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Liver Fluke Species Identification Isolated From Humans and Animals Using PCR-RFLP and DNA Sequencing

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ABSTRACT

Fasciola species are a member of flatworms belonging to the trematodes (flukes), commonly known as liver fluke, they are extremely pathogenic parasites that affect the liver of humans and animals, nowadays, most laboratories and research facilities use molecular-based techniques for identifying and describing *Fasciola* species. The molecular diagnostic markers such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and PCR-RFLP methods are accurate and more specific than the immunological and microscopical methods. The identification of the species of liver flukes will give a new clue for the treatment and control of fascioliasis. The aim, of this study, is to find the molecular characterization of *Fasciola* spp. isolated from humans and animals in Sulaimani city. The flukes were isolated from humans using endoscopic techniques and from slaughtered livestock at the new slaughterhouse of Sulaimani, 48 liver flukes were collected from different hosts; human (n = 3), cattle (n = 20), sheep (n = 20), and goats (n = 5) from October 2021 to April 2022. The uinversal primers ribosomal Deoxy Ribo Nuclic Acid (rDNA) were used, then the PCR products were subjected to restriction fragment polymorphism (RFLP) assay and The PCR Product was digested with restriction enzymes DraII, also the DNA sequencing was used for the PCR product of the primer Cytochrome Oxidase subuint 1 (*COX1*). The results of the PCR-RFLP of the 28s rDNA show the genetic polymorphisms among the flukes and two patterns of RFLP were observed *F. hepatica*, and *F. gigantica*, also the sequence analysis of the partial gene of the COX1 showed the isolated flukes belonged to *F. hepatica* and *F. gigantica* with some genetic variation, and the result of the sequences was deposited in the Gene Bank under the following Accession numbers; *F. gigantica* (OP718780 and OP718781) and *F. hepatica* (OP718782, OP718783, and OP718784). The present study concludes that *F. hepatica* and *F. gigantica* are both responsible for human and animal Fasciolasis in Kurdistan-Iraq, Therefore, RFLP techniques and DNA sequencing are a reliable, and differential method for species and genotyping identification of liver fluke.

Index Terms: *Fasciola hepatica*, *Fasciola gigantica*, Polymerase chain reaction, Restriction fragment length polymorphism, Liver fluke

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1. INTRODUCTION

The Fasciola species is an extremely pathogenic parasite that affects humans and animals and this disease has a dual impact on human health and economic losses [1], [2]. The parasite habitat is the livers and bile ducts of humans and animals.

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In livestock, it causes numerous financial losses, such as a decrease in milk, meat, and wool production [3]. This fluke directly affects livestock productivity through host mortality and partial or complete liver condemnation. Furthermore, it has an indirect effect on the host growth and feeding, which negatively reflect on the white and milk yield [4]. The effect of animal Fasciolasis on weight will be varied depending on the age group and poorer carcass quality indicators [5]. The human Fascioilasis which is known as a neglected disease is occurring in the area where the peoples eat raw water plants that may be contaminated with metacercaria [6]. Due to the epidemiological traits of *Fasciola hepatica* and *Fasciola gigantica* infections in humans, accurate and precise diagnoses are crucial for early uncomplicated treatment. The species of liver fluke have differential characteristics and aspects, also the different type of lymnaeid snails serve as the intermediate host of the liver flukes [7]. The clinical, pathological, and immunological diagnosis approaches are not efficient methods to differentiate between the species of liver fluke [7], [8]. Furthermore, the morphometric methods faced many limitations, the differentiation of *F. hepatica* and *F. gigantica* is currently based on implementing numerous molecular methods using various DNA markers [7]. Various molecular diagnostic tools, including polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (RFLP), and *rDNA* sequencing, have been used to find the genetic variation and species identification of *Fasciola* spp. [9], [10], [11], [12]. Furthermore, due to the shortcomings of the morphometric approach, many molecular strategies have been applied for genotyping and classifying liver flukes throughout the world. These strategies use various molecular markers [13]. A quick and straightforward PCR-RFLP experiment using the common restriction enzyme Dra II is presented to differentiate between species of liver flukes. Based on the 28S, rDNA gene sequence recently discovered the polymorphism among liver fluke populations [9], [14]. The rDNA region has been applied to establish the genetic polymorphism of *Fasciola* spp. in Spain. This revealed that heterozygous specimens had nucleotide variants [15]. In a new study, a genetically varied type of liver fluke was discovered, named *Fasciola intermediate*, it is morphologically similar to *F. gigantica* and *F. hepatica* [16]. The present study is an attempt to find the species of the liver flukes isolated from humans and animals using PCR-RFLP and DNA sequencing.

