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Original Article

Molecular Characterization of Liver Fluke Isolated from Sheep, Goat and Cattle in Sulaymaniyah, Iraq

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Abstract

Background: We aimed to determine species of liver fluke that predominately cause fascioliasis in sheep, goats, and cattle in the Sulaymaniyah Province, Iraq using the molecular technique of DNA sequencing and restriction fragment length polymorphism (RFLP).

Methods: The samples were collected from November 2021 to May 2022. The flukes were collected from infected livers of livestock at the slaughterhouse of Sulaymaniyah Governorate, Iraq. Overall, 205 flukes were collected from 56 hosts, cattle (n=22), sheep (n=28), and goats (n=6). The specific primers for FCOX1 and 28S rDNA gene amplification were used. The PCR products were subjected to restriction fragment polymorphism (RFLP) assay using *Hpy188III* and *Dra II* restriction enzymes, besides DNA sequencing.

Results: The results showed the genetic polymorphisms among the flukes. Three patterns of RFLP were observed *Fasciola hepatica, F. gigantica,* and *F. intermediate*, where 28 of them displayed *F. hepatica* (sheep, n=14, goat, n=3 and cattle, n= 11), whereas 24 samples displayed the *F. gigantica* (sheep, n=12, goat, n=3 and cattle, n= 9), and only four samples belonged to *F. intermediate* (sheep n=3 and cattle, n=1). In addition, the result of the ribosomal DNA (28S rDNA) sequencing confirmed that the isolated flukes belonged to *F. hepatica*, *F. gigantica* and *F. intermediate*.

Conclusion: All three main species are present in the study area and *F. hepatica* predominated among the animal species in this area also, our results concluded that PCR-RFLP is a rapid and reliable method for liver fluke species identification.

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Introduction

The liver fluke of the genus *Fasciola* is an extremely pathogenic parasite that affects humans and animals and causative agent of fascioliasis (1). Animal liver infection with *Fasciola* species results a large economic loss (2). This trematode directly affects livestock productivity through host mortality (in sheep) and partial or complete liver condemnation. In livestock, it causes numerous financial losses, such as a decrease in milk, meat, and wool production (3). The effects of *F. hepatica* infection on weight vary depending on the age group linked to lower weight and poorer carcass quality indicators (4). Lower growth and lower feed conversion rates in fattening cattle are examples of indirect losses in cattle, as are decreased milk yield and quality in dairy cattle (5).

While *F. gigantica* is found mostly in tropical regions, and *F. hepatica* is more commonly found in temperate regions (6). Due to the various modes of transmission and epidemiological traits of *F. hepatica* and *F. gigantica* infections in different hosts, differential diagnosis is crucial. Because each liver fluke species has unique evolutionary characteristics, and are transmitted by different lymnaeid snail (3, 4).

As far as is known, neither clinical nor pathological, nor immunological methods can be used to classify the *Fasciola* species. Serological results fail to distinguish between these species (5). As morphological methods, have too many limits. The differentiation of *F. hepatica* and *F. gigantica* is currently based on morphometric study or implementing numerous highly developed molecular methodologies using various molecular targets (7).

This disease is worldwide and the prevalence rate were varies depending on the control plan and environmental factors, in Iraq some studies were carried out to investigates the situation of animal and human fasciolosis. A recent study in Duhok indicates the prevalence of fasciolosis among goats and sheep were 6.3%, 8.9% respectively (8), also recently in Erbil province, high prevalence rate of Fasciolosis (7.28%) among sheep was reported (9).

Various molecular techniques, including Conventional PCR, Real-time PCR (qPCR), RFLP and PCR-RFLP of several genes (ribosomal), have been used to analyze the populations of *F. hepatica* (10-12). Also, due to the shortcomings of morphological methods, numerous molecular strategies have been created for differentiating *F. hepatica* and *F. gigantica* throughout East, South East, and South Asian countries. These strategies used different molecular targets (13). One of these goals is a quick and simple PCR-RFLP test using *Dra II* restriction enzyme to distinguish between two *Fasciola* species, which has been used by some researchers. It is based on a 618-bp-long 28S rRNA gene sequence recently used in South America, Europe, and Africa (14). In fact, by using a portion of the 28S rDNA region, it was possible to establish the genetic heterogeneity of *F. hepatica* isolates. This revealed that heterozygous specimens had nucleotide variation at one location (corresponding to the 105th nucleotide from the 28S rDNA) (7).

