

Submitted: 01/10/2023

Accepted: 15/12/2023

Published: 31/01/2024

Impact of ultrastructural and molecular identified *Babesiosoma* spp. in both Egyptian freshwater fishes (common carp and African catfish): Hematological, biochemical, and histopathological findings

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Abstract

Background: Babesiosomes are apicomplexan parasites of both marine and freshwater fish species.

Aim: In this study, we recorded the prevalence of *Babesiosoma* spp in two Egyptian freshwater fish species; the common carp and the African catfish with full pathological evaluation of the diseased condition, hematological and biochemical analysis of some parameters with exact recognition of the parasite with different methods.

Methods: Two hundred and forty fish blood samples from Al-Sharqiya and Al-Ismailia governorates from August 2022 to January 2023 followed by blood film examinations, performing electron microscopy and molecular detection of the parasite via polymerase chain reaction.

Results: The total infection prevalence was 63.75% with a higher prevalence observed among African catfish (42.5%) than Common carp (21.25%). Regarding hematologic parameters, the obtained results showed a significant decrease in the hematocrit values and a significant increase in the total leukocyte and lymphocyte values in both infected fish species. The serum ferritin, superoxide dismutase, and glutathione peroxidase were also significantly increased. However, the total iron binding capacity was significantly decreased. There was also a significant increase in the total serum bilirubin in the examined fish, all at ($p < 0.001$). Histopathologically, the lesions were more intense in the African catfish than the common carp but generally, the infected fish showed many changes with the gills being severely affected with pronounced hyperplasia of secondary lamellae with fusion and telangiectasis. The spleen, heart, and kidney are also affected.

Conclusion: Serious adverse effects on the health status of previously examined fishes infected with *Babesia* spp. were observed and detected by several diagnostic and descriptive tools. Histopathological, hematological, and biochemical studies give an idea of the extent of these changes which are largely fatal affecting the economic system depending on the fish industry.

Keywords: *Babesiosoma*, Egypt, Common carp, African catfish, Blood parasites.

Introduction

The importance of fish as a relatively cheap source of protein underscores the significance of maintaining fish farming conditions as healthy as possible (Soliman and Yacout, 2016). Parasitic infections can adversely affect high productivity in aquaculture, and they are considered bioindicators of water pollution. Among the possible parasitic infections in fish are blood parasites. The significance of these parasites lies in their detrimental pathological effects on their hosting fish (Davies and Johnston, 2000).

Babesiosomes are intraerythrocytic parasites of marine and freshwater fish (Bruno *et al.*, 2006). They belong to the family *Dactylosomatidae*. This family has two known genera: *Dactylosoma* and *Babesiosoma*. Members of the genus *Babesiosoma* are *Heteroxenous coccidians* that cycle between two hosts; an invertebrate, which is the main host, and a vertebrate which is an intermediate host (Barta, 1991). There are five known species in the *Babesiosoma* genus; *Babesiosoma Bettencourt*, *Babesiosoma mariae*, and *Babesiosoma tetragonis* which are recognized in fish hosts in addition to *Babesiosoma jahni* and *Babesiosoma stableri* which

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are described in amphibian hosts (Netherlands *et al.*, 2019).

The life cycles of only two babesiosomes have been investigated. These are *B. mariae* recognized in fish hosts in which a leech vector has been identified and *B. stableri* from a frog host in which also a leech vector has been identified (Barta, 1991; Negm-Eldin, 1998). The common feature in all described babesiosomes is that during merogony in the vertebrate host, they produce four merozoites in a cruciform or a rosette-shaped arrangement. One oocyst enclosed eight sporozoites during sporogony in their invertebrate host (Shahi *et al.*, 2013).

Much attention was drawn to the validation of the taxonomic affinities of these parasites which resulted in a scarcity of data regarding its prevalence and specific pathological effect in fish hosts. In this study, we are focusing light on the prevalence of babesiosomes in two species of Egyptian fresh-water fish; African catfish and Common carp, collected from Al-Sharqiya and Al-Ismailia governorates in the period between August 2022 and January 2023 and describing the hematological, biochemical, and histopathological changes in the hosting fish.

