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Original Article *Fish Diseases*

Incidence and molecular characterization of fungi and yeast isolated from cultured catfish and Nile tilapia

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ABSTRACT

Objective: To study the incidence and seasonal dynamics of different fungi affecting freshwater fishes in Lake Manzala as well as molecular identification of the isolated fungi.
***Animals*:** 300 Nile tilapia (*Oreochromis niloticus)* and 300 catfish (*Clarias gariepinus).*
***Design*:** Descriptive study.
***Procedures*:** Random samples of *Oreochromis niloticus* (*O. niloticus*) and *Clarias gariepinus (C. gariepinus*) were collected from Manzala fish farms. Clinical and postmortem examination of fish was applied. Isolation and identification of different fungi were performed by conventional methods. Furthermore, the molecular characterization of isolated fungi was carried out.
Results: *C. gariepinus* had a higher rate of infection with different fungal species than *O. niloticus*. *Aspergillus spp.* (*Aspergillus niger* and *Aspergillus flavus)* were the most fungal isolated from the examined fishes, followed by *Penicillium spp*. and *Candida albicans. Aspergillus* spp were detected in all seasons with a higher rate in summer and spring. *A. flavus*, *A. niger, Penicillium spp*. and *C.albicans* isolates were amplified from both *C. gariepinus* and *O. niloticus* at the specified molecular weight using PCR.
Conclusion and clinical relevance: Fungal infection affected the fish showing different external and internal lesions, all species of *Aspergillus* were found in all seasons with a high rate in, hot seasons, summer and spring. The Prevalence of *Penicillium* and *C. albicans* were also reported. All fungal isolates were identified on the phenotypic and molecular bases.

Keywords: *Fish*, mold, yeast, PCR, isolation and identification.

1. INTRODUCTION

Fish is one of the most important sources of animal protein available in the tropics and a widely accepted as a good source for the maintenance of body health. Fish proteins are of a high degree of digestibility and richness of lysine and sulfur-containing amino acids. Therefore it is suitable for complementing high carbohydrate diets, especially in the developed countries [1].

With more intensification to meet consumers' demand, a major problem faced the progress and growth of this sector that fish often succumb to infectious diseases. Infectious fish diseases not only caused by bacteria, viruses, or parasites but also could be mold- induced [2].

Fungal diseases are the result of the interaction of the pathogen, fish, and environment. Fish under intensive culture are continuously affected by water quality and management issues. Poor water quality , improper mangement, injured fish, or dead fish accompanied by large amounts of decomposing organic material are stressors that should be avoided to maintain fish health [3].

Molds grow over a temperature range of 10- 40℃, pH range of 4-8, humidity level greater than 62% and more than 12-13% moisture, while yeasts require free water [4].

Fish is mostly attacked by fungi due to changes in temperature, and water quality, which allow excessive zoospores to grow [5].

Molecular techniques have been increasingly employed to diagnose fish diseases. These techniques include polymerase chain reaction (PCR), restriction enzyme digestion, probe hybridization, in situ hybridization, and microarray. Molecular techniques are potentially faster and more sensitive than culture, serological, and histological methods that are traditionally used to identify fish pathogens [6].

Lake Manzala is the largest coastal shallow brackish lake in Egypt, It is extending between the Damietta Nile River branch and the Suez Canal with a maximum length of 50 km along the Mediterranean coast [7]. The northern boundary of the lake is connected with some narrow inlets such as El-Gamil canal with the sea. The eastern boundary is Suez Canal that is connected with the lake via El-Qabuty canal. Economically, Lake Manzala is considered as one of the most valuable fish sources in Egypt by about 36-50% of the total annual production of the Egyptian lakes. This contributes > 4.2% of the total country fish production; ca. 1.5 million tons [8]. Therefore, this work was planned to throw a spotlight on the prevalence and molecular identification of the fungal species isolated from Nile tilapia and catfish captured from Manzala fish farms.

2. MATERIALS AND METHODS

2.1. Naturally infected fish

A total number of 600 fishes, including 300 Nile tilapia and 300 Nile catfish, were collected from Manzala fish farms in Dakahlia Governorate, Egypt. The fish samples were collected during the period from April 2018 to March 2019. Fish were transported alive in a polyethylene bag to the laboratory of Animal Health Research Institute in Mansoura for mycological examination.

