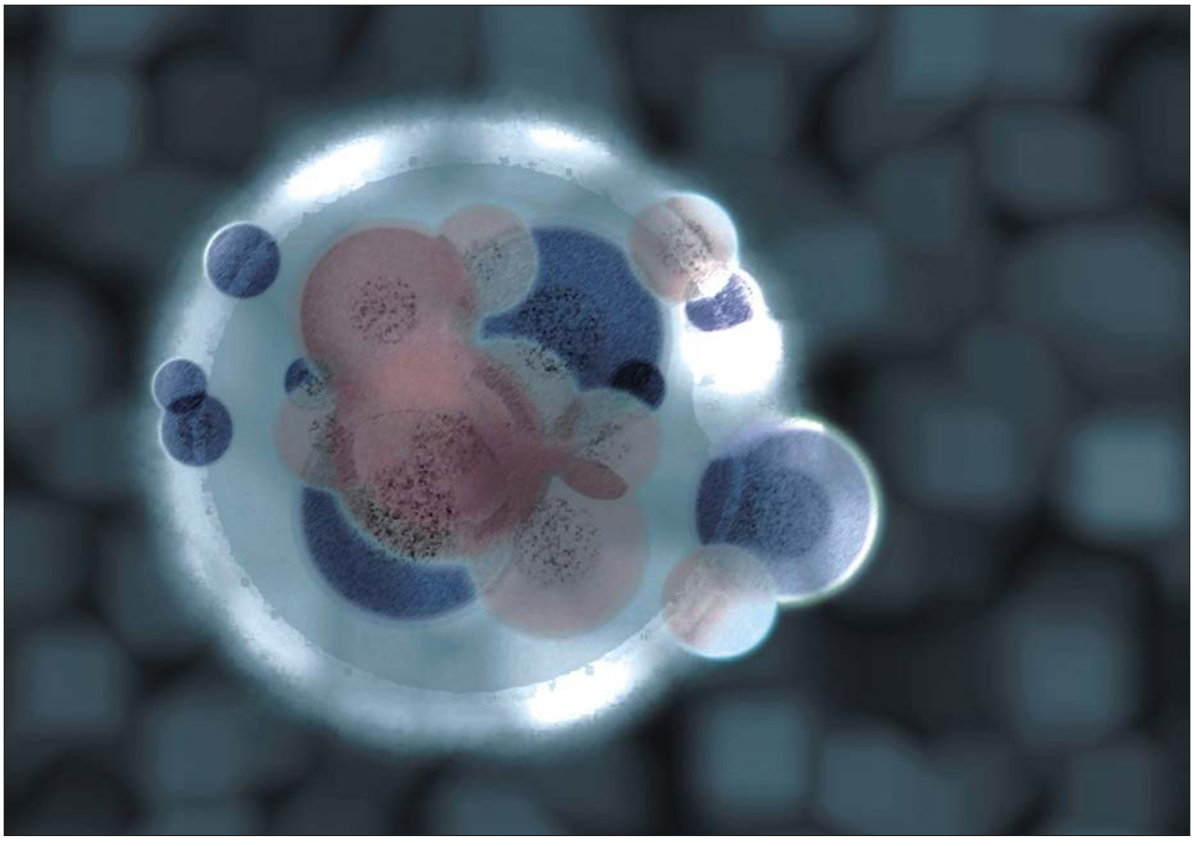

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Genetic Diagnosis of *Fasciola* Species Based on 18S *Ribosomal* DNA Sequences