Materials and Methods

Study area and fish sampling

Two hundred and forty live fish, 120 *Cyprinus carpio* (common carp) and 120 *Clarias gariepinus* (African catfish) had blood samples taken from their caudal peduncles. Local fishermen caught these fish for 6 months, from August 2022 to January 2023, from the Nile River branches in the Al-Sharqiya governorate and the Al-Ismailia lakes. The prevalence of the parasitic infection depending on the result of the blood film was calculated according to the formula given by Agbabiaka *et al.* (2017). Prevalence (%) = no. of infected fish/total no. of fish samples × 100.

Detection of *Babesiosoma* spp in blood films

Thin blood smears spread on grease-free glass slides were air-dried, fixed with 100% methanol for 2 minutes, and stained in a 1:20 dilution of Giemsa stain (10–20 minutes) (Kjemtrup and Conrad, 2000). Dried stained blood films were examined microscopically for the identification of intra-erythrocytic protozoa with its variable stages.

Molecular detection of *Babesiosoma* spp via polymerase chain reaction (PCR)

DNA extraction from blood samples ($n = 30$) was performed using the QIA amp DNA Blood Mini Kit (Qiagen, Germany, GmbH, Catalogue no. 51106) following the manufacturer's instructions.

The primers used for the detection of part of the 18SrRNA gene of *Babesia* spp., were: forward primer 5'-GTTTCTGMCCCATCAGCTTGAC-3' and reverse primer 5'-CAAGACAAAAGTCTGCTTGAAAC-3' and the amplicon size was 422 bp. The annealing temperature for the PCR run was 61°C and the reaction

was repeated for 45 cycles according to Hilpertshauer *et al.* (2006). The PCR product was run on an agarose gel electrophoresis according to Sambrook *et al.* (1989) and the gel was photographed by a gel documentation system.

Electron microscopy detection of *Babesiosoma* spp

Two pelleted red blood cell samples from morphologically confirmed positive fishes of both species were prepared for transmission electron microscopy (TEM) by immersing and fixing them in a modified Karnovsky (1965) solution, which was made up of 2.5% paraformaldehyde and 2.5% buffered glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4, and storing them overnight at 4°C. A three 15-minute wash in 0.1 M sodium phosphate buffer and 0.1 M sucrose was then performed on the pellet. 90 minutes were spent postfixing in 2% sodium phosphate-buffered osmium tetroxide (pH 7.4), followed by three 15-minute washes in 0.1 M sodium phosphate. After being dehydrated in increasing ethanol grades, the samples were mixed with acetone: Epon (2:1, followed by 1:1 and 1:2 for 30 minutes each). Finally, the pellet was left in an epon pure solution at 4°C for a whole night. The epon was reconstituted the following day and incubated for around 48 hours at 70°C to facilitate polymerization.

The pellets were cut using an ultramicrotome set to a section thickness of 50–100 nm. The sections were then rinsed to copper grids and post-converted, as per Reynolds's 1963 instructions: 10% uranyl acetate for 10 minutes, then 1% lead citrate for 5 minutes.

A TEM was used to examine the sections after they had dried for around 15 minutes. The EM Unit at Mansoura University in Egypt used a JEOL JEM-2100 to observe ultrathin sections at 160 kV.

Hematological and serum biochemical analyses

For hematological investigation, 25 blood samples were drawn from the fish that were being investigated and placed in tubes containing EDTA. The clear supernatant serum was carefully aspirated into dry, sterile, labeled vials after the serum was extracted by centrifugation at 3,000 rpm for 10 minutes. This method was employed for serum analysis in all labeled collected samples. Manual methods were used to measure the total leukocytic count, packed cell volume, hemoglobin concentration, and erythrocytic count (Blaxhall and Daisley, 1973). Differential leukocytic counts were calculated according to Cole (1986). The iron (Fe) and total iron binding capacity (TIBC/transferrin test), total bilirubin (direct and indirect) were measured in the sera according to Lobetti *et al.* (2000), superoxide dismutase (SOD), and Glutathione peroxidase (GPx) were assayed in sera according to Miller *et al.* (1993).