2. MATERIALS AND METHODS

2.1. Sample and Data Collection

The 48 liver flukes were collected from different hosts; humans (n = 3), cattle (n = 20), sheep (n = 20), and goats (n = 5). The data on human fascioliasis and the flukes was taken from the Kurdistan Center for Gastroenterology and Hepatology in Sulaimani. Furthermore, the animal data were collected in the new Sulaimani slaughterhouse from November 2021 to April 2022. The flukes were preserved in 70% ethanol, then transported to the laboratory of Sulaimani Polytechnic University and the genetic laboratory of the University of Human Development in Sulaimani Kurdistan-Iraq.

2.2. DNA Extraction and PCR

The DNA was extracted from the tissue of the isolated worm (whole body of the worm), the liver flukes were ground individually in an Eppendorf tube using micro-pistil, and the commercial DNA extraction kit (ADD prep, Tissue Genomic DNA extraction kit) was used. The extracted DNAs were stored at −20°C.

In the present study, two molecular approaches were followed RFLP and DNA sequencing, and two sets of primers were used Table 1. The 30 µL PCR reactions prepared to amplify the target region of the liver fluke DNA contained 2 µL of the target DNA (sample), 15 µL of master mix 2× (Amplicon, Skovlunde, Denmark), 1 µL of forwarding primer, 1 µL of reverse primer, and 11 µL deionized distilled water (ddH_2O), the conventional PCR was used (Thermocycler BIO-RAD iQ TM), the program of the thermo-cycler was set for both primers as follow; initial denaturation 5 min at 95°C followed by 35 cycles at 95°C for 50 s, 55°C for 40 s, and 72°C for 1 min with a final extension of 7 min at 72°C.

2.3. DNA Digestion by Restriction Enzyme

The detection of genetic variation and species identification of the liver fluke was achieved by a restriction fragment length polymorphism (RFLP) assay. The Dra II restriction enzyme (NEB, UK) is used for the digestion of the PCR product of the ribosomal 28s rDNA primer as described

Table 1: The nucleotide sequences of primers used in the present study

Primer name	Sequences of the primers	References
28S rDNA	F 5'-ACG TGA TTA CCC GCT GAA CT–3' R 5' –CTG AGA AAG TGC ACT GAC AAG–3'	[14]
FCOX1	F 5'–AAA TGC TTT GAG TGC TTG GTTG–3' R 5'–ATG AGC AAC CAC AAA CCA CG–3'	[15]

previously [14], The RFLP reactions contained 4 µL PCR product, 1 µL restriction enzymes, 5 µL buffers, and 30 µL ddH_2O. Then, the reaction mixture was incubated at 37°C for 15 min followed by heat-inactivated at 65°C for 20 min. While the PCR product of the *FCOX1* was subjected to DNA sequencing and the result of the DNA sequences was assembled, analyzed, aligned, and blasted in NCBI with the previously registered, the sequence editing software (Bioedit software) was used.

2.4. Gel Electrophoresis

The 5 µL of PCR product was run on 1.5% agarose gel, stained with 5 µL of safe Dye (ADDBIO INC), the gel electrophoresis was run at 84 V for 60 min and visualized under UV (Biobase- China) automatic gel imaging and analysis system in all amplifications. Furthermore, 5 µL of the digested PCR product was run on 2% agarose gels at 84 V for 90 min, 5 µL of safe Dye was added to the gel for DNA staining and visualized under a UV illuminator, also the DNA sequences were submitted to the Gene bank (NCBI) and got the accession numbers.

