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Original Article

Identification and Genetic Variation of *Fasciola* Species from Tabriz, North-Western Iran

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ABSTRACT

Background: Fascioliasis is considered as the most important helminthic infection of cattle and sheep. Traditional approaches using morphological and biologic characters cannot cause a certainty in the accurate and precise identification and intra-specific differences of *Fasciola* spp. In this study, we identified *Fasciola* species using ITS-1 marker and described genetic variation of each species of the parasite in isolates from Tabriz slaughterhouse in West Azerbaijan Province, north-western Iran.

Methods: Overall, 100 samples (50 from sheep and 50 from cattle) morphologically detected as *Fasciola* worms were studied for identification of *Fasciola* species by PCR-RFLP method and intra-species variation of the parasite using RAPD-PCR technique.

Results: A region of approximately 460bp in all samples was successfully amplified. There were no identifiable variations among the size of PCR products. Two and three fragments in samples correspond to *F. hepatica* and *F. gigantica* was seen, respectively, through PCR-RFLP method. No difference was seen in digestion pattern according to host (sheep or cattle). Different types of each species of the parasite was observed using RAPD-PCR technique.

Conclusion: We could have an estimate of frequency of *F. hepatica* and *F. gigantica* and different genotypes of the parasite in isolates from one locality in north-western of Iran. By extension of such studies in future to other animal hosts (buffalo and goat) and including more regions to sampling, the reliability of the results and their application for control programs in zoonotic diseases will be increased.

Keywords: *Fasciola hepatica*, *Fasciola gigantica*, PCR, Iran

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Introduction

Digenean trematodes of the genus *Fasciola* (Platyhelminthes: Trematoda: Digenea) have a global distribution and are the common liver flukes of a range of animals (1). A few ectopic cases of human fascioliasis, in non-hepatic sites such as thyroid, eyes, or skin, have been reported (2, 3). Recent documents estimate human infection by this parasite up to 17 million cases (4). In parts of Africa, such as Kenya, Zimbabwe and Zambia infection with *Fasciola* represents a major animal and human health problem (5-10) and, because of reduction in milk production, adverse effects on quality and quantity of fleece, this infection causing huge economic losses in the livestock, specially sheep and cattle (11). In tropical countries, fascioliasis is considered as the most important helminthic infection of cattle in prevalence of up to 90% (12). In mainland China, *F. hepatica* and *F. gigantica* have been reported from a range of mammals including buffalo, cattle, sheep, and goat (13-16). Fascioliasis is emerging as an important chronic disease of human in Gilan Province of Iran (17). In several outbreaks between 1989 and 1999, 7000 and 10000 human cases occurred in Gilan Province, respectively (18-22). There is no major macroscopic difference between two species of *Fasciola*, although *Fasciola gigantica* is longer and narrower, its cephalic cone is shorter, and ventral sucker is larger than *F. hepatica* (23).

Previously, it has been demonstrated that *F. hepatica* is mainly found in temperate areas, *F. gigantica* is mainly common in tropical localities, and both *F. hepatica* and *F. gigantica* may overlap in subtropical zones (24). Traditional approaches using morphological and biologic characters cannot cause a certainty in the accurate and precise identifica-

tion and intra-specific differences of *Fasciola* spp. (25).

Recently, using genetic approaches, a lot of information regarding the genetic characterization of *Fasciola* in many countries has been provided (26-28).

The low number of records on human infection with *F. gigantica* may be due to the lack of suitable tools to distinguish this species from *F. hepatica* (29). PCR methods using molecular markers such as first and/or second internal transcribed spacers (ITS-1, ITS-2) of ribosomal DNA (rDNA) can be used as a reliable tools for differentiation of *Fasciola* species in areas where two species overlapping (24,30). Several recent studies using the (ITS-1 and ITS-2) as genetic markers have identified a so-called "intermediate *Fasciola*" between *F. hepatica* and *F. gigantica* from Korea, Japan and China (25-32).

Different species of *Fasciola* including intermediate forms exist in Iran (33). In spite of several studies previously documented on distribution of *F. hepatica* and *F. gigantica* in Iran, there is not any documented information on the occurrence and genetic features of the parasite in north-western of the country. Therefore, the aim of this study was to genetic describe of the *Fasciola* spp. using ITS-1 marker by methods of PCR-RFLP and RAPD-PCR in isolates from Tabriz slaughterhouse in West Azerbaijan province, north-western Iran.

