



## RESEARCH ARTICLE

### Molecular Identification of *Fasciola* spp. Isolated from Domestic Animals Based on DNA Sequencing of the Nuclear Ribosomal ITS1 -ITS2 Markers, Kurdistan Region, Iraq

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#### ABSTRACT

Fascioliasis caused by *Fasciola* species (*Fasciola hepatica* and *Fasciola gigantica*) is considered as the most important helminthic infection of domestic animals in developing countries. The present study was performed during January to December 2019, in Biology Department, University of Zakho. Hundred adult's flukes of *Fasciola* spp. were isolated from bile ducts and gallbladder of cattle, sheep and goats slaughtered at different abattoirs (Duhok, Zakho, Shelidiza, Aqrah and Bardarash) in Duhok governorate, Kurdistan region, Iraq. Genomic DNA extraction was performed using a Genomic DNA Extraction kit, Jena Bioscience GmbH (Germany). Specific markers ITS1 and ITS2 ribosomal DNA were used for amplifying 480 and 550 bps fragments. DNA sequences have been used to characterize these liver flukes. The PCR products were separated by electrophoresis in 1.5% agarose gel, visualized by staining with RedSafe dye, and photographed. ITS-1 and ITS-2 markers were amplified successfully, the length of produced band for ITS-1 was 480 bp and for ITS-2 was 550 bp. Thirteen *Fasciola* spp. flukes isolated from cattle, sheep and goats from different districts were sequenced, from these thirteen PCR products, 7 amplicons were recognized as *Fasciola hepatica* and 6 amplicons as *Fasciola gigantica*. These sequences were deposited in GenBank under accession numbers: MW161261, MW084365, MW052601, MW084349, MW082585, MW084348, MW082830 for *F. hepatica* and MW161260, MW052602, MW084353, MW084350, MW082589, MW085034 for *F. gigantica*. This study confirms the prevalence of *Fasciola hepatica* and *Fasciola gigantica* in Iraq by using the ITS-1 and ITS-2 rDNA.

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#### INTRODUCTION

Fascioliasis is a neglected zoonotic disease caused by *Fasciola* species (*F. hepatica* and *F. gigantica*) and found in more than 50 countries where sheep and cattle are reared (Mehmood *et al.*, 2017; Mir *et al.*, 2018) *Fasciola* spp. are highly pathogenic and pose serious threat to livestock (Abbas *et al.*, 2020). Fascioliasis infect more than 250 million sheep and 300 million cattle worldwide besides, causing great impact on the economy, it affects around 2.6 million humans globally (Burden *et al.*, 1983; Ayele and Hiko, 2016). Two species of *Fasciola* causes Fascioliasis; *Fasciola hepatica* and *Fasciola gigantica* which can morphologically differentiate by their length, body shape and cephalic cone (Keiser and Utzinger, 2009). Adult flukes become mature and reproduce in the

biliary ducts of the host and discharge their eggs which reach the intestine with the bile to pass out with the feces (Ashrafi *et al.*, 2006; Rondelaud *et al.*, 2009). Egg embryonation occur once they reach the freshwater within two weeks approximately depending on environmental conditions, they hatch out to free living swimming miracidium which subsequently infects the snail host. In the snail, the miracidium develops to sporocyst; redia and cercaria (Rondelaud *et al.*, 2009). Thousands of cercariae are emerged from each infected snail they swim freely in the aquatic environment and then encyst as metacercarial stage on aquatic plants. Domestic animals acquire infection by ingesting encysted cercariae on these plants, in the definitive host, they excyst in the duodenum by the effect of gastric and intestinal secretions, then they penetrate the intestinal wall and transferred within the

peritoneal cavity to invade the liver parenchyma finally reaching the bile ducts of the infected animal (Burden *et al.*, 1983). When the adult flukes are established in the biliary ducts, chronic fascioliasis begin associated with anemia, fibrosis, biliary stasis and cholangitis (Lopez *et al.*, 2012). The morphological differentiation of both species is time consuming and due to overlapping in the measurements, such as the ratio of body length to width, the presence of cephalic cone, the size of both suckers (Rokni *et al.*, 2010, Hasanpour *et al.*, 2020). Modern molecular approaches were used to distinguish between the two species of *Fasciola*. Since it is crucial to differentiate between the two species due to variation in morphological characteristics and also new generations of *Fasciola* are raised from mating between the two species of *Fasciola* make it difficult for taxonomic classification (Walker *et al.*, 2012). Therefore, the golden method for discrimination between both species of *Fasciola* is the sequences analysis of the first Internal Transcribed Spacers (ITS-1) of 5.8S ribosomal ribonucleic acid (rRNA) and second Internal Transcribed Spacers (ITS-2) of 28S ribosomal ribonucleic acid (rRNA) (Marcilla *et al.*, 2002; Le *et al.*, 2008).

