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Incidence of Saprolegniasis in *Oreochromis niloticus* in El-Manzala fish farm with special reference to molecular diagnosis and histopathological alteration

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ABSTRACT

Saprolegniasis is considered one of the most important threatening disease problem facing aquaculture specially Nile tilapia *(Oreochromis niloticus*). This study aimed to conduct mycological study on saprolegniasis in El-Manzala fish farm in Dakahlia province. The incidence rate of saprolegniasis in El-Manzala fish farm was 90.9%. All strains isolated were morphologically classified in the genus *Saprolegnia.* They were identified as follow *S. diclina* oogonia are spherical or pyriform, with spherical central oospore and diclinous antheridial branches. *S.ferax* oogonia spherical with centric or subcentric oospore with no antherdial attachment. *S. australis* oogonia, spherical or pyriform, unpitted with spherical subcentral oospore and diclinous antheridial branches. PCR yielded a single specific and clear amplified band of suspected size 700bp corresponding to ITS genes.Histopathological examination of skin of naturally infected fish *O. niloticus* showed necrosis and desquamation in the superficial layer of epidermis with edema and heterophilic infiltration in the underlying dermis, in addition reddish to brownish fungal particles appeared on skin sections. Gills revealed congestion of lamellar blood capillaries, goblet cells metaplasia and desquamation of secondary lamellae.

Key words: *Nile tilapia, Saprolegnia,* Incidence, Histopathological.

INTRODUCTION

Aquaculture is growing rapidly worldwide with fish being the primary sources of animal protein in many countries. This fishery sector plays a significant role in food security through supplementation of food for developing countries (Kebede and Habtamu, 2016). Nile tilapia (*O. niloticus*) is one of the most important cultured freshwater fish worldwide including Egypt, due to its faster growth rate, resistance to adverse conditions, relatively low production cost, meat quality, high protein content, and consumer preference. Further, Nile tilapia is one of the most suitable spp for biological studies (Zaitseva et al., 2006; Zahran and Risha, 2013).

Infectious diseases, particularly mycotic diseases, still the major constraint affecting fish production sector with subsequent mortalities, economic losses and needing proper management in culture fisheries (Eli et al., 2011).One of the most crucial diseases affecting tilapia aquaculture, is saprolegniais. It is a mycotic disease usually known as water molds and caused by spp of *Saprolegnia. Saprolegnia* spp., are ubiquitous constituent of aquatic environment since they are saprophytic and obtains their nourishment by decomposing organic matter. They are primarily considered as a secondary invader associated with stressors. The disease is encountered in eggs, fry, fingerling and adult fishes (Roberts, 2012; Lone and Manohar, 2018).

Saprolegnia spp are usually implicated as the etiological agents, but their identification is sometimes troublesome and confusing (Zaki et al., 2008; Noor EL-Deen et al., 2010 and Zahran el al., 2017).Traditional methods for *Saprolegnia* identification are usually based on the patterns of asexual and sexual stages. The asexual characteristics have been most often used in identifying the mode of

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zoospore discharge, whereas the sexual characteristics, including oogonia, antheridia, antheridial origin, oospore and lipid droplet position in the oospore, have been used for species differentiation (Dieguez-Uribeondo´ et al., 2007; Ke et al., 2009; Shaheen et al., 2015).

Recently, using the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) genes in combination with morphological data has been suggested as a new tool for accurate spp identification (Eissa et al., 2013; Zahran et al., 2017). *Saprolegnia* infection results in histopathological alterations in skin and gills such as, destruction of epidermal cells and loss of the gill lamellae in severe cases associated with death (Ashour et al., 2017).Therefore the current research work was undertaken to investigate the incidence of *Saprolegnia* infection in cultured *O. niloticus* at El-Manzala fish farms in in Dakahlia province, identification of *Saprolegnia* spp infected *O. niloticus* by conventional and molecular methods, besides, the histopathological alteration associated with *Saprolegnia* infection.