Materials and Methods

The samples were collected from livers of sheep and cattle from Tabriz slaughterhouse. Then, they were washed with distilled water (DW) for three times and were transferred and preserved in 70% ethanol at room temperature for further use.

We used FTA tablets for DNA extraction as described previously (23). In brief, we crushed a portion of the parasite between two microscopic slides for 1 min with 300 µl sterile DW and placed 0.4 ml of the lysate on Whatman paper (FTA Elute cards, Tokyo, Japan) and dried it at room temperature as DNA cards until further process. For DNA extraction, one part of punched DNA cards (3mm in diameter) was vortexed 3 times in DW for 1 sec. Incubation of the paper punches at 95 °C for 20 min and centrifugation for 30 sec was the final processes of the DNA extraction. We stored the supernatants as DNA at -20 °C.

A set of primers including forward (fascF: 5'-ACC CGT GCT GAG AAG ACG-3') and reverse (fascR: 5'-CGA CGT ACG TGC AGT CCA-3') were used to amplify a 460bp DNA fragment in the ITS1 region of both *F. hepatica* and *F. gigantica* (23). The amplification condition was initial denaturation at 95 °C for 5 min followed by 30 cycles at 95 °C for 45 sec, 60 °C for 45 sec, 72 °C for 1 min, and final extension at 72 °C for 7 min. DW was used as negative control. 1.5% agarose was used for electrophoresis and the gels were stained with ethidium bromide (2ml/ml).

For PCR-RLFP analysis, 1.5 ml of 10x supplied buffer, five micro liters of PCR product and 0.5 microliter of Tas1(fermentas) enzyme were incubated at 37 °C for 2h. Separation of restricted fragments performed in 2% agarose gel in TBE buffer using ethidium bromide for staining.

Two primers including UBC90: GGG GGT TAG G and R151: GCT GTA GTG T was used for RAPD-PCR analysis. The thermal

cycling condition was as follows: 95 °C for 5 min, 40 cycles of 94 °C for 40 sec, 32 °C for 60 sec, 72 °C for 60 sec, and a final extension of 72 °C for 6 min. We used 2% agarose gel in TBE buffer for separation of fragments and ethidium bromide for staining the gel.

Results

A region of approximately 460bp in all samples was successfully amplified (Fig. 1) that was predictable. There were no identifiable variations among the samples based on the size of the PCR products (Fig. 1). No band was seen in negative controls.

After digestion of the PCR products with TAS-1, two patterns of digestion was seen by the enzyme (Fig. 2), one digestion site producing 2 fragments (about 150 bp and 310 bp) in 75 samples related to *F. hepatica* and two cutting sites producing 3 fragments (about 95bp, 150bp and 220bp) in 25 samples related to *F. gigantica* (23) (Fig. 2). No difference was seen in digestion pattern according to host (sheep or cattle) related to each parasite (Fig. 2).

Besides, intra-specific variation within *F. hepatica* and *F. gigantica* species were assessed by method of RAPD-PCR. The genomic DNA of all isolates was amplified by primers of UBC90 and R151. Results of two RAPD profiles are shown in (Fig. 3). Three types of *F. hepatica* and three types of *F. gigantica* were produced by primer UBC90. Using primer R151 two types of *F. hepatica* and of two types of *F. gigantica* were seen.

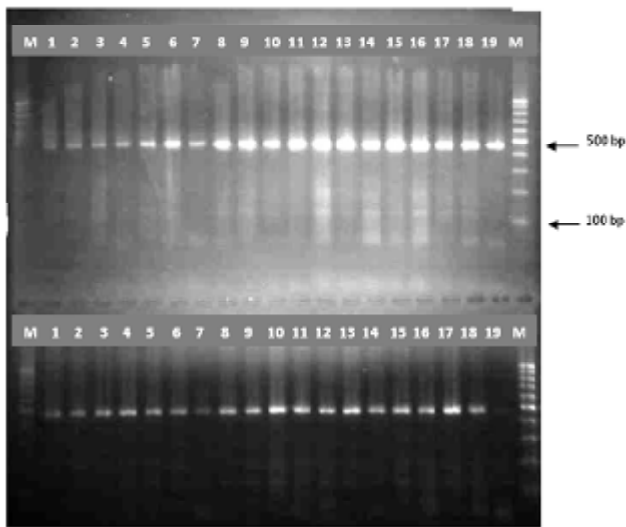


Fig. 1: Agarose gel electrophoresis of ITS1-PCR products. , Lane M 100-bp DNA ladder, lans 1-25 *F. hepatica*, lans 26-37 *F.gigantica*, lane C negative control

