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# Isolation and Molecular identification of Aspergillus species from Tor mahseer (*Tor tor)* in River Narmada

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**Abstract:**

The present study was carried out on 110 examined fishes, out of which 70 were diseased fish and 40 healthy. The samples were collected alive from River Narmada at different sites in Indore from March to November 2019. Grossly examined diseased fish revealed different sized ulcers, eroded fins, redness on the skin, eye turbidity, protruded anal and de-scaling of skin, especially in fish naturally infected *with Aspergillus, Saprolegnia, mucor, Penicillium* and *Alternaria*. Mycological examination revealed isolation of 9 genera of fungi from a total incidence of 260 fungal isolates, 210 fungal isolates from diseased fish and 50 isolates from an apparently healthy one. *Aspergillus flavus* and *Aspergillus niger* were the most predominant fungi isolated from either apparently healthy or even diseased internal organs and from external wounds of fish with Sabouraud Dextrose Agar (SDA) medium. Molecular identification was performed using the inter-transcribed spacer (ITS) gene for *Aspergillus*. Developed PCR assay specific primer detect *A. flavus* and *A. niger* showing clear bands at 595 and 600 bp molecular weight, respectively.

## Introduction:

Narmada, the oldest river system in India, also called the *Rewa* and "Life Line of Madhya Pradesh" is a river in central India that originates from Amarkantak in Madhya Pradesh, flows about 1163 kilometers and joins with the Gulf of Cambay on the Arabian Sea. It is the fifth largest river in India and also longest west flowing river of Indian peninsula. It is important for its huge contribution to the state of Madhya Pradesh in many ways. The river has a rich diversity of fresh water fishes including fish species like *Tor tor, Catla catla* (Ham) *Cirrihinus mrigala, Cyprinus carpio, Labeo rohita, Labeo calbasu, Labeo fimbriatus, Rita rita, Cirrihinus cirritosa, Cirrhinus reba, Labeo bata, Puntius choca, Mystus seenghala, Chanda nama* etc (D.Saini and KK Dube, 2017). After *Tor putitora*, the Tor mahseer *Tor tor* (Hamilton, 1822) is the most significant food and game fish in India (Hamilton, 1822). It constitutes an excellent fishery in the Narmada River in central India. It has also established itself in certain Indian lakes where this fish has been stocked (Desai, FAO 2003).

It is a known fact that the ecology and diversity of fishes is correlated with various physico-chemical and biological parameters of its environment, a series of dams were constructed to hold some of the water resources of Narmada river has led to some drastic changes in water quality, productivity, and aquatic flora and fauna of the river system. This has resulted in various types of diseases in different fish species inhibiting the river that has drastically influenced the productivity and distribution of different species of fishes.

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Fish is one of the key sources of animal protein available throughout the world and an extensively established as a good source for maintaining body health. However, with more intensification to meet consumer demand, a major problem faced by the progress of this sector is that fish often succumb to infectious diseases.

The primary causes for fungal infections are, the change in water temperature, decreased water quality, injury due to trauma or excessive handling, or another disease caused by bacteria or protozoan (Chauhan *et al*., 2014).

The oomycetes (fungi) are the second most damaging pathogens of freshwater fishes after bacteria. Fungal infections in fish are considered secondary infections. One of the most destructive oomycete pathogens of fish is *Aspergillus*. It is endemic to all freshwater habitats and is responsible for the decrease in natural fish populations. Infections caused by the species have been responsible for immense death in populations of fishes in the river. The disease symptoms include white or grey patches on the mouth, heads or fins (caudal, adipose and anal fins) of the body. This study reports the isolation and identification of fungal pathogens associated with *Tor tor* fishes in the Narmada River, Madhya Pradesh.

**Material and methods.**

A total of 110 sample fishes were caught alive from different sites of the Narmada river at Indore from March to November 2019. Water temperature was noted to be ranging from 29°C to 33°C. The fishes were transported immediately to the laboratory inside polyethylene bags. The fish were transferred in a well-aerated aquarium and were provided with artificial feed. The water temperature of the aquarium was maintained in a range varying from 18°C-20°C.

