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Molecular Study of *Cryptosporidium* spp. in Dogs from Southwest of Iran

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Abstract

Background: *Cryptosporidium* is a protozoan parasite that effects rodents, dogs, calves, humans, and cats. Infection with this parasite is known as cryptosporidiosis. *Cryptosporidium* spp. may induce clinical or subclinical signs in infected hosts. In the life cycle of this parasite infected dogs freely living in urban and rural areas of Khuzestan province are the definitive hosts that should be considered as a real problem in public health for humans.
Objectives: This study aimed at determining the frequency of cryptosporidiosis in dogs in southwest of Iran.
Methods: Overall, 350 fresh fecal samples were collected from domestic dogs living in 43 villages, from June 2012 to September 2013. All samples were investigated by Sheather's concentration method and fecal smears were stained with modified Ziehl-Neelsen followed by light microscope examination, and polymerase chain reaction (PCR).
Results: The results revealed that frequency of *Cryptosporidium* infection was 8% and 12.3%, using direct smear and molecular method, respectively.
Conclusions: The present findings indicated that domestic dog feces from southwest of Iran may contain zoonotic parasites such as *Cryptosporidium* spp. and may be a potential risk for humans and other animals, especially when they contaminate the environment. The role of dogs as source of human infection should be investigated by further studies.

Keywords: Sheather's, Modified Ziehl-Neelsen, PCR, Dogs, Iran, *Cryptosporidium* spp

1. Background

Cryptosporidium is a widespread zoonotic intestinal protozoan parasite belonging to the phylum Apicomplexa that contains 30 species with more than 50 genotypes and infects a wide range of vertebrate animals, including mammalians, avians, amphibians, reptiles, and fish species as well as humans by the fecal-oral route via ingestion of sporulated oocysts (1-3). *Cryptosporidium* spp. infects the epithelial cells of the gastrointestinal tract (primarily small intestine and colon) of hosts and may induce clinical or subclinical signs, including vomiting, diarrhea, abdominal pain, fever, anemia, anorexia, dermatitis, and loss of weight, yet, occasionally, some infected hosts may present no symptoms (4, 5).

Human cryptosporidiosis in immunocompetent individuals usually causes acute infection of the digestive system and self-limiting diarrhea, yet, in immunocompromised patients, such as people infected with Human Immunodeficiency Virus (HIV), people with malignancies, solid-organ transplants, and those on hemodialysis may suffer from severe diarrhea and dissemination to extra-intestinal sites, particularly the gall bladder, biliary tract, pancreas, and respiratory tract (6, 7).

Domestic dogs (Canis lupus familiaris) are generally considered as the first domesticated mammal from very early in human history (about 12,000 years ago) and are the most abundant species of carnivore around the world today, whoch are the definitive or reservoir hosts of more than 60 zoonotic parasites, such as *Cryptosporidium* spp. (8-10). Therefore, they are a real problem in public health for humans, particularly in villages and poorly marginalized communities of towns. There are 2 types of dogs in Iran including stray and owned dogs. Stray dogs often live freely in urban and rural areas, and the growing number of these animals in urban and rural residential areas of Iran and their easy access to public environments in order to obtain their nutritional needs from garbage may contaminate soil, food, and water with discharge of helminths eggs and protozoan oocysts, and consequently an increase in parasitic infections in humans and animals. Furthermore, if owned dogs, including shepherd dogs, police dogs, gardener dogs, and pet dogs, are infected by parasites, they can infect occupational groups, such as shepherds, police, gardeners and veterinarians or physicians. In 1983,

canine cryptosporidiosis was first reported by Fukushima and Helman (11) in a 3-month-old puppy, which was infected by Distemper disease.

Dogs become infected with the most common species/genotypes of *Cryptosporidium* spp., which are responsible for human cryptosporidiosis, including *Cryptosporidium canis*, *C. parvum*, *C. muris*, *C. felis*, and *C. meleagridis* by direct contact with infected animals (infected dogs and other animals such as ruminant, rodent, and/or ingestion of contaminated food or water from the environment) (12, 13). Therefore, these animals are 1 of the major sources of cryptosporidiosis, which causes the spread of this protozoan in the environment.

