Morphometric and Molecular Study of *Fasciola* Isolates from Ruminants in Iran

İran'da Geviş Getiren Hayvanlarda *Fasciola* Izolatlarının Morfometrik ve Moleküler Çalışması

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ABSTRACT

Objective: The purpose of the present study was morphometric and molecular characterization of *Fasciola* isolates from ruminants in Iran.
Methods: Flukes were collected from the livers of 54 naturally infected sheep and cattle. The proportion of body length to width (L/W) of each fresh fluke was measured using a digital caliper. We employed receiver operating characteristic (ROC) curve analysis to explore the reliability of L/W for differentiating the two species. Polymerase chain reaction (PCR)-sequencing was performed on ribosomal Internal Transcribed Spacers (ITS) and mitochondrial cytochrome c oxidase subunit 1 (cox1) genes. The sequences were then analyzed and phylogenetic relationships were investigated.
Result: Forty-eight out of 54 isolates (88.9%) were identified as *F. hepatica* and four isolates (7.4%) as *F. gigantica*. All the sheep isolates were *F. hepatica*, while 4 out of 10 cattle were infected with *F. gigantica*. The morphometric study revealed an L/W ratio of 1.2 to 6.5 in *Fasciola* isolates with significantly higher L/W ratio in *F. gigantica* ($p<0.00$). According to the ROC curve analysis, the L/W value of 3.55 was regarded as the critical value to discriminate between the two species.
Conclusions: Findings of the present study indicate the presence of both *Fasciola* species in southeastern Iran. The phylogenetic analysis revealed two different clades representing *F. hepatica* and F. gigantica. The two isolates in this study were described as *Fasciola* sp. The mitochondrial DNA of these isolates were similar to *F. hepatica*, while their ITS fragments were identical to *F. gigantica*.
Keywords: *Fasciola*, Morphometry, Iran, cox1, ITS
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ÖZ

Amaç: Bu çalışmanın amacı İran'daki geviş getiren hayvanlarda *Fasciola* izolatlarının morfometrik ve moleküler karakterizasyonunu yapmaktır.
Yöntemler: Doğal olarak enfekte olan 54 koyun ve sığırın karaciğerinden parazitler alındı. Her canlı parazitin vücut uzunluğunun genişliğine oranı (L/W) dijital kaliper ile ölçüldü. İki türü ayırt etmek için L/W güvenilirliğini bulmak için alıcı işletim karakteristik (ROC) eğrisi kullanıldı. Polimeraz zincir reaksiyon (PZR) dizilimi ribozomal iç transkripsiyonu aralayıcı (ITS) ve mitokondriyal Sitokrom c oksidaz altbirimi 1 (cox1) genlerinde yapıldı. Daha sonra dizilimler analiz edildi ve filogenetik ilişkiler araştırıldı.
Bulgular: Elli dört izolattan 48'inin (%88,9) *F. hepatica* ve 4'ünün (%7,4) *F. gigantica* olduğu belirlendi. On sığırdan 4'ü *F. gigantica* ile enfekte olurken, tüm koyun izolatları *F. hepatica* olarak bulundu. Morfometrik çalışmada, *Fasciola* izolatlarında 1,2/6,5 olarak tespit edilen L/W oranı, *F. gigantica* izolatlarından anlamlı ölçüde daha yüksekti. ROC eğrisi analizine göre, 3,55 L/W değeri iki türü ayırt etmek için kritik değer olarak kabul edildi.
Sonuç: Mevcut çalışmanın bulguları güneydoğu İran'da her iki *Fasciola* türünün varlığını göstermektedir. *Filogenetik* analiz *F. hepatica* ve *F. gigantica*'yi gösteren iki farklı türü ortaya koymuştur. Bu çalışmadaki iki izolat *Fasciola* sp. olarak tanımlandı. ITS fragmentleri *F. gigantica* ile aynı iken, bu izolatların mitokondriyal DNA'ları *F. hepatica*'ya benzerdi
Anahtar Kelimeler: *Fasciola*, Morfometri, İran, cox1, ITS
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INTRODUCTION

The two Fasciolid species *Fasciola hepatica* and *F. gigantica* are the agents of fasciolosis in animals and humans. The definitive host range of *Fasciola* is very wide with mainly herbivorous animals as well as several mammalian species including humans. The larval stages of the parasite develop in Lymnaeid snail as intermediate hosts (1).

