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Bacterial Species Identification after DNA Amplification with a Universal Primer Pair

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The diagnosis of bacterial infections can be difficult and time consuming. Rapid and reliable molecular triage of potentially infected patients, particularly the young and the elderly, would prevent unnecessary hospitalizations, reduce associated medical costs, and improve the quality of care. Polymerase chain reaction (PCR) amplification utilizing a universal bacterial primer pair, followed by hybridization with species-specific probes, would allow rapid identification of the presence or absence of bacterial DNA, along with an identification of the bacterial species present. Molecular microbiological analyses will require access to bacterial strain standards that can be catalogued and distributed to clinical laboratories. We amplified template DNA in filter paper spots containing boiled bacteria from 14 clinical isolates using a universal primer pair for the 16S ribosomal RNA (rDNA) coding sequence. Species-specific probes were hybridized to the amplification products for bacterial species identification. We conclude that template DNA can be identified with species-specific probes after universal bacterial amplification with a single primer pair. We also demonstrate a rapid and efficient method for the long-term storage and cataloguing of bacterial DNA for use in quality control at clinical laboratories adopting molecular diagnostic methodologies. We speculate that PCR amplification combined with species-specific probe hybridization not only will represent an improvement over culture-based methods in terms of speed, sensitivity, and cost, but will also allow for the identification of unculturable bacteria and emerging or reemerging pathogenic organisms.

Key Words: **ribosomal RNA, 16S; polymerase chain reaction (PCR); Bacterial identification; infectious agents, bacteria; molecular diagnostics; DNA amplification; amplification, DNA.**

Currently, the diagnosis of sepsis in immunocompromised individuals, including the young infant and the elderly patient, may pose a difficult task for clinicians (1–10). The evaluation of young, febrile patients requires a combination of clinical skill and laboratory support, particularly from the microbiology laboratory. Due to the time necessary for growth of cultures and the controversial discriminatory ability of clinical algorithms, a large number of patients without bacterial infections are admitted to the hospital to receive parenteral antibiotics (4–14). A rapid and reliable method for the identification of bacteria in blood and other body fluids would greatly decrease the number of hospitalizations and their attendant medical costs, while improving the quality of care (15–17).

Amplification of bacterial DNA using the polymerase chain reaction (PCR) (18) is both rapid and sensitive. There are two basic strategic approaches to PCR amplification of bacterial DNA in a clinical setting (17). The first strategy involves the use of a unique PCR primer pair for each bacterial species (19–25). This species-specific amplification strategy has been shown to be effective for the identification of a variety of organisms. However, this approach

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lacks the ability to determine definitely that bacterial infection is the cause of the patient's symptoms, since the absence of a PCR amplification product may simply mean that the primer pair for this patient's organism was not among those in the panel tested.

The second approach involves amplification with a universal bacterial primer pair, utilizing sequences that are found in all bacteria, but not in other organisms (15) This approach is valuable for initial evaluation of the presence of bacteria when the specific pathogen is unknown, as in "rule out sepsis," and, assuming appropriate sensitivity, a negative amplification reaction will indicate that no bacterial DNA is present. However, if a positive amplification is observed then it will indicate the presence of bacterial DNA, and additional work will be required to identify the specific organism.

Our group and others have shown that the 16S ribosomal RNA-coding sequences (16S rDNA) contain regions that are found in all bacteria and not in human DNA (15, 26). In addition, these regions flank species specific variable sequences (26–34). In this report, we demonstrate that PCR amplification with a single universal 16S rDNA primer pair allows species-specific identification of common pathogenic organisms by probe hybridization with an oligonucleotide designed uniquely for the individual bacterium. In addition, we describe a simple method for cataloguing and distributing bacterial isolates for molecular microbiological methods, involving spotting and drying boiled bacterial specimens on blotting paper.