Histopathological analysis

Freshly necropsied fish were used to obtain tissue samples from the gills, heart, spleen, and kidneys. The tissue samples were then preserved in 10% buffered neutral formalin, dried using increasing concentrations

Transmission electron microscopy

Some intra-erythrocytic stages as dividing form (merogony) and trophozoites stages of *Babesiosoma* spp. were observed by TEM within the cytoplasm of the host fish erythrocytes. The dividing form was characterized by pale-staining cytoplasm with numerous cytoplasmic organelles as numerous polyribosomes, mitochondria, rough endoplasmic reticulum, and a roughly spherical nucleus dark in color as in *C. carpio* (common carp) fish Figure 3A. This dividing form could be also observed in *C. garpinia* (African catfish) with the same constituents and even architecture in Figure 3B while trophozoites could be distinguished by their irregular appearance, cytoplasm contained more densely packed ribosomes and their nucleoplasm was more densely stained with the unique presence of the tubular food vacuole around endoplasmic reticulum (Fig. 3C).

Hematological picture and serum biochemical findings

Babesiosoma infection induced serious effects on the hematological findings of the infected examined fishes as demonstrated in Table 2. Our results declared significant decreases in values of RBCs, Hb, PCV, and MCV at $p < 0.001$ in infected fishes than that of un-infected ones. Significant decrease of MCH and MCHC in infected *C. carpio* at $p < 0.01$ and of MCHC in *Clarias garpinus* while a significant decrease in MCH value in *C. garpinus* at $p < 0.05$ was detected.

The leucocytic picture (leukogram) demonstrated in Table 3 had a significant increase in total leucocyte and lymphocyte values in both infected fish species at $p < 0.001$ when compared with noninfected fish. Neutrophils had a nonsignificant increase in infected *C. carpio* and a significant increase in *C. garpinus* at $p < 0.05$ while a significant increase of monocytes and eosinophil of infected *C. carpio* at $p < 0.001$ and $p < 0.01$ in *C. garpinus*. Basophil had a significant increase in both fish species at $p < 0.05$.

Biochemical findings included different parameters related to anemia as a result of erythrocytic damage at which significant increases of iron or Fe (ferritin), SOD, and GPx in both infected fish species at $p < 0.001$ while significant decrease in TIBC at $p < 0.001$ in both infected fishes species was detected and are shown in Table 4. Total bilirubin in both direct and indirect forms exhibited a significant increase in fishes at $p < 0.001$ except direct bilirubin in *C. carpio* revealed a significant increase in fishes at $p < 0.01$. These results are shown in Table 5.

Histopathological findings

The *Babesiosoma*-infected fish showed a myriad of pathological changes. The pathological changes did not change much between the two investigated species although it was more intense in the infected Common carp fishes. In Figures 4 and 5, we show representative photomicrographs of the most commonly observed histopathological changes in the infected common carp and the African catfish, respectively. The gills showed

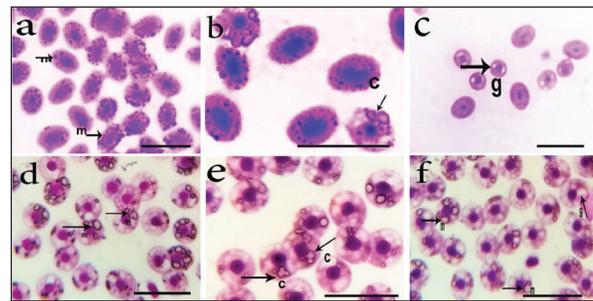


Fig. 1. Photomicrograph of *Babesiosoma* spp. in a Giemsa-stained blood film from both common carp (a, b, and c) and African catfish (d, e, and f) at 1,000 \times ; showing undivided meronts (m), cruciform meront (c) and elongated gamonts (g) scale bar = 10 μ m).

