Archive of SID

Tehran University of Medical Sciences Publication
http:// tums.ac.ir

Iranian J Parasitol

Open access Journal at
http:// ijpa.tums.ac.ir

Iranian Society of Parasitology
http:// isp.tums.ac.ir

Original Article

Detection and Molecular Characterization of *Cryptosporidium* species in Recreational Waters of Chaharmahal va Bakhtiyari Province of Iran using nested-PCR-RFLP

***K Manouchehri Naeini[1], M Asadi[1], M Hashemzade Chaleshtori[2]**

[1] *Department of Parasitology, Mycology and Entomology, School of Medicines, Shahr-e-kord University of Medical Science, Shahr-e-kord, Iran*
[2] *Cellular and Molecular Research Center, Shahr-e-kord University of Medical Sciences, Shahr-e-kord, Iran*

(Received 11 Nov 2010; accepted 18 Feb 2011)

ABSTRACT

Background: The aim of this study was to detect and characterize *Cryptosporidium* spp. in water samples collected from recreational ponds of Chaharmahal va Bakhtiyari Province of Iran .
Methods: Thirty water samples were collected from November 2009 to May 2010. Each sample contained 10 liters of water. We used the SSU rRNA-based PCR-RFLP technique.
Results: Out of thirty samples examined, 6 (20%) were positive for different *Cryptosporidium* spp. Restriction pattern analysis showed that *C. parvum* has been the most prevalent genotype, followed by *C. hominis* and *C. canis* , respectively. In this area, the higher prevalence of *C. parvum* compared with other genotypes is consistent with the distribution of cattle.
Conclusion: Farm animals, particularly cattle are the main source of cryptosporidial contamination for recreational waters in this area.

Keywords: *Recreational waters,* Cryptosporidium, *Genotyping, Nested-PCR-RFLP, Iran*

***Corresponding author**: **Phone**: +983813335635, **Email**: k_manouchehri@yahoo.com

www.SID.ir

Archive of SID

Introduction

Cryptosporidium is a coccidian parasite assigned to the phylum Apicomplexa. Initially, the parasite was considered host specific and species were named for 20 or more animal species from which they isolated. However, cross-transmission experiments revealed that more than one animal species could be infected by the same *Cryptosporidium* species (1-3).

There are currently 14 described species within the genus *Cryptosporidium* (4, 5) and more than 33 unique host-adapted genotypes, with only *C. hominis*, *C. hominis* monkey genotype, C. *parvum*, *C. muris*, *C. felis*, *C. meleagridis*, *C. canis*, *C. suis*, and *Cryptosporidium* cervine genotype demonstrated to cause infections in humans (6,7).

Contaminated water has been implicated as the source of infection in travelers and for various outbreaks in some countries, e.g., the United States. Spread of the parasite via sexual activity, aerosolization, fomites, and contaminated food has been suggested (8).

Unlike bacterial pathogens, *Cryptosporidium* oocysts are resistant to chlorine disinfection and can survive for days in treated recreational water venues (e.g., public and residential swimming pools and community and commercial water parks) (9).

Since low numbers of *C. parvum* oocysts are often found in the environment (6) and the number of oocysts required to cause infection is relatively low (10), a rapid and sensitive method is essential for the detection of *Cryptosporidium* oocysts in environmental samples, e.g. water sources.

For decades, microscopy was the sole method for detecting oocysts, first by direct fecal smears routinely stained, and later stained by IFA techniques. Immunofluorescence assay (IFA) which has been widely used for the detection of *Cryptosporidium* in water samples but these methods are time-consuming, labor intensive, and is subject to false positive and negative results (10). On the other hand, works based on detection of genus specific antigens on the surface of organism only provides detection on genus level (11, 12). Recently, genetic methods, which are based on detection of *Cryptosporidium* nucleic acid, by hybridization and amplification techniques such as PCR, have been developed (12, 13). Moreover, many genotyping tools have been used to differentiate between *Cryptosporidium* species in humans. The PCR primers of earlier tools were mostly based on antigenic, structural, housekeeping genes and unknown genomic fragments of *C. parvum*, and included various formats of detection and differentiation (14, 15). With few exceptions, most of these techniques can efficiently differentiate *C. parvum*, *C. hominis* and perhaps *C. meleagridis*, but are unlikely to amplify some of the more distant species (such as *C. canis*, *C. felis*, *C. muris* and *C. andersoni*). Therefore, these genotyping tools are mostly replaced by the genus-specific PCR-RFLP techniques based on the SSU rRNA gene, which have higher sensitivity and allow broad species detection (16-18).

