Denaturing HPLC for Identifying Bacteria

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William Hurtle, David Shoemaker¹, Erik Henchal¹, and David Norwood¹

Clinical Research Management, North Royalton, OH, and ¹United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA

ABSTRACT

*Denaturing HPLC (DHPLC) is used in a wide variety of genetic applications. Here we introduce a new application for this technique, the identification of bacteria. We combined the capability of DHPLC to detect sequence variation with the principles of rRNA genotyping analysis to develop a high-throughput method of identifying microorganisms. Thirty-nine bacterial species from a broad spectrum of genera were tested to determine if DHPLC could be used for identification. Most (36 of 39) species of bacteria had a unique peak profile that could be used as a molecular fingerprint. Furthermore, a blind panel of 65 different bacterial isolates was analyzed to demonstrate the diagnostic capability of this method to specifically identify *Yersinia pestis* and *Bacillus anthracis*. All the *Y. pestis* samples (10 of 10) and the majority of *B. anthracis* samples (12 of 14) were correctly identified. The procedure had an overall specificity of 100%, overall sensitivity of 91.7%, and a predictive value of 96.9%. The data suggest that DHPLC of products spanning regions of genetic variability will be a useful application for bacterial identification.*

INTRODUCTION

Denaturing HPLC (DHPLC) is a rapid and sensitive method for detecting genetic mutations (1,15). The technique consists of four parts: amplification by PCR, quantification, hybridization, and analysis of hybridized product. After PCR, heteroduplexes between wild-type and experimental products are formed during the hybridization step. Mismatches between the wild-type and mutant DNA sequences disrupt the structure of the heteroduplex, but DHPLC is capable of resolving these differences. Over the years, DHPLC has been employed in a wide range of applications, including nucleotide polymorphism analysis (3), gene mapping (13), analysis of genes (7), screening for mutations (8), analysis of primer extension products (6), and quantification of gene expression (5). Here we describe a new application for this technique—the identification of bacteria. Compared to current techniques of bacterial identification, DHPLC is quick, economical, and capable of screening large numbers of samples in a relatively short period of time.

In prokaryotes, the genes encoding rRNAs occur in tandem repeated arrays. Within the tandem array, each copy of the gene is identical or nearly identical. These genes are characterized by regions of high-sequence conservation and regions of sequence variability. Numerous reports have been published demonstrating the utility of analyzing these genes by PCR and other methods to distinguish different genera and species of bacteria (2,9,12,14).

We amplified a 320-bp segment of

the prokaryotic 16S rRNA gene using conserved primer sequences that spanned a region of genetic variability. Thirty-nine species were tested to determine if DHPLC peak profile analysis could be used for identification. We also tested the ability of this method to specifically identify two organisms that have a high potential to be used as agents in bioterrorism, *Yersinia pestis* and *Bacillus anthracis*. These two organisms were used as controls and tested against a panel of 65 bacterial strains.

MATERIALS AND METHODS

Primer Design

The primer set used in this study was designed by aligning the 16S rRNA gene from *Bacillus* sp. (GenBank® accession no. L29507), *Brucella melitensis* (LZ6166), *Enterococcus faecalis* (Y18293), *E. coli* (X80724), *Neisseria weaveri* (L10738), *Pseudomonas aeruginosa* (AJ249451), *Rickettsia rickettsii* (L36217), *Staphylococcus aureus* (L37597), and *Yersinia bercovieri* (X75281). The primer set UNIV1U 5'-ATTAGATACCCTGGTAGTCCACGC-3' (sense) and UNIV1L 5'-TTGCGGGACTTAACCCAAC-3' (antisense) was chosen from areas with a high degree of homology. The resultant 320-bp product spanned a region with a high degree of divergence. The primer set was analyzed with the software package Net Primer (Premier Biosoft, Palo Alto, CA, USA) for priming efficiency and synthesized on a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA).

