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Molecular differentiation of Turkey cattle isolates of *Fasciola hepatica* and *Fasciola gigantica*

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Summary

The most common and widespread liver flukes of the genus *Fasciola* are *Fasciola hepatica* and *F. gigantica*. Adults of both species occur in many domestic ruminants and in humans and can cause serious disease. The differential diagnosis of these flukes infection is very important because of their different transmission and epidemiological characteristics. A simple and rapid PCR-restriction fragment length polymorphism (RFLP) assay, using the common restriction enzymes *AluI* and *RsaI*, is described to distinguish between both fasciolid species. After the digestion of the mitochondrial cytochrome c oxidase 1 (CO1) PCR product with the restriction enzyme *AluI*, the RFLP profile obtained from *F. hepatica* revealed two fragments, whereas *F. gigantica* was not cut. The *RsaI* digestion generated two fragments from *F. gigantica*, whereas it did not cut the PCR product from *F. hepatica*. Results were confirmed with CO1 sequence analysis of both *F. hepatica* and *F. gigantica*. The present study suggests that the PCR-RFLP method described here can be used for the proper identification of *Fasciola* species.

Keywords: *Fasciola hepatica*; *Fasciola gigantica*; PCR-RFLP; cattle; Turkey

Introduction

Fasciolosis is an economically important disease of domestic livestock, in particular cattle and sheep, and occasionally man. The disease is caused by digenean trematodes of the genus *Fasciola*, commonly referred to as liver flukes. The two species most commonly implicated as the aetiological agents of fasciolosis are *F. hepatica* and *F. gigantica* (family Fasciolidae). *F. hepatica* has a worldwide distribution but predominates in temperate zones while *F. gigantica* is found on most continents, primarily in tropical regions (Andrews, 1999). Adlard *et al.* (1993), using the second internal transcribed spacer, demonstrated that the identity between *F. hepatica* and *F. gigantica* was 97.2 % and that between *F. hepatica* and *Fascioloides magna* was 86.8 %. They were also able to show that the *Fasciola* species from Japan was almost identical to *F. gigantica*. This work was confirmed by Hashimoto *et al*. (1997) who used the second internal transcribed spacer and mitochondrial cytochrome *c* oxidase subunit 1 (CO1) sequences to show that the Japanese *Fasciola* species was a strain of *F. gigantica*. Molecular biology techniques have enabled the identification of, and in particular the discrimination between, parasite species which are so closely related as to be difficult to distinguish by morphological methods (McManus & Bowles, 1996). These trematode species are involved in both animal and human. The geographical overlapping gives rise to many problems in the diagnosis. Traditional methods of identification of *Fasciola* species have relied on morphological characteristics of adults and eggs. Despite to this, it is usually difficult to accurately discriminate between *F. hepatica* and *F. gigantica* because of the many variations in their morphological characteristics. The differentiation becomes very difficult when dealing with small specimens. At the extremes of this morphological range, some resemble *F. hepatica*, whereas others resemble *F. gigantica*, with intermediate forms also occurring and involving phenomena such as abnormal gametogenesis, diploidy, triploidy and mixoploidy, parthenogenesis, and hybridization events between different genotypes (Mas-Coma & Bargues, 1997; Marcilla *et al.,* 2002). The two species can also be discriminated by DNA sequences of nuclear ribosomal internal transcribed spacer 1 (ITS-1), ITS-2, and 28S rRNA genes (Adlard *et al.,* 1993; Marcilla *et al.,* 2002; Itagaki & Tsutsumi, 1998; Itagaki *et al.,* 2005) and of mitochondrial NDI and CO1 genes (Itagaki *et al.,* 2005). There exists no detailed published information on the genotyping and differantiation of *F. hepatica* and/or *F. gigantica* Turkish isolates. Thus, a rapid and simple test for the differentiation of the two

Fasciola species is needed. The usefulness of molecular genetic techniques based on nuclear and mitochondrial DNA was emphasized while addressing problems of identification, characterization, and phylogeny of parasites. In the present study, PCR-restriction fragment length polymorphism (PCR-RFLP) patterns were analyzed for mitochondrial CO1 gene after digestion with two restriction enzymes (*AluI* and *RsaI*). In addition, nucleotide sequences of the mitochondrial CO1 gene were determined for *F. hepatica* and *F. gigantica* cattle isolates from Turkey to evaluate the phylogenetic relationships among them.

