Thesis Ref. No._____

CHARACTERIZATION OF LESIONS IN DISEASED FISHES; ISOLATION AND IDENTIFICATION OF *EDWARDSIELLA TARDA* FROM SELECTED LAKES AND PONDS OF CENTRAL ETHIOPIA

MVSc THESIS

BY

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MVSc THESIS

A thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Pathology

By

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August, 2021

Bishoftu, Ethiopia

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LIST OF ABBREVIATIONS

BHI	Brain Heart Infusion
DNA	Deoxyribonucleic Acid
H&E	Haematoxylin and Eosin
PCR	Polymerase Chain Reaction
SIM	Sulfur Indole and Motility Test Media
TSA	Tryptic Soya Agar
TSI	Triple sugar iron agar
XLD	Xylose Lysine Deoxycholate

ABSTRACT

Fish is a nutritious animal rich in protein and consumed by many peoples throughout the world. A cross-sectional study with purposive sampling was conducted from December 2020 to May 2021 with the objectives of lesion characterization in diseased fishes, isolation, and identification of *Edwardsiella tarda*. From Batu, Langano, and Babogaya lakes and ponds in Bishoftu, a total of sixty fishes were examined for gross lesions. Skin, muscle, gill, liver, kidney, spleen, and heart were collected for microscopic lesion characterization and *Edwardsiella tarda* isolation. Grossly, few lesions were observed. Out of 60 samples, 25% (n=15) were positive for *Edwardsiella tarda* by conventional PCR. A statistically significant difference ($P<0.05$) was observed in the frequency of identifying *E.tarda* between the three study areas and four fish species. After the histopathological process and staining, tissues were divided into two based on *E. tarda* confirmation. Gills from *Edwardsiella tarda* positive fishes revealed hyperplasia, focal necrosis, and swelling of the lamellar epithelium. Microscopic lesions in the liver include nuclear condensation, cytoplasmic vacuolation, and swelling of hepatocytes. Lesions in the hepatic blood vessels comprise severe hyperplasia of the endothelium, hyperemia, and elastic fibers in the intima of hepatic artery. Kidneys of *Edwardsiella tarda* positive fishes had suppurative interstitial nephritis, melano-macrophage deposition in interstitium, and interstitial hemosiderin deposition. Examination showed infiltration of inflammatory cells and focal area necrosis of the epidermis, lymphocyte infiltration in muscle, and accumulation of hemosiderin pigments in spleen. Microscopic lesions in *Edwardsiella tarda* negative fishes indicated significant changes like lamellar epithelial degeneration with vacuolation, and total necrosis of structure in gill, periportal hepatitis, and hepato-necrosis with vacuolation of liver, glomerulitis, multifocal tubulointerstitial nephritis, epithelial cells degeneration and cloudy swelling in kidney, dermal edema in skin and myocarditis with fibrosis of heart. Based on the study several lesions were identified from diseased fishes. Thus, further studies on lesion characterization in diseased fishes of different species with large sample sizes and study area should be conducted.

Keywords: *Edwardsiella tarda, Fish diseases, Lesion, Lakes*

1. INTRODUCTION

Fish are the most diverse groups of vertebrates dwelling in a variety of marine and freshwater habitats. Fisheries continue to be a significant source of food, nutrition, income, and livelihoods for people around the world (Kerie *et al*., 2019). Currently fish make up about 19% of the total protein consumption or over the 5% of proteins from both plants and animals origin (Dugenci and Candan, 2003). Fish consumption in sub-Saharan Africa is the world's lowest. Ethiopia's fish production is almost based on inland water bodies like lakes, reservoirs, and rivers (Sorsa *et al.,* 2019). Even if the country has an estimated fish production of 51,481 tonnes annually, national per capita fish consumption is 0.5 kilogram per year but the recommended quantity is between 12 and 17 kilogram per year (Martinus, 2020).

Infectious and non-infectious fish diseases are global problems and they affect freshwater, marine water, and cultured fish (Kebede and Habtamu, 2016). Infectious diseases are caused by pathogenic organisms present in the environment or carried by other fish and are contagious. Non-infectious diseases are non-contagious, caused by environmental problems, dietary deficiencies, or hereditary anomalies. Infectious diseases are broadly classified as parasitic, bacterial, viral, or fungal diseases (Nicky, 2004; Aly, 2013).

Fish are vulnerable to great hazards exerted by parasites and other disease-causing agents especially when they are stressed. Among organs susceptible to diseases the skin and gills are the most affected organs (Buller, 2014). Both opportunistic and obligate parasites are the most common cause of infectious diseases in fishes. Parasitic diseases of fish are most frequently caused by protozoa that live in the aquatic environment. A variety of protozoans infest the external part of fish causing irritation, weight loss, and eventually death. Fungal spores are abundant in the aquatic environment, although they rarely cause disease in healthy fish. Fungi colonize damaged exterior tissue of fish after they are infected with an external parasite, bacterial infection, or injured by handling, The areas appear to have a cottony growth

or appear as brown matted areas when the fish are removed from the water (Verma *et al*., 2010; Aly, 2013).

Endo-parasites of fish are common pathogens regularly reported to impose fish production in growing countries including Ethiopia. Trematodes, cestodes, nematodes, and acanthocephalans infect the internal organs of fish with their intermediate stages and sometimes encysting in various host tissues or most adults mainly affects the digestive systems of their hosts but few the circulatory systems (Hodda, 2007). But there are very limited studies concerning the economic and public health impact of endo-parasites in Ethiopia (Lemma, 2013; Gebremedhn and Tsegay, 2017).

The stress conditions like oxygen depletion, poor water quality and overstocking are the factors that stimulate bacterial pathogens in fish. Fish infected with a bacterial disease have hemorrhagic spots or ulcers along the body wall and around the eyes and mouth. Also have an enlarged, fluid-filled abdomen, and protruding eyes during internal infection that requires treatment with medicated feeds containing antibiotics. External bacterial infections result in erosion of skin and ulceration. *Columnaris* is an example of it which caused by rough handling. The lesions induced by infectious diseases, whether bacteria and /or viral should not be confirmed without a laboratory test (Verma *et al*., 2010).

One of the important bacterial diseases in fish is edwardsiellosis associated with *Edwardsiella spp* (Park *et al*., 2012). *E.tarda* bacterium is a known causative agent of a fish disease causing gangrene and septicemia lesions in different organs (Nakamura *et al*., 2013). The pathogenesis of *E. tarda* is multifactorial. The intestine and abraded skin are the most likely sites for penetration of the bacteria (Ali *et al*., 2014). *E. tarda* potential virulence factors include siderophores, cell adhesion factors, and cell invasion activity, and two types of hemolysin (Mohanty and Sahoo, 2007). Fish infected with *E. tarda* exhibit abnormal swimming behavior and floating near the water surface (Woo *et al*., 2011), loss of pigmentation, exophthalmia, the opacity of the eyes, swelling of the abdominal surface, petechial hemorrhage in fin and skin,

necrotizing small skin lesions and rectal hernia (Park *et al*., 2012; Yoo *et al*., 2019). It occurs sporadically in both fish and humans (Evans *et al*., 2011; Sorsa *et al.*, 2019).

In Ethiopia, major fish-borne bacterial and parasitic zoonoses were studied (Sharma *et al*., 2012; Sorsa *et al*., 2019). Studies on internal fish parasites were conducted in Lake Zeway (Lemma, 2013; Nigussu *et al*., 2017), in Lake Lugo (Hayke) (Amare *et al*., 2014), in Lake Charcher (Gebawo, 2014). Isolation of *E.tarda* from fishes of Lake Zeway and Langano by (Kebede and Habtamu, 2016) and Tana (Nuru, 2007) has been provided information about the diseases occurrence. However, no research has been conducted on the characteristics of lesions in diseased fish in Ethiopia.

Therefore, this study was designed with the objectives of:

- Characterizing lesions in diseased fishes from Batu, Langano, Babogaya lakes, and ponds in Bishoftu
- Isolating and identifying *E. tarda*

2. LITERATURE REVIEW

2.1 Common diseases of fishes

Fish are afflicted with various types of protozoan, fungal, bacterial, viral and helminth diseases. In many cases, they have proved to be a serious problem causing economic losses in the fishing industry and aquaculture (Imam and Dew, 2010). They cause epidemics and mortalities in fish farming and as fish culture gets more intensive and widespread, fish parasites infection is more likely to become increasingly major economic and health issues it reduces fish production by affecting the normal physiology of fish if left uncontrolled (Leveque *et al*., 2007).

2.1.1 Protozoan diseases

Ichthyophthiriasis multifiliis (I.multifilis) also referred to as Ich, is one of the most pathogenic protozoan parasites of fishes. *I.multifilis* is a ciliated parasite that infects different regions of the body externally. Mostly parasitizes the epithelial surface of fish and the mechanical trauma caused by the parasite act as a portal of entry for pathogens present in the water including *Aeromonas hydrophila* and *Edwardsiella ictaluri* (Almaw *et al*., 2014). This parasite causes simple hyperplasia of the epidermal cells around the site of infection causing postules formation. Whitish cysts on the skin, gill, and fin are symptoms of the diseases (Jhingran, 1991).

Costiasis is caused by *Costia necatrix*. Mostly affects skin, fins and gills. Indications of costiasis are the presence of a bluish coating on the skin of the fish and the presence of a large amount of mucus, highly affected body parts show red patches; the gills become brown. The parasite causes irritation and disturbs respiration (Mallik *et al*., 2015). Trichodiniasis is caused by a group of peritrichal ciliated protozoans. Clinically, fish show flashing, lethargy, an increase in mucus production causing a white to bluish haze on the skin (Robert and Moeller, 1999). Ulcers form on the skin and the fins fray. If the gills are damaged, the fish may suffer from severe respiratory distress. Histologically, masses of organisms are linked to the epidermis by adhesive

discs and denticles of the exoskeleton. Necrosis occurs in the underlying epithelial cells. There is secondary hyperplasia and hypertrophy of the gill epithelium (Jhingran, 1991).

Heteropolaria colisarum causes Epistylis (Red sore illness). It is mostly found in wild populations of scaled fish. Clinically, ulcers or cotton-like growths on the skin, scales, and spine that result in a red colored lesion. The lesion in *catfish* affects the spines and bones that reside behind the skin of the head and pectoral girdle. *Myxosporidians* constitute typical fish parasites known to produce cysts on different regions of the body and internal tissues and organs. Symptoms of this infestation include weakness, emaciation, rising of the scales along their posterior margins, falling of scales (Sharma *et al*., 2012).

2.1.2 Helminth diseases

Many parasitic worms infect fishes and cause great harm to them. *Dactylogyrus* and *Gyrodactylus* are the two common parasitic worms. *Dactylogyrus* attacks the gills, while gills and skin both are attacked by the infection of *Gyrodactylus*. Fish becomes less motile by the attack of these parasites, their fins start falling, the body becomes yellow and blood spots develop on their body. Delay in treatment may be lethal. Cyst of the metacercaria of the worm, *Posthodiplostomum cutiola* also causes "black spot disease" in fishes. Such black spots appear on the whole body, including eyes and mouth (OIE, 2009).

2.1.3 Fungal diseases of fish

Saprolegniasis, Branchiomycosis, and *Ichthyophonus* are the three most common fungal diseases of fish.

Saprolegniasis is caused by *Saprolegnia parasitica*. The fungus attacks the dead eggs and thereafter spread on the surrounding viable eggs resulting in their spoilage as well. This fungus often infects the fertilized eggs in hatching. Cotton-like growth on

the skin and fins of fishes. It begins as small, local infections and then spreads over the body (Jhingran, 1991; Rahaman *et al*., 2016). Branchiomycosis (Gill Rot) is caused by the fungi *Branchiomyces sanguinis*. Infect the gill tissue of fish gills appear striated or marbled with the pale areas representing infected and dying tissue (Klinger and Floyd, 1996; Patel *et al*., 2018).

The causative agents causing Icthyophonus Disease (Swinging Disease) are two species: *Ichthyophonus hoferi* and *I. Gasterophilum*. Fish with a mild to moderate infection will have no visible evidence of the disease's symptoms externally. In severe cases, infection under the skin as well as in muscular tissue can cause the skin to have a sandpaper texture. The curvature of the spine may be found in some fish. Internally, organs may be enlarged with white to grey-white lesions. The disease is called "swinging disease" because diseased fish shows curious swinging movements. It is a fungus, but it manifests itself internally. Primarily attacks the kidneys and liver. Symptoms of the disease include that fish become sluggish, lose balance and eventually show external cysts or sores (Blazer *et al*., 2002).

2.1.4 Common bacterial pathogens of fish

Examples of bacterial pathogens associated with fish include *Escherichia coli, Clostridium botulinum, Shigellady senteriae, Staphylococcus aurous, Listeria monocytogens, Edwardisiella species,* and *Salmonella*. The bacterial pathogens found naturally in the fish habitat are *Vibrio species,* Pseudomonades and *Aeromonas species.* Bacterial diseases are considered the main cause of high mortalities and economic losses among fish and fish farms. Escherichia species is a classic example of enteric bacteria causing gastroenteritis (Zhonghua *et al*., 2004).

E. coli and *Staphylococcus* species and sometimes *Enterococci* are commonly used as indices of hazardous conditions during the processing of fish (Ayalew *et al*., 2017). Aeromonads are virtually everywhere in the microbial environment. Pseudomonades are opportunistic gram-negative pathogens, naturally occurring in the aquatic environment (Taghreed, 2020). It is characterized by petechial hemorrhage, darkness

of the skin, detached scales, abdominal ascites, and exophthalmia (OIE, 2009). Mostly *A. hydrophila* and *P. fluorescens are* mentioned as a cause of fish disease known as hemorrhagic septicemia or bacterial septicemia both in experimental and natural infection are documented in the world (Almaw *et al*., 2014). Characterized by hemorrhaging at base of fins, eyes and vent, pale gills, exophthalmia externally and internal signs with bloody or ascitic fluid in a body cavity, hemorrhaging of internal organs, kidney soft, spleen and other organs pale (Meyers, 2000).