Human fasciolosis has been reported in many countries worldwide. It is estimated that 2.4 million people in more than 60 countries are infected, and the number of people at risk is more than 180 million throughout the world (1). *F. hepatica* infects more than 300 million cattle and 250 million sheep worldwide (2). *F. hepatica* and *F. gigantica* causes considerable economic losses to worldwide farming estimated as more than US$ 3.2 billion annually (3, 4). Human fascioliasis was sporadic in Iran until 1987 when an outbreak occurred in the northern province of Gilan and affected more than 10,000 people (5). The second outbreak occurred 10 years later and several thousand people were infected (6). The disease is also endemic in the countries of the region. In Erzurum, Turkey, out of 76 fecal samples collected from horses, 2.6% were found infected using flotation and sedimentation methods (7). Recent emerging focus of the disease has been documented in Yasuj, southwest of Iran (8).

Information about phenotype and molecular characterization of *Fasciola* is useful for accurate identification of the etiology of disease as well as for the prevention and control of fascioliasis in each endemic region (9, 10). The differentiation between species of *Fasciola* is important because the intermediate hosts for the two species are different with different ecological and biological characteristics. Additionally, the hybridization phenomenon occurs where both species co-exist. *Fasciola* forms intermediate between *F. hepatica* and *F. gigantica* have been reported from Asian countries, including Korea (11), Japan (12), Iran (13), China (14), and Vietnam (15, 16) as well as African countries including Egypt (17, 18).

Our understanding on the phenotypic and genetic features of *Fasciola* in southeastern Iran is limited. The purpose of this study was morphometric and genetic description of *Fasciola* isolates using nuclear and mitochondrial markers by Polymerase chain reaction (PCR) sequencing.

METHODS

Sample collection and morphometry

The research proposal has been approved by the Kerman University research of Medical Sciences Ethical Review. Infected livers from sheep and cattle were collected from abattoirs between June 2013 and September 2014. Fifty-four adult *Fasciola* parasites were collected from the bile ducts of infected cattle and sheep livers. Fresh worms were washed thrice in physiological saline. The flukes were placed on a slide, and body length and width were measured using a digital caliper; thereafter, the flukes were frozen in physiological saline for further molecular studies.

Molecular study

A small piece of the posterior parts of the flukes were cut and total DNA was extracted from individual flukes using High Pure PCR template purification kit (Roche, Heidelberg, Germany) according to the manufacturer's instructions. According to previous studies (12, 15), partial nuclear Internal Transcribed Spacers (ITS) and mitochondrial cytochrome c oxidase subunit 1 (cox1) regions were selected and corresponding primer sets (forward and reverse) were used as follows: ITS1 (660bp) ITS1F: TTGCGCTGATTACGTCCCTG and ITS1R: TTGGCTGCGCTCTTCATCGAC; ITS2 (530bp) 3Sf: GGTACCGGTGGATCACTCGGCTCGTG and BD2R: TATGCTTAAATTCAGCGGGT;.cox1 (500bp) Ita8: ACGTTGGATCATAAGCGTGT and Ita9: CCTCATCCAACATAACCTCT.

DNA fragments of each target region were amplified using PCR. The PCR thermal profiles were as follows: ITS1: 94°C for 2 min (initial denaturation) followed by 30 cycles of 94°C, 30 s (denaturation), 57°C, 30 s (annealing), and 72°C, 45 s (extension); ITS2: 94°C for 5 min (initial denaturation) followed by 30 cycles of 94°C, 1 min (denaturation), 50°C, 1 min (annealing), and 72°C, 1 min (extension); cox1: 94°C for 5 min (initial denaturation) followed by 30 cycles of 94°C, 1 min (denaturation), 60°C, 1 min (annealing), and 72°C, 30 s (extension).

