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Isolation and Molecular Identification of *Aspergillus* species from Cultured Nile Tilapia (*Oreochromis niloticus*)

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ABSTRACT

The present study was carried out on 120 examined cultured Nile tilapia, *Oreochromis niloticus* (*O. niloticus*) (80 diseased fish and 40 apparently healthy) were collected alive from private fish farms at Kafr El Sheikh Governorates from April to October 2018. Grossly, examined diseased *O. niloticus* revealed eroded fins, redness on the skin, congested protruded anal opening and sometimes darkness on skin, ulcers with different sizes, and eye turbidity especially in fish naturally infected with *Aspergillus*, *Fusarium* and *Penicillium*. Mycological examination revealed isolation of 7 Genera of fungi from total incidence of 300 fungal isolates; 240 fungal isolates from diseased *O. niloticus* and 60 isolates from apparently healthy one. *Aspergillus flavus* and *Aspergillus niger* were the most predominant fungi isolated from either apparently healthy or even diseased one mainly from internal organs. Molecular identification was performed using inter-transcribed spacer (ITS) gene for *A. flavus* and *A. niger*. Developed PCR assay specific primer detect *A. flavus* and *A. niger* showing clear bands at 595 and 600 bp molecular weight, respectively.

1. INTRODUCTION

Fish serves as the most significant source of human dietary protein around the world, particularly in African countries (Hussain et al., 2011). Natural fisheries are powerfully depleted, and the aquaculture end up basic plans to repay the need; providing a significant and principal protein source for human utilization (Mohamed et al., 2017). Aquaculture constitutes the greater part of the total fish production in the world and about 77% of the absolute Egyptian fish production (Mur, 2014 and FAO, 2016).

Nile tilapia (*Oreochromis niloticus*) is seen as a widely cultured species amongst the most pervasive freshwater fishes in Egypt as a result of their fast growth rate with great meat quality, marketable request, protection from illnesses and capacity to withstand unfavorable ecological conditions (Dawood et al., 2019 a, b).

The expansion of the aquaculture industry is exposed to a few biological stressors (Awad & Awaad, 2017). It was investigated that illness is a last result of a complex collaboration of the host, its condition and the pathogen itself where the ecological pressure is seen as an important actuating component viewed as a vital initiating element causing different diseases (El-Deep et al., 2019). Unfavorable aquaculture conditions can upgrade fish diseases transmission, particularly contagious fungal diseases, leading to decreased fish production and further economic losses (Ashour et al., 2017).

Fungal infections in fish are seen as secondary reason for infection; fungi influencing fishes are opportunistic and

assault the fishes just in the event that they are pushed or invulnerable bargained. Essential primary causes may be ascribed to temperature change, water quality issues, poor conditions, skin injury because of trauma or excessive handling, bacterial and/or parasitic diseases (Chauhan et al., 2014).

The most well-known widely recognized contagious fungal infection was the pathogenic oomycetes; which belong to the order Saprolegniales, including the genera Saprolegnia, Achlya and Aphanomyces. Saprolegniosis has noteworthy effect on freshwater biological systems by infecting different host organisms and their eggs (Ruthig, 2009; Eissa et al., 2013; Bassiouny et al., 2019).

Aspergillus sp. causes systematic diseases with high mortality rates in fish, whereby the diseases mostly occur through fish feed pollution (Urquhart et al., 2016) and the pathogenesis of *Aspergillus fumigatus* and *Aspergillus niger* have been accounted in freshwater fishes by Chauhan (2013).

The present study was carried out to shed light on the isolation and molecular identification of *Aspergillus* sp. among cultured Nile tilapia (*Oreochromis niloticus*) in Kafrelsheikh private fish farms.

2. MATERIAL AND METHODS

2.1. Fish

A total number of 120 examined cultured *O. niloticus* (80 diseased fish and 40 apparently healthy) were collected alive from private fish farms at Kafr El Sheikh Governorates from April to October 2018. The samples

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were collected with average body weight of 40±5 g. Alive fish were transported in double polyethylene bags; with ½ of its volume water pumped with pressured oxygen to the wet lab. of Fish Diseases and Management Department, Faculty of Veterinary Medicine, Kafr El-Sheikh University, Egypt and kept in well-prepared glass aquaria provided with adequate amounts of dechlorinated water with consistent aeration (Langdon & Jones, 2002).

2.2. Clinical & Postmortem examination

All collected fish were clinically examined to recognize any clinical abnormalities following McVicar (1982). Postmortem assessment of the internal organs was completed on sacrificed and freshly dead fish according to Austin & Austin (2012).

