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Utility of Pyrosequencing in Identifying Bacteria Directly from Positive Blood Culture Bottles▽

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Growth in liquid media is the gold standard for detecting microorganisms associated with bloodstream infections. The Gram stain provides the first clue as to the etiology of infection, with phenotypic identification completed 1 or 2 days later. Providing more detailed information than the Gram stain can impart, and in less time than subculturing, would allow the use of more directed empirical therapy and, thus, reduce the patient's exposure to unnecessary or ineffective antibiotics sooner. The study had two objectives, as follows: (i) to identify new targets to improve our ability to differentiate among certain enteric gram-negative rods or among certain *Streptococcus* species and (ii) to determine whether real-time PCR and pyrosequencing could as accurately identify organisms directly from positive blood culture bottles as culture-based methods. Two hundred and fifty-five consecutive positive blood culture bottles were included. The results showed a high level of agreement between the two approaches; of the 270 bacteria isolated from the 255 blood culture bottles, results for pyrosequencing and culture-based identifications were concordant for 264/270 (97.8%) bacteria with three failed sequences, and three sequences without match. Additionally, compared to the universal 16S rRNA gene target, the new 23S rRNA gene targets greatly improved our ability to differentiate among certain enteric gram-negative rods or among certain *Streptococcus* species. In conclusion, combining real-time PCR and pyrosequencing provided valuable information beyond that derived from the initial Gram stain and in less time than phenotypic culture-based identification. This strategy, if implemented, could result in a more directed empirical therapy in patients and would promote responsible antibiotic stewardship.

Growth in culture is the gold standard for detecting microorganisms present in the bloodstream (7, 24). Although automated blood culture systems have shortened the time needed to detect growth of an organism, we continue to rely heavily on the Gram stain result for the initial information about the organism's identity. This information is then provided to the healthcare team and used to determine the type of empirical therapy that will be ordered for the patient. It is common to start a patient on one or more broad-spectrum antimicrobial drugs while awaiting the culture-based identification and antimicrobial susceptibility test results. Unfortunately, phenotypic identification requires a minimum of 1 to 2 days to complete and another day to perform susceptibility testing. Having a faster way to classify the microorganism(s) present within positive blood culture bottles would allow tailoring of empirical antibiotic therapy and, thus, reduce the patient's exposure to ineffective or unnecessary antibiotic(s) while awaiting susceptibility testing results.

Sequence-based identification of PCR amplicons, targeting an rRNA gene(s), has proven to be useful for identifying many microorganisms and is becoming more commonplace in the clinical laboratory. Several sequence-based approaches have been successfully used to identify bacteria directly from positive blood culture bottles. Qian et al. successfully used the MicroSeq 500 kit (Perkin-Elmer Applied Biosystems, Foster City, CA), a commercially available method that sequences the first 527 bases of the amplified 16S rRNA gene, for this purpose (22). Turenne et al. used single-stranded conformation polymorphism analysis of PCR amplicons to distinguish between organisms (23), while Peters et al. used fluorescence in situ hybridization to identify pathogens out of positive blood cultures (19). Several investigators have used pyrosequencing (Biotage, Uppsala, Sweden) to identify numerous bacteria, yeasts, and fungi (9–11).

While most sequencing applications published to date test purified isolates for this purpose (2, 4, 8, 16–18, 26), others use clinical specimens. Kramski et al. successfully screened serum and urine specimens by reverse transcriptase PCR and pyrosequencing for hantavirus RNA (15). Kolak et al. screened sputum samples by PCR and pyrosequencing to identify bacterial flora from cystic fibrosis patients (14). Kobayashi et al. combined real-time PCR and pyrosequencing for the rapid identification of bacteria from specimens obtained from orthopedic surgeries, and they compared their results to those obtained by Gram staining and culture-based identification (13). Here, we describe using real-time PCR and pyrosequencing for identifying bacteria directly from positive blood culture bottles and compare those results to those obtained by culture-based identification. In this study, we also identified two different regions within the 23S rRNA gene that improved our ability to classify certain enteric gram-negative rods or certain *Streptococcus* species, compared to the previously described universal 16S rRNA gene target (11).

