JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2002, p. 3449–3454 Vol. 40, No. 9
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.9.3449–3454.2002

Quantitative Multiprobe PCR Assay for Simultaneous Detection and Identification to Species Level of Bacterial Pathogens

Samuel Yang,[1] Shin Lin,[1] Gabor D. Kelen,[1] Thomas C. Quinn,[1,2] James D. Dick,[1] Charlotte A. Gaydos,[1] and Richard E. Rothman[1]*

Department of Emergency Medicine, The Johns Hopkins University School of Medicine, Baltimore,[1] and National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda,[2] Maryland

Received 29 January 2002/Returned for modification 29 March 2002/Accepted 16 June 2002

We describe a novel adaptation of the TaqMan PCR assay which potentially allows for highly sensitive detection of any eubacterial species with simultaneous species identification. Our system relies on a unique multiprobe design in which a single set of highly conserved sequences encoded by the 16S rRNA gene serves as the primer pair and is used in combination with both an internal highly conserved sequence, the universal probe, and an internal variable region, the species-specific probe. A pre-PCR ultrafiltration step effectively decontaminates or removes background DNA. The TaqMan system described reliabAly detected 14 common bacterial species with a detection limit of 50 fg. Further, highly sensitive and specific pathogen detection was demonstrated with a prototype species-specific probe designed to detect *Staphylococcus aureus*. This assay has broad potential in the clinical arena for rapid and specific diagnosis of infectious diseases.

Currently, the standard method for diagnosing the presence of bacterial pathogens in clinical samples relies on culture techniques. However, active research is under way using new molecular methods to decrease detection time and increase assay sensitivity. PCR has emerged as the molecular method of choice in achieving these objectives. The utility of PCR and other molecular methods is evidenced by the recent guidelines issued by the NCCLS in 1999 encouraging the use of such methods in clinical laboratories performing bacterial identification assays (11).

To detect the presence of any bacterial pathogen in a clinical sample, primers annealing to regions of DNA conserved across a wide range of bacterial genomes have been employed. The design of such universal primers has often focused on the 16S rRNA gene (17). The presence of multiple copies of this gene within the bacterial genome facilitates its amplification by PCR. Further, sufficient sequence variability allows phylogenetic information to be attained for the purposes of microbial identification. However, up to the present, assays which provide for both universal detection and species identification require a second post-PCR processing step, which can be technically cumbersome and slow the time to reporting of results (9, 14).

Universal PCR-based bacterial detection systems have also been hampered by contamination issues. High sequence conservation of the DNA region chosen for PCR primer annealing coupled with the immense amplification power of PCR results in the amplification of exceedingly minor bacterial contaminants, leading to false positives. Attempts to decontaminate PCR materials have involved nearly all known methods to destroy DNA, including UV irradiation, 8-MOP treatment, and incubation with various enzymes, such as DNase, restriction enzymes, or both in combination (2, 4). Thus far, none of these methods has been shown to be entirely effective or reproducible.

Assessment of bacterial contamination can most reliably be made using real-time detection methods to characterize PCR amplification. Briefly, real-time PCR amplifications are reported by the cycle number at which the PCR product accumulates significantly over baseline levels, as detected by interaction with fluorogenic probes (C_T) (7). Aside from saving time and labor, this technique has been shown to be more objective and consistent than the traditional methods of amplification detection and starting template quantification involving gel electrophoresis (5, 13). With this more precise technique, Corless et al. found that most decontamination methods decreased PCR sensitivity (4). The implication of this finding was that the decontamination effect of the aforementioned methodologies could at least in part be explained by a retardation of the sensitivity of the PCR amplification system.

The quantitative capacity of real-time PCR has thus redefined the standards by which a decontamination method is measured. Not only will a particular method be required to yield negative for controls under the more precise probe-based real-time system but also the method must be shown to preserve the sensitivity of the PCR assay. In this report, we describe a multiprobe-based real-time PCR system involving the 16S rRNA gene, which allows for simultaneous detection of the presence of eubacterial DNA with species-specific discrimination. In addition, we report a decontamination method for the present PCR system which does not compromise detection sensitivity.

