Int J Enteric Pathog. 2016 May; 4(2):e33867. doi: 10.17795/ijep33867.

Published online 2016 May 1. Research Article

Comparative Study of *Campylobacter* spp. Isolated from Children With Gastroenteritis in Bahonar Hospital, Karaj, Using PCR and RFLP

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Received 2015 November 02; **Revised** 2016 January 13; **Accepted** 2016 January 19.

Abstract

Background: *Campylobacter* species are responsible for the majority of cases of food-borne gastroenteritis. The sources of the disease outbreaks are often contaminated water or milk, and consumption of undercooked poultry product is the main cause of sporadic campylobacteriosis cases.
Objectives: The aims of this study were to determine the prevalence of *Campylobacter* gastroenteritis in children and to differentiate the interfering species using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods at the Bahonar hospital in Karaj, Iran.
Patients and Methods: A total of 150 stool samples were collected from children under 10 years old during the summer of 2014. PCR was performed using genus- and species-specific primers and RFLP was done using *AluI* and *TasI* enzymes.
Results: The results showed the amplification of 400 and 491 bp segments and *Campylobacter* contamination in 30 (20%) samples; 5 out of 30 *Campylobacter* positive samples (16.66%) were identified as *C. jejuni*, 20 (66.66%) as *C. coli*, 3 (10%) as *C. jejuni* and *C. coli* (mixed infection), and 2 (6.66%) were identified as non-*jejuni*, non-*coli Campylobacter* using the PCR method. Following the evaluation of RFLP results, 7 positive samples (23.33%) showed the electrophoretic pattern of *C. jejuni*, 21 (70%) showed the electrophoretic pattern of *C. coli*, and 2 (6.6%) showed both of the patterns and mixed contamination with *jejuni* and *coli* species. The results of digestion with *TasI* did not show any *C. lari* or *C. upsaliensis* patterns.
Conclusions: The results of this study showed high percentage of *Campylobacter* contamination in the tested stool samples. The other surprising finding was the high rate of *Campylobacter coli* positive samples; the difference between the results of PCR using species-specific primers (*hipo* and *asp*) and the RFLP method (electrophoretic patterns) in some of the positive samples confirms the hypothesis of variations in nucleotide sequences of the *hipo* primer binding site in some Iranian isolates of *Campylobacters*.

Keywords: Children, Gastroenteritis, PCR, RFLP, *Campylobacter jejuni*, *C. coli*

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1. Background

Campylobacter species are responsible for the majority of food-borne gastroenteritis in many developed and developing countries. Infection by *Campylobacter* can result in severe problems outside the intestine such as Guillain-Barre syndrome (GBS). *Campylobacter* species are estimated to be responsible for 400 to 500 million cases of diarrhea each year worldwide. The incidence of diarrhea in children under five years of age is estimated at 40,000 to 100,000 cases yearly in developed countries. The sources of disease outbreaks are often contaminated water or milk, and consumption of undercooked poultry product is the main cause of sporadic campylobacteriosis cases (1-8). *Campylobacter* detection is based on culturing stool samples, which requires specific selective media and microaerophilic conditions at 37°C to 42°C for 48 hours. The sodium hippurate hydrolysis reaction is a culture-based biochemical test used to differentiate *Campylobacter jejuni* from *C. coli*. However, detection of *Campylobacter* using polymerase chain reaction (PCR) based methods is currently more common because of their high sensitivity and specificity and the above-mentioned difficulties and limitations of culture and biochemical methods (9, 10).

2. Objectives

According to the results of recent molecular surveys on *Campylobacter* gastroenteritis, variation in the nucleotide sequence of *hipo* and *asp* primer binding sites in Iranian isolates of *Campylobacter* is probable, and the aim of this study was to test this hypothesis by comparing the results of PCR (using the species-specific primers) and restriction fragment length polymorphism (RFLP) methods.

