Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2021.066

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

Ahmed B. Mohammed[1], Wijdan M.S. Mero[1,2*] and Chira A. Nerway[1]

[1]Department of Biology, Faculty of Science, University of Zakho, Iraq
[2]Department of Biology, College of Science, Nawroz University, Duhok, Iraq
*Corresponding author: wijdan.mero@uoz.edu.krd

ARTICLE HISTORY (21-275)

Received: June 28, 2021
Revised: August 09, 2021
Accepted: August 11, 2021
Published online: September 05, 2021

Key words:
Fasciola species
Genetic identification
ITS1
ITS2
Phylogenetic analysis

A B S T R A C T

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-1 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.

To Cite This Article: Mohammed AB, Mero WMS and Nerway CA, 2022. Molecular identification of *Fasciola* spp. isolated from domestic animals based on DNA sequencing of the nuclear ribosomal its1 -its2 markers, Kurdistan Region, Iraq. Pak Vet J, 42(2): 246-250. http://dx.doi.org/10.29261/pakvetj/2021.066

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 excysts 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; Raoof *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˚Cfor 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 ITSI and ITS2, this method was used to increase the efficiency of amplification with a range of annealing temperature (55-65ºC) 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.ncbinlm.nih.gov/BLAST). All sequences were cleaned up and aligned by BioEdite application. To construct a phylogenic 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 with10 QWSW00 replicates.

RESULTS

The concentration of DNA ranged from 11.7 to 185 ng/µl, 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 Fig. 1 and 2. Touch down PCR methods using both primer ITS1 and ITS2 PCR product were illustrated in Fig. 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* (Fig. 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 phylogenic tree was built by comparing the resulted sequences with their accession numbers with references from GenBank sequences of *F. hepatica* and *F. gigantica* (Fig. 4 and 5). The results from phylogenic 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 (MG569981and 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 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 Nigeria 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

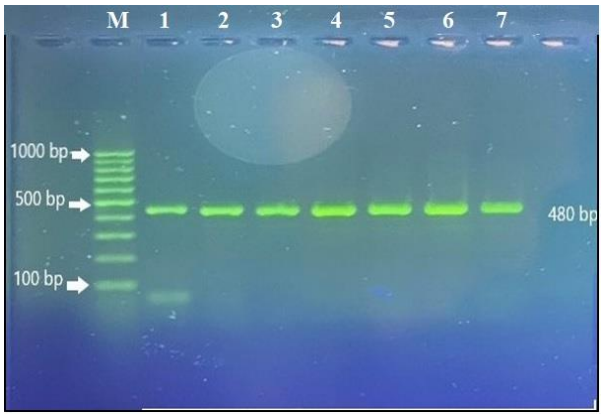

Fig. 1: Agarose gel (1.5%) product (size 480 bp) of DNA for 18S rDNA gene *Fasciola* spp. using species specific ITS1 primers: M: Ladder (100-1000 bp); 1 from gall bladder and 3 from bile duct of cattle (Duhok); 2 from gall bladder and 4 from bile ducts of sheep (Duhok); 5 from gall bladder of cattle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

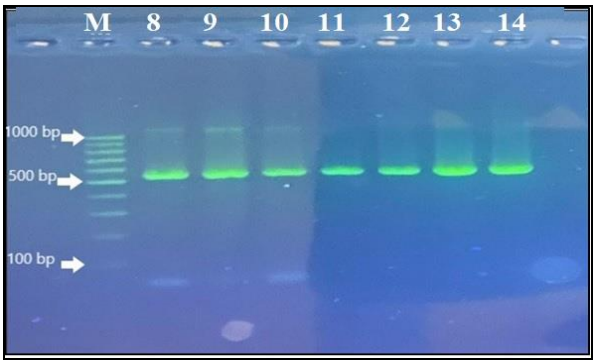

Fig. 2: Agarose gel (1.5%) PCR product (550 bp) of DNA for 18S rDNA gene of *Fasciola* species using species specific ITS2 primers: M: Ladder (100-1000 bp); 8 from gall bladder of cattle, 10 from bile ducts of goats, 9 from gall bladder and 11 from bile duct of sheep (Aqrah), 12 from gall bladder of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