3. RESULTS

Out of 48 liver flukes isolated from humans and animals, all PCR runs successfully amplified the suspected band (618 bp) for 28S rDNA primer and (836 bp) for the *FCOX1* primer (Figs. 1 and 2). Furthermore, for genetic characterization and species identification of liver flukes, DNA sequencing for *FCOX1* and RFLP were done.

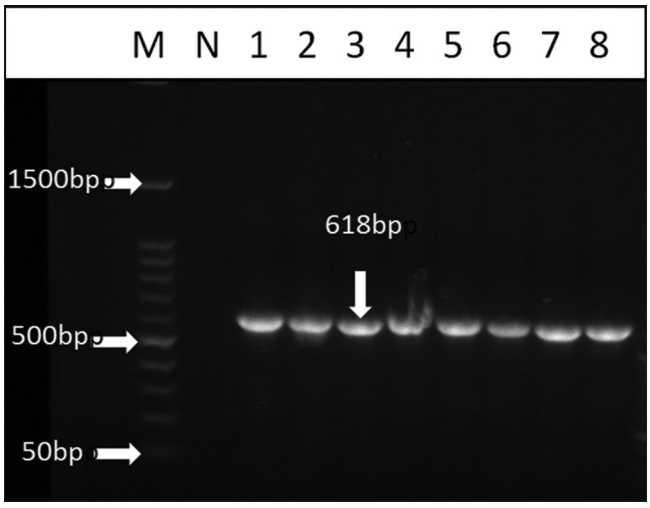

Fig. 1. Gel electrophoresis of the PCR product of the primer 28S rDNA. M= DNA ladder (50 bp), N = Negative control, 1–8 = The samples.

The result of RFLP analysis of the PCR product of the 28S rDNA primer (618 bp), which is digested by restriction enzyme (DraII) showed two patterns, the first one has two bands of (529 bp and 89 bp), while the other pattern is not digested and the 618bp band remains. Table 2 and Fig. 3, the first pattern belongs to *F. hepatica*, and the second is *F. gigantica* [15]. The DNA sequencing of the PCR product of the FCOX1 primers showed the genetic variation, and the result of the nucleotide analysis indicates the presence of *F. hepatica* and *F. gigantica* in the Kurdistan-Iraq, the results were deposited in the Genbank the following Accession numbers; (OP718780 and OP718781) and (OP718782, OP718783, and OP718784).

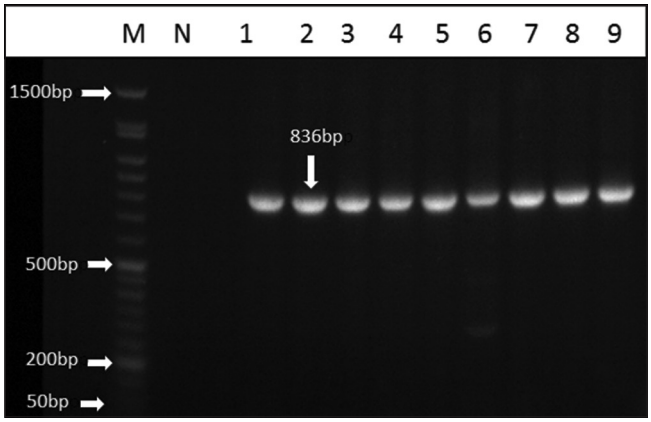

Fig. 2. Gel electrophoresis of the PCR product of the primer FCOX1. M = DNA ladder (50 bp), N = Negative control, 1–9 = The samples.

