



## MOLECULAR AND PHYLOGENETIC CHARACTERISATION OF *FASCIOLA* SPP. ISOLATED FROM CATTLE AND SHEEP IN SOUTHEASTERN IRAN

H. MIRAHMADI<sup>1,2</sup>, P. BIGLERI<sup>1,2</sup>, S. SEKANDARPOUR<sup>1,2</sup>,  
M. J. MODREK<sup>1,2</sup> & R. SHAFIEI<sup>3</sup>

<sup>1</sup>Infectious Disease and Tropical Medicine Research Center, Zahedan University of Medical Sciences, Zahedan, Iran; <sup>2</sup>Department of Parasitology and Mycology, Faculty of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran; <sup>3</sup>Vector-borne Diseases Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran

### Summary

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The objective of this study was to determine the genotype of *Fasciola* spp. in animal hosts from Zahedan, Sistan and Baluchestan province, southeastern Iran using PCR-RFLP. Overall, 50 and 43 adult *Fasciola* spp. were isolated from bile ducts of naturally infected cattle and sheep. PCR-RFLP with *RsaI* restriction enzyme and sequence analysis of the first nuclear ribosomal internal transcribed spacer (ITS 1) region from *Fasciola* spp. were used to conduct the study. RFLP pattern with *RsaI* produced 180 and 331 bp fragments in *F. gigantica* and amplicons of *F. hepatica* had a size of 77, 104 and 331 bp. 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* while cattle were infected with both species. The results of our study showed that *F. hepatica* and *F. gigantica* isolates were of common H1 and G1 haplotypes.

**Key words:** cattle, *Fasciola*, Iran, genotype, RFLP-PCR, sheep, Sistan and Baluchestan

### INTRODUCTION

Fasciolosis caused by the digenian liver fluke, *Fasciola hepatica* and *F. gigantica*, is a neglected emerging disease causing wellness and economic problems in many countries (Mas-Coma *et al.*, 2005). Food containing infected vegetables is the source of this parasitic infection (Mahami-

Oskouei *et al.*, 2011). Human and animal fasciolosis are a serious concern for public and animal health in Iran (Moghaddam *et al.*, 2004; Ashrafi *et al.*, 2006). Animal fasciolosis is common in pasture animals in most areas of Iran and its prevalence reaches up to 50% in some provinces es-

pecially in north of Iran (Moghaddam *et al.*, 2004; Rokni, 2008). During the past 20 years, human diseases were reported in some provinces of Iran and two serious human epidemics have been reported in the northern province of Gilan (Ashrafi *et al.*, 2006; Rokni, 2008).

Different morphological criteria between these two species – due to intermediate form existence in different Asian countries – have made accurate recognition difficult (Ashrafi *et al.*, 2006; Ichikawa & Itagaki, 2010). Molecular methods based on nuclear ribosomal (rDNA) and mitochondrial (mtDNA) genes are capable to differentiate *Fasciola* species (Itagaki *et al.*, 2005; Mas-Coma *et al.*, 2009; Shafiei *et al.*, 2014).

The province of Sistan and Baluchestan is located in the southeast of Iran. Previously, this province has not been known as an important endemic area for fasciolosis. Animal fasciolosis is a serious problem causing reduced production, especially in cattle and sheep (Rokni, 2008). Until now, no data are available about the prevalence of *Fasciola* spp. in this province. However, variations in phenotype

criteria, dissemination, host parasites, genotypes and intermediate forms were reported in Pakistan, in vicinity of the province (Itagaki *et al.*, 1998; Khan *et al.*, 2009). Therefore, it seems necessary to set a goal for recognition, prevention and control of the disease (Rokni *et al.*, 2010). It has been advocated to use PCR-RFLP on rDNA genes for this purpose (Marcilla *et al.*, 2002; Huang *et al.*, 2004; Rokni *et al.*, 2010; Shafiei *et al.*, 2013).