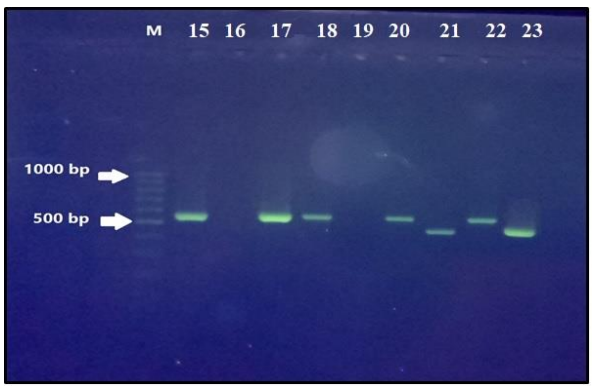

Fig. 3: Agarose gel electrophoresis (1.5%) using Touch Down PCR method with both primers ITS1 and ITS2 PCR products. Lane(M) DNA ladder, Lanes 15, 17, 18, 20 and 22 amplicon size 550 bps and Lanes 21 and 23 amplicon size 480 bps.

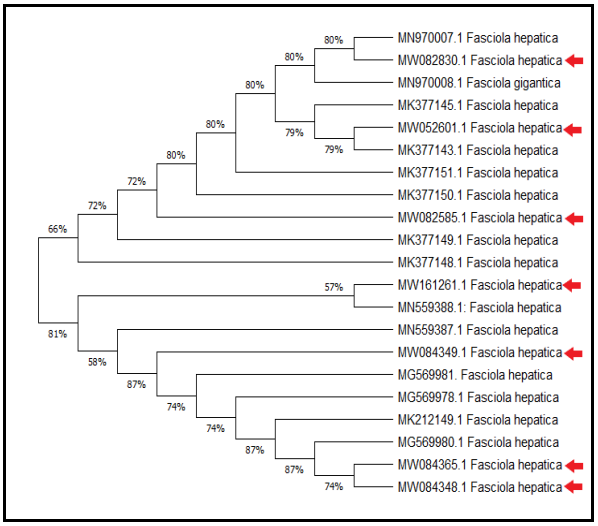

Fig. 4: Phylogenetic relationship of *Fasciola hepatica* isolated from sheep, goats and cattle. In Iraq and other isolated species in different countries. Phylogenic tree was aligned by using Muscle method associated with nucleotide substitution models (K2: Kimura's 2-parameter model). Tree was made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

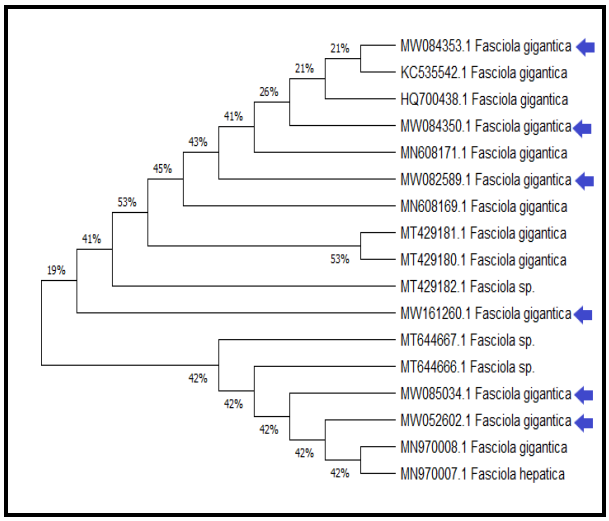

Fig. 5: Phylogenetic relationship of *Fasciola gigantica* isolated from sheep, goats and cattle. In Iraq and other isolated species in different countries. Phylogenic tree was aligned by using Muscle method associated with nucleotide substitution models (K2: Kimura's 2-parameter model). Tree was made 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 courtiers 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 *F. hepatica* and *F. 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 phylogenic 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.

REFERENCES

Abbas RZ, Zaman MA, Sindhu ZUD, *et al.*, 2020. Anthelmintic effects and toxicity analysis of herbal dewormer against the infection of *Haemonchus contortus* and *Fasciola hepatica* in goat. Pak Vet J 40:455-60.

Abdalnabi RA, 2012. Epidemiological study on *Fasciola hepatica* in children and animals at Babylon city. Al- Mustansiriyah J Sci 23:19-26.

