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# Pathogenicity of *Exophiala xenobiotica* isolated from infected Crocodile fish for Rabbit fish *Siganus rivulatus* (Forsskål, 1775)

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**Abstract**
In our study, we isolated 49 isolates belonging to three species and four genera from infected Crocodile fish (*Cymbacephalus beauforti*) collected from Hurghada, Red Sea. Of the few fungi isolated; (*Cladosporium cladosporioides, Aspergillus versicolor, Exophiala and Rhodotorula* sp.). The black yeast was identified by molecular sequence analysis of the internal transcript spacer (ITS) region of the ribosomal RNA gene. After sequencing the internal transcribed spacer domain, the fungus was identified as *Exophiala xenobiotica* and it was selected to infect an economical fish (Rabbit fish; *Siganus rivulatus*). Pathogenicity test showed a highly systematic infection of the liver and kidney of Rabbit fish. Histology revealed granulomatous infiltration containing septate, brown-colored dematiaceous hyphae in the liver and kidney while gills showed high damage with no lesion in the skin.

**Keywords:** *Exophiala xenobiotica,* pathogenicity, rabbit fish, crocodile fish

## Introduction
The *Siganidae* fish species have economic importance for the fishery production in several countries in the Indo-Pacific and the Middle East regions as food in many areas and colorful species are popular in the aquarium trade. The fast growth rate and shallow browsing habits of siganids make them ideal for aquaculture [1-4]. Siganus species as a group of fishes contribute about 56% of the total marine fishes landing in Egypt [5].

Fungal diseases are problematic in cultured fish and shellfish, their seeds, and sometimes wild marine animals [6]. Fungal spores are found in all fish ponds and poor water quality which lead to an increase in fungal infections [7]. In many instances, these agents are ubiquitous in nature and affect stressed, injured, or immunocompromised individuals. Some fungi are secondary or opportunistic invaders, but some are serious primary pathogens [8]. *Exophiala* is a genus of family Herpotrichiellaceae belongs to phylum *Ascomycota*, order *Chaetothyriales.* It is the main genus of black yeasts characterized by an annellidic mode of enteroblastic conidiogenesis [9]. Strains are showing high degrees of morphological diversity; most isolates initially grow as a mist to slimy due to yeast-like growth, brown to olivaceous black in color, later becoming velvety due to the development of short, aerial grayish hyphae. *Exophiala* is a genus of dematiaceous hyphomycetes whose taxonomy and nomenclature undergo constant revision [10, 11].

*Exophiala* species are generally considered widely distributed in the environment, including soil, decaying wood, plant material, polluted water and sewage. They are recovering at low frequency from low-nutrients or hydrocarbon-polluted environments. Infections by black yeast-like fungi in cold-blooded animals thus appear to be relatively frequent, at least in captive and farmed fish more than terrestrial animals [9, 12, 13]. *Exophiala* species are difficult to identify morphologically due to variable appearance [14]. In recent years diagnostics have expanded with molecular tools, Internal Transcribed Spacer (ITS) regions sequencing is a highly reliable tool for species identification of strains that are phylogenetically closely related to this black yeast species [15, 16].

During the last decades, the potential pathogenicity of black yeast infections in crustaceans, captive and farmed fish, amphibians, aquarium animals, and other cold-blooded vertebrates has increasingly been recognized as agents of subcutaneous phaeohyphomycosis [17-20].

Reports of black yeast-like infections in warm-blooded animals are relatively scant [6, 7], and these infections are mostly neurological, similar to infections in humans [21]. The presence of melanin and the ability to assimilate alkyl benzenes have been suggested to play an important role in pathogenicity and in evasion of the host defense [19].
*Exophiala xenobiotica*, first isolation was from soil polluted by volatile aromatic compounds [22]. The first case of *E. xenobiotica* infection in fish was reported in cultured striped jack fish [23]. It has the ability to grow in high concentrations of xenobiotics such as xylene, toluene, or creosote-treated utility poles, as well as to cause diseases [24]. The aim of the present work was designed to assess the pathogenicity of *Exophiala xenobiotica* recovered from diseased Red Sea fishes against Rabbit fish (*Siganus rivulatus*).

