Contact with poultry and animals increases risk of Campylobacter infections in adults of Ardabil province, Iran

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ORIGINAL ARTICLE

Contact with poultry and animals increases risk of *Campylobacter* infections in adults of Ardabil province, Iran

Reza Ranjbar* and Daryoush Babazadeh**

ABSTRACT

BACKGROUND
The acute gastroenteritis caused by campylobacteriosis is known as one of the common infectious diseases with worldwide distribution. The aim of this study was to detect *Campylobacter* species in stool samples by routine culturing and polymerase chain reaction (PCR) and explore the risk factors in adult subjects in East Azerbaijan province of Iran.

METHODS
A cross sectional study involving 1010 adult subjects, from whom stool samples were collected. Samples with inflammatory criteria like fecal leukocytes (WBC ≥5) were selected and isolated through fecal lactoferrin detection test. The WBC ≥5 and lactoferrin positive samples were selected for *Campylobacter* detection by culture and PCR methods. The *required* information consisting of gender, age, place of habitation, and contact with poultry and animals were asked and recorded. Chi-square test and prevalence ratio (PR) was used to analyze the data.

RESULTS
Of 1010 stool samples, 231 (22.9%) had WBC ≥5, and from these samples 58 (25.1%) were positive by culturing and 61 (26.4%) by PCR. Subjects having habitual contact with animals and poultry had increased risk of *Campylobacter* infections by 1.65 times compared with subjects without contact with animals and poultry (PR=1.65; 95% CI: 1.07-2.68).

CONCLUSIONS
Detection of *Campylobacter* infections by PCR was more sensitive in adults. Investigation of *Campylobacter* prevalence in Ardabil showed this bacterium should be viewed as one of the possible pathogens in inflammatory diarrheal cases. People having habitual contact with animals should check the health of the animals regularly and not consume food from suspected sources**.**

Keywords: *Campylobacter*, culture, lactoferrin, patient, PCR, prevalence

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INTRODUCTION

Infections of the gastrointestinal tract are strongly associated with morbidity and even mortality in children and elderly people.[1,2] Recently, food and water-borne outbreaks of gastrointestinal and diarrheal illness due to various microbial pathogens in Iran have been reported in many studies. [3-6] One of the most common and important causes of infectious diarrheal illness and acute gastroenteritis in adults and children with worldwide distribution is campylobacteriosis.[7] *Campylobacter* is well recognized as the leading cause of bacterial foodborne diarrheal disease worldwide.[8]

Campylobacteriosis is endemic in developing countries and the major sources of human infections are food and environmental contamination.[9] *Campylobacter* contamination can be the result of consumption of suspected animal sources of food and dairy products.[10] Environmental contamination can be caused by domestic animals and poultry (as natural reservoirs of *Campylobacter* species). [11] Poultry carcasses in stores that are not under sanitary monitoring and consumption of undercooked poultry meat are important causes of outbreaks.[12]

The occurrence of this disease is usually higher in older persons over 75 years of age, young children under 4 years of age and young adults who are 20-40 years old.[13,14] Person-to-person transmission of this disease is extremely rare.[15] Developing countries often do not have national surveillance programs for controlling this infection. According to various reports, the incidence of *Campylobacter* in developing countries is between 5 and 20 percent.[16]

A study showed that in rural households, the presence of poultry manure, uncovered litter in house yards and lack of barriers to keep animals out of houses have been identified as risk factors for *Campylobacter* infection in children.[17] Another study identified exposure to domestic animals as a sufficient risk factor for infection.[18]

The clinical symptoms of enteritis caused by *Campylobacter* species range from watery, non-bloody and non-inflammatory diarrhea to severe inflammatory diarrhea with abdominal pain and fever; however *C. jejuni* typically shows acute and self-limited signs.[19] Diagnosing *Campylobacter* infection will not be possible from routine clinical signs and usually there is co-infection with multiple pathogens such as *Escherichia* (*E. coli), Listeria monocytogenes, Staphylococcus aureus, Cronobacter sakazakii, Salmonella enterica, Vibrio cholera, and Shigella* and *Yersinia* species.[20-23]

