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COLLEGE OF VETERINARY MEDICINE AND ANIMAL SCIENCES

DEPARTMENT OF VETERINARY PATHOBIOLOGY

HAEMATOBIOCHEMICAL ALTERATIONS AND LESION CHARACTERIZATION CAUSED BY FASCIOLOSIS IN CATTLE SLAUGHTERED AT GONDAR ELFORA ABATTOIR, ETHIOPIA

MSc THESIS

By

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A thesis submitted to the Department of Veterinary Pathobiology, College of Veterinary Medicine and Animal Sciences, University of Gondar in Partial Fulfillment of the Requirements for the Degree of Master of Science in Veterinary Pathology

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LIST OF ABBREVIATIONS AND ACRONYMS

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
DLC	Differential leukocyte count
EDTA	Ethylene diamine tetraacetic acid
GOT	Glutamic oxalacetic transaminases
GPT	Glutamic pyruvic transaminase
HGB	Hemoglobin
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
PCV	Packed cell volume
RBC	Red blood cell
TEC	Total erythrocyte count
TLC	Total leukocyte count
WBC	White blood cell

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ABATRACT

An abattoir-based cross-sectional study was conducted from January to September 2023 on a total of one hundred (50 fasciola-infected and 50 non-infected) male apparently healthy local breed cattle to assess hematobiochemical alterations and lesion characterization caused by fasciolosis in cattle slaughtered at Gondar ELFORA abattoir, Ethiopia. The hematobiochemical study revealed significant mean reductions in hemoglobin (HGB), packed cell volume (PCV), total erythrocyte count (TEC), lymphocytes, monocytes, total protein, albumin and glucose, whereas elevations in total leucocyte count, eosinophils, neutrophils, aspartate aminotransferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP) in fasciola infected cattle. The erythrocyte indices revealed a microcytic hyperchromic anemia. Grossly, the fasciola-infected liver in the acute case showed hepatomegaly with rounded edges and juvenile flukes at the cut section of parenchyma, and in the chronic case, the liver was small in size and firm in consistency with a corrugated capsule and engorgement of the bile ducts with twisted flukes, Whereas microscopically, in the acute case, there were eosinophils infiltration, hemosiderin pigmentation and congestion around the central vein and sinusoids, and in the chronic case, there were proliferation of fibrous connective tissues and bile ducts, and metaplasia of columnar to cuboidal epithelial cells. The results of hematobiochemical alterations were also consistent with gross and microscopic findings, and cause a great impact on liver physiology and histology leads to high losses in meat and milk production. Hence, Hematobiochemical analysis should be used as complementary in diagnosis of bovine fasciolosis.

Keywords**:** *Abattoir, Cattle, Fasciolosis, Hematobiochemical alteration, Lesion characterization*

1. INTRODUCTION

Fasciolosis is a neglected zoonotic parasitic disease caused by *Fasciola hepatica* and *Fasciola gigantica,* which are more pathogenic to livestock and human health (Zang, 2023). Fasciola is commonly recognized as liver fluke and is characterized by acute and chronic inflammation of liver. As a sequel, submandibular edema and cirrhosis appear, followed by weight loss, anemia, hypoproteinemia, and anorexia. The larger fluke (*F. gigantica)* suppresses the host's immune system, making it more pathogenic and lethal (Yusuf *et al*., 2016; Ashoor and Wakid, 2023). All mammals are susceptible to these species; but cattle is the most susceptible one (Beesley *et al*., 2018).

Acute fasciolosis is generally characterized by the presence of immature flukes in the liver that destroy hepatic parenchyma and cause hemorrhage, extensive liver damage, and fibrinous deposits on the capsule. Then flukes enter the bile ducts. The chronic phase of fasciolosis occurs when flukes, once inside the bile ducts, extensively ingest blood, damage the mucosa, and cause anemia, hypoproteinemia and cirrhosis. The damaged bile ducts become enlarged or even cystic, and have thickened and fibrosis walls, and in cattle, they are usually calcified (Costa *et al*., 2022). Hence, measuring the hematological alteration is important to determine the hematological disorder and systemic diseases (Roland *et al*., 2014).

The liver performs a number of essential functions, and plays a very vital role in vertebrates. Hepatic damage has a variety of detrimental effects, and the metabolic functions of the liver are gradually reduced in fasciolosis (Gattani *et al*., 2018). As a result, biochemical molecules like blood glucose, liver-derived serum enzymes, serum proteins, and others are altered following injury and damage to hepatocytes. Liver enzymes are known to have intracellular action, and their levels in the blood are very low under normal conditions, but any increment in the systemic circulation is evidence of tissue damage (Yessuf *et al*., 2020). Hence, measuring the hematobiochemical alteration

is important to determine the hepatocellular damage induced by fasciolosis (Abd-Ellah *et al*., 2014; Gattani *et al*., 2018; Yessuf *et al*., 2020; Brahmbhatt *et al*., 2021).

Abattoirs are used as sources for information about disease epidemiology. Meat inspection at slaughterhouses, for hygienic quality, involves both antemortem and postmortem examinations. Post-mortem inspection is an essential step around which meat hygiene revolves since it provides information for the evaluation of clinical signs and pathological processes that affect the wholesomeness of meat (Raji *et al*., 2010; Suhair, 2013; Hassanin *et al*., 2017; Alsari *et al.*, 2017; Nasreldin and Zaki, 2020). In Ethiopia, most of the slaughtering takes place in the backyard (Mesfin and Mekonnen, 2014; AACCSA, 2015; Mekuriaw *et al*., 2016).

There have been several recent studies associated with lesion characterization based on gross pathological studies, and economic loss due to fasciollosis has been reported in several parts of the country (Dechasa *et al*., 2012; Meaza *et al*., 2017). Pathological with a serum biochemical study (Kitila and Megersa, 2014). However, there are very few detailed studies on hematobiochemical alterations with gross and microscopic lesion characterization in bovine fasciolosis (Uma, 2020). For this reason, this study will be a raw material to solve key questions on the hemobiochemical alterations and lesion characterization induced by fasciolosis and thus could help in policy formulation regarding the early diagnosis of animal fasciolosis in Ethiopia. Therefore, the present study was conducted with the following objectives:

- To characterize gross and microscopic lesions on cattle infected with fasciolosis.
- To determine and compare hematobiochemical profiles of *Fasciola*-infected and non-infected cattle.

2. LITERATURE REVIEW

2.1. Morphology of fasciolosis

The morphology of fasciola is helping us classify them at the species level. Different structures can be found in different *Fasciola* species. *Fasciola hepatica* is leaf-shaped with a broad and cone-shaped anterior projection. The tegument is armed with sharp spines. The young fluke at the time of entry into the liver is 1-2 millimetres (mm) in length and lancet-like when it has become fully mature in the bile ducts. The eggs have an indistinct operculum and develop only after the eggs have been laid (Michael, 2004). The *Fasciola* eggs have a yellowish-brown shell with a small knob at the posterior end. *Fasciola gigantica,* its length reachs up to 7.5cm, a narrow shoulder, a blunt posterior end, and an ovary longer with many branches, while *Fasciola hepatica,* reachs up to 3.5cm, has broad shoulders and a pointed posterior end. The eggs of *Fasciola gigantica* have an operculum with a measurement of 190 x 100 μm, while *Fasciola hepatica* eggs also have an operculum with a measurement of 150 x 90 μm (Taylor *et al*., 2007).

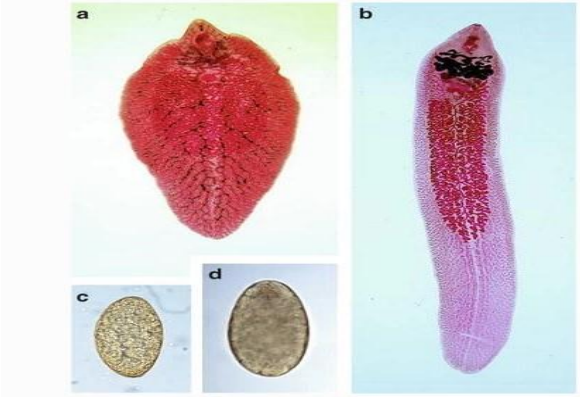


Figure 1: Adult and eggs of fasciola species: (a) *F. hepatica*: two prominent shoulders, converging margins, simple medial branches of the intestinal caeca, and smaller in size; *F. gigantica*: almost absence of shoulders, parallel lateral body borders, and larger in size; (c) the egg of *F.hepatica*: large, ovoid, operculated, bile-stained, unsegmented; (d) the egg of *F.gigantica*: larger in size but very similar to the egg of *F.hepatica* (Mas-Coma *et al*., 2014).

2.2. Etiology

Among the species of fasciola, *Fascila hepatica* and *Fasciola gigantica* are the most common in the country and cause fasciolosis in domestic animals, mainly ruminants, due to their indiscriminate feeding habits (Shafi, 2021). Disease progresses through four stages: an early incubation period of a few days to three months with little or no symptoms; a persistent phase with general clinical signs that is later changed to a latent phase with less known clinical signs; and finally, months to years later, an unending or chronic obstructive phase. In the chronic state, inflammation of the bile duct and gall bladder leads to gallstones and fibrosis (Rahman *et al*., 2017).

