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Original Article

Molecular Evidence of *Trichobilharzia* Species (Digenea: Schistosomatidae) in the Snails of *Lymnaea auricularia* from Urmia Suburb, North West Iran

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Abstract

Background: The present study was carried out to detect the infection of larval stages of *Trichobilharzia* species in the snail *Lymnaea auricularia* in northwestern Iran based on DNA analysis.

Methods: A total number of 320 snails of *L. auricularia* were sampled from four water-bodies located in the suburb of Urmia City, North West Iran, during May to November 2011. The snails were first microscopically inspected for the infection with larval stages of trematodes. Genomic DNA was extracted from the snails and PCR was performed to amplify a fragment of the ribosomal DNA of *Trichobilharzia* species in the infected snails.

Results: Microscopic examinations indicated that 11.25% (36 out of 320) of the snails were infected with larval stages of trematodes, while the PCR patterns showed a much higher infection rate (31.25%, 100/320). According to the PCR, the infections were caused by the larval stages of *T. szidati* (21.56%, 69/320) and *T. franki* (9.69%, 31/320) or both of them (8.44%, 27/320). The infected snails were observed in three out of the four studied sites. The highest infection rate in a single site was 50% (25/50). Only 7.81% (25 out of 320) of the infected snails were from the plain areas, while the remaining was from high altitudes.

Conclusion: Results of this study contribute the utility of the employed technique for quick and accurate detection of the infection with trichobilharzian species in their intermediate host snails, which may have potential zoonotic role in the region.

Introduction

Trichobilharzia (Weinland, 1858) is a genus of blood flukes of the family Schistosomatidae with more than 40 described species (1) infecting four families of freshwater snails and five orders of aquatic birds (2). Members of this genus may also cause cercarial dermatitis or swimmer's itch in human (3). The furcocercariae of several *Trichobilharzia* species including *Trichobilharzia franki* (4), *T. regenti* (5), *T. szidati* (6) (syn. of *T. ocellata*, La Valette, 1855) and *T. salmanticencis* (Simon-Martin and Simon-Vicente, 1999) have been recorded as the causative agents of human cercarial dermatitis in Europe (1, 7). Species of *Trichobilharzia* have been reported from various locations in all continents (8).

"Like all highly specialized trematodes, species of this group are characterized by high morphological similarity of adults and polymorphism of cercariae" (9). The accurate recognition of some developmental stages of these species is generally problematic due to their similarities to the other trematode groups. Although experimental infections have provided valuable data on schistosomatids life cycles, they have been associated with such limitations as the difficulties of isolating the intact adult trematodes from the infected birds (1, 10, 11). During the past few years, DNA analyses have helped scientists to clarify the systematics and status of the species within the genus *Trichobilharzia* (11-15). These analyses have also confirmed the role of molluscan as the intermediate hosts of these digenian trematodes (1, 16).

Trichobilharzia is the only genus of the family Schistosomatidae whose furcocercariae have morphologically been described from Iran (17). There also are a few reports of cercarial dermatitis caused by *Trichobilharzia* in north and south-west of Iran, typically in the areas where people swim in the rivers, canals and water reservoirs with uncovered bodies (16, 18).

Studying snails and intramolluscan stages of trematodes is important for the discovery of host-parasite interactions (19, 20) and prediction of the infection prevalence rates in a certain area (9, 21, 22). Active penetration of cercariae into their intermediate hosts is an important part of the life cycle of trematodes (2). The major snail families which are known as the intermediate hosts for *Trichobilharzia* are Lymnaeidae (Rafinesque, 1815), Planorbidae (Rafinesque, 1815), Pleuroceridae (Fischer, 1885) and Physidae (Fitzinger, 1833) (23, 24). *Trichobilharzia* is the only avian schistosome genus to use the snails of the family Lymnaeidae as an intermediate host (8) and has been isolated from several lymnaeid species. Neuhaus (6) reported the infection of *Lymnaea stagnalis* with *T. szidati* in Europe. *T. franki* parasitizes *Radix* (*Lymnaea*) *auricularia* (4), *R. ovata* (25) and exceptionally, *L. stagnalis* (14). Horák et al. (5) recorded the infection of *R. peregra* and *R. ovate* with *T. regent*.

