

Research Article

Histopathology of the fish infected with the epizootic ulcerative syndrome in Eastern Nepal

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Abstract

Epizootic ulcerative syndrome (EUS) is the major disease of fish which causes high mortality of fish in eastern Nepal since its initial outbreak. Of the 445 EUS infected fish samples, the suspected fungus was identified in eight spp. during the five years study period following standard methods to isolate, culture and identification. The sections of muscles, livers, and kidneys of infected fish samples showed the outbreak of EUS due to the presence of *Aphanomyces* spp. A complete loss of epidermis and the underlying musculature was replaced by granulomatous and inflammatory tissues. In some areas, myonecrosis and fungal hyphae, black stained with GMS, reflected a positivity for the presence of fungus. Pathogenicity test in *Heteropneustes fossilis* showed some lesions and 43.33% mortality when isolated zoospores were injected into experimental fish samples.

Keywords: Aseptate fungus, Fish samples, Granuloma, Hemorrhage, Zoospores

1 | Introduction

Among fish diseases, Epizootic ulcerative syndrome

(EUS) is the most lethal disease of complex nature involving certain fungi and bacteria in its later stages and probably some viruses as well (Chinabut 1995). This disease was specified as a seasonal epizootic condition of fresh water and estuarine warm water fish of multifaceted infectious etiology by the invasive *Aphanomyces* infection that later resulted in necrotizing ulcerative lesions and granulomatosis (DFID 1994). In Australia, the Fifth Symposium on Diseases in Asian Aquaculture, re-observed the causal factors known as *Aphanomyces invadans* or *A. piscida*. Besides these, parasites and Rhabdo viruses are also associated with exacting outbreaks, while secondarily gram-negative bacteria are usually allied with lesions due to EUS (FAO 2009; OIE 2013).

EUS is a disease of wild and farmed fish which initially appeared during summer months in farmed Ayu (*Plecoglossus altivelis*) in Japan in 1971 (Egusa & Masuda 1971). Miyazaki and Plumb (1985) reported that the

disease can affect the brain, liver, spleen, and kidney of infected fishes. EUS subsequently expanded to USA and Africa in 2006 (Mudenda 2012) affecting 130 species of fish in 26 countries as of now (Pradhan et al. 2018).

The socioeconomic status of the fish farmers and fish traders was badly affected by EUS in Bangladesh (Rahman et al. 1988). In Nepal, it caused a loss of about 15-20 % of total fish production during its initial outbreak in eastern Terai of Nepal, in February 1989 and afterward. Further, among the 37% of fish farms, the majority of cases (95%) were of EUS affected and national loss was about US\$ 114,000 compared to total loss (US\$ 120,000) from other fish diseases in Nepal (Phillips 1989; ADB/NACA 1991; 1995; Lilley et al. 2002). The intense rearing of fish through high stocking densities, use of artificial feed and fertilizer, and application of chemotherapeutic agents has prompted an increase in large-scale fish production. It also created physiological stress and an increased risk of a disease outbreak (Pillay 1996; McLean 1996). Further, increased people's unwillingness to turn towards aquaculture owing to the high risk of diseases- mainly EUS outbreaks and lack of knowledge about fish diseases (Callinan et al. 1999).

Some fish species like common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*), milk fish (*Chanos chanos*) (Lilley et al.1998), and European eel (*Anguilla anguilla*) (Oidtmann et al. 2008) are resistant to infection with *A. invadans*. Fish in natural habitats showcased abnormal swimming behavior with heads projected out of the water (Jhingran & Das 1990). In the primary stage of the disease, the infection generally started in the form of multiple inflammatory red spots on the body causing localized hemorrhage. In the advanced stage, the ulcers became deep, hemorrhagic, and necrotic leading to a black melanistic rim. In the final stages deep, hemorrhagic, necrotic ulcers were found in all parts of the body mainly in the head, abdomen, and peduncle of the fish. Pal and Pradhan (1990) observed a red spot on the skin of the fish without scales during the initial outbreak of EUS and ulcers developed badly underlying muscle layer later.

Since outbreaks of EUS occur in a cyclic manner when the temperature falls, especially after heavy rainfall, low alkalinity, and pH fluctuations in Asia Pacific regions (Roberts et al. 1986); more studies are required to get an accurate understanding of the role of various environmental risk factors responsible for EUS (Pradhan et al. 2014). Furthermore, no study so far has been carried out regarding the EUS of fish in Nepal. Therefore, the study aims to report EUS through

histopathological observations of muscles, livers, and kidneys of fish samples collected from eastern Nepal.

2 | Materials and methods

2.1 | Study area

Baidya fish farm (Site 1), Babiya Birta fish farm (Site 2), and Tarahara fish farm (Site 3) were selected in the diseased-prone areas of Eastern Nepal. Diseased fish were collected from these sites between 2010 to 2015.

