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.

Keywords:
Cryptosporidium andersoni, Cryptosporidium xiaoi, Iran, Wastewater

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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 anthropoontic 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. xiaoai are described from cattle and sheep with an age-related distribution (5, 7).

Infection with C. andersoni is often associated 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 microscope 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 automated 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).

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**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. xiaoii (Fig. 3). The most prevalent species was C. andersoni, which was detected in 21 samples (95.5%). C. xiaoii 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.

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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 K175408 |
| [32] SM2    | SWWTP4       | +         | C. andersoni K175409 |
| [33] SM3    | SWWTP4       | +         | C. andersoni K175410 |
| [34] SM4    | SWWTP4       | +         | C. andersoni K175411 |
| [35] SM5    | SWWTP4       | +         | C. andersoni K175412 |
| [36] SM6    | SWWTP4       | +         | C. andersoni K175413 |
| [37] SM7    | SWWTP4       | +         | C. andersoni K175414 |
| [38] SM8    | SWWTP4       | +         | C. andersoni K175415 |
| [39] SM9    | SWWTP4       | +         | C. andersoni K175416 |
| [40] SM10   | SWWTP4       | +         | C. andersoni K175417 |
| [41] SM11   | SWWTP4       | +         | C. andersoni K175418 |
| [42] SM12   | SWWTP4       | +         | Defaulted sequencing | -             |
| [43] SR1    | SWWTP5       | +         | C. andersoni K175419 |
| [44] SR2    | SWWTP5       | +         | C. andersoni K175420 |
| [45] SR3    | SWWTP5       | +         | C. andersoni K175421 |
| [46] SR4    | SWWTP5       | +         | C. xiaoi K175422 |
| [47] SR5    | SWWTP5       | +         | C. andersoni K175423 |
| [48] SR6    | SWWTP5       | +         | C. andersoni K175424 |
| [49] SR7    | SWWTP5       | +         | C. andersoni K175425 |
| [50] SR8    | SWWTP5       | +         | C. andersoni K175426 |
| [51] SR9    | SWWTP5       | +         | C. andersoni K175427 |
| [52] SR10   | SWWTP5       | +         | Defaulted sequencing | -             |
| [53] SR11   | SWWTP5       | +         | C. andersoni K175428 |
| [54] SR12   | SWWTP5       | +         | C. andersoni K175429 |

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 Shahrivar, 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. xiaoi 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

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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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