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Original Article

Detection of Infection with Larval Stages of *Ornithobilharzia turkestanicum* using PCR in Field-Collected Snails of *Lymnaea gedrosiana* from Northwestern Iran

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Abstract

Background: Infection with *Ornithobilharzia turkestanicum* has been reported in a wide range of animals worldwide. This study was undertaken to assess the utility of polymerase chain reaction (PCR), for detecting the infection with *O. turkestanicum* larvae stages in *Lymnaea gedrosiana*.

Methods: A total of 6,759 Lymnaeidae snails were collected from six aquatic habitats in West Azarbaijan, northwest Iran. Of these, the snails of *L. gedrosiana* were identified. To detect infected *L. gedrosiana* with the larval stages of *O. turkestanicum*, they were subjected for cercarial shedding and molecular examinations. The genomic DNA was extracted and PCR was performed to specifically amplify a fragment of the nuclear 28SrRNA gene of *O. turkestanicum*.

Results: Of all collected snails, 5.4% (365/6,759) were the snails of *L. gedrosiana*. The cercarial shedding method revealed that 23.56% (86/365) of the snails were infected. The PCR patterns confirmed that 28.77% (105/365) snails of *L. gedrosiana* were infected with larval stages of *O. turkestanicum*. The infected snails were observed in five studied sites. The highest infection rate (66.66%, 20/30) was recorded in the snails of Ghargologh in the northern part. Only 35.24% (37/105) of the infected snails were from the plain areas, whereas the remaining existed in high altitudes.

Conclusion: It was concluded PCR method could be an efficient and fast method for uncovering the actual rate of infection with larval stages of *O. turkestanicum* in the snails of *L. gedrosiana*. This method can be also useful for the domestic animals and public health management programs in the country.

Introduction

The dioecious trematode *Ornithobilharzia turkestanicum* (family, Schistosomatidae) is a well-documented parasitic fluke which lives in mesenteric veins of ruminants and other mammals (1, 2). It was formerly named as *Schistosoma turkestanicum*, but later assigned to the genus *Ornithobilharzia* (3). However, the species epithet was 'turkestanica', not *turkestanicum* (4). *O. turkestanicum* has been reported from different parts of Asia (5-7). The parasite is of great economic importance in Iran because of the losses in sheep meat and wool production and intestine processing by its damages (8, 9).

The phylum Gastropoda is comprised of about 28,000 aquatic and terrestrial snails' species worldwide (10). Snails of the family Lymnaeidae are of medical and veterinary importance since some 20 species in this family have been recognized to be potential transmitters of the schistosomatid trematodes (11-14). The miracidia of these trematodes infect their intermediate hosts, i.e. snails of the family Lymnaeidae, and then leave the snails to look for their definitive hosts which can be ruminants (1, 15), rodents, wild ungulates such as reindeer (*Rangifer tarandus*), red deer (*Cervus elaphus*) or roe deer (*Capreolus capreolus*) (16, 17). Several snail species and subspecies of the genus *Lymnaea* have been reported as the intermediate hosts of *O. turkestanicum*. These include *Lymnaea gedrosiana, L. ovata, L. lagotis, L. tenera, L. acuminata, L. peregra* and *L. auricularia rufescens* (18-20). Among the seven identified species of lymnaeid snails in Iran, the aquatic snail, *L. gedrosiana* (Annandale and Prashad, 1919) is the most widely distributed one throughout the country (14, 21, 22). It is a freshwater inhabitant which can be found in water bodies with diverse environmental conditions (23). In Iran, *L. gedrosiana* has been reported to be a preferred intermediate host for *O. turkestanicum* (24) and *Trichobilharzia* spp. (25). In Iran, the cases of animal schistosomosis by *O. turkestanicum* were reported for the first time in 1963 from Babolsar, northern Iran (26). Since then, it was also found in different parts of the country, i.e. the provinces of Fars (27), Khoozestan (28, 29), Tehran (30), and Mazandaran (31). Nevertheless, not many researches have aimed to study the causative agents of cercarial dermatitis in Iran. Adult helminths causing cercarial dermatitis have been reported from animal schistosomes, i.e. *Ornithobilharzia*, and bird hosts in southern and northern parts of Iran, respectively (27, 32). The furcocercariae of animal schistosomes generating cercarial dermatitis or swimmer's itch in the people working in the rice fields have been reported from northern (33) and southwestern Iran (25).

