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Identification through Culture and Molecular Methods of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter fetus* in Surface Waters in Rasht

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RK, JNJ, AL, RR, AD and AF designed the study, authors SM, AF, RA performed the statistical analysis, authors RK, AL, RR, DA and AF wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors RK, DA, JNJ, RR, SM and AF managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Backgrounds: As zoonotic infectious agents, *Campylobacter* spp. are important factors causing gastroenteritis in humans. Surveys show that the three strains; *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter fetus* play a major role in human infections. Identification of these infectious agents is valuable for sanitary control of disease transmission through water resources.

Objectives: The aim of this study was identification and molecular diagnosis of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter fetus* in surface waters in Rasht.

Materials and Methods: This cross-sectional study was conducted on 45 samples of surface water in Rasht collected according to water health guidelines. After culture and biochemical tests on collected samples, detection and identification of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter fetus* was done using sequence-specific amplification by Multiplex PCR. The results were subjected to statistical analysis using SPSS software.

Results: Out of 45 samples tested, 6 were positive in culture, four of which were identified as *Campylobacter jejuni* after biochemical tests. Using Multiplex PCR, 8 samples were positive, from which 3 were *Campylobacter jejuni*, 1 *Campylobacter coli* and 4 were positive for both *Campylobacter jejuni* and *Campylobacter coli*. All the samples did not yield *C. fetus.*

Conclusions: Multiplex PCR is regarded a diagnostic method with higher sensitivity and specificity than compared to methods for Campylobacter. The prevalence of *Campylobacter jejuni* and *Campylobacter coli* in surface waters in Rasht is considerable. Therefore, public health measures for the control of these organisms are recommended.

Keywords: Campylobacter jejuni; Campylobacter coli; Campylobacter fetus; multiplex PCR; molecular diagnosis; water.

1. INTRODUCTION

Campylobacter spp. are Gram-negative microaerophilic bacilli, which are important etiologic agents of gastroenteritis and would be considered as one of the risk factors of Guillain-Barré syndrome in humans [1]. Campylobacteriosis is a zoonosis with a global distribution. Infection sources for humans in the first place are pets harboring these bacteria. Several evidences support human infection by fecal-oral route as well as contaminated water and milk [2]. Guillain-Barré syndrome is caused by immune system attack to peripheral nerves. Symptoms of this disease include muscle weakness, numbness and sometimes paralysis [3]. Campylobacteriosis is now considered as an opportunistic infectious disease. The prevalence of this infection is higher among immunocompromised patients, especially AIDS patients or very young or old people [4]. Virulence mechanism of Campylobacteriosis is caused by enterotoxin and cytotoxin production, invasion of the intestinal wall and penetration into the sub mucosal layer of the intestinal wall, respectively [5]. Antibiotic resistance in *Campylobacter* species is a significant problem for clinicians. *Campylobacter* species in many parts of the world are highly resistant to trimethoprim, and there are also some reports of mild resistance to polymixin B and rifampicin [6-7]. Molecular screenings have shown that feces of wild and domestic animals are the source of water contamination with *Campylobacter* species. *Campylobacter jejuni* and *Campylobacter coli* play a major role in human *campylobacter* infections [8-9]. They have been isolated from running waters, rivers, turkey slaughterhouse, wastewater and even seawater. Water resources can function as *campylobacter* contamination sources and cause disease epidemics, especially during natural disasters. Information about transmitted infectious agents in water resources can be very important particularly in management of natural disasters such as floods and earthquakes [10].

Given the role of this disease, *campylobacter* screening in surface waters is of importance.

2. OBJECTIVES

The aim of this study was to determine the contamination of surface waters in the vicinity of Rasht with *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter fetus* using Multiplex PCR and bacterial culture.

3. MATERIALS AND METHODS

3.1 Rasht

Rasht is the capital of Gillan Province in north of IRAN with 37°16′ 51″ N, 49°34′59″ E coordinates.

3.2 Sampling

This is a cross-sectional study on 45 water samples collected from 8 rivers in the vicinity of Rasht (see Table 2). Two-liter sample was collected in a sterile container according to sampling standards in environmental health.

