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Original Article

Microscopic and Molecular Detection of *Cryptosporidium andersoni* and *Cryptosporidium xiaoi* in Wastewater Samples of Tehran Province, Iran

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Abstract

Background: As a waterborne pathogen, *Cryptosporidium* is one of the most common causes of gastroenteritis in human and hoofed livestock animals. This study aimed to investigate the distribution of *Cryptosporidium* spp. in human and livestock wastewaters in Iran, by the 18S rRNA sequence analysis.

Methods: A total of 54 raw wastewater samples collected from three urban treatment plants and two slaughterhouses during 2014-2015 in Tehran, Iran. The presence of the *Cryptosporidium* oocysts was assessed by immunofluorescence with monoclonal antibodies. To characterize the oocysts at the molecular level, the 18S rRNA gene of *Cryptosporidium* was PCR amplified and sequenced.

Results: Of the 54 wastewater samples examined, 34 (62.9%) were positive for *Cryptosporidium* oocysts using the IFA. Of these, 70.5% (24/34) were positive by PCR, that 91.6% (22/24) were successfully sequenced. The species of *C. andersoni* (95.4%) and *C. xiaoi* (4.6%) were detected in livestock wastewater samples.

Conclusion: *C. andersoni* was the major *Cryptosporidium* sp. found in the aquatic environmental wastewater samples. The high rate of detection of *C. andersoni* in domestic wastewater was probably the result of the predominancy of this species in cattle herds in Iran. The current study is the first report of *C. xiaoi* in Iran.

Introduction

Cryptosporidium spp. are common causes of gastroenteritis in human and a wide range of mammalian hosts (1). Oocyst shedding from livestock animals has been a contamination source for human cryptosporidiosis outbreaks (2).

Cryptosporidium spp., is a complex of morphologically similar but genetically different coccidian parasites that conventional methods are unable to detect and characterize the human-infecting species(3).

Since the genetic loci of *Cryptosporidium* differ in substitution rates, the resolution for parasite typing is different among loci. The most variable locus of 18S rRNA gene is traditionally used for genotype differentiation of *Cryptosporidium* species (4).

Cryptosporidium includes over 26 species (5), with *C. hominis*, which has anthroponotic transmission and *C. parvum*, which is zoonotic. Other species including *C. felis*, *C. meleagridis*, *C. canis*, *C. andersoni* and *C. suis* have been occasionally implicated in human illness (6). *C. bovis*, *C. ryanae, C. parvum*, *C. andersoni* and *C. xiaoi* are described from cattle and sheep with an age-related distribution (5, 7).

Infection with *C. andersoni* is often accosiated to reduced milk and weight gaining in dairy cattle and post weaned calves, respectively (8).

In Iran, most studies on *Cryptosporidium* have been limited to estimating the prevalence of species and genotypes in human and livestock faecal samples (9, 10), and few studies have been published regarding the detection of *Cryptosporidium* species and subtypes in the aquatic environmental samples (11, 12).

Molecular studies, on aquatic environmental samples, could contribute to a better insight on the origin of faecal contamination of surface waters and the possible zoonotic transmission of *Cryptosporidium*, thus, it is important to characterize the species of this parasite in wastewater.

Our study aimed at determining the species of *Cryptosporidium* in wastewater contaminated specifically by human and livestock faeces to elucidate the molecular epidemiology of these parasites in the environment.

Materials and Methods

Wastewater samples

Fifty four raw wastewater samples were collected from three urban wastewater treatment plants (WWTPs) and two slaughterhouses (SWWTPs) in Tehran, Iran. Two municipal plants were located in the west of the capital (WWTP1, Shahrak-e Ekbātān; WWTP2, Shahrak-e Gharb), and the third municipal plant (Tehran southern wastewater treatment plant: WWTP3) was located at the south of Shahr-e Ray, out of the development limit of Tehran City in the next 25 years. Two slaughterhouse wastewater treatment plants were located in one suburb area of Tehran: Meisam-robat-dam (SWWTP4) and Dam-pak (SWWTP5). Samples (≤5 l each) of untreated wastewater were collected once every month from December 2013 to November 2014.