MATERIALS AND METHODS

Sampling site

Fish farm at El- Manzala located within Dakahlia province was used for fish sampling. The rearing systems in the farm were semi intensive in earthen pond. Fish in farm fed twice daily at 3% of their body weight on commercial ration containing 30% crude protein. The source of water for El- Manzala farm from Bahr Haduth channel (mixture of agriculture and sewage waste). El-Manzala fish farm was suffered from high mortalities along with decreasing feed intake especially in shallow pond and at water inlet.The mortality increased especially with low heat bouts.

Fish sampling

One hundred and ten naturally infected Nile tilapia (*O. niloticus)* with saprolegniasis showing cotton wool like appearance were collected from El- Manzala fish farm. Fish body weight was 80±10 g were collected in plastic tank supplied with aerators during winter season and transported alive to Microbiology lab at animal health research institute –Mansoura branch for mycotic examination according to Noga (1996). Salinity was measured using salinometer and water temperature measured using glass thermometer. Water quality was measured (un-ionized ammonia, nitrite, nitrate, pH, and dissolved oxygen) via water quality test kits (Aquarium Pharmaceuticals, Inc.) (Mahboub, 2011).

Gross examination:

Cotton wool like lesion of saprolegniasis was visualized on skin, fins and gills. Any other clinical signs were examined using the method described by Noga (2010).Wet mount preparation technique followed by direct microscopic examination of skin, fins and gill mycotic lesion was carried out for preliminary identification. Briefly, lesions were scraped with a glass cover slip then placed on a glass slide contain drop of sterilized distilled water and examined by binuclear electric microscope (Optica, ST-30-2LF, Italy) at 10, 40 and 100x magnification for observing branched aseptated hyphae with zoosporangia.

Isolation and identification of *Saprolegnia* spp:

Isolation of *saprolegnia* spp was carried out according to Hatai and Egusa (1979). Briefly, parts of mycelial mats on skin, gills and fins were excised by sterilized scalpel and inoculated by sterilized mycological needles into GY agar (consist of 10 g of glucose (Al-Gomhoureia, Egypt), 2.5 g of yeast extract (Al-Gomhoureia Co, Egypt) and 15 g agar (Oxoid, USA) in 1 L of distilled water) supplemented with choloramphenicol (25mg/L) to prevent bacterial contamination; then medium was sterilized through autoclaving (Sturdy, SA230). Cultured plates were then incubated (Yamato, DX302) at 19±1°C and the growth was periodically checked for one week. Daily examination of cultures was

carried out macroscopically and later on microscopically. Pure isolates were then inoculated in GYE agar slant for further identification (Stueland et al., 2005).Morphological identifications were based on the characters of cultures such as growth appearance, texture, and methods of spore production (Frey et al., 1979). A tease mount method was used to examine *Saprolegnia* spp microscopically as follow; drop of sterile distilled water was placed on a clean glass slide with a small portion of the colony, with two dissecting needles, the mycelia mass was teased apart, covered with cover slip and examined under microscope (Optica, ST-30-2LF, Italy) (Dvorak and Atanasek, 1969).

Identification of sexual charactersistics:

Stimulation of *Saprolegnia* to make sexual organs; antheridia and oogonia, was carried out according to Zahran et al. (2016). Briefly, a 1 cm^2 diameter plug of the growing mycelial was placed in sterile tap water with 3-5 autoclaved hemp seeds and incubated at 19ºC. Examination of sexual characters on hemp seed cultures were done twice a week for 2 months by cutting part of mycelia and put on clean glass slide containing sterile tap water covered by cover slip then a drop of Canada balsam was used for preparation of permanent slides. Slides examined microscopically (Optica, ST-30-2LF, Italy) to monitor development of sexual structure. Finally, the isolated strains were identified according to Seymour (1970).