Site1 (S₁): Baidya Fish Farm, Tankisinwari, Morang is located at latitude 26°31'11.12"N and Longitude 87°16'25.64"E (Fig. 1). Site2 (S₂): Babiya Birta Fish Farm, Morang is located at latitude 26°30'23.85"N and Longitude 87°26'09.01" E. Site3 (S₃): Tarahara Fish Farm, Sunsari is located at latitude 26°42'05.77"N and Longitude 87°16'38.50"E.

2.2 | Collection of fish samples and fungal isolation

Fish samples were collected from different ponds in the Sunsari and Morang districts of eastern Nepal during the winter months of 2010 to 2015 using a cast net. The samples were used for histopathological study along with the isolation of fungi. Of the 445 naturally infected fish collected, 262 *Cirrhinus mrigala* (58.9%), 130 *Labeo*

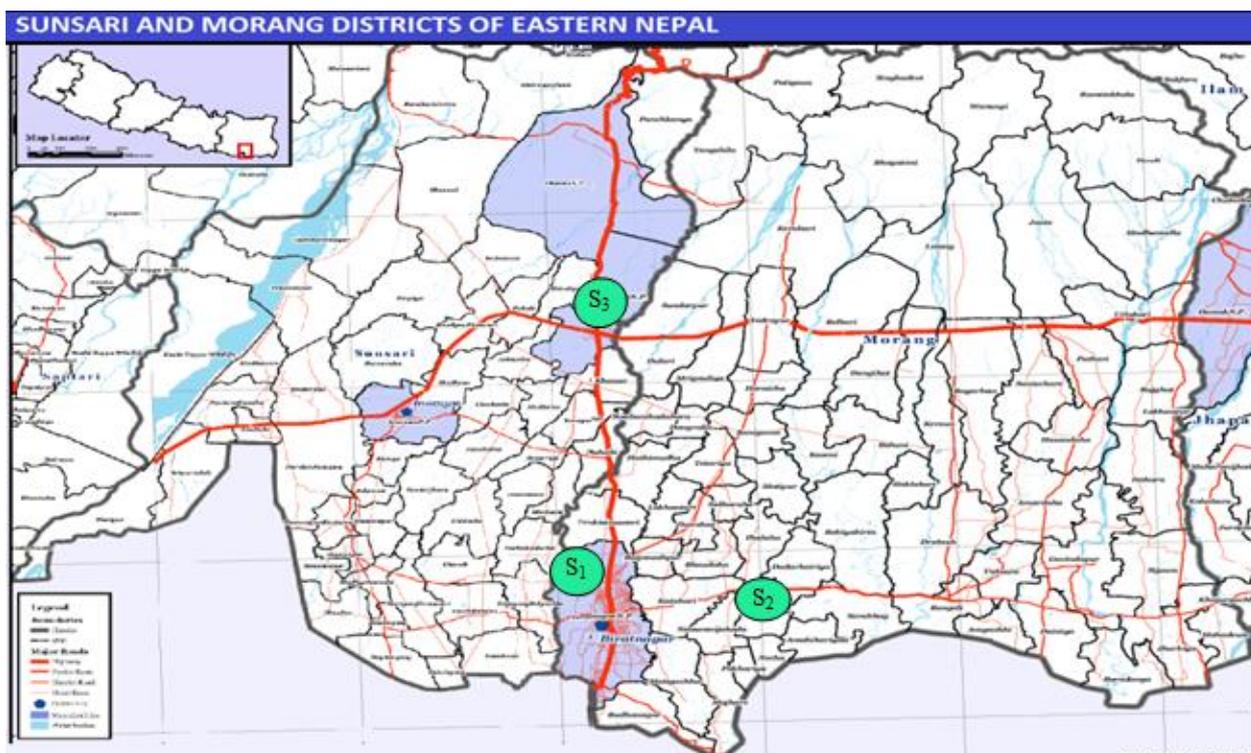


Figure 1. Map of Sunsari District showing sampling sites (1- 3) (Source: modified after OCHA, UN, Nepal, 2011)

rohita (29.20%), and 36 *Labeo bata* (8%) samples showing lesions on the body were sorted. Similarly, the remaining 3.9 % of fish species were *Catla catla*, *Channa* spp., *Puntius* spp., *Clarias batrachus*, *Heteropneustes fossilis*, *Mystus tengara*, and *Lepidocephalichthys guntea*.

2.3 | Fungal isolation, sporulation, and microscopic examination

Isolation of fungus was carried out following the methods described by Willoughby and Roberts (1994), Lilley et al. (1998), and Chinabut and Roberts (1999). In scaly fish, the scales around the periphery of slightly ulcerated lesions of infected fish were removed and underlying skin was seared with a red-hot spatula to sterilize the surface in a laminar flow cabinet. The underlying muscles of the lesion were then exposed by cutting the superficial tissues and excised into 4 mm pieces then placed on Petri dishes containing glucose peptone (GP) inoculated media were incubated at 25°C after examination under phase-contrast inverted microscope and the first transfer was made after six hours. The emerging hyphal tips were frequently transferred to fresh plates of GP medium containing antibiotics until the cultures were free of bacterial contamination. Isolates were then sub-cultured on GP agar and the transfer was given in every five days.

