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Short Communication

Molecular detection of the infection with *Fasciola hepatica* in field-collected snails of *Galba truncatula* and *Lymnaea stagnalis* from West Azarbaijan, Iran

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ABSTRACT

The liver fluke, *Fasciola hepatica*, is considered as the most common cause of fasciolosis in both domestic livestock and human. This study was carried out to detect the prevalence of the larval stages of *F. hepatica* in the snails *Galba truncatula* and *Lymnaea stagnalis* in West Azarbaijan, Iran. Snail collection was performed through searching 28 freshwater habitats from May to December 2010. Following the identification of the two snail species, polymerase chain reaction (PCR) was utilized to amplify the 28SrRNA gene of *F. hepatica* in the snails' tissues. The amplified DNA fragment was subjected to restriction fragment length polymorphism (RFLP) analysis. According to the RFLP patterns, 16.6% of the examined *G. truncatula* and 1.1% of *L. stagnalis* were infected by *F. hepatica*. While there was not detected infection with larval stages of *F. gigantica* in any examined snails. The RFLP analysis of 28SrRNA gene was proven to be a useful tool for detection of the infection and its transmission by the intermediate hosts, and can help with the establishment of suitable control programs against fasciolosis in livestock and human in any region of interest.

Keywords: *Fasciola hepatica*, *Galba truncatula*, *Lymnaea stagnalis,* PCR-RFLP, Iran

INTRODUCTION

The common liver fluke, *Fasciola hepatica* (Linnaeus, 1758; Trematoda: Fasciolidae), is the etiological agent of fasciolosis outbreak in both domestic livestock and human beings worldwide (Rim *et al* 1994). Fasciolosis is a vectorial disease transmitted by freshwater snails of the family Lymnaeidae (Mollusca: Gastropoda: Basommatophora) as intermediate hosts (Rognlie *et al* 1994). Similar to those reported from different parts of the world (Torgerson & Claxton 1999), the disease has been responsible for high morbidity rates in the domestic ruminants mainly due to the liver spoilage, reduced milk, meat and wool production and, as a result, great economic losses in the animal husbandry industry in Iran (Yakhchali & Ghobadi 2005). In Europe and some parts of Asia, the lymnaeid snail *Galba truncatula* (Müller 1774) has been considered as the main intermediate host of *F. hepatica* (Andrews, 1999;

Bargues *et al* 1997, Mas-Coma & Bargues 1997). This snail has later been introduced into South America (Bargues *et al* 1997, Mas-Coma *et al* 2001, Meunier *et al* 2001). *Lymnaea stagnalis* (Linnaeus 1774) is a pond snail with Holarctic distribution, occurring in Europe, Asia, North Africa and North America. Recently, eight trematode families including Fasciolidae have been isolated from *L. stagnalis* (Faltynkova *et al* 2007). In Iran, *G. truncatula* has a wide distribution range, while *L. stagnalis* is restricted to the west and northwest of the country (Imani Baran *et al* 2011, Mansoorian 2000a, Mansoorian 2000b, Massoud & Sadjadi 1980). Both *G. truncatula* and *L. stagnalis* were found to be susceptible to the Iranian isolates of *F. hepatica* in laboratory conditions (Massoud & Sadjadi 1980). Furthermore, studies have proved the capability of young *L. stagnalis* to transmit *F. hepatica* to its definite host (Massoud & Sadjadi 1980, Shahlapour 1996). The use of molecular tools for detection of infection with fascioloid parasites in lymnaeid snails has been helpful in providing information on the level of pasture contamination, an important issue in the control of fasciolosis in livestock (Cucher *et al* 2006). In this regard, several molecular studies have been carried out for the detection of infection with *Fasciola* species in the experimentally-infected or field-collected lymnaeid snails (Caron *et al* 2007, Magalhaes *et al* 2004, Relf *et al* 2009). In order to implement control measures against fasciolosis, it is crucial to determine the pattern by which the larval stages of *Fasciola* species are transmitted by lymnaeid snails. Additionally, collection of accurate data on the snails' infection rates is essential for estimating the potential infection risk of the ruminants in a region. The objective of the present study was to test the utility of a novel molecular method for detection of the infection with *F. hepatica* larvae in the field-collected snails of *G. truncatula* and *L. stagnalis* from West Azarbaijan Province, Iran.

