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Molecular Characterization of *Fasciola* Samples Using Sequences of Second Internal Transcribed Spacer-rDNA in Different Geographical Localities of Sistan and Balouchestan Province, Iran

Mahsa Shahbakhsh,[1] Reza Nabavi,[1,*] and Maryam Ganjali[1]

[1]Department of Pathobiology, Faculty of Veterinary Medicine, University of Zabol, Zabol, IR Iran

[*]*Corresponding author*: Reza Nabavi, Department of Pathobiology, Faculty of Veterinary Medicine, University of Zabol, Zabol, IR Iran. Tel: +98-5432235034, Fax: +98-5432235034, E-mail: rezanabavi@uoz.ac.ir

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Abstract

Background: The *Fasciola* trematodes are the most common liver flukes, living in a range of animals with global distribution and resulting in profound economic loss and public health challenges. Previous studies have indicated that the sequences of the second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA) provide reliable genetic markers for molecular systemic studies of *Fasciola*.
Objectives: The objective of the present study was to characterize *Fasciola* samples from different geographical regions of Sistan and Balouchestan province using sequences of second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA).
Materials and Methods: Twenty adult trematodes were collected from the livers of slaughtered infected cattle. Total genomic DNA was extracted and ITS-2 rDNA targets were amplified by polymerase chain reaction (PCR). All samples were sequenced and investigated using the ClustalW2 sequence alignment tool and MEGA software. The sequences of some Iranian and non-Iranian isolates were used for comparison, in order to evaluate the variation in sequence homology between geographically different trematode populations.
Results: The results of comparing the ITS-2 sequences with the BLAST GenBank database showed one type of sequence for *F. hepatica* and three different types of sequences for F. *gigantica* in the specimens.
Conclusions: The present study demonstrated that *Fasciola* samples from cattle in two geographical locations in Sistan and Balouchestan province represented no genetic diversity in *F. hepatica* and high genetic variation in *F. gigantica*.

Keywords: PCR, rDNA, Sistan and Balouchestan, ITS2, *Fasciola*

1. Background

The *Fasciola* trematodes are common liver flukes, living in a range of animals with global distribution and resulting in profound economic loss (1) and public health challenges (2-4). Human infection of *F. hepatica* and *F. gigantica* happens accidentally and occurs most frequently in sheep and cattle raising regions (5). In Iran, as a subtropical country, the distribution of these two species overlaps in most areas (6). In order to find the best understanding of fluke pathogenesis, life cycle and host-parasite relationship, proper identification of *Fasciola* species and strains is necessary. *F. hepatica* and *F. gigantica* can generally be identified based on morphology (6), but use of molecular methods could be helpful to distinguish intermediate types and subspecies. Previous studies have shown that the sequences of the second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA) provide reliable genetic markers for systemic molecular studies of *Fasciola* (7-12).

2. Objectives

Therefore, the objective of the present study was to characterize *Fasciola* samples from different geographical regions of Sistan and Balouchestan province using sequences of second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA). The present findings provide a foundation for further molecular studies on *F. hepatica* and *F. gigantica* in the province and have implications for a better understanding of the disease they cause.

3. Materials and Methods

3.1. Parasites

The study was carried out in the Zabol and Iranshahr districts of Sistan and Balouchestan province in the southeast of Iran (May 2014 to April 2015). Twenty adult trematodes were collected from the livers of slaughtered native cattle. All fluke specimens were washed several times in physiological saline (37°C) and identified morphologically as *Fasciola hepatica* and *Fasciola gigantica*, according to morphological keys and descriptions (13), and stored in 70% ethanol until the extraction of genomic DNA. The sample codes, host species and geographical origins are listed in Table 1.

3.2. DNA Extraction, PCR and Sequencing

Total genomic DNA was extracted from individual flukes using a MBST (molecular biological system transfer) DNA purification kit, according to the manufacturer's recommendations. All the DNA samples were stored at -20°C until further use. ITS-2 rDNA plus primer flanking 5.8S and 28S sequences was amplified by polymerase chain reaction (PCR) from genomic DNA using primers (forward: 5'-TCTTGAACGCATATTGCGGC-3') and (reverse: 5'-AGTTCAGCGGGTAATCACGT-3') (14). A thermocycler (eppendorf, mastercycler EP gradient S) was used to amplify 50 μL in 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 200 μM each of dNTP, 50 pmol of each primer, and 0.25 U Taq polymerase under the following conditions: 95°C for 5 minutes (initial denaturation), followed by 35 cycles at 95°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing), 72°C for 30 seconds (extension), and a final extension at 72°C for 7 minutes (15). One microliter of genomic DNA was used for each PCR reaction. Samples without genomic DNA were included in the PCR reaction as negative controls. An aliquot (5 μL) of each amplicon was examined on 1% agarose gel stained with ethidium bromide and photographed using a gel documentation system (Figure 1).

