundred microliters of EDTA-blood was added to 10 ml room temperature (RT) red blood cell lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, and 0.1% Triton X-100), mixed, and centrifuged at 6,000 relative centrifugal force (RCF) for 5 min at 4°C. The resulting supernatant was removed, and 100 μl of lysis buffer (5 mM guanidine-HCl, 100 mM Tris-HCl [pH 8.0]) was added to the pellet and vortexed. The resuspended cell pellet was transferred to another 2.0-ml tube containing 0.24 g of 0.1-mm zirconium silica beads (catalog no. 11079110zx; Biospec, Bartlesville, OK). Bead-based lysis of nucleated cells occurred by placing the 2.0-ml tube on a carrier (catalog no. 13000 V1; Mo Bio Laboratories, Inc., Carlsbad, CA) adapted to fit a vortexer and vortexed at high speed for 5 min at RT. After a brief spin in a microcentrifuge, 400 μl DNA-free water and 800 μl 99% benzyl alcohol were added to the tube containing beads and cell lysate, and the mixture was vortexed and left to sit undisturbed for 5 min at RT before centrifuging the tube for 5 min at 5,000 RCF. After centrifugation, the aqueous layer and any interface layer were retrieved and reextracted as above. The resulting aqueous layer was collected and volume estimated, so that 1/10 volume of 3 M sodium acetate (NaOAc), an equal volume of 99% isopropyl alcohol, and 1 μl of molecular-grade glycogen (catalog no. R0561; Life Technologies, Thermo Scientific, Pittsburgh, PA) were added, and the tube was vortexed. The tube was centrifuged at 13,000 RCF for 15 min at 4°C. The supernatant was removed and the resulting pellet washed in 100 μl 70% ethanol. The tube was recentrifuged at 13,000 RCF for 5 min at 4°C before the ethanol wash was removed, and the pellet was left to air dry for 15 min at RT. The final pellet was dissolved in 50 μl of 1× Tris-EDTA (TE) buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The

TABLE 1 Primer sequences used for analysis of bacterial rRNA genes[a]

Organism group	rRNA gene target	Real-time PCR primer set[a] (5′–3′)	Pyrosequencing primer(s)[b] (5′–3′)
Staphylococcus	16S	F^B, TGCCTAATACATGCAAGTCGAGCG R, GTTGCCTTGGTAAGCCGTTACCTT	GTGTTACTCACCCGTCCGCCGCTA
Streptococcus	23S	F^B, GCCTTTTGTAGAATGAACCGGCGA R, CGTTTGGAATTTCTCCGCTACCCA	TCACATGGTTTCGGGTCTA
Enteric	23S	F^1, CTAAGGCGAGGCCGAAAG F^2, CTAAGGCGAGGCTGAAAAG R^B, CTACCTGACCACCTGTGTCG	S^1, GGTTGTCCCGGTTTA S^2, GGTCGTCCCGGTTCA
Universal	16S	F^B, AACTGGAGGAAGGTGGGGAT R, AGGAGGTGATCCAACCGCA	TACAAGGCCCGGGAACGTATTCA

[a] F^B, biotinylated forward primer; R, reverse primer; R^B, biotinylated reverse primer; F^1 and F^2, degenerate forward primers used in combination.
[b] S^1 and S^2, degenerate pyrosequencing primers used in combination.

resulting DNA extract was stored at −20°C until analyzed by real-time PCR/pyrosequencing.

Beta-globin target amplification by real-time PCR. As a control measure to assess sample quality and adequacy, all DNA extracts were tested by PCR for a previously described 268-bp fragment of the human beta-globin gene, followed by melt curve analysis (11). Only those DNA extracts testing positive for the beta-globin target were further screened by real-time PCR for bacterial target(s).

Universal 16S rRNA gene target amplification by real-time PCR. A 15-bp region within the 380-bp amplicon generated using the Universal 16S rRNA gene primers can differentiate diverse groups of bacteria (12). Each 25-μl PCR master mix contained 5 μl of DNA extract, 10 μl of a 2.5× customized PCR master mix (catalog no. S-026-0250; Molzym GmbH & Co.KG, Bremen, Germany; final concentration, 3 mM $MgCl_2$), 2.5 μl 10× SYBR staining solution, 0.8 μl MolTaq 16S DNA polymerase, 5.7 μl DNA-free PCR grade water, and 0.5 μl each of the previously described Universal 16S rRNA gene primer pairs (10 μM) (Table 1) (12). Real-time PCR (95°C for 60 s followed by 40 cycles of 95°C for 20 s, 60°C for 60 s, and 72°C for 15 s) was carried out using a SmartCycler 2.0 (Cepheid Inc., Sunnyvale, CA).

