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### 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

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Received 16 Nov 2022

Accepted 18 Mar 2023

#### Keywords:

*Echinostoma*;  
Cercaria;  
Metacercaria;  
Freshwater snail;  
Iran

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#### 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 ITS1 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 ITS1 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.



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## Introduction

*Echinostomes* include the family Echinocazmide, Himastlidae and Echinostomatidae, 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. ebinatum* (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 fast, 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  $\mu\text{m}$ ) (<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'-CAGCIATGGTTCCTTAGATGTA-3'), C rev (5'-ATTCCATTATTCCATGCAAG-3') and, B for (5'-GCCAAGGATGTTTTCATTTGATCT-3'), B rev (5'-GAAACCGTCATTTGTAGCGCA -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 ITS1 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 ITS1 regions was constructed (19).

#### **Ethics approval**

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

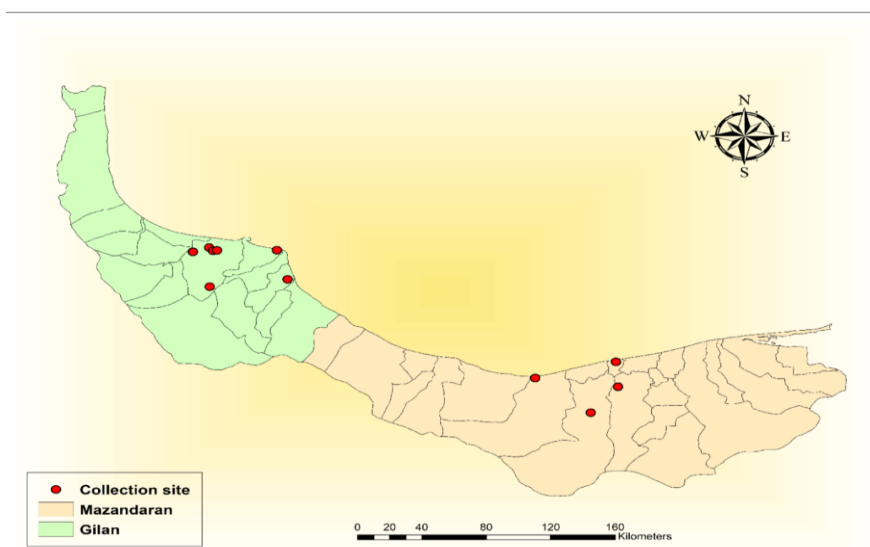
### **Results**

#### **Vector snails infected**

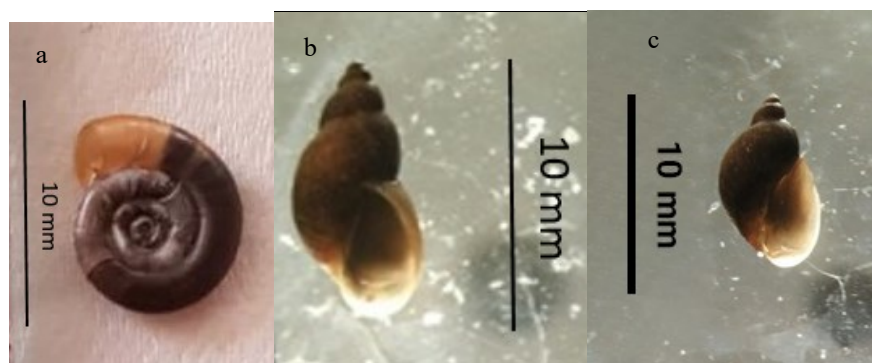
Larval stages of *Echinostoma* spp. were detected in natural freshwater snail populations sampled in seven of the 50 foci in northern Iran. Lymnaeidae and Planorbidae snails had acted as first and second intermediate hosts of *Echinostoma* sp. and *E. revolutum* complex in

the areas studied: *stagnicola* sp., *Radix* sp. and *Planorbis* sp. from Dehcola (52° 29' 8" N, 18° 38' 13" E), Sheikmahaleh (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 drawn 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)



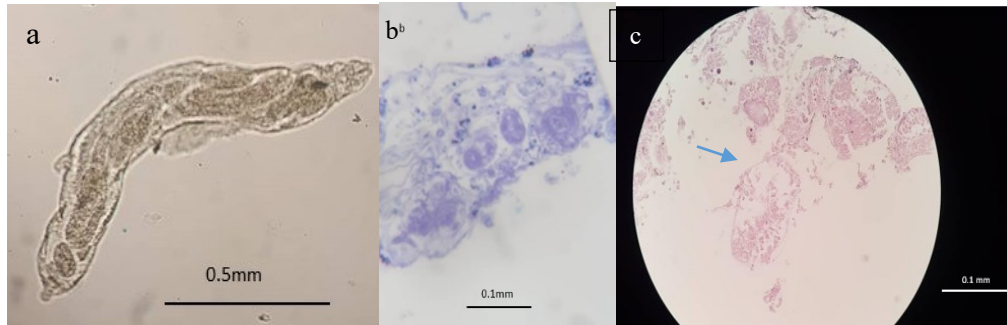
**Fig. 4:** *Echinostoma* spp., microphotographs of live cercaria. a) Body, ventral view; b and c) dorsal view showing para-oesophageal gland-cells and outlets (staining with neutral red); and d) head collar, ventral view showing angle and lateral spines (original)

