Iranian J Parasitol

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Original Article

Genotypic and Phenotypic Analysis of *Fasciola* Isolates

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(Received 5 Jan 2009; accepted 17 July 2009)

Abstract

Background: To identify the fasciolid species by morphometric and molecular methods in Zanjan, north-west of Iran.
Methods: Adult *Fasciola* worms (n=584) were obtained from cattle and sheep in Zanjan slaughterhouse in 2007. Living flukes were washed, then worms' images were taken by 3CCD camera and finally apical zone of each worm was obtained. Morphometric values such as body length, body width, body area, body perimeter and the distance between ventral sucker and posterior end of body were obtained using Auto-CAD image analysis software. Total gDNA was extracted from individual flukes by modified phenol-chloroform method. PCR amplification of ITS2 fragment was performed the isolated DNA samples and the amplicons were consequently subjected to RFLP assay and nucleotide sequencing to distinguish between fasciolid species.
Results: Mean of morphometric values in flukes from sheep was greater than those of cattle. Accordingly, the identified species included 31% *F. hepatica*-like, 7% *F. gigantica*-like and 62% intermediate forms. However, ITS2 fragment of 535 amplified specimens, showed no variation at the species-specific nucleotide sites 230, 340 and 341. The amplified fragment composed of partial 5.8S sequence (62bp), the complete ITS2 sequence (361bp) and partial 28S sequence (34bp). The nucleotide contents of ITS2 region were 69 A, 116 T, 81 C and 95 G and the average G+C content was approximately 49%. Comparing of ITS2 sequences with the BLAST GenBank database, also confirmed that all specimens were *F. hepatica*.
Conclusion: A simple and rapid PCR-RFLP assay can be used for distinguishing between these species.
Keywords*:* Fasciola, *Morphometric values, ITS2, PCR-RFLP, Restriction enzymes, Iran*

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Introduction

Fasciolosis has traditionally been considered as an important veterinary disease. However, increasing reports of human cases have changed epidemiological feature, markedly in recent years and caused further human health problems in the world (1). It has been an important zoonotic disease in Iran, and its causative agents, *Fasciola hepatica* and *F. gigantica* have different epidemiological characteristics in different areas. The infection has been endemic among domestic animals in most areas, with reports of sporadic human cases throughout the country for long time (2). Although the prevalence of fasciolosis among domestic animals in the southern part of Iran are higher than those from the northern part, the number of human reported cases is significantly higher in the northern provinces situated at the Caspian Sea and several large scale epidemics of fasciolosis, affecting thousands of individuals, have recently occurred in Gilan Province (3,4). In addition of co-existence of the above two fasciolids in livestock and the occurrence of intermediate forms, there are phenomena such as abnormal gametogenesis, diploidy, triploidy and mixoploidy as well as hybridization events between different genotypes. These all suggest a complicated figure of possible ways of circulation of the causal agents and make it difficult to identify the particular species involved (1). Proper identification of fasciolid species, followed by biological and ecological characterization, is important with concern to the planning for field control. Because there are many variations in morphological characteristics, exact distinguishing of fasciolid species is usually difficult by simple traditional microscopic measurements and, therefore, the morphometric characterization may be insufficient for the species identification (5). Moreover, the type of parasite infections in human cannot be classified based on clinical and pathological features or coprological and immunological diagnostic methods (6, 7).

Molecular approaches based on DNA analysis have been employed for genetic characterization and identification of morphologically similar parasites (8-10). Several haplotypes with different sequences at some loci, i.e. ribosomal internal transcribed spacer1 (ITS1), ITS2, mitochondrial NDI and COI genes, have been found in *Fasciola* forms (11-17). The PCR restriction fragment length polymorphism (PCR-RFLP) assays, base on the whole mtDNA and partial sequence of the large subunit (28S) of rRNA gene of the fasciolids were also utilized, recently (14, 16). However, given the variation observed in some fragments of mtDNA, differences in RFLP patterns even between quit closely related forms are likely to be present. Moreover, expected intraspecific variation was not seen at one nucleotide of 28S gene between the *F. gigantica* populations.