**Isolation and identification of the fungi from infected fish**

Samples were taken from different parts of the body of fish showing lesions using a sterile dissecting needle, according to Noga, (1993*)*. The samples were taken from the skin, gills, muscles, and internal organs and washed thoroughly with distilled water

These tissues were inoculated over plates containing media like Sabaroud's dextrose agar (SDA). Antibiotics including streptomycin and penicillin were added to the medium to check any bacterial growth. The inoculated plates were incubated at 25℃ and 37℃ for 3-5 days until fungal colonies developed (Whitman, 2004). Fungal Isolates were then used to make pure cultures by sub-culturing on SDA plates and transferred to the plates containing baits like soya seed with sterilized water.

**Isolation and identification of yeast species:**

Fungal colonies were examined with the help of a microscope. Identification of the morphological and physiological characteristics of the yeast isolates, including growth rate, surface texture, and color, was done according to Refai *et al.*, (2010). A small amount of fungal growth was placed on a glass slide with a drop of distilled water, the mycelial mass was carefully torn apart with two dissecting needles, and the slide was covered with a clean

cover slip before being examined under the microscope. The procedure outlined by Bassiouny *et al*.(2019) was used for the confirmation identification. In addition to that, staining of the fungal culture on the slide with lactophenol cotton blue (LPCB) for microscopic examination was performed.

**Molecular Identification:**

Mold identification using molecular techniques following the manufacturer's instructions, genomic DNA was isolated from a pure culture of fungal isolates using the DNA extraction Kit (Qiagen, Hilden, Germany). Fungal colonies were inoculated into SD broth transferred from SDA plates and incubated for one week at room temperature; then, 50 mg of cultured mycelium was centrifuged at 300xg for 10 minutes. Phosphate buffered saline (PBS) was used to wash the mycelia. The material was entirely mixed by vortexing after adding 20 l proteinase K, buffer AL, and centrifuged at > 6000 g (8000rpm) for 2 minutes. Buffer AW1 was added and spun for 2 minutes at > 6000 g (8000rpm), while buffer AW2 was added and centrifuged for 3 minutes at 20,000xg (14,000 rpm ). A fresh 2 ml microcentrifuge tube was used to house the spin column. DNA was eluted by pouring 200 µl of AE buffer into the middle of the spin column membrane, incubating for 2 minutes at room temperature, then centrifuging for 2 minutes at > 6000 g. (8000 rpm). Pre-designed oligonucleotide primers were used to amplify the ITS gene, ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) White *et al*. (1990).

A PCR reaction was set up with 6 µL of extracted DNA, 1 µL of forward and reverse primers (20 pmol), 12.5 µL of Emerald Amp GT PCR master mix (2x premix), and sterile water up to a final volume of 25 µL to amplify the DNA. The PCR reaction was carried out in a thermal cycler, with an initial cycle at 96°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 56°C for 50 seconds, and 72°C for 60 seconds and a final step of 72°C for 10 minutes of prolongation. PCR amplicons were extracted and analyzed using gel electrophoresis, which involved loading products onto a 1.5 percent agarose gel, imaging bands using a UV transilluminator at a wavelength of 302 nm, and staining with ethidium bromide.

## RESULTS AND DISCUSSION

Mycotic diseases of fish can be considered as one of the important affecting the decline of fresh water fishes observed in recent years. Thus there is a necessary requirement to isolate and identification of infectious moulds from affected fishes.

**Clinical & Postmortem examination** In the present study, the clinical signs of the diseased fishes including different sized ulcers, eroded fins, redness on the skin, eye turbidity, protruded anal and de-scaling of skin. Since fungal infections are considered as secondary invader pathogen, the ulcers on the skin must be the lytic action of primary bacterial infection as described earlier. (Meyer 1991). The extracellular components of moulds can cause redness on the skin, leading in severe clinical changes on the fish in the form of hemorrhagic spots as described earlier. (Bassiouny *et al*., 2019). The postmortem findings revealed an enlarged liver with significant hemorrhagic patches. This result may be attributed to either the

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fact that most mycotic diseases are secondary invaders following infections by bacterial pathogens, which is responsible for internal lesions, However, in recent years, this viewpoint has been disproved, as it has been discovered that it actively suppresses host immunity during the infection process. (Belmonte *et al*., 2014; de Bruijn *et al*.,2012; Minor *et al.*, 2014; Wawra *et al*., 2012).Thus It could also be the result of fungus producing extracellular chemicals that reduce hepatic competence, causing congestion in the liver and other internal organs. (Bassiouny *et al*., 2019; Ashraf A *et al*., 2020; van den Berg *et al.*, 2013).

