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Original Article

PCR-RFLP Analysis of 28 SrDNA for Specification of *Fasciola gigantica* (Cobbold, 1855) in the Infected *Lymnaea auricularia* (Linnaeus, 1785) Snails from Northwestern Iran

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Abstract

Background: Fasciolosis in livestock is a crucial concern in the globe, mainly due to its impact on human health. The aim of this study was to determine the rate of infection with *Fasciola gigantica* (Cobbold, 1855) larvae in the field-collected snails of *Lymnaea auricularia* (Linnaeus, 1785) from northwestern Iran using a molecular approach.

***Methods*:** A total of 6,759 pond snails were collected from 28 freshwater bodies in West Azarbaijan. PCR was performed to amplify a 618-bp fragment of the nuclear 28 SrRNA gene of *Fasciola*. The PCR products were digested by *Ava*II restriction enzyme to create restriction fragment length polymorphism (RFLP) patterns specific for the detection of *F. gigantica*.

***Results*:** Of the total collected snails 496 (7.34 %) were *L. auricularia*, among which 4.64% (23 out of 496) were infected with a *Fasciola* species according to the PCR analysis. Only 2.22% (11 out of 496) of the infected snails were from the mountainous areas. The highest *Fasciola* infection rate recorded in the snails of a single site was 1.81% (9 out of 496 snails). Based on the RFLP pattern, *F. gigantica* accounted for 2.42% of the infection rates in the study sites.

***Conclusion*:** Application of PCR-RFLP was proven to be a useful approach for valid and robust detection of the infection with *F. gigantica* in its intermediate host snails. These findings may therefore be applicable for establishment of the control programs against dissemination of the infection in different regions.

Introduction

Parasitic diseases are severe problems for human and veterinary medicine, while also affecting economy, agriculture and wildlife management. Fasciolosis is a cosmopolitan disease caused by the digenian trematodes of the family Fasciolidae. It has become as an issue of global concern especially because of its implication in public health (1). Snails of the family Lymnaeidae are of medical and veterinary importance, as several species of this family have been recognized to be the potential transmitters of fasciolid trematodes (2-5). Among the seven identified species of the lymnaeid snails from Iran, the eared pond snail, *Lymnaea auricularia* (Linnaeus, 1785), has been reported to be one of the most common pond snails in the country (6). It is a freshwater inhabitant which can be found in different water body types locating in diverse geographical conditions (7). *L. auricularia* is an intermediate host for *Fasciola gigantica* (Cobbold, 1855), the most important parasitic fluke infecting domestic ruminant of Iran (1, 8).

In the last few decades, knowledge of fasciolosis has revolutionized by improvement of the methods through which different parasitic species can be recognized (9). The invention of molecular techniques has led to faster and more accurate identification of the species, especially while infecting the host animals. DNA-based analyses have been applied to discriminate *Fasciola* species, or to detect their infection in lymnaeid snails (4, 5, 9, 10). However, very few studies have assessed the level of infection under field conditions (9). Determination of diversity and abundance of infection with fasciolid species in different lymnaeid snails has been the subject of several experimental studies in Iran (11-15). Recently, using molecular approaches in the study of adult *Fasciola* species have been considered by Iranian researchers (16, 17).

In this study we aimed to track the infection with larval stages of *F. gigantica* in field-collected snails of *L. auricularia* and to determine prevalence of the infection in North West of Iran, by applying restriction fragment length polymorphism (RFLP) analysis of the ribosomal 28SrDNA.

Materials and Methods

Snail sampling and identification

Over a period of eight months, from May to December 2010, field-collection of pond snails were undertaken from 28 freshwater bodies located in both mountainous and plain areas in northern, central and southern parts of the province of West Azarbaijan, northwest Iran (35°46´– 39°58´E and 44°3´–47°23´N) (Fig. 1). Ecological characteristics of the province have been described by Imani-Baran et al. (7). The collected snails of each habitat were placed individually into plastic screw-capped containers and transferred alive to the Laboratory of Malacology of Faculty of Veterinary Medicine, Urmia University. The snails were identified to the species level using the identification keys provided by Mansoorian (6) and Pfleger (18). The identity of the snail *L. auricularia* was further verified by Parasitology Museum of Faculty of Veterinary Medicine, Tehran University. The main environmental parameters, temperature, salinity and pH, of the sampling sites were measured on each sampling occasion.

Microscopic examination

The snails of the specie *L. auricularia* were preliminarily examined for the presence of the larval stages of *F. gigantica* by shedding method and/or microscopic inspection with and without snail crushing (19).

DNA extraction

For molecular analysis, the soft tissues of the snails were dissected, washed several times in 0.01M phosphate-buffered saline (PBS, pH 7.2) and stored at –20 ºC until DNA extrac-

tion. Genomic DNA was isolated by the modified phenol-chloroform method using cetyltrimethylammonium bromide (CTAB) at 60 ºC for 1 h (20).

