Real-Time Quantitative Broad-Range PCR Assay for Detection of the 16S rRNA Gene Followed by Sequencing for Species Identification

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Here we determined the analytical sensitivities of broad-range real-time PCR-based assays employing one of three different genomic DNA extraction protocols in combination with one of three different primer pairs targeting the 16S rRNA gene to detect a panel of 22 bacterial species. DNA extraction protocol III, using lysozyme, lysostaphin, and proteinase K, followed by PCR with the primer pair Bak11W/Bak2, giving amplicons of 796 bp in length, showed the best overall sensitivity, detecting DNA of 82% of the strains investigated at concentrations of $\leq 10^2$ CFU in water per reaction. DNA extraction protocols I and II, using less enzyme treatment, combined with other primer pairs giving shorter amplicons of 466 bp and 342 or 346 bp, respectively, were slightly more sensitive for the detection of gram-negative but less sensitive for the detection of gram-positive bacteria. The obstacle of detecting background DNA in blood samples spiked with bacteria was circumvented by introducing a broad-range hybridization probe, and this preserved the minimal detection limits observed in samples devoid of blood. Finally, sequencing of the amplicons generated using the primer pair Bak11W/Bak2 allowed species identification of the detected bacterial DNA. Thus, broad-spectrum PCR targeting the 16S rRNA gene in the quantitative real-time format can achieve an analytical sensitivity of 1 to 10 CFU per reaction in water, avoid detection of background DNA with the introduction of a broad-range probe, and generate amplicons that allow species identification of the detected bacterial DNA by sequencing. These prerequisites are important for its application to blood-containing patient samples.

Detection and identification of bacteria causing illness are essential to guide patient management including antimicrobial therapy. This is especially important when bacteria invade normally sterile body sites, such as blood, cerebrospinal fluid, pleural fluid, or synovial fluid (21). Detection of bacteria in these body sites reassures clinicians about the chosen empirical antimicrobial therapy, may help to streamline antibiotic treatment once the antibiotic sensitivity of the isolate has been assessed, and allows for prognostic information. Potential benefits are reductions in side effects of antimicrobial therapy, in treatment costs, and in selection of resistant bacterial strains (9). The current standard for the diagnosis of invasive bacterial infections is microscopic examination and culture of body fluids considered to be sterile in healthy subjects. Nevertheless, this approach is neither very fast nor optimally sensitive.

Microscopy, although rapid, requires a relatively large concentration of bacteria ($\geq 10^4$ CFU/ml) to become positive (3), and identification based on morphology is often not possible. Furthermore, application of microscopy to blood samples is cumbersome, insensitive, and therefore not part of routine diagnostics. Culture results may be available only after 24 h to 72 h. Moreover, culture results may be false negative when fastidious or culture-resistant bacteria are involved or when patient samples are obtained after antimicrobial therapy has started. In these situations, broad-range PCR could offer an

important benefit, as it can detect any kind of bacterial DNA present in a sample through targeting conserved bacterial sequences. In addition, sequencing of the amplicon generated by broad-range PCR allows subsequent identification of the organism, as the PCR product contains variable bacterial species-specific sequences. These sequences can be identified by comparison with known sequences deposited at GenBank or other databases (6, 25). Prerequisites for sufficient analytical sensitivity and specificity are (i) protocols for efficient extraction of bacterial genomic DNA, (ii) primer pairs designed to amplify bacterial DNA as broadly as possible and to generate amplicons sufficiently long for bacterial identification, and (iii) a probe avoiding detection of background DNA known to be present in some samples, e.g., blood (20).

Several broad-range PCR assays have been reported in the literature. The majority of these assays use primers targeting the 16S rRNA gene (6, 8, 10, 12, 13, 15, 17, 22–24), and a minority use primers targeting the 23S rRNA gene (7, 14, 22). Since broad-range PCR is more vulnerable to contamination than species-specific PCR (16), its adaptation to a real-time PCR-based format that does not require removal of samples from closed containers for sample transfer, reagent addition, or gel separation could offer advantages in this respect. Furthermore, real-time PCR allows quantification of the bacterial load. Until now, broad-range real-time PCR assays have rarely been devised to identify bacterial DNA detected in clinical samples (12).

The aims of the present study using real-time PCR-based assays were (i) to determine the lower detection limits of different bacterial genomic DNA extraction protocols followed by ampli-

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TABLE 1. Bacterial strains used for this study

Species	Source
<i>Actinomyces meyeri</i>	Clinical isolate
<i>Bacillus cereus</i>	Clinical isolate
<i>Bacteroides fragilis</i>	ATCC 25285
<i>Corynebacterium diphtheriae</i>	Clinical isolate
<i>Enterobacter aerogenes</i>	Clinical isolate
<i>Enterobacter cloacae</i>	ATCC 23355
<i>Enterococcus faecalis</i>	Clinical isolate
<i>Escherichia coli</i>	Clinical isolate
<i>Haemophilus influenzae</i> type b	Clinical isolate
<i>Klebsiella oxytoca</i>	Clinical isolate
<i>Klebsiella pneumoniae</i>	ATCC 700603
<i>Neisseria meningitidis</i> serogroup B.....	Clinical isolate
<i>Nocardia asteroides</i>	Clinical isolate
<i>Pseudomonas aeruginosa</i>	Clinical isolate
<i>Salmonella enterica</i> serovar Enteritidis	Clinical isolate
<i>Staphylococcus aureus</i>	Clinical isolate
<i>Staphylococcus epidermidis</i>	Clinical isolate
<i>Stenotrophomonas maltophilia</i>	Clinical isolate
<i>Streptococcus agalactiae</i>	Clinical isolate
<i>Streptococcus milleri</i>	Clinical isolate
<i>Streptococcus pneumoniae</i>	Clinical isolate
<i>Streptococcus pyogenes</i>	Clinical isolate

fication of 16S rRNA gene sequences whereby different broad-range primer pairs detecting a panel of 22 bacterial species are used, (ii) to evaluate if the assays are applicable to blood samples spiked with bacteria, and (iii) to investigate whether identification of detected bacterial DNA by sequencing of the amplicons generated by real-time PCR using probes is feasible.