2.3. Mycological examination :

2.3.1. Isolation of the fungus from diseased *O. niloticus*:

Mycological examination was carried out according to Noga, (1993). Specimens were taken from fish showing skin lesions under complete aseptic condition utilizing sterile dissecting needle from the skin, gills and internal organs (liver, kidneys) and inoculated into Sabaroud's dextrose agar (SDA) medium plates with expansion of chloramphenicol (CID) 50 mg/mL; to inhibit bacterial growth; and incubated at room temperature for 7-10 days (Whitman, 2004). All the positive molds cultures were purified by sub culturing on SDA plates. The mold growth was inspected for gross and micro morphological characteristics according to Collins & Lyne (1984).

2.3.2. Identification of different molds

Identification the morphological characters of plates including growth appearance of the cultures, rate of growth, texture and color of the surface and reverse side colonies according to Refai *et al.*, (2010). Preliminary recognition of molds was accomplished using wet mount preparation. A little bit of fungal growth was placed on a glass slide with drop of distilled water, the mycelial mass was gently teased a part with two dissecting needles, covered with a clean cover slide and inspected under the microscope.

The confirmatory identification was conducted utilizing the method described by Bassiouny *et al.* (2019). Briefly, a drop of lactophenol cotton blue dye was set on a clean glass slide; a portion of the fungal culture was transferred into the lactophenol cotton blue dye and teased with a 22 -gauge nichrome needle to separate the hyphae. The coverslip was placed on the preparation and inspected under low and high-power magnification with reduced light for identification using the microscope model Leica, Germany. Microscopic characteristic of fungi such as hyphae, conidial heads and arrangements of conidia were watched.

2.4. Molecular identification for molds

Genomic DNA was extracted from a pure culture of fungal isolates using the DNA extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's guidelines. Fungal colonies were inoculated into SD broth and incubated at room temperature for 7-10 days; then 55 mg from cultured mycelium was centrifuged at 300xg for 5 minutes. Mycelia were washed with sterile phosphate buffered saline (PBS). 20 µl proteinase K, buffer AL vortex and ethanol (96-100%) vortex were added and centrifuged at > 6000 ×g (8000rpm) for 1 min. Buffer AW1 was included and centrifuged at > 6000 ×g (8000rpm) for 1 min and buffer

AW2 was included and centrifuged for 3 min at 20,000xg (14,000 rpm). The spin column was transferred to a new 2 ml micro centrifuge tube. DNA was eluted by adding 200 µl buffer AE to the center of the spin column membrane, incubated for 1 min at room temperature (15-25 C) and centrifuged for 1 min at > 6000 ×g (8000 rpm). Oligonucleotide primers were planned according to White *et al.* (1990) for amplification of the ITS.

ITS1 (5 TCCGTAGGTGAACCTGCGG 3)

ITS4 (5 TCCTCCGCTTATTGATATGC 3)

To amplify the DNA, a PCR reaction was set up by including 6 µL of extracted DNA, 1 µL of forward and reverse primers (20 pmol), 12.5 µL Emerald Amp GT PCR master mix (2x premix) and sterile water up to a final volume of 25 µL. The PCR reaction was done in thermal cycler (Eppendorf, Hauppauge, New York) and incorporated an initial cycle at 94°C for 5 min, trailed by 35 cycles at 94°C for 30 sec, 56°C for 40 sec, 72°C for 50 sec, and a final prolongation step of 72°C for 10 min. Consequently, PCR amplicons were isolated and investigated by gel electrophoresis by loading products onto a 1.5 % agarose gel supplemented with 1% ethidium bromide (50 ng /mL) and picturing bands using an ultraviolet lamp at a wavelength of 300 nm.

3. RESULTS AND DISCUSSION

Mycotic diseases of fish are a genuine risk to the sustainability of aquaculture industry. The expanding recurrence of invasive fungal infection and the high mortality associated with dispersed fungal diseases have featured the requirement for quick identification of infectious molds from clinical samples.

1. Clinical & Postmortem examination

In the current study, the general signs of the clinically diseased *O. niloticus* appeared as eroded fins, redness on skin, congested protruded anal opening and sometimes darkness on skin, ulcers with different sizes, and eye turbidity. The ulcerative areas over the skin and fins erosion might be expected to the lytic action of primary bacterial infection as all fungal infections are considered as secondary invader pathogen; these results concurred with many authors (Chauhan, 2014 and Bassiouny *et al.*, 2019). Redness on the skin may be attributed to the extracellular products of molds resulting in severe symptomatic changes that appear on the fish in the form of hemorrhagic patches; the result agreed with (Bassiouny *et al.*, 2019) where hemorrhagic patches was observed in both examined diseased Nile tilapia and African catfish. Regarding the postmortem lesions, enlarged liver with moderate hemorrhagic spots were the most characteristic. This result may be ascribed to either the fact that most mycotic diseases are secondary invaders following fundamental systemic bacterial infection which is responsible mainly for this internal lesions and/or because of extracellular products produced by fungi that meddle with capacity of liver leading to congestion in internal organs particularly liver (El-Atta, 2008; Mohamed *et al.*, 2017; Bassiouny *et al.*, 2019 and Abd El Tawab *et al.*, 2020).

