Identification and characterization of *Clostridium perfringens* using single target DNA microarray chip

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Abstract

A DNA microarray method was developed to identify the presence of toxin genes: encoding beta toxin (*cpb*), epsilon toxin (*etx*), alpha toxin (*cpa*), and iota toxin (*iA*) in *Clostridium perfringens*. To build the DNA chip, each gene sequence was represented by one ~22-bp amino-modified oligonucleotide printed twice on aldehyde-coated slides. Multiplex PCR with Cy3 and Cy5-dCTP derivatized fluorescent nucleotides was used to label five genes and fluorescent probes were prepared. The PCR probes were denatured and single-strand-labeled DNAs were separated and purified using magnetic beads. The presence of toxin genes in *C. perfringens* was detected by hybridization of amplified ssDNA probes to oligonucleotides on the chip representing one target sequence of each toxin gene. The DNA chip was able to identify eight strains of *C. perfringens*. © 2003 Elsevier B.V. All rights reserved.

Keywords: *Clostridium perfringens*; Single target DNA microarray chip; Toxin genes

1. Introduction

*Clostridium perfringens* is considered to be one of the common microorganisms that causes human and veterinary diseases (Rood, 1998). Since *C. perfringens* cells and spores are normal inhabitants of gastrointestinal tracts of humans and animals, and the spores are quite common in soil, the presence of *C. perfringens* cells and spores in different food matrices is not sufficient indication of food contamination.

The virulence of *C. perfringens* is determined by its prolific toxin-producing ability, including enterotoxins. *C. perfringens* strains are divided into five toxin types (A, B, C, D and E) on the basis of production of four major lethal toxins: alpha, beta, epsilon, and iota (Rood, 1998). The alpha toxin encoded by the *cpa* gene is phospholipase C and it is produced by all *C. perfringens* types A, B, C, D, and E (Leslie et al., 1989). The beta toxin, encoded by the *cpb* gene, is produced by *C. perfringens* type B and C strains (Hunter et al., 1993). The epsilon toxin is encoded by the *etx* gene and it is produced by *C. perfringens* types B and C strains (Hunter et al., 1993). The biologically active iota toxin is a complex composed of two proteins and is produced by *C. perfringens* type E (Perelle et al., 1993). Enterotoxin E encoded by *cpe* is the only toxin that is not secreted from vegetative cells but it is produced during sporulation.
The cpe gene has been found in the chromosome and plasmid of C. perfringens isolated from human and domesticated livestock, respectively (Cornillot et al., 1995).

Pathogenic microorganisms are usually identified using biochemical and immunological markers and more recently by PCR. In the previous work, Meer and Songer (1997) were able to detect cpb, etx, cpe, cpa, and iA sequences in different C. perfringens isolates using the multiplex PCR method. In recent years, alternative approaches to identifying bacterial genes were used to replace PCR gel electrophoresis using a DNA microarray chip. The virulence or antibiotic genes are usually represented by 20- to 70-bp single strand oligonucleotides attached to a chemically treated (aldehyde) slide. The single strand DNA targets are printed on the chip using automation. Covalent bonding between the amino group attached to the single strand DNA targets and the aldehyde groups on the slide surface are established. The chip is then hybridized using fluorescent-labeled probes amplified from bacterial samples using multiplex PCR. The presence of the genes is detected by scanning the slide and fluorescent signals reveal the presence of the DNA sequence.

Building and optimizing a microarray chip that specifically identifies different isotypes of C. perfringens provides an attractive, alternative diagnostic strategy to PCR gel electrophoresis and is perhaps more feasible than radioisotope southern hybridization. In a previous work (Chizhikov et al., 2001), designing a DNA microarray chip was proven to be more specific offering less possibility of false negatives than gel electrophoresis. It is also less time-consuming and labor-intensive. In this work, we designed and built a DNA microarray chip to detect the gene sequences represented by one 22-bp single strand target of four secreted toxins (cpa, cpb, etx, and iA) and one enterotoxin (cpe).