METHODS AND MATERIALS

Sample Preperation

Clostridium perfringens, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Listeria monocytogenes, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhimurium, Shigella flexneri, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus pneumoniae, and *Streptococcus pyogenes* were obtained from clinical isolates of patient specimens. Bacteria were collected by centrifugation of liquid cultures and the pelleted organisms were placed in a boiling water bath for 10 min. Bromophenol blue (2.5 μL of a 0.25 mg/mL) was added to a 50-μL aliquot of each of the boiled bacterial samples. These colored samples were then spotted onto gel blot paper (No. GB002, Intermountain Scientific, Kaysville, UT), allowed to air dry, and stored at ambient temperature.

16S rDNA Amplification with Universal Primers

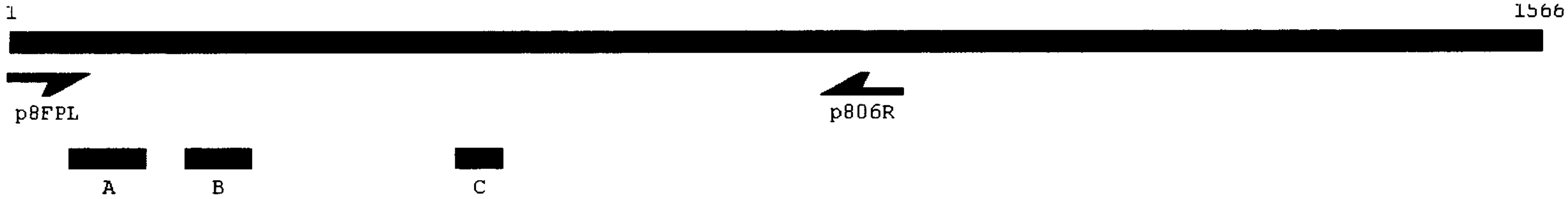
PCR amplification was performed with the highly conserved 16S rDNA primer pair, p8FPL and p806R (29, 31), using a modification of the procedure that we reported previously (15). Each PCR consisted of 10 μL of 2.5mM dNTPs (Perkin–Elmer, Norwalk, CT), 10 μL of *Taq* polymerase buffer (10×, Boehringer-Mannheim, Amsterdam, the Netherlands), 1 μL of each of the two primers (0.5 mM, synthesized on an ABI Model 394 DNA/RNA synthesizer, Foster City, CA), and *Taq* polymerase (8 units, Perkin–Elmer), brought to 100 μL with deionized water (Millipore Milli-Q, Bedford, MA) filtered through a 0.22-μm nylon filter, to which template was added as a 2-mm square cut from the dried blotting paper spot containing heat-killed bacteria. The reaction was then covered by 50 μL of mineral oil (Sigma, St. Louis, MO).

Amplification conditions were modified from the three temperature cycle, described previously (15), to a two-temperature cycle, with extension occurring during the temperature ramp between annealing and denaturation. PCR involved initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min and annealing at 55°C for 1 min and a final extension at 72°C for 7 min. The PCR products were then separated on a 0.8% SeaKem ME agarose (FMC BioProducts, Rockland, ME) gel containing 1 mM ethidium bromide for visualization on a UV light box.

Design of Species-Specific Probes

The species-specific probes were developed by examining the 16S rDNA sequences from a variety of bacterial species in GenEMBL, a nonoverlapping nucleotide sequence database. Sequences in the database were identified using the program Stringsearch (GCG, Madison, WI). Since complete 16S rDNA sequence data were not available in the database for *P. mirabilis* when these experiments were

FIG. 1. The diagram of the 16S rRNA gene illustrates the flanking of the divergent Region A, Region B, and Region C by the universal primer pair, p8FPL and p806R. The DNA sequence alignments of the divergent regions show the species-specific probes in bold italics.