Fin and tail rot diseases affect both adults and young fishes. The infection during its early stage appears as a white line on the margin of the fin, spreading and imparting frayed appearance to the appendage which eventually putrefies and disintegrates. The disease is contagious and causes considerable damage (Jhingran, 1991). Frayed fins and tails with a pale pinkish-white edge and some blood in the tissue (Rahaman *et al*., 2016). Ulcer disease (Columnaris disease) is caused by *Flexibactor columnaris* shows raised white plaques, often with reddish peripheral zone leading to hemorrhagic ulcers to deep lesions that expose underlying muscle or bone (Dror *et al*. 2006). Because of their chronicity, lesions are often secondarily infected with water molds, protozoa, and other bacteria (Jhingran, 1991). *Pseudomonas* and *Aeromonas* bacteria also cause pinky-white open wounds with a white edge and are occasionally secondarily infected by fungi and other bacteria. Extremely poor water quality or an abnormally high pH level. Minor scratches can become infected if conditions are poor (Rahaman *et al*., 2016).

Dropsy is usually triggered by poor water quality, especially the presence of ammonia and nitrite also drastic temperature changes, and improper nutrition. Can be caused by bacterial infection, viral infection, nutritional, metabolic, and osmoregulatory. In this condition accumulation of fluid inside the body cavity, scale protrusion, exophthalmic condition, and inflammation of the intestine and hemorrhagic ulcers occur on skin and fins. Bulging eyes, pale gills, the fish may stop eating and hang near the bottom or gasp for air at the top of the tanks and show signs of unbalanced swimming (Sharma *et al*., 2012; Rahaman *et al*., 2016).

Vibrio species *Vibrio anguillarum and Vibrio ordalii* cause a disease known as vibriosis. Result in hemorrhages in the eye, erythema at the base of fins, petechiae in skin and musculature, darkening of the dorsal surface, bloating, and open penetration of abdominal cavity, bleeding at the vent, pale gills. Internal signs include petechiae and hemorrhagic areas in internal organs and mesenteries, enlarged spleen and kidney, fluid in the gut (Meyers, 2000).

2.1.5 Edwardsiella species

The genus *Edwardsiella* was named after American microbiologist P.R. Edwards in 1965. Until 2013 the genus consisted of only 3 species due to its most similar biochemical characteristics, *E. tarda, E. ictaluri, and E. hoshinae*. But from recent studies, the genus consists of five species; *E. tarda, E.ictaluri, E. hoshinae, E. piscicida,* and *E. anguillarum* (Kerie *et al*., 2019). Ewing and his colleagues proposed a new species called *E. tarda* from human feces. It is a gram-negative, facultatively anaerobic, motile, peritrichously flagellated, rod-shaped bacterium (1µm in diameter and 2–3µm long) of the family Enterobacteriaceae, order Enterobacteriales, class Gammaproteobacteria and phylum Proteobacteria (Mohanty and Sahoo, 2007; Xu *et al*., 2014). The bacterium is photogenic and photosynthetic known to infect fish throughout the year, but it is usually highly contagious in high temperature or poor water quality. It is highly pathogenic when the temperature is between 20°C and 25°C than in low temperature below 15°C (Woo *et al*., 2011).

E.tarda can survive at 0–4% sodium chloride, pH 4.0–10.0, and temperatures ranging from 14–45°C. The biochemical characteristics of *E. tarda* are catalase-positive, cytochrome oxidase negative, production of indole and hydrogen sulfide, fermentation of glucose and reduction of nitrate to nitrite (Park *et al*., 2012), lysine positive, mannitol, dulcitol, sorbitol, inositol, xylose, rhamnose negative, alkaline slant, and acid but on Triple sugar iron Agar (Kebede and Habtamu, 2016). This bacterium can be isolated on Edwardsiella Isolation Media (EIM), Brain Heart Infusion (BHI), Tryptic Soya Agar (TSA), Xylose Lysine Deoxycholate (XLD), and MacConkey. On the mentioned culture media, *E.tarda* morphology is seen as small,

circular, raised, whitish with black center on XLD and pale on MacConkey agar, grow best at a temperature between 25°C-37°C, PH 7-8 and 0.5% NaCl (Wei and Musa, 2008).

E. tarda is a pathogen with an abroad host range that includes fish, birds, reptiles, and humans (Sun *et al*., 2012). It is the causative agent of edwardsiellosis and leads to extensive losses in many commercially important freshwater and marine fish (Kwon *et al*., 2006). *E tarda* infection is considered a dangerous septicaemic disease with high economic losses; the seriousness of *E. tarda* infection is its expanding fish host range. Even though *E. tarda* is a bacterium of fish, it infects humans posing public health threats; causing gastroenteritis, meningitis, liver and skin abscesses, and valvular endocarditis in a patient with acquired immune deficiency syndrome (Ibrahem *et al*., 2011). *E. tarda* infect a variety of fish species, including Japanese eel (*Anguilla japonica*), European eel (*Anguilla anguilla*), Japanese flounder (*Paralichthys olivaceus*), turbot (*Scophthalmus maximus*), yellowtail (*Seriola quinqueradiata*), red sea bream (*Pagrus major*), channel catfish (*Ictalurus punctatus*), and tilapia (*Oreochromis niloticus*) (Mohanty and Sahoo, 2007).

E. tarda survive in their host by consuming several important substances and abilities that serve as virulence factors in the host. Both avirulent and virulent *E. tarda* are able to adhere to, invade, and replicate in the epithelial papilloma (EPC) cell line using host microfilaments and protein tyrosine kinase. The gill, gastrointestinal tract, and body surface are the sites of entry of the virulent strain (Ling *et al*., 2001). Reports found that motility-related proteins, such as flagellin and autotransport adhesin, a fimbrial adhesin-like protein, are important for attachment and penetration into the epithelial cells of hosts. The ability of bacteria to acquire iron acquisition using the bacterial iron chelator, siderophore, is essential for the survival and replication of bacteria. E. tarda produces two kinds of hemolysin; one is a cell associated, iron-regulated hemolysin that is secreted as an extracellular protein (ECP) under iron-regulated conditions and the other is an extracellular hole forming hemolysin distinct from that is not regulated by iron (Srinivasa *et al*., 2003).

Edwardsiella tarda infected fish reveal vertical hanging, frothing, excess mucus secretion, listing, swollen abdomen, anorexia, fin, and tail rot, and reddish operculum. Gross pathology of fish affected by edwardsiellosis shows signs of spiraling movement and die with the mouth agape and opercula flared due to the development of anemia leading to oxygen insufficiency gross lesions on the skin, pale gills, tumefaction of the eye, excessive mucus secretion, scale erosion and ulcers, Swelling and bleeding of the anus leading to reddening. In peracute cases, there is congestion of the ventral parts of the body (Padros *et al.,* 2006). Internally, there are watery and bloody ascites in the abdominal cavity and congested liver, spleen, and kidney, hypertrophy of kidney and liver, internal organ tumors (Yoo *et al*., 2019).

The gross pathology lesion in fish diseases by different infectious diseases is not of much help in arriving at a diagnosis as many of these symptoms and signs match those of infection with other bacteria. In mild infections, the only manifestation of the disease is small cutaneous lesions (3–5 mm in diameter) on the posterolateral parts of the body. As the infection progresses, affected fish lose control over the posterior half of the body (Mohanty and Sahoo, 2007).

The histopathological features of Edwardsiellosis in fish kidneys are suppurative interstitial nephritis, renal blood vessel congestion, and multi-focal hemorrhages. Necrobiotic renal tubules changes as cloudy swelling (fig.1 C), hydropic degeneration and necrosis, ischemic type tubulopathy, hyperplastic hematopoietic tissue, rupture of the tubular basement membrane, hydropic dystrophy of nephritic cells, neutrophil infiltration, fibrinoid necrosis of nephritic tubules, hemosiderin deposition and edema. Lesions in liver are suppurative hepatitis, abscesses of various sizes, congestion of central veins. hepatocytic disorganization and disorientation of hepatic plates, hydropic degeneration of hepatic cells in which the cells swollen with irregular vacuoles in the cytoplasm, fatty changes in which the cells vacuolation in the cytoplasm, hepatic cells with signs of coagulative necrosis, increase in the melano-macrophages cells. Bacterial colonization and neutrophil and macrophage infiltration in the liver, spleen, and kidney (Darwish *et al*., 2000; Ibrahem *et al*., 2011).

Hypertrophy of the liver cells and enlargement of their nuclei (Miwa and Mano, 2000). Bacteria-laden phagocytes are found in the sinusoids of the anterior kidney, liver and spleen. Liquefaction and gaseous necrosis in the kidney, liver, spleen and body musculature leading to ulcer formation (Sahoo *et al.,* 2000). The liver sections reveal lymphocyte infiltration, dilation of hepatic sinusoids, expansion of space between hepatic sinusoids, and focal necrosis. Histopathological observations of muscle on infected fish reveal lymphocyte infiltration in muscle and focal necrosis, hyperplasia, edema, and swelling of the gill lamellar epithelium (fig.1 A and fig.1 B) (Abraham *et al*., 2015). The skin shows focal areas of necrosis in the epidermal layer forming erosions or necrosis, hyperplasia of epidermal cells including the mucus cells. Dermatitis with congested blood vessels through large areas of hemorrhages and inflammatory cells (Ibrahem *et al*., 2011).

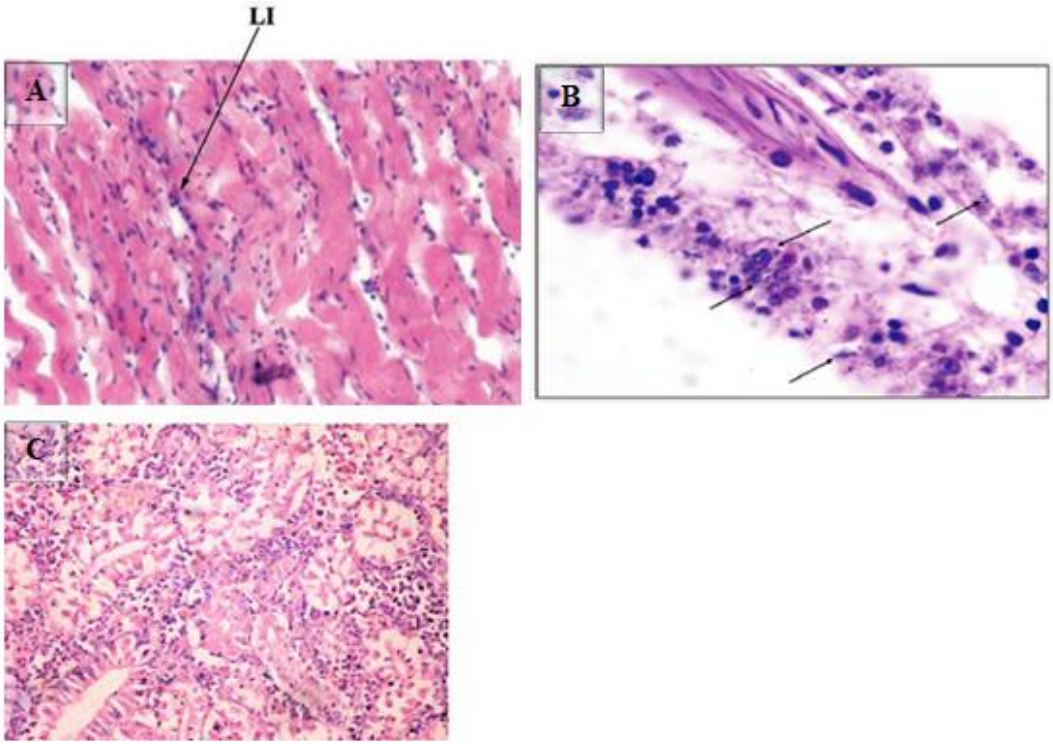

Figure : Histopathological characteristics of edwardsiellosis in muscle, gill, and kidney

(A) Muscle showing lymphocytic infiltration (LI) H&E 200X (Abraham *et al*., 2015); (B) Gill filament of *tilapia* hybrid. Numerous bacilli (arrows) invaded the epithelium of the filament, are edematous and necrotic and the lamellae are not visible. H&E; 100x (Garcia *et al*., 2012); (C) Kidney of *African catfish* experimentally infected by *E. tarda* showing necrobiotic changes in renal tubules. H&E 400x (Ibrahem *et al*., 2011).

2.1.6 Viral diseases

Viruses are responsible for a wide range of fish diseases. Some viruses produce tissue proliferation, whereas others cause degeneration. Although several diseases of a proliferative nature were reported to be of virus origin. Fish viral diseases cause large-scale mortality in fish and are very difficult to treat directly. One of the most effective ways to prevent viral infections is to avoid them. Viruses are typically easily spread from one culture system to another due to inappropriate handling or improper management and hence require easy and early diagnostic methods to detect infections (Sivasankar, 2017).

Spring viremia is caused by *Rhabdovirus carpio*. Common carp are the susceptible host species for the disease. The infected fish becomes black and develops masses on the skin and gills. Bleeding from scales, accumulation of fluid in the body and inflammation of the alimentary canal are some other symptoms of this disease. Fish pox is one of the earliest diseases of fish in which proliferative changes are involved. This disease is reported in European countries. The epidermis of the infected carp becomes proliferous and produces whitish opaque spots, which are why the lesions or blisters are formed on the skin (Sharma *et al*., 2012).

2.2 Diseases transmission in fish

One of the most common routes of disease transmission in aquaculture is direct contact. Fish disease spread by direct contact with water sources and fomites, via ingestion or indirectly through vectors. Entry occur through the skin, open wounds,

mucous membranes, or gills. Water sources also serve to transfer disease causing organisms that are contaminated by the urine, feces, reproductive fluids, and mucus of diseased fish. The movement of contaminated water during the transport of fish can spread pathogens to new locations (Dvorak, 2009).

A few fish pathogens have been found to spread via aerosols, sprays, or splashes between tanks, although less common and typically requiring proximity of sources. Moreover, vectors are living creatures, such as fish preying birds, snails or crustaceans, rodents that can spread pathogens and carry some fish pathogens in their feces or urine, contaminating the environment or fish feeds. People also serve as vectors, transferring pathogens to fish during handling. Furthermore, Disease organisms can be transmitted orally by consumption of contaminated feed, infected live or frozen fish, or cannibalism of dead or dying fish from the same unit. Ingestion of water contaminated with waste products from infected fish also serve as a transmission route (Kerie *et al*., 2019).

