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Biochemical Liver Functions and Molecular Identification of *Fasciola hepatica* from Experimentally Infected Rat Model

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Abstract

The current study was performed to evaluate the liver function status as well as molecular characterization of the recovered worms in rats experimentally infected with *F. hepatica*. Sixteen male Wister rats aged 30 days were randomly allocated into two groups ($n = 8$). The first group was infected orally with 15 viable encysted metacercaria of *F. hepatica* per animal. The other group was kept non-infected (control group). At zero time (before infection), the 2nd, 4th, 6th, 8th, 10th, 12th and 14th weeks post-infection (WPI), blood and serum samples were collected via puncture of retro-orbital plexus of veins from each rat. Serum enzyme level (AST and ALT) and total protein were measured, and the serum protein profile was carried out using agarose gel electrophoresis. During the period of the experiment, serum ALT and AST activities and serum total globulins significantly increased while serum total proteins and albumin markedly decreased in the infected group. On the 14th WPI, the data of the electropherogram showed that globulin fractions (α1-, β- and γ-globulin) levels were significantly increased while α2-globulin was markedly decreased in the infected group. The molecular analysis confirmed the amplification of the ITS1, ITS2 and NDI genes of *F. hepatica* recovered from the infected liver of rats with amplicon sizes of 630, 510 and 560 bp, respectively. Sequencing of the amplified ITS gene resulted in the determination of 3 strains (PP108836, PP108837, and PP108838). Also, analysis of the ITS2 gene resulted in obtaining 3 isolates under the accession numbers (PP109065, PP109066, and PP109067). In conclusion, fasciolosis in the rat

model is suitable for routine experimental infections and caused a pronounced liver dysfunction with discharging of the Fasciola eggs in the faeces and the development of adult stages in the bile ducts. Furthermore, molecular techniques are a sensitive tool for the identification and characterisation of the *Fasciola* parasite.

Keywords

Fasciola hepatica, Liver Functions, Serum Enzymes, Serum Protein Electrophoresis, Molecular Characterization

1. Introduction

Fasciolosis/liver rot is a worldwide neglected yet serious tropical zoonotic disease caused mainly by two species of trematodes *Fasciola hepatica* (*F. hepatica*) and *Fasciola gigantica* [1] [2]. The infection induces a major economic problem through a reduction in the growth and productivity of livestock animals, besides losses from the condemnation of liver in abattoirs during the inspection process [3]. The disease affects the livestock resulting in acute or sub-acute inflammation of the liver and bile ducts, subsequently, liver damage, submandibular oedema, reduced weight, anaemia, hypoalbuminemia, decreased milk gain, general intoxication, and mortality [4] [5].

The experimental animal model is widely employed instead of the definitive hosts to conduct biological studies, drug delivery and discovery in various parasitic diseases. Several animal models were developed to study the life cycle and other biological features of *F. hepatica* in snails and mammals [6]. The establishment of infection and completion of the life cycle for *F. hepatica* have been proven in many laboratory animals, such as rabbits, rats and mice [1] [4] [7]. Survival after infection and liver recovery is the challenge that hinders the experimental infection studies of *F. hepatica* and can determine the host of choice. The rat survived longer than other experimental animals (more than one year) and enabled the life cycle to continue till the adult stages with the possibility of liver recovery [6]. Accordingly, the rat is considered to be the more appropriate experimental animal for *F. hepatica* in terms of resilience and pathological reaction.

The migration of flukes causes defects and severe injuries to the liver tissue [8]. Serum protein concentration (albumin, and total globulins) and liver enzymes might be altered [8]. The assessment of serum proteins via electrophoretic pattern is considered an important laboratory tool. It is used in the detection and monitoring of many diseases, instead of the biochemical determination of the concentrations of albumin and globulins [9]. So, the determination of liver enzyme level and serum protein profile might be a proper indicator of liver infection.