Molecular identification:

Genomic DNA was extracted from 50 mg mycelia using ***Qiagen®, DNeasy kits*** according to the manufacturer's instructions. Specific identification was performed by sequencing the ITS-nrDNA region (Sandoval et al., 2014). The ITS-nrDNA region was amplified using the universal primers ITS1 and ITS4 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990). To amplify the DNA, a PCR reaction was prepared by adding 100 ng/µL of extracted DNA, 12.5 µL of Dream Taq Green PCR Master Mix (Thermo Scientific, USA), 2 µL of forward and reverse primers (100 pmol), and sterile water up to a final volume of 25 µL. The PCR reaction was carried out in thermal cycler (Techne, UK) and included an initial cycle at 94°C for 5 min, followed by 34 cycles at 94°C for 1 min, 50°C for 30 s, 72°C for 1 min, and a final elongation step of 72°C for 5 min. Subsequently, The PCR amplicons were visualized by gel electrophoresis on 1% agarose gel stained with GelRed® nucleic acid stain (Biotium, USA) in electrophoresis unit then take photo by digital camera (Huang et al., 2010).

Histopathological examination:

Tissue specimens of skin, and gills were taken from naturally infected fish, fixed in 10 % neutral buffered formalin and processed for dehydration with ascending grades of ethanol (Al-Gomhoureia Co., Egypt), cleared in xylol and samples were then embedded in paraffin wax. Sections of 5.0 microns thick were cut by microtome and mounted from water bath into clean glass slide and stained with Haematoxylin and Eosin (H&E) and Periodic acid Schiff (PAS) (Bancroft and Gamble, 2008).

RESULTS AND DISCUSSION

Clinical signs of naturally infected *O. niloticus* with *Saprolegnia:*

In the present study 110 *O. niloticus* were collected from El-Manzala. All fish were subjected to clinical examination for signs of saprolegniasis. Numbers of fish infected with *Saprolegnia* were 100 infected *O. niloticus* (90.9%) in Manzala fish farm (Table. 1).The infection rate was high in El- Manzala fish farm, this may be due to the water quality parameters such as salinity (0-0.5 g/L) and polluted water received to these fish farm containing both sewage waste and agriculture drainage water (Bahr Haduth drainage) **(**Bruno, 1999; Ali, 2005).

Similar results were reported by El Bahnsawy (2012) where incidence of

saprolegniasis in naturally infected *O. niloticus* and *Tilapia zilli* were higher in Manzala fish farm (90%, 75%) respectively than Gamasa fish farm (50%, 47.5%) respectively. Naturally infected fish exhibited signs of weakness, lethargy, off

Table (1): Incidence of saprolegniais among *O.niloticus* in El-Manzala fish farm.

Location	No of examined fish	No of infected fish	Incidence %
El-Manzala fish farms	110	100	90.9

Table (2): Incidence of *Saprolegnia* isolated from different organs of naturally infected *O.niloticus*

Organs	Skin	Fin	Gill	Total
No. of samples	100	100	100	300
No. of isolates	62	65	50	177
Intensity %	35.0	36.7	28.3	59

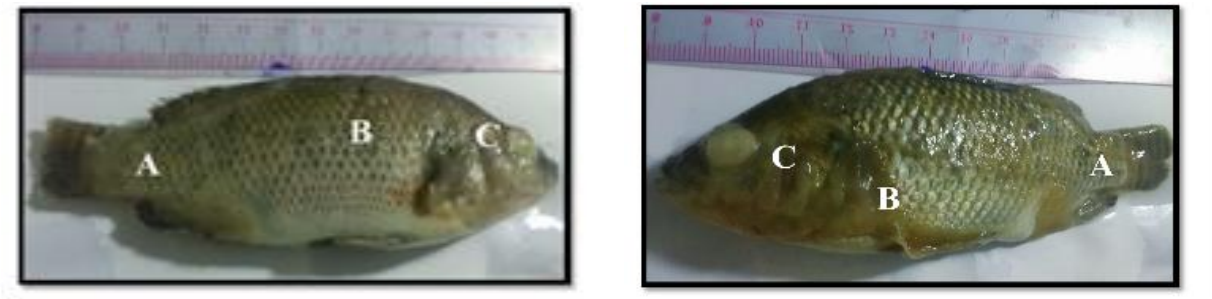

Plate1: Naturally infected *O. niloticus* with saprolegniasis, showing, A) cotton wool like masses invading caudal peduncle and loss of caudal fin; B) excessive growth of cotton wool like masses on lateral side of body and dorsal fin; C) cotton wool like masses on eye, around mouth and at isthmus region

