Molecular Probes for Diagnosis of Clinically Relevant Bacterial Infections in Blood Cultures[▽]

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Broad-range real-time PCR and sequencing of the 16S rRNA gene region is a widely known method for the detection and identification of bacteria in clinical samples. However, because of the need for sequencing, such identification of bacteria is time-consuming. The aim of our study was to develop a more rapid 16S real-time PCR-based identification assay using species- or genus-specific probes. The Gram-negative bacteria were divided into *Pseudomonas* species, *Pseudomonas aeruginosa*, *Escherichia coli*, and other Gram-negative species. Within the Gram-positive species, probes were designed for *Staphylococcus* species, *Staphylococcus aureus*, *Enterococcus* species, *Streptococcus* species, and *Streptococcus pneumoniae*. The assay also included a universal probe within the 16S rRNA gene region for the detection of all bacterial DNA. The assay was evaluated with a collection of 248 blood cultures. In this study, the universal probe and the probes targeting *Pseudomonas* spp., *P. aeruginosa*, *E. coli*, *Streptococcus* spp., *S. pneumoniae*, *Enterococcus* spp., and *Staphylococcus* spp. all had a sensitivity and specificity of 100%. The probe specific for *S. aureus* showed eight discrepancies, resulting in a sensitivity of 100% and a specificity of 93%. These data showed high agreement between conventional testing and our novel real-time PCR assay. Furthermore, this assay significantly reduced the time needed for identification. In conclusion, using pathogen-specific probes offers a faster alternative for pathogen detection and could improve the diagnosis of bloodstream infections.

Bloodstream infections are a major cause of death in the world and need a thorough and adequate therapeutic strategy. Inadequate antibiotic therapy is associated with higher mortality rates, the appearance of antibiotic resistance, and longer hospitalization lengths (14). Conventional identification and susceptibility testing have several limitations, such as lack of rapidity and sensitivity. The current gold standard, i.e., blood culture, usually requires 6 to 12 h of incubation before growth is detected and a further 24 to 48 h for the definitive identification of the infectious agent and its susceptibility to antibiotics (1, 9). Routine diagnostics already use molecular techniques for the direct detection of viral and bacterial pathogens. However, most in-house assays are targeted against one specific bacterium and/or virus and do not offer broad-range pathogen detection. Recently, several PCR assays have been developed targeting a panel of the most relevant bacterial and fungal bloodstream pathogens, which can be performed directly with blood, such as SeptiFast (Roche Diagnostics GmbH, Mannheim, Germany), SepsiTest (Molzym GmbH & Co. KG, Bremen, Germany), and VYOO (SIRS-Lab GmbH, Jena, Germany), or using positive blood cultures, such as the microarray-based system Prove-it Sepsis (Mobidiag, Helsinki, Finland).

As discussed in our previous work, direct detection in whole blood is hampered by several factors, such as the presence of PCR inhibitors and background DNA, low bacterial load, in-

sufficient sensitivity, and difficulty of establishing an assay capable of detecting a wide range of pathogens (5). In contrast, molecular testing of growth-positive blood cultures does not require highly sensitive assays because of the presence of a high bacterial load. Furthermore, until now, the use of culturing remains essential to determine the microorganism's antimicrobial profile. Therefore, the role of blood cultures remains important for the detection and identification of causative bacterial agents. Molecular testing of blood cultures, possibly in combination with conventional testing, could enable more rapid identification and, consequently, more rapid diagnosis and start of correct therapy. Molecular approaches such as broad-range real-time PCR and sequencing of the 16S rRNA gene region are widely known methods for the detection and identification of bacteria in clinical samples (4, 7, 10, 15). However, because of the need for sequencing, the identification of bacteria is time-consuming.

The aim of our study was to develop a more rapid 16S real-time PCR-based identification assay using species- or genus-specific probes. The assay is particularly intended for the identification of positive blood cultures, for which Gram staining results are known. In this proof-of-concept study, priority was given to the genera or species most frequently found in blood cultures and/or those that could direct the choice of a suitable antibiotic therapy. Therefore, we selected a panel of eight species- or genus-specific probes. The Gram-negative bacteria were divided into *Pseudomonas* spp., *Pseudomonas aeruginosa*, *Escherichia coli*, and other Gram-negative species. Within the Gram-positive species, probes were designed for *Staphylococcus* spp., *Staphylococcus aureus*, *Enterococcus* spp., *Streptococcus* spp., and *Streptococcus pneumoniae*.