Red blood cells and fecal leukocytes have been found in the majority of the stool samples of infected patients and the peripheral white blood cell (WBC) count is slightly elevated.[24]

Laboratory methods for diagnosing *Campylobacter* enteritis have been successfully developed, such as polymerase chain reaction (PCR), genotyping methods like ribotyping, pulsed-field gel electrophoresis and ELISA for detecting DNA and antigens in stool samples and also specific culturing of the organism from fecal specimens.[25-28] The ability of PCR to amplify minute amounts of specific microbial DNA sequences has made it a powerful molecular tool.[5,29] It is reported that multiplex PCR diagnostic tools are fast, inexpensive and sensitive for *Campylobacter* species.[30] The present study aimed to determine the prevalence of gastroenteritis related to *Campylobacter* species and explore the risk factors in adult patients of Ardabil province, Iran.

METHODS

Research design

A cross sectional study was conducted in Ardabil province from June to December 2016. Ardabil province is in Northwest Iran, having Ardabil City as its capital and largest city, and common borders with the provinces of Zanjan, East Azerbaijan, and Gilan, and the Republic of Azerbaijan.

Sample collection

A total of 1010 stool samples were collected from adult patients (517 men and 493 women aged 18-70 years) who were referred with acute diarrhea to the Central Laboratory of Ardabil, Bu-Ali Central Laboratory, Ardabil University of Medical Sciences, Ardabil, Iran. Patients who had consumed antibiotics before sampling, who were not satisfied with the investigation, were less than 18 years and more than 70 years old, and had a history of noninfectious diarrhea, were excluded from the study. The fresh samples with inflammatory criteria like fecal leukocytes (WBC ≥5 under a field of light microscope *x* 400) were selected and isolated through the fecal lactoferrin detection test as described in previous studies.[31] Lactoferrin detection in fecal samples has been confirmed for diagnosis of inflammatory bowel disease (IBD). Intestinal inflammation may be caused by bacterial infection or IBD and is directly related to the disease activity and severity.[31] The WBC ≥5 and lactoferrin positive samples were selected for *Campylobacter* detection by culture and PCR methods. The necessary information like age, gender, daily contact with animals (pets, exotic animals, poultry, small and large animals) and place of living was asked and recorded.

Culture of sample

The fresh samples were enriched on Campy-Thio medium (that is suggested as a holding medium for samples suspected to contain *Campylobacter* spp. when immediate inoculation cannot be performed) for 1-2 hours and then cultured on a *Campylobacter* selective agar (Merck, Germany), containing 5% defibrinated sheep blood, trimethoprim, polymyxin, and vancomycin for inhibiting growth of *E. coli, Pseudomonas, Pasteurella, Klebsiella, Shigella, Salmonella, Enterobacter, Streptococcus, Clostridium, Proteus* and *Corynebacterium*. The samples were incubated at 42°C for 48 hours in a microaerophilic atmosphere (85% N_2, 10% CO_2 and 5% O_2). Only the suspected colonies were examined by phase-contrast microscope and Gram staining technique for motility and morphology. For further investigation, the suspected colonies also were cultured on blood agar plates, and incubated under microaerophilic conditions at 42°C for 48 h. Hippurate hydrolysis, catalase activities, oxidase test, and also susceptibility to 30 μg discs of nalidixic acid and cephalothin (MAST Co., England) were used for confirming the results.

DNA extraction

DNG plus kit (Cinnagen, Iran) was used according to the commercial recommendations for extracting the DNA of samples.[32] The extracted DNA was stored at -20°C.