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Abstract: Genotypic analysis of 263 and 356 bp fragments of 18S rDNA obtained from *Fasciola hepatica* and *Fasciola gigantica* from Fars province using PCR-RFLP assay demonstrated that nucleotide sequences of *F. hepatica* from Iran differed to those reported in the other countries. PCR-RFLP bands profile using the DraI restriction enzyme differed markedly between *F. hepatica* and *F. gigantica* whereas, PCR-RFLP bands profile of *F. hepatica* and *F. gigantica* with restriction enzyme BfrI was similar together. The nucleotide sequencing results of 18S rDNA of *F. hepatica* and *F. gigantica* demonstrated 0.3% differences between Iranian *F. hepatica* and standard *F. hepatica* reported in genebank. This is the first time that molecular evidence had suggested the possible existence of an intermediate genotype of *Fasciola* in Iran, in addition to *F. hepatica* and *F. gigantica* as its 18S rDNA sequences were unique in that two different 18S rDNA sequences exist in the rDNA array within a single *Fasciola* worm. This micro heterogeneity is possibly due to sequence polymorphism among copies of the 18S rDNA array within the same worm. Based on our findings a PCR-RFLP should provide a valuable tool for the molecular identification and for studying the ecology, epidemiology and genetic structures of *F. hepatica* and *F. gigantica* especially in areas which both species co-exist, as Iran.

Key words: Genetic, diagnosis, 18S ribosomal DNA, *Fasciola hepatica*, *Fasciola gigantica*

INTRODUCTION

Fascioliasis is an important socio-economics disease caused by *Fasciola hepatica* and *Fasciola gigantica*. Besides its well known veterinary importance they are recognized also, as a serious public health problem (Mas-Coma *et al*., 1999, 2005). Human infection is estimated up to 17 million people (Hopkins, 1992). Importance of this zoonotic food-borne disease with a great impact on human development have been emphasized by WHO and other human health institutes, so, more recently Fascioliasis is added to the list of important helminthiasis (WHO, 1995; Anonymous, 2004). Whereas, in Europe, America and Oceania only *F. hepatica* is present, in Iran and many other areas of Asia and Africa both species co-exist (Mas-Coma *et al*., 1999; Rokni *et al*., 2002; Lotfy *et al*., 2002; Karimi, 2008). This geographical overlapping gives rise to many problems in the diagnosis, which finally remains classified as *Fasciola* sp. (Marcilla *et al*., 2002; Ashrafi *et al*., 2006). It is usually difficult to accurately discriminate between *F. hepatica* and *F. gigantica* because of many variations in the morphological characteristics. Moreover very experimental and field examination based on parasitological diagnosis, clinical, pathological and immunological analysis can not differentiate between *F. hepatica* and *F. gigantica* up to the present (El-Shabrawi *et al*., 1997; Hillyer, 1999; Mas-Coma *et al*., 2000). The low number of records of human infection with *F. gigantica* may be due to the lack of good tools to distinguish this species from *F. hepatica* (Marcilla *et al*., 2002). The differential diagnosis between *F. hepatica* and *F. gigantica* is very important, particular where overlapping observe, because of their different epidemiological diagnosis and characteristics the helmintic parasites (Karimi, 2008). Consequently a rapid and simple test for the differentiation of the two *Fasciola* species is needed. The usefulness of molecular genetic techniques based on nuclear and mitochondrial DNA was emphasized while addressing problems of identification characterization and phylogeny of parasites (Knox, 2004; Gasser, 2006). Choice of sequences not repeated multiple times in the genome may have the effect of limiting sensitivity. In this study, differential diagnosis was implied at DNA level using the PCR-RFLP technique. The high prevalence of Fascioliasis in the human and livestock populations of Iran and co-existence of *F. hepatica* and *F. gigantica* (thus proving the presence of intermediate forms) in all over the country provided basic principles for the present study, the aims of which were to characterize the Iranian *Fasciola* sp. using 18S rDNA sequence and to establish a molecular tool for the identification of *Fasciola* sp. from southern part of Iran using genetic markers in the 18S rDNA sequence.

MATERIALS AND METHODS

Parasites: Adult trematodes of *F. hepatica* (n = 20) and *F. gigantica* (n = 20) were collected from the liver of infected sheep and cattle in a slaughter house at Fars (Southern region of Iran) and Gilan (Northern region of Iran) provinces between February 2007 and February 2008. Individual flukes were washed extensively in physiological saline, identified morphologically to genus and/or species according to existing keys and descriptions (Ashrafi *et al*., 2006) and then frozen (-20°C) or fixed in 70% ethanol until extraction of genomic DNA.