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TABLE 1. Primers used for PCR amplification and pyrosequencing analysis of bacterial rRNA genes[a]

Target no.	rRNA gene target, bp positions (amplicon size) [reference strain]	Real-time PCR primers (5′–3′)	Pyrosequencing primer (5′–3′)
1	16S rRNA gene, 1155–1525 (371 bp) [*E. coli* Z83204]	F^B-AACTGGAGGAAGGTGGGGAT$_{1155–1174}$ R-AGGAGGTGATCCAACCGCA$_{1525–1507}$	TACAAGGCCCGGGAACGTATTC ACCG$_{1380–1355}$
2	16S rRNA gene, 16–258 (243 bp) [CoNS X70648]	F^B-TGCCTAATACATGCAAGTCGAGCG$_{16–39}$ R-GTTGCCTTGGTAAGCCGTTACCTT$_{258–235}$	GTGTTACTCACCCGTCCGCCGC TA$_{92–69}$
3	23S rRNA gene, 602–834 (233 bp) [GBS AB096754]	F^B-GCCTTTTGTAGAATGAACCGGGA$_{602–625}$ R-CGTTTGGAATTTCTCCGCTACCCA$_{834–811}$	TCACATGGTTTCGGGTCTA$_{727–709}$
4	23S rRNA gene, 1346–1625 (280 bp) [*E. coli* AJ278710]	F^1-CTAAGGCGAGGCCGAAAG$_{1346–1363}$ R^B-CTACCTGACCACCTGTGTCG$_{1606–1625}$	S^1-GGTTGTCCCGGTTTA$_{1464–1450}$
	81–360 (280 bp) [*P. mirabilis* U88708]	F^2-CTAAGGCGAGGCTGAAAAG$_{81–99}$ R^B-CTACCTGACCACCTGTGTCG$_{360–341}$	S^2-GGTCGTCCCGGTTCA$_{185–199}$

[a] Subscript numbers refer to the base pair numbering within the stated reference strain. F^B, biotinylated forward primer; R^B, biotinylated reverse primer; F^1 and F^2, degenerate forward PCR primers used in combination; S^1 and S^2, degenerate pyrosequencing primers used in combination.

MATERIALS AND METHODS

Study hospital setting. The Magee-Women's Hospital of the University of Pittsburgh Medical Center is a 400-bed, full-service women's hospital with a state-of-the-art 75-bed neonatal intensive care unit. The hospital has several specialty surgical services, including orthopedics, bariatrics, and urology. For this study, consecutive positive blood culture bottles were collected during 2008 from both infants and adults.

Bacterial strains. A total of 1,075 bacterial isolates was included in this study for assessing the usefulness of the two proposed 23S rRNA gene targets and consisted of 141 *Staphylococcus aureus*, 232 coagulase-negative *Staphylococcus* species (CoNS), 171 *Enterococcus* species, 107 *Streptococcus agalactiae* (GBS), 35 *Streptococcus pneumoniae*, 25 *Streptococcus mitis*, 74 viridans streptococci, 35 *Streptococcus pyogenes*, 76 *Escherichia coli*, 32 *Klebsiella oxytoca*, two *Citrobacter koseri*, 23 *Enterobacter faecalis*, 20 *Enterobacter faecium*, 10 *Citrobacter freundii*, five *Morganella morganii*, 20 *Proteus mirabilis*, two *Proteus vulgaris*, 53 *Klebsiella pneumoniae*, and 12 *Serratia marcescens* isolates. Most of these bacteria represent well-characterized clinical isolates, but several purchased ATCC reference strains used for quality control testing were also included. The bacterial isolates were identified by standard laboratory protocol using the MicroScan instrument and panels (Siemens Healthcare Diagnostics, Inc., Deerfield, IL) or the RapID STR system (Remel, Lenexa, KS) for identification of streptococci and/or individual biochemical reagents.

