Case Report

Identification of zoonotic *Cryptosporidium parvum* in fresh water ornamental fish: first report

Parisa Shahbazi[1*], Najmeh Sheikhzadeh[2], Ahmad Nematollahi[1], Javad Ashrafi Helan[1]

1- Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

2- Department of Food Hygiene and Aquatic Animals, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

[*]**Corresponding author**: *p.shahbazi@gmail.com*

(Received 1 August 2017, Accepted 25 September 2017)

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 described in more than 17 species of both fresh and saltwater fish with parasites located deep within and on the surface of the stomach or intestinal epithelium. To date, two important species of *Cryptosporidium* for public health, namely *C. parvum* and *C. hominis* have been identified in fish samples from cultured and wild freshwater and marine environments. In this study, 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 significant and suggests that the fish might be a good contamination indicator of water with sewage and agricultural run-off.

Key words: *Cryptosporidium parvum***,** ornamental fish**,** 18S rRNA, PCR.

Introduction

Cryptosporidium parvum (*C. parvum*), a protozoan parasite, is a primary etiologic agent of gastrointestinal illnesses in mammals and potentially lethal for humans and animals who are immunosuppressed (Fontaine and Guillot, 2003; Xiao and Feng, 2008). The infectious stage of *C. parvum* is a protozoan oocyst. At outside of its mammalian host, the oocyst is biologically dormant and does not replicate and increase its numbers. Various transmission routes, such as direct contact with infected persons (person-to-person transmission) or animals (zoonotic transmission) and ingestion of contaminated food (foodborne transmission) and water (waterborne transmission) have been reported worldwide (Xiao, 2010).

Current knowledge of the epidemiology, taxonomy and pathology, and host

specificity of *Cryptosporidium* species infecting fish species is limited. *Cryptosporidium* has been described in more than 17 species of both fresh and salt water fish with parasites located deep within and on the surface of the stomach or intestinal epithelium (Koinari et al., 2013). To date, two important species of *Cryptosporidium* for public health, namely *C. parvum* and *Cryptosporidium hominis* (*C. hominis*) have been identified in fish samples from cultured and wild fresh water and marine environments (Reid et al., 2010; Koinari et al., 2013). Considering the great commercial importance of the aquarium industry in all countries, zoonotic character of *C. parvum* and lack of knowledge about infection in cultured ornamental fish, this study was performed to analyze the distribution of zoonotic *C. parvum* in some aquarium fish species using Nested-PCR method and sequence analysis.

Materials and Methods

Sampling

A total of 100 fresh water ornamental fish, belonging to 22 species were collected from pet shops in different cities: Tehran, Tabriz, Zanjan and Shahindej, 25 samples from each city. On arrival in the laboratory, 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. 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 molecular screening.

Histology

Once the intestinal and stomach tissues were fixed, they were embedded in paraffin. Histological sections were prepared at 5 µm thickness and stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS). Sections were examined with an Olympus light microscope at 200 and 400 fold magnifications.

DNA extraction and Cryptosporidium genotyping

The preserved intestines and stomachs were washed five times with water to remove ethanol. The DNA was extracted from 25 mg of the intestine and stomach tissues using MBST-DNA extraction kit (Iran. Tehran) according to the manufacturer's instructions. All extracted DNA samples were stored at -20 °C until required for screening. All specimens were genotyped on the basis of the 18S rRNA gene by Nested-PCR method as previously described (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, that external primers amplify a 844bp fragment from genotype 1

and a 840-bp fragment from genotype 2 and Nested primers amplify a 593-bp fragment from genotype 1 and a 590-bp fragment from genotype 2 (Sturbaum et al., 2001).

Briefly, amplification of the 18S rRNA gene was carried out in 25 µL reaction volumes containing 2 µL of DNA templates, 12/5 µL of master mix (the main mixture with 2x concentration included Taq DNA Polymerase, dNTPs, MgCl2, PCR buffer) (CinnaGen Co. Iran) and 5-9 pmol of reverse and forward primers and purified, sterile water. The PCR parameters used in the external reaction included an initial denaturation at 95°C for 5 min followed by 40 cycles of 94°C for 45 s, 58°C for 75 s, and 72°C for 45s. Final extension was carried out at 72°C for 7 min. The nested-reaction parameters were the same except that 35 cycles were performed at an annealing temperature of 67°C and dehybridization, annealing, and extension time periods were 25 s each. PCRs were performed in a thermal cycler (MWG Biotech-Germany). To verify the results, 8 µL of each PCR products were mixed with 2 µL loading dye (5x) and were visualized and photographed on 1.2% agarose gels stained with ethidium bromide (0.5 µg/µl) following UV transillumination. The PCR products were purified using a PCR purification kit (MBST, Tehran, Iran) and were analyzed by sequencing (Bioneer, Korea). The sequence alignment was checked for sequencing accuracy using Bio Edit sequence Alignment and then were 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 *Cryptosporidium parvum* positive by Nested PCR (Fig 1). BLAST analysis revealed that this sequenced fragment was similar to the most of sequences of *Cryptosporidium parvum* 18S ribosomal RNA gene deposited in the GenBank (Fig 2). The greatest similarity is observed with *Cryptosporidium parvum* 18S ribosomal RNA gene, complete sequence under accession number AF222998.1; with Query Cover 100% and Identical Value 99%.