Materials and methods

Parasite materials

Adults of *F. hepatica* and *F. gigantica* were obtained from livers of naturally infected cattle taken from local abattoirs in Erzurum and Elazig provinces of Turkey, respectively. In a previous study (Balkaya & Simsek, 2010), a total of 2088 cattle were inspected for bile ducts of liver after slaughter in a local abattoir in Erzurum province and 439 (21 %) were found to be infected with *F. hepatica*. 15 adult flukes belonged to different hosts were used for DNA isolation. The *F. gigantica* isolate was obtained during the routine inspection of slaughtered cattle in a local abattoir of Elazig province. More than 80 flukes detected in the bile ducts and one of them was used for DNA isolation. Flukes washed extensively in PBS (pH 7.2) and subsequently fixed in 70 % ethanol and stored at -20 °C until used for DNA isolation.

The morphological differantiation of *F. hepatica* and *F. gigantica* were performed according to lineal biometric characters such as body length (BL), maximum body width (BW) and body perimeter (BP).

DNA isolation and amplification

Total genomic DNA (gDNA) was extracted from the apical zone of adult flukes using standard procedure (Sambrook *et al.,* 1989). Prior to DNA isolation, ethanol-fixed worm were cut from apical zone and washed 5 times with PBS. For the genomic DNA isolation, we used a com-mercially available DNA extraction kit (Promega, Wizard Genomic DNA Purification Kit) following additional proteinase-K (2 mg/ml) digestion in 56 °C for overnight. DNA concentration determined by spectrophotometer (Nano-Drop-ND1000) and the sample stored at -20 °C until use. Fragments of the mitochondrial CO1 gene were PCR-amplified as reported by Bowles *et al.* (1992) using the JB3/ JB4.5 primers (5′-TTTTTTGGGCATCCTGAGGTTT AT-3′/5′ TAAAGAAAGAACATAATGAAAATG3′). PCR amplification was performed in 50 µl volumes containing DNA (100 ng), 250 µM of each dNTP, 2.5 mM of $MgCl_2$, 20 pmol of each primers, 5 µl 10X PCR buffer and 1.25 U TaqDNA polymerase (MBI, Fermentas, Lithu-ania). The PCR conditions were: 5 min at 95 °C (initial denaturation), 35 cycles of 50 sec at 94 °C, 50 sec at 53 °C and 50 sec at 72 °C and finally 10 min at 72 °C (final extension). The PCR products were separated on agarose gel (1.5 %) and stained with ethidium bromide. Then the purified *Fasciola* mt-CO1 PCR products (5 µl) were digested directly with 5 units (1 µl) of restriction enzymes *AluI* and *RsaI* (Bioron, Ludwigshafen, Germany) in 20 µl for 2 h at 37 ºC. Restriction fragments were separated on 3 % agarose gel, stained with ethidium bromide and photographed upon transillumination. The restriction fragments were separated on 3 % agarose gels, stained with ethidium bromide and photographed. These restriction enzymes were chosen the results of restriction map of sequence. Lastly, the CO1 sequence was automatically obtained using a 377 ABI PRISM system (Applied Biosystems). Nucleotide sequence analysis was undertaken by BLAST algorithms and databases from the National Center for Biotechnology.

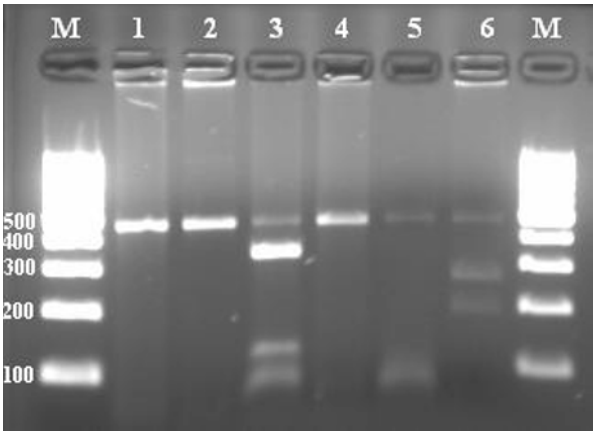

Figure 1. PCR and PCR-RFLP patterns of mitochondrial (mt) CO1 gene of the analyzed isolates. M: DNA marker (100 bp). mt-CO1 of the *F. hepatica* (1) and *F. gigantica* (2). PCR-RFLP patterns obtained from 3% agarose gel electrophoresis of *F.hepatica* after restriction enzyme digestion with *Alu*I (3), *Rsa*I (4). PCR-RFLP patterns of *F.gigantica* digestion with *Alu*I (5), *Rsa*I (6).

