

Original article

## Histopathological Alterations and Multidrug-Resistant Bacteria in *Mugil cephalus* from Polluted Tripoli Coastline, Libya

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### Abstract

The polluted coastal waters of Tripoli, Libya, driven by untreated sewage, pose significant health risks. This study investigated histopathological alterations in the kidneys and spleens, and isolated and identified multidrug-resistant bacteria from the kidneys of 20 *Mugil cephalus* specimens collected from anthropogenically stressed sites along the Tripoli coastline. Kidney tissues showed severe tubular necrosis, hyaline casts, glomerular atrophy, and interstitial mononuclear infiltration, while splenic tissues exhibited marked activation of the melanomacrophage centres (MMCs), vascular congestion, and multifocal granulomas. Bacteriological analysis of kidney homogenates detected growth in 60% of samples (mean  $4.2 \times 10^5$  CFU/g), predominantly multidrug-resistant *Enterobacteriaceae* (91.7% MDR), including *Proteus vulgaris* and *Enterobacter cloacae*. These findings demonstrate an association between antibiotic-resistant bacteria and chronic tissue damage, with MDR profiles indicating potential zoonotic transmission. The identified lesions may serve as bioindicators for monitoring drug-resistant microbes, underscoring the urgent need for wastewater treatment and surveillance to protect marine ecosystems and public health.

**Keywords.** *Mugil cephalus*, Histopathological Alterations, Multidrug-resistant Bacteria, Antimicrobial Resistance, One Health.

### Introduction

The coastline of Tripoli, Libya, is heavily polluted by intensive urban and industrial activities. This may introduce sub-lethal antibiotic concentrations into the environment, fostering the persistence of antibiotic-resistant bacterial strains [1–5]. The flathead grey mullet (*Mugil cephalus*), a species of considerable commercial importance and dietary relevance to local artisanal fisheries, is particularly susceptible to contamination because its benthivorous feeding increases exposure to sediment-bound pollutants and microorganisms [6–9]. This species may accumulate elevated levels of antibiotic-resistant bacteria (ARB), making it a valuable sentinel for monitoring drug-resistant pathogens and assessing histopathological impacts [10]. This issue is further complicated by the widespread presence of ARB in both environmental and healthcare contexts throughout Libya, thereby heightening zoonotic and public health concerns [11,12]. Histopathological analysis of the kidney and spleen effectively assesses systemic effects of ARB infections in teleost fish [13,14]. These critical immune and hematopoietic organs are primary targets for damage by resistant microbial strains, offering insight into lesion severity [15–17]. The kidney, essential for osmoregulation and excretion, is highly susceptible to blood-borne pathogens and toxins and typically exhibits tubular necrosis, glomerular atrophy, and hyaline casts [15,17–19].

The spleen, a secondary lymphoid organ, shows melanomacrophage centers (MMCs) proliferation and granuloma formation in response to persistent infection and stress [16,20–22]. Although histopathological changes in *Mugil cephalus* are commonly used to monitor coastal pollution [23,24], the specific role of ARB in driving these pathologies remains a critical area of investigation [25,26]. To bridge this gap, the present study investigates pathological alterations in the kidneys and spleens of *Mugil cephalus*, identifies multidrug-resistant bacteria from the kidneys, and evaluates the relationship between pathological lesions and bacterial contamination at anthropogenically stressed sites along the Tripoli coast, Libya.

### Materials and Methods

#### Sample Collection

Flathead grey mullet (n=20, *Mugil cephalus*) was collected from polluted coastal waters along the Tripoli coast, Libya. Fish were humanely euthanized according to ethical guidelines immediately before necropsy. Each specimen underwent a systematic macroscopic examination to identify external and internal clinical abnormalities.

#### Histopathological Examination

Kidney and spleen tissues were carefully excised and trimmed into small blocks (approximately 0.5 cm<sup>3</sup>). Samples were fixed in 10% neutral buffered formalin (NBF) for at least 48 hours at room temperature to

preserve the structural integrity of renal tubules and splenic ellipsoids. Following fixation, tissues were dehydrated in an ascending ethanol series (70%, 80%, 90%, 100%), cleared in two changes of xylene, and infiltrated with molten paraffin wax at 58°C. Paraffin-embedded blocks were sectioned at 4–5 µm thickness using a rotary microtome. Sections were mounted on glass slides and stained with Hematoxylin and Eosin (H&E) for routine morphological evaluation.