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Hence, a first indication about the causative microorganism is given after 2 h, while confirmation and precise identification can be achieved with sequencing. Consequently, multiple species can be detected in samples with polymicrobial infections. The present paper reports a retrospective study performed on blood cultures obtained from patients with suspected bloodstream infections. Results of this new multiprobe assay were compared with conventional blood culture findings.

MATERIALS AND METHODS

Clinical samples. A total of 248 blood cultures were collected at the Maastricht University Medical Center (MUMC; Maastricht, Netherlands). All samples were analyzed with standard conventional testing. Blood specimens drawn from patients suspected of having bloodstream infections were incubated in blood culture bottles (Plus Aerobic [product no. 442192] and Plus Anaerobic [product no. 442193]; BD Diagnostic Systems, Sparks, MD) and monitored for microbial growth in the Bactec 9240 automated blood culture device (BD). When growth was detected, Gram staining was performed. A small aliquot of each blood culture (1 ml) was requested for the novel molecular assay. Two separate assays were developed for Gram-negative and Gram-positive bacteria. Hence, further analysis was based on the results of Gram staining.

Conventional bacterial identification. Regarding the Gram-positive cocci (GPCs), to discern *Staphylococcus* spp. from other GPCs, a catalase test was performed by adding 1 colony to a drop of 3% H_2O_2. For the identification of *Staphylococcus* spp., catalase-positive strains were tested for coagulase and DNase production. If both tests were negative, the strain was identified as coagulase-negative *Staphylococcus* (CoNS). To discern *Enterococcus* spp. from other catalase-negative GPCs, bile esculin, Tellur diagnostic tablets (Rosco Diagnostica, Taastrup, Denmark), and an API 20 Strep test (bioMérieux SA, Marcy l'Etoile, France) were used, according to the manufacturer's guidelines. Optochin susceptibility (OXOID) was used to differentiate *Streptococcus pneumoniae* from the other viridans group streptococci, which were further identified by API 20 Strep. In the case of beta-hemolytic streptococci, latex agglutination was performed using the Prolex streptococcal grouping latex kit (product code PL.030; Bio Trading).

Bacterial strains. Reference strains were used to validate the specificity of the assay, including *S. aureus* ATCC 25923 and ATCC 29213, *Staphylococcus epidermidis* ATCC 12228 and ATCC 14990, *S. pneumoniae* ATCC 49619, *P. aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* BM 4147, and *Escherichia coli* ATCC 35218.

Multiprobe assay. An aliquot (0.1 ml) of blood culture was 1:100 diluted in 0.9% NaCl. Dilutions were centrifuged for 5 min at 12,000 rpm, and the bacterial pellet was resuspended in 100 μl nuclease-free water. The primers and the universal bacterial TaqMan probe have been described previously (8). The probes for *P. aeruginosa*, *Pseudomonas* spp., *E. coli*, *Staphylococcus* spp., *S. aureus*, *Enterococcus* spp., *Streptococcus* spp., and *S. pneumoniae* were designed by using the BLAST tool and ClustalW software. Multiple sequence alignments were made and are partly shown in Fig. 1. The designed probe sequences are given in Table 1. All primers and probes were tested for specificity and cross-reactions both manually and with use of the NCBI-BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). Primer and probe matrices were performed to determine optimal concentrations. Each test contained a 5-μl purified sample and a 20-μl reaction mixture. The reaction mixture contained 12.5 μl of TaqMan environmental master mix 2.0 (Applied Biosystems, Foster City, CA), 0.9 μM forward primer, 0.6 μM reverse primer, and 0.2 μM each probe. There were three separate reactions. The first reaction included the universal probe and the *P. aeruginosa* probe. The second reaction included the probes targeting *E. coli* and *Pseudomonas* spp. The third reaction included the *Staphylococcus* probe, the *S. aureus* probe, and the *Enterococcus* probe, the fourth and final reaction included the *Streptococcus* probe and the *S. pneumoniae* probe. Reactions were performed using the ABI Prism 7000 real-time PCR system (Applied Biosystems, Foster City, CA), and optimal thermal cycling conditions used were as follows: 2 min at 50°C, initial denaturation at 95°C for 15 min, 42 cycles of denaturation for 15 s at 95°C, and annealing at 60°C for 1 min. The cycle threshold (C_T) value, the cycle number at which amplicon fluorescence exceeded the preset detection threshold, was recorded for all samples. The cutoff value to consider a PCR result as positive was set to a C_T value of 35.

