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Original Article

Genotyping and Phylogenetic Analysis of *Fasciola* Spp. Isolated from Sheep and Cattle Using PCR-RFLP in Ardabil Province, Northwestern Iran

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Abstract

Background: The aim of this study was to detect the genotype of *Fasciola* spp. in Meshkin-Shahr, Ardabil Province, northwestern Iran in different hosts using PCR-RFLP.

Methods: The parasite hosts included cattle, and sheep. Overall, 70 adult flukes from livers of slaughtered animals were collected from the abattoirs of aforementioned area. The included 35 samples from infected sheep and 35 samples from 35 infected cattle. PCR–RFLP and sequence analysis of the first nuclear ribosomal internal transcribed spacer (ITS 1) region from *Fasciola* species were used to conduct the study.

Results: The fragment of approximately 700bp in all of the *Fasciola* samples was amplified. PCR products of ITS 1 were subjected for digestion by restriction enzyme. RsaI restriction enzyme was selected for RFLP method that caused the separation specifically of *Fasciola* species. Amplicons with the sequences of *F. hepatica* had a pattern of about 360, 100, and 60 bp band size, whereas *F. gigantica* worms had a profile of 360, 170, and 60 bp in size, respectively. Results based on PCR-RFLP analysis were confirmed by sequence analysis of representative ITS 1 amplicons. No hybrid forms were detected in the present study. All sheep were infected with *F. hepatica* but cattle were infected with both species.

Conclusion: Both species of *Fasciola* are present in Ardabil. The method described here can be valuable for identification of *Fasciola* species in endemic parts for fasciolosis, regions with intermediate species and in that overlapping distribution area.

Keywords: *Fasciola*, Genotyping, Fasciolosis, PCR, Iran

Introduction

Fascioliasis, caused by *Fasciola* spp., is one of the most zoonotic diseases with a global extension and of a significant concern in areas with suitable situations (1)." About 2.4-17 million cases of human fascioliasis are estimated in the world and 180 million exposed at risk of fascioliasis that demonstrates the importance of the disease (2)".

Two great epidemics of human fascioliasis with about 10,000 people infected in each case occurred in the north of Iran (3-5). According to WHO, fascioliasis is one of the most important diseases and parasitic infections transmitted by food (6). Considering the importance of fascioliasis in Iran, various studies especially in order to

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identify and genotyping of *Fasciola* seems indispensable.
Morphological features of adult worms and eggs are influenced by the different factors such as host type, age of parasite, fixation of the samples and intensity of the infection (7**)**.Distribution overlap of *F. hepatica* and *F. gigantica* in Asia and simultaneous infection with these two parasites in cattle in northern Iran and bug reporting interface is also introduced in other provinces of Iran (8). It is usually difficult to accurate differentiation between *Fasciola* species based on phenotype criteria (9-11). Today, molecular methods based on DNA analysis is a great impact in many fields, including identifying systematic parasitology, parasite infection, parasite epidemic, analysis of the genetic structure of parasite populations, such as genetic differences between the sexes, gene expression and drug resistance of and the vaccine.Therefore, it seems that the use of the accurate and reliable method for identification and differentiation of *Fasciola* species is necessary. For this purpose, PCR**-**RFLP based on 28s rRNA, 18s rRNA, ITS 1 or ITS 2 has been used (12-13).
In the present study, region between 18S and 28S (ITS 1, 5.8s,) of ribosomal DNA was used by PCR-RFLP method. Since, ITS 1 sequence was suitable genetic markers for genotyping, interaspecific variations, and phylogenetic studies of parasites (14); we used only ITS 1 for molecular identification of *Fasciola* species**.**The adjacent of Ardabil Province to endemic areas of fasciolosis persuaded us to conduct the present study (15).

Materials and Methods

Parasite

Overall, 70 *Fasciola* isolates were studied from two hosts in Ardabil Province, northwestern Iran (Fig. 1). These parasites were isolated from 35 infected sheep and 35 infected cattle. Individual worms were washed extensively in PBS buffer (37°C) and subsequently fixed in 20% ethanol and maintained at room temperature for several weeks until extraction of genomic DNA (Table 1).

