Experimental Parasitology 124 (2010) 209–213

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* using a simple PCR-restriction enzyme method

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ARTICLE INFO

Article history:
Received 1 June 2009
Received in revised form 13 September 2009
Accepted 14 September 2009
Available online 19 September 2009

Keywords:
Fasciola hepatica
Fasciola gigantica
PCR-restriction enzyme
Identification
TasI

ABSTRACT

Accurate morphological differentiation between the liver fluke species *Fasciola hepatica* and *Fasciola gigantica* is difficult. We evaluated PCR-restriction enzyme profiles of internal transcribed spacer 1 (ITS1) that could aid in their identification. Fifty *F. hepatica* and 30 *F. gigantica* specimens were collected from different hosts in three provinces of Iran. For DNA extraction, we crushed fragments of the worms between two glass slides as a new method to break down the cells. DNA from the crushed materials was then extracted with a conventional phenol–chloroform method and with the newly developed technique, commercial FTA cards. A primer pair was selected to amplify a 463-bp region of the ITS1 sequence. After sequencing 14 samples and *in silico* analysis, cutting sites of all known enzymes were predicted and TasI was selected as the enzyme that yielded the most informative profile. Crushing produced enough DNA for PCR amplification with both the phenol–chloroform and commercial FTA card method. The DNA extracted from all samples was successfully amplified and yielded a single sharp band of the expected size. Digestion of PCR products with TasI allowed us to distinguish the two species. In all samples, molecular identification was consistent with morphological identification. Our PCR-restriction enzyme profile is a simple, rapid and reliable method for differentiating *F. hepatica* and *F. gigantica*, and can be used for diagnostic and epidemiological purposes.

1. Introduction

The common liver flukes *Fasciola hepatica* and *Fasciola gigantica* are the etiological agents of fasciolosis, a cosmopolitan disease that affects both domestic livestock and humans. Species of the genus *Fasciola* also cause important economic losses due to liver spoilage, high morbidity rates and reduced production of milk, meat and wool (Mas-Coma et al., 2005). Iran has experienced two large outbreaks of fasciolosis in Northern provinces, with nearly 10,000 cases in each outbreak (Assmar et al., 1991; Rokni, 2008). Both *Fasciola* species in addition to probable intermediate forms are believed to exist in northern Iran (Moghaddam et al., 2004; Ashrafi et al., 2006).

Classically, the distinction between these two species has been based on morphological criteria. Adults of *F. gigantica* are longer but narrower, with a smaller shoulder, more anterior testes, larger ventral sucker and shorter cephalic cone compared to *F. hepatica*. In addition, the branching pattern of the caeca, ovary and testes is different in both species. The eggs are similar in shape, but are slightly larger in *F. gigantica* (160–190 × 70–90 μm) than in *F. hepatica* (130–150 × 60–85 μm) (Sahba et al., 1972; Muller, 2002). Nevertheless, accurate recognition of the two flukes is usually made difficult because of substantial variations in these morphological features. In addition, abnormal diploidy, triploidy and mixploid parthenogenesis as well as hybridization between different genotypes of *Fasciola* give rise to different forms of this genus (Mas-Coma et al., 2001). Because the intermediate host, epidemiological characteristics, control strategies and pattern of pathologic effects differ between the two species, it is essential to differentiate between *F. hepatica* and *F. gigantica* infection in humans.

A review of the literature shows that molecular DNA-based approaches have been used to distinguish between these species (Hashimoto et al., 1997; Marcilla et al., 2002; Huang et al., 2004). However, variations in the forms of these parasites that inhabit different countries mean that local and global needs for an accurate diagnostic method are not satisfied by available techniques. We therefore felt it would be helpful to develop a simple and reliable molecular method to identify *Fasciola* species.

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doi:10.1016/j.exppara.2009.09.015

In the present study, we aimed to characterize Iranian *Fasciola* flukes on the basis of the ITS1 sequence of the ribosomal DNA gene, and to establish a suitable PCR-restriction enzyme approach based on species-specific variations in ITS1 that would accurately differentiate between *F. hepatica* and *F. gigantica* in Iran and in other places where these parasites are a threat to human and animal health.