MATERIALS AND METHODS

Bacterial species and DNA isolation. Fifteen common pathogenic microorganisms, all of which were eubacterial except one, *Candida albicans*, were obtained from the clinical laboratory (Division of Medical Microbiology, Johns Hopkins School of Medicine, Baltimore, Md.). The species and American Type Culture Collection (ATCC) numbers are listed in Table 1. Microorganisms were grown in standard cultures, and DNA was extracted using the QIAamp DNA kit (Qiagen Corp., Santa Clarita, Calif.).

* Corresponding author. Mailing address: Department of Emergency Medicine, Johns Hopkins School of Medicine, Baltimore, MD 21205. Phone: (410) 614-5933. Fax: (410) 502-8881. E-mail: rrothman@jhmi.edu.

TABLE 1. Sensitivity of the Taqman assay using universal primers and probes

Isolated microorganisms	Isolate type or ATCC no.	Taqman PCR results
Staphylococcus aureus	29213	+
Staphylococcus hominis	Clinical isolate	+
Staphylococcus epidermidis	Clinical isolate	+
Streptococcus agalactiae	Clinical isolate	+
Streptococcus pneumoniae	49619	+
Klebsiella pneumoniae	990603	+
Listeria monocytogenes	Clinical isolate	+
Enterococcus faecalis	29212	+
Escherichia coli	25922	+
Proteus mirabilis	25933	+
Chlamydia pneumoniae	Clinical isolate	+
Neisseria gonorrhoeae	Clinical isolate	+
Neisseria meningitidis	Clinical isolate	+
Haemophilus influenzae (type A)	49247	+
Candida albicans	Clinical isolate	−

With regard to generating standard curves for starting DNA template quantification, *Staphylococcus aureus* (ATCC 29213) was grown in Luria-Bertani broth (Gibco/BRL Life Technologies Inc., Gaithersburg, Md.) at 37°C with continuous shaking to an optical density at 600 nm of 0.6. Equal aliquots were then plated to determine CFU and subjected to DNA extraction with the QIAamp DNA kit. The isolated DNAs were quantified based on optical density at 260 nm and then serially diluted. Analogous DNA isolation procedures were performed for three other strains of *S. aureus* (ATCC strains 02131, 15923, and 43300) as well as *Staphylococcus epidermidis* and *Staphylococcus hominis* to permit testing of both sensitivity and specificity of the SA probe.

Design of primers and probes. The 16S rRNA gene sequences from a variety of bacterial species were obtained from GenBank. Sequence data were obtained using the program Entrez (see the list below). The sequences were aligned using the program ClustalW from the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw.htm). Two regions of highly conserved sequences, separated by both an internal region of highly variable sequence and another adjacent internal region of highly conserved sequence, were selected as the universal primer annealing sites. The internal highly conserved and highly variable sequences were used as the annealing sites of conserved and species-specific TaqMan probes, respectively (Fig. 1).

The primers and TaqMan probes were designed according to the guidelines in the ABI Primer Express software program (PE Applied Biosystems, Foster City, Calif.). This program selects probes and primer sets with optimized melting temperatures, secondary structure, base composition, and amplicon lengths. The forward primer (p891F) and reverse primer (p1033R) amplify a fragment of 161 bp spanning nucleotides 891 to 1051 of the *S. aureus* 16S rRNA gene (Table 2). The universal TaqMan probe, or UniProbe, was labeled with the reporter dye VIC at the 5′ end, and the quencher dye TAMRA was labeled at the 3′ end and has the sequence which is the reverse complement of nucleotides 1002 to 1024 of the 16S rRNA gene (see list of sequence accession numbers below) (Table 2). *S. aureus*-specific probe, or SAProbe, was designed as the species-specific probe. The SAProbe was labeled with a different reporter dye, FAM, at the 5′ end and the same quencher dye at the 3′ end, with the sequence which spans nucleotides 946 to 976 of the *S. aureus* 16S rRNA gene (Table 2). The probes were designed to anneal to opposite strands of the template DNA. The primers and probes were manufactured by PE Applied Biosystems.