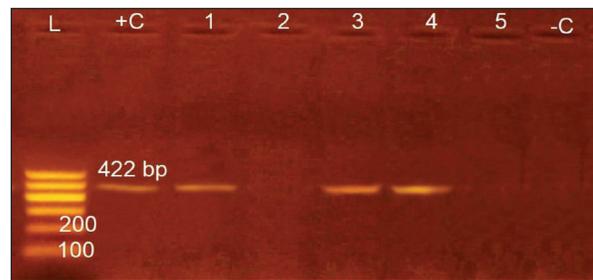


Fig. 2. Gel electrophoretic pattern of *Babesiosoma* spp. L: 200 bp DNA ladder. + C: Positive control - C: Negative control. Lanes (1, 3, 4): positive amplifications of *Babesiosoma* spp at 422 bp.

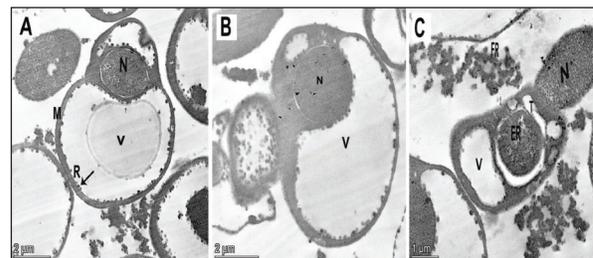


Fig. 3. Electron micrographs (TEM) of A) *C. carpio* (common carp) showing the dividing form of *Babesiosoma* spp. (N) nucleus, (R) ribosomes, and (V) vacuole (MB) membrane. B and C) *Clarias garpinus* (catfish) showing dividing form and trophozoite subsequently. (T) Tubular food vacuole and (ER) endoplasmic reticulum.

moderate to severe hyperplasia in the secondary lamellar epithelium with lamellar fusions and prominent telangiectasis. The hearts showed a notable thickening of the pericardium with serofibrinous exudate, dilated blood vessels, and leukocytic infiltration, whereas the myocardium exhibited intermuscular edema and partial muscular hyalinization. The spleen showed pronounced activation of the melanomacrophage

Table 2. Erythrogram (Mean ± S.E) of the infected fishes compared with control (n = 5).

parameter	Fish species <i>Cyprinus carpio</i>		Fish species <i>Clarias gariepinus</i>	
	Infected	Non-infected	Infected	Non-infected
RBC (10 ⁶ /mm ³)	2.11 ± 0.12***	3.70 ± 0.09	2.57 ± 0.18***	4.72 ± 0.14
Hb (g)	7.88 ± 0.29***	11.31 ± 0.30	8.31 ± 0.36***	13.59 ± 0.46
PCV	26.12 ± 0.99***	33.31 ± 0.40	26.98 ± 0.87***	36.6 ± 0.70
MCV (µm ³)	124.40 ± 3.78***	90.81 ± 1.16	106.32 ± 4.16 ***	77.66 ± 1.59
MCH (µg)	37.71 ± 0.81**	30.58 ± 0.22	32.65 ± 0.96*	28.79 ± 0.43
MCHC (%)	30.19 ± 0.34**	33.93 ± 0.49	30.77 ± 0.59**	37.14 ± 1.0

(*) Significant at p < 0.05, (**) Significant at p < 0.01, (***) Significant at p < 0.001.

