

***Cryptosporidium* genotypes and subtypes distribution in river water in Iran**

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ABSTRACT

Little is known about the diversity and public health significance of *Cryptosporidium* species in river waters in Iran. In the present study, we determined the genotype and subtype distribution of *Cryptosporidium* spp. in river water samples in Iran. A total of 49 surface water samples were collected from rivers and surface water in Guilan and Tehran provinces during 2009–2010. Water samples were filtrated through a 1.2- μ m pore size membrane filter or by Filta-Max filter followed by immunomagnetic separation or sucrose purification methods. Genotype and subtype of *Cryptosporidium* were identified by sequence analysis of the 18S rRNA and 60 kDa glycoprotein (gp60) genes, respectively. A total of 24 (48.97%) water samples were positive for *Cryptosporidium* species by the 18sRNA-based polymerase chain reaction (PCR)-sequencing technique. DNA sequencing revealed the presence of five species of *Cryptosporidium* (*C. parvum*, *C. hominis*, *C. muris*, *C. andersoni*, and *C. canis*) in the water samples of the study area and, to our knowledge, the first report of *C. muris* in Iran. The results of GP60 gene analysis showed that all *C. parvum* and *C. hominis* isolates belonged to the IId and Id subtype families, respectively. The investigated river water supplies were heavily contaminated by pathogenic species of *Cryptosporidium* from humans and livestock. There is potential risk of waterborne cryptosporidiosis in humans and animals.

Key words | *Cryptosporidium* spp., environmental samples, Iran, subtype

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INTRODUCTION

Cryptosporidium species are a major public health concern in both developing and industrialized countries and a major cause for morbidity and mortality in immunocompromised persons (Alves et al. 2006; Feng et al. 2009; Kotloff et al. 2013). Waterborne cryptosporidiosis has been reported worldwide and the role of water in disease transmission is now well recognized. The ingestion of oocyst-contaminated drinking water has led to a large number of outbreaks, mainly reported from North America, the United Kingdom, Japan, and Australia (Zhou et al. 2003; Karanis et al. 2007; Baldursson & Karanis 2011).

The use of molecular techniques in epidemiological studies has shown that five *Cryptosporidium* species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, and *C. canis*) are responsible for most human infections: two species, *C. hominis* and *C. parvum*, are the most common (Plutzer & Karanis 2009).

Some researchers have recently used highly discriminatory molecular techniques to characterize the transmission dynamics of *C. parvum* and *C. hominis* (Xiao et al. 2004; Sulaiman et al. 2005; Plutzer et al. 2008). One such tool is the sequence analysis of the 60 kDa glycoprotein (gp60) gene, which has high sensitivity and specificity and has been proven to be effective in studying the transmission of human cryptosporidiosis. Several subtype groups have been identified in these species: at least seven subtype groups in *C. hominis* (Ia–Ig), two zoonotic (IIa, IIc), and ten non-zoonotic subtype groups (IIb, IIc, IIe–III) in *C. parvum*, and six subtype groups in *C. meleagridis*. Within each subtype group, there are several subtypes primarily based on the number of

trinucleotide repeats coding for the amino acid serine (Alves et al. 2003; Sulaiman et al. 2005; Plutzer & Karanis 2009).

The distribution of *Cryptosporidium* species and subtypes in environmental water in Iran is unclear. The objective of this study was to identify the genotype and subtype distributions of *Cryptosporidium* spp. in river water in Iran.

METHODS

Geography and sample collection

Guilan province lies along the Caspian Sea and has a moderate, mild, and Mediterranean-like climate. Large parts of the province are mountainous, green, and forested (Table 1(a)). The Sefidrood is a river approximately 670 km long, rising in Guilan and flowing generally north-east to meet the Caspian Sea. The river is Iran's second longest river. Many pollutants such as rural, urban, and animal raw sewage threaten water supplies in Guilan.

Tehran province is located to the north of the central plateau of Iran. The province of Tehran is Iran's most densely populated region (Table 1(b)). The largest rivers of this province are the Karaj River and the Jajrood River. Bilaghan pond was constructed on the Karaj River. These rivers provide tap water for Tehran alongside agricultural development in Karaj.