Target-specific 16S rRNA gene or 23S rRNA gene amplification by real-time PCR. Samples positive for the Universal 16S rRNA gene target whose sequences were determined to be that of a *Staphylococcus* sp., *Streptococcus* sp., or enteric Gram-negative rod were further analyzed using a second set of primers as previously described to generate a more specific identification (13, 14). For these assays, each 25-μl PCR master mix contained 5 μl of DNA extract, 12.5 μl 2× SYBR Premix *Ex Taq* (catalog no. RR420A; TaKaRa Bio Inc., Shiga, Japan), 6.5 μl DNA-free PCR grade water, and 0.5 μl of each primer pair. Real-time PCR (95°C for 60 s followed by 40 cycles of 95°C for 20 s, 60°C for 60 s, and 72°C for 15 s) was carried out using a SmartCycler 2.0 (Cepheid Inc., Sunnyvale, CA).

Pyrosequencing of biotin-labeled PCR amplicons. The entire 25 μl of the biotinylated PCR mixture was sequenced using the PyroMark ID and PyroMark Q96 reagents (catalog no. 972804; Qiagen, Gaithersburg, MD) with the corresponding target-specific sequencing primer(s) as previously described (12–14). Using PyroMark Identifire v1.05.0 software, the identity of the sequences was generated, within seconds, after comparison with our extensive reference library, which includes validated sequences from previously identified clinical bacterial isolates, ATCC reference strains, and those found in NCBI GenBank using the BLAST algorithm. One hundred percent identity was required to make a genus or species level identification.

Calculating the time needed to communicate the Gram stain result or to enter the phenotypic identification into the LIS in cases of culture-confirmed bloodstream infection. Gram staining of culture fluid from newly detected blood culture bottles is routinely performed on all shifts. At GWU hospital, the clinical laboratory technologist calling the Gram stain result to the hospital unit documents both date and time in the hospital's LIS. The actual time interval, in hours, between when the blood was drawn for culture and when the Gram stain result was reported was calculated. This actual time was then compared to the theoretical time needed to obtain the Universal PCR/pyrosequencing result.

Similarly, the time interval, in days, between when the blood was drawn and when the final phenotypic identification was entered into the hospital's LIS was also determined; here the LIS records only the calendar date and not the exact time that the information was entered. The number of whole days required was then converted into hours and compared to the theoretical time in hours that was required to perform both Universal 16S rRNA gene and target-specific PCR/pyrosequencing assays and obtain the genotypic identification. The numbers of hours needed to generate the Gram stain result and to perform phenotypic identification were then compared separately to the corresponding PCR/pyrosequencing times using a one-sample Wilcoxon rank sum test and the molecular testing times as the reference. The two-sided *P* values are reported in the text.

RESULTS

Bead-based extraction of blood resulted in a high proportion of DNA extracts with an amplifiable beta-globin gene target. DNA was extracted directly from 1,130 blood specimens collected from participants being evaluated for BSI. A total of 99.1% (1,120/1,130) had positive beta-globin gene (268-bp) PCR results with the characteristic melting temperature (T_m) of 87.3°C (mean and median) after melt curve analysis. DNA was also extracted from 102 blood samples collected from an equal number of healthy donors who served as controls; 100% (102/102) of these DNA extracts had amplifiable beta-globin DNA and characteristic T_m. Together, 99.2% (1,222/1,232) of the DNA extracts had amplifiable beta-globin DNA with characteristic T_m using this bead-based extraction method.

PCR/pyrosequencing provided bacterial detection and identification directly from blood in over three-quarters of all cases of culture-proven BSI. Ninety-eight of the 126 (77.8%) culture-confirmed cases of BSI were detected and accurately classified from blood directly for the Universal 16S rRNA gene target, with an overall concordance of 96.9% (1,085/1,120). The clinical performance characteristics of the Universal 16S rRNA gene screening assay included the following: sensitivity, 77.8% (67.8 to 83.3); specificity, 99.3% (98.6 to 99.7); positive predictive value (PPV), 93.3% (86.5 to 97.2); and negative predictive value (NPV), 97.2% (95.8 to 98.0) (Table 2).