An agar plug containing actively growing mycelium was placed in a Petri dish containing GPY broth and was incubated for 4 days at about 20°C for sporulation of the fungus. The resulting mat was washed by sequential transfer through 5 Petri dishes containing autoclaved pond water and left overnight at 20°C. After about 12 hours motile secondary zoospores were observed under the microscope.

A portion of ulcerated tissue was taken and smeared on a clean glass slide. The smear was stained with Lactophenol cotton blue and was observed under the microscope. The fungal hyphae and sporangium from the pure culture maintained in GPYA were observed under the microscope after staining with cotton blue (OIE 2013).

2.4 | Histopathological techniques

Live fish samples were quickly put into a container of benzocaine solution (25 mg/L) for two minutes. Then the samples were taken out of the solution and pinned on a dissecting tray. The external lesions were first excised from the fish body with sterilized scissors and forceps and put in Bouin's fixative. The internal organs were carefully dissected out, cut into small pieces of 3

mm to 5 mm, and placed in fixative as early as possible. The volume of fixative was maintained always at least 20 times the volume of the tissues. The tissues were kept in fixative overnight.

The fixative was first washed out of the samples with 70% alcohol. The dehydration of tissue was done by passing through a graded series of alcohol solutions (70%, 90%, and 100%) (Schäperclaus 1986). After dehydration, the tissues were then placed in xylene and infiltrated with molten paraffin (melting point 58–60°C) and allowed to cool and harden.

The hardened paraffin-containing tissues were trimmed into rectangular blocks, mounted on a microtome and cut the sections (6µ thickness). The sections were placed on grease-free slides with albumin serving as an adhesive and properly stretched on warm distilled water. Then the slides were dried by keeping them overnight at room temperature in an incubator. Before staining, the stretched and dried sections were completely dewaxed by keeping them in xylene for 5–10 minutes.

2.5 | Procedures for haematoxylin–Eosin stain

After completely de-waxing, each slide with stretched section was kept in absolute alcohol for 5 minutes and passed through descending grades of alcohol (90%, 70%, 50%, and 30%) to water. Then the sections were stained with haematoxylin and washed thoroughly with water to remove the excess stain. Next, the sections were examined under a microscope to check the differentiation level. The sections were then passed through ascending grades of alcohol and stained with alcoholic eosin. The excess eosin was washed off by 90% alcohol and the tissues were further dehydrated in absolute alcohol, cleared in xylene, and mounted in DPX mounting medium.

2.6 | Grocott hexamine (methenamine) silver (GMS) staining for fungi

The section was hydrated through graded series of alcohols to water after de-waxing then oxidized in 5% aqueous chromium trioxide (Chromic acid) for 1 hour. The section was washed in tap water and rinsed in 1% sodium metabisulphite solution and rinsed in distilled water then placed in preheated (56°C) incubating solution in the dark for up to 1 hour. Afterward, it was rinsed well in distilled water and toned in 0.1% gold chloride for 4 minutes following which the section was rinsed in distilled water and fixed in 3% sodium thiosulphate for 5 minutes. It was finally counterstained

with Arzac's stain for 15-30 seconds, blotted, dehydrated, and mounted in DPX.

2.7 | Periodic Acid-Schiff stain (PAS)

The slides were slowly brought to water following downgrades of alcohol (100%, 90%, 70%, 50%, and 30%). Then the slides were kept in 10% periodic acid for 5-8 minutes and washed with running water for 3 minutes and subsequently with distilled water for 1 minute. Afterward, the slides were kept in Schiff's reagent for 15 minutes in dark and washed with tap water for 10 minutes then dried and mounted in Euparal.

2.8 | Fungus isolation, characterization, and pathogenicity test

In the culture, newly formed hyphae, which appeared after 6 hours of incubation at 23–25°C, were examined under an inverted phase-contrast microscope (CKII, Olympus). The growth of the hyphal tips was monitored routinely and next transferred after 24 hours. The pure culture was obtained after repeated transfer and finally transferred to GPA and GPYA for routine maintenance.

The cotton blue stained ulcer tissue revealed the presence of branched, aseptate fungus mycelium observed through the microscope in all samples. The mycelium of fungal isolate grown on GPA and GPYA were also branched, and aseptate but narrower than those found in ulcer tissue. It also showed the presence of terminal zoosporangia having a single row of zoospores. Identification of fungi was done by examining the asexual characteristics and particular characteristics of zoosporangia which were no wider than the hyphae.