To confirm the success of experimental infection of *F. hepatica* in laboratory animals, the shedding of eggs in the faeces and recovery of parasites from the infected liver is usually the golden diagnostic tools. However, molecular identi-

fication makes it possible to characterize the immature stage which would be difficult to differentiate by the morphological features [10]. The genetic characterization and adoption of genome sequencing techniques were employed using the nuclear ibosomal internal transcribed spacer (ITS1 and ITS2) and mitochondrial DNA markers such as NADH dehydrogenase I (NDI) genes [11] [12] [13]. Therefore, the current study was designed to assess the serum biochemical parameters and protein electrophoresis related to liver functions of rats experimentally infected with *Fasciola hepatica* during the 14 weeks post-infection (WPI). The recovered parasite stage from the infection was characterized based on molecular detection of ITS1, ITS2 and mitochondrial NDI genes adding to sequencing and phylogenetic analysis.

2. Material and Methods

2.1. Ethics Approval

All procedures for animals were reviewed and approved by the Institution ales Animal Care and Use Committee of the Institute of Experimental Morphology, Pathology and Anthropology with museum. /Permit number: 11 30127/.

Source of viable *Fasciola hepatica* metacercaria

The metacercaria were kindly provided by Institute of Experimental Morphology, BAS, Bulgaria from the experimentally cultivated snails (*G. truncatula*). The metacercaria were examined by light microscopy for viability whereas the excretory granules have existed in viable metacercaria [1] [14].

2.2. Experimental Design

The experiment was conducted on sixteen male Wistar albino rats-aged 30 days. On the 1st day of the experiment, the rats were orally infected with 15 viable *F. hepatica* encysted metacercariae per rat, suspended in de-chlorinated water, and passed through a stomach tube. Blood and faecal samples were collected and examined once biweekly from 0-time (before infection) till the 14th weeks post-infection. Fluke eggs detection was carried out using the Fluke finder technique to confirm successfull infection [15].

2.3. Blood Samples

At zero time (before infection) and then every 2 weeks post-infection (WPI) till the 14th WPI (the end of the experiment), blood samples were collected by puncture of the retro-orbital plexus of rats, left to clot and then centrifuged at 3000 rpm for 15 min for serum separation then stored at −20˚C for the biochemical parameters and serum and whole blood samples were stored at −80˚C.

2.4. Biochemical Analysis

2.4.1. Determination of Serum Enzymes and Protein Profile

The activity of alanine amino transaminase (ALT) and aspartate amino transaminase (AST) as well as the levels of total proteins and albumin in the serum of

rats were determined spectrophotometrically using Test kits purchased from Erba, Germany. Total globulins were calculated by subtracting the obtained value of albumin from the total proteins.

Serum protein electrophoresis was carried out at the zero-time (non-infected) and 14th WPI using HYDRAGEL7 B1 - B2 a semi-automated agarose gel electrophoresis (AGE) system-(Sebia, France) according to the manufacturer's instructions. The computer software Phoresis (Sebia, France) was used. The electrophoretic curves plus related quantitative specific protein levels for each sample were exhibited. The determination of relative protein levels within each fraction was estimated as the optical absorbance percentage (%).

2.4.2. Flukes Recovery

At the 14th week post infection, the examined rates were euthanized, and dissected where livers and gall bladders were examined carefully. Flukes were assembled and counted according to previous methods [16] [17].

2.4.3. DNA Extraction and Amplification

Six samples of *Fasciola hepatica* were collected from the infected rats. The total DNA was extracted from individual flukes using DNeasy Blood & Tissue Kits (Qiagen, USA). DNA fragments of each target region were amplified by PCR using 1.25 units of Taq polymerase (Promega, Madison, USA), 0.4 mM each of dATP, dTTP, dCTP and dGTP, 2 mM $MgCl_2$, each primer set (20 pmol/25 ml reaction mixture), and PCR buffer. The primer sets used to amplify the fragments are shown in **Table 1**.

Each PCR consisted of 30 cycles of denaturation at 98˚C for 10 s, annealing at 56˚C (for ITS) or 53˚C (for NDI) for 35 s, and extension at 72˚C for 50 s, with an initial denaturation step at 95˚C for 5 min and a final extension step at 68˚C for 10 min. PCR products were visualized by electrophoresis in 1.5% agarose gels [11].

