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Original Article

Identification of zoonotic *Cryptosporidium parvum* in freshwater ornamental fish

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Summary
Regarding the importance of zoonotic species of *Cryptosporidium* in humans and domestic animals, and the lack of information about the distribution of *Cryptosporidium* spp. especially the zoonotic species of aquarium ornamental fish with due attention to their relation to urban water sources and human owners, the present study was designed. *Cryptosporidium* has been demonstrated in more than 17 species with parasites presented deep within and on the surface of the intestinal and stomach epithelium. To date, two important species of *Cryptosporidium* for public health, namely *C. parvum* and *C. hominis* have been determined in fish specimens from cultured and wild freshwater, and marine environments. In this study, the identification of *Cryptosporidium* spp. and zoonotic *Cryptosporidium parvum* by histopathology and PCR amplification at the 18S rRNA locus among 100 freshwater ornamental fish were performed. Results showed that a total of 16 fish samples (16%) were identified as positive for *Cryptosporidium* spp. by histopathology. *C. parvum* was also found in two fish hosts (goldfish). The detection of zoonotic *C. parvum* in ornamental fish is considerable and suggests that the fish might be a good contamination indicator of water with sullage and agricultural run-off.
Keywords: *Cryptosporidium parvum***,** ornamental fish**,** 18S rRNA, PCR.

Introduction

Cryptosporidium parvum (*C. parvum*), a protozoan parasite, is well known as a primary etiologic cause of gastrointestinal illnesses in mammals and potentially lethal for immunosuppressed humans and animals (Fontaine and Guillot, 2003; Xiao and Feng, 2008). The oocysts are the infectious stage of *C. parvum*. At the outside of its mammalian host, the oocyst is biologically dormant and could not replicate and propagate its numbers. Different transmission routes have been detected worldwide for *C. parvum*, including a direct contact with infected persons known as person-to-person

transmission; animals that considered as zoonotic transmission; ingestion of contaminated food considered as foodborne transmission, and water known as waterborne transmission (Xiao, 2010).

Little information is available about the taxonomy, epidemiology, pathology, and host specificity of *Cryptosporidium* species infecting fish species. *Cryptosporidium* has been reported in more than 17 species of fresh and saltwater fish by parasites placed deep within and on the surface of the intestinal and stomach mucosa (Koinari et al., 2013). To date, two important species of *Cryptosporidium* for public health, namely *C. parvum* and *Cryptosporidium hominis* (*C. hominis*) have been detected in fish specimens from cultured and wild freshwater and marine environments (Reid et al., 2010; Koinari et al., 2013). Considering the great commercial importance of the aquarium industry in all countries, the zoonotic character of *C. parvum*, and lack of knowledge about infection in cultured ornamental fish, this study was conducted to analyze the distribution of zoonotic *C. parvum* in some aquarium fish species using Nested-PCR method and sequence analysis.

Materials and Methods

Sample collection

A total of 100 freshwater ornamental fish, belonging to 22 species, were collected from pet shops in different cities: Tehran, Tabriz, Zanjan, and Shahindej, 25 samples from each city. The samples were transferred to the laboratory and live fish were killed by immersion in a lethal dose of clove oil bath (50 µl L^{-1}) and dissected by a sterile scalpel blade. Then, the stomach and intestine segments were scraped off and placed in 10% buffered formalin for histological procedures. The remaining stomach and intestinal tissues were preserved in 70% ethanol for further molecular studies.

Histology

Once the intestinal and stomach tissues were fixed, they were routinely embedded in paraffin wax. Histological sections were prepared at 5 µm thickness, which stained by hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS). Sections were evaluated using a light microscope (Olympus, Japan) at 200 and 400 fold magnifications.