Sample processing

Raw wastewater samples were sieved through a polyester mesh of 50 (297 µm pore size), centrifuged (3000 × *g*, 15 min, 4°C) in a 4×500 ml-capacity-swinging-bucket rotor of a refrigerated centrifuge (Beckman, GS-6R Centrifuge), and the residues were clarified by centrifugal (water-ether) concentration procedure, as previously described (13-16). The final pellet was resuspended in 2 ml PBS.

Detection of oocysts with direct immunofluorescence assay

An aliquot of 50 µL of pellet was diluted (1:10–1:50) and placed onto a microscope slide with 8 mm diameter wells, air dried, fixed in acetone, and overlaid with 25 µL of fluorescein isothiocyanate (FITC)-conjugated anti *Cryptosporidium* oocysts monoclonal antibodies (Cellabs Diagnostics, Brookvale, Australia). The slides were incubated at 37 C in a humid chamber for 30 min. Any excess un-

bound FITC-antibody was removed by adding 50 mL of PBS to each well (left to stand for 5 min), and then excess PBS was aspirated. A drop (20 µL) of mounting medium (PBS:glycerol, 1:1 v/v) was added to each well, a coverslip was positioned on the top of each drop that was then scanned using micro scope fluorescence (Zeiss, Germany) at ×400 magnification. *Cryptosporidium* oocysts were identified by morphometric criteria including size, shape, and intensity of immunofluorescent assay staining.

DNA extraction and PCR amplification

DNA was extracted from each processed sample using an *AccuPrep*® stool DNA extraction kit (Bioneer, Daejeon, South Korea) according to the manufacturer's instructions. Nested PCR was also used to identify *Cryptosporidium* genus by amplification of the 18S rRNA gene (17).

Sequence analyses

All secondary PCR amplicons were purified using the *AccuPrep*® PCR purification kit (Bioneer, Daejeon, South Korea) and sequenced in both directions on an atumated DNA analyzer (ABI 3730 XL, Bioneer, South Korea). Sequences were edited manually in BioEdit software (http://www.mbio.ncsu.edu/BioEdit/page2.html), and aligned with reference sequences of *Cryptosporidium* from the GenBank database using the BLASTN software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for genotype identification. Creating multiple-sequence alignment and construction of a phylogenetic tree, were determined using Clustal W program and Neighbor-Joining (NJ) method under the nucleotide substitution model of Kimura 2-parameter in the MEGA V 6.0 software (18). The reliability of the NJ tree was assessed by the bootstrap method with 1,000 replications.

Results

Of the 54 raw wastewater samples examined, 34 samples (62.9%) were positive for *Cryptosporidium* oocysts using the IFA (Fig. 1). Of these, 70.5% (24/34) were positive by PCR, that 91.6% (22/24) were successfully sequenced (Fig. 2).