Figure 2. Natural EUS infected samples showing different stages of ulcer changed the normal architecture of the epidermis (*C. mrigala*, *C. striatus*, *L. bata*, *C. catla*, and *M. tetraodon*)

The *Aphanomyces* sp. was isolated from naturally infected fish (*Cirrhinus mrigala*) and inoculated into the healthy fish *Heteropneustes fossilis* weighing about 55-95 g collected from Itahari sub- metropolitan pond to test its pathogenicity. Those fish samples were acclimatized in the glass aquarium in the laboratory at a temperature of 25±1°C for 15 days. Then 10 fish samples were injected with a zoospore suspension of *Aphanomyces* sp. at the rate of 0.5 mL/100 g of body weight intramuscularly as described by Robert et al. (1993). The controlled set was injected with sterile saline solution (0.85% NaCl) @ 0.5 mL/100 g of body weight. Three sets of experiments were carried out simultaneously.

3 | Results

3.1 | Histopathological observations of diseased fish

Histopathological observations of muscles, livers, and kidneys of *Aphanomyces* sp. infected fish samples showed EUS (Figs. 2 & 3). Myonecrosis and back stained with GMS fungal hyphae showed the presence of *Aphanomyces* sp. The entire loss of epidermis and the underlying musculature replaced by granulomatous and inflammatory tissues were also visible. Histopathological observations of EUS-affected fish are shown in Figs. 4 and 5.

***Cirrhinus mrigala*:** The histological section of advanced lesions showing the complete loss of epidermis and the underlying musculature was replaced by granulomatous and inflammatory tissues. In some areas, myonecrosis and fungal hyphae, black stained with



Figure 3. Natural EUS infected *Puntius* sp. fish samples showing the different stages of ulcer changed the normal architecture of the epidermis