MATERIALS AND METHODS

Bacterial species. A panel of 22 (11 each of gram-positive and gram-negative) bacterial species, mainly those frequently isolated from normally sterile clinical samples from children in general or from children with febrile neutropenia, were obtained from the clinical laboratory (Division of Infectious Diseases, University Children's Hospital of Zurich, Zurich, Switzerland) or kindly provided by R. Zbinden (Institute of Medical Microbiology, University of Zurich, Zurich, Switzerland). The species and, where applicable, American Type Culture Collection (ATCC) numbers are listed in Table 1. The clinical isolates were identified by standard methods (18). Microorganisms were grown in standard cultures using sheep blood agar or chocolate agar. Between 24 h and 48 h after plating, four to six colonies of bacteria were resuspended in 0.9% NaCl solution and diluted until a McFarland standard of 0.5 was reached, representing approximately 10^8 CFU/ml. Starting from this concentration, 10-fold serial dilutions in physiological

saline were prepared. A volume of 100 μ l of each dilution was plated onto sheep blood agar or chocolate agar plates and then aerobically incubated overnight at 37°C to check the numbers of CFU. One-milliliter aliquots of the remainder of the dilutions were centrifuged at $20,000 \times g$ for 10 min, the supernatants discarded, and the pelleted bacteria stored at -80°C until further processing.

Detection of bacteria in spiked blood samples. For spiking of blood samples, *Staphylococcus aureus* and *Escherichia coli* were used. Pellets of 10^8 CFU were resuspended in 1 ml EDTA blood from a healthy adult, and a 10-fold dilution series using EDTA blood to dilute was prepared. To this bacterium-blood mixture, 2.5 ml erythrocyte lysis buffer (15.5 ml 1 M NH_4Cl , 1 ml 1 M KHCO_3 , 20 μ l 0.5 M EDTA, pH 8.0, and 100 ml *Limulus* amoebocyte lysate [LAL] reagent water) was added. Then, the mixture was incubated for 30 min on ice and shaken slightly by hand every 5 min. The mixture was centrifuged, the supernatant discarded, and the bacterial pellet stored at -80°C .

DNA extraction and purification. In order to compare their efficiencies, three different genomic DNA extraction and purification protocols were applied to the bacterial strains tested. For all three extraction methods, we started the extraction procedure from the same amount of bacterial pellet, i.e., the pellet of 1 ml of bacterial cell suspension with a McFarland standard of 0.5 as described above.

DNA extraction protocol I. DNA extraction protocol I was described earlier (8). In brief, a QIAamp DNA blood mini kit (QIAGEN, Basel, Switzerland) was used to extract bacterial DNA, with the following modifications to the instructions of the manufacturer. The bacterial pellet was resuspended in 200 μ l digestion buffer (50 mM Tris HCl, pH 8.5, 1 mM EDTA, 0.5% sodium dodecyl sulfate). The mixture was incubated at 55°C for 1 h after addition of proteinase K (19.2 mg/ml; Roche, Mannheim, Germany) to a final concentration of 0.4 mg/ml. The final elution volume was 100 μ l. For each PCR analysis, we used 1 μ l of this DNA extract.

DNA extraction protocol II. For DNA extraction protocol II, bacterial pellets were initially resuspended with 20 μ l TES buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl) and 5 U lysozyme (Ready Lyse; Epicenter, Madison, Wis.) and incubated for 12 h at room temperature. Then, protocol I was applied. The final elution volume was 100 μ l. For each PCR analysis, we used 1 μ l of this DNA extract.

DNA extraction protocol III. For DNA extraction protocol III, a Wizard SV genomic DNA purification system (Promega, Madison, Wis.) from the protocol Promega eNotes was used with the following modifications. The bacterial pellet was resuspended in 400 μ l enzymatic lysis solution (47 mM EDTA, 25 mg/ml lysozyme [Sigma, St. Louis, Mo.], 20 μ g/ml lysostaphin [Sigma]) and incubated at 37°C for 2 h. Then, proteinase K (19.2 mg/ml; Roche, Mannheim, Germany) was added to a final concentration of 0.4 mg/ml and the mixture was incubated at 55°C for 1 h. Nuclei Lysis solution (Promega) and RNase solution (Promega) were added, and after being mixed, the reaction solution was incubated at 80°C for 10 min. Further purification steps were done according to the instructions of the manufacturer. The final elution volume was 200 μ l. For each PCR analysis, we used 2 μ l of this DNA extract.

