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Histopathological Alteration and Molecular Detection of Gills Rot Fungus in Carp Fish

Zahraa Mustafa AL-Jumaa[1*], Shahbaa Khalil AL-Taee[2], Mohammed Tariq Jaber[3] and Aseel Mohammed Rahawi[4]

[1]Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

[2]Department of Pathology and Poultry Disease, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

[3]Forensic DNA Center for Research and Training, Al-Nahrain University, Baghdad, Iraq

[4]Department of Pathology and Poultry Disease, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

***Corresponding Author:** Zahraa Mustafa AL-Jumaa, **E-Mail:** sandy285@uomosul.edu.iq

ABSTRACT

Nowadays, diseases are a major concern in fish production, particularly in gills, which play a crucial role in blood oxygenation, acid-base balance, osmoregulation, and waste elimination. Fungal infections, particularly *Branchiomyces*, cause respiratory problems in fish. This study aims to determine the causative agent causing high mortality in common carp, *Cyprinus carpio*, in the middle Euphrates in Iraq. One hundred infected fish were sampled for microbial, histological, and molecular examination. Gill tissue was examined as well for histological alterations, and DNA was isolated from *Branchiomyces* and amplified with universal primers. Microscopically identifying *Branchiomyces spp*. using lactophenol staining, non-separated hyphae and spores (5–9 m in size) at various stages inside the structures of gill tissue were present. Histopathological examination revealed hyperplasia of epithelial cells and infusion of gill filaments, while primary gill filaments displayed severe hemorrhage and edema. Hyphae of *Branchiomyces* have been detected between necrotic and edematous myofiber. The ITS PCR products of the fungal isolate were found to be positive at 540 bp molecular weight. Economic losses can occur as a result of fish infections caused by *Branchiomyces*. Histopathological lesions in gill tissue indicated the presence of Branchiomyces infection, which was confirmed by molecular and microscopical examination.

Keywords: Branchiomycosis, Common carp fish, Histopathological alteration, ITS region.

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INTRODUCTION

The most widely farmed commercial freshwater fish in Iraq is the Common Carp, which is highly sensitive to a variety of water-borne pathogens **(Elsayed *et al.*, 2024)**. Although fungal pathogens have been responsible for a great deal of disease outbreaks, relatively little investigation has been done on them **(Eli *et al.*, 2011).**

Fish have a unique structure called a gill, which can serve as a good place for blood oxygenation depending on the quantity and size of it, as well as play a significant part in acid-base balance, osmoregulation, and waste excretion **(Muhammad *et al.*, 2022; Hussein *et al.*, 2024).** There are many other types of infections that can infect gills and cause respiratory distress; our investigation focused on one type of fungal infection, *Branchiomyces* **(Khoo *et al.*, 1998).** Many different types of freshwater fish are susceptible to Two oomycetes, *B. sanguinis* and *B. demigrans*, are responsible for an acute, localized fungal disease of the gills known as Branchiomycosis—gill rot **(Ramaiah, 2006)**. It's a big problem for commercial fisheries throughout a vast swath of the globe **(Eissa, 2024).** The disease is more prevalent in areas with a warmer climate **(Opiyo *et al.*, 2020).**

Most cases of branchiomycosis are characterized by respiratory symptoms, severe weakness, and significant mortality rates **(Ramaiah,**

2006; Sheikh and Mankodi, 2022). Both macroscopic examination of necrotic patches on affected gills and compression of affected gill tissue are important for the diagnosis of *Branchiomyces* **(Abdel-Latif *et al.*, 2020; Mahboub and Shaheen, 2021).** In addition, a confirming diagnosis can be made through the cultivation of *Branchiomyces spp*. by maintaining a pH of 5.8 in SDA media **(Al-Niaeem *et al.*, 2015).** Several studies show the presence of *Branchiomyces* hyphae inside the gill vessels, based on histological identification as an additional diagnostic technique **(Ibrahim, 2011)**. Both the vascular system and the extravascular tissues of the gills are rich in oomycete hyphae and spores. Intravascular hyphae elicit a mucosal immune response, but extravascular hyphae cause an inflammatory granulomatous reaction **(Flores-Lopes and Thomaz, 2011; FAO, 2020).**