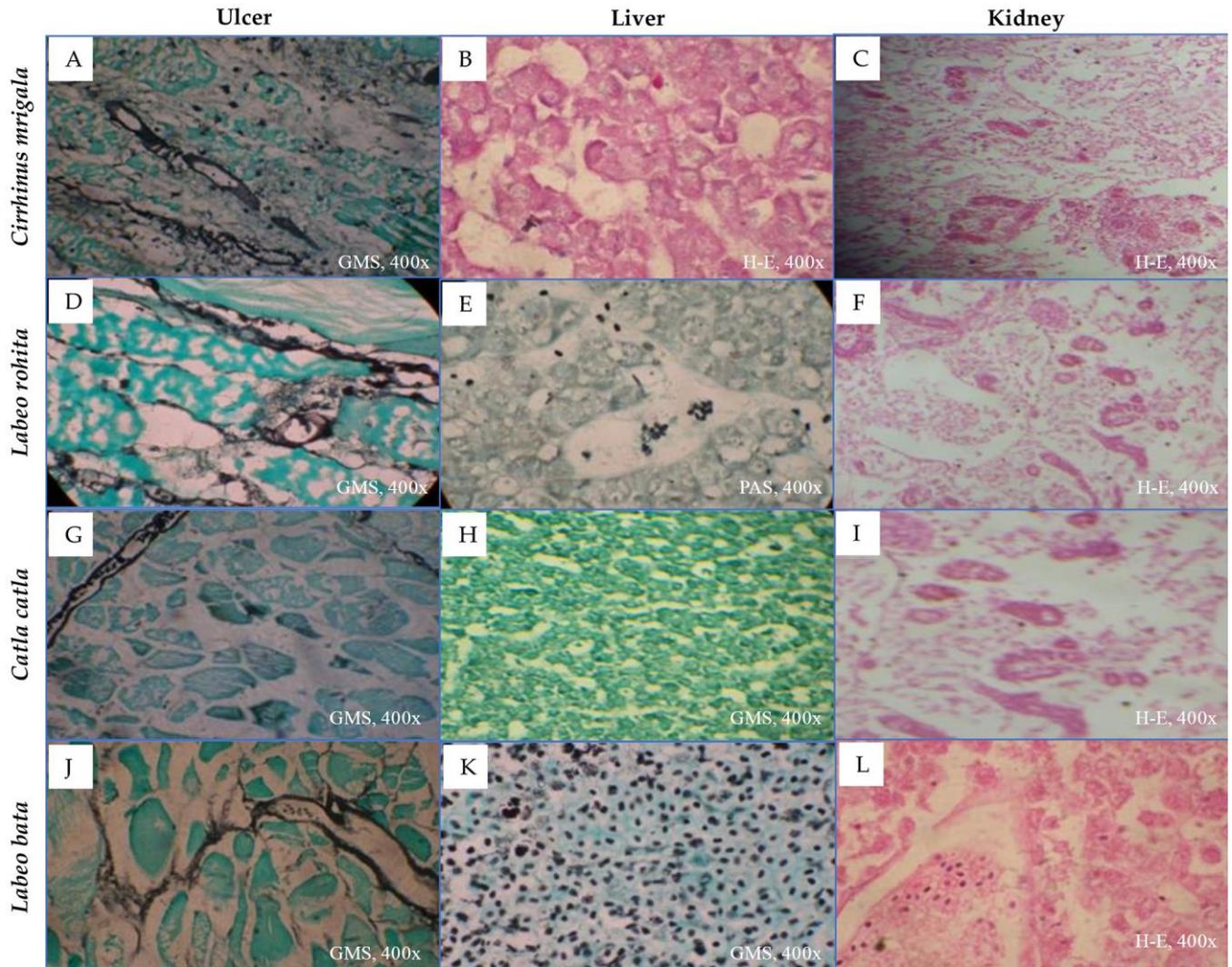


Figure 4. Histopathological observations of ulcer, liver and kidneys from diseased fish species. A-C, *Cirrhinus mrigala*; D-F, *Labeo rohita*; G-I, *Catla catla*; and, J-L, *Labeo bata*.

GMS, are often found. H-E stained section also shows the presence of fungus (Fig. 4A). The section of the liver shows degenerative changes and infiltration of blood capillaries. Necrotic changes, chord-like arrangement with enlarged sinusoids, and severely vacuolated hepatic cells can be observed in some areas whereas no fungi were detected (Fig. 4B). In the kidney section, necrotic changes and hemorrhages show some areas. Tubular degeneration and vacuolation of tubular cells can be seen but no evidence of the presence of fungi (Fig. 4C).

***Labeo rohita*:** In the section on the early stages of lesions, deterioration of the normal structure of the epidermis was observed. Advanced lesions showing complete loss of epidermis and the underlying musculature were replaced by granulomatous and inflammatory tissues. In some regions, myonecrosis can also be observed. Fungal hyphae can be seen in a section stained with H-E and GMS (Fig. 4D). The stained section

of liver showed degenerative changes and infiltration of blood capillaries. Necrotic changes, chord-like arrangement with enlarged sinusoids, and severely vacuolated hepatic cells can be observed in some areas. No evidence of the presence of fungi was found (Fig. 4E). Tubular breakage, tubular necrosis, vacuolation of tubular cells, and hemorrhages in some areas of the section of the kidney of naturally infected *Labeo rohita* can be observed. Fungi were not found in the section (Fig. 4F).

***Catla catla* :** In the section of early skin lesions, epithelial necrosis with hemorrhage from the underlying dermis can be observed. The epidermis at the margins of the ulcer is hyperplastic and thickened. In some regions, myonecrosis is also developed. Some aseptate invasive fungal hyphae were distinctly visible in a section stained with H-E and GMS (Figs. 2, 4G). The fungal invasion was not observed in the liver tissues. Degenerative changes and infiltration of blood