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3. Patients and Methods

3.1. Bacterial Strains and Stool Samples

C. jejuni RTCC 1097 and *C. coli* RTCC 1113 standard strains were prepared from Razi type culture collection (Razi vaccine and serum research institute). Children's stool specimens (n = 150) were collected from Bahonar hospital in Karaj from July to September 2014. These samples were collected from children under 10 years of age suffering from symptoms of gastroenteritis. Collected samples were stored at -20°C until DNA extraction.

3.2. DNA Extraction

DNA extraction from stool samples was performed by using CinnaPure DNA kits (CinnaGen, Iran) according to the manufacturer's recommended procedure. First, distilled water was added to the collection tube to provide a homogenous suspension. Then, 100 μL of this suspension was transferred to a sterile micro tube. In the next step, 100 μL pre-lysis buffer and 10 μL ributinase were added and shaken for a few minutes. Finally, the tube was placed for 45 minutes in a heater at 55°C, and every 10 minutes the sample was shaken again and transferred back to the heater. The obtained DNA was stored at -20°C.

3.3. Polymerase Chain Reaction

PCR was performed using primers targeting genes, namely *cadf* (genus-specific virulence gene), *23S rRNA* (genus-specific gene), *hipo* (hippuricase gene for *C. jejuni*) and *asp* (aspartokinase gene for *C. coli*). The primer sets are listed in Table 1 (Bioneer, South Korea). All extracted DNA from specimens was checked by genus-specific *cadF* primer, then positive samples were analyzed with *hipo* and asp primers to differentiate *jejuni* and *coli* species (Figure 1). All *cadf* positive samples were tested by PCR using *23S rRNA* (another *Campylobacter* genus-specific gene) primer, and the products were digested with *AluI* and *TasI* enzymes using the RFLP method (9-14). The PCR mixture in a total volume of 25 μL for each primer contained: 12.5 μL Taq Mix (2×) with 1× final concentration (DELTA life sciences), 1 μL reverse primer (10 μM), 1 μL forward primer (10 μM), 5.5 μL sterile water, and 5 μL template DNA. The PCR amplification was performed for 35 cycles for each set of primers, which have different thermal conditions as shown in Table 2. The amplified PCR products were stained by DNA safe stain and were visualized via a UV transilluminator after electrophoresis on 1.3% agarose gel.

Table 1. List of Oligonucleotide Primers used for PCR

Gene	Primer Sequences	Product Size, bp	Reference
***Cadf*, genus-specific**		400	(9)
F	5'TTG AAG GTA ATT TAG ATA TG 3'		
R	5'CTA ATA CCT AAA GTT GAA AC 3'		
***23S rRNA*, genus-specific**		491	(14)
F	THERM1: 5'ATT CCA ATA CCA ACA TTA GT 3'		
R	THERM4: 5'CTT CGC TAA TGC TAA CCC 3'		
***Hipo*, *C. jejuni*-specific species**		735	(9)
F	5'GAA GAG GGT TTG GGT GGT G 3'		
R	5'AGC TAG CTT CGC ATA ATA ACT TG 3'		
***Asp*, *C. coli*-specific species**		500	(9)
F	5'GGT ATG ATT TCT ACA AAG CGA G 3'		
R	5'ATA AAA GAC TAT CGT CGC GTG 3'		

3.4. PCR-RFLP Conditions

In this step, positive PCR products of *23S rRNA* were digested using two restriction enzymes, *AluI* and *TasI* (Thermo Scientific) in order to differentiate *Campylobacter* species (11, 13, 14). The RFLP mixture for a final *AluI* volume of 32 μL contained: 10 μL PCR product of *23S rRNA*, 1 μL restriction enzyme *AluI*, 2 μL (10×) tango buffer, and 18 μL nuclease-free water. Finally, the mixture was incubated at 37°C for 150 minutes. For *TasI*: 10 μL of PCR product of *23S rRNA*, 1 μL restriction enzyme *TasI*, 2 μL (10×) buffer B, and 18 μL nuclease-free water was incubated at 65°C for 4 hours after adding paraffin oil. Electrophoretic patterns obtained after digestion of the PCR-amplified DNA of the positive samples were analyzed on 1.7% agarose gel.

