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**Original Article**

# Evaluation of a Multiplex PCR Assay for the Identification of *Campylobacter jejuni* and *Campylobacter coli*

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**Background**: *Campylobacter jejuni* and *Campylobacter coli* are identified as the major causes of acute gastroenteritis in humans. Because of the fastidious nature of *Campylobacters*, many clinical laboratories fail to routinely culture them. The detection of *Campylobacter* spp. using molecular-based techniques can be useful for diagnostic and epidemiological applications. This study aimed to develop a multiplex PCR assay for the simultaneous detection of *C. jejuni* and *C. coli* strains from clinical specimens.
**Materials and Methods**: During a 19-month period, stool samples were collected from 980 children admitted to a hospital in Tehran, Iran and then examined. The samples were cultured on both Brucella agar and Modified Charcoal-Cefoperazone-Deoxycholate agar (mCCDA) media at 42°C for 48 h. To confirm suspected bacteria, Gram staining and other biochemical tests were carried out. Finally, after extracting DNA from pure cultures using the boiling method, the multiplex PCR assay was performed.
**Results:** The multiplex PCR assay showed that *Campylobacter* spp. can be detected using 400 bp target product of *cadF*. It can also accurately distinguish between *C. jejuni* and *C. coli* species with different bands of 735 bp and 500 bp using *hipO* and *asp* genes, respectively.
**Conclusions**: Results showed that the multiplex PCR assay can replace the biochemical assays for differentiating between *C. jejuni* and *C. coli* strains in a single-step PCR test.

*Keywords*: *Campylobacter jejuni*, *Campylobacter coli*, Multiplex PCR

## 1. Background

*Campylobacter* spp. is a common cause of acute gastroenteritis worldwide (1). Among various pathogenic *Campylobacter* species in humans, the thermophilic *Campylobacter jejuni* and *Campylobacter coli* are recognized as the major cause of human campylobacteriosis (2).

The major reservoir and source of *Campylobacters* for humans are poultry and poultry products. Undercooked poultry meat, unpasteurized milk, and water can be considered as the sources of human infection with *C. jejuni* and *C. coli* (3).

The gastrointestinal manifestations of campylobacteriosis disease include cramps, fever, myalgia, weight loss, acute watery or bloody diarrhea; however, the infection may result in severe extra-intestinal sequelae, especially acute immune-mediated neurologic complications such as Guillain-Barre and Miller Fisher syndromes, due to cross-reactivity between the bacterial lipooligosaccharides and nervous system gangliosides (4).

Isolation of *Campylobacters* using the culture method is considered the gold standard for campylobacteriosis disease diagnosis; however, it has several limitations because of the fastidious nature of *Campylobacters* (5). Furthermore, due to the phylogenetic relatedness of *C. jejuni* and *C. coli* species, the identification of *Campylobacter* at species level using biochemical assays is difficult (6). Therefore, other techniques such as PCR and real-time PCR are used as the methods for *Campylobacter* identification, showing a very high sensitivity (7-8).

Differentiation of *Campylobacter* spp. using molecular-based assays can be useful for rapid and specific detection and epidemiological applications. For this purpose, various genes including *fliY, cdt, asp, hipO , glyA, ceuE, cadF* are used (9).

The *asp* gene encodes aspartokinase enzyme and is highly specific for *C. coli*. Hippuricase is produced by the *hipO* gene in *C. jejuni* (a species-specific hippurate hydrolase enzyme), which is absent in *C. coli*. The *cadF* gene also encodes a fibronectin-binding protein that can promote the attachment of the microorganism to eukaryote cells. It has been described as a conserved and genus-specific gene (10-12).

## 2. Objectives

The aim of this study was to differentiate between *C. jejuni* and *C. coli* species using a set of specific primers for *asp*, *hipO,* and *cadF* genes in a multiplex PCR assay.