Region A

```
                 71                                                                                                150
C.perfringens    ....GATGAA GTT...TCTT CGGGAAACGG ATTAGCGGCG GACGGGTGAG TAACACGTGG GTAACCTGCC TCATAGAGTG
L.monocytogenes  AACGGAGGAA GAG...CTTG CTCTTCCAAA GTTAGTGGCG GACGGGTGAG TAACACGTGG GCAACCTGCC TGTAAGTTGG
S.aureus         AACGGACGAG AAG...CTTG CTTCTCTGAT GTTAGCGGCG GACGGGTGAG TAACACGTGG ATAACCTACC TATAAGACTG
S.epidermidis    AACAGACGAG GAG...CTTG CTCCTCTGAC GTTAGCGGCG GACGGGTGAG TAACACGTGG ATAACCTACC TATAAGACTG
S.agalactiae     .......... .......... .......... .........G AACGGGTGAG TAACGCGTAG GTAACCTNCC TCATAGCGGG
S.pneumoniae     .......... .......... .......... .........G AACGGGTGAG TAACGCGTAG GTAACCTGCC TGGTAGCGGG
S.pyogenes       .......... .......... .......... .........G AACGGGTGAG TNACGCGTAG GTAACCTGCC TNATAGCGGG
E.coli           GTAACAGGAA G.AAGCTTGC TCTTT.GCTG ACGAGTGGCG GACGGGTGAG TAATGTCTGG G.AAACTGCC TGATGGAGGG
H.influenzae     GTAGCAGG.A GAAAGCTTGC TTTCTTGCTG ACGAGTGGCG GACGGGTGAG TAATGCTTGG G.AATCTGGC TTATGGAGGG
K.pneumoniae     GTAGCACAGA G..AGCTTGC TC.TCGGGTG ACGAGCGGCG GACGGGTGAG TAATGTCTGG G.AAACTGCC TGATGGAGGG
P.mirabilis      GTAACAGGAA GAAAGCTTGC TTTCTTGCTG ACGAGCGGCG GACGGGTGAG TAATGTATGG G.GATCTGCC CGATAGAGGG
P.aeruginosa     GATGAAGGGA GCTTGCTCCT GGATTC.... ...AGCGGCG GACGGGTGAG TAATGCCTAG G.AATCTGCC TGATAGTGGG
S.typhimurium    GTAACAGGAA G.CAGCTTGC NC.TTCGCTN NNNAGTGGCG GACGGGTGAG TAATGTCTGG G.AAACTGCC TGATGGAGGG
S.flexneri       GTAACAGGAA G.CAGCTTGC TGTTTCGCTG ACGAGTGGCG GACGGGTGAG TAATGTCTGG G.AAACTGCC TGATGGAGGG
```

Region B

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                 181                                                                    250
C.perfringens    GCATAATGTT GAAAGATGGC ATCATCATTC AACCAAAGGA GCAAT..... .CCGCTATGA GATGGACCCA
L.monocytogenes  GAATGATARA RTGT.GGCGC ATGCCACGCT TTTGAAAGAT GGTTTCGGCT ATCGCTTACA GATGGGCCCN
S.aureus         GGATAATATT TTGA.ACCGC ATGGTTCAAA AGTGAAAGAC GGTCT.TGCT GTCACTTATA GATGGATCCG
S.epidermidis    GGATAATATA TTGA.ACCGC ATGGTTCAAT AGTGAAAGAC GGTTT.TGCT GTCACTTATA GATGGATCCG
S.agalactiae     GCATNAGAGT ANTT.AACNC ATGTNAGTNN TTTAAAAGGA GCAATTG..C TNCACTGTGA GATGGACCTN
S.pneumoniae     GCATAAGAGT AGAT.GTTGC ATGACATTTG CTTAAAAGGT GCACTTG..C ATCACTACCA GATGGACCTN
S.pyogenes       GCATAAGAGA GACT.AACGC ATGTNAGTNA TTTAAAAGGG GCAATTG..C TNCACTATGA GATGGACCTN
E.coli           GCATAACGTC GCAAGACCAA AGAGGGGGAC CTTCG..... .....GGCCT CTTGCCATCG GATGTGCCCA
H.influenzae     GCGTATTATC GGAAGATGAA AGTGCGGGAC TGAGA..... .....GGCCG CATGCCATAG GATGAGCCCA
K.pneumoniae     GCATAACGTC GCAAGACCAA AGTGGGGGAC CTTCG..... .....GGCCT CATGCCATCA GATGTGCCCA
P.mirabilis      GCATAATGTC TACGGACCAA AGCAGGGGCT CTTCG..... .....GACCT TGCACTATCG GATGAACCCA
P.aeruginosa     GCATACGTCC TGAGGGAGAA AGGGGGGGAT CCTCG..... .....GACCT CACGCTATCN GATGAGCCTA
S.typhimurium    GCATAACGTC GCAAGACCAA AGAGGGGGAC CTTCG..... .....GGCCT CTTGCCATCA GATGTGCCCA
S.flexneri       GCATAACGTC GCAAGACCAA AGAGGGGGAC CTTCG..... .....GGCCT CTTGCCATCG GATGTGCCCA
```

Region C