2.3. Epidemiology

Fasciolosis is found in marshy areas and is a limiting factor for bovine and ovine production. *Fasciolla hepatica*, which is a temperate species, is the most important trematode of domestic ruminants and one of the most common causes of liver fluke disease in temperate areas of the world (Garg *et al*., 2009). Thus, it is found in Southern and Northern America, Europe, Australia, and Africa. While, *Fasciolla gigantica* is very common and economically important, it is widely distributed in and around different tropical countries (Thrusfield, 1995). The development of *Fasciola hepatica* and *Fasciola gigantica* eggs, larval stages, and intermediate host snails in the environment is highly dependent on geo-climatic, ecological, and anthropogenic factors such as elevation, rainfall, temperature, evapotranspiration, moisture, vegetation, and soil type (Fatima *et al*., 2012; Brown *et al*., 2017).

The variations, prevalence, epidemiology, and ecology of the *Fasciola* species involved were reported by different authors in Ethiopia. They attributed the variation to different eco-climatic conditions like altitude, rainfall, temperature, and livestock management systems. Their work also identified the outstanding change that occurs during *Fasciola hepatica* infection in all host species (Terefe *et al*., 2012). The availability of suitable snail habitat is the main predisposing factor for the distribution of fasciollosis. Habitats

that are crucial for the intermammalian host of Fascia are mainly environments containing wet mud and free water, and they reside permanently in streams, ditches, and at the edges of small ponds. Fields with clumps of rushes are often suspect sites. A slightly acidic pH environment is optimal for Lymnaeidae truncatula, but excessively acid pH levels are detrimental (Wakuma, 2009).

2.4. Transmission and pathogenesis

The infestations and pathogenesis of fasciola are clearly indicated by following its developmental stages. When eggs laid by the adult parasite in the bile ducts of their hosts pass into the duodenum with the bile. The eggs then leave the host through the faeces (Zachary, 2017). The eggs then hatch to release the motile miracidium, which will then penetrate the intermediate host. The penetration is assisted by the secretion of histolytic glands. Miracidium is covered with small protoplasmic hairs (cilia), and it swims in water and cysts on pasture on which the host feeds (Taylor *et al*., 2016). The need to find a suitable host to penetrate is an urgent one, and those miracidia failing to do so generally die within 24 hours (Smith, 1994).

After penetrating the snail, the miracidium loses its cilia and becomes a sporocyst. The sporocyst divides and forms the redia (forum with sucker and primitive gut), and a fully mature redia passes through cercaria stages. On emerging from the snail, the cercaria attaches to the herbage and transforms into a metacercaria. Metacericariae can survive in wet grass and in shady places near water for years (Radositis *et al.,* 1994). When the metacercaria is ingested along with the contaminated vegetation by the definitive host, it enters the small intestine, releasing the young parasite, which penetrates the gut wall, enters the peritoneal cavity, and then migrates directly to the liver. Then, the parasite penetrates the liver and enters the bile ducts, where it matures into a fully adult parasite after about 3 months from the initial infection (Marquardt *et al*., 2000; Jones *et al.*, 2015). Ingestion of aquatic plants containing infectious larvae at various stages infects humans. The experimental study found that consuming raw liver dishes made from fresh liver

infected with juvenile flukes could infect humans (Duménigo *et al.*, 2000; Michael, 2004).

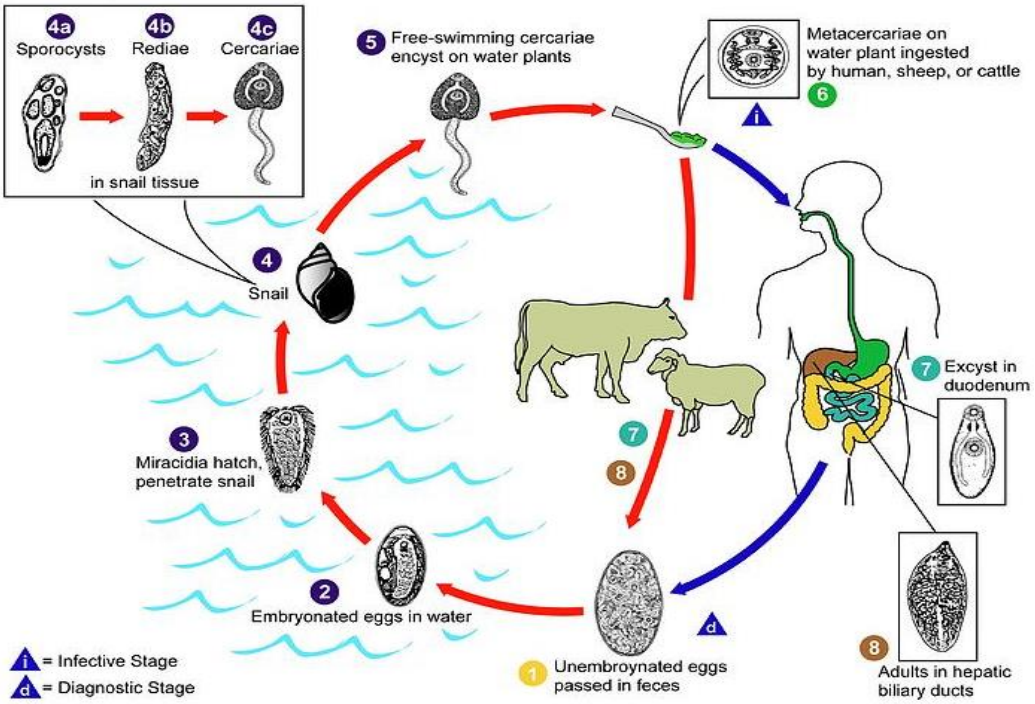


Figure 2: Life cycle of fasciola: Eggs are passed in the faeces (1), embryonate in water, and hatch into miracidia (2), which then infect the intermediate host (*Galba truncatula*) (4). In the snail, development occurs from sporocysts (4a), to rediae (4b), then to cercariae (4c); these are released from the snail (5) and subsequently encyst on vegetation as metacercariae, the infective stage (6). metacercariae are ingested by the definitive host (7), and parasite excysts and these juveniles migrate through the small intestine towards the liver and finally mature in the bile ducts (8) (Daramola, 2023).

2.5. Diagnosis

The diagnosis of bovine fasciolosis infection in live animals is usually based on prior knowledge of the epidemiology of the disease in a given environment, surveillance of clinical signs, grazing history, seasonal occurrence, and identification of snail habitats and the droppings of adult flukes examined from the faeces of animals, respectively. The presence of the *Fasciola* species in animals is confirmed by carrying out postmortem

examinations and laboratory examinations of faeces. The direct, reliable, and cost-effective diagnosis of fasciollosis is made through post-mortem examination of the liver of slaughtered animals (Abunnaet *et al*., 2010).

2.5.1. Clinical examination

Acute fasciolosis occurs rarely in cattle and is less common than the chronic form, causing hepatitis by the simultaneous migration of immature flukes (Alemu, 2019). It is responsible for wide-spread morbidity and mortality in cattle, characterized by weight loss, anemia, hypoproteinemia, subcutaneous edema, emaciation, and pale mucous membranes are typical signs (Radostits *et al*., 2000). The pressure caused by the load of parasites and lesions found in the liver results in pressing the vital organ of the thoracic cavity, and a grunting sound may appear upon animals moving downward (Cullen and Stalker, 2016). During chronic infection, fecal examination using the fecal smear and sedimentation technique may show oval, operculum, and yellow eggs (Marquradt *et al*., 2000; Webb and Cabada, 2018). They can be distinguished from the eggs of other flukes, especially the eggs of *Paramphistomum*. Egg of *Fasciola* species takes the color of bile, yellowish; while the egg of *Paramphistomum* species receives the color of methylene blue solution (Thrustfield, 1995).

2.5.2. Hematobiochemical examination

Hematologic analysis is not only relevant for diagnosing disorders of the hematologic system but is also helpful in the diagnosis of many organ and systemic diseases (Roland *et al*., 2014). Hematology analysis aids in diagnosing anaemia, certain cancers of the blood, inflammatory diseases, and monitoring blood loss and infection. Hence Hemoglobin concentration, packed cell volume, total erythrocyte count, total leucocyte count, differential leucocyte count, and calculating the red blood cell indices are very important to diagnose diseases caused by different pathogens like parasites, bacteria, viruses, and fungi (Jain, 1986).

Various biochemical attributes, namely blood glucose, creatinine, urea, and serum proteins, as well as the activity of certain serum enzymes such as Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), and gamma-glutamyl transferase (GGT), which increase following hepatic injury, can be measured for hepatic functions (Gattani *et al.*, 2018). The lesions in the liver are only partially a result of the mechanical action of liver fluke because the injury to the liver can be induced by parasite excretory products, decomposed parasite products, bile, and hepatic tissue. Hence, serum biochemical tests, including serum liver enzymes, are also helpful to assess the severity of hepatocellular injury and monitor the progress of the disease in ruminants (Lee *et al*., 2005; Hodzic *et al*., 2013).

Serum ALT and AST are the most sensitive indicators of hepatocellular injury. ALP, GGT, serum total proteins, and bilirubin are also used to evaluate the degree of cholestasis and synthetic capacity of the liver (Brahmbhatt *et al*., 2021). Total protein is another blood parameter used to measure liver synthetic capability because it is manufactured only in the liver (Elshahawy *et al*., 2021). The most important diagnostic methods to allocate the increment of leukocytes and the development of fibrogenesis are by measuring serum concentrations and using hematological methods as well (Adamu *et al*., 2019).

2.5.3. Gross and microscopic examination

Grossly, in acute fasciolosis, a fasciola-infected liver has an irregular outline, paleness, and a firm parenchyma. Liver fluke infestations and tracks where flukes migrate may be seen, and sometimes it is possible to find juvenile flukes in the liver parenchyma. The immature flukes are often so small that they are not easily discernible. Slicing a piece of liver and shackling it in water, permitting the flukes to settle to the bottom, most easily demonstrates those (Talukder *et al*., 2010).

