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Histopathologic Changes and Molecular Characterization of Fascioliasis (a Zoonotic Disease) among Slaughtered Livestock in Erbil and Halabja Abattoirs, Kurdistan Region-Iraq

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Abstract

Fascioliasis is a zoonotic disease, caused by the parasites Fasciola (F.) hepatica and F. gigantica, which poses a great threat to the ruminants in addition to humans in many countries, including Iraq. Hence, the study of this parasite and its deleterious impacts on host, morphologically, histopathologically, epidemiologically, and molecularly, is so important. Hence, the current study was designed to investigate this fluke of livestock in Erbil and Halabja slaughterhouses from August to October 2022. To meet the prerequisites of the study, 33 flukes were collected from the sheep and cattle at study sites. The samples were transported to the Zoology Research Laboratory, Salahaddin University-Erbil, Iraq. Some of them were used for morphological identification, and others were preserved in 96% ethanol and stored at -20°C. Subsequently, DNA was extracted. Conversely, pieces of the infested liver were fixed in 99% ethanol and 10% formalin before histopathologic examination. The parasite species were identified using traditional Polymerase Chain Reaction (PCR), and sequencing methods. Macroscopically, hepatomegaly was the main finding of the infestation. Among livestock, cattle exhibited the highest rate of morbidity, followed by goats and sheep. The amplified DNA region was 98–99%, matched to F. hepatica and F. gigantica sequences. It was also established that 28S rDNA sequencing combined with morphologic characteristics of Fasciola species can be applied as a salient indicator in the identification of flukes. The current study is reckoned as a comprehensive investigation regarding fascioliasis, because it covered the parasite epidemiology, morphology, and molecular identification, despite of histopathologic examination.

Keywords: Fascioliasis, Histopathology, Morphology, Molecular identification, Slaughtered Livestock.

Introduction

Fasciola (F.) hepatica and *F. gigantica* are common parasitic flatworms that infest small ruminants (ovine and caprine) and large ruminants (bovine) in various countries across the globe, including Iraq. Humans acquire the aforementioned liver flukes accidentally via water and food[1, 2], This digenean trematode belongs to the Phylum; *Platyhelminthes*, Class; *Trematoda*, and Family: *Fasciolidae*. Hepatic

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biliary ducts of the above-mentioned ruminants are predilection sites and habitats of the adult stage of the parasite, where sexual stages develop[3].

The parasite life cycle begins with the position of unembryonated eggs, which are released with the feces of previous definitive hosts to the aquatic environment, where eggs become embryonated. following that, the miracidia hatch and attack an appropriate snail intermediate host, such as *Lymnaea truncatula*. Within the snail, parasites undertake several asexual developmental stages, namely sporocysts, rediae, and cercariae. Eventually, the cercariae depart from the snail and encyst as metacercariae, which serve as the infective stage, on aquatic plants or various surfaces[4]. Mammals get infestation by ingesting plants holding infective stages. Humans acquire the infection by eating metacercariae-containing fresh plants[5, 6]. Post-ingestion, the encysted metacercaria are exposed to liberate the small form of trematodes in the first part of the small intestine then penetrate the wall of the intestine, the peritoneal membrane, and the hepatocytes into the bile channels, where they grow into mature flat worms[7]. The continuous irritation of bile ducts by adult worms induces severe inflammatory reactions[8]. Inflammatory white blood cells infiltration, primarily phagocytic cells, and lymphocytes with moderate counts of acidophils, an increasing number of cells producing fibrosis around portal regions, with metaplasia and fibrosis of bile canaliculi are other main indications of chronic inflammation[9]. Clinical signs include anemia, systemic toxicity, submandibular swelling, malnutrition, and mortality[10]. Fascioliasis can inadvertently spread to humans when people intake eggs or larval metacercaria, generated inside intermediate hosts[2, 6]. According to the WHO, fascioliasis is reckoned a re-emerging zoonotic disease; it was estimated that more than 2 million people harbored the infection in more than 70 countries around the world, and approximately 91 million people could be at risk of getting it[11]. Certain Asian countries, including Turkey, Iran, Iraq, Yemen, Saudi Arabia, Korea, Japan, India, and China, are being exposed to this zoonotic disease[12, 13]. Certain parasitological zoonotic studies are carried out on the livestock animals of Iraq other than fascioliasis[14, 15]. Based on their morphologic physical characteristics, *F. hepatica* and *F. gigantica* are differentiated from each other through the ratio between body width and length, and the distance between the distal end and the ventral sucker of the body[16, 17]. Regarding inflammation types, chronic, occasionally acute, or subacute inflammation of the hepatic and biliary tract, together with anemia, systemic toxicity, submandibular swelling, malnutrition, and mortality, is the primary symptom of the disease[10], it is considered a re-emerging neglected zoonotic disease[18]. This zoonotic disease affects several Asian countries, namely: Turkey, Iran, and Iraq[19-21].

For a tentative diagnosis, stool investigations, enzyme-linked immunosorbent assays (ELISA), and liver biopsies are recommended[22]. Serological, clinical, and parasitological diagnoses are unable to distinguish between these two species[23]. Conversely, it is difficult to separate *Fasciola* species morphologically, but a molecular assay based on the numerous nuclear genes and gene spacers' DNA sequences are considered a reliable tool for this purpose[4, 24]. Concerning this, the internal transcribed spacers (ITS1 and ITS2) found in ribosomal DNA (rDNA) as well as the 28S rDNA gene can be utilized to differentiate between *F. hepatica* and *F. gigantica*[25]. The ITS-2 sequence acts as a separator between the coding regions of 5.8S and 28S within the rDNA. Thus, it is crucial for the confirmation and genetic description of both *Fasciola* species. Moreover, the identification, phylogeny, inter- and intra-specific variations, and genotyping analyses of this parasite have previously used mitochondrial genes[16, 26]. Clarifying the morphological and molecular identification of *F. hepatica* and *F. gigantica* in the Kurdistan Region of Iraq was a goal of this study. Furthermore, the demonstration of the salient histopathological changes caused by liver fascioliasis in sheep and cattle is inevitable.

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Materials and Methods

Study Site and Sample Collection

The current research was carried out in the slaughterhouses of Erbil and Halabja provinces, the Kurdistan region of Iraq from August to October 2022. The survey included non-infected and infected-slaughtered animals. The sexes and species of animals in each province were precisely recorded on standardized data sheets. Liver pieces were squeezed to remove the flukes from the bile ducts and tissues as demonstrated in Fig. 1. Each isolated liver fluke was examined and classified on its size and shape. A combined sum of 33 flukes was collected from slaughtered animals at different abattoirs of Erbil and Halabja provinces. Flukes were preserved in 96% ethanol and placed in Eppendorf tubes, then labeled for proper identification and preserved at -20°C for morphologic and molecular identification. Also, 2 samples of liver were kept in 96% ethanol and 4 liver samples were stored in 10% formalin and deposited at 4°C for extra histopathologic examinations.