DNA sequencing. Samples with discrepant results were further analyzed with sequencing. The DNA was purified using the MSB PCRapace PCR cleanup kit (Invitek, Berlin, Germany) and dissolved in 30 μl NASBA H_2O. Cyclic sequencing was performed using BigDye 3.0 (Applied Biosystems, Foster City, CA). The resulting sequence product was purified and separated with the 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

RESULTS

Design of the species- or genus-specific probes. The specificity of the probes was evaluated using BLAST. *In silico* analysis revealed that nearly only cross-reactivity was found with not clinically relevant microorganisms such as *Geobacillus* spp. and *Lactobacillus plantarum*. *In silico* cross-reactivity with clinically relevant microorganisms was tested *in vitro* with reference strains. For example, the *in silico E. coli* probe cross-reacted with *Acinetobacter* spp. and *Enterobacter* spp. However, *in vitro* tests showed no positive signals for these microorganisms. Furthermore, cross-reactions were found with *Escherichia albertii* and *Shigella* spp. Based on the literature, these microorganisms occur only rarely in blood cultures, and therefore, the risk of false identification was minimal. In a few cases such as with the *Streptococcus* and the *Staphylococcus* probes, cross-reactivity with *Bacillus cereus* was found. The species conferring cross-reactivity in PCR were then sequenced to check for mismatches between the probe-target hybrids. Sequencing results showed that this occurred because of at least two mismatches at the binding site of both probes (data not shown). However, in all these cases, the cross-reacting fluorescent signals generated by the mismatched probes were weak compared to that of the positive template control and uncharacteristically appeared over 5 cycles later than signals from 100% matched probes (data not shown).

Evaluation of the multiprobe assay by testing clinical blood culture samples. From a total of 248 blood cultures, the presence or absence of bacterial DNA was determined in 232 growth-positive and 16 growth-negative samples using the universal probe, yielding a sensitivity and specificity of 100% (Table 2). Our multiprobe assay was performed after Gram staining of the positive blood cultures. Considering the growth-positive blood cultures, results from the multiprobe assay were in accordance with conventional identification in 222 cases (96%). The specific probes targeting *Pseudomonas* spp., *P. aeruginosa*, *E. coli*, *Streptococcus* spp., *S. pneumoniae*, and *Staphylococcus* spp. all had a sensitivity and specificity of 100%. The majority of Gram-positive blood cultures, 85 of 145 (59%), contained a coagulase-negative *Staphylococcus* species. In 31 blood cultures, the causative agent was identified as *S. aureus*. Regarding the Gram-positive staphylococcal blood cultures, the multiprobe assay was in conflict with culture results in nine cases. The nine discrepancies were further analyzed by coagulase testing, specific *S. aureus* real-time PCR, and sequencing (Table 3; Fig. 2). One out of nine cases, clinical isolate (CI) 7, tested coagulase positive and was positive for two targets specific for *S. aureus*, i.e., *femA* and *sa442*. Sequencing of the PCR product also confirmed the results of the multiprobe assay. The sequences of the eight other clinical isolates showed three mismatches with the reference *S. aureus* sequence, as shown in Fig. 2A. Hence, the remaining eight cases were confirmed as discordant. Consequently, sensitivity and specificity of 100% and 93%, respectively, and positive and

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Streptococcus pneumoniae    TCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGGAAGTCTGA 50
Streptococcus species       TCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGGAACCCTGA 50
Enterococcus species        TCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGA 50
Staphylococcus aureus       TCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGA 50 a
Staphylococcus species      TCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGA 50 a
Pseudomonas aeruginosa      TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGA 50
Pseudomonas species         TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGA 50
Escherichia coli            TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA 50 →
                            ****************** ****** **    ****** * **   ****

Streptococcus pneumoniae    CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGT 100
Streptococcus species       CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGT 100
Enterococcus species        CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGT 100 b
Staphylococcus aureus       CGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGT 100
Staphylococcus species      CGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAGCTCTGT 100
Pseudomonas aeruginosa      TCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTT 100 c
Pseudomonas species         TCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTT 100 c
Escherichia coli            TGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTT 100
                            *** * ******** *** ***** ***** * *****   ** *

Streptococcus pneumoniae    TGTAAGAGAAGAACGAGTGTGAGAGTGGAAAGTTCACACTGTGACGGTAT 150
Streptococcus species       TGTTAAGGAAGAACGAGTGTGAGAATGGAAAGTTCATACTGTGACGGTAC 150
Enterococcus species        TGTTAGAGAAGAACAAGGATGAGAGTAGAACGTTCATCCCTTGACGGTAT 150 b
Staphylococcus aureus       TATTAGGGAAGAACATATGTGTAAGTA-ACTGTGCACATCTTGACGGTAC 149 d
Staphylococcus species      TATTAGGGAAGAACAAACGTGTAAGTA-ACTGTGCACGTCTTGACGGTAC 149
Pseudomonas aeruginosa      AAGTTGGGAGGAA-GGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTAC 149 c
Pseudomonas species         AAGTTGGGAGGAA-GGGCAGTTACCTAATACGTAATTGTTTTGACGTTAC 149 c
Escherichia coli            CAGCGGGGAGGAA-GGGAGTAAAGTTAATACCTTTGCTCATTGACGTTAC 149 e
                            ** ***           *       *         ***** **

