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Original Article

Molecular Identification and Differentiation of *Fasciola* Isolates Using PCR- RFLP Method Based on Internal Transcribed Spacer (ITS1, 5.8S rDNA, ITS2)

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ABSTRACT

Background: In this study, we used both ITS1 and ITS2 for molecular identification of *Fasciola* species.

Methods: The region between 18S and 28S of ribosomal DNA was used in PCR-RFLP method for molecular identification of *Fasciola* species. Ninety trematodes of *Fasciola* were collected during abattoir inspection from livers of naturally infected sheep and cattle from Khorasan, East Azerbaijan, and Fars provinces in Iran. After DNA extraction, PCR was performed to amplify region ITS1, 5.8S rDNA, ITS2. To select a suitable restriction enzyme, we sequenced and analyzed the PCR products of *F. hepatica* and *F. gigantica* samples from sheep and cattle. Tsp509I fast digest restriction enzyme was selected for RFLP method that caused the separation specifically of *Fasciola* species.

Results: The fragment approximately 1000bp in all of the *Fasciola* samples was amplified and then digested with the Tsp509I restriction endonuclease. Seventy *F. hepatica* and 20 *F. gigantica* were identified of total 90 *Fasciola* isolates.

Conclusion: The new PCR-RFLP assay using Tsp509I restriction enzyme provides a simple, practical, fast, low cost, and reliable method for identification and differentiation of *Fasciola* isolates.

Keywords: *Fasciola hepatica*, *Fasciola gigantica*, ITS1, 5.8S rDNA, ITS2, PCR-RFLP, Iran

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Introduction

Fascioliasis, caused by *Fasciola* spp., is one of the most common parasitic diseases between humans and animals that in terms of health problems and great economic losses in various regions of the world, is of great grandness (1, 2). The role of *Fasciola* spp. in weight loss and therefore decrease the production of meat and other livestock as well as reduce fertility has been appraised already (3). *Fasciola* infection to cattle in some parts of Iran is very serious (4). In two last decades, some studies concerning of prevalence of animal fasciolosis have been carried out in different parts of Iran (5-7).

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 (3). Two great epidemics of human fascioliasis with about 10,000 people infected in each case occurred in the north of Iran (4, 8, 9).

As regards the health and economic importance of fascioliasis in Iran, various studies especially in order to identify and genotyping of *Fasciola* seems necessary. Morphological characteristics of adult worms and eggs are affected under the different factors such as host type, age of parasite, fixation of the samples and severity of infection (1). Due to the many variations in morphological characteristics, overlapping distribution, abnormal diploidy, triploidy and mixploidy, hybridization between various genotypes and likely intermediate forms, it is usually difficult to accurate differentiation between *Fasciola* species (10, 11). Also in human infections, clinical, parasitological, and serological findings do not distinguish these species (12). 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 has been used in some studies (13-17) based on 28s rRNA, 18s rRNA, ITS1 or ITS2. In this study, region between 18S and 28S (ITS1, 5.8s, ITS2) of ribosomal DNA was used by PCR-RFLP method. Since, ITS1 and ITS2 sequence was suitable genetic markers for genotyping, interaspecific variations, and phylogenetic studies of parasites (14, 18); we used both ITS1 and ITS2 for molecular identification of *Fasciola* species.

Materials and Methods

Parasite

Ninety trematodes of *Fasciola* were collected during abattoir inspection from livers of naturally infected sheep and cattle from Khorasan, East Azerbaijan, and Fars provinces in Iran (Table 1). All samples were washed in physiological saline, identified to the species level based on morphometric criteria according to standard taxonomic keys (19, 20) and subsequently fixed in 80% ethanol and stored at -80 °C until further use.

DNA extraction

Bioneer AccuPrep® kit was used for genomic DNA extraction of *Fasciola* Parasites. After removing the samples from -80 °C and squashing, DNA extraction was performed according to manufacturer instruction. To achieve the desired results, we used at least 4 h incubation time for *Fasciola* samples in 60 °C with lysis buffer and 40 µl of Proteinase K as a modified DNA extraction method.

