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. In terms of health problems and great economic losses in various regions of the world, it 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 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, interspecific 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
**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.

| Accession | Sequence Alignment |
|-----------|--------------------|
| HM746785  | AGCTCTGCGCGCTAG | 600 |
| HM746786  | GTGCTGGCTTTGGCAAACTGACCTCTGACGTGATCGTTACTCTCTTCCTGCTCGTTAATACCTTACATCTGCTGAGCTGCGACA  |
| HM746787  | ATCTATGGGTTCCCTAATAGTACCTGCGCCTGAGCTGCGACA  |
| HM746788  | ATCTATGGGTTCCCTAATAGTACCTGCGCCTGAGCTGCGACA  |

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 interspecific 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 BamHI 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, interspecific 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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