Polymerase chain reaction (PCR)

A fragment of 618-bp long of the 28S ribosomal RNA (rRNA) gene of *Fasciola* species was amplified by PCR following the procedure described by Marcilla et al. (15). The PCR was carried out in a 25µl reaction mixture and performed in a Bioer XP thermal cycler. Within the PCR products, those showing the band patterns corresponding to the molecular weight of 28SrRNA of *Fasciola* were considered as *Fasciola*-positive or infected, according which the infection rates were estimated. A PCR mixture excluding the DNA was used as negative control, while the mixture including the DNA of a known *Fasciola* isolate was considered as positive control.

RFLP analysis

To specify *F. gigantica*, a RFLP analysis was developed as of Marcilla et al. (15) using *Ava*II restriction enzyme. The restriction pattern was first predicted by using BioEdit software package (http://www.mbio. ncsu.edu). Digestion by *Ava*II was expected to produce three fragment sizes of, 27, 269 and 322bp from 28SrDNA of *F. gigantica.*

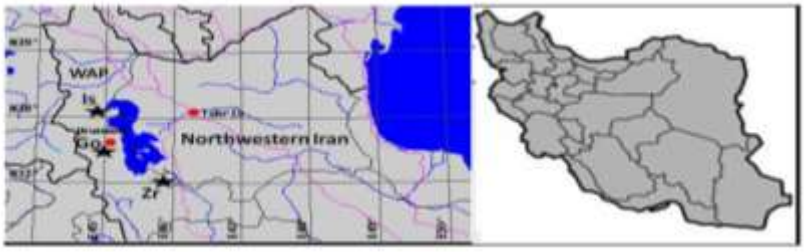

Fig. 1: Position of sampling sites and distribution of *Lymnaea auricularia* in West Azarbaijan Province (WAP), North West Iran. Go: Golestaneh; Is: Ismjondi: Zr: Zarineh-Roud

Results

A total of 6,759 freshwater snails were collected from the investigated water bodies. Of these, 496 (7.34%) were belonging to the species *L. auricularia* being found only in 3 out of 28 sites, i.e., in Golestaneh and Ismjondi in central part and Zarineh-Roud in southern part of West Azarbaijan (Fig. 1). Zarineh-Roud and Golestaneh were perennial water bodies with nearly the same salinity levels. Golestaneh had acidic water (pH=5.5) with an average temperature of 21°C, while Zarineh-Roud had neutral water (pH=7.5) with a quite higher temperature (34 ºC) than the two other sites. Ismjondi was a seasonal water body with acidic water (pH=6) and an average water temperature of 18 ºC (Table 1). The cercarial shedding and microscopic examination attested the presence of larval stages of digenian trematode species in *L. auricularia*. Based on the PCR analysis, 4.64% (23 out of 496) of the *L. auricularia* snails were infected with larval stages of *Fasciola* (Fig. 2). Only 2.22% (11 out of 496) of the infected snails were from mountainous areas; the remaining were distributed in low altitudes (Table 1). The highest infection rate was recorded in the snails from Golestaneh (2.22%, 11 out of 496 snails). The enzyme *Ava*II produced two restriction fragments of 269 and 322bp-long specifying the presence of *F. gigantica* in the infected snails. Based on the RFLP patterns, 2.42% (12 out of 496) of the *L. auricularia* snails were infected with *F. gigantica*, with highest infection rates recorded in Golestaneh (1.41%, 7 out of 496 snails) and Zarineh-Roud (1.01%, 5 out of 496 snails) (Fig. 3, Table 1).

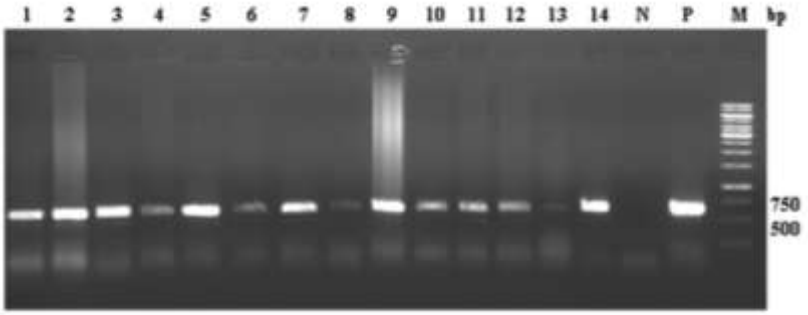

Fig. 2: PCR-amplified 618-bp-long 28SrRNA of *Fasciola* sp. in *Lymnaea auricularia* (Lanes 1-14). N: negative control; P: positive control; M: 250bp DNA size marker