DNA extraction and Cryptosporidium genotyping

The preserved tissues were washed five times by water to remove ethanol. The DNA was extracted from 25 mg of the intestine and stomach tissues using the MBST-DNA extraction kit (Iran. Tehran) according to the manufacturer's instructions. The extracted DNA samples were stored at -20 °C until needed for analyses. All specimens were genotyped based on the 18S rRNA gene by Nested-PCR method as previously presented (Xiao et al., 1999; Sturbaum et al., 2001) and sequencing (Guyot et al., 2001).

Nested PCR and Sequencing

A two-step nested PCR protocol was used to amplify the *Cryptosporidium parvum* 18S rRNA gene. The nested PCR primers

used for this study were designed as previously described (Sturbaum et al., 2001). In brief, the external primers amplify a 844-bp and a 840-bp fragments from genotype 1 and genotype 2, respectively. In addition, Nested primers amplify a 593-bp fragment from genotype 1 and a 590-bp fragment from genotype 2.

Briefly, amplification of the 18S rRNA gene was performed in 25 µL reaction volumes consisting of 2 µL of DNA templates, 12/5 µL of master mix (the main mixture with 2x concentration including Taq DNA Polymerase, dNTPs, MgCl2, PCR buffer) (CinnaGen Co. Iran), and 5-9 pmol of reverse and forward primers, and distilled water. Here, the PCR parameters used in the external reaction comprised an initial denaturation for 5 min at 95°C, which followed by 40 cycles of 94°C for 45 s, 58°C for 75 s, and 72°C for 45s. The final extension was subsequently conducted at 72°C for 7 min. The nested-reaction parameters were the same except that 35 cycles were carried out at an annealing temperature of 67°C and dehybridization, annealing, and extension periods were 25 s each. A thermal cycler (MWG Biotech-Germany) was used for all PCRs. To verify the findings, 8 µL of each PCR products were mixed with 2 µL loading dye (5x) and were evaluated and photographed on 1.2% agarose gels following UV transillumination. A PCR purification kit (MBST, Tehran, Iran) was used for purification of PCR products, which were analyzed by sequencing (Bioneer, Korea). The sequence alignment was checked for sequencing accuracy using Bio Edit sequence Alignment, and then was compared with sequences published in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http:// www. cbi. Ncbi.n/mnih. gov/ BLAST).

Results

Prevalence of C. parvum in ornamental fish hosts

Of the 100 samples screened during this study, 16 Cryptosporidium positives were detected by histology, giving an overall prevalence of 16%. Infected hosts were siamese fighting fish (n = 1), pearl gourami (n = 1), sailfin molly (n = 2), rosy barb (n = 1), platy (n = 1), altum angelfish (n = 1), electric yellow (n = 1), gold fish (n = 3), oscar (n = 4) and a slender rainbow (n = 1).

Identification of C. parvum in fish hosts at the 18S rRNA locus

Two fish samples (goldfish) out of all 100 fish samples were *C. parvum* positive by Nested PCR (Fig 1). BLAST analysis revealed that this sequenced fragment was similar to most of the sequences of the *C. parvum* 18S ribosomal RNA gene deposited in the GenBank (Fig 2). The most remarkable similarity is observed with the *C. parvum* 18S ribosomal RNA gene, complete sequence under accession number AF222998.1, with Query Cover 100% and Identical Value 99%.

