

**GENETICALLY CONFIRMED *FASCIOLA HEPATOGIGANTICA* N.SP.**

By

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**Abstract**

Identification of liver fluke species cannot be achieved by clinical, pathological, coprological or immunological methods. However, the differential diagnosis between *F. hepatica* and *F. gigantica* infection is very important because of their different pathological manifestations. Moreover, in countries where the two species co-exist, morphologically intermediate forms were reported. The present study aimed to identify these forms by the use of molecular characterization of DNA sequence.

Based on morphometric criteria, adults of *Fasciola hepatica*, *F. gigantica* and intermediate forms were collected from naturally infected sheep and cattle from various regions of Sohag Governorate. A simple and rapid new method (QIAamp DNA Mini Kit) was used to isolate DNA from the worms and their RELP patterns were obtained after digestion of the PCR products with AvaII restriction enzymes.

The result of a regular PCR experiment for the amplification of the selected 28S rDNA fragment with the designed primer set yielded identical 618- bp-long PCR products for the three types of *Fasciola* where the RFLP profile obtained from *F. hepatica* revealed four fragments of 628, 575, 165 and 95 bp, and *F. gigantica* generated three fragments corresponding to 628, 358 and 300 bp fragments whereas the intermediate forms revealed four fragments of 628, 541, 358 and 300 bp, which were similar to those of *F. gigantica* but with a distinctive fragment of 541.

These results confirmed that three species are present in our locality: *F. hepatica*, *F. gigantica* and an intermediate form which was named *F. hepatogigantica* n.sp. on basis of having few morphometric characters from *F. hepatica* (length and pattern of uterine coils) but genetically they were more related to *F. gigantica*.

**Key words:** DNA sequences, PCR-RELP, *Fasciola hepatogigantica* n.sp.

**Introduction**

Fasciolosis, caused by the digenetic trematodes of the genus *Fasciola*, is one of the most important plant-borne helminth infections of human and livestock in many parts of the world

(Mas-Coma *et al*, 2005, 2009; Roknil *et al*, 2010). It is a major zoonosis that causes a great deal of economic loss throughout the tropical region of the world (Gorokhov and Sergiev, 2008) and is an emerging public health prob-

lem in the Middle East (Alcaino and Apt, 1989; Hussein and Khalifa, 2010, Hussein *et al*, 2010).

Two species: *F. hepatica* and *F. gigantica* were recognized in humans and domestic farm animals. Since the second half of the last century, Price (1953) stated that mixed infections with the two species resulted in the emergence of flukes with intermediate morphological characters. At the extremes of their morphological range, some resemble *F. hepatica*, whereas others resemble *F. gigantica*, with intermediate forms occurring which may be involving the phenomena such as abnormal gametogenesis, diploidy, triploidy and mixoploidy, parthenogenesis, and hybridization events between different genotypes (Mas-Coma *et al*, 1997).

Understanding genetic structure and status of genetic variation of the parasite populations has important implications for epidemiology and effective control of fascioliasis (Ashrafi *et al*, 2006). On the other hand, intermediate forms between *F. hepatica* and *F. gigantica* have been morphologically recognized in most countries, (Ashrafi *et al*, 2006) and Arab Countries including Egypt (Allam *et al*, 2008; Hassan *et al*, 2008; Amor *et al*, 2011; El-Rahimy *et al*, 2012).

The identification of *Fasciola* species has been based solely on traditional morphological approaches. However, due to limitations of these methods, various molecular approaches have been developed and used for their identification and differentiation. These

molecular methods have raised questions and spurred debate on the recognition of the intermediate *Fasciola* as a hybrid/intergrossed form between *F. hepatica* and *F. gigantica* (Agatsuma *et al*, 2000; Ashrafi *et al*, 2006; Ichikawa and Itagaki, 2010).

Molecular techniques such as PCR and its variants are used for the diagnosis of parasitic diseases and the identification of parasites, for the development of specific antigens for serological tests and for studying the immune response in the patients. They provided a more specific method than conventionally employed methods in epidemiological studies (Rognlie *et al*, 1994; Heckerroth and Tenter, 1999; Mostafa *et al*, 2003).

Hence, the aim of this study was to try to differentiate between the three fasciolid worms encountered in sheep and cattle in Sohag through a simple and rapid PCR-restriction fragment length polymorphism (RFLP) assay, using the common restriction enzymes *Ava*II based on a 618-bp-long sequence of the 28S rRNA gene.

## Materials and Methods

Adult fasciolid worms were isolated from liver of naturally infected sheep and cattle collected from various regions of Sohag Governorate. *Fasciola hepatica* and *gigantica* were identified based on morphological criteria of adults (Sahba *et al*, 1972; Muller, 2002). Together with some intermediate doubtful samples were washed extensively in PBS (37°C) and subsequently fixed in 70% ethanol and main-

tained at 4°C until used for DNA extraction.