All the rivers investigated in the present study have different uses, including agriculture, industry, residential, and recreational activities. These rivers are popular weekend summer resorts. All the rivers mentioned above are polluted by human and animal discharges. Forty-nine

Table 1(a) | Geographic information about Guilan province

Total area	14,042 km ² (5,422 sq mi)
Population (2006)	
Total	2,404,861
Density	170/km ² (440/sq mi)
Rainfall	Generally around 1,400 mm
Humidity	Very high because of the marshy character of the coastal plains and can reach 90% in summer

Table 1(b) | Geographic information about Tehran province

Area	
Urban	730 km ² (280 sq mi)
Metro	1,274 km ² (492 sq mi)
Population (2012)	
Density	10,327.6/km ² (26,748.3/sq mi)
Urban	8,244,535
Metro	13,828,365
Precipitation	429 mm

water samples were collected during 2009–2010 from surface water in Guilan province (northern Iran) (29 samples) and Tehran province (20 samples), namely, the Kiashahr (five samples), Astaneh (four samples), Siahkal (four samples), Otaghvar (four samples), Lahijan lagoon (five samples), Amlash (three samples), and Polrooddam (four samples) in Guilan province (Figure 1) and River Karaj (five samples), Jajrood (eight samples), and Bilaghan (seven samples) in Tehran province (Figure 1).

Concentration of oocysts from water samples

Environmental water samples collected from river and surface water in Guilan were filtered through a 142-mm diameter cellulose acetate membrane filter with a pore size of 1.2 μm (Sartorius Co.) by means of a vacuum device. For recovery of particles, the filter was rinsed with 50 mL of 0.1% phosphate-buffered saline (PBS)-Tween 80. This process was repeated twice and particulates were concentrated by centrifugation at 3,000 g for 10 min, then the pellet was subjected to sucrose flotation. Also, Tehran water samples were filtered by Filta-Max filter. Briefly, 50-L untreated water samples were collected and filtered by Filta-Max *xpress* filters (IDEXX Corp., Westbrook, ME), with a flow

rate of 1 L/min. Filters were washed by automated filter elution system by adding 600 mL of PBS containing 0.01% Tween 20 (PBST) and following the manufacturer's instructions. The concentrated material was washed in 50 mL PBST by centrifugation at 1,500 g for 5 min. The supernatant was removed, and the pellet was subjected to sucrose flotation according to our previous study (Mahmoudi *et al.* 2013) or immunomagnetic separation (IMS) methods (Castro-Hermida *et al.* 2010; Mahmoudi *et al.* 2011).

Immunomagnetic separation

The IMS procedure was performed according to the manufacturer's instructions (Dynabeads G/C combo IMS kit; Dynal A.S., Oslo, Norway), with a few exceptions. Briefly, 10 mL of concentrate from a test sample was transferred into a screw-cap Leighton tube, and 1 mL of 10 \times SL buffer A, 1 mL of 10 \times SL buffer B, and 100 μL of the anti-*Cryptosporidium* bead conjugate were added. Oocysts were isolated by applying the magnetic particle concentrator A. The oocyst/cyst-bead complexes were washed in 1 mL of 1 \times SL buffer A, then transferred into an Eppendorf tube, and separated using a magnetic particle concentrator M and were used in DNA extraction.



Figure 1 | Map of Iran and rivers in Guilan province and Tehran province.

DNA extraction

DNA was extracted with the QIAamp DNA mini kit from the IMS-purified oocysts as recommended by Jiang et al. (2005). 180 µL of the animal tissue lysis (ATL) buffer from the QIAamp DNA minikit was added to the suspension, which was then subjected to 10 freeze–thaw cycles (1 min in liquid nitrogen and 1 min at 96 °C per cycle). The remaining procedures in DNA extraction followed recommendations by the manufacturer of the kit (Qiagen GmbH, Hilden, Germany).

Genotype and subtype analysis

Nested-polymerase chain reaction (PCR) assay amplified an 850 bp fragment of 18 rRNA gene specific for *Cryptosporidium* spp. (Mahmoudi et al. 2013). For subtyping of *C. parvum* and *C. hominis*, a fragment of about 400 bp of the gp60 gene was amplified by nested PCR as described previously (Abe et al. 2006). PCR products were purified using Centri-Sep spin columns (Princeton Separations, New Jersey, USA) and sequenced in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, NY, USA). All sequences were edited manually and analyzed with reference sequences using the GenRunner software (v. 3.05). Subtypes were named based on the number of trinucleotide repeats (TCA or TCG) coding for the amino acid serine (Sulaiman et al. 2005).