MATERIALS AND METHODS

Study area. The province of West Azarbaijan is located in North West of Iran (35°46´- 39°58´ and 44 °3´- 47 °23´) about 1300m above sea level. Climate of the province is largely influenced by the rainy winds of the Atlantic Ocean and Mediterranean Sea. The maximum temperature reaches 34 °C in July, while the temperature may be as low as –16 °C in January (Imani Baran *et al* 2011a). Annual precipitation varies between 300 and 800mm, with large monthly and yearly fluctuations. There are two main rainy seasons, the first from March to June and the second in October-November, in the region. There also are numerous water reservoirs with relatively appropriate environmental conditions which provide suitable habitats for pond snails.

Snail collection and identification. Field-collection of the pond snails was undertaken by searching 28 water bodies in West Azarbaijan over a period of eight months starting in May 2010. The amphibious snails of *G. truncatula* were taken from the littoral zone of shallow muddy marshes, streams, and water ditches. The aquatic snails of *L. stagnalis* were collected from perennial and seasonal water bodies with slow flowing or standing waters. The snails were sampled by using a standard flat wire mesh scoop (Caron *et al* 2007, Gutierrez *et al* 2005). On each sampling occasion, required data comprising type of water bodies, water temperature, salinity and pH were recorded. Collected snails of each habitat were placed separately into the 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 within the first 24 hours, using the identification keys provided by Mansoorian (1986) and Pfleger (1999). The identities of *G. truncatula* and *L. stagnalis* were confirmed by the Parasitology Museum of the Faculty of Veterinary Medicine of Tehran University.

Molecular procedures. The soft tissues of *G. truncatula* and *L. stagnalis* were dissected, washed in 0.01M phosphate-buffered saline (PBS, pH 7.2) and stored at –20 ºC. Genomic DNA was extracted by modified phenol-chloroform method using

cetyltrimethylammonium bromide (CTAB) at 60 ºC for 1 hour (Russell 2002).

A fragment of 618bp of the 28SrRNA gene of *Fasciola* sp. was amplified by polymerase chain reaction (PCR) using a universal primer set (sense: 5'-acgtgattacccgctgaact-3' and antisense: 5'-ctgagaaagtgcactgacaag-3') (Marcilla *et al* 2002). The PCR was carried out in a 25µl reaction mixture containing 2µl of the genomic DNA (diluted 1:30), 1.5U of *Taq* DNA polymerase (Fermentas, Germany), 50mM of each dNTPs (CinnaGen, Iran), 2mM of $MgCl_2$, 2.5µl of PCR reaction buffer (10×) and 0.2µM of each primer. The reaction was performed in a Bioer XP thermal cycler. The PCR mixtures were subjected to an initial denaturation step at 94 ºC for 3 min, followed by 30 cycles of 30s at 94 ºC, 30s at 60ºC and 60s at 72ºC, and a final extension step at 72ºC for 5 min. A volume of 10µl of each PCR product along with the positive (i.e. the PCR mixture including the known DNA sample of *F. hepatica*) and negative (i.e. the PCR mixture excluding the DNA) controls were analyzed by electrophoresis on 1.5% agarose gel (w/v) for approximately 1.5 hours at 90V, and visualized by staining with 1% ethidium bromide. To specify *F. hepatica*, a restriction fragment length polymorphism (RFLP) analysis was developed. The restriction enzyme *Dra*II was selected to specifically digest the DNA of *F. hepatica* producing the expected restriction fragments (BioEdit software package, http://www.mbio.ncsu.edu (Marcilla *et al* 2002)). For the restriction digestion, a total volume of 15µl of the digestion reaction containing 5µl of the PCR product, 1µl of the restriction enzyme, 1.5µ µl of enzyme buffer (Fermentas, Germany) and 8µl of ddH_2O was prepared. The reaction tubes were incubated at 37ºC for 16 hours. The digested PCR products were run on 2% agarose gel and visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

