**In Silico Analysis of the cadF Gene and Development of a Duplex Polymerase Chain Reaction for Species-Specific Identification of Campylobacter jejuni and Campylobacter coli**

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**Abstract**

**Background:** Campylobacteriosis is a zoonotic infectious disease caused by Campylobacter jejuni and C. coli. The cadF gene is considered as a genus-specific gene while other genes are mainly used for discrimination at the species level.

**Objectives:** This study aimed to analyze the cadF gene and to develop a duplex PCR assay for simultaneous detection of C. coli and C. jejuni, the two commonly encountered species.

**Materials and Methods:** In silico analysis of the cadF gene was carried out by several software and available online tools. A duplex PCR optimized with specific primers was used for detection and differentiation of both species. To evaluate specificity and sensitivity of the test, a panel of different Campylobacter spp., together with several intestinal bacterial pathogens was tested. The limit of detection (LOD) of method was determined using serial dilutions of standard genomes.

**Results:** The analysis of the full size cadF gene indicated variations in this gene, which can be used to differentiate C. jejuni and C. coli. The duplex PCR designed in this study showed that it could simultaneously detect and differentiate both C. jejuni and C. coli with product sizes of 737 bp and 461 bp, respectively. This assay, with 100% specificity and sensitivity, had a limit of detection (LOD) of about 14 and 0.7 µg/mL for C. jejuni and C. coli, respectively.

**Conclusions:** In silico analysis of the cadF full-gene showed variations between the two species that can be used as a molecular target for differentiating C. jejuni and C. coli in a single-step duplex-PCR assay with high specificity and sensitivity.

**Keywords:** In Silico, Duplex PCR, cadF, Campylobacter jejuni, C. coli

1. **Background**

*Campylobacter enteritis* is one of the most frequent foodborne infections worldwide (1). Thermophilic *C. jejuni* and *C. coli* have been recognized as the most common causes of bacterial diarrhea in humans, especially among children less than five years of age and young adults (2). Although, poultry and poultry products are important sources of Campylobacteriosis, yet the organism can be transmitted to humans via contact with other warm-blooded animals such as cattle, pigs, sheep, ostriches, shellfish, and pets (3, 4).

The symptoms of campylobacteriosis can vary from mild to severe complications, including abdominal pain, fever, myalgia and watery or bloody diarrhea. Although, in most cases the illness is self-limited and rarely fatal yet post-infectious acute immune-mediated neurologic complications such as Guillain-Barre syndrome and Miller Fisher syndrome can occur, which are the consequence of molecular mimicry between lipooligosaccharides (LOS) of bacterial cell wall and gangliosides in peripheral nerves of humans (5, 6). These complications can be prevented or lowered with rapid and accurate detection of etiological agents of the disease. Diagnosis of campylobacteriosis is performed through microbiological, molecular and serological tests. Culture is the gold standard of diagnosis of *C. coli* and *C. jejuni*; however, the culture conditions for detection of these fastidious bacteria are complicated and time consuming, which in some cases make the recovery of bacteria unsuccessful. Moreover, the emergence of viable but non-culturable (VBNC) phenotypes should not be ignored.

Differentiation of the two species is only performed through hippurate hydrolysis biochemical test or molecular-based detections (7-10). In molecular methods different genetic targets have been used for the detection Campylobacter species (e.g. *asp*, *hipO*, *ceuE*, *cadF*, 16SrRNA, 23S rRNA and *cdt*, *fur*, *glyA*, *cdtABC*, *ceuB-E* and *fhy*) (9). Among them, the *cadF* gene encodes a fibronectin-binding protein that promotes bacteria-host cell interaction and has
been described as a conserve and genus-specific gene. In most studies a fragment from this gene with a length of 400 bp is used for identification of Campylobacter spp. at the genus level (1, 11-15). There is no documented bioinformatics study on the cadF gene full-sequence analysis in C. jejuni and C. coli.

2. Objectives

The aim of this study was to analyze the cadF gene and to develop and evaluate a single-step duplex polymerase chain reaction (PCR) assay for simultaneous detection of C. coli and C. jejuni, the two commonly encountered species in human Campylobacteriosis.