DNA Extraction by a new method (QIAamp DNA Mini Kit): 1- 5mg of tissue sample were cut into small pieces and placed in 1.5 ml microcentrifuge tube then 180 µl of buffer ALT was added and 20 µl proteinase K, mixed by vortexing and incubated at 56° C until the tissue was completely lysed. 2- After centrifugation 200 µl Buffer ALT was added to the sample, mixed by pulse vortexing for 15 second and incubated at 70°C for 10 min.

Carefully, the mixture was applied to the QIAamp Mini Kit column (in 2 ml collection tube) without wetting the rim, centrifuged at 8000 rpm for 1min. The mixture was carefully applied to the QIAamp Mini Kit column and 500 µl buffer AWI was added, centrifuged at 8000 rpm for 1min.

The QIAamp Mini spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded and then 500µl buffer AW2 was carefully applied, centrifuged at full speed (14.000 rpm) for 3 min.

Carefully, the QIAamp Mini spin column was opened and 100µl buffer AE added, incubated at room temperature for 3 min, then centrifuged at 8000 rpm for 1 min, after which we incubated the last for 5 min with elution which increase the DNA yield.

Primer selection: After computerized study and comparison of nucleotide sequences in rDNA regions including 28S rDNA of different *Fasciola* species, fragment of 618 bp at the 5' end of the rDNA including 4 nucleotide dif-

ferences between all *Fasciola* forms, were selected and a PCR method to amplify it was developed for the amplification of this 618-bp-long fragment of the 28S rDNA gene, the primers 28F1 (5' ACGTGATTACCCGCTG-AACT3') and 28R600 (5' CTGAGA-AAGTGCACTGACAAG 3') were used (Marcilla *et al.*, 2002).

Each reaction mixture (total volume of 25µl) contained 1 µl of diluted (1:3 genomic DNA, 12.5µl 2x Promega master mix) and 0.2 µmol of each primer. The reaction mixture was amplified in a TPersonal thermal cycle (Biometra, Germany).

The samples were subjected to an initial denaturation step at 94°C for 5 min, followed by 45cycles of 30 s at 94°C, 30 s at 49°C, and 60 s at 72°C.

Finally, an extension step of 10 min at 72°C was used. Ten microlitres of PCR products were analyzed by electrophoresis on 1% (w/v) agarose gels. A 100-bp DNA ladder marker was included on each gel for base-pair comparisons. Gels were visualized by staining with ethidium bromide as described by Sambrook *et al.* (1989).

Restriction fragment length polymorphism analysis: Advantage was taken of the previous knowledge about the exact nucleotide differences between the two liver fluke species for choosing the appropriate, specific and common restriction enzymes *Ava*II to carry out the necessary restriction fragment length poly-morphism analysis, which would allow us to distinguish between the three encountered forms.

Specific restriction enzymes were

selected and lengths of resulting restriction fragments were predicted by means of the (BioDoc Analyzer Software). 12 microlitres of PCR products were completely digested with 15µl of *Ava*II (7.5 UI) restriction enzymes (Roche, Basel, Switzerland) at 37°C. The digested DNA was analyzed by

electrophoresis in 1% agarose gels and visualized by ethidium bromide staining.

## Results

The results are shown in table 1 and figures 1 & 2.

Table 1: Restriction fragment length polymorphism (RFLP) patterns (in bp for each restriction band) of the 628-bp-long PCR fragments from liver fluke species generated after digestion with restriction enzymes.

<i>Fasciola</i> species	Restriction enzyme <i>Ava</i> II
<i>F. gigantica</i>	628, 358 and 300
<i>F. hepatogigantica</i>	628, 541, 358 and 300
<i>Fasciola hepatica</i>	628, 575, 165 and 95