2.2. Clinical and postmortem examination:

Moribund and diseased fish were properly examined for any external clinical abnormalities and clinical alterations on the skin, scales, eyes, gills, abdomen, peduncle, fins, and abnormal behaviors. The postmortem examination was done on freshly dead fish to examine, all internal organs including liver, spleen, kidney and intestine, the clinical investigation and postmortem examination were carried out according to Austin and Austin [9].

2.3. Mycological examination

2.3.1. Isolation and identification of the fungi from infected fish

Samples were taken from fish showing skin lesions using a sterile dissecting needle from the skin, gills, and internal organs (liver, kidney). Collected specimens were inoculated into duplicate plates of SDA media (500mg of cycloheximide and 50 mg of chloramphenicol dissolved in 3 ml ethanol 95% were added to the media after autoclaving). The inoculated plates were incubated at 25°C and 37°C for 3-5 days. Pure isolates obtained from culture were preserved on Sabouraud's dextrose agar slants for further identification [10]. Fungal colonies were examined macroscopically for growth appearance, growth rate, the surface colony texture, surface colonies color, as well as the reverse side of the colonies were observed **[11]**. Additionally, a microscopic examination of the culture was carried out after staining with lactophenol cotton blue (LPCB) [12].

2.3.2. Isolation and identification of yeast species:

Yeast isolates were picked up with a sterile loop from preserved culture onto SDA slopes. Identification was carried out through the morphological and physiological properties of the yeast isolates and direct microscopic examination. For direct microscopic examination, a part of the colony was placed on a slide with one drop of distilled water, and covered with a coverslip and examined under high power magnification after staining with Gram-stain [13].

2.4. Molecular identification of fungal isolates:

DNA extraction using Qiagen extraction kit was performed according to the manufacturer instructions. For identification of fungal isolates, PCR was performed for identification of *A. flavus, A.niger, Penicillium spp.* and C.albicans *using* PEPO1–PEPO2, PEPI1–PEPI2, ITS1-ITS4, and LH1-LH2 primers respectively. Primer details were summarized in Table. 1. PCRs were performed in thermal cycler and the thermal conditions were described in Table 2. The amplified products were analyzed by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining using a UV transilluminator.

3. RESULTS

3.1. Clinical examination and postmortem lesions

The infected *C. gariepinus* catfish showed skin ulceration and scattered hemorrhagic patches on the ventral abdomen and mouth, while the external gross lesions of the examined Nile tilapia revealed darkened skin, severe congestion and sloughing of the tail fin with ascites, scale detachment and fins erosion.

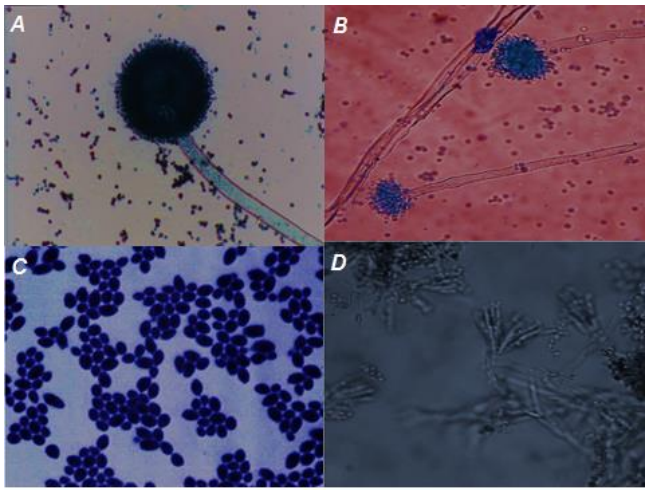

Figure 1. A) *A. niger*: the conidiophores are very long, smooth and the vesicles are very large and globes while the strigmata were biseriate, compact and radiate .The conidia are globes and smooth**. B) *A. flavus***: the conidiophores were long and rough. The vesicles are large and rounded. The strigmata are biseriate, loose, and radiate and gave rise to ovoid rough conidia. **C) *penicillium***:Septate hyphae with branched condiospores possessing metule with flask shape and the formed brush appearance strigmata (lactophenol blue staining/LPCB, × 400). **D) *C. albicanis***: small, oval, measuring 2-4 µm in diameter Yeast form, unicellular, reproduce by budding. Both yeast and pseudo-hyphae are gram positive. Encapsulated and diploid, also form true hyphae.