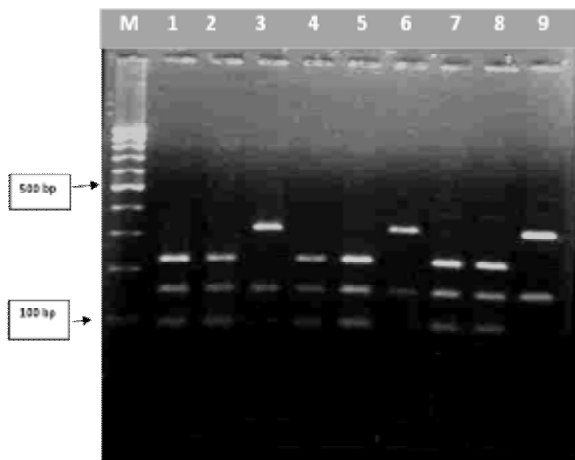


Fig. 2: Digestion of PCR products with TAS-1 enzyme: lanes 1,2,4,5,7,8 *F. gigantica*, Lanes 3,6,and 9 *F. hepatica* .Lane M 100 bp DNA marker

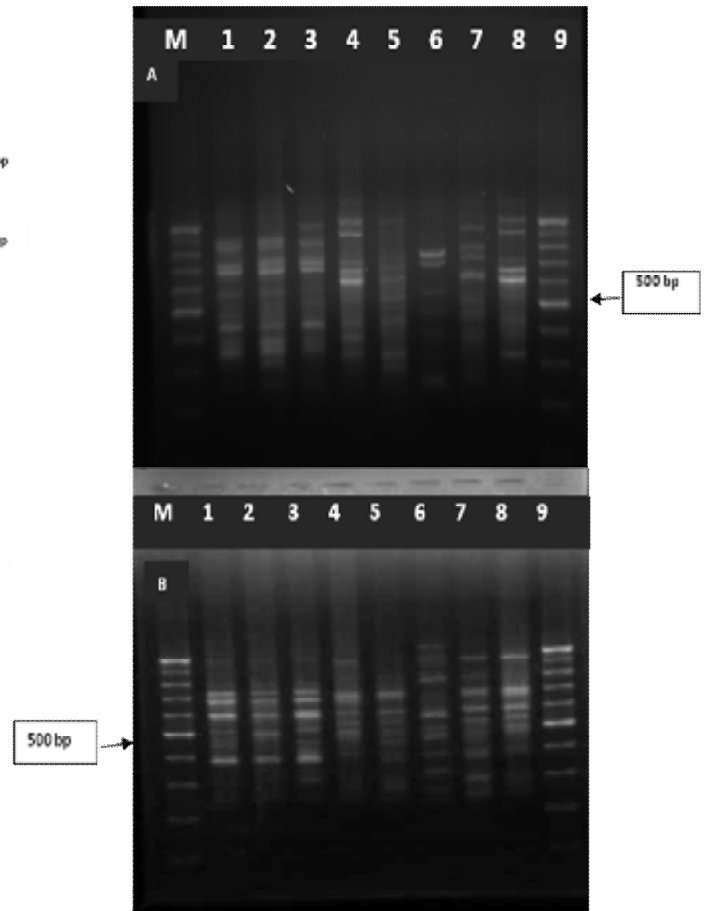


Fig. 3: Agarose gel electrophoresis of RAPD-PCR products of *Fasciola worms* isolates from Tabriz. A:RAPD-PCR patterns produced by primer UBC90, examples of three types of *F. hepatica* in lanes 1,4,5 and of three types of *F.gigantica* in lanes 6,7,8. Lane M 100 bp DNA marker. B:RAPD-PCR patterns produced by primer R151, examples of two types of *F. hepatica* in lanes 1,4 and of two types of *F. gigantica* in lanes 6,7

