Tehran University of Medical Sciences Publication
http://tums.ac.ir

Iran J Parasitol

Open access Journal at
http://ijpa.tums.ac.ir

Iranian Society of Parasitology
http://isp.tums.ac.ir

Original Article

The Larval Stages of *Echinostoma* spp. in Freshwater Snails as the First and Second Intermediate Hosts in Gilan and Mazandaran Provinces, Northern Iran

Mojgan Aryaeipour [1,2], Ramin Mazaheri Nezhad Fard [3], Mohammad Bagher Molai Rad [2], Majid Pirestani [4], Soheila Rouhani [5], Ahmad Daryani [6], Tina Asadi [7], *Shahabeddin Sarvi [6], *Mohammad Bagher Rokni [2]

1. *Student of Research Committee, Mazandaran University of Medical Sciences, Sari, Iran*
2. *Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
3. *Department of Medical Pathobiology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
4. *Parasitology and Entomology Department, Medical Sciences Faculty, Tarbiat Modares University, Tehran, Iran*
5. *Department of Medical Parasitology and Mycology, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
6. *Toxoplasmosis Research Center, Communicable Disease Institute, Mazandaran University of Medical Sciences, Sari, Iran*
7. *Comparative Zoology, Institute for Biology, Humboldt University of Berlin, Berlin, Germany*

Received 16 Nov 2022
Accepted 18 Mar 2023

Keywords:
Echinostoma;
Cercaria;
Metacercaria;
Freshwater snail;
Iran

***Correspondence Email:**
shahabesarvi@yahoo.com
roknimoh@tums.ac.ir

Abstract

Background: Identification of the larval stages of *Echinostoma* spp. in freshwater snails is an essential guide to continue monitoring the possibility of their transmission and the potential of echinostomiasis in areas where trematodes are the primary agent of parasitic diseases. The aim of this study was investigate *Echinostoma* using morphological and molecular techniques.

Methods: The study was conducted in Gilan and Mazandaran Provinces, northern Iran, from April 2019 to October 2021. Overall, 5300 freshwater snails were randomly collected and were identified using external shell morphology. Meanwhile, snails infected with trematodes were studied via shedding and dissecting methods. Larvae stages of *Echinostoma* were identified and the genomic DNA of the samples was extracted. The PCR amplification of the ITSI gene was carried out for 17 isolates and products were sequenced. Seven sequences were deposited in GenBank.

Results: Totally, 3.5% of snails containing three species (*Stagnicola* sp., *Radix* sp. and *Planorbis* sp.) were infected with two types of cercaria, *E. revolutum* with 37 and *Echinostoma* sp. with 45 spines in the collar. Moreover, 35% of the snails were infected with *Echinostoma* spp. metacercaria. Phylogenetic analysis illustrated that isolates were included in two ITSI haplogroups.

Conclusion: Results showed the potential hazard of a zoonotic parasite as *Echinostoma* in northern Iran. The potential of disease environmental relationship investigation and resource control optimization is necessary for effective disease prevention and health management.

Introduction

Echinostomes include the family Echinocazmide, Himastlide and Echinostomatide, as a large group of intestinal trematodes. Of the Echinostomidae family, *Echinostomes* are the most critical genus that causes zoonotic disease in humans, known as echinostomiasis (1). There are at least eight species that infect humans, including *Echinostoma revolutum, E. cinetorchis*, *E. echinatum* (needs confirmation), *E. lindoense, E. mekongi, E. miyagawai* (experimental), *E. paraensei* (from the coprolite of a human mummy) and *E. paraulum* (2).

The complex life cycle course of *Echinostomes* progresses with multi hosts. Fishes, reptiles, birds, and mammals play roles as their natural definitive hosts (2). Moreover, various species of freshwater snails play dual roles in the first and the second intermediate hosts of *Echinostomes*, namely, *Indoplanorbis* spp., *Gyraulus* spp., *Lymnaea* spp., *Pila* spp., *Viviparus* spp., *Filopaludina* spp. and *Bithynia* spp. as well as amphibians and fish as second intermediate hosts (3, 4).

In Indonesia and the Philippines, dogs and mice have been reported as reservoirs (5). Other parasitic infections reported from the snails mentioned above include fascioliasis, schistosomiasis, clonorchiasis, heterophyiasis, amphistomiasis and paragonimiasis (6). Accurately detecting species of parasites in different larval stages is very difficult morphologically and in some cases, impossible; however, molecular studies provide this capability field (7). Gilan and Mazandaran lay south of the Caspian Sea in northern Iran. Agricultural and animal husbandries in these areas result in the reproduction of intermediate host snails related to trematode parasites.

The present study was performed in the margin of the Caspian Sea by morphological and molecular methods to identify *Echinostomes* in the intermediate host snails. Results of this study can be a warning for the possibility of echinostomiasis in the study area.