2. Materials and methods

2.1. Parasite

Fifty *F. hepatica* and 30 *F. gigantica* specimens were collected during abattoir inspection from livers of sheep, goats, cattle and buffaloes from three provinces in Iran: Tehran, Khuzestan and West Azerbaijan (Table 1). The two species were identified based on morphometric criteria, and all doubtful samples were discarded (Sahba et al., 1972; Muller, 2002). The samples for analysis were washed in PBS for three times, fixed in 70% ethanol and stored at room temperature until further use.

2.2. DNA extraction

We used a new method to isolate DNA from the worms. Briefly, a random portion of each organism (2–5 mm^3) was removed and crushed between two microscopic slides for 1 min with 300 μl sterile distilled water (DW). Ten microliters of the lysate was placed on Whatman paper (FTA Elute Cards, Tokyo, Japan) and dried for a minimum of 3 h at room temperature, and the preparations were stored in the laboratory as a DNA archive until further analysis. For DNA extraction, one part of punched DNA cards (3 mm in diameter) was transferred to a tube containing 500 μl DW and vortexed 3 times for 1 s. The paper punches were transferred to a new tube containing 30 μl DW, incubated at 95 °C for 20 min, vortexed for a few seconds and centrifuged for 30 s to sediment the paper component. The supernatant was separated as DNA and stored at −20 °C for further processing. In addition, the lysate from each sample was subjected to DNA extraction using a conventional phenol–chloroform method with slight modifications (Sambrook et al., 1989). In the final step the DNA was eluted in 20 μl deionized distilled water (DDW) and frozen at −20 °C until the next step.

2.3. Primer selection

After computerized study and comparison of nucleotide sequences in ribosomal DNA regions including 18S rDNA, ITS1, 5.8S rDNA, ITS2 and 28S rDNA of different *Fasciola* species, forward (FascF: 5′-ACC GGT GCT GAG AAG ACG-3′) and reverse (FascR: 5′-CGA CGT ACG TGC AGT CCA-3′) primers were designed to amplify a 463-bp DNA fragment in the ITS1 region of both *F. hepatica* and *F. gigantica*. Primers were synthesized by CinnaGen Company (Tehran, Iran).

Table 1
Liver fluke and host species, and number of isolates and geographical origin.

Species	Host	No. of isolates	Province in Iran
F. hepatica	Sheep	30	Tehran
	Goat	1	Tehran
	Cattle	12	West Azerbaijan
	Buffalo	7	West Azerbaijan
F. gigantica	Sheep	1	Khuzestan
	Cattle	8	Khuzestan
	Buffalo	21	Khuzestan

2.4. PCR

Each PCR reaction contained 10.5 μl DDW, 12.5 μl of 2× premix (Ampliqon, Skovlunde, Denmark), 0.2 μM of each primer and 1 μl extracted DNA. The reaction mixture was amplified in a thermocycler (Corbett Research, Sydney, Australia) under the following conditions: 95 °C for 5 min as initial denaturation followed by 30 cycle at 95 °C for 45 s as denaturation, 60 °C for 45 s as annealing, 72 °C for 1 min as extension, and final extension at 72 °C for 7 min. Samples with 1 μl DDW instead of DNA were used as negative controls. Two microliters of the PCR product was analyzed by electrophoresis on 1.5% agarose gel in TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA) at 100 V for 1 h. Gels were stained with ethidium bromide (0.5 μg/ml). A 100-bp DNA ladder (Fermentas, Vilnius, Lithuania) was run to estimate the size of DNA in each gel. Gels were visualized and photographed on a transilluminator (UVItec, Cambridge, UK).

2.5. Sequencing and sequence analysis to select a suitable restriction enzyme

We sequenced 10 *F. hepatica* samples from sheep, goats, cattle and buffaloes and 4 *F. gigantica* samples from cattle. Sequencing was done with 25 μl PCR product using the forward primer. The results were analyzed with Blast, DNASIS (Hitachi, Tokyo, Japan) and ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) softwares. The resulting sequences were aligned with relevant sequences related to *Fasciola* spp. from other countries deposited in GenBank (Table 2). A consensus representative sequence containing 463 nucleotides from *F. hepatica* and *F. gigantica* was subjected to computerized cutting with almost all known restriction enzymes using DNASIS software.