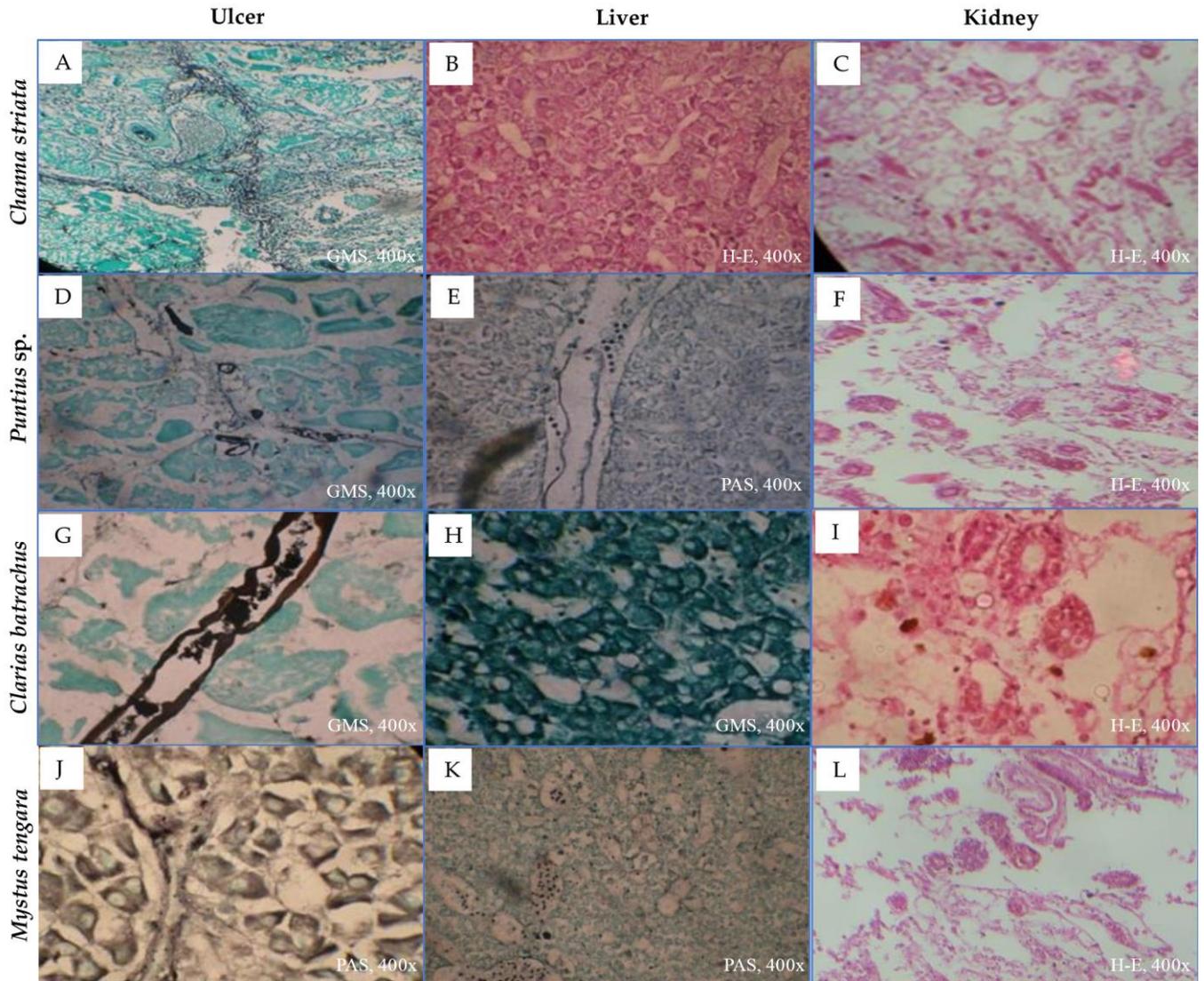


Figure 5. Histopathological observations of ulcer, liver and kidneys from diseased fish species. A-C, *Channa striata*; D-F, *Puntius sp.*; G-I, *Clarius batrachus*; and, J-L, *Mystus tengara*.

capillaries of the liver can be observed. Chord-like arrangement with enlarged sinusoids and highly vacuolated hepatic cells can also be observed (Fig. 4H). Renal tissues showed tubular and haematopoietic tissue degeneration along with hemorrhages in some areas of the section (Fig. 4I).

***Labeo bata*:** The section of deep ulcerated area displayed the complete loss of epidermis and the dermal layer lost its normal structural design and developed granulomas. Several non-septate hyphae can be observed in the dermis (Fig. 4J). Section of liver showing vacuolation, enlarged sinusoids, arrangement of hepatocytes in chord-like fashion, and infiltration of blood capillaries in some areas (Fig. 4K). Section of a kidney showing tubular breakage, tubular necrosis, vacuolation of tubular cells, and hemorrhages in some areas can be observed but fungus was absent (Fig. 4L).

***Channa striata*:** The initial lesions in the epidermis showed loss of its normal structure. In the advanced lesions, non-septate fungal hyphae can be frequently observed in the dermis and musculature. Changes can be seen in the formation of granuloma and myonecrosis (Figs. 2 and 5A). In the liver section, mild focal degenerative changes of hepatic cells can be seen and several hemorrhagic spots were seen. Vacuolation of hepatocytes with necrotic changes in some areas and infiltration of blood capillaries can be spotted but Fungi were not detected (Figure 5B). The noticeable important necrotic changes in specific haematopoietic areas, haemorrhages, and tubular vacuolation in the section of the kidney were also observed (Fig. 5C).

***Puntius sp.*:** The section of the ulcerated area showed a complete loss of epidermis and the lost normal structure of the dermal layer replaced by granulomas. Several non-septate fungal hyphae can be observed in the

dermis (Figs. 3 and 5D). The section of the liver of the naturally infected *Puntius* sp. showed vacuolation in the hepatocytes. Infiltration of blood capillaries can also be seen in some regions (Fig. 5E). Hemorrhages can be observed in some areas of the sections of the kidney. No fungal hyphae were detected. Tubular breakage, tubular necrosis, and vacuolation of tubular cells can also be observed (Fig. 5F).

***Clarias batrachus*:** The section of the early stages of lesions showed loss of the normal architecture of the epidermis and changed into advanced lesions. Complete loss of epidermis and the underlying musculature was replaced by granulomatous and inflammatory tissues. In some regions, myonecrosis was also developed. Fungal hyphae can be seen in a section stained with H-E and GMS (Fig. 5G). The liver section showed degenerative changes and infiltration of blood capillaries. In some areas, hepatic cells can be seen to have necrotic changes, chord-like arrangements with enlarged sinusoids, and severe vacuolation. There was no evidence of the presence of fungi (Fig. 5H). Tubular breakage, tubular necrosis, vacuolation of tubular cells, and haemorrhages were observed in some areas of the section of the kidney of naturally infected *Clarias batrachus*. Besides these, haemopoietic tissue degeneration can also be observed but fungi were not found (Fig. 5I).