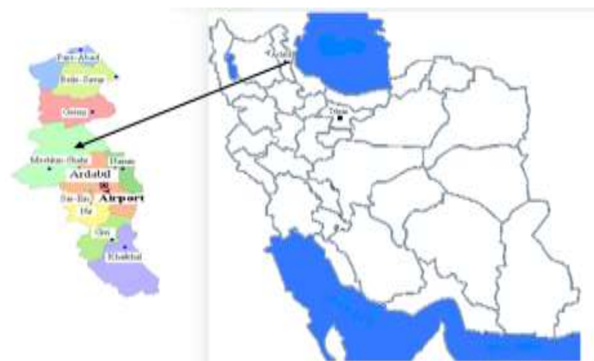

Fig. 1: Map of Iran (a) Ardabil Province, northwestern Iran (b)

DNA extraction

Approximately 10 mg tissue was removed from a portion of the lateral zone of adult flukes and crushed. The ethanol in each of these samples was allowed to evaporate for a few minutes and washed in distilled water for 3 times and squashing before the genomic DNA was extracted with (DNG™-PLUS) kit was used for genomic DNA extraction of *Fasciola* Parasites. The extracted DNA, split into aliquots, and stored at **-**20°C until used in the PCR.

Primer designing

We designed forward (5'-ACCGGTGCTGAGAAGACG-3') and Reverse (5'-CGACGTACGTGCAGTCCA-3') primers using Gene Runner and BLAST (http://www.ncbi.nlm.nih.gov/blast) software. The primers were synthesized by Bioneer Company (Korea).

PCR

To amplify a 700 bp region of the ITS 1 sequence, PCR was performed using a pair primer.
Total volume of reaction was 15µl containing 1.5µl DNA template, 5µl distilled water, 10pmol of each primers (Forward and Reverse), and 7.5µl master mix (amplicon). PCR amplification was performed in Eppendorf Mastercycler Gradient thermocycler . The temperature profile was as follows: an initiation of 95ºC for 5 min, followed by 30 cycles of 94ºC for 30s (denaturation), 60ºC for 30s (annealing), 72ºC for 30s (extension) and a final extension of 72ºC for 5min followed by cooling at 4ºC.

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Table 1: Position of variable bases and differences in sequences of ITS 1 region of *Fasciola* spp

Position Sample			ITS 1						
	Haplotype	Accession Number	59	68	186	276	370	448	468
F. hepatica	(H1)	KF982049	T	C	C	A	C	T	C
F. gigantica	(G2)	KF982046	C	T	T	T	T	A	T
F. gigantica	(G1)	KF982047	C	C	T	T	T	A	T

PCR products were electrophoresed on 1.5% (for ITS 1 PCR) agarose gel in TAE buffer at 60 V for 40 min and visualized by UV illumination (UVITEC) after ethidium bromide staining. To estimate the size of the amplicons, a 100bp DNA ladder (Fermentas) was used in gels.

Sequencing and analysis

To select a suitable restriction enzyme we sequenced PCR products of one *Fasciola* samples from sheep and one *Fasciola* samples from cattle. The sequences were analyzed and aligned using Blast (http://blast.ncbi.nlm.nih.gov/Blast) and multalin (http://multalin.toulose.inra.fr/ multalin) software in comparison with sequences of *Fasciola* ITS 1, previously published from other countries in GenBank. Nucleotides of 700bp from fasciolid species were subjected to comparison of restriction sites and selection the appropriate enzyme by Webcutter 2 using the following website: (http://bio.lundberg.gu.se/cutter2) software.

Restriction Fragment Length Polymorphism (RFLP)

Rsa I enzyme (Fermentas) was selected for RFLP method that caused the separation specifically of *Fasciola* species. To performance RFLP, 5 µl of *Fasciola* ITS 1 PCR product, 2.5 µl of supplied restriction enzyme buffer, 5 µl of restriction enzyme diluted, and D.W up to 25 µl were provided. According to the manufacturer instruction, the tubes were incubated at 37 °C for 7 h, to ensure full cutting of fragments. For analyzing the digestion products, 15 µl of each product in addition to 2 µl of loading buffer were electrophoresed on either 3% agarose gel in TAE buffer at 60 V for 40 min and visualized by UV illumination (UVITEC) after ethidium bromide staining. To estimate the size of the separation specifically of *Fasciola* species, a 100bp DNA ladder (Fermentas) was used in gels.

DNA Sequencing and Phylogenic Analysis

PCR products of ITS 1, of 20 isolates from two hosts (cattle and sheep) were sequenced .DNA Analyzers Sequencing, using the same primers which were used in the PCR.The sequences were aligned and compared with those of existing sequences from the region, related to Fasciola spp. available in the GenBank, using the BLASTprogram of NCBI GenBank. Multiple alignments were performed with data related to *Fasciola* spp. from Iran and other countries deposited in GenBank. A maximum likelihood tree was constructed using Blast (http://blast.ncbi.nlm.nih.gov/Blast).