When the blood supply to the gills is cut off or impaired, the filament tips die off. Researchers have found *B. demigrans* spores in the spaces between hyperplastic gill filaments and necrotic regions **(Patel *et al.*, 2018; Hussain and Kadhim, 2020).** Sporonts delivering spores can occasionally be seen in the channels of a gill **(Mahboub and Shaheen, 2021).** Since Branchiomycosis cannot be cured, infected fish cannot be safely transported to locations where it has not already been established. Those who find affected fish that have died should burn or bury them **(Khalil *et al.*, 2015; Sheikh and Mankodi, 2022).**

Particularly in central and southern Iraq, the effects of *Branchiomyces* on the local fish industry are severe. There are, however, insufficient investigations to fully characterize the pathogen in this area. Molecular and histological techniques were used in this investigation to help pinpoint the specific pathogen. By confirming and defining the environmental spread of this disease, this research will inform the creation of control measures **(Khalil *et al.*, 2015; Alfisyahrin *et al.*, 2019; Abdel-Latif *et al.*, 2020).**

MATERIALS AND METHODS

Ethical approval

This study did not require ethical approval; however, samples were obtained according to institutional and standard procedures.

The interval and geographical region

The infected fish have been collected from several locations using floating cages Al-Musayyib and Al-Saqlawiyah regions of the Middle Euphrates of Iraq. from October to December 2021.

Fish sampling

One hundred infected "*Cyprinus carpio*" common carp, each weighing 2 kilograms', were examined. Fish that showed symptoms of Branchiomycosis were sampled and taken to the laboratory at the **(F.DNA R.T.C.)** in polyethylene bags to maintain a sterile environment during travel. Samples were taken for histological and molecular analysis. Clinical examinations and pathological examinations were done on the obtained fish.

Mycological isolation

After removing the operculum and visually inspecting the gills of the obtained fish, we placed the gills from both sides in a container with an amount of regular salt water and examined them under a dissecting microscope. In order to detect spores and hyphae in gill filaments, Light microscopy with a high-power magnification lens (X 400) used to measure the diameters of hyphae and spores, as described in **(Ellis *et al.*, 2007; El-Bouhy *et al.*, 2014; Mondal *et al.*, 2023).**

Histopathological studies

After collecting infected gill, 24 hours of being fixed in 10% natural formalin buffered phosphate, being dried in increasingly strong concentrations of ethanol, being cleaned in xylene, and then being embedded in paraffin wax 50 degrees Celsius. A paraffin block was made, and then slices 5 microns thick were cut from it using a microtome. After being fixed on slides, tissue samples were stained with a combination of a hematoxylin and eosin stain and a specific PAS stain for identifying fungi in tissue **(Ali *et al.*, 2020).**

Molecular Technique

DNA extraction

Branchiomyces DNA was extracted from infected fish gills using a commercially available kit (Qiagene Extraction Kit). In short, DNA was extracted from obtained samples by following the manual's guidance. All DNA samples were frozen at -20 degrees centigrade until analysis.

Polymerase Chain Reaction

The ITS region was employed for the molecular characterization of *Branchiomyces*. The ITS1 sequence (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 sequence (5'-TCCTCGCTTATTGATATGC-3') were used as universal primers for the sequencing. BIONEER Co. (Korea) synthesized two sets of primers (forward and reverse) to specifically target the fungus. **Table 1** details the conditions for a PCR reaction with a 25µl reaction volume. A denaturing step at 95 degrees Celsius for 5 minutes was followed by 30 cycles of denaturing at 95 degrees Celsius for 20 seconds, annealing at 54 degrees Celsius for 30 seconds, and extending at 72 degrees Celsius for 5 minutes for the PCR. 2% agarose gel electrophoresis (Applied Biosystems, USA) with 2µl ethidium bromide in TBE buffer was used to examine all PCR results. In order to see the DNA bands, a UV transilluminator was used.