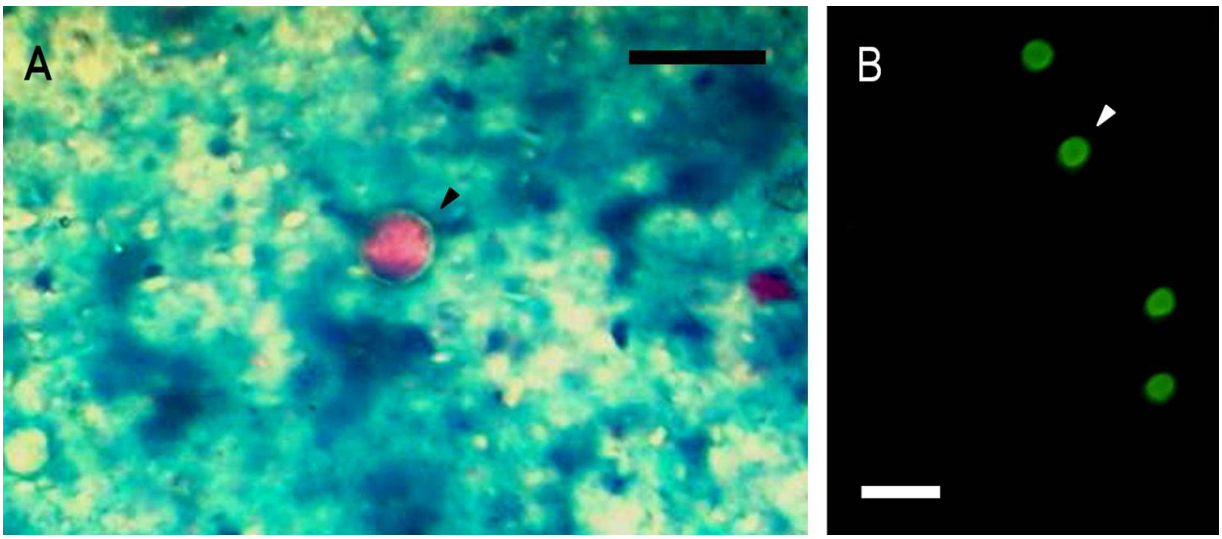

Fig. 1: *Cryptosporidium* oocysts detected in wastewater samples of the current study. Acid-fast staining (Panel A); IFA procedure, stained with mAb-conjugated FITC (Panel B) (bars showing 20 µm) (⊳ oocyst-like particles)

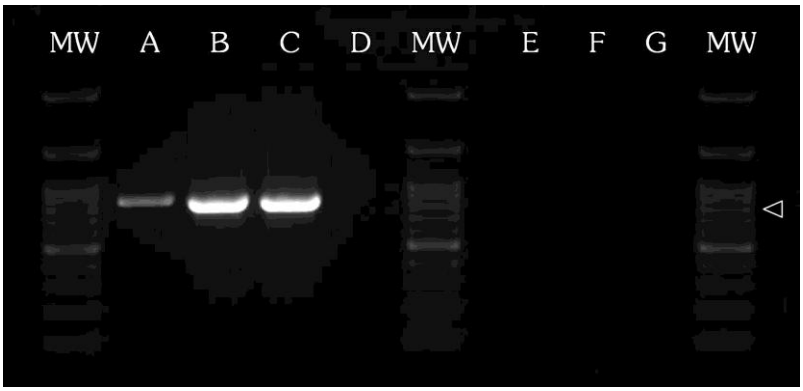

Fig. 2: PCR products on an ethidium bromide-stained 1% agarose gel. Column A, positive control (*C. parvum*); Columns B and C, *Cryptosporidium* spp. in domestic wastewater samples; Columns D-G, urban wastewater samples; ⊳ ~830 bp fragments; MW, 100 bp DNA ladder

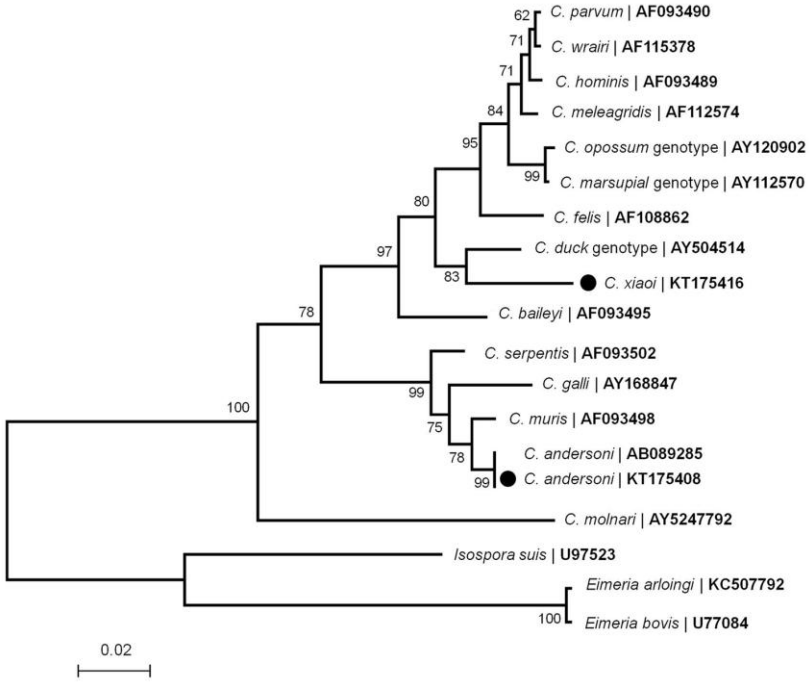

Fig. 3: Phylogenetic tree based on 18S sequences, constructed according to the NJ method, showing the position of *Cryptosporidium* species. *Isospora suis* and *Eimeria* spp. are used as outgroup. The percentage of replicate tree in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches

No amplification was observed for the 30 samples coming from the urban treatment plants, and PCR-positive samples were related to the slaughterhouse wastewaters. BLAST search of our 18S rRNA sequences (569-819 bp) against those previously published for other *Cryptosporidium* spp. revealed the highest similarity (99-100% homology) with those of *C. andersoni* and *C. xiaoi* (Fig. 3). The most prevalent species was *C. andersoni*, which was detected in 21 samples (95.5%). *C. xiaoi* was detected in one sample (4.5%). The nucleotide sequences described in this work have been deposited in the GenBank database under accession nos. KT175408 to KT175429.