Table 3. Leukogram (Mean ± S.E) of the infected fishes compared with control (n = 5).

parameter	Fish species <i>Cyprinus carpio</i>		Fish species <i>Clarias gariepinus</i>	
	Noninfected	Noninfected	Noninfected	Noninfected
WBC (10 ³ /mm ³)	12.49 ± 0.41***	8.10 ± 0.12	15.20 ± 0.21***	10.75 ± 0.29
Lymphocytes	9.78 ± 0.38***	5.91 ± 0.09	12.13 ± 0.16***	8.21 ± 0.21
neutrophil	1.78 ± 0.04 N.S	1.71 ± 0.02	1.90 ± 0.05*	1.72 ± 0.04
Monocytes	0.65 ± 0.018***	0.37 ± 0.016	0.92 ± 0.04**	0.66 ± 0.03
Eosinophil	0.26 ± 0.015***	0.10 ± 0.005	0.22 ± 0.02**	0.14 ± 0.01
Basophil	0.02 ± 0.002*	0.01 ± 0.002	0.03 ± 0.002*	0.02 ± 0.002

(*) Significant at p < 0.05, (**) Significant at p < 0.01, (***) Significant at p < 0.001. N.S = nonsignificant.

Table 4. Some serum biochemical and antioxidant parameters (Mean ± S.E) of the infected fish compared with control (n = 5).

Parameter	Fish species <i>Cyprinus carpio</i>		Fish species <i>Clarias gariepinus</i>	
	Infected	Noninfected	Infected	Noninfected
Ferritin (Fe)	41.81 ± 2.2***	17.11 ± 0.79	82.82 ± 4.37***	25.55 ± 1.4
IBC	0.47 ± 0.03***	3.12 ± 0.09	1.03 ± 0.07***	4.16 ± 0.13
SOD	127.4 ± 3.36***	38.42 ± 2.78	247.60 ± 3.8***	76.80 ± 2.2
GYs	147.6 ± 2.7***	55.85 ± 4.90	218.2 ± 4.35***	97 ± 2.45

(***) Significant at p < 0.001.

centers with marked expansion of the erythroid compartment and depletion of the lymphoid elements. The posterior kidneys showed vacuolation of the renal tubular epithelium, marked renal hematopoietic tissue hyperplasia, and mild activation of the melanomacrophage centers with a notable presence of hyaline casts inside some renal tubules.

Discussion

Babesiosis is considered one of the most important diseases in Egypt specifically in its acute form causing seriously recognized clinical manifestations and lowering productive performance (El-diasty *et al.*, 2017).

The identification of *Babesia* spp. depend on microscopical examination of blood films which varies with the examiner's proficiency and experience

but is considered a cheap and easy technique applied in all standard laboratories and even in the field for confirmation of acute cases (Lempereur *et al.*, 2017).

Our study documented the occurrence of *Babesiosoma* spp. in *C. carpio* (common carp) and *C. garpinus* (catfish) with a total prevalence of 63.75% which is considered a high prevalence compared with other international studies. This prevalence could not be nationally compared accurately because of unknown published local studies sharing the same geographical locations, fish species, and season of sample collection. Detection of most of the intraerythrocytic developmental stages of *Babesiosoma* spp. under the microscope was similar to those previously described by many authors (Barta, 1991; Lom and Dykova, 1992; Zulfiqar *et al.*, 2012; Shahi *et al.*, 2013). Hematological components have been developed for the evaluation of fish health

Table 5. Bilirubin parameters (Mean ± S.E) of the infected fish compared with control (n = 5)

Parameter	Fish <i>Cyprinus carpio</i>		<i>Clarias gariepinus</i>	
	Infected	Noninfected	Infected	Noninfected
Total bilirubin (T.B)	0.66 ± 0.02***	0.23 ± 0.02	1.22 ± 0.05***	0.48 ± 0.02
Direct bilirubin (D.B)	0.15 ± 0.01**	0.07 ± 0.01	0.29 ± 0.01***	0.15 ± 0.01
Indirect bilirubin (I.B)	0.51 ± 0.01***	0.16 ± 0.01	0.93 ± 0.05 ***	0.33 ± 0.01

(**) Significant at $p < 0.01$, (***) Significant at $p < 0.001$.