3. Materials and Methods

3.1. Alignment of cadF Sequences From GenBank

The cadF sequences from the complete genome of C. jejuni and C. coli were acquired from NCBI GenBank (http://www.ncbi.nlm.nih.gov/) (Table 1). Multiple alignments were performed using the CLC sequence viewer 7.6 software (CLC bio, Aarhus, Denmark).

3.2. In Silico Analysis of the cadF Gene

The conserved internal fragment (400 bp) of the cadF gene, reported by Konkel et al. (15) as a specific gene for detection of Campylobacter spp., was used as a reference sequence in this study. This fragment and other selected sequences from the full gene of cadF were subjected to in silico analysis with the online NEB cutter program (http://tools.neb.com/NEBcutter) to compare and select a proper restriction endonuclease for discriminating between C. jejuni and C. coli using analysis of enzymatic digestion pattern.

3.3. Designing a Duplex Polymerase Chain Reaction Assay for Specific Detection of Campylobacter jejuni and C. coli

The entire cadF sequence obtained from GenBank was robustly examined for the presence of intra-species conserved regions, which could differentiate inter-species. Universal forward primer, FU, (position 101 - 120) and reverse primer, R1, (position 478 - 497) were selected for the cadF gene, and were previously described by Konkel et al. (15). Other reverse primers, R2 (position 542 - 561) and R3 (position 818 - 837), were designed in this study using the Genrunner and CLC sequence viewer software (Table 2). Analysis of the designed primers was performed by the Primer-BLAST on NCBI (http://www.ncbi.nlm.nih.gov/). Schematic representation of the PCR amplification of fragments related to C. jejuni and C. coli in duplex PCR is shown in Figure 1. Oligonucleotide primers were synthesized by TAG Copenhagen (Denmark).

The duplex-PCR was carried out in a 25-μL reaction mixture, containing 10 ng of DNA template extracted by the boiling method, 2.5 μL PCR buffer 10X, 200 μM dNTP, 5 mM MgCl2, 0.1 μM of each primer, 1 unit of Taq DNA polymerase, and sterile deionized water (12, 14). Amplification conditions were 94°C for three minutes (one cycle), then denaturation at 94°C for 30 seconds, annealing at 43°C for 30 seconds and extension at 72°C for 30 seconds for 32 cycles in a thermocycler (Eppendorf, Hamburg, Germany). Finally, an additional extension step (five minutes, 72°C) was carried out.

Table 1. Campylobacter cadF Sequences Used in This Study

| Definition | Accession No. |
|------------|---------------|
| Campylobacter jejuni subsp. jejuni strain MTVDScj20, complete genome | CP008787.1 |
| Campylobacter jejuni subsp. jejuni 00-2538, complete genome | CP006707.2 |
| Campylobacter jejuni subsp. jejuni 00-2544, complete genome | CP006709.2 |
| Campylobacter jejuni subsp. jejuni PT14, complete genome | NC_018709.2 |
| Campylobacter jejuni subsp. jejuni NCIC 11168 complete genome | AL11168.1 |
| Campylobacter jejuni RM1221, complete genome | CP000025.1 |
| Campylobacter jejuni subsp. jejuni 81116, complete genome | CP000814.1 |
| Campylobacter coli RM2228 contig193, whole genome shotgun sequence | AAFL01000101.0 |
| Campylobacter coli RM1875, complete genome | CP007831.1 |
| Campylobacter coli 15-537360, complete genome | CP006702.1 |
| Campylobacter coli RM5611, complete genome | CP00779.1 |
| Campylobacter coli CVM N29710, complete genome | CP004066.1 |
| Campylobacter coli RM4661, complete genome | CP007181.1 |
| Campylobacter coli JV20 contig00034, whole genome shotgun sequence | AEER01000022.1 |
| Campylobacter coli JV20 genomic scaffold SCAFFOLD1, whole genome shotgun sequence | GL405235.1 |
### Table 2. Primer Sequences for The cadF Gene Used in the Duplex Polymerase Chain Reaction Assay

| Primer | Sequence (5' to 3') | Size of Product, bp | Target | Reference |
|--------|---------------------|--------------------|--------|-----------|
| FU     | TTGAAGGTAATTTAGATAG | 400                | Campylobacter spp. | (15)      |
| R1     | CTAATACCTAAAGTTGAAAC | 400                | Campylobacter spp. | (15)      |
| R2     | TTTAATACAACTTTTCTTG  | 461                | C. coli  | This study |
| R3     | ATATTITCAAGTTTATTAG  | 737                | C. jejuni | This study |