Different methods are used for detection of cryptosporidiosis, which are generally based on analysis of stool samples for identification of oocysts using microscopy with tinctorial and fluorescent stains (modified acid-fast, safranin methylene blue, and auramine-rhodamine), antigen detection (immunofluorescence and enzyme-linked immunosorbent assay (ELISA) or genome detection (Polymerase chain reaction (PCR) amplification) in stool samples (14). In addition, serological assays are used for epidemiological studies because specific antibody responses develop after both symptomatic and asymptomatic infection, especially for immunocompromised individuals (15), also considering the importance of zoonotic *Cryptosporidium* and the possibility of contamination of water and food with this parasite by infected animals.

2. Objectives

Due to the possible roles of dogs in parasite spreading rate, determination of frequency is necessary. Thus, the current study aimed at determining the frequency of cryptosporidiosis in dogs in southwest of Iran.

3. Methods

3.1. Study Area

The study was undertaken in the city of Ahvaz, the capital of Khuzestan Province, which covers approximately 63 238 km^2 and is located at 31° 3' longitude north and 48° 7' longitude east in southwest of Iran, bordering Iraq and the Persian Gulf (Figure 1). The climate of this area is generally hot and occasionally humid. Summer time temperatures exceed 52°C. This province is known to master the hottest temperatures on record for a populated city anywhere in the world (16).

Khuzestan is highlighted with green. Cities of this province are distinguished by colors. The map of Khuzestan province by Uwe Dering was highlighted by Dr. Blofeld.

3.2. Fecal Samples Collection

Villages of Khuzestan province were classified to 5 areas: east, west, north, south, and center. Then, 350 fresh fecal samples were collected from domestic dogs with different ages, according to their teeth, and were grouped in 3 groups, including puppies (< 1-year-old), young dogs (1 to 5 year-old), and old dogs (> 5-year-old), who were living in 43 villages (eas t = 8, west = 9, north = 9, south = 8, and center = 9 villages) from June 2012 to September 2013. In each geographical area, 70 fresh samples were collected from house dogs (owned dogs) and then they were placed in polyethylene bags, marked according to area and were separately carried to the laboratory and kept at 4°C, until processing

3.3. Fecal Examination

All samples were concentrated by sucrose flotation procedure (Sheather's method, with a specific gravity of 1.21), and thin smears of the concentrated layer of samples were then prepared on glass slides, air-dried, and fixed with methanol, and stained by modified Ziehl-Neelsen and investigated by a light microscope. Each slide was accessed at 1000 × magnification under oil emersion, and *Cryptosporidium* spp. was confirmed using morphological characteristic of oocysts. The positive samples were preserved in 2.5% potassium dichromate ($K_2Cr_2O_7$) and stored at 4°C until DNA extraction.

3.4. DNA Extraction

Approximately 200 μL of concentrated oocysts of each sample was added to a 2.0-mL eppendorf tube. The samples were pretreated by the freeze and thaw method by liquid nitrogen to break down the oocyst walls. Briefly, tubes were placed in liquid nitrogen for 15 minutes, and were then transferred to 100°C water bath for another 5 minutes. These steps were repeated for a total of 5 times. Next, the genomic DNA was purified using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, Korea), according to the manufacturer's instruction. DNA was eluted in 100 μL of elution buffer and stored at -20°C.

3.5. Polymerase Chain Reaction Amplification

The PCR protocol, based on the amplification of a specific sequence of the SSU rRNA gene, was used to detect *Cryptosporidium* by primers CryF: (5′-CTGACCTATCAGCTTTAGA- 3′) and CryR: (5′-GCTGAAGGAGTAAGGAACA- 3′), which produced a piece of DNA with a molecular weight of 720 bp (17). In order to perform the PCR reaction, AccuPower® PCR PreMix(Bioneer, Korea) was used, including Taq polymerase enzyme, dNTP, $MgCL_2$, reaction buffer, and tracking dye. In this step, 15 μL of deionized distilled water, 2.20 μL of extracted genomic