Recently, a SSU rRNA-based nested PCR-RFLP technique has been developed for the differentiation of *Cryptosporidium* species and *C. parvum* strains in clinical samples and water supplies (15, 17).

The work of Mohammadi et al. on Ardabil River water samples showed that among 30 samples were examined, 11 samples showed positive results. Restriction pattern analysis showed *C. andersony* as the most common species with 7 cases; followed by with 3 cases and *C. suis* with 1 case (18).

The objectives of the present study were to detect of *Cryptosporidium* spp. in Shahre-

www.SID.ir

Archive of SID

kord recreational water sources in Chaharmahal and Bakhtiari Province in Iran and to determine which species and genotype of the parasites are present, by means of a molecular assay because this area has 10% of water source of Iran and most outbreak of *Cryptosporidium* occur with waterborne transmission.

Material and Methods

Samples and Methods

Thirty water samples were collected from Choghakhor, Gandoman and Ben ponds from November 2009 to May 2010. Each sample contained 10 liters of water. To collect *Cryptosporidium* oocysts, samples were filtered through a membrane filter (pore size, 1.2 µm; Mililipore Corp., Bedford, Mass). The filter-trapped pellets were removed by a sterile scalpel blade and transported to a 1.5 mL microtube containing 2.5% potassium dichromate (18). To increase the oocysts recovery, several pellets from the same sample were collected in the microtubes. The tubes were stored at 4 ˚C for DNA extraction. All of the preserved samples were washed seven times with 1mL of PBS (pH= 7.3) and centrifuged at 14000 × g for 1 min (18).

Briefly, DNA was extracted through five freeze-thaw (-70˚C) cycles and purified by using QIAamp DNA Mini isolate columns (Qiagen, Germany).Molecular profiling of *Cryptosporidium* spp. was carried out by nested PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of the small ribosomal subunit RNA (18S rRNA) gene with primary and secondary primers as described by Xiao et al.

For restriction fragment analysis, 5 µl of the secondary PCR products was digested in a 20-µl reaction mixture containing 10 U of *Ssp*I (Fermentas, EU) (for species diagnosis) or 10 U of *Vsp*I (Fermentas, EU) (for genotyping of *C. parvum*) and 2 µl of the appropriate restriction buffer at 37°C for 6 h , under conditions recommended by the supplier. The digested products were fractionated on an 8.0% acrylamide gel and visualized after silver nitrate bromide staining. The species were characterized according to Xiao et al. (17).

Results

Out of the thirty water samples examined, six samples (20.0%) showed positive PCR amplification by nested PCR (Table 1).

Species diagnosis carried out by digesting the secondary PCR product with *Ssp*I (Fig. 1). *C. parvum* generated 3 visible bands of 448, 247, and 106 bp and generated 3 visible bands of 417, 254, and 105 bp (15). To differentiate human and bovine genotypes of *C. parvum*, the secondary PCR product was digested with *Vsp*I (Fig. 2).

The *C. parvum* produced two visible bands of 628 and 104 bp, whereas *C. parvum* human genotype produced two visible bands of 556 and 104 bp due to the presence of one additional *Vsp*I restriction site.

Table 1: *Cryptosporidium* spp. and genotypes in water samples from various recreational ponds

Location species	No. samples	Vol. filtered/sample(lit)	No. of positive samples	*Cryptosporidium* spp.
Gandoman	10	10	2.0	*C. hominis* *C. parvum*
Choghakhor	10	10	3.0	*C. parvum* *C. canis*
Ben	10	10	1.0	*C. parvum*

www.SID.ir

Archive of SID

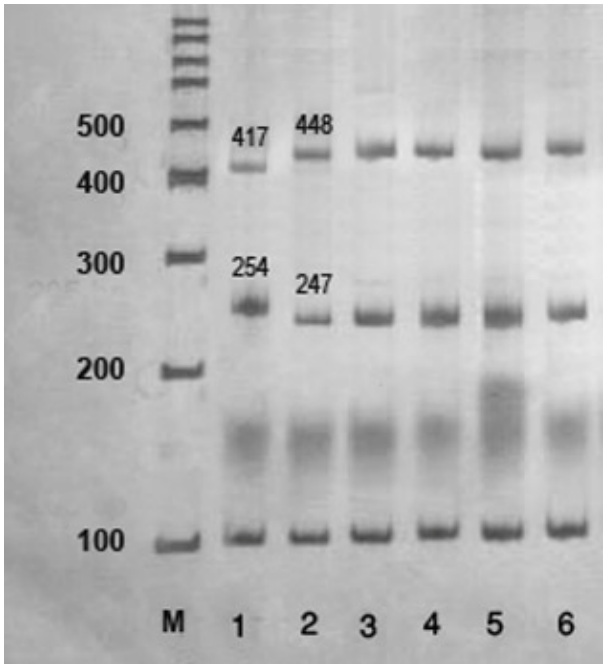

Fig.1: Differentiation of *Cryptosporidium* species and genotypes in recreational water samples with the small- subunit rRNA-based PCR-RFLP technique. Secondary PCR product was digested by *Ssp*I restriction enzyme. Lane M molecular weight marker, lane 1 *C. canis,* lane 2-6 *C. parvum*

