In silico oligonucleotide primer design for *Campylobacter jejuni* cytolethal distending toxin B gene amplification

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**Abstract**

Cytolethal distending toxin B (cdtB) is a genotoxin expressed by *Campylobacter jejuni*. cdtB is a DNase that induces DNA double-strand breaks (DSB) in the nucleus causing cell cycle arrest at the G2/M phase and apoptosis. This study aimed to design and analyze in silico PCR primer pairs to amplify cdtB gene of *C. jejuni*. Sequence of cdtB gene with accession number AY445094.1 was retrieved from GenBank NCBI and primer pairs were designed by using Primer-BLAST. Further analysis of primer quality related to self dimer, hairpin, repeat, and run was done by NetPrimer. The results showed that forward primer pair 3 (5’-AGCAAGTGGAGTGTTAGCGT-3’) and reverse primer pair 3 (5’-TTGGAGTGGCTGTTCTTGGT-3’) met requirements as an ideal primer set to amplify cdtB gene in the term of primer length, Tm and GC% with a product length of 103 bp. In addition, based on NetPrimer analysis results, this primer pair had no self dimer, hairpin, repeat, and run. It can be concluded that a primer set has been successfully designed to amplify cdtB gene of *C. jejuni*. However, a wet experiment is needed to run this primer set in the laboratory setting.

Keywords: *Campylobacter jejuni*, cdtB gene, in silico, primer.

**Introduction**

*Campylobacter jejuni* is known to be one of the most prevalent bacterial pathogens causing human gastroenteritis in the world. A disease caused by this pathogen is called campylobacteriosis. In developing countries, campylobacteriosis
in children under the age of 2 years are particularly frequent and sometimes resulting in death (1). Mainly, *C. jejuni* is well known for causing symptoms ranging from mild watery diarrhea to extreme neuropathy (2). Poultry (especially chicken and contaminated carcasses of raw chicken) is considered as a major source of human campylobacteriosis. Other sources such as raw milk, sheep, cattle, cats, pigs, dogs, vegetables and water have been identified as possible sources for *C. jejuni* (1,3–5).

In causing health problem in animal and human, *C. jejuni* can express various virulence factors. Cytolethal distending toxin (cdt), a genotoxin expressed by *C. jejuni*, consists of three subunits namely cdtA, cdtB, and cdtC. Specifically, cdtB is a DNase that has ability to induce DNA double-strand breaks (DSB) in the nucleus causing cell cycle arrest at the G2/M phase and apoptosis (6,7).

Breakthrough in molecular biology has brought rapid progress in the field of medicine. Molecular biology techniques such as polymerase chain reaction (PCR) can be used to detect the presence of genes that act as virulence factors of a pathogen. PCR which aims to amplify DNA consists of three stages. The initial stage of the amplification process is denaturation of the DNA strand, then the primer set attachment to the target DNA fragment (annealing) and the final stage is the extension process of the DNA sequence (8–10).

Oligonucleotide primers are a very important component in PCR techniques. A favorable primer is a primer that has specific properties that are expected to be able to amplify specific areas in the genome (11,12). This study aimed to design and analyze oligonucleotide primers in silico to amplify *C. jejuni* cdtB gene region.
Materials and Methods

Searching of *C. jejuni* cdtB gene sequence

*Campylobacter jejuni* cdtB gene sequence was obtained using nucleotide search menu from the online GenBank NCBI website (https://www.ncbi.nlm.nih.gov/genbank/). The gene sequence used in this study was *C. jejuni* cdt gene with accession number AY445094.1. The cdtB specific gene sequence was then exported into the FASTA format for the Primer-BLAST process.

Primer design

Primer candidates for cdtB gene amplification were generated using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). A file in FASTA format containing specific cdtB gene sequence was uploaded to Primer-BLAST and then several pairs of primers that might be used to amplify the cdtB gene were generated automatically.

Primer quality analysis

Ten primer pairs designed using Primer-BLAST were then analyzed further to obtain the most efficient primer pair. In the analysis phase, NetPrimer software (http://www.premierbiosoft.com/NetPrimer/AnalyzePrimer.jsp) was used to analyze self dimer, hairpin, repeat, and run.

