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 Ghargolgh 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 *L. gedrosiana*, *L. ovata*, *L. lagotis*, *L. tenera*, *L. acuminata*, *L. pergra* 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), Khouzestan (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

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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 1000 m above sea level) and plain areas of northern, central and southern parts of West Azerbaijan 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: Ghardgogoh; 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°C until the DNA extraction. The genomic DNA was isolated by the modified phenol-chloroform method (37) using cetyltrimethylammonium bromide (CTAB) at 60°C for 1 hr: 600 µl of 2x CTAB buffer (100 mM Tris-HCl, pH 8.0; 0.20 mM EDTA, pH 8; 1.4 M NaCl; 2% CTAB; 0.2% 2-mercaptoethanol) were added to 100 mg of the snail tissue. The mixture was incubated at 60°C for 60 min, vortex for 10 min and centrifuged at 14000 rpm for 15 min. In continue, 300 µl phenol and chloroform-isoamyl alcohol (24:1) was added to the aqueous part, shaken for 2 min and centrifuged at 14000 rpm for 15 min. The supernatant was transferred into a new tube and extracted with 600 µl of chloroform-isoamyl alcohol (24:1), followed by centrifugation at 14000 rpm for 15 min. In the next step, 0.7-fold volume of ethanol (EtOH) was added to the supernatant and the DNA was precipitated at -20°C overnight followed by centrifugation at 14000 rpm for 15 min. The EtOH was poured off and the DNA pellet was rinsed in 70% EtOH twice. The EtOH was poured off by centrifugation at 14000 rpm for 15 min and the pellet was dried at room temperature and finally, dissolved in 50 µl PCR water overnight.

**Polymerase chain reaction (PCR)**

Two specific primers (Ot-f: 5’-CCCTAG- TAACTGCGAGTCACAACAGG-3’ and Ot-r: 5’-GAGCAAGACAGCGAGATCTCACC-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 25 µl reaction containing 2 µl of the genomic DNA (diluted 1:30), 2.5U of *Ta*q DNA polymerase (Fermentas, Germany), 50 µM of each dNTPs (Cinnagen, Iran), 2 mM of MgCl2, 2.5 µl of PCR reaction buffer (10×) and 0.5 µM of each primer. The reaction was performed in a Bioer XP thermal cycler (China) and comprised. an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 60 sec, 57°C for 60 sec, and 72°C for 60 sec and finally, an extension step of 72°C for 5 min. A volume of 10 µl of each PCR product was analyzed by electrophoresis on 1% (w/v) agarose gel.
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. turkestanicum* 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 Gharogologh (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).

**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 |
|------------------|------------------------|------------------------|--------------------|--------------|
| Qarabaagh (N 45°03’ E38°04’) | 30                     | 16.66                 | Se     | Pe | M | Pl |
| Gharahaghaj (N 39°04’ E44°58’) | 75                     | 22.5                  | -      | +  | - | +  |
| Ghargolgh (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

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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. turkestanicum* 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 fascioloid 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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References