DNA

The DNA used in this study was from reference material obtained from recognized culture collections, commercial vendors, clinics, or unique entries from previous USAMRIID collections. The DNA was extracted using either Bactozol™ Kits (Molecular Research Center, Cincinnati, OH, USA) or a BioRobot 9604B (Qiagen, Valencia, CA, USA), according to the manufacturer's recommendations.

PCR

Each organism was amplified in 100- μ L reactions containing 1 μ M each primer, 40 mM each dNTP, 10 μ L 10 $\times$ PCR Buffer II, 5 U AmpliTaq Gold® (Applied Biosystems), and 8 μ L 25 mM MgCl₂ in molecular biology-grade water. Two hundred picograms of template DNA were used in each reaction. The amplification was performed with a PTC-100™ thermal cycler (MJ Research, Waltham, MA, USA). Cycling conditions were a 10-min preincubation at 95°C to activate the AmpliTaq Gold, followed by 30 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C, and a 10-min final extension at 72°C.

Quantification

PCR yields were quantitatively determined by reverse-phase HPLC using WAVE® software and a DNA Fragment Analysis System™ (both from Transgenomic, Omaha, NE, USA) consisting of a L-7100 pump, L-7200 thermoelectronic-type constant temperature sample rack, L-7200 auto sampler, L-7300 column oven, L-7400 UV detector (all from Hitachi, San Jose, CA, USA), and a L-761 on-line degasser (ECR, Kawaguchi-City, Saitama, Japan). The mobile phase was composed of buffer A containing 0.1 M triethylammonium acetate (Transgenomic), pH 7.0, and 0.025% HPLC-grade acetonitrile (Fisher Scientific, Fair Lawn, NJ, USA) and buffer B containing 0.1 M triethylammonium acetate, pH 7.0, and 25% acetonitrile. The analytical gradient used for quantification was 0 min for 48% A and 52% B; 0.5 min for 43% A and 57% B; 5 min for 34% A and 66% B; 5.1 min for 0% A and 100% B; 5.7 min

for 48% A and 52% B; and 8.2 min for 48% A and 52% B at 0.9 mL/min. The columns used for analysis were 50 $\times$ 4.4 mm (i.d.) DNASep® cartridges packed with nonporous polystyrene-divinylbenzene copolymer particles 2.1 $\pm$ 0.12 μ m in diameter. Fifteen microliters crude PCR product from each sample were injected onto the column.

Heteroduplex Formation

Hybridization reactions were performed using a PTC-100 thermal cycler in 200- μ L reactions containing 10 mM EDTA (Invitrogen, Carlsbad, CA, USA) and equimolar amounts of *B. anthracis* Sterne strain PCR product and experimental PCR product in molecular biology-grade water (Eppendorf®, Westbury, NY, USA). The volume of the crude PCR product used in the hybridization reactions was standardized to 20 000 U as determined by the Transgenomic software. Hybridization conditions were a 4-min preincubation at 95°C, followed by a cooling period from -1.5°C/min to 25°C over 45 min.

DHPLC Analysis

To determine if this primer set would produce unique peak profiles, 39 species of bacteria were analyzed by DHPLC using 15- μ L injections of hybridized sample at 57°C, 58°C, 59°C, 60°C, 61°C, and 62°C, with the analytical gradient of 0 min for 52% A and 48.0% B; 0.5 min for 47% A and 53% B; 5 min for 38% A and 62% B; 5.1 min for 0% A and 100% B; 5.7 min for 52% A and 48% B; and 8.2 min for 52% A and 48.0% B at 0.9 mL/min.

To determine the specificity of this method, a blind panel of 65 bacterial strains was analyzed at 57°C, 61°C, 62°C, and 63°C by using the analytical gradient, instrument, columns, and mobile phase previously described.