2.4.4. Sequencing and Phylogenetic Analysis

PCR products were purified from the gel using QIAquick Gel Extraction Kit (Qiagen, USA). The purified DNA was sent for direct sequencing. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [18]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis of ITS1 involved 20 nucleotide sequences while for the ITS2, the analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 524 and 335 positions in the final dataset of ITS1 and ITS2, respectively. While, *Babesia* sp. Hue-1 C8/2017/SL mitochondrial cox3 gene for cytochrome oxidase (Acc. No.: LC385890.1) were used as out of group. Evolutionary analyses were conducted in MEGA7 (www.megasoftware.net) [19].

Table 1. List of primers and their correspondence genes used in this study.

Gene name	Primer name	Primer sequence (5' - 3')	Annealing temperature. (˚C)	Expected amplicon size (bp)	Reference
ITS1	ITS1 forward	TTGCGCTGATTACGTCCCTG	56	630 bp	[11]
	ITS1 reverse	TTGGCTGCGCTCTTCATCGAC			
ITS2	ITS2 forward	TGTGTCGATGAAGAGCGCAG	56	510 bp	
	ITS2 reverse	TGGTTAGTTTCTTTTCCTCCGC			
NDI	NDI forward	AAGGATGTTGCTTTGTCGTGG	53	560 bp	
	NDI reverse	GGAGTACGGTTACATTCACA			

2.4.5. Statistical Analysis

The data were represented as mean ± standard error. The data were normally distributed. In biochemical parameters, the differences between the group of rats before (zero-time) and post-infected in different periods were tested for significance using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. However, the difference between the group of rats before (zero-time) and post-infected at the 14th WPI (protein fractions) was analyzed using Student *t*-test. The difference was considered significant at $P < 0.05$ level [20] using Statistical Package for Social Sciences (SPSS) software version 17 computer program (*SPSS Inc*, *Chicago*, *IL*, *USA*).

3. Results

3.1. *Fasciola hepatica* Faecal Egg Detection

The fasciolosis infection was confirmed by the application of the fluke finder technique. It was found that, the first appearance of *F. hepatica* eggs in the faeces of experimentally infected rats were at the 8th WPI.

3.2. Serum Biochemical Findings

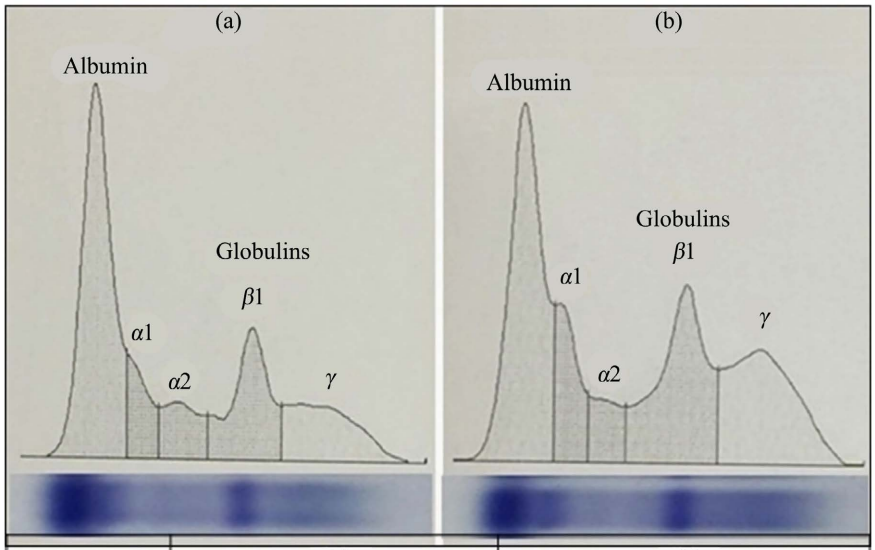
3.2.1. Serum Enzymes

In the present study, the activity of serum ALT has fluctuated in the infected group during the experimental periods which revealed a significant increase at the 14th WPI compared to non-infected rats at zero-time. While the serum AST activity significantly increased from the 2nd till the 12th WPI, and its activity was returned back to normal at the 14th WPI (**Table 2**).

3.2.2. Serum Protein Profile

Total serum proteins and albumin significantly decreased at the 8th and 10th WPI. But, a significant increase was recorded in serum globulins at the 12th and 14th WPI in comparison with non-infected rats at zero-time (**Table 2** and **Table 3**).