```
                 451                                         500
C.perfringens    TGTCTTTGGG GAAGA..... .......... .......... .TAATGACGG
L.monocytogenes  TGTTGTTAGA GAAGAACAAG GATAAGAGT. AACTGCTTGT CCCTTGACGG
S.aureus         TGTTATTAGG GAAGAACATA TGTGTAAGT. AACTGTGCAC ATCTTGACGG
S.epidermidis    TGTTATTAGG GAAGAACAAA TGTGTAAGT. AACTATGCAC GTCTTGACGG
S.agalactiae     TGTTGTTAGA GAAGAACGTT GGTAGGAGTG GAAAATCTAC CAAGTGACGG
S.pneumoniae     TGTTGTAAGA GAAGAACGAG TGTGAGAGTG GAAAGTTCAC ACTGTNACGG
S.pyogenes       TGTTGTTAGA GAAGAATGAT GGTGGGAGTG GAAAATCCAC CAAGTGACGG
E.coli           TTTCAGCGGG GAGGAAGGGA GTAAAGTTAA TACCTTTGCT CATTGACGTT
H.influenzae     TTTCGGTATT GAGGAAGGTT GATGTGTTAA TAGCACATCA AATTGACGTT
K.pneumoniae     TTTCAGCGGG GAGGAAGGCG ATGAGGTTAA TAACCTCATC GATTGACGTT
P.mirabilis      TTTCAGCGGG GAGGAAGGTG ATAAGGTTAA TACCCTTATC AATTGACGTT
P.aeruginosa     TTTAAGTTGG GAGGAAGGGC AGTAAGTTAA TACCTTGCTG TTTTGACGTT
S.typhimurium    TTTCAGCGGG GAGGAAGGTG TTGTGGTTAA TAACCGCAGC AATTGACGTT
S.flexneri       TTTCAGCGGG GAGGAAGGGA GTAAAGTTAA TACCTTTGCT CATTGACGTT
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initiated, the PCR product from that organism was sequenced with an ABI (Foster City, CA) Model 377 automated sequencer. Sequence data (with GenBank accession number shown in parentheses following the organism) for *C. perfringens* (M59103), *E. coli* (J01859), *H. influenzae* (M35019), *K. pneumoniae* (X87276), *L. monocytogenes* (M58822), *P. aeruginosa* (M34133), *S. typhimurium* (X80681), *S. flexneri* (X80679), *S. aureus* (L37597), *S. epidermidis* (L37605), *S. agalactiae* (X59032), *S. pneumoniae* (X58312), and *S. pyogenes* (X59029) were then retrieved from the database using the program Fetch (GCG). The sequences were then aligned using the program PileUp (GCG). The alignments were examined for regions 18–21 bp in length containing at least 1 bp difference from all other species for each of the species-specific probes. The probes were synthesized using the ABI Model 394 DNA/RNA synthesizer.

Hybridization with Species-Specific Probes

The species specific probes were end labeled with [γ-^{32}P]ATP using T4 kinase at 37°C for 45 min and 2×10^6 cpm/mL were added to the hybridization solution. Dot blots were prepared using 50 μL from a 100-μL mixture containing 40 μL of the PCR amplification products, 51 μL of deionized water (Millipore Milli-Q) filtered through a 0.45-μm nylon filter, 4 μL of 10 N NaOH, and 5 μL of 0.5 M EDTA. The dot blots were prehybridized with Church buffer (35) at 55°C for 1 h. The blots were hybridized at 55°C overnight with 10 mL of Church buffer, 200 μL of salmon sperm DNA (Gibco-BRL, Grand Island, NY) to block nonspecific binding and the radiolabeled probe. The blots were washed at 55°C for 10 min in Church wash buffer (35) and were washed again at 58°C for an additional 20 min if the counts remained too high when assayed by a hand held Geiger–Müller counter. The washed dot blots were autoradiographed using Kodak (Rochester, NY) X-OMAT AR film at −80°C for 24–72 h.