3.5. Sensitivity of PCR

Digestion of PCR products by *AluI* and *TasI* endonucleases yielded known electrophoretic patterns (the RFLP method confirms our PCR results) (15, 16).

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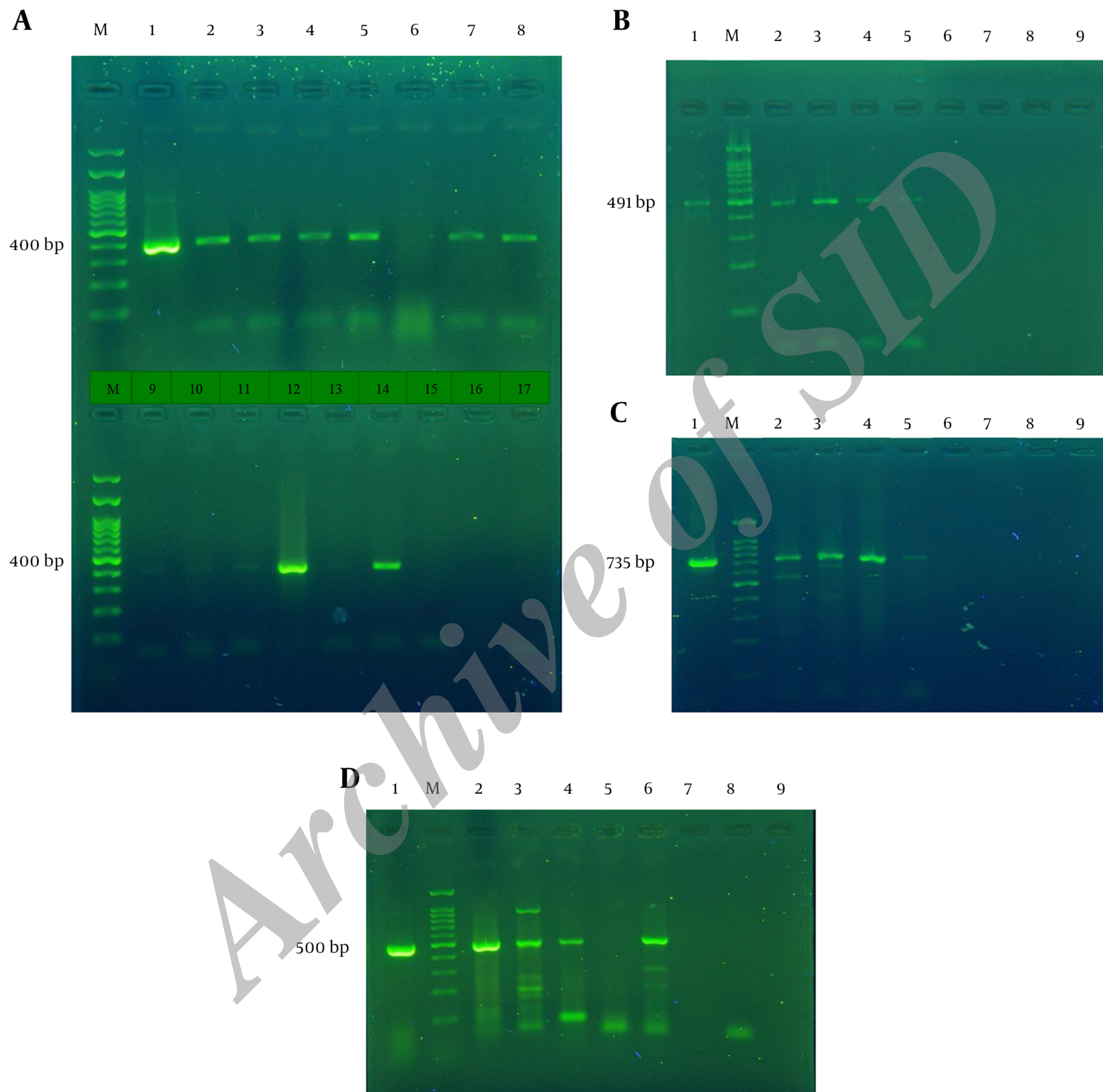
Figure 1. PCR Amplification Products of Samples for Identification and Speciation of *C. jejuni* and *C. coli*