Comparison of the partial sequences of the ITS2 fragment in several isolates of *F. hepatica* and *F. gigantica*, obtained from different countries, showed nucleotide variations at six positions (18-21). More recently, ITS2 sequences were used for characterization of *F. hepatica* in naturally infected lymnaeid snails in the mountainous areas of Talesh; north of Iran (22).

The present study was designed to investigate liver isolates of *Fasciola* worms from Zanjan, northwest of Iran, using morphometric method and molecular tools. We also determined the fasciolid species characteristics in this area to compare with the other identified forms presented from other geographical regions.

Materials & Methods

Fasciola worms were collected from the naturally infected livers of cattle and sheep in Zanjan slaughterhouse from March through May 2007. The living flukes were washed in PBS then incubated in PBS at 37 °C for at least 4 h to allow them to expel gut contents. After that, the flukes were placed between two microscopic slides and then adult worms were identified with a stereomicroscope by the presence of numerous eggs in the uterus. Images of adult worms were taken by 3CCD camera after they lost vigorous contractions. The morphometric values such as, body length (BL), body width (BW), body area (BA), body perimeter (BP) and the distance between ventral sucker and posterior end of body (VS-P) were obtained by AutoCAD image analysis software. Finally, apical zone of each adult worm was obtained and stored at -20^{O}C in 70% ethanol until genomic DNA (gDNA) extraction.

Total gDNA was extracted from obtained parts of individual flukes using modified phenol-chloroform method (23). Specimens were homogenized in 500 µl of lysis buffer (50 µM Tris-HCL, 25 µM NaCl and 5 µM EDTA) with glass beats (0.1-0.3 mm diameters). It was added with 50 µg of Proteinase K (Fermentas), 25 µl of 1% sodium dodecyl sulfate (SDS) and incubated at 57°C for 1 hour. The mixture was centrifuged at 12000 g for 5 min and the supernatant was extracted twice with phenol-chloroform and once with chloroform. The gDNA was precipitated by adding equal volume of 100% ethanol and 50% (v/v) 7.5 mM ammonium acetate. The pellet was subsequently washed with 70% ethanol, dried under vacuum condition and re-suspended in 50 µl ddH20. The quantity of protein and nucleic acid in extracted samples were evaluated by UV spectrophotometer (Biochrom, WPA, Biowave II, UK).

The ITS2 region was amplified in a Gradient-Palm-Cycler (Corbett research, Australia) using the isolated DNA samples, following optimization of PCR conditions. The oligonucleotides for PCR amplification, followed below, were designed using standard rDNA sequences available in GenBank with accession numbers: AB010974, AB010975, AB207148 and AB207149, forward primer, 5'–TCTTGAACGCATATTGCGGC-3' complementary to a conserved region of 5.8S rDNA; reverse primer, 5'-AGTTCAGCGGGTAATCACGT-3' complementary to a conserved region of 28S rDNA. The PCR reaction, in a total volume of 50 µl (prepared in 200 µl microtubes), contained 10 mM Tris-HCL (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 250 µM dNTPs (CinnaGen, Iran), 0.6 µM forward primer, 0.7 µM reverse primer, 1-2 pg DNA template and 2.5 Unites of Taq polymerase (Fermentas). The thermo profile consisted of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for one minute, annealing at 60°C for one minute and extension at 72°C for one minute, ended with one cycle of final extension at 72°C for 7 minutes.

The PCR amplicons, 7 µl each, mixed with 3 µl of loading buffer (bromophenol blue/xylene cyanole, Fermentas), were subjected to the gel electrophoresis on standard 1.5% agarose in Tris-Borate-EDTA buffer for approximately 1.5 hrs. at 84 V. The gels were stained with 1% Ethidium Bromide and the amplified DNA fragments were visualized by illumination with 254 nm wavelength using Uvidoc (Uvitec, Cambridge, UK).

