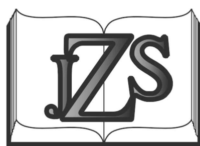

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Molecular characterization and phylogenetic analysis of *Fasciola* species in sheep and goats in Sulaymaniyah Province, Northern Iraq

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Abstract
Both *F. hepatica* and *F. gigantica* are considered as the main causes of human and animal fascioliasis that have much medical and economic importance worldwide. Nowadays, identification and description of Fasciola species using molecular-based techniques are critical and reliable approach in most laboratories and research centers. Thus, this research aimed to investigate the prevalence of fascioliasis and molecular characterization of isolated Fasciola species in sheep and goats in Sulaimaniyah province, Northern Iraq. Briefly, a total of 100 liver samples from slaughterhouse and 100 fecal samples from the animal field were collected from sheep and goats. In overall collected liver specimens, only 30 (15%) samples were found to be positive using polymerase chain reaction (PCR) assay, from which only 6 samples were selected for sequencing of the partial mitochondrial 28S rRNA gene and codon analysis. Simultaneously, the collected fecal samples were also analyzed using the Fecal Egg Count Reduction Test (FECRT), in which only 4 samples were positive. The results revealed that the identified four field sequences strains were *F. hepatica* and the other two field sequences were *F. gigantica.* In conclusion, we revealed that both *F. hepatica* and *F. gigantica* were distributed in Sulaimaniyah province and the mitochondrial 28S rRNA gene is confirmed as a potential biomarker in identifying various Fasciola species

Introduction

Liver fluke infection or fascioliasis in various animal species, especially in ruminants is mainly caused by *F. hepatica*, and *F. gigantica* while in humans the most predominant species to cause infection is *F. hepatica* [1]. Significant economic damages due to fertility disorders, incredible drop in animal growth and production, rejection of a huge numbers of infected livers during meat inspections, great morbidity and mortality, substantial cost of veterinary services for diagnosis, treatment and prevention makes fascioliasis to be counted as a critical disease worldwide especially in undeveloped countries including Iraq [1, 2].

F. hepatica accomplished its whole life cycle in dual major hosts including cattle as a definitive host, and the snail as an intermediate host, while the accidental host is a human being [3 - 5]. Several freshwater snails in the family Lymnaeidae serve as intermediate hosts. Undercooked or raw marine vegetables and drinking

fresh water polluted with the infective metacercariae are the main sources of human and animal are infection [6, 7].

Currently, the differentiation of *F. hepatica* and *F. gigantica* are based on the implementing of several highly developed molecular approaches, using different molecular targets since the morphological methods have too many limitations [8 - 10]. In Asian countries, recent reports proved the liver fluke infection of ruminants (cattle, sheep, and goat) including Iraq [11], Pakistan (Kashmir) [12], Saudi Arabia [13], Turkey [14], and Iran [15].

Therefore, the present researches designed to carry out the conventional PCR based analysis to determine the prevalence of fascioliasis in small ruminants in Sulaimaniyah province, and also for molecular differentiation between and *F. hepatica* and *F. gigantica.*

Materials and Methods

Sample collection

This study was carried out from February 2019 to August 2019. A total of 100 liver samples and 100 fecal specimens were collected from sheep and goats. Approximately, 10 g of fecal sample was obtained in a sterile way and placed in sterile plastic collecting caps, immediately transported to the laboratory at 4° C for Fecal Egg Count Reduction Test (FECRT) and subsequently preserved at - 20° C for DNA extraction, while part of liver tissues were collected in the slaughterhouse and transferred to the laboratory in a cold box for DNA extraction directly without delay.