2.6. Endonuclease digestion of PCR products to differentiate the species

Five microliters of *Fasciola* ITS1-PCR product, 8 μl DDW, 0.5 μl TasI (Fermentas) and 1.5 μl of 10× supplied buffer were incubated at 65 °C for 2.5 h. Restriction fragments were separated on 2% agarose gel in TBE buffer, stained with ethidium bromide and photographed.

3. Results

The primers we used in this study successfully amplified a region of approximately 460 bp in all *F. hepatica* and *F. gigantica* samples (Fig. 1). This size was always the same in both species and entirely consistent with the length predicted from sequence analysis to select primers (463 bp). There were no detectable variations among amplicons (Fig. 1). Negative controls produced no bands in any of the experiments. No overt differences were found between

Table 2
List of *Fasciola* species sequences used in this study.

Species	Accession numbers	
	Other studies	Present study
F. hepatica	AM900370, AM709649, AM709648, AM709647, AM709646, AM709645 AM709644, AM709643, AM709622, AM709621, AM709620, AM709614 AM709613, AM709612, M709610, AM709500, AM709499, AM709498 AM707030, AJ628432, AJ628431, AM900370	FJ756394, FJ756393, FJ756392, FJ756391, FJ756390, FJ756389, FJ756388, FJ756387, FJ756386, FJ756385
F. gigantica	AJ628425, AJ628043	FJ756398, FJ756397, FJ756396, FJ756395

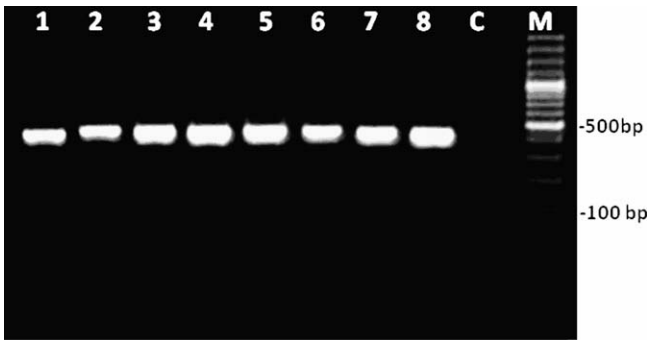

Fig. 1. Examples of agarose gel electrophoresis of ITS1–PCR products. Lanes 1–4 *F. hepatica* from cattle, Lane 5 *F. hepatica* from goat, Lanes 6–8 *F. hepatica* from buffalo, Lane C negative control, Lane M 100-bp DNA ladder.

the results obtained for DNA extraction with our method of mechanical crushing followed by elution with FTA Elute Cards on one hand and crushing followed by conventional phenol–chloroform on the other hand. One hundred percent of samples were successfully amplified using PCR in both methods.

Fourteen PCR products from different animals were sequenced. All isolates of each *F. hepatica* and *F. gigantica* species regardless of their host, yielded identical sequences. In other words, no intraspecies variation was found for the DNA target. We observed differences in five nucleotides between *F. hepatica* and *F. gigantica* (Fig. 2).

Inspection with computer software of the cutting sites of the nucleotides in the two species disclosed that some restriction enzymes were potentially useful for digestion with a view to distinguishing between the two species (Table 3). Based on a few nucleotide differences between fasciolid species, TasI was selected as one of the best enzymes for identification and differentiation of the two species and was used to digest all PCR products. Fig. 3 shows an example of the agarose gel electrophoresis of the PCR products after digestion with TasI. In light of the data in Table 3, this enzyme has one cutting site for *F. hepatica* and two cutting sites for *F. gigantica*, producing 2 fragments of 151 and 312 bp in the former and 3 fragments of 93, 151, and 219 bp in the latter species (Fig. 3). These sizes were consistent with those obtained by *in silico* sequencing analysis (Table 3).

Of 80 isolates examined, 50 samples were identified as *F. hepatica* and the remaining 30 as *F. gigantica*. No discrepancies were found between the results of restriction enzyme-PCR and morphological criteria.

4. Discussion

Some researchers believe that neither parasitological plus clinical tests nor immunological assays can differentiate between *F. hepatica* and *F. gigantica* (Marcilla et al., 2002). DNA-based methods to unequivocally identify flukes can confirm the results of morphometric studies and facilitate the task of differentiating between species because genotypic characters are not influenced by ecological and geographical factors. This makes DNA-based methods useful to identify unequivocally flukes, especially in regions where both species and possibly intermediate forms coexist.