In chronic fasciolosis, mature flukes can be found in the bile duct and are characterized by a reduced size of the liver and thickened bile ducts. The bile duct may protrude above

the surface of the liver, and cysts may be present due to blockage of the ducts with flukes. The mature flukes are leaf-shaped, greenish-brown, with a conical anterior end and shoulders. Calcification of the bile duct wall is a common finding in cattle (Marqurdt *et al*., 2000). Several types of fibrosis, such as post-necrotic scarring, ischemic fibrosis, and peribiliary fibrosis, may also present (Steyl, 2009). The flukes are also found in the wall of the gall bladder and cause bleeding in the gall bladder (Kardena *et al*., 2017).

Microscopically, in acute fasciolosis, severe congestion of central veins, hepatic sinusoids, and portal blood vessels will be observed. Hemorrhagic migrating tracts formed from degenerated hepatocytes and erythrocytes and infiltrated with eosinophils, macrophages, and lymphocytes will also be observed. Old parasitic tracts are represented by central necrotic areas surrounded by leucocyte infiltration, especially eosinophils, macrophages, and lymphocytes, together with connective tissue capsules and hemosiderin pigments. Moreover, severe eosinophilic cellular infiltration in the portal areas will be seen in cases of cattle and buffaloes (Borai *et al*., 2013; Adrien *et al*., 2013).

In chronic fasciolosis, hepatic cirrhosis is characterized by severe connective tissue proliferation infiltrated with mononuclear leucocytes in the presence of hepatocellular atrophy. Adult fasciola worms with desquamated epithelium are occasionally found inside the ductal lumen. Bile ducts revealed severe hyperplasia and desquamation of their epithelial cell lining with dystrophic calcification in cases of cattle and buffaloes, while this calcification will be associated with giant cell formation in cases of camels (Borai *et al*., 2013; Al-Mahmood and Al-Sabaawy, 2019). There are also thin collagen fibres, necrosis, and proliferation of collagen fibres, infiltration of inflammatory cells, hypertrophic epithelium, and epithelial hyperplasia in the mucosa of the gall bladder with short villi (Kardena *et al*., 2017). Cirrhosis is also found in chronic fasciolosis in cattle (Okoye *et al*., 2015).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted from January to September 2023 at Gondar ELFORA abattoir (a privately owned sheep, goats, and cattle abattoir), Gondar, which is the capital of the central Gondar administrative zone of Amhara regional state, located at 12° 36’ N, 37° 28' E latitude, and 12.6° N, 37.467° E longitude with an altitude range of 1800–2220 meters above sea level, 727 km away from Addis Ababa. It has an average annual rainfall of 880 to 1172 mm, with the highest rainfall during the wet season extending from September to September and the lowest rainfall during the short season, which extends from October to May. The average annual temperature and relative humidity are 20.3 °C and 55.7%, respectively (CSA, 2020). Now, the abattoir has slaughtered cattle for the local market and for the University of Gondar. During the study, on average, 25–30 cattle were slaughtered per week. Cattle slaughtered at the abattoir were purchased from different districts of the central Gondar zone.

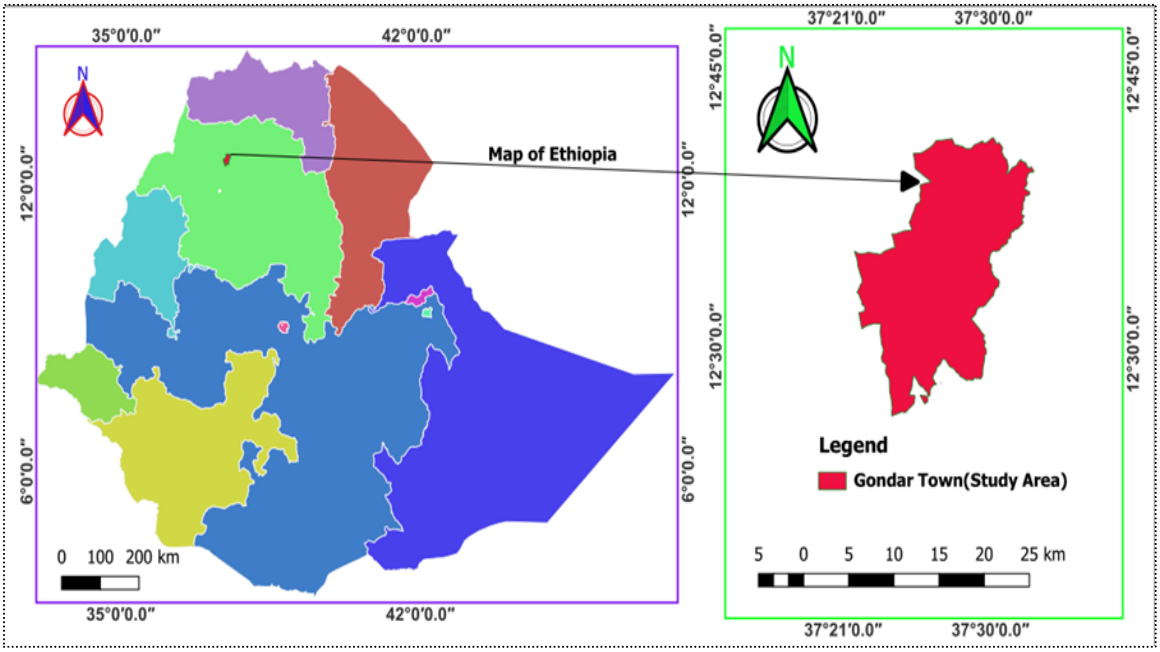


Figure 3: Map of the study area

3.2. Study animal

The study animals were male apparently healthy local breed cattle brought for slaughter for human consumption at Gondar ELFORA abattoirs. These cattle were raised in an extensive farming system, and transported from their origin to the abattoir by trucks and kept at lairage for one day.

3.3. Study design and sampling method

An abattoir-based cross-sectional study was conducted from January to September 2023 to assess the hemotobiochemical alterations and lesion characterization caused by fasciolosis in cattle slaughtered at the Gondar ELFORA abattoir**.** A total of one hundred (50 fasciola-infected and 50 non-infected) male apparently healthy local breed cattle were selected from the abattoir using the purposive sampling method.

3.4. Antemortem and postmortem inspection

Cattle brought for slaughtering purposes were subjected to antemortem inspection in the abattoir during the study period (Annex I**:** C). Blood samples were collected for hematology and biochemical analysis, and some fecal simples ware taken for fecal examination. The collected blood samples were categorized in the infected and control group after carrying out postmortem inspection.

Postmortem inspection was carried out by visual inspection, palpation, and systematic incision of the liver, bile ducts, and gallbladder of cattle (Annex I**:** D). The liver that showed gross lesions of fasciolosis with the presence of liver fluke in hepatic tissue was taken for histopathological examination, and some bile samples were also taken for bile examination. For further identification, Fasciola that appeared in the bile ducts and the gall bladder were harvested in normal saline as described by Javaregowda and Rani (2017).

3.5. Sample collection and transportation

A total of 200 blood samples: 100 from fasciola-infected cattle (50 for hematology analysis and 50 for serum biochemical analysis) and 100 from non-infected cattle (50 for hematology analysis control and 50 for serum biochemical analysis control) were collected from the jugular vein of these cattle after following the antemortem recording of animal parameters, and a total of 20 representative tissue samples were collected from the liver of fifty fasciola-infected cattle after conducting the postmortem inspection. 5 ml of the blood was collected in an EDTA-coated vacutainer tube for hematological analysis, and 5 ml of the blood sample was collected in a plain (anticoagulant-free) tube for serum biochemical analysis and placed in an ice pack containing ice box. Tissue samples were collected in the dimension of 1 cm^3 from the area showing gross lesions and placed in 10% neutral buffered formalin containing sampling bottle for histopathology examination as described by Al-Mahmood and Al-Sabaawy (2019). Finally, all samples were transported to the veterinary pathology laboratory of the University of Gondar.

3.6. Sample processing

3.6.1. Hematobiochemical analysis

The hematology analysis was conducted using EDTA coated blood samples, and for the late hematology analysis, samples were kept at +4°C. Packed cell volume (PCV), hemoglobin determination (HGB), total erythrocyte count (TEC), total leucocyte count (TLC), differential leukocyte count (DLC), and the calculated values: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were determined by using a Sysmex automated hematology analyzer (Annex II: C). Biochemical analysis was conducted by allowing the anticoagulant-free blood samples to stand in an undisturbed and slanted position for three to four hours, and retracting the clot to separate the serum. Then, the collected serum was stored at -20^{o}C in serum vials or centrifuge tubes, which were properly

capped and labeled as described by Yessuf *et al*. (2020). Finally, Serum activities of alanine aminotransferase (ALT/GPT), aspartate aminotransferase (AST/GOT), alkaline phosphatase (ALP), total protein, albumin, and glucose were measured in an automated clinical chemistry analyzer (Annex II: D). Standard commercial test kits from Human GmbH (Wiesbaden, Germany) were used in accordance with the manufacturer's instructions.