The objective of present study was to examine the utility of region between 18S and 28S, the first internal transcribed spacer (ITS1) of rDNA by PCR-RFLP for molecular identification of *Fasciola* spp. in Sistan and Baluchestan province, Iran.

## MATERIALS AND METHODS

### Study area

The province of Sistan and Baluchestan is one of the 31 Iranian provinces and is located in the southeastern part of the country, bordering Pakistan and Afghanistan (Fig. 1). The capital is Zahedan and the most prominent climatological phe-



Fig. 1. Map of Sistan and Baluchestan province, Iran.

nomenon of the region is the heat. Basically the climate of the province is characterised by long, hot and dry summers and short winters, whereas in the coastal region, near the Oman Sea, the weather is warm with a high percentage of humidity. Traditional farming and stock breeding of ruminants provide suitable conditions for transmission of fasciolosis in this area.

#### *Parasite*

A total of 50 and 43 adult *Fasciola* spp. was isolated from bile ducts of naturally infected cattle and sheep from two slaughterhouses at Zahedan. Individual worms were washed extensively in PBS buffer (37 °C) and subsequently fixed in 70% ethanol and maintained at room temperature for several weeks until extraction of genomic DNA.

#### *DNA extraction*

Approximately 10 mg tissue was removed from a portion of the lateral zone of adult flukes and then crushed. The ethanol was allowed to evaporate in each of samples for a few minutes and then washed 3 times in distilled water and squashed before the genomic DNA was extracted. Bioneer AccuPrep® kit was used for genomic DNA extraction of *Fasciola* parasites. The extracted DNA, split into aliquots was stored at -20 °C until used in the PCR.

#### *Polymerase chain reaction (PCR)*

To amplify a region of a 700 bp region of ITS1, PCR was performed using F: (5'-ACCGGTGCTGAGAAGACG-3') and R: (5'-CGACGTACGTGCAGTCCA-3') primers (Aryaeipour *et al.*, 2014). The total volume of reaction was 30 µL containing 3 µL DNA template, 15.5 µL distilled water, 10 pmol (1 µL) of each primers (Forward and Reverse), and 9.5 µL master

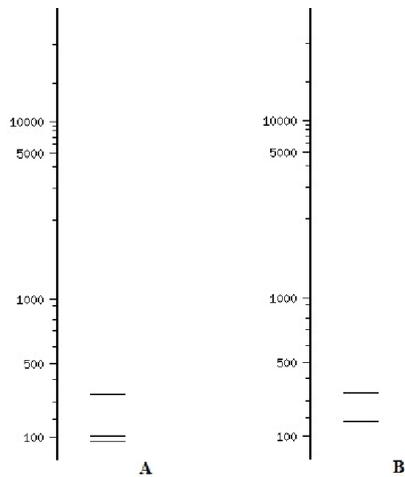
mix (amplicon). PCR amplification was performed in Eppendorf Mastercycler Gradient thermocycler. The temperature profile was as followed: an initiation of 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing), 72 °C for 30 s (extension) and a final extension of 72 °C for 5 min followed by cooling at 4 °C. The DNA product was sequenced and *Fasciola* species were identified.

#### *Restriction fragment length polymorphism (RFLP)*

After sequencing, using NEBcutter V2.0 software, the cutting sites of commercially available restriction enzymes on ITS1 sequences of *F. hepatica* and *F. gigantica* were assessed (Shafiei *et al.*, 2013). RsaI enzyme (Vivantis) was selected for RFLP method that caused the separation specifically of *Fasciola* species. To perform RFLP, 10 µL of *Fasciola* ITS1 PCR product, 2 µL of supplied restriction enzyme buffer, 2 µL of restriction enzyme diluted, and DD.H<sub>2</sub>O up to 30 µL were provided. As per manufacturer instruction, the tubes were incubated at 37 °C for 12 h, to ensure full cutting of fragments. For analysing the digestion products, 15 µL of each product in addition to 2 µL of loading buffer were run in 2% gel electrophoresis.

