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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*

Saeed Shams,[1] Bita Bakhshi,[1,*] and Tahereh Tohidi Moghadam[2]

[1]Department of Medical Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, IR Iran
[2]Department of Nanobiotechnology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, IR Iran

*Corresponding author: Bita Bakhshi, Department of Medical Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, IR Iran. Tel: +98-2182884558, Fax: +98-2182884555, E-mail: b.bakhshi@modares.ac.ir

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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 *fliY*) (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 $MgCl_2$, 0.1 μM of each primer, 1 unit of Taq DNA polymerase, and sterile deionized water (12, 14). Amplification conditions were 95°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

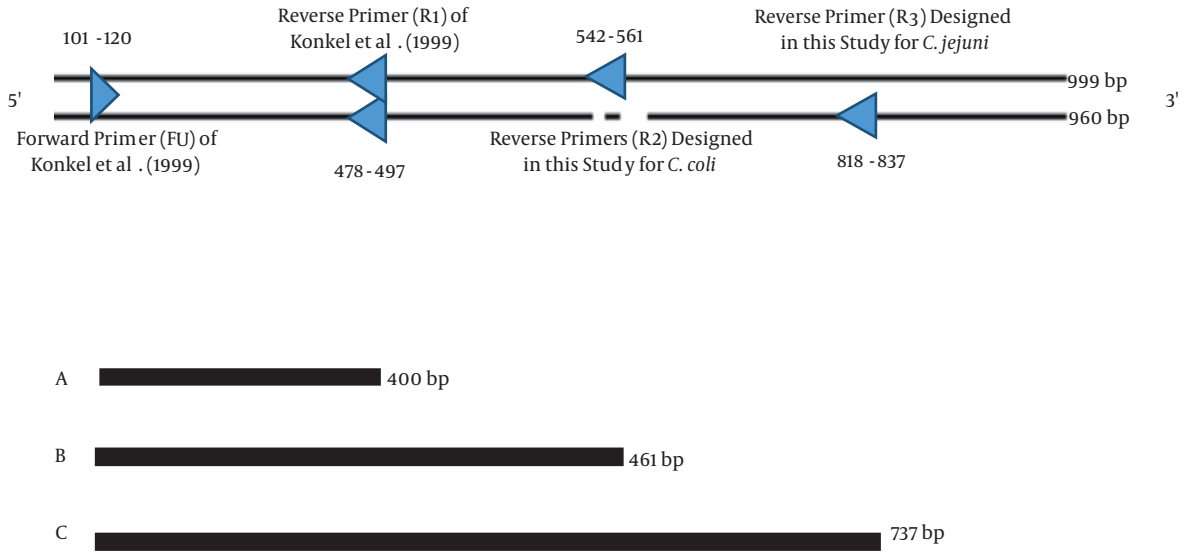
Definition	Accession No.
***Campylobacter jejuni* subsp. *jejuni* strain MTVDSCj20, complete genome**	CP008787.1
***Campylobacter jejuni* subsp. *jejuni* 00-2538, complete genome**	CP006707.2
***Campylobacter jejuni* subsp. *jejuni* 00-2544, complete genome**	CP006709.2
***Campylobacter jejuni* subsp. *jejuni* PT14, complete genome**	NC_018709.2
***Campylobacter jejuni* subsp. *jejuni* NCTC 11168 complete genome**	AL111168.1
***Campylobacter jejuni* RM1221, complete genome**	CP000025.1
***Campylobacter jejuni* subsp. *jejuni* 81116, complete genome**	CP000814.1
***Campylobacter coli* RM2228 cont193, whole genome shotgun sequence**	AAFL01000010.1
***Campylobacter coli* RM1875, complete genome**	CP007183.1
***Campylobacter coli* 15-537360, complete genome**	CP006702.1
***Campylobacter coli* RM5611, complete genome**	CP007179.1
***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[a]

Primer	Sequence (5' to 3')	Size of Product, bp	Target	Reference
FU	TTGAAGGTAATTTAGATATG	400	*Campylobacter* spp.	(15)
R1	CTAATACCTAAAGTTGAAAC	400	*Campylobacter* spp.	(15)
R2	TTTATTAACTACTTCTTTTG	461	*C. coli*	This study
R3	ATATTTTTCAAGTTCATTAG	737	*C. jejuni*	This study

[a]Annealing temperature is 43°C for all the primers.

