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Identification of pathogenic *Helicobacter* species by chaperonin-60 differentiation on plastic DNA arrays

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Abstract

A microarray method for bacterial species identification based on *cpn*60 and 16S rDNA hybridization was developed. Specific *cpn*60 or 16S rDNA oligonucleotides from various *Helicobacter* or *Campylobacter* species were printed and immobilized onto a proprietary plastic solid support. Using universal primers, fragments derived from either *cpn*60 or 16S rDNA genes from single isolates or from a complex human waste sludge DNA sample spiked with *Helicobacter pylori* were biotinylated and hybridized to the plastic slide. Subsequent querying with a streptavidin–horseradish peroxidase conjugate followed by color development using tetramethylbenzidine resulted in accurate *Helicobacter* species identification with no cross-hybridization to either the 16S rDNA or the *cpn*60 sequence of a closely related strain of *Campylobacter jejuni*. The combination of a nonfluorescence visual detection system with a polymer-based DNA microarray slide has resulted in a molecular tool that should prove useful in numerous applications requiring rapid, low-cost bacterial species identification.

Keywords: *Helicobacter*; *Campylobacter*; 16S rDNA; Microarray; Systematics; Chaperonin-60

The development of DNA microarray technology has greatly accelerated our understanding of the epigenetic responses of a living cell to alterations in its external environment through differential gene expression. A different application of microarray technology, which has started to see increasing application in only the past few years, is gene detection [1] and generally involves taxonomic identification [2,3], comparative or evolutionary genomics [4–6], and genotyping of microbial agents [7–9]. Although microarrays have been useful in microbial identification of single bacterial isolates, they have proven insufficiently sensitive for species identification in complex samples, in comparison to other molecular techniques like PCR [1]. An additional confounding factor in hybridization-based microbial identification using 16S rDNA is specificity, due to the conserved nature of this gene [10,11]. Yet another important limitation to the widespread usage of DNA microarrays is cost, as specialized glass slides, reagents, and fluorescent slide scanners are prohibitively expensive for widespread use. Altering array technology to lower costs significantly would allow a wider adoption of this approach by clinical laboratories as well as possible on-site testing for bacterial pathogens or other specific bacterial species in environmental samples like potable water.

To address these problems, we have developed a rapid method for specific bacterial species detection using carbodiimide-coated plastic slides as a solid support [12] for a microarray containing immobilized taxonomic DNA marker probes and a nonfluorescence visual detection protocol using biotin-labeled target DNA. To detect and identify strains at the species level, various housekeeping genes like RNA polymerases, gyrases, or

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16S–23S internal transcribed spacer region that possess greater sequence diversity than 16S can be used. In this study, the chaperonin-60 (*cpn*60) gene (also known as *gro*EL and *hsp*60) was chosen as it has been demonstrated to provide more discriminating power than 16S rDNA within genera such as *Streptococcus* and *Enterococcus* and has also proven useful for identifying organisms in complex microbial communities [13–15]. Two other positive attributes of *cpn*60 usage as a taxonomic marker include: (1) the availability of universal amplification primers and (2) accessibility, as it forms the second largest public data bank after 16S. This highly curated database (cpnDB), recently made available, contains no ambiguous sequence codes and covers a wide range of taxonomy [16].

Helicobacter species are large, tightly coiled helical rods known to cause various enteric, hepatic, and biliary disorders, with many species other than *Helicobacter pylori* being difficult to culture in vitro. Identification of *Helicobacter* species, and distinguishing them from their close taxonomic neighbor *Campylobacter*, is an arduous undertaking [17,18] and can often result in misidentification in clinical or veterinary settings [19]. Of more recent concern, various *Helicobacter* spp. are considered to be emerging pathogens having zoonotic potential [20]. Consequently, identifying clinically relevant *Helicobacter* species and their differentiation from the human *Campylobacter* pathogens, such as *Campylobacter jejuni*, assume even greater importance for a more accurate understanding of the actual prevalence of pathogenic strains and their proper clinical management and the role of zoonotic strains in human disease.