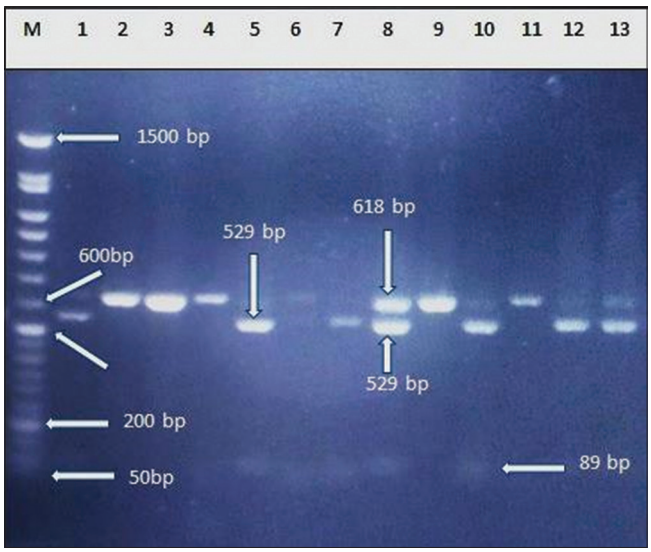

Fig. 3. Restriction fragment length polymorphism Patterns of PCR products of 28s rDNA primer digested with DraII enzyme: M = DNA Ladder (50 bp), 1–13 = The samples isolated from animals (1–11) and humans (12 and 13).

Table 2: Restriction fragment length polymorphism Patterns of PCR products of 28S rDNA primer digested with DraII enzyme

Host	No. of sample	Primer name	Restriction enzyme	PCR product	Restriction fragment (size)	Fasciola spp.
Human	3	28S rDNA	Dra II	618bp	529, 89 bp	*F. hepatica*
Sheep	20	28S rDNA	Dra II	618bp	529, 89 bp	*F. hepatica*
					618 bp	*F. gigantica*
Cattle	20	28S rDNA	Dra II	618bp	529, 89 bp	*F. hepatica*
					618 bp	*F. gigantica*
Goat	5	28S rDNA	Dra II	618bp	529, 89 bp	*F. hepatica*
					618 bp	*F. gigantica*

F. hepatica: Fasciola hepatica, PCR: Polymerase chain reaction

4. DISCUSSION

The present study is an attempt to evaluate two DNA markers to find the genetic characterization and species identification of liver flukes isolated from humans and animals, the RFLP and DNA sequencing were used for two DNA regions of the liver flukes. There are no adequate studies on the molecular and species identification of the liver fluke isolated from humans in Kurdistan Region – Iraq. In the present study, RFLP techniques were applied to identify the *Fasciola* spp. Many studies have been published in various countries throughout the world to identify the genetic polymorphism and species identification of this fluke, including Iraq, Iran, and Thailand [12], [13], [15]. In a study on the molecular diagnosis of *Fasciola hepatica* in livestock utilizing the COX1 gene in Erbil Province, Kurdistan – Iraq, PCR, and sequencing were used to identify the genetic variation among isolated liver fluke from animals they found genetic variation and they conclude that DNA sequencing is a differential technique for finding genetic variation and species identification using COX1 region, this finding in agreement with result [12]. In another study in Duhok governorate – Iraq, specifically used the ribosomal DNA markers; ITS1, and they found the common species that are responsible for animal fascioliasis are *F. hepatica* and *F. gigantica* this result agrees with the current finding [15].

Despite earlier studies, recent investigation on the prevalence of fascioliasis and molecular characterization of *Fasciola* spp. in sheep and goats was done in the Sulaymaniyah province of northern Iraq, using DNA markers and sequencing of the incomplete 28S rRNA gene and codon analysis, they found that *F. hepatica* represented the majority of the identified, followed by *F. gigantica* represented the other two field sequences, this study also supports the finding of the present study and they confirmed the Sulaymaniyah is the place to both *F. hepatica* and *F. gigantica*. the 28S rDNA is confirmed as a potential biomarker in identifying various *Fasciola* species, it was also used in the current study [9].