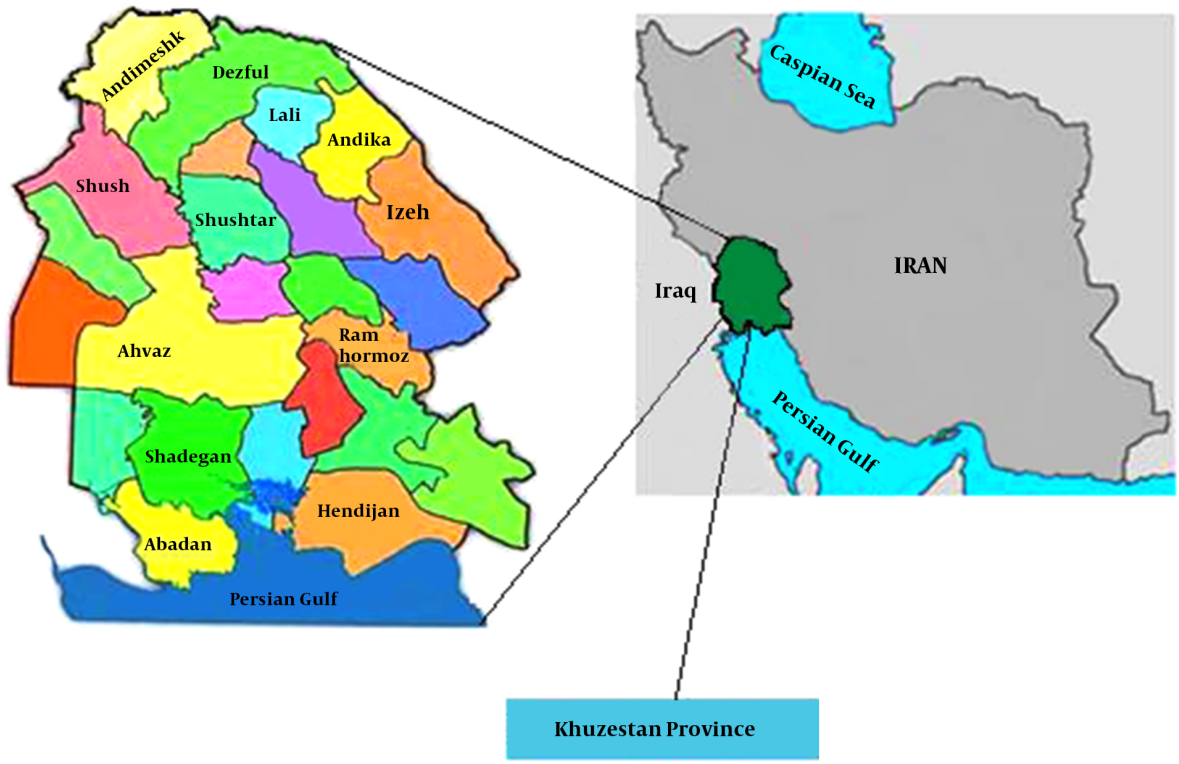


Figure 1. Map of Iran and Khuzestan Province

DNA (100 ng), and 1 μL of forward and reverse primers at 25 pmol were applied in a total volume of 25 μL. The PCR condition was as follows: predenaturation at 94°C for 4 minutes; denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute and extension at 72°C for 1 minute, followed by 30 cycles; final extension at 72°C for 5 minutes in a thermal cycler (Bio-Rad, Hercules, CA, US). The PCR product was analyzed by electrophoresis on 2% agarose gel in 1X TBE buffer and visualized using ethidium bromide staining on UV transilluminator.