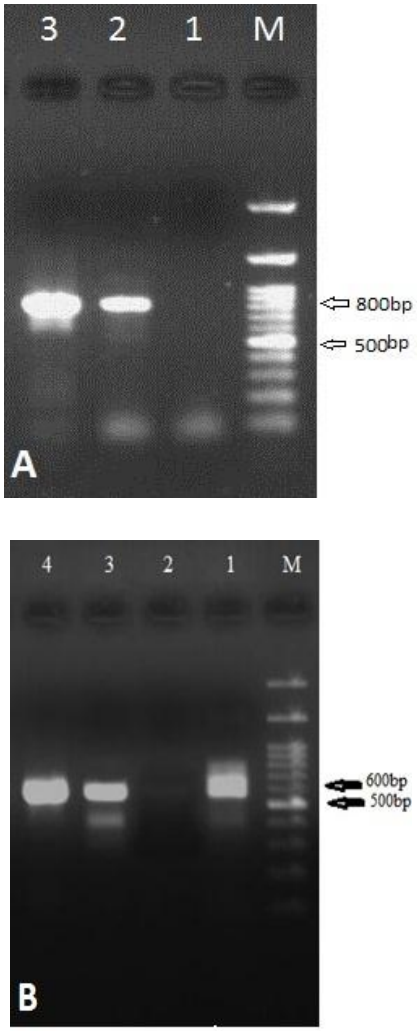

Fig. 1. A (1st run): Nested PCR amplification of a segment within the 18S rRNA of *Cryptosporidium* species; Lane 1: negative control, Lanes 2, 3: positive samples (840 bp product), Lane M: 100-bp molecular marker. B (2nd run): Lane 1: positive control, Lane 2: negative control, Lane 3, 4: positive samples (from the 1st run) (590bp product), Lane M: 100- bp molecular marker.

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Sample  1   TTATACGGTTAAACTGCGAATGGCTCATTATAACAGTTATAGTTTACTTGATAATC-TTT 59
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
C.parvum 73 TTATACGGTTAAACTGCGAATGGCTCATTATAACAGTTATAGTTTACTTGATAATCTTTT 132

Sample  60  ACTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAAACTCGACTTTAT 119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum133 ACTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAAACTCGACTTTAT 192

Sample  120 GGAAGGGTTGTATTTATTAGATAAAGAACCAATATAATTGGTGACTCATAATAACTTTAC 179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum193 GGAAGGGTTGTATTTATTAGATAAAGAACCAATATAATTGGTGACTCATAATAACTTTAC 252

Sample  180 GGATCACATTAAATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGG 239
            |||||||| | ||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum253 GGATCACAATTAATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGG 312

Sample  240 GTATTGGCCTACCGTGGCAATGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGG 299
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum313 GTATTGGCCGACCGTGGCAATGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGG 372

Sample  300 AGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCT 359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum373 AGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCT 432

Sample  360 AATACAGGGAGGTAGTGACAAGAAATAACAATACAGGACTTTTTGGTTTTGTAATTGGAA 419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum433 AATACAGGGAGGTAGTGACAAGAAATAACAATACAGGACTTTTTGGTTTTGTAATTGGAA 492

Sample  420 TGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGC 479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum493 TGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGC 552

Sample  480 CGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGT 539
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
C.parvum553 CGCGGTAATTCCAGCTTCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGT 612

Sample  540 TGGATTTCTGTTAATAATTTATATAAAATATTTTGATGAATATTTATATAA  590
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum613 TGGATTTCTGTTAATAATTTATATAAAATATTTTGATGAATATTTATATAA  663
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Fig. 2. BLAST analysis of the *Cryptosporidium* 18S-rRNA gene products from the nested PCR and compared with the *Cryptosporidium parvum* 18S-rRNA gene complete sequence from GenBank, (Accession No. AF222998.1.). Gaps are shown with dashes (-), and different bases are shown with highlights. The numbers to the right and left of the alignment show sequence positions for each subject.

Discussion

To date, little is known about the taxonomy, epidemiology, and pathology of *Cryptosporidium* isolates that can infect fish. In this regard, there have been some reports of *Cryptosporidium* spp. in wild and cultured freshwater and marine hosts (Alvarez-Pellitero et al. 2004).