Ai L, Dong SJ, Zhang WY, *et al.*, 2010. Specific PCR-based assays for the identification of *Fasciola* species: their development, evaluation and potential usefulness in prevalence surveys. Ann Trop Med Parasitol 104:65-72.

Al-Alo KZ, Albishtue AA and A-Dujaily AH, 2019. Retrospective Survey of Liver flukes in Sheep and Cattle based on Abattoir Data in Al-Najaf Province, Iraq". Acta Sci Vet Sci 1:02-8.

Alasaad S, Huang CQ, Li QY, *et al.*, 2007. Characterization of *Fasciola* samples from different host species and geographical location in Spain by sequence of internal transcribed spacers rDNA. J Parasitol Res 101:1245-50.

Al-Delemi JKA, 2005. Epidemiological and immunological study for *Fasciola gigantica* among cattle in Babylon province. Ph.D. Thesis, College of Veterinary Medicine, University of Baghdad, Iraq.

Al-Habbib WMS and Al-Zako SS, 1981. The effect of different temperatures on the development of intra-Molluscan Stages of *Fasciola gigantica*. J Ther Biol 6:373-7.

Aryaeipour M, Rouhani S, Bandehpour M, *et al*., 2014. Genotyping and phylogenetic analysis of Fasciola spp. isolated from sheep and cattle using PCR- RFLP in Ardabil province, northwestern Iran. Iranian J Pub Health 43:1364-71.

Ashrafi K, Valero M, Panova M, *et al*., 2006. Phenotypic analysis of adults of *Fasciola hepatica*, *Fasciola gigantica* and intermediate forms from the endemic region of Gilan, Iran Int J Parasitol 55:249-60.

Ashrafi K, Bargues MD, O'Neill S, *et al.,* 2014. Fascioliasis: a worldwide parasitic disease of importance in travel medicine. Travel Med Infect Dis 12:636-49.

Ayele M and Hiko A, 2016. Review on the Biology of *Fasciola* Parasites and the Epidemiology on Small Ruminants. Adv Life Sci Technol 48:19-27.

Ayoub MB, Wahba AA and Ibrahim M, 2015. Molecular characterization of *Fasciola* spp. in sheep and cattle. Animal Heal Res J 3:67-75.

Burden D, Bland A, Hammet N, *et al*., 1983. *Fasciola hepatica*: migration of newly excysted juveniles in resistant rats. Exp Parasitol 56:277-88.

Chaichanasak P, Ichikawa M, Sobhon P *et al*., 2012. Identification of *Fasciola* flukes in Thailand based on their spermatogenesis and nuclear ribosomal DNA, and their intraspecific relationships based on mitochondrial DNA. Parasitol 61:545-59.

Chamuah JK., Borkotoky D, Amenti K, *et al*., 2020. Molecular characterization and histopathological studies on Fasciola gigantica in Mithun (Bos frontalis). Indian J Anim Res 54:1012-7.

Erensoy A, Kuk S and Ozden M, 2009. Genetic Identification of *Fasciola hepatica* by ITS-2 sequence of nuclear ribosomal DNA in Turkey. J Parasitol Res 105:407-12.

Hamoo RN, Al-Rubaye FS and Mustafa NG, 2020. Genotyping study of *Fasciola gigantica* isolated from cattle in Aqrah city, Iraq. Iraqi J Vet Sci 34:123-7.

Hasanpour H, Falak, Naddaf SR, *et al*., 2020 Molecular characterization of *Fasciola* spp. from some parts of Iran. Iran J Public Health 49:157-66.

Ichikawa M and Itagaki T, 2012. Molecular analysis of a spermic *Fasciola* flukes From Korea on the basis of the nuclear ITS1 region and mitochondrial DNA markers and comparison with Japanese aspermic Fasciola flukes. J Vet Med Sci 74:899-904.

Keiser J and Utzinger J, 2009. Food-borne trematodiases. Clin Microbiol Rev 22:466-83.

Kordshooli MS, Solhjoo K, Armand B, *et al*., 2017. A reducing trend of fasciolosis in slaughtered animals based on abattoir data in South of Iran. Vet World 10:418-23.

Kostadiniva A, Georgieva S, Selbach C, *et al.,* 2013. New cryptic species of 'revolutum' group of Echinostoma (Digenea: Echinostomatidae) revealed by molecular and morphological data. Parasites and Vectors 6(64).