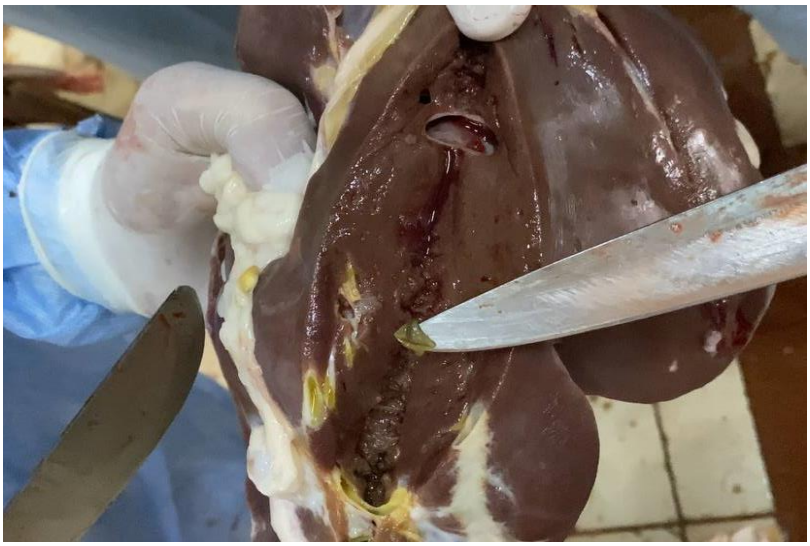

Figure 1. Pressed liver pieces with a sharp blade to pinch the flukes out from the canaliculi of the bile ducts and tissues.

Morphological Analysis

Depending on commonly used taxonomic keys, the liver fluke species were identified according to morphometric criteria[27, 28]. The flukes were embedded in boiled water to eliminate excess alcohol or formalin[16]. Any fluke with dimensions of up to 30 mm and 15 mm, and length–width ratio of 1.9–2.3 mm was classified as *F. hepatica*, whereas *F. gigantica* isolates, their dimensions reaching 75 mm long and 15 mm wide, with an average length–width ratio of 4.4–5.2 mm[16].

Molecular Identification

Extraction of DNA

Parts of *Fasciola* specimens were fragmented into small pieces using a sterilized scalpel and placed into microcentrifuge tubes with ethanol, then kept at -20°C. After that, samples were taken out from the refrigerator and the ethanol was discarded. The genomic DNA was extracted from the collected and sliced parasite using the PureLink® Genomic DNA kit instruction steps from Invitrogen (the USA). The quantity, quality, and purity of the DNA were assessed using a Nanodrop, specifically the K5600 Micro-Spectrophotometer. Subsequently, the DNA was stored at -20°C to facilitate subsequent examinations and PCR analysis.

PCR Amplification

Using two pairs of primers, the PCR was applied to amplify the 28S rDNA gene of each sample. The sequences of primers were recovered from the NCBI databases using the universal primers, forward sequence C1 arranged as 5'-ACCCGCTGAATTTAAGCAT-3' at location 25

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and reverse sequence C3 ordered as 5'-CTCTTCAGAGTACTTTTCAAC-3' at location 390[29]. Four tubes, each containing purified genomic DNA stored at a temperature of -20 °C were taken out from the refrigerator. These tubes were prepared for PCR cycles. Each of the 50 µl reactions consisted of a 2.5 µL DNA template, 2 µL 35× primer, with 1 µL of forward primer and 1 µL of reverse primer, 20.5 µL of double distilled water (ddH_2O) to reach the final volume and 25 µL of a master mix (GENEDIREX, KOREA). The master mix contained dNTPs, $MgCl_2$, and Taq polymerase. Subsequently, PCR amplification was conducted following the thermal cycling conditions outlined by Mucheka, Lamb [30]: The process commenced with an initial denaturation step at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds. Annealing was carried out at 51 °C for 45 seconds, and extension took place at 72 °C for 45 seconds. The final extension phase was performed for 5 minutes at 72 °C. Using sample combs to create sample wells, fragments were separated through electrophoresis in 2% agarose gel, it was prepared with dissolved 1g agarose powder in 50 ml TBE solution, with 3µL ethidium bromide, which is used to visualize the Fragments of DNA, 5 µL of each PCR product and a DNA ladder were loaded in the wells, at 100 V and 3 W for about 40 min, as a result, several bands are formed and are observed using a UV transilluminator.

The amplified DNA obtained from PCR subjected to the nucleotide sequence analyzer, An ABI 3130X located in SINGAPORE was used to determine the arrangement of 28S rDNA nucleotides. The parasite PCR fragments, serving as the DNA template, were extracted from the agarose gel and subsequently submitted to sequence-specific PCR amplification in Intergene Genetic Centre/ Ankara, Turkey.

Histopathological Studies

For the histological examination, 6 liver samples (2 preserved in 96% ethanol and 4 preserved in 10% formalin), 3 positives for fascioliasis and 3 controls were collected from cattle and sheep at different abattoirs in Erbil and Halabja provinces. The specimens were analyzed at LUAY Private Histopathologic Laboratory, Doctor's Street, Erbil. The specimens were fixed in 10% formalin for 25 days, then washed with 70% alcohol five times. Serial alcohols were used in ascending order from 90-100%, each was used for 1 hour for dehydration of the sample, then acetone was employed twice, each for 40 minutes. Following two rounds of xylene clearing, each lasting for an hour, the tissue samples were submerged in paraffin wax for 2 hours as exhibited in Fig. 2. Then, using a rotary microtome, the paraffin blocks were partitioned at 3 µm thicknesses. The pieces were placed on a hot plate that was usually regulated at 2 to 3°C under the melting point of wax following that, they were retained at 2 – 3°Cover the melting point of the wax. Tissue slices were subjected to staining with hematoxylin and eosin. Subsequently, the stained sections were carefully placed on glass slides and covered with dibutylpthalate polystyrene xylene (D.P.X.) to ensure the absence of air bubbles. The tissue slices were allowed to dry adequately for a period of 15 to 30 minutes before mounting[31].

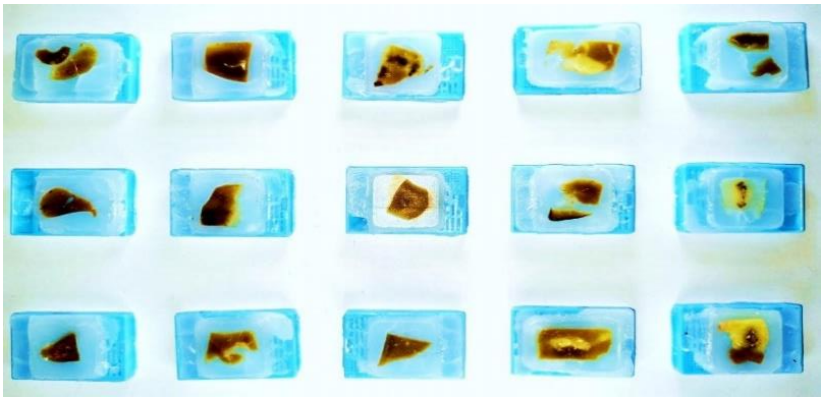

Figure 2. Prepared liver specimen for histopathological examination, which is embedded in paraffin wax blocks.

