

Molecular Identification of Animal *Fasciola* Isolates in Southwest of Iran

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Abstract: The aim of this study was to identify *Fasciola* isolates collected from natural livers of livestock by RFLP and sequencing in southwest of Iran. Morphological examination and biologic characters cannot cause a certainty in the accurate and precise identification and intra and inter-specific differences of *Fasciola* spp. In this study, we identified *Fasciola* species using 28S marker in isolates from Khuzestan slaughterhouse in south-western Iran. A number of 502 adult *Fasciola* worms were isolated from the natural infected livers of buffalo, cattle, sheep and goat in local slaughterhouses. Total genomic DNA was extracted from 70% ethanol stored flukes by extraction kit with some modification. The results indicated three hundred fourteen isolates out of 502(62.5%) identified as *F.gigantica* and 188(37.5%) as *F. hepatica*. All sequences of the different isolates showed high identity (99-100%) indicating no significant genetic variations in 28S rDNA. In conclusion, using 28S marker through PCR-RFLP method, without need to sequencing could discriminate easily between *F. hepatica* and *F. gigantica* and therefore could be very useful method to identify the causal of fasciolosis especially in areas where both *F. hepatica* and *F. gigantica* are coexist.

Key words: *Fasciola*; PCR-RFLP; sequencing; Iran.

INTRODUCTION

Fasciolosis or liver rot is a trematode helminthic disease caused by two impotent species of *Fasciola* including *Fasciola hepatica* Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1855 (Platyhelminthes: Trematoda: Digenea).

This infection is zoonotic and belongs to the plant-borne trematode. It is transmitted to the definitive host such as humans and other herbivorous mammals via contaminated water or green vegetables mostly watercress. The geographical distribution of these parasites is differs. In Europe, the Americas and Oceania only *F. hepatica* is a concern, whereas the distributions of both species overlap in many areas of Africa and Asia (Mas-Coma *et al.*, 2005). The parasite affected the liver of ruminates and chronic disease usually results in decreased production of meat, milk and wool (Black *et al.*, 1972; Coop *et al.*, 1977; Hope Cawdery *et al.*, 1977). It has been estimated that fasciolosis lost 2000 million dollars yearly in the world (Mungube *et al.*, 2006).

On top of that, human fascioliasis is increasing worldwide and has relaunched interest in fascioliasis. It is estimated that human infection up to 17 million people worldwide (Hopkins, 1992). Iran is endemic for the infection and especially the province of Gilan, have been huge increases in the incidence of human fascioliasis in recent years (Farag, 1998; Mas-Coma *et al.*, 1999).

Khuzestan Province with about 640,000 km² area and estimated population of 4,275,000 is located in Southwestern Iran, in the north of Persian Gulf. This location is specific field for the snail host (Mansoorian, 2001) and endemic for the infection which previous studies have been reported high infection rates of 91.4%, 49.2%, 29% and 11.2% in buffaloes, cattle, sheep and goats, respectively (Sabbaghian *et al.*, 1964; Sahba *et al.*, 1972).

Precise identification of *Fasciola* species has important implications for epidemiology and effective control of fasciolosis. The majority of the previous studies in this region were based on morphologic parameters (Sabbaghian *et al.*, 1964; Sahba *et al.*, 1972).

Because of many variations in morphological indices, this traditional approach is insufficient for discrimination of the fasciolosis causal species (Ashrafi *et al.*, 2006). It would be very important to know more about the appearance of fasciolosis in livestock and molecular characterization of the causative agents to control and limit of infection distribution in this region. Therefore, the aim of this study was to Identified and describe of the *Fasciola* spp. Using 28S marker by methods of PCR-RFLP and sequencing in isolates from Khuzestan slaughterhouses in southwest of Iran.

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MATERIAL AND METHODS

Sample Collection and Laboratory Methods:

Five hundred two of *Fasciola* samples collected from the naturally infected livers of 120 buffalo, cattle, sheep and goats in Southwest slaughterhouses from April 2010 to March 2011. The samples were washed in PBS then incubated in PBS at 37 °C for at least 4 h to allow them to expel gut contents.

The flukes were placed between two microscopic slides and then stained in carmine stain (Jeyathilakan, 2010).

All the samples morphologically detected as *Fasciola* worms were studied for identification of *Fasciola* species by PCR-RFLP and sequencing.