The majority of the studies on *Fasciola* species in Iraq focused on the development, the prevalence, epidemiological and immunological aspects of the parasite. These studies included the effect of different temperatures on the development of intra-Mollusca stages of *F. gigantica* in Nineveh governorate (Al-Habbib and Al-Zako, 1981), the prevalence of liver fluke among slaughtered animals in Al-Najaf abattoir (Al-Alo *et al.*, 2019), epidemiological and immunological study for *F. gigantica* among cattle in Babylon province (Al-Delemi, 2005) and epidemiological study on *F. hepatica* in children and animals in Babylon City (Abdalnabi, 2012).

The most common identification tool used for identification of *Fasciola* species was by morphological assay, although the differentiation between both species is difficult due to interindividual variation within morphological characters (Periago *et al.*, 2006; Ashrafi *et al.*, 2014; Mohammed *et al.*, 2016). Therefore, the proper identification of the species isolated from animals or humans is crucial (Rokni *et al.*, 2010). For more precise identification of *Fasciola* species, isolated from animals or humans several molecular methods have been used (Chaichanasak *et al.*, 2012; Ichikawa and Itagaki, 2012) among these, the sequencing of the first and second internal transcribed spacers (ITS-1 and ITS-2) for rDNA, and mtDNA provided reliable genetic markers for species level identification (Ayoub *et al.*, 2015; Chamuah *et al.*, 2019). Hence DNA detection by using PCR assay has been applied for differentiating between both species by targeting rDNA or mitochondrial sequences (Ai *et al.*, 2010; Le *et al.*, 2012; Kordshooli *et al.*, 2017).

Few molecular studies have been carried out on *Fasciola* spp. isolated from domestic animals in Kurdistan region and other parts of Iraq by using ITS1 and ITS2 as specific DNA primer (Mohammed *et al.*, 2016; Hamoo *et al.*, 2020; Raof *et al.*, 2020). The purpose of the current study is to designate the molecular characteristics of *Fasciola* spp. isolated from domestic animals in five districts (Duhok, Zakho, Shelidiza, Aqrah and Bardarash) of Duhok governorate, Kurdistan region/Iraq, and to

analyze the phylogenetic relationships by sequences of ITS-1 and ITS-2.

## MATERIALS AND METHODS

**Samples collection:** Hundred adult flukes of *Fasciola* spp. (67 *F. hepatica* and 33 *F. gigantica*) were isolated from the bile ducts and gall bladder of naturally infected sheep, goats and cattle slaughtered at different abattoirs in Duhok governorate during January, 2019 to December 2019. The flukes were removed by fine forceps to avoid any damage. Each fluke was washed several times with 0.9% normal saline, and then they were identified morphologically as *Fasciola hepatica* or *Fasciola gigantica* depending on the description keys (Periago *et al.*, 2006; Mohammed *et al.*, 2016). The isolated flukes were preserved in 70% ethanol and stored at 4°C for DNA extraction using Jena Bioscience GmbH (Germany).

**DNA extraction:** The genomic DNA was extracted from these flukes using Genomic DNA Extraction kit provided by Jena Bioscience GmbH (Germany) based on the guidelines. The DNA concentration and purity was checked by Nanodrop Spectrophotometer 2000 (Thermo Scientific, US).

The DNA was amplified through polymerase chain reaction (PCR), two pairs of primers were used each two from the known sequences of *F. hepatica* and *F. gigantica*. The sequences of primers were obtained from NCBI data base. The sequence of ITS1 was (Forward: 5' ACCGGTGCTGAGAAGACG'3 and Reverse: 5' CGACGTACGTGCAGTCCA '3) and the sequence of ITS2 was (Forward: 5' GGTACCGGTGGATCACTCG GCTCGTG'3 and Reverse: 5' TATGCTTAAATTCAGC GGGT'3).