2.3 Diagnostic techniques used in fish diseases

Disease diagnosis is the various procedures and techniques used to identify the nature of the disease and to incisively point out the primary and secondary pathogens involved (Mallik *et al*., 2015). Diagnosis of fish includes water quality testing, inspecting fish for signs of disease, color change, ventilation rate, swimming pattern and other behaviors, food intake, growth rate, presence of morphological abnormalities, injury and reproductive performance. Water quality analysis are tests that should be run in any fish disease it includes ammonia, nitrite, and pH and salinity in a marine. Oxygen and temperature are also part of this. History taking is useful to decide whether the problem is acute or chronic, help to eliminate some differentials (Wise *et al*., 2004).

Once history is taken, the fish should be closely examined physically for behavioral abnormalities sick fish often congregate together, separating themselves from their healthier cohorts. Weak fish in raceways with flowing water often found near the

water outlet. Different fish species inhabit different parts of the water column like in the surface, bottom, shoreline, etc and this position often changes with sickness. Extremely sick fish may be in dorsal or lateral recumbency. Sick fish also exhibit other behavioral signs, including staying near the surface of the water because of hypoxia, scraping the body or holding the fins close to the body because of parasite irritation, or showing various behavioral abnormalities because of nervous system involvement. Increased ventilation, as shown by broader and faster opercula opening and closing, indicate gill disease or a respiratory toxin (Noga, 2010).

2.3.1 Gross pathology

The melanin pigmentation in fish's skin is under neuroendocrine control and is affected by hormones, such as epinephrine. When fish are sick, maintenance of a normal pigmentation pattern presumably takes less precedence than homeostasis of more vital body functions. Thus, sick fish are often abnormally colored, compared with the healthier cohort (Noga, 2010). Local tissue irritation/damage, such as parasite feeding, chronic wounds, or healing wounds, can also induce focal color change which causes a change in the pigment cell distribution at that site. Reddening of the body is usually caused by hemorrhage, which results from systemic bacterial or viral infections. Skin thickening can also be caused by parasites or other Parasites or other irritating conditions, leading to whitish or bluish skin color (Wise *et al*., 2004). This change might be highly localized (pinpoint to larger foci) or cover nearly the entire body. Loss of fin tissue, resulting in eroded or irregular fins, most often results from poor water quality. Masses on the body may be due to parasite cysts or neoplasia (Agius and Roberts, 2003).

Abdominal swelling is most commonly caused by infectious peritonitis or by viral, bacterial, or parasitic but can also be caused by a metabolic disturbance like renal failure, neoplasia, obesity, or egg retention. It may also be a normal sign of sexual maturity in female fish that are ready to spawn. Chronically ill fish are often emaciated. This is evident by the loss of dorsal or back muscle, a concave abdomen, and enophthalmos. Eye lesions, such as exophthalmos are common in several

infectious diseases, including several viral and bacterial infections. Unilateral eye lesions often indicate a possible traumatic cause, especially in large fish. Many nutritional deficiencies are also associated with ocular pathology. Skeletal deformities especially of the vertebral column can have many causes, including hereditary factors, defective embryonic development, unsuitable water temperature, salinity fluctuation, environmental hypoxia, x - irradiation, ultraviolet radiation, ascorbate deficiency, parasitic infection, electric current, and certain toxins (Noga, 2010).

2.3.2 Histopathological methods

The microscopic examination of thin stained tissue sections is prepared to study their structural alterations in the case of histopathology, to find out changes that may be due to pathogens and disease (Mallik *et al*., 2015).

2.3.3 Bacteriological methods

Diagnostic tests facilitate the rapid identification of bacterial pathogens isolated from fish and their environment. Bacteriological diagnostic techniques in fish diseases include visual examination is the quickest diagnostic method for gross physical examination. But the symptoms associated with infections are common to many bacterial diseases that affect fish health. The microscopic examination involves the preparation of a smear from an affected area and placing it on a clean slide using a light microscope for examining the presence of bacterial pathogens. Bacterial culture and the traditional biochemical tests are time consuming (Baird *et al*., 2003).

Phenotypic test identification of many fish pathogenic bacteria can be carried out using various phenotypic tests. Polyclonal and monoclonal antibodies in a variety of formats are used for the rapid detection of an infectious agent. When fish are immunized with bacterial pathogens, they respond by producing antibodies to all antigens of the pathogens. Other methods are Latex Agglutination Test, Fluorescent Antibody Technique (FAT), Enzyme-Linked Immunosorbent Assay (ELISA),

Polymerase chain reaction (PCR), Nested PCR, Reverse transcriptase polymerase chain reaction (RT-PCR), Multiplex PCR (Mallik *et al*., 2015).

2.3.4 Molecular methods

Molecular techniques are potentially faster and more sensitive than culture, serology, and histology methods that are traditionally used to identify fish pathogens. Molecular techniques used to solve problems and increase the sensitivity and specificity of pathogen detection. Molecular approaches have been increasingly used to diagnose fish diseases over the last 15 years or so. Polymerase chain reaction (PCR), restriction enzyme digestion, probe hybridization, in situ hybridization, and microarray are examples of these techniques. Pathogens can be recognized in asymptomatic fish using molecular diagnostic techniques, allowing disease outbreak could be prevented. Thus antibiotic treatment can be reduced so that creation of antibiotic resistant bacteria may be eliminated (Altinok and Kurt, 2003).

2.4 Fish and Fishery in Ethiopia

Ethiopia has an estimated live water body of 7,334 km^2 of major lakes and reservoirs, and 275 km^2 of small water bodies, with 7,185 km of rivers within the country. The inland water body of Ethiopia is estimated to encompass about 7,400 km^2 of lakes and reservoirs and about 7,000 km of rivers (Cochrane *et al*., 2009). The country has only inland freshwater fisheries. It comprises Rift Valley lakes (such as lakes Chamo, Abaya and Batu), Lake Tana, Lake Hashenge, Baro and Tekeze Rivers. There is fishing in all these water bodies, but commercializing it is mainly concentrated around lakes such as Chamo, Batu (Southern Ethiopia) and Tana (North-Western Ethiopia). Hence, the existing role of a fishery is insignificant in the country's overall economy because the fishery sector in the country is far below its potential (Felegeselam, 2003). There are many rivers and lakes available in Ethiopia which used for fish production, but there is still a problem regarding fish production and productivity to increase the profit of private and GDP of the country (Kebede *et al*., 2017).

There are 180 different species of fish in Ethiopia and 30 of those are native to the country (FAO, 2003). The main commercial species contributing to the total landing are *Oreochromis niloticus, Labeo hori, Clarias gariepinus, Barbus species and Lates niloticus*. The main species are *Nile tilapia,* representing 60% of the catch. Besides, its captured fishery's importance, tilapia is one of the most important species for 21-century aquaculture stand is produced in more than 100 countries (FAO, 2012).

There are seven indigenous fish species in Lake Batu comprising *Barbus paludinosus, Garra dembecha, G. makiensis, Labeobarbus ethiopicus, L. intermedius, L. microterolepis, and Oreochromis niloticus.* Of these, *L. ethiopicus, G. makiensis, and L. microterolepis* were reported as endemic to the lake. The lake also harbors five exotic fish species (*Tilapia zillii, Cyprinus carpio, Carassius carassius, and Carassius auratus*) and *Clarias gariepinus* (Abera *et al*., 2018). And also the main fish species in lake Langano include *Barbus species, Clarias species, and Oreochronis niloticus*. Therefore, the lakes have several important fish species for fisheries (Kebede and Habtamu, 2016).

A parasitic, bacterial, protozoan and viral diseases of fish have great economic and public health importance particularly in the tropics. The disease is recognized as one of the most serious threats to the commercial success of aquaculture and a serious problem causing economic losses in the fishing industry and aquaculture (Wood and Dixon, 2002).

3. MATERIALS AND METHODS

3.1 Study Area

The study was conducted in Batu, Langano and Babogaya lakes and ponds in Bishoftu. Batu lake is located in Central Ethiopia in Oromia Regional State to the Eastern side of Batu town 163 km Southeast of the capital Addis Ababa. It lies in the Northern part of the rift valley between 7°51'N to 8°7'N and 38'43' E 38°57' E with an open water area of 422 km^2 and shoreline length of 137 km. The lake is fed by two major rivers, Ketar and Meki river and has one outflow in the South, Bulbula river which flows into Abiyata Lake. The lake has several inhabited islands including Tulu Gudo, Tsedecha, Funduro, Debresina, and Galila (Anonymous, 1999). Batu Lake is one of the Ethiopian lakes with an indigenous fishing population known for its aquaculture practice with fish ponds managed mainly by local communities. The major fish species in the lake include *Nile tilapia*, *Tilapia zilli*, *Catfish*, *Barbus* and *Carp* species (Yimer, 2000). There are many landing points around the lake from where fish is collected either by boat or trucks and brought to the major landing points adjoining Batu town (Kebede and Habtamu, 2016).

Lake Langanoo is located 200 km South of Addis Ababa lying between 7°36' N;38°45' E. It is 18 km long and 16 km wide with an open water area of 230 km^2, 7.5 km shoreline and 1600 km^2 catchments area. The main fish species in the lake include *Barbus* species, *Clarias* species and *Oreochronis niloticus* with the total annual catch of 1000 tones (LFDP, 1993).

Bishoftu town is located in the East Shewa zone at 47 km South of Addis Ababa. The town is located at 8°45′N longitude and 38°59′E latitude (Birhanu *et al*., 2017). Lake Babogaya is the volcanic Crater Lake found in the area of Bishoftu town. The Lake is a small, roughly circular and fairly deep lake found at an altitude of 1870 m and about 9°N latitude and 39°E longitude (Lemma, 2012).

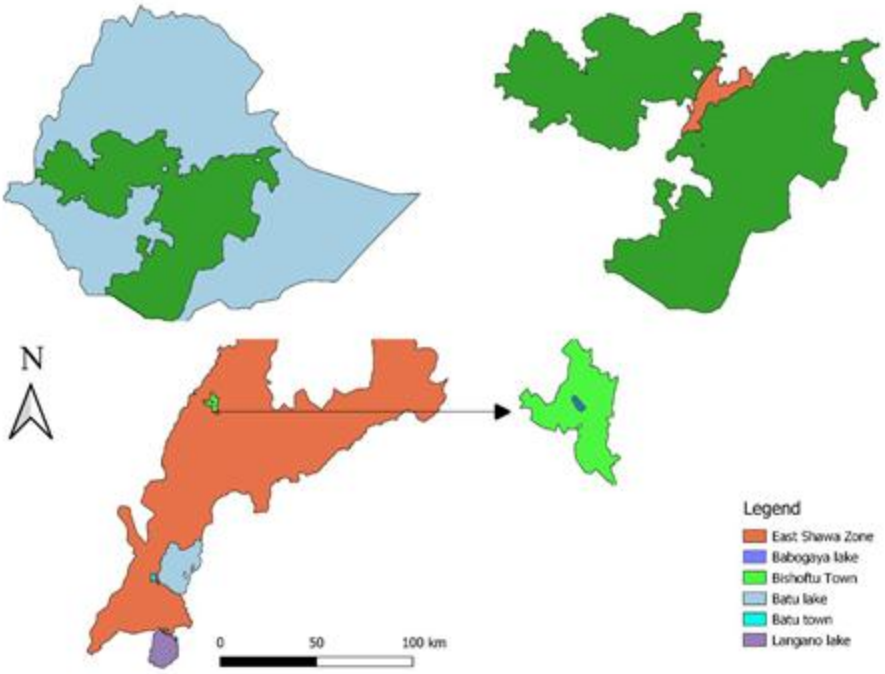

Figure : Map of study areas

3.2 Study Population

The study populations were different fish species from the selected lakes and ponds. Fish species harvested from study lakes and ponds were *Nile tilapia, Common carp, Crusian carp, and Catfish*. Beacause *Nile tilapia* is highly populated species in the study areas, it was the main part of the catch. They were physically examined for their species, sex and the data was recorded accordingly.

3.3 Study Design

A cross-sectional study design with purposive sampling of fishes showing any clinical signs was conducted from December 2020 to May 2021. Fishes showing clinical features were selected, physically visualized and palpated to characterize gross lesions.

3.4 Methods of sampling and sample processing

3.4.1 Gross examination and sample collection

During harvesting from lakes, fishes were examined for any abnormal signs. Fish with clinical signs were safely transported to the nearby laboratory in container with water. On arrival in laboratory, the fishes were put on a clean postmortem examination table and inspected for any visible gross lesions externally specifically lesions in gill and skin. The eye was covered with a towel, and euthanized humanely by decapitation, and followed by rapid destruction of the brain by pithing.

All necropsy and tissue sampling procedures were carried out under aseptic conditions with aseptic surgical instruments. An incision was made along the midline of the abdomen from the anus to the mouth. Another dissection was made from the anus to the lateral line which continued up to the gills cover to expose the internal organs (Kebede and Habtamu, 2016). The internal parts specifically muscle, liver, spleen, heart, and kidney of fish were visually observed for their consistency, texture and the findings were recorded. Then tissue samples were collected from skin, gill, muscle, liver, spleen, heart, and kidney in containers with 10 % neutral buffered formalin for histopathology and containers with sterile normal saline for *E.tarda* isolation (Appendix 1).

3.4.2 Sample transportation

The bottles containing the sample were kept in icebox with icepack until transported to Addis Ababa University College of Veterinary Medicine microbiology laboratory where bacterial isolation was conducted. Histopathologic work was done at National Animal Health Diagnosis Center (NAHDIC) pathology laboratory.