3.6.2. Histopathology examination

The well-fixed tissue samples were dehydrated by alcohol, later cleared by xylene, and impregnated, and embedded in paraffin wax, and then sectioned at five micrometers by rotary microtome and stained by routine hematoxylin and eosin stain (HE). Finally, the slides were mounted with dibutyl phthalate xylene (DPX) and coverslips were placed over them, and allowed to dry thoroughly before being examined under a light microscope at magnifications ranging from 10X to 100X, this allowing the microscopic alterations to be assessed and every microscopic lesion to be identified, described, and recorded (Annex II: E). Finally, photographs of the slides were taken for documentation as described by several authors (Talukder, 2007; Suvarna *et al*., 2018; Al-Mahmood and Al-Sabaawy, 2019; Madkour and Mohammed, 2021; Madkour and Kandyel, 2022; Madkour *et al*., 2022).

3.7. Analysis of data and statistical methods

The data obtained was arranged, checked, coded, entered into an Excel spread sheet (Microsoft Office Excel 2010); and analysed using STATA version 16. Significant differences between the haematological and biochemical parameters of the *Fasciola*-infected and non-infected samples were determined by t-test. Differences were considered as significant when $P<0.05$ and the results were expressed as means ±SE.

4. RESULTS

4.1. Hematobiochemical findings of fasciolosis

The mean values of hematology and serum biochemical parameters of fasciola infected and non-infected cattle, and the reference range are presented in Table 1 and Table 2, respectively. The mean of hemoglobin (HGB), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), total erythrocyte count (TEC), lymphocytes, monocytes, total protein, albumin and glucose values were lower, while the mean corpuscular hemoglobin concentration (MCHC), total leucocyte count (TLC), neutrophils, eosinophils, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) values were higher in the infected cattle as compared to the non-infected. However, the mean values of basophils were similar in both groups, and the mean values of hematobiochemical parameters of non-infected cattle were within the reference ranges. The statistical analysis revealed high significant differences between the mean of HGB, PCV, MCV, TEC, TLC, lymphocytes, monocytes, neutrophils, eosinophils, AST, ALT, ALP, TP, albumin and glucose values of the infected and non-infected cattle, and non-significant differences were also observed between the mean of MCH and MCHC values of the infected cattle and the non-infected cattle ($P<0.05$).

Table 1: Hematological parameters of fasciola-infected and non-infected cattle

Parameters	**Mean ± SE**		**Range**	**t-*value***	***P*-value**
	Infected (n=50)	**Non-infected (n=50)**			
HGB (g/dL)*	4.76 ± 0.18	9.76 ± 0.19	8-14	20.34	0.000
PCV (%)*	15.96 ± 0.62	34.98 ± 0.81	24-42	19.52	0.000
MCV (fL)*	49.10 ± 2.23	56.42 ± 1.59	40-60	2.87	0.005
MCH (pg)^{NS}	15.05 ± 0.88	15.78 ± 0.44	11-17	0.74	0.457
MCHC (%)^{NS}	33.18 ± 2.23	28.89 ± 1.07	28-36	2.00	0.0509
TEC ($\times 10^6/\mu L$)*	3.37 ± 0.10	6.29 ± 0.11	5-10	18.17	0.000

TLC ($\times10^3$/µL)*	14.35 ±0.18	6.41 ± 0.22	4-12	29.09	0.000
LYM ($\times10^3$/µl)*	1.66 ± 0.05	3.17 ± 0.17	2.5-7.5	8.79	0.000
MON ($x10^3$/µl)*	0.019 ± 0.001	0.79 ± 0.08	0.02-0.85	9.60	0.000
NEU ($x10^3$/µl)*	4.35 ± 0.21	2.52 ± 0.14	0.6-4	8.33	0.000
EOS ($x10^3$/µl)*	3.25 ± 0.135	0.53 ±0.037	0-2.4	18.19	0.000
BAS ($x10^3$/µl)^{NC}	0 ± 0	0 ± 0	0-0.2	-	-

The reference range adopted from Sirois and Hendrix (2015); HGB=Haemoglobin; PCV=Packed cell volume; TEC=Total erythrocyte count; MCV=Mean corpuscular volume; MCH=Mean corpuscular haemoglobin; MCHC=Mean corpuscular haemoglobin concentration; TLC=Total leukocyte count, LYM=Lymphocytes; MON= Monocytes; NEU=Neutrophils; EOS=Eosinophils; BAS=Basophils, *Significant at $P<0.05$, ^{NS}Non significant at $P<0.05$, ^{NC}No change between groups.

Table 2: Biochemical parameters of fasciola-infected and non-infected cattle

Parameters	**Mean ± SE**		**Range**	**t-value**	***P*-value**
	Infected (n=50)	**Non-infected (n=50)**			
AST (U/L)*	244.78 ± 5.63	108.52 ± 3.31	46-176	20.37	0.000
ALT (U/L)*	61.12 ±1.96	21.46 ± 0 .86	5-35	17.43	0.000
ALP (U/L)*	195.7 ± 3.11	69.14 ± 5.02	18-153	23.90	0.000
TP (g/dL)*	3.92 ± 0.17	6.51 ± 0.057	6-7.5	13.84	0.000
Albumin (g/dL)*	2.43 ± 0.086	3.79 ± 0 .035	3.4-4.3	14.03	0.000
Glucose (g/dL)*	25.14 ± 0.71	55.74 ± 1.42	37-79	19.22	0.000

The reference range adopted from Sirois and Hendrix (2015); ALT = Alanine aminotransferase; AST= Aspartate aminotransferase; ALP =Alkaline phosphatase; TP=Total protein, *Significant at $P<0.05$

4.2. Gross and microscopic lesions characteriztion of fasciolosis

Gross lesions: in the acute case, the liver was increased in size (hepatomegaly) with rounded edges due to inflammation of the parenchymal layer with pinpoint hemorrhages on the parietal surface. Some area of the parenchyma was pale, which was due to the necrosis, and there was a firm, rough and thick capsule with whitish or reddish discoloration within the parenchyma, and at the cut section, there was a juvenile fluke (Figure 4A). There were also congestion and engorgement of the gall bladder with bile, while dissection, there was a blackish brown exudate (Annex II**:** B2), and the mucosa was normal, and there was no fluke or egg in it (Figure 4B). While in the chronic case, the liver was small in size and firm in consistency with a corrugated capsule (Figure 5A), the bile duct engorgement (Figure 5B), while dissection, there were numerous mature and adult twisted flukes along with blackish brown exudate and giving the pipe stem appearance of the liver, and the gall bladder was decreased in size and its mucosa was thick and filled with flukes and ova (Figure 5C), The flukes collected from the bile ducts and gall bladder were identified as *Fasciola hepatica* and *Fasciola gigantica* (Figure 5D).

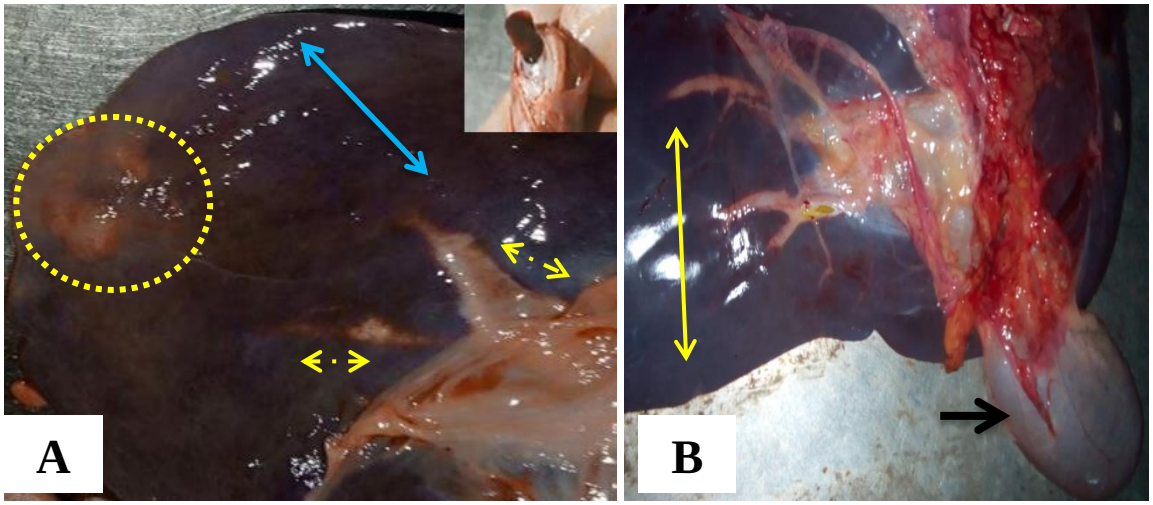


Figure 4: Gross lesion of acute fasciolosis: (A) Hepatomegaly with rounded edge and pinpoint hemorrhages (blue arrows) on the parietal surface, paleness in some areas (dotted arrows), a firm, rough, and thick capsule with whitish or reddish discoloration within parenchyma (dotted circle), juvenile fluke (inset) at the cut section; (B) Congestion (yellow arrows) and engorgement of gall bladder with bile (black arrow)

