The Assessment Of Mycotic Settlement Of Freshwater Fishes In Egypt

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Abstract: This study was carried out on 360 freshwater fishes (240 *Oreochromis* species and 120 *Clarias gariepinus*). They were collected from different governorates and during different seasons. Naturally infected fishes showed clinical abnormalities such as skin darkening, exophthalmia, corneal opacity, abdominal distention, ulceration of the skin and cotton wool like growths on various parts of the body. Fishes were then subjected to post mortem examination which revealed many abnormalities. Mycological examination revealed the isolation of 2081 fungal isolates from 150 diseased and 210 apparently healthy fish samples (1658 mould and 423 yeast isolates), of which 1334 were isolated from Oreochromis species and 747 isolates from Clarias gariepinus. Isolated moulds belonged to the following genera: Saprolegnia (4.2%), Aspergillus (43.0%), Fusarium (14.1%), Mucor (14), Penicillium (17.2), Rhizopus (4.8%), Scopulariopsis (1.2%), Paeciliomyces (1%) and Curvularia (0.4%). Yeasts isolated also from both fish species had the following incidence: Candida albicans (35.9 %), other Candida species (19.1%), Rhodotorula species (31.4%) and Torulopsis species (13.5%). Experimental infection with the most predominant fungi (Aspergillus flavus, Fusarium species and Candida albicans) was conducted to evaluate the pathogenicity of these isolates. Clinical pictures of experimentally infected fish were similar to those of natural infection. Inoculated fungi were re-isolated from different organs. Results were confirmed with histopathological examination, which revealed the presence of fungal hyphae and spores in different organs.
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Keywords: Mycotic infection, Oreochromis species, Clarias gariepinus, Moulds, Yeasts, Aspergillus, Fusarium, Candida, Penicillium.

1. Introduction

Many of the fungi that affect fishes are considered opportunists, attacking the fishes when they are stressed or immunocompromised because of unfavorable environmental conditions, or secondary to bacterial or viral infections, or when they have lost their mucus protection because of trauma or excessive handling (Roberts 1989 and Quiniou *et al*., 1998). Mycotic infections of fishes by Oomycetes are wide spread in freshwater and represent the most important fungal group affecting wild and cultured fishes. The *Saprolegniaceae*, in particular members of the genus *Saprolegnia*, are responsible for significant infections involving both living, dead fishes and eggs. Oomycetes are classical saprophytic opportunities, multiplying on fishes that are physically injured, stressed or infected (Pickering and Willoughby, 1982). Members of this group are generally considered agents of secondary infection arising from conditions such as bacterial infections, poor husbandry, and infestation by parasite and social interaction. However, there are several reports of Oomycetes as primary infectious agents of fishes (Pickering and Christie, 1980) and their eggs (Walser and Phelps, 1993). Moreover, there are other fungi that have been implicated in fish diseases. Some of the genera involved include *Aspergillus* (Salem *et al.,*1989b), *Fusarium* (Bisht *et al*., 2000), *Ichthyophonus* (Faisal *et al*., 1985), *Branchiomyces* (Easa 1984), *Phoma* (Hatai *et al*., 1986), *Paecilomyces* (Lightner *et al*., 1988), *Exophialia* (Langdon and MacDonald 1987), *Phialophora* (Ellis *et al*., 1983), *Rhizomucor* (Wolf and Smith 1999) and *Candida* (Neish and Hughes1980). Most of these are multiple case reports or single, and causing systemic disease with high mortality rates in fishes. The objective of this study was to determine the types of fungal pathogens affecting freshwater fishes specially those causing high mortality rates, elucidation of the incidence and distribution of such pathogens in *Oreochromis* species and *Clarias gariepinus*, studying the seasonal variations enhancing fungal diseases of fishes and determination of the pathogenicity of the most prevalent isolated fungi.