### 2. Materials and methods

#### 2.1. Bacterial strains and growth

*C. perfringens* strains used in this study are listed in Table 1. A trypsinase–peptone–glucose–yeast extract (TPGY) medium was used to grow the *Clostridium* strains. The TPGY medium was boiled for 10 min to eliminate dissolved air and cooled to room temperature before inoculation. The inoculated medium tubes were incubated at 35 °C aerobically for 16–24 h. Genomic DNA was isolated using a modification of the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) first by cold shocking the cells three times in dry ice followed by treatment of cells with 50 μl of 10 mg/ml lysozyme solution for 30 min at 37 °C. The Genomic *Clostridium* DNA was also isolated using the DNAzol Genomic DNA Isolation Kit (Molecular Research Center, Cincinnati, OH).

#### 2.2. Design of PCR primer and gene-specific oligonucleotide probes

Primers were adapted from Meer and Songer (1997) and 22-bp oligonucleotide target sequences were designed to complement the non-biotin-labeled strand. A homology search was done using Blast Genbank to confirm the uniqueness of the sequence. Each oligonucleotide sequence was attached to an amino group at the 5' end to allow covalent bonding with an aldehyde slide (the slides were chemically treated by uniform surface of reactive aldehyde groups that react to primary amines that are attached to the single strand nucleic acid).

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Strain Isotyped by the chip</th>
<th>Gene amplified</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 JGS 1984</td>
<td>B etx, cpb, cpb</td>
<td>(Meer and Songer, 1997)</td>
<td></td>
</tr>
<tr>
<td>2 JGS 1157 ATCC 129916</td>
<td>A cpa, cpe</td>
<td>(Meer and Songer, 1997)</td>
<td></td>
</tr>
<tr>
<td>3 153</td>
<td>A cpa, cpe</td>
<td>(Collie and McClane, 1998)</td>
<td></td>
</tr>
<tr>
<td>4 222</td>
<td>A cpa, cpe</td>
<td>(Collie and McClane, 1998)</td>
<td></td>
</tr>
<tr>
<td>5 E13</td>
<td>A cpa, cpe</td>
<td>(Duncan and Strong, 1969; Collie and McClane, 1998)</td>
<td></td>
</tr>
<tr>
<td>6 H6</td>
<td>Could not be isotyped</td>
<td>cpb, cpe</td>
<td>(Collie and McClane, 1998)</td>
</tr>
<tr>
<td>7 F4603</td>
<td>A cpa, cpe</td>
<td>(Collie and McClane, 1998)</td>
<td></td>
</tr>
<tr>
<td>8 F5603</td>
<td>A cpa, cpe</td>
<td>(Collie and McClane, 1998)</td>
<td></td>
</tr>
</tbody>
</table>

cpe*: The fragment appeared very weak in the gel electrophoresis using multiplex PCR but when the gene was amplified using single PCR a good yield was achieved.
Target sequence design for each gene was done by ArrayDesigner (Premier Biosoft International, Palo Alto, CA) software (Table 1). All DNA oligos (Table 3) were synthesized by the ABI model 394 and the Beckman model 1000 M oligonucleotide synthesizers using the standard cyanomethyl phosphoramidite chemistry at the Center of Biologics Evaluation and Research of the Food and Drug Administration. The biotin-labeled oligos were synthesized by Operon Technologies, Alameda, CA.

2.3. Microchip design and analysis

Microchips were printed using the contact micro-spotting robotic system, PixSys 5000 (Cartesian Technologies, Irvine, CA). The average size of the spot was 200–300 μm. The concentration of the oligonucleotides before printing was adjusted to 50 μM in 0.25 M acetic acid. Printed CSS-100 aldehyde slides (CEL Associates, Houston, TX) were incubated for 10 min at 75 °C to dry the samples. The printed slides were incubated for 5 min in fresh 25% solution of sodium borohydride (NaBH₄) in water. The slides were washed with 0.25% sodium dodecyl sulfate for 1 min, followed by two washings for 1 min in distilled water to remove unbound oligonucleotides.