3.4.3 Isolation of Edwardsiella tarda

Tissue samples from skin, gill, muscle, liver, heart, spleen and kidney of freshly killed fish were homogenized in sterile physiological saline. Then the homogenate taken by sterile pipette was dropped on tryptone soya broth and incubated at 37°C for 24 hours. The growth of bacteria was checked for turbidity and cloudy formation in the broth. From broth with turbid and cloudy appearance, a loopfull sample was streaked on xylose lysine deoxycholate agar (XLD) plate and incubated at 37°C for 24 hours. Colonies were characterized for morphology and those with morphological characteristics of *E.tarda* were further subcultured again on XLD plate and incubated at 37°C for 24 hours to get pure colonies. From the resulting pure culture, primary identification was done based on gram staining, motility, catalase and oxidase tests. Gram-negative, motile, short rods, catalase-positive and oxidase-negative were considered for further tests to confirm whether the colonies belong to *E. tarda*. Motility test was conducted using Sulfur Indole and Motility Test Media (SIM) by inoculating fresh colony into the medium and incubated at 37°C for 24 h. Outgrowth of bacteria from the stub line was seen (Zhou *et al*., 2016).

Secondary identification of isolates was conducted by biochemical tests to check the ability of the bacterium to utilize three sugars using Triple sugar iron agar (TSI), use of citrate as a source of carbon for metabolism, an amino acid, or an alcohol source in the medium and production of byproducts detected using appropriate indicators incorporated into the medium (Adanech and Temesgen, 2018) as showed in Appendix 2.

3.4.4 Molecular identification of Edwardsiella tarda

After biochemical confirmation, the isolates were preserved by tryptone soya broth with and without paraffin oil for further molecular identification. The PCR was done at National Veterinary Institute (NVI) molecular laboratory. *Edwardsiella tarda* species-specific primers were used. Briefly the genomic DNA from 17 isolates was extracted following the manufacturer's suggested protocols using the DNA isolation

kit which includes Taq polymerase, QIAGEN protease, AL buffer, AW1 buffer, AW2 buffer, alcohol and 1.5 ml microcentrifuge tube for extraction. Including the extraction control, 18 QIAamp spin column extractions were prepared according to the procedure explained in (Appendix 3). Next to DNA extraction master mix was prepared for molecular analysis using 3 µl of Nuclease free water, 2µl of *Edwardsiella tarda* forward (ETF) and *Edwardsiella tarda* reverse (ETR) from 20pmol of each primer ETF 5'CAGTGATAAAAAGGG GTGGA-3'and ETR 5'CTACACAGCAACGACAACG 3', 10µl of IQ super mix and 3µl of DNA template given a total of 20µl for one reaction and had a total of 500µl master mix for 25 reactions as the procedure described in (Appendix 4).

After the above two processes the PCR was optimized for scientifically studied thermal conditions of *Edwardsiella tarda* for amplification of the genome by varying temperature and exposure time; 95°C for 5 min of initial denaturation followed by 35 cycles of 95°C for 15 s of denaturation, 58°C for 15 s of Annealing, and 72°C for 15 s of elongation with a final extension step of 72°C for 5 min (Appendix 5). Agarose gel was prepared at 1.5% for observation of the amplified DNA. Four (4) µl of gel red was added with loading dye, PCR product and markers or ladder. Aliquots of each amplification reaction (5 µl) were electrophoresed through agarose gel by using a ladder having >1000 bp each ladder increase by 100 bp from the other, and visualized under ultraviolet light for the presence of the appropriately sized bands (ET: 114 bp) (Appendix 6) (Griffin *et al*., 2014).

3.4.5 Tissue processing and histopathology techniques

Paraffin method tissue processing and histological slide preparation of fish tissues were conducted according to the procedures given by Paul (2017). Briefly, tissues were trimmed and put into aplastic labeled cassettes and. processed using an automatic tissue processor. In a processor, the tissues were dehydrated by ascending concentrations of alcohol (70%, 80%, 95%, and 100%), were cleared by xylene and impregnated by molten paraffin. Tissue block was made and from each tissue block, 5µm sections were prepared using a microtome. The tissue ribbons were made to be

floated on a warm water bath at 45°c and the relaxed ribbons were collected on microscopic slides. The tissues ribbons were dewaxed by heat and xylene, hydrated in descending grades of alcohols (100%, 95%, and 70%), and finally stained by Haematoxylin and Eosin (H&E). The stained slides were dehydrated in ascending grades of alcohol (70%, 80%, 95%, and 100%), cleared by xylene and mounted using DPX (Dibutylphthalate Polystyrene Xylene) and examined under a microscope.

3.5 Data analysis

The data generated from field and laboratory investigations were recorded, screened and coded using a Microsoft Excel spreadsheet. The isolation and PCR results were analyzed using statistics such as frequencies and percentages. The association between the dependent variable (*Edwardsiella tarda* positivity on PCR) and independent variables (sex, fish species, and selected lakes) were analyzed by chi-square using R software. Descriptive statistics of median was used for characterization of lesions.

3.6 Ethical consideration

An ethical certificate with a Reference number of: VM/ERC/32/06/13/2021 was received from Animal Research Ethics and Review Committee of Addis Ababa University College of Veterinary Medicine and Agriculture (Appendix 8).

4. RESULTS

4.1 Gross lesion description

Figure : Gross lesions on different body parts of fishes

Clinically diseased fish were grossly characterized by red operculum (fig.3 A), swelling, congested vent and bleeding of the anus (blue arrow in fig.3 B), hemorrhage on ventral parts of body and swollen abdomen (black arrow in fig.3 B), tumefaction of eye (white arrow in fig.3 C), white spot on muscle (black fig.3 D) and pale gills (fig.3 E).

4.2 Bacteriological isolation of *E.tarda*

Samples comprising pulled skin, gill, muscle, kidney, spleen, heart, and liver were collected from 60 clinically diseased fishes. The samples were processed for *Edwardsiella tarda* isolation. The isolates appeared as small, whitish colonies with black center on Xylose Lysine Deoxycholate agar after 24 hrs of incubation at 37°C (fig.4).

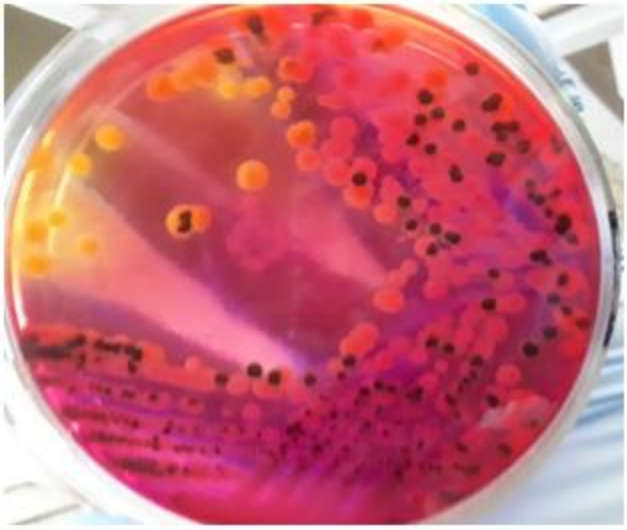

Figure : Whitish colonies with black center on XLD agar

Edwardsiella tarda suspected isolates were undertaken for further biochemical tests. Out of 60 samples collected from fish; samples showed characteristics of *Edwardsiella tarda* represented 28.3% (n=17) which were gram-negative short rods, motile, catalase-positive. In biochemical tests, isolates showed various characteristics in different tests as described in Table 1 (Appendix 2).

Table : Biochemical characteristics of the isolates

Biochemical Reaction	Results	Remarks
TSI	+	
Indole	+	
Lysine	+	
Methyl red(MR)	+	
Citrate(SCA)	_	Four isolates +
Voges Proskauer(VP)	_	
Motility	+	

4.3 Conventional PCR result of *E. tarda*

The electrophoresed result was read by ultra-violate light connected by the computer then *E.tarda* rest at 114bp (fig.5).

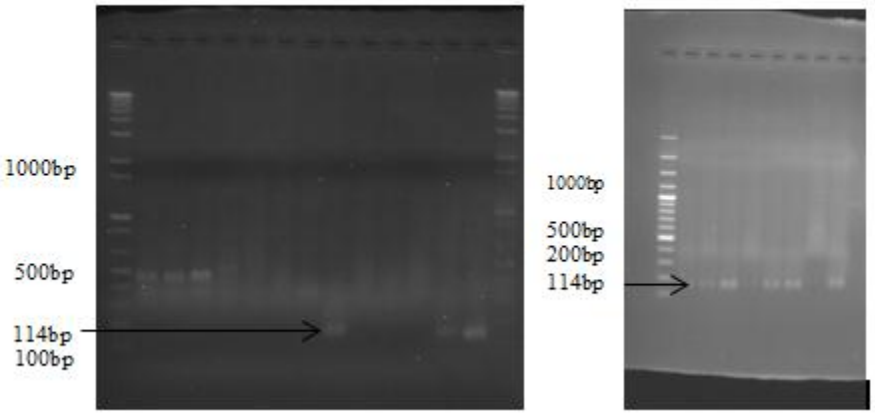

Figure : PCR result of *Edwardsiella tarda* at 114bp by conventional PCR

Out of 17 biochemically isolated samples, 88.2% (n=15) were deemed as *Edwardsiella tarda* positive by PCR which represented 25% of the total 60 samples.

About 34.6% (n=9) females and 17.65% (n=6) males were positive for *E.tarda*. Four species of fishes incorporated in the study from that about 78.3% (n=47) were *tilapia,* 6.7% (n=4) *crusian carp,* 6.7% (n=4) *common carp* and 8.3% (n=5) *catfishes.* There was a statistically significant difference (P<0.05) seen in the frequency of identifying *E.tarda* between the three study areas and four fish species. But, there was no statistically significant difference between the sex groups (P>0.05) as indicated in Table 2.

Table : Chi-square analysis of PCR results on the association of *E.tarda* with risk factors

Variables	Total	*E.tarda* positive	*E.tarda* Negative	X-squared	qchisq(0.95,1) or table value	P-value
Sex				1.448	3.841459	0.2289
Female	26	9	17			
Male	34	6	28			
Fish species				15.96	3.841459	0.001155
Catfish	5	4	1			
Crusian carp	4	3	1			
Common Carp	4	1	3			
Tilapia	47	7	40			
Study area				14.96	3.841459	0.000561
Batu	17	10	7			
Babogaya lake & ponds in Bishofitu	33	3	30			
Langano	10	2	8			

4.4 Microscopic lesions in *E.tarda* positive fishes

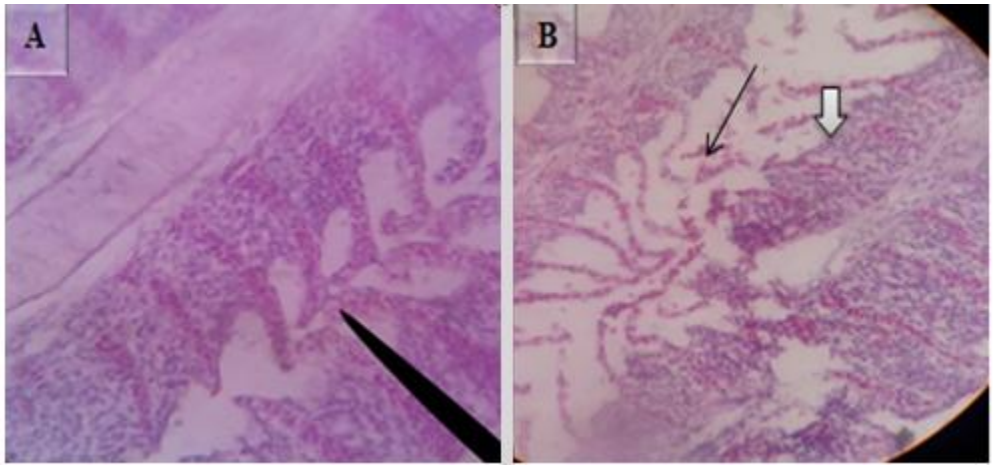

Figure : Microscopic lesions in gill of *E. tarda* affected fishes

Histopathologically, gills of *Edwardsiella tarda* positive fishes were characterized by hyperplasia of gill lamellae (fig.6 A), focal necrosis, and swelling of the lamellar epithelium (black arrow in fig.6 B) and (White arrow in fig.6 B). H&E, 100X (A) & 40X (B).

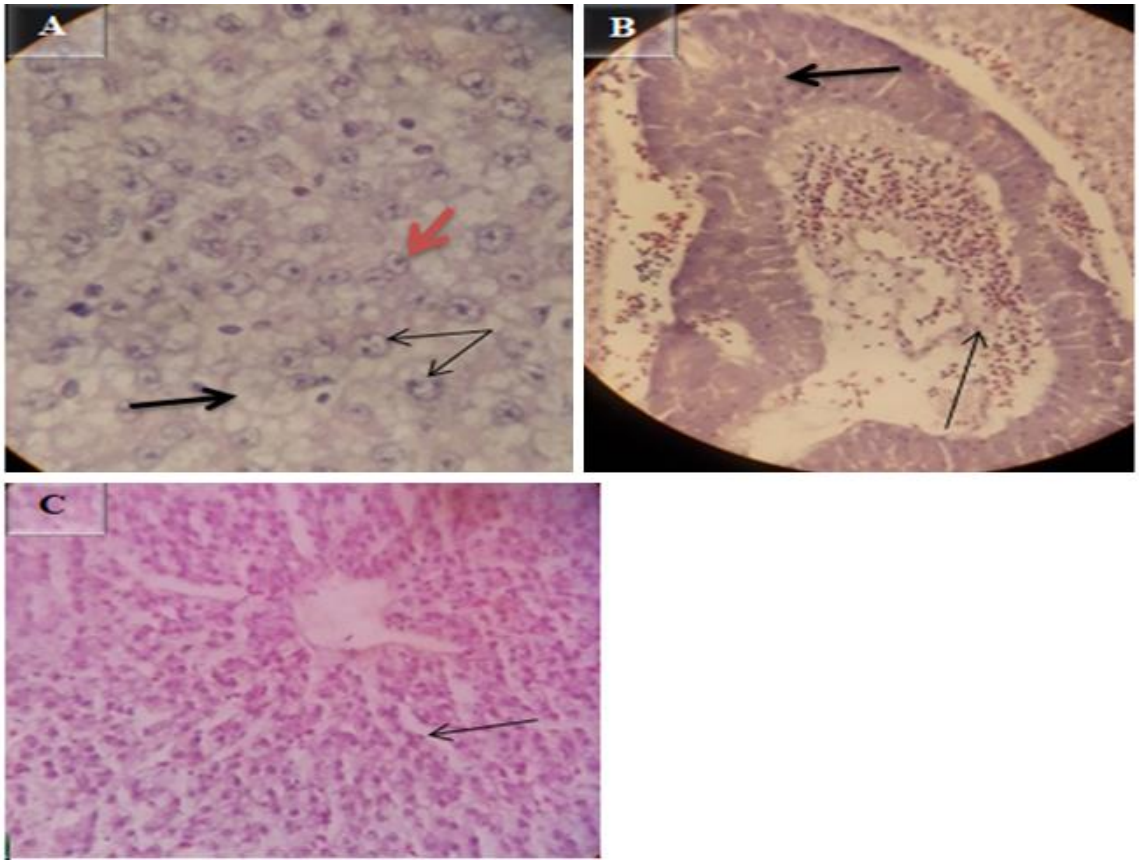

Figure : Microscopic lesions in liver of *E. tarda* affected fishes

The microscopic lesion in the liver of *Edwardsiella tarda* confirmed fish includes necrobiotic changes such as peracinary severe zonal hepatic necrosis with many hepatocytes with nuclear pyknosis or total nuclear loss (orange arrow in fig.7 A), and dilation of hepatic sinusoids (fig.7 C), swollen hepatocytes with peripheral pulled nucleus (double arrow in fig.7 A), hepatocytic cytoplasm vacuolation (thick black arrow in fig.7 A), Examination of blood vessel revealed severe hyperplasia of the endothelium (thick arrow in fig.7 B), and hyperemia and elastic fibers in the intima of hepatic artery with many layers of fibers in the arterial walls (thin arrow in fig.7 B). H&E at magnification power of 40X.