1. Malek, F.A. Snail-Transmitted Parasitic Diseases. CRC Press Inc., Boca Raton, FL. 1980. pp.179–307.
2. McGavin MD, Carlton WW, Zachary IF. Thomsonts special veterinary pathology. 3rd ed. Mosby, U.S.A. 2001.
3. Dutt SC, Srivastava HD. A revision of the genus Ornithobilharzia Odhner, 1912 (Trematoda: Schistosomatidae). In: Proceedings of the Indian Science Congress. 1955. p. 283.
4. Yamaguti S. A synoptical review of the histories of digenetic trematodes of vertebrates. Keigaku Publishing Co., Kyoto. 1975. pp. 384.
5. Witenberg G, Leng J. A case of natural infection of field rats with Ornithobilharzia turkestania. Refuah Veterinarith. 1966; 23:67–74.
6. Gurup N, Tinar R. The first case report on the occurrence of Ornithobilharzia turkestania (Skrjabin, 1913) in sheep in Turkey. Fınat Universitesi Veteriner Fakultesi Dergisi. 1982; 7:285–96.
7. Kumar F, De Burbure G. Schistosomes of animals and man in Asia. Helminthology. 1986; 55:469–80.
8. Massoud J. The pathology of Ornithobilharzia turkestania and Schistosoma bovis in cattle, sheep and goats in Iran. Trans R Soc Trop Med Hyg. 1971; 65: 431.
9. Ezzi A, Karimi GH, Gholami MR. Experimental Pathology of Ornithobilharzia turkestania in Sheep. Arch Razi Ins. 2004; 57:127-32.
10. Morton B. The population dynamics and expression sexuality in Balis schaplandi and Mononadis fulvescens (Mollusca: Gastropoda: Agloss) parasitic upon Arbaster typed (Echinodermata: Asteroidea). Malacologia. 1979; 18:327–46.
11. Hurtrez-Bousses S, Meunier C, Durand P, Renaud F. Dynamics of host parasite interactions: the example of population biology of the liver fluke (Fasciola hepatica). Microbiol Infect. 2001; 3: 841-49.
12. Lotfy WM, Brant SV, De Jong RJ, Le TH, Demiaszkiecz A, Rajapakse RPVJ, Perera VBVP, Laurens JR, Loker ES. Evolutionary origins, diversification, and biogeography of liver flukes (Digenea, Fasciolidae). Am J Trop Med Hyg. 2008; 79(2): 248–55.
13. Mera Y, Sierra RMY, Arrugas P, Cuervo P, Deis E, Sidoti L, Mas-Coma S, Bargues MD. Fasciolasis transmission by Lymnaea natricula confirmed by nuclear rDNA and mtDNA sequencing in Argentina. Vet Parasitol. 2009; 166: 73–9.
14. Imani-Baran A, Yakhchali M, Malekzadeh-Viayeh R. A study on geographical distribution and diversity of Lymnaeidae snails in West Azerbaijan Province, Iran. Veterinary Journal, Pajouhesh and Sazandegi. 2011; 82(4):53-63. (In Persian)
15. Al-Barrak NS, Wajdi N, Fadhil S. Observations on schistosomes parasitic in cattle in Iraq. Iraqi J Biol Sci. 1977; 5:69–81.
16. Skrjabin KI.Trematods of Animals and Man. Izdatelstvo Akademii Nauk SSSR, Moskva, 1951. pp. 320–30.
17. Zakhraylova YaN. The character and components of a natural focus of Ornithobilharzia turkestania in the Far-East. Voprosy Prirodnoj Ochagovosti Boleznii. 1978; 9:76–81.
18. Ghadirian E, Haghooghi N. The presence of snails of veterinary importance in Isfahan, Iran. Br Vet J. 1973; 129:1–3.
19. Kumar V. Studies on snail hosts of Ornithobilharzia turkestanicum (Skrjabin, 1913) Dutt and Srivastava, 1955 (Schistosomatidae: Trematoda) in India. Ann Soc Belg Med Trop. 1973; 53:17–23.
20. Massoud J. Observations on Lymnaea gedrosiana, the intermediate host of Ornithobilharzia turkestanicum in Khuzestan, Iran. J Helminthol. 1974; 48:133–38.
21. Mansoorian AB. A practical guideline for identification of Iranian freshwater snails. Iranian J Publ Health. 1986; 15(1-2): 41-53.
22. Imani-Baran A, Yakhchali M, Malekzadeh-Viayeh R, Paktarmani R. Molecular study for detecting the prevalence of Fasciola gigantica in field-collected snails of Radix gedrosiana (Pulmonata: Lymnaeidae) in northwestern Iran. Vet Parasitol. 2012; 89:374–77.
23. Hamburger J, Hoffman O, Kariuki HC, Muchiri EM, Ouma JH, Koech DK, Sturrock RF, King CH. Large-scale, polymerase chain reaction-based surveillance of Schistosoma haematobium

Available at: http://ijpa.tums.ac.ir
DNA in snails from transmission sites in coastal Kenya: a new tool for studying the dynamics of snail infection. Am J Trop Med Hyg. 2004; 71:76-73.