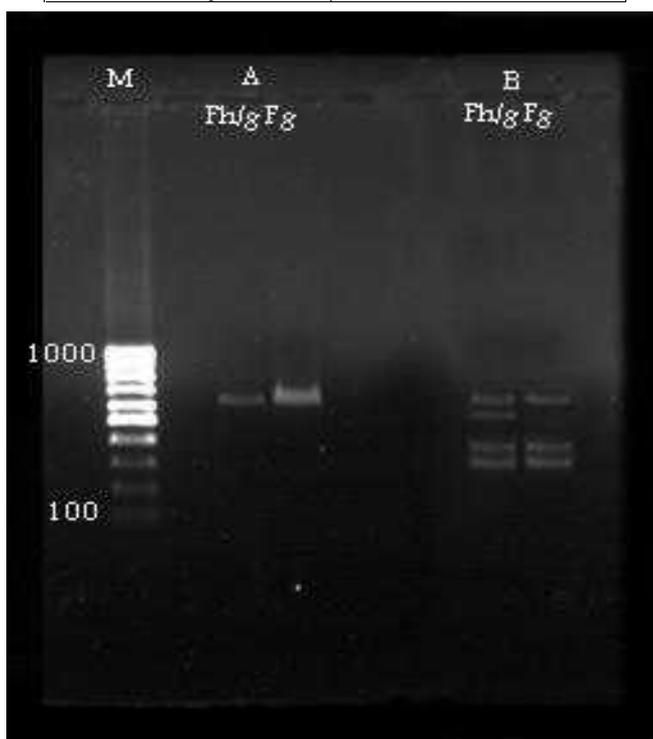


Figure 1: Restriction fragment length polymorphism (RFLP) patterns of PCR products of liver flukes after digestion with *Ava*II restriction enzymes.

Lane M: 100 bp DNA size marker:

Lanes A: Ethidium bromide staining pattern of the 648 bp-long PCR products from *F. hepatogigantica* (*Fhig*) and *F. gigantica* (*Fg*) DNA samples.

Lanes B: PCR products of the *F. hepatogigantica* (*Fhig*) and *F. gigantica* (*Fg*) after digestion with *Ava*II restriction enzyme respectively.

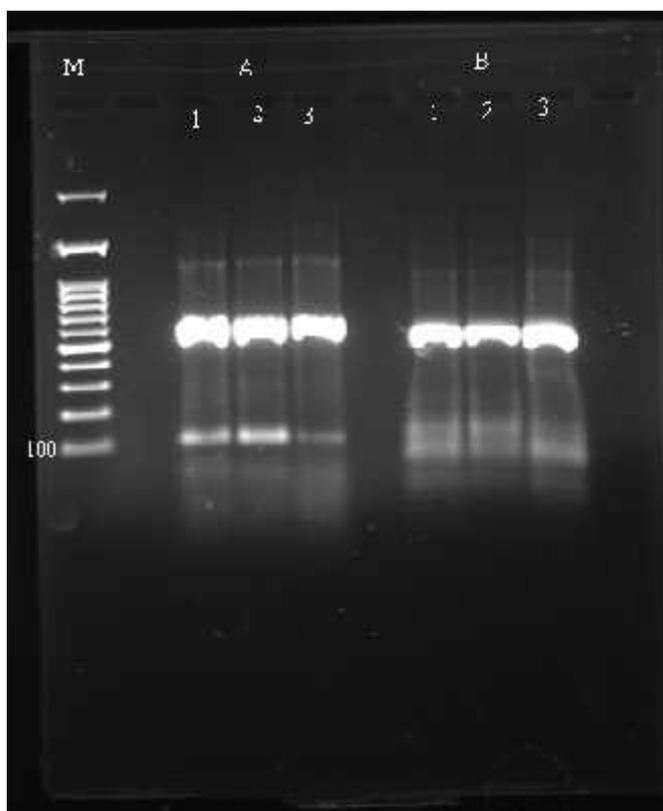


Figure 2: Restriction fragment length polymorphism (RFLP) patterns of PCR products of liver flukes after digestion with *Avall* restriction enzymes.

Lanes M: 100 bp DNA size marker:

Lanes A: Ethidium bromide staining pattern of the 648 bp-long PCR products from *F. hepatica* DNA samples.

Lanes B: PCR products of *F. hepatica* after digestion with *Avall* restriction enzyme.

### Discussion

A number of fasciolid species have been described within the genus *Fasciola*, but only two species, *Fasciola hepatica* and *Fasciola gigantica* are commonly recognized as taxonomically valid species occurring in domestic animals and humans (Yamaguti, 1958; Mas-Coma *et al*, 1999; 2005). However, the two species can have considerable intra-specific differences in morphological features and other biologic characters, which can cause uncertainty in their accurate identification through

the use of traditional approaches (Kendall, 1965; Itagaki, 1998). Moreover, recent experimental and field studies were in doubt that their definitive hosts may influence the morphometrics of adults and eggs (Valero *et al*, 2001).

It is very difficult to discriminate between *F. hepatica* and *F. gigantica* because of their many variations in their morphological characteristics although this is usually very important because of their different pathological effects. When the two species co-exist differentiation becomes very difficult,

sometimes impossible, when dealing with few worms (Marcilla *et al.*, 2002). The overlapping distributions of both species have become the basis of already longstanding controversy on the taxonomic identity of the *Fasciola* species occurring in Far and Middle East countries. At the extremes of their morphological range; some worms resemble *F. hepatica* whereas others resemble *F. gigantica* which lead to the assumption of the presence of different strains (Khalifa *et al.*, 1979; Hussein and Khalifa, 2010), intermediate forms (Lotfy *et al.*, 2002; Lin *et al.*, 2007; Saki *et al.*, 2011; Saira *et al.*, 2011), hybrid/intergrossed forms (Le *et al.*, 2008 and Thanh *et al.*, 2008), size races (Srimuzipo *et al.*, 2000) or genotypes (Ashrafi *et al.*, 2006; Ali *et al.*, 2010). Hence, it was a must to overcome this problem not only on morphometric basis but also by the study of the DNA pattern of the existing species.