Materials and Methods

Sampling procedures

Within two years of study (2019–2021) in Gilan and Mazandaran Provinces, northern Iran, 5300 freshwater snails were investigated (8). Snails randomly were collected using a strainer and pliers with a count per unit of time sampling method (9). Their geographical coordinates were recorded using a Global Positioning System (GPS) located in Gilan and Mazandaran provinces. The map of sampling locations was plotted using GIS Arc 10 software.

Identification of freshwater snails and examination for larval trematode infections

The snails were identified using taxonomic keys (10). Infected snails were distinguished and isolated. Larval species were preliminarily determined morphologically using a light microscope (Stereomicroscope Zeiss, Germany) and identified as the main type level (11). The hepatopancreas and gonads of snails were examined for the presence of redia and round cyst-like bodies.

Morphological analyses

For the initial identification of cercaria, vital dyes are used for live specimens. Larvae stage specimens preserved in 4% formalin solution were stained with trichrome and fall, dehydrated in a graded ethanol series, cleared with methyl salicylate and mounted on glass slides by Canada balsam. Invert and Light microscopy performed photos of the larval stages before and after being mounted. Image analysis software used measurements (in µm) (https://ostovaee.ir/fa/product/anix-image-analysis-software/). The sizes are given with ranges followed by means in parentheses. His-

tological analysis was performed on collected snails to detect the embryonic stages of larvae (12). Sections (5–6 µm thick) were stained with Mayer's hematoxylin or Erlich's hematoxylin-eosin.

Molecular analyses

DNA extraction

Each sample of cercaria, metacercaria and rediae was washed in distilled water (DW) three times and digested for 1–3 h at 56°C in 200 µl lysis buffer containing 10 mM Tris–HCl (pH 7.6), 20 mM NaCl, 100 mM EDTA (pH 8.0), 1% sarkosyl and 0.1 mg/ml proteinase K. Genomic DNA was extracted from the supernatant using Iranian DNGTM-PLUS Kit (*Cinacolon*, Iran) (13) and Add Prep Genomic DNA Kit (Product Code: 10023; AddBio, Korea) (14).

PCR amplification and sequencing

Polymerase chain reaction (PCR) amplification of partial fragment (~750 bp) of internal transcribed spacer region was carried out using two pairs of primers, A for (5'–CAGCTATGGTTCCTTAGATGTA-3'), C rev (5'–ATTCCATTATTCCATGCAAG-3') and, B for (5'–GCCAAGGATGTTTTCATTGATCT-3'), B rev (5'–GAAACCGTCATTGTAGCGCA -3') Bioneer, Korea (7). The total volume of the reaction was 25 µl containing 2 µl DNA template, 8.5 µl distilled water, 10 pmol of each primer (Forward and Reverse) and 12.5 µl master mix (Taq DNA Polymerase Master Mix RED; Amplicon, Denmark). PCR amplification was performed using Eppendorf Mastercycler Gradient Thermocycler, Germany. The temperature program was as follows: an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for the 30 s (denaturation), 49 °C for 30 s (annealing), 1 min at 72 °C (extension) and a final extension of 10 min at 72 °C. The PCR product was electrophoresed on a 1.2% agarose gel with added safe stain in TB buffer at 60 V for 40 min and visualized by UV illumination (UVITEC, UK). To estimate the size of the amplicons, a 50-bp DNA ladder (Thermo Fisher Scientific, USA) was used and deposited in GenBank. The PCR products were sequenced with the Sanger method using the same PCR primers. All resulting sequences were edited and trimmed by sequencer software version 5.4 (https://sequencher.software.informer.com/5.4/). The ITSI sequencing data was analyzed using the BLAST programs and databases of NCBI (http://blast.ncbi.nlm.nih.gov/Blast). Multiple sequence alignments were performed with the multalin program (http://multalin.toulouse.inra.fr/multalin/) and compared with the sequences present in the GenBank database.

Phylogenetic analysis

The phylogenetic tree and evolutionary analyses were performed on MEGA X software (15). The evolutionary history was induced utilizing the Neighbor-Joining method (16). Percentage of clustered replication trees (1000 replications) of new taxa related in the bootstrap test. (17). Developmental separations were computed using the Tamura-Nei method (18) and are in the units of the number of base substitutions per location. Distances were computed using the Tamura-Nei method (18). The haplotype network inferred by using haplotypes of ITSI regions was constructed (19).

Ethics approval

The Ethical Committee of Research, Tehran University of Medical Sciences, approved this study (no. 626).

Results

Vector snails infected

Larval stages of *Echinostoma* spp. were detected in natural freshwater snail populations sampled in seven of the 50 foci in northern Iran. Lymnaeidae and Planorbidae snails had acted as first and second intermediate hosts of *Echinostoma* sp. and *E. revolutum* complex in

the areas studied: *stagnicola* sp., *Radix* sp. and *Planorbis* sp. from Dehcola (52° 29' 8" N, 18° 38' 13" E), Sheikhmahaleh (52° 49' 46" N, 18° 42' 3" E), Dastak (52° 28' 0" N, 18° 19' 18" E), Chamkhaleh (52° 51' 14" N, 18° 38' 38" E), in Gilan Province and, Amol (52° 28' 58" N, 18° 19' 34" E), Noor (53° 36' 52" N, 19° 30' 39" E) Siacola (54° 10' 45" N, 16° 11' 15" E), in Mazandaran Province. The map of sampling locations and distribution of infected freshwater snails with *Echinostoma* spp. from the different foci was drowned with GIS Arc 10 software (Figs. 1 and 2).