2. Mycological examination

Mycological examination revealed the isolation of 300 fungal isolates; 240 and 60 isolates from 80 *O. niloticus* showing clinical signs and 40 apparently healthy *O. niloticus*, respectively from skin, gills, liver and kidneys (Table 1A & B). The high recurrence of mould isolates was in *O. niloticus* showing clinical signs; the result

agreed with Refai *et al.* (2010) and Bassiouny *et al.* (2019).

Table 1A Incidence of molds isolated from different organs of diseased *O. niloticus*

Molds	Skin & Fins		Gills	Liver	Kidneys
	No.	No.	No.	No.	No.
<i>Aphanomyces sp.</i>	8	8	0	0	0
<i>Aspergillus flavus</i>	58	0	10	28	20
<i>Aspergillus niger</i>	43	0	9	8	26
<i>Penicillium sp.</i>	27	11	7	9	0
<i>Fusarium sp.</i>	37	0	25	0	12
<i>Mucor sp.</i>	30	15	9	6	0
<i>Alternaria sp.</i>	37	0	0	22	15
Total	240	34	35	98	73

Table 1B Incidence of Molds isolated from different organs of apparently healthy *O. niloticus*

Molds	Skin & Fins		Gills	Liver	Kidney
	No.	No.	No.	No.	No.
<i>Aphanomyces sp.</i>	2	2	0	0	0
<i>Aspergillus flavus</i>	20	0	0	12	8
<i>Aspergillus niger</i>	15	0	2	8	5
<i>Penicillium sp.</i>	3	3	0	0	0
<i>Fusarium sp.</i>	7	0	7	0	0
<i>Mucor sp.</i>	8	3	4	1	0
<i>Alternaria sp.</i>	5	5	0	0	0
Total	60	13	13	21	13

A. flavus was the predominant among both diseased and healthy *O. niloticus* while *Aphanomyces sp.* was the most reduced isolates. This result disagrees with Bassiouny *et al.* (2019) where they recorded that *A. flavus* was the predominant among apparently healthy *O. niloticus* only. This might be credited to variable host susceptibility due to topographical distribution. However, the result is coincided with that reported by Abd El-Tawab *et al.* (2020).

3. Orphological identification of isolated molds

The morphological recognizable proof of molds revealed the presence of different moulds including: *Aphanomyces sp.*, *Aspergillus flavus*; *Aspergillus niger*; *Penicillium sp.*; *Fusarium sp.*, *Mucor sp.* and *Alternaria sp.* The most predominant isolated fungi was *A. flavus* among both diseased and healthy *O. niloticus* while *Aphanomyces sp.* was the most reduced isolates.

Strangely, *Aspergillus species* showed some variety inside similar genus. *Aspergillus flavus* (*A. flavus*) seemed smooth with several aerial growths; the shading changes from yellow to yellowish green by maturing; while microscopically, the conidiophores were long and harsh, the vesicles were rounded and huge and the stigmata were biseriatae, loose, and radiate giving rise to ovoid rough conidia (Fig. 1).

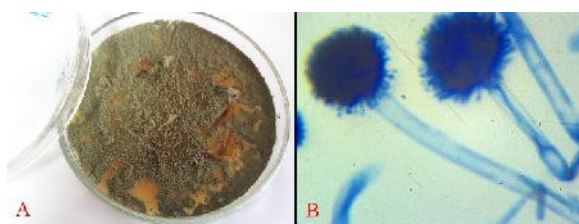


Fig. 1 *Aspergillus flavus* (A) Petri dish. Surface side. (B) hyphae stained with LCB showing characteristic typical head

Colonies of *Aspergillus niger* (*A. niger*) had dark shading with emanated edges with wooly surface, while microscopically had incredibly long, smooth and yellowish conidiophores, vesicles were extremely huge and globes

while the stigmata were biseriatae, compact and radiate and the conidia were globes and smooth (Fig. 2). The highest incidence within organs was observed to be in liver in most of *Aspergillus sp.* This may support the fact that Aspergillosis is a systemic disease. The result is similar to some previous papers (Diab, 2010; Refai *et al.*, 2010 and Abd El-Tawab *et al.* 2020).