With respect to protein electrophoresis at the 14th WPI, the data of electropherogram showed that globulin fractions (α1-, β- and γ-globulin) levels were significantly increased in the infected group, while the α2-globulin level was markedly decreased compared to non-infected rats at zero-time (**Figure 1**).



Parameters	(a)		(b)	
	%	g/dl	%	g/dl
Albumin	49.20	2.92	35.70	2.49
α1-globulin	9.80	0.58	10.70	0.75
α2-globulin	9.30	0.55	5.60	0.39
β-globulin	13.20	0.78	24. 60	1.71
γ-globulin	18.50	1.10	23.40	1.63
Total proteins	100.00	5.93	100.00	6.97
A/G ratio	0.97		0.56	

Figure 1. Agarose gel serum protein electrophoresis patterns and its scan representing: (a) rat before infected and (b) rat after infected with *Fasciola hepatica* at the 14th week post-infection.

Table 2. Serum enzymes and protein profile in rats before and after *Fasciola hepatica* infection during the experimental periods (Mean ± SE, N = 5).

Groups	Periods (WPI)	Parameters				
		ALT (IU/l)	AST (IU/l)	Total proteins (g/dl)	Albumin (g/dl)	Total globulins (g/dl)
Before infection	0	15.12 ± 0.74^{ab}	46.24 ± 1.56^{a}	6.30 ± 0.10^{d}	2.89 ± 0.04^{bc}	1.66 ± 0.08^{ab}
Post-infection	2	17.84 ± 1.65^{cd}	63.84 ± 3.67^{b}	5.88 ± 0.07^{cd}	3.03 ± 0.07^{c}	2.03 ± 0.07^{cd}
	4	13.96 ± 0.49^{a}	56.42 ± 1.91^{b}	5.18 ± 0.11^{b}	2.58 ± 0.09^{bc}	1.49 ± 0.12^{ab}
	6	16.87 ± 0.66^{bc}	56.42 ± 1.60^{b}	5.56 ± 0.18^{bc}	2.39 ± 0.13^{bc}	1.84 ± 0.08^{bc}
	8	12.51 ± 0.39^{a}	59.04 ± 1.41^{b}	4.47 ± 0.10^{a}	2.48 ± 0.03^{a}	1.32 ± 0.08^{a}
	10	15.01 ± 0.93^{ab}	57.73 ± 1.38^{b}	4.49 ± 0.30^{a}	2.63 ± 0.11^{ab}	1.30 ± 0.22^{a}
	12	14.83 ± 0.91^{ab}	61.66 ± 2.36^{b}	5.63 ± 0.28^{bc}	2.72 ± 0.15^{ab}	2.18 ± 0.17^{cd}
	14	19.90 ± 0.95^{d}	43.33 ± 3.36^{a}	5.99 ± 0.10^{cd}	2.90 ± 0.05^{bc}	2.30 ± 0.05^{d}

Means with different superscripts in the same column are significantly different at $P < 0.05$. ALT: Alanine aminotransferase. AST: Aspartate aminotransferase.

Table 3. Serum protein fractions (%) in rats before and after *Fasciola hepatica* infection (at the 14th week post-infection, Mean ± SE, N = 5).

	Normal rats at zero-time	Infected rats at 14th post-infection
Albumin	45.93 ± 1.17	35.47 ± 0.37***
*α*1-globulin	10.10 ± 0.40	12.55 ± 0.48**
*α*2-globulin	9.10 ± 0.14	7.32 ± 0.43*
β-globulin	17.30 ± 1.12	23.44 ± 0.41**
γ-globulin	17.57 ± 0.36	21.21 ± 0.75**

* = Significantly different compared to the normal control by *t*-student test, * = significant at $P < 0.05$. ** = Highly significant at $P < 0.01$. *** = very highly significant $P < 0.001$.

3.3. Molecular Detection

Identification of the obtained *Fasciola* species was conducted using PCR to amplify the specific ITS1, ITS2, NDI genes, respectively. These genes were successfully amplified at the expected sizes 630, 510, and 560 bp respectively as shown in Figure 2. Analysis of the obtained samples confirmed that the obtained worms were *Fasciola hepatica*. Based on the ITS1 gene, three isolates were obtained and uploaded to the NCBI database under the accession numbers (PP108836, PP108837, and PP108838) (Under release). Also, analysis of the ITS2 gene resulted in obtaining 3 isolates under the accession numbers (PP109065, PP109066, and PP109067) (under release).