Of a total of 6,759 pond snails collected from the 28 water bodies, 306 (4.53%) snails were belonging to the species *G. truncatula*, and 579 (8.57%) snails belonged to *L. stagnalis*. These two snail species were found in 10 sites located in northern (2), central (4) and southern (4) parts of West Azarbaijan (Figure 1). *G. truncatula* was found alive in the waters with temperatures ranging from 21 to 32.6 °C, salinity of 0.187-1.541g/l and pH of 5-9. *L. stagnalis* was living in the waters with temperatures of 17-28°C, salinity of 0.178-1.25 g/l and pH of 6-7.8 (Table 1). Based on our molecular examination, infection with *Fasciola* was observed in only 2 out of the 10 sites where the two snail species were present (Table 1). Results of the PCR (P) showed that 50% (153/306) of the examined *G. truncatula* and 2.5% (15/579) of the *L. stagnalis* snails were infected with a *Fasciola* species (Figure 2, Table 1). According to the restriction patterns of the RFLP (R) analysis, 16.6% of the *G. truncatula* and 1.1% of the *L. stagnalis* snails were found to be infected with the larval stages of *F. hepatica* (Figure 3, Table 1). While examined snails were found not to be infected with larval stages of *F. gigantica*. Maximum infection rates with *F. hepatica* were 16.6% in *G. truncatula* sampled from Dolatabad (37 °15' N and 44 °51' E) and 1.1% in *L. stagnalis* from Jabalkandi (45 °53' N and 37 °15'E). Determination of the prevalence of infection with *Fasciola* larvae and the role of the lymnaeid snails in their transmission is of great importance because of its relevance to the domestic livestock and human health (Rognlie *et al* 1994). Results of this study confirmed the presence of the infection with *F. hepatica* larvae in the examined field-collected snails from northwestern Iran. Though *F. hepatica* is reported to be prevalent in the domestic ruminants from different parts of Iran (Yakhchali & Ghobadi 2005), experimental studies on its transmission route are rare (Massoud & Sadjadi 1980, Shahlapour 1996). Molluscs of the genera *Lymnaea* and *Galba* serve as the intermediate hosts for the common liver fluke, *F. hepatica*, worldwide (Rognlie *et al* 1994), and *G. truncatula* has been found to be the main intermediate host snail species for the Iranian *F. hepatica* (Ashrafi *et al* 2007). However, this snail is not the only transmitter of fasciolosis to the domestic ruminants, as the disease has been detected in

the areas where *G. truncatula* is lacking (Abrous *et al* 1996). In contrast, the examined snails were not infected with larval stages of *F. gigantica* in the region. This finding was in line with Ashrafi et al. (2004) who noted that *Lmnaea gedrosiana* is preferred intermediate host for *F. gigantica* in Iran. The infected snails were geographically distributed over the central part of the province of West Azarbaijan where almost same environmental conditions were predominant. Such conditions seem to be suitable for both *F. hepatica* and its hosts such as *G. truncatula* and *L. stagnalis* snails. Hence, fasciolosis may outbreak following seasonal variations in the environmental conditions (Mas-Coma & Bargues 1997, Yakhchali & Ghobadi 2005). However, some snail species and probably their infectious agents can withstand different environmental conditions. For instance, our study showed that *G. truncatula* can spread out over the habitats with wide range of pH levels. It is important to note that even though some areas, e.g. Dolatabad and Jabalkandi, are identified as the hot spots for *Fasciola* infection in the domestic ruminants, the percentage of the infected snails harboring larval stages of *F. hepatica* in these areas was relatively low. Comparable rates of infection with *F. hepatica* in the host snails have been reported from different parts of the world. The infection rates were found to be 11.4% in Spain (Manga-Gonzalez *et al* 1991), 5.1% in France (Mage *et al* 2002), 4% in Algeria (Mekroud *et al* 2004) and 7% in Switzerland (Schweizer *et al* 2007). Although this study witnessed a low prevalence rate for *F. hepatica* larvae in *G. truncatula*, this low fasciolid prevalence in the examined snails seems to be sufficient for incidence of major infections in the domestic ruminants of the region. Furthermore, the prevalence of the infection with *F. hepatica* in the domestic ruminants of northwestern Iran was reported to be higher than that with *F. gigantica* (Ghobadi & Yakhchali 2003, Yakhchali & Ghobadi 2005). In the last few decades, 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 (Lotfy *et al* 2008; McGarry et al 2007, Mera y Sierra *et al* 2009). Determination of diversity and abundance of infection with fasciolid species in different lymnaeid snails has been the subject of several experimental studies in Iran (Athari *et al* 2006, Farahnak & Essalat 2003, Imani-Baran *et al* 2011). Recently, using molecular approaches in the study of adult *Fasciola* species have been considered by Iranian researchers (Mahami-Oskouei *et al* 2011, Shahbazi *et al* 2011). However, there is no previous molecular study on the epidemiology of *F. hepatica* in field-collected lymnaeid snails of Iran. This could therefore be considered as the first report in Iran in which the infection rate of a *Fasciola* species and its natural transmission was estimated by application of a novel molecular tool, i.e. the RFLP of 28SrRNA gene of *Fasciola* sp. It is worth noticing that the expected fragment of 27bp as a result of the digestion of 28SrRNA gene in *F. hepatica* by *Dra*II enzyme (Marcilla *et al* 2002) was not detectable in our study. This might be because of the quality of the DNA or more importantly, due to the genotypic differences between *Fasciola* isolates from different countries, which requires further studies to be clarified. Using molecular techniques for valid detection of infection with various trematode species is crucial not only due to its association with public health, but also because of its significant contribution to the livestock production and national economy. On the other hand, prevalence of animal fasciolosis as a major concern in several Iranian provinces makes such studies worthwhile. So that, this prompt and specific approach for detecting *F. hepatica* infection in the snails can be useful for establishment of suitable control programs against the domestic animals fasciolosis.

