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Original Article

Nuclear Ribosomal DNA ITS-2 Sequence Characterization of *Fasciola hepatica* and *Galba truncatula*

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Abstract

Background: Human fascioliasis is an important health problem in the province of Gilan, at the Caspian Sea, Iran. There is the overlapping of both fasciolid species, *Fasciola hepatica* and *F. gigantica*. Recent studies on both domestic animal and lymnaeid infection furnished evidence suggesting that *F. gigantica* and *Radix gedrosiana* may be the main fasciolid and lymnaeid involved in the disease in that province, controversy still being there concerning the presence and importance of *F. hepatica* and other lymnaeid species. The present paper includes the results of studies on *Galba truncatula* and the first finding of natural infection by *F. hepatica* in Gilan proved by molecular studies.

Methods: Snail collections were carried out in summer, when their populations present the highest densities. Surveys on lymnaeids furnished the finding of a lymnaeid snail infected by trematode rediae and cercariae in the mountains of Talesh, in the Asalem district, western Gilan.

Results: Nuclear ribosomal DNA ITS-2 sequences proved that they were *F. hepatica* and *G. truncatula*. The liver fluke ITS-2 sequence was identical to that of *F. hepatica* from Spain and the Northern Bolivian Altiplano and that of *G. truncatula* to the haplotype H-2 known in Portugal, Spain, France and The Netherlands.

Conclusion: This genetic characterization suggests that both may be also involved in human fascioliasis infection in Gilan.

Keywords: Fasciola hepatica, *Iinfection, rDNA ITS-2 sequences, Iran*

Introduction

Fascioliasis is an important disease caused by two digenetic trematode species of the genus *Fasciola* Linnaeus, 1758 (Trematoda: Fasciolidae): *F. hepatica* (Linnaeus, 1758) and *F. gigantica* Cobbold, 1855. Whereas in Europe, the Americas and Oceania only *F. hepatica* is concerned, the distributions of both species overlap in many areas of Africa and Asia (1). *F. hepatica* is believed to be of European origin, with *G. truncatula* as the original intermediate host species (2). In Europe it has been even found in prehistoric human populations of the Stone Age, living at the end of the Mesolithic period, 5000-5100 yr ago and the Neolithic, a period marked by the domestication of animals and the development of agriculture, among other characteristics (3). *F. hepatica* has succeeded in expanding from the European original geographical area up to actually colonize the five continents (1).

Throughout its large geographical distribution, *F. hepatica* is a well-known veterinary problem. Moreover, studies carried out in recent years have shown it to be an important public health problem as well (4-7). Human cases have been reported in 51 countries of the five continents (8) with severe symptoms and pathology being observed (1, 4, 7, 9) we know that fasciolosis can no longer be considered merely as a secondary zoonotic disease but must be considered to be an im-

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portant human parasitic disease (6, 7). Recent papers estimate human infection up to 2.4 million (10) or even up to 17 million people (11).
Several areas in Central and South America, Europe, Africa and Asia have recently shown to be human endemic areas, ranging from hypo- to hyperendemic (6). These areas present a very wide spectrum of epidemiological characteristics related to the very wide diversity of environments. Such diversity is emphasized by only mentioning that fasciolosis is unique in being capable to give rise to human hyperendemic areas from below sea level (as in the Gilan province, besides the Caspian Sea, in Iran) up to the very high altitude (as in the Andean altiplanos and valleys of Bolivia, Peru and Venezuela). No other vector-borne disease presents such a wide altitudinal range (12). At present, fasciolosis by *F. hepatica* is the vector-borne parasitic disease presenting the widest latitudinal, longitudinal and altitudinal distribution known (12).
In Asia, we know today that in given regions human fasciolosis is an important health problem, as in the Near East countries surrounding the Caspian Sea. Iran, with epidemics affecting up to 10,000 subjects in Gilan province, in the zone around Rasht and Bandar-e Anzali (13) but also with cases in neighboring provinces as Mazandaran (14, 15) is the country from which the present knowledge on human fascioliasis is larger.
In Gilan there is the overlapping of both fasciolid species, and adult flukes belonging to both species are usually found in the liver of the same livestock individual. Recent studies on both domestic animal and lymnaeid infection furnished evidence suggesting that *F. gigantica* and *R. gedrosiana* may be the main fasciolid and lymnaeid involved in the disease in that province, controversy still being there concerning the presence and importance of *F. hepatica* and other lymnaeid species (13).
The present paper includes the results of studies on *G. truncatula* and the first finding of natural infection by *F. hepatica* in Gilan proved by molecular studies.