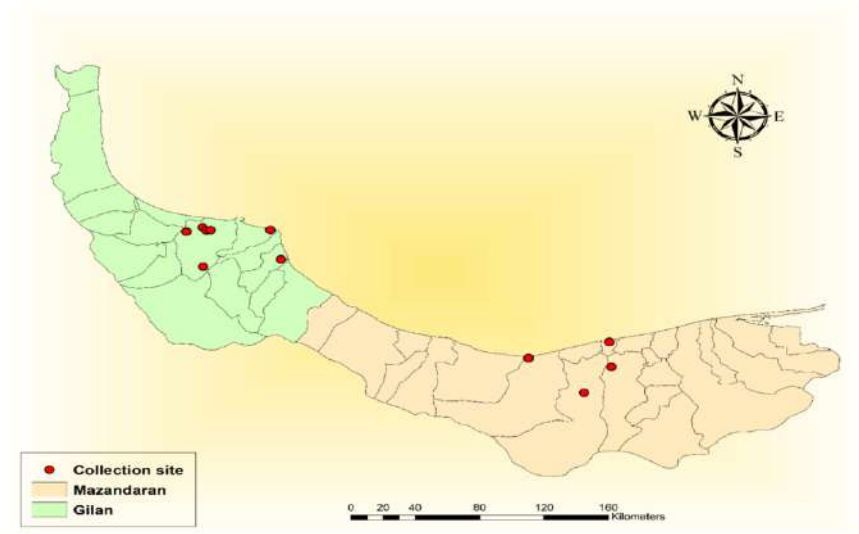

Fig. 1: Distribution of infected freshwater snails with *Echinostoma* spp. from northern Iran using GIS Arc 10 software

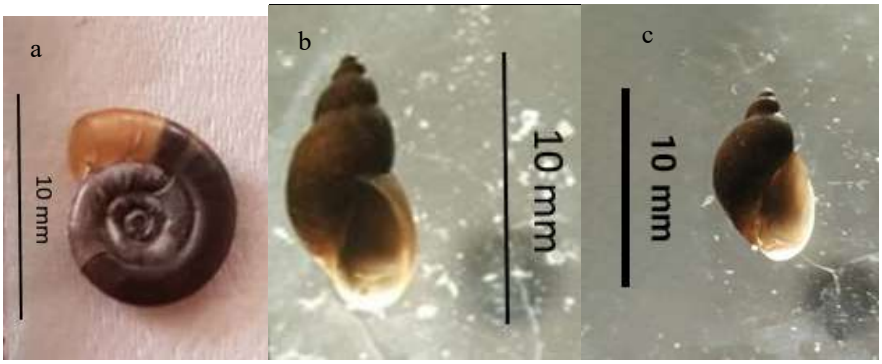

Fig. 2: Vector snails of *Echinostoma* sp. a, *Planorbis* sp.; b, *Stagnicola* sp.; and c, *Radix* sp.

Emerging larval forms

Nearly 3.5% of the snails (*Stagnicola* sp., *Radix* sp. and *Planorbis* sp.) were infected with *Echinostoma* cercaria. Prevalence of *Echinostoma* metacercaria, were higher than cercaria (30–40%).

Morphologic characteristics of larval Echinostoma spp.

According to the keys, a detailed examination of cercarial morphology allowed us to identify two types of *Echinostomes* cercaria of the isolated samples in Gilan and Mazandaran (Figs. 3, 4). One type be-

longs to the *E. revolutum*, distinguished by the cercaria features: (i) 37 collar spines and (ii) tail with a tip form. Another type belongs to *Echinostoma* sp. with (i) 45 collar spines and (ii) a tail with a rounded end (Fig. 3). The bodies were covered with very fine spines. Two prominent suckers were present. Twelve small peripheral gland cells of the esophagus with excretory ducts around the oral sucker were seen in *Echinostoma* spp. (Fig. 4) (20). Morphometry of 20 cercariae of *Echinostoma* spp. was done. The measurements below are in micrometers. Each cercaria possesses a spindle-shaped body, ovoid to triangular, tapering to the anterior part (collar), 30–80 (55) long by 40–70 (55) wide. The length of the body cercaria was 120–330 (175) long by 30–120 (75) wide. The tail was long and thin, 140–420 (280) long by 30–40 (35) wide. The length of each cercaria was 260–750 (505). The ventral sucker was post-equatorially 30–60 (45) in length. The sizes are given with ranges followed by means in parentheses. The ratio of body length to tail length (BL/TL), 0.77 (0.65), body length to width (BL/BW) 2.5 (2.3) and tail length to body length (TL/BL) 1.3 (1.5) were calculated. The ratio sizes are given in *E. revolutum* followed by *Echinostoma* sp. in parentheses (Fig. 3). Comparing the measured data collected for live cercaria revealed that *E. revolutum* had a shorter tail.