Results
This in silico study focuses on designing oligonucleotide primers using the Primer-BLAST application for cdtB gene amplification. Sequence of cdtB gene which was a complete sequence of cdt gene consisting of cdtA, cdtB, and cdtC of *C. jejuni* was obtained through GenBank NCBI with accession number AY445094.1. Primer-BLAST software generated ten primer pair candidates to amplify *C. jejuni* cdtB gene in the 804-1601 region of the complete DNA sequence of cdt gene (Figure 1).

Ten primer pairs designed by Primer-BLAST showed varying length, Tm, GC%, self complementarity, self 3’ complementarity, and product length (Table 1). The shortest sequence primer was reverse primer pair 9 with 19 nt then the rest had length 20 and 21 nt. Furthermore, Tm of each primer pair possessed a difference not exceeding 5°C with temperatures ranging from 58.61 to 60.37°C. Analysis of self complementarity and self 3’ complementarity exhibited different number but interestingly only primer pair 3 had the lowest number of self complementarity and no self 3’ complementarity.

| Primer Pair 1 | Length | Tm  | GC%  | Self compl. | Self 3’ compl. |
|---------------|--------|-----|------|-------------|----------------|
| F: AATTTGCAAGGCTCATCCGC | 20 | 59.83 | 50.00 | 6.00 | 2.00 |
| R: GGTTGTTCCACCTTGTTGCAC | 20 | 59.90 | 55.00 | 5.00 | 3.00 |
| Product Length 164 bp | | | | | |

| Primer Pair 2 | Length | Tm  | GC%  | Self compl. | Self 3’ compl. |
|---------------|--------|-----|------|-------------|----------------|
| F: TCTCGCGTTGATGTAGGAGC | 20 | 59.90 | 55.00 | 4.00 | 2.00 |
| R: AATCGCGAGCTAAAGCGGTG | 20 | 59.55 | 50.00 | 4.00 | 3.00 |
| Product Length 423 bp | | | | | |

| Primer Pair 3 | Length | Tm  | GC%  | Self compl. | Self 3’ compl. |
|---------------|--------|-----|------|-------------|----------------|
| F: AGCGACTGGAGGTAGCGT | 20 | 59.61 | 50.00 | 2.00 | 0.00 |
| R: TTTGAGCTGGCTTTCTTGT | 20 | 59.45 | 50.00 | 2.00 | 0.00 |
| Product Length 103 bp | | | | | |
Cont. Table 1.

| Primer Pair  | Sequence (5'->3') | Length | Tm  | GC%  | Self compl. | Self 3' compl. |
|--------------|------------------|--------|-----|------|-------------|----------------|
| **Primer Pair 4** | F: TAGTGCAACTCAAGCAAGCG | 20 | 59.13 | 50.00 | 4.00 | 2.00 |
|              | R: AAATCGCAGCTAAAGCGGTG | 21 | 60.14 | 47.62 | 4.00 | 3.00 |
|              | **Product Length 98 bp** | | | | | |
| **Primer Pair 5** | F: GGAATTTGCAAGGCTCATCCG | 21 | 60.20 | 52.38 | 6.00 | 2.00 |
|              | R: AGGGGTGGCTCCACTTACA | 20 | 58.85 | 50.00 | 3.00 | 0.00 |
|              | **Product Length 81 bp** | | | | | |
| **Primer Pair 6** | F: CAAGCGGAGGGACTCTTGATT | 21 | 60.07 | 52.38 | 4.00 | 2.00 |
|              | F: ATCGCAGCTAAAAGCGGTG | 20 | 61.37 | 55.00 | 4.00 | 1.00 |
|              | **Product Length 82 bp** | | | | | |
| **Primer Pair 7** | F: CTAGTGCAACTCAAGCAAGCG | 21 | 60.14 | 52.38 | 4.00 | 2.00 |
|              | R: AAAATCGCAGCTAAAAGCGGTG | 21 | 59.46 | 42.86 | 4.00 | 2.00 |
|              | **Product Length 100 bp** | | | | | |
| **Primer Pair 8** | F: CTCGCGTTGATGTAGGAGCTA | 21 | 59.67 | 52.38 | 4.00 | 3.00 |
|              | R: TCACGGTTAAAATCCCCTGCT | 21 | 59.65 | 47.62 | 4.00 | 0.00 |
|              | **Product Length 259 bp** | | | | | |
| **Primer Pair 9** | F: AGCAGGGGATTTTAACCGTGAA | 21 | 59.65 | 47.62 | 4.00 | 3.00 |
|              | R: CCGCTTGCTTGAGTGCAC | 19 | 60.37 | 57.89 | 4.00 | 2.00 |
|              | **Product Length 108 bp** | | | | | |
| **Primer Pair 10** | F: ATGTGCAACAAGGTGGAACAC | 21 | 59.59 | 47.62 | 5.00 | 3.00 |
|              | R: TAGCTCCTACATCAACGCGA | 20 | 58.61 | 50.00 | 4.00 | 2.00 |
|              | **Product Length 108 bp** | | | | | |

