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Isolation, Molecular Detection, and Risk Factors of Campylobacter Infection From Companion Dogs

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Abstract

Background: *Campylobacter* is an organism that is usually associated with diarrhea in pet animals and humans, as well as other domestic, wild, and laboratory animals.

Objective: The aim of the present survey was the isolation, molecular detection, and risk factors of *Campylobacter* infection from companion dogs referred to the Veterinary Hospital of Ahvaz district, the South-West of Iran.

Materials and Methods: Rectal swabs were examined by culture and polymerase chain reaction (PCR) methods from 122 companion dogs (52 diarrheic and 70 clinically healthy). Several risk factors were reviewed, including age, gender, breed, nutrition status, and lifestyle.

Results: The results showed that only five samples (4.1%) were positive for *Campylobacter* spp. in the culture method. *Campylobacter* spp. was detected in 18 out of 122 dogs by the PCR, yielding an overall prevalence of 14.8%. The most prevalent species of *Campylobacter* among the referred dogs were *C. coli* (38.89%) and *C. jejuni* (33.33%). A lower prevalence was found for *C. upsaliensis* (11.11%) and *C. lari* (5.55%). Concurrent infections were observed in two cases of *C. upsaliensis* + *C. lari* (5.55%) and *C. coli* + *C. lari* (5.55%). No significant difference was noted between healthy (11.43%) and diarrheic (19.23%) dogs ($P>0.05$). Eventually, age, gender, breed, nutrition status, and lifestyle had no significant effect on *Campylobacter* infection ($P>0.05$).

Conclusion: Although the prevalence of *Campylobacter* was moderate in the dog population of Ahvaz district, these bacteria can constitute a public health hazard because of the frequent presence of *Campylobacter* species in the feces.

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Background

Campylobacter spp. are thin, curved, Gram-negative rods that are found in singular, pairs, or chains with three to five spirals. The cells may be S- or gull-shaped when joined together. *Campylobacter* species are microaerophilic and have a single, none sheathed polar flagellum.

There are more than 30 different species and subspecies of *Campylobacter* in humans and animals.[1] *Campylobacter jejuni* is the organism which is typically associated with diarrhea in dogs, cats, and humans, as well as other domestic, wild, and laboratory animals. *C. coli* and other intestinal *Campylobacters*, *C. upsaliensis*, *C. helveticus*, and *C. lari* have been isolated from asymptomatic and diarrheic dogs and cats.[2,3]

Dogs may be more sensitive to clinical diseases when stressed by hospitalization, simultaneous disease, pregnancy, traveling, or surgery.[2,4] The majority of dogs show subclinical infection but some of them will develop mild to moderate enteritis. Acute *Campylobacteriosis*, which extends in puppies and some adult dogs, is present by mucus-laden, watery, bloody or bile-streaked diarrhea, anorexia, dehydration, abdominal pain, and occasional vomiting. In many cases, dogs are asymptomatic carriers of *Campylobacter* species and play a significant role in the epidemiology of *Campylobacteriosis* and *Campylobacter* spp. in animals and humans. Particularly, many dogs live as free in urban and rural areas and have access to other animals that increases the risk of public health.[2]

The prevalence of different species of *Campylobacter* spp. in animals varies and depends on the age, animal species, housing (e.g., kennel or shelter), and the presence of associated disease or infection with other enteropathogenic bacteria, the sampling season, geographic region, and the study design.[5-7] For example, the prevalence of *C. jejuni* is significantly greater in dogs younger than six months old and those living in high-

density regions for long periods, and in the autumn months. *C. jejuni* has been isolated from 29% and 21% of diarrheic dogs and cats, respectively, compared with 4% of clinically healthy dogs and cats. In other studies, the isolation rate varies from zero to 50%.[2,7] The results of the conducted surveys in Iran showed a relatively high prevalence of *Campylobacteriosis* in dogs and cats. For example, from a total of 100 dogs and cats, 39 cases were infected with *Campylobacter* in a research study in Tehran.[8]

Campylobacter spp. is one of the most prevalent bacteria causing gastroenteritis in humans. Poultry and pet animals are the most important reservoirs for human infection. Risk factors for infection include nutrition with undercooked or contaminated meat products (especially poultry), consuming contaminated or unpasteurized milk and dairy products, drinking water from contaminated supplies, foreign travel, and contact with contaminated pets.[5,9,10] The people who are in close contact, living or working with pet animals (especially young children), and the immunocompromised patient must be aware of the risks of *Campylobacteriosis*. It is recommended that these people comply with sanitary measures when dealing with puppies or kittens and the pets with gastroenteritis signs and obtaining a new pet from a centralized housing environment.[11,12] Moreover, people using raw meat-based diets for pet animals must consider the potential risk of infection which is associated with this manner.[7,13]