Design of primer pairs and probe. The sequences of the complete genome or of the 16S rRNA gene of all 22 bacterial species listed above were retrieved from GenBank. Three broad-range primer pairs targeting the 16S rRNA gene reported in the literature (8, 10, 17, 19) (Table 2) were validated using Clone Manager Suite 7 software (Scientific & Educational Software, Cary, North Caro-

TABLE 2. Oligonucleotide primers and TaqMan fluorescent probe sequences used for real-time PCR assays

Primer or probe name	Primer sense	Sequence (5'→3')	Position (bp) of 16S rRNA gene		Amplification product (bp) for <i>S. aureus</i> / <i>E. coli</i>	Reference(s)
			<i>S. aureus</i> (X68417) ^a	<i>E. coli</i> ATCC 25922 (X80724) ^a		
Bak11W	Forward	AGTTTGATC(A/C)TGGCTCAG	11–28	1–18	804/797	8
Bak2	Reverse	GGACTAC(C/T/A)AGGGTATCTAAT	795–814	778–797		8
Taf	Forward	TCCTACGGGAGGCAGCAGT	348–367	331–349	467/467	17, 19
Tar	Reverse	GGACTACCAGGGTATCTAATCCTGTT	789–814	772–797		17, 19
16SFa	Forward	GCTCAGATTGAACGCTGG		13–30	345/338	10
16SFb	Forward	GCTCAGGA(C/T)GAACGCTGG	23–40			10
16SR	Reverse	TACTGCTGCCTCCCGTA	351–367	343–349		10
Tap ^b		CGTATTACCGCGGCTGCTGGCAC	523–545	506–528		17, 19, 24

^a X68417 and X80724 are GenBank accession numbers.

^b Fluorophore and quencher, 5' 6-carboxyfluorescein and 3' 6-carboxytetramethylrhodamine.

TABLE 3. Numbers of cycles and temperature settings for PCR

Primer pair	Stage no.	No. of cycles	Temp (°C)	Time
Bak11W/Bak2	1	45	50	10 min
	2		95	10 min
	3		95	1 min
			50	1 min
			72	1 min
Tar/Taf	1	40	50	2 min
	2		95	10 min
	3		95	15 s
			60	1 min
16SFa/16SFb/16SR	1	35	50	10 min
	2		94	3 min
	3		94	30 s
			63	1 min
			72	1 min

lina). The published broad-range probe Tap (24) (Table 2) was originally designed to be used with the primer pair Taf/Tar, but it was confirmed in GenBank that the Tap sequence also lies within the amplicon defined by the primer pair Bak11W/Bak2.

Real-time PCR. The primer pairs specific for conserved DNA sequences encoding the 16S rRNA gene used are listed in Table 2. SYBR green PCR mix (Applied Biosystems, Cheshire, United Kingdom) consisted of 2.5 μ l SYBR green buffer, 3 μ l 25 mM MgCl₂, 2.5 μ l 12.5 mM deoxynucleoside triphosphate, 0.25 μ l Amp Erase, 0.13 μ l AmpliTaq Gold (5 U/ml), 2 μ l of each primer at a concentration of 10 mM (Microsynth, Balgach, Switzerland), 1 or 2 μ l of DNA extract (for protocols I and II, 1 μ l; for protocol III, 2 μ l), and LAL reagent water (Cambrex, Verviers, Belgium) to a final volume of 25 μ l. PCR was performed using an ABI PRISM 7700 sequence detector (Applied Biosystems). The numbers of cycles and the temperature settings used for the different primer pairs are indicated in Table 3. For the real-time PCR with the fluorogenic probe TaqMan universal PCR master mix, No AmpErase UNG (Applied Biosystems) was used with 0.5 μ M of each primer and 0.25 μ M of the probe, 2 μ l DNA, and LAL reagent water (Cambrex). Each PCR analysis was performed in duplicate.

The correct size of the PCR product from each assay was verified by running an amplified sample from each reaction tube on agarose gels stained with ethidium bromide.

Sensitivity, detection range, and specificity. To determine the detection range, we prepared a 10-fold dilution series from 10⁸ CFU/ml to 10⁰ CFU/ml. The arbitrary definition for a detectable concentration was a cycle threshold (C_T) value of 3 C_T values lower than the mean C_T value from the negative template control. This definition was chosen to have at least a 10-fold-higher concentration of detectable DNA in the positive samples than in the negative template control samples.

Sequencing. A BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, Calif.) was used to sequence the amplification products from *S. aureus* and *E. coli* generated following DNA extraction protocol III and PCR using the primer pair Bak11W/Bak2. Cycle sequencing (96°C for 1 min, followed by 30 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min) was performed with either Bak11W or Bak2. Sequencing products were purified by manual sodium acetate-ethanol precipitation. Following resuspension in 20 μ l LAL reagent water (Cambrex), the samples were analyzed with an ABI Prism 310 genetic analyzer (PerkinElmer, Boston, Mass.).

Bacterial identification. Database analysis was done in a two-step procedure as described previously (1). A first search was performed with the FASTA algorithm of the GCG Wisconsin software package (Accelrys). All positions showing differences from the best scoring reference sequence were visually inspected in the electropherogram, and the sequence was corrected if necessary. Then, a second search was done using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). Undetermined nucleotides in the determined sequence or the reference sequence were counted as matches. For each sequence, only the highest-scoring and, if applicable, the next-highest-scoring species were recorded.

RESULTS

Sensitivity of the PCR assay depends on DNA extraction protocol. In a first set of experiments, we determined the sensitivity of the broad-spectrum real-time PCR assay using genomic DNA extracted according to DNA extraction protocol I and amplified with the primer pair Bak11W/Bak2, as described by Goldenberger et al. (8). Targets were *S. aureus* as a representative of gram-positive bacteria and *E. coli* as a representative of gram-negative bacteria. Application of the real-time PCR assay to serial dilutions of the bacteria revealed that *S. aureus* could be detected only at concentrations of $\geq 10^3$ CFU per PCR (Fig. 1A) and *E. coli* at concentrations of $\geq 10^2$ CFU per PCR (Fig. 1B). Since the efficiency of DNA extraction from bacterial cells is one major determinant of the sensitivity of assays designed to detect bacterial DNA, we conducted a series of experiments aimed at an improvement of the extraction procedure, i.e., DNA extraction protocols II and III (see Materials and Methods) were used. Extraction protocol II resulted in no improvement in the sensitivity to detect *S. aureus* (Fig. 1C) or *E. coli* (Fig. 1D). By contrast, extraction protocol III allowed the detection of *S. aureus* and *E. coli* at concentrations as low as 10¹ CFU per PCR (Fig. 1E and F). These results suggested that extraction protocol III was superior to extraction protocols I and II in disrupting the bacterial cell wall, especially of gram-positive bacteria, allowing release of bacterial DNA and at the same time not significantly reducing its detection.

