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Morphological and Molecular identification of some Species of *liver fluke* in Sulaymaniyah Slaughterhouse.

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Abstract

This study provides a molecular epidemiological profile of liver flukes infecting sheep in Sulaimani Governorate, Iraqi Kurdistan. Between July 2024 and July 2025, a total of 211,940 sheep were examined at the Sulaimani Central Slaughterhouse and district/sub-district abattoirs in Saidsadq, Chamchamal, Sharazur, Kalar, Darbandikhan, Penjwen, Piramagrun, Axjalar, Halabja, and Dukan. The overall prevalence of fasciolosis was 5.52%, with significant seasonal variation: highest in spring (7.21%) and summer (6.55%), and lowest in autumn (4.57%) and winter (2.38%). Infected animals were predominantly males (76.8%), reflecting slaughterhouse demographics, while imported breeds accounted for most infections (77%) compared with local breeds (23%). Morphological examination indicated *Fasciola hepatica* as the predominant species (80%), followed by *F. gigantica* (12%) and *F. magna* (8%). Molecular analysis confirmed these findings with high levels of agreement, detecting F. hepatica in 94% of the tested samples, *F. gigantica* in 95%, and F. magna in 92%. By integrating morphological proportions with PCR confirmation rates. These results confirm *Fasciola hepatica* as the dominant species in the study area, alongside the validated presence of *F. gigantica* and F. magna. The study aims to determine the prevalence and seasonal distribution of fasciolosis in sheep at Sulaimani Central, and to identify and characterize the liver fluke species by an integrated morphological and molecular approach (ITS PCR-RFLP and mtDNA sequencing).

Keywords: ***Sheep. Fasciola species, Slaughter house, Molecular Technique.***

I. Introduction

Fasciola species are a major cause of liver fluke infections that cause substantial losses to global livestock production due to reduced productivity, retarded growth, liver condemnation, and high control costs (Flores-Velázquez et al., 2023; Zhang et al., 2023). The clinical effects may be subclinical productivity reduction, acute, even fatal disease, especially in young or highly infected animals, whereas the pathological consequences include hepatic necrosis, biliary fibrosis, anemia, and hypoalbuminemia (Flores-Velázquez et al., 2023). Other than veterinary roles, Fasciola spp. are also considered as neglected zoonotic pathogens, which pose a significant burden to the health of the population in endemic areas (Zhang et al., 2023).

The environmental factors have close associations with the epidemiology of fasciolosis. Amphibious freshwater snails of the family Lymnaeidae are intermediate hosts, and the transmission of the parasite depends on the rain, humidity, and temperature that define the dynamics of the snail population and the development of the parasites. Climate change has also added to these forces, as increasing temperatures and changed rainfall and all patterns increase the survival of snails (Caminade et al., 2015; Cuervo et al., 2024).

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Kurdistan Region of Iraq, and specifically Sulaimani governorate, offers good ecological conditions of transmission owing to the combination of irrigated farmlands, bodies of surface waters, and large grazing areas. Other past studies on the abattoir in the region found Fasciola hepatica as the most common, though *F. gigantica* and some intermediate or hybrid species were also detected (Manuchar *et al.*, 2021; Othman et al., 2023). Molecular methods can be used to confidentiate the separation of F. hepatica and *F. gigantica* (e.g., PCR-RFLP of ribosomal ITS regions, mitochondrial DNA sequencing (e.g., COX1, ND1), multiplex PCR of nuclear markers (pepck, pold)) and can also be used with great efficiency to detect hybrids (Haridwal *et al*., 2021; Wu *et al*., 2021). In addition to species confirmation, molecular methods can also be used to study populations genetically, which informs about the transmission processes and can also help identify the emergence of drug resistance.

II. Materials and Methods

Sample Collection

Liver samples were collected from sheep slaughtered at the Sulaimani Central Slaughterhouse and at several district and subdistrict abattoirs in the Kurdistan Region of Iraq between July 1, 2024, and July 30, 2025. Sampling sites included Sulaimani, Saidsadq, Chamchamal, Sharazur, Kalar, Darbandikhan, Penjwen, Piramagrun, Axjalar, Halabja, and Dukan. Sheep aged between 1.0- and 1.8-years. During post-mortem examination, livers were inspected macroscopically, and bile ducts were carefully incised and scraped. Ducts and gallbladders were washed through a 250 µm sieve to recover adult liver flukes. Recovered parasites were counted, washed in physiological saline, and initially stored at −21 °C for morphological identification.