A PCR-RFLP marker was developed to distinguish specifically between *F. hepatica* and *F. gigantica* based on nucleotide differences detected in ITS2 region of rRNA gene. Two restriction enzymes, *PagI* (Fermentas) and *BamHI* (Fermentas) were selected by BioEdit (http://www.mbio.ncsu.edu) soft-

ware package. Comparison of the restriction maps of two fasciolid species revealed that the enzymes cut only ITS2 fragment of *F. gigantica* at 230 and 340 nucleotide positions and digested products are expected to contain 230, 110 and 20 bps. For restriction digestion, a total volume of 20 µl, including 10 µl of ITS2 PCR product added with 10 U of *PagI* with 2 µl of 10X Buffer 0 (Fermentas) or 10 U of *BamHI* with 2 µl of 10X Buffer *BamHI* (Fermentas) and 7 µl of dd-H_2O. The tubes were incubated at 37°C for 16 hrs, according to the manufacture's instruction to ensure full cutting of fragments. For analyzing the digestion products, 5 µl of each product in addition to 2 µl of loading buffer were run in gel electrophoresis.

Nucleotide sequencing of the PCR products from 13 worm isolates was also carried out for a better comparative resolution of the ITS2 regions amplified from the fasciolid DNA isolates. The PCR products separated by electrophoresis on 1.5% agarose gels were excised with scalpel. DNA was recovered and prepared using a DNA gel extraction kit (Qiagen) according to the manufacturer's instructions. Sequencing was done by cycle sequencing on an ABI 3730 DNA analyzer (Applied Biosystems) using the above described primers. All PCR products were sequenced in duplicate in both directions by Kawsar Biotech Company (http://www.kawsar.ir). Template DNAs were retained at -70 °C in the Laboratory of Molecular Systematic at the Department of Parasitology, Zanjan University of Medical Sciences, as voucher species.

Nucleotide sequences were edited and aligned by MUSCLE (24) and BioEdit softwares. Similarity with other sequences available in the GenBank database was assessed using BLAST (http://www.ncbi.nlm.nih.gov). The generated nucleotide sequences were submitted to the GenBank and respective accessing numbers assigned.

Morphometric data processing was carried out with SPSS version 12 software. Species identification was done by BL, BW, VS-P indices and BL/BW ratios according previous studies (2, 5, 25, 26). Significance of means of morphometric parameters in the hosts were performed using Student's *t*-test or by comparing 95% intervals. Proportion of fasciolid infection in the hosts was compared by χ^2 test. $p < 0.05$ was regarded as significant.

Results

The infection with fasciolid worms found in the present study, was 4.85% (39) and 1.12% (13) in the slaughtered cattle and sheep, respectively, with a significant higher rate in cattle (P=0.0001). A total number of 586 fasciolid worms were obtained from infected livers, 96% of which were adults. Mean and range of morphometric values in 538 adult flukes isolated from sheep and cattle are shown in Table1. Means of morphometric values in flukes from sheep were greater than those of cattle. However, the measured range values denoted a wide overlap between the isolates from the two animal species. Considering the general shape and the index of BL/BW, the identified worm isolates grouped into 31% *F. hepatica* like, 7% *F. gigantica* like and 62% intermediate forms.

Genomic DNA was extracted from 416 and 116 *Fasciola* isolates from cattle and sheep respectively. The PCR amplification generated a fragment of approximately 457 bps from all parasite DNA samples, as it was expected. No product was amplified from the PCR negative control reactions (with the absence of DNA template).

The RFLP patterns corresponding to the *Fasciola* species were obtained after diges-

tion of the PCR products with *BamHI* and *PagI* restriction enzymes. There was no evidence of restriction digestion in the ITS2 RFLP patterns, indicative of no variations at the nucleotide positions 230, 340 and 341 that are specific to the *F. hepatica* species (Fig. 1).