Fecal Egg Count Reduction Test (FECRT)

To visualize and count the number of Fasciola eggs per gram (epg) of feces, a sedimentation method was used. Briefly, the fecal sample (10 g) was mixed thoroughly and diluted with 100 ml of water [16]. Then, poured into 3 sieves of 38 mm, 150 mm and 500 mm in which they stacked with the smallest aperture at the bottom and largest at the top to allow slow pass of fecal water. Later on, thorough washing with water ran clear from the bottom sieve was done, then the 500 mm sieve was removed and washing through the remaining 2 sieves was repeated. Next, the 150 mm sieve was removed and the retentive on the surface of the 38 mm sieve was washed out and the remaining contents back washed into a 500 ml beaker. The beaker was topped up with water and left to stand for exactly 4 minutes, then the supernatant was poured off leaving approximately 100 ml of sediment and then the beakers were refilled with water and left to stand for another 4 minutes. This step was repeatedly done until supernatant became cloudless and then was poured off to 100 ml carefully without loss and the remaining content was transferred into a large square petri dish. About 4 drops of methylene blue were added and the number of eggs counted using a dissecting microscope [17]. Finally, the number of eggs per gram of feces (epg) was calculated by dividing the total number of eggs by 10.

Sample preparation and DNA extraction

Regarding the fecal samples, the specimens were vortexed well in phosphate-buffered saline (1 ml of 0.1 M PBS with pH 7) then DNA was extracted using a DNA extraction kit (Genaid, Co, Korea) based to the manufacturer's instructions without change. Whereas, DNA extracted from liver samples using (Genet-bio, Korea) exactly according to the manufacturer protocol.

Oligonucleotide primer and DNA amplification

The fragmented 618bp of the partial mitochondrial 28S rRNA gene from each sample was amplified using PCR assay with the primer forward sequence (5-ACGTGATTACCCGCTGAACT-3) and reverse sequence (5-CTGAGAAAGTGCACTGACAAG-3) [18, 19]. The PCR Premix kit provided a complete system for fast, high yield and reliable single-tube PCR (Genet-bio, Korea). The reactions were conducted in 0.2 ml PCR tube with a final volume of 20 µl that contained 10 µl supreme script PCR premix, 5.0 µl DNA, 1.0 µl forward, 1.0 µl reverse primers (10 pmol from each), and 3.0 µl ultra-pure water. The conventional PCR machine (Hercuvan, USA) was set as an initial denaturation at 95° C/10 minutes; 40 cycles of 95° C/40 seconds; annealing at 58° C/45 seconds, and extension at 72 °C/45 seconds and a final extension at 72° C/10

minutes. Next, 5 µl of each amplified DNA sample was loaded on to a 1.2% agarose gel in TBE buffer at 60 V for 60 minutes. The gels stained with safe dye (Eurx-poland), visualized and photographed using a transilluminator (UVITEC، UK). To estimate the size of the amplicons (618 bp), a 100 bp DNA ladder (Genaid. Co., Korea), was used in gels.

Sequencing of the PCR products

To confirm the PCR results, gene sequencing was conducted using 25 µl of the amplified PCR product of two positive samples with both direction primers were sent for sequencing using the Sanger sequencing method (Macrogen, South Korea). The resulted products were submitted into NCBI/ GenBank with the accession number of MN326120-MN326125.

Phylogenetic tree and sequence analysis

The phylogenetic tree was constructed based on partial mitochondrial 28S rRNA gene. The CLUSTALW program was used to conduct sequence homology and multiple sequences alignment at the nucleotide and amino acid levels [20]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X employing the neighbor-joining (NJ) method [21].

Results

In this current study, out of 200 screened samples, only 30 samples were positive including 22 liver and 8 fecal samples using PCR assay (Table1 and Figure1). From which, only 6 samples were selected for sequencing of the partial mitochondrial 28S rRNA gene and codon analysis. Only 4 of the fecal samples were positive using FECRT.