**DNA amplification using PCR:** The PCR amplification reaction was performed in 40 µl reaction tube consisting approximately of 20 µl Crystal Hot Start DNA Master Mix (2X), 4 µl for each forward and reverse primer, 4 µl of DNA template and 8 µl of nuclease free water for each of the primers ITS1 and ITS2. The amplification was performed using thermocycler (Gene AMP PCR system 9700 Thermocycler). The PCR cycling protocol for ITS 1 was as follows; one cycle of initial denaturation at 95 °C for 5 min; then 30 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1.5 min; and followed by one cycle of final extension at 72°C for 7 min (Aryaeipour *et al.*, 2014). PCR cycling protocol for ITS-2 was as follows; one cycle of initial denaturation at 94°C for 5 min; then 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec; and followed by one cycle of final extension at 72°C for 7 min (Phalee and Wongsawand, 2014).

Also, touch down PCR program was performed for 30 samples by using both primer ITS1 and ITS2, this method was used to increase the efficiency of amplification with a range of annealing temperature (55-65°C) to reduce the primer dimers formation and to eliminate the nonspecific bands of primer to DNA template.

PCR products were run on 1.5% (w/v) agarose gel in 100 ml of 1X TBE buffer stained with RedSafe Dye at 45 Volt for 15 min then 85 Volt for 60 min. To each gel, A 100-1000 bps DNA ladder was added as references ladder for determining the band size. The gel was viewed using UV Transilluminator to confirm amplification.

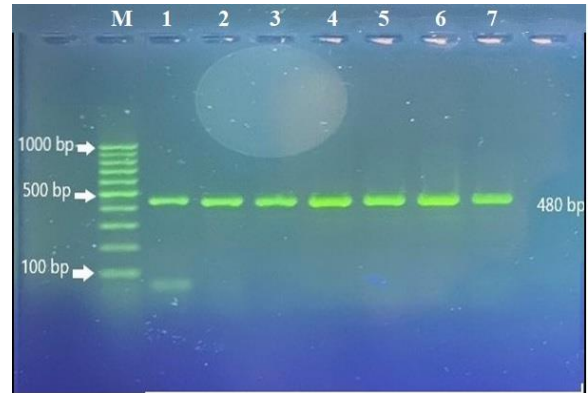
**DNA sequencing:** Thirteen amplified PCR products of *Fasciola* species were selected based on different abattoirs (Duhok, Zakho, Aqrah, Bardarash and Shelidiza) types of animals (cattle, sheep and goats), and location (bile ducts or gall bladder) they were submitted to MacroGen Company (Korea) for DNA sequencing and for sequence analysis database such as Basic Local Alignment Search Tool (BLAST) was applied to identify the obtained DNA sequences (<http://www.ncbi.nlm.nih.gov/BLAST>). All sequences were cleaned up and aligned by BioEdit application. To construct a phylogenetic tree for the obtained specimens, MEGA Software Neighbor Joining methods and Kimura's 2-parameter model were used for analysis (Kumar *et al.*, 2018), the branches robustnesses were measured by bootstrap analysis with 10 QWSW00 replicates.

## RESULTS

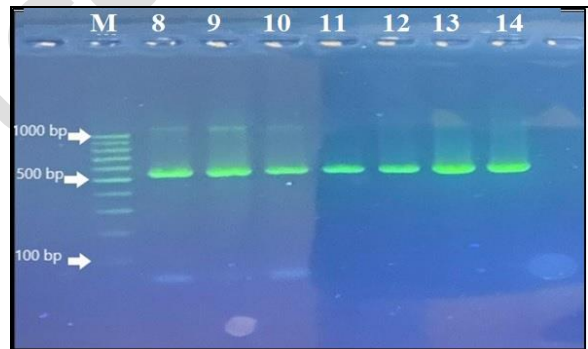
The concentration of DNA ranged from 11.7 to 185 ng/ $\mu$ l, while the purity was 1.8-2.0 measured at A260/A280 nm. The amplified DNA of *F. hepatica* and *F. gigantica* generated DNA fragments of 480 and 550 bps, respectively as shown in Figures (1 and 2). Touch down PCR methods using both primer ITS1 and ITS2 PCR product were illustrated in Figure (3). The PCR products using internal transcribed spacer 1 (ITS1) revealed 480 bps DNA fragment for *F. hepatica* and *F. gigantica*. While PCR amplification of internal transcribed spacer 2 (ITS2) revealed 550 bps fragments for *F. hepatica* and *F. gigantica* (Figures 1 and 2). From these, thirteen PCR products were sent for sequencing, 7 amplicons recognized as *Fasciola hepatica* and 6 amplicons as *Fasciola gigantica*. The resulting accession numbers and their sequences were compared to most related sequences of *Fasciola* spp. The phylogenetic tree was built by comparing the resulted sequences with their accession numbers with references from GenBank sequences of *F. hepatica* and *F. gigantica* (Figures 4 and 5). The results from phylogenetic tree confirm that the results obtained by PCR, subsequently sequenced belonging to the same species are clustered together. The Blast analysis of ITS1 and ITS2 rDNA showed homology to references obtained from GenBank. Concerning *Fasciola hepatica*, the obtained sequences share 100% homology to Vietnamese isolates (MN970008 and MN970007), 99.7% to Iranian isolates (MK377151, MK377150, MK377145 and MK377143), 99.5% to Mexicans and Saudi Arabians isolates (MG569981 and MK212149) and 98.7% to Iranian and Saudi Arabian isolates (MK377149, MK377148, MN559388 and MN559387). The sequences analysis of *Fasciola gigantica* shares 100% homologies to Vietnamese isolates (MN97000 and MN9700078), 99.7% to Nigerian isolates (MT644667 and MT644666), 98.1% to Vietnamese isolates (MT429180) 97% to Chinese and Indian isolates

