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Morphological and molecular study of *Fasciola* spp. in sheep in Alkut city

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Abstract

Liver fluke disease or fasciolosis is one of the most important helminth infections of ruminants in the world caused by *Fasciola* spp.Therefore, this study was conducted to investigate and identify the fasciolid species by morphometric and molecular methods in Alkut city. Adult *Fasciola* worms (n=79) were obtained from sheep in different condition, ages, and sex from slaughterhouse. Morphometrically, the liver worms collected from the sheep were stained with alum carmine stain and results revealed the occurrence of *F. hepatica*. The PCR amplification 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. Seventy nine samples collected from 360 sheep, were identified subsequently based on genetic markers:nuclear ribosomal internal transcribed spacer 2 (ITS2). This study showed the intermediate form of *Fasciola*in Iraq. We concluded that morphometric examination alone is not sufficient and reliable in the species-specific identification and differentiation of *Fasciola* isolates. Threfore, to get more conclusive, molecular identification assay using PCR and further confirmation by sequencing is highly recommended.

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Introduction

Liver fluke disease or fasciolosis, or fascioliasis, is one of the most important helminth infections of ruminants in the world caused by digeneantrematodes belonged to the Phylum Platyhelminthes of Class Trematoda, and the genus *Fasciola* spp. which are a common liver flukes and the common species are *F. gigantica* and *F. hepatica* (Massoud *et al.,* 2012). The disease also known as liver not or distomatosis.

Mas-Coma *et al.* (2005) recorded that the *F. hepatica* can be found in temperate zones and *F. gigantica* in tropical areas, and that both species may be overlapped in subtropical zones. Fasciolosis is an example of an emerging/re-emerging human parasitic disease in the Caspian area (Iran and neighbouring), Andean countries (Bolivia, Peru, Chile, Ecuador), northern Africa (Egypt), the Caribbean area (Cuba) western Europe (Portugal, France and Spain) and countries) (Mas-Coma, 2004). 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 (Mas-Coma *et al.*, 2005). Furthermore, hybridization / introgression phenomena might take place where both species coexist(Periago *et al.,* 2008).*F. hepatica* and *F. gigantica* can generally be distinguished on the basis of their morphology (Ashrafi *et al.*, 2006). Fasciolids are identified primarily on differencein body shape and size of adults, with the smaller *F. hepatica* exhibiting wide and defined shoulders comparedto the slender *F. gigantica* having less defined shoulders and shorter cephalic cones (Khan, 2004). The use of molecular methods and markers are useful to distinguish intermediate forms and between species (Marcilla *et al.,* 2002).The first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rDNA) which occurs between the 18S, 5.8S, and 28S coding regions, have been used for diagnostic purposes at the level of species (Tandon *et al.,* 2007; Prasad *et al.,* 2008). The present study was aimed to Identify the morphology of *Fasciolasp* in Akut city and to use the PCR-RFLP technique for detection and identification of *Fasciola* spp. in addition to determin the sequencing of Fasciola spp.

Materials and methods

Species identification

Hepatic flukes recovered from each infected livers during the study were morphologically identified and recorded as *Fasciola* infections as well using the size parameters described by Soulsby, (1982).

Morphological examination

The identified worm isolates grouped into *F. hepatica* like. The body length (BL) and Body width (BW) were measured by using caliper as Shown in the Fig. (1).

Molecular analysis

Genomic DNA was isolated from all samples according to the protocol Relia Prep™ Blood gDNAMiniprep System, Promega.

Primers of the study

Amplification of the DNA was performed as described by Ghavami *et al.,*(2009) using a pair primer to amplify a 457 bp region of the ITS2 sequence. Forward primer:5.8S rDNA (5-TCTTGAACGCATATTGCGGC-3_) and Reverse primer : 28S rDNA (5_ AGTTCAGCGGGTAATCACGT-3_).

Restriction Fragment Length Polymorphism (RFLP)

The PCR-RFLP technique was performed in this study to distinguish specifically between both Fasciolid sp. (*F. gigantica* and *F. hepatica)* based on nucleotide differences detected in ITS2 region of rRNA gene. The restriction enzyme BamHI was selected. In comparison of the restriction maps of two fasciolid species shown that *BamHI* enzyme cuts only ITS2 fragment of *F. gigantica*at (230 and 340) nucleotide positions and digested products are expected to comprise 230, 110 and 20 bps(Ghavami *et*

al., 2009).