Ethics

I hereby declare all ethical standards have been respected in preparation of the article.

Table 1. Prevalence and the infection rates of *Fasciola* (detected by PCR, P) and *Fasciola hepatica* (detected by RFLP, R) in the snails *Galba truncatula* and *Lymnaea stagnalis* in different water bodies in North West Iran (n=885).

Location	No. of examined snails		Infection rate (*n/N*, %)				Water body-type		Area feature		Environmental parameters		
			Lt		Ls								
	Gt	Ls	P	R	P	R	Se	Pe	M	Pl	T (°C)	Sal (g/l)	pH
Barbaranbalanj (37°20' N and 45°09'E)	108	-	0	0	0	0	+	-	+	-	21	0.187	5
Dolatabad (37°15' N and 44°51'E)	115	-	50	16.6	0	0	+	-	+	-	27	0.187	5.5
Gargolugh (39°15' N and 45°08'E)	-	30	0	0	0	0	-	+	+	-	18	1.44	6.5
Islamabad (36°59' N and 45°25'E)	-	170	0	0	0	0	+	-	-	+	28	0.562	6
Jabalkandi (45°53' N and 37°15'E)	-	203	0	0	2.5	1.1	+	-	-	+	18	0.612	7.8
Kanikur (39°19' N and 44°54'E)	-	98	0	0	0	0	-	+	+	-	17	0.673	7.5
Khankandi (36°53' N and 46°07'E)	-	54	0	0	0	0	-	+	-	+	27	1.25	7
Moshirabad (36°52' N and 46°15'E)	-	24	0	0	0	0	-	+	-	+	25	0.178	6
Osalu (37°42' N and 45°13'E)	75	-	0	0	0	0	+	-	-	+	23	0.512	6.5
Shurgul (37°44' N and 45°04'E)	8	-	0	0	0	0	-	+	-	+	32.6	1.541	9
Total	306	579	50	16.6	2.5	1.1							

Gt, *Galba truncatula*; Ls, *Lymnaea stagnalis*; P and R, infection rates obtained by PCR and RFLP, respectively; Se, seasonal; Pe, perennial; M, montainous; Pl, plain; Sal, salinity; T, temperature.