Nucleotide sequences of ITS2 PCR products, representative to the fasciolid isolates from different morphometric patterns, confirmed the absence of restriction sites, 5' G↓GATCC 3' and 5' T↓CATGA 3' for the purposed enzymes on the positions 230, 340 and 341 of ITS2 fragments (Fig. 2).

Three samples of 30 sheep isolates (15.67 – 19.88 mm in VS-P and 2.5- 2.6 in BL/BW ratio) and 10 samples of 50 cattle isolates (29.7 – 32.91 mm in VS-P and 3.7 – 4.5 in BL/BW ratio) were selected randomly. The PCR products of the selected samples were sequenced in both directions. The generated sequences are available in the GenBank, with the accession numbers EU391412 to EU391424.

The nucleotide content of the amplified fragment was 85(19%) adenine (A), 141(30%) thymine (T), 108(24%) cytosine (C) and 123(27%) guanine (G) with the average G+C content of 50.5%. The amplified fragment composed of partial 5.8S sequence of 62bp, the complete ITS2 sequence of 361bp and partial 28S sequence of 34bp (Fig. 2). The nucleotide contents of the ITS2 region included, 69 A, 116 T, 81 C and 95 G with the average G+C content of 49%. Comparing of ITS2 sequences with the BLAST GenBank database conformed that all specimens were *F. hepatica* (Fig. 2).

Table 1: Comparative morphometric data of *Fasciola* worms isolated from liver of cattle and sheep in Zanjan

Morphometric criteria	Cattle		Sheep	
	Range	**Mean (95 % cf)**	**Range**	**Mean (95 % cf)**
Body length, BL (mm)	11.01-48.64	23.89±0.39	18.59-42.79	32.20±0.91
Body width, BW (mm)	4.46-15.91	10.13±0.20	7.34-17.99	12.65±0.39
Body area, BA (mm^2)	19.85-451.35	170.91±2.23	140.71-458.86	284.02±13.85
VS-P* (mm)	0.75-42.71	21.31±0.47	15.67-39.76	29.26±0.88
Body perimeter, BP (mm)	22.60-111.65	55.21±1.07	48.30-115.58	73.95±1.96
BL/BW ratio	1.01-6.02	2.42±0.07	1.61-4.29	2.6±0.9
BL/ VS-P ratio	0.55-1.37	1.12±0.01	0.99-1.19	1.10±0.01

*VS-P = Distance between ventral sucker and posterior end of body.

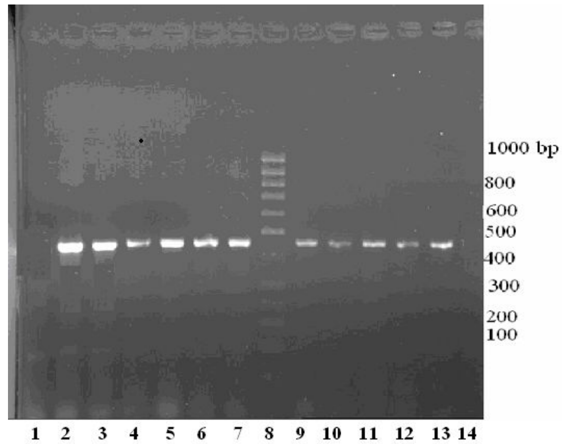

Fig. 1: Gel electrophoresis of PCR and PCR-RFLP products of ribosomal DNA ITS2 region of fasciolid flukes isolated from cattle and sheep in Zanjan, Iran. Lanes 1 and 14, negative controls; Lanes 2-7, representative of *Fasciola* isolates PCR products; Lanes 9-13, RFLP patterns representative of PCR products after digestion with *BamHI* (Lanes 9-10) and *PagI* (Lanes 11-13) restriction enzymes; Lane 8 is 100 bps DNA size marker