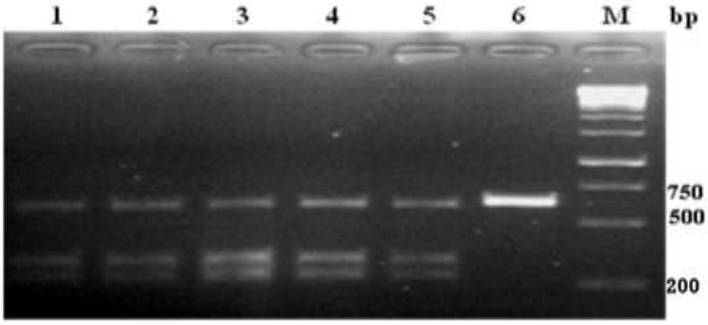

Fig. 3: RFLP patterns of the PCR products digested by *Ava*II restriction enzyme. Lanes 1-5: two restricted fragments of 269 and 322bp-long specific for *Fasciola gigantica* in the infected *Lymnaea auricularia* snails. Lane 6: a *Fasciola* isolate other than *F. gigantica*. M: 250bp DNA size marker

Table 1: Characteristics of the sampling sites and prevalence rates of *Fasciola* spp. (by PCR) and *Fasciola gigantica* (by RFLP) in *Lymnaea auricularia* snails

Location	No. of examined snails	Prevalence (*n*, %)		Types of water bodies		Area feature		Environmental parameters		
				Se	Pe	M	Pl	T (°C)	Sal (g/l)	pH
		PCR	RFLP							
Golestaneh (45°53' N and 37°15' E)	318	2.22	1.41	-	+	+	-	21	0.198	5.5
Ismjondi (37°59' N and 45°02' E)	105	1.41	0.00	+	-	-	+	18	0.44	6
Zarineh-Roud (36°49' N and 46°05' E)	73	1.01	1.01	-	+	-	+	34	0.178	7.5
Total	496	4.64	2.42							

M: Montainous, Pl: Plain, Pe: perennial, Sal: salinity, Se: seasonal, T: temperature

Discussion

The use of nucleic acid techniques in the diagnosis of parasitic infection has become increasingly widespread. This trend is found to be extremely beneficial as, for instance, using molecular approaches has resulted in determining significantly higher and more accurate

infection rates than the traditional methods (9). In this study, the presence of the developmental stages of *F. gigantica* in *L. auricularia* snails from North West of Iran was confirmed by using the PCR-RFLP analysis. This analysis has been used for discriminating the *Fasciola* species from different parts of the world by targeting diverse gene regions including 18SrDNA, ITS1, 5.8SrDNA, ITS2 and 28SrDNA (15, 16, 21-24). Ribosomal DNA is available in high copy number and contains variable sequences flanked by more conserved sections. It is, therefore, considered as a suitable genetic marker for genotyping, intraspecific variations and phylogenetic studies of organisms at different taxonomic levels (21, 23). Rokni et al. (24) could distinguish *F. gigantica* isolates from buffalos of Khuzestan, South West Iran by RFLP analysis of the ITS1 region. They also suggested that digestion of ITS1 is a reliable method for differentiating *F. gigantica* from its congener species, *F. hepatica*. Mahami-Oskouei et al. (16) by restriction digestion of the ribosomal DNA spanning ITS1, 5.8SrDNA and ITS2 discriminated *F. gigantica* and *F. hepatica* isolated from sheep and cattle of three Iranian provinces. It was also realized from this study that the restriction enzyme *Ava*II is able to generate restricted DNA fragments from 28SrDNA based on which *F. gigantica* originating from distinct geographical locations can unambiguously be distinguished from the rest of *Fasciola* species. In a similar study, Saki et al. (25) using the digestion pattern of 28SrDNA generated by *Ava*II enzyme verified the infection with *F. gigantica* in the liver of different livestock from southwest Iran.

Fasciolid infection rates obtained in the nature is usually lower than those observed in laboratory exposures (9). Although *L. auricularia* has been experimentally known as a potential vector of *F. gigantica* in Iran (26, 27), a comparison cannot be made on the prevalence of the infection or the host preference of the parasite between localities, as no similar previous report is available. Similarly, there is no data on the infection rate in *L. auricularia* with *F. gigantica* obtained via a molecular approach. Microscopic examination by Ashrafi et al. (11) revealed that only 0.35% of the snails *L. gedrosiana* collected from north of Iran were infected by *F. gigantica*.

Occurrence of both lymnaeid snails and the larval stages of fasciolid trematodes have been shown to be highly dependent on climatic and environmental conditions (28). Furthermore, Boray (29) and Al-Kubaisee and Altaif (30) noted that the infection rates of *F. gigantica* in *L. auricularia* were associated with the geographic origin of the parasite. Adequate precipitation and water temperatures higher than 18°C in the studied region, during early spring and mid autumn, provide suitable conditions for the transmission of *F. gigantica*. The relationship between prevalence of fasciolosis and climate change has been well-documented (31).

In Iran, fasciolosis first came to the attention some 50 years ago (32). Two major fasciolosis outbreaks infected several thousands of people in Iran (11, 16). Results of this study highlight the role of *L. auricularia* as a potent transmitter of fasciolosis to animals and humans, which should be taken into account in the development of control programs against the disease.

Conclusion

It is recommended that both traditional and molecular methods should be used to achieve a more comprehensive understanding of the epidemiological situation of *Fasciola* species and to assess their host preferences in a given area (33). This will provide useful information on the level of pasture contamination and consequently, prevent high fasciolosis outbreaks.

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