Bacterial isolate crude cell lysate preparation. Crude cell lysates were prepared from each of the above-mentioned bacterial isolates for PCR analysis. Using a sterile pipette tip, cells from a well-isolated bacterial colony were transferred to a 1.5-ml Eppendorf tube containing 200 μl of lysis buffer (10 mM Trish-HCl [pH 8.0], 0.5% Brij-35) and 0.24 g of 0.1-mm zirconium beads (catalog no. 11079101z; Biospec Products, Bartlesville, OK). The contents of the tube were vortexed for 5 min, followed by a quick spin to settle the beads out of solution.

Blood culture bottle fluid DNA preparation. Discarded, deidentified Bactec 9240 blood culture bottles, which were resin-containing aerobic bottles, lytic anaerobic bottles, or PedsPlus bottles, were included in this study. The specimen preparation procedure uses a protocol described by Fredricks and Relman (6).

Real-time PCR. Four individual PCR assays were set up with each specimen analyzed. These specimens included the crude cell lysates made from bacterial isolates and purified DNA from positive blood culture bottles. The primers used in each PCR assay are described in Table 1. Each master mix contained 0.5 μl each of 10 μM concentrations of forward and reverse primers (IDT, Coralville, IA), 5 μl of purified DNA prepared from fluid removed from the blood culture bottle or 1 μl of crude cell lysate prepared from isolated bacteria, and 12.5 μl of 2× SYBR TakaRa PreMix *Taq* polymerase master mix (catalog no. RR039B; Fisher Scientific, Pittsburgh, PA). The PCR cycling conditions consisted of 95°C for 1 min, followed by 40 cycles of 95°C for 20 s, 60°C for 1 min, and 72°C for 15 s. The three positive controls consisted of intact *E. coli*, *S. aureus*, and GBS isolates, while the two negative controls consisted of a no-template control and fluid from a discarded, de-identified blood culture bottle containing no growth. All of these controls were included in each PCR run.

Pyrosequencing. All PCR-positive reactions were analyzed by pyrosequencing as previously described (11). The sequencing primer(s) used for each individual pyrosequencing reaction is listed in Table 1. The dispensation order used when sequencing the two 16S rRNA gene target amplicons and the 23S rRNA gene target amplicon (bp 602 to 834 in reference strain GBS AB096754) was 11(ACTG), while the dispensation order used for the 23S rRNA gene target amplicon (bp 1346 to 1625 in reference strain *E. coli* AJ278710) was AGC12(CTGA).

The PSQ 96 SQA software determined the base sequences and assessed the quality of the pyrograms, which is based on a combination of actual peak height, relative peak height, and background noise. The sequences generated were analyzed using Identifire 1.0.5.0 software that aligns the input sequences with those present in a reference library. The reference library consists of validated sequences generated by us from previously identified clinical isolates, ATCC reference strains, and known sequences within the National Center for Biotechnology Information (NCBI) GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Assessment of two 23S rRNA gene targets. Our previous work (11) describes two regions within the 16S rRNA gene that are useful for classifying bacteria by PCR and pyrosequencing; one is a universal target (target 1 [Table 1]) that provides broad-based classification of most medically relevant bacteria, while the other is a region that differentiates *S. aureus* from CoNS (target 2 [Table 1]). However, neither of these 16S rRNA gene targets is helpful for differentiating among enteric gram-negative rods or among *Streptococcus* species. To that end, we interrogated the 23S rRNA gene and found two regions—one that contained sequence variability among many of the enteric gram-negative rods associated with bloodstream infections (target 4 [Table 1]), and another that contained sequence variability among certain *Streptococcus* species (target 3 [Table 1]).