Sequencing and phylogenetic construction

The PCR products were sequenced using ABI 3730XL capillary machine (Macrogen Inc., South Korea). The GenBank Blast program was used for ITS and CO1 comparisons. Sequences were analyzed using Bioedit software and aligned with published sequences of different Digenean helminthes using the ClustalW multiple alignment program. The sequences were entered in the MEGA® for constructing phylogenetic trees using the maximum likelihood approach. Branch support was given using 1000 bootstrap replicates in MEGA®. Specific identification was confirmed by comparison with known sequences of the corresponding species in GenBank.

Statistical analysis

A digital caliper was used to calculate the length (L), width (W), and the ratio of L/W. The mean and standard deviations were calculated for the dataset. For analyzing the significance of difference between morphometric characters, the Student t-test was used with a p value <0.05 as a threshold of significance.

Polymerase chain reaction analysis was used to distinguish between *F. hepatica* and *F. gigantica*, and the results were considered gold standard. We applied receiver operating characteristic (ROC) curve statistical analysis to explore whether L/W can be used as a surrogate for PCR. In particular, we aimed to determine a cut off with high sensitivity and specificity. We used the Itagaki et al (16) dataset as a test set to assess the external validity of our results. We applied the optimal cut off derived from our data to Itagaki et al (16) dataset. Results are presented in terms of sensitivity and specificity.

RESULTS

Fifty-four *Fasciola* isolates from sheep and cattle were collected from three geographical locations, i.e., Kerman (33 sheep and 1 cattle), Jiroft (8 cattle), and Karaj (11 sheep and 1 cattle).

Morphometric findings

The results of the morphometric study are summarized in Table 1. The range of L/W was 1.2 to 6.5 in *Fasciola* parasites. According to the molecular study, a significant difference in L/W data

were documented between *F. gigantica* (4.1–6.5) and *F. hepatica* (1.2–2.7; p<0.05). According to the ROC curve analysis, we found an L/W value of 3.55 as critical to discriminate between the two species (Table 2). Hence, we postulated that all the isolates with an L/W value >3.55 are *F. gigantica* with 100% sensitivity and specificity. When we applied this cut off to the Itagaki et al (16) dataset, sensitivity and specificity values were 90% and 85%, respectively. As shown in Table 1, a significant difference in the body length and width as well as L/W was recorded between the two species of *Fasciola* (p<0.05).

Molecular findings

Molecular analyses revealed that 48 and 4 isolates of *Fasciola* had the sequences identical to *F. hepatica* and *F. gigantica*. The molecular analysis of the isolates showed 11, 6, and 3 haplotypes for mitochondrial, ITS1 and ITS2 regions, respectively. Phylogenetic rDNA analysis of the specimens showed two sister clades representing *F. hepatica* (ITS-Fh) and *F. gigantica* (ITS-Fg). The phylogenetic analysis revealed two different clades representing *F. hepatica* and *F. gigantica* (Figure 1, 2). The two isolates in this study were described as *Fasciola sp*. The mitochondrial DNA of these isolates were similar to *F. hepatica*, while their ITS fragments were identical to *F. gigantica*.

Table 1. Morphometric characters of adult *Fasciola* isolates from ruminants in Iran

	Length (L), mm Range (mean±SD)	Width (W), mm Range (mean±SD)	L/W Range (mean±SD)
F. hepatica	4-30.2 (20.1±4.6)	3.7-13.8 (9.8±1.9)	1.2-2.7 (2.0±0.42)
F. gigantica	6.8-27.9 (36.2±4.3)	5.5-7.1 (6.5±0.73)	4.1-6.5 (5.4±0.99)
Fasciola sp	8.4-27.1 (2.7±0.91)	9.2-10.6 (9.9±0.98)	2.9-3 (2.9±0.014)

DISCUSSION

Data on the genetic characteristics of *Fasciola* species in the southeast of Iran are limited. In the present study, adult specimens of *Fasciola* infecting sheep and cattle from different localities were characterized by sequencing rDNA ITS regions as well as mitochondrial cox1 genes. In addition, the morphometric features of the isolates were studied.