DNA Isolation and Amplification:

Genomic DNA was isolated from the apical zone of adult flukes using the Accu Prep DNA extraction kit (Bioneer Co., Korea) according to the manufacturer's instructions with some modification. For amplification of the 618-bp-long fragment of the 28S rRNA gene, the primers 28F1 (5' ACGTGATTACCCGCTGAACT 3') and 28R600 (5' CTGAGAAAGTGCACTGACAAG 3') were used according to previous study (Marcilla *et al.*, 2002). The ready to use Bioneer, Accupower PCR premix, 96 tubes, 0.2ml, 20µlrxn (Bioneer Co., Korea) with some modification such as addition of DMSO (Dimethyl Sulfoxide) and Betaine was used as PCR reaction. The PCR reactions were conducted in a Corbett Thermocycler (Corbett Research, Australia) under the following conditions: 94 °C for 3 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 60 s at 72 °C. Finally, a primer extension step of 5 min at 72 °C was used. Fifteen microlitres of PCR products were analysed by electrophoresis on 1.5% (w/v) agarose gels. One hundred bp molecular weight marker (Fermentase life science, Germany) was included on each gel for base-pair comparisons. One and a half percent Gel was visualized by staining with ethidium bromide. Then the PCR products were digested using restriction enzymes *Ava*II and *Dra*II in 5h at 37°C and the digested products were analyzed on 2% agarose gel. To control of RFLP results and assessment of 28S rDNA nucleotide polymorphism, randomly 15 samples of PCR products were chosen and submitted to MacroGen Co. in Korea for sequencing using both forward and reverse PCR primers.

RESULTS AND DISCUSSIONS

Our finding showed both *Fasciola hepatica* and *Fasciola gigantica* among 502 samples isolated from naturally infected cattle, sheep, buffalo and goat in southwest of Iran. Morphologic compartments showed multiple dimensions of *Fasciola* sp. from same or deferent herbivorous hosts. Amplification of the 28S rDNA fragment revealed 618 bp long PCR products for all samples of different regions and different hosts (Fig. 1). PCR- restriction fragment length polymorphism (RFLP) bands profile of *F. hepatica* and *F. gigantica* with restriction enzyme *Ava* II and *Dra* II showed in Fig 2 and 3. The results of PCR products digestion with *Ava* II and *Dra* II are presented in Table 1. Accordingly, three hundred fourteen out of 502(62.5%) samples were detected as *F. gigantica* and 188(37.5%) as *F. hepatica* (Table 2). In this regard 90.6% of *Fasciola* isolated from buffalo and 68.75% of *Fasciola* isolated from sheep were detected as *F. gigantica* and *F. hepatica* respectively. The nucleotide sequences generated from the sequenced samples were aligned with the published sequences of JF323865.1 as *Fasciola gigantica* isolate Izatnagar and AB553694.1 as *Fasciola* sp. The results showed high homology with JF323865.1 but considerable nucleotide substitutions with AB553694.1 (Fig. 4).

Table 1: The visualized restriction fragment length polymorphism (RFLP) patterns of the 618bp-long PCR fragments from isolated liver flukes generated after digestion with restriction enzymes.

Species	<i>Ava</i> II	<i>Dra</i> II
<i>F. hepatica</i>	529bp	529 bp,90 bp
<i>F. gigantica</i>	322 bp, 269 bp	619 bp

Table 2: Frequency of *Fasciola hepatica* and *Fasciola gigantica* identified by PCR RFLP and sequencing in different animal hosts slaughtered in southwest of Iran.

Livestock	No of isolates	<i>F. gigantica</i>	<i>F. hepatica</i>
Goat	40 /502	19 (47.5%)	21(52.5%)
Sheep	176 /502	55 (31.25%)	121(68.75%)
Cattle	211 /502	172 (81.5%)	39(18.5%)
Buffalo	75 /502	68 (90.6%)	7(9.4%)
Total	502	314 (62.5%)	188(37.5%)

The highest percentage of *F. gigantica* was detected in Buffalo isolates and the highest percentage of *F. hepatica* was detected in sheep isolates.

