

Detection and Identification of *Cryptosporidium* species in Water Samples from a River in Ardabil City, Northwestern Iran

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Abstract: *Cryptosporidium* is an opportunistic parasite typically associated with large waterborne outbreaks. Surface waters contaminated with human and animal feces serve as main source for epidemic spread of *Cryptosporidium* parasites. In this study, we used a small-subunit rRNA-based PCR-Restriction Fragment Length Polymorphism (RFLP) technique to determine the prevalence and to characterize human-infective species of *Cryptosporidium* parasites in water samples collected from a stream in Ardabil city in Iran. Among 30 samples examined, 11 samples showed positive results. Restriction pattern analysis showed *C. andersoni* as the most common species with 7 cases; followed by *C. parvum*, bovine genotype, with 3 cases and *C. suis* with 1 case. The results indicated that PCR-RFLP technique provides an applicable and feasible method for detection and identification of *Cryptosporidium* oocysts in environmental water samples. The results, furthermore, demonstrated that wildlife is the major source of *Cryptosporidium* oocysts in surface water resources in the study region.

Key word: *Cryptosporidium* species, PCR-RFLP, water, detection, identification, Iran

INTRODUCTION

Cryptosporidium parasites are prevalent causes of long-lasting and life-threatening diarrheal diseases among immunocompromised patients (Leoni *et al.*, 2006; Meamar *et al.*, 2006; Cama *et al.*, 2006; Gatei *et al.*, 2003). *Cryptosporidium* sp. colonize human and animals (Hunter and Thompson, 2005; Thompson *et al.*, 2005) and cause mild diarrhea in healthy people especially in children (Hamedi *et al.*, 2005; Nath *et al.*, 1999). Five species of *Cryptosporidium* parasites including *C. parvum* human genotype or genotype II (previously known as *C. hominis*), *C. parvum* bovine genotype or genotype I, *C. parvum* dog genotype, *C. meleagridis*, *C. felis* and *C. suis* (pig genotype) have been found in human so far (Sunnotel *et al.*, 2006; Xiao *et al.*, 2002a). Molecular typing tools have indicated that two human and bovine genotypes of *C. parvum* are responsible for the most outbreaks (Caccio, 2005; Sulaiman *et al.*, 1998). Outbreaks of cryptosporidiosis occur as consequences of human and animal feces contaminated water consumption (Brandonisio, 2006; Fayer, 2004; Rush *et al.*, 1990; Thompson *et al.*, 2005). Resistance to chlorine

disinfectants (Korich *et al.*, 1990; King and Monis, 2006) and small infectious dose of *Cryptosporidium* parasites (Chappell *et al.*, 2006) has made them a potential hazard to water supplies; such that *Cryptosporidium* sp. can be important public health concern.

Recent molecular characterization of *Cryptosporidium* from wildlife indicated that most animals are infected with host adopted species or genotypes (Xiao *et al.*, 2002b). Thus identification of human-infective parasites is the mainstay in epidemiologic studies of *Cryptosporidium*.

Immunofluorescence assay was extensively used for the identification of *Cryptosporidium* in environmental and clinical samples (Lechevallier *et al.*, 1995; Stibbs and Ongerth, 1986). This method works based on detection of genus specific antigens on the surface of organism and only provides detection on genus level (Yu *et al.*, 2002). PCR technique together with other genetic tools, such as Restriction Fragment Length Polymorphism Technique (RFLP) has successfully been used in detection and differentiation of *Cryptosporidium* genotypes in water samples (Xiao *et al.*, 2000; Jiang *et al.*, 2005; Quintero-Betancourt *et al.*, 2003).

In the present study, we used SSUrRNA-based nested PCR-RFLP technique to characterize and determine distribution of human-infective species and genotypes of *Cryptosporidium* parasites in water samples collected from the Balikhli river in Ardabil city in Northwest part of Iran.