*Annealing temperature is 43°C for all the primers.*

### 3.4. Limit of Detection, Sensitivity and Specificity of the Duplex Polymerase Chain Reaction

Limit of detection (LOD) of the amplification assay was evaluated using serial 10-fold dilutions of genomes with initial concentrations of 140 (C. jejuni) and 7 (C. coli) µg/mL. A total of 20 clinical and environmental *Campylobacter* isolates were examined for further evaluation of the sensitivity. Specificity of the test was evaluated using genomic DNA from standard and isolated clinical strains of other enteric non-*Campylobacter* bacterial pathogens (Table 3). Sensitivity and specificity were calculated according to the following Equations (16):  

\[
\text{Sensitivity} = \frac{\text{(number of positive isolates, as determined by duplex PCR)}}{\text{(total number of positive isolates as determined by three genes (cadF / hipO / asp) PCR}}} \times 100
\]

\[
\text{Specificity} = \frac{\text{(number of negative isolates, as determined by three genes (cadF / hipO / asp) PCR)}}{\text{(total number of negative isolates, as determined by PCR)\times 100}}}
\]
Table 3. List of Bacteria Used for the Determination of Specificity and Sensitivity of \textit{cadF} Targeted Species-Specific Duplex Polymerase Chain Reaction$^a$

| Organism Name               | Strain Name   | Amplification with Newly Designed Primers |
|-----------------------------|---------------|------------------------------------------|
| \textit{Shigella sonnei}    | ATCC 25931    | negative                                  |
| \textit{Shigella flexneri}  | ATCC 12022    | negative                                  |
| \textit{Shigella boydii}    | ATCC 8700     | negative                                  |
| \textit{Shigella dysenteriae}| ATCC 13313    | negative                                  |
| \textit{Aeromonas hydrophila}| ATCC 7966     | negative                                  |
| \textit{Enterobacter aerogenes}| ATCC 13048   | negative                                  |
| \textit{Vibrio cholerae}    | ATCC 39315    | negative                                  |
| \textit{Enteropathogenic Escherichia coli}| ATCC 43887 | negative                                  |
| \textit{Escherichia coli} O157:H7| ATCC 35150   | negative                                  |
| \textit{Enteroinvasive Escherichia coli}| ATCC 43893 | negative                                  |
| \textit{Enterogaugreggative Escherichia coli}| ATCC 35780 | negative                                  |
| \textit{Enterotoxigenic Escherichia coli}| ATCC 35401 | negative                                  |
| \textit{Salmonella typhimurium}| ATCC 29946   | negative                                  |
| \textit{Salmonella typhi}   | ATCC 19431    | negative                                  |
| \textit{Campylobacter jejuni}| ATCC 29428    | positive                                  |
| \textit{Campylobacter coli} | ATCC 43478    | positive                                  |
| \textit{Campylobacter coli} | Isolate 1     | positive                                  |
| \textit{Campylobacter coli} | Isolate 2     | positive                                  |
| \textit{Campylobacter coli} | Isolate 3     | positive                                  |
| \textit{Campylobacter coli} | Isolate 4     | positive                                  |
| \textit{Campylobacter coli} | Isolate 5     | positive                                  |
| \textit{Campylobacter coli} | Isolate 6     | positive                                  |
| \textit{Campylobacter coli} | Isolate 7     | positive                                  |
| \textit{Campylobacter coli} | Isolate 8     | positive                                  |
| \textit{Campylobacter coli} | Isolate 9     | positive                                  |
| \textit{Campylobacter jejuni}| Isolate 1     | positive                                  |
| \textit{Campylobacter jejuni}| Isolate 2     | positive                                  |
| \textit{Campylobacter jejuni}| Isolate 3     | positive                                  |
| \textit{Campylobacter jejuni}| Isolate 4     | positive                                  |
| \textit{Campylobacter jejuni}| Isolate 5     | positive                                  |
| \textit{Campylobacter jejuni}| Isolate 6     | positive                                  |
| \textit{Campylobacter jejuni}| Isolate 7     | positive                                  |
| \textit{Campylobacter jejuni}| Isolate 8     | positive                                  |
| \textit{Campylobacter jejuni}| Isolate 9     | positive                                  |
| \textit{Campylobacter jejuni}| Isolate 10    | positive                                  |
| \textit{Campylobacter jejuni}| Isolate 11    | positive                                  |

$^a$Source of isolations is clinical.