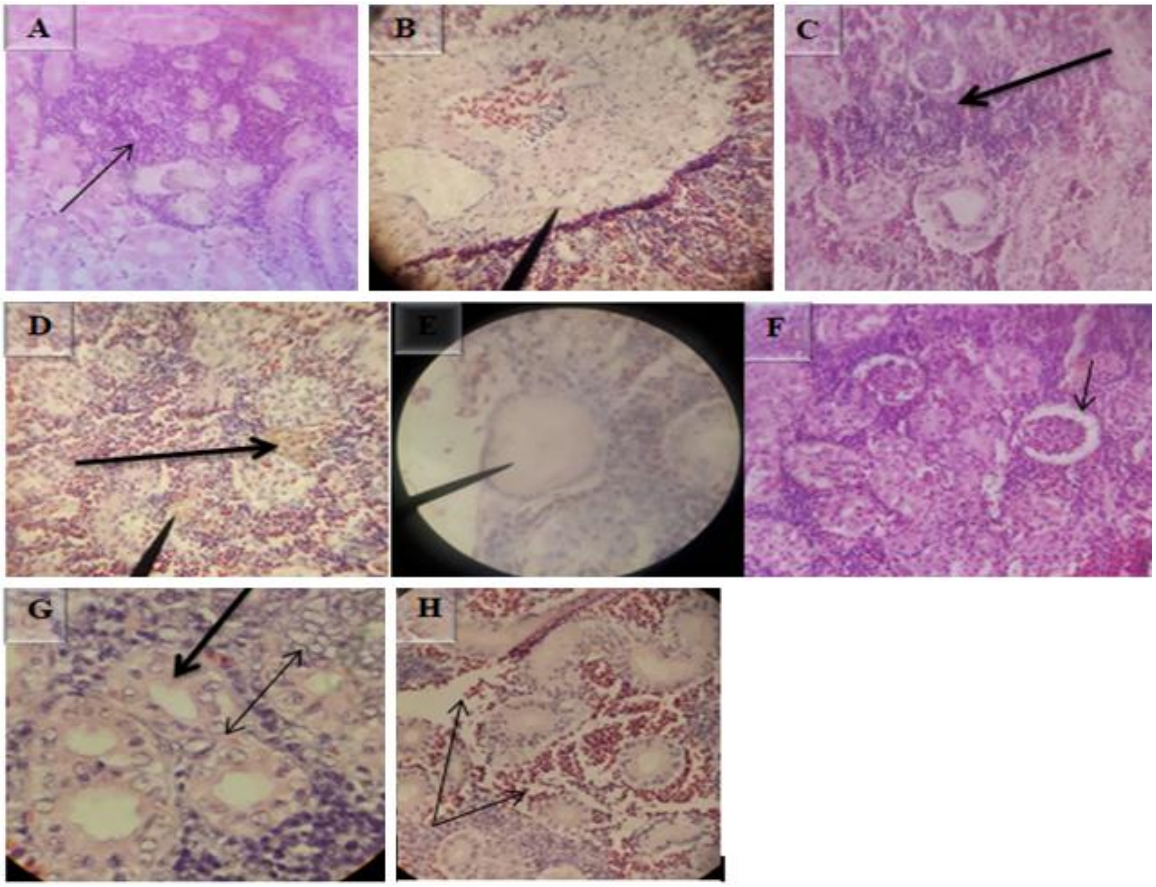

Figure : Microscopic lesions in kidney of *E. tarda* affected fishes

Histopathological examination of *Edwardsiella tarda* positive kidney revealed suppurative interstitial nephritis (fig.8 A), necrosis with fibrosis of the interstitium (fig.8 B), interstitial hemosiderin deposition (fig.8 D), and edema (fig.8 E). Melano-macrophage deposition in the kidney interstitium (fig.8 C), dilation of bowman's space due to loss of the epithelium (fig.8 F), hypertrophy of renal tubules (thick arrow in fig 8 G), tubular cytoplasmic degeneration with vacuolation (double arrow in fig.8 G) and congestion of renal blood vessels with multi-focal hemorrhages (fig.8 H). H&E,10X (A),40X (C, D, F & H) and 100X (B, E & G).

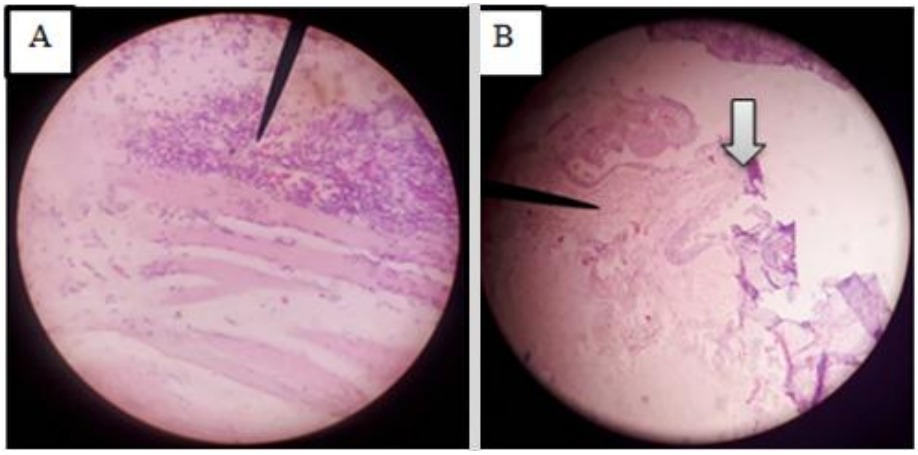

Figure : Microscopic lesions in muscle and skin of *E.tarda* affected fishes

Microscopic lesions in muscle of *Edwardsiella tarda* positive fish revealed lymphocyte infiltration (fig. 9 A) and lesions in skin include severe dermatitis with infiltration of inflammatory cells into epidermis, focal areas of necrosis in epidermal layer and necrosis of the epidermal layer (fig.9 B). H&E (40X).

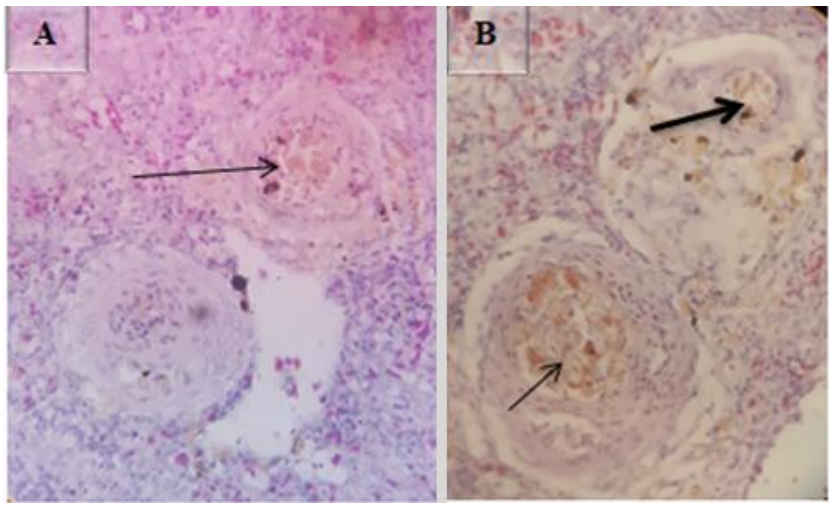

Figure : Microscopic lesions in spleen of *E. tarda* affected fishes

Microscopic lesions in the spleen include excessive accumulation of hemosiderin pigments around periarterial sheath (thin arrow in fig.10 A & B) and necrotic depletion of the lymphoid tissue in the spleen (thick arrow in fig.10 B). H&E (40X).

4.5 Microscopic lesions in *E. tarda* negative fishes

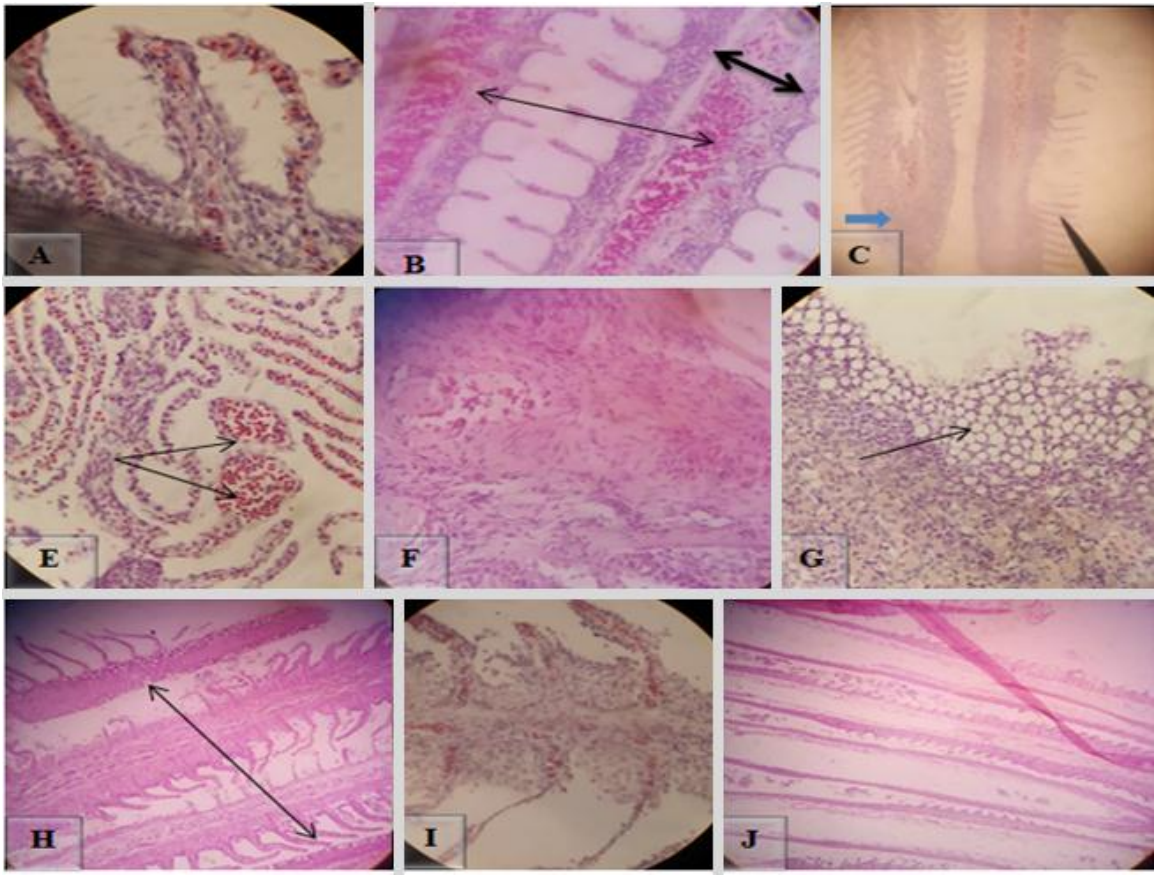

Figure : Photomicrograph showing microscopic alterations in gill

Microscopic changes observed in gill were lamellae with dilated marginal channel and dilation of central venous with blood congestion (fig.11 A and B), lamellar cell hyperplasia (blue arrow in fig.11 C), telangiectasis (fig.11 E), lamellar epithelial necrosis, and lifting (black arrow in fig.11 C). The most frequent microscopic changes in gill were fibrosis in lamellar epithelium, epithelial degeneration with vacuolation, necrosis with severe inflammatory cell infiltration of lamellar epithelium

and total necrosis of the gill structure (fig.11 F, G, H, I and J). H&E, 100X (A, B & F) and 40X (C, E, G, H, I & J).