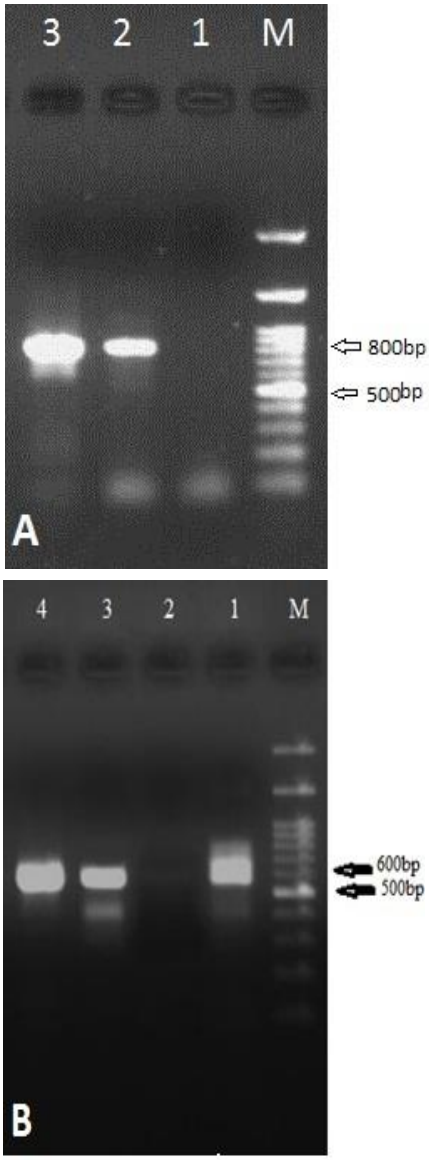

Fig. 1. A (1st run): Nested PCR amplification of a segment within the 18S rRNA of *Cryptosporidium* species; Lane 1: negative control, Lanes 2, 3: positive samples (840 bp product), Lane M: 100-bp molecular marker. B (2nd run): Lane 1: positive control, Lane 2: negative control, Lane 3, 4: positive samples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

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

Sample   60  ACTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAAACTCGACTTTAT 119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum 133 ACTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAAACTCGACTTTAT 192

Sample   120 GGAAGGGTTGTATTTATTAGATAAAGAACCAATATAATTGGTGACTCATAATAACTTTAC 179
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum 193 GGAAGGGTTGTATTTATTAGATAAAGAACCAATATAATTGGTGACTCATAATAACTTTAC 252

Sample   180 GGATCACATTAAATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGG 239
             |||||||| | ||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum 253 GGATCACAATTAATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGG 312

Sample   240 GTATTGGCCTACCGTGGCAATGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGG 299
             ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum 313 GTATTGGCCGACCGTGGCAATGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGG 372

Sample   300 AGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCT 359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum 373 AGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCT 432

Sample   360 AATACAGGGAGGTAGTGACAAGAAATAACAATACAGGACTTTTTGGTTTTGTAATTGGAA 419
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum 433 AATACAGGGAGGTAGTGACAAGAAATAACAATACAGGACTTTTTGGTTTTGTAATTGGAA 492

Sample   420 TGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGC 479
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum 493 TGAGTTAAGTATAAACCCCTTTACAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGC 552

Sample   480 CGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGT 539
             |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
C.parvum 553 CGCGGTAATTCCAGCTTCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGT 612