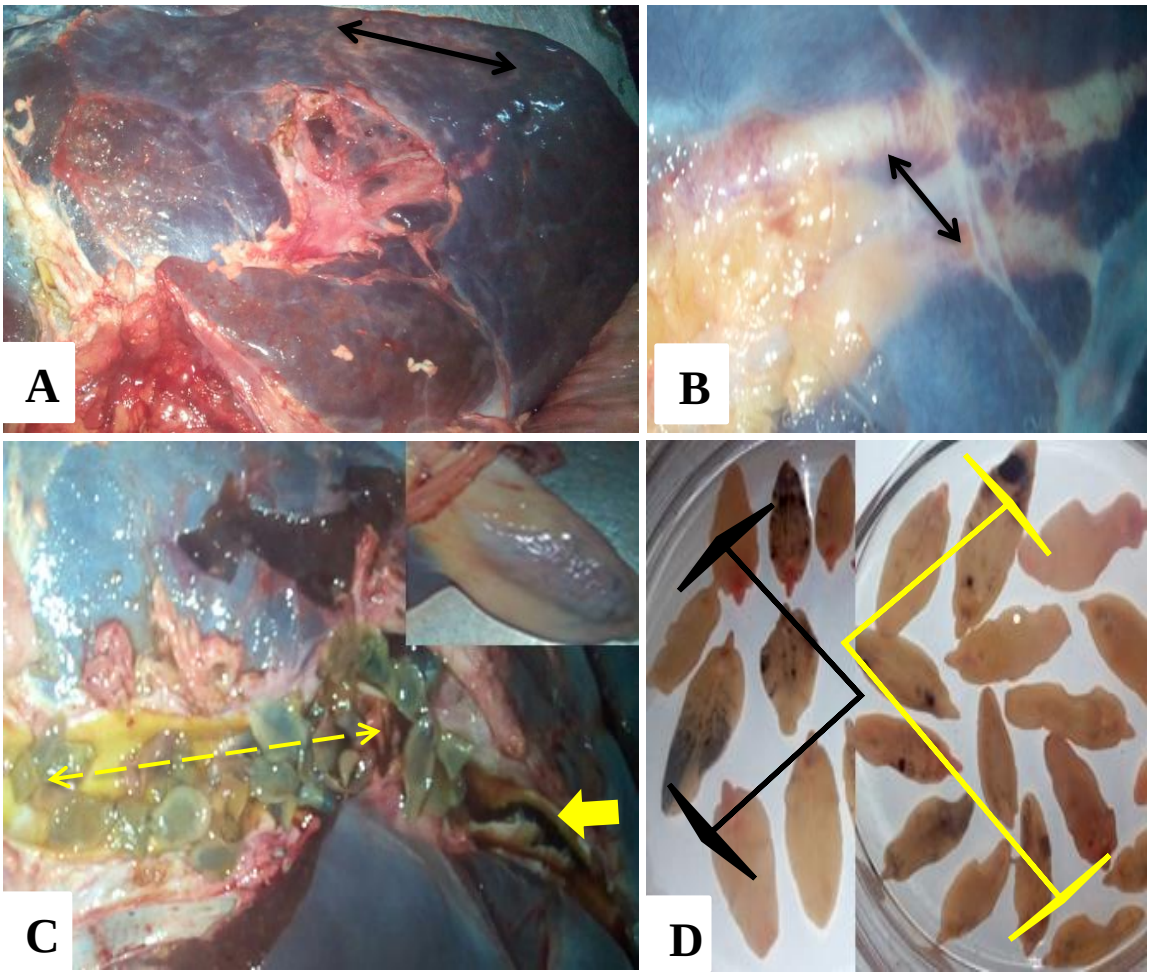
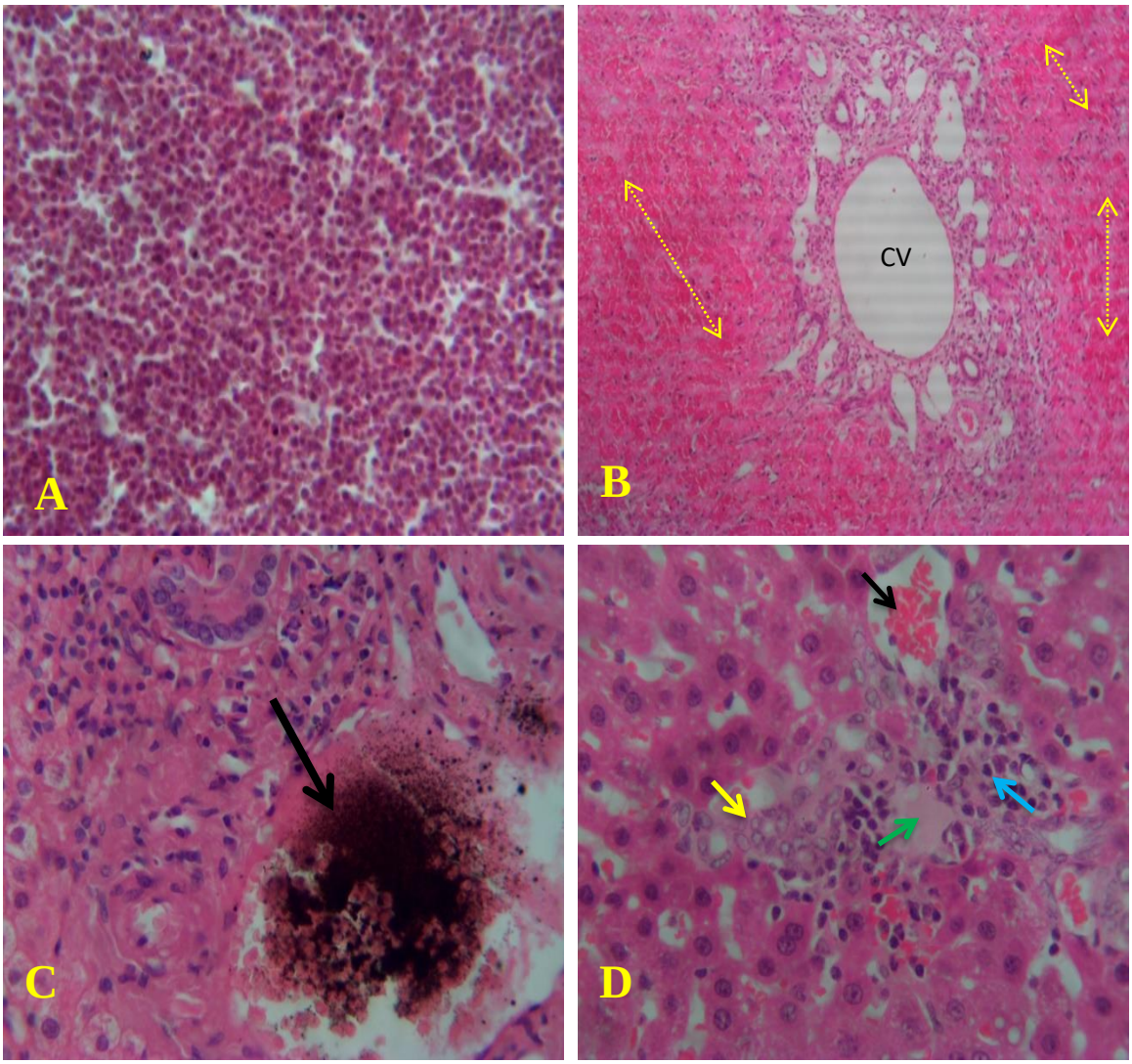


Figure 5: Gross lesion of chronic fasciolosis: (A) Liver was small in size and firm in consistency with a corrugated capsule (arrows); (B) Engorged bile ducts (arrows); (C) mature and immature numerous twisted flukes (lined arrows) along with a blackish brown exudate (yellow arrow), and the gall bladder (inset) was decreased in size; (D) *F. hepatica* (black image) and *F. gigantica* (yellow image)

Microscopic lesions: in acute case, there were excessive eosinophil infiltration in migratory tracts (Figure 6A), congestion around the central vein due to dilation of central vein and sinusoids and engorged with a large number of RBCs (Figure 6B), hemosiderin pigmentation (Figure 6C), a necrotic area with RBCs engorgement surrounded by degenerating swollen hepatocytes and inflammatory cells (Figure 6D), Coagulative necrosis in the migratory tracts and surrounded by large clear vacuole, pyknosis and karyolysis and karyorrhesis within the cytoplasm (Figure 6E), Swollen hepatocytes with

collapsed cytoplasm, hyperplasia of fibrocytes and congested blood vessels at the portal area (Figure 6F) were recorded. In the chronic case, there were neutrophils infiltration within the sinusoidal capillaries and among necrotic hepatocytes (Figure 7A), proliferation of fibrous connective tissues with fibrosis and hemorrhage (Figure 7B), fatty changes along with metaplasia of columnar epithelial cells and bile duct proliferation (Figure 7C), metaplasia of columnar to cuboidal epithelial cells, and metaplasia of columnar epithelial cells (Figure 7D).