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Photomicrographs and Measurements

Samsung Galaxy S21 FE Digital Camera with 12 Mega Pixels was used for taking photos. The dimensions of *Fasciola* species were measured with an ordinary ruler and ocular-stage micrometer, as well as Image J software applied for scale-bar images.

Results and Discussion

Epidemiological Study

The findings of this investigation revealed that sheep are the most slaughtered ruminants in the abattoir in Erbil as compared to goats and cattle. Considering the rate of fascioliasis among slaughtered animals, the highest morbidity rate was 5.6% in cattle, followed by 5.0% in goats, and 2.0% in sheep, as displayed in Table 1.

The epidemiology of helminth infections is thought to be a complicated process comprising an association between both the morbidity rate and the host's immunity. In temperate and tropical regions, young animals are highly susceptible to severe infections as compared to older animals. This variation may be due to the paucity of immunity in young animals. Nevertheless, variations in the occurrence of the disease through different regions have been noted, particularly in tropical areas where prolonged hot and dry periods are prevalent, as well as in other tropical regions with a short or nonexistent dry season[32, 33].

Table 1. The total slaughtered animals infected with fascioliasis and morbidity rates for both genders in the Erbil abattoir during August – October 2022.

One year examination	Slaughtered animal	Examined male	Examined female	Fascioliasis	Rate of infection %	Total number
August	Cattle	17784	1917	1116	5.6	19704
to	Sheep	121187	13068	2739	2	134255
October 2022	Goat	710	106	41	5	816
	Total	139681	15091	3896	2.5	154772

Regarding the number of monthly-slaughtered animals and morbidity rates, details are demonstrated in Table 2, that was taken from Erbil slaughtered house. The data revealed that the highest number of slaughtered animals (14584) was in July 2021, while the lowest number of slaughtered animals (10052) was in September 2020.

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Table 2. The monthly distribution of liver fluke infections among slaughtered animals in Erbil abattoir from September 2020 to August 2021.

Monthly Examination	Slaughtered animal	Examined Male	Examined Female	Fascioliasis	Rate of Infection %	Total Number
September 2020	Cattle	1159	131	90	6.9	1290
	Sheep	8868	852	205	2.1	9720
	Goat	25	6	1	3.2	31
	Total	10052	989	296	2.4	11041
October 2020	Cattle	1124	118	105	8.4	1242
	Sheep	9052	1008	218	2.1	10060
	Goat	25	4	1	3.4	29
	Total	10201	1130	324	2.8	11331
November 2020	Cattle	1319	138	127	8.7	1457
	Sheep	9278	1153	249	2.3	10431
	Goat	39	4	1	2.3	43
	Total	10636	1295	377	3.1	11931
December 2020	Cattle	1328	139	93	6.3	1467
	Sheep	9070	1141	194	1.8	10211
	Goat	33	3	2	5.5	36
	Total	10431	1283	289	2.4	11714
January 2021	Cattle	1357	148	87	5.7	1505
	Sheep	8989	1147	183	1.8	10136
	Goat	32	4	1	2.7	36
	Total	10378	1299	271	2.3	11677
February 2021	Cattle	1474	158	95	5.8	1632
	Sheep	9571	1052	219	2	10623
	Goat	49	7	3	5.3	56
	Total	11094	1217	317	2.5	12311
March 2021	Cattle	1492	154	91	5.5	1646
	Sheep	9651	1025	231	2.1	10676
	Goat	129	24	7	4.5	153
	Total	11272	1203	329	2.6	12475
April 2021	Cattle	1564	184	93	5.3	1748
	Sheep	9494	1028	263	2.4	10522
	Goat	91	15	4	3.7	106
	Total	11149	1227	360	2.9	12376
May 2021	Cattle	1773	169	89	4.5	1942
	Sheep	12235	1031	272	2	13266
	Goat	74	11	5	5.8	85
	Total	14082	1211	366	2.3	15293
June 2021	Cattle	1917	218	83	3.8	2135
	Sheep	12084	1272	293	2.1	13356
	Goat	72	9	3	4.1	81
	Total	14073	1499	379	2.4	15572
July 2021	Cattle	1868	211	80	3.8	2079
	Sheep	12633	1254	248	1.7	13887
	Goat	83	11	7	7.4	94
	Total	14584	1476	335	2	16060
August 2021	Cattle	1409	149	83	5.3	1558
	Sheep	10262	1105	209	1.8	11367
	Goat	58	8	6	9	66
	Total	11729	1262	298	2.2	12991

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In addition to the number of slaughtered animals, the data encompassed other parameters, such as the difference in the susceptibility of slaughtered males and females to infection with fascioliasis. It was demonstrated in the aforementioned Table that the highest rate of infection with fascioliasis was reported among goats during August 2021 with a prevalence of 9.0%, whereas the lower rate was 1.7% among inspected sheep in July 2021. In contrast to our survey, the study of Piri *et al* [32], carried out in Iran, showed that sheep and goats had lower rates of infection.

According to a study conducted in Mazandaran, northern Iran[34], *F. hepatica* is more prevalent in sheep than goats, which does not match the findings of the current study. The infection rate with *F. hepatica* in sheep for the current investigation was lower than that carried out in Abu-Ghraib district, and Kirkuk province, which found 12.73%, and 72% respectively, using different diagnostic techniques[35, 36]. Although the current study outcome was lower than the rates obtained in Iran, which was 32%[37]. In contrast, the prevalence was lower than 7.5 % reported in Turkey[38].

The Morphological Study

Based on the dimensional measuring of body length/width/ratio, as shown in Fig. 3, morphological measurements categorized the samples as *F. hepatica* and *F. gigantica*. Fig. 3A represents an adult whole mount of *F. hepatica* cleared by acetone, while Fig. 3B shows formalin preserved *F. gigantica* adult sample. From Fig. 3C, which was well preserved in formalin, demonstrations of oral sucker (OS) and ventral sucker (VS) can be seen. The two male organs are represented as multi-lobed testes (T), which are located in the body's medial region and are shown in Fig. 3D. On the other hand, Fig. 3E and F demonstrate the additional specific morphological traits, which are comprised of the major intestinal caeca (I), which diverges from the pharynx (Ph), mouth (M), the genital pore (GP), the ventral sucker (or acetabulum, VS), the main two vitelline glands (VG), one on each side, the single branched ovary (O), also on the left side of the fluke, and delivers the egg (E) through the oviduct (Od) to the ootype, in which each oocyte collaborates with vitelline cells, and an egg-shell (fully completed eggs) as indicated in Fig. 3G and H and reach the proximal folds of the uterus (U), where they are initially kept until the newer ova arrive from behind then push them forward to reach the genital hole.