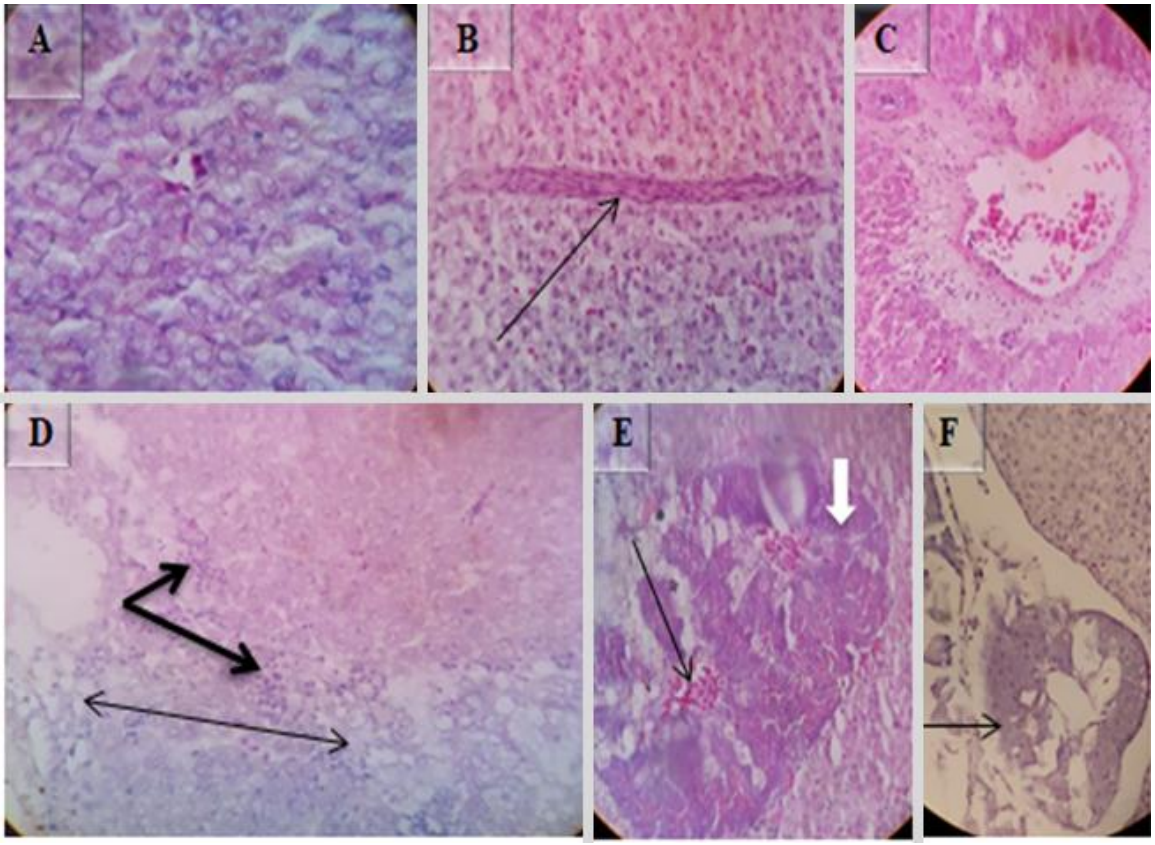

Figure : Photomicrograph showing microscopic alterations in liver

Microscopic examination of liver exhibited centrilobular hepatic necrosis (fig.12 A), congestion of sinusoid (fig.12 B), periportal hepato-necrosis with necrotized hepatocytes around the portal area with infiltration of inflammatory cells (fig.12 C), periportal hepatitis, and infiltration of inflammatory cells (thick arrow in fig.12 D), multifocal hepato-necrosis with vacuolation (thin arrow in fig.12 D). Microscopic lesions observed in bile duct were biliary duct hyperplasia (white arrow in fig.12 E), infiltration of inflammatory cells into sub-epithelial lamina (black arrow in fig.12 E), and in some cases loss of bile duct epithelium (fig 12 F). H&E, 40X (A, C,E &F) and 10X (B & D).

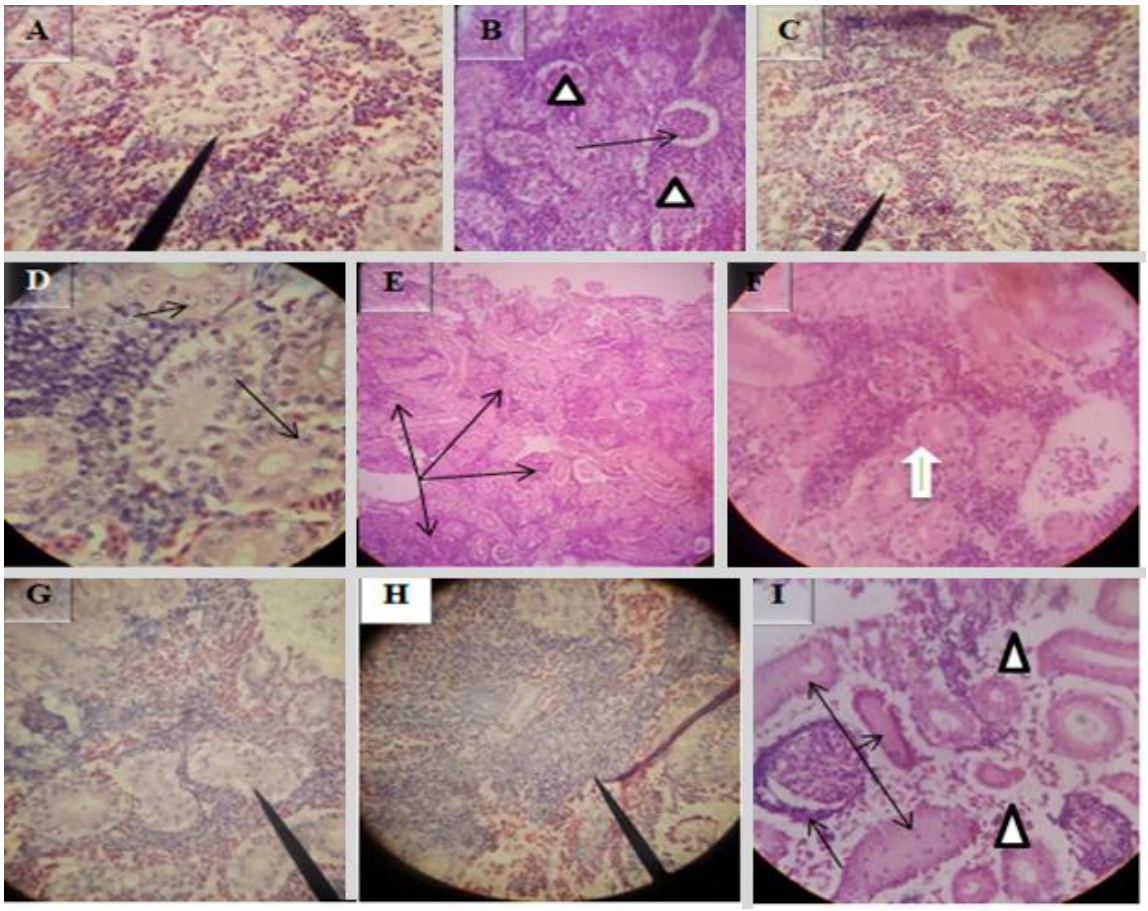

Figure : Photomicrograph showing microscopic alterations in kidney

Various microscopic abnormalities were observed in the kidneys. The most frequent changes found in the glomerulus were glomerular expansion resulting in absence of Bowman's space (fig.13 A), glomerulitis (arrowheads in fig.13 B), destruction of glomerular capillaries (arrow in fig.13 B), inflammatory cell infiltrated around glomerulus (arrow in fig.13 I). In the tubules the most frequent alterations were tubuloepithelial necrosis with epithelial nucleus loss and vacuolation (fig.13 C), hypertrophy of tubular cell nucleus (black arrow in fig.13 D), epithelial cells degeneration (white arrow in fig.13 F), the tubular epithelium lost nucleus some with pyknotic nucleus others with total loss of epithelium (fig.13 G), degeneration of epithelium and the damaged epithelium form hyaline casts in the tubules (three arrows in fig.13 I). There was severe multifocal tubulointerstitial nephritis with inflammatory cells infiltration (arrows in fig.13 E), the interstitial space infiltrated by

inflammatory cells (fig.13 H), total loss of kidney interstitium (arrowheads in fig.13 I), H&E, 100X (D), 40X (A, B, C, F, G, H & I) and 10X (E).

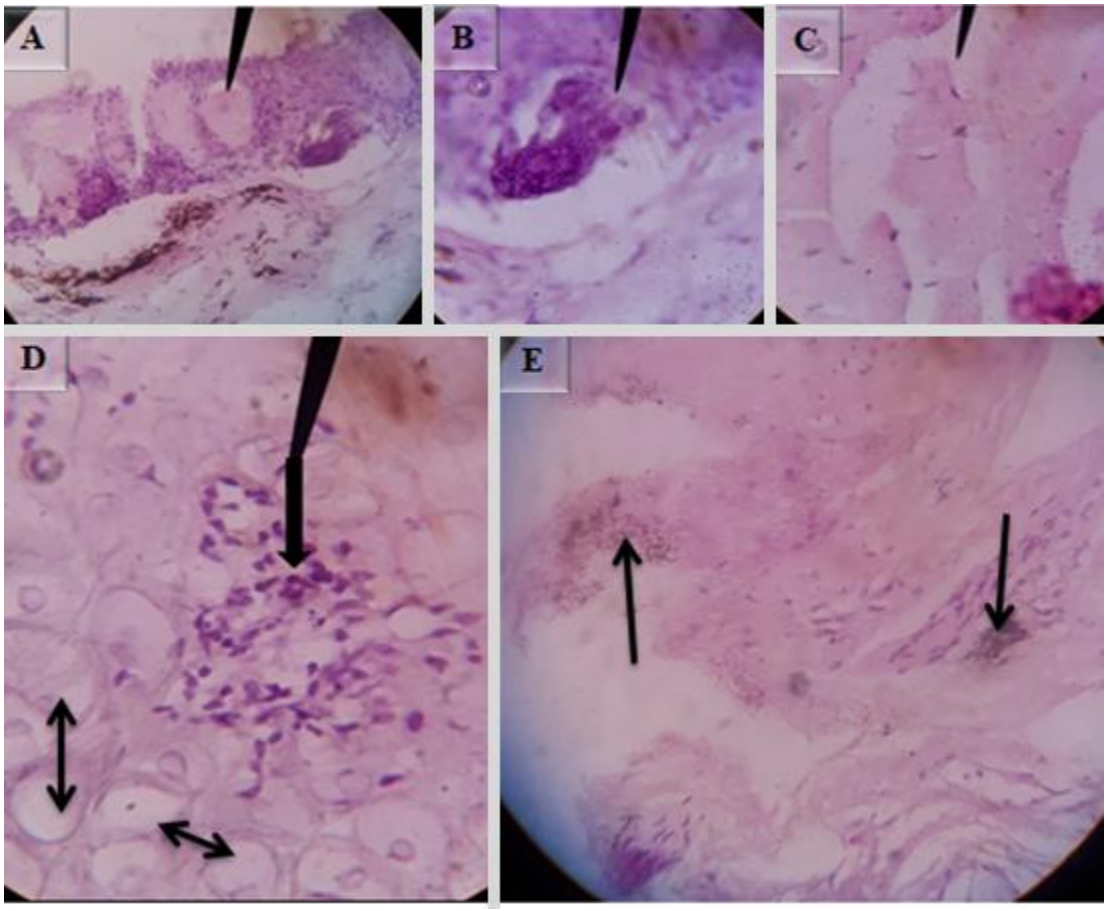

Figure : Photomicrograph showing microscopic alterations in the skin

The microscopic alterations seen in the skin were hyperplastic superficial mucus gland with mucus accumulation (fig.14 A), dermal edema (fig.14 C), focal inflammatory nodules in the dermis (single arrow in fig.14 D), dermal cell degeneration, and vacuolation (double arrows in fig.14 D), melanin deposition in the dermal region (fig.14 E). In some cases cross sectional parasitic cut was observed in the epidermis (fig.14 B). H&E, 100X (B, C , D & E) and 40X (A).

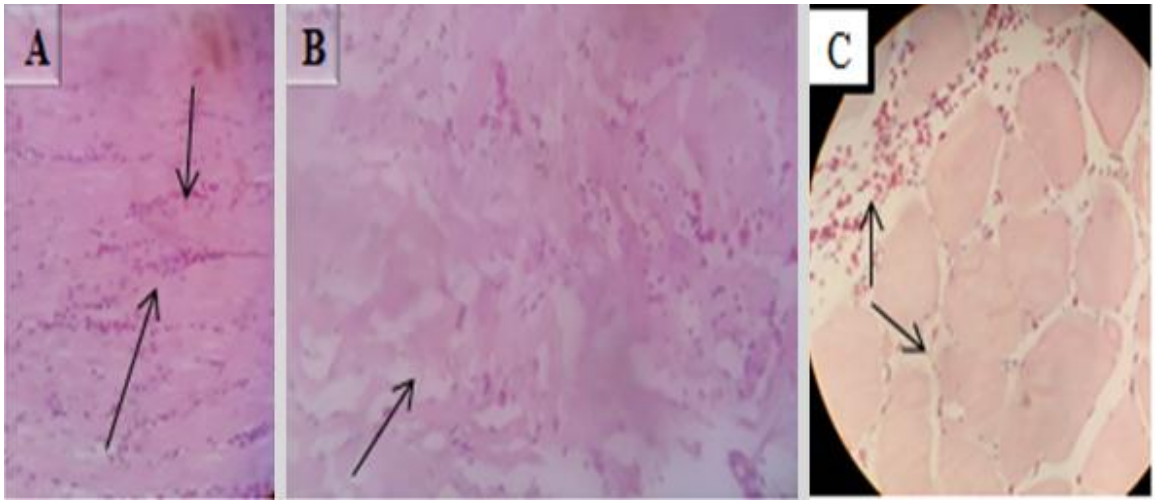

Figure : Photomicrograph showing microscopic alterations of muscle

Muscle showed inflammatory myositis with inflammatory cells infiltrated into the endomysium (fig.15 A), capillaries in endomysium were dilated and full of RBC's, and also there were multifocal hemorrhages (fig.15 B and C). H&E, 40X (B & C) and 10X (A).