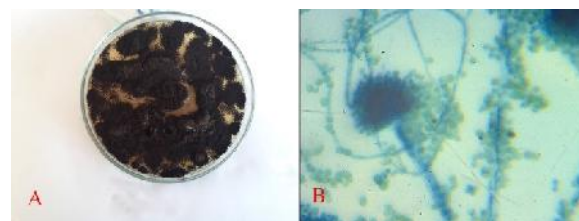


Fig. 2 *Aspergillus niger* (A) Petri dish Surface side. (B) hyphae stained with LCB showing characteristic round head with black conidia

4. Molecular identification

Molecular identification (Polymerase Chain Reaction) using inter-transcribed spacer (ITS) gene, which is a universal gene, is one of the most significant ways to deal with various fungal infections (Eissa *et al.*, 2013). Accordingly, in the current study, molecular identification was performed using ITS gene for the most prevalent isolates among either diseased or apparently healthy fish. Two isolates of *Aspergillus flavus* and *Aspergillus niger* isolates were used. Developed PCR assay specific primer detect not only *Aspergillus flavus* demonstrating a clear band at 595 bp molecular weight (Fig. 3), but also *Aspergillus niger* showing clear bands at 600 bp molecular weight, respectively (Fig. 4). The current acquired outcome is actually like Henry *et al.*, (2000) where *Aspergillus flavus* and *Aspergillus niger* delivered a reasonable band at 595 bp and 599 bp, respectively. These variations may be attributed to various tested primers sequences and PCR protocol as a result of the different amplification cycles (Afzali *et al.*, 2015 and Youssef, 2017).

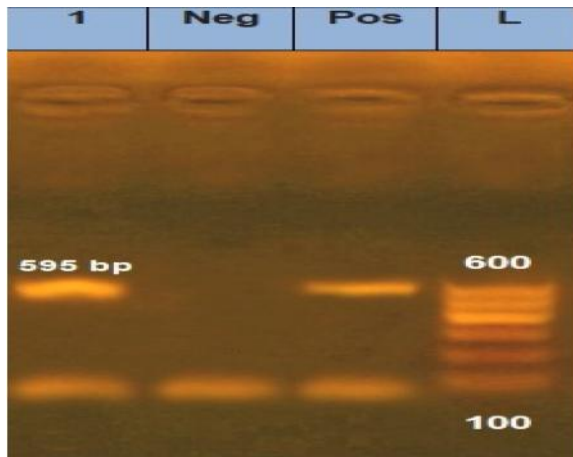


Fig. 3 Agarose gel electrophoresis for PCR products representing amplification of 595 bp of inter-transcribed spacer (ITS) gene in *Aspergillus flavus*. Lane 1: 100 bp DNA Ladder. Pos.: is control positive. Neg.: is control Negative. 1: positive sample for *Aspergillus flavus*

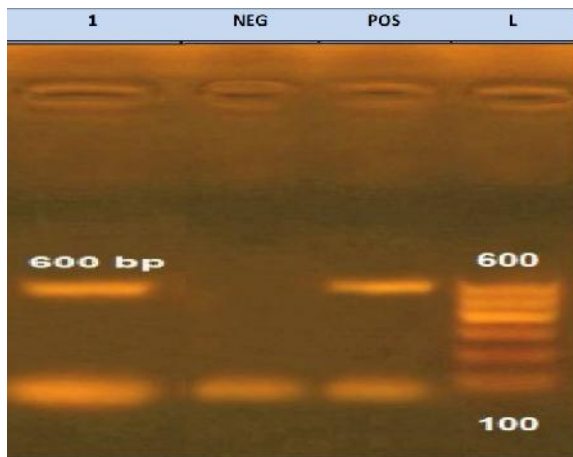


Fig. 4 Agarose gel electrophoresis for PCR products representing amplification of 600 bp of inter-transcribed spacer (ITS) gene in *Aspergillus niger*. Lane 1: 100 bp DNA Ladder. Pos.: is control positive. Neg.: is control Negative. 1: positive sample for *Aspergillus niger*

4. CONCLUSION

From the current study, it could be presumed that *Aspergillus flavus*, *Aspergillus niger* could be isolated from both clinically diseased and apparently healthy *O. niloticus* mainly from internal organs supporting the fact that Aspergillosis is a systemic disease. Briefly, although most fungi are considered to be typical mycoflora, but they still can cause infections to fish. Therefore, it is suggested that proper health management practices should be adapted while rearing fish, so that the chances of fungal infection can be limited.

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