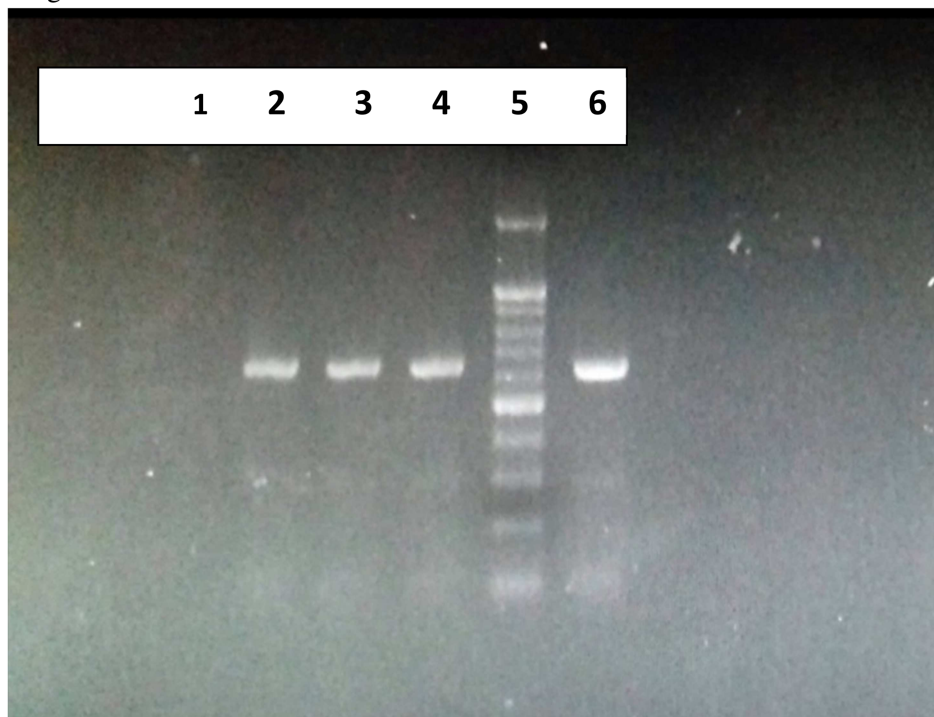

Figure 1. Agarose gel electrophoresis of PCR amplification products. Lane 1-3: Positive samples (618 bp), Lane 4: 100 bp ladder as molecular size DNA marker, Lane 5: positive control (618 bp), and Lane 6: Control negative.

Phylogenetic tree and sequences analysis

Only 6 Fasciola species sequences identified in this study, in which 16 reference sequences of the mitochondrial 28S rRNA gene were studied in this research. The sequences created from various continents including Europe, United States, Asia, and Africa countries. The mitochondrial 28S rRNA gene sequences formed 3 distinct groups (group I–group III). Four field sequences from this research grouped within the Group1 branch, together with the *F. hepatica* from Poland, Bulgaria, and Saudi Arabia strains which indicated that those four field sequences (SFH1-SFH4) strains were *F. hepatica* species. The two outstanding

field sequences categorized within the Group II branch with *F. gigantica* sequences from Iran, Senegal, and Indian strains (Figure 2) which indicated that these two field sequences (SFG1 & SFG2) strains were *F. gigantica* species.

On the other hand, identities of the 4 field sequences of *F. hepatica* and other *F. hepatica* sequences were compared with six 28s rRNA sequences from different countries (Table 2), in which the 4 sequences from this study showed nucleotide identities ranged between 99.16-99.66% between them. The four *F. hepatica* sequences were mostly analogous to the sequences from Iran and Saudi Arabia with identities of 99.45% and 99.33%, respectively (Table 2). Similarly, when the two field sequences of *F. gigantica* were compared with 5 other reference sequences of *F. gigantica*, the highest identities were exhibited with the Indian strain ranged from 99.45-99.63% (Table 3). Partial sequence of mitochondrial 28S rRNA gene of six fields isolated aligned and compared with the reference strains for sequence analysis 28S rRNA sequences revealed limited mutation between them (only one or two nucleotide changed) (Figure 3).

Table 1. Examined fecal and liver from sheep and goats with the positive and negative results using PCR assay.

Sample	No.	Animals species	Positive	Negative
Liver	100	Sheep and goat	22	78
Fecal	100	Sheep and goat	8	92
Total	200		30	170

Table 2. Percentage of specimen identity with *Fasciola hepatica* gene for 28S rRNA, partial sequence isolate.