2. Material and Methods

A total number of 360 fish were observed for their behavior, external lesions prior to autopsy. Then they were killed and examined. The examination included external changes as well as examination of internal organs. Wet mount preparation of fish samples were commonly made in 10% KOH. A simple stain such as lactophenol cotton blue was used. The preparation was examined microscopically after about 30 minutes for fungal elements. Mycological examination was done according to and (1993 Noga). Identification of moulds was carried out according to Refai (1987). Identification of yeasts: Plates were examined microscopically for the presence of chlamydospores, arthrospores and blastospores (Refai, 1987) and the scheme of identification of yeasts given by Terrence (1971). Urease test (Cruickshank *et al*., 1975). Suspected *Candida* species were scratched onto rice or corn meal agar for pseudohyphae and chlamydospores production (Larone, 1987) and a confirmatory identification was carried out by germ tube test (Martin, 1979).

Histopathological examination:

Tissue samples were prepared according to Roberts 1989. and stained by periodic acid Schiff´s (PAS) and GMS (Sheehan and Hrapchak 1980) .

Experimental infection:

A total of 120 *Oreochromis* species with 30-40 g average body weight were used. They were divided into four equal groups (each one contained 30 fish). Each group were subdivided into three sub-groups, each contained 10 fish.

Preparation of spores suspension for experimental infection: Inocula were prepared by spreading 5 ml of sterile phosphate buffer saline over the plates containing 7- 10 day old pure cultures of *Aspergillus flavus* and *Fusarium* sp. The spores were harvested by gentle washing of the surface of the colonies with sterile loop, then transferred aseptically to sterile flasks. Two drops of tween 80 were added to avoid clumping of spores in case of *Aspergillus flavus* group. Spores were counted by aid of haemocytometer and suspension was diluted to reach 9X 10^4 spores/ml for both *Aspergillus flavus* and *Fusarium* sp.

Preparation of yeast suspension for experimental infection: A loopfull of one day old pure yeast culture of *Candida albicans* was added to test tube containing 5 ml of sterile phosphate buffer saline and mixed gently to reach equal distribution. Spores were counted by using haemocytometer then suspension was adjusted to reach $2x10^3$ Candida spores per ml.

3. Results and Discussion

Mycological examination revealed the isolation of 2081 fungal isolates from 150 diseased and 210 apparently healthy fish samples, of which 1334 were isolated from *Oreochromis* species and 747 isolates from *Clarias gariepinus.* identification of fungi into yeasts and moulds revealed that the percentage of moulds was slightly higher in *Oreochromis* species (80.5%) in comparison to that in *Clarias gariepinus* (78.2 %). On other hand, the rate of yeast isolates per fish was slightly higher in *Clarias gariepinus*. Isolated moulds belonged to the following genera: Saprolegnia, Aspergillus, Fusarium, Mucor, Penicillium, Rhizopus, Scopulariopsis, Paeciliomyces and Curvularia. The same fungal isolates were reported by Abdel Alim (1992) and khalil (1993).

The Incidence of moulds in diseased and apparently healthy fishes were recorded in (Fig.1&2), also the incidences of isolated moulds from different organs of *Oreochromis* species (Fig.3) and *Clarias gariepinus* (Fig.4) were detected. Seasonal incidences were seen in (Fig. 5). As these isolates were recovered from apparently healthy and clinically diseased *Oreochromis* species and *Clarias gariepinus* This was expected, as almost all these fungi were categorized by Shaheen (1986) as normal mycoflora. This does not mean that they cannot produce disease. They can better be considered as opportunistic fungi (Refai, 1987) as many of them possess virulence factors, which enable them to cause diseases (Refai *et al.,* 2004), particulary under favourable predisposing condition. Regarding to seasonal incidence *Saprolegnia* species were isolated with high incidenc in Winter, followed by early Spring and late Autumn. This result agrees with Naguib (1994)), who stated that the seasonal variations play an important role in spreading of the Saprolegnia infection among freshwater fishes especially during late Autumn, Winter and early Spring, where the water temperature was low.