Sensitivity of the PCR assay depends on the choice of primer pairs. The length of the PCR product may be relevant for the sensitivity and detection range of a real-time PCR assay. Therefore, we tested and compared three primer pairs resulting in amplicons of different lengths (Table 2) on a dilution series of *S. aureus* and *E. coli* as representative strains for gram-positive and gram-negative bacteria, respectively, following extraction of genomic DNA with extraction protocol III. As presented in Fig. 2, the C_T values for the negative template controls using primer pair Bak11W/Bak2 (amplicon length, 796 bp) were higher than those for the primer pairs Taf/Tar (amplicon length, 466 bp) and 16SFa/16SFb/16SR (amplicon length, 346 bp). Thus, the primer pairs resulting in shorter amplicons showed lower C_T values for the negative template controls than the primer pair resulting in the longest amplicons. In turn, using the primer pair resulting in the longest amplicon allowed detection of *S. aureus* and *E. coli* at lower concentrations and within a larger linear detection range than using the primer pairs resulting in the shorter amplicons (Fig. 2). These results suggested that the combination of DNA extraction protocol III and the use of the primer pair Bak11W/Bak2 could result in the optimal sensitivity among the extraction and primer pair combinations investigated.

Analytical sensitivities of three different DNA extraction protocols followed by real-time PCR assays using three different primer pairs. Since the three DNA extraction protocols and the three primer pairs used in the previous experiments could possibly perform in a different way when applied to bacterial species other than *S. aureus* and *E. coli*, we next assessed the analytical sensitivities of the nine combinations of the three different primer pairs following the three different DNA extraction protocols for 22 bacterial strains (Table 4).

FIG. 1. Comparison of analytical sensitivities of the broad-spectrum real-time PCR assays in water using primer pair Bak11W/Bak2 following extraction of genomic DNA using three different extraction protocols. (A and B) Extraction protocol I. (C and D) Extraction protocol II. (E and F) Extraction protocol III. (A, C, and E) Detection of *S. aureus*. (B, D, and F) Detection of *E. coli*. Results shown are the means and standard deviations of three independent experiments. SYBR green was included in the PCR mix to allow for detection of amplicons. The dashed lines are drawn at the C_T of the negative control minus 3 C_T values, defining the lower limit of detection.

Use of the primer pair Bak11W/Bak2 following DNA extraction protocol I resulted in detection of 14 (64%) of the 22 strains at concentrations of $\leq 10^2$ CFU per PCR, following extraction protocol II in detection of 16 (73%) strains, and following extraction protocol III in detection of 18 (82%) strains. By contrast, the use of the primer pair Taf/Tar resulted in the detection of 10 (46%), 16 (73%), and 6 (27.3%) of the 22 strains following their extraction at concentrations of $\leq 10^2$

CFU per PCR using DNA extraction protocols I, II, and III, respectively. The primer pair 16SFa/16SFb/16SR detected one (5%), two (9%), and none (0%) of the 22 tested bacterial strains at concentrations of $\leq 10^2$ CFU per PCR when using DNA extraction protocols I, II, and III, respectively. Whereas DNA extraction protocol III proved to be somewhat superior than DNA extraction protocols I and II for the detection of gram-positive bacteria, DNA extraction protocols I and II were

FIG. 2. Comparison of analytical sensitivities of the broad-spectrum real-time PCR assays in water using three different primer pairs following extraction of genomic DNA by protocol III. Results shown are the means and standard deviations of three independent experiments. SYBR green was included in the PCR mix to allow for detection of amplicons. The dashed lines are drawn at the C_T of the negative control of a given primer pair minus 3 C_T values, defining the lower limit of detection of this primer pair.

superior than DNA extraction protocol III for the detection of gram-negative bacteria at concentrations of $\leq 10^2$ CFU per PCR when using the primer pair Bak11W/Bak2. Nevertheless, DNA extraction protocol III followed by PCR using the primer pair Bak11W/Bak2 showed the best overall sensitivity for bacteria at concentrations of $\leq 10^2$ CFU per PCR (Table 4). Thus, we used DNA extraction protocol III and the primer pair Bak11W/Bak2 in the subsequent experiments.

Sensitivity of the PCR assay in blood samples spiked with bacteria. Since a broad-spectrum real-time PCR assay should also serve to diagnose bloodstream infections, we assessed the sensitivity of the assay when applied to whole blood. For this purpose, whole blood was spiked in two separate experiments with serial dilutions of *S. aureus* and *E. coli*, respectively, prior to DNA extraction using protocol III and subsequent PCR using primer pair Bak11W/Bak2, the combination that had

shown the best sensitivity for the detection of bacteria at concentrations of $\leq 10^2$ per PCR, and final detection using SYBR green. The results were compared with the results obtained using LAL water instead of blood. As shown in Fig. 3, PCR targeting 16S rRNA in blood samples not experimentally mixed with bacteria resulted in positive signals at around 13 C_T values below the C_T values obtained for LAL reagent water not mixed with bacteria (27 versus 40). PCR with whole-blood samples mixed with *S. aureus* or *E. coli* showed C_T values that were similar for all bacterial concentrations of $\leq 10^4$ CFU per PCR and interpreted as negative, i.e., less than 3 U below the negative control. These results indicated that, when the concentration of the bacteria introduced into the blood samples was reduced below a certain level, broad-spectrum PCR detected nonspecific DNA sequences interfering with detection of DNA sequences from the bacteria introduced. In conse-