Multiplex PCR assay

Strains of *C. jejuni* ATCC 29428 and *C. coli* ATCC 33559 were prepared at Tabriz University of Medical Sciences, Tabriz, Iran, as positive controls. The target genes *hipO* and *asp* were targeted for *C. jejuni* and *C. coli* respectively. The oligonucleotide primers of the study are presented in Table 1. The reaction mixture consisted of 25 μl multiplex master mix (Qiagen, Iran), 0.5 μl *asp* primer (50 pmol μl^{-1}), 1.0 μl *hip* primer (100 pmol μl^{-1}), 0.5 μl bovine serum albumin [BSA] (10 mg/ml) (Promega, Iran), 4.5 μl eluted DNA (0.1 μg) and sterile water to a final volume of 50 μl. The PCR amplification cycle used was heat denaturation at 95°C for 10 min, 35 cycles with denaturation at 94°C for 50 seconds, annealing at 55°C for 40 seconds, extension at 72°C for 50 seconds, and a final extension at 72°C for 4 min. All PCR products were analyzed by gel electrophoresis on 1.8% agarose gel and stained with 0.1 μg/ml ethidium bromide for visualization. The predicted product size for *hipO* primer was 735 bp and for *asp* primer 500 bp.[33,34]

Ethical clearance

The present research was carried out after ethical approval of the Research Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran under the ethics code no. 5/4/7518 according

Table 1. Primers used for amplification of target genes of *Campylobacter* species isolated from adult subjects

Target genes	Primer Sequences	Product Size (bp)	References
asp (*C. coli*)	F-5' GGTATGATTTCTACAAAGCGAG-3'	500	(2027)
	R-5'ATAAAAGACTATCGTCGCGTG-3'		
hipO (*C. jejuni*)	F-5' GAAGAGGGTTTGGGTGGT-3'	735	(26)
	R-5'-AGCTAGCCTCGCATAATAACTTG-3'		

to the Declaration of Helsinki (http://www.ufrgs.br/HCPA/gppg/helsin5.htm).

Statistical analysis

Data analysis was carried out using Statistical Package for Social Sciences (SPSS) software (version 16, Chicago, USA). The relationship of the prevalence of Campylobacter with other quality variables was computed by chi-squared test and prevalence ratio. P values less than 0.05 were reported as statistically significant.

RESULTS

A total of 711 out of 1010 samples were non-inflammatory diarrheal cases (WBC less than 5) and 299 samples were inflammatory cases (WBC more than 5). All inflammatory samples were tested for lactoferrin and 231 cases were positive. The prevalence of *Campylobacter* species obtained by PCR and culture methods was 6% and 5.7%, respectively. Sixty one (26.4%) out of 231 lactoferrin positive samples were positive for *Campylobacter* infection by multiplex PCR and 58 (25.1%) samples were positive by specific culture. There was no significant correlation between different ages and prevalence of *Campylobacter* (p=0.977). The highest prevalence of *Campylobacter* species was seen in 18-30 years old patients (culture results: 6.3% and PCR results: 6.6%) and the lowest number of positive samples was found in 60-70 years old patients (culture results: 5.7% and PCR results: 5.7%). The average contamination rates per case were 6% and 5.7% among all collected samples which were indicated by PCR and specific culture methods respectively. The highest contamination rate was recorded in the age range of 18-30 years (6.6%) and the lowest contamination rate was recorded in 40-70 year old patients (5.7%) (Table 2). There was no significant correlation between gender and prevalence of *Campylobacter* (p≥0.05). The contamination of females (culture results: 5.5% and PCR results: 5.7%) was insignificantly lower than that of males (culture results: 6.0% and PCR results: 6.4%) (p=0.638). The contamination rate in male patients (6.3%) was higher than in females (5.7%) (Table 2).

Table 2. Relationship of age and gender with *Campylobacter* species detected by culture and PCR (n=1010)

Variables	Culture			PCR			C/P (%)
	positive (n,%)	negative (n,%)	p	positive (n,%)	negative (n,%)	p	
Age (years)			0.992			0.977	
18-30	18 (6.3)	249 (93.7)		19 (6.6)	249 (93.4)		6.6
30-40	15 (5.6)	252 (94.4)		16 (6.0)	236 (94.0)		6.0
40-50	12 (5.3)	216 (94.7)		13 (5.7)	203 (94.3)		5.7
50-60	8 (5.7)	133 (94.3)		8 (5.7)	133 (94.3)		5.7
60-70	5 (5.7)	83 (94.3)		5 (5.7)	83 (94.3)		5.7
Gender			0.772			0.638	
Male	31 (6.0)	486 (94.0)		33 (6.4)	484 (93.6)		6.3
Female	27 (5.5)	466 (94.5)		28 (5.7)	465 (94.3)		5.7