Streptococcus pneumoniae    CTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT 200 f-g
Streptococcus species       TTAACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT 200 f-g
Enterococcus species        CTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT 200 g
Staphylococcus aureus       CTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT 199 g
Staphylococcus species      CTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT 199 g
Pseudomonas aeruginosa      CAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGA 199 g
Pseudomonas species         CGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAG 199 g
Escherichia coli            CCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG 199 g
                            *  ***  ********  ********************

Streptococcus pneumoniae    AGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGG 250
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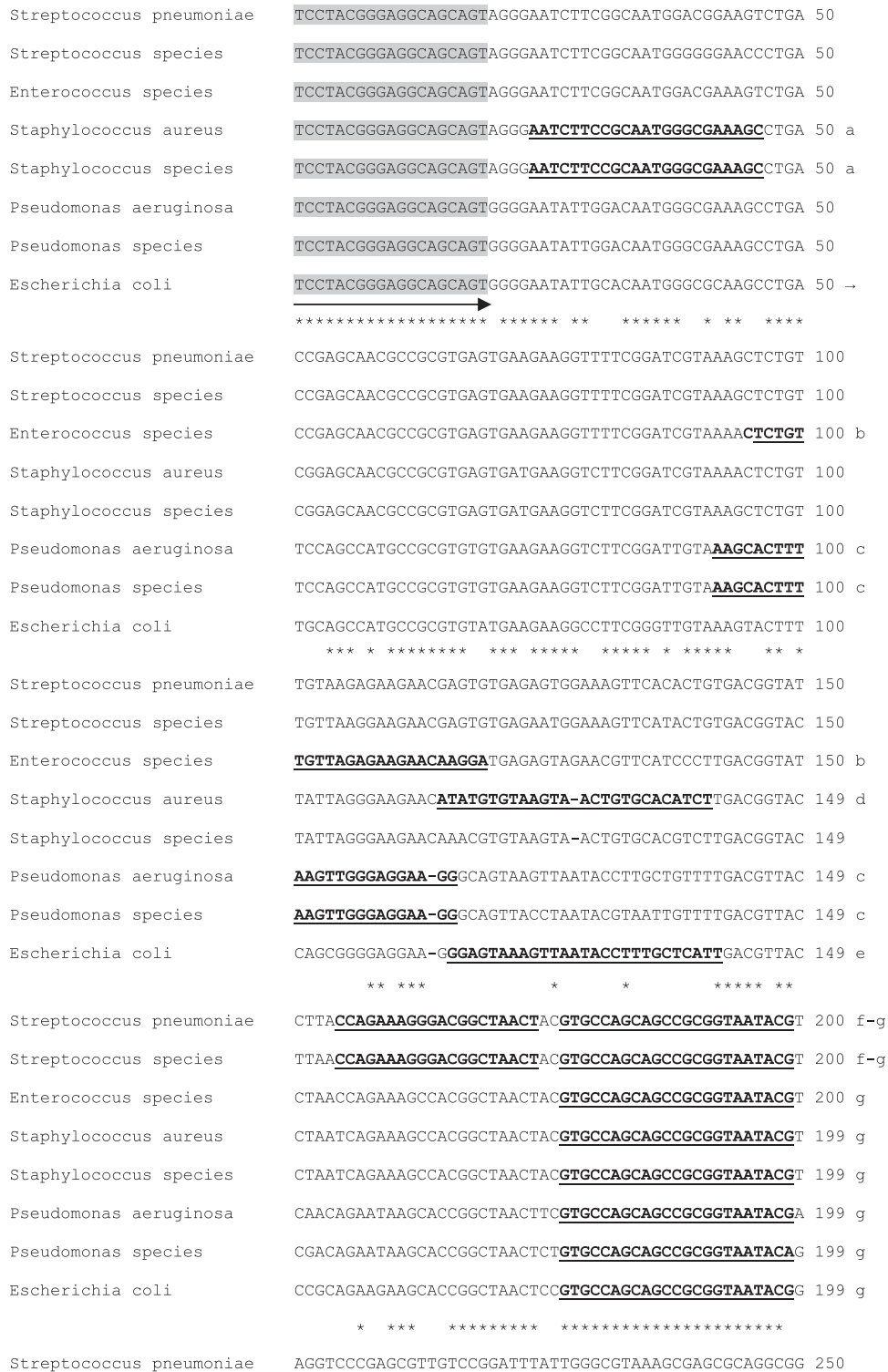
FIG. 1. Multiple sequence alignments of the amplified part of the bacterial 16S rRNA gene. Sequences of the bacteria included in the multiprobe assay are shown. The arrows represent the forward and reverse primer targeting the 16S rRNA gene. The sequences of the probes are in boldface and underlined. a, *Staphylococcus* probe; b, *Enterococcus* probe; c, *Pseudomonas* probe; d, *S. aureus* probe; e, *E. coli* probe; f, *Streptococcus* probe; g, universal probe; h, *S. pneumoniae* probe; i, *P. aeruginosa* probe.