Polymerase chain reaction (PCR) and quantitative PCR are the current methods of choice for detection and identification of *Campylobacter* and *Helicobacter* species [21]. Despite the high sensitivity of the PCR method based on 16S rRNA gene sequences, problems in *Helicobacter* identification can occur due to rRNA gene sequence variation caused by intervening sequences, which, depending on the location, can produce altered fragment sizes or, worse, false negatives [17]. Quantitative PCR assays have had some success in identifying *H. hepaticus* and *H. pylori* [22,23].

In this study we present a rapid, accurate, low-cost means of identifying specific bacterial isolates at the species level, which may have great potential diagnostic or environmental applications.

Results

Taxonomic 16S rDNA and cpn60 amplicon hybridization

A typical 16S rDNA and *cpn*60 hybridization experiment carried out at 50°C on 50-mer probe chips is shown in Fig. 1. By using the positive control spots as an orientation grid, two sets of spots that hybridized with a biotinylated *H. pylori* 16S rDNA PCR fragment were detected in the array (Fig. 1A). As expected, the upper pair of positive spots corresponded to the *H. pylori* 16S rDNA probe and the lower pair corresponded to the general *Helicobacter* genus 16S rDNA. When the *cpn*60 amplicon from *H. pylori* was amplified, biotinylated, and hybridized to the chip, a strong signal (Fig. 1B) corresponding to the *H. pylori cpn*60 probe was observed. In both hybridizations, absolute probe–target specificity was observed as neither the *Campylobacter* 16S rDNA probe nor any of the other printed *Helicobacter* species *cpn*60 probes showed any evidence of cross-hybridization.

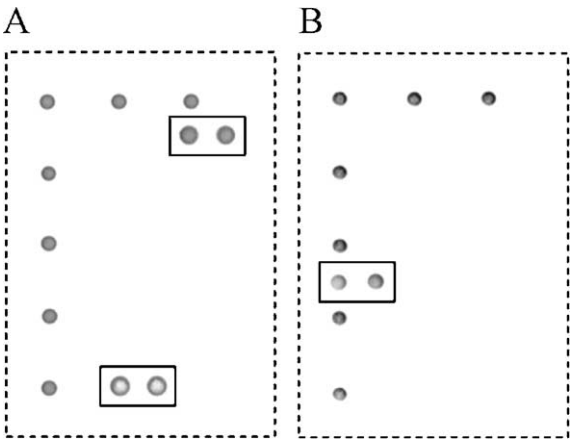

Fig. 1. Typical hybridization experiments using 16S rDNA or *cpn*60 biotinylated amplicons. After hybridizing 100 ng of biotinylated (A) 16S rDNA or (B) *cpn*60 amplicons, the plastic slides were washed and positively hybridized spots revealed by HRP-based color development. After drying at room temperature, the slides were scanned on a desktop scanner. Positive 16S rDNA or *cpn*60 spots are marked by an open box. All other spots represent the positive control oligonucleotide and are used for orientation.

Effect of oligonucleotide length on hybridization sensitivity and specificity

In conventional fluorescent dye-based microarrays, increasing oligonucleotide probe length generally results in stronger hybridization signals [24] with a corresponding decrease in hybridization specificity. To ascertain whether the same paradigm exists with the plastic arrays, 100 ng of biotin-labeled *cpn*60 amplicon from *H. winghamensis* was hybridized to either a 50- or a 70-mer plastic chip at various temperatures. As shown in Fig. 2, after hybridization at 50°C, the 50-mer chip produced a specific signal for *H. winghamensis* with no observable cross-hybridization and with a signal strength comparable to that of the positive control. At the same temperature, the 70-mer chip produced a stronger *H. winghamensis* probe signal relative to the positive control; however, strong cross-hybridization signals were observed with *H. canadensis* and *H. pullorum*. These nonspecific signals decreased with increasing hybridization temperature. Although differences in the probe melting temperatures were small (72°C for the 70-mer versus 69°C for the 50-mer), an increase in hybridization temperature of 10–15°C was generally required to eliminate cross-hybridization. Since smaller probes generally result in increased specificity and since signal intensity was comparable between the 50- and the 70-mer probes, the 50-mer chip was used for all subsequent experiments.

Species specificity of array probes

A series of *Helicobacter* and *Campylobacter* amplicon (16S rDNA and *cpn*60) hybridizations was performed on the 50-mer