Out of the total examined fish samples collected from Manzala fish farms, 121 fish were tested positive for fungal detection in a prevalence rate of 20.1%. The isolation rate of fungi from catfish was higher (22.3%) than that of ~~*O. niloticus*~~ Nile tilapia (18 %). The frequency of *A. niger*, *A. flavus, Penicillium spp*. and *C. albicans* in *C.gariepinus* and *O. niloticus* was (17.9, 16.6 %), (14.9, 12.9 %), (13.4, 11.1 %) and (8.9, 11.1 %) respectively.

Regarding the frequency of *A. niger*, *A. flavus, Penicillium spp*. and *C. albicans* in relation to different seasons, in autumn, the prevalence rate was (13.3 &14.2%), (6.6 &7.1%), (13.3 & 7.1 %), and (13.3 & 21.4 %) from *C.gariepinus* and *O.niloticus,* respectively. While, in spring, the prevalence of *A. niger, A. flavus, Penicillium spp.* in *C. gariepinus,* and *O.niloticus* was (21.4 & 18.1 %), (28.5 & 27.2 %) and (21.4 & 18.1 %) respectively, meanwhile *C. albicans* couldn't be detected. The prevalence of *A. niger, A. flavus,* and *C.albicans* during the summer season was (30 &20%), (20 &10 %), and (10 & 10%) for both *C. gariepinus* and *O. nilotiaus* respectively.

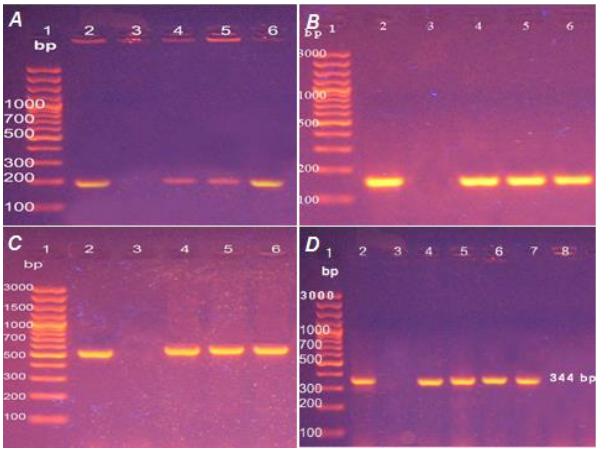

Figure 2. Agarose gel electrophoresis of positive amplicons of **A)** ***A. niger*** (150 pb): Lane 1: 100 bp DNA Ladder, Lane 2, 4, 5, 6 positive *A. niger* isolates. **B)** ***A. flavus*** (200 pb): Lane 1: 100 bp DNA ladder, Lane 2,4,5,6 positive *A. flavus*, isolates. **C)** ***penicillium*** (550 pb): Lane 1: 100 bp DNA ladder, Lane 2,4,5,6: positive *penicillium* isolates. **D)** ***C. albicanis*** (344 pb): Lane 1: 100 bp DNA ladder, Lane 2, 4,5,6,7 positive *C. albicans* isolates.

Table 1. Primers used in PCR detection of fungal isolates.

Species	Primer pairs	Sequence (5'→3')	Amplicon size (bp)	References
A. flavus	F: PEPO1 R: PEPO2	CGACGTCTACAAGCCTTCTGGAAA CAGCAGACCGTCATTGTTCTTGTC	~200 bp	[38]
A. niger	F: PEPI1 R: PEPI2	CCAGTACGTGGTCTTCAACTC CTATTGTACCTTGTTGCTTCGGCG	~150 bp	
Candida albicanis	F: LH1 R: LH2	AGC CACAAC AAC AAC AAC AAC TCT TTGAGA AGG ATC TTT CCA TTG ATG	344 bp	[39]
penicillum	F:ITS1 R: ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTTATTGATATG	variable	[40]

Table 2. PCR thermal conditions for *A. favus, A. niger, C. albicanis* and *penicillium.*

