

Molecular Detection and Speciation of *Campylobacter* Species in Children With Gastroenteritis Using Polymerase Chain Reaction in Bahonar Hospital of Karaj City

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Background: *Campylobacter* spp. is recognized as one of the important bacterial agents of food borne diseases worldwide. *C. jejuni* and *C. coli* are the most frequently reported species causing bacterial gastrointestinal disorders in developed and some of the developing countries.

Objectives: The goals of this survey were to detect and to differentiate *C. jejuni* and *C. coli* species in stool specimens and to determine the frequency of *Campylobacter* gastroenteritis in children referred to Bahonar hospital in Karaj city using polymerase chain reaction (PCR).

Patients and Methods: One hundred sixty stool specimens were collected from neonates and children under 8 years old during August to October of 2009. PCR was optimized to amplify a 400 bp fragment of the *cadF* gene of *Campylobacter* genus in the clinical specimens, then multiplex PCR optimization was carried out using *hipO* and *asp* primers. *C. jejuni* RTCC 1097 and *C. coli* RTCC 1113 reference strains were used as positive controls and only the PCR positive specimens were examined by this method.

Results: The results revealed the amplification of a 400 bp DNA fragment and *Campylobacter* contamination in 11.3 % of the specimens. Of 18 PCR positive specimens examined by duplex PCR method, 4 (22.2%) were identified as *C. jejuni*, 2 (11.1%) as *C. coli*, 3 (16.6%) as mixed infection with both species and 9 (50% of the positive specimens) were identified as non *coli* – non *jejuni* *Campylobacter*. The sensitivity of the single PCR method at the DNA level was determined to be 100 pg and the specificity of the method was determined using 6 other important bacterial agents of gastrointestinal diseases.

Conclusions: Although the findings of this survey confirmed the presence of *C. jejuni* and *C. coli* species in half of the positive specimens, the probable role of the other species of *Campylobacter* in children gastroenteritis was unexpected.

Keywords: *Campylobacter*; Gastroenteritis; Iran; Multiplex Polymerase Chain Reaction

1. Background

Campylobacter spp. is recognized as one of the important bacterial agents of food borne diseases worldwide. *C. jejuni* and *C. coli* are the most frequently reported species causing bacterial gastrointestinal disorders in developed and some of the developing countries (1-3). Infection with above mentioned species is characterized by the sudden onset of fever, abdominal cramps, diarrhea, dysentery, presence of fecal leukocytes and in a few percent of the cases can result in post infectious sequelae such as Guillain-Barre syndrome. There are many probable sources of infection with *C. jejuni* and *C. coli*, as they are members of the normal gut flora in a wide range of birds and mammals. Large-scale outbreaks of human *Campylobacteriosis* are rare and are usually associated with the consumption of polluted water or unpasteurized milk. Sporadic cases of *Campylobacteriosis* are more common and due to the consumption of undercooked chicken (1). The results of

the limited published molecular surveys on *Campylobacter* infections in Iran confirmed the major role of *jejuni* and *coli* species in the cases of *Campylobacteriosis* (4).

2. Objectives

According to the specific geographical and social situation of Karaj which is a large city and very close to Tehran, limited published surveys about the *Campylobacter* gastroenteritis are available in this region. Therefore, the goals of this survey were to detect and to differentiate *C. jejuni* and *C. coli* in stool specimens and to determine the frequency of *Campylobacter* gastroenteritis in children referred to Bahonar hospital in Karaj city using PCR

3. Patients and Methods

3.1. Clinical Samples

One hundred sixty stool specimens were collected from

neonates and children under 8 years old with gastrointestinal disorders referred to Bahaonar hospital of Karaj city during August to October of 2009. All the collected specimens were stored at -20°C until the time of DNA extraction.

3.2. Bacterial Strains

The reference strains of bacteria used in this survey were as follows: *Campylobacter jejuni* RTCC 1097 and *Campylobacter coli* RTCC 1113 (as positive controls of PCR assays) and *Salmonella enterica* serovar Enteritidis RTCC 1621, *E.coli* RTCC 1161, *Shigella dysenteriae* PTCC 1188, *Proteus vulgaris* RTCC 1463, *Klebsiella pneumoniae* RTCC and *Bacillus cereus* RTCC 1068 (were used to investigate the specificity of PCR). All the reference strains were purchased from Razi Type Culture Collection (Razi Vaccine and Serum Research Institute) except the *Shigella dysenteriae* PTCC 1188 which were Prepared from Persian Type Culture Collection (Iranian Research Organization for Science and Technology).

3.3. DNA Extraction

DNA of the specimens were extracted using DNG plus kit (Cinnagen) according to the manufacturer's recommended procedure.