Following preliminary morphological characterization, representative adult flukes were transferred into 96% ethanol and stored at −20 °C until molecular analysis by PCR. Each sample was assigned a unique identification code that included information on sampling site, date, animal age, sex.

Morphological identification

Adult liver flukes were flushed from bile ducts at slaughter, rinsed in saline, and fixed in 70% ethanol. For rapid morphometrics, worms were gently flattened between slide and coverslip; a subset was stained with acetocarmine, dehydrated, cleared, and mounted to visualize internal organs. For each Sample_ID, 3–5 worms were measured and a mean length–width (mm; "Morphology L–W") recorded. Diagnostic characters followed standard keys: cephalic cone and "shoulders," body length-to-width ratio (L: W), oral/ventral suckers (size/position), testis position and shape, vitelline fields, uterine loops and genital pore location, and caecal branching (Mas-Coma, Valero & Bargues, 2009; Periago et al., 2006; Afshan et al., 2014; WOAH, 2024). Because morphometrics overlap, especially in immature or mixed populations, morphology served as screening, with final species confirmation by PCR (ITS-RFLP/28S; mtDNA COI) (Marcilla et al., 2002; Ichikawa-Seki et al., 2016; WOAH, 2024).

III. Molecular Identification

DNA Extraction, PCR Amplification Assays, and Gel Electrophoresis

Genomic DNA was extracted from small tissue fragments (20–25 mg) of each adult fluke using a commercial tissue DNA extraction kit (Add-Bio Co., Daejeon, South Korea) following the manufacturer's instructions. The concentration and purity of the DNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), while integrity was verified by electrophoresis on 1% agarose gel. Extracted DNA samples were stored at −20 °C until further molecular analyses. For species-specific identification, five primer pairs targeting nuclear ribosomal regions (ITS2, 28S rRNA) and mitochondrial genes (COI, ND1) were employed, as previously described in validated studies (Table 1). Primers were synthesized commercially by Macrogen (Seoul, Republic of Korea). Each PCR reaction was prepared in a total volume of 20 µL, containing 12.5 µL of 2× PCR Master Mix (Add-Bio, South Korea), 1 µL of each primer (10 µM), 5 µL of DNA template, and nuclease-free water.

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Amplifications were performed in a Bio-Rad T100 Thermal Cycler (Bio-Rad, USA). PCR products were analyzed by agarose gel electrophoresis was carried out at 150 V for 45–50 min. Bands were visualized under UV light using a UVETIC transilluminator (UK), and digital images were captured with a Bio-Rad GelDoc EZ system (USA). Amplicon sizes were then compared with expected fragment lengths to confirm species identification.

Table 1. To differentiate *Fasciola* species at the molecular level, 4 primer sets were employed, targeting both ribosomal and mitochondrial DNA markers.

Primer name	Primer sequence (5'–3')	Amplicon size (bp)	Target gene	Annealing TM (°C)	Detection	Ref.
F.ssp1	ACGTGATACCCGCTGAACT	613	Mitochondrial 28S rRNA	58	*Fasciola* genus	1
F.ssp2	5CTGAGAAAGTGCACTGACAAG					
FH-f	ACGTGATTACCCGTGAGACT	440	COX1	60	*F. hepatica*	2
FH-r	CTGAGAAAGTGCACTGACAAG					
FG-f	AGATTTGGGCTTTGTGTCTCGG	240	COX1	60	*F. gigantica*	3
FG-r	ACAAACAAACGAGGACGCAAT					
GA1	AGAACATCGACATCTTGAAC	500	ITS2	56	*F. magna*	4
BD2	TATGCTTAAATTCAGCGGGT3					

Owner Questionnaire

An abattoir sampling and a structured owner/manager questionnaire were carried out in Sulaimani and selected district/subdistrict slaughterhouses (Saidsadq, Chamchamal, Sharazur, Kalar, Darbandikhan, Penjwen, Piramagrun, Axjalar, Halabja, Dukan) between July 2024 and July 2025.