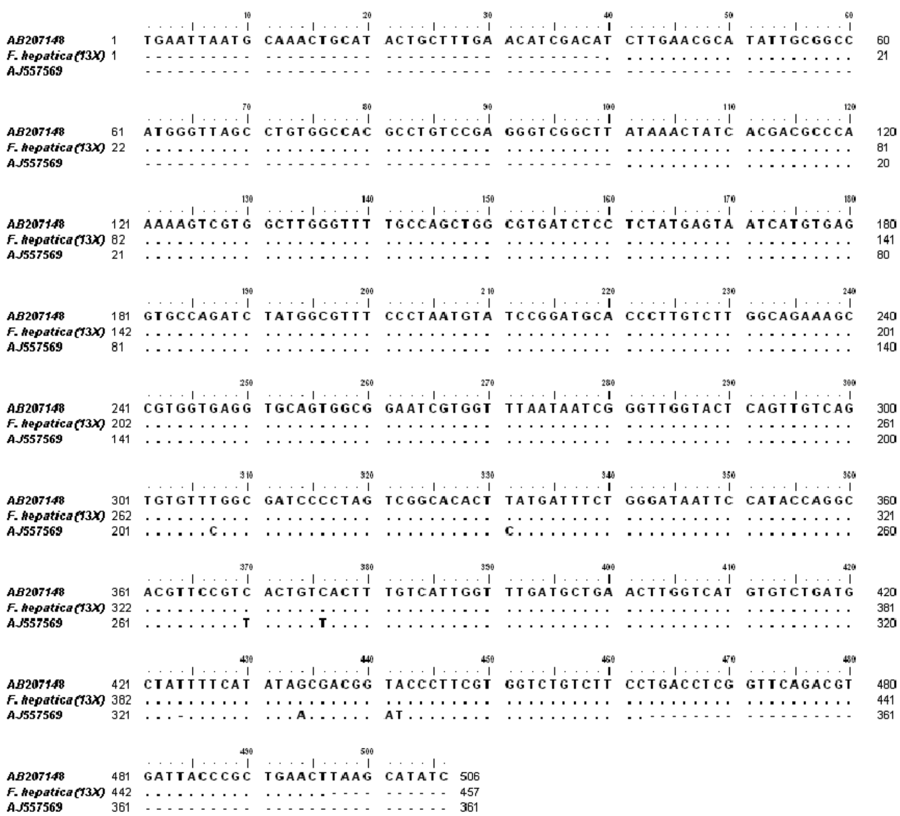

Fig. 2: Sequence alignment of partial 5.8S, complete ITS2 and partial 28S genes of the ribosomal DNA. The upper is standard strain of *Fasciola hepatica*, the 2nd representative of 13 identical sequences generated from *Fasciola hepatica* isolates of the present study and 3rd sequences belong to the standard strain of *F. gigantica*. (•) Indicates identity with the same sequence and (-) indicates indel

Discussion

The present study showed remarkable differences in morphometric indices among the *Fasciola* liver flukes isolated from animals in Zanjan. Considering the variations observed within the BL, BW and VS-P parameters in the studied samples, they are likely to be grouped into *F. hepatica* like*, F. gigantica* like and *Fasciola* intermediate forms. However, the ITS2-RFLP patterns and sequences of the PCR products confirmed that all of the worms were *F. hepatica.* In fact, traditional morphometric indices seem to be insufficient for the aim of species-specific diagnosis of *Fasciola* isolates.

The ITS2 region compared among the studied fasciolids was highly conserved with no intraspecific variation. The ITS2 sequences did not vary in length (361bp) or nucleotide composition when compared between different isolates. This is in complete agreement with the results of the previous studies (18-22). Like some other studies, our PCR target included ITS2 region and partial 5.8S and 28S genes, but the primers we designed for amplifying and sequencing, resulted in a better amplification as visualized on the gel with lack of primer dimmer and sharp banding patterns. This can potentially provide a simple and rapid molecular assay for identification of fasciolid species in future.