There are many different methods for the diagnosis of *Campylobacteriosis*, including direct microscopic examinations, culture, serologic test (e.g., enzyme-linked immunosorbent assay), and molecular identification (e.g., polymerase chain reaction, PCR). Direct microscopic examination (dark-field or phase-contrast microscopy) alone is insufficient and thus further examination is needed to confirm *Campylobacteriosis*.[6] *Campylobacter* spp. are fastidious and slow growth, hence, their isolation heavily relies on the applied procedure in this regard.[6] Additionally, the phylogenetic alliance of *Campylobacter* with *Arcobacter* and *Helicobacter* spp., as well as the recognition of several *Campylobacter* spp. has limited the discriminatory power of culture and phenotypic methods, and thus they are not considered suitable for true *Campylobacter* spp. Diagnosis.[14] On the other hand, molecular assays (PCR) are becoming increasingly available for diagnosing *Campylobacter* infections in dogs and cats since they are easy, rapid, and sensitive and allow the speciation of isolates from culture or infected tissues. Molecular methods permit rapid identification and prevail problems with culture, growth situation, reduced viability of bacteria, and bacterial contamination. Molecular techniques are regarded as the gold standard for *Campylobacter* genus and species identification and are valuable in epidemiological research, especially in *Campylobacter* strain-typing.[10,15] Specific primers can be used for 23SrRNA as internal controls for recognizing closely related genera *Campylobacter*, *Arcobacter*, and *Helicobacter*. These organisms can also be rapidly identified on clinical isolates using the PCR- restriction fragment length polymorphism analysis of the 16 SrRNA gene.[16]

Unfortunately, based on our knowledge, no survey has so far investigated *Campylobacteriosis* in the dogs of Ahvaz district. Furthermore, epidemiological research with the molecular diagnostic method is needed to determine the range and role of *Campylobacter* spp. in pet animals, and their zoonotic significance, especially in this area. Therefore, the present study aimed to focus on the isolation and molecular characterization of *Campylobacter* spp. in companion dogs in the Ahvaz region, South-West of Iran.

Materials and Methods

Sample Population

A cross-sectional survey was conducted in the Ahvaz region, South-West of Iran from November 2016 to September 2017. One hundred twenty-two dogs (52 diarrheic and 70 clinically healthy dogs) were sampled, and only those dogs not recently been treated with antibiotics or glucocorticoids were included in the study. The companion dogs were referred to the Veterinary Teaching Hospital of Shahid Chamran University of Ahvaz for vaccination, health care, and different diseases including diarrhea as requested by their owners. All parameters were recorded including age, gender, breed, clinical signs, nutrition status, lifestyle (open or close environment), and vaccination history. The studied dogs were divided into two groups based on their age (less and higher than one year old). In some cases, sedative drugs such as ketamine with a dosage of 15 mg/kg and acepromazine 0.15 mg/kg were injected intramuscularly. Fresh fecal specimens from the dogs were examined by culture and PCR methods. The swab samples were collected from the rectum of the studied dog population. Two simultaneous swabs were taken of every dog. A swab was used for culture and transported in an Amies transport medium and was sent on ice to the laboratory of veterinary microbiology as soon as possible (less than 1 hour). The other sample was kept at -20°C for DNA extraction from the stool.

Culture Method

Supplemented Preston enrichment broth (Himedia, India) including 5% (*v*/v) defibrinated lysed horse blood, sodium pyruvate (0.125 g/500 mL), ferrous sulfate (0.125 g/500 mL), Polymyxin B (250 000 units/500 mL), rifampicin (0.005 g/500 mL), trimethoprim (0.005 g/500 mL), and amphotericin B (0.005 g/500 mL) was used for the enrichment of the specimens for 24 hours at 37°C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2).[17] Then, a loop full of enriched broth was streaked onto Preston *Campylobacter* selective agar (Himedia;

India) including 7% (*v*/v) defibrinated lysed horse blood, sodium pyruvate (0.125 g/500 mL), ferrous sulfate (0.125 g/500 mL), polymyxin B (250 000 units/500 mL), rifampicin (0.005 g/500 mL), trimethoprim (0.005 g/500 mL), and amphotericin B (0.005 g/500 mL). Next, they were incubated for 48-72 hours under microaerophilic conditions.[17] Suspect colonies were purified by streaking onto blood agar and presumptively identified by Gram- and dilute carbol fuchsin staining (DCF), biochemical tests (catalase and oxidase), and phase-contrast microscopy according to standard procedures.[3] Compatible colonies were confirmed by the PCR.