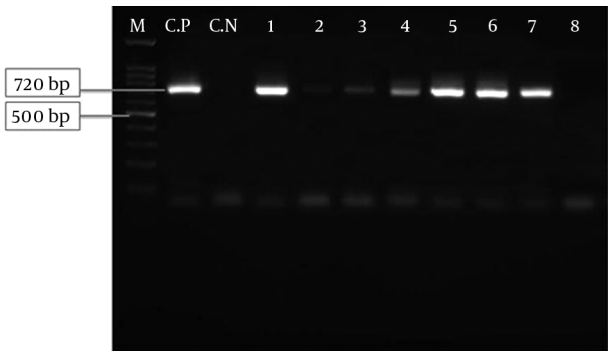
4. Results

The frequency of *Cryptosporidium* infection in dogs by PCR was 12.3% (95% CI: 8.86% to 15.75%). Also the frequency of *Cryptosporidium* infection in dogs using staining and molecular methods were 8% (28/350) and 12.3% (43/350), respectively (Figure 2). In this study a comparison was made between gender, age, and geographical areas of dogs to assess the existence of *Cryptosporidium* spp. Investigation results indicated that the frequency of *Cryptosporidium* spp. infection in stools collected from villages in different geographical areas of Khuzestan province including east, west, north, south, and center were 4.28%, 12.85%, 11.42%, 24.28%, and 10%, respectively. The statistical analysis showed a significant relationship between geographical areas and frequency of *Cryptosporidium* spp. in dogs ($P < 0.05$). The results showed that there was no statistically significant difference among gender and age groups as indicated by staining and PCR methods for diagnosis of *Cryptosporidium* in females and males ($P > 0.05$) (Table 1).

5. Discussion

Cryptosporidiosis is a zoonotic protozoal disease, which is reported in animals and humans with worldwide distribution in more than 106 countries, especially in developing countries (18, 19), and may cause gastrointestinal problems such as diarrhea in immunocompromised and immunocompetent people and even the environment (5). Molecular epidemiological investigations strongly suggest that zoonotic species and genotypes of *Cryptosporidium* play an important role in cryptosporidiosis and were mentioned as a risk factor for human cryptosporidiosis (20). A single oocyst is sufficient to produce

Figure 2. Polymerase Chain Reaction of *Cryptosporidium* spp. in Dog Feces Samples



M, 100 bp molecular marker; C.P, positive control; C.N, negative control; 1 - 7 lines, positive samples; line 8, negative sample.

Table 1. Frequency of *Cryptosporidium* spp. in Different Regions of Khuzestan Province[a]

Factor	Diagnostic Technique	
	Staining	PCR
Gender		
Male	17/215 (7.9)	26/215 (12)
Female	11/135 (8.14)	17/135 (12.6)
Age, y		
< 1	2/76 (2.63)	4/76 (5.26)
1 - 5	17/199 (8.54)	28/199 (14.07)
> 5	9/75 (12)	11/75 (14.67)
Geographical areas[b]		
East	2/70 (2.86)	3/70 (4.29)
West	6/70 (8.57)	9/70 (12.86)
North	5/70 (7.14)	7/70 (10)
South	10/70 (14.28)	17/70 (24.3)
Center	5/70 (7.14)	7/70 (10)
Positive	28 (8)	43 (12.3)
Negative	307	322
Total	350	350

[a]Values are expressed as No. (%).
[b]The frequency of *Cryptosporidium* spp. in dogs indicated a statistically significant relationship with geographical areas, according to diagnostic techniques ($P < 0.05$).

infection and disease in susceptible hosts (21). Dogs are often considered faithful friends and intimate companions of humans from very early in human history that can act as definitive or reservoir hosts for a large number of zoonosis parasitic diseases of parasitic zoonoses, such as *Taenia* sp., *Echinococcus* sp., *Toxocara canis*, *Giardia* spp., and *Cryptosporidium* spp. (22). Dogs are vertebrate animals that are infected with *Cryptosporidium* spp. in the wildlife and represent a potentially significant source of environmental contamination and reservoir of the disease for domestic livestock and humans, due to transmission of the infection through close contact with infected dogs (21).

Epidemiological studies on *Cryptosporidium* infection indicated that the prevalence of *Cryptosporidium* spp. in dogs is very different in various countries (from 0% to 52.7%) (23-26); a prevalence of 1.4% in the Czech Republic (27), 2.1% in Thailand (28), 2.4% in Brazil (29), 3.9% in Japan (30), 4.1% in Northern Spain (31), 18.5% in Nigeria (32), and 52.7% in Romania. This difference depends on factors, such as geographical location, the number of dogs, status of animals ownership, existence and number of other hosts correlated with dogs, including domestic animals (such as cattle, horses, sheep, goats, and pigs), species of *Cryptosporidium*, sampling protocols, anthelmintic use, and diagnostic techniques (33, 34).