There has been confusion in the past due to the existence of intermediate morphological characteristics between *F. hepatica* and *F. gigantica* (7). According to earlier research, *F. intermediate* form was discovered to have similar unclear-morphological characteristics with *F. gigantica* and *F. hepatica*, size, and shape (15). Additionally, parthenogenic (aspermic) *Fasciola* flukes originally appeared in China due to hybridization events between *F. hepatica* and *F. gigantica.* It has persisted without extinction to the present, are the third causative *Fasciola* forms (16).

The main objective of this investigation was to determine the common cause of fascioliasis in sheep, goats, and cattle in the Sulaymaniyah Province using the molecular technique (PCR-RFLP).

Materials and Methods

Sample collection

Totally, 205 liver flukes were collected from 56 infected livestock animals, including, cattle (n=22), sheep (n=28), and goats (n=6) in the Sulaimani new slaughterhouse (Qragol abattoir). The flukes were delivered to the laboratory of Sulaymaniyah Polytechnic University and the genetic laboratory of MLS at the University of UHD in Sulaimani Kurdistan-Iraq, where they were preserved in 70% ethanol and stored at room temperature until use.

DNA extraction

Genomic DNA was extracted from the *Fasciola* fluke after grinding each fluke individually in an Eppendorf tube. DNA extraction was performed by a commercial DNA extraction kit for tissue (Addprep Genomic DNA extraction kit, ADD-BIO INC. Korea) according to the manufacturer's instructions. The extracted DNAs were stored at room temperature until used.

The current study ethically was approved by the ethical committee of the technical college of health and medical technology under the ethical aprovmrnt letter number: MLD 0050.

PCR

Two sets of primers were used in this study, the first set is FCOX1 primer that was designed to amplify 836 bp fragment, and the sequences of the primers were (F 5'–AAA TGC TTT GAG TGC TTG GTTG–3' and R 5'–ATG AGC AAC CAC AAA CCA CG–3') (15). The 30 µl of the PCR reactions contained 2 µl of the template DNA, 15 µl of master mix (Amplicon, Skovlunde, Denmark), 1 µl of each primer, and 11 distilled water (DDW) to the final volume. The condition of the PCR for an initial denaturation of 5 min at 95 °C followed by 35 cycles of 95 °C for 50 sec, 55 °C for 40 sec, and 72 °C for 1 min with a final extension of 7 min at 72 °C.

The other set of primers were 28S rDNA with the following sequences (F 5'-ACG TGA TTA CCC GCT GAA CT–3') and (R 5 –CTG AGA AAG TGC ACT GAC AAG–3'), this primer is designed to amplify the 618 bp amplicon. (14), The 30 µl reactions contained 2 µl of the template DNA, 15 µl of master mix (Amplicon, Skovlunde, Denmark), 1 µl each primer, and 11 µl distilled water (DDW). PCR amplification was programmed for an initial denaturation of 5 min at 95 °C followed by 35x cycles of 95 °C for 50 sec, 56 °C for 30 sec, and 72 °C for 1 min with a final extension of 7 min at 72 °C.

Gel electrophoresis

Three µl of PCR product were run on 1.5% gel, stained with 5 µl of safe stain (Safe gel Dye, ADDBIO INC. Korea). The gel electrophoresis was run at 84 V for 90 min and visualized under UV (Biobase- China) automatic gel imaging and analysis system.

DNA digestion by restriction enzyme

The detection of genetic polymorphism and *Fasciola* species (*F. hepatica* and *F. gigantica*) was performed by a restriction fragment polymorphism (RFLP) assay using the *Hpy188III* enzyme (NEB, UK) as described (15). The reactions contained 4 µl of PCR product of COX1, 1 µl of the enzyme, 5 µl of the buffer, and 30 µl DDW. The mixture was incubated at 37 °C for 1 hr. and heat-inactivated at 65°C for 20 minutes, followed by electrophoresis on 2% agarose gels staining with 5 µl of safe stain and the patterns compared to 50 bp DNA ladder (Sina Clon).