Ninety-four of the 98 specimens contained *Staphylococcus* species, *Streptococcus* species, and/or enteric Gram-negative rod(s) and were further screened by PCR/pyrosequencing for a more specific target; either a different region of the 16S rRNA gene for differentiating *Staphylococcus* spp. or one of two 23S rRNA gene targets for differentiating *Streptococcus* spp. or enteric Gram-neg-

TABLE 2 Comparison of the initial Universal 16S rRNA gene PCR/pyrosequencing results to Bactec 9240 blood culture results, with analytical performance characteristics[a]

Molecular method result	Culture result (no. of samples)		Total no.
	Positive	Negative	
Positive	98[b]	7	105
Negative	28	987	1,015
Total	126	994	1,120

[a] Analytical performance characteristics (95% CI) of the initial testing using the Universal 16S rRNA gene PCR/pyrosequencing approach compared to standard culture included an overall concordance of 96.9% (1,085/1,120), sensitivity of 77.8% (69.5 to 84.7), specificity of 99.3% (98.6 to 99.7), PPV of 93.3% (86.8 to 97.3), and NPV of 97.2% (96.0 to 98.2). Ten of the total 1,130 blood samples collected were beta-globin negative and as such were not tested for 16S rRNA gene by PCR.
[b] Two of the 98 culture-confirmed specimens correctly identified as *Streptococcus* species using the Universal 16S rRNA gene PCR/pyrosequencing approach were misclassified when using a *Streptococcus* species-specific 23S rRNA gene target.

ative rods. Here 92/94 (97.9%) correctly matched with the corresponding culture results; two specimens correctly identified as a *Streptococcus* sp. using the Universal 16S rRNA gene PCR assay were misidentified when tested by PCR/pyrosequencing using the 23S rRNA gene *Streptococcus*-specific target; one was identified as a member of group G streptococci (GGS) and the other as an *Enterococcus* sp., while both isolates were identified as members of group C streptococci (GCS) by culture. DNA was extracted from the 2 culture isolates, and real-time PCR/pyrosequencing was performed; the molecular results from the culture isolates confirmed the phenotypic identity obtained in the clinical laboratory.

By molecular screening, the 7 PCR-positive, culture-negative discordant specimens were identified as either *Bacillus* sp. or coagulase-negative *Staphylococcus* sp. (CoNS) (2 each), or 1 each of viridans group streptococci, *Streptococcus pneumoniae*, and *Enterobacter aerogenes*. Review of the electronic medical records revealed no positive culture results of any kind from these 5 participants during their admission in which *Bacillus* sp., CoNS, or viridans group streptococci were detected using PCR/pyrosequencing. These false-positive molecular-method-based results were consistent with possible skin contaminants occurring during the blood draw. In contrast, the remaining 2 patients in which *S. pneumoniae* or *E. aerogenes* was detected by PCR/pyrosequencing did have earlier positive culture results consistent with these molecular test results; *S. pneumoniae* was cultured from an earlier sputum specimen, and *E. aerogenes* (>100,000 CFU/ml) was isolated from an earlier urine specimen.

DNA from the 28 discordant PCR-negative, culture-positive samples were retested in duplicate for both the Universal 16S rRNA gene target and the appropriate specific target. Upon retesting, both duplicates of the two rRNA gene targets tested in 9 of the 28 (32.1%) specimens generated sequences whose identities agreed with culture results; these 9 bacteria included 1 each of *E. faecalis*, GCS, *Citrobacter freundii*, and *Klebsiella pneumoniae*, 2 of CoNS, and 3 of *Escherichia coli*. After retesting, the overall concordance was 97.7% (1,094/1,120), with analytical performance characteristics (95% CI) of 84.7% (77.1 to 90.5) for sensitivity, 99.3% (98.6 to 99.7) for specificity, 93.8% (87.6 to 97.5) for PPV, and 98.1% (97.1 to 98.9) for NPV.

The remaining 19 PCR-negative, culture-positive discordant specimens tested in duplicate were found to be repeatedly negative by PCR/pyrosequencing for the Universal 16S rRNA gene target. The bacteria isolated from culture included 1 each of CoNS, *S. pneumoniae*, *Streptococcus pyogenes*, GCS, *Proteus mirabilis*, *Stenotrophomonas maltophilia*, or *Bacillus* species (not *anthracis*), 2 of *K. pneumoniae*, 3 each of viridans group streptococci and *Pseudomonas aeruginosa*, and 6 of *E. coli*. DNA was extracted from each of these cultured isolates, and real-time PCR/pyrosequencing was performed and as such ruled out any issues surrounding primer/probe recognition.