According to the morphometric criteria proposed by Periago *et al.* (2006) for the differentiation of liver fluke adult fasciolids, the results of their study indicated the presence of *F. hepatica*, *F. gigantica* and intermediate forms in Egypt. Although the fasciolids can be characterized according to their morphometric techniques (Ashrafi *et al.*, 2006), yet flukes with intermediate morphological characteristics can cause confusion (Terasaki *et al.*, 1982; Itagaki *et al.*, 2005a).

Although some species of *Fasciola* can be distinguished by iso-enzyme analysis, such approaches are time consuming and require specialist skills (Mc-Garry *et al.*, 2007). Thus, there

was unfortunately neither a direct coprological nor an indirect immunological test available for their identification. Specific differentiation can only be more accurately made by molecular tools (Periago *et al.*, 2008). Over the last two decades, several PCR-based approaches, including PCR-linked restriction fragment length polymorphism (PCR-RFLP), PCR-linked single-strand co-information polymorphism (PCR-SSCP) and specific PCR assays have been developed for the accurate identification of *Fasciola* spp. (Marcilla *et al.*, 2002, Huang *et al.*, 2004; Lin *et al.*, 2007, Roknil *et al.*, 2010; Alasaad *et al.*, 2011).

Previous studies in Japan (Itagaki *et al.*, 1998; Itagaki *et al.*, 2005b) using the first and second internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA (rDNA) demonstrated that the Japanese form of *Fasciola* represented both *F. hepatica* and *F. gigantica*, and the authors suggested that this form may be a hybrid between *F. hepatica* and *F. gigantica*. Furthermore, studies of Korean *Fasciola* specimens, using ITS-1, ITS-2 and the D2 region of the 28S rDNA as genetic markers, demonstrated that the Korean liver flukes were divided into *F. hepatica*, *F. gigantica* and intermediate forms (Agatsuma *et al.*, 2000; Itagaki *et al.*, 2005).

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. Based on the sequences of the second internal-transcribed spacers

(ITS-2) within the parasites' nuclear ribosomal DNA (rDNA) in China allowed the identification and differentiation of *F. hepatica*, *F. gigantica* and an intermediate form with no amplicons produced from heterogenous DNA samples (Ali *et al*, 2010)

The results of studies in Japan, Korea, China, Iran, Viet Nam and Egypt indicated that an 'intermediate' *Fasciola*, which may be a hybrid between *F. hepatica* and *F. gigantica*, is present in these countries (Itagaki *et al*, 1998; Agatsuma *et al*, 2000; Huang *et al*, 2004; Itagaki *et al*, 2005a, b; Ashrafi *et al*, 2006; Lin *et al*, 2007; Periago *et al*, 2008). Moreover, Karimi (2008) used 18S rDNA-RFLP and sequencing, and reported the first molecular evidence of an intermediate genotype of *Fasciola* in Fars province, southern Iran.

Fascolid species 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 *Ava*II and *Dra*II. These sequences revealed a few nucleotide differences between the two species without intraspecific variations within species (Marcilla *et al*, 2002).

In the present study, a rapid and simple method was developed to differentiate *Fasciola* species by PCR-RFLP assay. Adults of *F. gigantica*, *F. hepatica* and intermediate forms were isolated from livers of naturally infected animals (cow, sheep and buffaloes) collected from various regions of Sohag Governorate. We used a new method to isolate DNA from the worm (QIAamp DNA Mini Kit) and Restriction

fragment length polymorphism (RFLP) analysis of PCR products of liver flukes using *Ava*II restriction enzymes. The result of regular PCR experiment for the amplification of the selected 28S rDNA fragment with the designed primer set yielded identical 618-bp-long PCR products for all types of *Fasciola* encountered. However, the RELP analysis indicated the presence of three different species. Using the same restriction enzymes, Mohammad *et al*. (2009) differentiated between *F. hepatica* and *F. gigantica* without mentioning the presence of intermediate forms.

### Conclusion

The present results confirmed that three species of *Fasciola* are present in our locality: *F. hepatica*, *F. gigantica* and an intermediate forms which were named *F. hepatogigantica* n. sp. on basis of having few morphometric characters from *F. hepatica* (length and pattern of uterine coils) but genetically, they were more related to *F. gigantica*.

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