The *EcoO109I* (*Dra II*) enzyme (NEB, UK) is used for the digestion of the PCR product of the 28s rDNA primer as described (14) . The reactions contained 4 µl of PCR product, 1 µl of the enzyme, 5 µl of the buffer, and 30µl DDW. The mixture was incubated at 37 °C for 15 min and heat-inactivated at 65°C for 20 minutes, followed by electrophoresis on 2.5% agarose gels and stained with 5µl of safe dye and the patterns compared to 50 bp DNA ladder (Sina Clon).

DNA sequencing

The PCR product of the primer 28S rDNA was selected for DNA sequencing according to the polymorphism found in the RFLP marker, the samples were sent to a Korean Company (Macrogen. Korea) for 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 7.1) was used.

Results

Out of 56 samples isolated from different hosts, all the samples amplified the target sequences successfully for primer FCOX1 (836 bp), and 28S rDNA primer yielded 618 bp (Fig. 1A and 1B).

The result of RFLP for each PCR product showed a genetic polymorphism for the isolated fluke. Three patterns were observed, *F. hepatica*, *F. gigantica*, and *F. intermediate*. Twenty-six samples belonged to *F. hepatica*, while 21 and 4 samples belonged to *F. gigantica* and *F. intermediate*, respectively.

The (COX1) PCR product digestion generated two patterns (Fig. 2): one comprised three bands of approximately 420, 378,29 bp *F. hepatica*, and

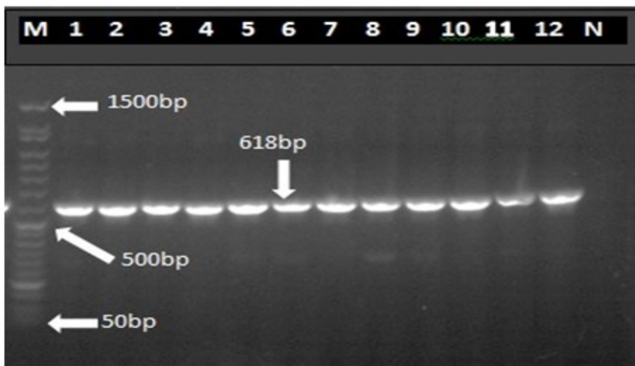
the other four bands of 378,317,103,29 bp indicating *F. intermediate* (11).

While the PCR product of the primer 28S rDNA was digested with *DraII* restriction Enzyme (Fig. 3) generated two patterns, one comprising two bands of approximately 529, 89 bp *F. hepatica* and the other band of 618 bp of *F. gigantica* (14). The results of RFLP are presented in Table 1.

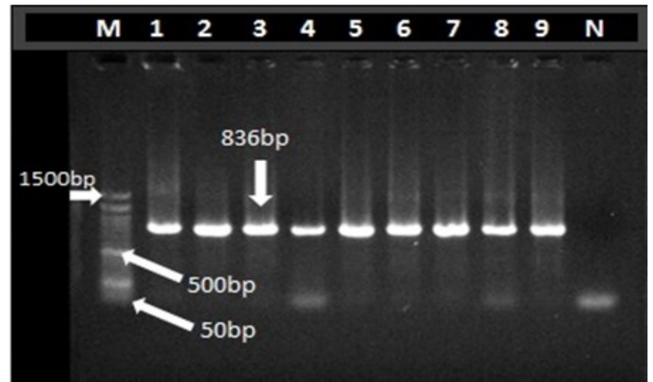
The predominant species of liver flukes among animals was *F. hepatica* 51.8% followed by *F. gigantica* 42.8% and *F. intermediate* 5.4% (Table 2).

The result of the DNA sequencing of the 28S rDNA indicates the same species which found through RFLP and the DNA sequences were submitted to the GenBank and get the following accession numbers: *F. hepatica* (OR676757, OR676758, OR676759, OR676760, OR676761 and OR676762), *F. gigantica* (OR676763, OR676764, OR676765, OR676766 and OR676768) and the OR676767 recorded as an intermediate form of *Fasciola species.*

A slight genetic variation was found in some regions of the isolated liver fluke (Fig. 4 and 5).