A, lanes M: 100 bp ladder; lane 1: positive control for *cadf* PCR product; lanes 2 - 5, 7 - 12, 14: *Campylobacter*; lane 17: negative control; B, lanes M: 100 bp ladder; lane 1: positive control of *23S rRNA* PCR product; lanes 2 - 5: *Campylobacter*; lane 7: negative control; C, lane M: 100 bp ladder; lane 1: positive control for hipo PCR product; lanes 2 - 5: *C. jejuni*; lane 7: negative control; D, lane M: 100 bp ladder; lane 1: positive control for *asp* PCR product; lanes 2 - 4, 6: *C. coli*; lane 8: negative control.

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Table 2. Reagents and Thermal Conditions of PCR

Primer	Primary Denaturation	Denaturation	Annealing	Extension	Number of Cycles	Final Extension
cadf	95°C, 5 minutes	95°C, 30 seconds	50°C, 1 minute	72°C, 1 minute	35	72°C, 10 minutes
23S rRNA	95°C, 5 minutes	95°C, 30 seconds	54°C, 45 seconds	72°C, 1 minute	35	72°C, 10 minutes
hipo	95°C, 5 minutes	95°C, 30 seconds	64°C, 40 seconds	72°C, 80 seconds	35	72°C, 7 minutes
asp	5°C, 5 minutes	95°C, 30 seconds	54°C, 45 seconds	72°C, 1 minute	35	72°C, 10 minutes

4. Results

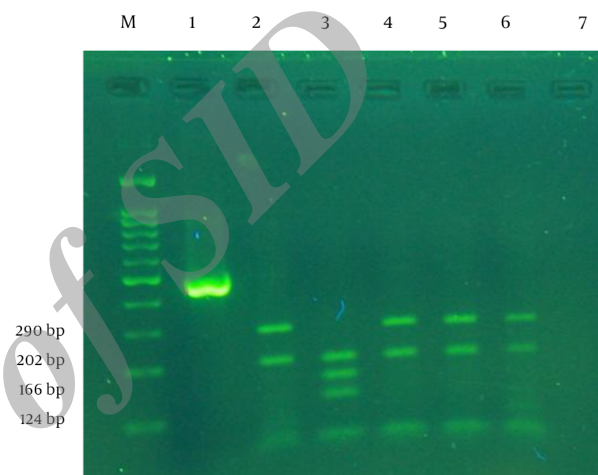
Evaluation of the PCR products showed amplification of 400 and 491 bp genus-specific segments in 30 out of 150 tested samples. The results of PCR using species-specific primers and RFLP methods for speciation of *Campylobacter* in positive samples are shown in Table 3, Figures 2 and 3. Analysis using the t-test showed a significantly higher rate of infection in children under 1 year of age (P = 0.37), but there was no statistically significant difference between the rates of infection in male and female groups (Tables 4 and 5).

Table 3. PCR and RFLP Results[a,b]

Campylobacter Species	Methods	
	PCR	RFLP
C. jejuni	5 (16.66)	7 (23.33)
C. coli	20 (66.66)	21 (70)
C. jejuni and *C. coli*	3 (10)	2 (6.66)
Non *jejuni* and non *coli*	2 (6.66)	0 (0)

[a]Did not identify any *C. lari* and *C. upsaliensis*.
[b]Values are expressed as No. (%).

Figure 2. Electrophoretic Patterns of Positive Samples Following Digestion With *AluI*



Lanes M: 100 bp ladder; lane 1: positive control for *23S rRNA* PCR product; lanes 2, 4 - 6: *C. coli*; lane 3: *C. jejuni*; lane 7: negative control.