TABLE 4. Synopsis of analytical sensitivities of the real-time broad-range PCR assays in relation to primer pairs and extraction protocols used

Strain	Detection limit (CFU/PCR)								
	DNA extraction protocol I			DNA extraction protocol II			DNA extraction protocol III		
	Bak11W/ Bak2	Taf/Tar	16SFa/16SFb/ 16SR	Bak11W/ Bak2	Taf/Tar	16SFa/16SFb/ 16SR	Bak11W/ Bak2	Taf/Tar	16SFa/16SFb/ 16SR
Gram-positive strains									
<i>Actinomyces meyeri</i>	10 ³	10 ⁴	10 ⁶	10 ³	10 ²	10 ⁴	10 ¹	10 ³	10 ⁶
<i>Bacillus cereus</i>	1	10 ⁴	10 ⁶	10 ²	10 ²	10 ⁴	1	10 ³	10 ⁴
<i>Corynebacterium diphtheriae</i>	10 ³	10 ²	10 ⁶	10 ⁴	10 ¹	10 ⁶	1	10 ²	10 ⁶
<i>Enterococcus faecalis</i>	10 ³	10 ⁴	10 ⁶	10 ²	10 ²	10 ⁴	10 ²	10 ³	10 ⁶
<i>Nocardia asteroides</i>	10 ²	10 ¹	10 ⁶	>10 ⁶	10 ⁴	>10 ⁶	10 ⁵	10 ⁴	10 ⁶
<i>Staphylococcus aureus</i>	10 ³	10 ⁴	10 ⁶	10 ⁴	10 ⁴	10 ⁶	10 ²	10 ²	10 ⁴
<i>Staphylococcus epidermidis</i>	10 ³	10 ⁴	10 ⁶	10 ⁴	10 ⁴	10 ⁴	1	10 ⁴	10 ⁴
<i>Streptococcus agalactiae</i>	10 ³	10 ¹	10 ⁶	10 ²	10 ³	10 ⁶	10 ²	10 ³	10 ⁴
<i>Streptococcus milleri</i>	10 ²	10 ³	10 ⁶	10 ²	10 ²	10 ⁴	10 ¹	10 ⁴	10 ⁴
<i>Streptococcus pneumoniae</i>	10 ³	10 ³	10 ⁶	10 ²	10 ²	10 ⁴	10 ¹	10 ⁴	10 ⁴
<i>Streptococcus pyogenes</i>	10 ³	10 ³	10 ⁶	10 ²	10 ³	10 ⁴	10 ²	10 ²	10 ⁴
Gram-negative strains									
<i>Bacteroides fragilis</i>	1	10 ¹	10 ⁶	1	1	10 ⁶	10 ²	10 ⁴	10 ⁶
<i>Enterobacter aerogenes</i>	1	10 ²	10 ⁴	10 ²	10 ¹	10 ²	10 ²	10 ²	10 ⁴
<i>Enterobacter cloacae</i>	10 ²	10 ²	10 ⁴	10 ²	1	10 ²	10 ⁴	10 ³	10 ⁶
<i>Escherichia coli</i>	10 ²	10 ³	10 ⁴	10 ²	10 ²	10 ⁴	1	10 ²	10 ⁴
<i>Haemophilus influenzae</i> type b	10 ²	10 ²	10 ⁴	1	10 ¹	10 ⁴	10 ²	10 ²	10 ⁴
<i>Klebsiella oxytoca</i>	10 ¹	10 ²	10 ⁴	10 ²	10 ²	10 ⁴	1	10 ³	10 ⁴
<i>Klebsiella pneumoniae</i>	10 ²	10 ³	10 ⁴	10 ²	10 ²	10 ⁴	10 ⁴	10 ³	10 ⁴
<i>Neisseria meningitidis</i> serogroup B	10 ²	10 ²	10 ⁴	10 ²	10 ²	10 ⁴	10 ²	10 ³	10 ⁴
<i>Pseudomonas aeruginosa</i>	1	10 ²	10 ⁴	10 ²	10 ³	10 ⁴	10 ²	10 ⁴	10 ⁴
<i>Salmonella enterica</i> serovar Enteritidis	1	10 ³	10 ²	10 ²	10 ²	10 ⁴	10 ¹	10 ⁴	10 ⁶
<i>Stenotrophomonas maltophilia</i>	10 ²	10 ³	10 ⁴	10 ²	10 ²	10 ⁴	10 ³	10 ⁴	10 ⁴

quence, the analytical sensitivity of the assay applied to blood samples was diminished by at least 2 to 3 orders of magnitude. The presence of background DNA in blood has been reported previously (20). To exclude external bacterial contamination in the negative blood controls, we sequenced the amplification products obtained in these samples. The nucleotide sequences did not match with any known bacterial sequence or with human DNA (data not shown).

Sensitivity of the PCR assay in blood samples spiked with bacteria by using a TaqMan probe. To overcome the hurdle of background DNA detection, we introduced a TaqMan probe replacing SYBR green to detect amplicons. The probe used was originally designed for the primer pair Taf/Tar (Table 2). A GenBank evaluation confirmed that this probe had a position inside amplicons from the primer pair Bak11W/Bak2. Replacement of SYBR green with the TaqMan probe resulted in C_T values for blood samples not spiked with bacteria of ≥ 40 (Fig. 4). This indicated that the probe did not detect amplified background DNA in blood samples. Furthermore, the C_T values for *S. aureus* and *E. coli*, respectively, were similar in whole blood and saline for bacterial concentrations of >10 CFU per PCR (Fig. 4). Thus, the introduction of the TaqMan probe prevented the detection of background DNA and maintained the sensitivity of the assay.