## 3. Materials and Methods

### *3.1. Sampling and growth conditions*

During a 19-month period (June 2012-January 2014), 980 children with intestinal signs were admitted to one of the major hospitals in Tehran, Iran. 750 suspected cases to campylobacteriosis were included in this study. After collecting the samples, the stool specimens using Carry-Blair Transport media (Micro Media, Hungary) were transferred to the microbiological laboratory and then cultured immediately on Brucella agar (Merck, Germany) with 5% sheep blood and Modified Charcoal–Cefoperazone-Deoxycholate agar (mCCDA) (Merck, Germany). The plates were incubated at 42°C for 48 h under microaerobic conditions in a sealed jar using gas packs (Merck, Germany). Both of the media were supplemented with vancomycin, polymyxin B, and trimethoprim (pH 7.2±0.2). Suspected colonies to *Campylobacter* spp. were confirmed using Gram staining and spiral morphology, catalase and oxidase positive, nitrate reduction positive, and indoxyl acetate hydrolysis positive. For differenting between them at species level, hippurate hydrolysis test positive (*C. jejuni*) and negative (*C. coli*) as well as susceptiblity to nalidixic acid (*C. coli*) were used.

*3.2. DNA extraction and multiplex PCR assay*

DNA extraction from pure cultures of *Campylobacter* isolates was performed by the boiling method in 200 µL sterile distilled water and boiled for 15 min at 100°C. Suspension was then centrifuged at 13,000 g at room temperature for 10 min, and the supernatant was used as template DNA in multiple PCR assay.

The multiplex PCR was carried out within a thermal cycler (Eppendorf, Germany) in a 25-µL reaction mixture containing 10 mg of DNA template, 2.5 µL PCR buffer 10X, 200 µM dNTP, 5 mM MgCl2, 0.1 µM of specific primers (Table 1), 1 unit of Taq DNA polymerase and sterile deionized water.

Amplification conditions for the three genes (*asp*, *hipO,* and *cadF*) were as follow: 95°C for 5 min, followed by 30 amplification cycles; denaturation at 95°C for 45 seconds, annealing at 48°C for 30 seconds, and extension at 72°C for 30 seconds. Finally, an additional extension step (5 min, 72°C) was performed. Finally, amplicons were electrophoresed on 1% agarose gel. *C. coli* ATCC 43478 and *C. jejuni* subsp. *jejuni* ATCC 29428 were used as reference strains.

## 4. Results

Among studied population, 750 children had bacterial systemic and gastrointestinal symptoms including fever, abdominal pain, vomiting, diarrhea with high WBC and RBC count. From a total of 750 cases, 48.5 and 51.5% were male and female, respectively, with an average age of ~6 years. According to the culture results, the number of *Campylobacter* positive cases was 35, in which 33 cases (94 %) were infected by *C. jejuni,* and 2 cases (6 %) were infected by *C. coli*. About 50% of the bacteria were isolated by each of the media (Brucella agar and mCCDA). Most of the positive cases occurred during the summer season from July to September.

The multiplex PCR assay showed that *Campylobacter* spp. can be detected using 400 bp target product of *cadF*. It can also accurately distinguish between *C. jejuni* and *C. coli* species with different bands of 735 bp and 500 bp using *hipO* and *asp* genes, respectively (Fig.1).

## 5. Discussion

*Campylobacter* species have currently been reported as the most common causes of acute bacterial gastroenteritis worldwide both in developing and developed countries. Some studies showed that gastrointestinal infection caused by *C. jejuni* leads to the high levels of morbidity and mortality. About 2 million cases are annually reported to be infected by *C. jejuni* with a mortality rate greater than 2,000 people (13).

Although biochemical tests such as hippurate hydrolysis are useful for the differentiation of *Campylobacters* at the species level, they could also be used for the identification of hippurate hydrolysis-negative strains of *C. jejuni* and false-positive isolates (14). Another disadvantage of conventional tests is that they are extremely time consuming. In this study, the time required for the identification of *Campylobacter* at the species level from a sample was approximately 4 days.

In addition, the accurate differentiation between two *Campylobacter* species is important for the following treatment of the disease in human. For example erythromycin is used for treating *C. jejuni* infection, while *C. coli* strains are resistant to this antibiotic (6). Therefore, an accurate molecular method can be easily used for clinical diagnosis and appropriate treatment of the disease.