PCR master mix and fluorogenic-probe-based PCR (TaqMan assay). Reactions were performed in 50-μl volumes in 0.5-ml optical-grade PCR tubes (PE-Applied Biosystems). PCR master mix was prepared from the TaqMan core reagent kit (PE-Applied Biosystems). The master mix was comprised of 200 μM (each) dATP, dGTP, dUTP, and dCTP, 0.5 U of AmpErase uracil-DNA glycosylase (UNG), 2.5 mM $MgCl_2$, 1× TaqMan buffer A, 900 nM concentrations of each primer, and 100 nM concentrations of each fluorescence-labeled probe (UniProbe and/or SAProbe). Template DNA, 2 U of AmpliTaq Gold DNA polymerase (PE-Applied Biosystems), and water were added to give a final volume of 50 μl for each sample. The fluorogenic-probe-based PCR, or TaqMan assay, was performed using the ABI 7700 sequence detection system (PE-Applied Biosystems). The cycling conditions used were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min each. All PCRs were performed in triplicate.

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.

Ultrafiltration of the PCR mix. An ultrafiltration step, using the Amicon Microcon YM-100 centrifugal filter device (Millipore Corporation, Bedford, Mass.) was utilized for filtering the PCR mix prior to addition of template DNA. The PCR mix that underwent ultrafiltration included the PCR master mix and AmpliTaq Gold DNA polymerase. This filtration device prevents the passage of potential contaminating double-stranded DNA of 125 bp or greater. The PCR mix was spun through the YM-100 device at 100 × *g* for 30 min.

Post-PCR analysis. Amplification data were analyzed by the SDS software (PE-Applied Biosystems), which calculates ΔR_n using the equation $R_n(+) - R_n(-)$. $R_n(+)$ is the emission intensity of the reporter divided by the emission intensity of the quencher at any given time, whereas $R_n(-)$ is the value of $R_n(+)$ prior to PCR amplification. Thus, ΔR_n indicates the magnitude of the signal generated. The threshold cycle, or C_T, is the cycle at which a statistically significant increase in ΔR_n is first detected. The C_T is inversely proportional to the starting amount of target DNA. Amplification plots were generated by plotting ΔR_n versus C_T (5, 15).

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences determined in this study are as follows: *S. aureus*, AF015929; *S. hominis*, AY030318; *Enterococcus faecalis*, AJ276460; *S. epidermidis*, L37605; *Streptococcus pneumoniae,* X58312; *Mycoplasma pneumoniae*, AF132741; *Escherichia coli*, AF233451; *Haemophilus influenzae*, AF224306; *Legionella pneumoniae*, M59157; *Neisseria meningitidis*, AF059671; *Rickettsia rickettsii*, U11021; *Borrelia burgdorferi*, AF091368; *Bacillus anthracis*, AF290552; *Yersinia pestis*, AF366383; *Proteus mirabilis*, AF008582; and *Klebsiella pneumoniae*, AF228919.

RESULTS

Sensitivity of Universal TaqMan PCR. The sensitivity of the primers and probes used for universal amplification of eubacterial 16S rRNA gene was first assessed with genomic DNA extracts from 14 different bacterial species. An isolate from *Candida albicans* was used as a negative control (Table 1). In each PCR assay, 5 ng of purified DNA was used. The assay's positivity was determined by examination of the amplification plot (C_T versus ΔR_n) generated by the sequence detection software (Fig. 1). All 14 bacterial species were correctly amplified and detected, with C_T values in the range of 19.2 to 21.8. No amplification ($C_T > 40$) was detected when DNA isolated from *C. albicans* was used. The assay results were further verified by subjecting reaction products to gel electrophoresis, with visualization of bands of the expected size (162 bp) (data not shown).

FIG. 1. Design of primers and probes. The forward (P891F) and reverse (P1033R) primers anneal to highly conserved regions of the 16S rRNA gene. An internal highly conserved region was selected as the annealing site of the UniProbe, and the other internal region of highly variable sequence was selected as the annealing sites of a prototype species-specific SAProbe.