DNA isolation and enzymatic amplification: Genomic DNA was isolated from the apical end of adult flukes. After of cutting this part and place into labeled DNA free microcentrifuge tubes. DNA was extracted and purified using DNA extraction kit (MBST, Iran) according to manufacture protocol. The DNA concentration was estimated spectrophotometrically by reading absorbance at 260 nm and the purity of samples was examined OD 260 nm/OD 280 nm. Additionally the DNA was analyzed by electrophoresis on a 1.5% agarose gel in TBE buffer (0.095 M Tris-Borate, 0.001 M EDTA). The gels were stained with ethidium bromide and the DNA was visualized using an UV transilluminator (Karimi *et al*., 2008). DNA samples were stored at -20°C until further use.

The 18S rDNA molecule is highly conserved in both *F. hepatica* and *F. gigantica*, with no intraspecific variation and only a few interspecific nucleotide differences (Karimi, 2008). Basing on this, a fragment of 263 and 356 bp of the 18S rRNA gene, including 2 nucleotide differences between *F. hepatica* and *F. gigantica* were selected and PCR methods to amplify them were developed by Marcilla *et al*. (2002). The first amplification was done on the 263 bp fragment of 18S rRNA gene using the forward primer DraI-sense: (5'- CATATGCTTGTCTCAGAGATTAAGCC - 3') and reverse primer DraI-Antisense (5'-CGATCAGTGAAGTTATCCAGAGTC-3') and a second amplification was done on the 356 bp fragment of this genomic part using the forward primer BfrI-sense (5'- CGAAGACGATCAGATACCGTC-3') and reverse primerBfrI-antisense(5'-AGCAGGCCAGAGTCTCGTTC-3'), (Karimi, 2008). PCR reactions (total volume of 100 μL) containing 20 ng of genomic DNA, 10 μL PCR buffer 10X (Cinnagen Company, Iran), 0.2 mM dNTPs each, 1.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase (Cinnagen Company, Iran) and 0.2 μM of each primers (Cinnagen Company, Iran): in a thermocycler (MWV-Germany) under the following conditions: 95°C for 5 min (initial denaturation), followed by 35 cycles of 94°C, 1 min (denaturation), 60°C, 45 sec (annealing), 72°C, 1 min (extension) and a final extension of 72°C for 10 min. Samples without genomic DNA were included in each amplification run as negative controls. An aliquot (10 μL) of each amplicon was examined on 1.5% agarose-TBE (65 mM Tris-HCl, 22.5 mM boric acid and 1.25 mM EDTA, pH 8.0) gels stained with ethidium bromide and photographed upon transillumination. The 100 bp DNA ladder marker (GeneRuler 100 bp DNA ladder plus, Fermentas Company) was used to estimate the sizes of the 18S rDNA amplicons.

Purification, sequencing and analysis of the *Fasciola* 18S rDNA PCR product: Representative PCR products were purified using spin columns (PCR purification kit, MBST, Iran) and the purified PCR products were sent to Kawsar Biotech Company (Tehran-Iran) for sequencing using Genetic Analyzer 3130, automated DNA sequencer (Applied biosystems USA).

The 5' and 3' ends of the *Fasciola* 18S rDNA sequences were determined by comparison with previously published *Fasciola* 18S rDNA sequences (Turbeville *et al*., 1992; Fernandez *et al*., 1998) and the sequences were aligned manually. Pairwise comparisons were made of the level of sequence differences (D) using the formula D = 1-M/L (Chilton *et al*., 1995), where, M is the No. of alignment positions at which the two sequences have a base in common and L the total No. of alignment positions over which the two sequences are compared. Restriction maps of the *Fasciola* 18S rDNA sequences were determined for a range of common enzymes using the MacVector program (Version 4.1, Kodak).