Furthermore, many studies from different areas demonstrate that molecular approaches are applicable to discriminate *Fasciola* species, the commonly used restriction enzymes Ava II and Dra II, based on a sequence of the 28S rRNA gene, *COX1, ITS1, and ITS2* [15], [16], [17], [18]. They concluded that RFLP is rapid and easy for species identification of liver flukes, and they support current results that indicated the incubation period of DNA digestion by the restriction enzymes can be shortened, also different enzymes can be used in future studies to find the most applicable enzyme to discriminate all three species of *Fasciola*.

Despite the use of genetic markers for *Fasciola* species identification, the immunological markers were done to find the efficacy and applicability to discriminate the species of liver flukes, a study from Duhok, Kurdistan – Iraq demonstrated the effectiveness of an enzyme-linked immunosorbent assay (ELISA) in infected sheep with *Fasciola* spp. the direct examination visual inspection of the liver and immunological assay was followed [7], the result shows the infection rate among animals at a high rate while the species of liver flukes cannot be accurately diagnosed immunologically due to morphologically high similarity, this make researches to adapt molecular markers exactly PCR-PFLP which is the dependable approach for the detection of *Fasciola* species.

5. CONCLUSION

The our finding concluded that all species of *Fasciola* were found in studied area and *F. hepatica* is the common species responsible of human infection. Furthermore, the present study concludes the use of molecular markers PCR-RFLP with different restriction enzymes for species identification and genetic variation is rapid, easy, and reliable. Furthermore, nucleotide sequencing can be used for species identification

and genotyping while the nucleotide sequence analysis and editing will make mistakes or substation of the nucleotide base pair and the result may be not accurate as in the PCR-RFLP which is depend on the restriction side and it will be highly specific.

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REFERENCES

[1] Ö. Y. Çelik and B. A. Çelik. "Investigation of the prevalence of *Fasciola hepatica* in small ruminants in the Siirt region, Turkey". *Iranian Journal of Parasitology*, vol. 13 no. 4, pp. 627-631, 2018.

[2] K. Piri, M. Saidijam, A. Maghsood, M. Matini and M. Fallah. "Prevalence of animal Fasciolosis and specification of *Fasciola* spp. isolated from sheep, goats and cattle, by molecular method: Hamadan province, West of Iran". *Iranian Journal of Parasitology*, vol. 13, no. 4, pp. 524-531, 2018.

[3] R. A. da Costa, L. G. Corbellini, E. Castro-Janer and F. Riet-Correa. "Evaluation of losses in carcasses of cattle naturally infected with *Fasciola hepatica*: Effects on weight by age range and on carcass quality parameters". *International Journal of Parasitology*, vol. 49, no. 11, pp. 867-872, 2019.

[4] J. C. Pinilla, A. A. F. Muñoz and N. U. Delgado. "Prevalence and risk factors associated with liver fluke *Fasciola hepatica* in cattle and sheep in three municipalities in the Colombian Northeastern Mountains". *Veterinary Parasitology, Regional Studies and Reports*, vol. 19, p. 100364, 2020.

[5] N. A. Ouchene-Khelifi, N. Ouchene, H. Dahmani, A. Dahmani, M. Sadi and M. Douifi. "Fasciolosis due to *Fasciola hepatica* in ruminants in abattoirs and its economic impact in two regions in Algeria". *Tropical Biomedicine*, vol. 35, no.1, pp. 181-187. 2018.

[6] Y. Zhang, H. Xu, Y. Liu, J. Kang, H. Chen, Z. Wang and D. Cai. "Case report : Fascioliasis *Hepatica* precisely diagnosed by metagenomic next-generation sequencing and treated with albendazole". *Frontiers in Medicine* (*Lausanne*), vol. 8, p. 773145, 2021.

[7] A. A. Meerkhan and A. H. Razak. "The differences between direct examination and enzyme linked immunosorbent assay (ELISA) test, during the diagnosis of fasciolosis in Jaundiced slaughtered sheep in Duhok Abattoir, Kurdistan region of Iraq". *International Journal of Chemical, Environmemtal and Biological Sciences*, vol. 1, no. 5, pp. 707-709, 2013.