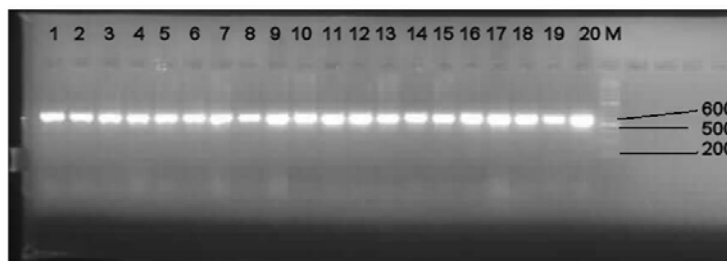


Fig.1: Gel electrophoresis of PCR products of ribosomal DNA 28s region of fasciolid flukes isolated from Livestock in Khuzestan, Iran. Lane M, 100 bp DNA size marker; Lanes 1-20,618-bp-long PCR products of *Fasciola hepatica* and *F. gigantica*.



Fig. 2: Restriction fragment length polymorphism (RFLP) Patterns of PCR products of Khuzestan, Iran liver flukes after digestion with *AvaII* enzyme: Lane M , 100 bp DNA size marker; Lanes 1-3,5-7,9,10,12,13,16 and18-20 PCR products of *F. gigantica* and Lanes 4,8,11,14,15,17and 21-23 PCR products of *F. hepatica* after digestion with *AvaII*.

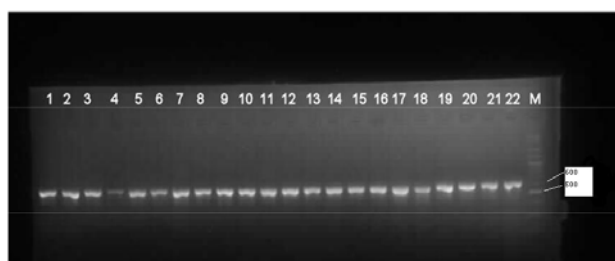


Fig. 3: Restriction fragment length polymorphism (RFLP) Patterns of PCR products of Khuzestan, Iran liver flukes after digestion with *DraII* enzyme: Lane M , 100 bp DNA size marker; Lanes 1,3,4,6,8,12,14 and19-22 PCR products of *F. gigantica* and Lanes 2,5,7,9,10,11,13 and 15-18 PCR products of *F. hepatica* after digestion with *DraII*.

Fig. 4.