In Iran, snails of the genera *Lymnaea* and *Planorbis* have been reported to be the main intermediate hosts of the causative agents of cercarial dermatitis (7, 18). However, to date, no molecular analysis has been performed for detection of infection with *Trichobilharzia* species in the field-collected lymnaeid snails of the country. We aimed to apply a molecular approach to recognize if the snail *L. auricularia* collected from North West Iran was parasitized with *Trichobilharzia* species.

Materials and Methods

Snail collection and examination

Field-collection of the lymnaeid snail *L. auricularia* was performed in four freshwater- bodies located in the suburb of Urmia City, Northwest Iran (Fig. 1) from May to November 2011. The water bodies are located in both mountainous and plain areas. The snails were randomly collected and transferred alive to the

Laboratory of Malacology of Faculty of Veterinary Medicine, Urmia University. *L. auricularia* was identified according to the standard keys (26, 27), and its identity was verified by Parasitology Museum of Faculty of Veterinary Medicine, Tehran University. The snails were preliminarily examined for the presence of the larval stages of trematodes by shedding method and microscopic inspection with and without snail crushing (28).

Fig. 1: Map of West Azarbaijan Province in northwestern Iran and the positions of the study sites. 1. Jabalkandi; 2. Golestaneh; 3. Tazehkand; 4. Osalu

DNA extraction

The soft tissues of *L. auricularia* were dissected, washed several times in 0.01M phosphate-buffered saline (PBS, pH 7.2) and stored at –20 ºC until the DNA extraction. The genomic DNA was extracted by the modified phenol-chloroform method using cetyltrimethylammonium bromide (CTAB) at 60 ºC for 1 hr (29).

Polymerase chain reaction (PCR)

A fragment of the ribosomal DNA of *Trichobilharzia* sp., spanning the sequences of internal transcribed spacers 1 and 2 (ITS1, ITS2), and 5.8S, 18S and 28S ribosomal RNA (rRNA) gene regions, was amplified using the specific primers *its5Trem* (5'-GGAAGTAAAAGTCGTAACAAGG-3') and *its4Trem* (5'-TCCTCCGCTTATTGATATGC-3') (12). The PCR was performed in 25µl reaction containing 4 µl of the genomic DNA, 2.5U of *Taq* DNA polymerase (Fermentas, Germany), 50 µM of each dNTPs (CinnaGen, Iran), 2 mM of $MgCl_2$, 2.5µl of 10× PCR buffer and 0.5µM of each primer. The reaction was run in a Bioer XP thermal cycler (China) and comprised an initial DNA denaturation step at 95 ºC for 5min, followed by 35 cycles of DNA denaturation at 95ºC for 60s, primer annealing at 50 ºC for 45s, primer extension at 72 ºC for 2 min, and a final extension at 72 ºC for 10 min. A volume of 10 µl of each PCR product was analyzed by electrophoresis on 1% agarose gel at 120V for about 30 min. The gel was visualized by staining with ethidium bromide. The snail samples showing the band patterns corresponding to the gene regions of *Trichobilharzia* species were considered as infected.

Results

A total number of 320 *L. auricularia* snails were collected from the investigated water bodies. The cercarial shedding and microscopic examination showed that 11.25% (36 out of 320) of the *L. auricularia* snails were infected with the larval stages of digenian trematodes.

The PCR amplified different lengths of the ribosomal DNA of *Trichobilharzia* in the examined snails. The amplified gene fragments comprised a 1330bp-long section representing the entire ITS1, ITS2, 5.8S, 18S and 28SrRNA

gene regions of *T. szidati*, a 745bp-long ITS1 fragment of *T. szidati*, sections of 966bp and 322bp in length of the ITS1 and ITS2 gene regions of *T. franki*, respectively and a total length of 477bp of ITS1 and 5.8SrDNA of *T. franki* (Fig. 2). Based on the PCR patterns, 31.25% (100 out of 320) of the *R. auricularia* snails were infected with two *Trichobilharzia* species, i.e. *T. szidati* (21.56%, 69/320) and *T. franki* (9.69%, 31/320). Mixed infection with both *Trichobilharzia* species was also found in 8.44% (27/320) of the infected snails.