3.3. Sequencing and Data Analysis

All samples were sequenced in both directions by Pishgam biotechnology company. Multiple alignments of the ITS-2 sequences of the present sequences were then used to compare and calculate similarity scores. In this step, ITS-2 sequences of some *Fasciola* isolates from other parts of Iran and other countries around the world were also included. The ClustalW2 sequence alignment tool was used for all alignments and calculation of similarity scores (16). The phylogenetic trees were constructed based upon the ITS-2 sequences from Sistan and Balouchestan province using the maximum parsimony (MP) and distance methods, namely, neighbor-joining in MEGA 6.0 (17-19). Branch support was given using 1,000 bootstrap replicates in MEGA (18).

4. Results

The fragments of amplified DNA were 456 - 457 bp long. Next, the ITS-2, PCR products were subjected to direct sequencing and one type of sequence for *F. hepatica* and three different types of sequences for *F. gigantica* were obtained. These sequences were deposited in GenBank under accession number (f.h. Zabol1 KT033696, f.g. Zabol KT223394, f.g. Iranshahr 1 KT223395, f.g. Iranshahr 2 KT223396 and f.g. Iranshahr 3 KT223397). The sequences were composed of partial 5.8S sequences of 63 bp, the complete ITS-2 sequences of 362 bp and partial 28S sequence of 75 bp for *Fasciola* samples. Table 2 lists the nucleotide variation at five variable sequence positions in the ITS-2 of the *Fasciolidae* species from different geographical regions that have been analyzed in the present study.

Table 1. Geographical Locations and Host Origins of *Fasciola* Samples Used in This Study[a]

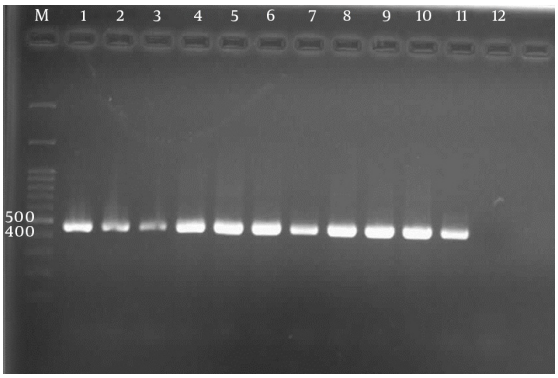
Sample Codes	Parasite	Geographical Origin	Genotype
1	*F. hepatica*	Zabol	f.h. Zabol
2	*F. hepatica*	Zabol	f.h. Zabol
3	*F. hepatica*	Zabol	f.h. Zabol
4	*F. gigantica*	Zabol	f.g. Zabol
5	*F. hepatica*	Zabol	f.h. Zabol
6	*F. gigantica*	Zabol	f.g. Zabol
7	*F. hepatica*	Zabol	f.h. Zabol
8	*F. hepatica*	Zabol	f.h. Zabol
9	*F. hepatica*	Zabol	f.h. Zabol
10	*F. hepatica*	Zabol	f.h. Zabol
11	*F. gigantica*	Iranshahr	f.g. Iranshahr 1
12	*F. gigantica*	Iranshahr	f.g. Iranshahr 1
13	*F. gigantica*	Iranshahr	f.g. Iranshahr 1
14	*F. gigantica*	Iranshahr	f.g. Iranshahr 2
15	*F. gigantica*	Iranshahr	f.g. Iranshahr 1
16	*F. gigantica*	Iranshahr	f.g. Iranshahr1
17	*F. gigantica*	Iranshahr	f.g. Iranshahr 3
18	*F. gigantica*	Iranshahr	f.g. Iranshahr 1
19	*F. gigantica*	Iranshahr	f.g. Iranshahr 1

[a]Host: cattle.

Also, Table 2 shows the second and third sequences of *F. gigantica* from Iranshahr (f.g. Iranshahr 2, f.g. Iranshahr 3) that have a variation with *F. gigantica* from India, China and *F. gigantica* Iranshahr 1 and other geographical locations of Iran. The sequences that were obtained (ITS-2 rDNA of *F. hepatica* and *F. gigantica*) were compared with sequences of other *F. hepatica* and *F. gigantica* from GenBank using the ClustalW2 tree building method.

In this study, primary sequence analysis revealed a close relationship between the query sequence (*F. hepatica* from Zabol) and isolates of *F. hepatica* from China, Ireland, Ecuador, Egypt, Turkey, Austria and other geographical locations in Iran (Zanjan, Shiraz, Gachsaran) and a close relationship between *F. gigantica* samples and *F. gigantica* from China and India (Figure 2). The BLAST hit results indicated that our query ITS-2 sequences were identical to the sequences of various geographical isolates of *F. hepatica* and *F. gigantica*.

Figure 1. Agarose Gel Electrophoresis of ITS-2 PCR Products of Representative *Fasciola* Samples



Lanes 1 - 11 represent *Fasciola* samples from sheep and cattle from different geographical locations (Table 1). Lane 12 represents the negative control. M is a DNA size marker (ordinate values in bp).