5. Discussion

Campylobacter spp., especially *C. jejuni* and *C. coli*, are recognized as one of the major bacterial agents of food-borne gastroenteritis worldwide over recent decades (1). Culture-based methods have a number of limitations for detection of *Campylobacter* spp. in human fecal samples, such as the requirement for 48 hours incubation to isolate the organisms (10). Therefore, PCR-based methods have been developed for detection of *Campylobacter* spp. in different specimens, including fecal samples (11, 13, 14). The main source of *Campylobacter* disease outbreaks is associated with consumption of contaminated water or milk and consumption of undercooked food; in particular, poultry products are the main cause of sporadic Campylobacteriosis cases (1-8). Contamination of milk with *Campylobacter* spp. is often seen after pasteurization or due to poor pasteurization; for example, milk bottles that were pecked by jackdaws and magpies, contaminating free school milk, were a probable source of human *Campylobacter* infection in Great Britain in an outbreak affecting around 2500 children (6). Particular groups of people, such as infants, children, pregnant women, and individuals with immunodeficiency, are more at risk of developing campylobacteriosis (9, 11, 17-21).

Many PCR-based molecular surveys have been performed on *Campylobacter* contamination in different clinical samples during the last two decades. Al Amri et al. (9) evaluated 114 culture-positive stool specimens (54 human and 60 chicken) using multiplex PCR. According to the results, 70 (61.4%) were identified as *C. jejuni*, 35 (30.7%) as *C. coli*, and 9 (7.9%) as a mixed infection with *C. jejuni* and *C. coli*. In addition, two culture-negative samples were identified as *C. jejuni*. This study showed that multiplex PCR is a highly sensitive and specific tool for direct detection and identification of *Campylobacters* spp. in stool samples.

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Table 4. *Campylobacter* spp. Contamination of Stool Specimens in Different Age Groups

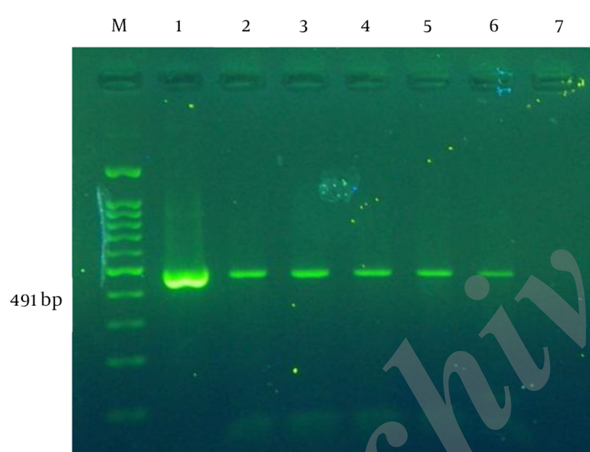
Age Group	*C. jejuni*	*C. coli*	Mixed Infection	Other Species	Total, No. (%)
Under 1 year	3	12	2	0	17 (24.28)
1 - 2 years	2	2	0	0	4 (12.5)
Over 2 years	2	7	0	0	9 (18.75)

Table 5. *Campylobacter* spp. Contamination of Stool Specimens by Gender Groups[a]

Gender	*C. jejuni*	*C. coli*	Mixed Infection	Other Species	Total
Male (n = 91)	5 (5.49)	12 (13.18)	2 (2.19)	0	19 (20.87)
Female (n = 59)	2 (3.38)	9 (15.25)	0	0	11 (18.64)

[a]Values are expressed as No. (%).

Figure 3. Electrophoretic Patterns of Positive Samples Following Digestion With *TasI*



Lanes M: 100 bp ladder; lane 1: positive control for *23S rRNA* PCR product; lanes 2 - 6: *23S rRNA*; lane 7: negative control. There were no restriction fragments by digestion with *TasI*.