3.4. Single PCR

PCR optimization was performed to amplify a 400 bp segment of the *cad F* gene of *Campylobacter* genus in the clinical specimens, the primer set used as described previously (5) was: *cadF* (F 5'-TTG AAG GTA ATT TAG ATA TG-3' and R 5'-CTA ATA CCT AAA GTT GAA AC-3'), the PCR protocol was performed in reaction mixture at a total volume of 25 µL, consisting of 2.5 µL of 10X PCR buffer, 0.5 µL of dNTPs (10 mM), 0.75 µL of MgCl₂, 1.5 µL (10 pmol) of each of forward and reverse *cad F* primers, 1 µL of template (DNA), 17 µL of sterile water and 0.25 µL of Taq DNA Polymerase (5 unit / µL). The PCR amplification cycle included primary denaturation at 95°C for 6 minutes, followed by 38 cycles of denaturation at 95°C for 45 seconds, annealing at 50°C for 1 minute and extension at 72°C for 1 minute. The final stage was an extension cycle at 72°C for 10 minutes. PCR cycles were performed in an Applied Biosystems thermal cycler.

3.5. Multiplex (Duplex) PCR

Multiplex (duplex) PCR was optimized using the previously described primer sets (6) including : *hipO* (*jejuni* species specific): (F 5'-GAA GAG GGT TTG GGT GGT G-3' and

R 5'-AGC TAGCIT CGC ATA ATA ACT TG-3') yielding a 735 bp product and *asp* (*coli* species specific) : (F 5'-GGT ATG ATT TCT ACA AAG CGA G-3' and R 5'-ATA AAAGAC TAT CGT CGC GTG-3') yielding a 500 bp product. In all experiments *C. jejuni* RTCC 1097 and *C. coli* RTCC 1113 reference strains were used as positive controls and only the PCR positive samples were tested by this technique, the amounts of the reagents of the duplex PCR was the same as single PCR method and the temperature profile included primary denaturation at 96°C for 6 minutes, followed by 35 cycles of denaturation at 95°C for 40 seconds, annealing at 56°C for 45 seconds and extension at 72°C for 1 minute. The final stage was an extension cycle at 72°C for 10 minutes. The amplified PCR products were stained with DNA Safe Stain (Cinnagen) and visualized with a UV transilluminator. The length of PCR amplicons were estimated by comparison with 100 bp DNA molecular markers and the amplified control strains.

3.6. Sensitivity and Specificity of PCR

The DNA concentration was measured and then serial dilutions (1: 10, 1: 100, 1: 1000 and 1: 10000) were used together with the concentrated DNA to determine the minimal amount of DNA detectable by the single PCR protocol. Specificity of the test was determined using the positive controls and 6 other bacteria such as important agents of gastrointestinal disorders and some enterics that are found in stool specimens normally including: *Salmonella enterica* serovar Enteritidis, *E. coli*, *Shigella dysenteriae*, *Proteus vulgaris*, *Klebsiella pneumonia*.

4. Results

Evaluation of the single PCR products by agarose gel electrophoresis revealed the amplification of a 400 bp fragment and *Campylobacter* contamination in 11.3 % of the stool specimens (Figure 1). Of 18 PCR positive specimens evaluated by duplex PCR technique, 4 (22.2%) were detected as *C. jejuni*, 2 (11.1%) as *C. coli*, 3 (16.6%) as mixed infection with both species and 9 (50% of the positive samples) were detected as non *coli* - non *jejuni* *Campylobacter* species (Figure 2). The sensitivity of the single PCR method at the DNA level was determined to be 100 pg and the specificity of the test was determined using 6 other important bacterial agents of gastrointestinal disorders and found to be positive only for *Campylobacter* spp. Using t - test showed the significantly higher rate of the infection in under 2 years age groups (P = 0.01) but there was no statistically significant difference between the rates of the infection in male and female groups (Tables 1 and 2).

Table 1. *Campylobacter* spp. Contamination of Stool Specimens in the Gender Groups ^a

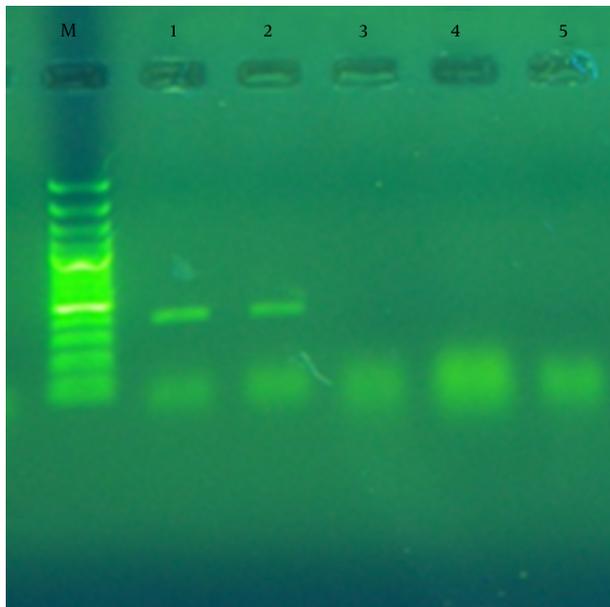
Gender	<i>C. jejuni</i>	<i>C.coli</i>	Mixed Infection	Other Species	Total
Male (n=93)	2 (2.2)	1 (1.1)	3 (3.2)	6 (6.5)	12 (12.9)
Female (n=67)	2 (3)	1 (1.5)	0 (0)	3 (4.5)	6 (9)