TABLE 2. Oligonucleotide sequences of primers and probes used in the study

Oligonucleotide	Sequence (5′ → 3′)[a]	Position[b] (bp)	Fluorophores	T_m[c] (°C)
Forward primer (P891F)	TGGAGCATGTGGTTTAATTCGA	891–912		59.1
Reverse primer (P1033R)	TGCGGGACTTAACCCAACA	1051–1033		58.6
UniProbe	CACGAGCTGACGACARCCATGCA	1024–1002	VIC, TAMRA	67.3/69.3
SAProbe	CCTTTGACAACTCTAGAGATAGAGCCTTCCC	946–976	FAM, TAMRA	65.3

[a] Sequences used for alignment were from the following strains: *S. aureus, S. hominis, E. faecalis, S. epidermidis, E. faecalis, S. pneumoniae, M. pneumoniae, E. coli, H. influenzae, L. pneumoniae, N. meningitidis, R. rickettsii, B. burgdorferi, B. anthracis, Y. pestis, P. mirabilis,* and *K. pneumoniae.* See Materials and Methods for sequence accession numbers.
[b] Nucleotide position based on *S. aureus* sequences.
[c] T_m, melting temperature.

Theoretical detection limit of TaqMan PCR. The detection limit of the TaqMan assay was determined by amplifying serial dilutions of eubacterial DNA. The minimal detection limit of the TaqMan system was defined as the amount of template DNA at which the relationship between C_T and starting template DNA became nonlinear. Serial dilutions of *S. aureus* DNA (50 ng to 5 fg) were added to PCRs with universal primers (p891F plus p1033R) and probe (UniProbe). The results are shown in Table 3. The standard curve in which C_T values were plotted against starting template DNA is linear from 50 ng to 5 pg (Fig. 2). At DNA levels below 5 pg, this relationship became nonlinear, and the C_Ts were similar to the C_T of the no-template control (NTC). This suggested the presence of contaminating eubacterial DNA in the NTC. The minimal detection limit of the assay was thus 5 pg of *S. aureus* DNA.

As an effort to improve the detection limit of the assay, we implemented a prefiltration step for the PCR mix prior to the addition of template DNA, in addition to the conventional precautionary measures used for reducing contaminating or carryover DNA present in PCR reagents. The filtration device retains contaminating DNA but allows for passage of all components of the PCR mix, including primers, probes, *Taq* polymerase, and UNG. Addition of this prefiltration step increased the C_T of NTC to 40, effectively reducing the amount of contaminating DNA (Table 3). C_Ts at DNA levels (50 ng to 5 pg) remained comparable with or without the prefiltration step. Furthermore, the C_T values of starting DNA template below 5 pg and those of that greater than 5 pg all fell on the same line, with an r^2 value of 0.998. (Fig. 2).

The efficiency (E) of the prefiltered PCR amplification was calculated to be 1.94 (maximum = 2) based on the following equation: $E = e^{(-1/\text{slope})}$.

The minimum detection limit of the assay with prefiltration was 50 fg of *S. aureus* DNA (Table 3). Based on the size of the *S. aureus* genome, which is approximately 2,750 kbp, 50 fg of *S. aureus* DNA is equivalent to approximately 15 genomes or CFU. This was calculated as follows: 2,750 kb is equal to 1.8×10^6 g/mol; division of this value by Avogadro's number, 6×10^{23}, yields 3 fg per *S. aureus* genome. Comparable results were derived empirically based on counting CFU on plates.

TABLE 3. Detection limit of the TaqMan assay with or without prefiltration

Sample type	C_T for template DNA concn (~ equivalent CFU)[a] of:								
	NTC	50 ng (10^7)	5 ng (10^6)	500 pg (10^5)	50 pg (10^4)	5 pg (10^3)	500 fg (10^2)	50 fg (10^1)	5 fg
(−) Filter	31.5	16.2	19.1	22.5	25.8	29.5	31.6	31.3	
(+) Filter	40	16.8	19.6	22.8	26.2	30.0	33.7	37.8	>40

[a] Mean values, based on triplicate samples.