Results and discussion

The mean morphological characteristics for *F. hepatica* were 19.1 mm, 10.4 mm and 53.2 mm for BL, BW and BP, respectively. However, these rates for *F. gigantica* were 43.6 mm, 9.7 mm and 93.8 mm for BL, BW and BP.

The CO1-PCR with the JB3 and JB4.5 primers yielded of 446 bp amplification products with all samples analysed (Figure 1). In total 16 of *F. hepatica* and 1 of *F. gigantica* isolates were examined by PCR-RFLP analysis of the CO1 region of mt-DNA using two restriction endonucleases (*AluI* and *RsaI*) and the RFLP patterns of cattle isolates were presented in Figure 1. The different band patterns generated after digestion and used to differentiate between the two species. After the digestion of the PCR product with the restriction enzyme *AluI*, the RFLP profile obtained from *F. hepatica* revealed two fragments of approximately 330 and 110 bp, whereas *F. gigantica* was not cut (Figure 1). The *RsaI* digestion generated two fragments of approximately 190 bp and 280 bp from *F. gigantica*, whereas it did not cut the PCR product from *F. hepatica* (Figure 1). The corresponding sequences of *F. hepatica* and *F. gigantica* have been deposited in the

GenBank™ databases under the accesion numbers were GQ121276 and GQ121277, respectively. The alignment of these sequences with published sequence results (GQ231549 and GQ231548) for *F. hepatica* is presented in Figure 2 and for *F. gigantica* (AJ628033 and AJ628022) is shown in Figure 3. According to the alignment results, there was 100 % identity for *F. hepatica* isolate. While this identity rate was 98 % for *F. gigantica* isolate.

Sheep and cattle are heavily infected with liver flukes in Turkey with prevalence of up to 60 % by ELISA (Simsek *et al.,* 2007) and 22.1 % (303/1370) by postmortem inspection (unpublished data). Toparlak *et al*. (1989), examined 495 cattle livers and found as 50.3 % with *F. hepatica* and 1.8 % with *F. gigantica* in east of Turkey. Human infection has been documented in Turkey as 1.8 % in Van province (Yilmaz & Godekmerdan, 2004) by stool examination and 3.01 % in Antalya (Turhan *et al.,* 2006) and between 0.9 and 6.1 % by serology in Isparta provinces (Demirci *et al.,* 2003), mediterranean region of Turkey. However, there were no detailed published information as to whether both *F. hepatica* and *F. gigantica* occur in human in Turkey and their molecular differentiation. Erensoy *et al.* (2009), to determine the phylogenetic location of *F. hepatica* of Turkey origin based on ITS-2 rDNA molecular data, adult *F. hepatica* trematodes collected from the liver naturally infected sheep from different geographical locations in Turkey and ITS-2 rDNA sequenced, and compared with published sequences. They found that, primary sequence analysis revealed a close relationship between the query sequence (from Turkey) isolates of *F. hepatica* from China, France, Spain, Australia and isolates of *Fasciola* sp. from China. Different molecular techniques have been used for the characterization of liver fukes. Concerning the mitochondrial DNA, part of the mt-DNA of *F. hepatica*