Fig. 3: Cercaria fixed in 4% formaldehyde solution. a) *E. revolutum*, 37 collar spines (MW829605.1); and b) *Echinostoma* sp., 45 collar spines (MZ436902) (original)

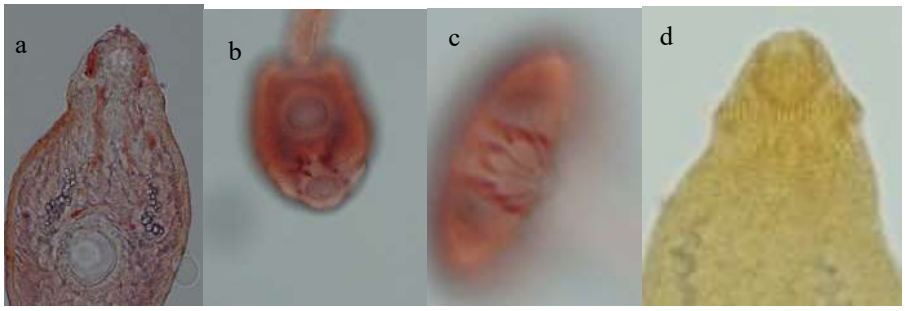

Fig. 4: *Echinostoma* spp., microphotographs of live cercaria. a) Body, ventral view; b and c) dorsal view showing para-oesophageal gland-cells and outlets (staining with neutral red); and d) head collar, ventral view showing angle and lateral spines (original)

Daughter rediae intramolluscan stages developing in Lymnaeidae snails with multiple live cercarial forms had a fusiform body. Ten daughter rediae measured 0.96–1.5 long (1.2) by 0.22–0.28 wide (0.25), pharynx 0.059–0.107 (0.081) by 0.057–0.104 (0.078) wide (Fig. 5).

Histological sections of mother sporocysts and rediae were collected from the digestive gland of the host snail containing germ cells, germ balls and cercarial embryos.

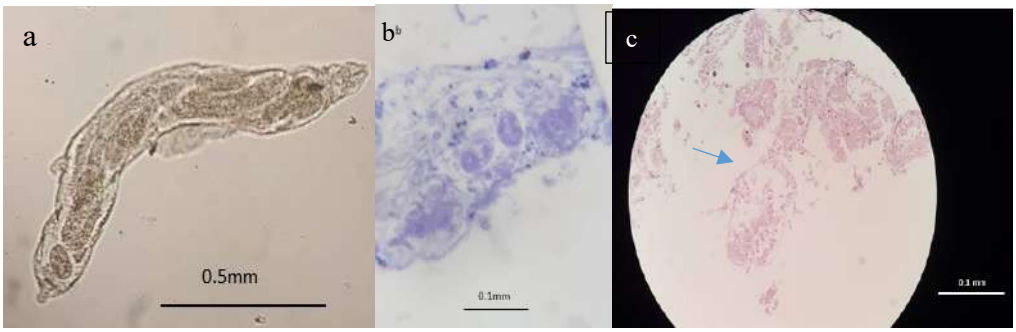

Fig. 5: a) Daughter rediae of *E. revolutum* developing in Lymnaeid snails with cercaria inside; b) histological sections of mother sporocysts; and c) histological sections of *E. revolutum* inside of rediae (MW829611.1) (original)

Metacercaria of *E. revelutum*, folded within a transparent cyst were collected from dissecting snails, morphologically spherical, 150-170 µm in diameter, with a thin wall (n = 10). Spines and large excretory granules were visible. Cysts collected from dissecting snails were clumped together in the pericardial (Fig. 6). Fig. 6 (b) shows the development of an *Echinostome* larva released from an encysted form.

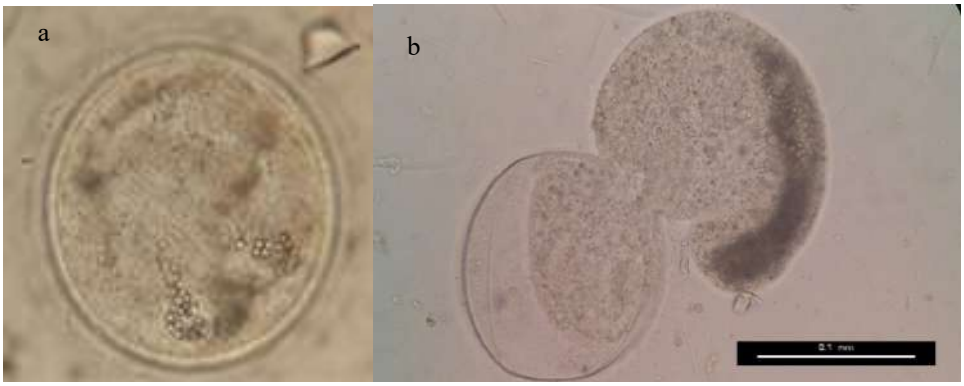

Fig. 6: Metacercaria of *E. revolutum* (150 µm in diameter). a) Spines arrangement and excretory vesicles in metacercaria (MZ417523.1); and b) development of an *Echinostoma* larva released from an encysted form (original)