Microscopic examination is the most frequently-used technique to detect the larval stages of trematodes in the snails (34). However, this technique has low sensitivity and/or specificity because of the difficulties in detection and differentiation of the trematodes larvae (35). For this reason, recent studies for discerning the experimental or natural infections with schistosomatid larvae in lymnaeid snails have employed molecular tools. All previous reports of the schistosomatid infections in the field-collected snails from Iran have been made based on the detection of infection by cercarial shedding method (20, 29, 32).

In this study, it was aimed to assess the utility of a molecular approach, polymerase chain reaction (PCR), for detecting the prevalence of the infection with *O. turkestanicum* larvae stages in the field-collected snails of *L. gedrosiana* from northwest Iran.

Materials and Methods

Snail collection

This study was carried out in the province of West Azarbaijan, northwestern Iran (35°46´–39°58´E and 44°3´–47°23´N), where the existence of plenty of water bodies and reservoirs

with relatively appropriate environmental conditions provides suitable habitats for freshwater snails (14).

Field-collection of the lymnaeid snails was undertaken in six freshwater bodies located in both mountainous (altitudes over 1000m above sea level) and plain areas of northern, central and southern parts of West Azarbaijan over a period of eight months from May to December 2010 (Fig.1). The collected snails of each site were placed individually into the plastic screw-capped containers and transferred alive to the Laboratory of Malacology of Faculty of Veterinary Medicine, Urmia University. Of 6,759 collected lymnaeid snails, the snails of *L. gedrosiana* were identified using the identification keys provided by Mansoorian (21) and Pfleger (36). The identities were then verified by the Parasitology Museum of the Faculty of Veterinary Medicine of Tehran University.

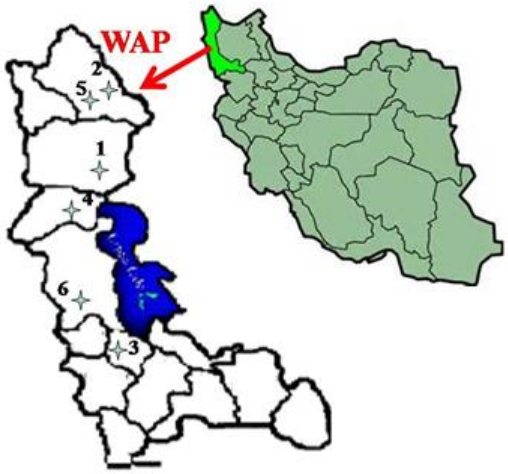

Fig.1: Map of West Azerbaijan Province, northwestern Iran (WAP) and the study sites. 1: Shabanlu; 2: Marganlar; 3: Shorgol; 4: Qarabaagh; 5: Ghargologh; 6: Gharahaghaj