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

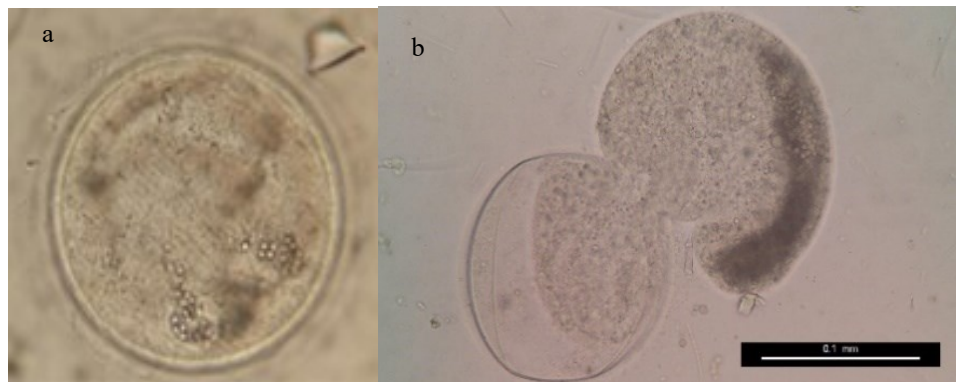
gland of the host snail containing germ cells, germ balls and cercarial embryos.



**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. revolutum*, folded within a transparent cyst were collected from dissecting snails, morphologically spherical, 150-170  $\mu\text{m}$  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 *Echinostoma* larva released from an encysted form.



**Fig. 6:** Metacercaria of *E. revolutum* (150  $\mu\text{m}$  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 ITS1, 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 extrac-

tion 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 ITS1. 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.

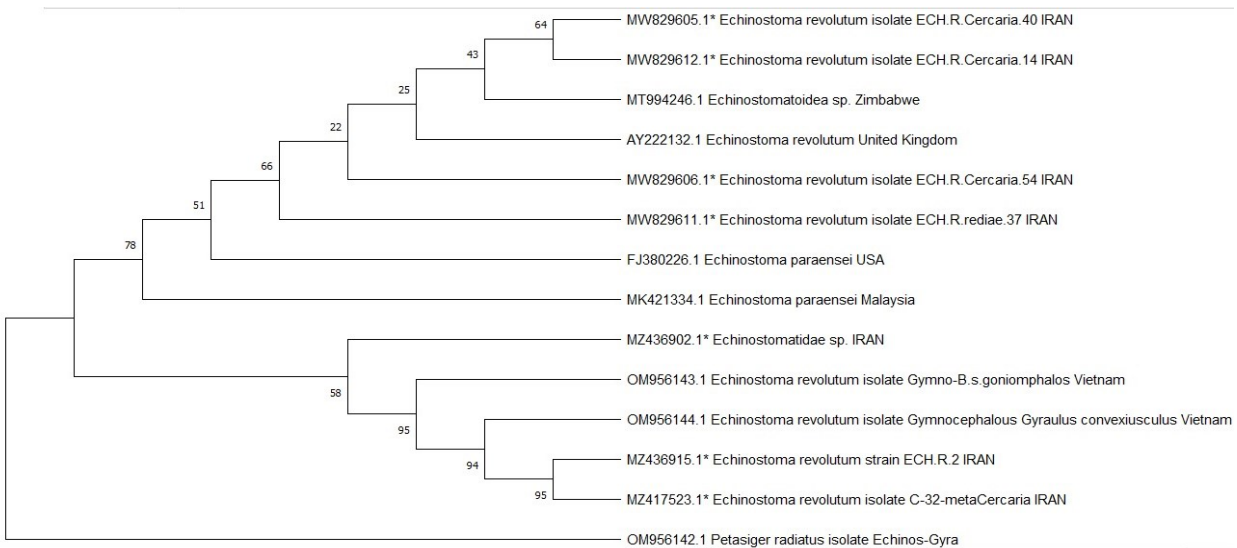
Two ITSI haplogroups were detected including seven haplotype, four in haplotype 1 and three in haplotype 2. The phylogenetic

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.
<i>E. revolutum</i>	Cercaria	<i>Stagnicola</i> sp.	52° 29' 8" N, 18° 38' 13" E	MW829606.1*
	Rediae	<i>Stagnicola</i> sp.	52° 51' 14" N, 18° 38' 38" E	MW829611.1*
	Cercaria	<i>Stagnicola</i> sp.	52° 49' 46" N, 18° 42' 3" E	MW829612.1*
	Cercaria	<i>Radix</i> sp.	52° 28' 0" N, 18° 19' 18" E	MW829605.1*
<i>E. paraensei</i>	Adult	<i>Mesocricetus auratus</i>	U.K	AY222132.1
	-----	-----	Malaysia	MK421334.1
	Adult	Hamster	USA	FJ380226.1
	Cercaria	<i>Biomphalaria pfeifferi</i>	Zimbabwe	MT994246.1
<i>E. revolutum</i>	Rediae	<i>Planorbis</i> sp.	52° 28' 0" N, 18° 19' 18" E	MZ436915.1*
	Metacercaria	<i>Radix</i> sp.	52° 29' 8" N, 18° 38' 13" E	MZ417523.1*
			Vietnam	OM956143.1
			Vietnam	OM956144.1
<i>Echinostoma</i> sp	Cercaria	<i>Radix</i> 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 parasitological 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, esophagus 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. revolutum* were seen (35). In addition, the metacercaria of *E. revolutum* was clumped together in the pericardial, morphologically spherical, 150.0–170.0  $\mu\text{m}$  in diameter with a bilayered wall. Chantima from Thailand reported that Metacercaria of *E. revolutum* was spherical and 136.0–195.0  $\mu\text{m}$  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 ITS1 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. Aryaiepour) 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.

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