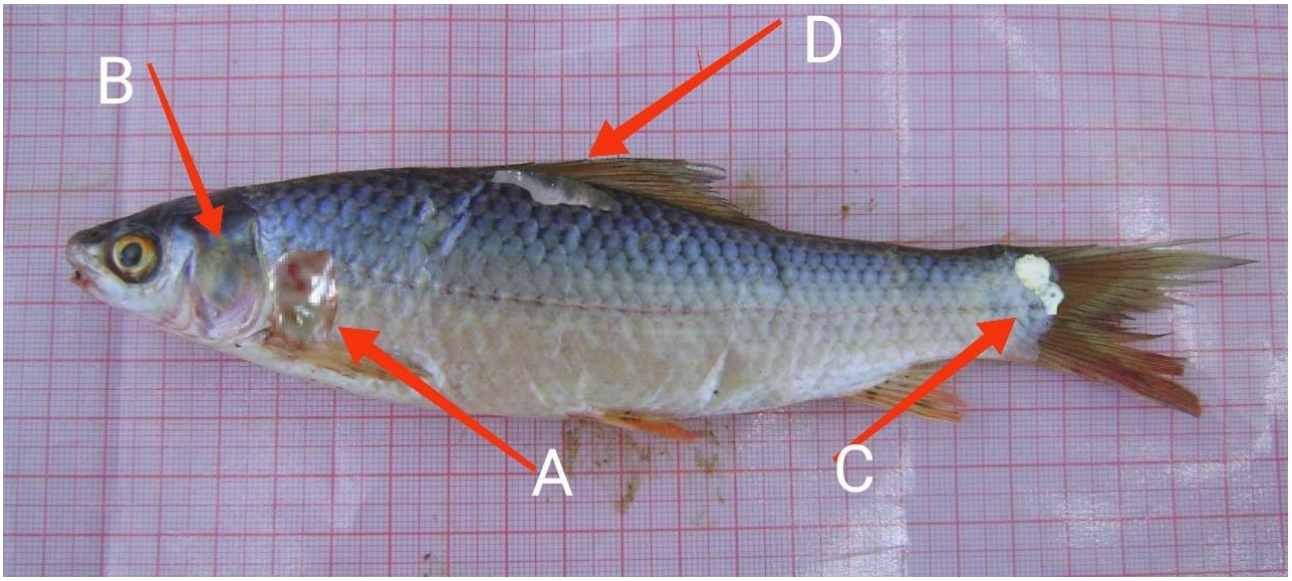


Fig.1 Photograph showing *Tor tor* infected with *Aspergillus sp.* A) Body ulcer B) Damaged gills C,D) Fungus on tail fins and tail

**Mycological examination**: Mycological examination revealed the isolation of 360 fungal isolates; 210 from diseased and 50 from healthy ones, from different parts of the body including skin, fins, gills, kidneys and liver. The high recurrence of isolates were evident in *Tor tor* showing clinical signs; with the result *A. flavus* was the most prominent among both diseased and healthy *Tor tor* while Aphanomyces sp. was the least prevalent isolates. The result is coincided with that reported by Abd El-Tawab *et al*. (2020).

**Morphological identification of isolated molds**

Isolated moulds were morphologically identified. Molds such as *Aspergillus flavus, Aspergillus niger, Aphanomyces sp., Penicillium sp., Fusarium sp., Mucor sp., and Alternaria sp*. were discovered through morphological identification. *A. flavus* was the most commonly isolated fungus in both diseased and healthy fish, while *Aphanomyces* sp. was the least common isolate. Within the genus *Aspergillus*, there was some variation. In *Aspergillus flavus (A. flavus),* The conidiophores were long and harsh, the vesicles were rounded and huge, and the stigmata were biseriate, loose, and radiate, giving rise to ovoid rough conidia. Microscopically, the conidiophores were long and harsh, the vesicles were rounded and huge, and the stigmata were biseriate, loose, and radiate, giving rise to ovoid rough conidia (Fig. 2A). *Aspergillus niger (A. niger)* colonies featured black colouring, emanated edges, and a woolly surface, Microscopically, the conidiophores were exceptionally long, smooth, and

