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Original Article

Molecular Characterization of Animal *Fasciola* spp. Isolates from Kermanshah, Western Iran

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Abstract

Background: We evaluated the genetic diversity of samples identified morphologically as *Fasciola* spp. from sheep, cattle and goat from Kermanshah Province, western Iran using PCR-RFLP method.

Methods: We used PCR-RFLP analysis of ribosomal ITS1 fragment using RsaI restriction enzyme to investigate the genetic characteristics of *Fasciola* species obtained from different hosts (16 sheep, 28 cattle, 4 goats). The species of *Fasciola* were confirmed by sequencing the 700 bp region of ribosomal ITS1 gene.

Results: In Kermanshah, *F. hepatica* was present in 96% of the samples, *F. gigantica* was found only in two cattle sample. No hybrid forms were detected in the present study.

Conclusion: Our results contribute to clarify the dark spots of *Fasciola* genotyping in different parts of Iran.

Keywords: *Fasciola hepatica*, *Fasciola gigantica*, Genotype, PCR, Iran

Introduction

Fascioliasis in livestock and humans is caused by two of the most socioeconomically important trematodes: *F. hepatica* Linnaeus, 1758 and *F. gigantica* Cobbold, 1855, respectively (1). A number of freshwater snails of the family Lymnaeidae serve as intermediate hosts. Humans and animals are infected by eating aquatic vegetables or drinking water contaminated with the infective metacercariae (2).

Human fascioliasis is a foodborne disease, included in the WHO list of neglected tropical diseases (NTD) (3). More than 180 million people are at risk of fascioliasis with 2.4–17 million individuals infected (2). *Fasciola hepatica* appears mainly in Europe, the Americas and Oceania, and *F. gigantica* occurs mainly in Africa and Asia (1). The distributions of both species overlap in many countries of Africa and Asia and leads to many problems as for identification (4).

The differentiation of *Fasciola* species is essential because their intermediate host, epidemiological characteristics, control strategies, host specificity, drug susceptibility and virulence is different (5).

Although fasciolid species can generally be distinguished by morphological characters, but both fasciolid species are polymorphic with many factors affecting the morphology and leading to considerable taxonomic confusion (6).

Nowadays morphological methods are incomplete and imperfect and various molecular and genetic techniques based on DNA analyses have been employed for distinguish species confirmation and genetic structuring of parasitic populations (6, 7). Reaction-restriction fragment length polymorphisms (PCR-RFLP) assay is very rapid, simple, inexpensive, easy to perform and appropriate method for distinguish of *F. hepatica* from *F. gigantica* (8-10). However, for more detailed and complete interpretation of the results consequent sequence analysis of target DNA is recommended.

In Iran, the prevalence of fascioliasis is 0.1% to 91.4% in various livestock (11). Ectopic infection with fascioliasis in non-hepatic sites such as the thyroid, eye or skin, have been reported from Iran (12, 13).

Kermanshah Province is located in western of Iran. This province is divided into three distinct portions: Warm, moderate and cold climates and suitable pastures for traditional rearing of domestic animals. *F. hepatica* and *F. gigantica* have been reported from a range of mammals including buffalo, cattle, sheep and goat based on morphological features in this province. A small outbreak of 17 cases of human fascioliasis happened in the province in 1998 and verified a new emerging focus of human fascioliasis in western part of the country (14, 15).

The aim of this study was to identify and differentiate *Fasciola* flukes by PCR-RFLP of ITS1 analysis for epidemiological applications especially in regions where the two species overlap in Kermanshah Province. Besides, we analyzed their phylogenetic relationship with populations from other countries.

Materials and Methods

Parasite

Adults of *F. hepatica* and *F. gigantic* were collected from the bile ducts of different livestock at slaughterhouses in Kermanshah, Iran from September 2014 to September 2015. Flukes were washed in saline solution and subsequently stored in 70% ethanol until required for PCR and sequencing.