MATERIALS AND METHODS

Water samples and samples processing: Total thirty water samples were collected from the Balikhli river. This river originates from Sabalan mountains, passes about 100 km through farmlands and several villages, then crosses Ardabil city and joins to the Aras river in the borderline between Iran and republic of Azerbaijan and finally drains into Caspian Sea. Samples were collected during April 2006, when the river has the largest volume of water. In total 30 samples were collected, of which each contained volume of 30 L water. To collect the oocysts, samples were passed through an Envirochek filter (Pall Gelman Laboratory, Ann Arbor, Mich, USA). The trapped pellets on the backwash of the filters were collected by a scalpel in a 1.5 mL tub, containing 2.5% potassium dichromate (Coupe *et al.*, 2005). To maximize oocysts recovery, several pellets collected from same sample were pooled together in a 1.5 mL tub. The pellets were stored at 4°C for DNA extraction and further study.

DNA extraction: To remove the potassium dichromate and other possible PCR inhibitors, the pellets were extensively washed with 1 mL of PBS and centrifuged at 12000×g for 4 min to recollect them. This process was repeated for seven times. The pellets then were subjected to 8 freeze-thaw cycles and DNA extraction carried out by using CTAB method (Frederick *et al.*, 1999). The extracted DNA was dissolved in 30 mL of TBE buffer.

PCR-restriction fragment length polymorphism: The species of *Cryptosporidium* oocysts in water samples were identified by using a small-subunit rRNA-based nested PCR described previously (Xiao *et al.*, 2001, 2000). For primary PCR, oligonucleotid primers: 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTTCCTTCGAAACAGGA-3' with expected amplicon size of 1325 bp was used. The PCR amplification reaction mixtures contained 100 µM of each deoxynucleoside triphosphate, 0.5 µM of each forward and reverse primer, 3 mM MgCl₂, 2.5 U of *Taq* DNA polymerase, 2.5×PCR reaction buffer (Fermentase, Lithuania), 0.4 µg bovine serum albumin (Sigma, USA) and four different volumes of DNA template (1.5, 2, 2.5 and

3 µL) in total volume of 50 µL reaction mixtures. Cycling parameters were 4 min at 94°C hot start, (initial heat activation step), followed by 35 cycles of 45s at 94°C, 1 min at 52°C and 45s at 72°C, with a final extension of 7 min at 72°C. In addition DNA templates extracted from unwashed samples were also subjected to polymerase chain reaction. Both positive (*Cryptosporidium* DNA, a gift from Dr. Meamar, Tehran University of Medical Sciences) and negative controls (No template DNA) were included in each PCR to validate results. The false negative PCR results were ruled out by adding the *Cryptosporidium* DNA in samples produced negative results.

The secondary PCR was performed using oligonucleotid primers: 5'-GGAAGGTTGTATTTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3' according to previous works (Xiao *et al.*, 2000, 2001). Depending on the species and genotypes this primer set amplifies a range of 826-bp to 864-bp amplicon size (Xiao *et al.*, 2000, 2004). The reaction mixture contained 100 µM of each deoxynucleoside triphosphate, 0.5 µM of each forward and reverse primer, 2 mM MgCl₂, 2.5 U of *Taq* polymerase, 2.5×PCR reaction buffer and 2 µL DNA template (primary PCR product). The amplification condition was identical to the primary PCR except that the annealing temperature was 55°C.

To differentiate *Cryptosporidium* sp. and *C. parvum* genotypes, the RFLP analysis were performed by digesting secondary PCR product with *SspI* and *VspI* (Fermentase, Lithuania) restriction enzymes under condition recommended by the supplier. *C. andersony* and *C. muris* species were further differentiated by digestion with *DdeI* (Biolabs, New England) restriction enzyme under manufacture recommendation (Xiao *et al.*, 2000, 2001). Digested products were separated on a 2% agarose gel and visualized by etidium bromide staining and recorded by UV transillumination (Frederick *et al.*, 1999). The species were characterized according to previously published restriction patterns (Xiao *et al.*, 2004) and for confirmation of the species, the restriction pattern of secondary PCR product compared with patterns produced by digestion of known *Cryptosporidium* species DNA, *C. parvum* and *C. andersony* (a gift from Dr. Meamar, Tehran University of Medical Sciences).