yellowish, the vesicles were enormous and globes, the stigmata were biseriate, compact, and radiating, and the conidia were globes and smooth (Fig. 2B). In most cases of *Aspergillus sp.*, the highest incidence was found in the liver. This could imply that Aspergillosis is a systemic infection. The outcome is comparable to that of some prior studies (Diab, 2010; Refai *et al*., 2010 and Abd El-Tawab *et al.* 2020).

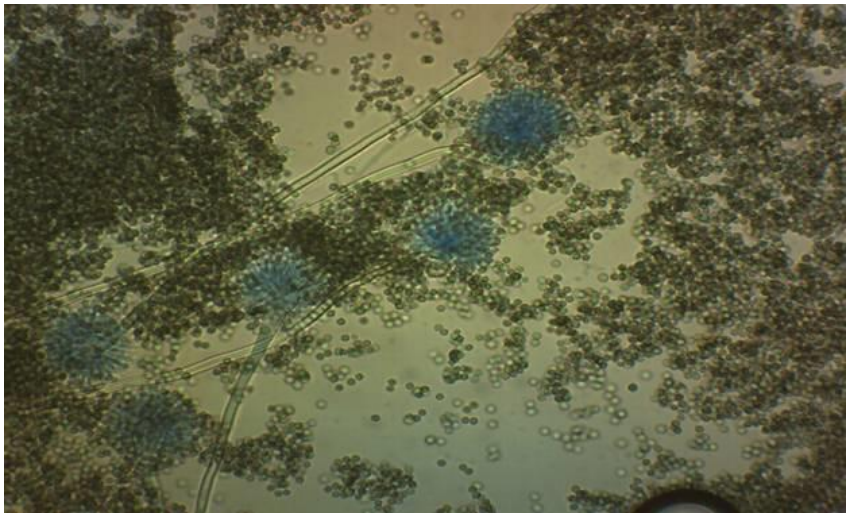

Fig 2. A). Showing photomicrograph of *Aspergillus flavus* (400X)

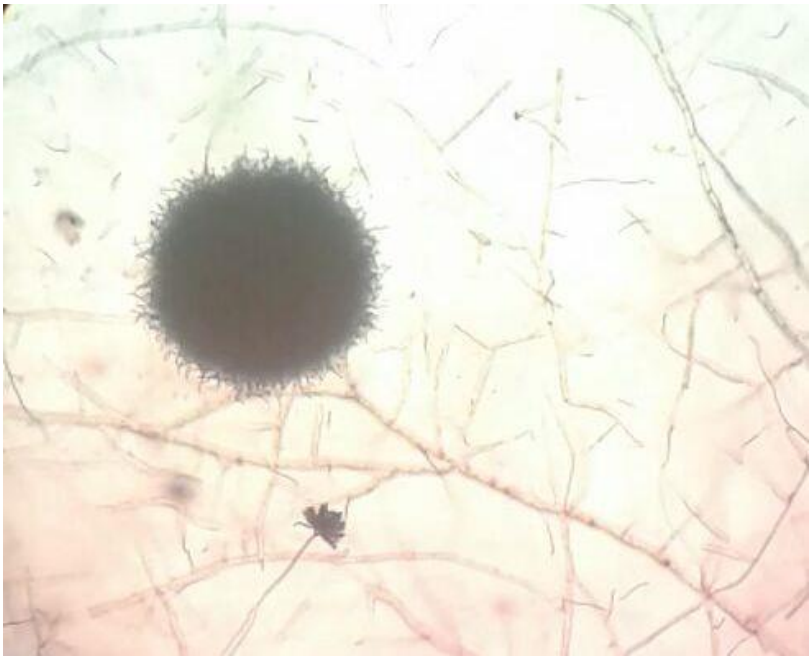

Fig 2. B) Showing photomicrograph of *Aspergillus niger* (400X)