PCR-linked restriction fragment length polymorphism (PCR-RFLP): Purified *F. hepatica* and *F. gigantica* 18S rDNA PCR products (21.5 μL) were digested directly with 20 units (1 μL) of restriction enzyme DraI and BfrI, respectively in 25 μL for 1 h at 37°C (Fermentas). Then a volume of 25 μL of restricted samples were mixed with 5 μL of loading buffer and transferred onto a 1.5% agarose gel together with a 100 bp DNA ladder marker (Fermentas) for fragment size determination. DNA fragments were thereafter separated by horizontal electrophoresis in 0.5X TBE buffer at 100 V for 1.5 h. The gel was stained using ethidium bromide staining and then photographed upon transillumination.

RESULTS

PCR amplification, sequencing and analysis of *Fasciola* 18S rDNA: The trematode specimens from Fars, Southern region of Iran were identified as *F. hepatica* and

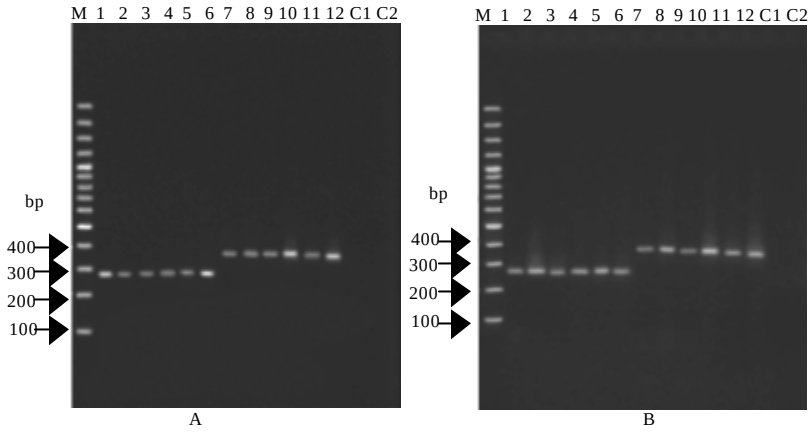

Fig. 1: Analysis of 18S rDNA PCR products of *Fasciola* by agarose gel electrophoresis. Lane M represents 100 bp (base pair) DNA ladder plus marker. Lanes 1-3 and 7-9 represent *F. hepatica* and lanes 4-6 and 10-12 represent *F. gigantica*. Lanes 1-6 and 7-12 represent amplicons with primers DraI and primers BfrI respectively. Photograph A and B represent flukes from sheep and cattle respectively. Lane C1 and C2 represent no DNA control with primers DraI and BfrI, respectively

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Query  1    CATATGCTTGTCTCAGAGATTAAGCCATGCATGTCTAAGTACATACCTTTAAACGGTGAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5    CATATGCTTGTCTCAGAGATTAAGCCATGCATGTCTAAGTACATACCTTGAAACGGTGAA  64

Query  61   ACCGCGAATGGCTCATTAAATCAGCTATGGTTCCTTGGATCGTACATACTACATGGATAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  121  CTGTAGTAATTCTAGAGCTAATACATGCCACTATGCCCTGACCCGCGAGGGAACGGGTGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125  CTGTAGTAATTCTAGAGCTAATACATGCCACTATGCCCTGACCCGCGAGGGAACGGGTGG  184

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Query  241  ACTCTGGATAACTTCACTGATCG
            |||||||||||||||||||||||
Sbjct  245  ACTCTGGATAACTTCACTGATCG
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Fig. 2: Comparison of nucleotide sequences of 263 bp fragment of 18S rDNA obtained from Iranian *F. hepatica* and *F. gigantica*. Query row represent sequence of *F. hepatica* subjected to *F. gigantica*. Under line high lighted represent site of nucleotide difference detectible with DraI restriction enzyme

F. gigantica according to morphological criteria. Genomic DNA was isolated from 20 individuals of *F. hepatica* and 20 specimens of *F. gigantica* from sheep and cattle for comparative purposes. As expected, a fragment of approximately 263 bp and 356 bp length was amplified from each parasite gDNA and in no case was product amplified from No-DNA sample (Fig. 1).