Table 1: Final PCR volume composition

Content	Amount (µL)
Mstermix 2.5X	10
Forward primer	1
Reverse primer	1
$MgCl_2$	1.5
DDW	6.5
Template DNA 50 ng/µL	5
Total volume	25

RESULTS

The affected fish showed signs of respiratory abnormalities, such as lethargy, gasping for oxygen above the waters, and fast movement of the operculum. Postmortem examination revealed no obvious indications of disease in the dead fish, but a marbled or striped pattern in the gills, indicating infection and dead tissue that has begun to necrotize, as shown in **Fig. 1.**

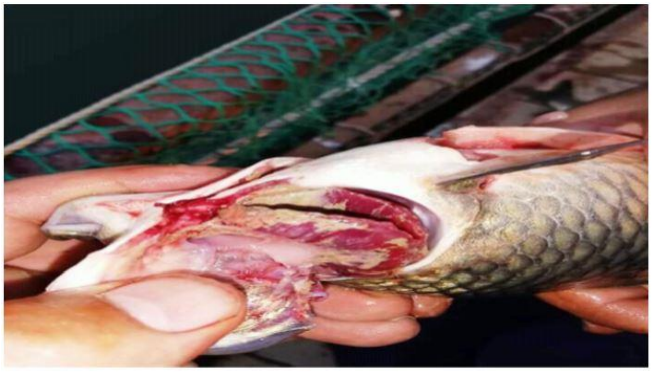

Fig. 1: Macroscopic examination of fish infected with *Branchiomyces Spp*. reveals gills marbling appearance.

The spores were easily visible upon close inspection of the mounted gills under a light microscope. Fungal smears with lactophenol cotton blue stain exhibits non-sseparated hyphae spores (**Fig. 2).**

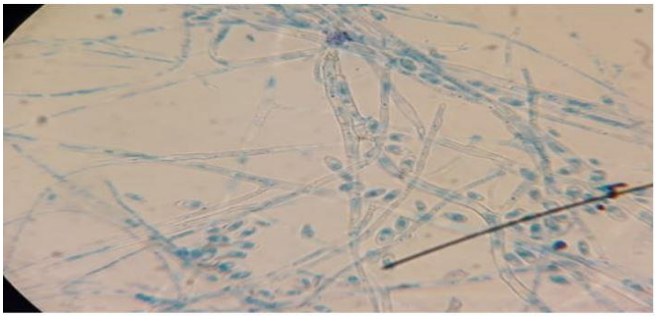

Fig. 2: Microscopic examination of fungal non-separated hyphae and spore stained with Lactophenol cotton blue stain 100X.

Upon histopathological inspection, the infected gill showed fusion of mainly lamellae and heavy growth of the gill filament, as well as the presence of many spores and uniseptate hyphae. As a result of the spores and hyphae blocking, hemostasizing, and thrombosing the blood vessels of the gill, large swaths of the gill filaments died and turned brown. Large, macrophage-like cells formed when they dispersed into the surrounding tissue, and their spread led to the formation of these cells with a marginal, flattened nucleus. Epithelial cells that had proliferated into a thicker layer arranged themselves in concentric rings around the fungal hyphae. The hyphal wall appeared to be encased in a thin layer of homogeneous matrix, the result of cellular necrosis in the intermediate area **Fig. 3-5.**

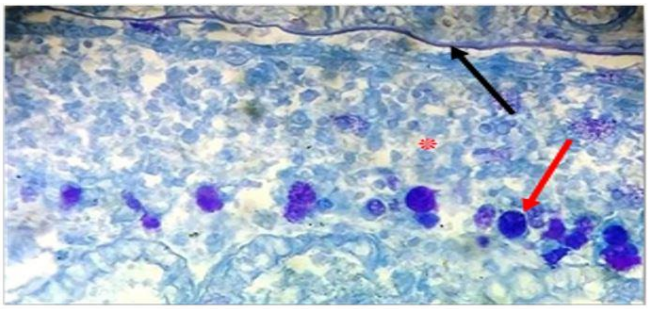

Fig. 3: Histological section of infected gill revealing necrotic gill tissue (*) with present fungal hyphae (black row) and spores (red row) H and E stain 100X.