3.3 Culture of Bacteria

The samples were cultured in Preston broth containing supplements, and were incubated under microaerophilic conditions (using type c gas pack) at 42°C. After 48 hours, 100µl of Preston broth was transferred to charcoal agar medium. The plates were incubated under microaerophilic conditions at 42°C. After 48 hours, suspected colonies were subject to biochemical tests, and microscopic slides were prepared and evaluated.

3.4 Biochemical Tests

Biochemical tests were conducted on positive culture samples to confirm the presence of *Campylobacter* species. Isolates were identified by conventional methods, that is, Oxidase, catalase and hippurate hydrolysis tests [9].

3.5 DNA Extraction

Three ml of an overnight culture of each *Campylobacter* species in preston broth were centrifuged at 9000 RPM for 10 min. Genomic DNA of the *Campylobacter* species were extracted using a DNA extraction Kit (Roche, Germany) according to the manufacturer's instruction. The supernatant containing the DNA was transferred into a clean tube and stored at -20°C until used for PCR.

3.6 Primer Designing

Specific primers for the target genes to detect *Campylobacter jejuni*, *Campylobacter fetus* and *Campylobacter coli* were designed for *hipO*, *sal A* and *glyA* genes, respectively. Primers specific for *Campylobacter jejuni*, *C. fetus* and *C. coli* were synthesized by Takapoozist Company (Table 1).

3.7 Multiplex PCR

Multiplex PCR mixture in one reaction was prepared by using a total volume of 25 µL containing 0.5 µM of each primer (three pairs), 2,5 µL PCR buffer with concentration 10×, 1 mM $MgCl_2$, 200 µM dNTPs (Fermentas, Lithuania) , 2 U Taq DNA polymerase (Fermentas, Lithuania) and 1 µL DNA template. The multiplex PCR was carried out through 35 cycles following a pre-heat step at 95°C for 5min. Each cycle consisted of denaturation at 95°C for 15s, annealing at 56°C for 1min, and extension at 72°C for 1min. After the 35 cycles, samples were maintained at 72°C for 5 min. Sterile distilled water was included in each PCR assay as a negative control. The amplified DNA was separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination.

4. RESULTS

In this study to detect *Campylobacter* species by Multiplex PCR, 45 surface water samples collected from the vicinity of Rasht (Iran) were examined. Out of 45 samples under study, 6 became positive in culture. Biochemical tests in this study showed that all the positive samples were also positive for oxidase and catalase tests. Out of 6(13.33%) samples positive in culture, 4 were positive for hippurate hydrolysis, and were identified as *Campylobacter jejuni* (Table 2).

5. DISCUSSION

The main pathogenic *Campylobacter* species in humans include *Campylobacter jejuni*, *Campylobacter fetus* and *Campylobacter coli*. *Campylobacter jejuni* has been raised as the most common cause of bacterial gastroenteritis in developed countries. *Campylobacter jejuni* was reported as an infectious agent in Guillain Barre Syndrome for the first time in 1982, and shortly afterwards several reports were published in confirmation of it. *Campylobacter coli* is the second most common cause of *Campylobacter* gastroenteritis after *Campylobacter jejuni*. Unlike other species of *Campylobacter*, *C.fetus* is involved in extra intestinal infections like infectious abortion, infectious arthritis, abscess, meningitis and endocarditis [11]. During infection in human societies, stool culture is the gold standard to diagnose campylobacteriosis. The disadvantages of culture method include high expense, prolonged course and lower sensitivity in comparison to molecular methods. *Campylobacter*, which is transmitted through water and food contaminated by the fecal matter has become the most common zoonosis in European Union. In the study of Van Dyke in Canada, 12.8% of the water samples from southern Ontario were positive for Campylobacter in culture. Using molecular methods, 69.8% of the samples were positive in the above study [12]. The results were consistent