#### *DNA sequencing and phylogenetic analysis*

PCR products of ITS1 from eight isolates, six samples from *F. gigantica* from cattle and two samples from *F. hepatica* of each host (cattle, sheep) were purified from the agarose gel, using PCR purification kit (Bioneer, Korea), and sequenced from both directions (Applied Biosystems, DNA Analyzers Sequencing, Bioneer, Korea, Sanger method), using the same primers which were used in the PCR. Multiple alignments were performed with data re-



**Fig. 2.** Cutting sites of RsaI restriction enzyme on A) *F. hepatica*, B) *F. gigantica*.

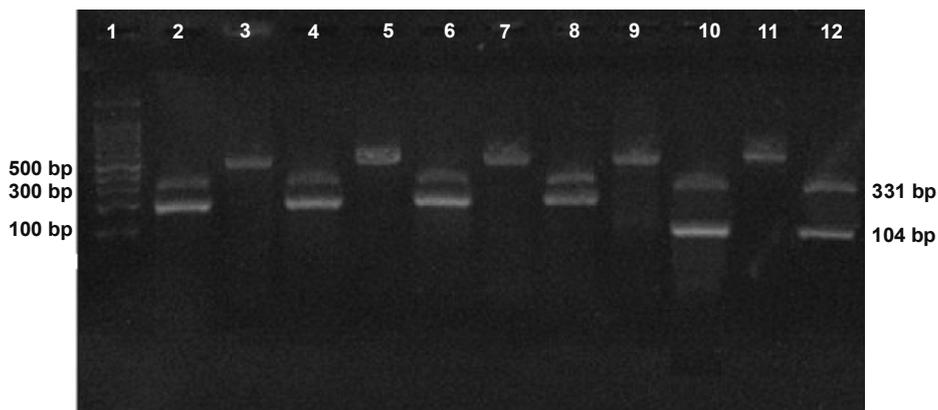
lated to *Fasciola* spp. from Iran and other countries deposited in Gen-Bank using BioEdit Sequence Alignment Editor Vr. 7.2.5 software. A Neighbor-Joining tree was constructed using the MEGA 5.0 software. Bootstrap analyses (using 1,000 replicates) were carried out to determine the robustness of the finding (Shafiei *et al.*, 2014).

## RESULTS

Genomic DNA from 93 isolates of *Fasciola* was extracted and eight of them confirmed by sequencing. All amplified products of *Fasciola* were digested with the RsaI restriction endonuclease and two species were distinguished. The RFLP pattern results showed that out of 50 *Fasciola* isolates of cattle, 3 isolates (6%) were *F. hepatica* and 47 (94%) had a pattern corresponding to *F. gigantica*. All 43 isolates (100%) from sheep had a RFLP pattern corresponding to *F. hepatica*.