In some previous studies, only one gene was used for differentiating between *C. jejuni* and *C. coli* strains. Klena et al. (2004) used divergent and conserved regions of *lpxA* gene of the lipid A, encoding a UDP-*N*- acetyl glucosamine acyl transferase, to differentiate between *C. coli, C. jejuni, C. lari,* and *C. upsaliensis* strains using a multiplex PCR. In another study conducted by Shams et al. (2016), conserved regions of the *cadF* gene were used for the detection and differentiation between *C. jejuni* and *C. coli* species. In another study, Gonzalez et al. (1997) also discriminated between both *C. jejuni* and *C. coli* species using the *ceuE* gene (7, 15-16).

Our study is similar to Al Amri et al. (2007) who used a combination of the genus-specific virulence gene (*cadF*) together with the hippuricase gene (*C. jejuni*) and the aspartokinase gene (*C. coli*), respectively. The same PCR assay was developed to detect isolated strains from Iranian patients (10). Other genes have also been used for distinguishing *Campylobacter* spp. by multiplex PCR method. For example, Cloak et al. (2002) developed a multiplex PCR method using the *cadF* gene of pathogenic *Campylobacter* spp and a specific but undefined gene of *C. jejuni* and the *ceuE* gene of *C. coli*. The *lpxA, hipO,* and *glyA* genes were used by Adzitey et al. (2011) to differentiate between *C. jejuni* and *C. coli* species. In another study, *ceuE*, *cadF,* and oxidoreductase subunit genes were used for the differentiation purpose (6, 17-18).

In all mentioned studies the multiplex PCRs were able to amplify products with different sizes which can be concurrently visualized on the agarose gel without extra reaction and electrophoresis. Thus, it could be considered as a significant advantage over single-species identification systems for the evaluation of the disease in a large number of clinical specimens.

## 6. Conclusion

The designed multiplex PCR assay in this study is a sensitive and specific tool which can be useful for the rapid and simultaneous detection and differentiation of *C. jejuni* and *C. coli* species in clinical settings.

## Conflict of Interest

The authors declare they have no conflict of interest.

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## Authors' Contributions

All of authors contribute to this study.

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**Table 1. Nucleotide sequences of the primers used in the multiplex PCR assay.**

| Primer | Sequence ( 5' to 3' ) | Size of product (bp) | Targets | | Annealing temperature (°C) |
|---|---|---|---|---|---|
| | | | Gene | Bacteria | |
| ***cadF* -F**<br>***cadF* -R** | TTGAAGGTAATTTAGATATG<br>CTAATACCTAAAGTTGAAAC | 400 | *cadF* | *C. jejuni* & *C. coli* | 43 |
| **asp-F**<br>**asp-R** | GGTATGATTTCTACAAAGCGAG<br>ATAAAAGACTATCGTCGCGTG | 500 | *asp* | *C. coli* | 43 |
| ***hipO* -F**<br>***hipO* -R** | GAAGAGGGTTTGGGTGGTG<br>AGCTAGCTTCGCATAATAACTTG | 735 | *hipO* | *C. jejuni* | 43 |

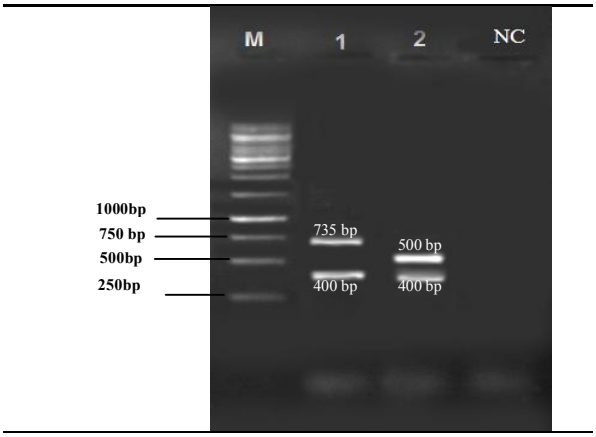


**Fig. 1:** Gel electrophoresis of the multiplex PCR. Lane 1, 400 bp fragment of the *cadF* gene of *Campylobacter* spp. and 735 bp target of *hipO* ; lane 2, 400-bp fragment of the *cadF* gene of *Campylobacter* spp. and 500 bp fragment of *asp* gene; lane NC, Negative Control, lane M, 1 kb molecular weight marker.

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