PCR = polymerase chain reaction; C/P = contaminated based on PCR results/all subjects with certain age

Table 3. Relationship of subjects' contact with animals and poultry and place of habitation with *Campylobacter* species detected by culture and PCR (n=1010)

Variables	Culture				PCR				C/P (%)
	positive n (%)	negative n (%)	PR	95% CI	positive n (%)	negative n (%)	PR	95% CI	
Contact with animals and poultry			1.69	1.03-2.79			1.65	1.07-2.68	
Yes	27 (7.9)	316 (92.1)			28 (8.2)	315 (91.8)			8.2
No	31 (4.6)	636 (95.4)			33 (4.9)	634 (95.1)			5.0
Contact with animals			1.38	0.80-2.35			1.28	0.75– 2.18	
Yes	18 (7.2)	231 (92.8)			18 (7.2)	231 (92.8)			7.2
No	40 (5.3)	721 (94.7)			43 (5.7)	718 (94.3)			5.6
Place of habitation			0.71	0.43-1.17			0.69	0.43-1.13	
City	28 (4.9)	544 (95.1)			29 (5.1)	544 (94.9)			5.3
Village	30 (6.8)	408 (93.2)			32 (7.3)	406 (92.7)			7.3

PCR = polymerase chain reaction; C/P = contaminated based on PCR results/all subjects with certain age; PR= prevalence ratio

The results of *Campylobacter* prevalence in patients with daily contact with animals and poultry (343 patients), in patients with daily contact with animals but not poultry (249 patients) and in patients without any contact with animals (667 patients) are shown in Table 3. In subjects who had habitual contact with animals and poultry the risk of *Campylobacter* infections increased by 1.65 times compared to subjects who had no contact with animals and poultry (PR=1.65;95% CI:1.07-2.68).There was no significant correlation between contact with animals and prevalence of *Campylobacter* in the collected samples (PR=1.28; 95% CI:0.75-2.18) (Table 3). The lowest contamination rate among all patients was seen in patients who had no contact with animals (5%) and the higher contamination rates were seen respectively in patients who had daily contact with domestic animals and poultry (8.2%) and in patients who had daily contact with animals but not poultry (7.2%).

The rate of *Campylobacter* infection in patients who were referred from villages and cities was indicated by fecal culture and PCR-positive infections as shown in Table 3. There was no significant correlation between living in city or village and prevalence of *Campylobacter* (PR=0.69;95% CI 0.43-1.13). The contamination rates in rural and urban patients were 7.3% and 5.1% respectively.

DISCUSSION

Investigation of *Campylobacter* prevalence in Ardabil showed that this bacterium should be viewed as one of the possible pathogens in inflammatory diarrheal cases. The 6% and 5.7% prevalence of *Campylobacter* species in Ardabil province was obtained in the present research by PCR and culture methods, respectively. Previous findings in some cities of Iran indicated 12.4% in Semnan,[3] 8% in Tehran,[35] 10% in Hamedan [36] and 6.4% in Zanjan.[37] Differences in prevalence rates probably are related to pathogenic agents or living conditions in different regions.

The prevalence of *C. jejuni* and *C. coli* and mixed infections among all patients with gastroenteritis were in agreement with previous findings of researchers who detected *Campylobacter* by multiplex PCR method and reported a higher prevalence of *C. jejuni* than *C. coli*.[38,39]

Rapid detection of *Campylobacter* species is extremely important to ensure food and water safety. Multiplex PCR is one of the possible and trustworthy molecular methods for indicating the prevalence of bacterial diseases and especially *Campylobacter* infection in a single sample.[29, 30] There are other possible methods for detection of *Campylobacter* infection like specific culture under specific conditions.[16] The *hipO* and *asp*

genes were targeted in the present investigation, since the *hipO* gene has been represented as the most widely validated gene for the identification of *C. jejuni* and is highly conserved in *C. jejuni* strains,[26] while the *asp* gene is highly specific for *C. coli* and encodes aspartokinase.[34] Although several researches have targeted the rRNA genes for genus and species identification of *Campylobacter* they might have found a lower specificity due to the high level of conservation among closely related species.[26]