## Materials and Methods

### Samples collection:

During the period from October 2014 to September 2015,samples of Crocodile fishes (*Cymbacephalus beauforti*) exposed to environmental stress in polluted earthen pond (10 m L, 4m W and 1.5 m D) at the National Institute of Oceanography and Fisheries (NIOF), Hurghada (27 ˚ 17' 3'' N and 33 ˚ 46'21'' E) were collected.

### Fungal isolation

Fungi from infected fishes were isolated on both Potato Dextrose (PDA) agar containing potato extract, 4.0g; Dextrose, 20.0g; Agar, 20.0g; seawater 1000 mL) and Peptone Yeast Extract, Sea Water (PYGS) agar containing 0.125% peptone, 0.125% yeast extract, 3% glucose and 1.2% agar in artificial sea water) at 28 °C for 14 days.

### Morphological identification

Isolated fungi were identified according to morphological features, cultural characteristics such as pigmentation of the mycelium. Identification was accomplished using appropriate taxonomic techniques [22, 23, 25].

**Molecular identification:** Sequencing analysis was performed at Solgent Co., Ltd Bio industry development site. Resultant sequences were compared with available sequences at the National Center for biotechnology information using the neighbor-joining tree of the isolate and close taxa were constructed based on sequences obtained for the ITS regions of the ribosomal gene cluster.

**Pathogenicity test:** Sixty Rabbit fish (*Siganus rivulatus*) samples were caught with round wire fish trap and left to acclimate in inter aquaria for 15 days. Twenty healthy fish samples were used in the pathogenicity test for a period of three weeks. Five fish samples were arbitrarily assigned to either a control or test tank[9]. Tanks were static, containing 70 liter seawater each and equipped with a foam filter for biological filtration and an air stone. Fish samples were fed a commercial diet once daily and 10% water changes were performed daily to avoid build-up of organic matter. Water temperature, pH and salinity were monitored daily using (HANNA Multiparameter HI 9828 HANNA Company, Rhode Island, US).

**Inoculum preparation:** To prepare fungal inoculum for pathogenicity test, *Exophiala xenobiotica* was subcultured on potato dextrose agar (PDA) at 28 °C for 10-15 days, and then flooding with 3 ml of sterile distilled water [27]. Using a sterile loop, the surface gently scraped and the suspension was allowed to stand for 5 min to permit large particles to settle out. Conidial concentrations were manually counted in 0.1 ml of the suspension using (Neubauer hemocytometer Boeckol Co. Scientific equipment, Germany). 0.3 ml of the inoculum was then drawn syringes and stored at 4 °C until use.

**Fish inoculation:** Fish samples were inoculated with 0.1 ml suspension containing approximately $1x10^6$ conidia/ml. Inoculations were administered by intramuscular injection, control groups inoculated with sterile distilled water in the same manner to serve as controls [9]. The skin surface was rubbed using a sterile swab, and the inoculum was painted onto the abraded skin surface using a cotton-tipped culture swab soaked in the 0.1ml inoculum, allowing a contact time of about 30 seconds.

**Microbiology isolation:** After death or euthanasia according to CCAC guidelines [28] all fish samples were weighed and the total length was measured. A swab of the exposed muscle, gills, liver and kidney was taken using a sterile cotton-tipped swab and streaked onto plates of PDA with chloramphenicol incubated at 28 °C examined twice weekly for month to detect olivaceous to grey-black, velvety mold growth, subcultures of the isolates were made on plates of potato dextrose agar (PDA) using the slide culture technique, mounted in lactophenol cotton blue and examined under the microscope.

**Histopathology:** Sections of the exposed muscle, gills, liver, and kidney were collected and fixed in buffered neutral formalin 10% for histopathologic examination according to Nyaoke *et al.* [14] Hematoxylin &eosin stain, Periodic acid-Schiff stain and Giemsa stain used to detect fungal effect on several tissues.

## Results

Forty-nine isolates comprised 3 species of fungi belonging to 4 genera were isolated from infected Crocodile fish (*Cymbacephalus beauforti*) collected from Hurghada, Red Sea. Of all these, *Exophiala xenobiotica* was the most dominant species (42.8 % of total isolates), followed by *Aspergillus versicolor* (24.5 %), *Rhodotorula* sp. (16.3 %), and *Cladosporum cladosporioides* (14.2 %) (Table1).