RESULTS

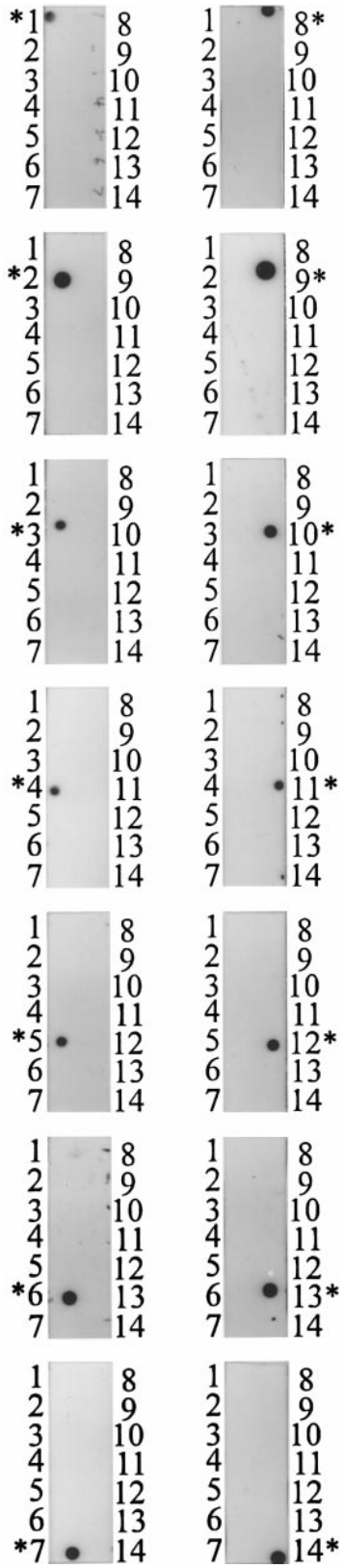
The universal bacterial primer pair, p8FPL and p806R, successfully amplified DNA from the colored blotter paper spots of killed bacteria using the two-temperature PCR. The two-temperature PCR reduced the time required for amplification by at least 1 h and produced no detectable loss of signal compared with the three-temperature cycle reported previously (15) when visualized by UV light on a 0.8% ME agarose gel stained with ethidium bromide (data not shown). Specimens stored as dried spots of boiled organisms were stable for at least 1 year.

The sequence of the *P. mirabilis* PCR product was determined and deposited into GenBank (Accession No. 245420). This sequence along with the 13 other 16S rDNA sequences from GenEMBL, when aligned and examined, were found to contain regions suitable for the development of species-specific probes. These probes, all 18–20 bp in length, contained at least 1 bp difference from all other sequences examined and fell into three distinct variable regions of the amplified portion of the 16S rDNA that we have designated Regions A, B, and C (Fig. 1). The probes, when labeled and hybridized to dot blots containing a PCR product from each of the 14 organisms, bound only to the corresponding product in every case (Fig. 2) indicating that these probes, as predicted by the alignments, were indeed species specific within this set of templates.

DISCUSSION

In this study we confirm the extremely robust amplification of the universal bacterial primer pair, p8FPL and p806R, to amplify the 16S rDNA from clinical isolates. The PCR cycle and, therefore, the overall time required for amplification can be reduced significantly by elimination of a step dedicated to extension, with no apparent loss of signal. Previously, we have shown this primer pair is specific for bacterial DNA and does not amplify human DNA (15). These features would allow this primer pair to be utilized with clinical samples, in which human DNA would certainly be present.

