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Identification of *Cryptosporidium* oocyst and *Giardia* cyst in of the samples of raw surface water of Kan River in Tehran

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ABSTRACT

Background: *Giardia* and *Cryptosporidium* are considered the most important causal agents of non-bloody diarrhea, especially among primary school children, in many countries, including Iran. Many rivers are contaminated with *Cryptosporidium* oocyst and *Giardia lamblia* cysts due to domestic wastewater or farm wastewater and also the living of rodents on their margins. The present study aims to evaluate Kan River contamination with *Cryptosporidium* oocyst and *Giardia* cyst in Tehran by molecular method.

Materials and Methods: Sampling was conducted from different parts of the Kan River in different seasons in 2019. Firstly, the smear has been prepared from sediments after filtering the water and collecting the sediment, stained with trichrome and acid-fast methods, and finally examined microscopically. Then, they were amplified with the Nested-PCR method by using the specific primers: the giardian gene from *Giardia* and the 18s rRNA gene from *Cryptosporidium*. Positive samples were sequenced, and a phylogenetic tree was drawn.

Results: 12 suspected samples of *Giardia* cyst and 2 suspected samples of *Cryptosporidium* oocyst were detected, but only 4 samples infected with *Giardia lamblia* were molecularly found, and no *Cryptosporidium* infection was observed. In terms of genotype, the identified *Giardia* was 100% consistent with human isolates of genotype B.

Conclusion: The presence of *Giardia lamblia* cysts in the water of the Kan River indicates the contamination of this river by human-contaminating parasites.

Keywords: *Giardia*, *Cryptosporidium*, Kan River, Tehran.

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1. Introduction

*Giardia*sis is a small intestinal parasitic infection with worldwide incidence that is created in humans by unicellular flagellates called *Giardia lamblia*. This unicellular flagellate is currently the most common unicellular parasite in many countries, including Iran, and is also considered one of the most important causes of non-bloody diarrhea, especially in primary school children (1-3). This parasite not only impairs the absorption of nutrients through multiple mechanisms but also competes with its host by using nutrients (4-5). Since the mental growth and fertility of humans occur at the primary school age, prevention and treatment of this disease are very necessary.

On the other hand, *Cryptosporidium* is a coccidial protozoan that can be found throughout the environment and is usually associated with animwaste. s. Human infection occurs throthe ingestiontion of oocysts of the parasite in contaminated water or other substances. The latency is 4 to 14 days, and the symptoms last 10 to 15 days (6). *The Giardia* parasite is often found in the feces of humans, beavers, mice, and dogs. Also, cattle's feces are known as the primary and basic origin of *Cryptosporidium*. However, these parasites have been found in humans and other animals. Water resources are contaminated due to the entry of feces with infected parasites. If refinement processes are inadequate and defective, the drinking water may contain a sufficient number of parasites that can cause diseases. Direct contact with infected humans or animals's feces, eating contaminated food, or accidental drinking of contaminated recycled water are other contaminating sources. The rivers may be infected with *Cryptosporidium* oocyst and *Giardia lamblia* due to the entry of domestic wastewater or the wastewater of animal husbandries, as well as rodents living on the margins. This contamination may be transmitted to humans and animals. So far, several studies have been done in Iran and other countries around the world about the contamination of rivers with pathogenic microbial and parasitic organisms (7–23).

A huge amount of agricultural products in the west of Tehran are produced from the Kan Valley; therefore, the contamination of this water can result in the contamination of farmers and local people with *Giardia lamblia* and *Cryptosporidium*. In addition, a part of the West of Tehran's drinking water is supplied from the Kan River, so the health of its water is so important for human consumption. The present study aims to investigate the contamination of the Kan River with *Cryptosporidium* oocysts and *Giardia* cysts using molecular methods.