Materials and Methods

Snail sampling Snail collections were carried out in summer, when snail populations present the highest densities. The water bodies were surveyed in mountainous areas of the province of Gilan, Iran, including mainly springs, shallow running water and small streams from different sources. Snails were most often found in small springs with very shallow and clean water coming out from the ground and originating small streams. Snails were collected using a soft forceps, put in plastic screw capped containers including natural water with some small pieces of lettuce for snail feeding and taken to the laboratory for examination.

Detection of snail infection Once in the laboratory, some snails were put in glass Petri-dishes containing natural water and a piece of lettuce for cercarial shedding. The rest of the snails, with a shell length of more than 5 mm, were crushed between two glass slides, under a dissection microscope and examined for the presence of fasciolid larval stages. The positive specimens were transferred into glass Petri-dishes containing natural water for metacercarial formation. The metacercariae and other larval stages found were put in 90% alcohol for molecular studies. Some snails from the same population were also fixed in 90% alcohol for molecular studies.

Molecular techniques

F. hepatica larval stages washed extensively in PBS (37 °C) and subsequently fixed in 70% ethanol and maintained at 4 °C for several weeks. The fixed samples were used for DNA extraction according to the phenol-chloroform method (16). DNA extraction was performed according to BARGUES & MAS-COMA (17).
The fragment corresponding to the complete, second internal transcribed spacer of the nuclear ribosomal DNA (ITS-2) of each trematode and lymnaeid were amplified by the Polymerase Chain Reaction (PCR) using 4-6 µl of genomic DNA for each 50 µl PCR reaction, according to methods outlined previously (18). The PCR amplification was performed using primers designed in con-

served positions of 5.8S and 28S rRNA genes of several eukaryote Metazoa species.

Amplifications were generated in a GeneAmp PCR system 9600 (Perkin Elmer, Norwalk, CT, USA), by 30 cycles of 30 sec at 94 °C, 30 s at 50 °C and 1 min at 72 °C, preceded by 30 s at 94 °C and followed by 7 min at 72 °C. Ten microliters of the reaction mixture were examined by 1% agarose gel electrophoresis, followed by ethidium bromide staining.

Primers and nucleotides were removed from PCR products by purification on Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's protocol and resuspended in 50 µl of 10 mM TE buffer (pH 7.6). The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm.

Sequencing was performed on both strands by the dideoxy chain-termination method, and with the Taq dye-terminator chemistry kit for ABI PRISM 377 (Perkin Elmer, Foster City, CA), using PCR primers.

For sequence alignment the CLUSTAL-W version 1.8 (THOMPSON, HIGGINS & GIBSON, 1994) was used (19).

Results

Geographical origin of the finding More than two hundred lymnaeid snails from the above-mentioned mountainous area were collected. Small snails of less than 5 mm were used for experimental infections and larger ones of more than 5 mm for studying natural infections. A total of 30 snails were placed in separated Petri dishes for cercarial shedding and another 43 lymnaeid individuals were crushed for the search of fasciolid larval stages. Those snails located in Petri dishes were followed until death and none of them shed cercariae. Of the 43 crushed snails, only one showed to be infected with trematode rediae including cercariae inside.

The infected lymnaeid was collected in a snail population located in a place with pastures called Taklamestan (latitude: 37,537; longitude: 48,789) in the mountains of Talesh, in the Asalem district near the Talesh district, western Gilan, on July 31, 2003. The lymnaeid population showed a density of 15-20 lymnaeids per square meter. The place is located at an altitude of 1800 m, where pastures are mainly inhabited by sheep and goats, with cattle in fewer numbers. In the place of collection, the water had a temperature of 18° C, a pH of 6-6.5, and showed dense vegetation forming small water covers. There was only one species of lymnaeid snail in the water body.