Bacteria within polymicrobial BSIs were difficult to identify by PCR/pyrosequencing. In this study, 12 blood culture bottles contained polymicrobial growth; 8 were drawn in the ICU and 4 in the ED. Table 3 lists the 25 bacteria cultured from these bottles and the success of PCR/pyrosequencing to detect and identify them. The Universal 16S rRNA gene screen was able to classify bacteria from polymicrobial infections when the bacteria were both from the same genus (e.g., in Table 3, numbers 1 to 3 and 6), but not when they were from different genera (e.g., in Table 3, numbers 4, 5, 7, and 9); in these cases, sequences may have been generated, but no identity was assigned. In contrast, the target-

TABLE 3 Comparison of results of standard blood culture and PCR/pyrosequencing to detect bacteria from polymicrobial BSIs[a]

ID no.	Culture ID	Universal 16S rRNA gene ID	Target-specific 16S rRNA gene ID[b]
1^{ED}	*S. aureus*, CoNS	*Staphylococcus* sp.	*S. aureus* and CoNS[1]
2^{ICU}	*S. aureus*, CoNS	*Staphylococcus* sp.	*S. aureus* and CoNS[1]
3^{ICU}	*S. aureus*, CoNS	*Staphylococcus* sp.	*S. aureus*[1]
4^{ICU}	CoNS, *E. faecalis*	Sequence but no ID[c]	CoNS,[1] *E. faecalis*[2]
5^{ED}	CoNS, viridans group streptococci	Sequence but no ID[c]	CoNS,[1] viridans group streptococci[2]
6^{ED}	GGS, viridans group streptococci	*Streptococcus sp.*	GGS[2]
7^{ICU}	*E. faecalis*, *P. aeruginosa*	Sequence but no ID[c]	Negative result
8^{ICU}	GCS, *E. aerogenes*	Enteric Gram-negative rod II	GGS[2], *E. aerogenes*[3]
9^{ICU}	*E. faecalis*, *K. pneumoniae*	Sequence but no ID[c]	*E. faecalis*[2]
10^{ICU}	GFS, *E. coli*	Negative result	Negative result[2,3]
11^{ICU}	*K. pneumoniae*, *P. mirabilis*, viridans group streptococci	Negative result	Negative result[2,3]
12^{ED}	K. *pneumoniae*, *S. maltophilia*	Enteric Gram-negative rod II	*K. pneumoniae*[3]

[a] ID, identification; ED, emergency department; ICU, intensive care unit; CoNS, coagulase-negative *Staphylococcus* sp.; GFS, group F *Streptococcus*.
[b] Misidentification of bacteria occurred using the *Streptococcus*-specific 23S rRNA gene target, with correct classification of *Streptococcus* sp. using the Universal 16S rRNA gene target. The more-specific rRNA gene targets tested are followed by superscript numbers: 1, 16S rRNA gene *Staphylococcus*-specific target; 2, 23S rRNA gene *Streptococcus*-specific target; 3, 23S rRNA gene enteric Gram-negative rod-specific target.
[c] A pyrogram containing readable sequence was generated, but when the sequence was analyzed using PyroMark Identifire v1.05.0 software, no identification was made.

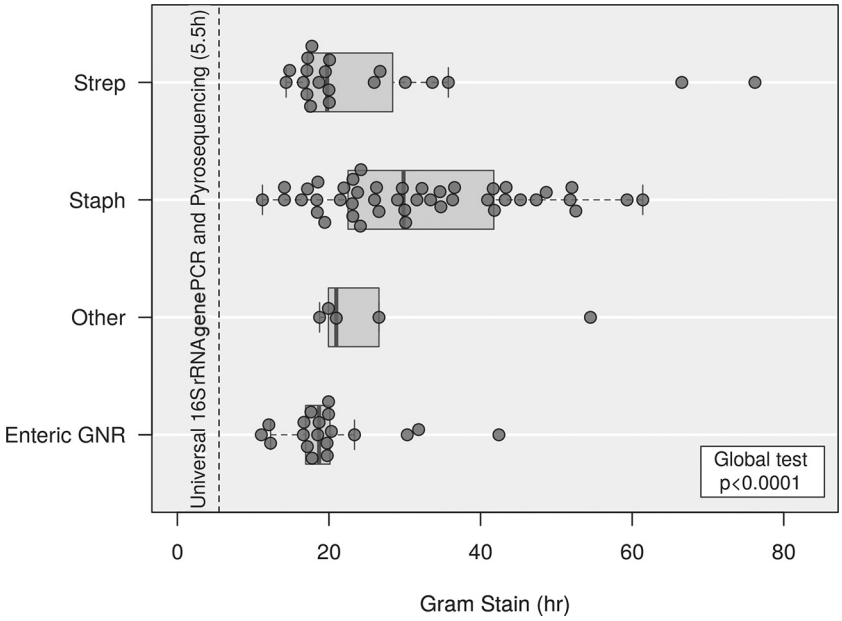

FIG 1 Comparison of the time it took to have the Gram stain results ($n = 103$) called (box plots) and the time that would have been needed for availability of the initial 16S rRNA gene PCR screen (dotted line) for the concordant positives, subdivided by microorganism type.