Molecular characterization

Molecular characterization of sequences from ITSI, 5.8S region was collected from ten cercaria, five metacercaria and two rediae isolated from the intermediate host identified as *Stagnicola* sp., *Radix* sp. and *Planorbis* sp. Both of DNGTM-PLUS Kit and of Add Prep Genomic DNA Kit was suitable for the extraction of genomic DNA from larvae stages of Trematode. After optimizing the PCR protocol, all *Echinostom* spp. isolates successfully demonstrated amplifying about 700-bp target band for the ITSI. The sequences were aligned and compared with the sequences available in GenBank. The seven sequences are listed in Table 1 to show the corresponding accession numbers submitted

to GenBank and the original province of each isolate.

Two ITSI haplogroups were detected including seven haplotype, four in haplotype 1 and three in haplotype 2. The phylogenetic tree was constructed based on ITSI to evaluate the haplotypes recovered in this study and retrieved sequences from other world regions (Fig. 7).

Table 1: Isolates of the *Echinostoma* spp. collected in this study and another regions in the world

Species	*Stage*	*Host*	*Location*	*Accession no.*
E. revolutum	Cercaria	*Stagnicola* sp.	52° 29' 8" N, 18° 38' 13" E	MW829606.1*
	Rediae	*Stagnicola* sp.	52° 51' 14" N, 18° 38' 38" E	MW829611.1*
	Cercaria	*Stagnicola* sp.	52° 49' 46" N, 18° 42' 3" E	MW829612.1*
	Cercaria	*Radix* sp.	52° 28' 0" N, 18° 19' 18" E	MW829605.1*
E. paraensei	Adult	*Mesocricetus auratus*	U.K	AY222132.1
		-------	Malaysia	MK421334.1
	Adult	Hamster	USA	FJ380226.1
	Cercaria	*Biomphalaria pfeifferi*	Zimbabwe	MT994246.1
E. revolutum	Reriae	*Planorbis* sp.	52° 28' 0" N, 18° 19' 18" E	MZ436915.1*
	Metacercaria	*Radix* sp.	52° 29' 8" N, 18° 38' 13" E	MZ417523.1*
			Vietnam	OM956143.1
			Vietnam	OM956144.1
Echinostoma sp	Cercaria	*Radix* sp.	52° 51' 14" N, 18° 38' 38" E	MZ436902.1*

* Isolates of the *Echinostoma* spp. in Iran

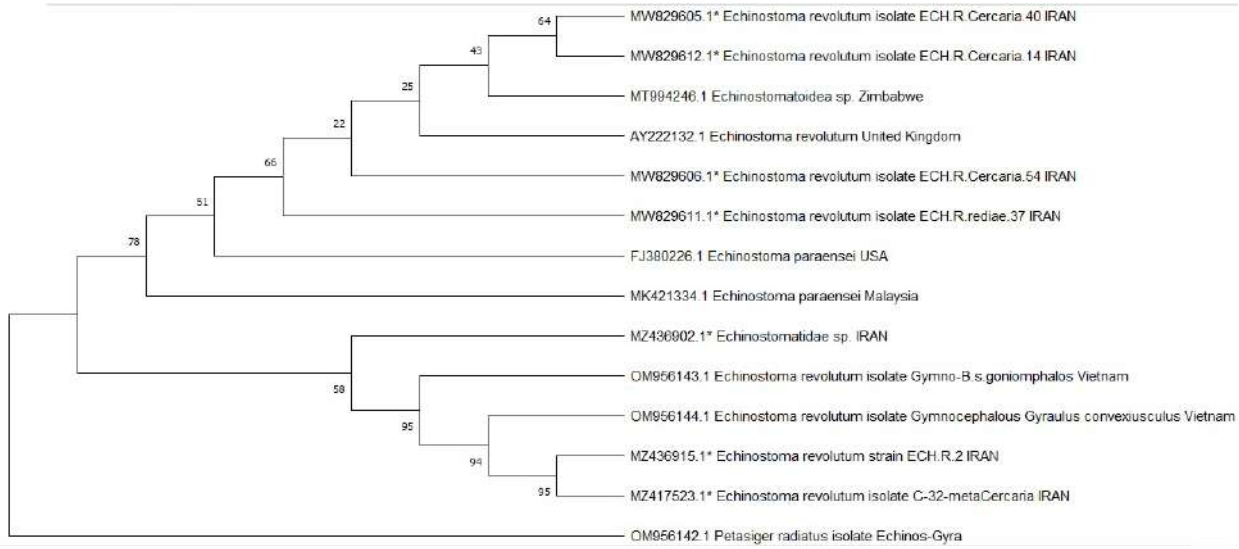

Fig. 7: Phylogenetic tree of *Echinostoma* spp. isolates collected in this study based on ITSI gene sequences and constructed tree using Tamura 3-parameter model by MEGA X software. Bootstrap was set as 1000 replication