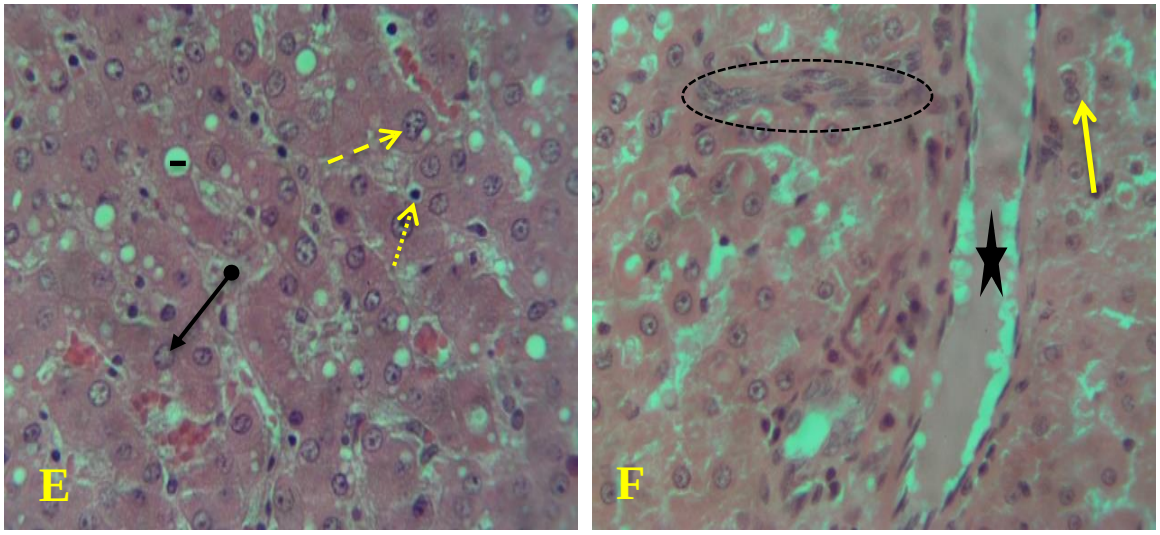
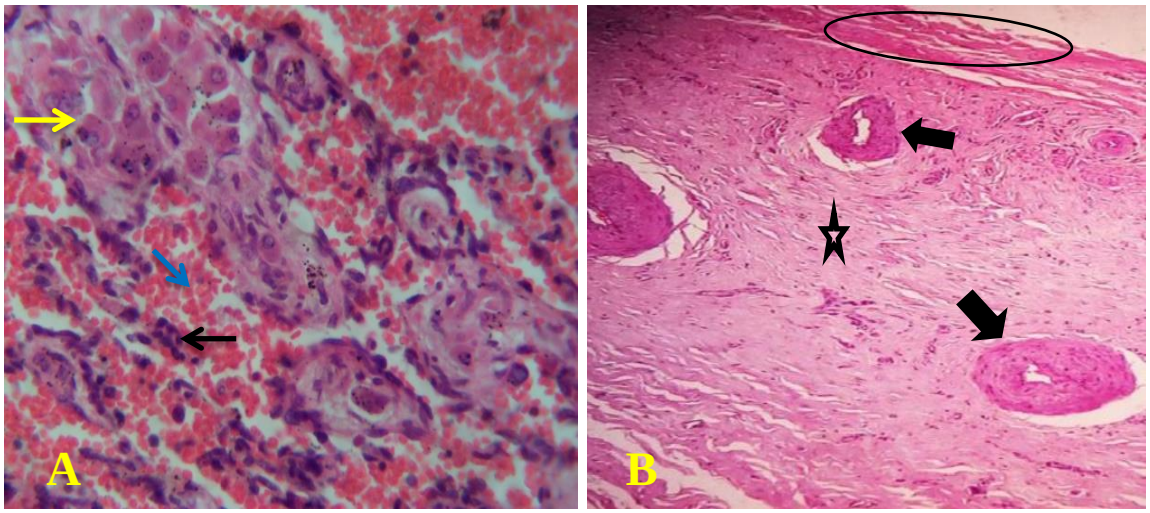


Figure 6: Microscopic lesion of acute fasciolosis**:** (A) excessive eosinophil infiltration in migratory tracts; (B) congestion around the central vein (CV) with RBCs engorgement (doted arrows), 10X; (C) hemosiderin pigmentation (arrow); (D) a necrotic area (green arrow) with RBCs engorgement (black arrow) surrounded by degenerating swollen hepatocytes (yellow arrows) and inflammatory cells (blue arrow); (E) Coagulative necrosis (arrow tail) in the migratory tracts and surrounded by large clear vacuole (line), pyknosis (doted arrow), and karyolysis (arrow head) and karyorrhesis (lined arrow) within the cytoplasm; (F) swollen hepatocytes with collapsed cytoplasm (arrow), hyperplasia of fibrocytes (circle) and congested blood vessels around at the portal area (star); H&E. 40X.



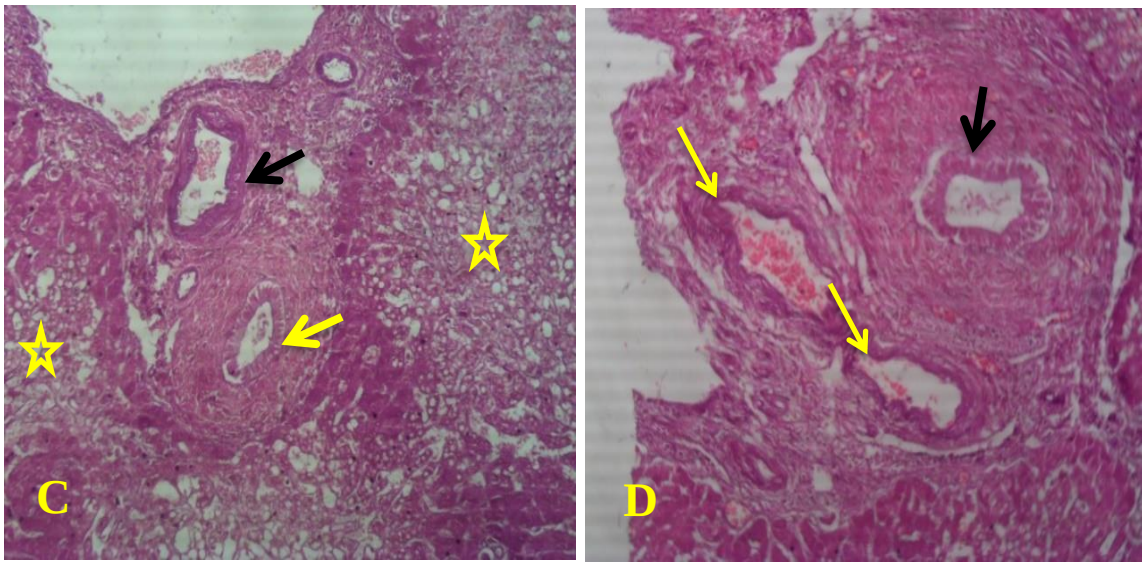


Figure 7: Microscopic lesion of chronic fasciolosis**:** (A) neutrophils infiltration (black arrow) within the sinusoidal capillaries (blue arrow) and among necrotic hepatocytes (yellow arrow); (B) proliferation of fibrous connective tissues (arrows) with fibrosis (star) and hemorrhage (circle); (C) fatty changes (stars) along with metaplasia of columnar epithelial cells (black arrow) and bile duct proliferation (yellow arrow); (D) metaplasia of columnar to cuboidal epithelial cells (yellow arrows) and metaplasia of columnar epithelial cells (black arrow); H&E. 40X.

5. DISCUTION

The diagnosis of bovine fasciolosis was made based on hematobiochemical findings, and gross and microscopic lesions. According to Brahmbhatt *et al*. (2021), hematobiochemical parameters are non-specific but important indicators for the assessment of the proper functioning of the internal organs of animals. Here, the analysis of hematobiochemical mean values of fasciola infected cattle showed high significant reductions in hemoglobin (HGB), packed cell volume (PCV), mean corpuscular volume (MCV), total erythrocyte count (TEC), lymphocytes, monocytes, total protein, albumin and glucose, and elevations in total leucocyte count, eosinophils, neutrophils, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), and a non-significant reduction in mean corpuscular hemoglobin (MCH) and elevation in mean corpuscular hemoglobin concentration (MCHC) as compared to the non-infected cattle. The mean values of basophils were similar in both groups, and the mean values of hematobiochemical parameters of non-infected cattle were within the reference ranges. These findings were in agreement with the reports of other researchers (Hossain *et al*., 2006; Singh *et al*., 2011; Abd-Ellah *et al*., 2014; Gattani *et al*., 2018; Uma, 2020; Brahmbhatt *et al*., 2021).

Reductions in PCV, TEC, HGB and MCV, and elevation in MCHC indicate a microcytic hyperchromic anaemia, which is characterised by low MCV and high MCHC values. This might be due to the loss of blood from haemorrhages caused by the extensive migration of the immature flukes through the liver parenchyma and bile ducts, and the blood sucking habit of an adult fluke (Brahmbhatt *et al*., 2021). Maskur *et al*. (2022) reported significant reductions in PCV, HGB and TEC mean values, and stated that the reduction is due to an infestation of liver flukes in which young flukes can burrow and destroy hepatic cells and lead to haemorrhage (blood loss anemia), which is associated with abnormal iron metabolism due to chronic invasion and the migration of immature flukes inside the liver parenchyma, and the hepatocyte may be unable to produce the normal count of red blood cells. Similar findings of PCV, TEC and HGB values were also recorded by other researchers (Van-Wyk *et al*., 2012; Egbu *et al*., 2013; Gattani *et*

al., 2018; Ngetich *et al*., 2019; Brahmbhatt *et al*., 2021). Similarly, Ahmed *et al*. (2006), Matanovi *et al*. (2007) and Yesuf *et al*. (2020) reported significant reductions in PCV, HGB and TEC values in fasciola infected sheep.

A significant reduction in the mean of MCV, non-significant reduction in the mean of MCH and elevation in the mean of MCHC values were almost in agreement with Taimur *et al*. (1993), who observed non-significant reductions in the mean of MCV, MCH and MCHC values in fasciola infected cattle. Similarly, high significant reductions in the mean of MCV, MCH and MCHC values were reported by Yesuf *et al*. (2020) in fasciola infected sheep. Coppo *et al*. (2011) observed non-significant reduction in the mean of MCV and elevations in the mean of MCH and MCHC values in fasciola infected cattle. The variations might be due to ecological and nutritional differences that highly affect the haematological profiles (Yesuf *et al*., 2020).