Sequencing of PCR Products

The PCR product was purified using a QIAquick™ spin column (Qiagen). Sequencing reactions were 20 μ L containing 2.5 μ m primer, 45 ng PCR product, 1 μ L BigDye™ (Applied Biosystems), and molecular biology-grade water. Sequence cycling was per-

formed on a PTC-100 thermal cycler. The sequence cycling conditions were 30 s preincubation at 85°C, followed by 25 cycles of 10 s at 96°C, 5 s at 50°C, 4 min at 60°C, followed by a 10-min final extension at 60°C. The sequencing reactions were purified on Centri Sep columns (Princeton Separations, Aldelphia, NJ, USA), and sequencing was performed on an ABI PRISM™ 377 DNA sequencer, according to the manufacturer's recommendations.

RESULTS AND DISCUSSION

A primer set capable of amplifying a broad range of microorganisms was designed by aligning the 16S RNA sequences of several bacteria from different genera. We found two regions of high conservation that spanned a region with relatively high sequence variability of about 320 bp. This primer set amplified all but two of the organisms used in this study, *Bacteroides distasonis* and *Clostridium botulinum*.

From previous work in our laboratory with the *vrrA* region in *B. anthracis*, we noticed that distinct mutations produced unique DHPLC peak profiles (data not shown). Recently, reports have been published demonstrating that DHPLC can be useful in identifying mutations (4, 10, 11). To see if it was possible to obtain unique peak profiles for each microorganism, we tested 39 species of bacteria from 24 genera (Table 1) over a temperature range of 57°C–62°C using *B. anthracis* Sterne as our heteroduplex driver.

We chose *B. anthracis* Sterne as our heteroduplex driver because of our familiarity with the organism from our previous work with the *vrrA* region. In addition, preliminary testing indicated that the product produced from the UNIV1U and UNIV1L primer set would hybridize with products from species highly divergent from *B. anthracis* Sterne. Given that *B. anthracis* Sterne hybridized to widely divergent species, we can speculate that any species of bacteria may be used as a heteroduplex driver using the UNIV1U and UNIV1L primers.

To ensure the reproducibility of peak profiles, before heteroduplex formation we quantified our PCR products with HPLC by measuring the area un-

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Table 1. List of Organisms Used in This Study for the Initial Proof of Concept

Genus	Species
<i>Acineobacter</i>	<i>baumanni</i>
<i>Alcaligenes</i>	<i>xylosoxldans</i>
<i>Bacillus</i>	<i>anthracis</i> , <i>cereus</i> , <i>thurigiensis</i> , <i>coagulans</i> , <i>macerans</i> , <i>popilliae</i> , and <i>subtilis</i> var <i>niger</i>
<i>Bordetella</i>	<i>bronchiseptica</i>
<i>Brucella</i>	<i>melitensis</i>
<i>Comamonas</i>	<i>acidivarns</i>
<i>Corynebacterium</i>	<i>sp.</i>
<i>Enterococcus</i>	<i>durans</i> and <i>faecalis</i>
<i>Escherichia</i>	<i>coli</i>
<i>Haemophilus</i>	<i>influenzae</i>
<i>Klebsiella</i>	<i>pneumoniae</i>
<i>Moraxella</i>	<i>cattaharlis</i>
<i>Neisseria</i>	<i>lactamica</i>
<i>Proteus</i>	<i>vulgaris</i>
<i>Providencia</i>	<i>stuartii</i>
<i>Pseudomonas</i>	<i>aeruginosa</i>
<i>Ralstonia</i>	<i>picketti</i>
<i>Salmonella</i>	<i>enteritidis</i>
<i>Serratia</i>	<i>odorifera</i>
<i>Shigella</i>	<i>flexneri</i> and <i>sonnei</i>
<i>Staphylococcus</i>	<i>aureus</i> , <i>hominis</i> , <i>saprophyticus</i> , and <i>epidermidis</i>
<i>Streptococcus</i>	<i>pnumoniae</i> and <i>pyogenes</i>
<i>Strenotropho- monas</i>	<i>maltophilia</i>
<i>Yersinia</i>	<i>enterocolitica</i> , <i>kristensenii</i> , <i>fredericksenii</i> , and <i>pseudo- tuberculosis</i>

der the peaks. Our quantification procedure compensated for any variations in PCR yields. Overlays of the 39 species showed that 36 of them had unique peak profiles (Figures 1 and 2). Some species had unique profiles at all temperatures, whereas others were distin-