Discussion

Echinostoma is a food-borne intestinal trematode that needs one or two snail hosts to complete its life cycle (5). Various types of food animals, including freshwater fish, amphibians and freshwater snails, play roles in human parasitical infections (2). Human echinostomiasis is an endemic disease, mainly in low-income countries in Southeast Asia and the Far East (1). However, the level of at-risk population elevates because of infection, geographical border development and factors such as international markets, expanding transport systems improvement, new diets and nourishment in developed countries and demographic changes (1). Almost, Snail-borne infections are emerging as a common disease, mainly due to their chronic nature (21). Of the reported cases of human echinostomiasis, one was a 62-year-old man with gastrointestinal symptoms in Nepal (22). The other was an unusual case of echinostomiasis without gastrointestinal symptoms (23). In addition, in India, a three-year-old boy had reported severe anemia, gastrointestinal disorders, fish observation in the diet and no travel history (24). Due to the prolonged latent phase, short acute phase, asymptomatic manifestations and the clinical symptoms resemblance to other intestinal worms determining of time and place of infection will be challenging (25).

Despite the importance of this matter, echinostomiasis has been ignored in Iran for many years. Only during a study in Mazandaran Provinces (2015), three cases of echinostomiasis were reported from patients admitted to rehabilitation wards (26). As previously stated, the similarity of symptoms and ova of the parasite to *Fasciola* sp. makes the disease hidden, especially in areas where fascioliasis is native such as study areas (27, 28). In previous studies, *Echinostome* cercaria was reported in Viviparidae, Planorbidae, Lymnaeidae and thiaridae snails (29–31). Salahi-Moghaddam et al (32) and Sharif et al (33) from Mazandaran reported *Echinostoma* cercaria in *L. palustris* and *R. auricularia,* naturally, by Systematic key in Iran. This study identified two types of cercaria that characteristically revealed a prominent head collar with 37 and 45 collar spines as *E. revolutum* and *Echinostoma* sp. from *Stagnicola* sp., *Radix* sp. and *Planorbis* sp., snails, which serve the first and second intermediate (33).

Collar spines are arranged in double alternating rows. Lateral-group spines were more prominent than other head-collar spines. Other features of cercaria-included suckers, per pharynx, excretory vesicles, excretory canal, tail features, esophagous and peripheral glands of the esophagus (34). A comparison of metric data collected for cercaria shows high morphological diversity of *Echinostoma* spp. cercaria. In mother sporocysts and daughter rediae, developing in the Lymnaeid snails host and their histological sections, germ cells, germ balls and cercarial embryos of *E. revelutum* were seen (35). In addition, the metacercaria of E. revelutum was clumped together in the pericardial, morphologically spherical, 150.0–170.0 µm in diameter with a bilayered wall. Chantima from Thailand reported that Metacercaria of *E. revelutum* was spherical and 136.0–195.0 µm in diameter (36).

To confirm the specificity of the larval stage of *Echinostoma* spp., collected naturally, molecular methods have been carried out on Iranian isolates for the first time. Similar to a report of simultaneous infection of the snail with several species of trematode in a previous study (8), use of several larvae at the extraction stage can lead to errors in sequencing results. Sequences of the ITS1, 5.8S were compared to published sequences. Based on the results of this study, phylogenetic analysis indicated that the 14 isolates (haplotypes) were set in two ITSI haplogroups as well as an out-group. Haplotype 1 consisted of four isolates of *E. revolutum* (MW829606.1, MW829611.1, MW829612.1) in *Stagnicola* spp. and (MW829605.1) in *Radix* spp., showing partial homology with GenBank sequences of adult isolates in Mesocricetus from the United Kingdom (AY222132.1), larvae isolate of *Echinostoma paraensei* in *B. pfeifferi* from Zimbabwe (MT994246.1) and adult isolates in hamster from USA (FJ380226.1) and (MK421334.1) from

Malaysia. Haplotype 2 included rediae isolate (MZ436915) in *Planorbis* spp., cercaria isolate (MZ436902.1) and metacercaria isolate (MZ417523.1) in *Radix* spp., showing partial homology with GenBank sequences of larvae isolates in *Gyraulus* (OM956144.1) and *Goniomphalus* (OM956143.1) from Vietnam. It seems *Echinostoma* represents diverse haplotypes and needs further verification using further isolates from various regions of Iran to investigate its genetic variations. Sixteen species of Echinostoma are currently acknowledged as valid worldwide (37).