Molecular classification of the trematode larval stages

The complete rDNA ITS-2 sequence of the rediae and cercariae was 364 bp long, with a 48.3% GC content (Fig 1). The comparison with the ITS-2 sequences of trematodes available in the GenBank showed that this sequence is identical to that of *F. hepatica* from Spain and the Northern Bolivian Altiplano (Accession Number AJ272053). In the 364-bp-long alignment, no nucleotide difference was found when comparing with the haplotypes of this liver fluke species, thus proving that the larval stages found in Gilan belong to *F. hepatica*. In the same alignment, five nucleotide differences were detected when comparing with *F. gigantica* (positions 210, 234, 273, 279 and 337).

Molecular classification of the lymnaeid host individual The complete rDNA ITS-2 sequence of the lymnaeid snails collected in the same population was 401 bp long, with a 59.1% GC content (Fig 2). This sequence is identical to that of *G. truncatula* populations of the haplotype H-2 known in Portugal, Spain, France and the Netherlands and available in the GenBank (Accession Number AJ296271), proving that the Iranian material belong to this lymnaeid species.

In the 401-bp-long alignment, the lymnaeid sequence of Gilan differs from the remaining haplotype (H-1) present in Europe and also in Morocco by only one C/T transition (C/T) in position 149 (Accession Number AJ243017) and from that known from the Northern Bolivian Altiplano by one transversion (G/T) in position 55 (Accession Number AJ272051).

Fig.1: Sequences of the second internal transcribed spacer of the nuclear ribosomal DNA (rDNA ITS-2) of *Fasciola hepatica* from Gilan

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1     GTTATAAACT ATCACGACGC CCAAAAAGTC GTGGCTTGGG TTTTGCCAGC
TGGCGTGATC
61    TCCTCTATGA GTAATCATGT GAGGTGCCAG ATCTATGGCG TTTCCCTAAT
GTATCCGGAT
121   GCACCCTTGT CTTGGCAGAA AGCCGTGGTG AGGTGCAGTG GCGGAATCGT
GGTTTAATAA
181   TCGGGTTGGT ACTCAGTTGT CAGTGTGTTT GGCGATCCCC TAGTCGGCAC
ACTTATGATT
241   TCTGGGATAA TTCCATACCA GGCACGTTCC GTCACTGTCA CTTTGTCATT
GGTTTGATGC
301   TGAACTTGGT CATGTGTCTG ATGCTATTTT CATATAGCGA CGGTACCCTT
CGTGGTCTGT
361   CTTC
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Fig. 2: Sequences of the second internal transcribed spacer of the nuclear ribosomal DNA (rDNA ITS-2) of *Galba truncatula* from Gilan

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1     GCTAGTCACA AAGCATTCGT GTCCTTGCAG CTCTCGCAAA AACCGAAGCC
TTGCGGCGTG
61    AGCTCTCACG CTGCTCGGCG ATGGTTGGAT ACGCCCTGGA CCCTCGCGGC
CAAAGCTGTC
121   GTTGCCTGCT CGGCGGCGAC GGTGACGGCC CCGTGGTCTT AAGCGCAAGC
CGCGCCGTTG
181   TCCGTTCATC TCGTAACGTC TTCGACGCTG CCCTGCTCTT GGCGGCCTGT
CCGTTTTCTC
241   TACCGCCAGG CAGGACCCGG CTCGCTTACT TTATTTATTA TCGTGGCGTT
CTCGGGCCTG
301   CAGTCCATGG CATCGCAGCT CGTGGGTGGA GAACAAGGGG CTCTAAGACG
CTACGTGGTC
361 GGCGCCCGTC GTTGAATGAA ACATTATTTG TTTCTTTTCT C
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Discussion

The two species *F. hepatica* and *F. gigantica* are present in fasciolosis endemic areas in Iran. The main disease problematic is known in the Gilan province, at the Caspian Sea, at the northwestern area of Iran, where high fasciolosis prevalence in livestock and human infections are known since long ago (20). Additionally, during the 80s and the 90s several large epidemics, including thousands of human cases, were reported in Gilan (21-28). Recently, human fasciolosis cases and an animal endemic situation have also been described in the neighboring, Caspian province of Mazandaran (14).