3.4. Phylogenetic Analysis

Phylogenetic analysis of the partially amplified ITS1 amplicons cleared the presence of a high similarity percentage between the obtained 3 isolates and the confirmed uploaded sequences of *F. hepatica* obtained from the different countries. The obtained isolate (accession number: PP108836) was closely related to *F. hepatica* which was previously isolated in Iran during 2021 (Accession number: MZ614980.1 and MZ614981.1) with a percentage of 100%. While the other obtained 2 isolates (Accession number: PP108837, and PP108838) are more related to each other rather than the isolate (accession number: PP108836) but more related to the *Fasciola* strains (accession number: MF969009.1) obtained in Iran during 2017 and (accession number: OP787141.1) obtained in Saudi Arabia during 2022 with a percentage of 99.63% (Figure 3).

Sequencing and the phylogenetic analysis of the ITS2 gene for the 3 obtained strains supported the previously obtained results with minor differences as the obtained strains with accession numbers WPP109065, PP109066, and PP109067 were very close to each other with a percentage of more than 99%. All of the strains were closely related to the strains isolated in Switzerland during 2018 (accession number: MK321597.1, MK321598.1, MK321599.1) (Figure 4).

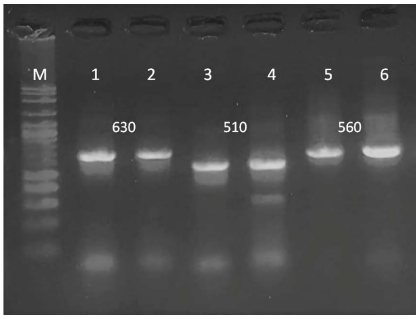


Figure 2. Molecular characterization of the recovered *F. hepatica*. M: 100 bp marker; L1, L2: The amplified PCR products of ITS1 gene; L3, L4: The amplified PCR products of ITS2 gene and L5, L6: The amplified PCR products of NDI gene.

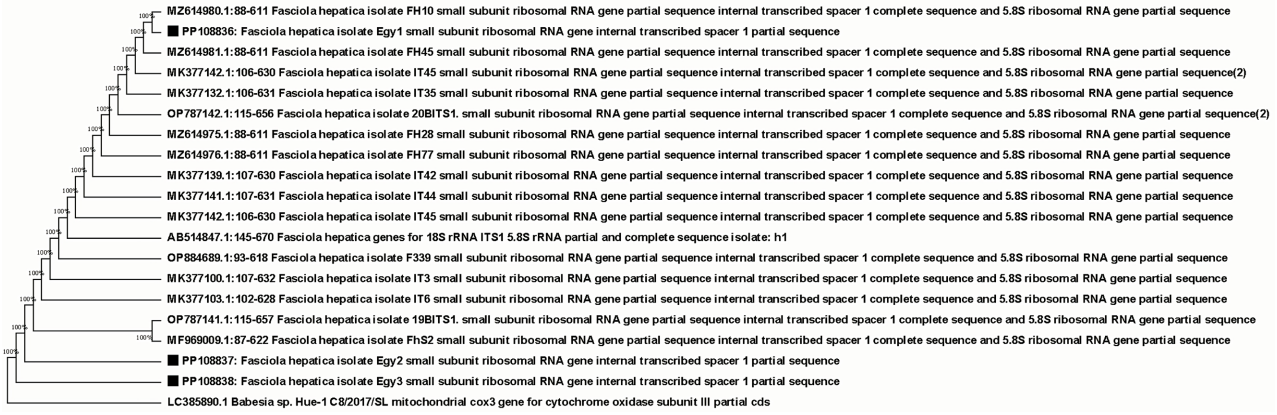


Figure 3. The cladogram of the obtained *Fasciola hepatica* strains based on the ITS1 gene. The maximum likelihood option of the MEGAX software was used to have the tree. The confidence level of the NJ tree was assessed by bootstrapping using 1000 replicates. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 524 positions in the final dataset. The black squares represent the obtained *Fasciola hepatica* sequences of the current study.