with our study in terms of higher sensitivity of molecular detection method relative to culture. Using molecular methods with higher sensitivity can lead to higher results in the detection of microorganisms. The highest level of *C.jejuni* contamination has been reported in water samples of Idaho ponds by Dungan et al. using Real time PCR [13]. Minimal contamination by *C.jejuni* has been reported by Meinersmann et al in samples collected from Upper Oconee River Watershed in Georgia with 7.5% rate of contamination [14]. Contamination of surface water sources with *Campylobacter* species all year round shows that the highest level of contamination is seen in autumn and winter. Accordingly, in this study, surface water samples were collected in the mentioned seasons [15]. It seems that domestic and wild animals can spread the infectious agent by contamination of water sources with species of *Campylobacter*. *Campylobacter* spp. in Iran have been isolated from such animals as cows, sheep, chickens, cats, sheepdogs, pigeons and squirrels [16-17]. Few studies have been conducted to identify and isolate *Campylobacter* from surface waters in our country. In the study of Ghane et al, *Campylobacter jejuni* was isolated from the Caspian Sea waters during the four seasons using culture and PCR [18]. They also reported 36.92% prevalence rate of *Campylobacter* spp. in the rivers of Gillan and Mazandaran Provinces, which was higher than our study. This difference seems to be due to the geographic region, because we did sampling from the rivers in the vicinity of Rasht, where there are fewer domestic and wild animals [19]. Food resources of plant origin can also be contaminated with *Campylobacter* species, also contamination of edible mushroom species with *Campylobacter* has been reported in Iran [20]. In other countries, there are also reports of *Campylobacter* contamination in fruits and vegetables [21-22]. In other studies, prevalence of *Campylobacter* in fresh vegetables and fruits products with a 95% confidence level [22]. Pollution of water resources with environmental waste can upset the natural ecosystem of resident microorganisms. Desirable pH of *Campylobacter* spp. in 6.5-7.5 range. In pH values below 5 and above 9, Campylobacter spp.are progressively inactivated. The pH measurement of river resources under consideration in this study indicated that the water resources of Rasht can act as reservoirs of Campylobacter strains. In this study, *hipO*, *sal A* and *glyA* chromosomal gens were used to detect *Campylobacter jejuni*, *Campylobacter fetus* and *Campylobacter coli*, respectively. *hip O* gene causes *Campylobacter jejuni* to be hippurate positive, and is absent in other species of *Campylobacter*. It is a chromosomal gene, and is easier to isolate due to genome stability during DNA extraction process. On the other hand, this region is specific to *Campylobacter jejuni*, and has a high level of specificity in the diagnosis. One of the main tenets of the Multiplex PCR is proximity of annealing temperature of selected primers. Multiplex PCR primers designed in this study had similar annealing temperatures. Proliferation of *Campylobacter fetus* along with *Campylobacter jejuni* and *Campylobacter coli* was done with two objectives in this study. Our objective was to set up a multiplex PCR method for simultaneous detection of *C. jejuni*, *C. coli* and *C. fetus.* This is because numerous patients with Campylobacteriosis are referred to health centers in our country with reports of infection with *C. fetus* [23]. Animal husbandry is practiced in traditional form in many areas of Rasht, and the livestock freely graze in pastures and along the rivers. There is no reference for isolation of *Campylobacter fetus* from surface waters in the literature, and the identity of *Campylobacter* strains isolated from water in a series of studies is unknown. The authors believed that there is a risk of surface water contamination in Rasht with *Campylobacter fetus*, but eventually it became clear that all the samples were negative for *Campylobacter fetus*. In this regard, the ecological evaluation for *C. fetus* because of the likelihood of its existence in surface water samples is recommended.

Table 1. Primers specific for *Campylobacter jejuni*, *C. fetus* and *C. coli*

Species	Primer oligonucleotids	Gene	Primer type	Product size
C. jejuni	GAAGAGGGTTTGGGTGGTG	*hip O*	Forward	735bp
	AGCTAGCTTCGCATAATAACTTG		Reverse	
C. coli	AAGGCGTTTATGCTGCACTT	*gly A*	Forward	344bp
	AATGGACTTGGATGCTCACC		Reverse	
C. fetus	GGCTGCCGCTACTAAACTTG	*sal A*	Forward	228bp
	GCCGGTGAAAGCAGTTATCGT		Reverse	