Accession No.	Country	DNA identities (%)	Phylogenetic Group
HM369290	Bulgaria	97.45 - 97.63	Group I
MF099788	Australia	99.16 - 99.33	Group I
AY222244	Saudi Arabia	99.16 - 99.33	Group I
HM369315	Poland	98.69 - 99.00	Group I
HM369300	Bulgaria	98.88 - 99.25	Group I
AB674557	Iran	98.69 - 99.45	Group I

Table 3. Percentage of specimen identity with *F. gigantica* gene for 28S rRNA, partial sequence isolate.

Accession No.	Country	DNA identities (%)	Phylogenetic Group
AJ439739	Island	98.91 - 98.73	Group II
AY222245	Senegal	99.16 - 99.32	Group II
JF323866	Kashmir	99.27 - 99.45	Group II
MF099787	Vietnam	99.32 - 99.44	Group II
JF323865	India	99.45 - 99.63	Group II

Furthermore, sequence analysis for both intra-species and interspecies genetic distance was analyzed and results exhibited that the intraspecific variability among individuals of both species ranged between 0.2 and 1.6% while the interspecific diversity between *F. hepatica* and *F. gigantic* was 0.8 and 1.3%.

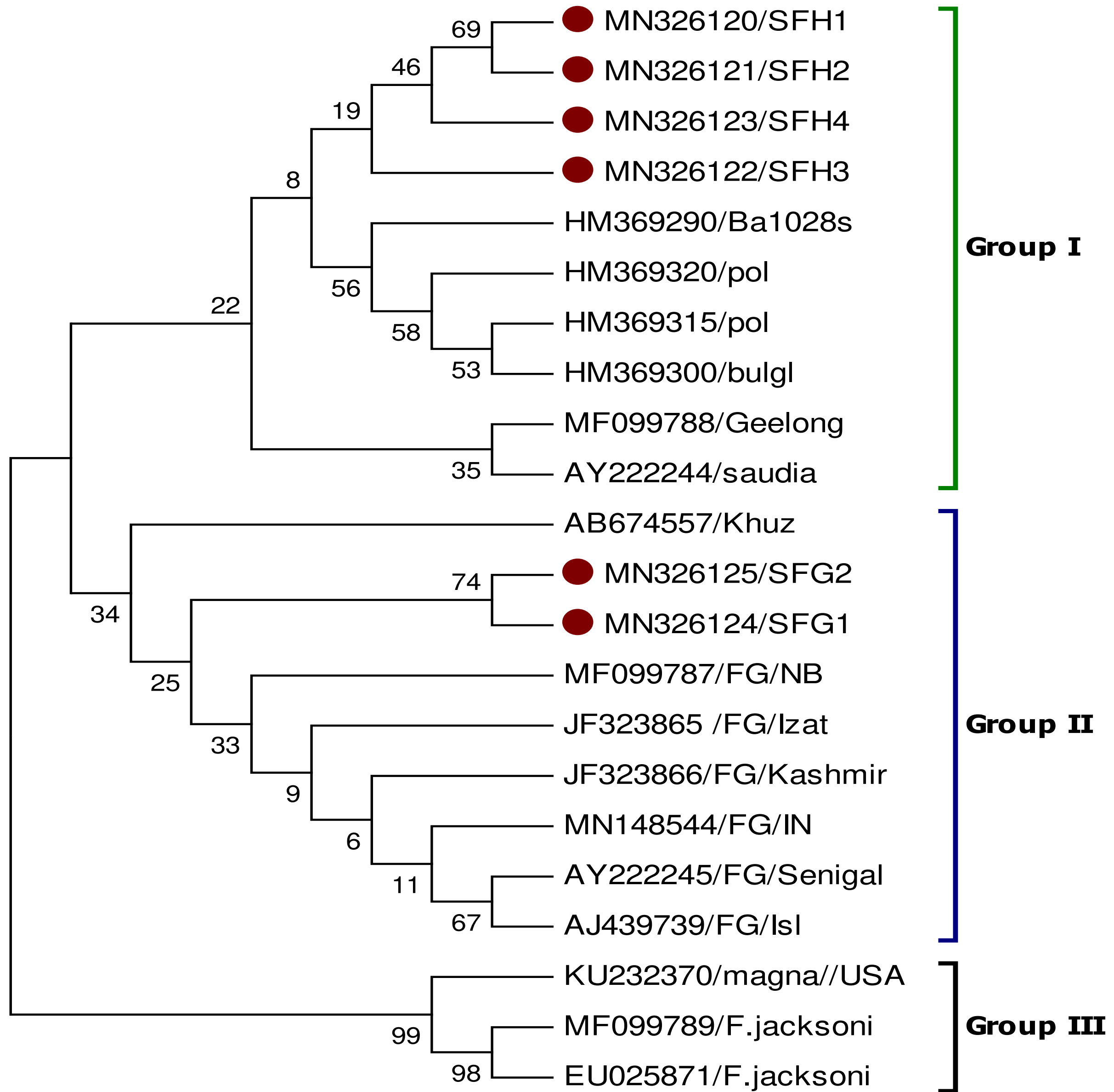

Figure 2. Neighbor-joining tree (Mega-x-version) constructed using Fasciola species of partial sequences mitochondrial 28s rRNA gene and the reference sequences of different countries.