Sequencing of amplicons to identify bacterial species in the samples. Finally, we wondered whether sequencing of amplicons generated by PCR following extraction of genomic DNA by extraction protocol III and using the primer pair Bak11W/Bak2 for amplification allowed the identification of the bacterial species present in the original samples. Both primers

Bak11W and Bak2 were used to sequence the amplicons from both ends. For *S. aureus*, $\geq 91\%$ sequence agreement was found at concentrations of $\geq 10^3$ CFU per PCR, at least with one of the two sequencing primers, and for *E. coli*, $>90\%$ agreement at concentrations of ≥ 10 CFU per PCR (Table 5).

DISCUSSION

In this study we evaluated three DNA extraction protocols followed by broad-range real-time PCR assays using three different primer pairs targeting the 16S rRNA gene in the detection of a panel of 22 bacterial species. Our results show that the broad-range real-time PCR assays described herein detected as few as 1 to 10 CFU in water per reaction. The introduction of a hydrolysis probe with a fluorochrome prevented the detection of interfering bacterial background DNA in blood samples. The length of the amplicons generated by real-time PCR was sufficient to allow for identification of the bacteria present in the samples by sequencing.

The minimal detection limits of our broad-range real-time PCR assays following DNA extraction protocols that used different amounts of enzymes varied between 10 and 10^6 CFU per PCR for the bacterial species investigated. The extraction protocol III described here, which uses lysozyme, lysostaphin, and proteinase K, combined with PCR using the primer pair Bak11W/Bak2 and resulting in amplicons of 796 bp in length showed the best overall analytical sensitivity for the detection of bacteria in water at concentrations of $\leq 10^2$ per PCR, with a $>80\%$ detection rate. For selected bacterial species, including the gram-positive species *Bacillus cereus*, *Corynebacterium*

FIG. 3. Analytical detection ranges and sensitivities of the broad-spectrum real-time PCR assays using the primer pair Bak11W/Bak2 following DNA extraction protocol III from blood samples spiked with bacteria. SYBR green was used for detection. (A) Detection of *S. aureus*. (B) Detection of *E. coli*. Results shown are the means and standard deviations of three independent experiments.

diphtheriae, *Staphylococcus epidermidis*, *Streptococcus milleri*, and *Streptococcus pneumoniae* and the gram-negative species *E. coli* and *Klebsiella oxytoca*, the minimal detection limit was as low as 1 CFU per PCR. Thus, the primer set resulting in the longest amplicons was overall the most sensitive. This may

seem counterintuitive as shorter amplicons should amplify more efficiently than the long amplicon. Shorter amplicons, however, showed lower C_T values than the longest amplicons when negative template samples were tested and thus reduced the analytical sensitivity. Nevertheless, depending on the bac-

FIG. 4. Analytical detection ranges and sensitivities of the broad-spectrum real-time PCR assays using the primer pair Bak11W/Bak2 following DNA extraction protocol III from blood samples spiked with bacteria. A TaqMan probe was used for detection. (A) Detection of *S. aureus*. (B) Detection of *E. coli*. Results shown are the means and standard deviations of three independent experiments.

TABLE 5. Sequencing of amplicons from templates with different concentrations and similarities to the template species

Template species	Concn (CFU/PCR)	Bak11W			Bak2		
		Position (bp) of analyzed fragment ^a	% Similarity to template species	Next species (% similarity) ^b	Position (bp) of analyzed fragment ^a	% Similarity to template species	Next species (% similarity) ^b
<i>S. aureus</i>	10 ⁵	52–273	95	Only <i>S. aureus</i> ^c	1–580	92	Only <i>S. aureus</i> ^c
	10 ⁴	90–233	93	Only <i>S. aureus</i> ^c	1–608	93	Only <i>S. aureus</i> ^c
		133–204	97	Only <i>S. aureus</i> ^c	120–391	93	Only <i>S. aureus</i> ^c
		82–260	88	Only <i>S. aureus</i> ^c	1–611	85	<i>Staphylococcus pseudintermedius</i> sp. nov. (85)
	10 ²	157–197	100	Only <i>S. aureus</i> ^c			
		1–579	No <i>S. aureus</i>	<i>E. coli</i> (88)	1–659	No <i>S. aureus</i>	<i>Enterobacter agglomerans</i> (87)
<i>E. coli</i>	10	1–566	No <i>S. aureus</i>	<i>E. coli</i> (85)	1–563	No <i>S. aureus</i>	<i>Serratia marcescens</i> (90)
	10 ⁵	1–566	90	<i>Serratia odorifera</i> (90)	109–225	97	<i>Shigella boydii</i> (98)
	10 ⁴	130–505	93	<i>Shigella boydii</i> (93)	151–217	91	<i>Shigella boydii</i> (92)
	10 ³	77–444	93	<i>Shigella boydii</i> (93)	188–238	92	<i>Serratia</i> sp. (92)
	10 ²	1–566	94	<i>Shigella boydii</i> (94)	122–210	97	<i>Shigella boydii</i> (97)
	10	122–309	86	<i>Shigella boydii</i> (92)	1–605	95	<i>Serratia</i> sp. (96)

^a A fragment of the whole sequence with the best sequencing quality was analyzed.

^b First species not being *S. aureus* or *E. coli*, respectively.