negative predictive values of 79% and 100%, respectively, were achieved when sequencing was considered the gold standard. The probe specific for *Enterococcus* spp. showed one conflicting result. One blood culture was determined to contain *Aero-coccus viridans*, while our multiprobe assay identified the infectious agent as an *Enterococcus* sp. (Table 3). Further analysis revealed that the partial sequence of CI 10 was completely similar to the sequence of a reference *Enterococcus*

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Streptococcus species       AGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGG 250

Enterococcus species        AGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGG 250

Staphylococcus aureus       AGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGG 249

Staphylococcus species      AGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGG 249

Pseudomonas aeruginosa      AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGG 249

Pseudomonas species         AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGG 249

Escherichia coli            AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGG 249
                            ***    * ******   **** *** ************  ** *** **

Streptococcus pneumoniae    TTAGATAAGTCTGAAGTTAAAGGCTG**TGGCTTAACCATAGTAGGCT-TTG** 299 h

Streptococcus species       TTAGAAAAGTCTGAAGTGAAAGGCAGTGGCTCAACCATTGTAGGCT-TTG 299

Enterococcus species        TTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTG 300

Staphylococcus aureus       TTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTG 299

Staphylococcus species      TTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTG 299

Pseudomonas aeruginosa      TTCAGCAAGTTGGATGTGAAATCGCCGGGCTCAACCTGGGAACTGCAT**CC** 299 i

Pseudomonas species         TTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTC 299

Escherichia coli            TTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCT 299
                            **     ****  ** ** ***      **** ****    *         *

Streptococcus pneumoniae    **G**AAACTGTTTAACTTGAGTGCAAGAGGGGAGAGTGGAAT-CCATGTGTAG 348 h

Streptococcus species       GAAACTGTTTAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAG 349

Enterococcus species        GAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAG 350

Staphylococcus aureus       GAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAG 349

Staphylococcus species      GAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAG 349

Pseudomonas aeruginosa      **AAAACTACTGAGCTAGAGTACG**GTAGAGGGTGGTGGAATT-CCTGTGTAG 348 i

Pseudomonas species         AAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAG 349

Escherichia coli            GATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAG 349
                             * ***       ** ****     ** **   ** ****  *  ******

Streptococcus pneumoniae    CGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCT 398

Streptococcus species       CGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCT 399

Enterococcus species        CGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCT 400

Staphylococcus aureus       CGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGGACTTT 399

Staphylococcus species      CGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGGCTTT 399

Pseudomonas aeruginosa      CGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCAC 398

Pseudomonas species         CGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCAC 399

Escherichia coli            CGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCC 399
                            *********** *** **  * ***** *** ******** *** *

Streptococcus pneumoniae    CTGGCTTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCA|AACAGGAT| 448

Streptococcus species       CTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGGGAGCG|AACAGGAT| 449
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FIG. 1—*Continued*.

strain, as shown in Fig. 2B. Comparison of the reference *A. viridans* and *Enterococcus* strains showed five mismatches. Consequently, the probe specific for *Enterococcus* spp. yielded a sensitivity and specificity of 100% when sequencing was considered the gold standard.

DISCUSSION

New strategies for the detection and identification of bacterial pathogens in blood are continuously under investigation worldwide. Current diagnostic tools are hampered by several

TABLE 1. Probes designed for use in multiplex PCR

Probe	Sequence (5′–3′)[a]
Pseudomonas species	NED-CCTTCCTCCCAACTTAAAGTGCTT-MGB
P. aeruginosa	JOE-CCAAAACTACTGAGCTAGAGTACG-BHQ1
E. coli	JOE-GGAGTAAAGTTAATACCTTTGCTCATT-BHQ1
Staphylococcus species	NED-AATCTTCCGCAATGGGCGAAAGC-MGB
S. aureus	FAM-AGATGTGCACAGTTACTTACACATAT-BHQ1
Enterococcus species	JOE-TCCTTGTTCTTCTCTAACAACAGAG-BHQ1
Streptococcus species	NED-CCAGAAAGGGACSGCTAACT-MGB
S. pneumoniae	JOE-CCAAAGCCTACTATGGTTAAGCCA-BHQ1