Elevations in TLC, eosinophils and neutrophils in infected cattle indicate leukocytosis, eosinophilia and neutrophilia, respectively, whereas reductions in lymphocytes and monocytes indicate lymphocytopenia and monocytopenia, respectively. These findings were almost in agreement with the results of other researchers (Taimur *et al*., 1993; Egbu *et al*., 2013; Brahmbhatt *et al.,* 2021). Similarly, Ganguly *et al*. (2016) reported significant leukocytosis, neutropenia and lymphocytopenia, and non-significant eosinophilia and monocytosis in fasciola-infected sheep. Yesuf *et al*. (2020) reported significant leukocytosis and eosinophilia, and non-significant neutropenia, lymphocytosis and monocytopenia in fasciola-infected sheep. El-Aziem Hashem and Mohamed (2017) also reported significant elevations in leukocytosis, eosinophilia and neutrophilia in fasciola-infected cattle.

Elevation in TLC indicates the presence of white blood cells responses to protect against liver flukes (Maskur *et al*., 2022). Egbu *et al*. (2013 also reported that the changes in the differential counts is a means of body defense against fasciola obstructive effects, or due to the toxin-mediated lesion of the bone marrow. Eosinophilia might be due to the body's defense mechanism against parasitic infection (Yesuf *et al*., 2020), or due to the

inactivation of the parasite by freeing cytotoxic molecules from the surface of the worm body (Piedrafita *et al*., 2001). Neutrophilia might be a result of secondary bacterial infections caused by migration of young flukes through the biliary parenchyma (Taimur *et al*., 1993), or due to the phagocytic action of neutrophils, which secrete lytic substances to degrade parasite cuticular portions, and lymphocytopenia might be due to its involvement in immune mechanisms and the increase in neutrophil value (Katre *et al*., 2020).

Elevations in the activity of AST, ALT and ALP, and reductions in the activity of TP, albumin and glucose in Fasciola infected cattle were also observed by other researchers (Singh *et al.*, 2011; Abd-Ellah *et al*., 2014; Kitila and Megersa, 2014; Gattani *et al*., 2018; Uma, 2020; Brahmbhatt *et al*., 2021). According to Brahmbhatt *et al*. (2021), the elevation of AST and ALT mean values are generally observed in fasciola infection because this fluke causes damage to the liver in the way of their migration, leading to mobilization of inflammatory cells, causing fibrosis and necrosis with the release and increased activity of the enzymes. Hodzic *et al*. (2013) also reported elevated values of ALT and AST in fasciola-infected sheep and stated the elevation is due to hepatocellular necrosis and degenerative changes produced by the migration of juvenile flukes through the liver parenchyma. The elevation of ALP indicates the degree of cholestasis and synthetic capacity of the liver (Coppo *et al*., 2011; Brahmbhatt *et al*., 2021).

Reductions in protein and albumin indicate hypoproteinemia and hypoalbuminemia, respectively. This might be due to the damage or death of hepatocytes by the flukes, which leads to impaired total protein and albumin synthesis, and the reduction in glucose level indicates hypoglycemia due to the inhibition of hepatic glycogenic pathways as a result of fluke migration leads to hepatocyte damage (Hossain *et al*., 2006; Gatani *et al*., 2018; Yesuf *et al*., 2020).

Gross and microscopic lesions were typical indicators of acute and chronic bovine fasciolosis. In the present study, macroscopic changes observed in the acute case were an increase in the size of the liver (hepatomegaly) with rounded edges due to inflammation

of the parenchymal layer with pinpoint hemorrhages on the parietal surface, congestion, paleness in some areas of the parenchyma due to necrosis, a firm, rough, and thick capsule with whitish or reddish discoloration within the parenchyma and while dissection, juvenile flukes were observed. These lesions were similarly observed by Ahmedullah *et al*. (2007) and Sayed *et al*. (2008) in buffalo, Metwally *et al*. (2009) in sheep, Borai *et al*. (2013) in farm animals, Kitila and Megersa (2014) in cattle and sheep, Okoye *et al*. (2015) and Mohamed *et al*. (2021) in cattle. In the current study of acute fasciolosis, engorgement of the gall bladder with bile was recorded. Similarly, Islam *et al*. (2016) also reported a distended gall bladder with bile in a fasciola-infected goat, but they didn't mention any abnormality about its distention. Adrien *et al*. (2013), who studied acute fasciolosis in cattle, and observed an enlarged gallbladder with thick and edematous wall along with several trematodes. However, in the current study, at the cut section of the engorged bladder, there was a blackish-brown exudate (Annex II**:** B2), the mucosa was normal, and there was no fluke or ovum or egg in it. The absence of ova or eggs was checked by wet smear microscopic examination, and similarly, fluke's eggs were not found from the fecal sample either; this might be due to the absence of eggs in the acute infection or before 10 to 12 weeks (Urquhart *et al*., 1996). Therefore, the bladder and bile duct abnormalities were not recorded in the current study of acute fasciolosis; this change might be due to breed variation.

In the chronic case, the liver was small in size and firm in consistency with a corrugated capsule. Similar lesions were found by Borai *et al*. (2013) in farm animals and Kitila and Megersa (2014) in cattle and sheep. In this study, the bile duct was engorged, and while dissecting the engorged duct, both immature and adult twisted flukes were found along with blackish brown exudate, and giving the pipe stem appearance of the liver. These lesions were similarly observed by Salmo *et al*. (2014) and Mohamed *et al*. (2021) in cattle, and Islam *et al*. (2016) in goats. In the present study of chronic fasciolosis, the gall bladder was decreased in size and filled with flukes and ova or eggs, and its mucosa was thick and edematous. Similarly, Adrien *et al*. (2013) and Kardena *et al*. (2017) reported thick bladder mucosa with fluke infestation. But, they reported nothing about the size decrement of bladder. The size decrement of the bladder in chronic fasciolosis might be

due to bile synthesis impairment and blockage of the bile duct by flukes. Moreover, the ova were confirmed by wet smear microscopic examination (Annex II**:** B). Similarly, fluke's egg was detected from the fecal sample (Annex II**:** A). This is because the immature flukes become adult flukes after 10 to 12 weeks or 3 months and come back to the bile duct to start laying, then the laid eggs go to the bladder and duodenum with bile, and the ova or eggs appear in both the bile-containing bladder and the fecal-containing intestine (Urquhart *et al*., 1996). The *F. gigantica* and *F. hepatica* found in the bile duct and gall bladder were identified on the basis of morphology (Soulsby, 1986).

Microscopic changes observed in the acute case were excessive eosinophil infiltration in the migratory tracts, congestion around the central vein due to dilation of the central vein and sinusoids, engorgement with a large number of RBCs and hemosiderin pigmentation, a necrotic area with RBC engorgement surrounded by degenerating swollen hepatocytes and inflammatory cells, coagulative necrosis in the migratory tracts and surrounded by a large clear vacuole, pyknosis, karyolysis and karyorrhesis within the cytoplasm, and swollen hepatocytes with collapsed cytoplasm, hyperplasia of fibrocytes and congested blood vessels around the portal area were also found. These findings were almost similar with the findings of other researchers (Borai *et al*., 2013; Salmo, 2014; Al-Mahmood and Al-Sabaawy, 2019; Mohamed *et al*., 2021; Ashoor and Wakid, 2023). Borai *et al*. (2013) reported severe congestion of central veins, hepatic sinusoids and portal blood vessels, and hemorrhagic migrating tracts formed from degenerated hepatocytes and erythrocytes, infiltrated with eosinophils, macrophages and lymphocytes, and hemosiderin pigments in fasciola infected sheep and cattle. Salmo (2014) reported hepatocyte swelling, eosinophil and neutrophil infiltration, fatty changes in which clear vacuoles appeared in the cytoplasm with peripherally located nuclei, and congestion due to dilation of the central vein and sinusoids with a large number of RBC engorgements in fasciola-infected cattle. Mohamed *et al*. (2021) reported hepatocyte swelling, eosinophil and neutrophil infiltration, fatty changes in which clear vacuoles appeared in the cytoplasm with peripherally located nuclei, and congestion due to dilation of the central vein and sinusoids engorged with a large number of RBCs in fasciola-infected cattle. The findings reported by Mohamed *et al*. (2021) are in line with the results reported by Salmo (2014).

Al-Mahmood and Al-Sabaawy (2019) reported infiltrations of inflammatory cells, congested blood vessels in the portal area, vacuolar degeneration with coagulative necrosis around the central vein, hyperplasia of fibrocytes, and hemosiderin pigmentation in fasciola-infected cattle. Ashoor and Wakid (2023) reported hemosiderin pigmentation with marked fibrous connective tissue proliferation and degenerated hepatocytes with dark nuclei in fasciola infected sheep. The results reported by Ashoor and Wakid (2023) are almost in concordance with the findings of Al-Mahmood and Al-Sabaawy (2019).

In the chronic case, there were neutrophil infiltrations within the sinusoidal capillaries and among necrotic hepatocytes. Salmo (2014) also reported neutrophil infiltrations within the sinusoidal capillaries and among necrotic hepatocytes. In this study, there were also proliferation of fibrous connective tissues and bile ducts with fibrosis and hemorrhage, fatty changes along with metaplasia of columnar epithelial cells, and metaplasia of columnar to cuboidal epithelial cells. These lesions were almost in concordance with the lesions found by Salmo (2014), Al-Mahmood and Al-Sabaawy (2019), Uma (2020) and Mohamed *et al*. (2021) in cattle, Borai *et al*. (2013) in farm animals, and Ashoor and Wakid (2023) in sheep. Salmo (2014) and Ashoor and Wakid (2023) reported proliferation of fibrous connective tissue and bile ducts. Al-Mahmood and Al-Sabaawy (2019) and Uma (2020) reported metaplasia of columnar to cuboidal epithelial cells. Borai *et al*. (2013) reported bile ducts hyperplasia and desquamation of their epithelial cell lining in fasciola infected cattle and buffalos. The microscopic lesions found by Al-Mahmood and Al-Sabaawy (2019) and Uma (2020) are almost in line with the findings reported by Salmo (2014) and Ashoor and Wakid (2023).