Results

Genomic DNA was extracted from 70 isolates, which could amplify a fragment approximately 700bp in all of the samples (Fig. 2). Negative control did not produce any band on the gels.All amplified products of *Fasciola* were digested with the Rsa I restriction enzyme. The band patterns of the fragments digested with Rsa I were accurately distinguished among the two forms of *Fasciola*.

PCR-RFLP Analysis

Based on RFLP pattern, from 70 *Fasciola* isolates, 4 isolates (11.4%) from cattle had a RFLP pattern corresponding to *F. hepatica*, 31 isolates (88.6%) from cattle had a RFLP pattern corresponding to *F. gigantica*.35 isolates (100%) from sheep had a RFLP pattern corresponding to *F. hepatica*.

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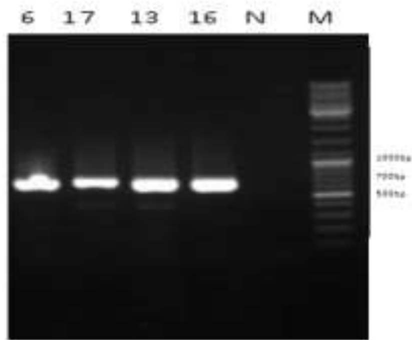

Fig. 2: PCR pattern of *Fasciola*. Lane M: 100bp DNA ladder, Lanes 6and 17: *Fasciola* from cattle, Lane 13 and Lane 16: *Fasciola* from sheep, Lanes N: Control negative

RFLP pattern from *F. hepatica*, which had 5 cutting sites, were predicted to be separated into fragments of 367, 104, 68,59, 54, and 28 bp in the amplicons**.** while 5 fragments were produced by 4 cutting sites from *F. gigantica* including 367, 172, 59, 54, and 28 bp in the amplicons (Fig. 3, 4). The band of 60 bp was thought to include the three fragments of 68 bp (only *F. hepatica*), 59 and 54 bp, and the small fragment of 28 bp was not detected on agarose gels. These findings showed that the band patterns between the amplicons with the sequences of *F. hepatica* and *F. gigantica* were evidently different.

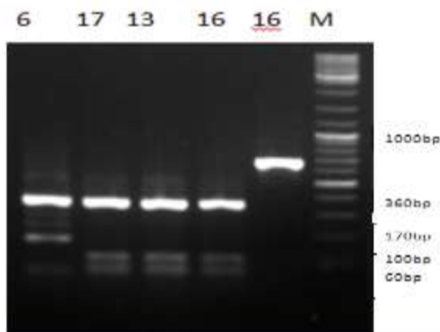

Fig. 3: PCR-RFLP pattern of *Fasciola* after digestion with Rsa I restriction enzyme. Lane M: 100bp DNA ladder, Lanes 6: *F. gigantica* from cattle, Lane 17 *F. hepatica* from cattle, Lane 3: *F. hepatica* from sheep, Lanes16: *F. hepatica* from sheep after and before digestion with Rsa I restriction enzyme

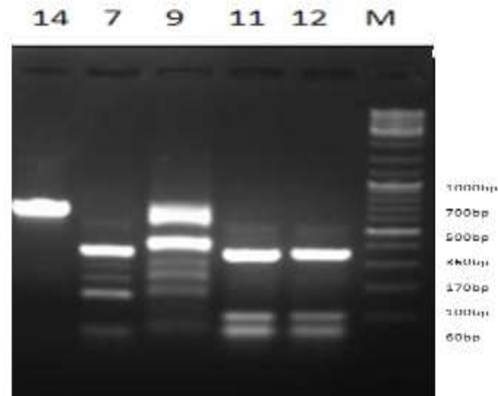

Fig. 4: PCR-RFLP pattern of *Fasciola* after digestion with Rsa I restriction enzyme. Lane M: 100bp DNA ladder, Lanes 7 F. *gigantic*, from cattle, Lane 9: PCR amplicons (upper) of *F. gigantic* and their restriction fragments (lower) produced by the PCR–RFLP method.from cattle, Lane 11 and 12: *F. hepatica* from sheep, Lane 14: *F. gigantica* from cattle before digestion with Rsa I restriction enzyme

The sequences of 700 bp ITS 1 of the flukes were analyzed and aligned with those of available sequences in GenBank using multalin (http://multalin.toulose.inra.fr/ multalin) software in comparison (Fig. 5, 6). Alignment of the sequences of ITS 1 showed DNA variable sites in which nucleotides at the position of 59, 68, 186, 276, 370,448, and 468 were single-base substituted resulting in segregation of the specimens (Table 1). Some sequences of *F. hepatica* and *F. gigantica* were analyzed and deposited in GenBank (Table 2). Ninety-hundred percent similarities were obtained in comparison of these sequences with all available data of *Fasciola* spp. in GenBank.