Infected dogs with cryptosporidiosis shed oocysts with their feces, which can contaminate environment. *Cryptosporidium* spp. oocysts are resistant to harsh environmental conditions and can be well preserved under cold and wet environments. In addition, these are very resistant to the most common disinfectants, therefore, can contaminate water, and there is a potential risk for areas with a large dog population (35-37). Prevalence of *Cryptosporidium* spp. in geographical regions of Khuzestan province, southwest of Iran, is variable and it seems that the prevalence of this protozoa in south of the province is higher than other areas (38-40).

The current findings may be due to the following reasons; southern provinces have a high temperature and humid weather conditions, life style of people regarding consumption of seafood more than other regions, and birds immigration to south of Khuzestan province, which may be carriers of infection. In Iran, people who live in villages because of lifestyle and closely related agricultural and animal husbandry sources are exposed to zoonotic pathogenesis microorganisms, such as parasitic zoonoses. Therefore, the potential for zoonotic transmission from domestic animals such as dogs, that are reservoirs via environmental contamination, is of increasing concern.

In the current study, *Cryptosporidium* spp. oocysts were identified in 8% (28/350) and 12.3% (43/350) of samples examined using staining and molecular methods, respectively. This rate is more than the results of some previous studies, which were carried out about cryptosporidiosis in dogs of different areas of Iran. Bahrami et al. (41) reported 7.04% infection in stray dogs of Ilam using the Ziehl-Neelsen staining method. In another study conducted in the Southeast of Iran (Kerman), prevalence of *Cryptosporid-*

ium spp. was 2% using the formalin ether sedimentation and modified Ziehl-Neelsen staining technique. In addition, Gharekhani (42) reported that 3.8% of the infections among pet dogs in Hamedan in Western Iran were infected with *Cryptosporidium* spp. by formalin-ether and modified Ziehl-Neelsen technique.

The study of prevalence of gastrointestinal parasites of pet dogs in Tehran (Central Iran) and Urmia (Northwest of Iran) indicated that 1.6% and 2.9% of animals were infected with *Cryptosporidium* spp., respectively (43, 44). In another study, Mosallanejad et al. (45) investigated the prevalence of *C. parvum* in urban and rural dogs of Ahvaz district by using antigenic detection and modified Ziehl-Neelsen staining methods, the results of which indicated 4.3% and 2.5% of dogs were infected, as indicated by ELISA and staining methods. Also, the infection had greater prevalence in rural dogs (6.4%) in comparison with urban dogs (2.17%). The results were in agreement with other studies in different parts of the world, such as Bahía Blanca of Argentina that indicated 14.7% of dogs were infected with *Cryptosporidium* spp. (9). The results of the current study showed that the frequency of *Cryptosporidium* spp. in feces of dogs in villages of Ahvaz was high.

Despite the results of the current study, in some researches the frequency of *Cryptosporidium* infection was higher in young dogs (21, 31, 33, 46). Some studies in comparison to the current study indicated that infection rates in female dogs were higher than male dogs, which may be due to reduced immunity at certain periods in female physiologic cycle (32). Other investigations in Iran indicated that female dogs had more infection than male dogs. Bahrami et al. and Gharekhani reported that the prevalence of *Cryptosporidium* in female dogs of Ilam and Hamedan were more than male dogs (41, 42). Also, Mirzaei showed that the prevalence of *Cryptosporidium* in female dogs was higher than male dogs in Kerman (34). Although the role of infected dogs in transmission of *Cryptosporidium* infection to humans is not exactly clear, yet, *C. canis* can be infect immunocompromised patients. Also, a small number of zoonotic *Cryptosporidium* including *C. parvum*, *C. muris*, and *C. meleagridis* can infect dogs (12).

The free entrance of stray dogs to public places of villages and existence of owned dogs, such as shepherd dogs in houses has caused defecation in different areas of villages and may contaminate soil, food or water. Therefore, it can be a potential hazard for humans and domestic animals and this phenomenon is important in public health and livestock husbandry.

In conclusion, control programs including, public educational activities, regarding risk of parasite transmission, and role of animals such as dogs in parasite distribution should be considered. It is suggested to conduct control programs, including education for people about cryptosporidiosis and the potential transmission of this protozoan to humans and animals, prevention of free entrance of stray dogs in public places and houses, also collection and hygienic disposal of dogs feces. In addition, determining the frequency and treating cryptosporidiosis in owner dogs should be done by veterinarians or physicians.

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