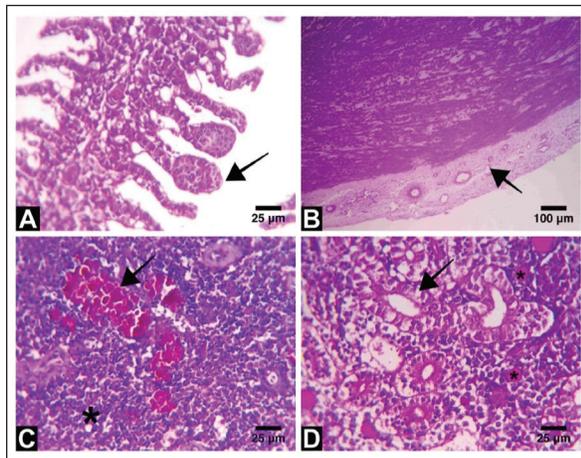


Fig. 4. Representative photomicrographs of H&E stained tissue sections of *Babesiosoma* spp infected common carp fish. A) Tissue section from the gills showing mild hyperplasia of the secondary lamellar epithelium and prominent telangiectasis (arrow). B) Heart section showing marked pericarditis where the epicardium is diffusely thickened by eosinophilic serofibrinous exudate with dilated blood vessels and leukocytic infiltration (arrow). C) Splenic tissue section showing pronounced activation of the melanomacrophage centers (arrow) and notable depletion of the lymphoid compartment with relative expansion of the erythroid compartment (asterisk). D) Tissue section from the posterior kidney showing vacuolation and degeneration in some renal tubules (arrow) with the presence of hyaline casts in others (asterisk). Scale Bar: 25 µm in A, C, and D, 100 µm in B.

conditions. Blood is considered the most reliable and easier indicator to demonstrate the general condition of the animal body. Therefore, in the presence of blood infection, blood parameters can serve as a standard biomarker (bioindicator) to determine diseased conditions and metabolic disturbances in fish (Celik, 2004).

Both light and electron microscopic examinations corroborated the acquired results, which showed that the primary merogonic cycle produces merozoites that either enter a subsequent primary cycle of development or start a secondary merogony. The progeny of secondary merogony, known as Barta and Desser (1986), either replicate secondary merogony or form gamonts.

According to Guimarães *et al.* (2003), who reported that the trophozoites' plasma membrane invasions enclosed trace amounts of cell hemoglobin, trophozoites are distinguished by their tubular feeding structure, which is formed by the simultaneous invasion of the erythrocyte and parasite plasma membranes.

PCR testing is a quick and very accurate method of identifying certain genetic alterations and parasite infectious illnesses. The examination tests work by finding the DNA of *Babesia* in a sample with higher sensitivity, specificity, and accuracy values than microscopic examination as mixed infection may represent false results, particularly with microscopy (Souza *et al.*, 2016). Thus, molecular diagnosis should be utilized to routinely diagnose blood-borne diseases because of the insufficiency of clinical, light microscopy, or hematological findings alone which cannot provide a reliable diagnosis (Gad *et al.*, 2023). It has been demonstrated that the PCR is an extremely sensitive and specific method, especially for identifying carriers of babesiosis (Zulfiqar *et al.*, 2012). There are numerous established and real-time PCR tests that can be used to identify *Babesia* species in tick and vertebrate hosts.

According to Wang *et al.* (2015), these methods are typically more sensitive than microscopy. Depending on the size and target gene, they may allow phylogenetic analysis and/or identification to the genus or species level. *Babesia* species, even those of similar size and infecting the same animal host, exhibit far greater diversity, according to more recent molecular studies that use gene sequencing and phylogenetic analyses. These studies have reinforced the significance of using molecular techniques for species identification by demonstrating that *Babesia* spp. actual groups into several discrete clades that separate what appear to be identical parasites by light microscopy (Criado-Fornelio *et al.*, 2004; Schnittger *et al.*, 2012; Yabsley and Shock, 2013).