Table 2. List of Organisms Used in the Blind Panel to Evaluate DHPLC as a Method for Identifying Microorganisms and Results of the Blind Panel

Species	Strains	ID	Species	Strains	ID
<i>Acineobacter baumannii</i>	1	0	<i>P. stuartii</i>	1	0
<i>B. anthracis</i>	14	12	<i>P. aeruginosa</i>	1	0
<i>B. cereus</i>	2	0	<i>Ralstonia picketti</i>	1	0
<i>B. coagulans</i>	1	0	<i>S. enteritidis</i>	1	0
<i>B. macerans</i>	1	0	<i>S. odorifera</i>	1	0
<i>B. megaterium</i>	1	0	<i>S. flexneri</i>	1	0
<i>B. popilliae</i>	1	0	<i>S. sonnei</i>	1	0
<i>B. subtilis</i> var <i>niger</i>	1	0	<i>S. aureus</i>	2	0
<i>B. thurigiensis</i>	1	0	<i>S. epidermidis</i>	1	0
<i>B. bronchiseptica</i>	1	0	<i>S. hominis</i>	1	0
<i>C. botulinium</i>	1	0	<i>S. saprophyticus</i>	1	0
<i>C. acidivarns</i>	1	0	<i>S. pneumoniae</i>	1	0
<i>Corynebacterium</i> sp.	1	0	<i>S. pyogenes</i>	1	0
<i>E. durans</i>	1	0	<i>Y. enterocolitica</i>	2	0
<i>E. faecalis</i>	2	0	<i>Y. frederiksenii</i>	1	0
<i>E. coli</i>	1	0	<i>Y. kristensenii</i>	1	0
<i>H. influenzae</i>	1	0	<i>Y. pestis</i>	10	10
<i>K. Pneumoniae</i>	1	0	<i>Y. pseudotuberculosis</i>	1	0
<i>Neisseria lactamica</i>	1	0	<i>Y. ruckeri</i>	1	0
<i>P. mirabilis</i>	1	0			
	Total No. of Positives on Panel	Total No. of Negatives on Panel	Total Identified	False Negatives	False Positives
<i>B. anthracis</i>	14	51	12	2	0
<i>Y. pestis</i>	10	55	10	0	0
TOTAL	24	41	22	2	0
Organism	Specificity ^a	Sensitivity ^b	Predictive Value ^c		
<i>B. anthracis</i>	100.0%	85.7%			
<i>Y. pestis</i>	100.0%	100.0%			
TOTAL	100.0%	91.7%	96.9%		
The technique had a specificity of 100%, sensitivity of 91.7%, and a predictive value of 96.9%. All of the (10 of 10) <i>Y. pestis</i> samples and most (12 of 14) of the <i>B. anthracis</i> samples were correctly identified.					
^a Specificity, percentage of true negative samples called negative					
^b Sensitivity, percentage of true positive samples called positive					
^c Predictive value, percentage of all 65 samples called correctly					

guishable at one temperature. Three organisms, *P. aeruginosa*, *N. lactamica*, and *E. coli*, had similar peak profiles at all temperatures. For those species in which multiple strains were tested, DHPLC peak profiles were consistent among strains at all temperatures. The only exception to this was *S. aureus*; both strains tested had unique profiles at all temperatures. The data indicate that the technique is capable of identi-

fying most bacteria to the species level and some bacteria to the strain level.