2.4. Single PCR amplification of five C. perfringens toxins

The PCR mixture (50 μl) contained 4 mM MgCl₂, 200 μM (each) deoxynucleoside triphosphate, 24 pmol primer, 50 ng of DNA template, 1:10 diluted enzyme buffer, and 2 U of Taq polymerase (Sigma, St. Louis, MO). Following an initial incubation at 94 °C for 1.5 min, target amplification was achieved by 35 cycles at 94 °C for 1.5 min, 50 °C for 1 min, 72 °C for 1 min, and terminated with a cycle of 72 °C for 10-min incubation.

2.5. Multiplex PCR and synthesis of fluorescence-labeled probes

Each 50 μl reaction contained 2 units of Klen taq DNA polymerase (Sigma) in 1 × PCR buffer supplemented with 4 mM MgCl₂ and 200 μM each of four deoxynucleoside triphosphate. Amplification was performed for 35 cycles using the same conditions as single PCR amplification. The PCR products were analyzed by 2% agarose gel electrophoresis. Cy5-dCTP and Cy3-dCTP (Amersham Biosciences, Piscataway, NJ) fluorescent probes were generated using the same PCR conditions.

2.6. Microarray hybridization

Single strand fluorescent DNA was prepared using GenoPrep Streptavidin Beads (GenoVision, West Chester, PA) according to the manufacturer’s protocol. Single strand DNA product was additionally purified using Centri-Sep columns (Princeton Separation, Adelphia, NJ). The purified probes were dried by vacuum centrifugation. The labeled single strand probes were resuspended in 2 μl of H₂O and 2 μl of hybridization solution (Chizhikov et al., 2001). One-microliter total reaction was added to the spots and covered with a cover slide. The hybridization was conducted in an Arrayit hybridization chamber (Tel-Chem International, Sunnyvale, CA) and kept in a 40 °C waterbath for 30 min. The chip was washed with 6 × SSC for 1 min twice, 2 × SSC for 1 min twice, 1 × SSC for 1 min once, and the slide air-dried.

2.7. Scanning of microarray

Fluorescent images of the microarray were generated by scanning the slides by using a ScanArray 4000 (Perkin Elmer, Sunnyvale, CA). The fluorescent signals from each spot were measured and compared using Quant Array software (Perkin Elmer, Irvine, CA). Analysis of collected data from the slide was performed on the basis of total fluorescent intensities measured from a fixed circular area of each oligonucleotide spot. Signals with fluorescent intensities greater or equal to those observed at the mixed target spots were considered to be positive. Mixed target sequence spots consisted of an equimolar combination of each target oligo (5 μM).

3. Results and discussion

3.1. Labeling efficiency of the probes using PCR and different DNA templates

Five different virulence genes (cbe, etx, cpe, cpa, and iA) were used as representatives of the toxin
producing genes in *C. perfringens*. Eight different *C. perfringens* strains were used in this study (Table 1). The forward primers were biotin labeled at the 5' end to separate the complementary strands that were used to complement the target sequences located on the chip. The location of the genes, the target sequences, and the length of the PCR fragments are described in Table 2. PCR was used to label the DNA probes of each gene with Cy3 and the amplified fragments corresponded with predicted fragment sizes (655 bp for *etx*, 442 bp for *iA*, 325 bp for *cpa*, 232 bp for *cpe*, and 196 bp for *cpb*) (Fig. 1). Because the amplification of the *cpb* was weak, the reaction was repeated using asymmetric PCR (the reverse primer was five-fold concentrated as the forward primer) resulting in good amplification yield. Similar results were obtained when the five toxins were labeled with Cy5 (data not shown).