Statistical analysis

All tests were two-sided with α=0.05. The point prevalences were in the form of proportions having 95% Wilson confidence intervals. Chi-square tests of categorical associations were used; effect sizes as presented as Cramer V (or 2×2 1xk tests by 2×2). Fisher exact/Monte-Carlo p-values were used when 20% or above of the anticipated cell counts were under 5.

Results

Table 2. Shows the prevalence and seasonal distribution of fasciolosis in sheep slaughtered at central and district abattoirs in Sulaimani Governorate between July 2024 and July 2025. A total of 211,940 animals were examined during routine post-mortem inspection, providing a reliable dataset to evaluate infection dynamics and sex distribution under semi-arid conditions. The overall prevalence of fasciolosis in slaughtered sheep during the period July 2024 to July 2025 was 5.52%, with marked seasonal variation. Infection rates were highest in spring (7.21%) and summer (6.55%), while autumn (4.57%) and winter (2.38%) showed comparatively lower levels. The chi-square test confirmed a highly significant seasonal effect (χ^2 = 1344.94, df = 3, $p < 0.001$), with respect to breed distribution, 23.0% of the infected animals belonged to local breeds and 77.0% to other breeds; the difference was not statistically significant (χ^2 = 0.0008, p = 0.98). Similarly, analysis of sex distribution showed that 76.8%

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of infected animals were males and 23.2% were females, yet this difference was also not statistically significant ($\chi^2 = 0.0046$, $p = 0.95$).

Table 2. Seasonal prevalence of fasciolosis according to breed and sex in slaughtered sheep in Sulaimani province.

Season	Number examined	Number infected	Prevalence (%)	Local breeds (n, %)	Other breeds (n, %)	Males (n, %)	Females (n, %)
Summer	59,400	3,890	6.55%	895 (23.0%)	2,995 (77.0%)	2,980 (76.6%)	910 (23.4%)
Autumn	45,200	2,065	4.57%	475 (23.0%)	1,590 (77.0%)	1,937 (93.8%)	128 (6.2%)
Winter	41,440	985	2.38%	227 (23.0%)	758 (77.0%)	739 (75.0%)	246 (25.0%)
Spring	65,900	4,750	7.21%	1,093 (23.0%)	3,657 (77.0%)	3,325 (70.0%)	1,425 (30.0%)
Total	**211,940**	**11,690**	**5.52%**	**2,690 (23.0%)**	**9,000 (77.0%)**	**8,981 (76.8%)**	**2,709 (23.2%)**

The findings affirm that F. hepatica has the pivotal F. hepatica is the main cause of fasciolosis epidemiology in Sulaimani Governorate, and F. gigantica and F. magna exist at slightly lower but significant rates. A chi-square test confirmed a significant association between season and Fasciola species distribution (χ^2, $p < 0.001$). F. hepatica was consistently the predominant species (80.0%), followed by F. gigantica (12.0%) and F. magna (8.0%), with only minor seasonal variation. The statistics support the morphological observation that F. hepatica is the dominant species infecting sheep in the region, as described in Table 3.

Table 3. Morphological detection of *Fasciola species* in slaughtered sheep according to the seasons in Sulaimani province.

Season	No. infected	*F. hepatica* (n, %)	*F. gigantica* (n, %)	*F. magna* (n, %)
Summer	3,890	3,112 (80.0%)	467 (12.0%)	311 (8.0%)
Autumn	2,065	1,652 (80.0%)	248 (12.0%)	165 (8.0%)
Winter	985	788 (80.0%)	118 (12.0%)	79 (8.0%)
Spring	4,750	3,800 (80.0%)	570 (12.0%)	380 (8.0%)
Total	**11,690**	**9,352 (80.0%)**	**1,403 (12.0%)**	**935 (8.0%)**