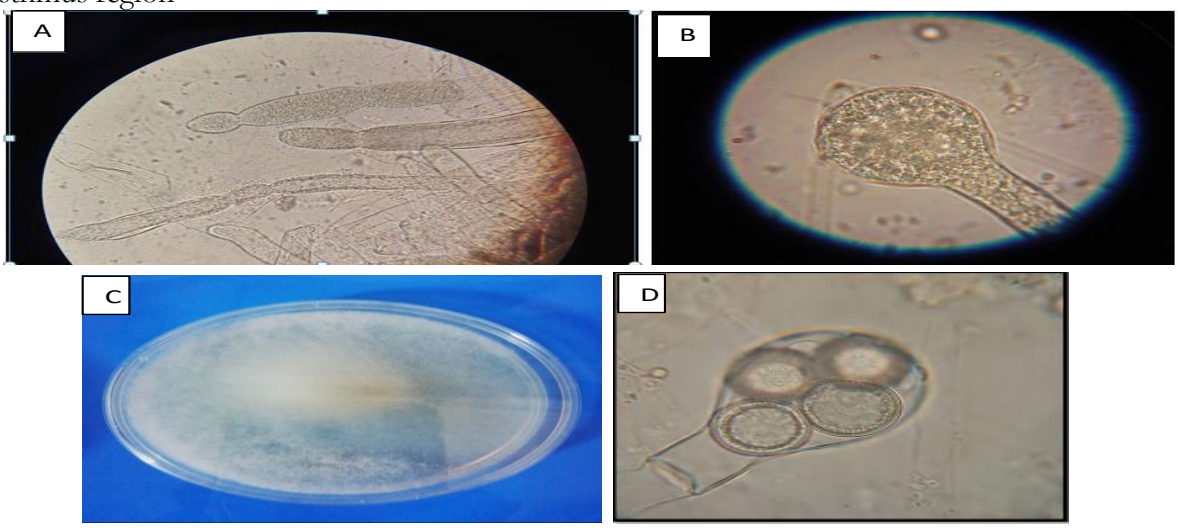

Plate2: A. Wet mount of *O. niloticus* infected with *Saprolegnia* spp, showing aseptated hyphae. B. Tip of hyphae are ended by zoosporangium which filled by sporangiospores low (40x) and high (100x) power magnification. C. *Saprolegnia* spp. on GYA which appear as white cottony mycelium after 3day of incubation at 19 °C. D. Main morphological characteristics of the sexual structures of isolated *Saprolegnia* spp. from infected *O. niloticus*.

food and loss of equilibrium with absence of physiological reflexes. The main characteristic lesions of saprolegniasis were appearance of cotton wool like patches on all fins, eyes, head, isthmus region; and mouth of fish. Cotton wool lesions were in form of white to gray or brown color due to mud. Additionally, unilateral or bilateral cloudy or opacity of the eye, loss of scales and sever ulceration in severe cases were evident on several parts of the body surface studies reported similar signs including loss of scales and sever ulceration in severe cases on several parts of the body surface; these signs result in osmoregulatory failure and respiratory distress from fungal growth on gills causing death of fish (Van West, 2006; El Ashram et al., 2007; Abou- El Atta, 2008; Mahfouz et al., 2019).