Currently fecal lactoferrin has majored as one of the important factors for investigating fecal samples suspected of bacterial infections.[37] Acute bacterial infections in the gastrointestinal system or severe bowel diseases will increase fecal lactoferrin. It is reported that the fecal lactoferrin value was is higher in patients with *Campylobacter* (10.32 μg/g) and *Salmonella* (11.17 μg/g).[40] The results of the present study revealed the presence of *Campylobacter* species in 26.4% and 25.1% of lactoferrin positive samples as determined by PCR and specific culture methods respectively.

The present results revealed that the age and gender of adult patients are not effective factors in the prevalence of campylobacteriosis. However a higher contamination rate was seen in younger patients than in older patients. These findings are in accordance with the previous report of Samia et al. from Egypt who indicated that gender is not an effective factor in the prevalence of this infection and that younger patients had more infections with *Campylobacter* species.[39] However some researchers believed that the occurrence of this disease was higher in young children, young adults and oldest elderly patients.[2,13,14]

As gender and age did not have a major role in morbidity in the present study, the living conditions and location of habitations were not significantly associated with the causes of contamination. However, patients who lived in villages and had daily contact with animals or poultry showed a higher prevalence ratio of disease than patients who lived in the cities. Regardless of the present findings, it is reported that the gastroenteritis prevalence and contamination rate of *Campylobacter* species isolated from patients who lived in urban areas were higher than in patients from rural areas, which is confirmed by PCR and lactoferrin assay.[37] There are various reports about the prevalence of gastroenteritis in relation to campylobacteriosis in rural and urban patients that indicated different prevalences of infections.[13]

The presence of *Campylobacter* species in stool samples and egg shells of poultry as the normal flora has been demonstrated.[41,42] In a 10-year study in Switzerland (from 2002 to 2012) the prevalence of *Campylobacter* in animals was indicated and it has been found that chicken are the most common reservoir/source of *Campylobacter* infection, with average contamination of 70.9%, whereas cattle have been named as the second most common source, with a rate of 19.3%, while dogs and pigs were other sources with rates of 8.6% and 1.2% respectively.[11] The presence of *Campylobacter* species as normal flora in stool and eggs of poultry is one of the major causes that might affect the prevalence of campylobacteriosis in patients.[38] Also there are some reports that found this pathogen in the normal flora of other animals.[43] Regardless of present findings, in two studies the possible effect of poultry on incidence of *Campylobacter* disease was denied; but consumption of unpasteurized milk and ingestion of under cooked chicken were significantly associated with infection.[12,39] Reducing the number of *Campylobacter* in poultry carcasses will greatly decrease the risk of infection in consumers.[26,44] One of suggested methods for controlling the *Campylobacter* species as normal flora in poultry is using feed additives like probiotics and synbiotics. It is reported that a mixture of fructooligosaccharides and a galactooligosaccharide plus one *Bifidobacterium* strain (*B. longum* subsp. *longum* PCB133) can significantly reduce the *C. jejuni* concentration in poultry feces.[45]

There were several limitations in our study. Some limitations are inherent in the structure of a cross sectional study, in which the association between risk factors and *Campylobacter* infection cannot be explained as a cause and effect. The risk associations determined in this study are most appropriately understood as relative indicators of risk. A larger prospective epidemiological study with active case finding might circumvent the limitations of the present study design.

CONCLUSIONS

In this study, investigation of *Campylobacter* prevalence showed this bacterium should be viewed as one of the possible and important pathogens in inflammatory diarrheal cases. Persons who have habitual contact with animals and poultry are at increased risk of *Campylobacter* infections and should check the health of the animals regularly and not consume food from suspected sources.

CONFLICT OF INTEREST

All authors declare to have no conflict of interest.

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CONTRIBUTION

RR contributed to the design of the project, prepared the laboratory equipment for PCR and performed the practical procedure of this method. DB contributed to the design of the project and was responsible for sample collection, the practical procedure of culturing the fecal samples and writing of the manuscript. All authors read and approved the final manuscript.

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