PCR

To amplify region (ITS1, 5.8S rDNA, ITS2), PCR was performed using BD1 (forward; 5′-GTCGTAACAAGGTTTCCGTA-3′) and BD2 (reverse; 5′-TATGCTTAAATT-

CAGCGGGT-3′) primers (21). PCR reaction were performed in a total volume of 20 µl contained 1 µl DNA template, 10 mM Tris-HCl(PH=9), 250 µM dNTP, 30 mM KCl, 1.5 mM MgCl2, 1 U Taq DNA Polymerase and 10 Pmol of each primer in a thermocycler (BioRad®) under the following conditions: 95 °C for 5 min as initial denaturation, followed by 30 cycles of 95 °C for 30 s (denaturation), 61.6 °C for 30 s (annealing), 72 °C for 30 s (extension) and final extension of 72 °C for 7 min. For detection of PCR results, 5 µl of the PCR product was examined on 1.5% agarose gel in TAE buffer at 80 V for 45 min. The gels stained with ethidium bromide, visualized, and photographed using a transilluminator (UVITEC). 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 4 *Fasciola* samples from sheep and cattle. The sequences were analyzed and aligned using Blast (http://blast.ncbi.nlm.nih.gov/Blast) and ClustalW (http://www.ebi.ac.uk/Tools/clustalw) software in comparison with sequences of *Fasciola* ITS1, 5.8S, ITS2 previously published from other countries in GenBank. Nucleotides of 1000bp 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)

Tsp509I fast digest restriction enzyme (Fermentas®) was selected for RFLP method that caused the separation specifically of *Fasciola* species. To performance RFLP, 3 µl of *Fasciola* ITS1, 5.8S, ITS2 PCR product, 1 µl of supplied restriction enzyme buffer, 0.5 µl of restriction enzyme, and 10.5 µl DDW in total volume 15 µl were incubated at 65 °C for 1 h. Digestion products were analyzed and photographed on 2.5% agarose gel.

Results

In this investigation, 90 *Fasciola* isolates were studied from two hosts and three geographical regions of Iran. Seventy *F. hepatica* and 20 *F.gigantica* were identified (Table 1). Genomic DNA was extracted from 90 isolates, which could amplify a fragment approximately 1000bp in all of the samples. Negative control did not produce any band on the gels.

All amplified products of *Fasciola* were digested with the Tsp509I restriction endonuclease. After digestion, the PCR-RFLP profile was obtained from *F. hepatica* and *F. gigantica*. RFLP pattern from *F. hepatica*, which had 3 cutting sites, produced 102, 171, 343 and 427 bp fragments while 5 fragments were produced by 4 cutting sites from *F. gigantica* including 102, 171, 208, 219 and 343 bp without 427 bp fragment (Fig. 1).

ITS1, 5.8S rDNA, ITS2 sequence of *F. hepatica* and *F. gigantica* were analyzed and deposited in GenBank (Accession numbers: **HM746785**-**HM746788**). Ninety-nine percent similarities were obtained in comparison of these sequences with all available data of *Fasciola* spp. in GenBank (Fig. 2). Restriction sites of the nucleotides in the *Fasciola* species were studied by computer software for selection a suitable enzyme. Accordingly, Tsp509I was selected as one of the best restriction enzyme for differentiation between *Fasciola* isolates.

Table 1: Number of *Fasciola* isolates and their geographical origin

Location	Host	Species	Number of isolates
Khorasan	Sheep	*F. hepatica*	10
Khorasan	Sheep	*F. gigantica*	5
Khorasan	Cattle	*F. hepatica*	15
Fars	Sheep	*F. hepatica*	15
Fars	Cattle	*F. gigantica*	15
East Azerbaijan	Sheep	*F. hepatica*	15
East Azerbaijan	Cattle	*F. hepatica*	15