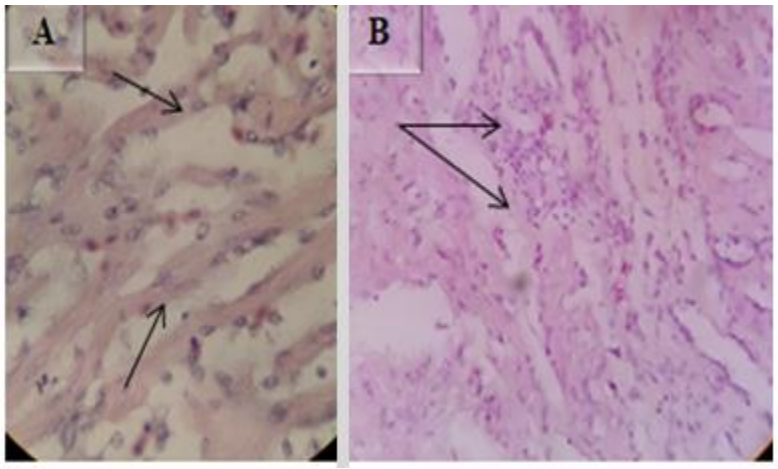

Figure : Photomicrograph showing microscopic alterations in the heart

Microscopic lesions in the cardiac muscle were severe chronic myocarditis with fibrosis (fig.16 A) and heavy infiltration of inflammatory cells (fig.16 B). H&E 40X.

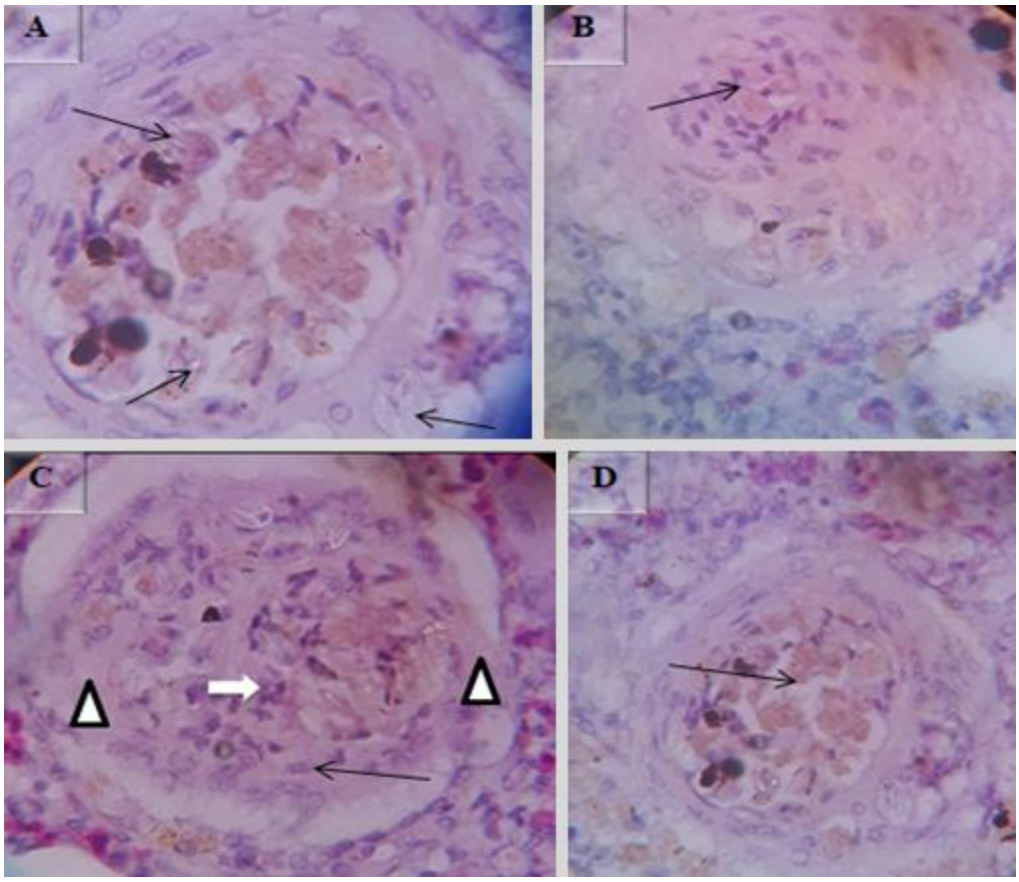

Figure : Photomicrograph showing microscopic alterations in the spleen

In some cases the spleen showed protozoan parasites with peer shaped sporocysts (fig.17 A), Surrounding these protozoan parasites there were typical multifocal granulomatous lesions (arrow in fig.17 B and white arrow in fig.17 C) with central necrosis (fig.17 D) followed by lymphocytic layers (arrowheads in fig.17 C) into which are intermingled by epitheloid macrophages (black arrow in fig.17 C). H&E (100X).

5. DISCUSSION

The fishery sector plays a significant role in food security through the supplementation of food for developing countries (Dugenci and Candan, 2003). One of the problems of the fishery sector in both natural environments and in culture is the disease which has a serious impact on fish (Wood and Dixon, 2002). In the present study the microscopic lesions in diseased fishes were characterized, *E. tarda* was bacteriologically isolated and molecularly categorized. Grossly examined lesions in this study like pale gills, tumefaction of eye and red operculum, swelling and bleeding of the anus, hemorrhage on ventral parts of body, swollen abdomen and congested vent agrees with report undertaken in South Korea by Oh *et al*. (2020) and his collaborators described: "upon visual inspection of the diseased fish, there were no significant external lesions that are commonly observed, but proptosis was evident, which is a typical clinical symptom of bacterial infection". As compared to the reports of studies conducted in India by Abraham *et al*. (2015) and Hoque *et al*. (2020) most of the gross lesions were indications of bacterial diseases.

According to Ibrahem *et al*. (2011) the gross lesions caused by edwardsiellosis from experimentally infected Nile tilapia and African catfish in Egypt were described as scale detachment and pale discoloration, severe edematous swelling at the site of injection, swollen abdomen with yellowish ascetic fluid, protruded hemorrhagic anus with opaqueness eyes. Internally, both Catfish and Nile tilapia showed severe hemorrhagic enteritis with adhesion between organs, the body cavity was filled with abundant yellowish mucoid fluid; in *catfish,* the liver showed multiple tiny white foci while the intestine contained thick white opaque mucus. The current study doesn't observed most gross lesions due to the possible reasons of variation in way and severity of infection, fish species and environmental factors.

In this study, *E. tarda* was isolated from pulled samples of skin, gill, muscle, kidney, spleen, heart, and liver of 60 diseased fishes indicating that 17 fishes (28.3 %) tested positive by colony characteristics and biochemical tests and 15 (25%) were confirmed positive through PCR test. The study showed a significant association between fish

species and *E. tarda* which is contrary to the previous report by Kebede and Habtamu (2016) it may be due to the small sample size used in the recent study which may not be sufficient enough to show such differences. But there was no significant differences in the frequency of identifying *E. tarda* between sex groups which agree with the earlier studies by Kebede and Habtamu (2016), Adanech and Temesgen (2018) and also support the agreement with other works were both sex are equal chance of being infected by *E.tarda* (Yu *et al*., 2004; Savan *et al*., 2005).

Tissues of *E. tarda* confirmed fishes were also further processed for microscopic lesion characterization. The lesions in gill were agreed with the previous findings by Darwish *et al*. (2000) and Abraham *et al*. (2015) who studied experimental infection with *Edwardsiella tarda* in channel catfish and pathology of *Edwardsiella tarda* infection in African catfish, *Clarias gariepinus* revealed necrosis, hyperplasia, and swelling of lamellar epithelium. These changes could reduce the surface area for effective respiration, which severely stresses fish, or can even lead to death from lack of oxygen.

The findings in liver and kidney were comparable with studies reported by Miwa and Mano (2000) stated infection with *Edwardsiella tarda* causes hypertrophy of liver cells and enlargement of their nuclei in the Japanese flounder Paralichthys olivaceus, Abraham *et al*. (2015), indicated focal necrosis with lymphocyte infiltration and expansion of space between hepatic sinusoids in liver and neutrophil infiltration, hemosiderin deposition, fibrinoid necrosis of nephretic tubules, and edema in kidney of *E. tarda* infected catfish. Comparable with lesions reported by Darwish *et al*. (2000) suppurative interstitial nephritis and increased melanomacrophage deposition in interstitium were observed in the kidney and hepatitis in liver. According to Agius and Roberts (2003) melano-macrophage centers, also known as macrophage aggregates, are distinctive groupings of pigment-containing cells. It usually contains a variety of pigments, including melanin, and these increase in range and volume in older fish or the presence of cachectic disease. Melano-macrophage centers act as focal depositories for resistant intracellular bacteria.

Similar lesions were reported by Hoque *et al*. (2020) who studied on examination of lesions in *Pangasianodon hypophthalmus* with *E. tarda* revealed dilation of Bowman's space, hypertrophy, necrosis of glomerulus and tubular epithelium leading to complete necrosis and fibrosis of renal tubules leading to loss of their normal shape. Vacuolation due to degeneration of cytoplasm and different necrobiotic changes such as cellular and cloudy swelling degeneration of renal tubules in the kidney and blood congestion and necrobiotic changes such as nuclear pyknosis in liver. Accord with observations of Ibrahem *et al*. (2011) microscopic lesion in kidney showed congestion of renal blood vessels with multi-focal hemorrhages.

Edwardsiella tarda confirmed fish showed lymphocytic infiltration in muscle and severe dermatitis with infiltration of inflammatory cells into epidermis, focal areas of necrosis in epidermal layer of skin were similar with investigations reported by Ibrahem *et al*. (2011) and Abraham *et al*. (2015). Similar to Darwish *et al* (2000) stated earlier the spleen revealed an accumulation of haemosiderin pigments these occur due to massive hemolysis of the red blood cells engulfed by macrophages with the presence of hyperemia and necrotic changes.

Microscopic lesions in the gill, skin, muscle, kidney, liver, spleen and heart of *E.tarda* negative fishes were also studied in the present investigation. Microscopic changes seen in gill agreed with the previous study reported by Camargo and Martinez (2007) who studied histopathology of a Neotropical fish caged in an urban stream included dilation of the marginal channel, hyperplasia of the epithelial cells with lifting of the lamellar epithelium and blood congestion. This study is also in agreement with reports of microscopic changes in fish exposed to aluminum and lead acetate by Hadi and Alwan (2012) and Mustafa *et al*. (2017) showed lamellar epithelial necrosis and telangiectasis in the gill. The alterations not reported in other studies but frequently seen in these findings were fibrosis in lamellar epithelium, epithelial degeneration with vacuolation, and total necrosis of the gill structure.

The organ most linked with the detoxification and biotransformation process is the liver, and due to its function, position and blood supply (Van der Oost *et al*., 2003),

it is also one of the organs most affected by contaminants in the water (Rodrigues and Fanta, 1998). Microscopic lesions in liver of *E. tarda* negative fish were agreed with the report by Mustafa *et al*. (2017) who studied on fish exposed to Lead Acetate exhibited various alterations include centrilobular hepatic necrosis and multi-focal hepato-necrosis with vacuolation. Pacheco and Santos (2002) described increased vacuolization of the hepatocytes as a signal of degenerative process that suggests metabolic damage, possibly related to exposure to contaminated water.

As Kotob *et al*. (2017) stated that the necrosis in the fish liver can be caused by several factors such as bio-agents (viruses, fungi, bacteria, and parasites), and blood transport disorder that affects the blood supply to particular tissues. All of these disturbances can trigger specific damage to the cell. Congestion of sinusoid and hepatitis with infiltration of inflammatory cells were seen in the study which supported by Naeemi *et al*. (2013) and Maftuch *et al*. (2018). Periportal hepato-necrosis with necrotized hepatocytes around the portal area and infiltration with inflammatory cells, periportal hepatitis and lesions observed in bile duct were biliary duct hyperplasia, infiltration of inflammatory cells into sub-epithelial lamina and loss of bile duct epithelium were uniquely reported in the study.

The recent microscopic alterations demonstrated in the kidney were supported by the earlier reports of Camargo and Martinez (2007) and Hadi and Alwan (2012) indicated changes including glomerular expansion causing absence of Bowman's space, tubuloepithelial necrosis and cloudy swelling, hypertrophy of tubular cell nucleus and hyaline droplet degeneration of *Prochilodus lineatus* fish caged into organic and inorganic pollutants and freshwater fish *Tilapia zillii* exposed to aluminum. The tubular epithelium lost nucleus some with pyknotic nucleus others with total loss of epithelium, the interstitial space infiltrated by inflammatory cells, degeneration of epithelium. The finding is comparable with observations of Naeemi *et al*. (2013), Mustafa *et al*. (2017) and Fernandes *et al*. (2019).

The study reported by Maftuch *et al*. (2018), Koi carp due to the infection of Myxobolus sp. parasite revealed inflammatory myositis with infiltration of inflammatory cells in muscle and necrosis and hyaline degeneration in kidney was related with the current finding.

Microscopic changes seen in skin, heart, and spleen that uniquely reported in this study were hyperplastic superficial mucus gland with mucus accumulation, dermal edema, focal inflammatory nodules in the dermis, dermal cell degeneration and vacuolation, melanin deposition in the dermal region, cross sectional parasitic cut in the epidermis, severe chronic myocarditis with fibrosis and the spleen showed protozoan parasites with peer shaped sporocysts, surrounding these protozoan parasites there were typical multifocal granulomatous lesions with central necrosis followed by lymphocytic layers into which are intermingled by epitheloid macrophage. No earlier study in fish found to compare the findings.

6. CONCLUSION AND RECOMMENDATIONS

In the present investigation four species of fishes were collected from study lakes and ponds according to the clinical signs. *Edwardsiella tarda* was bacteriologically isolated, molecularly categorized and microscopic lesions were characterized from *E. tarda* positive and negative fishes. In the study, non-significant gross lesions of external and internal organs were demonstrated and the most frequent microscopic changes were fibrosis in lamellar epithelium, epithelial degeneration with vacuolation, necrosis with severe inflammatory cell infiltration of lamellar epithelium, and total necrosis in gill, periportal hepato-necrosis in liver, tubuloepithelial necrosis with epithelial nucleus loss and vacuolation and glumerulitis in kidney. Also there were microscopic lesions uniquely reported in the study.

Based on the above conclusion the following recommendations are suggested

- ✓ Further studies comprising different species of fish with a large sample size and study area should be conducted.
- ✓ Since different arrays of lesions by combined infection have been isolated works with the trial of isolating all micro-organisms and chemicals that may trigger lesion formation should be implemented.
- ✓ Characterization of lesions should be used as the fundamental tool in the diagnosis of fish diseases.
- ✓ Since *E.tarda* is important fish and human diseases attention should be given for it.