Figure 1. Position of Primers for Developing the Duplex Polymerase Chain Reaction Assay



Black thin lines of 999 and 960 bp are related to the *cadF* gene of *C. coli* and *C. jejuni*, respectively. The discontinuity is related to deletion in *C. jejuni*. A, produced a 400-bp fragment from both *C. jejuni* and *C. coli* (as the positive control of the assay); B, produced a 461-bp fragment from *C. coli*; C, produced a 737-bp fragment from *C. jejuni*.

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):

$$\text{Sensitivity} = \frac{(\text{number of positive isolates, as determined by duplex PCR})}{(\text{total number of positive isolatesas determined by three genes } (cadF / hipO / asp) \text{ PCR})} \times 100 \quad (1)$$

$$\text{Specificity} = \frac{(\text{number of negative isolates, as determined PCR})}{(\text{total number of negative isolates, as determined by three genes, } (cadF / hipO / asp) \text{ PCR})} \times 100 \quad (2)$$

Table 3. List of Bacteria Used for the Determination of Specificity and Sensitivity of *cadF* Targeted Species-Specific Duplex Polymerase Chain Reaction[a]

Organism Name	Strain Name	Amplification with Newly Designed Primers
Shigella sonnei	ATCC 25931	negative
Shigella flexneri	ATCC 12022	negative
Shigella boydii	ATCC 8700	negative
Shigella dysenteriae	ATCC 13313	negative
Aeromonas hydrophila	ATCC 7966	negative
Enterobacter aerogenes	ATCC 13048	negative
Vibrio cholerae	ATCC 39315	negative
Enteropathogenic Escherichia coli	ATCC 43887	negative
***Escherichia coli* O157:H7**	ATCC 35150	negative
Enteroinvasive Escherichia coli	ATCC 43893	negative
Enteroaggregative Escherichia coli	ATCC 33780	negative
Enterotoxigenic Escherichia coli	ATCC 35401	negative
Salmonella typhimurium	ATCC 29946	negative
Salmonella typhi	ATCC 19430	negative
Campylobacter jejuni	ATCC 29428	positive
Campylobacter coli	ATCC 43478	positive
Campylobacter coli	Isolate 1	positive
Campylobacter coli	Isolate 2	positive
Campylobacter coli	Isolate 3	positive
Campylobacter coli	Isolate 4	positive
Campylobacter coli	Isolate 5	positive
Campylobacter coli	Isolate 6	positive
Campylobacter coli	Isolate 7	positive
Campylobacter coli	Isolate 8	positive
Campylobacter coli	Isolate 9	positive
Campylobacter jejuni	Isolate 1	positive
Campylobacter jejuni	Isolate 2	positive
Campylobacter jejuni	Isolate 3	positive
Campylobacter jejuni	Isolate 4	positive
Campylobacter jejuni	Isolate 5	positive
Campylobacter jejuni	Isolate 6	positive
Campylobacter jejuni	Isolate 7	positive
Campylobacter jejuni	Isolate 8	positive
Campylobacter jejuni	Isolate 9	positive
Campylobacter jejuni	Isolate 10	positive
Campylobacter jejuni	Isolate 11	positive