Amplified DNA	Initial denaturation °C/minutes	Actual cycles °C/second	Final extension °C/minutes
A. favus and A. niger	94/5	38 cycles of Denaturation: 94/60 Annealing: 59/60 Extension: 72/90	72/5
C. albicans	92/3	35 cycles of Denaturation: 92/60 Annealing: 55/60 Extension: 72/60	72/10
Penicillium spp.	94/4	35 cycles of Denaturation: 94/60 Annealing: 56/60 Extension: 72/60	72/10

3.2. Morphological characters of the isolated fungi

A. niger characterized by long conidiophores, large vesicle, and biseriate, compact radiated strigmata, and conidia are globes and smooth. *A. flavus* has long and rough conidiophore, large and rounded vesicle, and are biseriate, loose, and radiate strigmata and gave rise to rough ovoid conidia. While *Penicillium spp.* showed aseptate hyphae with branched conidiospores possessing metule with flask shape and formed brush appearance Strigmata. However, *C. albicans* is small, oval budding yeast cells measuring 2-4 µm in diameter (Figure 1).

3.3. Molecular identification of isolated fungi

The *A. flavus* (n=3) were successfully amplified, producing a 200 bp amplicon (Figure 2). *A. niger* (n=3) strains were amplified, yielding fragment size of 150 bp (Figure 3). *Penicillium spp.* isolates (n= 4) were amplified at a 550 bp amplicon size (Figure 4). For identification of *C. albicanis* (n=4), a product size of 344bp was successfully amplified from all the tested isolates using one pair of primers targeting the integrin-like protein alpha-INT1p (Figure 5).

4. DISCUSSION

The clinical examination of the infected fish, *C. gariepinus,* revealed skin ulceration and scattered hemorrhagic patches on the ventral abdomen and mouth. These symptoms may be attributed to the toxins secreted by molds and yeasts causing severe symptomatic changes that appeared on the fish in the

form of hemorrhagic patches, ascites and destruction and degeneration of the gills; the results agreed with that of previous reports [5], [14] and [15]

The external gross lesions of the examined *O. niloticus* revealed darkened skin, severe congestion, and sloughing of the tail fin with ascites, scale detachment, fins erosion, and eye opacity. These results are in agreements with [14,16]. Gills with sever congestion considered as secondary invader pathogen; these results agreed with many authors [15, 17-19].

Fish mortalities may be due to either blindness, which consequently disables fish to feed or due to the fungal growth over gills causing suffocation. The ulcerative areas over the skin may be attributed to the lytic action of primary bacterial infection since fungal infections are considered as secondary invader pathogens to the bacterial ones. These results were supported by many previous studies [15, 18,19].

In the current study, out of the whole examined fish, only 121 fish were infected with different fungi at a rate of 20.1%. *C. gariepinus* recorded a higher incidence (22.3%) than *O. niloticus* (18 %). In contrast to our results, a higher frequency of fungi was recorded in *O. niloticus* by Diab [14]. Also Refai et al. [20] and Mahfouz et al. [15] recorded higher incidence in *O. niloticus* (80.5 %& 56.8 %) than *C. gariepinus* (78.2 % & 43.1 %), respectively . The variation of the infection rate might be due to the scale-less nature of the catfish skin, as suggested by Hussien et al. [21], who reported that scales covered the fish skin act as a physical barrier against external pathogens.

The incidence of the isolated fungi from both species of fishes *C. gariepinus* and *O. niloticus* was as follow; *A. niger* (17.9 & 14.8 %), *A. flavus* (15.2 & 12.1%), *Penicillium spp.*(11.9 &12.9 %) and *Candida albicans* (8.9 & 9.2 %) respectively. Similarly, a similar prevalence of *Penicillium spp* (17.2%*)* and a higher prevalence of *Candida spp.* (35.9%*)* were reported previously [22]. However, a higher prevalence of *Penicillium spp.* **(**70%) was recorded by Hassan et al. [23], additionally, A higher prevalence of *Aspergillus spp*. (90 %) and *Candida spp*. (70 %) from El- Wadi El- Gadid and El Fayome governorates was reported previously [24].