Many malacological studies on the freshwater mollusks fauna of Iran have been performed since long ago, several including the northern parts of the country, at the Caspian Sea shore (15, 29-36). It is well known that the two fasciolid species show different lymnaeid snail host specificity: *F. hepatica* is mainly transmitted by species of the *Galba/Fossaria* group, whereas *F. gigantica* is above all transmitted by species belonging to the *Radix* group (37). *G. truncatula* is the main in-

termediate snail host species of *F. hepatica* where present (1, 18, 37) and experimentally it appears to also be very susceptible for Iranian *F. hepatica* isolates (38). However, studies in Iran have already proved the capacity of *R. gedrosiana* to transmit both *F. gigantica* (38, 39) and *F. hepatica* (40) so that the question about which species is transmitting Iranian *F. hepatica* in nature was still open. Moreover, recent studies suggest that *F. gigantica* may be the predominant fasciolid species in Gilan (13) a fact supported by both morphology of the liver flukes found in livers of slaughtered livestock and the wide spread of *R. gedrosiana* throughout the endemic areas.

The results obtained in the present study confirm that both *F. hepatica* and *G. truncatula* are present in Gilan. Moreover, results show that they are linked one another in the transmission of the disease in spite of the very low larval stage prevalence detected in *G. truncatula* individuals (only one snail found infected). At any rate, very low fasciolid infection rates in lymnaeid snails appear to be the common situation even in high endemic areas. Thus, studies have shown that very low snail infection rates were sufficient to produce major infections in mammalian hosts in Australia and Louisiana (41, 42) and in Morocco only two infected snails were found during a 3-year period study (43).

Ribosomal DNA sequences show that *F. hepatica* from Iran is genetically identical to that present in Spain and the Northern Bolivian Altiplano at least at ITS-2 level (18). Taking into account that in Spain this liver fluke species have been reported in many human cases (8) and that the Northern Bolivian Altiplano appears to be the highest human fasciolosis endemic area known (44) the capacity of Iranian *F. hepatica* to be an important human pathogen is evident.

Concerning the intermediate host, Iranian *G. truncatula* appears to be genetically identical to the haplotype H-2 of this lymnaeid species known in Western Europe (Portugal, Spain, France and The Netherlands), namely the European region where more human cases of fascioliasis have been diagnosed (6, 45).

All this does not fit well with the recent results suggesting that *F. gigantica* and *R. gedrosiana* are the most widespread of both fasciolids and of the lymnaeid species in Gilan, respectively (13). Additional studies are needed to clarify the geographical distribution and prevalences of the two fasciolids and the different lymnaeid species to ascertain the real epidemiological situation in the Gilan human endemic area.

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The authors declare that they have no conflict of Interests.