2. Material and methods

2-1. Kan River

The Kan River, or Sooleghan River, is 33 km in length and originates from the Tochal Mountains, passes through Tehran, and is dried in the south of Tehran. This is now the most watery river that passes through Tehran. This river is the wateriest river in Tehran, with a length of 33 km and a flow rate of 2700 liters per second. Also, about 5000000 cubic meters of water from the river is transferred to Chitgar Lake in District 22.

2-2. Sampling method:

At first, 10 points were marked, respectively, from the downstream to the upstream of the river. Five liters of water were collected in 5-liter containers from each point. The operation was conducted in four seasons of the year, and eventually 200 liters of water were collected from 10 different points. Then, the water was filtered by 0.2-micron Whitman paper filters. The filter papers were soaked in disposable glasses and left to soak for 24 hours. Then, the water from each glass was poured into separate tubes and centrifuged. After that, the supernatant was discharged, and the sediment from the bottom of the tube was placed on the lam for microscopic examination of the *Giardia* and *Cryptosporidium* by the following two methods.

2-3. Microscopic examination:

Trichrome staining and acid-fast staining methods were used to observe *Giardia* cysts and *Cryptosporidium*, respectively.

2-4. DNA Extraction by the cTAB Method:

The sediment was dissolved in 100 µl of deionized distilled water, and the DNA of the parasites was extracted by the cTAB method.

2–5. Nested PCR

A nested PCR method based on the designed primer was used for the amplification of the 18S rRNA gene of *Cryptosporidium* and the giardian gene of *Giardia*. The primers for the 18S rRNA gene of *Cryptosporidium* for nested PCR were AWA722F: 5'-agtgcttaaagcaggcaactg -3 '; and AWA1235R: 5'-cgttaacggaattaaccaga -3 '. The expected internal fragment was 655–667 bp (depending on the species). AWA995F: 5'-tagagattggaggttgttcct -3'; AWA995 R: 5'-ctccaccaactaagaacggcc -3'.. The expected external fragment is 429–455 bp.

The primary PCR contained 2 µl DNA in a 20 µl reaction, DNA (5 µl), 20 picomol forward and reverse primer (2 µl), 2X Master Mix (8 µl), and distilled water (5 µl). The secondary PCR was designed by proliferation from the product of primary PCR in a final volume of 15 µl, DNA (2 µl), 20 picomol forward and reverse primer (2 µl), 2X master mix (8 µl), and distilled water (3 µl). After mixing the above compounds, it was spun down for a few seconds, and the following protocol was used according to the thermocycler program: Primary PCR includes primary denaturation at 95 °C for 5 minutes; 25–35 cycles at 94 °C for 30 seconds; 68 °C for 1 minute; 72 °C for 30 seconds; and annealing at 72 °C for 10 minutes. Secondary PCR includes primary denaturation at 95 °C for 1 minute, 25–35 cycles at 94 °C for 30 seconds, 60 °C for 1 minute, and 72 °C for 30 seconds, and annealing was done at 72 °C for 10 minutes.

The amplification of the giardin gene by the Nested PCR for *Giardia intestinalis* was done using primers MAH433F: 5'-cataacgacgccatcgcggctctcaggaa -3 '; MAH592R: 5 '-ttagtgctttgtgaccatcga -3'. The expected internal fragment was 218 bp. As well as MAH658F: 5'-aagtgcgtcaacgagcagct -3'; MAH658 R: 5'-ttagtgctttgtgaccatcga -3'. The expected external fragment was 171 bp.

After mixing the PCR compounds, it was spun down for a few seconds, and the following protocol was used according to the thermocycler program:

Primary PCR includes primary denaturation at 95 °C for 5 minutes, 35 cycles at 94 °C for 60 seconds, at 60 °C for 1 minute, at 72 °C for 30 seconds, and annealing at 72 °C for 10 minutes. Secondary PCR includes primary denaturation at 94 °C for 10 minutes, 35 cycles at 94 °C for 30 seconds, 60 °C for 1 minute, and 72 °C for 30 seconds, and annealing was done at 72 °C for 10 minutes. After the timeout, 6 µl of the PCR product was electrophoresed by 1% agarose gel stained with safe stain, and then imaging was conducted under short-wave UV. The size of the PCR product with the commercial ladder of Sinacolon Company was evaluated.