^c Among all entries listed.

terial species, the use of DNA extraction protocol I or II in combination with the same or other primer pairs in too many instances yielded lower minimal detection limits than extraction protocol III combined with the primer pair Bak11W/Bak2. This was especially the case for gram-negative species. These results suggest that the combination of DNA extraction protocol and primer pair determines analytical sensitivity. Indeed, currently there seems to be no DNA extraction protocol that has the same effectiveness for both gram-positive and gram-negative bacteria. Most studies of broad-spectrum PCR assays have reported the use of commercial kits, enzyme treatment, freezing and thawing or boiling, mechanical disruption, or a combination of these methods (7, 10, 12, 13, 17, 19, 24, 26) for DNA extraction. Minimal detection limits were determined only for *S. aureus* or *E. coli* or both and were in the range between 10 and 10³ CFU or CFU equivalents per PCR (7, 10, 12, 13, 17, 19, 24, 26). Thus, the minimal detection limit of our assays for these two bacterial species was among the lowest reported so far. Our results further highlight the fact that the combinations of bacterial DNA extraction protocols and primer pairs are differently suited for detection of different bacterial species. This needs to be taken into account when evaluating the analytical sensitivities of broad-range PCR assays and calls for the determination of minimal limits of sensitivity for a panel of bacterial species. Nevertheless, the performance of the assays presented here needs to be studied with clinical samples before the assays can be introduced into routine diagnostics.

The introduction of a broad-range probe targeting a highly conserved sequence within the sequence amplified by the broad-range primer pairs prevented the detection of background DNA in blood samples spiked with bacteria and protected the sensitivity of the assay when applied to bacteria diluted in saline. The application of broad-range PCR to blood specimens has yielded results suggesting the presence of bacterial DNA in healthy individuals (20). Recently, the use of a broad-range probe proved to be extremely useful in avoiding the detection of background DNA (19, 26). The fluorescein-tagged probe Tap used in this study was originally described

for the primer pair Taf/Tar (19). We confirmed by a GenBank evaluation that this probe had a position inside amplicons from the primer pair Bak11W/Bak2. Yang et al. reported an elegant innovation, i.e., the combination of broad-range primer pairs with a broad-range probe and a species-specific probe that circumvents nonspecific DNA detection while conserving the sensitivity and the specificity of the assay (26). At present, this system is limited by the number of fluorophores commercially available and the discriminatory power of the detection instruments. Therefore, such a multiprobe real-time PCR assay can at present be designed only for a very restricted number of bacterial species.

A major observation of this study was that a primer pair generating amplicons of more than 500 bp could be used in a real-time PCR format. The amplification products of 804 bp for *S. aureus* and 797 bp for *E. coli* generated by the primer pair Bak11W/Bak2 allowed identification of the bacterial species present in the sample by sequencing. Species identification by sequencing required at least 10 to 10³ bacterial CFU in the original PCR sample (Table 5), which was 1 to 2 orders of magnitude higher than the analytical sensitivity of the assay per reaction in water. It should be noted that *Shigella* shares a very high DNA homology with *E. coli* and should be included in the same species (18). Further, species differentiation within *Enterobacteriaceae* based on sequencing is generally difficult due to high sequence homologies (18). In this context, it needs to be stressed that the maximum length of determined sequences was 540 bp. Thus, since sequencing was performed from both ends the sequenced portions were not identical. This explains why, depending on the sequencing direction, the sequences did not match. Nevertheless, the assay reported here represents a remarkable step towards improvement of the identification of the bacterial culprit when using broad-range real-time PCR assays. This is of considerable clinical interest given the limited possibilities mentioned in designing a multiprobe real-time PCR.

In this study we made no attempts to decontaminate PCR materials but used highly purified reagents such as AmpliTaq Gold and LAL reagent water. Broad-range PCR assays are more vulnerable to contamination than species-specific PCRs.

Taq DNA polymerases are frequently reported as an important source of contaminating bacterial DNA (2, 4). In agarose gel electrophoresis detection systems, several approaches, including UV irradiation, 8-methoxypsoralen treatment, DNase treatment, and restriction endonuclease treatment, have successfully overcome this problem (11). However, most decontamination procedures also affect the sensitivity of a broad-range PCR when a sensitive detection system, such as TaqMan, is used (5). One exception to this is the pre-PCR ultrafiltration step recently described by Yang et al. (26). In our assays, the negative template control showed C_T values between 38 and 40, suggesting the absence of contaminating eubacterial DNA in that control. This and our arbitrary definition of detectable bacterial DNA concentration set at 3 C_T values below the C_T value of the negative template control, i.e., detection of DNA only when present at least at concentrations 10-fold higher than that in the negative control, did not induce us to perform an ultrafiltration of the PCR master mix. Nevertheless, we used the purified AmpliTaq Gold DNA polymerase (26). The omission of decontamination methods might be risky in a clinical or research laboratory.

In conclusion, the DNA extraction protocol III presented here combined with the broad-range primer pair Bak11W/Bak2 plus the Tap probe validated in a real-time PCR format is suitable for the detection and quantification of bacteria not only in otherwise-sterile body fluids but also in blood samples and in addition allows for bacterial speciation of the detected DNA by sequencing. The time required from start to finish of the 16S rRNA detection is 8 to 9 h, and the time required for sequencing is 3 to 4 h. Thus, results can be obtained within 1 to 2 working days. Therefore, the quantitative broad-range PCR assay reported here not only may serve as a tool for an improved etiologic diagnosis, for pathogenetic studies, and for monitoring of invasive bacterial infections but also may be used to identify bacterial contamination of blood products.