^a Data are presented as No.(%).

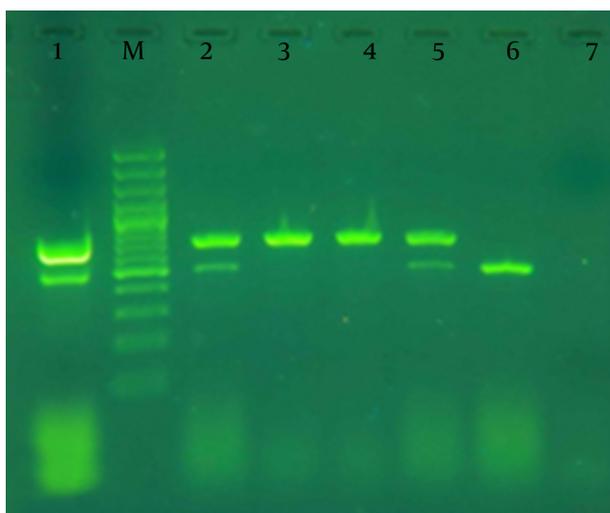
Table 2. *Campylobacter* spp. Contamination of Stool Specimens in Different Age Groups

Age Groups	<i>C. jejuni</i>	<i>C. coli</i>	Mixed Infection	Other Species	Total ^a
Under 1 year	2	2	2	5	11 (21.6)
1 - 2 years	2	0	1	2	5 (10.9)
Over 2 years	0	0	0	2	2 (4.4)

^a Data are presented as No.(%).

Figure 1. Agarose Gel Electrophoresis of Single PCR Products

M: 100 bp DNA ladder, lane 1: positive control, lane 2: *Campylobacter* positive sample (400 bp product), Lanes 3, 4: negative samples, lane 5: negative control.

Figure 2. Agarose Gel Electrophoresis of Multiplex PCR

M, 100 bp DNA ladder, lane 1: positive controls of *C. jejuni* and *C. coli*, lanes 2 and 5: mixed infections, lanes 3 and 4: *C. jejuni* (735 bp product), lane 6: *C. coli* (500 bp product), lane 7: negative control

5. Discussion

Campylobacter species, primarily *C. jejuni* and *C. coli*, are recognized as important bacterial agents of gastroenteritis in human (1, 3) and domestic animals especially poultry, livestock and companion animals (7). The goals of this survey were to detect and to differentiate *C. jejuni* and *C. coli* species in stool specimens and to determine the frequency of *Campylobacter* gastroenteritis in children referred to Bahonar hospital in Karaj city using PCR.

In this survey primers against three genes of *Campylobacter* spp. including *cadF* (genus - specific virulence gene), *hipO* (hippuricase gene for *C. jejuni*) and *asp* (aspartokinase gene for *C. coli*) were used. These genes and primers have been studied independently and reported on by other workers and all of them, especially the *cadF* gene, are highly conserved among isolates of different sources (1, 5, 6, 8-10).

The results point to the suitability of the PCR based assays especially multiplex PCR as sensitive methods for rapid and direct detection and simultaneous speciation of *Campylobacter* spp. in clinical specimens (according to fastidious growth requirements and limited biochemical tests for conventional detection of these bacteria). Our findings in this survey confirmed the presence of *C. jejuni* and *C. coli* in 50% of the positive samples, but the probable role of the other species of *Campylobacter* in children gastroenteritis is unexpected, although hippuricase and aspartokinase genes which were the target of the species specific primers used in this survey are highly conserved (6), the low rate of the infection with *C. jejuni* and *C. coli* comparing with the results of the other researches may be due to the variations in the binding sites of these primers in some of our isolates. Of course more surveys using different primers specific for the other species of *Campylobacter* genus like *fetus*, *laridis*, *upsaliensis*, *hyointestinalis*, restriction fragments length polymorphism (RFLP) assay and sequencing of the single PCR products (*Campylobacter* positive samples of this survey) seem to be necessary for molecular characterization of these isolates and are going to be done.

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Authors' Contributions

All authors have participated in this research.

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