**Molecular identification Molecular identification**

One of the most important techniques to deal with diverse fungal diseases is to use PCR with the inter-transcribed spacer (ITS) gene, which is a universal gene (Eissa *et al*., 2013). As a result, the most common isolates among ill or apparently healthy fish were molecularly

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identified using the ITS gene in the current investigation. Two isolates of *Aspergillus flavus* and two isolates of *Aspergillus niger* were used. Not only does the developed PCR assay particular primer detect *Aspergillus flavus*, which shows a distinct band at 595 bp molecular weight (Fig. 3A), but it also detects *Aspergillus niger*, which shows clear bands at 600 bp molecular weight (Fig. 3B). The current result is similar to Henry *et al*., (2000), who found that *Aspergillus flavus* and *Aspergillus niger* produced a respectable band at 595 and 599 bp, respectively. As a result of the diverse amplification cycles, these variances may be attributed to several tested primer sequences and PCR procedure (Afzali *et al*., 2015 and Youssef *et al*., 2015).

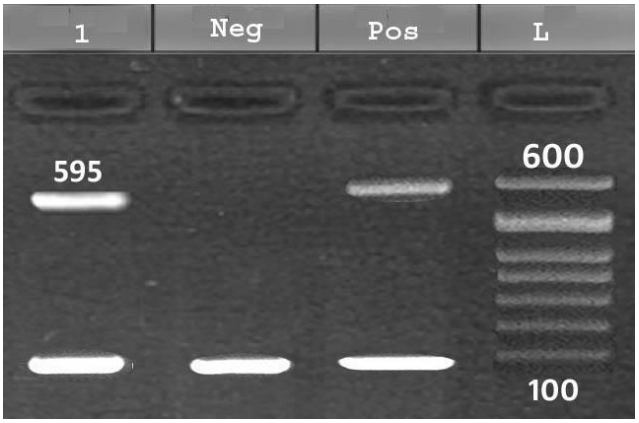
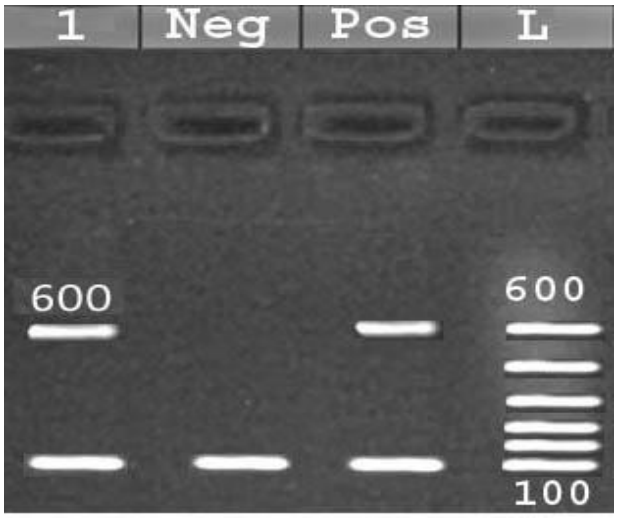



Fig 3. A) PCR results reflecting amplification of 595 bp of the inter-transcribed spacer (ITS) gene in *Aspergillus flavus* on an agarose gel electrophoresis. Lane 1 contains a 100-bp DNA ladder. Pos. : indicates that the control is positive. Neg. for control negative. 1: shows that sample that is positive for *Aspergillus flavus*




B) PCR results reflecting amplification of 600 bp of the inter-transcribed spacer (ITS) gene in *Aspergillus niger* on an agarose gel electrophoresis. Lane 1 contains a 100-bp DNA ladder. Pos: indicates that the control is positive. Negative: the control is negative. 1: *Aspergillus niger* positive sample

## Conclusion

*Aspergillus flavus* and *Aspergillus niger* were identified from both clinically afflicted and apparently healthy fishes from external body surface and internal organs, indicating that Aspergillosis is a systemic illness, according to the current study. In a nutshell, even though most fungi are classified as typical mycoflora, they can nevertheless infect fish. As a result, it is recommended that suitable health management procedures be adopted when rearing fish to reduce the risk of fungal infection.

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