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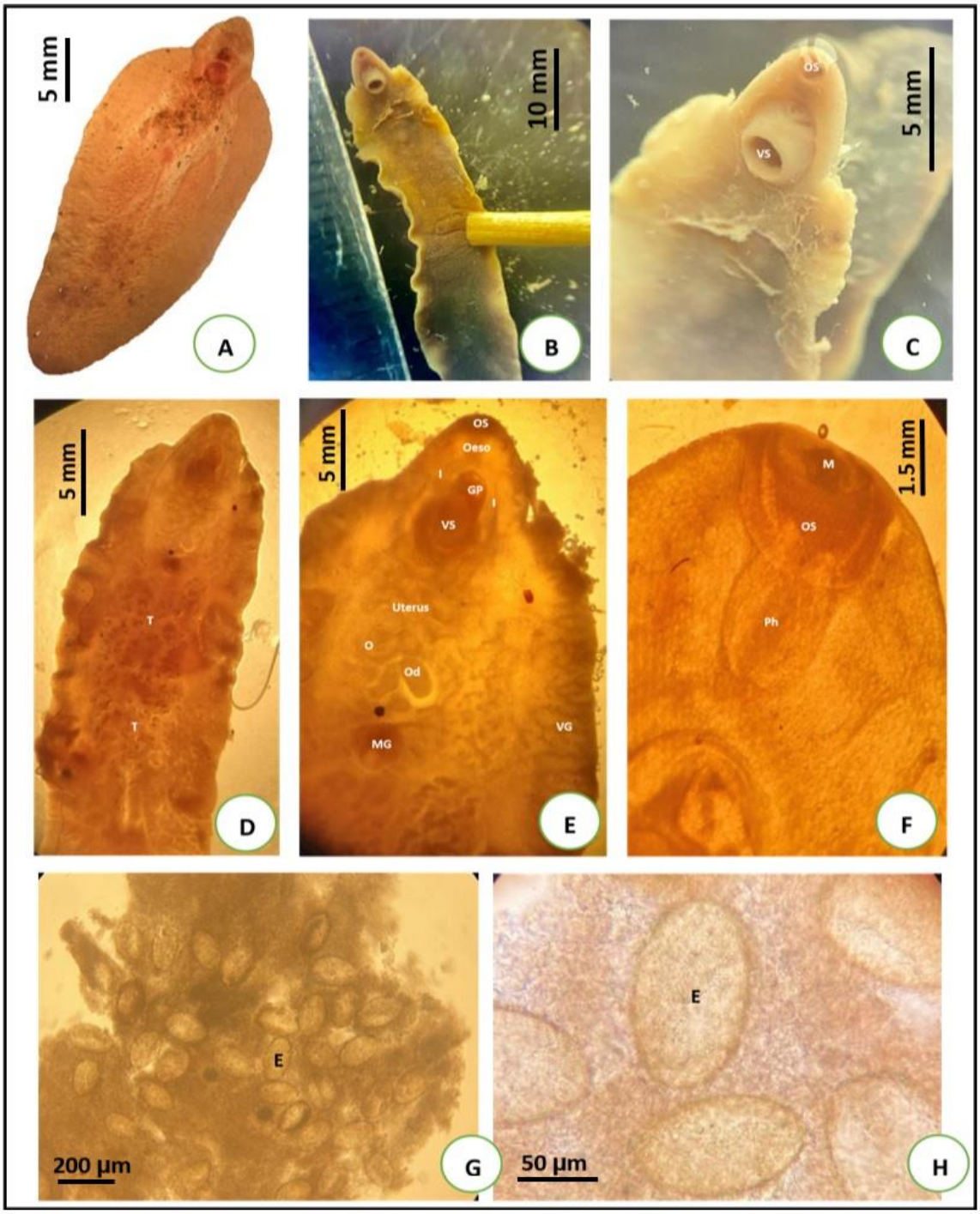

Figure 3. From A-H included different stages of *Fasciola* species: fresh and fixed sample mounted.

A: Photomicrograph of F. hepatica adult.

B: Photomicrograph of F. gigantica adult.

C: The Anterior view of adult Fasciola species, showing oral and ventral suckers (OS and VS).

D: Photomicrograph of the anterior and middle parts of F. gigantica, showing testes of the male reproductive organs.

E: Magnified photomicrograph of F. gigantica anterior end, showing female reproductive organs.

F: The Anterior end of F. gigantica, showing mouth, oral sucker and pharynx.

G; H: Operculated egg of Fasciola species.

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The main two intestinal caeca (I) split off from the pharynx (Ph) behind the oral sucker (OS). The situation of the genital pore is marked (GP), the ventral sucker (or acetabulum, VS). The median region of the fluke's body is taken up by the two extensively lobed testes (T). Two bilaterally situated vitelline glands (VG). On the left side of the fluke, a single branching ovary (O) delivers oocytes or eggs, which then connect with vitelline cells at the ootype. At this point, an eggshell begins to form around the mass cells. Mehlis's glands are present (MG) developed eggs enter the proximal coils of the uterus (U) and are temporarily stored there before being sent towards the placenta.

For *F. hepatica* and *F. gigantica*, the typical length/width and corresponding standard deviation (SD) were 21.16 ± 4.29/10.53 ± 1.80 mm and 39.61 ± 1.09/10.44 ± 1.59 mm, respectively. The results additionally demonstrated that the key factor in morphological classifying 33 described samples as either *F. hepatica* or *F. gigantica*. Due to their distinct modes of transmission and epidemiological traits, the differential diagnosis of *F. hepatica* and *F. gigantica* infection in both people and animals are extremely important[39]. For follow-up investigation, infection control, and treatments, it is essential to determine the species of the genus of such parasites. Morphological approaches cannot provide the precise identification of *Fasciola* species due to numerous differences in morphological characters[16]. Several investigations were conducted to determine the species of *Fasciola*. To differentiate between species, the morphological characteristics of adult worms and eggs are typically employed. However, this method can be influenced by various factors, such as the age of the parasite and the preservation method used for the sample[40], the phenotypic appearance of *Fasciola* spp. can also exhibit multiple variations, leading to difficulties in accurate identification and causing confusion among researchers[41], As a result, there has been an increasing reliance on both morphometric and molecular techniques to effectively differentiate between species[42].

Molecular Study

The genomic DNA was extracted from four *Fasciola* individuals representing two different host species (cattle and sheep) and two geographical regions in Erbil and Halabja, Kurdistan Region, Iraq. It was found that the designed forward (F) and reverse (R) primers were universal for the expected fragment of 365 bp as the 28S rDNA. This amplicon was observed in the studied samples as a visible amplicon with a 365 bp size as shown in Fig. 4.