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Clinical findings of *Oreochromis* species inoculated with *Aspergillus flavus*, *Fusarium* species and *Candida albicans* revealed that exophthalmia (Photo.21), skin darkening (Photo.22), cotton wool-like growth on various parts of the body (Photo.23&24), moderate abdominal distention (Photo.25) and corneal opacity and haemorrhages allover the body surface (Photo.26). These resultes are supported by Marzouk *et al*.(2003).

Postmortem finding revealed congestion and ulceration of gills (Photo.27), haemorrhagic abdominal fluids, necrotic foci within liver and distention of gall bladder (Photo.28), multiple nodules within spleen (Photo.29) and severe intestinal congestion (Photo.30) were also observed. On the other hand, no clinical or postmortem changes were detected on fish groups maintained at 18ºC. These findings are in agreement with those of Refai *et al.* (1987).

It can be concluded from the results obtained in the present work that, though most fungi isolated from fishes are considered by several authors as normal mycoflora, yet we could prove in the present study that many fungi can cause natural infections. This was confirmed by histopathological reactions characteristic of fungal infection in naturaly infected fishes, and the presence of fungal elements in the lesions. This was substantiated also by experimental infection of fish that induced similar findings as the natural infection, i.e. a clear application of Koch's postulate. This conclusion should direct our attention to the possible role of fungi in affecting fishes industry.

The pathological changes and the fungal elements in tissue sections in naturally infected fishes of various organs are described under each of the following photos (31-39)., stained by either PAS or GMS stains.

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Table (3): Type, average body weight of fish, spores concentration per ml, dose, route of inoculation and temperature.

Fish	Body weight	Number of fish in each subgroup	Inoculated fungi	Dose	Conc.	Route	Temp.	References
Tilapia sp.	30-40 g	10 10	*Aspergillus flavus*	0.2ml 0.2ml	$9x10^4$ $9x10^4$	I.P I.M	18°C	Olufemi *et al.* (1983)
		5 5	Normal saline	0.2ml 0.2ml	_ _	I.P I.M		
Tilapia sp.	30-40 g	10 10	*Aspergillus flavus*	0.2ml 0.2ml	$9x10^4$ $9x10^4$	I.P I.M	26°C	Olufemi *et al.* (1983)
		5 5	Normal saline	0.2ml 0.2ml	_ _	I.P I.M		
Tilapia sp.	30-40 g	10 10	Fusarium	0.2ml 0.2ml	$9x10^4$ $9x10^4$	I.P I.M	22°C	Muhvich *et al.* (1989)
		5 5	Normal saline	0.2ml 0.2ml	_ _	I.P I.M		
Tilapia sp.	30-40 g	10 10	*Candida albicans*	0.2ml 0.2ml	$2x10^3$ $2x10^3$	I.P I.M	22°C	Faisal *et al.* (1986)
		5 5	Normal saline	0.2ml 0.2ml	_ _	I.P I.M		