[a] NED, fluorescent label (Applied Biosystems); MGB, minor groove binder; JOE, 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein; BHQ1, black hole quencher 1; FAM, 6-carboxyfluorescein.

factors, such as a lack of sensitivity, a long time to results, the presence of PCR inhibitory compounds in certain sample materials, and the occurrence of fastidious and nonculturable microorganisms. Rapid universal 16S rRNA gene-based PCR is often combined with sequence analysis, and a considerable number of commercial assays have been generated and tested recently, i.e., SeptiFast (Roche Diagnostics GmbH, Mannheim, Germany), Prove-it Sepsis (Mobidiag, Helsinki,

TABLE 2. Bacterial isolates in 232 positive blood cultures and results from universal and specific real-time PCR

Pathogen	Result of blood culture (no. [%])[a]								
	UNI	PSEU	PSEUAE	ECOLI	STAPH	STAU	ENTE	STREPT	STREPN
Gram-negative pathogens									
Acinetobacter lwoffii/haemolyticus	1 (100)	0	0	0	NA	NA	NA	NA	NA
Bacteroides fragilis	2 (100)	0	0	0	NA	NA	NA	NA	NA
Citrobacter koseri	1 (100)	0	0	0	NA	NA	NA	NA	NA
Enterobacter cloacae	3 (100)	0	0	0	NA	NA	NA	NA	NA
Escherichia coli	43 (100)	0	0	43	NA	NA	NA	NA	NA
Gram-negative rod	2 (100)	0	0	0	NA	NA	NA	NA	NA
Klebsiella oxytoca	5 (100)	0	0	0	NA	NA	NA	NA	NA
Klebsiella pneumoniae	7 (100)	0	0	0	NA	NA	NA	NA	NA
Moraxella catarrhalis	1 (100)	0	0	0	NA	NA	NA	NA	NA
Morganella morganii	1 (100)	0	0	0	NA	NA	NA	NA	NA
Neisseria meningitidis	1 (100)	0	0	0	NA	NA	NA	NA	NA
Prevotella buccae	1 (100)	0	0	0	NA	NA	NA	NA	NA
Proteus mirabilis	2 (100)	0	0	0	NA	NA	NA	NA	NA
Pseudomonas aeruginosa	10 (100)	10	10	0	NA	NA	NA	NA	NA
Pseudomonas oryzihabitans	1 (100)	1	0	0	NA	NA	NA	NA	NA
Serratia marcescens	4 (100)	0	0	0	NA	NA	NA	NA	NA
Gram-positive pathogens									
Aerococcus viridans	1 (100)	NA	NA	NA	0	0	1	0	0
Coagulase-negative *Staphylococcus* spp.	85 (100)	NA	NA	NA	85	9	0	0	0
Corynebacterium spp.	2 (100)	NA	NA	NA	0	0	0	0	0
Enterococcus avium	1 (100)	NA	NA	NA	0	0	1	0	0
Enterococcus faecalis	3 (100)	NA	NA	NA	0	0	3	0	0
Enterococcus faecium	4 (100)	NA	NA	NA	0	0	4	0	0
Lactobacillus spp.	1 (100)	NA	NA	NA	0	0	0	0	0
Propionibacterium acnes	1 (100)	NA	NA	NA	0	0	0	0	0
Staphylococcus aureus	31 (100)	NA	NA	NA	31	31	0	0	0
Streptococcus agalactiae	1 (100)	NA	NA	NA	0	0	0	1	0
Streptococcus group A and/or D	2 (100)	NA	NA	NA	0	0	0	2	0
Streptococcus milleri	3 (100)	NA	NA	NA	0	0	0	3	0
Streptococcus oralis	1 (100)	NA	NA	NA	0	0	0	1	0
Streptococcus pneumoniae	6 (100)	NA	NA	NA	0	0	0	6	6
Streptococcus pyogenes	1 (100)	NA	NA	NA	0	0	0	1	0
Streptococcus sanguis	1 (100)	NA	NA	NA	0	0	0	1	0
Mixed									
Alcaligenes faecalis + *Propionibacterium* spp.	1 (100)	0	0	0	0	0	0	0	0
Klebsiella oxytoca + *Serratia marcescens*	1 (100)	0	0	0	NA	NA	NA	NA	NA
Streptococcus salivarius + *Streptococcus viridans*	1 (100)	NA	NA	NA	0	0	0	1	0
Totals									
Concordant		11	10	43	116	31	8	16	6
Discordant		0	0	0	0	9	1	0	0

[a] UNI, universal probe; PSEU, *Pseudomonas* spp.; PSEUAE, *P. aeruginosa*; ECOLI, *E. coli*; STAPH, *Staphylococcus* spp.; STAU, *S. aureus*; ENTE, *Enterococcus* spp.; STREPT, *Streptococcus* spp.; STREPN, *S. pneumoniae*; NA, not applicable.