RESULTS AND DISCUSSION

PCR amplification: SSUrRNA-based nested PCR method has been found to be more sensitive and specific in detection of *Cryptosporidium* in water (Xiao *et al.*, 2000; Jiang *et al.*, 2005; Quintero-Betancourt *et al.*, 2003) and

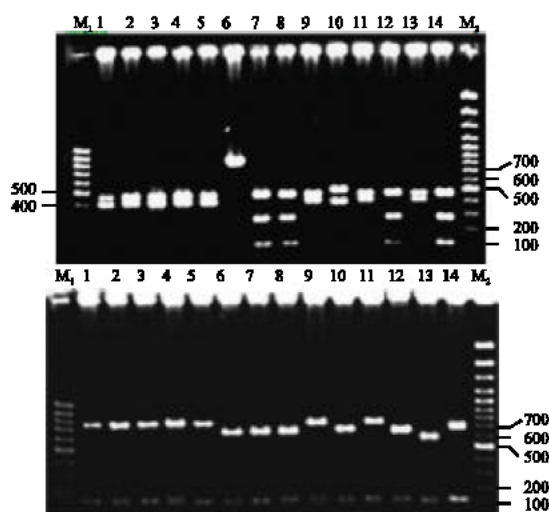


Fig. 1: Differentiation of *Cryptosporidium* species and genotypes in surface water samples with the small-subunit rRNA-based PCR-restriction fragment polymorphism technique. Secondary PCR product was digested by *SspI* and *VspI* restriction enzymes. Digestion of secondary PCR product with *SspI* (upper panel). Lanes 1 through 5, 9 and 11, *C. andersoni/muris* (385 bp and 448 bp); Lanes 7, 8 and 12, *C. parvum* (449 bp, 254 bp and 110 bp); lane 6, undigested secondary PCR products; Lanes 13 and 14, digestion products of known *C. andersoni/muris* and *C. parvum* species respectively and lanes M₁ and M₂ 50 bp and 100 bp DNA ladders, respectively. Digestion of secondary PCR product with *VspI* (lower panel). Lanes 1 through 5, 9 and 11, *C. andersoni/muris* (703 bp, 102 bp); lanes 6 through 8, *C. parvum* bovine genotype (628 bp, 104 bp), lane 10 *C. suis* (632 bp, 104 bp); lanes 12, 13 and 14, digestion products of known *C. andersoni/muris*, *C. parvum* human genotype and *C. parvum* bovine genotype respectively and lanes M₁ and M₂ 50 bp and 100 bp DNA ladders

human or animal fecal samples (Meamar *et al.*, 2006; Guyot *et al.*, 2001; Sulaiman *et al.*, 2005). In our study out of total 30 water samples examined, 11 (36.66%) samples produced positive PCR amplification by nested PCR. The positive results rates obtained in this study is lower than the other similar studies. For example Xiao *et al.* (2000) and Jiang *et al.* (2005) produced high rate of positive PCR results, 88.4, 93.1%, respectively. Beside the original geographical spread of *Cryptosporidium* oocysts, some technical difficulties may affect the PCR amplification rate

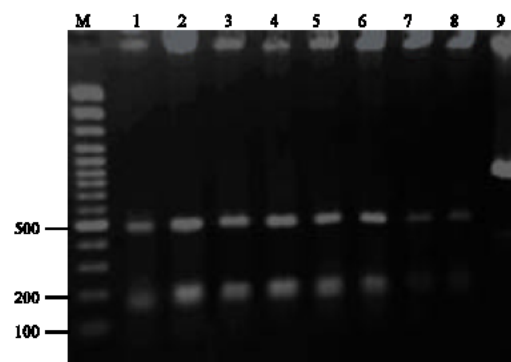


Fig. 2: Differentiation of *C. andersoni* and *C. muris* with digestion of secondary PCR product with *DdeI*. Lanes 2 through 8, *C. andersoni* (470 bp, 186 bp, 156 bp); lane 1, known *C. andersoni* species; lane 9 undigested secondary PCR product and lane M 100 bp DNA ladder