The amplified products were subsequently subjected to gel electrophoresis. Visualization of the bands under UV irradiation confirmed the expected amplicon size (data not shown).

Multiprobe assay. In order to demonstrate the assay's ability to simultaneously detect the presence of any eubacterial DNA as well as one or more species of interest within a single reaction tube, a prototype species-specific probe for *S. aureus* (SAProbe) was designed. The SAProbe was labeled with a different reporter fluorophore so that its signal could be distinguished from that of the universal probe. Real-time PCR assays were performed with the universal primer set, UniProbe, and SAProbe in a single reaction mix. Template DNA samples from each of three closely related *Staphylococcus* species, *S. aureus*, *S. epidermidis*, and *S. hominis*, were tested in different reactions. For *S. aureus*, four strains were tested (ATCC 02131, 15923, 29213, and 43300). The assay correctly detected the presence of eubacterial DNA in all samples under

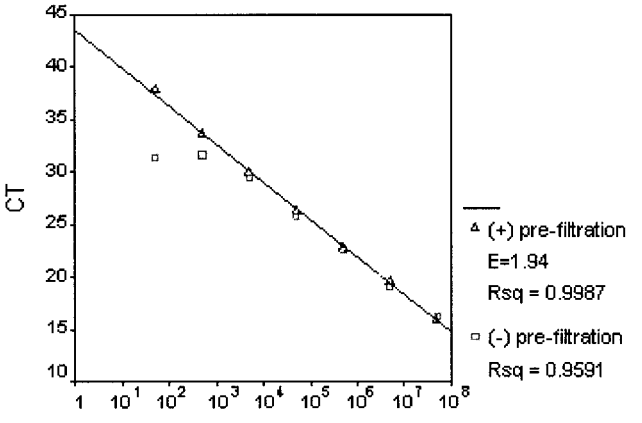

FIG. 2. Inverse linear relationship of C_T versus starting template DNA with and without prefiltration. C_Ts were generated from PCRs with starting template *S. aureus* DNA serially diluted 1:10 from 50 ng to 5 fg. In the nonprefiltration curve (squares), nonlinearity is encountered at starting template DNA quantities below 5 pg, suggesting background eubacterial DNA contamination of this amount. With prefiltration (triangle), the detection limit was extended to 50 fg.

TABLE 4. Sensitivity and specificity of the multiprobing PCR assay

Probe	Result for:						
	NTC	*S. aureus* (ATCC 29213)	*S. aureus* (ATCC 02131)	*S. aureus* (ATCC 15923)	*S. aureus* (ATCC 43300)	*S. epi*[a,b]	*S. hominis*[b]
VIC-UniProbe	−	+	+	+	+	+	+
FAM-SAProbe	−	+	+	+	+	−	−

[a] *S. epidermidis.*
[b] Refer to Table 1 for ATCC strain.

the VIC dye layer. When the detection system was reconfigured to detect the FAM dye layer in the same reaction tubes, only the reaction containing the various strains of *S. aureus* DNA yielded a positive signal (Table 4).

For further experiments involving *S. aureus*, strain 29213 was used. PCR results for reactions using UniProbe versus those for reactions using SAProbe showed no significant differences in C_T values for amplifications with equivalent amounts of *S. aureus* DNA (Table 5). Detection equivalence is also described in Fig. 3 with standard curves in which C_Ts are plotted against starting template DNA. The similar slopes of the two lines indicate equal efficiencies; the coinciding extrapolated axis intercepts indicate equal detection limits.

PCR results obtained by using UniProbe with serial dilutions of *S. epidermidis* template DNA yielded results similar to those obtained with *S. aureus* template DNA. Specifically, plots of C_Ts against starting template DNA revealed almost equivalent amplification efficiencies between the two (Fig. 4). PCR results obtained by using SAProbe with serial dilutions of *S. epidermidis* template DNA gave no detection (Table 5).