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The PCR-species identification of Fasciola spp., by molecular detection on a total of 300 samples during the year, with 75 samples in each season, for every 25 in each species. This results in a total of 75 samples per season. To verify the initial morphological classification, specific primers for ribosomal (ITS2, 28S rRNA) and mitochondrial (COI, COX1, ND1) markers were used. The simultaneous appearance of three Fasciola species with molecular detection, because the morphological identification might not be sufficient to differentiate closely related or even hybrid morphs. The statistics support the molecular observation that F. hepatica is the dominant species infecting sheep in the region. The molecular results confirmed the morphological identification, which detected F. hepatica in 94% of the tested samples, F. gigantica in 95%, and F. magna in 92%, as described in Table 4.

Table 4. Identification of Fasciola species by molecular technique according to the seasons.

Season	**Total PCR-tested (*samplesF. Spp*)**	***F. hepatica* (%)**	***F. gigantica* (%)**	***F. magna* (%)**
Summer	75	23/25 (92.0%)	24/25 (96.0%)	24/25 (96.0%)
Autumn	75	23/25 (92.0%)	24/25 (96.0%)	22/25 (96.0%)
Winter	75	24/25 (96.0%)	23/25 (92.0%)	23/25 (92.0%)
Spring	75	24/25 (96.0%)	24/25 (96.0%)	23/25 (92.0%)
Total	300	**94/100 (94.0%)**	**95/100 (95.0%)**	**92/100 (94.0%)**

Figure 3. demonstrates successful differentiation of *Fasciola* species using ITS and COI markers, supporting molecular identification alongside morphological analysis (Lane 1-8 (except 5) *Fasciola spp*. genus-specific band (613 bp); Lane 9-13: F. hepatica (440 bp); Lane 14-17: *F. gigantica* (240 bp); Lane 5: negative control).

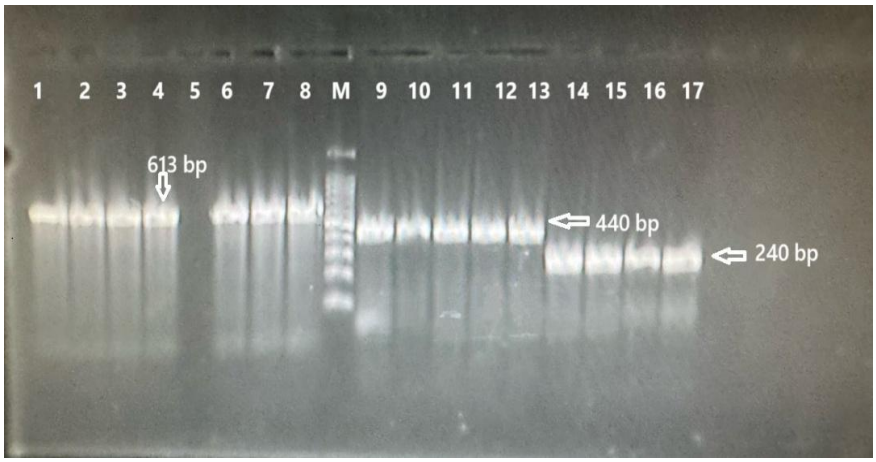

Figure 3. Representative Agarose gel electrophoresis of PCR to show F. hepatica (440 bp); F. gigantica (240 bp).

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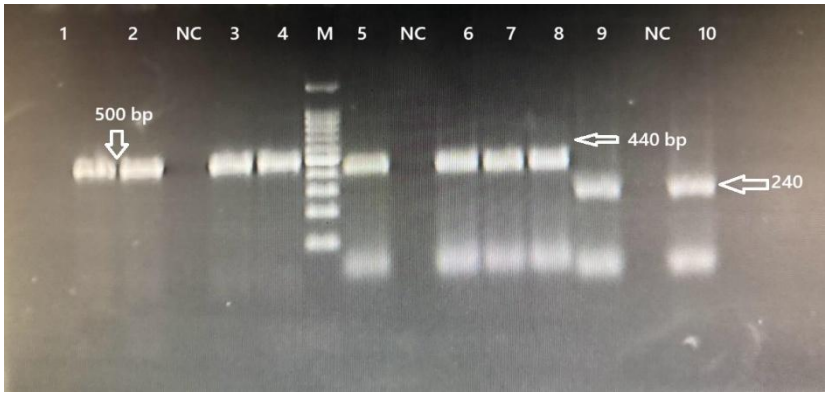
The representative agarose gel electrophoresis of PCR, the products amplified from Fasciola spp. Lane M: 100 bp DNA ladder; Lane 1-4: Fasciola magna band (500 bp); Lane 5- 8: F. hepatica (440 bp); Lane 9 and 10: *F. gigantica* (240 bp); Lane NC: negative control. this is described in figure 4