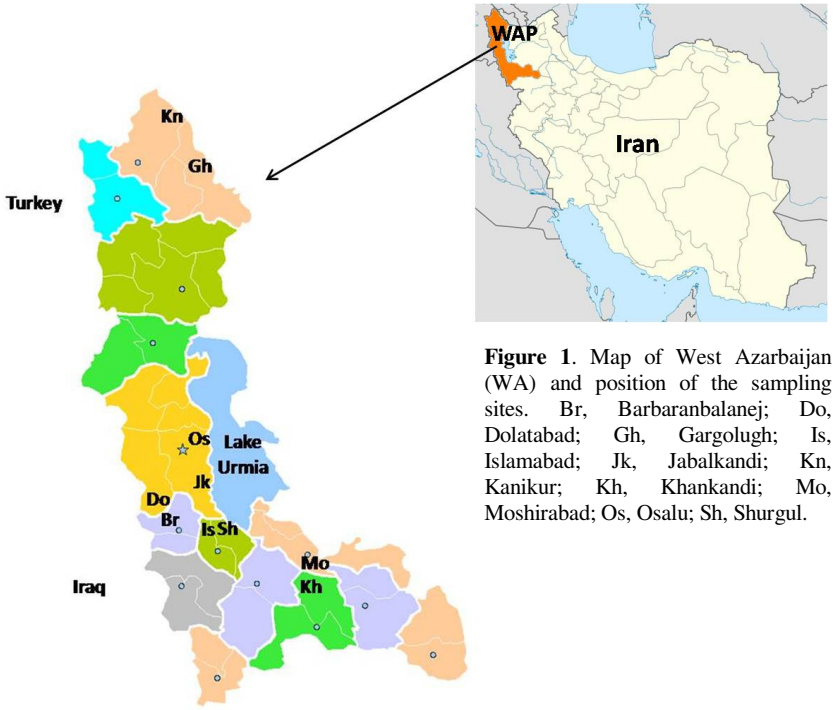

Figure 1. Map of West Azarbaijan (WA) and position of the sampling sites. Br, Barbaranbalanej; Do, Dolatabad; Gh, Gargolugh; Is, Islamabad; Jk, Jabalkandi; Kn, Kanikur; Kh, Khankandi; Mo, Moshirabad; Os, Osalu; Sh, Shurgul.

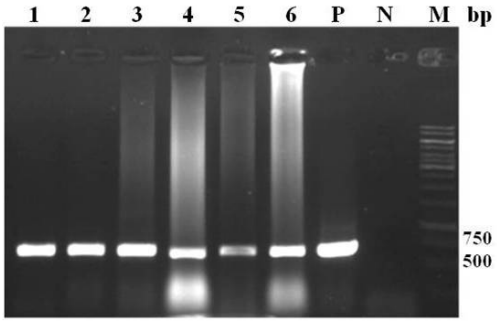

Figure 2. Agarose gel electrophoresis of the PCR products representing the infection of *Galba truncatula* (Lanes 1-3) and *Lymnaea stagnalis* (Lanes 4-6) with the larval stages of *Fasciola* sp. Lane P, positive control (*Fasciola* sp.); Lane N, negative control; Lane M, 250bp DNA size marker.

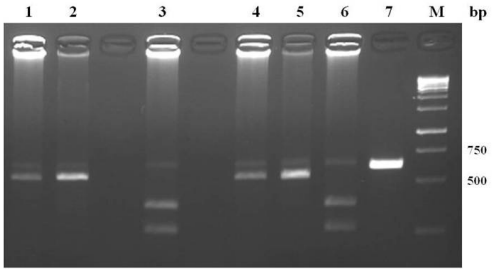

Figure 3. The PCR-RFLP pattern of the 28SrRNA gene of *Fasciola hepatica* in the infected snails. The infected snails are *Galba truncatula* (Lanes 1, 2) and *Lymnaea stagnalis* (Lanes 4, 5). Lane 7: *Dra*II and Lanes 3, 6: *Ava*II (positive controls, *Fasciola gigantica*). Lane M, 250bp DNA size marker.

Conflict of Interest

Hereby, I declare "no conflict of interest exists" regarding submitted article.

References

Abrous, M., Rondelaud D., and Dreyfuss G. (1996). Paramphistomum daubneyi and Fasciola hepatica: the effect of dual infection on prevalence and cercarial shedding in preadult Lymnaea glabra. *The Journal of parasitology* 82:1026-9.

Andrews, S.J. (1999) The life cycle of Fasciola hepatica, CABI Publishing, UK.

Ashrafi, A., Massoud J., Holakuei K., Mahmoodi M., and Joafshani M.A. (2004). Evidence suggesting that Fasciola gigantica may be the most prevalent causal agent of fasciolosis in Northern Iran. *Iranian Journal of Public Health* 33:31–37.

Ashrafi, K., Massoud J., Holakouie Naieni K., Jo-Afshani M.A., Mahmoodi M., Ebadati N., Rezvani S.M., Artigas P., Bargues M.D., and Mas-Coma S. (2007). Nuclear Ribosomal DNA ITS-2 Sequence characterization of Fasciola hepatica and Galba truncatula. *Iranian Journal of Public Health* 3:42-49.