To date, various molecular methods and DNA markers have been used to identify fasciolids. Comparisons of nucleotide sequences of mitochondrial NADH dehydrogenase 1 (nad1) and cytochrome *c* oxidase subunit 1 (cox1) genes suggested that Japanese triploid forms of *Fasciola* were nearly identical to *F. gigantica* but different from *F. hepatica* (Itagaki and Tsutsumi, 1998). PCR-restriction fragment length polymorphism (RFLP) assays have been used in some studies of *Fasciola* (Hashimoto et al., 1997; Marcilla et al., 2002; Itagaki et al., 2005b; Lin et al., 2007). Fascolid species from liver fluke populations in South America, Europe and Africa were distinguished based on RFLP patterns obtained with a 618-bp sequence of the 28S rRNA gene, using restriction enzymes AvaII and DraII. These sequences revealed a few nucleotide differences between two species but no intraspecific variations within species (Marcilla et al., 2002).

ITS1 and ITS2 sequences from rDNA provide reliable genetic markers for systematic molecular studies of parasites and interspecific variations (Huang et al., 2004), and these markers have been used to identify fasciolid species. ITS2 sequences of *F. hepatica* from Spanish and Bolivian flukes showed no nucleotide variations and were identical, but comparisons with ITS2 sequences of *F. hepatica* from other geographical regions showed a few nucleotide

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F.hepatica      AGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTGAAAATCTACTC
F.gigantica     AGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTGAAAATCTACTC
                ************************************************************

F.hepatica      TCACACAAGCGATACACGTGTGACCGTCATGTCATGCGATAAAAATTTGCGGACGGCTAT
F.gigantica     TTACACAAGCGATACACGTGTGACCGTCATGTCATGCGATAAAAATTTGCGGACGGCTAT
                * ************************************************************

F.hepatica      GCCTGGCTCATTGAGGTCACAGCATATCCGAACACTGATGGGGTGCCTACCTGTATGATA
F.gigantica     GCCTGGCTCATTGAGGTCACAGCATATCCGATCACTGATGGGGTGCCTACCTGTATGATA
                ******************************** ****************************

F.hepatica      CTCCGATGGTATGCTTGCGTCTCTCGGGGCGCTTGTCCAAGCCAGGAGAACGGGTTGTAC
F.gigantica     CTCCGATGGTATGCTTGCGTCTCTCGGGGCGCTTGTCCAAGCCAGGAGAACGGGTTGTAC
                ************************************************************

F.hepatica      TGCCACGATTGGTAGTGCTAGGCTTAAAGAGGAGATTTGGGCTACGGCCCTGCTCCCGCC
F.gigantica     TGCCATGATTGGTAGTGCTAGGCTTAAAGAGGAGATTTGGGCTACGGCCCTGCTCCCGCC
                ***** ********************************************************

F.hepatica      CTATGAACTGTTTCATTACTACATTTACACTGTTAAAGTGGTACTGAATGGCTTGCCATT
F.gigantica     CTATGAACTGTTTCATTACTACAATTACACTGTTAAAGTGGTATTGAATGGCTTGCCATT
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Fig. 2. Alignment of two representative ITS1 partial sequences of *F. hepatica* and *F. gigantica*. Five nucleotide differences (gaps in the rows of asterisks) between the two species allowed us to choose enzymes to differentiate the species.

Table 3
Restriction enzymes suitable for differentiation of *F. hepatica* and *F. gigantica.*

Enzyme name	Recognition sequence	Restriction site		Fragment size after digestion	
		F. hepatica	*F. gigantica*	*F. hepatica*	*F. gigantica*
AfaI	gt!ac	286, 390, 458	286, 458	286, 104, 68, 5	286, 172, 5
BfucI	!gatc	28, 85	28, 85, 197	28,57,378	28, 57, 112, 266
FatI	!catg	135, 140, 242	135, 140, 291, 425	135, 5, 102, 221	135, 5, 151, 134, 38
TasI	!aatt	151	151, 370	151, 312	151, 219, 93