2-6. Molecular Analysis

Totally, 4 isolates were sent to Pishgam Company (Iran) for sequencing. The results of the sequencing were analyzed by Portable-Sequenccher 4.1.4 software. In order to assess the genetic diversity among the *Giardia* isolates recorded in this study and the isolates previously recorded in Genbank, multiple alignments were performed, and the phylogenetic tree was drawn using the neighbor-joining algorithm in MEGA 6.0 software.

3. Results

3-1. Microscopic examination results

After microscopic examination, 12 samples suspected of *Giardia* and 2 samples suspected of *Cryptosporidium* contamination were detected.

3-2. Molecular examination results

It was found in the Nested PCR test that 4 out of 12 suspected samples were contaminated with *Giardia*, but there was no contamination with *Cryptosporidium* in any of the suspected samples.

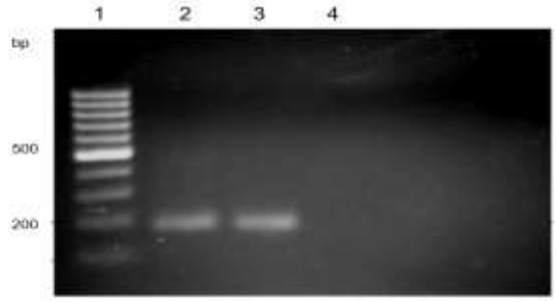

Fig 1. Electrophoresis gel of a DNA fragment of the Giardia gene with external primers; column 1 contains a kb DNA ladder; columns 2 and 3 are *Giardia* positive samples; and column 4 is the negative control.

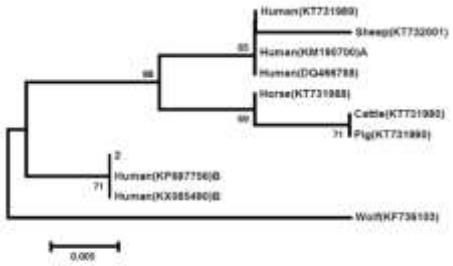

Fig 2. Phylogenetic relationships of *Giardia* genotypes inferred by the neighbor-joining algorithm.

4. Discussion

In this study, 4 positive samples for *Giardia* were detected with molecular methods out of 40 water samples collected from the Kan River. Also, no *cryptosporidium* infection was observed in the collected samples. In comparison with similar studies in Iran, Mohammadi Qalebin et al. (2007) studied *Cryptosporidium* species in the water resources of Ardabil Province using the PCR-RFLP method. In this study, 200

water samples were taken from 10 sites. Then, positive samples for *Cryptosporidium* were detected by PCR, and finally, the species of the positive samples were determined by the RFLP method. Among 8 water samples with a positive Nested-PCR result, 5 samples were related to *Cryptosporidium andersoni*, two were related to *Cryptosporidium parvum* bovine genotype, and one sample was consistent with *Cryptosporidium* pig genotype (7).

Shahsavari Sari and Ismaili Sari (2007) determined the microbial contamination of the Haraz River and allowed applications of river water according to international standards. The water samples were analyzed in terms of microbial contamination and according to the available indicators of fecal coliform. The results showed that the amount of contamination in the river downstream was higher than upstream, and the microbial contamination of the Haraz River in the spring at all stations was higher than in other seasons. The results also indicated that the mean level of *E. coli* in the Haraz River was higher than international standards determined for most uses due to the various domestic, urban, agricultural, and population wastewaters. Thus, the river's water quality is not healthy (8).