### **Bacteriological Identification and Antimicrobial Susceptibility Testing**

Bacteriological analysis was performed exclusively on kidney tissue, as the teleost kidney serves as the primary lymphoid and hematopoietic organ. Under strict aseptic conditions, the fish surface was disinfected with 70% ethanol. Kidney tissue (0.5 g) was aseptically excised, homogenized in sterile PBS (pH 7.2) at 1:10 w/v, and serially diluted ( $10^{-1}$  to  $10^{-6}$ ). Aliquots (100 µL) were spread-plated in triplicate on TCBS agar and blood agar. Plates were incubated aerobically at 25–30°C for 24–48 h, and isolated colonies were selected and further characterized phenotypically. For this step, only one isolated colony from a chosen plate was picked when similar colonial growth was observed for each sample, and the sample was then subjected to Gram staining, motility, oxidase, and catalase testing. In cases of multiple growth, two colonies were picked; however, all plates in the current study showed a similar pattern of a single type of colonial growth.

Definitive identification to genus and species level and antimicrobial susceptibility to antimicrobials was performed using the BD Phoenix automated identification and susceptibility testing system (PAMS, BD Biosciences, Sparks, MD, U.S.A.). Antimicrobial susceptibility profiles of confirmed isolates were interpreted using the Phoenix system. Multidrug resistance (MDR) was defined as resistance to at least one agent in three or more antimicrobial classes.

### **Statistical analysis**

Spearman's rank correlation coefficient was used to assess the association between bacterial load and histopathological lesion severity. A  $p$ -value < 0.05 was considered statistically significant.

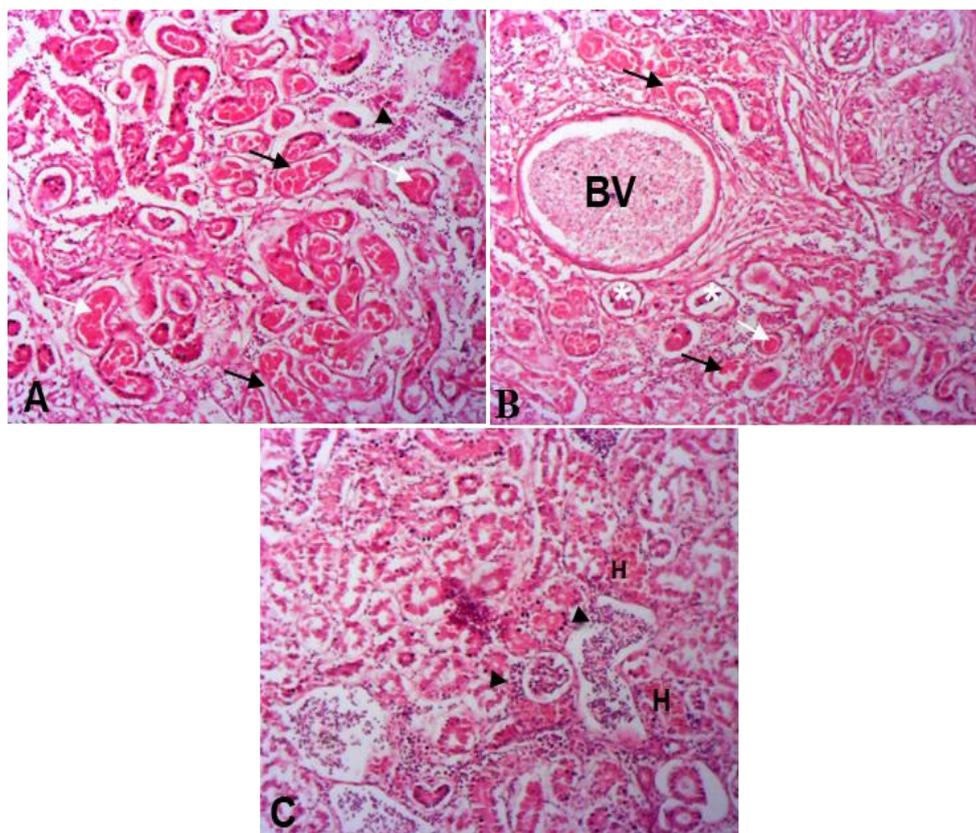
## **Results**

### **Macroscopic and Histological examination**

In the current study, 85% (17/20) of *Mugil cephalus* examined exhibited significant macroscopic and pathological changes. External examination revealed mild lordosis and gill marbling. Internal necropsy consistently showed widespread hemorrhage across multiple organs (Figure 1). In several specimens, paleness of the gills and liver was also noted, suggesting chronic anemia or advanced organ failure. Histologically, the renal tubules revealed severe vacuolar degeneration, epithelial desquamation, and necrosis with intraluminal hyaline cast formation (Fig. 2A and B). Some glomeruli appeared atrophied, with widened Bowman's spaces (Figure 2B). The interstitial tissue showed mild to moderate mononuclear cell infiltration, with vascular congestion and hemorrhage (Figure 2A, B, and C).