Table 2. Bacterial culture and multiplex PCR for *Campylobacter jejuni, C. fetus* and *C. coli*

Sample number	Sample site	Sample water PH	Bacteria culture	Biochimestry metode			Multiplex PCR		
				Oxidase	Catalase	Hippurate	*C. jejoni*	*C. coli*	*C. fetus*
R3	Way pir bazar-river tash 1	**7**	**+**	**+**	**+**	**+**	**+**	**-**	**-**
R8	River bridge taleshan 1	6.5	+	+	+	+	+	+	-
R17	River barband 2	6	+	+	+	-	-	+	-
R20	River ghobak 2	6	-	-	-	-	+	+	-
R22	Ghomam river	6.5	+	+	+	+	+	+	-
R29	Lagoon eynak 1	6	+	+	+	+	+	+	-
R34	River pesyghan 2	6.5	+	+	+	+	+	-	-
R45	River syah sofian 2	6	-	-	-	-	+	-	-

Table 3. Culture and PCR test result

PCR & Culture test		PCR campylobacter		Total
		Negative	Positive	
Negative	% within Culture test	94.9%	5.1%	100.0%
	% within PCR	100.0%	25.0%	86.7%
	Count	37	2	39
Positive	% within Culture test	0.0%	100.0%	100.0%
	% within PCR	0.0%	75.0%	13.3%
	Count	0	6	6
Total	% within Culture test	82.2%	17.8%	100.0%
	% within PCR	100%	100%	100%
	Count	37	8	45

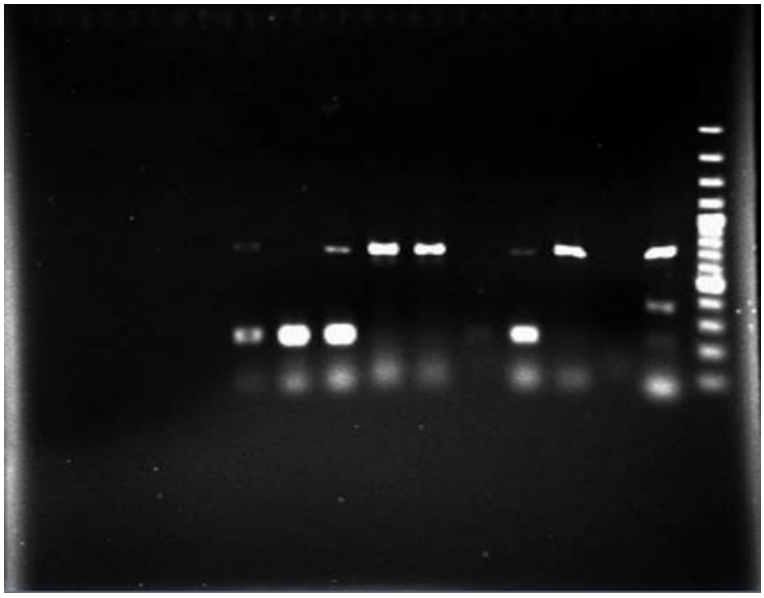

Fig. 1. From right to left: Lane 1, DNA molecular size marker (100 bp ladder; Sinaclon), lane 2, 4, 5, 7, 8, 9, 10, 11 were positive in Multiplex PCR test

Out of 45 surface water samples in this study, 8 (17.77%) were positive in Multiplex PCR test (Fig. 1); From 8 positive samples, 3 were positive for *C. jejuni,* 1 was positive for *C. coli* and 4 were positive for both *C. jejuni* and *C. Coli.* None of the samples was positive for *C. fetus* (Table 3).

6. CONCLUSION

In developed countries, molecular techniques have been developed for detection of *Campylobacter* species, and are now commercially available. In this study, Multiplex PCR method was used to quickly and effectively identify slow growing organisms of *Campylobacter jejuni*, *C. coli* and *C. fetus* in surface water samples. *Campylobacter* detection in clinical laboratories in Iran is based on phenotypic tests. Multiplex PCR based molecular techniques can replace culture methods for the detection of *Campylobacter* in clinical specimens. Therefore, the use and localization of Multiplex PCR is suggested in clinical laboratories.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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