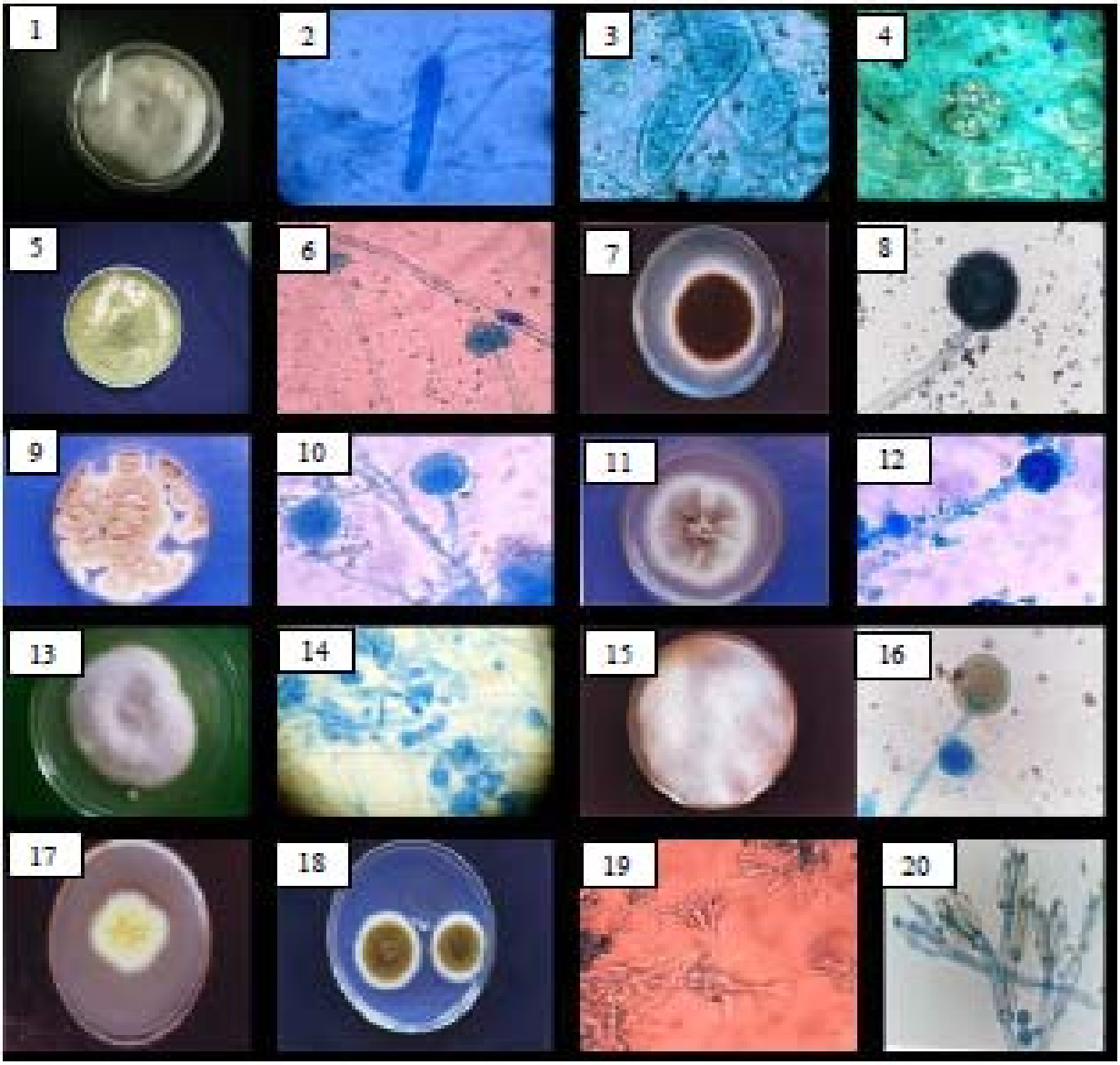

Photo. (1): A colony of *Saprolegnia* species with the characteristic cotton- wool like growth. **Photo. (2):** Non-septated broad hyphae of *Saprolegnia* species (X 200). **Photo. (3&4):** Different stages of reproductive structures of *Saprolegnia* species on hemp seeds (X 400). **Photo. (5):** Colonies of *Aspergillus flavus* on SDA, one weak old. **Photo. (6):** Typical heads *Aspergillus flavus* (X 400). **Photo. (7):** A colony of *Aspergillus niger* on SDA. **Photo. (8):** *Aspergillus niger* showing characteristic round head with black conidia (X 400). **Photo. (9):** Colonies of *Aspergillus terreus* on SDA. **Photo. (10):** *Aspergillus terreus* with small hemispherical vesicle (X 400). **Photo. (11):** A colony of *Aspergillus fumigatus* on SDA. **Photo. (12):** *Aspergillus fumigatus* with columnar head (X400). **Photo. (13):** A colony of *Fusarium* species on SDA with rose pigments on the center. **Photo. (14):** *Fusarium* species with characteristic slender, multicelled conidia (X 200). **Photo. (15):** Colonies of *Mucor* species showing spread over the surface of SDA. **Photo. (16):** Round sporangia of *Mucor* species containing sporangiospores (X 400). **Photo. (17):** *Penicillium* species on SDA with different colour and texture. **Photo. (18):** *Penicillium* species showing brush- like arrangement of fruiting head "A" (X400) and "B" (X 200). **Photo. (19):** *Rhizopus* species colony on SDA showing dens woolly mycelia. Sporangia are seen as small black dots. **Photo. (20):** *Rhizopus* species showing long, branched Sporangiophores and terminate with rhizoids (X200).