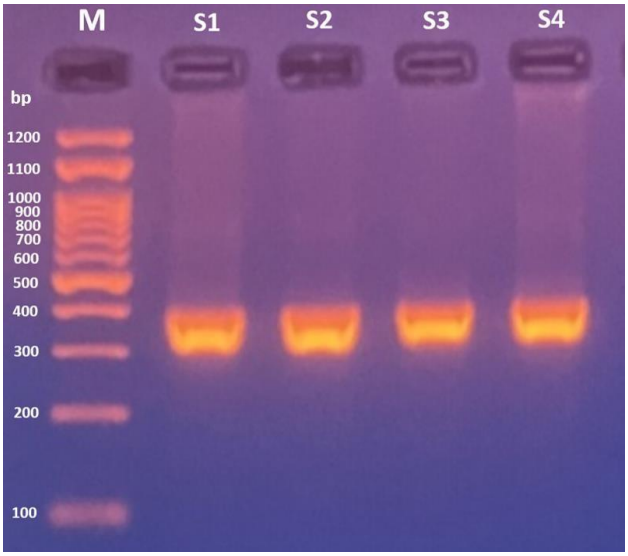

Figure 4. One percent of agarose gel electrophoresis with loading 3 µl of extracted genomic DNA. S1-S4 represented sample number one to number4. The displayed bands considered as 28S rDNA primer for isolated liver flukes the position 360 bp, using standard molecular DNA ladder (M = 100 bp Marker) for comparison.

The sequence from extracted DNA of *F. hepatica* and *F. gigantica* was 28S rDNA of 339 bp (amplified fragment was 365 bp, while after sequencing 11-26 miss-nucleotides were excluded accordingly, related to improving the quality of sequencing analysis) as shown in Fig. 5A-D, after that, the sequences put to the BLAST then compared with the other stored species of *Fasciola* sequences from GenBank, the top score sequence similarity searching to identify homologous sequences and relative mutations for liver fluke databases.

The collection of information from different specimens deposited in GenBank. The BLAST results are constructed via alignment sequences. Regarding the query sequences, which was represented as forward sequence, showed 98% identical to *F. hepatica*, whereas, reverse sequence showed 99% identical to *F. hepatica*. On the other

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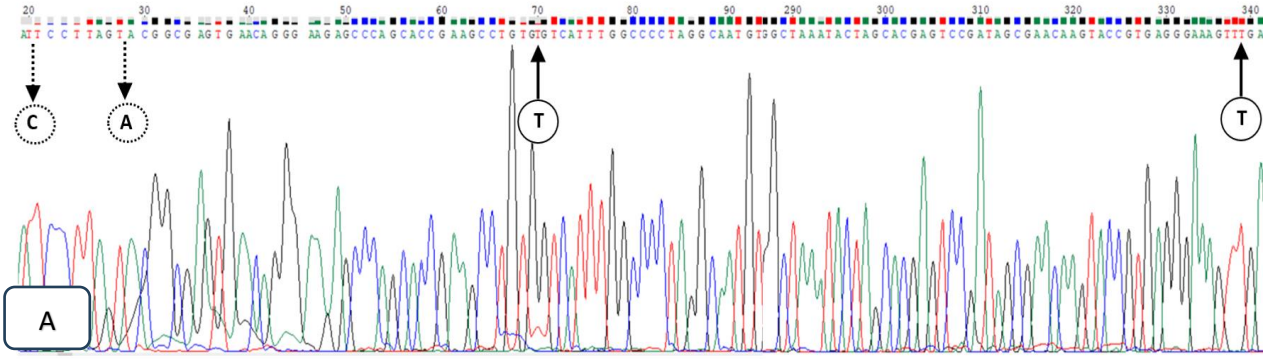
hand, the query forward sequence was 97% identical to *F. gigantica*, whereas, reverse sequence exhibited 99% identical to *F. gigantica*.

After the alignments of the sequences, the following mutations associated with the polymorphism of the query sequences were detected. From the DNA forward sequence of *F. hepatica*, at site 22 there was a gap representing the deletion of Cytosine (C), after that, there was another gap at site 30 which resulted from missing Adenine (A). The last deletion mutation was demonstrated at site 351 with Thymine (T). On the other hand, there was a single transition substitution mutation at site 349, which was C substituted with T and another transversion substitution at site 347, which was A converted with T. Additionally, 3 insertion mutations were reported at sites 70, 338 and 348, which were T, A and T respectively. According to the results of the reverse query DNA sequence, only a single deletion mutation with the Cytosine was determined at site 22.

Regarding the DNA forward sequence of *F. gigantica*, at site 15 there was a gap representing the deletion of Cytosine (C), after that, there were several gaps at sites 23, 31, and 42, which resulted in missing Adenine (A). There were two transition substitution mutations at sites 333 and 343: A substituted with G and C substituted with T respectively. Furthermore, the transversion substitution occurred at site 344, which was represented as C converted with A. Additionally, the last insertion mutation was reported at sites 347 and 349 with Cytosine (C). On the other hand, and according to the results of the reverse query DNA sequence of *F. gigantica*, only single insertion and single deletion mutations with C were determined at sites 7 and 20 respectively.

According to Imani Baran, Cheraghi Saray [43]. Molecular methods have been regarded as more accurate when compared to other conventional techniques for identifying *Fasciola* species. Furthermore, the variation among *Fasciola* species regarding to life cycle and species-specific intermediate hosts is also recognized in many studies[44-46]. Several researches together constructed on nuclear and mitochondrial DNA sequences have shown that *F. hepatica*, is the commonest and greatest prevalent species in temperate areas, whereas *F. gigantica* is found in tropical countries of Africa[26, 46].

The polymorphism of *F. hepatica* encountered in locating its snail host or in tissue migration in its vertebrate host, as well as variations in host preference among parasite populations, may be related to the high polymorphism reported in *F. hepatica*[47]. The development of novel anthelmintics that target the parasite's neuromuscular system may be especially relevant to this polymorphism[48]