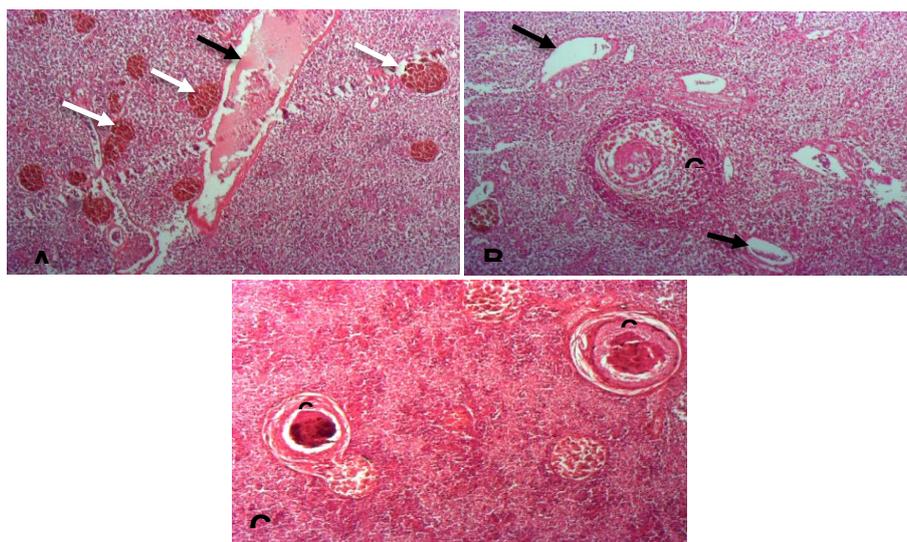


**Figure 1. Naturally infected *Mugil cephalus* fish showing widespread hemorrhage across multiple organs. (A) liver (black arrow). (B) swim bladder (black arrow). (C) kidney (white arrow).**



**Figure 2.** Photomicrographs of the kidney tissue of infected *Mugil cephalus* fish showing (A and B) severe vacuolar degeneration and necrosis of the renal tubular epithelium (black arrows) with hyaline casts in tubular lumen (white arrows); (B) glomerular degeneration (asterisks); (A, B and C) mild to moderate interstitial mononuclear cell infiltration (arrowheads) with vascular congestion (BV) and hemorrhage (H) (H&E stain: A, B & C  $\times 100$ ).

Microscopic examination of the spleen of infected *Mugil cephalus* revealed severe vascular congestion and marked activation of melanomacrophage centers (MMCs) (Fig. 3A & B). In some splenic tissues, multifocal granulomas, each with a central necrotic area enclosed by a fibrous capsule, were also observed (Figure.3B & C).



**Figure 3.** Photomicrographs of the spleen tissue of infected *Mugil cephalus* fish showing (A) marked activation of melano-macrophage centers (white arrows); (A and B) dilation and congestion of splenic blood vessels (black arrows); (B and C) multiple splenic granulomas consisting of a central necrotic area (G) encapsulated by a fibrous layer (G) (H&E stain: A, B & C  $\times 40$ ).

## **Bacteriological Analysis and Antimicrobial Susceptibility Testing**

### **Bacteriological Identification**

Bacterial growth was detected in kidney homogenates from 60% (12/20) of *Mugil cephalus* samples, with a mean bacterial load of  $4.2 \times 10^5$  CFU/g tissue (range:  $2.1 \times 10^3$  to  $8.7 \times 10^6$  CFU/g). A total of 12 distinct isolates were recovered and identified by phenotypic characterization and the automated Phoenix microbiology system. The predominant species was *Proteus vulgaris* (n=4; 33.3%), followed by *Enterobacter cloacae* (n=3; 25.0%) and *Citrobacter freundii* (n=2; 16.7%). Other identified *Enterobacteriaceae* include *Hafnia alvei*, *Kluyvera intermedia*, and *Klebsiella oxytoca* (n=1 each; 8.3%). Higher bacterial loads ( $>10^5$  CFU/g) were significantly associated with severe histopathological lesions in the kidney (e.g., tubular necrosis and hyaline casts; Spearman's rho = 0.72, p<0.05), indicating a positive correlation between pathogen burden and tissue damage.