**Table 1:** Numbers of isolated fungi from skin and gills of infected Crocodile fish (*Cymbacephalus beauforti*) samples

| Strains / Source | Skin | Gills |
|---|---|---|
| ***E. xenobiotica*** | 21 | - |
| ***C. cladosporioides*** | 7 | - |
| ***A. versicolor*** | - | 12 |
| ***Rhodotorula sp.*** | - | 8 |

### *Exophiala xenobiotica* characterization

**Morphology:** Colonies on Potato dextrose agar (PDA) and Peptone yeast extract, sea water agar (PYGS) showed an olivaceous black colony, later becoming velvety due to the development of short, aerial grayish hyphae restricted to olivaceous black. Conidiogenous cells were a lemon-shaped to fusiform and conidia adhering in small groups, subhyaline, obovoidal shape (Figure 1).

**Internal transcribed spacer (ITS) sequencing:** *Exophiala xenobiotica* was identified based on molecular identification

techniques using amplified fragments of ITS and D1/D2 domains of large subunit sequence with 98 % sequence homology with the strain type (Figure 2).

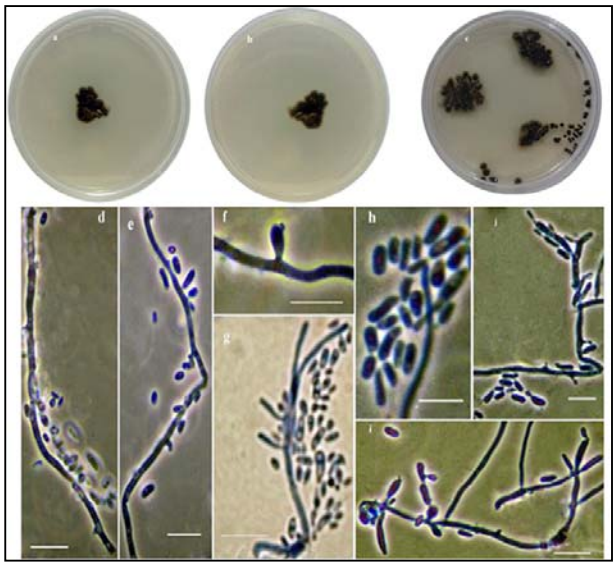

**Fig 1:** *Exophiala xenobiotica* isotated from some Red Sea Crocodile fish (a) Colony surface on PDA, (b) reverse side in PDA and (c) colonies in PYGSafter 7 days at 28 °C (lactophenol cotton blue); (d, e) conidiophores; (f to g) phialophora-like conidial state, phialides and conidia accumulated in balls at apices and tending to slide down (i, j) conidiogenous cells Bars= 10 µm

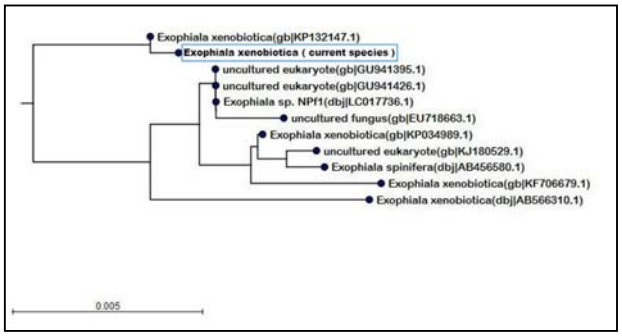


**Fig 2:** Neighbour-joining phylogenetic tree based on ITS sequences showing the distance of isolated strains with the nearest species of *Exophiala xenobiotica*. Bootstrap percentage values as obtained from 50 resamplings of the date set are given at the nodes of the tree. Bar 0.005 substitutions per nucleotide position.

## Pathogenicity

The inoculated Rabbit fish samples started to die on the 14th day after inoculation. Fish became darker and lethargic, with erratic and abnormal swimming behavior. Round yellow to white granulomas were present in visceral organs like liver and kidney with prominent enlargement of the posterior kidney.

**Fungal isolation:** To confirm that *Exophiala xenobiotica* was responsible for the pathogenicity, the fungus reisolated from experimental infected Rabbit fish on PDA from all infected organs. The results showed the ability of the fungus to infect liver and kidney with a range (8/10) and in gills was (6/10) while in muscles were (2/10) (Table 2). There was no growth on plates for control samples.