Restriction sites of the nucleotides in the *Fasciola* species were studied by computer software for selection a suitable enzyme. Accordingly, Rsa I was selected as one of the best restriction enzyme for differentiation between *F. hepatica* and *F. gigantica* which showed effect on GTAC nucleotides.

Phylogenic tree

Phylogenic Tree of ITS 1 showed that flukes were scattered as pure *F. hepatica* and *F. gigantica* clades, suggesting that two genotypes of *Fasciola* are able to infect animals and probably human in Northwestern of Iran (Fig. 7).

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Fig. 5: Sequence alignment of the ITSI, region from *Fasciola hepatica (*KF982049*)* and *Fasciola gigantic* (KF982046 & KF982047) of Ardabil Province, Iran

Fig. 6: Alignment of ITSI sequence of *F. hepatica* (GQ925431.1& AB514861.1) and *F. gigantica* (JF295001.1) deposited in GenBank with *F. hepatica (*KF982049) and *F. gigantica* (KF982046& KF982047) of Ardabil Province, Iran

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Table 2: Profile of *Fasciola* Spp. used in this study

Specimen code	species	Haplotyp	Host	ITS 1 types		
				Sequence analysis	Accession no.	PCR-RFLP Analysis
ADS1	*F.hepatica*	H1	Sheep	*F.hepatica*	KF982049	*F.hepatica*
ADS8	*F.hepatica*	H1	Sheep	*F.hepatica*	KF982045	*F.hepatica*
ADS11	*F.hepatica*	H1	Sheep	*F.hepatica*	KJ728732	*F.hepatica*
ADS16	*F.hepatica*	H1	Sheep	*F.hepatica*	KJ728734	*F.hepatica*
ADS25	*F.hepatica*	H1	Sheep	*F.hepatica*	KJ728735	*F.hepatica*
ADS18	*F.hepatica*	H1	Sheep	*F.hepatica*	KJ728736	*F.hepatica*
ADC45	*F.gigantica*	G1	Cattle	*F.gigantica*	KF982047	*F.gigantica*
ADC47	*F.hepatica*	H1	Cattle	*F.hepatica*	KF982048	*F.hepatica*
ADC51	*F.gigantica*	G2	Cattle	*F.gigantica*	KF982046	*F.gigantica*
ADC53	*F.gigantica*	G1	Cattle	*F.gigantica*	KJ728737	*F.gigantica*
ADC57	*F.gigantica*	G1	Cattle	*F.gigantica*	KJ728738	*F.gigantica*