A sequence of 263 and 354 bp were obtained for each of the 4 specimens representing *F. hepatica* and *F. gigantica* from sheep and cattle amplified with primers DraI and BfrI, while a 263 bp sequence was obtained from *F. hepatica* and *F. gigantica* specimens of sheep and cattle amplified with primers DraI whereas 356 bp fragment produced with primers BfrI in all specimens. Automated sequencing of the amplicons composed of partial 18S rDNA sequences of 263 and 354 bp. While, there was no variation in length or composition of the 18S rDNA sequences among multiple specimens from sheep and cattle but sequences difference including 2 nucleotides were detected between specimens from *F. hepatica* and *F. gigantica* (Fig. 2, 3). While, no variation in length or composition of the two 18S rDNA sequences was detected among the two sequenced specimens of sheep and cattle, the consensus in the 356 bp fragment of 18S

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Query  1081  CGAAGACGATCAGATACCGTCCTAGTTCTGACCATAAACGATGCCAACTGACGATCCGTG  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084  CGAAGACGATCAGATACCGTCCTAGTTCTGACCATAAACGATGCCAACTGACGATCCGTG  1143

Query  1141  GTGGTTCTTTATGTGTCCCCACGGGCAGTCCCCGGGAAACCCTTAAGTCTTTGGGCTCCG  1200
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Sbjct  1144  GTGGTTCTTTATGTGTCCCCACGGGCAGTCCCCGGGAAACCTTTAAGTCTTTGGGCTCCG  1203

Query  1201  GGGGAAGTATGGTTGCAAAGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGAG  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1204  GGGGAAGTATGGTTGCAAAGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGAG  1263

Query  1261  TGGAGCCTGCGGCTTAATTCGACTCAACACGGGAAAACTCACCCGGCCCGGACACTGTGA  1320
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Query  1321  GGATTGACAGATTGATAGCTCTTTCTTGATTCGGTGGTTGGTGGTGCATGGCCGTTCTTA  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1324  GGATTGACAGATTGATAGCTCTTTCTTGATTCGGTGGTTGGTGGTGCATGGCCGTTCTTA  1383

Query  1381  GTTGGTGGAGCGATTTGTCTGGTTAATTCCGATAACGAACGAGACTCTGGCCTGCT
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1384  GTTGGTGGAGCGATTTGTCTGGTTAATTCCGATAACGAACGAGACTCTGGCCTGCT
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Fig. 3: Comparison of nucleotide sequences of 356 bp fragment of 18S rDNA obtained from Iranian *F. hepatica* and standard *F. hepatica*. Query row represent sequence of Iranian *F. hepatica* subjected to standard *F. hepatica* (Accession No. AJ004969). Under line high lighted represent site of the nucleotide difference between two samples detectible with BfrI restriction enzyme

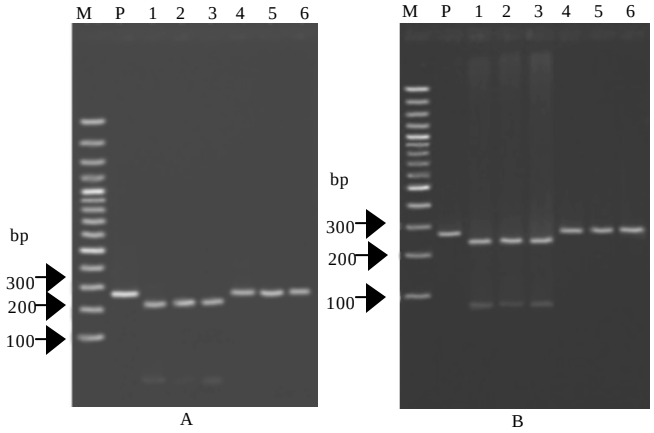

Fig. 4: Identification of *F. hepatica* and *F. gigantica* from different host PCR-linked restriction fragment length polymorphism analysis of the 18S rDNA products using endonuclease DraI. Lane M represent marker and lane P represent template PCR products with primers DraI (263 bp in sizes). Lanes 1-3 and 4-6 represent PCR-RFLP pattern of *F. hepatica* and *F. gigantica*, respectively. Photograph A and B represent *Fasciola* specimens from sheep and cattle, respectively

