ORIGINAL PAPER

Molecular detection and characterization of *Cryptosporidium* spp. in the sewage-contaminated rivers entering Bandar-e Anzali Lagoon in Guilan Province, Iran

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Abstract

Waterborne cryptosporidiosis in river water is considered to be an important source of *Cryptosporidium* oocysts in most developing countries across the world. In the present study, 20 water samples were collected from Zarjoob and Goharrood rivers contaminated with wastewater in the province of Guilan, Iran. The samples were filtrated through a membrane filter (pore size: 1.2 µm), then the sucrose clarification and molecular genotyping methods were also carried out. In total, 12 out of 20 the samples were positive for *Cryptosporidium* species based on the 18S RNA-based polymerase chain reaction (PCR) and sequencing techniques. In addition, *C. parvum, C. muris, C. homnis*, and *C. canis* were detected in the samples studied. According to the results, the river water resources were polluted with the pathogenic species of *Cryptosporidium* with human and animal sources; this may result in an increase in the potential risk of waterborne cryptosporidiosis. Moreover, the long-term use of sewage-contaminated river water to irrigate crops and agriculture farms could be a major threat to local residents.

Keywords: Bacterial isolation, Water resources, Sewage-contaminated rivers, Guilan Province

Introduction

Cryptosporidiosis is a prevalent diarrheal disease in developing and industrialized countries. Molecular studies have helped scientists to recognize the transmission of this parasite to individuals, and the popular health significance of Cryptosporidium spp.1 Currently, waterborne cryptosporidiosis is known to cause numerous disease outbreaks mainly in the North America, United Kingdom, Japan, and Australia.[2-4]

Cryptosporidium oocysts have been detected in surface waters throughout Asia.[5] A minimum of 165 water-associated outbreaks of cryptosporidiosis were describe globally since the beginning of the last century until 2004[6] while 120 outbreaks were reported during 2004 until 2010.[7] There are several types of *Cryptosporidium* spp., while only some species (*C. parvum* and *C. hominis*) are considered to be responsible for most of the associated human infections.[8]

In Iran, the epidemiology of *Cryptosporidium* species in environmental

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resources remains unclear, and constant monitoring is required in this regard. The present study aimed to provide an update on the information regarding the distribution of *Cryptosporidium* spp. in the rivers located in Guilan Province, Iran and propose an approach to recognize the origin of *Cryptosporidium* oocysts in the contaminated rivers in the study area.

Materials and Methods

Study area and sample collection

In total, 20 water samples were collected from the Zarjoob and Goharrood rivers located the province of Guilan, Iran. This province is situated beside the Caspian Sea coastline and has a moderate and Mediterranean weather. The Zarjoob and Goharrood rivers are the two branches of the Sefidrood River (the second longest river in Iran), which originates in the city of Rasht and flows through the Bandar-e Anzali Lagoon. Unfortunately, these areas are threatened by numerous pollutants, such as rural, urban, and agricultural runoff.[9] In addition, the water of these sewage-contaminated rivers is used by animals and for the irrigation of crops and agriculture farm by the local residence.

Separation of oocysts from the water samples

In this study, 20 water samples (5 L each) were collected from the depth of ~10-20 cm in Zarjoob and Goharrood rivers, which flow across the city of Rasht. The samples were taken in places where the rivers exited the city. Any contaminants originating from the upstream and midstream may pollute the downstream as well.

At the next step, the samples were filtrated through a cellulose acetate membrane filter (diameter: 142 mm, pore size: 1.2 μm). The filter was washed twice with 0.1% phosphate buffered saline-Tween 80 (50 mL), and the material was concentrated via centrifugation at 3,000 rpm for 10 min. For oocyst concentration and purification, the sediment pellet was subjected to sucrose flotation[10] and the supernatant was subjected to the molecular technique.