Incidence of infection: The incidence of saprolegniasis in different organs is presented in Table (2) as follow, 36.7%

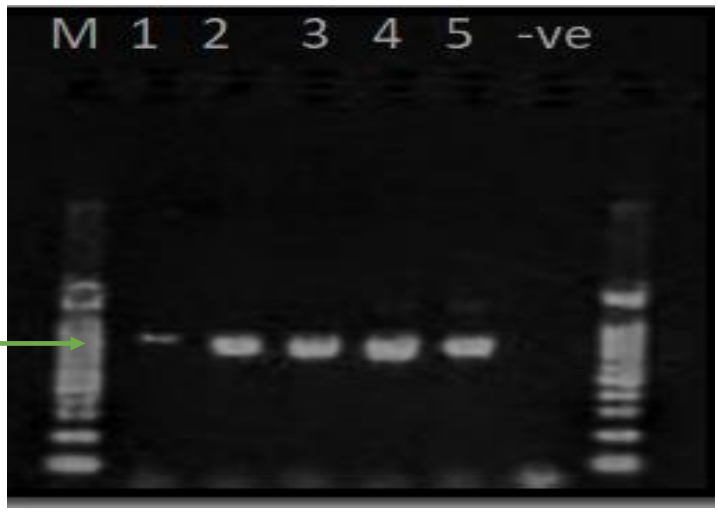

Fig. 3: Agarose gel electrophoresis of products showing the results from PCR amplification of genomic DNA from *Saprolegnia* spp. *M*= DNA marker, Lane 1-5= *Saprolegnia* isolates, lane -ve= -ve control. +ve isolates yielded (700) using primers specific for ITS.

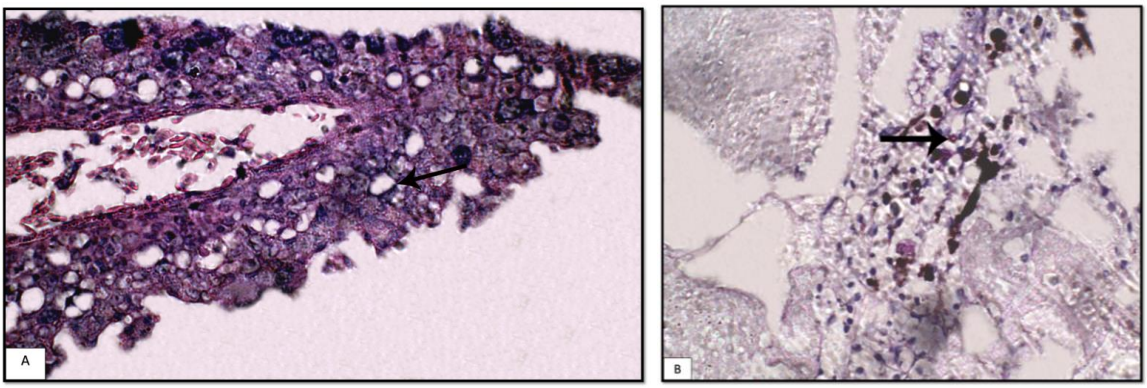

Plate 6: A. Gill section of naturally infected *O. niloticus* with *Saprolegnia* spp, showing congestion of lamellar blood capillaries, goblet cells metaplasia (arrow) and desquamation of secondary lamellae (H&E, 400x). B. Skin section of naturally infected *O. niloticus* with *Saprolegnia* spp, showing marked edema, heterophilic infiltration and reddish to brownish stained sporulated form of fungi (arrow) (PAS, 400x).