### **Antimicrobial Susceptibility Testing**

Antimicrobial susceptibility testing showed that 91.7% (11/12) of isolates exhibited a multidrug-resistant (MDR) phenotype. Universal resistance (100%) was observed for imipenem (IPM), cephalothin (KF), cefuroxime (CXM), ampicillin (AMP), and tigecycline (TGC). Additionally, 91.7% of isolates showed co-resistance to critical antibiotics, including third- and fourth-generation cephalosporins (e.g., ceftriaxone [CRO] and cefepime [FEP]) and aminoglycosides (e.g., amikacin [AK] and gentamicin [CN]). One *Proteus vulgaris* isolate had a narrower resistance profile, primarily limited to carbapenems and early-generation cephalosporins. Minimum inhibitory concentration (MIC) values for key aquaculture antibiotics—oxytetracycline (OTC) and amoxicillin (AMX) in all MDR strains were MIC  $\geq 32$   $\mu\text{g/mL}$  for OTC;  $\geq 64$   $\mu\text{g/mL}$  for AMX.

## **Discussion**

The high prevalence of lesions found in this study (85%) aligns with the findings of Bukha et al. [4], who identified a direct correlation between untreated wastewater discharge along the Western Tripoli coast and systemic bacterial pathologies in local fish populations. The severe histopathological alterations observed in the studied kidneys and spleens of *Mugil cephalus* from the Tripoli coast clearly reflect the deleterious impact of the local marine environment. The presence of intraluminal hyaline casts and severe tubular necrosis indicates a profound loss of renal integrity. This damage likely results from the systemic circulation of bacterial exotoxins, a phenomenon documented in *Mugil cephalus* populations, where pathogens such as *Vibrio* spp. produce extracellular products—hemolysins and cytotoxins—that cause invasive tissue damage [27]. These findings align with those of Magouz et al. [28] and Shahid et al. [17], who reported that hyaline droplet degeneration in teleost kidneys is a definitive marker of protein leakage following glomerular filtration failure. In the Tripoli coast context, this damage is likely exacerbated by antibiotic-resistant bacteria, which further compromise the fish's immune and physiological integrity [29].

Similar renal and splenic congestion has been reported in *Mugil cephalus* along the Libyan coast, often associated with seasonal bacterial outbreaks [29]. Furthermore, acute coagulative necrosis in fish is a known consequence of bacterial toxins that target cell membranes, causing vascular injury and subsequent organ failure [30]. Unlike sensitive strains, the multidrug-resistant *Enterobacteriaceae* isolated in this study (e.g., *Proteus vulgaris*, *Enterobacter cloacae*) persist longer in host tissues, resulting in a chronic inflammatory state, as evidenced by atrophied glomeruli and widened Bowman's spaces.

Pronounced activation of melanomacrophage centers (MMCs) and the development of multifocal splenic granulomas indicate a shift from acute to chronic pathogenesis. While MCCs are normal components of the teleost immune system, their massive proliferation is a documented response to cumulative stress from untreated municipal sewage and resistant pathogens [20,31]. The presence of fibrous-encapsulated granulomas is particularly significant; it suggests that the *Mugil cephalus* immune system, unable to clear antibiotic-resistant pathogens through standard phagocytosis, attempted to sequester the bacteria within a necrotic core [32,33]. Similar granulomatous responses have been reported in *Mugil cephalus* from contaminated Mediterranean sites near Benghazi [29]. These findings are corroborated by recent environmental assessments [34], which show that untreated wastewater discharge along the Tripoli coastline introduces a complex mix of chemical and microbial contaminants. This intersection of chemical pollution and microbial resistance creates a unique pathological profile characterized by chronic tissue inflammation and necrotic lesions that severely compromise the health of Libyan marine resources and pose risks to public health [29,34].

The isolation of members of the *Enterobacteriaceae* family from *Mugil cephalus* in this study highlights a significant concern about the microbiological quality of these fish. Species such as *Proteus vulgaris*, *Enterobacter cloacae*, and *Klebsiella oxytoca* are often considered opportunistic pathogens in aquaculture, capable of causing systemic infections under stressful environmental conditions or high organic loads [35, 36]. Furthermore, previous studies have documented that *Enterobacter* species can progress to septicemia when the host immune system is compromised by poor water quality [37]. The presence of *Citrobacter freundii* and *Klebsiella oxytoca* is noteworthy; while common in aquatic environments, they are also recognized as indicators of faecal contamination [38]. Their recovery from *Mugil cephalus*—a species

frequently consumed by humans—suggests a potential risk of foodborne illness if the fish are not handled or cooked properly [39]. Given that *Mugil cephalus* is a bottom feeder, it is highly susceptible to pathogen accumulation from contaminated sediments, thereby acting as a bridge for zoonotic transmission [40].