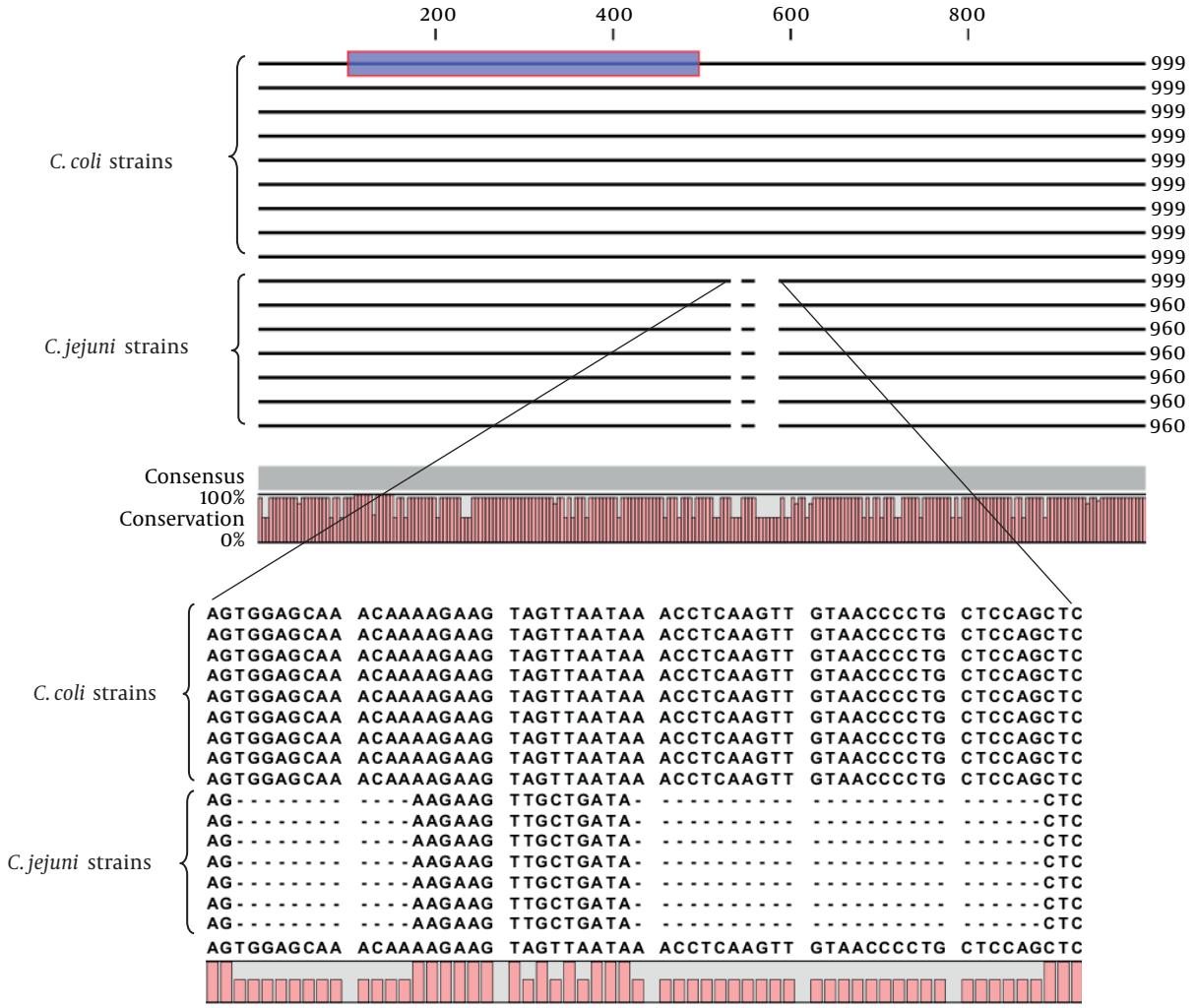
[a]Source of isolations is clinical.

4. Results

4.1. In Silico Analysis of the cadF Gene

The length of the *cadF* sequences extracted from complete genome of *C. jejuni* and *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 *cadF* gene in both species was located after the *rpsI* gene, which coded for a 30S ribosomal protein. Although there were some nucleotide variations along the sequence of *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 *C. jejuni* (Figure 2). The results of the BLAST analysis of *cadF* gene showed an average sequence identity of 98.5% and 94% among *C. jejuni* and *C. coli* strains, respectively. The identity between the two species was also estimated as 88%, approximately (Table 4).

Figure 2. Analysis and Multiple Alignment of the *cadF* Complete Gene of *Campylobacter jejuni* (960 bp) and *C. coli* (999 bp) by the CLC Software



Dashes in the lower box indicate deleted nucleotides in *C. jejuni*. The blue box is the conserved 400-bp internal region introduced by Konkel et al.

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

	Values
***Campylobacter* spp.**	
C. jejuni[a]	
C + G	305 (31.8)
A + T	655 (68.2)
C. coli[a]	
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

[a]Value are presented as No. (%).

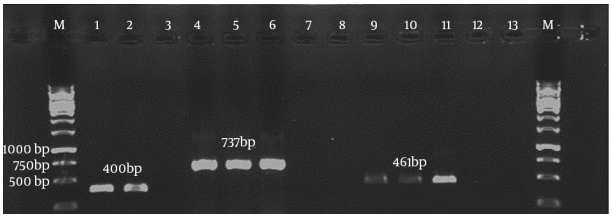
The analysis of the *cadF* gene 400-bp product sequence (Figure 2), introduced by Konkel et al. via the NEBcutter online web site, indicated that this part of the gene is conserved and there are no commercially proper restriction enzymes to produce fragments with good intervals to differentiate between *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-

terial species. The assay showed limit of detection (LOD) of 14 and 0.7 μg/mL (approximately equal to 7×10^9 and 3×10^8 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 hippuricase 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 designed 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 *glyA* 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 (R_2 and R_3) 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 7×10^9 and 3×10^8 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 2×10^5 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* 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

of the manuscript: Saeed Shams; critical revision of the manuscript: Bita Bakhshi.

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