Abbreviation: F = Forward primer; R = Reverse primer; Self compl.= Self complementarity; Self 3' compl.= Self 3' complementarity.
Oligonucleotide primer sequences obtained from Primer-BLAST were then analyzed further to identify the most efficient primer. Analysis to identify self dimer, hairpin, repeat, and run was done using NetPrimer. All analyzed primers showed variable result in the term of self dimer, hairpin, and run but all primer possessed no repeat. Interestingly, primer pair 3 had no self dimer, hairpin, repeat, and run. In addition, NetPrimer also gave rating regarding to primer quality. The range of primer rating was about 80 to 100 and primer pair 3 was the highest rating among all Primer-BLAST-designed primers (Table 2).
Table 2. Results of oligonucleotide primer analysis using NetPrimer

| Primer Pair 1 | Sequence (5'->3') | Self Dimer | Hairpin | Repeat (# of pairs) | Run (# of bases) | Rating |
|---------------|------------------|------------|---------|---------------------|------------------|--------|
|               | F: AATTTCGAAGGCTCATCCGC | 2          | None    | None                | 3 (TTT)          | 80     |
|               | R: GGTGTTCCACCTTGGTGCAC | 3          | 1       | None                | None             | 83     |

Cont. Table 2.

| Primer Pair 2 | Sequence (5'->3') | Self Dimer | Hairpin | Repeat (# of pairs) | Run (# of bases) | Rating |
|---------------|------------------|------------|---------|---------------------|------------------|--------|
|               | F: TCTCGCGTTGATGTAGGAGC | 2          | None    | None                | None             | 81     |
|               | R: AATCGCAGCTAAAAAGCGGTG | 2          | 1       | None                | 4 (AAAA)         | 80     |

| Primer Pair 3 | Sequence (5'->3') | Self Dimer | Hairpin | Repeat (# of pairs) | Run (# of bases) | Rating |
|---------------|------------------|------------|---------|---------------------|------------------|--------|
|               | F: AGCAAGTGAGTGTAGCGT | None      | None    | None                | None             | 100    |
|               | R: TTGAGTGGTGTGTTCCTTGGT | None      | None    | None                | None             | 100    |

| Primer Pair 4 | Sequence (5'->3') | Self Dimer | Hairpin | Repeat (# of pairs) | Run (# of bases) | Rating |
|---------------|------------------|------------|---------|---------------------|------------------|--------|
|               | F: TAGTGCAACTCAAGCAAGCG | 1          | 1       | None                | None             | 85     |
|               | R: AAATCGCAGCTAAAAAGCGGTG | 2          | 1       | None                | 3 (AAA) and 4 (AAAA) | 80     |

| Primer Pair 5 | Sequence (5'->3') | Self Dimer | Hairpin | Repeat (# of pairs) | Run (# of bases) | Rating |
|---------------|------------------|------------|---------|---------------------|------------------|--------|
|               | F: GGAATTTGGAGGCTCATCCCG | 3          | 1       | None                | 3 (TTT)          | 80     |
|               | R: AGGGGGTTGCTCCACTTACA | None      | None    | None                | 4 (GGGG) and 3 (TTT) | 100    |