RESULTS

PCR-sequencing was performed to detect and identify *Cryptosporidium* oocysts at species and subtype levels. The distribution of genotypes, subtype families, and subtypes of *Cryptosporidium* species in river water samples is shown in Table 2. Of the 49 river water samples examined in this study, 24 (48.97%) were positive for *Cryptosporidium* species by the 18S rRNA-based PCR technique. The sequences obtained were aligned with those available in GenBank.

DNA sequencing of PCR products revealed the presence of the following five species of *Cryptosporidium* (*C. parvum*, *C. hominis*, *C. muris*, *C. andersoni*, and *C. canis*) in the samples. *Cryptosporidium parvum* was the most common

species (being detected in 11 samples, or 45.8% of all PCR-positive samples), followed by *C. hominis* (seven samples, or 29.2% of all PCR-positive samples), *C. muris* (three samples, or 12.5% of all PCR-positive samples), *C. andersoni* (two samples, or 8.3% of all PCR-positive samples), and *C. canis* (one sample, or 4.2% of all PCR-positive samples). The five sequences from the five isolates have been deposited in the GenBank with these accession numbers [KJ162056 (*C. muris*), KJ162057 (*C. muris*), KJ162058 (*C. parvum*), KJ162059 (*C. andersoni*), and KJ162060 (*C. andersoni*)].

The result of GP60 sequence analysis showed that the *C. parvum* and *C. hominis* detected belonged to the IId and Id subtype families, respectively. Three subtypes were detected within the subtype family IId, including IIdA15G1 (8/11), IIdA20G1a (2/11), and IIdA18G1 (1/11). In contrast, only one subtype (IdA20) was detected within the subtype family Id.

DISCUSSION

In this study, *Cryptosporidium* spp. were identified by PCR in 24 (49%) water samples. *C. parvum* was the most frequently species found (11/24; 45.8%), followed by *C. hominis* (7/24; 29.2%), *C. muris* (3/24; 16.6%), *C. andersoni* (2/24; 8.3%), and *C. canis* (1/24, 4.2%). A mixture of *Cryptosporidium* species was not found in any samples during this study. Previously, few studies have characterized the distribution of *Cryptosporidium* species in water samples in Iran. Manouchehri Naeini et al. (2011) analyzed 30 water samples by small-subunit (SSU) rRNA-based polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and showed that *C. parvum* was the most prevalent genotype, followed by *C. hominis* and *C. canis*, respectively. In a comprehensive study by Keshavarz et al. (2009), Iranian cattle were mainly infected with *C. parvum* (72.6%), *C. andersoni* (17.7%), and *C. bovis* (7.8%). In contrast, *C. parvum* was the predominant species in children with diarrhea and AIDS patients in other studies (Meamar et al. 2006; Keshavarz et al. 2008).

Some studies have also shown a frequent occurrence of *C. hominis* in children or AIDS patients (Pirestani et al. 2008; Zavvar et al. 2008). Thus, both cattle and humans

Table 2 | The distribution of genotypes, subtype families, and subtypes of *Cryptosporidium* species in river water samples