***Mystus tengara*:** The section of the ulcerated area showed a complete loss of epidermis. The normal structure of the dermal layer was lost and replaced by granulomas. Several non-septate fungal hyphae were observed in the dermis. Granuloma formation and myonecrosis were prominent in the centre of the ulcer (Fig. 5J). The section of the liver can be seen vacuolation in the hepatocytes and in some regions the hepatocytes are arranged in a chord-like arrangement with enlarged sinusoids. Infiltration of blood capillaries can be also seen in some regions (Fig. 5K). Hemorrhages can be observed in some areas of the sections of the kidney but no evidence of fungal hyphae. Tubular breakage, tubular necrosis, and vacuolation of tubular cells can be observed in the section of the kidney (Fig. 5L).

Test of pathogenicity in *Heteropneustes fossilis* showed some lesions and 43.33% mortality when isolated zoospores were injected into experimental fish samples (Table 1). A single row of primary zoospores found within the zoosporangia is also shown in Supporting information (S 1-4 of A₁-A₄).

The fungal isolates grew slowly in culture media from 25-30°C but did not grow at 37°C. *Aphanomyces* sp. was observed only in muscle samples and isolated from ulcer tissues of *Cirrhinus mrigala*, *Channa striatus*, *Labeo rohita*, *Labeo bata*, *Catla catla*, *Mystus* sp., *Puntius* sp., and *Clarias batrachus*.

3.2 | Pathogenicity test of isolated fungus *Aphanomyces* sp. in *Heteropneustes fossilis*

Healthy fish samples were injected with a zoospore suspension of *Aphanomyces* sp. at the rate of 0.5 mL/100 g of body weight intramuscularly showed a red spot at the site of injection after 48 hrs of inoculation. Then the red spot increased in size and the ulcer developed after 72 hrs. Among treated 30 fish, 13 (43.33%) fish died, six developed moderate ulcer and 11 developed advanced ulcers (Table 1) of mortality was recorded during 15 days of observation. In the control set of fish, no ulcer formation and mortality were observed.

4 | Discussion

In this study, ulcerated muscles of all EUS infected fish showed loss of epidermis and granuloma and myonecrosis where several non-septate hyphae were observed (Figs. 4A, 4D, 4G, 4J, 5A, 5D, 5G and 5J) but in sections of livers and kidneys, no fungal hyphae were seen. Sections of livers in all infected fish showed more or less similar degenerative changes and infiltration of blood capillaries and necrotic changes, enlarged sinusoids, and vacuolated hepatic cells (Figs. 4B, 4E, 4H, 4K, 5B, 5E, 5H and 5K). Sections of kidneys also showed similar pathological changes like tubular breakage and necrosis, vacuolation of tubular cells, and haemorrhages (Figs. 4C, 4F, 4I, 4L, 5C, 5F, 5I and 5L).

Table 1. Percentage mortality and nature of ulcer formation in *Heteropneustes fossilis* injected intramuscularly with saline suspensions of *Aphanomyces* sp. zoospores from *Cirrhinus mrigala* (A₁).

	No. of fish	No. of fish dead	Nature of ulcer		Mortality
			Moderate (erosion in the epidermis)	Advanced (necrotic)	
Control	30	0	0	0	0
Saline suspension of <i>Aphanomyces</i> sp.	30	13	6	11	43.33%

A histopathological study by Vishwanath et al. (1998) found that the fungal invasion and associated pathology are not confined to the dermal ulcer regions, but fungus invades and proliferates in tissues away from the site of dermal ulcers and even penetrates across the vertebral septum. The fungus invades the body cavity and develops mycotic granulomas in all visceral organs. Chinabut et al. (1995) observed the infections of the muscle tissues that led to the formation of mycotic epithelioid granulomas. But such granulomas are not always associated with the infection in the experiment in European catfish (Oidtmann et al. 2008).

Pathogenic *Aphanomyces* sp. was isolated by several workers from the EUS-affected fish (Paclibare et al. 1994; Willoughby et al. 1994; Callinan et al. 1995a; Lilley & Roberts 1997; Lumanlan-Mayo et al. 1997) which was later named as *A. invadaris* (Willoughby et al. 1994). The protein banding profiles (Callinan et al. 1995b; Lilley et al. 1997b), growth characteristics (Lilley & Roberts 1997), and genetic fingerprinting techniques (Lilley et al. 1997a) showed that various *Aphanomyces* sp. isolated by various workers were alike and *A. invadaris* was renamed to *A. invadans* (David & Kirk 1997). Kar and Upadhyaya (1998) revealed that the fungus caused mainly increased fibrosis and chronic inflammatory cell infiltration in muscle; fatty degeneration of the hepatocytes surrounding the portal triads and occasional infiltration by mononuclear cells in the liver. Furthermore, an aseptate fungus capable of multiplication by budding within the tissue of affected fish exerts pathological changes as observed by many workers (Vishwanath et al. 1997, 1998; Blazer et al. 1999; Chinabut & Roberts 1999; Oidtmann et al. 2008) in histopathology of EUS affected fish.