Sample   540 TGGATTTCTGTTAATAATTTATATAAAATATTTTGATGAATATTTATATAA  590
             ||||||||||||||||||||||||||||||||||||||||||||||||||
C.parvum 613 TGGATTTCTGTTAATAATTTATATAAAATATTTTGATGAATATTTATATAA  663
```

Fig. 2. BLAST analysis of the *Cryptosporidium* 18S-rRNA gene products from the nested PCR and compared with *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

In the present study, the overall prevalence of *Cryptosporidium* spp. Determined by histology was 16%, which was nearly similar to prevalence of 10.5% for *Cryptosporidium* reported in ornamental fish by molecular studies (Zanguee et al., 2010). Previous studies in fish species have reported a different prevalence 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 higher prevalence (10-100%), mostly among juvenile fish (Alvarez-Pellitero et al., 2004; Sitja-Bobadilla et al., 2005; Murphy et al., 2009). A previous study monitored *Cryptosporidium* in aquarium fish of different species in Western Australia and found that the great prevalence of *Cryptosporidium* in ornamental fish was likely associated with crowded environment of the aquarium tanks and the frequent 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*), gold fish (*C. auratus auratus*), pearl gourami (*T. leerii*), siamese fighting fish (*B. splendens*), slender rainbow (*M. gracilis*) and electric yellow (*L. caeruleus*). This is also the first study in which *C. parvum* has been identified in goldfish.

The pathogenesis of the *Cryptosporidium* species identified in the present study is unknown. However, some affected fish exhibited 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 detected in the intestine with emaciation in one gold fish while the other one was apparently healthy. Since some affected ornamental fish were apparently healthy it seems that *Cryptosporidium* species, namely *C. parvum* represent actual or mechanical infections remain to be determined as the oocysts might pass through rather than infecting these fish.

In the present 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 cultured freshwater (Nile tilapia), wild freshwater

(silver barb) and a marine (mackerel scad) fish. The detection of zoonotic and livestock species of *Cryptosporidium* in ornamental fish could be due to waterborne contamination with human and animal waste (Reid et al., 2010). Millions of oocysts of *C. parvum* can be released from infected humans and animals into the environment and 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 probability of infection was nearly one for urban anglers while fishing and consuming caught fish (Roberts et al., 2007). On the other hand, the zoonotic *C. parvum* in ornamental fish can constitute a major 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 affect the native freshwater fish through competition, predation and the introduction of diseases (Zanguee et al., 2010).

Conclusions

The current knowledge of epidemiology, taxonomy, and pathology and host specificity of *Cryptosporidium* species infecting fish species is limited. 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.

Acknowledgments

The first author is grateful to Research affairs of University of Tabriz, Iran, for supporting financially.

References

Alvarez-Pellitero P., Quiroga M.I., Sitja-Bobadilla A., Redondo M.J., Palen-zuela O., Padros F., and Nieto J.M. (2004). *Cryptosporidium scophthalmi* n. sp. (Apicomplexa: Cryptosporidiidae) from cultured turbot *Scophthalmus maximus*. Light and electron microscope description and histopathological study. *Diseases of Aquatic Organisms*, 62, pp. 133–145.

Fontaine M. and Guillot E. (2003). An immunomagnetic separation–real-time PCR method for quantification of *Cryptosporidium parvum* in water samples. *Journal of Microbiology Meth*, 54, pp. 29– 36.

Guyot K., Follet-Dumoulin A., Lelievre E., Sarfati C., Rabodonirina M., Nevez G. and Dei-Cas E. (2001). Molecular characterization of *Cryptosporidium* isolates obtained from humans in France. *Journal of Clinical Microbiology*, 39, pp. 3472–3480.

Koinari M., Karl S., Ng-Hublin J., Lymbery A.J. and Ryan U.M. (2013). Identification of novel and zoonotic *Cryptosporidium*

species in fish from Papua New Guinea. *Veterinary Parasitology*, 198, pp. 1-9.

Morine M., Yang R., Ng J., Kueh S., Lymbery A.J. and Ryan U.M. (2012). Additional novel *Cryptosporidium* genotypes in ornamental fishes. *Veterinary Parasitology*, 190, pp. 578–582.

Murphy B.G., Bradway D., Walsh T., Sanders G.E. and Snekvik K. (2009). Gastric cryptosporidiosis in freshwater angelfish (*Pterophyllum scalare*). *Journal of Veterinary Diagnosis Investigation*, 21, pp. 722–727.

Reid A., Lymbery A., Ng J., Tweedle S. and Ryan U. (2010). Identification of novel and zoonotic *Cryptosporidium* species in marine fish. *Veterinary Parasitology*, 168, pp. 190–195.

Roberts J.D., Silbergeld E.K. and Graczyk T. (2007). A probabilistic risk assessment of *Cryptosporidium* exposure among Baltimore urban anglers. *Journal of Toxicology and Environmental Health*, 70, pp. 1568–1576.

Sitja-Bobadilla A., Padros F., Aguilera C. and Alvarez-Pellitero P. (2005). Epidemiology of *Cryptosporidium molnari* in Spanish gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.) cultures: from hatchery to market size. *Applied Environmental Microbiology*, 71, pp. 131–139.

Shahbazi P., Shayan P., Ebrahimzade E. and Rahbari S. (2009). Specific Egg Yolk Antibody against Recombinant *Cryptosporidium parvum* P23 Protein. *Iranian Journal of Parasitology*, 4(3), pp. 15-24

Sturbaum G.D., Reed C., Hoover P.J., Jost B.H., Marshall M.M. and Sterling C.R. (2001). Species-specific, Nested PCR-restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocytes. *Applied Environmental Microbiology*, 67(6), pp. 2665-2668.

Xiao L., Escalante L., Yang C., Sulaiman I., Escalante A.A., Montali R.J., Fayer R. and Lal A.A. (1999). Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. *Applied Environmental Microbiology*, 65, pp. 1578–1583.

Xiao L. and Feng Y. (2008). Zoonotic cryptosporidiosis. *FEMS Immunology and Medical Microbiology*, 52(3), pp. 309-23.

Xiao L. (2010). Molecular epidemiology of cryptosporidiosis: an update. *Experimental Parasitology*, 124, pp. 80-89.

Zanguee N., Lymbery J.A., Lau J., Suzuki A., Yang R., Ng J. and Ryan U.M. (2010). Identification of novel *Cryptosporidium* species in aquarium fish. *Veterinary Parasitology*, 174, pp. 43–48.