rDNA sequences of *F. hepatica* was different from those of specimens from gene bank of accession numbers AJ004969 and X56041 in that one variable sequence positions were polymorphic, with the differences of bases from the 18S rDNA sequences representing specimens from gene bank and that from Iran (Fig. 3). But 18S rDNA sequences of *F. gigantica* specimens of Fars were similar to standard *F. gigantica* reported in genebank (Accession No. AJ011942 and AJ004804).

Characterization of *F. hepatica* and *F. gigantica* by PCR-RFLP: Based on the restriction maps generated for the 18S rDNA sequences of the *F. hepatica* and *F. gigantica* (not shown), the restriction endonucleases DraI and BfrI were selected for the delineation of the *F. hepatica* and *F. gigantica* by PCR-RFLP. The undigested 18S rDNA PCR product of primers DraI 263 bp in length and with primers BfrI was 356 bp (Fig. 1). When the 18S rDNA PCR products were digested with DraI

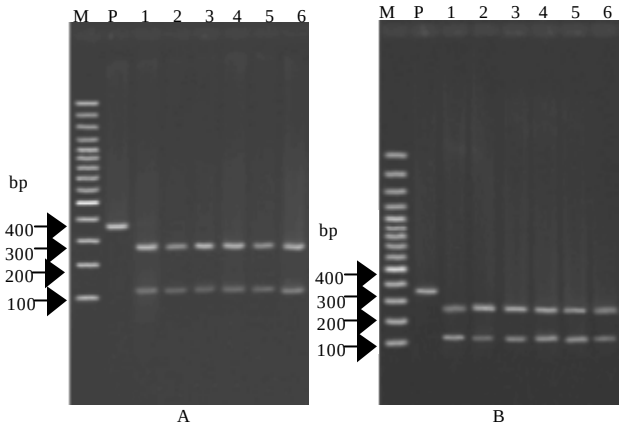

Fig. 5: Identification of *F. hepatica* and *F. gigantica* from different host PCR-linked restriction fragment length polymorphism analysis of the 18S rDNA products using endonuclease BfrI. Lane M represent marker and lane P represents template PCR products with primer BfrI (356 bp in sizes). Lanes 1-3 and 4-6 represents *F. hepatica* and *F. gigantica*, respectively. Photograph A and B represent *Fasciola* from sheep and cattle, respectively

restriction enzyme, two bands of approximately 50 and 213 bp were produced for *F. hepatica* samples from sheep and cattle (small sizes of the 50 bp band was not clearly visualize) but *F. gigantica* remained undigested (Fig. 4). PCR-RFLP profiles with restriction enzyme BfrI, consisted of two similar DNA bands of approximately 102 and 254 bp in length from both *F. hepatica* and *F. gigantica* samples of sheep and cattle (Fig. 5).

To assess variation in restriction patterns among individuals of *Fasciola*, the 18S rDNA was amplified from 40 individuals from sheep and cattle and the amplicons were then subjected to RFLP analysis with these two restriction endonucleases, as expected, no variation in restriction pattern was detected among multiple individuals from the same fluke, consistent with sequence data. The sizes of the digestion fragments were in accordance with calculations based on the restriction maps, except the co-migrating fragments.