The parasitic rate of *Echinostoma* spp. was 3.4% using releasing cercaria technique and 30–40% using dissection technique for metacercaria infection. Salahi-Moghaddam reported 1.2% from Mazandaran (32) and Imani, 5.19% from West Azerbaijan (38), parasitized rate of *Echinostoma* spp. using releasing cercaria in Iran. The finding of the larval stages of *E. revolutum* in this research shows that eating raw or partially cooked fish and drinking contaminated water can be possible sources of human infection (39). The phenomenon of cercarial encystment in the presence of human gastric juice has been suggested as a possible mechanism for causing echinostomiasis (40). Uninterrupted defecation and night soil (wastes collected from the toilets) for fertilizing fishponds can cause infection (41). Serious health education should be provided and necessary training about diet and environmental behaviors should be given. Results can be helpful for the control and prevention program (42).

Conclusion

Microscopists must know essentials of detecting the parasite's eggs. The ultimate goal is to change people's behavior towards consuming raw and grilled aquatic products, untreated spring water and unsanitary disposal of human excreta in these areas.

Acknowledgements

This study was part of the Ph.D. thesis of the first author (M. Aryaeipour) that was supported financially by a grant from Mazandaran University of Medical Science (no. 7741) as a joint project with the Research Center for Endemic Parasites of Iran, Tehran University of Medical Sciences, Tehran, Iran (51185-211-3-99).

Conflict of Interest

The authors declare no conflict of interest.

References

1. Toledo R, Esteban J. An update on human echinostomiasis. Trans R Soc Trop Med Hyg. 2016;110(1):37-45.
2. Chai J-Y. Human intestinal flukes. Springer; 2019.
3. Soldánová M, Selbach C, Sures B, Kostadinova A, Pérez-del-Olmo A. Larval trematode communities in *Radix auricularia* and *Lymnaea stagnalis* in a reservoir system of the ruhr river. Parasit Vectors. 2010;3:56.
4. Faltýnková A, Našincová V, Kablásková L. Larval trematodes (digenea) of planorbid snails (gastropoda: Pulmonata) in central europe: A survey of species and key to their identification. Syst. Parasitol. 2008;69(3):155-178.
5. Ewens W.J, Warren J, Fried B, Toledo R. The Biology of Echinostomes: From the Molecule to the Community. 2009. Springer New York.
6. Owiny MO, Obonyo MO, Gatongi PM, Fèvre EM. Prevalence and spatial distribution of trematode cercariae in vector snails within different agro-ecological zones in western kenya, 2016. Pan Afr Med J. 2019;32:142.
7. Routtu J, Grunberg D, Izhar R,et al. Selective and universal primers for trematode barcoding in freshwater snails. Parasitol Res. 2014;113(7):2535-2540.
8. Aryaiepour M, Mansoorian AB, Rad MBM,et al. Contamination of vector snails with the larval stages of trematodes in selected areas in northern iran. Iran J Public Health. 2022;51(6):1400-1410.