The bacterial isolates from *Mugil cephalus* exhibited multidrug-resistant (MDR) profiles across several critical antibiotic classes, indicating a significant environmental reservoir of resistance genes along the Tripoli coast. Notably, carbapenem resistance (to imipenem, meropenem, and ertapenem) is particularly alarming, as these "gold standard" agents are typically reserved for severe Gram-negative infections [41]. Furthermore, variable resistance to third-generation (ceftriaxone) and fourth-generation (cefepime) cephalosporins suggests the presence of extended-spectrum beta-lactamases (ESBLs), which compromise the efficacy of these essential human therapeutics [42]. Most critically, resistance to colistin and tigecycline—classified by the WHO [43] as "highest priority critically important antimicrobials"—underscores the penetration of last-resort resistance into the marine food chain, posing a profound risk to public health. However, it is important to acknowledge that phenotypic detection of colistin resistance using automated systems like Phoenix may require validation. Definitive confirmation of polymyxin resistance typically necessitates broth microdilution for Minimum Inhibitory Concentration (MIC) determination, as recommended by standard guidelines, to rule out potential discrepancies inherent to some automated platforms.

The widespread occurrence of these profiles suggests that *Mugil cephalus* may serve as a reservoir for antimicrobial resistance genes (ARGs) in the marine ecosystem, likely driven by runoff of veterinary or human pharmaceuticals into coastal habitats. Identifying less common species, such as *Kluyvera intermedia* and *Hafnia alvei*, adds depth to understanding microbial diversity within the Mugilid family. *Hafnia alvei*, specifically, is an emerging pathogen associated with "brown blood disease" and hemorrhagic septicemia [35]. Its presence warrants further investigation, particularly given its correlation with vascular congestion and hemorrhage observed in histological sections.

The presence of multifocal granulomas in splenic tissue indicates a chronic immune response. As Roberts [44] noted, such lesions represent protective sequestration of pathogens by the host's cellular immune system and often appear when the innate response fails to achieve rapid clearance. In the Mediterranean basin, this chronic inflammatory profile is frequently observed in *Mugil cephalus* inhabiting anthropogenically stressed environments [32]. Specifically, along the Tripoli and Benghazi coasts, the prevalence of these granulomatous alterations is increasingly linked to the synergy between heavy-metal accumulation and multidrug-resistant *Vibrio* species [29]. The activation and proliferation of MMCs serve as a reliable bio-indicator of environmental stress and bacterial load [22], effectively bridging the gap between the persistent microbiota isolated from the Tripoli coast and the host's chronic pathological condition [44]. The high prevalence of bacterial isolates in the kidney and the observed lesions in the spleen are linked to the host's ecological niche. As a commercially vital species in Libya, *Mugil cephalus* is highly sensitive to fluctuations in coastal ecosystems [45]. Its bottom-feeding behavior facilitates constant contact with contaminated sediments and water along the Tripoli port [10], which explains the systemic entry of the pathogens identified in this study. The striking symmetry between the AMR profiles observed in *Mugil cephalus* and those reported in clinical isolates from Libyan national hospitals [12,46] suggests a direct epidemiological link between the municipal sewage system and the coastal food chain. Generic fecal coliforms do not merely contaminate the coastline but are actively seeded with high-priority clinical pathogens, creating a closed-loop transmission system back to the human population. The uniformity of resistance patterns across genera suggests environmental selection pressure from antibiotic-laden sewage. Notably, isolates from fish with severe splenic granulomas showed higher resistance to last-resort agents (e.g., colistin, meropenem), and the MDR index correlated positively with lesion severity score ( $\rho = 0.68$ ,  $p < 0.05$ ), indicating potential contribution of MDR bacteria to the observed chronic inflammatory responses.

## Conclusion

This study shows severe histopathological changes in the kidneys and spleens of *Mugil cephalus* from Tripoli's polluted coastal waters, indicating significant systemic damage and immune exhaustion due to persistent infection. The isolation of multidrug-resistant *Enterobacteriaceae* suggests an association with worsened tissue damage. Urgent wastewater management and surveillance, using these renal and splenic markers as bioindicators, are necessary to reduce the spread of AMR and protect ecosystems and public health.

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## Conflict of interest

The authors declare no conflict of interest.

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