DISCUSSION

Fascioliasis has shown to be a greatest food borne parasitic disease in Iran. Southern regions of Iran (as a Fars and Khuzestan provinces) appear to be the important endemic areas including most human and animal cases. In these southern endemic areas of human fascioliasis in Iran, environmental characteristics favour liver fluke transmission as well as lymnaeid presence (Mas-Coma *et al*., 2005) and both *F. hepatica* and *F. gigantica* are present simultaneously in individual cattle and buffaloes (Sahba *et al*., 1972). High prevalence of fascioliasis in livestock and human from Iran has been worth mentioning. Moreover, at the end of the 1980 s and during the 1990s several large epidemics, including thousands of human cases, were reported that appear to the largest epidemics of human fascioliasis throughout the world (Moghaddam *et al*., 2004).

Overlapping distribution of both *F. hepatica* and *F. gigantica* has even become the basis of an already long controversy on the taxonomic identity of these species occurring in some countries, especially Iran, Egypt, Japan, Taiwan, the Philippines and Korea, in which a wide range of morphological types is detected. At the extremes of this morphological range, some resemble *F. hepatica*, whereas others resemble *F. gigantica* with intermediate forms also occurring and involving phenomena such as abnormal gametogenesis, diploidy, triploidy and mixoploidy, parthenogenesis and hybridization events between different genotypes (Mas-Coma *et al*., 2005).

Existence of intermediate forms of *Fasciola* from countries as Iran caused difficulty and confusion in the identification of specimens using parasitological, immunological and pathological analysis (Ashrafi *et al*., 2006; Periago *et al*., 2008). In the present study, samples of the *Fasciola* were characterized using well-defined 18S rDNA sequence because previous studies have shown that rDNA sequence provides reliable genetic markers for the accurate differentiation and identification of *Fasciola* species (Zurita *et al*., 1988; Barker *et al*., 1993; Hashimoto *et al*., 1997; Itagaki and Tsutsumi, 1998; Blair, 2005; Nolan and Cribb, 2005). Using restriction maps of the *ribosomal* genes, demonstrated that a *Fasciola* isolated from Japan was identified to *F. gigantica* but different from *F. hepatica*. No intraspecific variations in the restriction endonuclease maps of *F. hepatica* or

F. gigantica were detected, but length heterogeneity was noted in the intergenic spacer even within individual worms (Blair and McManus, 1989). Difference were detected in the 28S rDNA gene of *F. hepatica* in sheep and *F. gigantica* in cattle, but were not intraspecific variation performed. Another study demonstrate that individual cows infected by numerous genetically different liver flukes (Karimi *et al*., 2008). Differences among nucleotide sequences of ITS_2 (internal transcribed spacer 2) fragment of the rDNA gene obtained from *F. hepatica* and *F. gigantica* were shown, besides proved that ITS_2 sequence is identical for *F. hepatica*, which differ in various geographic origins (Mas-Coma *et al*., 2005). Analysis of the ITS2 and d_2 regions were found to be polymorphic; that is, out of five *Fasciola*, two possessed a *F. gigantica*-type sequence, one, a *F. hepatica*-type sequence and two possessed sequences of both types indicating an existence of different alleles at the loci. It should be noted that these variations of the ITS_2 and d_2 regions co-occur at the same individual worms (Agatsuma *et al*., 2000). The rDNA sequence of *Fasciola* sp. from Japan matched closely that of *F. gigantica* and demonstrates variability in nucleotide sequence within the ITS2 region which allows discrimination between species of fasciolidae. Sequence divergence between *F. hepatica* and *F. gigantica* was 2.8% but intraspecific sequence divergence was negligible (Adlard *et al*., 1993). Using PCR-RFLP assay, with restriction enzymes AvaII and DraII, was described to distinguish between both fasciolid species. It was based on a 618-bp-long sequence of the 28S rRNA gene besides this sequence showed a few nucleotide differences between both fasciolids and no intraspecific variations within each species (Marcilla *et al*., 2002).