6. LIMITATON OF THE STUDY

Due to time and financial limitations, other secondary complications induced by fasciolosis were not considered in this study.

7. CONCULUTION AND RECOMMENDATIONS

This study attempted to assess the hematobiochemical, and gross and microscopic lesion alterations induced by bovine fasciolosis. In this study, there were significant and non-significant differences between the hematobiochemical profiles of fasciola-infected and non-infected cattle. Grossly, in the acute case, there was a juvenile fluke in the parenchyma, and in the chronic case, there were cholangitis and atrophic bladder, which was due to the occurrence of secondary infection by immature and adult flukes. Therefore, many lesions that have been reported by several researchers are non-fluke lesions. While microscopically, in the acute case, there were eosinophils infiltration and congestion around the central vein and sinusoids, and in the chronic case, there were proliferation of fibrous connective tissues and bile ducts and metaplasia of columnar to cuboidal epithelial cells. Some fecal and bile samples were also examined to strengthen this study. The results of hematobiochemical alterations were also consistent with gross and microscopic findings, and cause a great impact on liver physiology and histology leads to high losses in meat and milk production.

Based on the above conclusion, the following recommendations were forwarded:

- ✓ Hematobiochemical analysis should be used as complementary in diagnosis of bovine fasciolosis.
- ✓ Bacteriological examination along with microscopic lesion alterations induced by fasciolosis should be studied.

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9. ANNEXES

Annex I: Abattoir work memo

A) Veterinary medicine graduate students were doing their abattoir experience with the investigator.

B) Cattle brought to slaughter at Gondar ELFORA abattoir were rested in the lairage.

C) Antemortem inspection

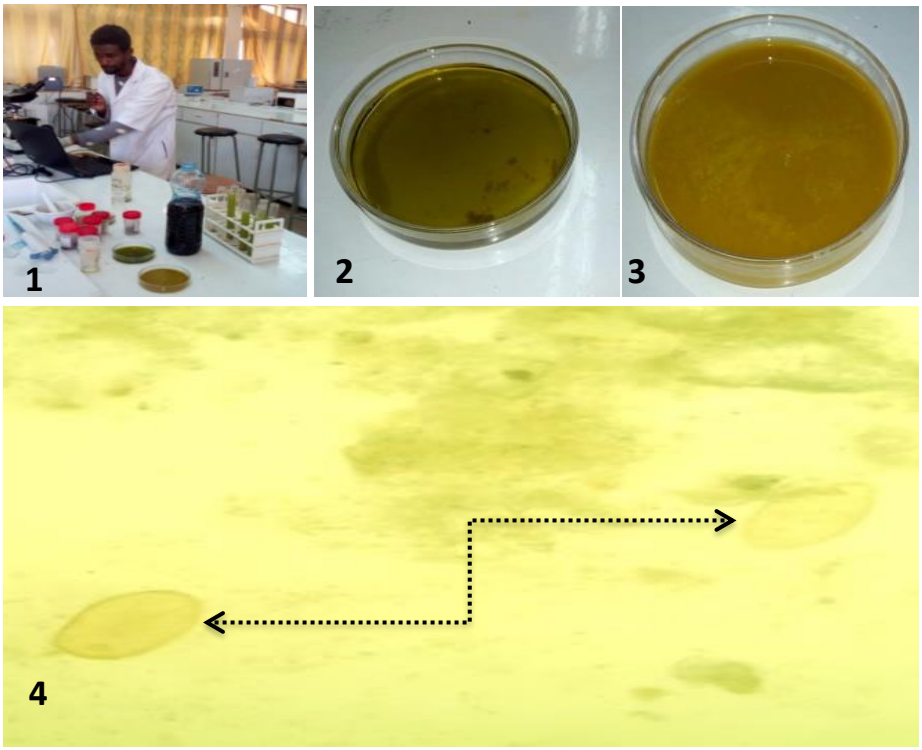
D) Postmortem inspection

Annex II: Laboratory work memo

A) Fecal examination

(1) Microscopic examination; (2) egg of fluke (arrow)

B) Bile examination



(1) Microscopic examination; (2) bile of bladder in acute case; (3) bile of bladder in chronic case; (4) eggs/ova of fluke (dotted arrows).

C) Set-up of an automated hematology analyzer machine

The automated machine was adjusted, and the sample code numbers were typed. The EDTA-coated blood samples were getting clothed on the sample prop. The instrument by itself pipetted an adequate volume of blood, and the results were displayed on screen.

D) Set-up of an automated clinical chemistry analyzer machine

After the selection of tests from the test menu, the program was set, and the sample code numbers were typed on the machine. After calibration, adequate controls and serum were placed in the sample cup in an appropriate order, and enough working reagents were poured into reagent bottles. The instrument by itself pipetted the programmed sample volume and working reagent, and after incubation, the formed color absorbance was read at the appropriate wavelength, and the results were displayed on screen.

E) Histopathology technique (Talukder, 2007)

Principle: Diseases that have gross pathological lesions can be revealed histopathologically.

Materials and reagents:

- ✓ Gloves, Microscope
- ✓ DPX
- ✓ Slide drying bench
- ✓ Chopping/Trimming board
- ✓ Automatic tissue processor
- ✓ Frosted microscope slide
- ✓ Different ascending grades of alcohol
- ✓ Diseased tissue sample
- ✓ Tissue cassette
- ✓ Paraffin section mounting path
- ✓ Incubator/ Dry oven
- ✓ Scalpel handler & blades
- ✓ Hematoxylin and Eosin dye
- ✓ Embedding rings
- ✓ Paraffin wax
- ✓ Pencil/ Permanent marker
- ✓ 10% Buffered Formalin
- ✓ Microtome
- ✓ Wax dispenser
- ✓ Forceps
- ✓ Staining jars
- ✓ Base mold
- ✓ Refrigerator
- ✓ Zylene
- ✓ 1 % Acid alcohol
- ✓ Coverslips

1. Tissues were resized to a dimension of 1 cm3 from the area showing gross lesions, placed in the sampling bottle containing 10% neutral buffered formalin, and labeled.
2. After fixing them in 10% neutral buffered formalin for 72 hours, tissues were placed in the labeled tissue cassettes, washed in tab water for 3 hours, and placed in the tissue basket. The tissue cassette-containing tissue basket was then placed on the automated tissue processor's basket holder and dehydrated as follows:

Alcohol (70%) - 1 hr.	Alcohol (100%) - 1 hr.
Alcohol (80%) - 1 hr.	Alcohol (100%) - 1 hr.
Alcohol (90%) - 1 hr.	Alcohol (100%) - 1 hr.
Alcohol (96%) - 1hr.	Alcohol (100%) - 1 hr.

3. The tissues were cleared with xylene I and II for one hour each.
4. Then, the tissues were impregnated with molten paraffin I and II for two hours each.
5. Embedding was done by pouring some melted molten paraffin on processed tissue samples containing base molds and embedding rings.
6. Then these melted molten paraffin-filled base molds and embedding rings were placed on the ice-containing tray.
7. Finally, blocks containing embedding rings were removed from the base molds.
8. Sectioning was done by placing blocks on the microtome's block holder and cutting at five micrometers, and ribbons were placed in a 45^{o}C-adjusted tissue bath.
9. The ribbons were taken by clean slides, and the ribbon containing slides were placed on slide drying bench. Then, finally, ribbon-containing slides were stained as follows:

Xylene I&II – 2 min each	Dewaxing	Xylene I&II – 1 min each	Clearing
Alcohol (100%) -2 min	Hydration	Alcohol (100%) - 1min	Dehydration
Alcohol (90%) - 2 min		Alcohol (90%) - 1 min	
Alcohol (80%) - 2 min		Alcohol (80%) - 1 min	
Alcohol (70%) -2min		Alcohol (70%) - 1 min	
Alcohol (50%) -2 min		Alcohol (50%) - 1 min	
Tap water - 1 min		Tap water – 30 sec	
Hematoxylin - 10 min →	Nuclear staining	Eosin - 5 min →	Cytoplasm staining
Tap water – 5 min		Tap water - 30 sec	
1% acid alcohol- 15 sec →	Deferencetion		

10. Stained slides were mounted by DPX solution, and coverslips were placed over them, and finally, they were examined under a light microscope at different magnification powers.

Pictorially, it was illustrated as follows:

I. Tissue processing (1–4): (1) leveling the tissue containing tissue cassettes; (2) programming; (3) placing the tissue cassette containing tissue basket on the automatic tissue processor's tissue basket hanger and starting the program after closing the lid.

II. Embedding (5–7): (1) flaming the base mold to prevent precooling; (2) filling of melted wax over the processed tissue containing base mold and embedding ring; (3) freezing to remove the block from the mold.

III. Sectioning (8): (1) tiny section cutting; (2) placing the ribbon in warm water containing tissue bath to avoid shrinkage of the ribbon or tiny section.

IV. Staining (9): (1) flaming to facilitate deparaffinazation or dewaxing in xylene; (2) placing ribbon-containing slides in different chemicals.

V. Mounting (10): (1) pouring the DPX solution on the slide and covering with coverslips; (2) documentation (saving the pictures on the computer).