[8] D. N. Anh, L. T. Anh, L. Q. Tuan, N. D. Bac, T. V. Tien, V. T. B. Phuong, T. T. Duong, N. K. Luc and N. B. Quang. "Identification of *Fasciola species* isolates from Nghe an province, Vietnam, based on ITS1 sequence of ribosomal DNA using a simple PCR-RFLP method". *Journal of Parasitology and Research*, vol. 20, no. 2018, p. 2958026, 2018.

[9] H. S. Rahman, H. Marif, M. O. B. Sheikh and A. M. Ahmed. "Molecular characterization and phylogenetic analysis of *Fasciola* species in sheep and goats in Sulaymaniyah province, Northern Iraq". *Journal of Zankoy Sulaimani Part A*, vol. 22, pp. 297-305, 2020.

[10] D. Teofanova, V. Kantzoura, S. Walker, G. Radoslavov, P. Hristov, G. Theodoropoulos, I. Bankov and A. Trudgett. "Genetic diversity of liver flukes (*Fasciola hepatica*) from Eastern Europe". *Infection Genetics and Evolution*, vol. 11, no. 1, pp. 109-115, 2011.

[11] A. E. Laatamna, M. Tashiro, Z. Zokbi, Y. Chibout, S. Megrane, F. Mebarka and M. Ichikawa-Seki. "Molecular characterization and phylogenetic analysis of *Fasciola hepatica* from high-plateau and steppe areas in Algeria". *Parasitology International*, vol. 80, 2021, p. 102234.

[12] M. J. Muhammad and Z. I. Hassan. "Molecular diagnosis of *Fasciola hepatica* in livestock using cox1 gene in Erbil province-Kurdistan region/Iraq". *Zanco Journal of Pure and Applied Sciences*, vol. 33, no. 4, pp. 36-42. 2021.

[13] M. Aryaeipour, S. Rouhani, M. Bandehpour, H. Mirahmadi, B. Kazemi and M. B. Rokni. "Genotyping and phylogenetic analysis of *Fasciola* spp. isolated from sheep and cattle using PCR-RFLP in Ardabil province, Northwestern Iran". *Iranian Journal of Public Health*, vol. 43, No. 10, pp. 1364-1371, 2014.

[14] A. Marcilla, M. D. Bargues and S. Mas-Coma. "A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*". *Molecular and Cellular Probes*, vol. 16, no. 5, pp. 327-333, 2002.

[15] P. Siribat, P. Dekumyoy, C. Komalamisra, S. Sumruayphol and U. Thaenkham. "Molecular identification of *Fasciola* spp. -representative samples from Thailand based on PCR-RFLP". *Journal of Tropical Medicine and Parasitology*, vol. 41, no. 1, pp. 1-7, 2018.

[16] S. Farjallah, B. Ben Slimane, C. M. Piras, N. Amor, G. Garippa and P. Merella. "Molecular characterization of *Fasciola hepatica* from Sardinia based on sequence analysis of genomic and mitochondrial gene markers". *Experimental Parasitology*, vol. 135, no. 3, pp. 471-478, 2013.

[17] S. Kasahara Y. Ohari, S. Jin, M. Calvopina, H. Takagi, H. Sugiyama and T. Itagaki. "Molecular characterization revealed *Fasciola* specimens in Ecuador are all *Fasciola hepatica*, none at all of *Fasciola gigantica* or parthenogenic *Fasciola* species". *Parasitology Inernational*, vol. 80, p. 102215, 2021.

[18] A. Saadatnia, K. Solhjoo, M. H. Davami, S. Raeghi and A. Abolghazi. "Molecular identification of *Fasciola* isolated from the liver of meat animals in Fars province, Iran". *Journal of Parasitology Research*, vol. 2022, p. 4291230, 2022.