(A)



(B)

Fig. 1: (A) Gel electrophoresis of PCR products of ribosomal DNA primer. M= DNA ladder, 1-4 samples isolated from cattle, 5-9 samples isolated from sheep, 10-12 samples isolated from goat, N= negative control. (B) Gel electrophoresis of PCR products of FCOX1 primer. M= DNA ladder, 1-3 samples isolated from cattle, 4-7 samples isolated from sheep, 8-9 samples isolated from goat, N= negative control

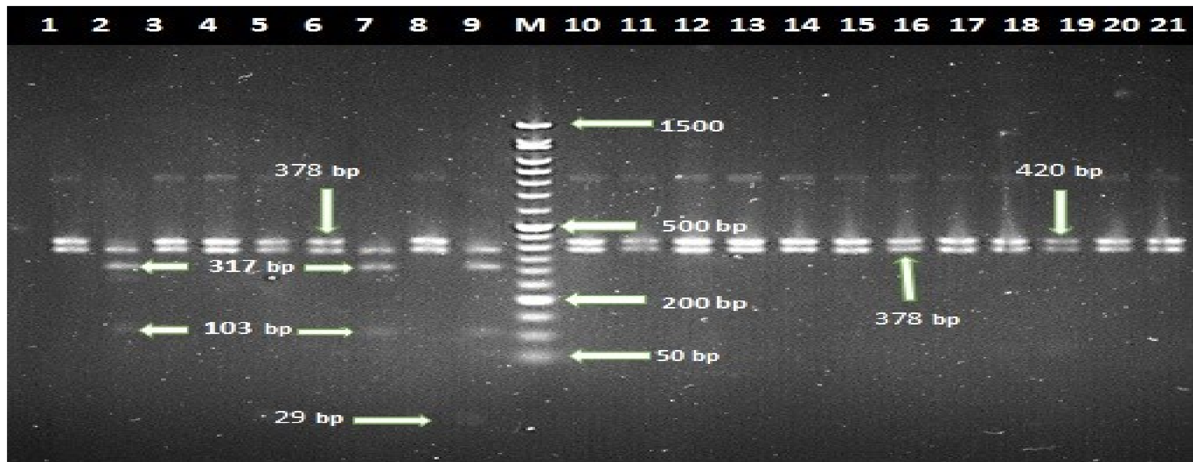


Fig. 2: Restriction fragment length polymorphism (RFLP) Patterns of PCR products of FCOX1 primer digested with Hpy188III enzyme: M= DNA Ladder, 1-9= the samples isolated from sheep, 10-15= samples isolated from cattle and 16-20 = samples isolated from goat

Fig. 3: Restriction fragment length polymorphism (RFLP) Patterns of PCR products of 28s rDNA primer digested with DraII enzyme: M= DNA Ladder, 1-7= the samples isolated from sheep, 8-15=the samples isolated from cattle, 16-20= the samples isolated from goat

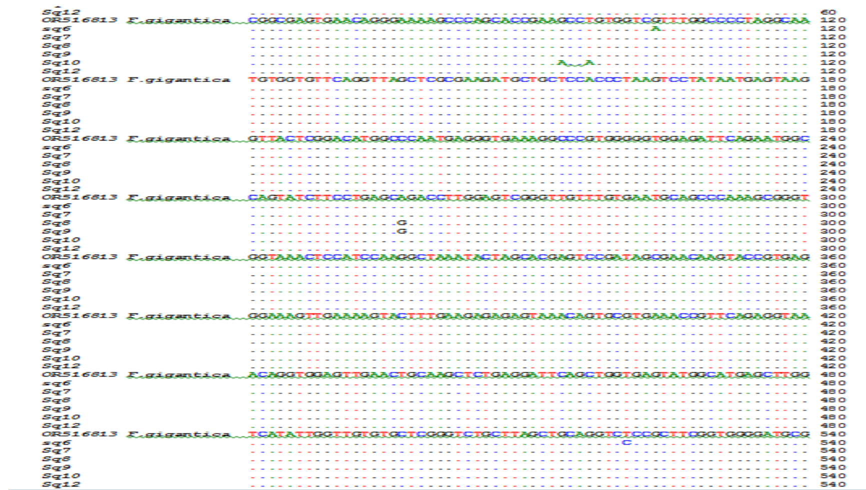


Fig. 4: The partial sequence alignment of the PCR product of 28S rDNA show the genetic variation among F. hepatica and intermediate form (sq6,7,8, 9 and 12= F. hepatica and sq10 = intermediate form)