Table 2 illustrates the sequence patterns obtained from testing 255 previously identified enteric gram-negative rods with the primers described in Table 1 (target 4). The results illustrated the usefulness of this region for differentiating between *E. coli*, *K. oxytoca*, *K. pneumoniae*, *P. mirabilis*, *P. vulgaris*, *S. marcescens*, *M. morganii*, and *C. koseri*. The *Enterobacter* species and *C. freundii* isolates had identical sequences and therefore could not be differentiated from each other, but they were distinguishable from the other enteric gram-negative rods analyzed (Table 2). No amplicons were generated when using this primer set for any of the 373 *Staphylococcus* sp. or 447 *Streptococcus* sp. isolates tested (data not shown).

Table 3 illustrates the sequence patterns obtain from 447 previously identified streptococcal isolates using the primers described in Table 1 (target 3). The result showed the utility of this region for differentiating between *S. pyogenes*, GBS, and

TABLE 2. Sequence patterns of the first 21 bases of DNA generated by pyrosequencing using primers targeting a region within the 23S rRNA gene informative for enteric gram-negative rods

Organism	No. of isolates tested	DNA sequence (5′–3′)[a]
E. coli	76	AGC GTG TAG GCT GAT TTT CCA
K. oxytoca	32	AGC ATG TAG GCG GAT GTT CCA
K. pneumoniae	53	AGC ATG TAG GCT GAT TGT CCA
P. mirabilis	20	AGC AGG TAG GCA GAG TGA TTA
P. vulgaris	2	AGC ATG TAG GCA GAG TGA TTA
S. marcescens	12	AGC GTG TAG GGG GTG TGA CCT
M. morganii	5	AGC GTG CAG GTG GAC TGA CCA
C. koseri	2	AGC GTG TAG GTG TGT GCT CCA
Enterobacter species	43	AGC ATG TAG GCG GAG GTT CCA
C. freundii	10	AGC ATG TAG GCG GAG GTT CCA

[a] Primers for the 23S rRNA gene target 4 of Table 1.

the *Enterococcus* spp. The highly diverse group of viridans streptococci demonstrated variability within its group that was distinguishable from the other *Streptococcus* species analyzed. *S. pneumoniae* and *S. mitis* had identical sequences and therefore could not be differentiated from each other but, as a group, were distinguishable from the other *Streptococcus* species. No amplicons were generated using this primer set for any of the 255 enteric gram-negative rods tested (data not shown). A unique amplicon was generated using this primer set for the 373 staphylococcal isolates tested (5′-CGA CCA AAT ACT CAA CGC CCT-3′) that was distinguishable from those generated for all of the streptococcal isolates.

Positive blood culture bottles with single isolates. A total of 270 bacteria were isolated from these 255 positive blood culture bottles; 242 bottles (94.9%) contained a single bacterial type, while 13 bottles (5.1%) contained polymicrobial growth. Table 4 illustrates the results for 242 blood culture bottles containing a single organism. Compared to culture-based identification, pyrosequencing accurately classified 239/242 (98.8%) of these organisms. The three discordant results were as follows: pyrosequencing reaction repeatedly failed to generate sequencing data for amplicons generated for one viridans streptococcus isolate and two *S. pyogenes* isolates. As noted above, the sequence generated for the *S. pneumoniae* could not be distinguished from that of *Streptococcus mitis*, and neither could the *Enterobacter* species be distinguished from *C. freundii*.

TABLE 3. Sequence patterns of the first 21 bases of DNA generated by pyrosequencing using primers targeting a region within the 23S rRNA gene informative for certain *Streptococcus* species