DNA Extraction and PCR

DNA was extracted from stool samples by using a DNA extraction kit (AccuPrep® Stool DNA Extraction Kit, Bioneer) according to the manual of the kit. In the present survey, specific primers were used for the detection of *Campylobacter* genus and the species of *C. Jejuni*, *C. coli*, *C. Lari*, and *C. Upsaliensis* in the obtained fecal samples and the isolates in the culture by the multiple PCR technique. The sequences of primers are presented in Table 1.

The multiplex PCR was performed in a 50 µL reaction mixture containing master mix (25 µL, including Tris-HCl pH: 8.5, $(NH4)_2S0_4$, 2 mM Mg Cl_2, 0.2% Tween 20, 0.4 mM dNTPs, 0.2 units/µL Ampliqon Taq DNA polymerase, and inert red dye; Ampliqon, Denmark), each primer (1 µL = 0.4 mM; Bioneer, South Korea), distilled water (13 µL), and template DNA (2 µL). A reaction with DNA of *C. jejuni* and *C. coli* and a reaction without the template DNA were used as positive and negative controls, respectively. The PCR program was performed in a thermal cycler (Eppendorf, Germany) as initial denaturation at 95°C for 15 minutes, 35 cycles of 30 seconds at 95°C, 90 seconds at 58°C, and 60 seconds at 72°C, followed by a final extension at 72°C for 7 minutes.[18] Amplification products were separated on 1.5% agarose gel containing safe stains (Cinnagen, Iran).

Statistical Analysis

The studied dogs were grouped by age, gender, breed, nutrition status, clinical signs (diarrheic and none-diarrheic), and lifestyle (open or close environment) to determine whether these factors were associated with *Campylobacter* infection by Chi-square test, Fisher's exact test, and Z test. Statistical analyses were performed using SPSS (Version 16.0; SPSS Inc., Chicago, USA). Logistic regression was applied to calculate odds ratios, and differences were considered statistically significant when $P \leq 0.05$.

Results

In the present study, 122 dogs were tested for the presence of *Campylobacter* spp. using culture and PCR techniques. The breed distribution of the studied dogs was mixed (30.3%), Terrier (25.4%), Doberman Pinscher and German Shepherd (9.8%), Spitz (5.7%), Siberian Husky and Shitzu (4.9%), Rottweiler (4.1%), and other breeds (4.9%), respectively (Table 2). The age of the studied dogs was between one month and five years (median 1.9 years). Overall, fewer samples were positive by culture compared to the direct PCR. Among the 122 samples, only 5 samples (4.1%) were positive in the culture. All the suspected *Campylobacter* isolates were confirmed as *Campylobacter* by the PCR and were confirmed as *C. coli* by specific primers for the species. *Campylobacter* spp. was detected in 18 of the 122 dogs in the PCR, yielding an overall prevalence of 14.8%. The most prevalent species of *Campylobacter* among the dogs were *C. coli* (38.89%) and *C. jejuni* (33.33%). A lower prevalence was observed for *C. upsaliensis* (11.11%) and *C. lari* (5.55%). Concurrent infections were observed in two cases, namely, *C. upsaliensis* + *C. lari* (5.55%) and *C. coli* + *C. lari* (5.55%). Figure 1 displays representative bands of the *Campylobacter* genus and species by the multiplex PCR method.

The prevalence of *Campylobacter* in the studied dogs by PCR was 14.8% (95% CI: 21.3-8.%, 18 out of 122 cases).