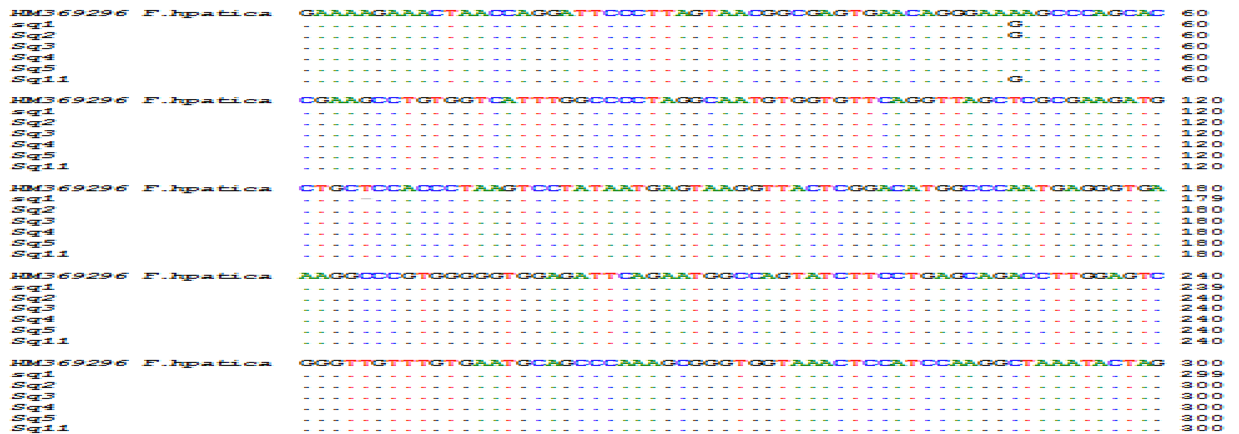


Fig. 5: The partial sequence alignment of the PCR product of 28S rDNA show the genetic variation among *F. gigantica* (sq1,2,3, 4,5 and 11= the samples belong to *F. gigantica*)

Table 1: The PCR product digestion with the Hpy188III & Dra II enzyme

Host	*Primer name*	*Restriction enzyme*	*PCR product*	*Restriction Fragment (size)*	*Fasciola Spp.*
Sheep	FCOX1	Hpy188III	836bp	420, 378,29 bp	*F. hepatica*
	F28s rDNA	Dra II	618bp	529, 89 bp	*F. hepatica*
	F28s rDNA	Dra II	618bp	618 bp	*F. gigantica*
	FCOX1	Hpy188III	836bp	378,317,103,29 bp	*F. intermediate*
Cattle	FCOX1	Hpy188III	836bp	420, 378,29 bp	*F. hepatica*
	F28s rDNA	Dra II	618bp	529, 89 bp	*F. hepatica*
	F28s rDNA	Dra II	618bp	618 bp	*F. gigantica*
Goat	FCOX1	Hpy188III	836bp	420, 378,29 bp	*F. hepatica*
	F28s rDNA	Dra II	618bp	529, 89 bp	*F. hepatica*
	F28s rDNA	Dra II	618bp	618 bp	*F. gigantica*

Table 2: The percentage liver flukes species according to the host

Host	*The No. of sample*	*Fasciola species*		
		F. hepatica **No. (%)**	*F. gigantica* **No. (%)**	*F. intermediate* **No. (%)**
Sheep	28	14 (25)	11(19.6)	3 (5.4)
Cattle	22	11(19.6)	10(17.8)	1(1.8)
goat	6	4 (7.1)	2(3.6)	0
Total	56	28 (50)	24 (42.8)	4 (7.2)

Discussion

In the present study molecular markers (COX1 and 28s rDNA) were used to find the genetic polymorphism of *Fasciola species* isolated from domestic animals in the Sulaymaniyah Province. There are few studies on the molecular identification of the *Fasciola spp*. from Kurdistan, Iraq. Specifically, in the Sulaymaniyah Province of Kurdistan, this is the first study performed on the identification of *Fasciola* spp. using RFLP.