Number	Code of sample	Place		Concentration method	Water volume in L	NTU	Species	Subtype family	Subtype
		Province	River						
1	124	Guilan	Kiashahr	SF	13	9	<i>C. parvum</i>	IId	IIdA18G1
2	125	Guilan	Kiashahr	SF	8	5.5	<i>C. muris</i>	–	–
3	126	Guilan	Kiashahr	SF	12.5	4	<i>C. parvum</i>	IId	IIdA15G1
4	127	Guilan	Astaneh	IMS	18	8	<i>C. parvum</i>	IId	IIdA20G1a
5	128	Guilan	Astaneh	IMS	18	8	<i>C. hominis</i>	Id	IdA20
6	129	Guilan	Astaneh	SF	24	4	<i>C. hominis</i>	Id	IdA20
7	132	Guilan	Lahijan lagoon	IMS	21	2	<i>C. hominis</i>	Id	IdA20
8	133	Guilan	Lahijan lagoon	IMS	12	1	<i>C. andersoni</i>	IId	–
9	134	Guilan	Lahijan lagoon	SF	7	1.6	<i>C. parvum</i>	IId	IIdA15G1
10	140	Guilan	Siahkal	IMS	16	2	<i>C. hominis</i>	Id	IdA20
11	143	Guilan	Siahkal	SF	10	5	<i>C. parvum</i>	IId	IIdA20G1a
12	150	Guilan	Otaghvar (Langrood)	SF	3	3	<i>C. muris</i>	–	–
13	151	Guilan	Polrood Dam (Roodsar)	SF	25	2	<i>C. parvum</i>	IId	IIdA15G1
14	152	Guilan	Polrood Dam (Roodsar)	SF	30	4	<i>C. hominis</i>	Id	IdA20
15	106	Guilan	Siahkal	SF	4	15.6	<i>C. canis</i>	–	–
16	117	Guilan	Siahkal	SF	8	20	<i>C. andersoni</i>	–	–
17	102	Tehran	Karaj	IMS	2	10.7	<i>C. hominis</i>	Id	IdA20
18	103	Tehran	Jajrood	IMS	6	75.6	<i>C. parvum</i>	IId	IIdA15G1
19	105	Tehran	Jajrood	IMS	2.5	20.3	<i>C. parvum</i>	IId	IIdA15G1
20	114	Tehran	Jajrood	SF	6	122	<i>C. hominis</i>	Id	IdA20
21	123	Tehran	Bilaghan	IMS	50	8.6	<i>C. parvum</i>	IId	IIdA15G1
22	137	Tehran	Bilaghan	IMS	50	1.7	<i>C. parvum</i>	IId	IIdA15G1
23	153	Tehran	Bilaghan	IMS	50	2	<i>C. parvum</i>	IId	IIdA15G1
24	115	Tehran	Jajrood	SF	3.5	12.9	<i>C. muris</i>	–	–

SF: sucrose flotation; IMS: immunomagnetic separation; NTU: nephelometric turbidity units.

appear to be the major sources of *Cryptosporidium* spp. in river waters in Iran. Thus far, no reports have described the occurrence of *C. muris* in any human or animal samples in Iran. However, *C. muris* is a well-known rodent parasite (Chalmers et al. 1997), indicating that these animals are also a source of *Cryptosporidium* oocysts in river water in Iran.

PCR and sequencing analysis of the GP60 gene was successful for 22 water samples containing *C. parvum* (11 samples) and *C. hominis* (seven samples). IdA20 was the only subtype of all *C. hominis* identified in water samples.

This subtype was previously identified in one Iranian patient infected with *C. hominis* (Nazemalhosseini-Mojarad et al. 2011a). Within *C. parvum*, we found three subtypes belonging to one subtype family (IId). More than half of the *C. parvum* samples (72.2%) had IIdA15G1 (8/11), two (18.2%) had IIdA20G1a (2/11), and one had IIdA18G1. All three subtypes in water samples have also been identified in humans in Iran (Nazemalhosseini-Mojarad et al. 2011a, b; Taghipour et al. 2011). Subtype IIdA15G1 was also identified in two calves (Nazemalhosseini-Mojarad et al. 2011a; Nazemalhosseini-Mojarad et al. 2012). These

results underline the importance of possible waterborne and zoonotic transmission in cryptosporidiosis epidemiology in Iran.

CONCLUSIONS

There is no report of waterborne cryptosporidiosis outbreaks in Iran. However, the identification of *C. parvum* and *C. hominis* subtypes that can infect humans suggests that there is potential for waterborne transmission of cryptosporidiosis in Iran. Therefore, measures should be developed to reduce the transport of (oo)cysts to water supplies and their transmission to humans, especially in the rivers of Tehran province that provide tap water for Tehran city.

ACKNOWLEDGMENTS

We would like to express our appreciation to Mrs Jahantab, Mrs Raesiyani, Mrs Parvar, and Isaia Sotiriadou for their kind cooperation and assistance. This work was supported and funded by the Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences and Tehran Province Water & Wastewater Co. (TPWW), and by the host University of the author P. Karanis in Germany, Cologne.

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First received 5 December 2013; accepted in revised form 31 August 2014. Available online 1 November 2014