Table 1: *Cryptosporidium* spp. in wastewater samples

Sample code	Sampling site	IFA assay	18S rRNA-SEQ	Accession nos
[1] UE1	WWTP1	+	No DNA amplification	-
[2] UE2	WWTP1	Not detected	-	-
[3] UE3	WWTP1	Not detected	-	-
[4] UE4	WWTP1	Not detected	-	-
[5] UE5	WWTP1	+	No DNA amplification	-
[6] UE6	WWTP1	Not detected	-	-
[7] UE7	WWTP1	Not detected	-	-
[8] UE8	WWTP1	+	No DNA amplification	-
[9] UE9	WWTP1	Not detected	-	-
[10] UE10	WWTP1	+	No DNA amplification	-
[11] UE11	WWTP1	Not detected	-	-
[12] UE12	WWTP1	Not detected	-	-
[13] US1	WWTP2	+	No DNA amplification	-
[14] US2	WWTP2	+	No DNA amplification	-
[15] US3	WWTP2	Not detected	-	-
[16] US4	WWTP2	Not detected	-	-
[17] US5	WWTP2	Not detected	-	-
[18] US6	WWTP2	Not detected	-	-
[19] US7	WWTP2	Not detected	-	-
[20] US8	WWTP2	Not detected	-	-
[21] US9	WWTP2	Not detected	-	-
[22] US10	WWTP2	+	No DNA amplification	-
[23] US11	WWTP2	Not detected	-	-
[24] US12	WWTP2	Not detected	-	-
[25] UG7	WWTP3	+	No DNA amplification	-
[26] UG8	WWTP3	+	No DNA amplification	-
[27] UG9	WWTP3	Not detected	-	-
[28] UG10	WWTP3	Not detected	-	-
[29] UG11	WWTP3	Not detected	-	-
[30] UG12	WWTP3	+	No DNA amplification	-
[31] SM1	SWWTP4	+	*C. andersoni*	KT175408
[32] SM2	SWWTP4	+	*C. andersoni*	KT175409
[33] SM3	SWWTP4	+	*C. andersoni*	KT175410
[34] SM4	SWWTP4	+	*C. andersoni*	KT175411
[35] SM5	SWWTP4	+	*C. andersoni*	KT175412
[36] SM6	SWWTP4	+	*C. andersoni*	KT176413
[37] SM7	SWWTP4	+	*C. andersoni*	KT175414
[38] SM8	SWWTP4	+	*C. andersoni*	KT175415
[39] SM9	SWWTP4	+	*C. andersoni*	KT175416
[40] SM10	SWWTP4	+	*C. andersoni*	KT175417
[41] SM11	SWWTP4	+	*C. andersoni*	KT175418
[42] SM12	SWWTP4	+	Defaulted sequencing	-
[43] SR1	SWWTP5	+	*C. andersoni*	KT175419
[44] SR2	SWWTP5	+	*C. andersoni*	KT175420
[45] SR3	SWWTP5	+	*C. andersoni*	KT175421
[46] SR4	SWWTP5	+	*C. xiaoi*	KT175422
[47] SR5	SWWTP5	+	*C. andersoni*	KT175423
[48] SR6	SWWTP5	+	*C. andersoni*	KT175424
[49] SR7	SWWTP5	+	*C. andersoni*	KT175425
[50] SR8	SWWTP5	+	*C. andersoni*	KT175426
[51] SR9	SWWTP5	+	*C. andersoni*	KT175427
[52] SR10	SWWTP5	+	Defaulted sequencing	-
[53] SR11	SWWTP5	+	*C. andersoni*	KT175428
[54] SR12	SWWTP5	+	*C. andersoni*	KT175429

WWTPs, urban wastewater treatment plants; SWWTPs, slaughterhouse wastewater treatment plants