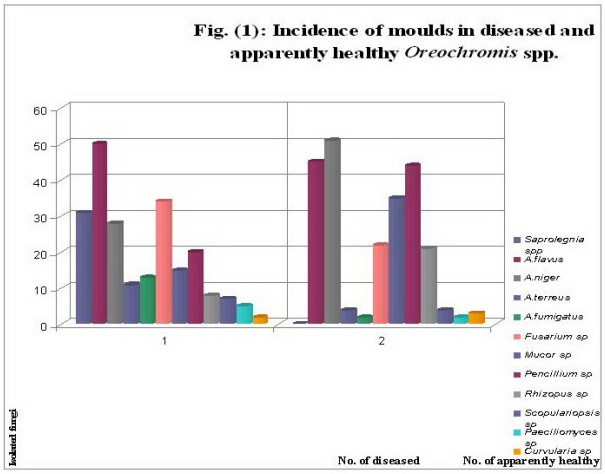
Fig. (1): Incidence of moulds in diseased and apparently healthy Oreochromis spp.
Isolated fungi
No. of diseased
No. of apparently healthy
Saprolegnia spp
A.flavus
A.niger
A.terreus
A.fumigatus
Fusarium sp
Mucor sp
Penicillium sp
Rhizopus sp
Scopulariopsis sp
Paeciliomyces sp
Curvularia sp

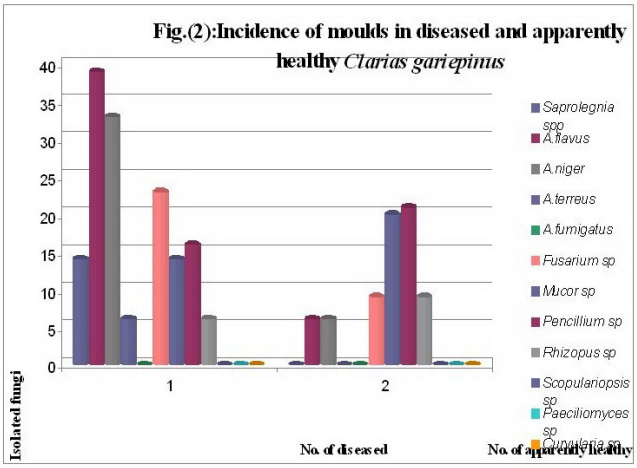
Fig.(2):Incidence of moulds in diseased and apparently healthy Clarias gariepinus
Isolated fungi
No. of diseased
No. of apparently healthy
Saprolegnia spp
A.flavus
A.niger
A.terreus
A.fumigatus
Fusarium sp
Mucor sp
Penicillium sp
Rhizopus sp
Scopulariopsis sp
Paeciliomyces sp
Curvularia sp