Among other blood indices, our hematological data for fish with babesiosis showed a significant decrease in the erythrocytic count, hemoglobin concentration, and packed cell volume. All of this might be caused by the parasites' direct destruction of the erythrocytes in infected fish as opposed to healthy fish. These outcomes were consistent with those of Mahmoud *et al.* (2015). Moreover, the parasites merely serve as a stressor; during the early stages of stress, catecholamine is

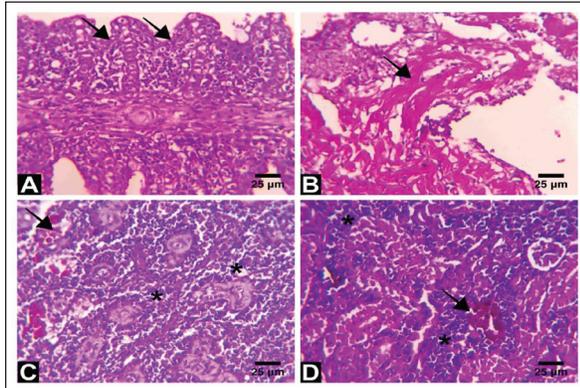


Fig. 5. Representative photomicrographs of H&E stained tissue sections of a *Babesiosoma* spp.-infected African catfish. A) Tissue section from the gills showing extensive hyperplasia of the secondary lamellar epithelium and adhesion of the secondary lamellae (arrows). B) Myocardium section showing intermuscular edema and partial muscular hyalinization (arrow). C) Tissue section from the spleen showing mild activation of the melanomacrophage centers (arrow) and marked depletion of the lymphoid elements (asterisks). D) Section from the posterior kidney showing renal hematopoietic tissue hyperplasia (asterisks) with the presence of pigmented macrophage aggregates (arrow) and mild degeneration of the renal tubules. Scale Bar: 25 µm.

released, changing the packed cell volume and possibly causing red blood cells to be released from the spleen (Wells and Weber, 1990) or swell as a result of fluid entering the intracellular compartment (Chiocchia and Motais, 1989). Due to their correlation with anemia, a few biochemical indicators including bilirubin, iron, and TIBC (measured by the Transferrin test) were examined. Our findings for these parameters matched those of El-diasty *et al.* (2017).

Important antioxidant markers like GPx and SOD were also impacted by blood parasite infection as a reaction to stressful situations and oxidative stress brought on by excessive reactive oxygen species (ROS) production (Naiel *et al.*, 2021). The initial line of defense is represented by SOD, which breaks down superoxide into hydrogen peroxide and oxygen (Li *et al.*, 2015), whereas CAT catalyzes the breakdown of hydrogen peroxide (Wang *et al.*, 2017). Excessive ROS can cause lipid peroxidation, which can result in membrane damage and cell death, in addition to its detrimental effects on cellular proteins and DNA (Jiao *et al.*, 2019). The histopathological changes described in *Babesiosoma* spp., infected fish corroborates the histological changes reported in other *Babesia*-infected species where the kidneys showed vacuolation and degeneration of renal epithelium and hyaline cylinders in the tubular lumen, the spleen exhibited the presence of aggregates melanomacrophages and depletion of the lymphoid elements (Habela *et al.*, 1991; El-diasty *et al.*, 2017; Jasik *et al.*, 2023). Pericarditis was described

(Habela *et al.*, 1991) in Merino sheep experimentally infected by *Babesia ovis* and myocardial pathology is frequently reported in canine babesiosis (Lobetti, 2005; Petra *et al.*, 2018). Moreover, changes in gills reflect hypoxia associated with anemia due to parasitism (Gilmour and Perry, 2018).

Acknowledgment

The authors thank Prof. Dr. Nashwa Abdelrazik Abbasa, Central lab. For Fish Research, for her help in sample collection and first recognition via blood.

Authors' contribution

AAA and REM contributed to the idea, design of the research project, and supervision. SAG and REM contributed to practical application as well as the data collection, MAA, and REM writing the paper after the result preparation and supervision. AAA and NAR critical revision of the essential intellectual content with supervision. The submitted article has been reviewed and approved by all authors.

Competing interests

The authors declare no competing interests.

Funding

The authors did not receive any financial support from anywhere

Data availability

All data used were available and have been included in the manuscript.

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