Discussion

Cryptosporidium oocysts in aquatic environmental samples are generally identified by IFA after concentration using methods such as flotation or immunomagnetic separation (IMS) methods.. However, microscopic evaluations have been only applied for detection of infecting-oocysts of *Cryptosporidium* spp., while are not able to identify infectivity of the waterborne oocysts of the parasite (19). Thus, molecular methods have proven to be useful for the identification and classification of *Cryptosporidium* oocysts in order to overcome the limitations of these traditional procedures. In this study, nested PCR assay targeting the 18S rRNA was carried out for *Cryptosporidium* to determine the genus in the wastewater samples.

In the present study, the positive rate of PCR was lower than that of IFA. Similar issues where the positive rate of PCR is lower than that of IFA have also been reported in the previous studies (19, 20). The efficiency of amplification technique could be reduced by the presence of inhibitory substances in wastewater samples, such as humic and fulvic acids, which are coexisted with DNA and inhibit PCR amplification (19).

The high rate of detection of *C. andersoni* in domestic wastewater samples is in line with the previous theory that mature cattle are more likely to be infected with *C. andersoni* (22).

C. andersoni is a gastric *Cryptosporidium* parasite of juvenile and adult cattle. Other *Cryptosporidium* species reported to infect the farm animals, such as *C. parvum* and *C. bovis*, were not found here. This was expected, because *C. parvum* is most common in pre-weaned calves until two months of age (7) with diarrhea that are not usually slaughtered in abattoir. In Japan Koyama et al. (22) described the distribution of *Cryptosporidium* in 325 faecal samples from pre-slaughtered adult cattle in a slaughterhouse, stating that the five adult cattle were found to be positive for *C. andersoni* Kawatabi strain, and *C. parvum* was not found.

In Milwaukee, *C. andersoni* was major *Cryptosporidium* sp. found in urban wastewater, probably the result of animal slaughterhouses and/or of animal feces using to fertilize parks (21).

Ayed and colleagues reported the presence of *C. parvum, C. muris, C. andersoni, C. hominis, C. ubiquitum*, *C. meleagridis* and avian genotype II in raw and treated wastewater samples from 18 urban treatment plants. *C. andersoni* was the most prevalent species (23).

In another study, Fallah and colleagues genotyped 11 slaughterhouse sewage samples in Iran (Tabriz) by PCR-RFLP analysis of the 18S rRNA gene, and classified majority of the samples (64%) as *C. andersoni* (24).

Pirestani and colleagues (9) described the distribution of *C. parvum* genotypes in 59 human and bovine clinical faecal samples in Shahriyar, Iran, stating that the genotype 1 or *C. hominis* (in human samples) and genotype 2 or *C. parvum* (in human and bovine samples) were identified.

C. parvum and *C. hominis* are the most prevalent species causing disease in humans. In the present study, the microscopic examination resulted in positive results for 33.3% of samples coming from the urban treatment plants. No DNA amplification observed in these samples, probably the result of low numbers of oocysts and consequently the low amount of DNA.

This work is the first report of *C. xiaoi* in Iran. Oocysts of *C. xiaoi*, had previously been known as the *Cryptosporidium bovis*-like genotype or as *C. bovis* originated from sheep in Spain, Tunisia, United Kingdom, and the United States are recorded as such in GenBank (EU408314-EU408317, EU327318-EU327320, EF362478, EF514234, DQ991389, and EF158461) (25).

The detection of *Cryptosporidium* species in samples collected in slaughterhouses or farms may provide information about the potential risk for public health, especially if livestock raw wastes are directly released into surface water that is subsequently used for drinking

water production or recreational activities. At last, studies concerning animal restricted species could also contribute to their evaluation as indicators of the origin of faecal contamination in environmental samples.

Conclusion

Few published reports on the distribution of *Cryptosporidium* species in environmental samples are available in Iran, and the present study provides new data on the prevalence of genotypes of this parasite in urban and livestock wastewaters. Indeed, molecular methodologies are helpful tools to aid to understand the epidemiology of *Cryptosporidium* species in the environment. Further studies, simultaneously in a larger series of environmental and faecal samples, could contribute to a better insight on the origin of faecal contamination in surface waters, and the possible zoonotic transmission of these waterborne parasites.

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