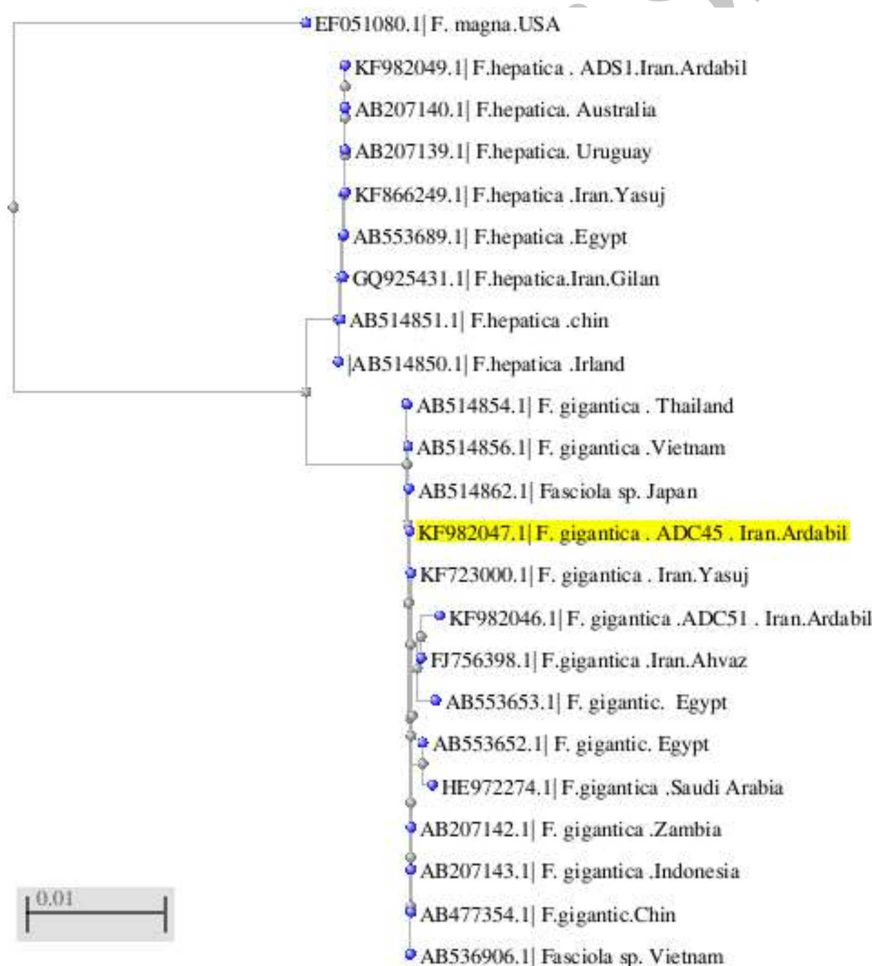

Fig. 7: Phylogenetic relationship of ITS 1 sequences of isolates of *Fasciola hepatica* and *F. gigantica* from Iran using Blast (http://blast.ncbi.nlm.nih.gov/Blast). Fasciolides magna (AN: EF051080) was used as the out group

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Discussion

In this study, we used 700bp region contained ITS1, 18s rRNA for identification and differentiation of *Fasciola* isolates by PCR-RFLP method. Moreover, both *F. hepatica* and *F. gigantic* samples were digested with Rsa I restriction enzyme, which showed different RFLP patterns. PCR-RFLP has been used in different studies in Iran to differentiate *Fasciola* spp. (8-16-18).

To detect the rate of similarity or difference of *Fasciola* samples, altogether 11 samples of both *F. hepatica* and *F. gigantica* were sequenced. Accordingly, it was showed that there was about 1.5% difference among some nucleotides. Karimi et al. showed that using Bfrl enzyme there was similar pattern for both aforementioned parasites, while using Dral different profiles are obvious (8). Rokni et al. using TasI for ITS1 region showed differentiation between both species (19). Saki et al., using AvaII and DraII for differentiation between both species (16). In 2013, Shafiei et al.in PCR-RFLP method was used to specifically distinguish *F. hepatica* from *F. gigantica* in ITS1 with Rsa I enzyme and in IST2 with MSPI and KpnI (20). In their study no intermediate forms were seen. In 2009, Ghavami et al. reported a digested pattern of 330, 340 and 241 bp in F. hepatica using BamHI and PagI enzymes on ITS 2 gene (17). As for studies in other countries, Shalabi et al. used RAPD-PCR to differentiate *F. hepatica* and *F. gigantica* among imported sheep to Saudi Arabia from Sudan. They reported one intermediate case as well (21). In China, Huang et al. using ITS 2 and HSP9211 plus RcaI enzymes has differentiated both species besides intermediate cases. They recommended the first enzyme as better choice (14). In 2010, Madoka Ichikawa used PCR-RFLP method to specifically distinguish *F. hepatica* from *F. gigantica* in ITS1 with Rsa I enzyme (22).

Ashrafi et al. used ITS2 nucleotide sequencing and showed two species of F. hepatica and F. gigantica in Guilan, northern Iran (23). Accordingly *F. hepatica* in that region was similar to Europe *F. hepatica* and *F. gigatinca* was similar to Burkina Faso isolate in terms of above mentioned method. The authors believed a kind of intermediate isolate in Guilan (23).

Using phylogenetic tree, we could detect a close relationship of our isolates with those of other countries. As mentioned in tables and figures already, we detected two haplotypes of *F. gigantica* as G1 and G2. It is worth mentioning that recently a hypothesis has been proposed that this *F. gigantica* might be considered as separate species (24). Our findings showed that all isolates belonging to F. hepatica were of common H1 haplotype. Figure 6 shows that this haplotype has been reported already from Yasuj and Guilan from Iran, as well as from Egypt, Ireland, China and Australia as well. Besides, haplotype G1 of *F. gigantica* has been reported from Egypt, Vietnam, Indonesia, China, and Yasuj plus Guilan from Iran. Haplotype G2 is reported from Iran and Egypt as well as Saudi Arabia (All references in Fig. 7). As mentioned already no intermediate isolates were detected in our study.

The limitation of our study is that more samples should have been collected to be sure of the deduction as much as possible. The strength might be told that this is for the first time that this study has been conducted in this area in its kind.

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Conclusion

Comparing *F. hepatica* and *F. gigantica* sequences showed 90-100% homology between our samples with those originated from other regions available in GenBank, but comparing *F. hepatica* and *F. gigantica* with each other showed a few nucleotide differences.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

Acknowledgments

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