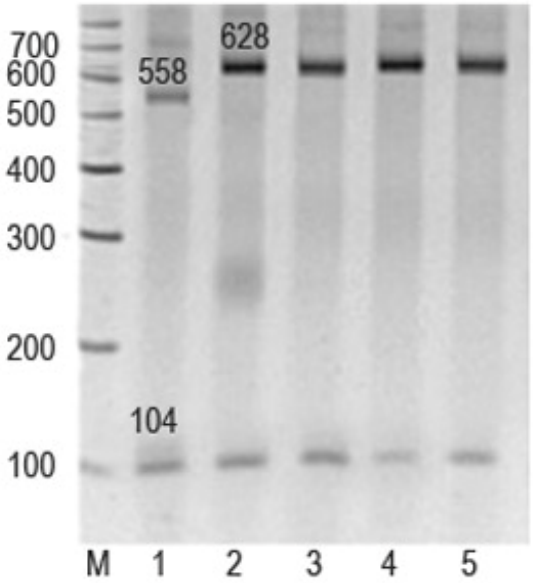

Fig.2: Differentiation of *Cryptosporidium* species and genotypes in recreational water samples with the small- subunit rRNA-based PCR-RFLP technique. Secondary PCR product was digested by *Vsp*I restriction enzyme. Lane M molecular weight marker, lane 1 *C. hominis,* lane 2-5 *C. parvum*

www.SID.ir

Archive of SID

Discussion

The rate of positive results obtained in this study (20%) is nearly similar to Karanis et al. (18.1%) (19) but differ from a number of such studies. For example Xiao *et al.* (2000) (17) found a higher rate of positive PCR results, 88.4%. A number of factors, including climatic conditions, locations of sampling, number, and volumes of samples examined, number and diversity of animals in the areas, type of ecosystems, season, and some of technical difficulties for the recovery of *Cyptosporidium* oocysts from the water samples may contribute in these variations. There is usually a strong association between the occurrence of cryptosporidiosis and the rainy season in tropical areas, or the cooler months in dry areas. Thus, the transmission of cryptosporidiosis in developing countries is probably different from that in the industrialized nations (20). One major obstacle is the presence of PCR inhibitors in water. In recent studies, PCR inhibitors are removed by Immunomagnetic Separation (IMS) (6). Immunomagnetic separation (IMS) has increased oocyst recoveries from water concentrates (21). The benefits of IMS, in capturing oocysts from crude samples and concentrating and processing them in a buffer free of PCR inhibitors, increases the sensitivity of detection (22), and this approach has been used to genotype oocysts in water and foods by many workers (23, 24) In the present study, we removed the inhibitors by repeated washing of collected oocysts with PBS buffer. Repeated washings might decrease the recovery rate of *Cryptosporidium* oocysts in the samples and reduce the final DNA concentration and the sensitivity of the PCR (6).

Five *Cryptosporidium* spp. have been responsible for most of human *Cryptosporidial* infections in developing countries; including *C. hominis*, *C. parvum*, *C. meleagridis*, *C. canis* and *C. felis*. They were initially found in otherwise healthy children in Peru in a longitudinal cohort study using a SSU rRNA-based genotyping tool (25), but have also recently been found in diarrheic children in Kenya (26). In most developing countries studied, *C. parvum* and *C. hominis* are responsible for more than 90% of human cases of cryptosporidiosis. Our findings indicated that the water sources were mainly contaminated by animal species of *Cryptosporidium*. In Iran, among seven isolates of *Cryptosporidium* 4 isolates were known as *C. parvum* (27). As might be expected, the presence of several villages and farmlands near the study areas with a significant number of domestic animals ,particularly cattle are consistent with our results. In Botswana, in early 2006 after heavy rain, thousands of cryptosporidiosis cases and several hundreds of deaths in a number of districts occurred. Both *C. parvum* and *C. hominis* were identified in infected people, with the former responsible for more cases (20). Bovine and human genotypes of *C. parvum* commonly infect human, farm and wild animals. It is well established that farm animals and human sewage discharge are generally considered the major sources of surface water contaminations (28). In addition, the work of Keshavarz et al. showed that *C. parvum* was common genotype in this study (29).