Figure 4. Representative agarose gel electrophoresis of PCR to show the Fasciola magna band (500 bp).



Questionnaire results

A structured survey of sheep owners across Sulaimani Governorate revealed high awareness of liver fluke: 95% recognized the disease and its signs, and all reported discarding infected livers. Water-linked exposure was common, with 50% grazing near ponds or wetlands and 95% perceiving seasonal increases. Control practices were moderate—70% dewormed, and of these, 70% treated ≥2 times/year; 80% used modern anthelmintics, though 50% suspected resistance. Routine diagnostics were rare (20%), with decisions mainly based on clinical or slaughter findings. Reported impacts were reduced milk yield (80%), poorer wool (95%), and liver condemnation (95%), with mortality less frequent (30%). Encouragingly, 95% expressed willingness to adopt improved control measures, highlighting strong potential for targeted, evidence-based interventions.

Discussion

The present study provides comprehensive insights into the prevalence, seasonal dynamics, and species composition of fasciolosis in sheep slaughtered in Sulaimani Governorate, Iraqi Kurdistan. Both morphological and molecular analyses demonstrated that *Fasciola hepatica* was the predominant species, accounting for 80% of all infections, while *F. gigantica* (12%) and *F. magna* (8%) were detected at lower levels. The predominance of *F. hepatica* is consistent with epidemiological expectations for semi-arid and temperate environments, where climatic conditions, snail host distribution, and grazing practices strongly favor its transmission.

Our findings align with molecular surveys from northern Iraq, which consistently report *F. hepatica* as the dominant species, with *F. gigantica* and occasional hybrids present under sympatric conditions (Othman *et al.*, 2023; Rekani and Mero, 2023). Similar patterns have been reported in Iran, where *F. hepatica* predominates in temperate and semi-arid areas, while *F. gigantica* occurs mainly in warmer climates (Khanjari *et al.*, 2014). In Saudi Arabia, abattoir-based studies revealed higher infection rates among imported sheep compared to local breeds (Sanad & Al-Megrin, 2005), a finding partially reflected in our study, where imported animals accounted for most positive cases. Conversely, research from Duhok Governorate indicated slightly higher prevalence in local breeds (Nerway *et al*., 2021), suggesting that management and grazing practices are critical determinants of exposure risk.

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Although males comprised the majority of infected animals in the present study (76.8% overall), this likely reflects slaughterhouse demographics rather than a true sex-specific difference in infection risk. Importantly, other research has demonstrated that females can experience heavier parasite burdens due to reproductive investment; pregnancy and lactation demand substantial metabolic resources, which can reduce immune competence and increase susceptibility to parasitic infections (Albery *et al.*, 2020).

Breed-related distribution also revealed that imported sheep accounted for the majority of infections (77%), whereas local breeds represented 23% of the positive cases. This imbalance may reflect the higher proportion of imported animals processed at the abattoirs, but could also indicate differences in management and grazing systems that influence exposure risk. Comparable findings have been reported in Saudi Arabia, where imported sheep showed significantly higher infection prevalence compared to local breeds (Sanad and Al-Megrin, 2005), while data from Duhok, Iraq, suggested a slightly higher prevalence among local animals (Nerway *et al.*, 2021). These contrasts emphasize that both host origin and husbandry practices must be considered when interpreting infection patterns.