Finally, PCRs using both probes with a constant amount of *S. epidermidis* template DNA and serial dilutions of *S. aureus* template DNA were performed. SAProbe results coincided with the titration in which only *S. aureus* DNA was serially diluted. UniProbe results differed, however. At concentrations in which *S. aureus* DNA predominated over that of *S. epidermidis*, the C_Ts of the UniProbe results were comparable with those of the SAProbe results. As the amount of *S. aureus* DNA was diluted and the *S. epidermidis* DNA became predominant, the C_Ts leveled off at a value corresponding to the constant amount of *S. epidermidis* DNA added to each reaction tube (Table 5).

DISCUSSION

Current methods of universal detection with species identification include PCR amplification with a universal primer set followed by performance of species identification assays, such as oligonucleotide array, restriction digestion, or sequencing (1, 6, 8, 12). Another variation has been to universally amplify cultured clinical samples and then subject the amplified product to hybridization using different sets of specific probes (10). Regardless of the methodology, virtually all techniques for universal detection and species identification of bacteria have thus far involved at least two sequential steps.

With the probe-based PCR system described here, both steps can for the first time be accomplished simultaneously. The probe-based PCR system we have devised is comprised of a universal primer set, a universal probe, and a species-specific probe. In this way, detection of amplification and extraction of sequence information from amplicons can be performed within the confines of the PCR run, eliminating the need for post-PCR manipulations. This innovation reduces overall assay time to about 2 h or less, depending on the PCR instrument used, while conserving the sensitivity and specificity of the assay.

The only present limitation of this system is inherent in the number of fluorophores commercially available and the discriminatory power of the detection instrument itself, which presently can simultaneously differentiate up to four different fluorophores in a single tube (15). Thus, the number of species-specific probes which may be included in an individual reaction (in addition to the universal probe) is restricted.

Simultaneous detection and/or species identification of microorganisms in a given sample has been reported recently with the multiplexing technique, with multiple sets of species-specific primer pairs and probes corresponding to different amplification targets (3). Our novel PCR design in which multiple probes, including a universally conserved one, exploit regions within the same amplification target does confer several advantages over multiplexing. First, possible competition between multiple PCR primer pairs is avoided with multiprobing, which involves only a single pair of primers. Moreover, when technologies advance to allow for more fluorophores, expansion of the multiprobe system for detection of other templates will require only the addition of another probe. This is in contrast to multiplexing, in which both new primers and probes will have to be added to the reaction mixture. In that circum-

TABLE 5. Comparison of multiprobing PCR results in single versus dual infection

Probe	C_T of the following sample types in multiprobe PCR[a]											
	SA				SE				200 pg of SE + SA			
	50 ng	500 pg	5 pg	50 fg	20 ng	200 pg	2 pg	20 fg	50 ng	500 pg	5 pg	50 fg
VIC-UniProbe	16.8	22.8	30.0	37.8	18.1	24.8	32.9	38.2	16.6	22.8	24.1	25.0
FAM-SAProbe	17.4	23.7	31.1	>40	>40	>40	>40	>40	17.2	23.1	32.0	>40