(HQ700438 and KC535542), 93.5% to Nigerian isolates (MW082589 and MN608169) and 91.8% to Vietnamese isolates (MT429182 and MT429181).

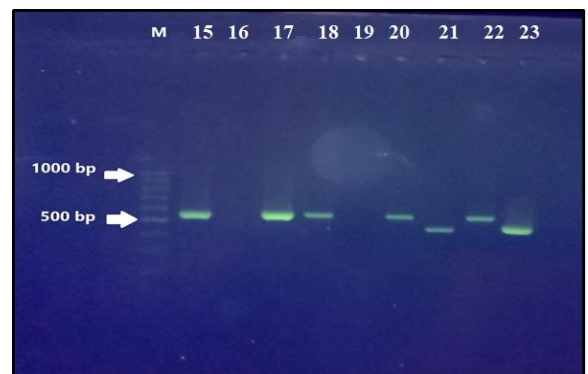
The phylogenetic tree of *Fasciola hepatica* showed high relatedness among isolates of the same species, they were generally assigned into two main clusters; three belonged to the first cluster and four sequences belonged to the second cluster (Figure 4). The phylogenetic tree of *Fasciola gigantica* showed high relatedness among



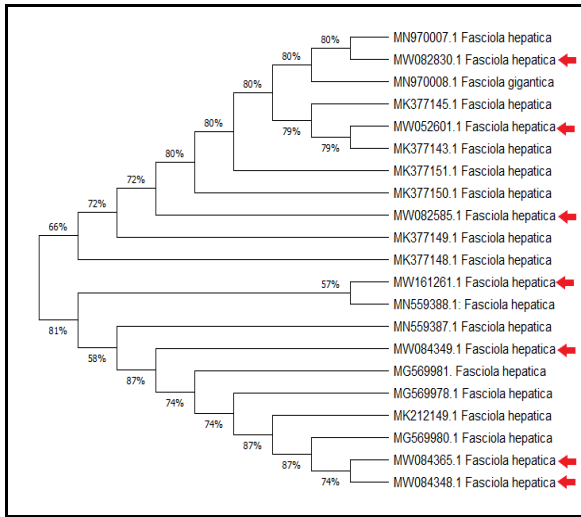
**Fig. 1:** Agarose gel (1.5%) product (size 480 bp) of DNA for 18S rDNA gene *Fasciola* spp. using species specific ITS1 primers: M: Ladder (100-1000 bp); 1 from gall bladder and 3 from bile duct of cattle (Duhok); 2 from gall bladder and 4 from bile ducts of sheep (Duhok); 5 from gall bladder of cattle (Zakho); 6 from bile duct of goats and 7 from bile ducts of sheep (Shelidiza).



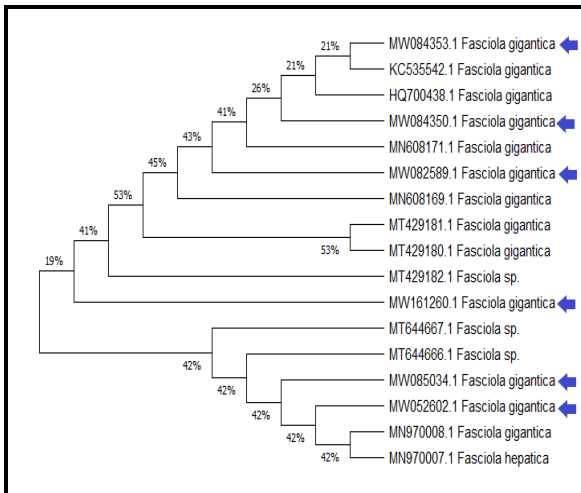
**Fig. 2:** Agarose gel (1.5%) PCR product (550 bp) of DNA for 18S rDNA gene of *Fasciola* species using species specific ITS2 primers: M: Ladder (100-1000 bp); 8 from gall bladder of cattle, 10 from bile ducts of goats, 9 from gall bladder and 11 from bile duct of sheep (Aqrah), 12 from gall bladder of sheep and 14 from bile duct (Zakho) and 13 from bile ducts of sheep (Bardarash).