Cryptosporidium parvum is a coccidian protozoan that has zoonotic significance. Because of the broad host spectrum of this pathogen, inefficiency of the common drinking water treatment methods, and the lack of reliable therapy in humans, zoonotic genotypes of *Cryptosporidium* spp. are considered to be among the major threats found in water supply systems (30). From the perspective of human health, cattle have often been implicated as a source of zoonotic

www.SID.ir

Cryptosporidium species. Risk of human infection has been based on physical contact with cattle, contamination of fresh fruits and vegetables with manure, and manure runoff from farms into drinking water supplies (28).
Information on the source of *C. parvum* contamination is necessary for effective evaluation and selection of management practices for reducing *C. parvum* contamination of the surface water and the risk of Cryptosporidisis (28). Differences have been observed among endemic areas in the proportion of infections due to each species (20). Thus, identification of species and genotype of *Cryptosporidium* spp. is of public health importance.
In summary, the SSU rRNA-based PCR-RFLP technique has the potential to differentiate among *Cryptosporidium* spp. and to assess the sources of *Cryptosporidium* parasites in environmental water samples. Results of the present study suggest that farm animals, particularly cattle are a major source of contamination of recreational waters with *Cryptosporidium* oocyst. Extensive genotyping of the parasite in various water sources and environmental settings (feral, rural, urban, and recreational) is essential to have a better knowledge of *Cryptosporidium* spp. distribution. The results of the present study can help public health care systems in prevention and management of cryptosporidiosis.

Acknowledgments

The work reported in this paper was supported by the Cellular and Molecular Research Center, Shahr-e-kord University of Medical Sciences. We thank Dr. B. Mohammadi Ghalebin for his useful suggestions and Dr. S. Bahrami for providing positive controls for this research. The authors declare that there is no conflict of interests.

References

1. Hart CA. Cryptosporidiosis. In: Gilles HM, editor. Protozoal Diseases. London: Arnold; 1999. p. 592-610.
2. McDonald V, Kelly MP. Intestinal coccidia: cryptosporidiosis, isosporiasis, cyclosporiasis. 2005 In: Cox FEG, Wakelin D, Gillespie SH, editors. Despommier D.D. Toply and Wilsonn's, editors. Microbiology and Microbial Infections, Parasitology. London: Hodder Arnold Ltd; Reprinted 2007. p. 399-420.
3. Morgan UM , Xiao L, Fayer R, Lal AA, Thompson RC. Variation in *Cryptosporidium* towards a taxonomic revision of the genus. Int J Parasitol. 1999; 29:1733-51.
4. Ryan UM, Monis P, Enemark HL, Sulaiman I, Samarasinghe B, Read C, Buddle R, Robertson I, Zhou L, Thompson RCA, Xiao L. *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (*Sus scrofa)*. J Parasitol. 2004;90:769-73.
5. Xiao L, Fayer R, Ryan U, Upton SJ. *Cryptosporidium* taxonomy: recent advances and implications for public health. Clin Microbio Rev. 2004;17:72-97.
6. Rose JB. Environmental ecology of *Cryptosporidium* and public health implications. Annu Rev Public Health. 2004;18:135-161.
7. Smith HV, Rose JB. Waterborne cryptosporidiosis: current status. Parasitol Today.1998; 14:14-22.
8. Korich DG, Mead JR, Madore MS, Sinclair NA, Sterling CR. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl Environ Microbiol. 1990;56:1423-28.