4. Results

4.1. In Silico Analysis of the \textit{cadF} Gene

The length of the \textit{cadF} sequences extracted from complete genome of \textit{C. jejuni} and \textit{C. coli} was 960 (with C + G 31.8\% and A + T 68.2\%) and 999 bp (with C + G 34\% and A + T 66\%), respectively. The \textit{cadF} gene in both species was located after the \textit{rpsl} gene, which coded for a 30S ribosomal protein. Although there were some nucleotide variations along the sequence of \textit{cadF} between the two species, yet the main difference was related to the 39-bp deletion in the positions of 533 - 544 and 560 - 586 of \textit{C. jejuni} (Figure 2). The results of the BLAST analysis of \textit{cadF} gene showed an average sequence identity of 98.5\% and 94\% among \textit{C. jejuni} and \textit{C. coli} strains, respectively. The identity between the two species was also estimated as 88\%, approximately (Table 4).
The analysis of the cadF gene 400-bp product sequence (Figure 2), introduced by Konkel et al. via the NEBcutter online web site, indicated that this part of the gene is conserved and there are no commercially proper restriction enzymes to produce fragments with good intervals to differentiate between C. jejuni and C. coli (15). The selected segments of the cadF gene in this study were theoretically appropriate for enzymatic digestions and the produced fragments could be used for species differentiation.

4.2. Design and Evaluation of a Species-Specific Duplex Polymerase Chain Reaction Assay

Duplex PCR showed that it could simultaneously detect both C. jejuni and C. coli with product sizes of 737 bp and 461 bp, respectively (Figure 3). Specificity and sensitivity of the duplex PCR assay was determined to be 100% with exclusive amplification for C. jejuni and C. coli, while this result was negative for other non-Campylobacter enteric bac-

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Table 4. The Distribution of Nucleotides and the Percentage Identity of the cadF Gene

| Campylobacter spp. | Values |
|--------------------|--------|
| **C. jejuni**<sup>a</sup> | **C + G** | 305 (31.8) |
|                    | **A + T** | 655 (68.2) |
| **C. coli**<sup>a</sup> | **C + G** | 340 (34) |
|                    | **A + T** | 660 (66) |

| Identity between strains, % |
|-----------------------------|
| C. jejuni and C. jejuni     | 97 - 100 |
| C. coli and C. coli         | 88 - 100 |
| C. jejuni and C. coli       | 85 - 91  |

<sup>a</sup>Value are presented as No. (%).
terial species. The assay showed limit of detection (LOD) of 14 and 0.7 µg/mL (approximately equal to $7 \times 10^8$ and $3 \times 10^8$ copy number) for *C. jejuni* and *C. coli*, respectively.

**Figure 3.** Agarose Gel Electrophoresis of the Duplex Polymerase Chain Reaction Assay with Specific Primers

Lanes 1 and 2, 400-bp fragment of the cadF gene of *Campylobacter jejuni* and *Campylobacter coli*, respectively as positive controls; lanes 4 - 6, 737 bp fragment of *C. jejuni*; lanes 9 - 11, 461 bp fragment of *C. coli*; lanes 3, 7, 8, 12 and 13, negative controls; lane M, 1 kb molecular weight marker.