The ratio of body length and width (BL/BW) has been considered one of the practical criteria for discrimination between *F. hepatica* and *F. gigantica* (17). In the present study, the L/W ratio was proved as a sensitive and specific index for discriminating *F. hepatica* and *F. gigantica* isolates. We used the Itagaki et al (16) dataset based on molecular tools to test our morphometric model in a set of *Fasciola* isolates that were positively identified by rDNA as well as mitochondrial sequencing. The ROC curve analysis was conducted for the first time to find a discrimination threshold with the highest sensitivity and specificity for distinguishing *Fasciola* species. Most morphometric data have not been tested on an independent dataset. In the present study, the external validity of the threshold has been independently calculated based on the Itagaki et al (16) dataset. The threshold of 3.55 was perfectly matched on both datasets using ROC curve modeling with an adequate sensitivity and specificity of 90% and 85%, respectively. This indicates that all the isolates with an L/W value of >3.55 are *F. gigantica*.

Table 2. Sensitivity and specificity values of the length to width (L/W) ratio of adult *Fasciola* isolates for discriminating *F. hepatica* and *F. gigantica* using receiver operating characteristic (ROC) curve analysis. The optimal threshold for sensitivity and specificity of L/W was assessed. Maximum sensitivity and specificity values were obtained at a threshold of 3.55

L/W	Sensitivity	1 - Specificity	L/W	Sensitivity	1 - Specificity
.000	1.000	1.000	2.285	1.000	.420
1.100	1.000	.980	2.310	1.000	.300
1.250	1.000	.960	2.345	1.000	.280
1.350	1.000	.900	2.385	1.000	.260
1.450	1.000	.880	2.435	1.000	.220
1.505	1.000	.860	2.485	1.000	.200
1.555	1.000	.840	2.535	1.000	.100
1.650	1.000	.820	2.635	1.000	.080
1.750	1.000	.780	2.720	1.000	.060
1.815	1.000	.720	2.820	1.000	.040
1.865	1.000	.700	2.950	1.000	.020
1.950	1.000	.640	3.550	1.000	.000
2.030	1.000	.580	4.750	.750	.000
2.080	1.000	.560	5.550	.500	.000
2.155	1.000	.480	6.100	.250	.000
2.230	1.000	.460	7.500	.000	.000
2.260	1.000	.440			