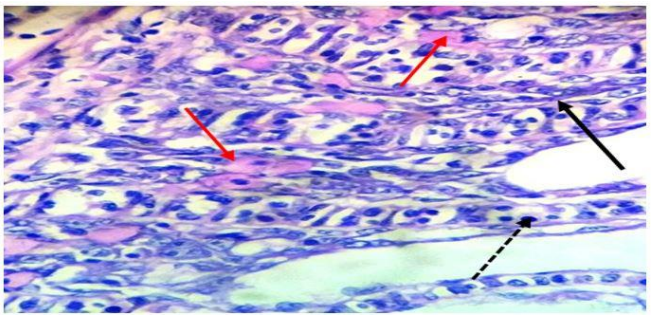

Fig. 4: Histological section of infected gill revealing adhesion of gill filament (black row), vacuolar degeneration of pillar cells (black dot row), and hyperplasia of mucus cells (red row) H and E stain 100X.

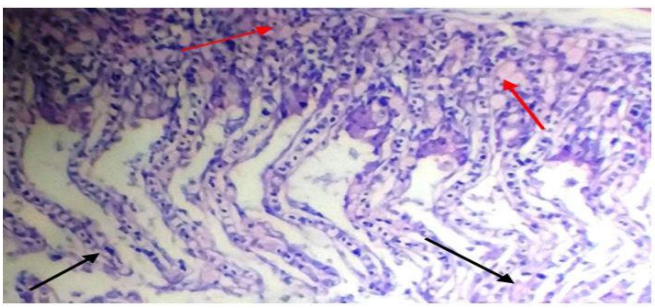

Fig. 5: Histological section of gill of fish infected with *B. sanguinis* reveals Hyperplasia of mucus cells (red row) necrosis in primary gill filament (black row) H and E stain 100X.

Using universal primers, the isolated DNA was amplified so that a molecular identification of *Branchiomyces spp*. The amplification PCR results revealed the target identified 540 bp DNA fragment **Fig. 6,** which points to the fungus *Branchiomyces* as the source. All of the samples have been examined, and all results was positive.

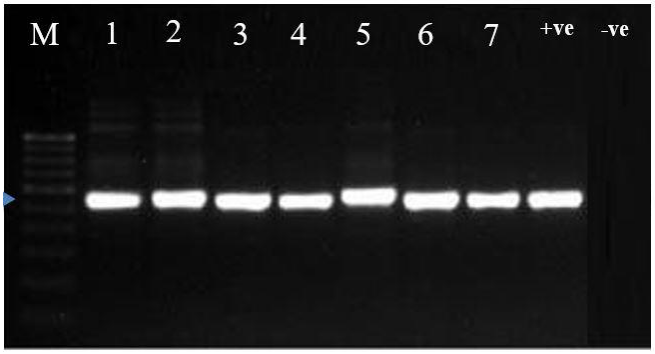

Fig. 6: ITS PCR products of fungal isolates on 2% (w/v) agarose gel at 540bp M: represent Marker 100 – 1000 bp; Samples (1 – 7): represent positive samples.
+ve: represent control positive. -ve: represent control negative.

DISCUSSION

Diseases can affect fish just like they can affect any other species. Branchiomycosis is a fungal disease that is feared by fish farmers everywhere, but notably those that raise carp **(Ramaiah, 2006)** in enclosed environments. Branchiomycosis, often known as "Gill Rot," is a fungal problem caused by the fungus *Branchiomyces*. Two species, *B. sanguinis* and *B. demigrans*, are typically found together; both of them produce hyphae **(Khalil, *et al.*, 2015).** Field observation focuses on case history, clinical, and gross indications of disease, while laboratory diagnosis relies mostly on proving the spores and hyphae of the fungi via separation in the culture technique and histological investigation **(Ganguly *et al.*, 2016; Adeshina *et al.*, 2019; Mondal *et al.*, 2023).**

The current study confirms previous reports **(Khalil *et al.*, 2015; Ganguly *et al.*, 2016)** that infected fish exhibit respiratory disorder symptoms such as rapid operculum movement and gasping at the surface of the water, and that infected pathognomic lesions on the gills cause them to appear striated or marbled. **(Yanong, 2003).**

The diameters of the hyphae of *Branchiomyces spp*. range from 12 to 20 µm, while the diameters of the spores range from 5 to 7 µm. These fungal measurements are in conformity with those provided by **El-Bouhy *et al.*, (2014)** which are attributed to *B. sanguinis*.