specific assays had partial success if the bacteria present in the specimen were from different genera (e.g., in Table 3, numbers 4, 5, and 8). Differentiating bacteria of the same genus within mixed infections was partially successful only when both were *Staphylococcus* spp. but not when the bacteria were from another genus. Jordan et al. described the presence of 4 individual single nucleotide polymorphisms within the 23-bp sequence that differentiates *Staphylococcus aureus* from CoNS (13). In this study, we were successful at detecting and identifying both *S. aureus* and CoNS in 2 of 3 specimens from polymicrobial BSIs (Table 3).

The molecular test results could have been available before either the Gram stain result was called to the hospital unit or the phenotypic identification was entered into the LIS. Incubating blood cultures are continually monitored for microorganism growth, and when it is detected, fluid is removed from the blood culture bottle and a smear made and Gram stained. The Gram stain result, including morphology, is the first piece of information concerning the identity of the potential pathogen provided to the hospital unit. In this study, the actual mean time interval between when the sample was drawn and when the Gram stain result was called was 27.9 h (standard deviation [SD], ±13.6 h). Of the 126 culture-confirmed cases of BSI from this study, Gram stain call times were documented for 103 (81.7%). The time to call the Gram stain was significantly longer than the theoretical ~5.5 h needed to generate the Universal 16S rRNA gene PCR/pyrosequencing screening result directly from blood ($P < 0.0001$) (Fig. 1).

The time needed to generate the final phenotypic identification was also compared to the theoretical time of ~7.5 h required to extract the DNA and complete sequential PCR/pyrosequencing for both the initial Universal 16S rRNA gene target and target-specific (16S rRNA gene or 23S rRNA gene) assay. These times differed significantly ($P < 0.0001$), with an average difference of 74.1 h.

DISCUSSION

In this study, we compared a molecular approach for screening blood directly for bacteria to conventional blood culture. Overall, greater than 99% of all extracts screened would have generated reportable results based on the beta-globin PCR results. In ~5.5 h, the theoretical time needed to complete DNA extraction and Universal 16S rRNA gene PCR/pyrosequencing, we were able to provide more specific information to over three-quarters of all cases of culture-confirmed BSI than was provided by the Gram stain result, which was available on average in 27.9 ± 13.6 h. As a research lab, although we did not run all specimens immediately, the potential exists to screen specimens within this ~5.5-h time frame in a clinical setting that has round-the-clock coverage for stat testing, wherein Gram staining of positive blood culture bottles occurs in real time.

This study illustrated the importance of testing extracts in duplicate, which resulted in an improved analytical sensitivity of ~85%, rather than ~78% (Table 2) when testing was performed in singleton. Based on Poisson's distribution, the probability of detecting bacteria directly from blood, where it is common to find only ~1 to 10 CFU/ml, will be greater if testing more than one aliquot of the sample, and that is what we found when testing additional aliquots of the original DNA extracts of the discordant specimens. The limit of detection of this molecular approach using whole blood is 40 CFU/ml for *E. coli* and 50 CFU/ml for group B *Streptococcus* (15).

Moving forward, we realize that testing in duplicate is not a viable option, due to the increased costs. So to improve assay sensitivity, larger blood volumes (1 to 5 ml) will need to be extracted and screened (16). However, using a larger blood sample will require a different extraction approach, one that eliminates much of the human genomic material present in whole blood. The Molzyme MolYsis Complete5 DNA extraction kit (Molzym

GmbH & Co., KG, Bremen, Germany) uses a selective lysis technique removing most human white blood cell DNA before lysing bacterial cells for DNA extraction. We found that when using this extraction technique with 1 ml whole blood in a well-characterized *S. aureus*-induced sepsis model in canines, we are able to detect more culture-confirmed blood samples than by our current bead-based extraction method, which uses 0.5 ml (17).