There was no significant relationship between age and infection ($P > 0.05$). The average age and standard deviation of infected and non-infected dogs with *Campylobacter* were 9.06±6.49 and 10.55±7.78, respectively, which were not statistically significant ($P > 0.050$). The relative frequency of positive cases was 12.30% and 2.5% in dogs under and above one year old, respectively (Table 2). The odd of infection in 1-year-old dogs and younger was 1.75

Table 1. Nucleotide Sequence of Primer, Target Gene, and Product Size for Detecting *Campylobacter* Genus and Species

Gene	Genus/Species	Sequence	Size	Reference
16SrRNA	*Campylobacter*	F:5′- GGATGACACTTTTCGGAGC -3′ R: 5′- CATTGTAGCACGTGTGTC -3′	816	18
Aspartokinase	*C. coli*	F:5′- GGTATGATTTCTACAAAGCGAG -3′ R: 5′- ATAAAAGACTATCGTCGCGTG -3′	502	
C. jejuni	*oxidoreductase*	F:5′- CAAATAAAGTTAGAGGTAGAATGT-3′ R: 5′- CCATAAGCACTAGCTAGCTGAT -3′	161	
C. lari	*glyA*	F:5′- TAGAGAGATAGCAAAAGAGA -3′ R: 5′- TACACATAATAATCCCACCC -3′	251	
lpxA	*C. upsaliensis*	F:5′- CGATGATGTGCAAATTGAAGC -3′ R: 5′- TTCTAGCCCCTTGCTTGATG -3′	86	

Table 2. Distribution of Absolute and Relative Frequency of *Campylobacter* Infection in the Population of Dogs Referred to the Veterinary Hospital of Ahvaz Based on Age, Gender, Breed, Health Conditions, Living-, and Nutrition status

Parameter		Negative	Positive	Total
		Absolute (Relative %)	Absolute (Relative %)	Absolute (Relative %)
Age	≤ 1 year	77 (63.1)	15 (12.3)	92 (75.4)
	> 1 year	27 (22.1)	3 (2.5)	30 (24.6)
	Total	104 (85.2)	18 (14.8)	122 (100)
Gender	Female	53 (85.5)	9 (14.5)	62 (50.8)
	Male	51 (85)	9 (15)	60 (49.2)
	Total	104 (85.2)	18 (14.8)	122 (100)
Breed	Mixed	30 (24.59)	7 (5.75)	37 (30.3)
	Terrier	25 (20.5)	6 (4.92)	31 (25.4)
	Doberman Pinscher	11 (9)	1 (0.82)	12 (9.8)
	German Shepherd	12 (9.84)	0 (0)	12 (9.8)
	Spitz	7 (5.74)	0 (0)	7 (5.7)
	Siberian Husky	5 (4.1)	1(0.82)	6 (4.9)
	Shitzu	6 (4.92)	0 (0)	6 (4.9)
	Ruth Weiler	2 (1.64)	3 (2.49)	5 (4.1)
	unknown	6 (4.92)	0 (0)	6 (4.9)
	Total	104 (85.2)	18 (14.8)	122 (100)
Health conditions	Diarrheic	42 (34.4)	10 (8.2)	52 (42.6)
	Healthy	62 (50.8)	8 (6.6)	70 (57.4)
	Total	104 (85.2)	18 (14.8)	122 (100)
Living status	Indoor	45 (36.9)	8 (6.6)	53 (43.4)
	Outdoor	59 (48.3)	10 (8.2)	69 (56.6)
	Total	104 (85.2)	18 (14.8)	122 (100)
Nutrition status	Cooked food	70 (57.3)	12 (9.9)	82 (67.2)
	Raw food	34 (27.9)	6 (4.9)	40 (32.8)
	Total	104 (85.2)	18 (14.8)	122 (100)

times greater compared to dogs older than 1 year (95% CI: 0.15-2.12). Furthermore, 1.1% of fluctuation in infection was justified by age.

The relative frequency of positive cases in male and female dogs was approximately equal (Table 2). The infection was not associated with gender ($P > 0.05$). Based on the univariate logistic regression, the odds of infection in males was 1.039 compared to that in females (95% CI: 0.38-2.82). Further, 0.008% of fluctuation in infection was justified by gender.

The prevalence of infection was higher in mixed breeds (5.75%) in comparison with other breeds (Table 2), nevertheless, the difference was not significant ($P > 0.05$). In addition, 6.6% of fluctuation in infection was justified by the breed.

The prevalence of infection in diarrheal and healthy dogs was 8.2% and 6.6%, respectively (Table 2), and no significant difference was noted between the dogs in this regard ($P > 0.05$). The univariate logistic regression demonstrated that the odds of infection in diarrheic dogs was 1.85 compared to none-diarrheic (95% CI: 0.67-5.06). Furthermore, 2.1% of fluctuations in infection were justified by gastrointestinal status (diarrheic or none-diarrheic).