[a] SA, *S. aureus;* SE, *S. epidermidis.*

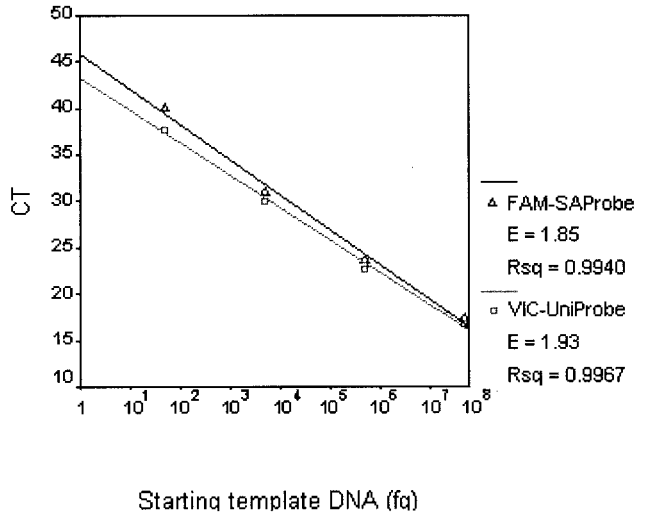

FIG. 3. Comparison of the standard curves for *S. aureus* DNA using UniProbe and SAProbe. PCRs generating the C_Ts for the curves contained both UniProbe and SAProbe, as well as *S. aureus* starting template DNA, which was serially diluted 1:100 from 50 ng to 50 fg. That the lines corresponding to UniProbe (square) and SAProbe (triangle) nearly overlay one another indicates comparable amplification efficiencies and detection limits for the different probes used in the same reaction mixtures.

stance, the new primers may not amplify under the original PCR conditions, which will thus require optimization studies to accommodate all the primer pairs. Finally, even if suitable conditions are ultimately attained, amplification efficiencies may be altered such that standard curves for starting template

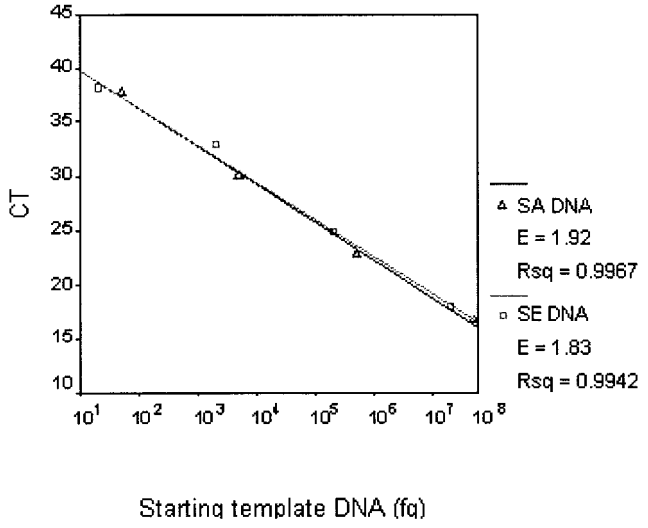

FIG. 4. Comparison of the standard curves for *S. aureus* and *S. epidermidis* DNA obtained by using UniProbe. PCRs generating the C_Ts for the curves contained UniProbe and either *S. aureus* or *S. epidermidis* starting template DNA. *S. aureus* DNA was serially diluted 1:100 from 50 ng to 50 fg, whereas *S. epidermidis* DNA was diluted from 20 ng to 20 fg. The near equivalency of the lines indicates that UniProbe has equal detection capacity irrespective of whether *S. aureus* DNA (triangle) or *S. epidermidis* DNA (square) is used as the starting template.

quantification will need to be rederived. Since multiprobing involves only a single target region, quantification of starting template will in principle rely on only one standard curve, if all probes are designed with a similar melting temperature.

With regard to contamination, residual bacterial DNA from various sources has historically prevented widespread use of universal primer sets in PCR-based assays. In our experience, pretreatment of PCR reagents with restriction enzymes followed by heat inactivation did eliminate amplification in the negative controls. However, C_T values for positive controls from runs with pretreated reagents were consistently greater than those without pretreatment (data not shown). Whether these findings reflected residual restriction enzyme activity even after heat inactivation, or systemic inhibition of the PCR system by the addition of restriction enzyme, is unknown.

The contamination problem was eventually resolved by passing PCR reagents through Microcon YM-100 centrifugal filter devices (Millipore Corporation). Of note, these filters allow decontamination of all PCR reagents, including UNG, *Taq* polymerase, primers, and probes, which was not possible using other methods, such as DNase treatment. Although Centricons have been employed for decontamination purposes in the past, heretofore their adequacy in the context of real-time PCR systems had not been studied (16). With prefiltration, the PCR efficiency of the system was not reduced. In addition, with significant reduction in background contamination, the prefiltration step improved the minimum detection limit of the assay from 5,000 to 50 fg of *S. aureus* DNA. The mean C_T of the negative control was 40. In our experience, the C_T values of negative controls, although consistently above 35, were variable. These results were not unexpected, since greater sampling errors are encountered at low starting template concentrations (4).