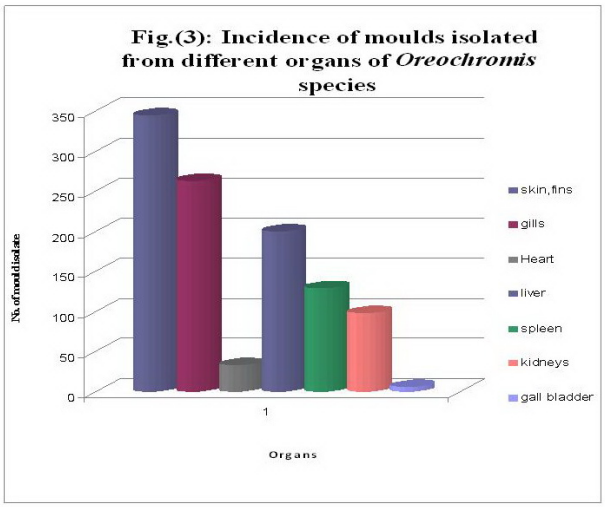
Fig.(3): Incidence of moulds isolated from different organs of Oreochromis species
No. of moulds isolate
Organs
skin,fins
gills
Heart
liver
spleen
kidneys
gall bladder

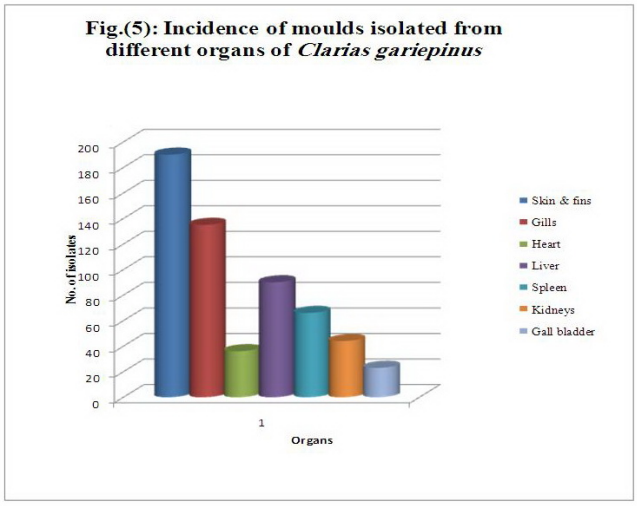
Fig.(5): Incidence of moulds isolated from different organs of Clarias gariepinus
No. of isolates
Organs
Skin & fins
Gills
Heart
Liver
Spleen
Kidneys
Gall bladder

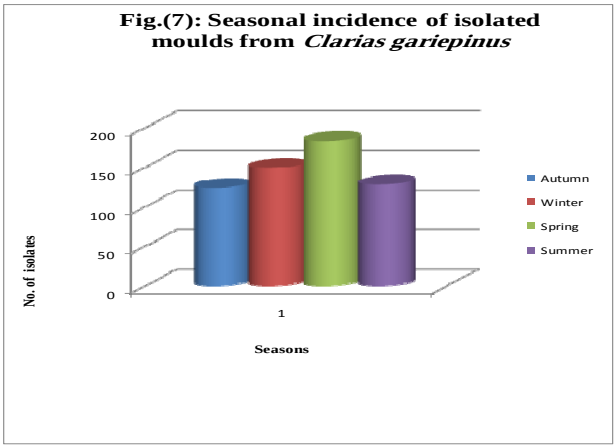
Fig.(7): Seasonal incidence of isolated moulds from Clarias gariepinus
No. of isolates
Seasons
Autumn
Winter
Spring
Summer