Species	No. of isolates tested	DNA sequence (5′–3′)[a]
Streptococcus species		
S. agalactiae	107	CAA $^{C}_{T}$AT GAT ACT ATG GCG CCC
S. pyogenes	35	CAA CAT GAT ACT AAA TCG CCC
S. pneumoniae	35	CG$^{T}_{A}$ CAT GAT ACT AA$^{T}_{G}$ TCG CCC
S. mitis	25	CG$^{T}_{A}$ CAT GAT ACT AA$^{T}_{G}$ TCG CCC
Enterococcus species	171	CGA C$^{T}_{A}$A CAT ACT CA$^{A}_{T}$ TCG CCC
Viridans group streptococcus	74	CGT CAT GAT ACT AAT GCG CCC CGA CAT GAT ACT AAT GCG CCC CGA CAT AAT ACT AAA GCG CCC CGT CAA GTA ACT GTC GCC CCC CAA CAT AAT ACT AAA ACG CCC CAT CAT GAT ACT AAA GCG CCC CGA CAT AAT ACT ATT GCG CCC

[a] Primers for the 23S rRNA gene target 3 (Table 1). Superscript/subscript notation indicates the variability seen within that single base position for the non-viridans group streptococcus isolates.

TABLE 4. Comparing results of pyrosequencing and the culture-based method for identifying bacteria from 242 blood culture bottles containing a single organism

Organism[a]	No. of isolates with result for (% agreement with culture):	
	Culture	Pyrosequencing
CoNS	81	81 (100)
S. aureus	33	33 (100)
Viridans group streptococcus	17	16 (94.1)[b]
E. faecalis (*Enterococcus* sp.)	12	12 (100)
E. faecium (*Enterococcus* sp.)	7	7 (100)
S. pyogenes	9	7 (77.8)[c]
GBS	6	6 (100)
S. pneumoniae (*S. pneumoniae* or *S. mitis*)	5	5 (100)
E. coli	40	40 (100)
K. pneumoniae	13	13 (100)
E. aerogenes (*Enterobacter* species or *C. freundii*)	3	3 (100)
E. cloacae (*Enterobacter* species or *C. freundii*)	3	3 (100)
S. marcescens	4	4 (100)
K. oxytoca	1	1 (100)
C. freundii (*Enterobacter* species or *C. freundii*)	1	1 (100)
Pseudomonas aeruginosa	3	3 (100)
Haemophilus influenza (*Haemophilus* sp.)	4	4 (100)

[a] Organism(s) in parentheses refers to results for pyrosequencing only.
[b] Sequencing reaction repeatedly failed for one isolate.
[c] Sequencing reaction repeatedly failed for two isolates.

Positive blood culture bottles with multiple organisms. Thirteen of 255 (5.1%) positive blood culture bottles contained more than one organism when subcultured. Table 5 compares the results of pyrosequencing with those of culture-based identification of the 28 bacterial isolates from 13 bottles. Pyrosequencing accurately classified 19/28 organisms, while 6/28 bacterial isolates, namely two *E. coli*, two *Enterobacter cloacae*, one *Enterobacter aerogenes*, and one *S. mitis* isolate, were broadly categorized only as either enteric group 1 (five gram-negative rods) or *Streptococcus* species (one *S. mitis* isolate), based solely on the sequences generated from the universal 16S rRNA gene target (target 1 [Table 1]) and not from the 23S rRNA gene target (target 4 [Table 1]). No matches were found for 3/28 organisms, including for two CoNS isolates and one *S. mitis* isolate.

DISCUSSION

The results illustrated that the two new 23S rRNA gene targets greatly improved our ability to differentiate among certain enteric gram-negative rods associated with bloodstream infections or certain *Streptococcus* species compared to the universal 16S rRNA gene target previously described (11). Rather than generating the less-specific classifications of "enteric gram-negative rod" or "*Streptococcus* species" with the universal 16S rRNA gene target, the two 23S rRNA gene targets provided more-specific identifications. We also demonstrated that PCR and pyrosequencing could be used to as accurately identify organisms directly from positive blood culture bottles as culture-based methods. Although the sequence

TABLE 5. Comparing results of pyrosequencing and culture-based method for identifying bacteria from the blood culture bottles with polymicrobial growth