A second fragment with around ~500 bp appeared in Lane 5, Fig. 1 when *cpe* was amplified and labeled. Since DNA chip technology relies on hybridization of DNA sequences and not on fragment size which is one of the limitations of multiplex PCR, this problem was not a concern.

### 3.2. Labeling efficiency of the probes using multiplex PCR and different DNA templates

When multiple primers were used to conduct multiplex PCR using different fluorescent dye, Cy5 labeling exhibited good yield for *etx*, *cpa*, and *cpb* fragments using template B (JGS 1984) (Fig. 2A, Lane 2). Using templates E13, F5603, and F4903 with Cy5 multiplex PCR revealed a good amplification for *cpa* and *cpe* fragments, (Fig. 2A, Lanes 4, 6, and 7). Similarly, template AE (JGS 1157) exhibited good yield for *cpa* and *cpe* using Cy3 multiplex PCR amplification (Fig. 2B, Lane 2). Templates 153 and 222 showed good amplification for the *cpe* fragment and poor amplification yield for *cpa* fragments, (Fig. 2B, Lanes 3 and 4). Multiplex PCR Cy3 amplification showed good yield for *cpa* and *cpe* (very weak) using H6 template and an extra fragment around 400 bp appeared as nonspecific amplification (Lane 5, Fig. 2A). Although all of these results were consistent with the strain genotype (Table 1), it appears that different templates showed different amplification and labeling efficiency. One possible reason might be related to template impurity.

While Meer and Songer (1997) were able to amplify all the five genes of *C. perfringens* using multiplex PCR, it was important to amplify and label all of the five probes using Cy3 or Cy5 without inhibiting interaction. Fig. 2A (Lane 3) reveals that the fragments of all five toxins were amplified and labeled success-

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### Table 2

<table>
<thead>
<tr>
<th>Target gene</th>
<th>GenBank accession number</th>
<th>Primers</th>
<th>PCR product size (bp)</th>
<th>Location of the primer</th>
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<tbody>
<tr>
<td><em>cpb</em></td>
<td>L13198</td>
<td>F: -5' biotin</td>
<td>195</td>
<td>871–891</td>
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<tr>
<td></td>
<td></td>
<td>R:</td>
<td></td>
<td>1066–1045</td>
</tr>
<tr>
<td><em>etx</em></td>
<td>M95206</td>
<td>F: -5' biotin</td>
<td>655</td>
<td>258–277</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:</td>
<td></td>
<td>913–893</td>
</tr>
<tr>
<td><em>cpe</em></td>
<td>X81849</td>
<td>F: -5' biotin</td>
<td>232</td>
<td>193–212</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:</td>
<td></td>
<td>425–407</td>
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<tr>
<td><em>cpa</em></td>
<td>X13608</td>
<td>F: -5' biotin</td>
<td>325</td>
<td>1280–1299</td>
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<td></td>
<td></td>
<td>R:</td>
<td></td>
<td>1605–1585</td>
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<tr>
<td><em>iA</em></td>
<td>X73562</td>
<td>F: -5' biotin</td>
<td>442</td>
<td>1739–1758</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:</td>
<td></td>
<td>2181–2161</td>
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</table>

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**Fig. 1.** Cy3 Polymerase chain reaction labeling and amplification of toxin genes *C. perfringens* using biotin forward primers and non-biotin reverse primers specific for each fragment, Lane 1: 100-bp molecular size DNA marker (TaKaRa Bio., Pittsburgh, PA), Lane 2: etx 655 bp, Lane 3: iA 442 bp, Lane 4: cpa 325 bp, Lane 5: cpe 232 bp, Lane 6: cpe 196 bp, Lane 7: negative control—no template with etx forward and reverse primers.
fully using multiplex PCR and these probes were used in hybridizing the gene chip in later experiments.