Previous reports pertaining to the capability of DHPLC to detect mutations have focused on relatively subtle changes in the DNA structure. Sequencing of the 320-bp product revealed that many organisms have numerous and sizable deletions and insertions when compared to *B. anthracis* Sterne. To determine if consistent results could be

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Figure 1. The unique peak profiles of (A) *B. anthracis* Sterne, (B) *R. picketti*, (C) *S. enteritidis*, (D) *S. flexneri*, (E) *S. sonnei*, and (F) *S. pyogenes* at 62°C. The bacteria used in this study were distinguished from each other based on their unique profiles at a given temperature. *B. anthracis* Sterne is a homoduplex. *R. picketti*, *S. enteritidis*, *S. flexneri*, *S. sonnei*, and *S. pyogenes* are homoduplexes, with *B. anthracis* Sterne as the heteroduplex driver.

Figure 2. Peak profiles of (A) *Y. enterocolitica* and *S. sonnei* at 62°C. (B) Overlay of *Y. enterocolitica* and *S. sonnei* at 62°C. (C) *B. anthracis* Sterne and *S. enteritidis* at 62°C. (D) Overlay of *B. anthracis* Sterne and *S. enteritidis* at 62°C. Some organisms had similar peak profiles. These organisms were distinguished from each other by overlaying chromatograms.

obtained when analyzing organisms of great sequence divergence, we did three injections of each of the 39 organisms on four different days. One organism tested, *E. coli*, gave us inconsistent results at 61°C. Interestingly, this was the only organism that presented this problem and only at this one temperature.

We also observed differences in peak profiles from column to column. While each column gave us highly reproducible results, there were differences in peak profiles between columns (Figure 3). In addition, on any given column, the relative retention time between peaks increased as the number of injections on the column increased (Figure 4, A and B). However, even after injecting 4000 samples on a column, we were still able to get reproducible results on a single run of four organisms at six different temperatures with three injections of each organism at each temperature (Figure 4, C and D).

To evaluate DHPLC as a method of identifying bacteria, we tested two control organisms, *Y. pestis* and *B. anthracis*, against a blinded panel of 65 organisms (Table 2). Of the 10 *Y. pestis*

samples on the panel, 10 were identified correctly. Of the 14 *B. anthracis* strains, 12 strains were identified correctly. There were no false positives. Of the two false negatives, one was the result of a quantification miscalculation. The other was a *B. anthracis* strain with a unique peak profile. Given the high degree of specificity, these results (Table 2) indicate that DHPLC can be used to screen a large number of samples for organisms of interest.

With the capability of DHPLC to detect variations in DNA sequences, its high throughput, and the utility of rRNA genes in identifying bacteria, it may be possible to refine further this method of identifying microorganisms, allowing for the screening of a large number of samples relatively quickly. It may be possible to analyze additional regions of an rRNA gene of variability for increased identification at the species level. Furthermore, specific regions of genetic variability within species could be exploited for strain typing. DHPLC appears to be a promising technique for identifying and genetically characterizing bacteria at the molecular level without sequencing DNA.

Figure 3. Various columns with *B. anthracis* Sterne at 61°C. Each column tested had variations in retention times, relative retention times, number of peaks, and peak shape.

Figure 4. The increase in relative retention time between the two peaks of *B. anthracis* Sterne at 62°C after 370 and 3450 runs. The relative retention time between the two peaks of *B. anthracis* Sterne at (A) 370 runs was 0.30 min, and at (B) 3450 runs it was 0.35 min. Overlays of *B. anthracis* Sterne at (C) 370–373 runs and (D) 3450–3453 runs. Although the relative retention time between the two peaks increased, peak profiles within a given run are highly reproducible.

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Address correspondence to:

Dr. David Norwood
United States Army Medical Research Institute
of Infectious Diseases
Diagnostic Systems Division
1425 Porter Street
Fort Detrick, MD 21702, USA
e-mail: david.norwood@det.amedd.army.mil

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