Kumar S, Stecher G, Li M, *et al.,* 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biol Evol 35:1547-9.

Le TH, De NV, Agatsuma T, *et al*., 2008. Human fascioliasis and the presence of hybrid/introgressed forms of *Fasciola hepatica* and *Fasciola gigantica* in Vietnam. Int J Parasitol 38:725-30.

Le TH, Nguyen KT, Nguyen NT, *et al.,* 2012. Development and evaluation of a single-step duplex PCR for simultaneous detection of *Fasciola hepatica* and *Fasciola gigantica* (family Fasciolidae, class Trematoda, phylum Platyhelminthes). J Clin Microbiol 50:2720-6.

Lopez M, White AC and Cabada MM, 2012. Burden of *Fasciola hepatica* infection among children from Paucartambo in Cusco, Peru. The Am J Trop Med Hyg 86:481-5.

Marcilla A, Bargues MD and Mas-Coma S, 2002. A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *F. gigantica*. Mol Cell Probes 16:327-33.

Mas-Coma MS, Bargues MD and Valero MA, 2005. Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol 35:1255-78.

Mehmood KH, Zhang AJ, Sabir RZ, *et al*., 2017. A review on epidemiology, global prevalence and economical losses of fasciolosis in ruminants. Microb Pathog 109: 253-262.

Mir S, Dabirzadeh M, Ronki MB, *et al*., 2018. Identification and Phylogenetic Classification of *Fasciola* species Isolated from Sheep and Cattle by PCR-RFLP in Zabol, in Sistan and Baluchistan Province, Southeast Iran. Iran J Public Health 48:934-42.

Mohammed A, Mustafa A, Issa A, *et al*., 2016. Molecular Characterization of *Fasciola* spp. Isolated from the Gallbladder of Infected Cattle in Duhok Province, Kurdistan Region Iraq. Sci J Uni Zakho 4:37-42.

Periago MV, Valero MA, Panova M, *et al*., 2006. Phenotypic comparison of allopatric populations of *Fasciola hepatica* and *Fasciola gigantica* from European and African bovines using a computer image analysis system (CIAS). Parasitol Res 99:368-78.

Periago MV, Valero MA, Elsayed M, *et al*., 2008. First phenotypic description of *Fasciola hepatica/ Fasciola gigantica* inter mediate from the human endemic area of the Nile Delta, Egypt. Infection. Gen Evol 8:51-8.

Phalee A and Wongsawad C, 2014. Prevalence of infection and molecular confirmation by using ITS-2 region of *Fasciola gigantica* found in domestic cattle from Chiang Mai province, Thailand. Asian Pacific J Trop Med 7:207-11.

Prasad PK, Tandon V, Biswal DK, *et al.,* 2008. Molecular identification of Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae) based on ribosomal internal transcribed spacer regions). J Parasitol Res 103:1247-55.

Prasad PK, Tandon V, Biswal DK, *et al*., 2009. Use of sequence motifs as barcodes and secondary structures of internal transcribed spacer 2(ITS-2, rDNA) for dentification of the Indians liver fluke, *Fasciola* (Trematoda: Fasciolidae). Bioinformation pp:314-20.

Raoof HS, Marif HF, Rahman HS, *et al*., 2020. Molecular characterization and phylogenetic analysis of *Fasciola* species in sheep and goats in Sulaymaniyah Province, Northern Iraq. J Zankoy Sulaimaniyah 22:298-306.

Rokni MB; Mirhendi H, Mizani A, *et al.,* 2010. Identification and differentiation of *Fasciola hepatica* and *F. gigantica* using a simple PCR-restriction enzyme method. Exp Parasitol 124:209-13.

Rondelaud D, Belfaiza M, Vignoles P, *et al.,* 2009. Redial generations of *Fasciola hepatica*: a review. J Helminthol 83:245-54.

Semyenova S, Morozova EV, Vasilyev VA, *et al*., 2005. Polymorphism of internal transcribed spacer 2 (ITS-2) sequences and genetic relationships between *F. hepatica* and *F. gigantica*. Acta Parasitol 50:240-3.

Walker SM, Prodohl P, Hoey EM, *et al.,* 2012. Substantial genetic divergence between morphologically indistinguishable populations of *Fasciola* suggests the possibility of cryptic speciation. Int J Parasitol 42:1193-9.