Standard Sequencing

PCR products were send for Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation – Korea. The results were received by email then analyzed using genious software. Analysis of data determined the sequence variation between samples of specific gene after amplification.

Results

Morphological examination results

Most of fasciolid worms that were obtained from infected livers were adults. Considering the general shape and the indices of BL/BW, the identified worm isolates grouped into *F. hepatica* like, showed that the mean of the body length (BL) ranged from 17 to 44 mm and the body width (BW) from 7.2 to 15.4 mm. The microsopical examination results of stained showed that the worms were leaf like and grayish brown in color flattened in their shape, and have morphological similarity to *F. hepatica* characteristics such as: broad and prominent shoulders and shorter body length Fig 2, smooth tegument Fig.3, pointed, smoothposterior end Fig. 4.

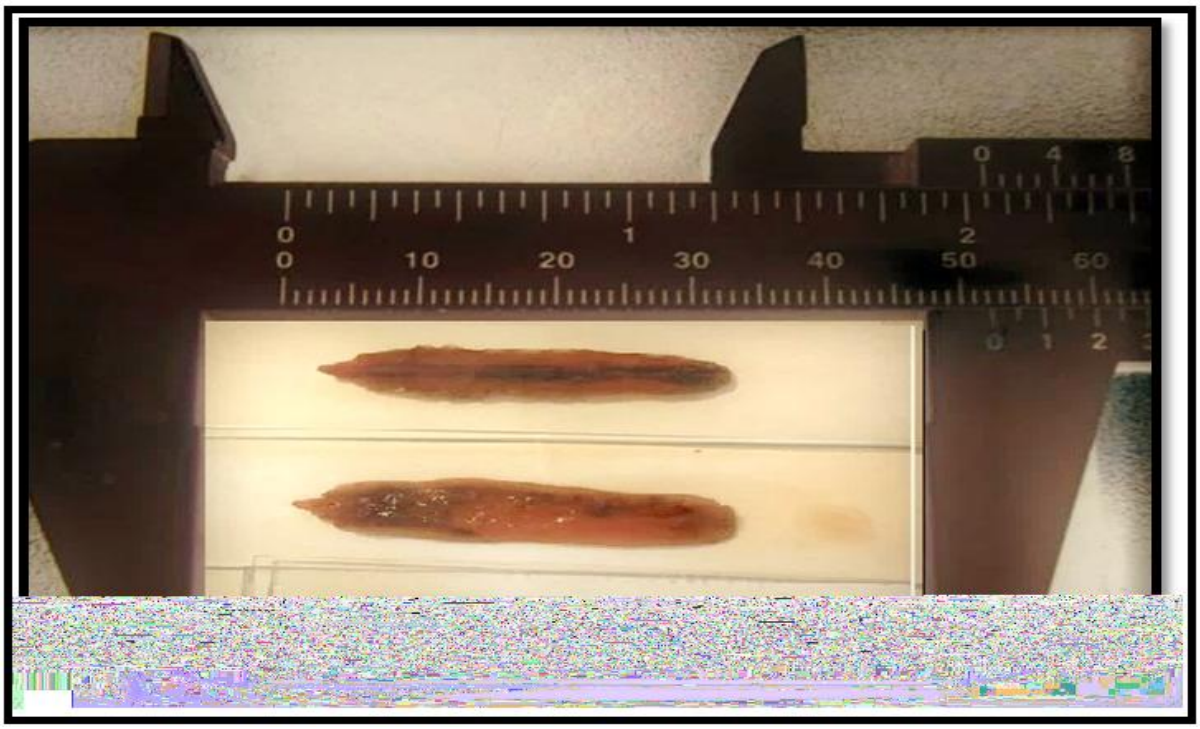

Fig. 1. *Fasciola* samples collected from infected sheep the worms were measured by using a caliper.

Molecular Diagnosis

Molecular detection of genus (Fasciola) by conventional PCR

The results of the PCR technique detection were shown in the Fig. 5.

Restriction Fragment Length Polymorphism (RFLP)

ITS2 – RFLP patterns of local fasciola isolates indicated that they all were identical to *F. hepatica* species that showed no restriction sites. However, nucleotide sequencing of the PCR products confirmed that there were no restriction sites for the *BamHI* restriction enzymes in the sequencing of the suspected isolates (Fig. 6).