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3a1G      CCTATAATGAGTAAGGTTACTCGGACATGGGCCAATGAGGGTGAAAGGCCCGTGGGGGTG
4A1G      CCTATAATGAGTAAGGTTACTCGGACATGGGCCAATGAGGGTGAAAGGCCCGTGGGGGTG
2a1.G     CCTATAATGAGTAAGGTTACTCGGACATGGGCCAATGAGGGTGAAAGGCCCGTGGGGGTG
5A1G      CCTATAATGAGTAAGGTTACTCGGACATGGGCCAATGAGGGTGAAAGGCCCGTGGGGGTG
JF323865  CCTATAATGAGTAAGGTTACTCGGACATGGGCCAATGAGGGTGAAAGGCCCGTGGGGGTG
AB553694  CGTGGCTTGGGTTTGGCAGCTGG-CGTGATCTCCTCTATGAGTAAT-CATGTGAGGTGC
* * * * *
3a1G      GAGATTGAGAATGGCCAGTATCTTCCTGAGCGGACCTTGGAGTCGGGTGTGTTGTGAATG
4A1G      GAGATTGAGAATGGCCAGTATCTTCCTGAGCGGACCTTGGAGTCGGGTGTGTTGTGAATG
2a1.G     GAGATTGAGAATGGCCAGTATCTTCCTGAGCGGACCTTGGAGTCGGGTGTGTTGTGAATG
5A1G      GAGATTGAGAATGGCCAGTATCTTCCTGAGCGGACCTTGGAGTCGGGTGTGTTGTGAATG
JF323865.1g GAGATTGAGAATGGCCAGTATCTTCCTGAGCGGACCTTGGAGTCGGGTGTGTTGTGAATG
AB553694.1G CAGATCTATGGCG-----TTTCCCTAATGTATCCGGATGCACCCCTGTCTTGGCAGA
* * * * *
3a1G      CAGCCCAAAGCGGGTGGTAAACTCCATCCAAAGGCTAAATACTAGCAGGATCCGATAGCG
4A1G      CAGCCCAAAGCGGGTGGTAAACTCCATCCAAAGGCTAAATACTAGCAGGATCCGATAGCG
2a1.G     CAGCCCAAAGCGGGTGGTAAACTCCATCCAAAGGCTAAATACTAGCAGGATCCGATAGCG
5A1G      CAGCCCAAAGCGGGTGGTAAACTCCATCCAAAGGCTAAATACTAGCAGGATCCGATAGCG
JF323865.1g CAGCCCAAAGCGGGTGGTAAACTCCATCCAAAGGCTAAATACTAGCAGGATCCGATAGCG
AB553694.1G AAGCGTGGTGAGGTGCAGTGGCGGAATCGTGGTTTAAATAAT-----CGGGTT-GGTACTC
* * * * *
3a1G      AACAAGTACCGTGAGGGGAAAGTTGAAAAGTACTTTGAAAGAGAGAGTAAACAGTGCCTGAA
4A1G      AACAAGTACCGTGAGGGGAAAGTTGAAAAGTACTTTGAAAGAGAGAGTAAACAGTGCCTGAA
2a1.G     AACAAGTACCGTGAGGGGAAAGTTGAAAAGTACTTTGAAAGAGAGAGTAAACAGTGCCTGAA
5A1G      AACAAGTACCGTGAGGGGAAAGTTGAAAAGTACTTTGAAAGAGAGAGTAAACAGTGCCTGAA
JF323865.1g AACAAGTACCGTGAGGGGAAAGTTGAAAAGTACTTTGAAAGAGAGAGTAAACAGTGCCTGAA
AB553694.1G AGTTGTGAGTGTG-----TTTGGCGATCCCTAGTCCGACACTCATGATTCTGGG
* * * * *
3a1G      ACCGTTTCAGAGGTAAACAGGTGGAGTTGAACTGCAAGCTC-TGAGGATTGAGTGGTGAG
4A1G      ACCGTTTCAGAGGTAAACAGGTGGAGTTGAACTGCAAGCTC-TGAGGATTGAGTGGTGAG
2a1.G     ACCGTTTCAGAGGTAAACAGGTGGAGTTGAACTGCAAGCTC-TGAGGATTGAGTGGTGAG
5A1G      ACCGTTTCAGAGGTAAACAGGTGGAGTTGAACTGCAAGCTC-TGAGGATTGAGTGGTGAG
JF323865.1g ACCGTTTCAGAGGTAAACAGGTGGAGTTGAACTGCAAGCTC-TGAGGATTGAGTGGTGAG
AB553694.1G ATATTTCATACCCAGGCACGTTCCGTT--ACTGTTACTTTGTCATTGGTTTATGCTGAA
* * * * *
3a1G      TATGGC-ATGAGCTTGGTCATATTGGTTGTGTGCTCGGGTCTGCTTAGCTGCAGGCTCTCC
4A1G      TATGGC-ATGAGCTTGGTCATATTGGTTGTGTGCTCGGGTCTGCTTAGCTGCAGGCTCTCC
2a1.G     TATGGC-ATGAGCTTGGTCATATTGGTTGTGTGCTCGGGTCTGCTTAGCTGCAGGCTCTCC
5A1G      TATGGC-ATGAGCTTGGTCATATTGGTTGTGTGCTCGGGTCTGCTTAGCTGCAGGCTCTCC
JF323865.1g TATGGC-ATGAGCTTGGTCATATTGGTTGTGTGCTCGGGTCTGCTTAGCTGCAGGCTCTCC
AB553694.1G CTGGYCATGTGTCTGATGCTATTTCATATA-ACGACGGTACCCCTTCGTGGTCTGTCTTC
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Fig. 4: Sequence alignment of partial 28S genes of the ribosomal DNA.3a1G,4A1G,2a1.G and 5A1G representative of identical sequences generated from *F. gigantica* isolates of the present study, JF323865 and AB553694 are *F. gigantica* and *Fasciola* sp. respectively Genbanke isolates. (*) Indicates identity with the same sequence.

Discussion:

Correct identification of fasciolosis causal agents has important implications for studying population biology, epidemiology and therefore the effective control and manages of the diseases they cause.

Several studies have used DNA-based molecular techniques for genetic characterization and differentiation of *Fasciola* isolates (Mas-Coma *et al.*, 2005; Lin *et al.*, 2007; Farjallah *et al.*, 2009). To our knowledge there have been no comprehensive study using molecular approaches to differentiate animal *Fasciola* isolates in southwest of Iran. This study was conducted to discriminate between *F.hepatica* and *F. gigantica* isolates of different livestock from different geographical locations in Southwest of Iran, using 28S sequencing and PCR-RFLP.