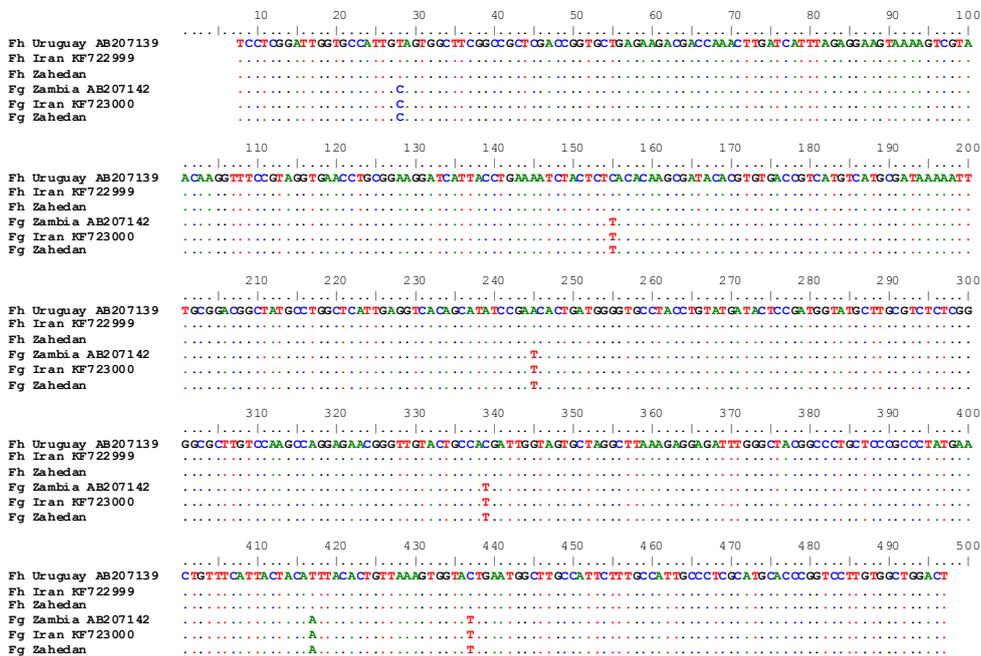
Based on NEBcutter pattern, RsaI which showed effect on GTAC nucleotides in the amplicons of *F. gigantica* had one cutting site and the digested DNA fragments were 180 and 331 bp whereas in the amplicons of *F. hepatica* with two cutting sites, the digested DNA fragments were 77, 104 and 331 bp (Fig. 2). However, the resultant bands of the fragments were about 180 bp in *F. gigantica* and 104 bp in *F. hepatica* (Fig. 3). The other band was not detected on agarose gels.

Complete sequences of 700 bp ITS1 of the flukes were aligned with those of available sequences in GenBank. Ninety-



**Fig. 3.** PCR-RFLP pattern of *Fasciola* after digestion with RsaI restriction enzyme.

Lane 1: 100 bp DNA ladder; lanes 2, 4, 6, 8: *F. gigantica* from cattle; lane 10: *F. hepatica* from sheep; lane 12: *F. hepatica* from cattle; lanes 3, 5, 7, 9: DNA *Fasciola* without restriction enzyme.



**Fig. 4.** Alignment of ITS1 sequence of *F. hepatica* (AB207139, KF722999) and *F. gigantica* (AB207142, KF723000), with *F. gigantica* and *F. hepatica* of Sistan and Baluchestan province, Iran

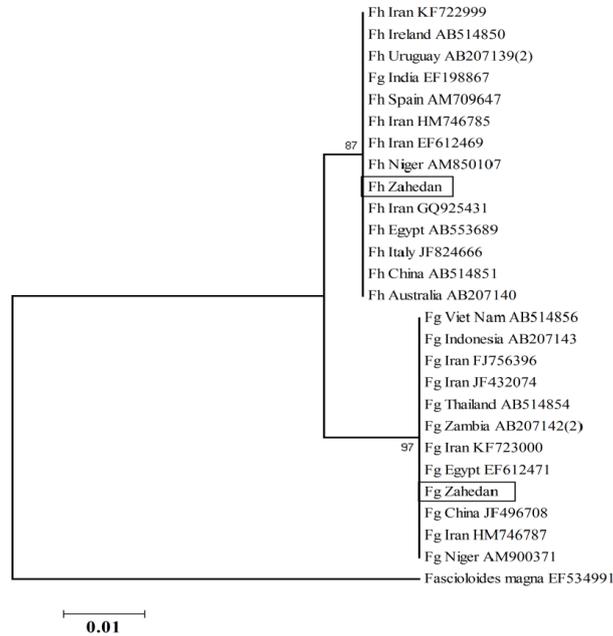
hundred percent similarities were obtained when comparing these sequences with all available data of *Fasciola* spp. in GenBank. The sequences of *F. hepatica* from cattle and sheep and the sequences between *F. gigantica* had complete similarity. Alignment of the sequences of ITS1 showed six DNA variable sites in which nucleotides at the position of 28, 155, 255, 339, 417 and 437 were single-base substituted resulting in segregation of the specimens into two different groups (genotypes). The main differences between *F. gigantica* and *F. hepatica* were the single-base substitution of T>C at nucleotide site of 28, C>T at the sites of 155, 339, and 437, A>T at the site of 255 and T>A at the site of 417 (Fig. 4).