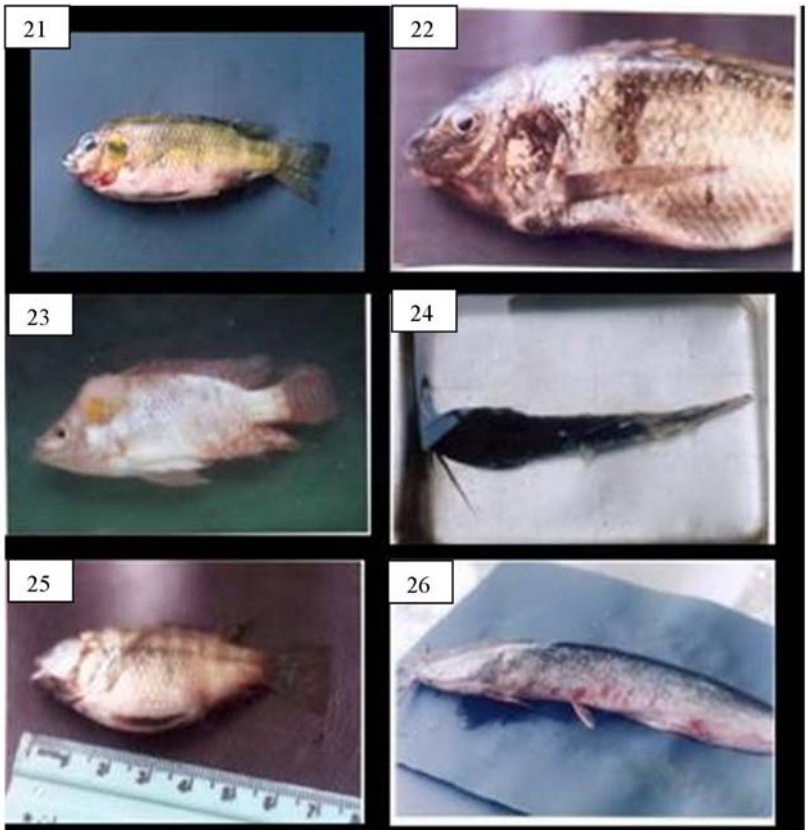

Photo. (21): *Oreochromis* species showing exophthalmia. **Photo. (22):** *Oreochromis* species showing skin darkening. **Photo. (23&24):** *Oreochromis* species and *Clarias gariepinus* showing cotton wool-like growth on various parts of the body. **Photo. (25):** *Oreochromis* species showing ascitis. **Photo. (26):** *Clarias gariepinus* showing haemorrhages allover the body surface.

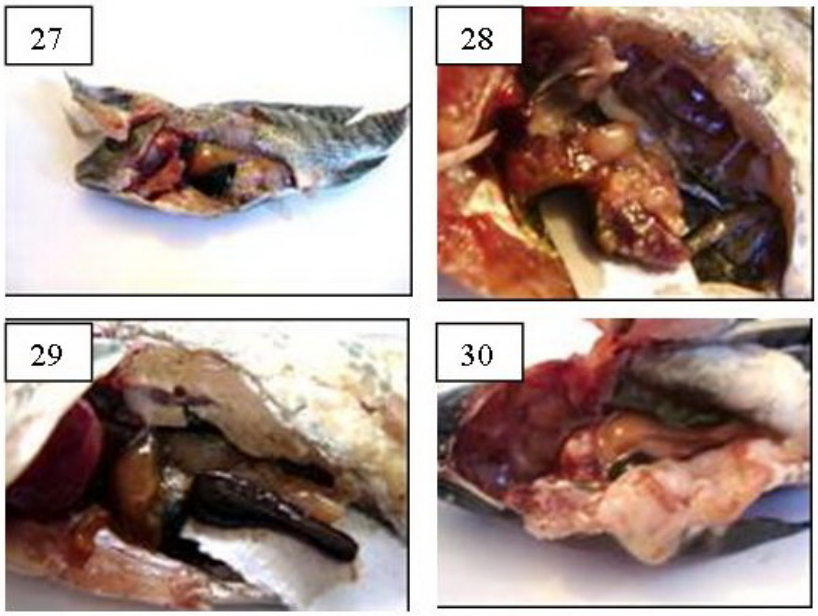

Photo. (27): Liver of *Oreochromis* species showing necrotic foci with distention of gall bladder. **Photo. (28):** Spleen of *Oreochromis* species showing multiple nodules **Photo. (29):** *Oreochromis* species showing severe enteritis. **Photo. (30):** *Oreochromis* species showing severe enlargement of spleen.