| Primer Pair 6 | Sequence (5'->3') | Self Dimer | Hairpin | Repeat (# of pairs) | Run (# of bases) | Rating |
|---------------|------------------|------------|---------|---------------------|------------------|--------|
|               | F: CAAGCGGAGGGACTTCTTGGATT | 1          | 1       | None                | 3 (GGG)          | 85     |
|               | R: ATCGCAGCTAAAAAGCGGTG | 1          | 1       | None                | 4 (AAAA)         | 84     |

| Primer Pair 7 | Sequence (5'->3') | Self Dimer | Hairpin | Repeat (# of pairs) | Run (# of bases) | Rating |
|---------------|------------------|------------|---------|---------------------|------------------|--------|
The self-dimer owned by each primer had different positions and values of ΔG and some primers had more than one self-dimer as in the forward primer pair 1 (5'-AATTTGCAAGGCTCATCCGC-3') which showed two self-dimers with different positions with ΔG values of -10.94 and -5.36 kcal/mol (Figure 2A). The results of the analysis on the hairpin also showed varying results for each primer but in this study, two hairpins were identified in reverse primer pair 7 (5'-AAAATCGCAGCTAAAAGCGGT-3') with ΔG values of -0.54 and -3.65 kcal/mol (Figure 2B). Furthermore, the number of run of each primer varied greatly. For example run analysis, reverse primer pair 7 possessed two AAAA runs at the start base number 1 and 13 (Figure 2C).
Figure 2  Primer quality analysis using NetPrimer. Two self dimers were found in forward primer pair 1 with ΔG values of -10.94 and -5.36 kcal/mol (A). Two hairpins were found in reverse primer pair 7 with ΔG values of -0.54 and -3.65 kcal/mol (B). Two runs AAAA on bases 1-4 and 13-16 were identified in reverse primer pair 7.
Discussion

The primer design is the first step which determines the performance of DNA amplification using the PCR method (13). Things that need to be considered in the selection of a primer set include the length of the primer, melting temperature (Tm), GC content and bond at the 3 'end. The length of oligonucleotide primer ranges from 18-30 nt. Primers which have nucleotide length of more than 30 nt will cause the unspecific primer attachment. The second characteristic to consider in primary selection is Tm. A favorable primer set has a Tm difference of around 5°C. This is intended to prevent a decrease in the amplification process. The percentage between bases G and C also needs to be considered because the content of the number of bases G and C is related to Tm of a primer (14). Ideally, a primer has a percentage of G and C around 40-60%. Other criteria for ideal primers are having low number of self dimer, hairpin, repeat and run (15,16).

Analysis of forward primer pair 3 (5'-AGCAAGTGAGTGTAGCGT-3') and reverse primer pair 3 (5'-TTGGAGTGGCTGTTCTTGGT-3') showed a product length of 103 bp which was in position 109-211 of C. jejuni cdtB gene (Figure 3). Analysis results showed that primer pair 3 was the ideal primer pair to amplify C. jejuni cdtB gene because this primer set met the criteria for the length of nucleotides, small Tm temperature difference (0.16°C), GC% by 50%, and had no self dimer, hairpin, repeat, and run. In addition, NetPrimer analysis exhibited the primer pair 3 had the highest rating value of 100.
In silico study is an essential computational prediction in oligonucleotide primer design. The primer set needs also to be run through a series of optimizations in the laboratory. Primer candidate optimization involves optimization of primer concentration and optimization in annealing temperature (Ta) by PCR gradient. In addition, optimization of the PCR reaction is also important to check the minimum detection and quantification of nucleic acids in the reaction. All optimization stages require work in a wet laboratory to achieve excellent PCR results (16).

**Conclusion**

Oligonucleotide primer set for *C. jejuni* cdtB gene amplification has been successfully designed. Primer pair 3 designed in this study has fulfilled the requirements as a favorable primer set in the term of primer length, Tm and GC% with a product length of 103 bp. In addition, based on NetPrimer analysis, primer pair 3 does not have self dimer, hairpin, repeat, and run. However, a wet experiment is needed to run this primer set in laboratory setting.
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