DNA extraction and PCR

DNA was extracted from the purified oocysts using the QIAamp DNA Mini Kit as recommended previously[11] with some modification including ten freeze-thaw cycles (1 min in -196 °C and 1 min at 96 °C per cycle). The procedure continued until DNA extraction, which was performed based on the instructions by the manufacturer (Qiagen GmbH, Hilden, Germany). In addition, the nested PCR assay was used to amplify a 435bp fragment of the *Cryptosporidium* spp.-gene-specific 18S rRNA using the CPB-DIAGF/R and N-DIAGF2/R2 primers.[12,13]

In accordance with previous studies, each PCR reaction was performed in 50 microliters,[12,13] and the conditions of the first amplification were as follows: one cycle at 95 °C for 15 min, 35 cycles at 94 °C for 30 sec, 68 °C for 60 sec, and 72 °C for 30 sec, one cycle at 72 °C for 10 min, with the final hold at 4 °C. The set up of the second amplification was similar to the first procedure, with the exception of the annealing parameters, which changed to 60 °C for 60 sec. It is notable that both the positive negative controls were included in the amplifications.

The PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide (0.6 mg/mL) and imagine using a gel documentation instrument. Following that, the PCR products were sequenced using an ABI 3730XL DNA analyzer. All the sequences were edited and the genotype was identified through the comparison of the accessible *Cryptosporidium* DNA sequences in GenBank by using the Chromas software.

Results and Discussion

According to the current research, the studied rivers were contaminated with

Cryptosporidium oocysts. In total, 12 out of 20 water samples (60%) were positive for *Cryptosporidium* spp. Some studies have detected *Cryptosporidium* oocysts in 24-100% of surface and wastewater samples collected from other regions in the world.[14,15]

The genotyping of *Cryptosporidium* isolates contributed to the evaluation of the origin and human-infective potential of oocysts in polluted water. In the present study, the genotyping of the isolates revealed the presence of *C. parvum, C. muris, C. homnis,* and *C. canis*. Similarly, some studies have identified the species in untreated water in Iran.[16-18] *C. hominis* and *C. parvum* have been most common in contaminated river water as detected by PCR-based techniques worldwide. Our findings support the previous studies regarding the complexity of *Cryptosporidium* isolates in untreated domestic wastewater.[19-21]

Although *C. hominis, C. parvum,* and *C. canis* are all accountable for human infections, *C. hominis* and *C. parvum* have been shown to be more prevalent in this regard.[22] In addition, *C. parvum* is considered to be the main species in humans with diarrhea and AIDS worldwide.[23-26] *C. hominis* is a more important cause of infections than *C. parvum* in humans in developing nations according to genotyping researchs.[27-32] In the United Kingdom, various European regions, and New Zealand, *C. parvum* has been reported to be as common as *C. hominis* in human infections.[33-37] The variations in spread of *Cryptosporidium* genotypes in human are due to the differences in the infection sources.[1]

According to the literature, *C. muris* mostly infects rodents[38] while *C. canis* is considered to be a parasite in canines.[39] These species were the next most frequent genotypes detected in the present study. Furthermore, *C. canis* is responsible for human infections.[22,39] Consistent with our findings, *C. canis* and *C. muris* have been detected in raw water and wastewater from other parts of world.[1,35]

C. parvum has also been identified in domestic livestock and other animals, as well as humans, while *C. homonis* has been exclusively observed in the isolates from humans. Our findings highlighted the potential risk caused by waterborne cryptosporidiosis due to irrigation using contaminated river water. Therefore, the monitoring of *Cryptosporidium* in water sources is critical considering its public health implications.

Conclusion

According to the results, the river water samples were contaminated with *Cryptosporidium* oocysts. Therefore, it is hazardous for the local population to use the sewage-contaminated river water for the irrigation of crops and rice farms, and the subsequent transfer of the water to the food chain must also be prevented.

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Authors' Contributions

M. R. M: data collection, analysis, drafting and revision of the paper.

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