Numerous studies on the identification of *Fasciola* species have been published in various countries throughout the world, including Ecuador,Iran, Spain, India, Japan, Korea, and Turkey (17- 20). These studies aimed to distinguish between *F. hepatica* and *F. gigantica*. Among those few studies in Sulaymaniyah and Kurdistan region, a study was carried out on the molecular diagnosis of *F. hepatica* in livestock in Erbil Province (5). They extracted DNA from 52 liver flukes from different animals (22 sheep, 3 goats, and 27 cattle). The prevalence rates for fascioliasis showed 7.28%, 3.37%, and 10.19%, for sheep, goats, and cattle respectively. The prevalence rate was indicated greater in female cattle (5.28%) than in male cattle (4.91%) (5). Another study was conducted on 100 adult *Fasciola* spp. flukes that were isolated from cattle, sheep, and goats that were butchered at various abattoirs (Duhok, Zakho, Shelidiza, Aqrah, and Bardarash) in the Duhok governorate, Kurdistan region, Iraq, in 2019 at the University of Zakho. They specifically used the ribosomal DNA markers ITS1 and ITS2 for amplifying 480 and 550 bps fragments, and the liver flukes have been described using DNA sequences. The ITS-1 band was 480 bp, while the ITS-2 band was 550 bp. Sequencing was done on thirteen *Fascio-*

la spp. flukes that had been isolated from cattle, sheep, and goats in various districts. Of these thirteen PCR results, seven were identified as *F. hepatica* and six as *F. gigantica* (19).

Despite earlier studies, a new investigation of the prevalence of Fascioliasis and molecular characterization of isolated *Fasciola* species in sheep and goats in the Sulaymaniyah province of northern Iraq has been done. 100 liver sample has been obtained from the abattoir, 6 of these samples were chosen for sequencing of the incomplete mitochondrial 28s rDNA gene. They found that *F. hepatica* represented four of the identified field sequence, whereas *F. gigantica* represented the other two samples (12). This study support our finding using DNA sequence analysis.

In addition, research from other countries demonstrates that it is possible to discriminate among the *Fasciola* species using a PCR-RFLP experiment using the commonly used restriction enzymes *Ava II* and *Dra II*. It is based on a sequence and restriction site of the 28S rDNA gene (14) also the origin of the different species of the liver flukes were studied to find the environmental factors on the geographically distribution of this parasite (20).

Despite the use of genetic tests for *Fasciola* species, a study from the Duhok district of Kurdistan demonstrates the effectiveness of an ELISA in sheep infected with *F. hepatica*. In 2012, they examined 232 jaundiced sheep out of the 5208 sheep slaughtered at the Duhok abattoir (regardless of their gender) by direct examination (looking for adult flukes in the bile duct) and by ELISA, and they discovered an increased ratio in specific months (21). According to our concluded information, the PCR-PFLP has been demonstrated to be an accurate and dependable approach for the detection of *Fasciola* species.

Regarding the DNA sequencing of the partial genes were used by many researchers and it can be dependable for species identification and genetic variation of liver flukes (7). In another study in Iran, the liver flukes have been described using DNA sequences. The ITS-1 band was 480 bp, while the ITS-2 band was 550 bp. Sequencing was done on thirteen *Fasciola spp.* flukes that had been isolated from cattle, sheep, and goats in various districts of these 30 PCR results, 7 were identified as *Fasciola hepatica* and 6 as *Fasciola gigantica* this finding supports our finding (22). Despite earlier studies, a new investigation of the prevalence of fascioliasis and molecular characterization of isolated *Fasciola species* in sheep and goats in the Sulaimani Province of northern Iraq has been done. On hundred liver samples have been obtained from the abattoir, 6 of these samples were chosen for sequencing of the partial mitochondrial 28S rDNA gene. They support our result they indicates the presence of both *F. hepatica* and *F. gigantica* (12), and according to our results and the studies done in Sulaiamnia Province, *F. hepatica* is the predominant one. Therefore, further field studies to verify these results are suggested.

Conclusion

This study indicated the common species of liver fluke worm among animals in Sulaimani-Kurdistan are *F. gigantica* and *F. hepatica*. PCR-RFLP using *HPY088111* can be used for the identification and differentiation of *F. hepatica* and *F. intermediate*.

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Conflict of Interests

The authors of this study confirmed that we have no kind of conflict of interest.

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