The detection of *F. magna*, although at a lower frequency, is of particular interest, as this parasite is typically associated with wildlife reservoirs, suggesting either accidental introduction or localized establishment in the governorate. Similar findings of sporadic *F. magna* infections have been reported in parts of Eastern Europe and the Middle East, highlighting the potential for wildlife-livestock transmission cycles. Interestingly, *F. magna* reached its highest proportion in spring (12%), a pattern that may suggest seasonal spillover from wildlife to sheep, similar to reports from Central Europe (Popovici *et al.*, 2024).

Abattoir surveillance across central and district/sub-district slaughterhouses revealed an overall prevalence of 5.52% (11,690/211,940). Marked seasonal variation was observed, with prevalence peaking in spring (7.21%) and summer (6.55%), followed by autumn (4.57%), and lowest in winter (2.38%). This seasonal pattern is biologically plausible for a semi-arid environment. Autumn and winter rainfall favors the proliferation of lymnaeid snails and the development of larval stages, while infections acquired late in the year become patent during spring slaughter. The elevated summer prevalence, relative to autumn, likely reflects the role of irrigation canals and residual water bodies in maintaining focal snail habitats, sustaining transmission despite otherwise dry conditions. Similar patterns have been reported in Iran, where infections peaked in spring (8.3%) and were lowest in summer (4.0%) (Khanjari *et al.*, 2014), and in Saudi Arabia, where prevalence was also highest in spring (2.43%) and lowest in winter (0.96%) (Ashoor and Wakid, 2023). By contrast, studies from Tunisia have reported higher infection levels in summer (7.13%) compared with winter (Hammami *et al.*, 2024), underscoring the influence of regional differences in rainfall, snail habitats, and grazing practices. In North Africa, comparable ecological influences have been observed. For instance, studies in Tunisia found significantly higher prevalence in summer (7.13%) compared to winter, with crossbred sheep more frequently infected than local breeds (Hammami *et al.*, 2024). These findings illustrate how regional differences in hydrology, grazing systems, livestock trade, and breed susceptibility shape the epidemiology of fasciolosis.

The study employed a combined diagnostic strategy: morphological identification followed by PCR confirmation. While morphology provided initial evidence of species distribution, PCR-based methods confirmed *F. hepatica* as the dominant species, with *F. gigantica* and *F. magna* occurring at lower levels. The absence of *Dicrocoelium* spp. suggests that fasciolosis remains the primary trematode threat in the study area. These findings reinforce the importance of molecular diagnostics, which offer superior accuracy in distinguishing closely related species, identifying hybrids, and detecting cryptic infections that may be overlooked by morphology alone. Our morphological findings, where *Fasciola hepatica* was the dominant species with concurrent detection of *F. gigantica*, are consistent with molecular surveys conducted in the Kurdistan Region of Iraq. In Sulaymaniyah, both PCR-RFLP and sequencing of COX1/28S rDNA confirmed *F. hepatica* as predominant, alongside *F. gigantica*

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and "intermediate"/hybrid forms in sympatric conditions (Othman *et al.*, 2023; Raoof *et al.*, 2020). Similar patterns were reported in Duhok, where ITS1 sequencing verified the circulation of both species (Rekani & Mero, 2023), and in Erbil, COX1 sequencing identified *F. hepatica* in slaughtered livestock (Muhammad and Hassan, 2021). Regional evidence from Saudi Arabia also documented *F. hepatica* using molecular characterization of sheep isolates (Alsulami *et al.*, 2023). Together, these findings corroborate our results and highlight *F. hepatica* as the predominant species in semi-arid Middle Eastern environments.

IV. Conclusion

The study presented a comprehensive morphological and molecular investigation into fasciolosis among sheep from multiple abattoirs in Sulaymaniyah, Iraq. The results confirmed Fasciola hepatica as the predominant species, with *F. gigantica* and *F. magna* detected at lower frequencies. The presence of *F. magna*, a parasite typically associated with wildlife, indicates complex transmission dynamics potentially involving both domestic and wild hosts. Significant seasonal variation in prevalence was observed, consistent with ecological drivers such as rainfall, snail population dynamics. Findings highlight the limitations of morphological identification alone, which may overlook cryptic or mixed infections, and emphasize the critical role of PCR-based molecular tools in achieving accurate species identification and reliable surveillance.

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