The sequence analysis

In this study, partial sequence of mitochondrial 5.8S rRNA gene was used as a marker for molecular barcoding of Fasciola collected from sheep to determine the diversity prevalence of Fasciola species among sheep in alkut city. Genotype identification was done by comparing with available *Fasciola* DNA sequences in the GenBank based on sequence analysis of 5.8s rRNA region (457 bp).

Fig. 2. Adult Stage of *Fasciolaspp*stained with alum carmine, The anterior end with the cephalic cone 2 shoulders and converging margins (10X).

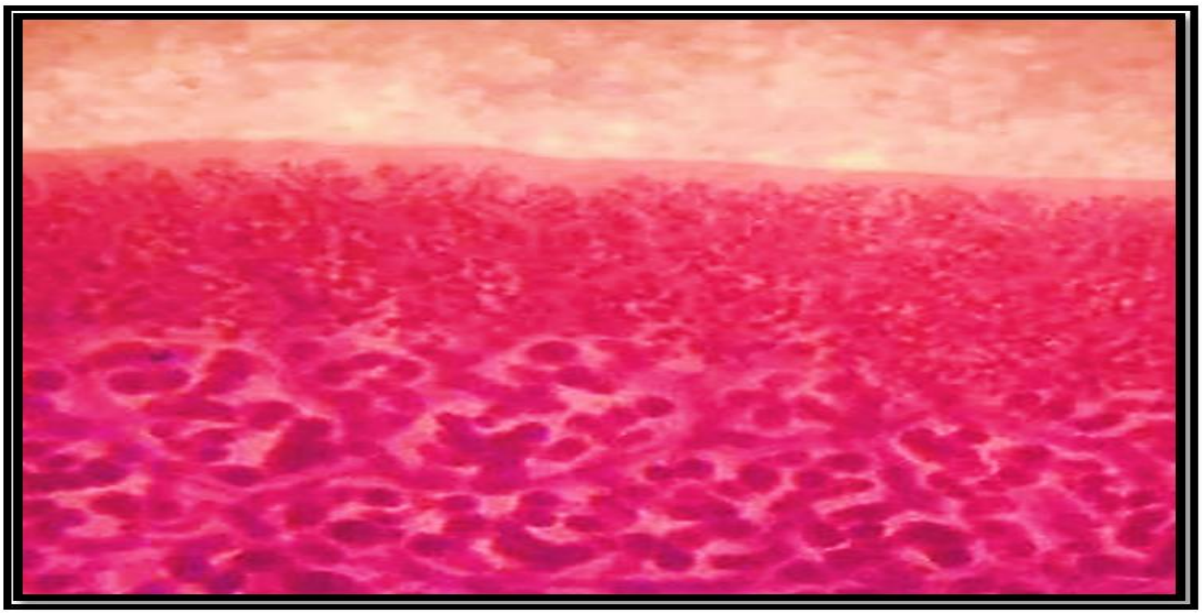

Fig. 3. Isolated *Fasciolaspp*stained with alum carmine, was showed smooth tegument (10X).

The Sequence alignment showed that isolated samples have complementary match in 99% with *Fasciola gigantica* and match in 98% with *Fasciola hepatica*.The local *fasciola* species were submitted in NCBI-Genbank database to get the Gene bank accession number for our fasciola isolates for the first time in Iraq with the accession no. : MK034874.1.

The phylogenetic tree indicated a new *Fasciola spp*. strain in Iraq which was approuch to Turkish and Iranian strains more than other strains such as Egypt, China, Japan and India (Fig. 7).

Discussion

Characterization of the causative agent of fasciolosis in each geographic region has been a major global concern from both veterinary medicine and public health points of view. For several years, the disease was considered to be caused by either of the two-fasciolid species, *F. hepatica* and *F. gigantica*. Later,

it was found that at least one intermediate form or likely yet-undescribed taxonomic units within the genus *Fasciola* are also responsible for fasciolosis worldwide (Lin *et al.,* 2007). Different *Fasciola* forms can also vary morphologically depending on their host species, so that the differences among the specimens from different hosts are even far greater than those usually observed between species of other flatworms (Dosay-Akbluta, 2005).

Fig. 4. The posterior end of isolated *Fasciola spp*.