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8. APPENDICES

Appendix : Sample collection

- ✓ Fishes were examined for any external lesions
- ✓ Putted on water containing container and transported for examination
- ✓ The eye covered by towel and decapitation was performed to kill the fish
- ✓ The fish opened along the midline of the abdomen from the anus to the mouth aseptically using sterile scalpel blade with holder, scissor and forceps for further characterization of the lesions.
- ✓ Then pulled sample of skin, gill ,muscle, liver and kidney were collected in sterile container containing sterile saline for bacteriology and preserved in icebox containing icepack and samples including spleen and heart as addition were collected by container 10% neutral buffered formalin and preserved at room temperature until histopathological processing

A) during selection of diseased fishes harvested from lake Langano B) exposure of internal organs

Appendix : Isolation procedures

- ✓ The sample was homogenized by physiological saline, poured in tryptone soya broth and incubated at 37°C for 24hr

  1litre distilled water is for 56.8gm
- ✓ XLD media was dissolved in distilled water

✓ Shaked and boiled with frequent agitation to dissolve completely

✓ Don't autoclave

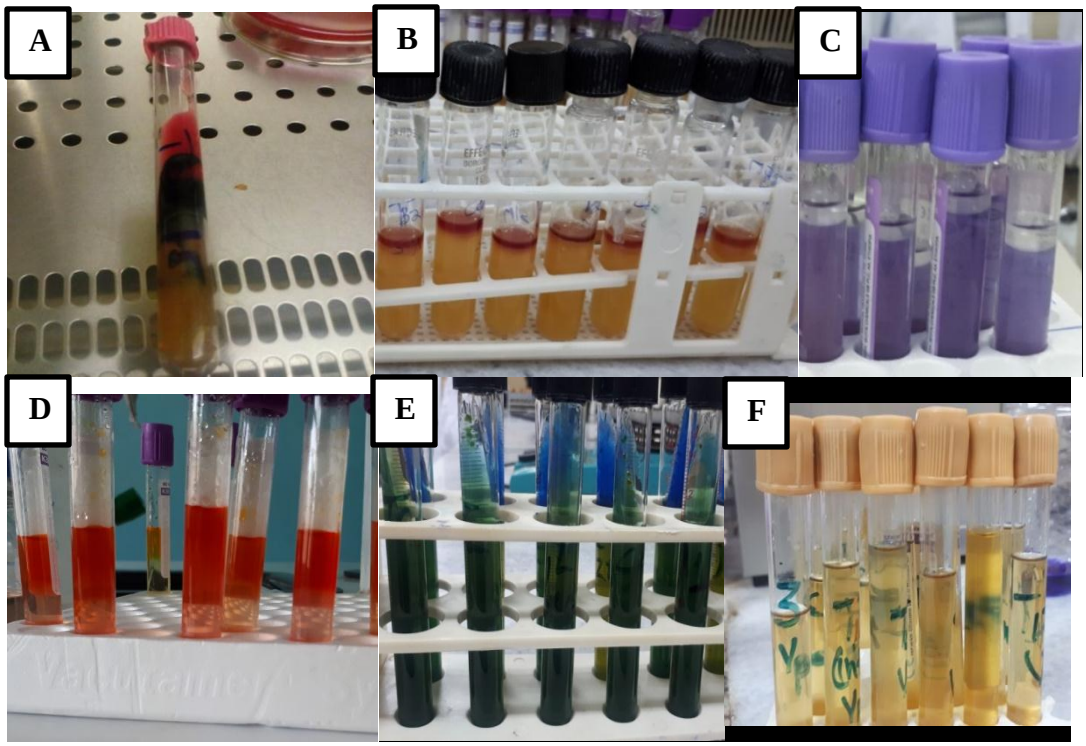
✓ Cooled and then dispensed on petri plates

✓ Bacteria striked on XLD agar and incubated at 37°C for 24hr

✓ The colony resembling the needed bacteria selected and subcultured on XLD agar at 37°C for 24hr to get the pure colony

✓ The pure colony then cultured on nutrient agar for biochemichal tests

✓ Six biochemichal tests were done (IMVIC tests including TSI and lysine decarboxylation test)



(A) K/A and H2S production on TSI (B).Red ring formation on SIM media by addition of kovac's reagent (C)Red on MR-VP media for methyl red (D)Purple on lysine decarboxylase (E) Do not utilize citrate remain greenish (F) No color change for VP test

Appendix : DNA extraction procedures

✓ Isolated bacterial sample was submitted to National Veterinary Institute molecular(NVI) laboratory with Tryptone soya broth with and without paraffin oil

✓ Refrigerated samples and reagents from the kit were brought to room temperature before starting

✓ 200µl of sample was added to microcentrifuge tube

✓ 200µl of buffer AL was added to the sample and mixed by pulse-vortex for 15sec.

✓ 20µl of QIAGEN protease was pipetted into the bottom of a 1.5ml microcentrifuge tube.

✓ Incubated at 56°C for 10min.

✓ The 1.5 ml microcentrifuge tube was centrifuged to remove drops from the inside of the lid.

✓ 200µl of 97% ethanol was added to the sample and mix again by pulse- vortexing then centrifuged to remove drops from the inside of the lid.

✓ The mixture was carefully transferred from the above step to the QIAamp spin column containing silica (in a 2ml collection tube)without wetting the rim, closed the cap and centrifuged at 8000rpmfor 1 min.

✓ The QIAamp spin column was placed in a clean 2ml collection tube and the tube containing the filtrate was discarded.

✓ The QIAamp spin column was carefully opened and 500µl of buffered AW1 was added without wetting the rim.The cap was closed and centrifuged at 8000rpm for 1min.The QIAamp spin column in a clean 2ml collection tube was placed and the collection tube containing the filtrate was discarded

✓ QIAamp spin column was opened carefully and 500µl buffer AW2 was added without wetting the rim then closed and centrifuged at full speed (14000rpm)for 3 min.

✓ As an optional step to eliminate the possible buffer AW2, to wash and clear the alcohol from the nucleic acid the QIAamp spin column was placed in a new 2ml collection tube and the filtrate was discarded. Centrifuged at full speed for 1min.

✓ QIAamp spin column was placed in a clean 1.5ml microfuge tube and the filtrate was discarded.

✓ QIAamp spin column was opened and 200µl Buffer AE was added to deattach the bond between silica and nucleic acid then incubated for 5min at room temperature and centrifuged at 8000rpm for 1min.

During DNA extraction in DNA extraction room

Appendix : Master mix preparation

✓ 3µl of nuclease free water was added for one reaction ;total of 75µl nuclease free water was added for 25 reaction.

✓ 2µl of ETF and ETR primers added for one reaction with total of 50µl ETF and 50µl ETR for 25 reaction

✓ 10µl IQ Super mix was added for areaction and 250µl for 25 reaction

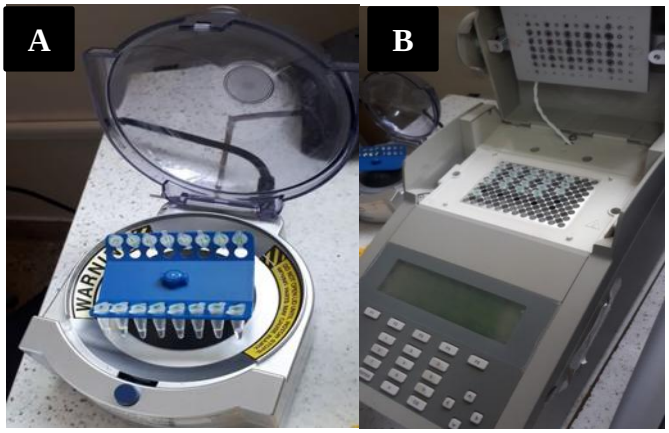
✓ 3µl of template was added for areaction with total volume of 20µl for one reaction

Appendix : DNA amplification

✓ The mixtures were centrifuged and putted on a PCR machine

✓ The PCR was optimized for initial denaturation step at 95°C for 5minutes, for 1 cycle

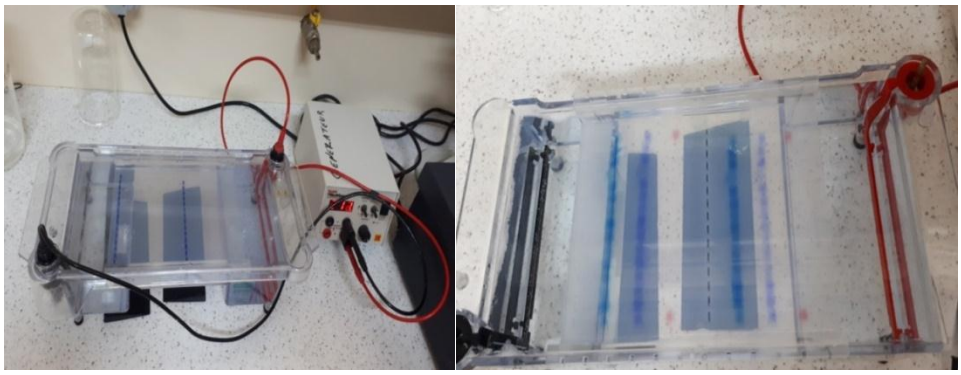
- ✓ Denaturation step at 95°C for 15seconds, Annealing at 58°C for 15sec, Elongation steps at 72°C for 15 sec ,for 35 cycles
- ✓ Final elongation at 72°C for 5minutes,for 1 cycle
- ✓ Then putted at 4°C unti the machine is off



A) Centrifugation of the mixed template with the master mix B) Preparation of PCR for amplification

Appendix : Agarose gel preparation and gel electrophoresis

- ✓ 1.5% of Agarose gel was prepared
- ✓ 4µl of Gel red with loading dye,10µl of PCR product and markers or ladder were added
- ✓ Electrophoresis was runned for 1:20 hour at 120V
- ✓ Then the result was readed by using UV-light
- ✓ It was around 114bp for *E.tarda*



Gel electrophoresis

Appendix : Histopathological procedures

1. Preparation of Formalin

- Buffered neutral formalin 10%:

  37 – 40 % Formalin ---------- 100.0 ml

  Distilled water ---------- 900.0 ml

  Sodium phosphate monobasic ---------- 4.0 gm

  Sodium phosphate dibasic (anhydrous) ------ 6.5 gm

2.Trimming tissue: Tissues samples were trimmed and put in to plastic tissue cassettes and then processed using an automatic tissue processer.

3. Fixation of tissue by 10% formaldehyde (Formalin-I for 2 hours & Formalin-II for hours).

4. Tissue processing: dehydration, clearing and impregnating

- Dehydrating tissue by using increasing strength of alcohol; e.g. 70%, 95% and 100%.

Dehydration 70% alcohol 1 hour

95% alcohol 1 hour

100% alcohol I 1 hour

100% alcohol II 2 hour

100% alcohol III 2 hours

- Clearing of tissue by Xylene

Xylene I 1.30 hours

Xylene II 1.30 hours

Xylene III…..1.30 hours

- Impregnation tissue with Paraffin wax

Removal of the clearing reagents, by substitution, as the paraffin penetrates the tissue with use of, paraffin baths. Allowed to occur at melting point temperature of paraffin wax, which is 56 – 580C or 54-60oC. Volume of wax should be about 25-30 times the volume of tissues

Paraffin wax I 2 hours

Paraffin wax II 3 hours

5. Embedding or Blocking: Tissue specimens embedded with paraffin wax named as tissue blocks were removed from the machine. **–** the orientation of tissue in melted paraffin, which when solidified provides a firm medium for keeping intact all parts of the tissue when sections are cut by microtome sharpest knife.

- ✓ impregnated tissues are placed in a mould with their labels
- ✓ then fresh melted wax is poured in it and allowed to settle and solidify
- ✓ Once the block has cooled sufficiently to form a surface skin it should be immersed in cold water to cool it rapidly**.**

6. Section: sectioning of tissue in to 4- 5 micron thickness using a semi-automatic microtome machine, tissue ribbons were spread on warm water bath and tissues were attached to albumenized glass slides. Then the slides were incubated in incubator at 60oC to avoid paraffin wax. The sectioned tissues were then deparaffinised in three changes of xylene (Xylene-I, Xylene-II & Xylene-III), and then rehydrated in descending grades of alcohol (100% I, 100% II, 100% III, 95 %, &70 %).

7. Staining: staining with Haematoxylin and Eosin to give colour for sectioned tissue.

Staining procedure:

- ✓ Put the sections fixed on slides in xylene (xylene for 5 minutes and xylene for 5 minutes.
- ✓ Then transfer to absolute alcohol (100% for 3 minutes, 100% for 3 minutes and 100% for 3 minutes).
- ✓ Transfer to 95% alcohol for 3 minutes.
- ✓ Place in 70% alcohol for 3 minutes.
- ✓ Rinse the slide in running tape water for 1 minute and put in Haematoxylin for 10-15 minutes.
- ✓ Rinse in running tape water
- ✓ Counter stain with eosin (3 dips).
- ✓ Rinse in running tape water.
- ✓ Dehydrated in ascending grades of alcohol1 (70 % 3 dips, 95 % 3 dips, 100% I for 3 minutes, 100% II for 3 minutes & 100% III for 3 minutes)
- ✓ Cleared it in xylene (Xylene-I for 5 minutes , Xylene-II for 5 minutes & XyleneIII for 5 minutes and mounted with DPX or Canada balsam.
- ✓ Microscopic examination: stained slide is examined under microscope at 4x, 10x, 40x and 100 x magnification for the presence of microscopic lesions and finally photographs of the slides were taken

Appendix : Ethical certificate

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
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Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/32/06/13/2021

Name of Applicant: **Rediet Wolde (DVM, MVSc fellow)**

Address: Department of pathology and Parasitology, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Pathological lesion characterization in diseases fish; isolation and identification of Edwardsiella tarda in selected lakes and ponds of central Ethiopia*

Date of application:	**December, 2020**
Nature of the project:	**Mildly invasive**
Target animal species:	**Fish**
Number of animals involved:	**60**
Study area:	**Central Ethiopia**

Minutes No. and date of review: **VM/ERC/06/13/021, 28/03/2021**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary

Getachew Terefe (DVM, PhD, Professor)
Chairman

Signature

Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

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