24. Motamedi GhR, Ghorashi SA, Paykari H, Dalimi AH, Salehi Tabar R, Motamedi N, Karimi GhR. Detection of Ornithobilharzia turkestanicum cercaria (trematoda) by nested-PCR in intermediate host snail, Lymnaea gedrosiana. Arch Razi Inst. 2008; 63(2):35-40.

25. Farahnak A, Essalat MA. A study on cercarial dermatitis in Khoozestan Province, Southwestern Iran. BMC Public Health. 2003; 3: 35-8.

26. Dawood M. Dezful bilharziasis pilot project. 3rd quarterly report. 1963.

27. Maleki M, Khodokaram A, Oryan A, Aslani M, Housseinzadeh SA, Sadjadi SM. Pathological findings in ornithobilharziasis in the herds of sheep and goat of Assyrians in Fars Province, Iran. Res Reconstruc. 1994; 24:143.

28. Arfaa F, Sabbaghian H, Ale-Dawood H. Studies on Ornithobilharzia turkestanicum (Skrjabin, 1913). Ann Parasitol Hum Comp. 1994; 40:45-50.

29. Massoud J. Studies on the schistosomes of domestic animals in Iran: Observations on Ornithobilharzia turkestanicum (Skrjabin, 1913) in Khuzestan. J Helminthol. 1973; 47(2):165-80.

30. Eslami A, Rad MN, Salehi MR, Faiz A. Trematode infection of the liver of ruminants in Tehran Abattoir. Journal of Tehran Faculty of Veterinary Medicine, University of Tehran. 1976; 32 (1-4): 21-7.

31. Eslami A, Sarafrazi M, Hassani TA. Current status of ovine Ornithobilharzia turkestanicum infection in Juybar, Mazandaran. Pajohesh and Sazandegi. 1998; 35:106-7.

32. Athari A., Gohar-Dehi SH, Rostami M, Jalilian MD. Determination of definitive and intermediate hosts of cercarial dermatitis-producing agents in Northern Iran. Arc Iranian Med. 2006; 9:11-5.

33. Sahba GH, Malek EA. Dermatitis caused by cercariae of Ornithobilharzia turkestanicum in the Caspian Sea area of Iran. Am J Trop Med Hyg. 1979; 28(5):912-3.

34. Kaplan RM, Dame JB, Reddy GR, Courtney CH. The prevalence of Fasciola hepatica in its snail intermediate host determined by DNA probe assay. Int J Parasitol. 1997; 27: 1585–93.

35. Caron Y, Rondeaud D, Losson B. The detection and quantification of a digenean infection in the snail host with special emphasis on Fasciola sp. Parasitol Res. 2008; 103:735–44.

36. Pfleger V. A field guide in colour to molluscs. Aventinum Nakladatelstyi, S.T.O., Polygrafía, Prague, Czech Republic. 1999. pp.28-9.

37. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd Ed. Cold Spring Harbor Laboratory Press, New York, USA. 2002.

38. Majoros G, Dan A, Erdely K. A natural focus of the blood fluke Ornithobilharzia turkestanica (Skrjabin, 1913) (Trematoda: Schistosomatidae) in red deer (Cervus elaphus) in Hungary. Vet Parasitol. 170; 218–23.

39. Moghaddam AS, Massoud J, Mahmoodi M, Mahvi AH, Periago MV, Artigas P, Fuentes MV, Bargues MD, Mas-Corra S. Human and animal Fascioliasis in Mazandaran province northern Iran. Parasitol Res. 2004; 94(1):61-9.

40. Eslami A. Veterinary helminthology: Trematoda. 3rd ed. Tehran University Publisher, Tehran, Iran. 2006; pp. 165-70.

Available at: http://ijpa.tums.ac.ir