www.SID.ir

Archive of SID

9. LeChevallier MW, Norton WD, Lee RG. Occurrence of *Giardia* and *Cryptosporidium* spp. in surface water supplies. Appl Environ Microbiol. 1991;57: 2610-16.
10. Yu JR, O'Hara SP, Lin JL, Dailey ME, Cain G, Lin JL. A common oocysts surface antigen of *Cryptosporidium* recognized by monoclonal antibodies. Parasitol Res. 2002;88:412-20.
11. Johnson DW, Pieniazek NJ, Griffin DW, Misener L, Rose JB. Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. Appl Environ Microbiol. 1995;61:3849-55.
12. Laxer MA, Timblin BK, Patel RJ. DNA sequences for the specific detection of *Cryptosporidium parvum* by the polym-erase chain reaction. Am J Trop Med Hyg. 1991;45:688-94.
13. Xiao L. Cryptosporidiosis: an update in molecular epidemiology. Curr Opin Infect Dis. 2004;17:483–90.
14. Cacciò SM, Molecular epidemiology of human cryptosporidiosis, Parasitologia. 2005; 47(2):185-92.
15. Xiao L, Escalante L, Yang CF, Sulaiman I, Escalante AA, Montali RJ, Fayer R, Lal AA. Phylogenetic analysis of *Crypto-sporidium* parasites based on the small-subunit rRNA gene locus. Appl Environ Microbiol. 1999; 65:1578-1583.
16. Fayer R, Santín M,DumitruMacarisin D. *Cryptosporidium ubiquitum* n. sp. in animals and humans. Vet Parasitol. 2010;172: 23-32.
17. Xiao L, Alderisio K, Limor J, Royer M, Lal AA. Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool. Appl Environ Microbiol. 2000; 66:5492-8.
18. Ghalebin BM, Fallah E, Asgharzadeh M, Kazaemi AH, Arzanlou M. Detection and Identification of *Cryptosporidium* species in Water Samples from a River in Ardabil City, Northwestern Iran. Res J Bio Sci. 2007;2(4):498-502.
19. Karanis P, Sotiriadou I, Kartashev V, Kourenti C, Tsvetkova N and Stojanova K, Occurrence of *Giardia* and *Cryptosporidium* in water supplies of Russia and Bulgaria. Env Res. 2006; 102(3): 260-271.
20. Xiao L. Molecular epidemiology of human cryptosporidiosis in developing countries. In: Ortega-Pierres G, Cacciò S., Fayer R, Mank TG, Smith HV, Thompson RCA, editors. *Giardia* and *Cryptosporidium* from molecules to disease. UK: CAB International; 2009. p.51-62.
21. Smith HV, Campbell BM, Peet J. The DWI-licensed Inter-laboratory *Cryptosporidium* Proficiency Scheme (*CRYPTS*). In: Hoyle BG, editor. *Regulatory Cryptosporidium:11 Months On*. Royal Society of Chemistry, Institution of Water Officers and Society of Chemical Industry; 2001. Paper 3-4.
22. Smith HV. Detection of *Cryptosporidium* and *Giardia* in water. In: Pickup RW, Saunders JR, editors. Molecular Approaches to Environmental Microbiology. London:Ellis-Horwood; 1996. p.195–225.
23. Nichols RAB, Campbell BM, Smith HV. Identification of *Cryptosporidium* spp. oocysts in United Kingdom non-carbonated natural mineral waters and drinking waters by using a modified nested PCR-RFLP assay. App and Env Micro. 2003;69:4183–9.
24. Xiao L, Singh A, Limor J, Graczyk TK, Gradus S, Lal AA. Molecular characterisation of *Cryptosporidium* oocysts in samples of raw surface water and wastewater. Applied and Environ-

www.SID.ir

Archive of SID

mental Microbiology. 2001;67, 1097–1101.
25. Xiao L, Bern C, Limor J, Sulaiman I, Roberts J, Checkley W, Cabrera L, Gilman RH, Lal AA. Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru Journal of Infectious Diseases 2001;183:492–7.
26. Gatei W, Wamae CN, Mbae C, Waruru A, Mulinge E, Waithera T, Gatika SM, Kamwati SK, Revathi G, Hart CA. Cryptosporidiosis: prevalence,genotype analysis, and symptoms associated with infections in children in Kenya. Am J Trop Med and Hyg. 2006;75:78–82.
27. Meamar AR, Guyot K, Certad G, Dei-Cas E, Mohraz M, Mohebali M, Mohammad K, Mehbod AA, Rezaie S, Rezaian M. Molecular characterization of *Crypto-sporidium* isolates from humans and animals in Iran. App and Env Micro. 2007;73, 1033–1035.
28. Xiao L, Lal AA, Jiang J. Detection and differentiatation of *Cryptosporidium* oocysts in water by PCR-RFLP, In the Public Health Microbiology, Method and Protocols, In: John FT, Ruget S, Alicia L. Otawa:Nj: Humana Press; 2004. p.163-267.
29. Keshavarz A, Haghighi A, Athari A, Kazemi B, Abadi A, Mojarrad EN, Prevalence and molecular characterization of bovine *Cryptosporidium* in Qazvin province, Iran. Vet Par. 2009; 3-4:316-318.
30. Mohammed HO, Wade SE . The Risk of Zoonotic Genotypes of *Cryptosporidium* spp. in Watersheds. In: Ortega-Pierres G, Cacciò SM, Fayer R, Mank TG, Smith HV, Thompson RCA, editors. *Giardia* and *Cryptosporidium* from molecules to disease. UK: CAB International; 2009. p.123-132.

www.SID.ir