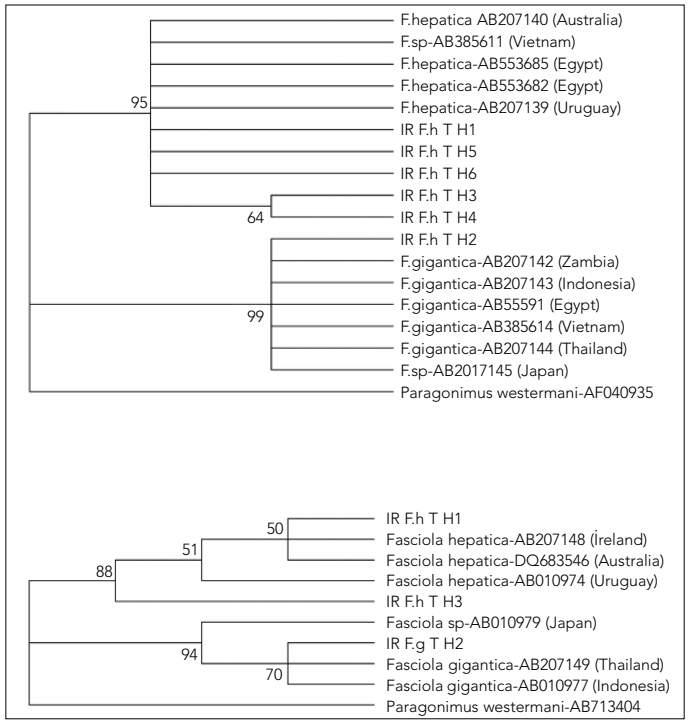

Figure 1. Genetic relationships of *Fasciola* species from ruminants in Iran and reference species (*F. gigantica*, *F. hepatica*, and *Fasciola sp.*) from previous studies based on nuclear ITS-1 (upper) and ITS2 (lower) markers were estimated using the maximum likelihood approach. Phylogenetic trees were obtained using MEGA 4.0 with bootstrap values of 1000 replicates set for maximum likelihood. There were six haplotypes for ITS1 region including H1 (KT921263–KT921265), H2 (KT921274, KT921278), H3 (KT921266), H4 (KT921267), H5 (KT921268), and H6 (KT9 21269) and three haplotypes for ITS2, including H1 (KT921270–KT921272), H2 (KT921275), and H3 (KT921273)

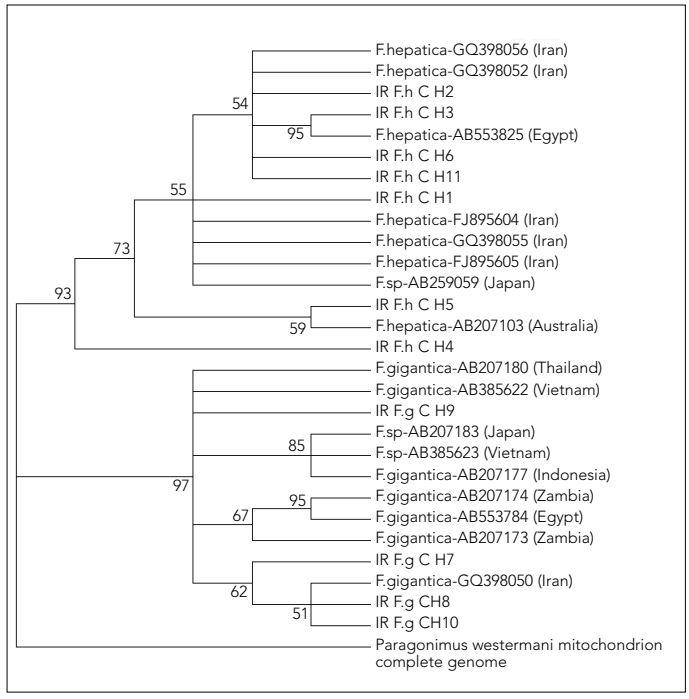

Figure 2. Genetic relationships of Fasciola species from ruminants in Iran and reference species (*F. gigantica, F. hepatica*, and *Fasciola spp.*) from previous studies based on cox1 gene marker estimated using the maximum likelihood approach. Phylogenetic trees were obtained using MEGA 4.0 with bootstrap values of 1000 replicates set for maximum likelihood. There were 11 haplotypes for cox1 region, including H1 (KT893716–KT893717), H2 (KT893718–KT893720), H3 (KT893721–KT893722), H4 (KT921276), H5 (KT893723), H6 (KT893724), H7 (KT893712), H8 (KT893713), H9 (KT893714), H10 (KT893715), and H11 (KT893725)

Based on the molecular results, 88.9% and 7.4% of the isolates were identified as *F. hepatica* and *F. gigantica*, respectively. *F. hepatica* has been the dominant species responsible for fascioliasis in ruminants in the world. Studies in Iran have shown that *F. hepatica* and *F. gigantica* are more prevalent in sheep and cattle, respectively. Recent studies in Iran showed that 15 out of 45 (33.3%) cattle isolates were *F. gigantica* compared to only 11.1% of 45 sheep isolates (19). Rokni et al (20) showed that 30 out of 31 sheep (96.8%) were *F. hepatica*. One study in Niger showed 66.7% of 12 cattle isolates were *F. gigantica* (21). In Tanzania, all the Fasciola isolates from 41 sheep were *F. hepatica* (22). Another study in Spain showed all of the isolates from cattle were *F. hepatica* (23). In the present study, none of the 44 sheep isolates were *F. gigantica* implicating a host preference of this species toward cattle.