Concerning the seasonal dynamics of infection, winter season showed a high rate of infection in both *C. gariepinus* (31.1 %) and *O. niloticus* (23.1%), followed by autumn (25 &19.4 %) spring (17.2 &15.4 %) and summer (14.2&13.1%). These results were supported by Moeller [25], who mentioned that the most epizootic infection occurs when temperatures are below the optimal temperature range for fish species and might be due to sudden changes in temperature [26] and [27] as observed during the autumn season in the present study.

Aspergillus spp. was found all over the year with high incidence (30 &20%) in both *C. gariepinus* and *O. niloticus,* respectively, in summer (hot season). The infection rate for *A. niger* and *A. flavus* during the summer season was (20 &10 %), (10 & 30 %), respectively, in both examined fish species, Same findings were supported in many previous studies [14, 20,28]. The prevalence of *Penicillium spp.* was high in summer and spring, which in agreement with Mahfouz et al. [15]. *C.albicans* was isolated during all seasons but not detected in spring. *C.albicans* recorded high incidence in autumn (13.3 & 21.4 %) followed by winter (10.7 & 10 .5%) and spring (10 & 10 %) in both examined fish species, in line with Mahfouz et al. [15]**.**

Concerning the results of the molecular identification of Aspergillus *spp*., *A. flavus* (n=3) produced a 200 bp amplicon. *A. flavus* strains were successfully amplified by the PEPO1–PEPO2 primer set. *A. niger* (n=3) strains were amplified by the specific primer pair (PEPI1–PEPI2), yielding fragment in size (150 bp) PCR products. The obtained results were comparable to that recorded by Logotheti et al. [29] who mentioned that the multiplex PCR assay for the discrimination of the most frequent *Aspergillus* pathogens, *A. flavus and A. niger* through distinct amplicons of 200 bp, and 150 bp respectively, derived from the rDNA gene of *A. terreus* and the aspergillopepsin genes of the remaining species.

The molecular detection of *Penicillium spp.*, all isolates amplified using primers ITS1 and ITS4 to a single band of about 550 bp except two species, *Penicillium* variable showed slight increases band about 565 bp in size, whereas *Penicillium rugulosum* showed a band of size 540 bp, which was lowest in our study. Similarly, Tiwari et al. [31], stated that the consensus primers ITS1 and ITS4 were used to amplify a region of the rDNA gene repeat unit. A recent study of *Penicillium* isolates based on the banding pattern of the ITS region has shown a more similarity among different species of *Penicillium*.

For *C. albicans*, one pair of primers (LH1- LH2) was selected for specific identification of this species. In the same line, Lim and Do-Hyun [32] identified 26 isolates of *C. albicans* from clinical samples yielding the same product size of 344 bp, while about 15 clinical isolates couldn't be amplified. The PCR system using 344 bp αINT1 as a target is more specific and rapid than the conventional culture method, and the sensitive detection method is applicable to clinical diagnosis of *C. albicans* infections**.**

Many studies reported that the ITS2 showed considerable interspecies variability to identify 99.7% of yeasts and 100% of molds to the species level, while ITS1 had an identification accuracy of 96.8%–100% for yeasts and 100% for molds [33]. Further, PCR-RFLP assays have been used to identify the isolated yeast species as was successfully done [34]

Yang et al. [35] stated that phenotypic methods are valuable in the identification of yeasts into genera, but these methods take more time. Molecular methods through the amplification of (internal transcribed spacer) ITS1-ITS2 regions of fungal rRNA, followed by RFLP-PCR using Msp1 restriction enzyme allowed simple, rapid, cost-effective, sensitive, and accurate identification of the phenotypically.

Conclusion

Fungal infection affected the fish showing different external and internal lesions, all species of *Aspergillus* were found in all seasons with a high rate in, hot seasons, summer and spring. Prevalences of Penicillium and C. albicans were also reported. All fungal isolates were identified on the phenotypic and molecular bases.

Conflict of interest statement

The authors declare no conflict of interest

Research Ethics Committee Permission

The current research work was executed according to animal care regulations approved by o the Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University.

Authors´ contributions

O.M.H. conducted the procedure of research and drafted the MS; R.S.A. supervised the study. V.H.Z. supervised, reviewing and editing the MS.

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