Finally, although the probes used in our real-time PCR system were empirically found to be specific for the organisms tested, there exists the theoretical possibility that the assay will yield a false-positive result when applied more broadly. This is an inherent limitation of design, based on the extent of the sequence search carried out, which in turn is dictated by the intended clinical application of the assay. As such, future studies may require more exhaustive bioinformatic analysis followed by even broader clinical validation of specificity. Nevertheless, the concept of multiprobing in a genomic region comprised of conserved and highly variable stretches remains a valid technique.

In conclusion, we have devised a rapid, highly sensitive and specific molecular assay which allows for the simultaneous detection, quantification, and species identification of bacterial organisms. Further development in the technology of species-specific probes will theoretically expand the number of organisms which can be identified using this method, and future research will address this. In principle, the methodology described here could be coupled with DNA extraction protocols using clinical samples derived from any bodily fluid or tissue and thereby provide an assay for diagnosing numerous diseases of infectious etiology. Such an addition to the clinicians' diagnostic armamentarium will better equip physicians to carry out expedited risk assessment and targeted antibiotic treatment.

ACKNOWLEDGMENTS

This study was partly funded by the National Institutes of Health [3 M01RR00052-39-5(S1)] and an Emergency Medicine Foundation Center Grant.

REFERENCES

1. **Anthony, R. M., T. J. Brown, and G. L. French.** 2001. DNA array technology and diagnostic microbiology. Expert Rev. Mol. Diagn. **1:**30–38.
2. **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.
3. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, A. J. Fox, and E. B. Kaczmarski.** 2001. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J. Clin. Microbiol. **39:**1553–1558.
4. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, E. B. Kaczmarski, and A. J. Fox.** 2000. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. J. Clin. Microbiol. **38:**1747–1752.
5. **Gerard, C. J., K. Olsson, R. Ramanathan, C. Reading, and E. G. Hanania.** 1998. Improved quantitation of minimal residual disease in multiple myeloma using real-time polymerase chain reaction and plasmid-DNA complementarity determining region III standards. Cancer Res. **58:**3957–3964.
6. **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.
7. **Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams.** 1996. Real time quantitative PCR. Genome Res. **6:**986–994.
8. **Lu, J. J., C. L. Perng, S. Y. Lee, and C. C. Wan.** 2000. Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. J. Clin. Microbiol. **38:**2076–2080.
9. **Martinez, G., J. Harel, and M. Gottschalk.** 2001. Specific detection by PCR of Streptococcus agalactiae in milk. Can. J. Vet. Res. **65:**68–72.
10. **McCabe, K. M., Y. H. Zhang, B. L. Huang, E. A. Wagar, and E. R. McCabe.** 1999. Bacterial species identification after DNA amplification with a universal primer pair. Mol. Genet. Metab. **66:**205–211.
11. **NCCLS.** 1999. Molecular diagnostic methods for infectious diseases. Approved guideline MM3-A. NCCLS, Wayne, Pa.
12. **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.
13. **Schmittgen, T. D., B. A. Zakrajsek, A. G. Mills, V. Gorn, M. J. Singer, and M. W. Reed.** 2000. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. Anal. Biochem. **285:**194–204.
14. **Shang, S., Z. Chen, and X. Yu.** 2001. Detection of bacterial DNA by PCR and reverse hybridization in the 16S rRNA gene with particular reference to neonatal septicemia. Acta Paediatr. **90:**179–183.
15. **Vet, J. A., A. R. Majithia, S. A. Marras, S. Tyagi, S. Dube, B. J. Poiesz, and F. R. Kramer.** 1999. Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc. Natl. Acad. Sci. USA **96:**6394–6399.
16. **Wages, J. M., Jr., D. Cai, and A. K. Fowler.** 1994. Removal of contaminating DNA from PCR reagents by ultrafiltration. BioTechniques **16:**1014–1017.
17. **Wilson, K. H., R. B. Blitchington, and R. C. Greene.** 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J. Clin. Microbiol. **28:**1942–1946.