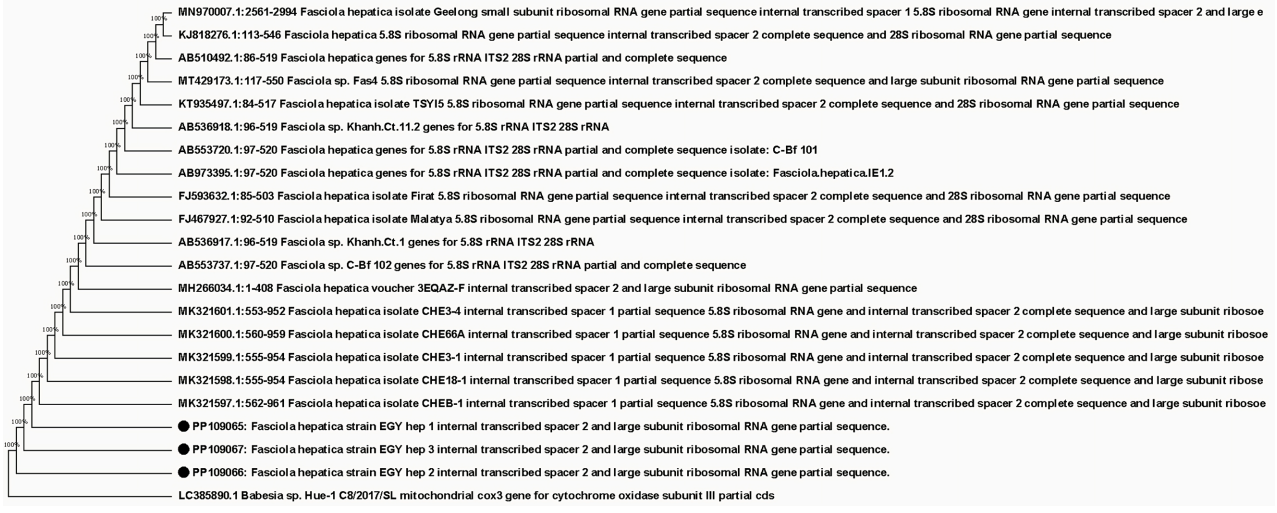


Figure 4. The cladogram of the obtained *Fasciola hepatica* strains based on the ITS2 gene. The maximum likelihood option of the MEGAX software was used to have the tree. The confidence level of the NJ tree was assessed by bootstrapping using 1000 replicates. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 335 positions in the final dataset. The black circles represent the obtained *Fasciola hepatica* sequences of the current study.

4. Discussion

Fasciolosis is a serious parasitic zoonotic disease of great economic importance [1] [21]. Several studies highlighted the adverse effect of *Fasciola* infections on animal health and production [22] [23] [24] [25]. One of the challenges in *Fasciola*'s research studies on the pathogenesis of the disease or therapeutic efficacy of candidate drugs is the limited availability of animal models to conduct the experimental infections with the ability to tolerate and survive after the infection and showing the pathological condition typically occurs in definite hosts. The success of the animal model in *Fasciola hepatica* infections is based on the survival index of the lab animal, parasitic migration, shedding of the egg in the face, development of mature stages in the liver and consequent liver dysfunction. With respect to this objective, it was found that the infection was successfully established in Wister rats with shedding of the typical *F. Hepatica* eggs in the faeces of experimentally infected rats at the 8th WPI and the worms could develop and mature to the adult stages. In accordance with the present results, previous studies have demonstrated that eggs were detected in the feces after 9 weeks of infection with the development of metacercaria into infective worms when the rat was used as an animal model [6].