Only these fungi can infect the gills, which produces localized damage to the gills that is referred to as "gill rot." This condition leads to acute signs of respiratory illness and significant mortality owing to anoxia, and the results are comparable to those that have been documented **(Flores-Lopes and Thomaz, 2011; Mishra *et al.*, 2017; Hussain and Kadhim, 2020).** The work confirms that fungal spores and hyphae infect gill blood vessels, gill arches, and the base of the primary lamellae, leading to an infarctive necrosis of the gill that blocks blood flow and leads to thrombosis (hence the term "gangrenous bronchitis") **(El-Bouhy *et al.*, 2014; Adah *et al.*, 2023).** These findings are in agreement with those of **Mishra *et al.*, (2018),** who reported the presence of infections of *Branchiomyces* in carp in Europe**.** *Branchiomyces* is a fungus that can be extremely harmful to fish and is difficult to treat.

The gill histology of affected fish reveals extensive proliferation of gill filaments, spores, and non-septated hyphae, as well as the fusion of primary lamellae. These hyphae hold sporangium-like bodies with multiple nuclei, as well as sporonts that have been divided into uninucleate or binucleate bodies at different developmental stages **(Kumar *et al.*, 2020).** These findings corroborate those showing that some fungal sporonts leave behind necrotic residues of congestion and localized damage to the epithelial layer **(Adeshina *et al.*, 2019; FAO, 2020; Hussain and Kadhim, 2020).**

This study detects *Branchiomyces spp.*, which is supported by PCR successfully confirming that an infection with *Branchiomyces* has occurred. **Tandel *et al.*, (2020)** non-coding ITS areas. Primers for the amplification of fungal sequences are readily available, which contributes to the widespread use of ITS1 of rRNA as a genetic marker. ITS1 sequences have relatively high levels of sequence diversity **(Belbahri *et al.*, 2008; Rahman and Sarowar, 2016).** These codes are located between two coding regions, the 18S and 28S genes. To confirm infection with *Branchiomyces*, molecular analysis of such regions was used in common carp *C. carpio* **(Belbahri *et al.*, 2008).**

Poor environmental conditions are one of the most important contributors to the induction of branchiomycosis in fish that are naturally afflicted with the disease. The outbreak of the disease is caused by environmental factors such as the high temperatures that occur during the summer, as well as poor management practices such as the improper transport and treatment of fish that are brought in from other ponds. **Opiyo *et al.*, (2020),** Consequently, in accordance with **Belbahri *et al.*, (2008),** this helped speed up conditions ideal for the spread of this fungus. Bioassay results and the interpretation of such data can be complicated by water quality characteristics such as temperature, dissolved

oxygen levels, nitrogenous wastes, and so on, as well as bacterial and other pollutants**. (Ganguly *et al.*, 2016; Mishra *et al.*, 2017; Ali *et al.*, 2020),** so it's important to take appropriate quality control measures for these variables when investigating mycotic diseases.

CONCLUSIONS

It can be concluded that common carp tissue infected with Branchiomyces spp. has been identified molecularly. This is supported by histological findings of lesions in the gills of diseased fish. To better design a mycotic infection control program to reduce or completely eliminate economic losses, more research is required to better understand the environmental distribution of infected fungal-like pathogens and other related mycotic pathogens in Iraqi fish farms.

Authors' Contributions

All authors took part in reading and editing the paper; ZMA and SKI were responsible for collection of samples and experimental designing MTM analyzed, interpreted, and drafted the report. The final manuscript was read and approved by all writers.

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Conflict of interest

The authors declare that they have no conflict of interest.

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