Athari, A., Gohar-Dehi S.H., Rostami M., and Jalilian M.D. (2006). Determination of definitive and intermediate hosts of cercarial dematitis-producing agents in Northern Iran. *Archives of Iranian Medicine* 9:11-15.

Bargues, M.D., Mangold A.J., Munoz-Antoli C., Pointier J.P., and Mas-Coma S. (1997). SSU rDNA characterization of lymnaeid snails transmitting human fascioliasis in South and Central America. *The Journal of parasitology* 83:1086-92.

Caron, Y., Lasri S., and Losson B. (2007). Fasciola hepatica: An assessment on the vectorial capacity of Radix labiata and R. balthica commonly found in Belgium. Veterinary *Parasitology* 4:95-103.

Cucher, M.A., Carnevale S., Prepelitchi L., Labbe J.H., and Wisnivesky-Colli C. (2006). PCR diagnosis of Fasciola hepatica in field-collected Lymnaea columella and Lymnaea viatrix snails. *Veterinary Parasitol*ogy 137:74-82.

Faltynkova, A., Nasincova V., and Kablaskova L. (2007). Larval trematodes (Digenea) of the great pond snail, Lymnaea stagnalis (L.), (Gastropoda, Pulmonata) in Central Europe: a survey of species and key to their identification. *Parasite* 14:39-51.

Farahnak, A., and Essalat M.A. (2003). A study on cercarial dermatitis in Khoozestan province, southwestern Iran. *BMC Public Health* 3:35-38.

Ghobadi, K., and Yakhchali M. (2003). Determination of frequency of helminthes infection rate of liver and economical lost. *Journal of Faculty of Veterinary Medicine, Tehran University* 58:19-20.

Gutierrez, A., Hernandez D.F., and Sanchez J. (2005). Variation of snail's abundance in two water bodies harboring strains of Pseudosuccinea columella resistant and susceptible to Fasciola hepatica miracidial infection, in Pinar del Rio Province, Cuba. *Memorias do Instituto Oswaldo Cruz* 100:725-7.

Imani-Baran, A., Yakhchali M., Malekzadeh-Viayeh R., and Farhangpajuh F. (2011). Prevalence of cercariae infection in Lymnaea auricularia (Linnaeus, 1758) in North West of Iran. *Veterinary Research Forum* 2:121-127.

Imani Baran, A., Yakhchali M., and MalekzadehViayeh R. (2011). A study on geographical distribution and diversity of Lymnaeidae snails in West Azerbaijan Province, Iran. Veterinary Journal, Pajouhesh and Sazandegi 82:53-63.

Sambrook, J. and Russell D.W. (2002) Molecular cloning: a laboratory manual. 3 ed. Cold Spring Harbor Laboratory Press, New York.

Lotfy, W.M., Brant S.V., DeJong R.J., Le T.H., Demiaszkiewicz A., Rajapakse R.P., Perera V.B., Laursen J.R., and Loker E.S. (2008). Evolutionary origins, diversification, and biogeography of liver flukes (Digenea, Fasciolidae). *The American journal of tropical medicine and hygiene* 79:248-55.

Magalhaes, K.G., Passos L.K., and Carvalho Odos S. (2004). Detection of Lymnaea columella infection by Fasciola hepatica through Multiplex-PCR. *Memorias do Instituto Oswaldo Cruz* 99:421-4.

Mage, C., Bourgne H., Toullieu J.M., Rondelaud D., and Dreyfuss G. (2002). Fasciola hepatica and Paramphistomum daubneyi: changes in prevalences of natural infections in cattle and in Lymnaea truncatula from central France over the past 12 years. *Veterinary research* 33:439-47.

Mahami-Oskouei, M., Dalimi A., Forouzandeh-Moghadam M., and Rokni M.B. (2011). Molecular identification and differentiation of Fasciola isolates using PCR- RFLP method based on internal transcribed spacer (ITS1, 5.8S rDNA, ITS2). *Iranian Journal of Parasitology* 6:35-42.

Mansoorian, A.B. (1986). A practical guidline for identification of Iranian freshwater snails. *Iranian Journal of Public Health* 15:41-53.