Genomic DNA Extraction and PCR-RFLP analysis

Genomic DNA was extracted from the adult flukes using DNGTM-plus Kit (CinnaGen, Iran) following manufacturer's recommendations. The ITS1 region was amplified using two primers forward (5'- TTGCGCTGATTACGTCCCTG -3') and Reverse (5'- TTGGCTGCGCTCTTCATCGAC -3 '). The primers were synthesized by Bioneer Company (Korea). PCR amplifications were performed in 15 µl reactions containing of 7.5 µl master mix (Amplicon), 0.5 µl of each primers (5 pmol) (Forward and Reverse), 1.5 µl genomic DNA, and 5 µl distilled water. The reactions were performed as follows: pre-denaturation at 95 ºC for 5 min, 30 cycles of denaturation at 94 ºC for 30 sec, annealing at 50 ºC for 30 sec, elongation at 72 ºC for 30 s, followed by a final extension at 72 ºC for 5 min. The PCR products were separated in 1.5 % agarose gel using Simply Safe (Eurx, Poland). A PCR-RFLP method was used to distinguish specifically *F. hepatica* from *F. gigantica* in ITS1with RsaI enzyme (9). To confirm results PCR-RFLP, a fragment of 700 bp from the ITS1 gene was sequenced for seven cases. Sequencing was performed in forward direction using Sanger sequencing (Macrogen Inc. Korea, ABI3730XL).

Data analysis

Sequences were performed using Bioedit Sequence Alignment Editor, version 5.0.9 (Hall, 1999). Then resulted sequences aligned against nr database using BLAST 2.0 to find the closest entries. Multiple sequence alignment was carried out using the ClustalW with default parameters; alignments were visualized using jalview software. Phylogenetic analysis was performed using MEGA 6 package with neighbor joining method and default parameters.

Results

PCR-RFLP Analysis

Overall, 48 adult flukes were collected from 28 cattle, 16 sheep, and 4 goats from the abattoirs of Kermanshah Province.

PCR product of ITS1 resulted in a fragment size of 700 bp. Amplicons were subsequently digested using the Rsa I restriction enzyme. This approach yielded the identification of two genetically distinct banding patterns belonging to the genus *Fasciola*: the *hepatica* genotype displays five bands of about 367, 104, 68, 59, 54, and 28 bp, the *gigantica* genotype shows five bands of about 367, 172, 59, 54, and 28 bp.

Based on RFLP pattern, from 48 *Fasciola* isolates, 4 isolates (100%) from goat had a RFLP pattern corresponding to *F. hepatica*, 27 (96%) and 1 (4%) isolates from cattle had an RFLP pattern corresponding *F. hepatica* and *F. gigantica*, respectively. Fifteen isolates (94%) and 1 isolates (6%) from sheep had RFLP pattern corresponding to *F. hepatica* and *F. gigantica*, respectively (Fig. 1).

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. 2). There was no nucleotide variation in the ITS-1 rDNA sequences among the *F. hepatica* and *F. gigantica* samples examined. Some sequences of *F. hepatica* and *F. gigantica* were analyzed and deposited in GenBank (Table 1). One-hundred percent similarities were obtained in comparison of these sequences with all available data of *Fasciola* spp. in GenBank.