Two isolates have been reported as *Fasciola sp*. in this study. The intermediate forms of *Fasciola* have been reported from Asian countries, including Japan, Vietnam, Korea, and China. Mitochondrial DNA sequence analysis of two isolates showed identical results with *F. hepatica*; however, rDNA sequencing identified them as *F. gigantica*. These results were in strong agreement with the morphometric results. The length to width analysis of 48 isolates sequenced as *F. hepatica* showed an average ratio of 2, while four *F. gigantica* isolates showed a mean value of 5.4. The corresponding L/W value for the two *Fasciola sp*. isolates was 2.9. These results are consistent with those of a study by Nguyen et al (2) wherein the ratio ranges of L/W values were 1.29–2.80 in *F. hepatica* and 3.4–6.8 in *F. gigantica* (2). Watanabe (24) reported L/W as 2 in *F. hepatica* and >3 in *F. gigantica*. An intermediate L/W ratio of 3.1±0.5 was reported in aspermic triploid *Fasciola* specimens from Japan (25). According to this finding, the two *Fasciola* sp. isolates of the present study may be considered as hybrid forms of *F. hepatica/F. gigantica*.

As shown in other studies, the level of diversity in mitochondrial cox1 was significantly higher than that in the nuclear ITS (26). In the present study, eleven cox1 haplotypes were found, while 6 ITS1 and 3 ITS2 haplotypes were identified in 54 isolates. It has been previously documented that the ITS regions are more conserved than the mitochondrial regions (18).

The molecular analysis showed 7 haplotypes within 50 isolates of *F. hepatica* and 4 haplotypes in 4 isolates of *F. gigantica*. Amer et al (18) found 19 haplotypes in 31 *F. gigantica* isolates, while 14 haplotypes were found in 34 *F. hepatica* isolates. The findings indicated that the genetic variation within *F. gigantica* is higher than that in *F. hepatica* in mitochondrial regions (18). The size of cattle liver, larger diameter of bile ducts, intensity of infection in cattle, and the number of the parasites in the bovine hepatic bile

ducts may contribute to greater genetic exchange and higher intraspecific variation within *F. gigantica* in cattle.

Two different clades were produced using phylogenetic analysis of cox1 gene representing *F. hepatica* and *F. gigantica*. The dendrogram topology showed that the two species split apart (Figure 2). The isolates were divided into three sister clusters in *F. hepatica* and two clusters in *F. gigantica*. The two *Fasciola* sp. isolates were located between the two species with more resemblance to *F. hepatica* isolates in a same clade with an intermediate form of *Fasciola* sp. from Japan (27). The haplotypes 2, 6, and 3 were remarkably similar to *F. hepatica* isolates from the northern province of Gilan (28) and from Egypt (18). The haplotypes 7, 8, and 10 have shown to be clustered with cattle isolates of *F. gigantica* from the southwestern city of Ahvaz (28).

The phylogenetic analysis of the ITS regions produced a well-defined dendrogram representing two distinct clades for *F. hepatica* and *F. gigantica* isolates. ITS-1 haplotypes 1, 3, 4, 5, and 6 were remarkably similar to *F. hepatica* isolates from Egypt (18), Uruguay, and Australia (29). Two out of three isolates within haplotype 2 have shown to be *Fasciola sp.* and located in a same cluster with *Fasciola* spp from Japan (29) and *F. gigantica* from Zambia, Indonesia and Thailand, Egypt (18), and Vietnam (16).

CONCLUSION

Accurate identification of *Fasciola* species is fundamental to any control measures. Information provided in this study indicated the presence of both *Fasciola* species as well as the intermediate forms in southeastern Iran. However, more molecular investigations are required on a wider variety of isolates from different host species and geographical locations. The background information for more comprehensive molecular and morphological studies on fascioliasis in the region has been provided in the present study.

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