Morphologic compartment of this study revealed multiple sizes among separated isolates. In agreement, the studies in the area where *F. hepatica* and *F. gigantica* co-exist showed the existence of phenotypic variations in adult flukes (Ashrafi *et al.*, 2006; Periago *et al.*, 2008).

As discussed by previous studies morphometric parameters can be depend on intensity of infection, host species, parasite live stage and immune reactions due to a possible previous exposure to the infection (Mas-Coma *et al.*, 2005; Lotfy *et al.*, 2002; Valero *et al.*, 2001).

Although we have found different sizes among isolates with same hosts, however using molecular method revealed these differences were due to different stages of the parasites life cycle.

Morphometric analysis showed 3 types such as *F. gigantica* like, *F. hepatica* like and intermediate forms. Nevertheless the analysis of 28S sequences by RFLP and sequencing revealed two *F.gigantica* and *F. hepatica* and there was no third intermediate species suggesting no eligibility of traditional morphologic approach for this

diagnostic purpose. This finding corresponded with previous studies by Ali *et al.*, (2008) in Niger. However, this is not supported by the studies performed by Alasaad *et al.*, (2008) in Spain and Ghavami *et al.*, (2009) in Northwest of Iran, in which only *Fasciola hepatica* was reported.

Also the results of present study was differed from Ichikawa *et al.*, (2010), Le *et al.*, (2008), Lin *et al.*, (2007) and Hashimoto *et al.*, (1997) who found intermediate species of *Fasciola* in their studies.

The result of the present study for PCR amplification of 28S fragment showed complete conformity with Marcilla *et al.*, (2002) who generated 618 bp long PCR products of *F. hepatica* and *F. gigantica*. In this study to overcome of PCR limitations such as unwanted bands we explored the use of DMSO and betaine in PCR reaction. Though we found both additives extremely enhanced target product specificity and yield during PCR amplification.

This PCR-based method could distinguish the samples at the level of *Fasciola* genus and to clear the *Fasciola* species, digestion of 28S sequences using the common restriction enzymes *AvaII* and *DraII* was required.

By this PCR- RFLP, based approach and sequencing we could have successfully estimate of frequency of *F. hepatica* and *F. gigantica* and Genotyping of the parasite in isolates from one locality in Southwestern Iran. The *AvaII* could digest the 28S rRNA sequences of two *F. hepatica* and *F. gigantica* easily and produced 322 and 269 bp visualized bands from *F. gigantica* and 529 from *F. hepatica*. Restriction of the PCR Products by *DraII* generated two fragments approximately 529 bp and 90 bp from *F. hepatica*, whereas PCR products from *F. gigantica* remained intact. Nonetheless no variation in RFLP patterns was observed among different isolates of PCR products from *F. gigantica* and *F. hepatica* suggesting no intraspecific variations in the maps of *F. hepatica* or of *F. gigantica*. This result has complete consistency with the previous study (Marcilla *et al.*, 2002). To confirm the RFLP results and to nucleotides analysis of the 28S sequences, 15 of PCR productions were sequenced and subjected to intra- and inter- isolates compartments.

There was no variation in length of the 28S sequences among multiple specimens collected from different hosts and different geographical regions of southwest of Iran. Comparison of the obtained 28S sequences with that of *Fasciola hepatica* and *Fasciola gigantica* from elsewhere revealed that the *Fasciola* samples examined represent two species, namely *F. hepatica* and *F. gigantica*. The comparison revealed of high identity of southwest of Iran sequences with JF323865.1 (*Fasciola gigantica* isolates from GenBank sequence database) and high nucleotides substitution with AB553694.1 (*Fasciola* sp isolates from GenBank sequence database). These results can be due to the targeted genes that was 28S for first and 5.8S rRNA, ITS2, 28S rRNA for the second respectively.

The limitation of this study was no access to confident slaughtered livestock source information to determine the dominant *Fasciola* species in some districts of the province.

In conclusion, using 28S marker through PCR- RFLP method, without need to sequencing could have an estimate of *F. hepatica* and *F. gigantica* in geographical areas such as Iran, where both *F. hepatica* and *F. gigantica* appear to be coexisting and other methods could not discriminate between them.

ACKNOWLEDGEMENTS

The authors are very grateful to Khuzestan Slaughterhouse for kindly assistance in providing infected livers. This study is conducted and financially supported by grants No: 89105 from Ahvaz Jundishapur Medical sciences. The authors declare that there is no conflict of interest in this paper.

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