of the samples. One major obstacle is the presence of PCR inhibitors in water. In recent studies PCR inhibitors were removed by Immunomagnetic Separation (IMS) method (Ochiai *et al.*, 2005; Yakup and Stadterman, 2004). In present study we removed the inhibitors by repeated washing of collected oocysts with PBS buffer. Before extensive washing process PCR did not amplify any product in samples but washed samples successfully produced positive PCR results. However, repeated washing might lead to possible loss of oocysts and decrease the final DNA concentration. This method might reduce the sensitivity of the PCR, so the results may not be realistic and at low oocysts density in samples it might give false negative results. As Xiao *et al.* (2006) showed amplification rate of the samples is affected by the volume of DNA used in PCR.

Restriction pattern analysis: Digestion of secondary PCR products with *VspI* and *SspI* showed the presence of *C. andersoni/muris*, *C. parvum* bovine genotype and *C. suis* (Fig. 1). Digestions with the mentioned enzymes produce the identical patterns for the *C. andersoni* and *C. muris* species (Fig. 1). They were differentiated by digesting secondary PCR products with *DdeI* (Xiao *et al.*, 2000, 2001). Digestion of *C. andersoni* yields four bands at 20, 156, 186 and 470 bp and *C. muris* yields five bands at 20, 156, 186, 224 and 247 bp. Digestion patterns of *C. andersoni* and *C. muris* give three and four visible bands on agarose gel, respectively (Xiao *et al.*, 2004). The results showed all strains belong to *C. andersoni* no *C. muris* species was detected (Fig. 2). *C. andersoni* was prominent genotype with 7 (23 %) cases followed by *C. parvum* bovine genotype with 3 (10 %).

cases and *C. suis* (pig genotype) with 1(3/33 %) case. The results were not out of the mind because before entering into the city, the river crosses the farmlands and several villages that the main occupation of the people in those regions is agriculture and animal husbandry. These species commonly infect farm and wild animals. It is well established that human and animals are two major source of *Cryptosporidium* oocyst contamination of surface waters (Brandonisio, 2006; Thompson *et al.*, 2005; Fayer, 2004). *C. andersoni* and *C. parvum* bovine genotype were the most common species characterized respectively. The higher prevalence of these species is consistent with the geographic spread of their hosts. The cattle and bovine are domestic animals grass around the river that the water samples were collected. The source of isolated *C. suis* (pig genotype) could be from wildlife because there is no pig farm in the region.

Public health importance of isolated species: From public health importance view, among the identified *Cryptosporidium* parasites, *C. parvum* bovine genotype is potentially human-infective and may contribute as a public health concern (Chappell *et al.*, 2006; Sterling, 2000; Xiao *et al.*, 2002). Human specific species were not found in this study. It is in contrast with our previous study that revealed high prevalence of human-infective *Cryptosporidium* oocysts in stool samples from children with diarrhea (M. Arzanlou, unpublished data). The absence of human specific genotypes of *Cryptosporidium* parasites in this study may be attributed to the swage discharge system of the city. The city doesn't have sewerage system and the swages are drained by cesspools so the effluences of waste water into the river and presence of human specific genotypes are less probable.

CONCLUSION

In conclusion, results of this study indicated the importance of zoonotic cycle of *Cryptosporidium* parasites in contamination of surface water resources in the study region. As this study is the first report of *Cryptosporidium* parasites in water samples from Iran, further studies are needed to characterize the full genetic nature of parasites from human, animal and other water and environmental samples to identify the source of contamination and human-infective potential of species. The results of this study might help public health care systems in management of cryptosporidiosis.

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