References

1. Mas-Coma S, Bargues MD (1997). Human liver flukes: a review. *Res Rev Parasitol*, 57(3-4):145-218.
2. Oviedo JA, Bargues MD, Mas-Coma (1995). Lymnaeid snails in the human fascioliasis high endemic zone of the Northern Bolivian Altiplano. *Res Rev Parasitol*, 55(1):35-43.
3. Mas-Coma S (2003). The 9th Chamlong-Tranakchit Harinasuta Lecture-Human fascioliasis: epidemiological patterns in human endemic areas of South America, Africa and Asia. In: 4th Seminar on Food- and Water-borne Parasitic Zoonoses, 2nd International Meeting on Gnathostomiasis, and Joint International Tropical Medicine Meeting 2003 (4th FBPZ & JITMM 2003, 2-4 Dec. 2003, Siam City Hotel), Bangkok, Thailand: 44-60.
4. Chen MG, Mott KE (1990). Progress in assessment of morbidity due to *Fasciola hepatica* infection: a review of recent literature. *Trop Dis Bull*, 87(4):R1-R38.
5. World Health Organization (1995). Control of foodborne trematode infections. *WHO Tech Rep Series*, World Health Organization, Geneva, 849:1-157.
6. Mas-Coma S, Esteban JG, Bargues MD (1999a). Epidemiology of human fascioliasis: a review and proposed new classification. *Bull WHO*, 77(4):340-46.
7. Mas-Coma S, Bargues MD, Esteban JG (1999). Human Fasciolosis. In: *Fasciolosis*. Ed, JP Dalton. CAB International Publishing. Wallingford, Oxon, UK, pp.411-34.
8. Esteban JG, Bargues MD, Mas-Coma S (1998). Geographical distribution, diagnosis and treatment of human fascioliasis: a review. *Res Rev Parasitol*, 58(1):13-42.
9. Mas-Coma S, Bargues MD, Marty AM, Neafie RC (2000). Hepatic Trematodiases. In: *Pathology of Infectious Diseases*, Eds, WM Meyers, RC Neafie, AM Marty & DJ Wear. Armed Forces Institute of Pathology and American Registry of Pathology, Washington D.C, pp.69-92.
10. Rim HJ, Farag HF, Sornmani S, Cross JH (1994). Food-borne trematodes: ignored or emerging? *Parasitol Today*, 10(6):207-9.
11. Hopkins DR (1992). Homing in on helminths. *Am J Trop Med Hyg*, 46:626-34.
12. Mas-Coma S, Bargues MD, Valero MA, Fuentes MV (2003). Adaptation capacities of *Fasciola hepatica* and their relationships with human fascioliasis: from below sea level up to the very high altitude. In: *Taxonomy, Ecology and Evolution of Metazoan Parasites.* Eds, C. Combes & J. Jourdane. Presses Universitaires de Perpignan, Perpignan, pp.81-123.
13. Ashrafi K, Massoud J, Holakouie Naieni K, Mahmoodi M, Jo-Afshani MA, Valero MA, Fuentes MV, et al. (2004). Evidence suggesting that *Fasciola gigantica* may be the most prevalent causal agent of fascioliasis in the endemic province of Gilan, northern Iran. *Iranian J Pub Health*, 33(4):31-7.
14. Moghaddam AS, Massoud J, Mahmoodi M, Mahvi AH, Periago MV, Artigas P, Fuentes MV, Bargues MD, Mas-Coma S (2004a). Human and animal fascioliasis in Mazandaran province, northern Iran. *Parasitol Res*, 94(1):61-9.
15. Moghadam AS, Massoud J, Mahmoodi M, Khoubbane M, Artigas P, Periago MV, Fuentes MV, Bargues MD, Mas-Coma S (2004b). Distributional outline of lymnaeid snails (Gastropoda) in the fascioliasis endemic area of Mazandaran, Iran. *Acta Parasitol*, 49(2):145-52.
16. Sambrook J, Fritsch EF, Maniatis T (1989). *Molecular Cloning, a Laboratory Manual*. 2nd ed. Cold Spring Harbor, New York, USA, p.1647.
17. Bargues MD, Mas-Coma S (1997). Phylogenetic analysis of lymnaeid snails based on 18S rDNA sequences. *Mol Biol Evol*, 14 (5):569-77.