Organism[b]	No. of isolates with result for[a]:	
	Culture	Pyrosequencing
CoNS	7	5 (2)
S. aureus	2	2
Viridans group streptococci	4	4
S. mitis (*Streptococcus* sp.)[c]	2	1 (1)
GBS	2	2
E. cloacae (enteric group 1)[c]	2	2
E. faecium (*Enterococcus* sp.)	2	2
E. faecalis (*Enterococcus* sp.)	2	2
E. coli (enteric group 1)[c]	2	2
E. aerogenes (enteric group 1)[c]	1	1
K. oxytoca	1	1
Pseudomonas aeruginosa	1	1

[a] Number in parentheses indicates the number of isolates for which no match was obtained.
[b] Organism(s) in parentheses refers to results for pyrosequencing only.
[c] Identification based solely on pyrosequencing data obtained from the universal 16S rRNA gene target 1 of Table 1.

variability within the 23S rRNA gene targets did not provide species-level identification for all bacteria, it did provide genus-level identification for most other organisms associated with bloodstream infection. Certain highly related bacteria, e.g., *S. pneumoniae* and *S. mitis*, or *Enterobacter* species and *C. freundii* were not differentiated using this target; DNA targets outside of the rRNA genes will most likely be needed for this purpose (3, 9, 10, 20, 21, 25).

The overall agreement between pyrosequencing and culture-based identification methods was high (97.8% [264/270]). In blood culture bottles with a single organism isolated, concordance was even higher (98.8% [239/242]). In blood culture bottles having more than one organism present, pyrosequencing had more limited success (89.3% [25/28]). The most likely explanation for this lower rate of agreement may be the differences in bacterial loads of the organisms present in a bottle, as these purified isolates from mixed infections were successfully amplified and pyrosequenced using the primers described here (data not shown). The rates of sequencing failures and no matches were low (1% each). Like other investigators (5, 12, 22), we also found this approach to be useful in confirming false-positive blood culture results when Gram staining and subculture onto solid media yielded no organisms (data not shown). It is a good idea to incorporate more than one ribosomal gene target into the testing algorithm, as it increases the number of DNA sequences one has to analyze, which can provide a cross check on the data generated and increase your confidence in the accuracy of the organism's identification, if concordance occurs.

One limitation to this approach was the occasional presence of background signal sequences from uninoculated Bactec blood culture bottles when using the 23S rRNA gene target primer set that was useful in differentiating among certain *Streptococcus* species. We sometimes got sequence patterns generated from these uninoculated bottles that matched that of either *Streptococcus sanguinis* (oral flora) or the viridans streptococcus group. The frequency of this phenomenon varied with the lot number of bottles being used and has been described by others as well (1). This finding demonstrates the difficulty in using rRNA gene targets in molecular-based testing and one that will require evaluation of each lot of blood culture bottles for this nonviable background DNA before being put into use.

Ultimately, our goal is to evaluate these four rRNA gene targets directly on whole-blood samples collected from patients being evaluated for bloodstream infection. However, at this time, we felt it was important to begin our assessment of these two new 23S rRNA gene targets screening fluids from positive blood culture bottles as a way to build confidence in these targets. For the greatest benefit to the patient, this type of testing needs to be available on a 24/7 basis, and the information must be acted upon in a timely manner. To accomplish this, we need protocols or platforms that are easier to perform and interpret. Future emphasis needs to be placed on identifying simpler, yet efficient, whole-blood extraction methods that will purify bacterial DNA that is found in low abundance away from human genomic DNA, which is found in high abundance. Just as importantly, more gene targets that can help predict antibiotic resistance need to be identified. Physicians will need to have confidence in these newer approaches before they will be willing to act on the information and tailor empirical antibiotic therapy sooner than is currently being done. Implementing more-rapid diagnostic testing for bloodstream infection is a very worthwhile goal and would promote antibiotic stewardship by reducing a patient's exposure to unnecessary or ineffective antibiotics, but it is one that will ultimately take time, education, and significant amounts of data to change a physician's practice.

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