The phylogenetic tree of ITS 1 showed that flukes were scattered as pure *F. hepatica* and *F. gigantica* clusters, suggest-

ing that two genotypes of *Fasciola* are able to infect animals and probably humans in southeastern of Iran (Fig. 5).

## DISCUSSION

DNA analysis is an appropriate plan for understanding the different species of *Fasciola* and performance of control procedures in an endemic area (Itagaki *et al.*, 2005; Peng *et al.*, 2009; Shafiei *et al.*, 2014). RFLP assay is a major method for detection of two species of *Fasciola*. In this study we used a reliable, rapid and truthful method based on ITS1-RFLP assay on *Fasciola* species with RsaI restriction enzyme to show two different patterns for both *Fasciola* species in Sistan and Balichestan province. Our study did not detect any mixed patterns in this re-



**Fig. 5.** Phylogenetic relationship of ITS1 sequences of isolates of *F. hepatica* and *F. gigantica* from Iran using Neighbor-Joining method. *Fasciolooides magna* (AN: EF534991) was used as the out group.

gion. The result of our study was similar to that of Ichikawa (2010) which recommended the first *RsaI* restriction enzyme in ITS1 to specifically distinguish *F. hepatica* from *F. gigantica* (Ichikawa & Itagaki, 2010).

The RFLP pattern was utilised for accurate differentiation between *Fasciola* species in different studies in Iran (Karimi, 2008; Saki *et al.*, 2011; Shafiei *et al.*, 2013). Shafiei *et al.* (2013) used *RsaI* enzyme in RFLP-PCR method to specifically distinguish *F. hepatica* and *F. gigantica* in ITS1 region. They did not report intermediate form in their study. Aryaeipour *et al.* (2014) showed that *RsaI* restriction enzyme was an appropriate enzyme to differentiate both species. The *TasI* enzyme was used on ITS1 region in RFLP method and differentiation between both species was reported (Rokni *et al.*,

2010). In another study, Karimi (2008) utilised *BfrI* and *DraI* enzymes in 18S DNA region in RFLP method and showed that *BfrI* could differentiate two aforementioned parasites while *DraI* could not differentiate any profile (Karimi, 2008). It was also shown that *AvaII* and *DraII* restriction enzymes in 28S DNA are suitable for differentiation between both species (Saki *et al.*, 2011). In a study conducted by Ghavami *et al.* (2009) *BamHI* and *PagI* restriction enzymes on ITS2 gene showed specificity for *F. hepatica* identification and had no effect on *F. gigantica* (Ghavami *et al.*, 2009).

The results of our study were consistent with most of previous studies and demonstrated polymorphism of two species of *Fasciola* in ITS1 region with six variable nucleotides sites (Itagaki *et al.*, 2005; Peng *et al.*, 2009; Aryaeipour *et al.*,

2014; Shafiei *et al.*, 2014). The phylogenetic tree with high bootstrap showed a close relationship of our isolates with those sequences registered in Genbank from other regions of the world. According to the phylogenetic tree, isolates belonging to *F. hepatica* and *F. gigantica* of our study as mentioned in other regions of Iran were of the common H1 and G1 haplotypes.

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#### Correspondence:

Dr. Mohammad Jafari Modrek  
Infectious Diseases and Tropical  
Medicine Research Center,  
Zahedan University of Medical Sciences,  
Zahedan, Iran,  
tel: +98-9155415930,  
e-mail: mjmodrek@gmail.com

Dr. Reza Shafiei  
Vector-borne Diseases Research Center,  
North Khorasan University of Medical Sciences,  
Bojnurd, Iran,  
tel: +98-9157029331,  
e-mail: reza\_shafi@yahoo.com