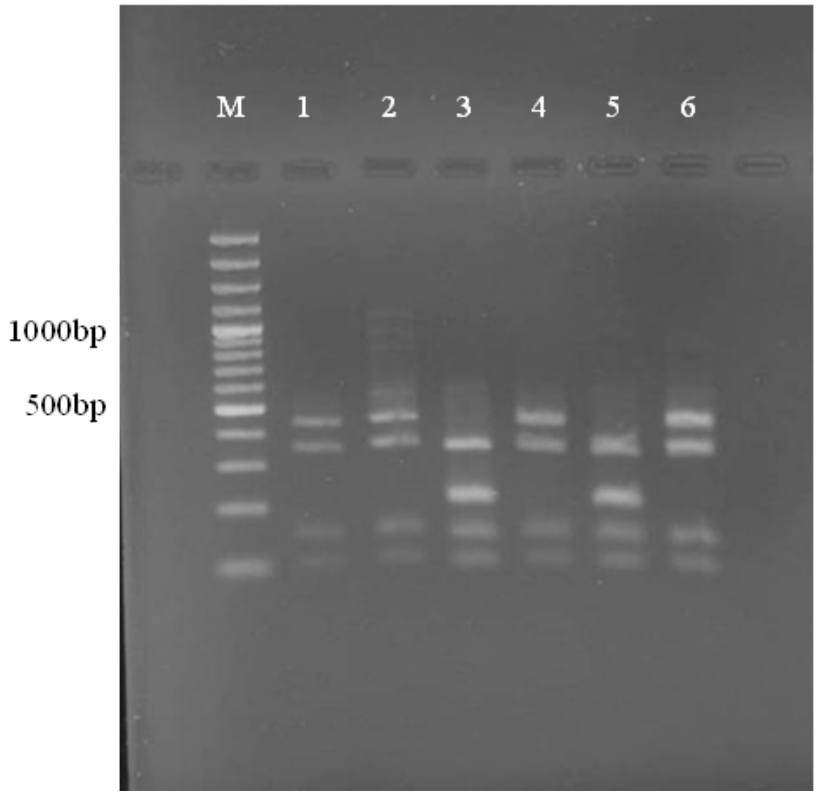

Fig. 1: PCR-RFLP pattern of *Fasciola* after digestion with Tsp509I restriction enzyme. Lane M: 100bp DNA ladder, Lanes 1 and 2: *F. hepatica* from sheep, Lane 3: *F. gigantica* from sheep, Lanes 4 and 6: *F. hepatica* from cattle, Lane 5: *F. gigantica* from cattle

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HM746785        TACTCTCACACAAGCGATACACGTGTGACCGTCATGTCATGCGATAAAAATTTGCGGACG 60
HM746786        ------------------------------------------------------------ 60
HM746787        ------T----------------------------------------------------- 60
HM746788        ------T----------------------------------------------------- 60
                ****** ************************************************** 

HM746785        GCTATGCCTGGCTCATTGAGGTCACAGCATATCCGAACACTGATGGGGTGCCTACCTGTA 120
HM746786        ------------------------------------------------------------ 120
HM746787        -----------------------------------T------------------------ 120
HM746788        -----------------------------------T------------------------ 120
                ********************************** ************************ 

HM746785        TGATACTCCGATGGTATGCTTGCGTCTCTCGGGGCGCTTGTCCAAGCCAGGAGAACGGGT 180
HM746786        ------------------------------------------------------------ 180
HM746787        ------------------------------------------------------------ 180
HM746788        ------------------------------------------------------------ 180
                ************************************************************ 

HM746785        TGTACTGCCACGATTGGTAGTGCTAGGCTTAAAGAGGAGATTTGGGCTACGGCCCTGCTC 240
HM746786        ------------------------------------------------------------ 240
HM746787        ----------T------------------------------------------------- 240
HM746788        ----------T------------------------------------------------- 240
                ********** ********************************************** 

HM746785        CCGCCCTATGAACTGTTTCATTACTACATTTACACTGTTAAAGTGGTACTGAATGGCTTG 300
HM746786        ------------------------------------------------------------ 300
HM746787        ----------------------------A-------------------T----------- 300
HM746788        ----------------------------A-------------------T----------- 300
                **************************** ****************** *********** 

HM746785        CCATTCTTTGCCATTGCCCTCGCATGCACCCGGTCCTTGTGGCTGGACTGCACGTACGTC 360
HM746786        ------------------------------------------------------------ 360
HM746787        ------------------------------------------------------------ 360
HM746788        ------------------------------------------------------------ 360
                ************************************************************ 

HM746785        GCCCGGCGGTGCCTATCCCGGGTTGGACTGATAACCTGGTCTTTGACCATACGTACAACT 420
HM746786        ------------------------------------------------------------ 420
HM746787        ------------------------------------------------------------ 420
HM746788        ------------------------------------------------------------ 420
                ************************************************************ 

HM746785        CTGAACGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGCAGCCAACTGTGTGAATTA 480
HM746786        ------------------------------------------------------------ 480
HM746787        ------------------------------------------------------------ 480
HM746788        ------------------------------------------------------------ 480
                ************************************************************ 

HM746785        ATGCAAACTGCATACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCCATGGGTT 540
HM746786        ------------------------------------------------------------ 540
HM746787        ------------------------------------------------------------ 540
HM746788        ------------------------------------------------------------ 540
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HM746785        AGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCACGACGCCCAAAAAGTC 600
HM746786        ------------------------------------------------------------ 600
HM746787        ------------------------------------------------------------ 600
HM746788        ------------------------------------------------------------ 600
                ************************************************************

HM746785        GTGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCTCTATGAGTAATCATGTGAGGTGCCAG 660
HM746786        ------------------------------------------------------------ 660
HM746787        ------------------------------------------------------------ 660
HM746788        ------------------------------------------------------------ 660
                ************************************************************

HM746785        ATCTATGGCGTTTCCCTAATGTATCCGGATGCACCCTTGTCTTGGCAGAAAGCCGTGGTG 720
HM746786        ------------------------------------------------------------ 720
HM746787        ------------------------------------------------------------ 720
HM746788        ------------------------------------------------------------ 720
                ************************************************************