TABLE 3. Clinical isolates with discordant blood culture
and PCR results

CI[a]	Result of blood culture[a]			
	Specific probe(s)	*femA/sa442*	Coagulase activity	Sequence
1	STAPH, STAU	N/N	N	Unidentified
2	STAPH, STAU	N/N	N	*Staphylococcus hominis*
3	STAPH, STAU	N/N	N	*S. hominis*
4	STAPH, STAU	N/N	N	*Staphylococcus pasteuri*
5	STAPH, STAU	N/N	N	*S. hominis*
6	STAPH, STAU	N/N	N	*Staphylococcus schleiferi*
7	STAPH, STAU	P/P	P	*S. aureus*
8	STAPH, STAU	N/N	N	*S. pasteuri*
9	STAPH, STAU	N/N	N	*S. epidermidis*
10	ENTE	NA	NA	*Enterococcus* spp.

[a] Clinical isolates (CI) 1 to 9 are CoNS species, while CI 10 is *A. viridans*. STAPH, *Staphylococcus* spp.; STAU, *S. aureus;* ENTE, *Enterococcus* spp; N, negative; P, positive; NA, not applicable.

Finland), and SepsiTest (Molzym GmbH & Co. KG, Bremen, Germany). The latter includes a universal 16S rRNA gene-based PCR assay combined with sequence analysis, while the two other commercial assays use a panel of probes for the detection of a range of bacterial and mycotic pathogens. Virtually all studies conducted with the SeptiFast and SepsiTest assays found similar results, i.e., an overall agreement of 77 to 83% (2, 3, 6, 12, 13, 15, 16). Both assays are validated for whole-blood samples and can be completed in 4 to 6 h. Tissari et al. performed an observational study comparing conventional culture with Prove-it Sepsis. The assay, based on a microarray platform and performed with blood cultures, had a clinical sensitivity of 94.7% and a specificity of 98.8% (11).

In our study, the 16S rRNA gene was used for the design of eight species- or genus-specific probes. The sequence similarities found in *in silico* analysis (BLAST) were derived mainly from not clinically relevant microorganisms such as *Geobacil-*
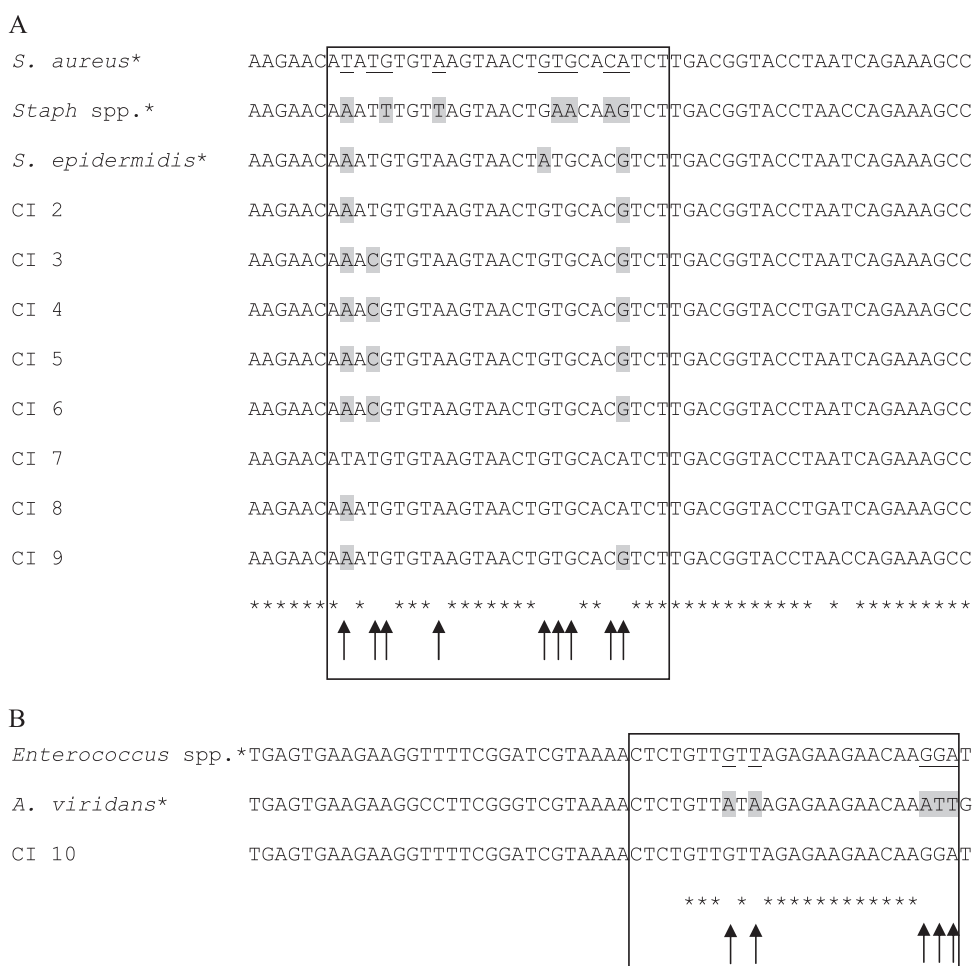