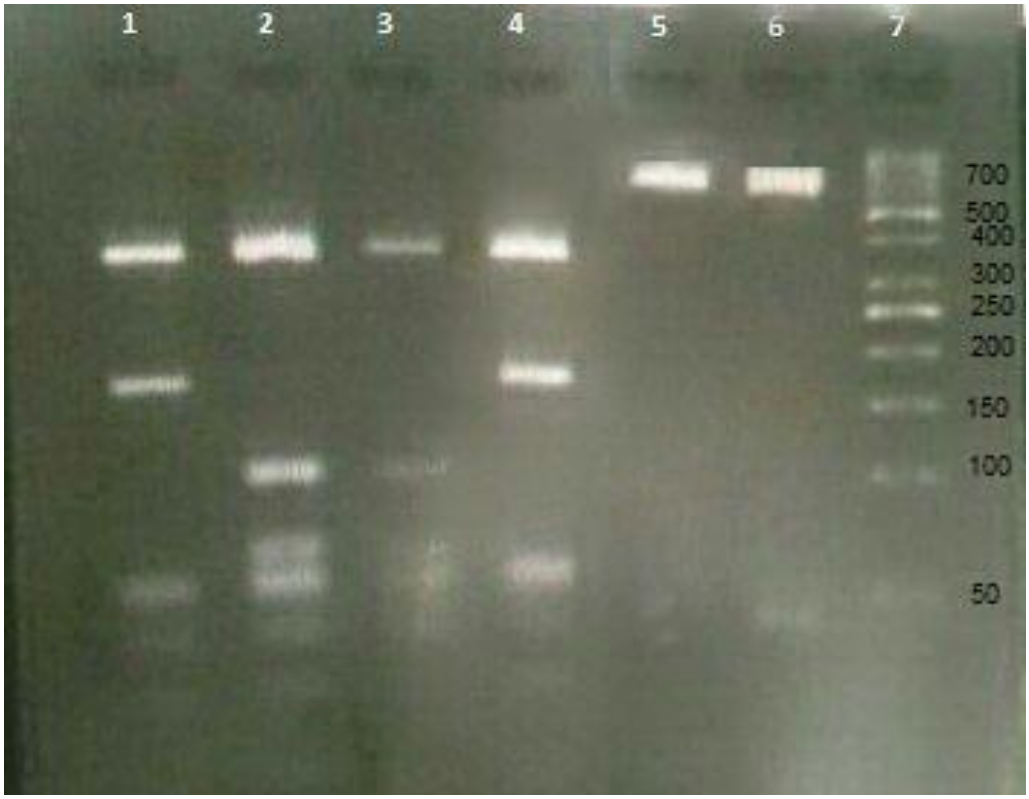

Fig. 1: PCR-RFLP pattern of *Fasciola* after digestion with Rsa I restriction enzyme. Lane 7: 50bp DNA ladder, Lane 1: *F. gigantica* from cattle, Lane 2: *F. hepatica* from cattle, Lane 3: *F. hepatica* from sheep, Lanes 4: *F. gigantica* from sheep after digestion with Rsa I restriction enzyme. Lane 5: *F. hepatica* from cattle before digestion with Rsa I restriction enzyme, Lane 6: *F. gigantica* from sheep before digestion with Rsa I restriction enzyme

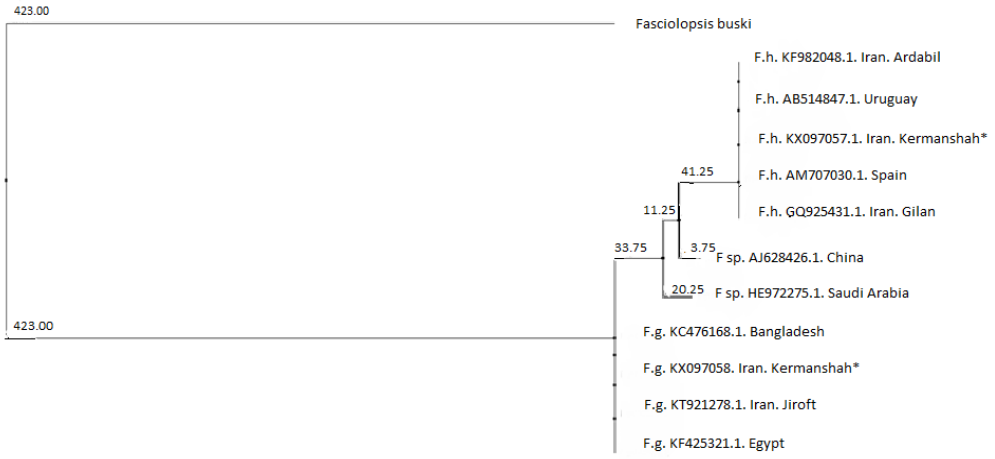

Fig. 2: Phylogenetic trees were obtained by using MEGA 6.0 (Tamura 3- parameter model) with bootstrap values of 1000 replicates set for neighbor-joining. *Fasciolopsis buski* was used as the out group. * Sequences from specimens isolated in different hosts from our study

Table 1: Sequencing profile of *Fasciola* Spp.