(Plate. 1). These results are consistent with El Ashram et al. (2007); Zaki et al. (2008); Abou- El Atta (2008); Earle and Hintz (2014) and Mahfouz et al. (2019). Other

from fin, 35.0 % from skin and 28.3 % from gill. Similar results were reported by El Bahnsawy (2012). Within other studies conducted by Ashour et al. (2017) and

Mahfouz et al. (2019), *Saprolegnia* was highly isolated from skin followed by fins then gills. This could be attributed to various factors such as water parameters at the time of outbreaks such as temperature, the health status of fish, most importantly the density load of zoospores in water (Liu et al., 2017). Additionally, the mucus quality and quantity at different parts of the body might have a role in saprolegniasis (Noga, 1993), since it is well known that mucus concentration is lower on the fins than the remaining sites on the body and thus allowing higher percentage of zoospores to be attached on fins rather than the rest of body (Richards and Pickering, 1978). *Saprolegnia* infection often start at the head and fin then spread to all body surface (Van west, 2006).

Mycological identification:

Asexual Identification:

Wet mount preparation of skin, fins and gills lesions revealed masses of mature and immature sporangia filled with large number of sporangiospore, the hyphae appeared broad profusely branched aseptated, these morphological findings were characteristics of *Saprolegnia* .spp (Plate 2A). The fungal isolates appeared as long hairs with white cottony color (Plate 2C). These Growing young mycelia were examined microscopically every day. Upon examination, it was noted that the vegetative thallas is tubular, non-septate, multinucleate, variably branched and with transparent hyphae. Zoospores, active and motile, were discharged through an exit apical pore of the sporangium one by one in rapid succession. Our isolates were identified as aquatic fungi belonging to the genus *Saprolegnia* based on the type of zoospore emergence from zoosporangia (Plate 2B). This is consistent with other studies confirmed similar type of zoospore release from the zoosporangia (Seymour, 1970 and Steciow, 2003).

Sexual Identification:

Morphological characteristics of the sexual structures of isolated *Saprolegnia* spp from infected *O. niloticus* as follow *S. diclina* oogonia abundant, spherical or pyriform, unpitted with spherical central oospore and diclinous antheridial branches (antheridium and oogonium in different hyphae) with no bundle of long hair on secondary cyst (Plate 2D). Our findings were consistent with Fregeneda-Grandes et al. (2007), who reported similar sexual characters in different *Saprolegnia* spp

Molecular identification:

The amplified patterns obtained by PCR with tested *Saprolegnia* strains showing that all isolates were positively reacted to the ITS1 and ITS4 gene primers. Each strain gave almost a common band with the same molecular weight observed in the different strains. All isolates yielded a single band of amplified product at 700 bp corresponding to ITS genes as shown in (Fig. 3).Using such tool in the molecular identification based on the ITS region showed the advantage rather than depending only on several typical morphological features involving asexual and sexual reproductive organs, identification based on morphological characteristics of their sexual structures is not always possible (Dieguez-Uribeondo et al., 2007; Eissa et al., 2013; Zahran et al., 2017).

Histopathological examination of naturally infected fish:

The skin of naturally infected *O. niloticus* showed necrosis and desquamation in the superficial layer of epidermis, while the underlying dermis showed marked edema and heterophilic infiltration. Reddish to brownish fungal particles appeared on skin sections stained with PAS (Plate 6A). Gills displayed congestion of lamellar blood capillaries, goblet cells metaplasia and desquamation of secondary lamellae by H&E stain. (Plate 6B) .Similar histopathological alterations were reported in other studies (El Genaidy el al., 2004; El-Ashram et al., 2007; Abou El Atta, 2008; Salih and Mustafa, 2017; Hamad and Mustafa, 2018; Mahfouz et al., 2019). These histological variable degenerative effects of saprolegniasis on tissues were attributed to the secretion of hydrolytic proteins into the

extracellular space such as glycosyl hydrolases leading to breaking down cell wall components and thus allow entry into host tissues (Wawra et al., 2012).

CONCLUSION

The present results indicate saprolegniasis is a devastating mycotic disease, affecting freshwater fish. Pollutants and stressors play a key role in reducing fish immunity render them more sustainable to saprolegniasis. Using molecular identification tool based on the ITS region showed the advantage rather than depending only on several typical morphological features involving asexual and sexual reproductive organs as identification based on morphological characteristics of their sexual structures is not always possible.

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