The determination of liver enzyme activities may be used as valuable markers for the diagnosis of different stages of *F. hepatica* infection. *Fasciola* infection has two distinct phases; parenchymal and ductular phases. During the initial parenchymal phase, the activities of serum liver enzymes were markedly elevated as a reason for juvenile flukes' migration through liver parenchyma that caused damage to hepatic cells [26]. But, their serum levels were normalized during the ductular phase due to the resolution of parenchymal lesions. Gonzalo-Orden *et al.* [27] demonstrated that the measurement of serum activity of AST gives good information about the discrimination between two phases of *Fasciola* infection which returned to a normal level on the 12th WPI. This is in accordance with the present finding which illustrated that the serum activity of AST significantly increased from the 2nd to the 12th WPI and then progressively decreased at the 14th WPI indicating the ductular phase of *Fasciola* infection has begun. This finding was inconsistent with the result of *Fasciola* egg detection which illustrated that *Fasciola* eggs were detected in faeces from the 8th WPI to the end of the experiment. So, it could be said that the ductular phase began at the 8th WPI. The discrepancy between the result of *Fasciola* egg detection and AST activity may be attributed to the delayed regeneration of hepatic tissue after the invasive phase ended. On the other side, serum ALT activities insignificantly fluctuated during the experimental periods. However, a significant increase in its activity was only recorded at the 14thWPI. This finding agrees with the result of Mert *et al.* [28]. The intensity of liver damage depends on the number of invading flukes that subsequently rely on the dose of metacercariae. In this study, each rat was experimentally infected with 15 *F. hepatica* metacercariae which is considered below the standard infection doses used in rats. This may be explained by the mild elevation of AST activity (about 1.3-fold) during the study [29].

In the current study, the total serum protein level was significantly reduced in *F. hepatica* infected rats as a consequence of albumin reduction which constitutes about 35% to 50% of total serum proteins in rodents [30]. The decreased level of serum albumin may be due to inhibition of its synthesis [31] as the flukes reside in the liver. Protein electrophoresis is a technique that changes in their constituents give early and valuable diagnostic information. Serum globulins revealed a significant increase in the infected rats which, in this study, was mainly associated with the increase of the β- and γ-globulins. These increases may be attributed to the increased levels of one or more of their main individual proteins such as acute-phase proteins, complement, and immunoglobulin that are mainly included during infection and inflammation [32].

It was recorded that molecular techniques are the best method to genetically identify the parasites especially those with similar appearance [33]. Accordingly, to characterize the recovered parasite from the experimental infection, the nuclear ribosomal internal transcribed spacer (ITS1 and ITS2) and mitochondrial NADH dehydrogenase I (NDI) genes were amplified and resulted in amplicon sizes of 630, 510 and 560 bp using specific primers, this results adding to the sequences analysis confirmed the presense of *Fasciola hepatica* which commonly found in different world areas [34] [35]. It is worth noting that these genes have been used extensively for characterisation of *Fasciola* species especially when combined with the restriction fragment length (RFLP) technique [36]. The phylogenetic analysis of Fasciola species was based on a sequence of internal transcribed spacer (ITS1 and ITS2) genes than NADH dehydrogenase I (NDI) gene attributed to the higher substitution rate of mitochondrial DNA than nuclear ribosomal DNA makes the latter suitable for identification and taxonomy of parasites [13]. Therefore, molecular techniques offer some advantages over morphological based identification in terms of accuracy and applicability.

5. Conclusion

Fasciolosis had marked adverse effects on the liver function of the infected animals that might be threatening the health and productivity of the host. The infection with *F. hepatica* had the capacity to negatively alter the normal level of liver serum enzymes and protein profiles indicating hepatic lesions and malfunction. The levels of liver serum enzymes and protein profile are considered helpful as biomarkers that aid in the assessment of the animal status health. The recovered worms were successfully characterised based on the ITS1, ITS2, and NDI genes. Furthermore, the sequence analysis confirmed the obtained results. Consequently, it could be concluded that the rat is an appropriate experimental animal for *F. hepatica* in terms of resilience and pathological reaction. Furthermore, molecular techniques are sensitive techniques to identify and characterise the *Fasciola* parasite.

Conflicts of Interest

The authors declare no potential conflicts of interest concerning the research,

authorship, and publication of this paper.

Author Contributions

OMK designated, supervised and directed the experiment. MG carried out the experimental infection to provide metacercaria. OMK, EBA, HAS, THA, NMFH and MAH conducted the laboratory work. OMK, EBA, SMN, EMA, MAH and THA, analysed and discussed the data of result. OMK, EBA, SMN, NMFH, MAH and THA implemented writing the manuscript. OMK, EBA, HAS, NMFH, THA, and EMA revised and reviewed the manuscript for publication. All authors read and approved the final manuscript.

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