The infected snails were geographically distributed over three out of the four study sites. The highest infection rate in a single site (50%, 25 out of 50) was observed in Osalu, while the lowest infection (11.76%) was recorded in Golestaneh (Fig. 1). The rate of infection in plain areas was 7.81% (25 out of 320), whereas the remaining infected snails (23.43%, 75/320) were found in the water bodies located in high altitudes (Table 1).

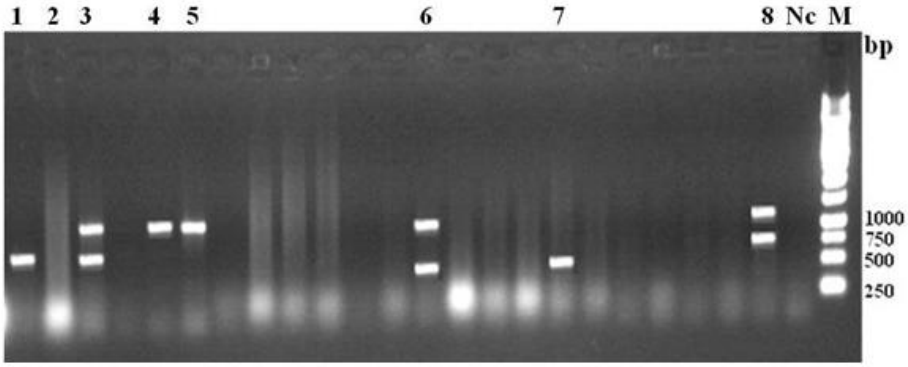

Fig. 2: Results of the PCR for amplification of ribosomal DNA of *Trichobilharzia* species in the infected *Lymnaea auricularia* snails. The amplified fragments comprise a 966bp-long section of the ITS1 of *Trichobilharzia franki* (Lanes 2, 3, 4, 5), a fragment of 477bp representing the ITS2 and 5.8SrDNA of *T. franki* (Lanes 1, 2, 6), a 322bp-long ITS2 fragment of *T. franki* (Lane 5), an amplicon of 1330bp in length covering entire ITS1, ITS2, 5.8S, 18S and 28SrRNA gene regions of *T. szidati*, and 745bp-long ITS1 sequence of *T. szidati* (Lane 7). Nc: Negative control. M: 250bp DNA size marker

Table 1: Rates and geographic distribution of the infection of *Lymnaea auricularia* with larval stages of trematodes (microscopic observation) and *Trichobilharzia* species (PCR analysis) (n=320)

Study site	Microscopic observation (%)		Molecular analysis (%)			Water body type		Site position	
	Examined	Infected	Ts	Tf	Mi	S	P	M	Pl
Golestaneh (N45°53'E37°15')	85	11.76	1.9	1.3	0.65	-	+	+	-
Jabalkandi (N37°22' E45°15')	45	0	0	0	0	+	-	-	+
Osalu (N37°42' E 45°13')	50	25	4.38	3.44	2.19	+	-	-	+
Tazehkand (N37°43' E 45°37')	140	46.43	15.31	5	5.63	+	-	+	-
Total	320	31.25	21.56	9.69	8.44				

Ts: *Trichobilharzia szidati*, Tf: *T. franki*, Mi: mixed infection, S: seasonal, P: perennial, M: mountainous, Pl: plain

Discussion

The taxonomy of trichobilharzia trematodes is a yet-unresolved parasitological issue mainly due to their complicated life cycle, the difficulties of their isolation and morphological recognition and lack of information on their developmental stages (1, 21). Among the several traditional and modern techniques, used for the screening of digenian infection in their intermediate and definite hosts (30), PCR-based analyses have had significant contribution to the accurate and specific estimation of the infection (5, 8, 9, 20). Genetic analysis is particularly beneficial for specific diagnosis of *Trichobilharzia* species as their cercariae are indistinguishable even by detailed examinations such as electron microscopy (12). However, very few studies have used molecular tools for detection of naturally infected snails with trematodes (30).