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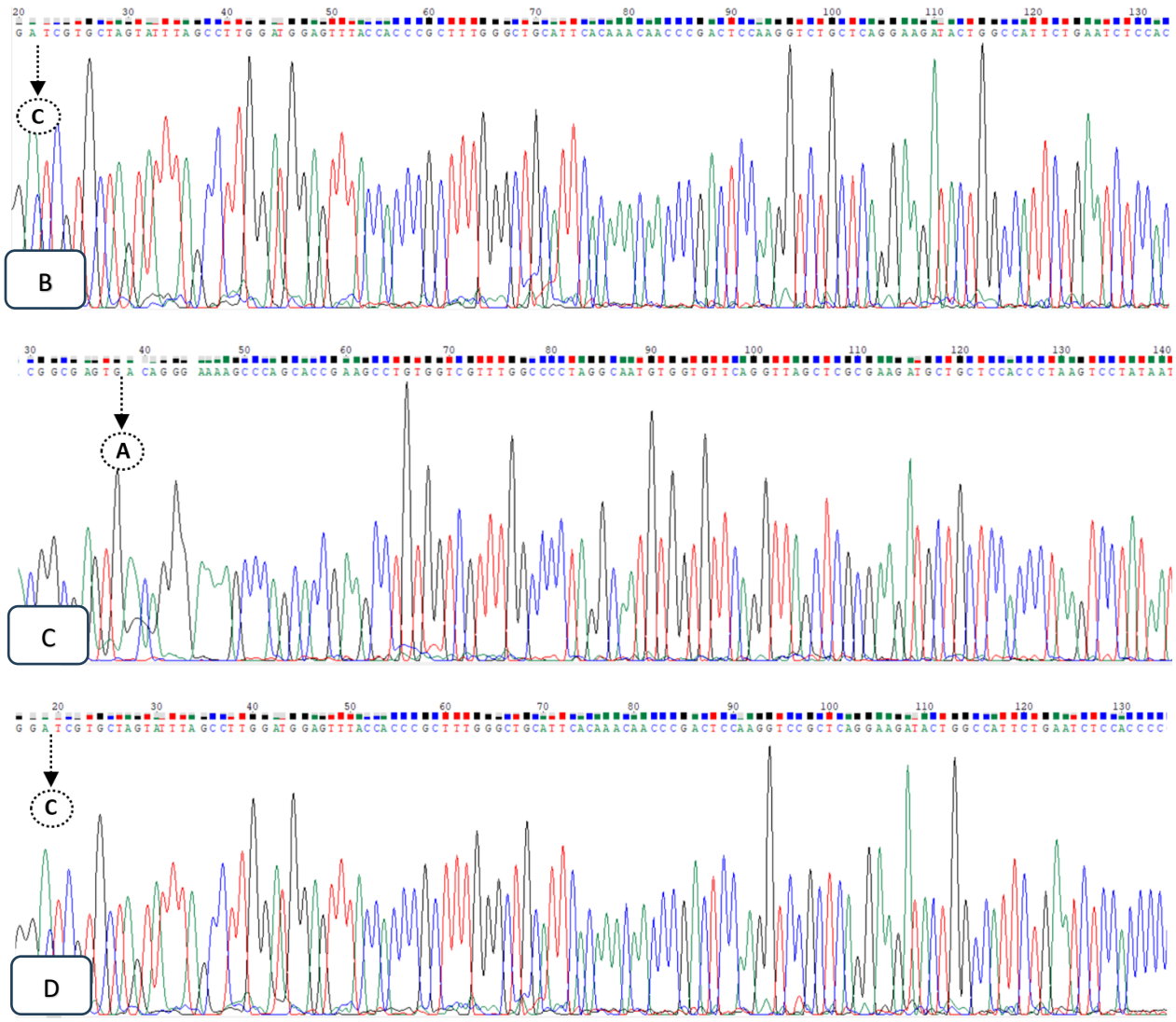

Figure 5. The chromatogram of the sequenced PCR product, the results extracted from 28S rDNA *Fasciola* species representing nucleotide polymorphism (upward black arrow represents the insertion of nucleotide, downward discrete arrow represents deletion of nucleotide).

A The Forward sequence of F. hepatica ***B The Reverse sequence of F. hepatica.***
C The Forward sequence of F. gigantica ***D The Reverse sequence of F. gigantica.***

Histopathological Study

Gross Hepatopathology

The liver exhibited notably gross pathology, including an enlargement in size (hepatomegaly) as observed in Fig. 6A. This was accompanied by bleeding and inflammatory responses occurring within the liver tissue parenchyma, as shown in Fig. 6B. There were several pathologic lesions on the parietal surface of the liver, including congestion, firm, whitish patches within the parenchyma assumed to be fibrosis, in addition to an abscess with calcification in certain cases and demonstrated in Fig. 6C. Engorgement of the bile in the conduit was one of the major biochemical abnormalities in the biliary duct. Prominent in the dissected sections, there were visibly enlarged and fibrotic bile ducts that were obstructed by twisted flukes. Blackish-brown exudates were noticed in the bile ducts of Fig. 6D.

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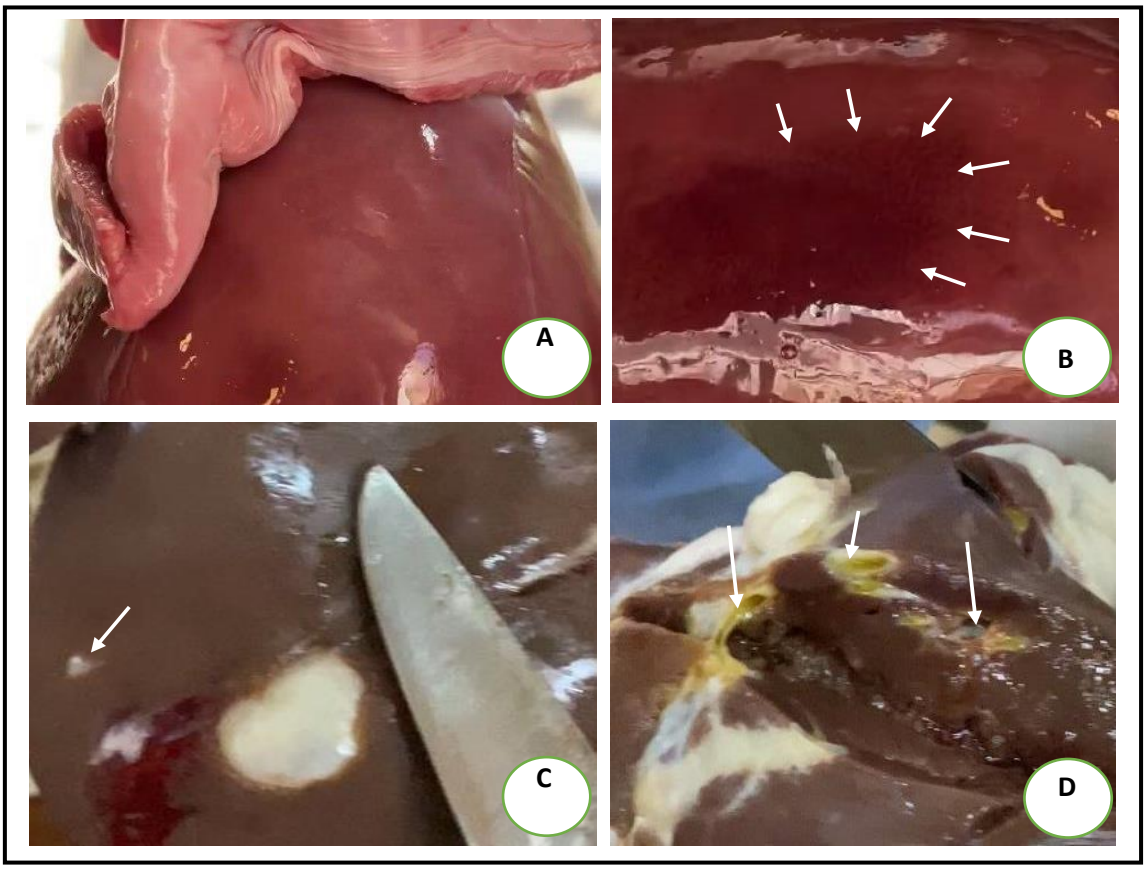