FIG. 2. Partial sequence alignments of the 16S rRNA gene, derived from the clinical isolates with discordant results. (A) Region where the probe specific for *S. aureus* binds. The sequences of the eight discrepancies are shown in the rectangle. The nucleotides underlined in the rectangle represent the nucleotides which distinguish *S. aureus* from *Staphylococcus* (*Staph*) spp. The sequence alignment of clinical isolate (CI) 7 is the exact reverse complement of the *S. aureus* probe sequence, which implicates that this isolate, which was identified as CoNS, is in fact *S. aureus*. CI 1 was not shown because the sequence could not be determined. GenBank accession numbers of reference strains are as follows: *S. aureus*, FN433596; *Staphylococcus* spp., GQ222246; and *S. epidermidis*, GQ911565. (B) Region where the probe specific for *Enterococcus* spp. binds. The nucleotides underlined in the rectangle represent the nucleotides which distinguish *Enterococcus* spp. from *A. viridans*. Conventional testing identified CI 10 as *Aerococcus viridans*, whereas our assay and sequencing classified it as an *Enterococcus* sp. GenBank accession numbers of reference strains are as follows: *Enterococcus* spp., AB489105; *A. viridans*, M58797. ∗, reference sequences.

lus spp., *Paenibacillus* spp., *L. plantarum*, and *Lactobacillus fermentum*. Remaining similarities were, if available, tested *in vitro* with reference strains. A limited number of microorganisms showed cross-reactivity. For example, streptococcal and staphylococcal probes weakly cross-reacted with *Bacillus cereus*. In these cases, sequencing determined the correct identification. Additionally, as the assay is always preceded by Gram staining, in most cases, morphology data obtained by Gram staining was in contradiction with these false-positive signals and targeted the samples for confirmatory sequencing.

Our multiprobe assay was designed for use with blood culture material and can be completed within 2 h. Use of whole-blood samples could significantly improve the turnaround time of our assay. However, since culturing still remains essential to determine the microorganism's antimicrobial profile, we chose to use blood cultures instead of whole blood. In this study, we found an overall agreement of 97% between conventional testing and our multiprobe assay. One of the strengths of our approach is that the assay can be extended by adding more probes for other bacterial pathogens to the identification panel. The grouping of Gram-negative and Gram-positive bacteria was based on the identifications needed for antimicrobial susceptibility testing. The assay was designed to use a universal probe for detection instead of Sybr green, because it is known that Sybr green can generate false-positive signals because of the presence of background human DNA or the formation of primer-dimers.

Analysis of the conflicting results showed that, in some cases, our assay was in accordance with sequencing results. The reference sequence derived from a *Staphylococcus* sp. and a *S. epidermidis* strain showed seven and three mismatches, respectively, with the reference sequence derived from an *S. aureus* strain. The sequences derived from the discrepant clinical isolates only showed three or fewer mismatches with the *S. aureus* sequence. This indicated that within the group of staphylococci, more homologous sequences can cause false positives because of the less efficient binding capacity of the *S. aureus*-specific probe.

Overall, these results showed strong agreement between conventional testing and our novel, real-time PCR assay. Furthermore, this assay significantly reduced the time needed for identification in comparison to that of routine diagnostics. In conclusion, using pathogen-specific probes offers a faster alternative for pathogen detection and could improve the diagnosis of bloodstream infections. The assay will be implemented in a clinical trial investigating the impact of earlier pathogen identification in combination with susceptibility testing with the choice of therapy.

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