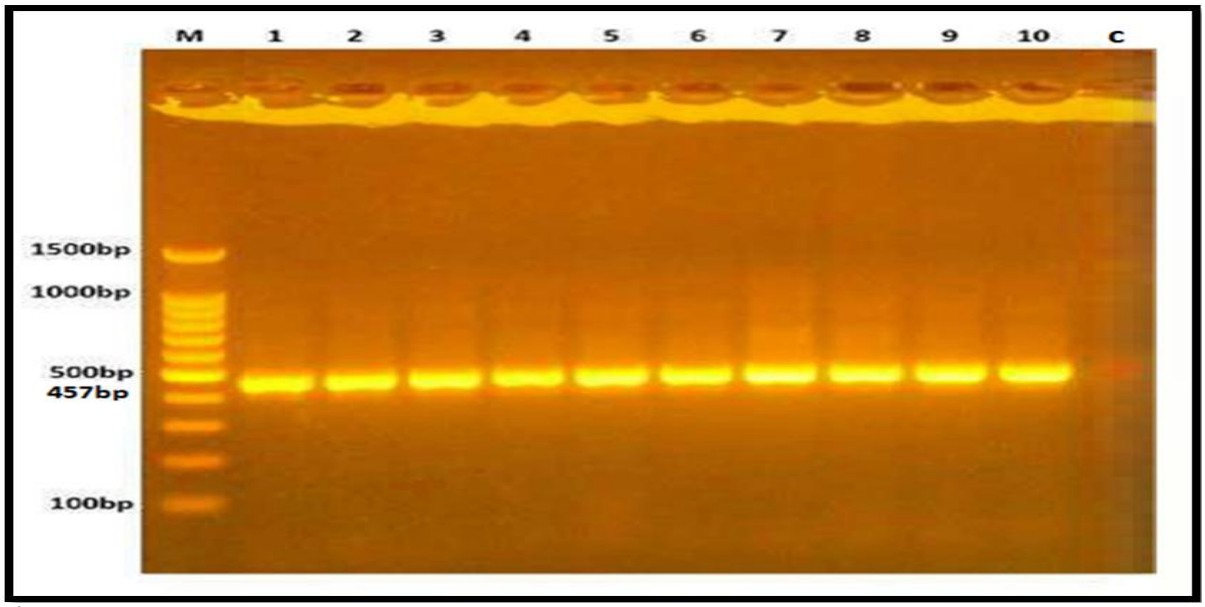

Fig. 5. Results of the presence of the specific gene ribosomal DNA ITS-2 region of fasciolid Liver flukes isolated from sheep were fractionated on 1% agarose gel electrophoresis stained with Eth.Br. Lane M :100bp DNA size marker. lane C : negative control.

The results in this study showed that the adult liver flukes are leaf like and grayish brown in color flattened in its morphology that was agreed with Radostitis *et al.*, (2000). Considering the general shape and the index of BL/BW, in present study the identified worm isolates grouped into *F. hepatica* like, with mean measured Body length, BL (17 to 44 mm) and Body width, BW (mm) (7.2 to 15.4 mm) that

results in agreement with De Vera *et al.* (2009) and Ghavami *et al.* (2009).

Some researchers believe that neither parasitological plus clinical tests nor immunological assays can differentiate between *F.hepatica*and *F. gigantica* (Marcilla *et al.,* 2002). Various studies were developed to identify Fasciola types. Of which, morphological characteristics of adult worms and eggs is used but this method of identification is affected by different factors including parasite age and good sample fixation (Mas-Coma, 2004).

Due to the presence of many similarities in morphological characteristics, differentiation between Fasciola species using classical morphological taxonomy become difficult (ItagakiandTsutsumi, 1998; Mas-Coma *et al.,* 1999)*F. hepatica* and *F. gigantica*are reproducing sexually through fertilization by mating with other individuals or by self-fertilization (Massoud *et al.,* 2012).