Discussion

There are several studies have exhibited that *Fasciola* parasites are the causative agents of fascioliasis in animal and human in Iran (3,17-22). Identification of the parasite spe-

cies in most of the studies have been done based on the morphologic and morphometric criteria. Because of morphological similarities between *Fasciola* species, and, due to overlap in the values of most measurements, speciation of the parasite based on morphologic and morphometric criteria cannot be decisive, and there have been some difficulties in definite identification of *Fasciola* isolates, including differentiation of *F. hepatica* and *F. gigantica* where both species, and possibly intermediate forms, coexist (15, 16, 23). At present, genetic methods using molecular markers have an increasing importance in epidemiology of parasites, especially when there is not a reliable method to morphologically distinguish of them (15, 16, 23).

Among different genetic markers are used to identify *Fasciola* species (27), the first internal transcribed spacer (ITS-1) of the rDNA has been used several times for *Fasciola* classification. This marker provides reliable genetic data for systematic molecular studies and intra-specific variations of the parasite (30). Using specific ITS-1, it demonstrated that, *F. hepatica* is only common species in some areas of Spain (34). By use of ITS-1 marker in Japan, it has been revealed that intermediate species are more similar to *F. gigantica* than *F. hepatica*. (28). In the present study, we could not find intermediate species of *Fasciola* parasite. Similar findings have been reported from Iran previously (23). Using 18s rDNA marker through PCR-RFLP method, the first intermediate genotype of *Fasciola* was reported from Fars province, Southern Iran (35). By sequencing of ITS's-2 marker, it was revealed that isolates of the parasite in infected snails from Northern provinces of Iran were *F. hepatica* (36). No intermediate species of *Fasciola* was seen using ITS-1 and 5.8s rRNA marker in Nigeria (37).

We assessed intra-specific variation within *F. hepatica* and *F. gigantica* species by method

of RAPD-PCR. Amplification of small parts of the genome of an organism can be performed by RAPD-PCR method. This technique has a great potential for describing genetic features of organisms, performing molecular epidemiology and population studies and revealing genetic relatedness of infectious agents (7, 38, 39).

In the present study, the primers produced different DNA fragments in size according to the two species of the worm. Three patterns of genomic DNA by primer UBC90 was found in both *F. hepatica* and *F. gigantica*. By using primer R151, we found two patterns of amplification in both of the parasites. The patterns of amplification of DNA of each parasite with both primers were similar in sheep and cattle, demonstrating that these patterns were not specific for cattle and sheep originated *F. hepatica* and *F. gigantica*, whereas, in some cases, different patterns by the same primers in different hosts (sheep and buffalo) were reported in isolates from other areas of the country, previously (23). This means the homogeneity of the parasite in isolates in our study is more than former report. Based on the sequencing of ITS-2 marker complete homogeneity was also reported among isolates of the parasite from Mexico, Australia, and Malaysia (27). Studies have been performed in China (27) and Spain using ITS-1 marker showed that the sequence heterogeneity was not related to host species and/or geographical origins of the isolates (28).

As has been showed previously (23), we observed that DNA extraction method using FTA Elute card is easy, without special equipment need and is economical and the DNA prepared from FTA Elute card can be preserved for a long time.

According to the results of this study we emphasize again that the PCR-RFLP method can be useful tool for epidemiological surveys on both human and livestock fas-

cioliasis and should be considered for identification of *Fasciola* species in geographical areas such as Iran, where both *F. hepatica* and *F. gigantica* appear to be coexist and clinical, immunological / pathological methods and morphological features do not permit a discrimination between them at veterinary fields with overlapping distribution areas.

In conclusion, in the present study, using ITS-1 marker through PCR- RFLP and RAPD-PCR methods, we could have an estimate of *F. hepatica* and *F. gigantica* variability and different genotypes in isolates from one locality in north- western of Iran. Although we could show a relative intra-species heterogeneity in the isolated parasites in one locality of the country, but by extension of such studies in future to other animal hosts (buffalo and goat) and including more regions to sampling, the reliability of the results and their application for control programs in zoonotic diseases will be increased.

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