**Table 2:** Number (out of 10 samples) percentage and frequency of occurrence fungal isolates from exposed and control of fish samples from kidney, liver, gills identified.

| Organ/ fungal occurrence | Kidney | Liver | Gills | Muscle |
|---|---|---|---|---|
| Occurrence | 8 | 6 | 6 | |
| Percentage | 80% | 60% | 60% | 20% |

**Histopathology:** All tested fish samples showed a darker color with black spots on fish skin, but no gross or histologic lesions were identified along the skin surface (Figure 3). Both kidney and liver sections contained regions of histiocytic granulomatous infiltration containing septate and brown-colored dematiaceous hyphae (6/10) for kidney and liver tissues in exposed fish samples. Infected liver tissues showed irregular arrangements of hepatocytes; blood congestion, hemorrhage and necrosis were also detected in the hepatic tissues of most tested fish. Small aggregates of pigmented liver cells appeared in two samples due to environmental stress condition during fungal infection. The gills of this fish showed low frequency, random distribution of respiratory epithelial cells containing granulomatous and mats of fungal hyphae penetrating gill lamellae (4/10) with necrosis infected gill tissue (Figures 4, 5).

**Fig 3:** (a) natural infected Crocodile fish with mouse lesion, (b) experimental infected rabbit become darker with different black spots on the skin with no gross lesions.

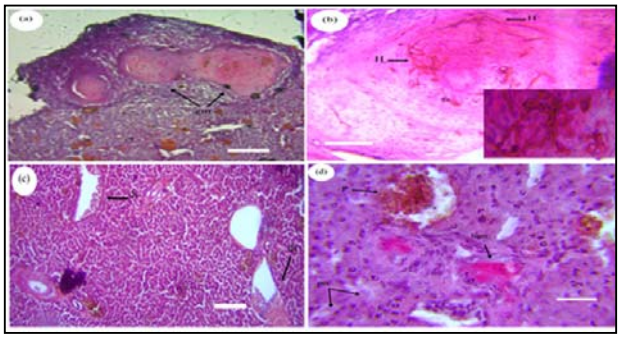

**Fig 4:** (a) Photomicrograph of Rabbit fish liver tissue infected by *Exophiala xenobiotica* showing Granulomatous (Gen) inflammation with melanized fungal hyphae. (b) Higher magnification of the liver granuloma show septate cylindrical fungal hyphae (H) Periodic acid Schiff Stain (PAS). Scale bar =25 µm. (c) Infected liver associated with necrosis and an influx of inflammatory cells and blood congestion (ds) Hematoxylin &eosin stain. Scale bar =20 µm. (d) Infected liver tissue with hemorrhage (hem), necrosis (nec) pigmented cells due to environmental stress. Periodic acid-Schiff Stain (PAS). Scale bar =25 µm

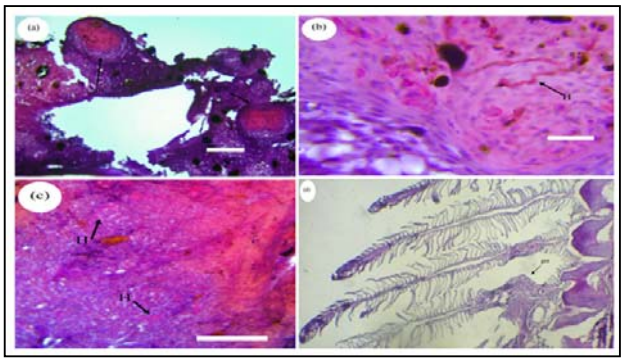

**Fig 5:** (a) Photomicrograph of kidney granuloma inflammation (grn) and hyphal elements. (b) Higher magnification of the affected kidney showing melanized intralesional fungal hyphae (H). Scale bar =25 µm. (c) Infected kidney show spread of fungal hyphae. Periodic acid-Schiff Stain (PAS). Scale bar =50 µm (H). (d) Microscopic image of the gill with granulomatous (grn) and mats of fungal hyphae penetrating gill lamellae. Giemsa stain.

## Discussion

The genus *Exophiala* has been reported as pathogenic in fish and other aquatic animal population [29, 30]. *Exophiala xenobiotica* is described, a sergeant of the *Exophiala jeanselmei* complex. It is morphologically very similar to *E. jeanselmei* with less melanized conidiogenous cells [22, 31].