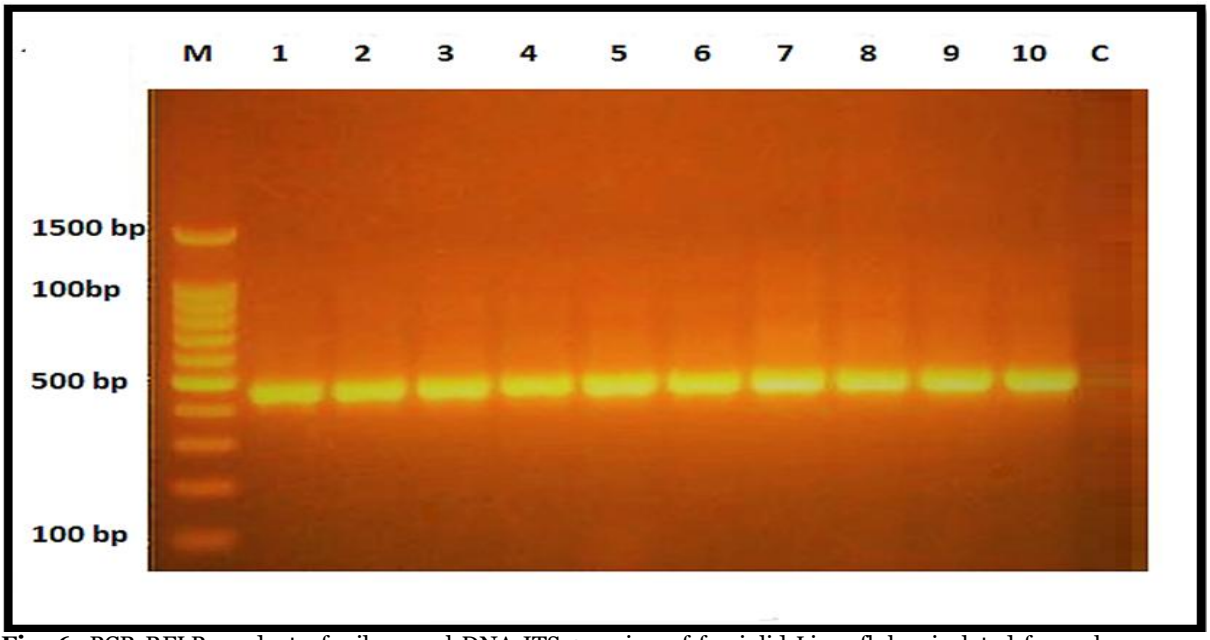

Fig. 6. PCR-RFLP productsof ribosomal DNA ITS-2 region of fasciolid Liver flukes isolated from sheep were fractionated on 1% agarose gel electrophoresis stained with Eth.Br.after digestion with *BamHI* enzyme. Lane M :100bp DNA size marker. lane C : negative contro.l.

As a result of difficulties in differentiating between two species of Fasciola morphologically, molecular methods using a polymerase chain reaction (PCR) based on the DNA sequences of several nuclear genes and gene spacers (Yuan *et al.,* 2016).

Also, mitochondrial genes such as mitochondrial 5.8S rRNA gene have been previously developed for precise identification, genotyping, intera-specific and inter-specific variations, and phylogenetic studies of these parasites (Nguyen *et al.,* 2009; Chamuah *et al.,* 2016).

El-Rahimy*et al.*(2012) supported the current findings as they concluded that conventional morphological and metric assessments were not useful for differentiation between *F. gigantica* and *F. hepatica* due to extensive overlap in the relative ranges.

For specific identification, the present study recommended genotyping using RFLP-PCR which gave consistent results and clear differentiation between the two species.

This makes DNA-based methods useful to identify unequivocally flukes, especially in regions where both species and possibly intermediate forms coexist **(**Rokni *et al.,* 2010).