Fermer et al. (11) reported on the PCR–RFLP method using THERM1 and THERM4 primers and *AluI* and *Tsp5091* restriction enzymes as a high-potential technique for detection and speciation of thermophilic *Campylobacters* (*C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*). Following molecular analysis of 118 *Campylobacter* isolates from human and veterinary clinical samples, they identified 95.5% of human isolates as *C. jejuni*, 3% as *C. coli*, and 1.5% as *C. upsaliensis*. Steinhauserova et al. (14) reported on the PCR-RFLP method using THERM1 and THERM4 primers and *AluI* and *Tsp5091* restriction enzymes as a high-potential technique for detection and speciation of thermophilic *Campylobacters* (*C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*). Following molecular analysis of 204 *Campylobacter* isolates from human, dog, and pig clinical samples, they identified 64.4% of human isolates as *C. jejuni* and 35.6% as *C. coli*.

In this study PCR using genus-specific primers (*cadf* and *23S rRNA*) was performed to determine the frequency of *Campylobacter* contamination in the cases of children with gastroenteritis in Bahonar hospital, Karaj, Iran, and PCR using species-specific primers (*hipo* for *C. jejuni* and *asp* for *C. coli*) and RFLP were carried out to differentiate the interfering species. The results of PCR using both genus-specific primers (*cadf* and *23S rRNA*) revealed *Campylobacter* contamination in 20% of tested stool samples, which is relatively high rate compared to the results of most other published surveys, i.e., those performed by Bessede et al. (2), Zaghloul et al. (3), Lawson et al. (10), Samosornsuk et al. (22), and Harzandi et al. (15). The higher frequency of *Campylobacter* gastroenteritis could be due to increased consumption of undercooked poultry products in recent years as well as the high sensitivity of molecular techniques in comparison with culture-based methods. *Cadf*, *hipo*, and asp genes have all been the targets of primers used in much independent research. Although these genes are reported to be highly conserved among isolates from food and clinical samples (9), according to the results of recent molecular surveys on *Campylobacters* (15, 16) variation in the sequence of *hipo* and *asp* primer binding sites in Iranian isolates is probable, and the aim of this study was to test this hypothesis. Comparing the results of PCR and RFLP using *AluI* RE in the speciation of positive samples (Table 3) confirmed the above-mentioned hypothesis. It can be concluded that the lack of amplification of species-specific segments in two of seven *C. jejuni* isolates (detected based on *jejuni* species-specific electrophoretic pattern, yielding 202, 166, and 124 bp segments) is due to differences in the nucleotide sequence of the *hipo* primer

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binding site in these isolates compared with the standard strain. Another finding supporting the hypothesis is that increasing the annealing temperature using a gradient thermal cycler weakened the corresponding species-specific bands in some of the positive samples.

Both PCR (with *hipo* and *asp* primers) and RFLP results showed remarkable and high percentages of *C. coli* contamination in tested samples, which is in contrast to the results of most other studies. Of course, Wardak et al. (17) in Poland and Ronveaux et al. (18) in Belgium reported similar findings. High frequency of *C. coli* could be due to transmission of the bacterium from person to person as well as a common source of infection at the same time and in the same area. None of the *Campylobacter*-positive samples were identified as *C. lari* or *C. upsaliensis* using the RFLP method, which shows low contamination with these species in the cases of gastroenteritis in children. The results of this study showed a high percentage of *Campylobacter* contamination in the tested stool samples. The other surprising finding was the high rate of *Campylobactercoli*-positive samples. In addition, the difference between the results of PCR using species-specific primers (*hipo* and *asp*) and the RFLP method (electrophoretic patterns) in some of the positive samples confirms the hypothesis of variations in the nucleotide sequences of the *hipo* primer binding site in some Iranian isolates of *Campylobacters*.

Acknowledgments

The authors thank the staff of the microbiology laboratory of Karaj islamic azad university, especially Mr. Bakhtiari, for their help and technical support in this study.

Footnote

Funding/Support: The authors thank the staff of microbiology laboratory of Karaj Islamic Azad university, especially Mr. Bakhtiari, for their help and technical support in this study.

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