5. Discussion

*Campylobacter jejuni* and *C. coli* are now recognized as important causes of acute bacterial diarrhea in most countries. The isolation and discrimination of *C. jejuni* and *C. coli* by biochemical tests at the species level is limited and laborious, thus there is a crucial need to develop a sensitive, validated and rapid DNA-based method for detection of *Campylobacter* at the species level (10, 17). In some studies, multiple genes have been used for distinguishing *C. jejuni* and *C. coli*. Al Amri et al. (2007) developed a multiplex PCR assay using the combination of a genus-specific virulence gene (*cadF*) together with hippurase and aspartokinase genes (*asp*) for species-specific identification of *C. jejuni* and *C. coli*, respectively (11). In a study by Cloak and Fratamico (2002), a multiplex PCR was developed for differentiation of *C. jejuni* and *C. coli* by means of *cadF* and *ceuE* genes. In another work by Adzitey and Corry (2011), *lpxA*, *hipO* and *gryA* genes were used for differentiating *C. jejuni* and *C. coli* species. The study of Nayak et al. (2005) was also designed with *cadF*, *ceuE* and oxidoreductase subunit genes as fragments of 400-bp conserved region in *Campylobacter* spp. 894-bp specific for *C. coli* and 160-bp specific for *C. jejuni*, respectively (14, 18, 19).

Our duplex PCR method was developed only with the *cadF* gene and the specificity and sensitivity of novel reverse primers (R1 and R2) in association with a previously described forward primer (FU) was studied. The PCR assay designed in this work showed 100% sensitivity and specificity while no amplification product was seen for the genomic DNA from non-*Campylobacter* enteric bacteria. One applicable advantage of this newly designed duplex PCR assay is that the amplified products are of different sizes, which can be concurrently visualized on agarose gel without the need to duplicate the reaction or further electrophoresis and sequencing. In a similar study, Klena et al. used divergence and conservation regions of *lpxA* to develop a robust PCR assay. They differentiated *C. coli*, *C. jejuni*, *C. lari* and *C. upsaliensis* using multiplex PCR with the lipid A gene *lpxA*, encoding a UDP-N-acetyl glucosamine acyl transferase. Another work similar to our research was the study of Gonzalez et al. They discriminated *C. jejuni* and *C. coli* by using *ceuE* gene diversity (approximately 13%) between two species (20, 21).

The lowest concentration of genomic DNA for detection of *C. jejuni* and *C. coli* was 14 and 0.7 µg/mL (approximately equal to $7 \times 10^8$ and $3 \times 10^8$ copy number), respectively. These LODs are almost comparable with the study conducted by Wisessombat et al., in which the sensitivity of the multiplex PCR for the detection of *Campylobacter* spp. was $2 \times 10^5$ CFU/PCR (22). Another study indicated that the colony multiplex PCR sensitivity range for *C. jejuni* and *C. coli* was $10^8$ to $10^{13}$ and $10^6$ to $10^{13}$ CFU/mL, respectively (23).

The bioinformatics data analysis of the 400-bp internal section of the *cadF* introduced by Konkel et al. which has been used by many investigators for genus-specific detection of *Campylobacter* spp. showed that this fragment is highly conserved among *C. jejuni* and *C. coli* strains and is significantly validated for the identification of both species. It seems that there is a concomitant general misjudged belief that the *cadF* full-gene is genus-specific. Our analysis of total *cadF* sequence revealed that other than single-nucleotide variations between two bacteria, an approximately 4% deletion has occurred in the *cadF* sequence of *C. jejuni* compared with *C. coli*, which could be useful for our work. The intra-species identity level among *C. jejuni* and *C. coli* strains was about 98.5% and 94%, respectively. The identity level was approximately 88% between the two species. These results were similar to the report of Konkel et al. with 87% identity between *C. jejuni* and *C. coli* strains and 98.6% among *C. jejuni* strains, individually (15).

There are several articles about the PCR-RFLP method for the differentiation of *Campylobacter* spp. using genes other than *cadF* (24, 25). Although the restriction pattern of enzymatic digestion of the 400-bp fragment introduced by Konkel et al. is not suitable for separation of the two species, yet the enzymatic digestion of the full-length gene may be useful for differentiation and clinical diagnosis of *C. jejuni* and *C. coli* (15) The *cadF* full-gene has some variations in its sequence and length between species, which can be beneficial for developing a duplex PCR. The designed PCR assay in this study is highly sensitive and specific and provides an accurate, inexpensive, sensitive and specific tool for rapid and simultaneous detection and differentiation of *C. coli* and *C. jejuni* in clinical settings.

**Footnotes**

**Authors’ Contribution:** Study concept, design analysis and interpretation of data: Bita Bakhshi, Tahereh Tohidi Moghadam and Saeed Shams; performance and drafting
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