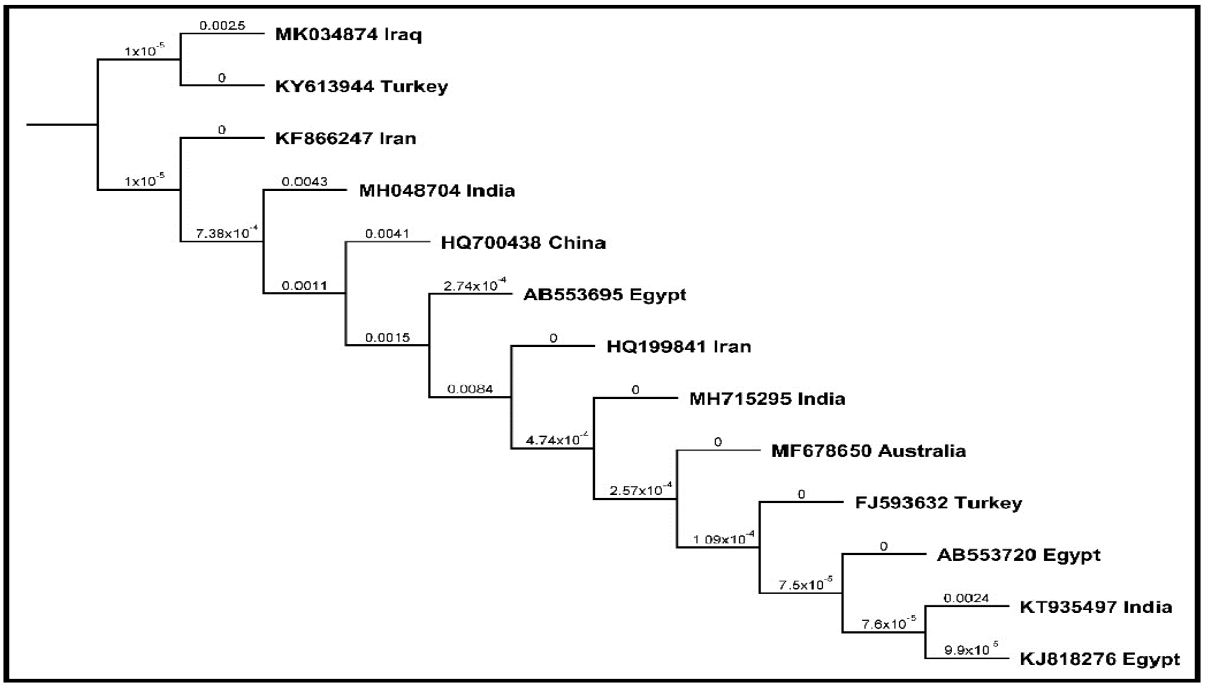

Fig. 7. Results of Phylogenitic tree of local *Fasciola spp*. isolates by the program Mega v.6. 457 bp based on 5.8S rRNA.

PCR-restriction fragment length polymorphism (RFLP) assays have been used in some studies of Fasciola(Itagaki *et al.,* 2005;Lin *et al.,* 2007). Both species of *F. hepatica* and *F. gigantica*were recognized in humanand domestic farm animals. Therefore, understanding genetic structure and status of genetic variation of these parasite populations has important consequences forepidemiology and effective control of fascioliasis (Ashrafi *et al.,* 2006).

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.

In our study, we used 5.8S and ITS2 for identification and differentiation of f*asciola*isolates by PCR-RFLP method. In addition to the two classical species of Fasciola, two intermediate or hybrid forms of Fasciola have been reported mostly from several Asian countries, based on analysis of nuclear and mitochondrial sequences. The first intermediate form bears the nuclear ribosomal sequence of *F. hepatica*, but has the mitochondrial sequence of *F. gigantica*(Le *et al.,* 2008; Peng *et al.,* 2009). The other form possesses typical nuclear ribosomal sequences (either ITS1 or ITS2) of both species in the same worm (Nguyen *et al.,* 2012).

These results led to the suggestion that interspecific cross-hybridization between the two species may have occurred (Agatsuma *et al.,* 2000).

The ITS2 restriction enzyme pattern obtained with *BamHI* seems to be a reliable, fast and straight forward criterion for differentiating between *F. hepatica* and *F. gigantica*. In this study used DNA extraction, then the detection genus *Fasciola* byusing PCR technique and for distinguish between both species usingrestriction enzyme *(BamHI*), accordingly were detected as *F.hepatica* in agree with Ghavami *et al.,* (2009)who conducted that*BamHI* and *PagI* restriction enzymes on ITS2 gene showed specificity for *F. hepatica* identification and had no effect on *F. gigantica*.Although nucleotide sequencing of the PCR products confirmed that there were no restriction sites for the *BamHI*restriction enzymes in the sequencing. The Sequence alignment showed that our isolated samples had complementary match in 99% with *Fasciolagigantica* and match in 98% with *Fasciola hepatica*.

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 (Ghavami *et al.,* 2009).

The possibility that intermediate forms of *Fasciola* spp. exist was revealedin the present study. When our results compare with morphological examination it could be vary because these results present *F. hepatica* in all samples. this vary may cause in such case because of different answers : 1) poor in molecular information of Iraqi isolates that shared with data base. 2) Isolated samples may have different taxonomy that need more study to fix it. The phylogenetictree with high bootstrap showed aclose relationship of our isolates with those sequences registered in Genbank *in* 99% with *F. gigantica*and 98% with *F. hepatica* from other regions of the world. Accordingto the phylogenetic tree, isolates belonging is closely approach to the Turkish and Iranian isolates.

We concluded that morphometric examination alone is not sufficient and reliable in the species-specific identification and differentiation of *Fasciola* isolates. Threfore, to get more conclusive, molecular identification assay using PCR and further confirmation by sequencing is highly recommended.

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