18. Mas-Coma S, Funatsu IR, Bargues MD (2001). *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology*, 123:S115-S27.
19. Thopson JD, Higgins DG, Gibson TJ (1994). Improving the sensitivity and progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res*, 22:4673-680.
20. Sabokbar RD (1960). Geographical distribution of *Fasciola hepatica* and its relation with human distomatosis. *J Med Fac Tehran Univ*, 17:251-60.
21. Massoud J (1990). Fascioliasis outbreak of man and drug test (Triclabendazol) in Caspian littoral, northern part of Iran, 1989. *Bull Soc Fr Parasitol*, 8(Suppl. 1):438.
22. Massoud J (1993). Present status of human fascioliasis in Iran. In: *Food-borne trematodes*, *World Health Organization*, WHO Manual, Manila, Mimeogr. Rep. Sch/SG/93 w, p.19.
23. Pourtaghva M, Shafi A, Saberi A, Bahar K, Solaymanlou F (1990). Fasciolase en Iran. *Bull Soc Fr Parasitol*. 8(Suppl. 1): 404.
24. Yadegari D, Forghan-Parast K, Assmar M (1990). Investigation of an epidemic of fascioliasis in North of Iran. *Bull Soc Fr Parasitol*. 8(suppl. 2):868.
25. Yadegari D, Talaie H, Massoud J (1999). Clinical trial of Triclabendazole on human fascioliasis: long term follow up. *Med J Islamic R Iran*, 13:89-91.
26. Assmar M, Milaninia A, Amir-Khani A, Yadegari D, Forghan-Parast K, Nahravanian H, Piazak N (1991). Seroepidemiological investigation of fascioliasis in northern Iran, *Med J Islamic R Iran*, 5:23-7.
27. Forghan-Parast K, Yadegari D, Assmar M (1994). Study of clinical epidemiology of fascioliasis in Gilan, *J Med Fac Gilan Univ Med Sci Iran*, 2(6&7):4-11.
28. Yadegari D, Talaie H (1996). Six years follow-up of triclabendazole in human fascioliasis. In: 7th Iranian Congress of Internal Medicine (16-19 May 1996), Shahid Beheshti University of Medical Sciences, Abstracts book, p.167.
29. Issel A (1866). Catalogo dei molluschi raccolti dalla Missione Italiana in Persia aggiuntavi la descrizione delle specie nuove o poco note. *Memorie della Reale Accademia delle Scienze di Torino, Serie Seconda*, 23:387-442.
30. Forcart L (1935). Die Mollusken der nordpersischen Provinz Masenderan und ihre tiergeographische Bedeutung. *Archive für Naturgeschichte*, Leipzig, NF, 4(3):404-47.
31. Starmühlner F, Edlauer Ä (1957). Ergebnisse des österreichischen Iran-Expedition 1949/50. Beiträge zur Kenntnis der Molluskenfauna des Iran. *Knochyliologische Bestimmungen und Beschreibungen. Sitzberichte der Oesterreichischen Akademie der Wissenschaften*, Wien, Mathem.-Naturwiss. Klasse, Abteilung I, 166(9-10):435-94.
32. Starmühlener F (1961). Ein kleine Molluskenausbeute aus Nord- und Ostiran. *Sitzberichte der Oesterreichischen Akademie der Wissenschaften, Wien, Mathem-Naturwiss.* Klasse, Abteilung I, 170(3-4): 89-99.
33. Starmühlener F (1965). Ein weiterer Beitrag zur Wassermolluskenfauna des Iran. *Sitzberichte der Oesterreichischen Akademie der Wissenschaften,* Wien, Mathem.-Naturwiss. Klasse, Abteilung I, 174(5-6):171-84.
34. Eliazian M, Tamiji Y, Akbar-Zadeh M, Hagh-Nazari J (1979). Snails from the northern parts of Iran (Caspian Sea). *Archives of the Institute of Razi*, 31:29-36.
35. Mansoorian A (1994). Freshwater snails of Iran. Scientific Publication, School of Public Health and Public Health Research, Tehran, Iran, *Technical Series* No. 2145/1374.
36. Mansoorian A (2000). Some freshwater snails from Northern Iran. *Iranian J Pub Health*, 29(1-4):77-82.
37. Bargues MD, Vigo M, Horak P, Dvorak J, Patzner RA, Pointier JP, Jackiewicz M, Meier-Brook C, Mas-Coma S (2001).

European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. *Inf Gen Evol*, 1(2):85-107.

38. Massoud J, Sadjadi S (1980). Susceptibility of different species of Lymnaea snails to miracidia of *Fasciola gigantica* and *F. hepatica* in Iran. *J Helminthol*, 54(3): 201-2.
39. Sahba GH, Arfaa F, Farahmandian I, Jalali H (1972). Animal fascioliasis in Khuzestan, southwestern Iran. *J Parasitol*, 58(4):712-16.
40. Arfaa F, Movafagh K, Mahdavi M (1969). *Lymnaea gedrosiana*, an intermediate host of *Fasciola hepatica* in Iran. *J Parasitol*, 55(1):134-35.
41. Boray JC, Happich FA, Andrews JC (1969). The epidemiology of fascioliasis in two representative endemic regions of Australia. *Australian Vet J*, 45:549-53.
42. Malone JB, Loyacano AF, Hugh-Jones ME, Cortman KC (1984). A three-year study on seasonal transmission and control of *Fasciola hepatica* of cattle in Louisiana. *Preventive Vet Med*, 3:131-41.
43. Khallaayoune KH, Stromberg BE, Dakkak A, Malane JB (1991). Seasonal dynamics of *Fasciola hepatica* burdens in grazing Timahdit sheep in Morocco. *Int J Parasitol*, 21(3):307-14.
44. Mas-Coma (2004). Human fascioliasis. In: *World Health Organization, Waterborne Zoonoses*: *Identification, Causes and Control*. Eds, JA Cotruvo, A Dufour, G Rees, J Bartram, R Carr, DO Cliver, GFCraun, R Fayer, VPJ Gannon, IWA Publishing, London, UK, pp.305-22.
45. Mas-Coma S, Angles R, Esteban JG, Bargues MD, Buchon P, Franken M, Strauss W (1999). The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. *Trop Med Int Health*, 4(6):454-67.