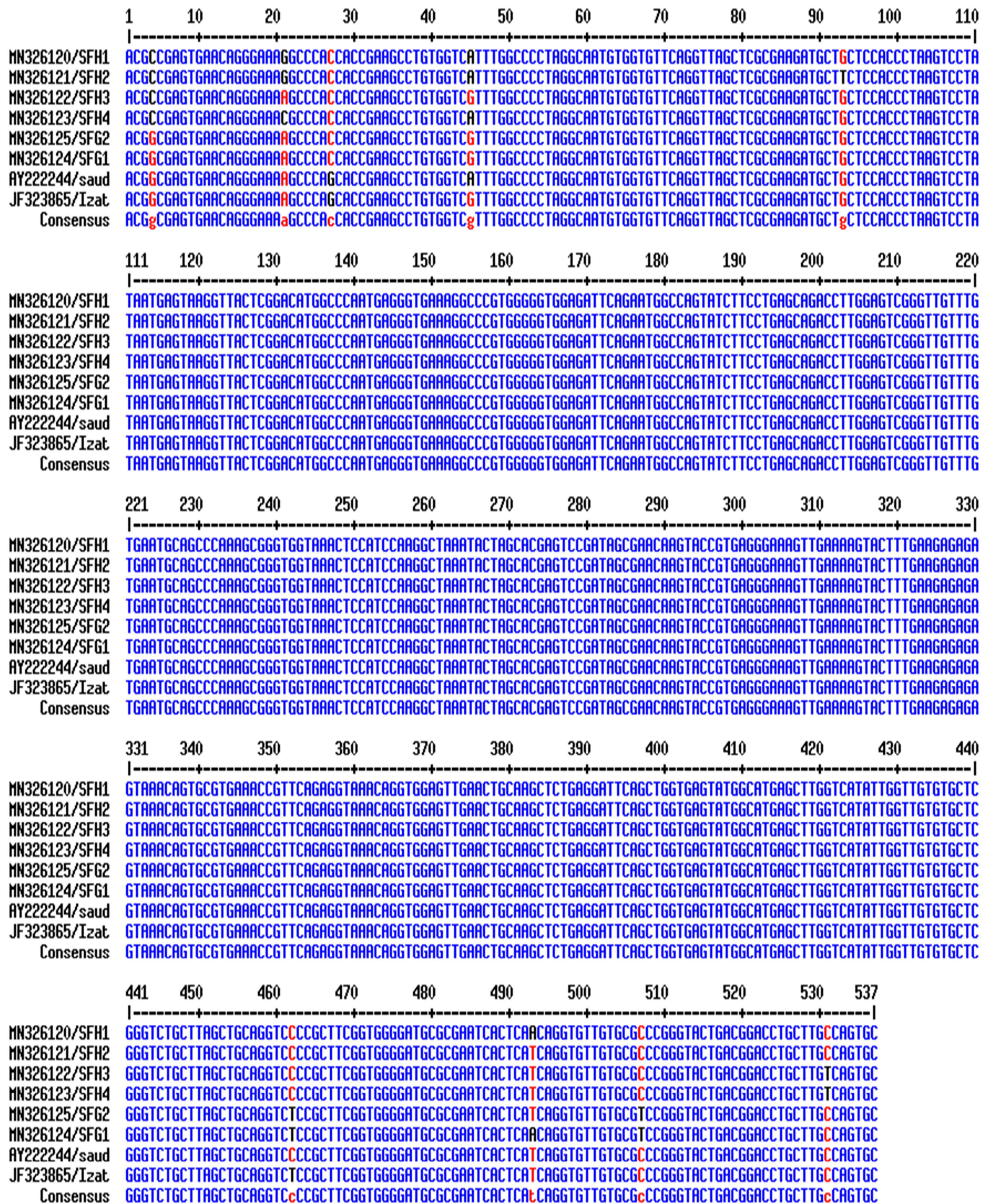

Figure 3. Alignment of the partial sequence of mitochondrial 28S rRNA gene of six fields isolated with the references isolated *F. hepatica* and *F. gingentica* accession number (AY222244 and JF323865), respectively.

Discussion

The traditional identification methods are based on discrimination of morphology between *F. hepatica* and *F. gigantica* species. The recent technical approaches include molecular approach in comparison with other diagnostic methods for Fasciola parasites that have been more accurate investigated by Marcilla [22]. PCR based on mitochondrial 28S rRNA gene is a reliable tool to differentiate *F. hepatica* from *F. gigantica* [23, 24]. In the present study, the phylogenetic tree revealed that *F. hepatica* makes a separate group from *F. gigantica* due to difference in few nucleotides in DNA sequences, also phylogenetic tree presented grades of

intra and interspecific variability between individuals of the same species and among species of the same genera. However, in multiple sequences alignment of a field isolate sequences revealed that 4 Fasciola species were *F. hepatica* and 2 were *F. gigantica.*

F. hepatica mostly similar sequences from Iran and Saudi Arabia, but *F. gigantica* mostly identity with Indian strain, these results indicated for multiple sources of parasite infection. Besides the genetic relationship between sequences of the current study and different countries, it may be related to the legal and illegal trade of export animals from different original regions.