Our molecular analysis discovered the infection of the natural populations of the snail *R. auricularia* with the species of the genus *Trichobilharzia* in the studied region. Diverse lengths of the ribosomal DNA of these trematode species were amplified in the infected snails using the specific primer set. The amplified fragments correspond to different ribosomal DNA regions of two *Trichobilharzia* species, *T. szidati* and *T. franki* (12). Thus, based on the analysis, at least two species of the genus *Trichobilharzia* are present in the studied region, which can infect the snails and eventually, their definite hosts, i.e., birds and human. The sequences of ITS1 and ITS2 fragments of the ribosomal DNA have been found to be suitable tools for species identification in bird schistosomes of the genus *Trichobilharzia* (11, 13-15). However, for more accurate characterization of the trichobilharzian trematodes; a description of their entire life cycle, developmental stages and host-parasite interactions also needs to be obtained (12).

This is the first record of the infection with *Trichobilharzia* in the field-collected *R. auricularia* snails in Iran. The earlier studies have mainly focused on the epidemiology of human schistosomiasis in the country. Sahba and Malek (31) investigated cercarial dermatitis in the Iranian coastal areas of the Caspian Sea. *Schistosoma haematobium* was found to be the causative agent of human schistosomiasis in Southwest Iran (18, 31, 32). 1.1% of the examined people in Southwest Iran had the clinical signs of cercarial dermatitis (18). They also estimated a 2.1% infection rate in the *L. gedrosiana* snails with the furcocercariae of *Trichobilharzia* species. Athari et al. (17) isolated adult schistosomes from the water birds in northern Iran. Karamian et al. (33) discovered the infection with schistosome furcocercariae in the snail *Melanoides tuberculata* from Khuzestan, South Iran by PCR-amplification and sequencing of the ribosomal DNA of the parasites.

Although in the study of Semyenova et al. (9) cercariae of different genetic attributes were found in individual snails, a remarkable biological feature in *Trichobilharzia* species is their differential host preference for snail species (1,12). For instance, while the infection rate of some snail species with *Trichobilharzia* is reported to be as low as 0.3% (34), it exceeded 5% in *L. auricularia* (10). In Europe, nearly all *Trichobilharzia* species use lymnaeid snails as the intermediate hosts, while in North America they favor both lymnaeid and physid snails (8). Kock (25) suggested that the intermediate host spectrum of *T. szidati* is limited to the snails *L. stagnalis* and *Stagnicola palustris*. Rudolfová et al. (14) isolated the European *T. ocellate* (a syn. of *T. szidati*) from *L. stagnalis*. *T. franki* was also isolated from *L. auricularia* (13) and unexpectedly, from *L. stagnalis* (14). Snails of the family Lymnaeidae are distributed worldwide (8). *L. auricularia* is reported to be the second most common lymnaeid snail after *L. gedrosiana* in West Azarbaijan, Northwest Iran (26, 35). The infection of *L. auricularia* with cercariae of the digenian trematodes of the genus *Fasciola* was reported by Imani-Baran et al. (22). The broad distribution of this snail and its adaptation to the local environmental

conditions in northwestern Iran has made it a potent transmitter of the avian schistosomes in this part of the country. Northwest Iran has a Mediterranean climate comprising two rainy seasons, the first from March to May and the second in October-November; with plenty of water bodies, which well suited, for the completion of the life cycle of the trematodes such as *Trichobilharzia* species. Furthermore, with regard to the high cercarial production capacity of schistosomes (36), it is predictable that the infected snails can spread these parasites across a wide range of natural habitats. In addition, the seasonal variations in the activities of the snails and the avifauna are the important factors affecting the rate of the infection with *Trichobilharzia* species.

Conclusion

The implemented molecular technique in this study is a fast, simple and accurate way of uncovering the infection with digenian trematodes in their intermediate and final hosts. This will eventually assist in the estimation of the infection rates in a definite region even if no apparent clue of the infection would be available. Results of our molecular analysis witness the relatively high rates of infection with both *T. szidati* and *T. franki* in the *L. auricularia* snails in Northwest Iran. These results can provide a baseline for further studies and can be useful in the control programs against these parasitic trematodes.

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The authors declare that there is no conflict of interest.

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