The relative frequency of positive cases in dogs living in open environments was higher than that of those living indoors, which was not statistically significant ($P > 0.05$, Table 2). The odds of infection in dogs living outdoors was 1.04 times greater than that of those living indoors (95% CI: 0.38-2.87) and it justified 0.01% of fluctuation infection.

The relative frequency of positive cases in dogs fed with raw food was higher compared to those fed with cooked foods, which was not statistically significant ($P > 0.05$, Table 2). The odds of infection in dogs fed with raw food was 1.029 times greater in comparison with those fed with cooked food (95% CI: 0.356-2.978). Moreover, 0.008% of fluctuation in infection was justified by nutrition status.

Discussion

Campylobacteriosis is an important zoonotic disease, and the companion and stray dogs are important in the epidemiology of the disease. The infected dogs can be concerned with disease transmission to other

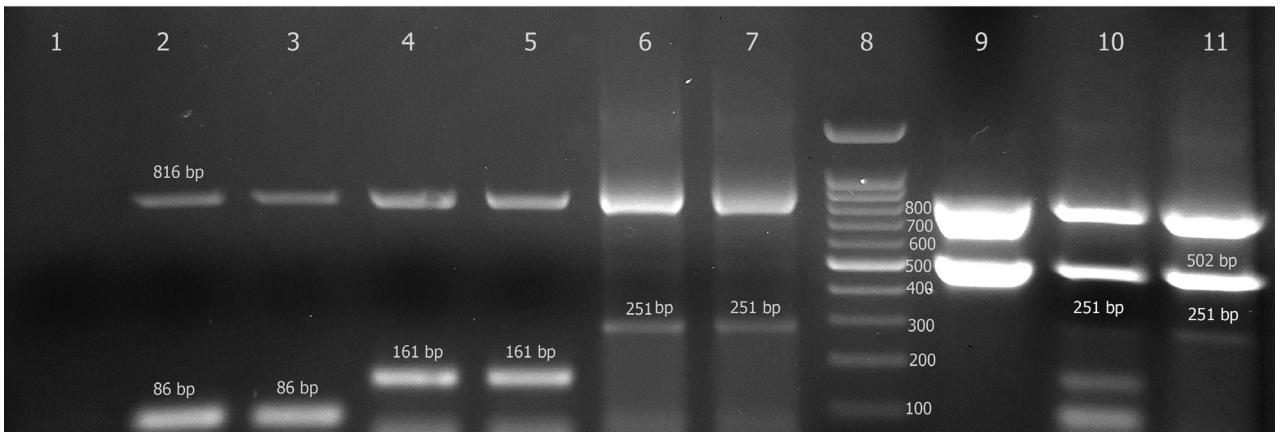

Figure 1. PCR Detection of the *Campylobacter* Genus and Species. *Note*. PCR: Polymerase chain reaction. The well No. 1 negative control. The wells No. 2 and 3: Positive sample and positive control of *C. upsaliensis,* respectively. The wells No. 4 and 5: Positive sample and positive control of *C. jejuni,* respectively. The wells No. 6 and 7: Positive sample and positive control of *C. Lari,* respectively. The well No. 9: 100 bp ladder. The well No. 9: Positive sample of *C. coli.* The well No. 10 multiplex PCR on four positive controls of *Campylobacter upsaliensis, jejuni, lari,* and *coli*. The well No. 11: The sample had concurrent infection with two species of *C. Lari* and *C. coli*.

animals while there is a risk of infection transmission to humans. The study on infectious diseases such as *Campylobacteriosis* is compulsory due to the increasing tendency of people to keep dogs in the home.[2] Given that the dogs can be actually as one of the main reservoirs of pathogenic *Campylobacter* spp., it is necessary to increase information about the epidemiology of the disease in these animals. The present study revealed that 14.8% of companion dogs were positive for *Campylobacter* infection by the PCR in the Ahvaz region, South-West of Iran. Rectal swab samples were examined by culture and PCR methods. Molecular techniques are considered to be the gold standard for the trustworthy identification of the species of *Campylobacter* and are valuable in epidemiological studies in humans and animals[2] although there have been a few studies based on molecular methods for *Campylobacter* species in pets in Iran. The direct PCR was found to be more sensitive compared to the culture method for the detection of *Campylobacter* species. The findings are consistent with those of other studies.[19-21]