Nyaoke (2010) has studied the effect of *Exophiala* sp. Nov. on Chinook salmon in saltwater aquaria at 10 °C and 15 °C and noted that it was highly pathogenic. The exposed salmon had extensive cutaneous ulcers (13/20), myconecrosis (17/20), and renal necrosis (12/20) [9]. Also, fungal cultures yielded grey – black velvety isolates from (8/10) muscle and kidney cultures of salmon at 15 °C, and from (9/10) muscle and (3/10) kidney of salmon cultures of salmon at 10 °C. This in contrast to a study in Japanese flounder by Kurata *et al.* [32] in which contact to the inoculum on abraded skin was found to induce erosive lesions of the epidermis on histologic examination, and hyphae were present in the superficial dermis.

Munchan *et al.* [23] have examined *Exophiala* infection in cultured striped Jack, *pseudocaranx dentex*, in Japan 2005. They observed that mortalities continued for a month. Diseased fish showed swelling of the abdomen and kidney distension. Histology revealed abundant fungal hyphae and conidia in gills, heart and kidney.

Kurata *et al.* [32] have tested novel *Exophiala* on Japanese Flounder *paralichthys olivaceus* and noticed that fungal hyphae extended laterally in the dermis, and were absent from the epidermis and musculature of the skin lesions and kidneys of the diseased fish. An inflammatory response with granuloma occurred in the dermis. Experimental infection reproduced hyphae extension and infiltration of inflammatory cells in the dermis of the flounder, confirming the pathogenicity of the fungus.

In studying on some dematiaceous fungi; which have been known to cause systematic mycosis of marine fish were identified as a pathogen that spreads its hyphae into the internal organs [33-38]. Systemic mycosis due to infection with an *Exophiala* – like fungus has been described in other marine fish [39-41].

*Exophiala xenobiotica* used in this study was highly pathogenic to Rabbit fish (*siganus Rivulatus*) first mortality happened by day 14 after inoculation. Chinook salmon inoculated with a novel *Exophiala* species in salt water at 15 °C die by day 23 post-inoculation [9]. Japanese flounder kept at 20 °C, all fish survived the duration of the experiments (36 days) and gross lesions consisting of raising foci in the skin with necrosis of underlying muscle were identified in only 2 of 10 fish injected intramuscularly [32]. Juvenile rainbow trout were only maintained for 12 days post-inoculation [42].

No gross and microscopic lesions were seen on the body surface of fish contact exposure of *Exophiala xenobiotica* as in Atlantic halibut. Overy *et al.* [43] have examined a total of seven fish caused with systemic mycosis by *Exophiala angulospora* and none of the fish showed evidence of significant external lesions to the body proper, in Chinook salmon [9]. Also *Exophiala* species failed to produce lesions in juvenile rainbow trout, and no fungus was re-isolated from swabs of the kidney [42]. In contrast, Japanese flounder found to induce erosive lesions of the epidermis on histologic examination, and hyphae were present in the superficial dermis [32]. Removal of the mucus layer is thought to remove the intrinsic innate immunity of fish and thus promote the establishment of disease [44].

In our study fungus was isolated from swabs of infected organs and identified from (8/10) liver and kidney, (6/10) of gills and (2/10) muscles of infected fish, while the rate of growth, colonial and microscopic morphology of these isolates were very similar, Fish with mycotic granulomatosis shows some pathological characteristics, especially, appearance of ulcer lesion on some parts of the body surface of infected fish and formation of granuloma around the invaded hyphae in the muscle [32]. Kidney sections of Atlantic Halibut fish showed locally extensive necrotizing granulomatous infiltration of the renal interstitium and ureters of the rostral trunk kidney, with a marked fungal colonization of both the ureter lumen and adjacent peritubular interstitial tissue [43]. In the current study, different infected fish tissues show mycotic granulomatosis (4/10) gills and (6/10) liver and kidney, granulomatous infiltration containing septate, brown-colored dematiaceous hyphae clear in both liver and kidney.

## In conclusion

The results of this study revealed that *Exophiala xenobiotica* isolated from Red Sea infected fish. Future studies are required to use other routes of inoculation as intraperitoneal injection and bath immersion and extend to fish in freshwater may be useful in reproducing the ulcerative skin lesions. Gills suggested as route natural infection of striped jack in Japan is passing by the fish circulatory system as fungal hyphae present in the gills. These results can be used in future investigations of host-pathogen interaction, innate immunity and treatment of disease.

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