DNA extraction

The soft tissues of the snails belonging to the specie *L. gedrosiana* were dissected, washed several times in 0.01 M phosphate-buffered saline (PBS, pH 7.2), and stored at –20 ºC until the DNA extraction. The genomic DNA was isolated by the modified phenol-chloroform method (37) using cetyltrimethylammonium bromide (CTAB) at 60 ºC for 1 hr: 600µl of 2x CTAB buffer (100 mM Tris-HCl, pH 8.0; 0.20 mM EDTA, pH 8; 1.4M NaCl; 2% CTAB; 0.2% 2-mercaptoethanol) were added to 100mg of the snail tissue. The mixture was incubated at 60 ºC for 60 min, vortex for 10min and centrifuged at 14000rpm for 15min. In continue, 300µl phenol and chloroform-isoamyl alcohol (24:1) was added to the aqueous part, shaken for 2min and centrifuged at 14000rpm for 15min. The supernatant was transferred into a new tube and extracted with 600µl of chloroform-isoamyl alcohol (24:1), followed by centrifugation at 14000rpm for 15min. In the next step, 0.7-fold volume of ethanol (EtOH) was added to the supernatant and the DNA was precipitated at -20ºC overnight followed by centrifugation at 14000rpm for 15min. The EtOH was poured off and the DNA pellet was rinsed in 70% EtOH twice. The EtOH was poured off by centrifugation at 14000rpm for 15min and the pellet was dried at room temperature and finally, dissolved in 50µl PCR water overnight.

Polymerase chain reaction (PCR)

Two specific primers (Ot-f: 5'-CCTTAG-TAACTGCGAGTCAACAGG-3' and Ot-r: 5'-GAGCAAGACAGCAGGATCTCACC-3') were used to amplify a fragment of the 28SrRNA gene of *O. turkestanicum* in the *L. gedrosiana* tissues (38). The PCR was carried out in 25µl reaction containing 2µl of the genomic DNA (diluted 1:30), 2.5U of *Taq* DNA polymerase (Fermentas, Germany), 50µM of each dNTPs (CinnaGen, Iran), 2mM of $MgCl_2$, 2.5µl of PCR reaction buffer (10×) and 0.5µM of each primer. The reaction was performed in a Bioer XP thermal cycler (China) and comprised. an initial denaturation step at 94ºC for 5min, followed by 35 cycles of 94 ºC for 60s, 57 ºC for 60s, and 72 ºC for 60s and finally, an extension step of 72ºC for 5min. A volume of 10µl of each PCR product was analyzed by electrophoresis on 1% (w/v) agarose

gel for about 1.5h at 90V. The gel was visualized by staining with 1% ethidium bromide. The snail samples showing the band patterns corresponding to the 28SrRNA gene of *O. turkestanikum* were considered as infected.

Results

A total of 6,759 Lymnaeidae freshwater snails were collected from the investigated water bodies. Of these, 5.4% (365/6,759) were the lymnaeid snails of *L. gedrosiana*. The cercarial shedding and microscopic examination showed that 23.56% (86 out of 365) of the *L. gedrosiana* snails were infected with the larval stages of digenian trematode.

The PCR patterns confirmed that 28.77% (105/365) snails of *L. gedrosiana* were infected with larvae stages of *O. turkestanicum* (Fig. 2). Geographically, the infected snails were distributed over five out of the six study areas. The maximum infection rate was for Ghargologh (66.66%, 20/30) located in northern part of West Azarbaijan, while the minimum infection was recorded in Qarabaagh (16.66%, 5/30), a water body in the central part of the province (Fig.1). Only 35.24% (37 out of 105) of the infected snails were from the plain areas; the remaining were distributed in high altitudes (Table 1).

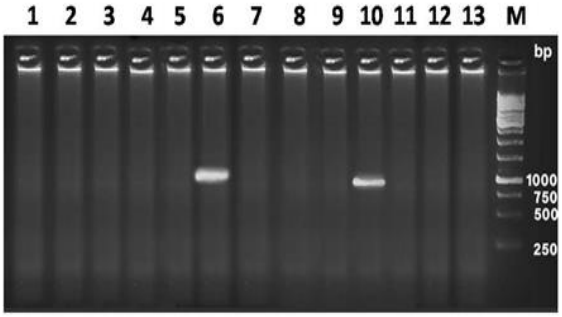

Fig. 2: Agarose gel electrophoresis of the PCR products. Lanes 6 and 10: amplified 28SrRNA gene of *Ornithobilharzia turkestanicum* in the infected *Lymnaea gedrosiana*. Negative control (Lane 13); M: 250bp DNA size marker