The fungus was isolated from EUS-affected *C. mrigala* and formed sporangia at the hyphal tip not wider than hyphae in GPY medium and balls of spores were noticed at the tip of the sporangium at 25°C. From these characteristics, it appeared that the isolated fungus was *Aphanomyces* sp. Experimentally, the fungal zoospores induced ulcers in healthy *H. fossilis* and produced typical granulomas in the dermis and underlying musculature.

Roberts et al. (1993) reported that fish affected with EUS in different countries throughout South and Southeast Asia showed a persistent presence of morphologically typical fungus within the lesion in a survey. When the mycelium from pathogenic isolates of *Aphanomyces* sp. was inoculated below the dermis of healthy fish, it caused an inflammatory response and spread into the tissues inducing brutal myonecrosis with chronic

epithelial effect. Chinabut et al. (1995) inoculated snakeheads; *C. striata*, with a spore suspension of *Aphanomyces* sp. isolated from EUS-affected fish in Southeast Asia. In the early stages of the disease, degenerative changes were observed in all samples, but inflammatory infiltrate was much more marked in fish kept at 26°C and 31°C while fish kept at 19°C developed a severe invasive myonecrosis with limited macrophage response. The mortalities in the fish kept at 19°C were 40% higher than in the group of fish kept at 26°C and 31°C (12%). It explains the mortalities from EUS occurring when water temperatures become low.

Blazer et al. (1999) reported *Aphanomyces* sp. as a cause of ulcerative skin lesion of Atlantic menhaden both by histopathologically and PCR test (Lilley et al., 2003). Vogelbein et al. (2001) reported that ulcers of menhaden harboured a deeply invasive, highly pathogenic fungus now known to be *A. invadans*. On the contrary, Afzali et al. (2013) isolated and identified freshwater fungi species from the Malaysian natural water bodies and fish farms. When intramuscular injection of the *Aphanomyces* spp. isolates to the *Trichogaster microlepis* showed neither mortality nor signs of EUS, Afzali concluded that all the *Aphanomyces* isolates were non-pathogenic. However, the section of ulcer of fungal zoospore inoculated into *H. fossilis* showed typical granulomatous changes very similar to the naturally infected fish and mortality was 43.33% (Table 1) in this study.

5 | Conclusions

The histopathological study confirmed that the causative agent of EUS was the fungus *Aphanomyces* sp. and the mortality rate of fish from the outbreak of the EUS was higher compared to the other fish diseases in Nepal.

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Authors' contributions

Thapa, G. B. performed the field and laboratory works, and prepared the manuscript. Pal, J. conceived the concept, designed the research, supervised research work, aided in interpreting the findings and improved the manuscript.

Conflicts of interest

Authors declare no conflict of interest.

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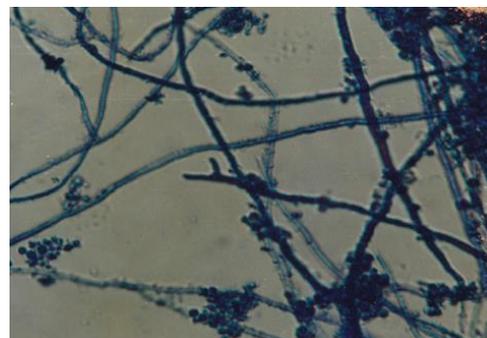
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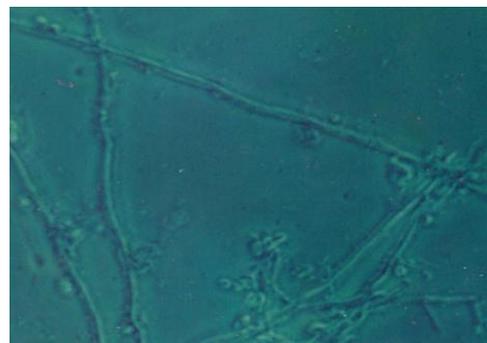
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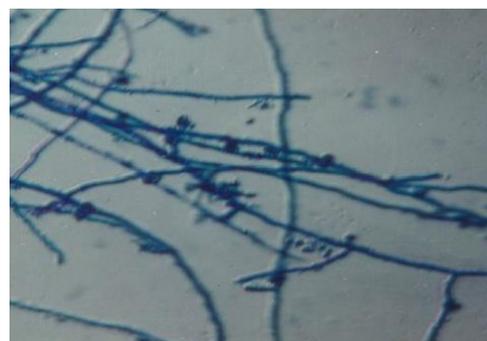
S1. Zoosporangia of *Aphanomyces* sp. from ulcer of natural infected *Cirrhinus mrigala*



S2. Zoosporangia of *Aphanomyces* sp. from ulcer of natural infected *Catla catla*.



S3. Zoosporangia of *Aphanomyces* sp. from natural infected *Labeo bata*.



S4. Zoosporangia of *Aphanomyces* sp. from naturally infected *Puntius* sp.