In the present study the 263 and 354 bp fragments of 18S rDNA gene of the *F. gigantica* were no variation in length or composition among multiple specimens from other part of world. When the restriction endonuclease DraI was used, the 18S rDNA PCR products of *F. gigantica* from sheep and cattle remained undigested as they lacked restriction site for DraI whereas, *F. hepatica* from sheep and cattle produced two bands of approximately 50 and 213 bp in sizes (Fig. 4). The 354 bp fragment of 18S rDNA PCR products of both *F. hepatica* and *F. gigantica* from sheep and cattle after digested with restriction enzyme BfrI produced two similar bands of approximately 102 and 254 bp length (Fig. 5). Similar restriction maps of *F. hepatica* and *F. gigantica* with BfrI restriction enzyme supported by PCR product sequencing as similar nucleotide sequences of 356 bp fragments of 18S rRNA gene between these flukes detected. The 18S rDNA sequences obtained from the present study were compared with Turbeville *et al*. (1992) and Fernandez *et al*. (1998) and it was found that the part of 18S rDNA sequence with 263 bp length of *F. hepatica* and *F. gigantica* from sheep and cattle from Fars continent was identical to that of *F. hepatica* and *F. gigantica* from elsewhere (Fig. 2), whereas according to Fig. 3, the 356 bp fragment of 18S rDNA nucleotide sequence differences of 0.3% (1/356) was detected between *F. hepatica* from Fars province to *F. hepatica* published previously on the other part of the world (Turbeville *et al*., 1992; Fernandez *et al*., 1998).

This study showed that the 18S rDNA sequences of *F. hepatica* from sheep and cattle from southern regions of Iran were quite unique in that two different 18S rDNA sequences exist in the rDNA array of a worm. One of the sequences was identical to that of *F. hepatica* and the other was almost identical to that of *F. gigantica* in that nucleotides at one polymorphic position represent *F. gigantica*. This micro heterogeneity is possibly due to sequence polymorphism among copies of the 18S rDNA array within the same worm. The consensus 18S rDNA sequence of *Fasciola* appears to be a mixture of that of *F. hepatica* and *F. gigantica* with the presence of the bases of both *F. hepatica* and *F. gigantica* 18S rDNA sequences at one variable sequence positions. This finding was also consistent to some extent with result of morphological identification as specimens were atypical of either *F. hepatica* or *F. gigantica* morphologically. These results indicated that *Fasciola* from Fars may represent an intermediate genotype or even a hybrid between *F. hepatica* and *F. gigantica*, although there are other possible explanations to the existence of sequence polymorphism in the 18S rDNA array of *Fasciola* from Fars, such as introgression or lineage sorting and retention of ancestral polymorphism (Dr. Robin Gasser, personal communications), these findings are supported by the results of the previous study (Agatsoma *et al*., 2000; Karimi, 2008). This is the first time that molecular evidence had suggested the possible existence of an intermediate genotype of *Fasciola* in Iran, in addition to *F. hepatica* and *F. gigantica*. The nucleotide variation in the 18S rDNA sequences of the *Fasciola* sp. resulted in differences in restriction sites for endonuclease BfrI. Hence, a PCR-RFLP assay was used to characterize and differentiate the *Fasciola* sp.

The PCR-RFLP method described here can be used for the proper identification of *Fasciola* species and become a helpful tool for the specific diagnosis of the liver fluke causal agent of fascioliasis in human and animal subjects in geographical areas such as Iran, where, both *F. hepatica* and *F. gigantica* appear to be sympatric and clinical, pathological, coprological and immunological

analyses do not allow a differentiation between them. It might also be a useful alternative to distinguish between the two *Fasciola* species at veterinary level in those overlapping distribution areas in which they often coexist in the liver of the same domestic animal and morphological characteristics of the fluke adult stage are tedious, time consuming and sometimes not sufficient for the necessary specific classification. Moreover the 18S rDNA characterization of the *Fasciola* sp. and the establishment of a PCR-RFLP assay for their delineation have important implications for studying the population genetic structures and ecology of these *Fasciola* species and may also have implications for the diagnosis and control of Fascioliasis of humans and ruminants. Further studies are warranted to clarify the nature of intermediate genotype in Iran using other genetic makers.

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