Herati et al. (2013) examined the Karon River's water quality in the region of Mollasani in Ahvaz using the water quality index of the National Institutes of Health (WQI), Oregon (OWQI), and Canada (CWQI) due to their higher performance. The results showed that changes in water quality index between the stations and during the study period are statistically insignificant. Also, the WQI index showed the best results among other indicators (9).

Kalantari et al. (2009) examined the groundwater microbial contamination of the East Dezful in Khuzestan province. 17 samples of surface water and groundwater were taken, and chemical and biological analysis was performed to check the source and scope of contamination. The results showed that surface and ground water resources in a large part of the area are contaminated. In addition to the concentration of salts, bacteria such as *Shigella, Salmonella paratyphi, Proteus vulgaris, E. coli, Enterobacter, Staphylococcus aureus, Staphylococcus epidermidis,* and protozoa such as *Giardia* and *Cryptosporidium* have been observed in water resources (10).

Hassani et al. (2010) examined the microbial contamination of groundwater resources in rural areas of Islamshahr. The microbiological quality of drinking water in the area was examined by twice sampling and testing each sample well. It was found that there is contamination with total coliform in some villages. In terms of contamination with fecal coliform, there was nothing but a case that it was related to the Islamabad Village (12).

Fallah et al. (2013) searched for *Giardia* cysts and *Cryptosporidium* oocysts in drinking water resources in Hamadan. The resource of raw water, the water treated by two main water treatment plants, and six drinking water distribution tanks in Hamadan were examined using two methods, namely immunofluorescence and eosin staining. The results showed that the average amounts of *Giardia* and *Cryptosporidium* oocysts observed by the

immunofluorescence method in 100 liters of treated water were 25% for each; these values were also 2.5 and 1.5 in 100 liters of raw water. The level of living *Giardia* cysts and *Cryptosporidium* oocysts observed by the eosin method in 100 liters of treated water was 0.00, and these values were 0.5 and 0.1 in 100 liters of raw water, respectively (18).

Dalimi et al. (2014) examined the *Cryptosporidium* species isolated from humans using PCR-RFLP in Tehran. In this study, a total of 922 samples of human stool were collected in the first stage from the patients admitted to Ali Asghar, Mofid, and Imam Khomeini hospitals in Tehran. The samples were changed, stained, and detected by the acid-fast method. In the second stage, the DNA of the positive samples was extracted, and the 15s rRNA gene was proliferated using the Nested-PCR method. Then, the PCR product was cut by the inhibitor enzyme Vsp1 in order to determine the species. Finally, the PCR product was sequenced. 10 samples stained with the Ziehl-Neelsen method were detected as positive (1.06%). All of these samples were confirmed by the nested-PCR method. The amplified fragment (835 bp) of the 15s rRNA gene was cut with the enzyme Vsp1. After enzymatic cut on 2.5% agarose gel, 10 samples were detected as *Cryptosporidium parvum* based on enzymatic cut pattern and sequencing, one sample as Anderson *Cryptosporidium*, and one sample as *Cryptosporidium hominis*. It turned out that, though parvum is known as the common species in humans, people may also become infected with hominis, according to Anderson (21).

Many studies have been done in other countries in this field; some of them are mentioned below.

In a study conducted by Xiao (2001) on 41 samples of surface water from different regions of the United States and 38 samples of wastewater from Milwaukee, Wisconsin, *Cryptosporidium* was isolated from 25 samples of surface water and 10 samples of wastewater. In this study, human and bovine *Cryptosporidium parvum* were the dominant genotypes in the samples of surface water, while the dominant species in the samples of wastewater was *Cryptosporidium andersoni* (23).

Conclusion: According to the research results, the presence of *Giardia lamblia* cyst in the water of the Kan River indicates the contamination of this river by human-contaminating parasites.

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Conflict of interest

We declare that we have no conflict of interest.

Data availability statement

The data that supports the findings of this study are available from the corresponding author, upon reasonable request.

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