Specimen code	Species	Haplotype	Host	Sequence analysis	ITS 1 types Accession no.	PCR-RFLP Analysis
0029	*F. hepatica*	H1	Cattle	*F. hepatica*	KX179506	*F. hepatica*
001	*F. gigantica*	G1	Sheep	*F. gigantica*	KX179507	*F. gigantica*
002	*F. hepatica*	H1	Cattle	*F. hepatica*	KX179508	*F. hepatica*
0017	*F. hepatica*	H1	Cattle	*F. hepatica*	KX097057	*F. hepatica*
0027	*F. gigantica*	G1	Cattle	*F. gigantica*	KX097058	*F. gigantica*
0047	*F. hepatica*	H1	Cattle	*F. hepatica*	KX097059	*F. hepatica*
0013	*F. hepatica*	H1	Sheep	*F. hepatica*	KX097060	*F. hepatica*

Phylogenic tree

The length of the trimmed aligned ITS 1 sequence was 350 base pairs. Phylogenetic analysis of *Fasciola* samples based on the ITS1 region revealed 2 haplotypes (1 *F. gigantica* and 1 *F. hepatica*) (Figure 2). Therefore suggesting that two species of *Fasciola* are able to infect domesticated animals and probably human in west of Iran. Some sequences of *F. hepatica* and *F. gigantica* in our study were analyzed and deposited in GenBank (Table 1).

Discussion

The present paper provides additional information on the molecular epidemiology of fascioliasis in Kermanshah regions. Characterization of the population genetic variability of *Fasciola* species is useful as it can help disease surveillance, diagnosis and control of the parasite.

The prevalence of fasciolosis in Kermanshah has been reported to be 0.25% in livestock (16).

Molecular characterization using a PCR-RFLP approach on a nuclear marker has revealed that *F. hepatica* was more prevalent than *F. gigantica* in Kermanshah, confirming previous studies that *F. hepatica* is found in temperate areas while *F. gigantica* mainly in tropical zones (9). In tropical regions when both species are present, *F. gigantica* is commonly endemic in lower areas and *F. hepatica* is endemic in the highlands (17, 18).

The distribution of the two species overlaps in some countries and the presence of hybrid form of *Fasciola* has been reported from livestock or human in Asian countries, such as Iran (19), Thailand (20), Vietnam (21), and Pakistan (22). Phenotypic differences of the liver fluke have been recognized, suggesting hybridization of the two species. This indicates that intermediate forms may exist within our study region, although none has been found in this study.

Analyses based on the nuclear markers such as ITS 1 and 2 are useful for species confirmation, whereas mitochondrial markers such as cytochrome c oxidase (CO1) and nicotinamide dinucleotide dehydrogenase subunit-1 (ND1) are more variable because its mutation rate is often fast and can be used to distinguish closely related species and populations of *Fasciola*.

Our findings showed that all isolates belonging to *F. hepatica* and *F. gigantica* were of common H1 and G1 haplotype, respectively but the comparisons with ITS1 sequences of *F. hepatica* and *F. gigantica* from other localities showed nucleotide differences at least in one position.

Haplotype G1 of *F. hepatica* has been reported already from Ardabil (9) and Guilan (23) from Iran, as well as from Uruguay (24), Egypt (25) and Saudi Arabia (26) as well. Besides, haplotype G1 of *F. gigantica* has been reported from Zambia (24), Egypt (27), Thailand (28), plus Ardabil from Iran (9).

Two species *Fasciola* require a particular snail as an intermediate host in order to complete their life cycle. The main intermediate host of *F. hepatica* in Iran is *Lymnaea truncatula* while the main intermediate host of *F. gigantica* is *L. gedrosiana* (11). The distribution of *Fasciola* species is determined by the ecological characteristics such as population dynamics, anthropophylic characteristics, type of water bodies the availability of the snail intermediate hosts (29).

Conclusion

Lack of data on the susceptibility of potential intermediate hosts to *Fasciola* infection in the present study makes it difficult to determine the role of these snails in the transmission of parasite.

Complementary studies are needed to determine the prevalence of Fasciola species and to determine the presence of the parasite in its snail hosts risk area. We cannot forget that the correct identification of lymnaeidae is a key element to detect possible transmission foci and to estimate the infection risk.

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.

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