With the increased availability of molecular infectious disease testing in more clinical laboratories, physicians see the value and impact that same-day test results can have for their patients. However, achieving round-the-clock molecular testing in more hospitals for critical infections like BSI will be disruptive, requiring increased staffing to handle more on-demand testing of single specimens rather than performing once-a-day batch testing of specimens. This less efficient approach will increase costs but has the potential to provide important information, more rapidly, about the pathogen's identity to promote antimicrobial stewardship, with the potential of improving patient outcome as well.

Detecting and identifying potential pathogens in significantly less time than standard culture practices could permit clinicians to better utilize their hospital's antibiogram when choosing which antibiotic(s) to either continue or discontinue in their patients. Having a genotypic identification based on the 16S rRNA gene pyrosequencing results, which differentiate diverse groups of bacteria (e.g., differentiating *Staphylococcus* spp. from *Streptococcus* spp. from enteric Gram-negative rods, etc.) in ~5.5 h after the blood draw that a specimen contains may allow for more-rapid tailoring of the initial antibiotic(s) therapy on which a patient was started and ultimately reduce selective pressures on bacteria that result in antibiotic resistance. Unfortunately, the exact time of when the phenotypic identification of pathogens was entered into the hospital LIS was not available. Therefore, assuming that results were entered into the LIS at random times throughout each day, using a full 24 h to calculate the time required for phenotypic identification will have overestimated the time taken to provide this result.

It should be noted that using MALDI-TOF to identify microorganisms from positive blood culture bottles could have shortened by 24 h the time needed compared to phenotypic identification and thus would have generated a significantly shorter time for when the results had been entered into the hospital's LIS (18). Unfortunately, the clinical laboratory performing the phenotypic identification of the cultured isolates did not have access to this technology. However, using MALDI-TOF still requires waiting for the blood culture bottle to turn positive before the testing can be performed.

The strengths of this study included equal representation of patients from the ED and the ICU, which tested our assays' abilities to identify a broader range of bacterial pathogens: those associated with both community-acquired and health care-associated BSI. In addition, the research samples were collected during the very same blood draw procedure as the blood cultures to which research samples were compared, and the DNA was extracted in a timely manner. We did not experience an excessive number of PCR-positive, culture-negative results, which may reflect a lower use of antibiotics prior to blood collection, but this is only conjecture, as we did not have access to this information during the study. Lastly, this study included a healthy control group for which we successfully demonstrated a lack of false-positive molecular results.

There were limitations to this study. For one, the Universal 16S rRNA gene primers do not recognize well all DNA targets from anaerobic bacteria (our unpublished data). In addition, only a single blood sample per participant per admission was collected and screened for 16S rRNA genes and compared to the results of the matched blood culture set, when in fact sepsis guidelines recommend that 2 or 3 sets of blood culture be collected due to the fact that a single blood culture is only 65 to 75% sensitive. Also, we did not extract DNA from research specimens immediately after the blood draw occurred, which could have allowed for some bacterial growth to occur at room temperature, the temperature at which the specimens were held until we started the DNA extraction process.

This molecular approach did not provide genotypic antimicrobial susceptibility information. It should be noted that identification alone, without antimicrobial susceptibility testing, would not impact clinical care to the same extent as having both sets of results. Therefore, moving forward, this critical component must be included when screening blood for BSI. Lastly, polymicrobial BSIs, containing different genera of bacteria, were problematic when using the Universal 16S rRNA gene target and required using target-specific PCR assays in an attempt to identify the bacteria. This was certainly the case for 1 of the 2 misidentifications seen, with GGS being identified genotypically as a GCS (Table 3).

Our approach, like that of other molecular tests using blood directly, is not a replacement for blood culturing but rather a supplemental test to be used when attempting to diagnose BSIs. We estimated that the average cost to screen a single sample for the 16S rRNA gene target was $21.74 for reagents, with 3.5 h of hands-on time, including 2 h for extraction, 0.5 h for real-time PCR, and 1 h for pyrosequencing. Although it would certainly have saved time in identifying the bacteria if all four rRNA gene targets were screened simultaneously, the cost of this approach would be prohibitive, as the vast majority of all blood specimens screened are negative for bacteria. Lastly, although performing stat molecular-method-based testing will be disruptive to laboratory workflow, one must consider the benefits of being able to detect bacterial pathogens directly from blood in a significant percentage of all culture-confirmed cases of BSIs and weigh those benefits against the increased costs associated with adding tests rather than just substituting one test for another and the staffing demands that go with rolling out this testing around the clock.

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