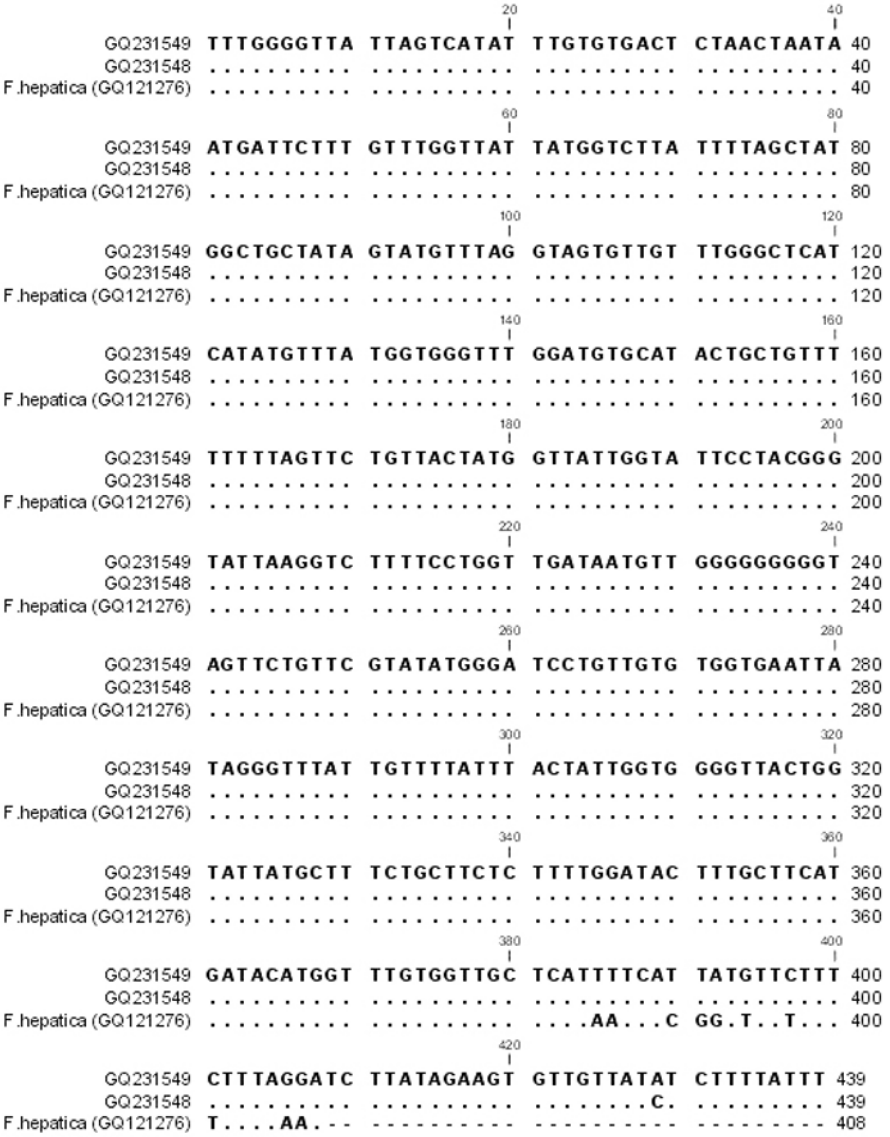

Figure 2. Nucleotide sequences of a fragment (446 bp) of mitochondrial cytochrome *c* oxidase subunite 1 (CO1) for 2 isolates of *Fasciola hepatica* analyzed in the study aligned with the published (GenBank TM/EBI Data Bank Accession No. GQ121276) CO1 sequence of the *F. hepatica* as a reference. A dot indicates a nucleotide that is conserved relative to the published sequence.