Table 2. Comparison of Nucleotide Variations at Seven Positions in the ITS-2 Sequences of *F.hepatica* and *F.gigantica* from Different Geographical Locations

Taxon	Five Variable Positions in the ITS-2 Sequences							Accession Number	Geographical Location
	87	259	268	358	388	401	408		
Fasciola hepatica	_	_	T	C	T	G	T	Present study (KT033696)	Zabol
	_	_	T	C	T	G	T	KF866250	Iran-Gachsaran
	_	_	T	C	T	G	T	JF432072	Iran-Shiraz
	_	_	T	C	T	G	T	EU391412	Iran-Zanjan
	_	_	T	C	T	G	T	EU391424	Iran-Zanjan
	_	_	T	C	T	G	T	DQ683546	Austria
	_	_	T	C	T	G	T	KJ789364	China
	_	_	T	C	T	G	T	LC056930	Ecuador
	_	_	T	C	T	G	T	LC056929	Ecuador
	_	_	T	C	T	G	T	AB510492	Egypt
	_	_	T	C	T	G	T	KJ81828276	Egypt
	_	_	T	C	T	G	T	AB973396	Ireland
	_	_	T	C	T	G	T	FJ459806	Turkey
	_	_	T	C	T	G	T	JN585288	Turkey
Fasciola gigantica	_	_	C	_	C	T	_	Present study (KT223394)	Zabol
	_	_	C	_	C	T	_	Present study (KT223395)	Iranshahr 1
		G	C	_	C	T	_	Present study (KT223396)	Iranshahr 2
	G	_	C	_	C	T	_	Present study (KT223397)	Iranshahr 3
	_	_	T	_	C	C	_	KF866247	Iran-Gachsaran
	_	_	T	_	T	T	T	JF432073	Iran-Shiraz
	_	_	C	_	C	T	_	EF027103	India
	_	_	C	_	C	T	_	KC535542	India
	_	_	C	_	C	T	_	KF667375	India
	_	_	C	_	C	T	_	KF667382	India
	_	_	C	_	C	T	_	JF496711	China

Figure 2. Phylogenetic Relationship Between *Fasciolidae* Species From Different Geographical Locations

H, *Fasciola hepatica*; G, *Fasciola gigantica*. The present specimen (Sample codes 1 - 20) descriptions are presented in Table 1.

5. Discussion

Previous studies have characterized *Fasciola* from different countries and different regions of Iran using genetic approaches (15, 20-25). However, before the present study, there have been no molecular investigations regarding the *Fasciola* species from Sistan and Balouchestan province.

Therefore, the objective of the present study was to characterize *Fasciola* samples from different geographical locations of Sistan and Balouchestan province, using sequences

of second internal transcribed spacer of ribosomal DNA, because this sequence has been shown to provide specific molecular markers for the identification of *Fasciola hepatica* and *Fasciola gigantica* and the intermediate forms (15, 26).

The present results confirmed many previous findings around the world that have indicated the ITS-2 target is convenient for detection of the *Fasciola* species and subspecies (8, 12).

F.hepatica and *F. gigantica* have been detected accurately in all specimens and morphologically intermediate forms. Two *Fasciola* flukes (11 and 12) were collected from Iranshahr and identified morphologically as *F. hepatica*, however, comparative sequence analysis specified them as *F. gigantica*. In the same study in Zanjan, Rahimi et al. (2009) showed that 7% of isolates were *F. gigantica*, based on the morphometric markers. However, ITS2-RFLP patterns and sequence analysis indicated that they were all identical to the *F. hepatica* species (14). Based on such overlaps between the two species, which could be observed anywhere in the world, it is suggested that molecular approaches be used for accurate identification (26).

In the present study one type of sequence for *F. hepatica* and three different types of sequences for *F. gigantica* have been detected. In Turkey, Erensoy et al. (2009) detected just one sequence for *F. hepatica* in all studied flukes (5). In addition, Ali et al. (2008) detected one type for *F.* hepatica and one type for *F. gigantica* in Niger (12). The variation of genetic types in *F. gigantica* in the present study is more than in previous studies in other geographical locations and could be due to the entrance of exotic cattle from India and Pakistan to the province, as well as the occurrence of *Fasciola* contamination in native flocks with exotic strains.

In this study, primary sequence analysis revealed a close relationship between the query sequence (*F. hepatica* from Zabol) and isolates of *F. hepatica* from China, Ecuador, Egypt, Turkey, Austria and other geographical location of Iran (Zanjan, Shiraz, Gachsaran). Also, a close relationship between *F. gigantica* samples and isolates of *F. gigantica* from China and India has been detected (Figure 2).

The BLAST hit results showed that our query ITS-2 sequences were much more similar to the sequences of various geographical isolates of *F. hepatica* and *F. gigantica*, in addition to some *Fasciola* spp. In conclusion, the present study demonstrated that *Fasciola* samples from cattle in two geographical locations in Sistan and Balouchestan province represented no genetic diversity in *F. hepatica* and high genetic variation in *F. gigantica*. The results of the present study provide the foundation for further studies on *F. hepatica* and *F. gigantica* in Sistan and Balouchestan province and have implications for the diagnosis and control of the disease they cause.

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Footnotes

Authors' Contribution: All authors participated equally in this article, especially in design, work, statistical analysis and manuscript writing.

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