In the current study, the overall prevalence of *Cryptosporidium* spp. determined by histology was 16%, which was nearly similar to the prevalence of 10.5% for *Cryptosporidium* reported in ornamental fish by molecular studies (Zanguee et al., 2010). Previous studies in fish species have presented a different incidence of infection with *Cryptosporidium*. A lower prevalence of 0.8% (Reid et al., 2010), 1.14% (Koinari et al, 2013), and 3.5% (Morine et al., 2012) in different fish groups have been reported. Others have reported a higher prevalence (10-100%), frequently in juvenile fish (Alvarez-Pellitero et al., 2004; Sitja-Bobadilla et al., 2005; Murphy et al., 2009). A previous study monitored *Cryptosporidium* in various species of aquarium fish in Western Australia and found that the great prevalence of *Cryptosporidium* in fish was likely associated with a crowded environment of the aquarium tanks and the successive introduction of new species (Zanguee et al., 2010). New fish host for *Cryptosporidium* sp. were identified as; sailfin molly (*P. latipinna*), rosy barb (*P. conchonius*), platy (*X. maculatus*), goldfish (*C. auratus auratus*), pearl gourami (*T. leerii*), siamese fighting fish (*B. splendens*), slender rainbow (*M. gracilis*) and electric yellow (*L. caeruleus*). Of note, this is the first study in which *C. parvum* has been identified in goldfish.

Clinical signs of gastrointestinal cryptosporidiosis are different in piscine hosts. Some time, no clinical or pathological symptoms have been detected (Alvarez-Pellitero et al. 2004). However, in some infected fish, loose of appetite, regurgitation of food, atrophy of skeletal muscle, weight loose, and tucked abdomen have been found (Hooveret al., 1981; Gratzek, 1993; Camus and Lo´pez, 1996). The pathogenesis of the *Cryptosporidium* species detected in the present study is unknown. However, some affected fish showed variable levels of listless, fin rot, emaciation, and fin clamping. Six of the affected fish had concomitant coccidian and fungal infections. Meanwhile, other ornamental fish examined in the present study appeared well. *C. parvum* in goldfish was also identified in the intestine with emaciation in one goldfish while the other one was clinically healthy. Since some affected ornamental fish were clinically healthy, it seems that *Cryptosporidium* species, namely *C. parvum*, represent true or mechanical infections that remain to be revealed as the oocysts might pass through rather than infecting these fish.

In the current study, the zoonotic *C. parvum* in two goldfish samples was detected

which is of significance to public health. This is the first study in which *C. parvum* has been identified in freshwater ornamental fish. In two recent studies, the zoonotic *C. parvum* was detected in different fish species. Reid et al (2010) identified *C. parvum* in marine whiting (*Sillago vittata*). Koinari et al (2013) also found *C. parvum* subtypes IIaA14G2R1, IIaA15G2R1and IIaA19G4R1 in a marine (mackerel scad), wild freshwater (silver barb), and cultured freshwater (Nile tilapia) fish. However, the first assessment to experimentally infect fish with *C. parvum* was unsuccessful (Graczyk et al. 1996a). The identification of livestock and zoonotic species of *Cryptosporidium* in ornamental fish could be due to waterborne contamination with animal and human waste (Reid et al., 2010). Millions of oocysts of *C. parvum* can be released from infected animals and humans into the environment, which they can contaminate soil, food, water, or surfaces contaminated with the feces harboring oocysts (Shahbazi et al., 2009). Even though there is no evidence for transmission of *Cryptosporidium* from fish to human, in a previous study, the mean possibility of infection was nearly one for urban fisherman while fishing and eating caught fish (Roberts et al., 2007). On the other hand, the zoonotic *C. parvum* in ornamental fish can establish a significant threat to endemic and threatened freshwater fish fauna as most freshwater ornamental fish species are released into waterways, either accidentally or deliberately, which can adversely impact on the native freshwater fish by competition, predation, and the introduction of diseases (Zanguee et al., 2010).

Conclusion

The current data of taxonomy, epidemiology, pathology, and host specificity of *Cryptosporidium* species contaminating fish species is restricted. The detection of zoonotic *Cryptosporidium parvum* in ornamental fish is significant and suggests that fish may be a good contamination indicator of water with sewage and agricultural run-off.

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