3.3. Chip hybridization using labeled probes for quality control

Because of the difficulty of locating the spots in the chip during and after hybridization as has been reported by different investigators (Chizhikov et al., 2002; Volokhov et al., 2002), the chip was designed to have mixed targets of the five genes surrounding five columns and two rows. Fig. 3A shows the quality control (QC) and the design of the printed chip. Each target sequence was printed twice from a microtiter well plate with a 50-µM final DNA concentration. The mixed target sequences (unboxed spots) of the five toxins were prepared from a microtiter well plate with 5 µM of each synthesized oligo.

Quality control hybridization was used to test the consistency and irregularities of printing using multiplex labeled probes on the chip. Labeled Cy5 probes of five genes (Fig. 2A, Lane 3) were mixed together and hybridized to the chip. All the target sequences of the designated spots located on the chip were hybridized successfully to the probes indicating a good printing.

3.4. Chip hybridization using labeled probes by PCR

Fig. 3B shows five Cy3 labeled probes cpb, etx, cpe, cpa, and iA hybridized to the two spots of each target sequence without any cross hybridization. The cpb-labeled Cy3 probe binds to spots 3a and 3b, the etx-labeled Cy3 probe binds to spots 4a and 4b, the cpe-labeled Cy3 probe binds to spots 5a and 5b, the cpe-labeled Cy3 probe binds to spots 6a and 6b, and the iA-labeled Cy3 probe binds to spots 7a and 7b. As it was expected, weak hybridization to the mixed target control spots was observed due to the low concentration of the targets (5 µM).

The hybridization of the DNA microarray chip to the labeled cpe probe exhibited more specificity than gel electrophoresis when a ∼ 500-bp unspecified fragment of cpe in the gel (Fig. 1, Lane 5) did not cause any cross hybridization in the chip. The ability of the labeled fragments representing each gene to hybridize to the predicted spots without any cross hybridization reveals that the chip design and fabrication were successful and the chip is ready to be tested for the multiplex labeled probes that were amplified from different C. perfringens templates.

3.5. Chip hybridization using labeled probes by multiplex PCR

In the multiplex labeled probe in Fig. 3C, the Cy5-labeled probe of strain B (JGS 1984) hybridized to
In the figure, Cy5-labeled probes of all the toxins using multiplex PCR were hybridized to the chip. Each spot represents hybridized probe to target sequence of the represented gene. The name and the location of each spot are shown as follows: cph spots: 3a, 3b; etx spots: 4a, 4b; cpe spots: 5a, 5b; cpa spots: 6a, 6b; iA spots: 7a, 7b. All the unmarked spots are mixed target sequences of the five labeled genes with the final concentration 5 μM. (B) PCR Cy3-labeled probe of the five toxins hybridized to the chip. (C) PCR Cy5 multiplex labeled probes using different templates from strain B (JGS 1984), E13, F5603, and F4903 hybridized to the chip.

Cpb (spots 3a, 3b), etx (spots 4a, 4b), and cpa (spots 6a, 6b) target sequences. The templates E13, F5603, and F4903 multiplex Cy5 probes hybridized to cpe (spots 5a, 5b) and cpa (spots 6a, 6b; Fig. 3C), whereas templates AE (JGS 1157), 153, and 222 Cy3-labeled multiplex probes showed good hybridization to cpa (spots 6a, 6b). The template 153 multiplex probe showed strong hybridization to cpe as well. Weak hybridization to cpe (spots 5a, 5b) appeared from the probe amplified using templates AE (JGS 1157) and 222 (Fig. 3D).