Table 1: The prevalence and geographic distribution of *Lymnaea gedrosiana* infected with larval stages of *Ornithobilharzia turkestanicum* in northwest Iran (n=365)

Location	No. of examined snails	*Prevalence (*n*/*N*, %)	Type of Water body		Area feature	
			Se	Pe	M	Pl
Qarabaagh (N 45°03' E38°04')	30	16.66	-	+	-	+
Gharahaghaj (N 39°04' E44°58')	75	22.5	-	+	-	+
Ghargologh (N 39°15' E45°08')	30	66.66	-	+	+	-
Marganlar (N 39°07' E44°58')	100	47.61	-	+	+	-
Shabanlu (N 38°26' E44°54')	15	0	-	+	+	-
Shorgol (N 37°44' E45°04')	115	30.43	-	+	-	+
Total	365	28.77				

Notes: M, Monainous; Pl, Plain; Pe, perennial; Se, seasonal. /* Based on the prevalence rates obtained by PCR method

Discussion

The expansion of the parasites with indirect life cycles, such as *O. turkestanicum*, is facilitated where an adequate number of intermediate and definite host species coexist (38). In the present study, *L. gedrosiana* showed to be common snail specie throughout the studied region living in perennial and typically acidic waters. According to Moghaddam et al. (39) and Mansoorian (21), *L. gedrosiana* is the most widely distributed lymnaeid snail throughout Iran. Earlier, Imani-Baran et al. (14) also reported a high abundance and wide distribution of this snail in West Azarbaijan. This province is characterized by flat lands, numerous stagnant water catchments and irrigation canals covered by aquatic vegetation and agricultural tradition. These together with having average annual temperatures higher than 20˚C, adequate rainfall and humidity and intense population of domestic ruminants set the scene for the transmission of digenian trematodes by the snails. This scenario can hold true for the infection of *L. gedrosiana* with *O. turkestanicum*.

This is the first study in Iran in which the infection of field-collected *L. gedrosiana* snails with larval stages of the digenian trematode *O. turkestanicum* was discovered by molecular examination. The application of molecular methods can give an accurate estimation of infection with a certain disease-causing organism. This is not only correct the underestimations made by the traditional methods, can also uncover the infections which could not be detected by the classical manners, especially with the larval stages of trematodes. The utility of molecular approaches for studying the epidemiology of *O. turkestanikum* was confirmed in this study.

The animal schistosomes including *O. turkestanicum* have shown high infection rates and wide distribution over some Iranian provinces, so that their prevalence rates ranged between 35% and 100% in sheep and goats (39). However, the prevalence of infection with *O. turkestanicum* larvae in *L. gedrosiana* observed in the current study was relatively low. In accordance with the results of this study, Majoros et al. (38) reported very low infection rates with fasciolid larval stages in the lymnaeid snails of northern Iran.

Determination of the seasonal distribution of *L. gedrosiana* as the intermediate host of *O. turkestanicum* is of great importance. According to Eslami (40), there is a seasonal variation in *O. turkestanicum* infection in the ruminants of Iran, and this is directly related to the abundance and incidence of the native snails. Thus, infection with *O. turkestanicum* may outbreak following a seasonal variation (40). Such outbreaks have been frequently reported in spring and fall. The prevalence of *O. turkestanicum* in small ruminant is more important in fall than in spring. Furthermore, the incidence of the infection in a range of the host animals with season-dependent life cycles may play an important role in persistence of the infection in the livestock of the region.

Conclusion

From the results of the current study, it was confirmed that the snail *L. gedrosiana* can be considered as a potent transmitter of *O. turkestanicum* to the domestic ruminants and humans. This should be taken into consideration in the development of control programs against the infection. Further studies should be directed to understand the extent to which the infection rates in the snails affect the degree of infection in the domestic ruminants and human beings in each part of the region. It is also recommended that both traditional and PCR methods should be used to better understanding of the epidemiological situation of the infection in a given area.

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