**Fig. 3:** Agarose gel electrophoresis (1.5%) using Touch Down PCR method with both primers ITS1 and ITS2 PCR products. Lane (M) DNA ladder, Lanes 15, 17, 18, 20 and 22 amplicon size 550 bps and Lanes 16, 19, 21 and 23 amplicon size 480 bps.



**Fig. 4:** Phylogenetic relationship of *Fasciola hepatica* isolated from sheep, goats and cattle. In Iraq and other isolated species in different countries. Phylogenetic tree was aligned by using Muscle method associated with nucleotide substitution models (K2: Kimura's 2-parameter model). Tree was made by using MEGAX VERSION 10 with bootstrap value of 1000 replicates for neighbor joining.



**Fig. 5:** Phylogenetic relationship of *Fasciola gigantica* isolated from sheep, goats and cattle. In Iraq and other isolated species in different countries. Phylogenetic tree was aligned by using Muscle method associated with nucleotide substitution models (K2: Kimura's 2-parameter model). Tree was made by using MEGAX VERSION 10 with bootstrap value of 1000 replicates for neighbor joining.

isolates of the same species, they were generally assigned into two main clusters; most of them belonged to the first cluster (four sequences) except *Fasciola gigantica* (MW161260) clustered as monophyletic among the first cluster and the remaining two sequences belonged to the second cluster (Figure 5).

## DISCUSSION

DNA -based molecular methods in comparison with others diagnostic methods for identification of *Fasciola* species are accurate and reliable. Therefore, various DNA markers are needed to identify *Fasciola* spp. such as ITS1 and ITS2. The sequencing results of ITS 1 and ITS 2-region revealed that both *F. hepatica* and *F. gigantica* exist in Duhok province. Notably, this study represents the first accurate one conducted in Duhok province using

sequencing analysis of the PCR products of ITS1 and ITS 2. Both marker ITS-1 and ITS-2 are coding the sequences between 18S, 5.8S, and 28S coding regions, since their highly conserved and repeatable sequences, is used in molecular studies (Prasad *et al.*, 2008; Prasad *et al.*, 2009; Kostadiniva *et al.*, 2013). Several studies on identification of *Fasciola* species have been reported in different countries worldwide, such as Japan, Korea, Spain, India and Turkey to discriminate between *F. hepatica* and *F. gigantica* (Semyenova *et al.*, 2005; Alasaad *et al.*, 2007; Erensoy *et al.*, 2009). Although, morphological and immunological assays were used to differentiate between *Fasciola hepatica* and *Fasciola gigantica* species, the morphological characters of eggs are very similar, hence it is difficult to differentiate between both species (Periago *et al.*, 2008). Molecular tool was used for specific differentiation between the two species (Ashrafi *et al.*, 2006, Periago *et al.*, 2008). The characterization of intermediate host (snail) is misunderstanding because it can occur especially in area where both species of *Fasciola* are prevalent, where hybrids interbreed most present (Periago *et al.*, 2008). Hence, molecular methods are very valuable for useful identification and as diagnostic tools on genetic variation of the *Fasciola* species (Mas-Coma *et al.*, 2005).

**Conclusions:** The molecular technique in addition to phylogenetic analysis of *Fasciola* species in the current study revealed that the parasite isolated from sheep, cattle and goats recognized as *F. hepatica* and *F. gigantica*. The morphological and genetic features of *Fasciola* species represented in this study is considered as suitable tool to obtain accurate and basic information necessary for control of fascioliasis among domestic animals as well as human. Besides understanding the genetic variation and genetic structure within species of *Fasciola* and their dynamic transmission in Iraq and other neighboring countries.

**Authors contribution:** ChAN conducted the experiments, prepared the tables and graphs, and wrote the draft of the manuscript. ABM and WMSM designed and supervised the project and reviewed the article.

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