The prevalence of infection in diarrheal and healthy dogs was 8.2% and 6.6%, respectively. The other studies reported *Campylobacter* infection rates of 4.81-51.1%.[5,22-27] The variation in the rate of infection may rely on the sample size, age, diet, and gastrointestinal status (diarrheal or healthy) of the studied dogs, different diagnosis methods, and geographical location, as well as the season of sampling. The most prevalent species of *Campylobacter* among the dogs were *C. coli* (38.89%) and *C. jejuni* (33.33%). A lower prevalence was observed for *C. upsaliensis* (11.11%) and *C. lari* (5.55%). Dogs and cats are the main reservoirs of *C. jejuni* and *C. upsaliensis.*[28] Engvall et al, Hald et al, and Wieland et al reported that the majority of bacterial isolates from dogs were *C upsaliensis.*[22,29,30] Workman et al detected a predominant prevalence for *C jejuni* in dogs.[31] The importance of these *Campylobacter* species, especially *C. jejuni* in humans is transmission to humans through sick or carrier dogs. Domestic dogs are usually in close contact with family, especially children. The infection of 11.42% of healthy dogs with *Campylobacter* showed that healthy dogs (even without diarrhea) could be a threat to humans as a carrier of *Campylobacter* spp. Concurrent infections were observed in two cases (*C. upsaliensis* + *C. lari* and *C. coli* + *C. lari*). Similar mixed infections of *Campylobacter* species were reported of dogs in previous studies.[22,30]

In this study, the relative frequency of positive cases was more in dogs under one-year-old. Although there was no significant relationship between age and infection, several surveys have emphasized that dogs less than one year old were most likely to be infected with *Campylobacter.*[2,16,22,24-26,32] It may be because of the weakness of the puppies and the low level of immunity in young dogs compared to adults.[22,30]

Although *Campylobacter* was more prevalent in male compared to female dogs, the statistical analysis showed no significant difference, implying that *Campylobacter* exposure has no gender predilection in dogs, which is consistent with the results of a previous study.[33] In the case of the breed, although the prevalence of infection was higher in mixed breeds in comparison with other breeds, the difference was not significant, which corroborates with the findings of another study.[34]

The prevalence of *Campylobacter* infection in dogs living indoor or outdoor is a risk factor for infection. In addition, its incidence in dogs living in kennels is higher compared to single-household pets due to the high density of dogs and increased communication between the dogs.[5,22,31] Nevertheless, in the present study, lifestyle was not significant between the two groups, which is consistent with the results of Andrzejewska et al.[26]

The previous research revealed that the probability of *Campylobacter* infection in dogs fed with raw meat is more than those fed with cooked food. The dogs can

be infected with raw meat from poultry, wild birds, pigs, and other animals.[2,26] However nutrition status was not statistically significant in the studied dogs.

Campylobacter species can be found in dogs and cats with gastrointestinal symptoms as an opportunistic infection and may act as a primary or secondary pathogen. Several studies reported that the infection rate to *Campylobacter* was higher in diarrheic dogs[24,25,27,35] while the true relation between gastrointestinal status in animals and the presence of *Campylobacter* remains uncertain.[23,36] However, no significant difference was noted among healthy and diarrheic dogs in this study, which matches the findings of Harrus et al[2] and Sandberg et al.[37] Systemic signs of fever, lethargy, vomiting, and weight loss were not observed in the infected dogs.

Campylobacter coli and *C. jejuni* were commonly isolated from the feces of companion dogs in the Ahvaz district. Veterinarians should encourage good hand hygiene for the owners of pets because carriage may be prolonged. In conclusion, the PCR is a sensitive and specific procedure for the detection of *Campylobacter*. This study highlights the necessity of using rapid and effective diagnostic techniques for screening healthy and diarrheic dogs.

Conclusion

Our results showed that *Campylobacter* is a specific infection and appears to be endemic in dogs in this area. In addition, it is a zoonotic pathogen, thus the prompt treatment of the infected dogs is suggested to prevent human disease. Testing programs for the diagnosis and the prohibition of contact are the most effective preventative ways between sick and healthy animals. Considering that the vaccine is unavailable for *Campylobacter* infection, the only ways for the prevention of the disease are observing the principle of hygiene and avoiding contact with feces, especially stray dogs. Finally, further studies will be necessary for various areas to survey the overall epidemiological status of campylobacteriosis in dog populations.

Authors' Contributions

Experiment design, experiment conduct, data interpretation and manuscript writing - review & editing by DG and BM; Visualization, Investigation and Methology by RA; literature review, Investigation , laboratory performance and practice by MF.

Ethical Approval

We hereby declare that all ethical standards have been respected in
the experiment and preparation of the article.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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