Mansoorian, A.B. (2000a). Some freshwater Gastropods from Baluchestan and Sistan province, south-east Iran. *Journal of the Faculty of Veterinary Medicine, tehran University* 55:49-51.

Mansoorian, A.B. (2000b). Some freshwater Gastropods from Kermanshah province, western Iran. *Journal of the Faculty of Veterinary Medicine, Tehran University* 55:85-87.

Marcilla, A., Bargues M.D., and Mas-Coma S. (2002). A PCR-RFLP assay for the distinction between Fasciola hepatica and Fasciola gigantica. *Molecular and Cellular Probes* 16:327-333.

Mas-Coma, S., and Bargues M.D. (1997). Human liver flukes: a review. *Research and Reviews in Parasitology* 57:145-218.

Mas-Coma, S., Funatsu I.R., and Bargues M.D. (2001). Fasciola hepatica and lymnaeid snails occurring at very high altitude in South America. *Parasitology* 123:115-127.

Massoud, J., and Sadjadi S. (1980). Susceptibility of different species of Lymnaea snails to miracidia of Fasciola gigantica and F. hepatica in Iran. *Journal of helminthology* 54:201-202.

McGarry, J.W., Ortiz P.L., Hodgkinson J.E., Goreish I., and Williams D.J.L. (2007). PCR-based differentiation of Fasciola species (Trematoda: Fasciolidae), using primers based on RAPD derived sequences. *Annals of tropical medicine and parasitology* 101:415-421.

Mekroud, A., Benakhala A., Vignoles P., Rondelaud D., and Dreyfuss G. (2004). Preliminary studies on the prevalences of natural fasciolosis in cattle, sheep, and the host snail (Galba truncatula) in north-eastern Algeria. *Parasitology Research* 92:502-505.

Mera y Sierra, R., Artigas P., Cuervo P., Deis E., Sidoti L., Mas-Coma S., and Bargues M.D. (2009). Fascioliasis transmission by Lymnaea neotropica confirmed by nuclear rDNA and mtDNA sequencing in Argentina. *Veterinary Parasitol*ogy 166:73-9.

Meunier, C.C., Tirard S., Hurtrez-Bousses P., Durand M.D., Bargues M.D., Mas-Coma S., Pointier J.P., Jourdane J., and Renaud F. (2001). Lack of molluskan host diversity and the transmission of emerging disease in Bolivia. *Molecular Ecology* 10:1333-1340.

Pfleger, V. (1999) A field guide in colour to molluscs Aventinum Nakladatelstyi, S.T.O., Polygrafia, Prague, Czech Republic.

Relf, V., Good B., McCarthy E., and De Waal T. (2009). Evidence of Fasciola hepatica infection in Radix peregra and a mollusc of the family Succineidae in Ireland. *Veterinary Parasitology* 163:152-155.

Rim H.J., Farag H.F., Sornmani S., and Cross J.H. (1994). Food-borne trematodes: ignored or emerging?. *Parasitology Today* 10:207-209.

Rognlie M.C., Dimke L.L., and Knapp S.E. (1994). Detection of Fasciola hepatica in infected intermediate hosts using RT-PCR. *Journal of Parasitology* 80:748-755.

Schweizer, G., Meli M.L., Torgerson P.R., Lutz H., Deplazes P., and Braun U. (2007). Prevalence of Fasciola hepatica in the intermediate host Lymnaea truncatula detected by real time TaqMan PCR in populations from 70 Swiss farms with cattle husbandry. *Veterinary Parasitology* 150:164-9.

Shahbazi, A., Akbarimoghaddam M., Izadi S., Ghazanchaii A., Jalali N., and Bazmani A. (2011). Identification and genetic variation of Fasciola species from Tabriz, North-Western Iran. *Iranian Journal of Parasitology* 6:52-59.

Shahlapour, A.A. (1996). A survey on the role of fascioliasis intermediate host in disease. *Veterinary Journal, Pajouhesh and Sazandegi* 30:121-124.

Torgerson, P., and Claxton J. (1999) Epidemiology and control CABI Publishing, UK.

Yakhchali, M., and Ghobadi K. (2005). A survey on helminthes infection of liver and economic loss in slaughtered sheep of Urmia slaughterhouse, Iran. *Iranian Veterinary Journal* 11:60-65.