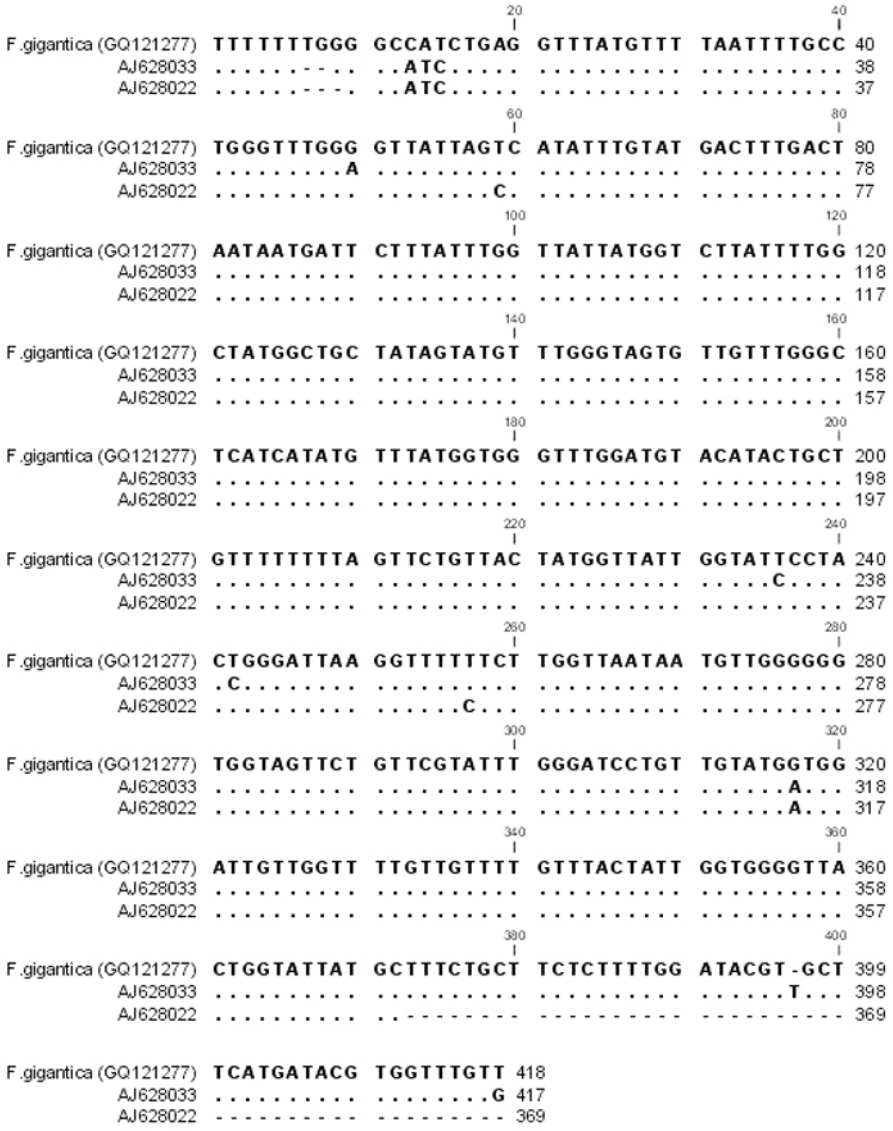

Figure 3. Nucleotide sequences of a fragment (446 bp) of mitochondrial cytochrome *c* oxidase subunite 1 (CO1) for 2 isolates of *Fasciola gigantica* analyzed in the study aligned with the published (GenBank TM/EBI Data Bank Accession No. GQ121277) CO1 sequence of the *F. gigantica* as a reference. A dot indicates a nucleotide that is conserved relative to the published sequence.

was sequenced and showed length heterogeneity, suggesting length differences among individual mitochondrial genomes (Marcilla *et al.,* 2002).

In the present study, the mt-DNA CO1 gene digestion patterns differed markedly between *F. hepatica* and *F. gigantica*. Given the variation observed within the short region of the CO1 gene sequenced, there are likely to be considerable differences in RFLP patterns. Restriction fragment length polymorphism patterns were analysed for the whole mt-DNA of *F. hepatica* from Australia, *F. gigantica* from Malaysia and *Fasciola* sp. from Japan after digestion with *HinfI*, *MspI* and *RsaI* endonucleases (Hashimoto *et al.,* 1997). The mt-DNA digestion patterns differed markedly between the three fasciolids. For each enzyme there were some bands specific for each geographical isolate, the Japanese *Fasciola sp*. sharing more bands with *F. gigantica* than with *F. hepatica*. However, given the variation observed within the short region of the CO1 gene sequenced, there are likely to be considerable differences in RFLP patterns even between quite closely related forms. Moreover, intraspecific variation was found at one nucleotide site between different specimens from the same *F. gigantica* population from Malaysia (Hashimoto *et al.,* 1997). Concerning ribosomal DNA, restriction endonuclease maps of the rRNA genes were distinct for *F. hepatica* and *F. gigantica*, Japanese *Fasciola sp*. being identical in restriction map to *F. gigantica*. No intraspecific variations in the maps of *F. hepatica* or of *F. gigantica* were detected, but length heterogeneity was noted in the intergenic spacer, even within individual worms (Blair & McManus, 1989). The ITS-2 of the rDNA has been used several times for liver flukes differentiation (Itagaki & Tsutsumi, 1998; Andrews, 1999; Huang *et al.,* 2004). One nucleotide difference in a 263 bp long ITS-2 fragment between the *F. hepatica* population from Mexico and those from Australia, Hungary and New Zealand, and

no difference in the 213 bp long ITS-2 fragment compared between *F. gigantica* from Indonesia and Malaysia were found (Andrews, 1999).

The nucleotide sequences were obtained for mt-CO1 gene were obtained of adult *F. hepatica* and *F. gigantica* and were compared with sequences of other species in GenBank. The BLAST hit results shown that our query mt-CO1 sequences were much more similar to the sequences of various geographical isolates of *F. hepatica* and *F. gigantica*.

In this study, we present a new approach of molecular differentiation of fasciolid flukes by using distinct endonucleases from previous work in PCR-RFLP of mitochondrial CO1 gene region. The nucleotide variation in the CO1 sequences of the cattle isolates of *F. hepatica* and *F. gigantica* resulted in difference in restriction sites for endonucleases *AluI* and *RsaI*. Consistent with CO1 sequence data, *F. hepatica* and *F. gigantica* could be differentiated from one another unequivocally using either *AluI* or *RsaI* based on their unique restriction banding patterns.

The PCR-RFLP method described here can be used for the proper identification of *Fasciola* species. Moreover, this method may be very useful for epidemiological surveys on both human and livestock fascioliosis in high endemic regions.

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