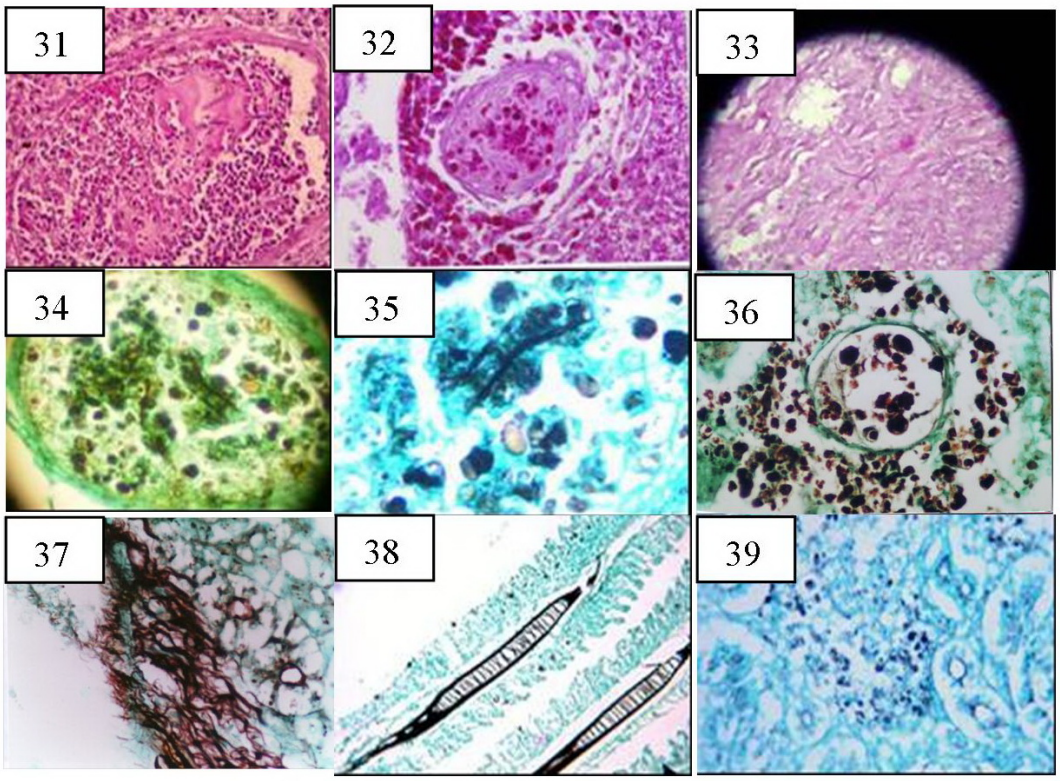

Photo. (31): Spleen section stained with PAS (X400) showing a granuloma formed of epithelioid cells and macrophages surrounded with fibroblasts and fibrous connective tissue capsule. Fungal hyphae appear within the granuloma. **Photo. (32):** Spleen section stained with PAS (X400) showing granuloma consists of epitheloid cells, macrophages and surrounded with connective tissue capsule. Large number of fungal spores appear within and surrounding granuloma.**Photo. (33):** Liver section showing fungal hyphae between the hepatocytes stained with PAS (X200). **Photo. (34):** Liver section stained by GMS (X400) showing granuloma consists of aggregation of epithelioid cells, macrophages and fibrous connective tissue capsule. Fungal hyphae and spores appear within granuloma. **Photo. (35):** Liver section stained by GMS (X 1000) showing fungal hyphae and spores between the hepatic tissue. **Photo. (36):** Spleen section stained by GMS (X 400) showing focal aggregation of spores surrounded with proliferating fibroblasts and fibrous connective tissue in between. **Photo. (37):** Kidney section stained by GMS (X 400) showing hyphal threads in between the interstitial tissues with marked severe degenerative changes in the tubular epithelium. **Photo. (38):** Gills section stained by GMS (X 400) showing yeast cells investing necrosed areas of epithelial lining the secondary lamellae. **Photo. (39):** Kidney section stained by GMS (X 400) showing yeast cells investing the interstitial tissues.

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