Fecal egg count reduction test is not as precise as PCR, that is why the number of positive samples was less using this technique or another reason that point out decrease or absence of eggs in the fecal samples was might be the owners gave anthelmintic to their animals very often to eliminate the internal parasite.

According to the results of other studies, the prevalence of fascioliasis was 15%. In Egypt, depending on fecal inspection, the prevalence has been assessed as 12.70% in sheep and goats [25], while in Pakistan the prevalence of the fascioliasis recorded was 28.75% [26***],*** and in Iran was 0.1% to 91.4% in various livestock [15].

These differences in the prevalence of *F. hepatica* reported from various studies may be due to differences in resistance to infection, grazing habits and breed of the hosts. In the study conducted previously [26], the prevalence of *F. hepatica* in goats was significantly affected by the age and body weight of the animal and the results showed that as the weight and age of the animal increases the parasitic infection decreases. The results of our study also verified the higher infection rate at a younger age and in lower weight animals.

Conclusion

Identification of Fasciola species based on the sequences of mitochondrial 28S rRNA genes can be useful in a further trail to formulate appropriate control strategies and decrease the economic loss due to fascioliasis in livestock. The findings of this study indicated that *F. hepatica* was the dominant species in animals of the studied area. Therefore, further field studies to verify these results are suggested.

Conflict of Interest

The authors disclose no conflict of interest related to this conducted study.

Acknoledgments

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