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REFERENCES

1. Bosshard, P. P., A. Kronenberg, R. Zbinden, C. Ruef, E. C. Bottger, and M. Altwegg. 2003. Etiologic diagnosis of infective endocarditis by broad-range polymerase chain reaction: a 3-year experience. *Clin. Infect. Dis.* **37**:167–172.
2. Böttger, E. C. 1990. Frequent contamination of *Taq* polymerase with DNA. *Clin. Chem.* **36**:1258–1259.
3. Brun-Buisson, C., M. Fartoukh, E. Lechapt, S. Honore, J. R. Zahar, C. Cerf, and B. Maitre. 2005. Contribution of blinded, protected quantitative specimens to the diagnostic and therapeutic management of ventilator-associated pneumonia. *Chest* **128**:533–544.
4. Carroll, N. M., P. Adamson, and N. Okhravi. 1999. Elimination of bacterial DNA from *Taq* DNA polymerases by restriction endonuclease digestion. *J. Clin. Microbiol.* **37**:3402–3404.
5. Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, E. B. Kaczmarek, and A. J. Fox. 2000. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. *J. Clin. Microbiol.* **38**:1747–1752.
6. Drancourt, M., C. Bollet, R. Carlioz, R. Martelin, J. P. Gayral, and D. Raoult. 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J. Clin. Microbiol.* **38**:3623–3630.
7. Dreier, J., M. Stormer, and K. Kleesiek. 2004. Two novel real-time reverse transcriptase PCR assays for rapid detection of bacterial contamination in platelet concentrates. *J. Clin. Microbiol.* **42**:4759–4764.
8. Goldenberger, D., A. Kunzli, P. Vogt, R. Zbinden, and M. Altwegg. 1997. Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. *J. Clin. Microbiol.* **35**:2733–2739.
9. Gould, I. M. 2002. Antibiotic policies and control of resistance. *Curr. Opin. Infect. Dis.* **15**:395–400.
10. Harris, K. A., and J. C. Hartley. 2003. Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service. *J. Med. Microbiol.* **52**:685–691.
11. Heininger, A., M. Binder, A. Ellinger, K. Botzenhart, K. Unertl, and G. Doring. 2003. DNase pretreatment of master mix reagents improves the validity of universal 16S rRNA gene PCR results. *J. Clin. Microbiol.* **41**:1763–1765.
12. Klaschik, S., L. E. Lehmann, A. Raadts, M. Book, J. Gebel, A. Hoeft, and F. Stuber. 2004. Detection and differentiation of in vitro-spiked bacteria by real-time PCR and melting-curve analysis. *J. Clin. Microbiol.* **42**:512–517.
13. Klaschik, S., L. E. Lehmann, A. Raadts, M. Book, A. Hoeft, and F. Stuber. 2002. Real-time PCR for detection and differentiation of gram-positive and gram-negative bacteria. *J. Clin. Microbiol.* **40**:4304–4307.
14. Kotilainen, P., J. Jalava, O. Meurman, O. P. Lehtonen, E. Rintala, O. P. Seppala, E. Eerola, and S. Nikkari. 1998. Diagnosis of meningococcal meningitis by broad-range bacterial PCR with cerebrospinal fluid. *J. Clin. Microbiol.* **36**:2205–2209.
15. Ley, B. E., C. J. Linton, D. M. Bennett, H. Jalal, A. B. Foot, and M. R. Millar. 1998. Detection of bacteraemia in patients with fever and neutropenia using 16S rRNA gene amplification by polymerase chain reaction. *Eur. J. Clin. Microbiol. Infect. Dis.* **17**:247–253.
16. Millar, B. C., J. Xu, and J. E. Moore. 2002. Risk assessment models and contamination management: implications for broad-range ribosomal DNA PCR as a diagnostic tool in medical microbiology. *J. Clin. Microbiol.* **40**:1575–1580.
17. Mohammadi, T., H. W. Reesink, C. M. Vandenbroucke-Grauls, and P. H. Savelkoul. 2003. Optimization of real-time PCR assay for rapid and sensitive detection of eubacterial 16S ribosomal DNA in platelet concentrates. *J. Clin. Microbiol.* **41**:4796–4798.
18. Murray, P. R., E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Tenover (ed.). 1999. *Manual of clinical microbiology*, 7th ed. ASM Press, Washington, D.C.
19. Nadkarni, M. A., F. E. Martin, N. A. Jacques, and N. Hunter. 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* **148**:257–266.
20. Nikkari, S., I. J. McLaughlin, W. Bi, D. E. Dodge, and D. A. Relman. 2001. Does blood of healthy subjects contain bacterial ribosomal DNA? *J. Clin. Microbiol.* **39**:1956–1959.
21. Peters, R. P., M. A. van Agtmael, S. A. Danner, P. H. Savelkoul, and C. M. Vandenbroucke-Grauls. 2004. New developments in the diagnosis of blood-stream infections. *Lancet Infect. Dis.* **4**:751–760.
22. Rantakokko-Jalava, K., S. Nikkari, J. Jalava, E. Eerola, M. Skurnik, O. Meurman, O. Ruuskanen, A. Alanen, E. Kotilainen, P. Toivanen, and P. Kotilainen. 2000. Direct amplification of rRNA genes in diagnosis of bacterial infections. *J. Clin. Microbiol.* **38**:32–39.
23. Schuurman, T., R. F. De Boer, A. M. Kooistra-Smid, and A. A. Van Zwet. 2004. Prospective study of use of PCR amplification and sequencing of 16S ribosomal DNA from cerebrospinal fluid for diagnosis of bacterial meningitis in a clinical setting. *J. Clin. Microbiol.* **42**:734–740.
24. Warwick, S., M. Wilks, E. Hennessy, J. Powell-Tuck, M. Small, J. Sharp, and M. R. Millar. 2004. Use of quantitative 16S ribosomal DNA detection for diagnosis of central vascular catheter-associated bacterial infection. *J. Clin. Microbiol.* **42**:1402–1408.
25. Wilson, K. H., R. B. Blichington, and R. C. Greene. 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *J. Clin. Microbiol.* **28**:1942–1946.
26. Yang, S., S. Lin, G. D. Kelen, T. C. Quinn, J. D. Dick, C. A. Gaydos, and R. E. Rothman. 2002. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. *J. Clin. Microbiol.* **40**:3449–3454.