Our group and others (15, 26–34) have shown that, following universal bacterial amplification, the template organism can be identified using species-specific probes designed by computer comparisons of sequence data. For organisms in which complete sequence data are not available, the PCR product can be sequenced and the sequence information can be used for the design of new probes. In addition, since amplification is performed using 16S rDNA primer sequences found in all bacteria, initially unidentifiable PCR products can be sequenced to allow for the identification of the pathogen causing the bacterial infection. The ability to sequence bacterial PCR products and tailor-make these new probes for any bacterium will allow not only for routine identification of common pathogenic organisms, but also



for the identification of emerging or reemerging bacteria, for which no sequence data may exist.

Molecular microbiological methods will require known standards for quality assurance, in contrast with classical culture-based approaches that identify the organism on the basis of recognized growth and metabolic characteristics. Probe hybridization following PCR amplification is fundamentally a biochemical methodology, and, therefore, the results from unknown specimens should be compared with control DNA samples for which the identity of the organism is known and the quality is standardized and certified. A complete collection of all possible organisms meeting these criteria might be difficult for the routine clinical microbiology laboratory to accumulate and maintain. Fortunately, since the molecular genetic approach is biochemical in nature, living organisms are not required. In this report, we demonstrate that boiled bacterial specimens can be spotted onto blotter paper, and the DNA in these specimens will remain sufficiently stable to permit PCR amplification for at least 1 year. A dye, bromophenol blue, can be added to permit recognition of the position of the killed bacterial sample on the paper; this dye does not interfere with the PCR amplification. Therefore, this simple and labor-efficient approach will permit centralized development of catalogued specimen libraries that can be shipped to clinical laboratories to serve as standardized control samples. This method of bacterial DNA storage will offer the same advantages afforded by the dried blood spots on filter paper blotters of the type used for newborn screening (36, 37), simplicity of preparation, stability of the DNA analyte, and ease of shipment and analysis. A commercial paper product impregnated with chemicals to stabilize the DNA may improve the shelf-life of the dried bacterial specimens (FTA, Fitzco, Inc.; Maple Plain, MN; http://www.fitzcoinc.com/fta.htm).

Further computer analysis of the probe designed using the *P. mirabilis* sequence indicates that it may also detect *Proteus vulgaris*. Of two 16S rDNA se-

FIG. 2. This series of dot blots shows the specificity of the species-specific probes. For each dot blot, the only PCR product to which the probe bound was the species for which the probe was designed. The template species being probed for is indicated by an asterisk. The template species are: (1) *E. coli;* (2) *H. influenzae;* (3) *K. pneumoniae;* (4) *P. mirabilis;* (5) *P. aeruginosa;* (6) *S. typhimurium;* (7) *S. flexneri;* (8) *C. perfringens;* (9) *L. monocytogenes;* (10) *S. aureus;* (11) *S. epidermidis;* (12) *S. pyogenes;* (13) *S. pneumoniae;* (14) *S. agalactiae.*

quences of *P. vulgaris* examined in GenEMBL, J01874 and X07652, the latter contains the *Proteus* species probe, while the other sequence differs by one base pair. Extensive sequencing of the rDNAs will be required to evaluate the effectiveness of species or strain specific probes at the individual base pair level.

Other approaches to distinguish between phenotypically or genetically similar organisms examine the 16S–23S spacer regions of bacterial genomes (38–43). While some of the analysis is probe based (38, 42), much of the work centers on electrophoretic banding patterns of PCR products from the various bacterial templates (39–42). As sequencing technology improves and costs decrease, sequence-based approaches to bacterial diagnosis will become clinically viable (17).

We describe amplification of bacterial 16S rDNA with a universal primer pair followed by probe hybridization for species-specific identification of bacterial pathogens. We speculate that advances in molecular genetic technology will allow improvements in speed and sensitivity (17). Molecular triage of sepsis will eventually improve the diagnosis and management of patients with the nonspecific signs and symptoms of possible bacterial infection in a wide variety of clinical settings. PCR with unique and universal primer pairs will represent complementary molecular microbiological methods to reduce unnecessary hospitalizations and antibiotic use, improving the care of patients.

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