9. Olivier L, Schneiderman M. A method for estimating the density of aquatic snail populations. Exp Parasitol. 1956;5(2):109-117.
10. Glöer P, Pešić V. The freshwater snails (gastropoda) of Iran, with descriptions of two new genera and eight new species. ZooKeys. 2012:11-61.
11. Yamaguti, Satyu. A Synoptical Review of Life Histories of Digenetic Trematodes of Vertebrates : with Special Reference to the Morphology of Their Larval Forms. [S.l.]: Yugaku -sha , 1975.
12. Ataev GL, Dobrovolskij AA, Fournier A, Jourdane J. Migration and development of mother sporocysts of *Echinostoma caproni* (digenea: Echinostomatidae). J Parasitol. 1997;83(3):444-453.
13. Aryaeipour M, Rouhani S, Bandehpour M, Mirahmadi H, Kazemi B, Rokni MB. Genotyping and phylogenetic analysis of *Fasciola* spp. Isolated from sheep and cattle using pcr-rflp in ardabil province, northwestern IRAN. Iran J Public Health. 2014; 43(10): 1364-1371.
14. Alizadeh G, Aryaeipour M, Mohebali M, Mowlavi GR, Raissi V, Rokni MB. Evaluation of semi-nested pcr compared with indirect-elisa to diagnose human fasciolosis. Iran J Public Health. 2022;51(3):686-694.
15. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. Mega x: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018; 35(6):1547-1549.
16. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4(4):406-425.
17. Shimodaira H, Hasegawa M. Consel: For assessing the confidence of phylogenetic tree selection. Bioinformatics. 2001;17(12):1246-1247.
18. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 1993;10(3):512-526.
19. Bandelt H-J, Forster P, Röhl A. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol. 1999;16(1):37-48.
20. Faltýnková A, Našincová V, Kablásková L. Larval trematodes (digenea) of the great pond snail, *Lymnaea stagnalis* (l.),(gastropoda, pulmonata) in central europe: A survey of species and key to their identification. Parasite. 2007;14(1):39-51.
21. Bozorgomid A, Rouhani S, Harandi MF, Ichikawa-Seki M, Raeghi S. Genetic diversity and distribution of *Fasciola hepatica* haplotypes in Iran: Molecular and phylogenetic studies. Vet Parasitol Reg Stud Reports. 2020;19:100359.
22. Sah R, Khadka S, Hamal R, Poudyal S. Human echinostomiasis: A case report. BMC Res Notes. 2018;11(1):17.
23. Khanna V, Tilak K, Mukim Y, Khanna R. An unusual case of echinostomiasis in a retropositive patient: A case report. Hum Parasit Dis. 2016;8:43.
24. Khanna V, Ashraf AA, Khanna R. Echinostomiasis in a child with severe anemia. Trop Parasitol. 2019;9(1):54-56.
25. Graczyk TK, Fried B. Echinostomiasis: A common but forgotten food-borne disease. Am J Trop Med Hyg. 1998;58(4):501-504.
26. Ahmadi M, Beigom Kia E, Rezaeian M, Hosseini M, Kamranrashani B, Tarighi F. Prevalence of strongyloides stercoralis and other intestinal parasites in rehabilitation centers in mazandaran province, northern iran. Journal of Mazandaran University of Medical Sciences. 2015;25:1-7.
27. Aryaeipour M, Kia EB, Heidari Z, Talaie ZS, Rokni MB. Serological study of human fasciolosis in patients referring to the school of public health, Tehran University of Medical Sciences, Tehran, Iran during 2008–2014. Iran J Parasitol. 2015;10(4):517-522.
28. Hosseini-Safa A, Rokni MB, Mosawi SH, et al. High-resolution melting analysis as an appropriate method to differentiate between fasciola hepatica and F. Gigantica. Iran J Public Health. 2019;48(3):501-507.
29. Chantima K, Suk-Ueng K, Kampan M. Freshwater snail diversity in mae lao agricultural basin (Chiang Rai, Thailand) with a focus on larval trematode infections. Korean J Parasitol. 2018;56(3):247-257.
30. Anucherngchai S, Tejangkura T, Chontananarth T. Epidemiological situation and molecular identification of cercarial stage in freshwater snails in chao-phraya basin, central Thailand. Asian Pac J Trop Biomed. 2016;6:539-545.

31. Krailas D, Namchote S, Koonchornboon T, Dechruksa W, Boonmekam D. Trematodes obtained from the Thiarid freshwater snail melanoides tuberculata (müller, 1774) as vector of human infections in Thailand. Zoosyst Evol. 2014;90:57.
32. Salahi-Moghaddam A, Mahvi AH, Mowlavi G, Hoseini-Chegini A, Massoud J. Parasitological study on *Lymnaea palustris* and its ecological survey by gis in mazandaran province. Pathobiol Res. 2009;11:65-71.
33. Sharif M, Daryani A, Karimi S. A faunistic survey of cercariae isolated from lymnaeid snails in. Pak J Biol Sci. 2010;13(4):158-163.
34. Yamaguti S. Synopsis of digenetic trematodes of vertebrates. Vols I and II. 1971. Keigaku Publishing Co.
35. Ataev GL, Coustau C. Cellular response to echinostoma caproni infection in *Biomphalaria glabrata* strains selected for susceptibility/resistance. Dev Comp Immunol. 1999;23(3):187-198.
36. Chantima K, Chai J-Y, Wongsawad C. Echinostoma revolutum: Freshwater snails as the second intermediate hosts in chiang mai, Thailand. Korean J Parasitol. 2013;51(2):183-189.
37. Georgieva S, Selbach C, Faltýnková A, et al. New cryptic species of the 'revolutum'group of echinostoma (digenea: Echinostomatidae) revealed by molecular and morphological data. Parasit Vectors. 2013;6:64.
38. ImaniBaran A. Survey of population changes and cercarial infection of lymnaea spp freshwater snails. In Miyandoab region, west azarbaijan province in 2010. Journal of Rafsanjan University of Medical Sciences. 2014;13:581-596.
39. Xiao X, Lu D, Wang T,et al. Studies on mode of human infection with *Echinochasmus liliputanus*. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi= Chinese journal of parasitology and parasitic diseases. 1995;13(3):197-199.
40. Chai JY, Sohn WM, Na BK, Van De N. Echinostoma revolutum: Metacercariae in Filopaludina snails from Nam Dinh Province, Vietnam and adults from experimental hamsters. Korean J Parasitol. 2011;49(4):449-455.
41. Tenorio JCB, Molina EC. Monsters in our food: Foodborne trematodiasis in the philippines and beyond. Veterinary Integrative Sciences. 2021;19:467-485.
42. Haveland-Smith R, Combes R. Screening of food dyes for genotoxic activity. Food Cosmet Toxicol. 1980;18(3):215-221.