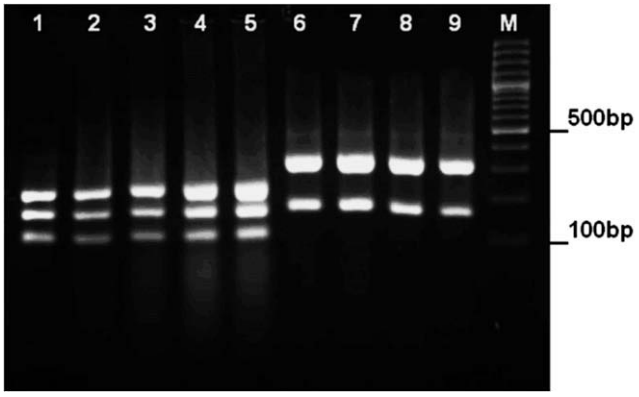

Fig. 3. The pattern of PCR products after digestion with TasI: Lanes 1–5 are *F. gigantica* from buffalo, Lanes 6–9 are *F. hepatica* from sheep, Lane M 100-bp DNA ladder.

differences in at least one position. No differences in nucleotides were found between Bolivian and Spanish flukes in the 433-bp ITS1 sequence (Mas-Coma et al., 2001). RFLP patterns of 361–362 bp segments of the ITS2 sequence obtained with restriction enzyme Hsp92II were compared in *Fasciola* species from France and China (Sichuan, Guangxi and Heilongjiang provinces). This work identified *Fasciola* samples from France and Sichuan as *F. hepatica* and those from Guangxi as *F. gigantica,* whereas other samples from Heilongjiang province represented an intermediate genotype whose RFLP pattern showed a combination of features of *F. hepatica* and *F. gigantica.* The ITS2 sequences were unique in that two different ITS2 sequences were present in the rDNA array of *Fasciola* samples from mainland China, in addition to the sequences found in *F. hepatica* and *F. gigantica* (Huang et al., 2004). ITS1 and ITS2 sequencing of cattle isolates from India showed that all isolates but one was *F. gigantica* (Prasad et al., 2008).

McGarry and colleagues described PCR assays to differentiate *Fasciola* species using two primer sets based on RAPD-derived sequences (McGarry et al., 2007). In Iran, Ashrafi and colleagues used ITS2 sequencing and reported that the larval stage of *Fasciola* in infected snails in Gilan province belonged to *F. hepatica* (Ashrafi et al., 2007). Recently, Karimi used 18S rDNA–RFLP and sequencing, and reported the first molecular evidence of an intermediate genotype of *Fasciola* in Fars province, southern Iran (Karimi, 2008). However, in our study, all 80 ITS1–RFLP patterns were identified as either *F. hepatica* or *F. gigantica,* and no mixed patterns were seen. In addition, all 14 sequenced samples were identified unambiguously.

In the present study, we used ITS1 as a target for PCR-RFLP to develop an accurate method to distinguish these two parasites. The ITS1 restriction enzyme pattern obtained with TasI seems to be a reliable, fast and straightforward criterion for differentiating between *F. hepatica* and *F. gigantica.* The procedure is simple, yields unequivocal results, and can be done in half a day. This method is especially suitable for screening when a large number of *Fasciola* isolates need to be identified. We found no intraspecific nucleotide variations in the 463-bp fragment of ITS1rDNA gene sequenced, regardless of the host species and geographical origin. Sequencing 14 isolates of *F. hepatica* and *F. gigantica* showed 100% homology between our samples and those from other regions available in GenBank, but the occasional nucleotide differences we found between the two fasciolid species were useful in distinguishing between them.

We evaluated the results of our new crushing method after phenol–chloroform extraction and after application on FTA Elute Card extraction. Crushing was efficient in both methods in addition to its advantages in terms of economy in time and expense. More to the point, the DNA obtained from FTA Elute Card can be preserved for later use even after prolonged periods without the need for special archiving equipment.

In conclusion, the method described here is valuable for the identification of *Fasciola* species in parts of the world where liver flukes are endemic and in areas where species distributions overlap. The PCR-restriction enzyme method is a potentially useful tool to identify fasciolid flukes and aid epidemiological research in humans and domestic animals in the aria that fasciolosis is endemic.

Acknowledgments

We thank the Isfahan Research Station of Tehran University of Medical Science for their kind provision of materials, tools and laboratory work used in this study. We also thank K. Shashok (AuthorAID in the Eastern Mediterranean) for improving the use of English in the paper. The authors declare that they have no conflicts of interest.

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