Figure 6. Macroscopic description of infected sheep liver with Fascioliasis.
A. Hepatomegaly ***B. The congestion and Hemorrhages***
C. Necrosis and Fibrosis ***D. Bile duct filled with blackish brown exudates.***

The liver is the most important organ for an animal's health, reproduction, and production. The liver is associated with many disorders, either directly or indirectly, and liver dysfunction interferes with metabolic functions essential for optimal health and productivity[49]. Animals with fasciolosis suffer liver damage as a consequence of the mechanical or chemical actions of the parasite or as a result of the inflammatory and immunological reactions of the host. Infectious metacercaria induces mechanical harm as they pass through the liver capsule and hepatic tissue[8].

The objective of this research was to evaluate the gross clinical and histological alterations that occur in the livers of sheep and cattle following infection with *F. hepatica* and *F. gigantica*. Gross pathology indicated an enlargement of the liver, which is related to immune response, alterations in the hepatocytes, and congestion on the liver's cell surface. These findings match the observations of Ashoor & Wakid [50], Hepatomegaly and congestion on the liver's surface were reported in persistent fasciolosis from livestock. The bleeding sites on the skin reflected the young parasites' portal of entry into the liver structure[8, 51]. It may also occur as a result of inflammatory reactions and immature fluke migration into the liver parenchyma. Moreover, multiple pale areas of the liver due to necrosis or injured tissue occurred. Congestion and rigid whitening patches within the parenchyma, referred to as fibrosis, were seen in certain instances. These findings are similar in some ways to those of Navarro & Uzal [52], who found also pale and anemic discoloration, as well as swelling and several necrotic areas in the liver's impacted areas. Additionally, Al-Mahmood & Al-Sabaawy [53] reported similar findings. Researchers also documented bile duct engorgement. The bile ducts were found to be occluded with a dark brown discharge. It's possible that adult flukes in the bile duct produce constant irritation, resulting in

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hyperplastic proliferation and severe ductular fibrosis[8, 50].

Histopathological Examination

In the liver and bile duct sections of cattle infected with *Fasciola* species, many histological changes were found, which occurred in various degrees based on the severity of the infection as compared to the non-infected sheep or cattle livers, in contrast to the normal structures in the non-infected sheep or cattle liver can be seen in Fig. 7. The infiltration of fibroblasts and mononuclear leukocytes (chronic inflammation) in the area originally invaded by immature flukes was a characteristic of the histological changes in cattle liver caused by fasciolosis. Clear regions with superficially located nuclei appeared in the cytosol of the liver cell, which had suffered fatty change.

Similarly, liver sections exhibited atrophy and necrosis of liver cells as a result of long-lasting fasciolosis. The hepatocyte was significantly damaged as a result of the parasite's migration through the liver and parenchyma tissue. The vacuolar disintegration of liver cells was attributed to the disruption of their cell walls, distorted nuclei, and the infiltration of cytosolic contents into the sinusoids. This demonstrates the acute infection or parenchymal phase, in addition to the significant pathological impacts it has. In some situations, periportal vein, cellular infiltration with eosinophils, macrophages, and lymphocytes was seen along with the breakdown of enlarged hepatocytes. With the entrance of mononuclear inflammatory cells and extensive fibrous connective tissue proliferation in the gateway zone, pressure atrophy of hepatocytes close to the fibrosis zone was observed. This sometimes develops along with the formation of pleomorphic large cells. The modification of the hepatocytes was employed to categorize some components. However, additional sections similarly demonstrated hyperplasia of the bile ducts together with persistent fibrous cholangitis and an accumulation of many inflammatory cells, principally mononuclear cells and eosinophils. Yellowish-brown concretions representing the aggregation of bile pigment in the bile canaliculi or enlarged bile ducts were found. Portal triaditis (inflammation of liver triads and neighbor connective tissue) was similarly detected. Inflammation of the liver's triads and the surrounding connective tissue was also noted as portal triaditis. A few sections of the migratory tract had lymphocytic infiltration. The liver's histological alterations from fascioliasis were marked by the infiltration of eosinophils, fibroblasts, and lymphocytes into the area previously penetrated by immature flukes. The flukes' protease production results in long-lasting stimulation and recruitment into the hepatic cells, which promotes hemorrhages that cause tissue damage. The current results are in line with previous studies that demonstrated immature liver flukes induced bleeding, irritation, and cellular inflammatory responses as they moved across the tissue[8, 54].