The results of this study show that the sheep isolated flukes are larger than those of cattle are. As mentioned by previous researchers (1, 2, 5, 25, 26) the comparison of the size of developmental different stages of *F. hepatica* between two different host species, indicating that the morphometric patterns are dependent to the host species. As it is discussed by Valero *et al.* (26), several mammalian species may serve as definitive hosts for *F hepatica,* but there is remarkable variation in susceptibility and induced pathogenicity of this parasite in different host species. Moreover, sheep show low resistance, whereas cattle show medium resistance to the parasite.

In the present study, the size of flukes isolated from both sheep and cattle found to be similar to the isolates of Gilan (north of Iran) and Nile (Delta of Egypt) populations (5, 25). However, they were bigger than the Bolivian (Altiplano highlands) isolates (26). Likewise, the study of Nile *Fasciola* isolate, morphometric indices of flukes in present study are obtained from live worms. Nevertheless, in the study of Gilan and Altiplano populations they are taken from fixed and mounted specimens. Moreover, fixing and mounting of specimens may affect some parameters the significant differences of these populations should be arisen from ecological issues. It seems that the ecologic conditions can be affected by altitude of the endemic areas. In our study, adult worms recovered from animals maintained in geographical areas with altitude between 1000 to 1800m, where, the isolates from Altiplano gathered from areas with 3800 to 4100m altitude. In the high altitude environment, oxygen and air density as well as temperature and humidity are low. These factors influence on the vertebrate and those borne flukes. Therefore, living at high altitude shows different morphological and physiological characteristics from those inhabiting at low altitude. These variations may influence the development of a liver fluke, its haematophagous and its tissue migration, although it cannot be disregarded that this may be attributed to some extent to intraspecific variability. Therefore, further research is needed to ascertain the association of morphometric and biological differences.

According to the previous studies, the amount of fasciolid cecum, ovary and testis branches could aid the differentiation of two species (2). Moreover, the BL, BW, VS-P and BL/BW ratios are the most important

indices for the above aim. It is believed that complete morphometric description and allomethric analysis of adults *F. hepatica* and *F. gigantica*, VS-P (8.86-27.08 mm in *F. hepatica,* 26.28-50.09 in *F. gigantica*) and BL/BW (1.29-2.8 in *F. hepatica,* 3.4-6.73 in *F. gigantica*), are useful tools for the specific differentiation of the two fasciolid species (5). Our results showed that 7% of isolates were *F. gigantica* like, based on the morphometric indices. However, ITS2 – RFLP patterns and sequence analysis of 10 representative cattle isolates indicated that they all were identical to *F. hepatica* species.

The limitation of ITS2-RFLP in the present study is that a standard strain of *F. gigantica* was not included in the PCR-RFLP technique due to unavailability. However, nucleotide sequencing of the PCR products confirmed that there were no restriction sites for the *BamHI* and *PagI* restriction enzymes in the sequencing of the investigated isolates resembling *F. gigantica* based on morphometric indices.

The phenotype of a living organism is an extremely complex dynamical system. Morphometric differences of body parts of fasciolids can be influenced by intensity of infection, host species, age and immune reactions due to a possible previous exposure to the infection.

It could be concluded that, when any morphometric measurement was considered, a great overlap between the species was observed. Due to the presence of intra-species morphometric differences and considerable overlaps of these indices between the two species, morphometric measurements alone are insufficient for differential diagnosis of the fasciolid species.

Acknowledgements

The authors are very grateful to Dr. A. Biglari for his assistance, to Zanjan Veterinary Center and Zanjan Slaughterhouse for providing infected livers. We also acknowledge Dr. A. Fazaeli for his critical reading of the manuscript and all reviewers for their constructive comments on the draft manuscript. This work was founded in part by Deputy for Research at Zanjan University of Medical Sciences. The authors declare that they have no conflicts of interest.

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