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آموزش مهارت های کاربردي در تدوين و چاپ مقاله
Detection and Molecular Characterization of Cryptosporidium species in Recreational Waters of Chaharmahal va Bakhtiyari Province of Iran using nested-PCR-RFLP

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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

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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. meleagris, 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. meleagris, 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-
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 SspI (Fermentas, EU) (for species diagnosis) or 10 U of VspI (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 SspI (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 VspI (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 VspI restriction site.

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

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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.
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 polymerase 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, DumitrMacarisin 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 (CRY-PTS). 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 Enviro-
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, Deicas E, Mohraz M, Mohebali M, Mohammad K, Mehbod AA, Rezaie S, Rezaian M. Molecular characterization of Cryptosporidium isolates from humans and animals in Iran. App and Env Micro. 2007;73, 1033–1035.

28. Xiao L, Lal AA, Jiang J. Detection and differentiation 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, Haghhighi 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.
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