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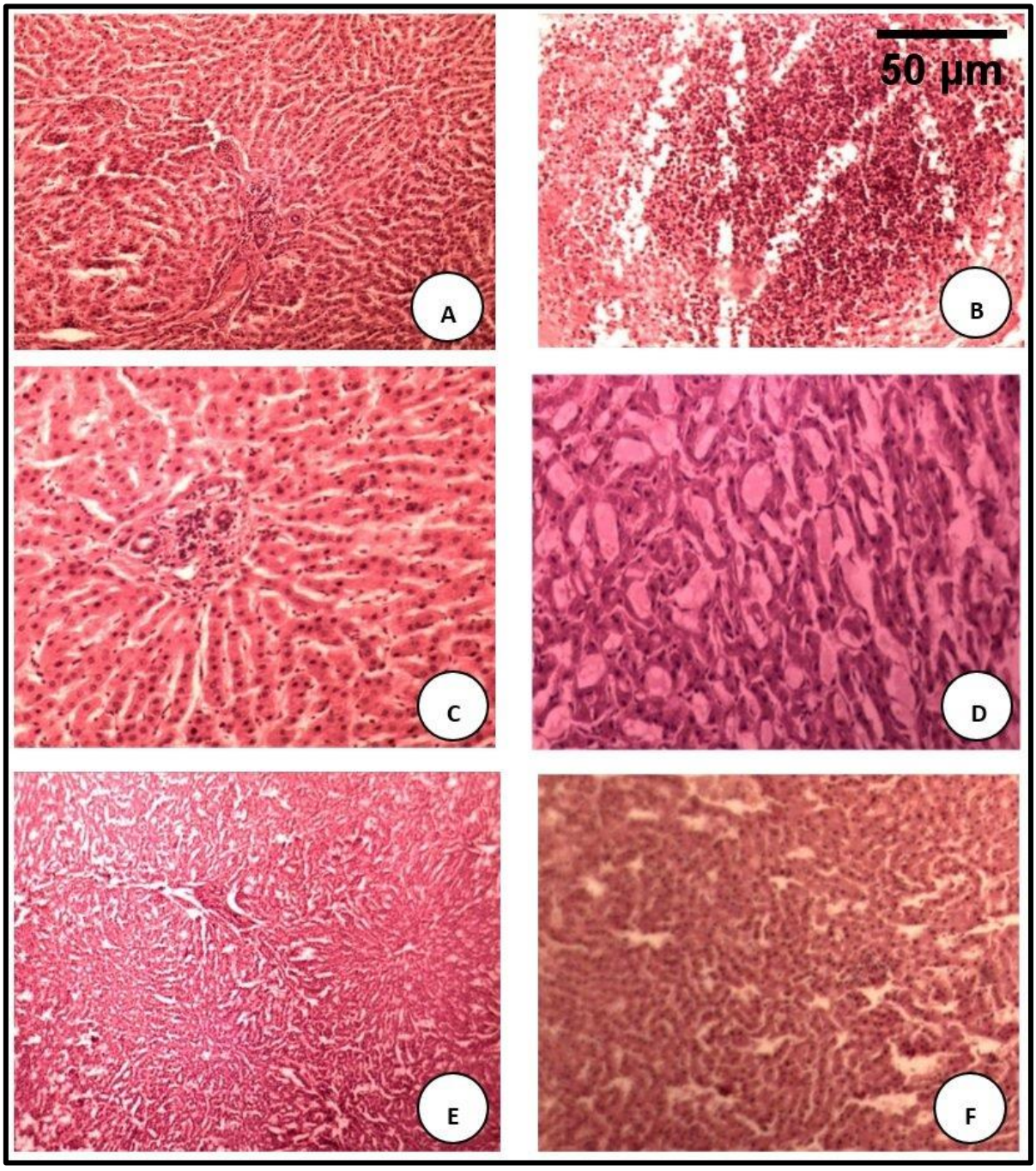

Figure 7. Histopathological sections of the liver. Hematoxylin-Eosin stains were used.

A. The mononuclear leukocyte infiltrations and hepatocyte necrosis of infected cattle's liver with Fasciola spp.

B. The liver of infected cattle with Fasciola* spp. *Experiences infiltrations of inflammatory cells, particularly macrophages.

C. The presence of inflammatory cells, particularly macrophages and eosinophils, along with tissue fibrosis around the portal area, can be observed in the liver of cattle infected with Fasciola spp.

D. Cattle infected with Fasciola spp. exhibit fatty alteration and the presence of large clear vacuoles.

E. The uninfected cattle's liver.

F. The uninfected sheep liver.

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Conclusion

Based on the findings of the present epidemiological study, it can be concluded that fascioliasis is widespread among sheep, goats, and cattle in Erbil and Halabja provinces of the Kurdistan Region of Iraq. This parasitic infection poses a significant barrier to the development of livestock resources and economic growth in the region. The identification of *Fasciola hepatica* and *F. gigantica* through the analysis of 28S rDNA sequences can provide valuable insights for designing effective control measures and minimizing economic losses in the field of animal husbandry. Furthermore, the identification of specific point mutations in the sequenced DNA allows researchers to gain a more comprehensive understanding of the genetic characterizations of the local *Fasciola* species.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee at University of Salahaddin.

Authors' Contribution Statement

Q. M.K. K.: Research title suggestion and proposal, laboratory techniques, parasites morphology-based identification, histological preparation, manuscript draft writing and revising.
R.A. Kh.: Molecular part techniques (including DNA amplification and sequencing) of the parasite.
M. L. R. and L. N. N.: Sample and data collection from the slaughter houses, histopathological section preparation with making tabulation of the results and the parasite photo taking.

Supplemental Files

- Supplement 1: Appendix

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التغيرات النسيجية والتوصيف الجزيئي لداء المتورقات (مرض حيواني المنشأ) بين الماشية المذبوحة في مجازر أربيل وحلبجة، إقليم كوردستان – العراق

قارەمان مامه خضر كۆیی ، ڕۆژگار عبدالله خیلانی ، محمود لقمان رحمن، ليزا نعمان نصرالدين

قسم علوم الحياة، كلية العلوم، جامعة الصلاح الدين، أربيل، العراق.

الخلاصة

داء حلزون كبد الاغنام (المتورقات) الناجم عن *Fasciola (F.) hepatica* و *F. gigantica* مرض معد يصيب الإنسان والحيوانات المجترة في العديد من دول العالم، بما في ذلك العراق. إن دراسة هذه الطفيليات، وتأثيراتها الضارة على مختلف المضائف، شكليًا ، نسيجيا ، وبائيًا وجزيئيًا مهمة جدًا. لهذا السبب تم تصميم البحث الحالي للتحقق من المثقوبة الكبدية في الماشية التي تم ذبحها في مسالخ مدينة أربيل وحلبجة من شهر اب إلى شهر تشرين الاول ٢٠٢٢. حيث تم جمع ٣٣ مثقوبة كبدية من القنوات الصفراوية للحيوانات. نقلت العينات إلى مختبر أبحاث علم الحيوان، جامعة صلاح الدين- أربيل. تم استخدام بعض العينات لتحديد العينة مظهريا، في حين تم حفظ بعضها في ٩٦ ٪ من الإيثانول وتخزينها عند -20 م° .ثم تم استخلاص الحمض النووي لتحديد العينة جزيئيا. من ناحية أخرى، تم تثبيت قطع من كبد المجترات المصابة في ٩٩٪ إيثانول و ١٠٪ فورمالين قبل استخدامها في فحص الأنسجة. تم التعرف على أنواع هذه الطفيليات باستخدام الوحدة الفرعية 28SrDNA ، وتفاعل البوليميراز المتسلسل التقليدي (PCR)، وطرق التسلسل. بالفحص المجهري كان تضخم الكبد هو النتيجة الرئيسية للاصابات. كانت أعلى نسبة للالصابة بين الأبقار ٥،٦٪ ، يليها الماعز ٥،٠٪ ، والأغنام ٢،٠٪. بينت النتائج وجود تطابقا بنسبة ٩٨-٩٩٪ مطابقة لسلسلةمن بنك الجينات ل *F. hepatica* و *F. gigantica*. ثبت أيضًا أن تسلسل 28SrDNA-PCR جنبًا إلى جنب مع الخصائص الشكلية لأنواع *Fasciola* يمكن تطبيقه كمؤشر بارز في تحديد هذة المثقوبات. تعتبر الدراسة الحالية بمثابة تحقيق شامل لداء المتورقات في إقليم كردستان.

الكلمات المفتاحية: داء المتورقات، أمراض الأنسجة، علم التشكل، التحديد الجزيئي، الماشية المذبوحة.