The C. perfringens H6 template (Fig. 2A, Lane 5) showed no hybridization to any target sequences, despite observed amplified fragments (cpa, 325 bp;
The PCR labeling was conducted several times and with two different fluorescent dyes Cy3 and Cy5 and no hybridization was detected using template H6 suggesting that the genes might have allelic variations. Further sequence analysis of the PCR product of \textit{cpa} for the H6 template indicated a mismatch from C to A at the complementary target sequence probe AGCAT-GAGT-(C/A)-ATAGTTGGGATG (Table 3). Ginter et al. (1996) have reported that the alpha toxin showed sequence variation from the \textit{cpa} gene isolated from \textit{C. perfringens} NCTC 8237 (Tsutsui et al., 1995; Ginter et al., 1996). The inability of the chip to reveal the presence of \textit{cpe} might be related to weak labeling and amplification with bulky molecules of Cy3 and Cy5. Another possibility is the presence of allelic variation in the binding region of the PCR primers. This will give PCR amplification with a less stringent annealing temperature but it will interfere in the hybridization of the chip. Perhaps one way to overcome this limitation is to design multiple target sequences to represent each gene and this would circumvent the problem during hybridization experiments.

3.6. Sensitivity of chip hybridization

Target sensitivity represents the minimum molar concentration of the targets in the hybridization solution that is required to exceed the detection threshold of the sensor after hybridization and washing steps are complete (Call et al., 2001). It is quite possible that 5 \(\mu\)M, the minimum concentration of the target sequence in the unboxed spots, could be the minimum concentration in single PCR labeling and hybridization. Call et al. (2001) have found that after 35 amplification cycles of labeling reaction (biotin-labeled bases) using different dilutions of \textit{E. coli} templates (1 fg to 100 ng), the probes had sufficient molar concentration to saturate and hybridize to the chip using the streptavidin-alkaline phosphatase method.

All the template concentrations which were used in PCR amplification were 50 ng per reaction in single and multiplex PCR using Cy3 and Cy5 labeling. Although template concentrations are acceptable for a single PCR reaction, multiplex PCR is more prone to low yield because of primer interactions and a bulky Cy3 or Cy5-dCTP base for the Taq DNA polymerase in PCR. In addition, multiplex Cy5 labeling showed homogenous spot intensity compared to multiplex Cy3 labeling (Fig. 3C and D) hybridization. Although the multiplex PCR amplification probe reaction for strain AE (JGS 1157) had good yield as it appeared in gel electrophoresis (Fig. 2B), the hybridization reaction to the chip (Fig. 3D) gave a weak reaction. This was the opposite in strain 153 where the electrophoresis gel PCR band amplification was weak (Fig. 2B) and the hybridized spots in the chip had a strong reaction (Fig. 3D). This indicates that more quantitative studies should be conducted to explore the relation between probe amplification and probe hybridization. In addition, experiments to assay the increase of the template labeling using different primer concentrations should be conducted in the future to optimize the labeling reactions using multiplex PCR Cy3 labeling.

Only one template out of eight could not be identified by the chip using the multiplex PCR probe (\textit{C. perfringens} template H6); and this represents 87\% reliability. The next goal will be building a \textit{C. perfringens} chip that has six targets representing each gene to overcome the problems of allelic point mutation. A universal DNA labeling technique is currently under development that would be able to use random heximers for labeling the whole DNA genome. Finally, hybridization using a DNA microarray chip proved to be more specific, with no cross hybridization, and required less time.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide target sequence amino link modified</th>
<th>Location of target sequence</th>
<th>Annealing temperature (°C)</th>
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<tbody>
<tr>
<td>\textit{cpb}</td>
<td>ACAGACAGATCATCATCATTCAACCTCT</td>
<td>926–947</td>
<td>51</td>
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<tr>
<td>\textit{ets}</td>
<td>AGTTGAATTAGTGAGAAACCA</td>
<td>518–535</td>
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<tr>
<td>\textit{cpe}</td>
<td>GGAACCCTCAGTAGATTTCAGT</td>
<td>213–234</td>
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</tr>
<tr>
<td>\textit{cpa}</td>
<td>AGCATGAGTCATAGTTGGGATG</td>
<td>1493–1514</td>
<td>52.9</td>
</tr>
<tr>
<td>\textit{iA}</td>
<td>TGAGTCCTCCAGAGAAATTTGC</td>
<td>1869–1890</td>
<td>52.9</td>
</tr>
</tbody>
</table>
Acknowledgements

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References