HM746785        AGGTGCAGTGGCGGAATCGTGGTTTAATAATCGGGTTGGTACTCAGTTGTCAGTGTGTTT 780
HM746786        ------------------------------------------------------------ 780
HM746787        ------------------------------------------------------------C 780
HM746788        ------------------------------------------------------------C 780
                ************************************************************

HM746785        GGCGATCCCCTAGTCGGCACACTTATGATTTCTGGGATAATTCCATACCAGGCACGTTCC 840
HM746786        ------------------------------------------------------------ 840
HM746787        ----------------------C------------------------------------- 840
HM746788        ----------------------C------------------------------------- 840
                ********************** ************************************

HM746785        GTCACTGTCACTTTGTCATTGGTTTGATGC.TGAACTTGGTCATGTGTCTGATGCTATTT 899
HM746786        ------------------------------.---------------------------- 899
HM746787        --T-----T--------------------C---------------------------- 900
HM746788        --T-----T--------------------.---------------------------- 899
                ** ***** ********************* ****************************

HM746785        TCTATATAGCGACGG.TACCCTT.CGT 924
HM746786        ---------------.-------T--- 925
HM746787        .-------A------C-------.--- 925
HM746788        .-------A------.-------.--- 923
                 ******* ****** ******* ***
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Fig. 2: Sequence alignment of the ITS1, 5.8S rDNA, ITS2 region from *Fasciola hepatica* and *Fasciola gigantica*. Accession numbers: HM746785, HM746786 (*F. hepatica*) and HM746787, HM746788 (*F. gigantica*) are sequences that have been deposited in GenBank from Iran

Discussion

Differentiation between species of *Fasciola* according to life cycle and species-specific intermediate host is necessary (1). DNA-based methods in comparison with other diagnostic methods for *Fasciola* parasites have more accuracy (13). In this study, a rapid and simple method was developed to differentiate *Fasciola* species by PCR-RFLP assay. This method was used in some studies for identification of *Fasciola* based on 28s rRNA, 18s rRNA, ITS1 and ITS2 (13-17). AvaII and DraII restriction enzymes were

used for RFLP method based on 618 bp sequence of the 28s rRNA gene, but no interaspecific variations were detected in this sequence because there were a few nucleotide differences between *Fasciola* species (13). 361-362 bp of the ITS2 sequence of *Fasciola* samples from France and China were compared by PCR-RFLP assay with Hsp92II restriction enzyme (14). In Iran, RFLP patterns of *Fasciola hepatica* and *Fasciola gigantica* from Fars province based on 263 and 356 bp fragments of 18s rDNA using DraI and BfrI restriction enzymes showed that BfrI restriction enzyme was obtained similar bands profile of *F.hepatica* and *F.gigantica* whereas, restriction enzyme DraI can be created to differentiate between two species of *Fasciola* (15). No evidence of restriction digestion in RFLP patterns of the ITS2 sequence of *Fasciola hepatica* samples was seen from Zanjan obtained with BamH1 and PagI restriction enzymes (16). In another study, *Fasciola* samples from Tehran, West Azerbaijan and Khuzestan provinces were identified by PCR-restriction enzyme method based on 463 bp region of the ITS1 sequence with restriction enzyme TasI (17). Various studies have indicated that, Internal Transcribed Spacer (ITS1 and ITS2) sequence was suitable genetic markers for genotyping, interaspecific variations, and phylogenetic studies of parasites (14, 18). In the previous studies, only ITS1 or ITS2 were used for differentiation of *Fasciola* based on RFLP assay (14, 16, 17). In our study, we used 1000bp region contained ITS1, 5.8S and ITS2 for identification and differentiation of *Fasciola* isolates by PCR-RFLP method. Indeed, we designed the PCR-RFLP assay for differentiation of *Fasciola* using both ITS1 and ITS2 regions. On the other hand, ITS1 and ITS2 of *Fasciola* samples amplified at one PCR reaction, which used for sequencing to provide other studies such as phylogeny and genotyping. Moreover, both *F. hepatica* and *F. gigantica* samples were digested with Tsp509I restriction enzyme, which showed different RFLP patterns.

According to our result, all ninety *Fasciola* samples from three different geographical regions (Khorasan, Fars and East Azerbaijan provinces) and two different hosts (sheep and cattle) were identified as either *F. hepatica* or *F. gigantica* by PCR-RFLP. In conclusion, the new PCR-RFLP assay using Tsp509I restriction enzyme provides a simple, practical, fast, low cost, and reliable method for identification and differentiation of *Fasciola* isolates.

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