Efficacy of Polymerase Chain Reaction in Diagnosis of Saproleniosis in *Oreochromis Niloticus.*

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Abstract:

The present study was carried out on 100 cultured Nile tilapia *(O. niloticus)* of different ages were collected from farms of Central Laboratory for Aquaculture Research (Abbassa, Abou Hammad, Sharkia, Egypt) during period from late November, 2014 to march, 2015. The collected fish samples were studies for presence of fungal infection. Fish were clinically showed respiratory manifestation, erratic movement, loss of equilibrium, off food and also showed whit to gray patches of filamentous mycelium "cotton like" on fish skin, around head, dorsal and caudal fins, gills, on the muscular layer and eye causing unilateral or bilateral eye opacity. Internally, the diseased fish showed, pale gills, dark and enlarged liver, kidney and intestine free from any food. The collected samples were suspected to mycological and biochemical identification which initially suggestive of Saprolegnia spp. The tradionally identification was supported by using Polymerase Chain Reaction (PCR) which very important rapid and accurate method to identify Saprolegnia from other water mold. Also, in this study, the antifungal effect of ethanolic extract of *Ruta graveolens*(natural herbal plant) on Saprolegnia was studied in vitro by disk and well diffusion methods. Further by trials, iodine was an effective antifungal disinfectant for infected fish.

Key words: *O.niloticus, Saprolegnia spp., Ruta graveolens.*

Introduction:

Now aquaculture represents more than 30% of total fish production for human consumption ***(Delgado et al., 2003)*** and in the following years, aquaculture will become the greatest source for increasing fish production in the world ***(Van West, 2006).*** This increase threated by appearance of many diseases ***(Murray and peeler, 2005; Moran and Fofana, 2007).*** Water mold *(Saprolegnia spp.)* considers one of the most prominent causes of diseases in comparable to bacterial and see lice infection ***(Meyer, 1991;***

Noga, 1993; Costello, 2006). Saprolegnia is a fish pathogenic Oomycete, belongs to Saprolegniales order and known to infect a wide range of fish, amphibians, and crustaceans ***(Van West et al., 2008).*** It causes Saprolegniosis, a disease characterized by visible white or grey patches of filamentous mycelium on the body and fins of freshwater fish ***(Van West, 2006; Schornack et al, 2009).*** Outbreaks of Saprolegniosis are particularly catastrophic at lower water temperatures. Thereby, most of Saprolegnia associated mortalities are confined to late autumn, winter and early spring seasons **(*Bly et al., 1992 and Abou El Atta, 2008).*** Saprolegnia cause the same symptoms, growth of "cotton like" mycelium in embryonic stages **(*Fernandez-Beneitez et al., 2008; Rezinciuc et al., 2014).*** Globally, *Saprolegnia spp.* is responsible for at least 10% of annual economic losses in fish production ***(Hussein and Hatai, 2002; Philips et al., 2008; Robertson et al., 2009; Van den Berg et al., 2013).*** In other cases, the losses reach up to 50% of total annual fish production ***(Bly et al., 1992; Van West, 2006; Bruno et al., 2011).*** In Egypt, Saprolenia cause mass kills in cultured tilapia during winter season ***(Zaki et al., 2008).***

The using of these traditional criteria for identification of Saprolenia often difficult and also contributed to miss-identification of isolates ***(Dieguez-Uribeondo et al., 2007).*** Recently, molecular tools such PCR coupled with partial sequencing of inter transcribed spacer (ITS) gene are the most important tools to distinguish *S. parasitica* from other *Saprolegnia spp.* ***(Ke et al., 2009).***

The routine application of disinfectants is a commonly used procedure during egg incubation at fish hatcheries worldwide **(*Khomvilai et al., 2005; Niska et al., 2009)*** and Saproleniasis was controlled with malachite green but bunch of literature has confirmed that malachite green is a potential carcinogen, teratogen and mutagen. Hence, it has been banned for usage in aquaculture by FDA **(*Forneris et al., 2003; Culp et al., 2002; Gieseker et al., 2006).*** This ban has necessitated the search for acceptable safe\efficient alternative antifungal agent to be used. This work aimed to identify aquatic fungi affecting Nile tilapia by different mycological methods and PCR technique another aim and show some trials to control with *Ruta Graveolens* extract and iodine to control Saprolegnia growth in vitro.

Material and Methods:

A total number of 100 diseased Nile tilapia *(Oreochromis niloticus),* showed skin lesions were collected randomly from farms of Central Laboratory for Aquaculture Research during period from late November, 2014 to march, 2016,

with average 70±5 gm. body weight and length 12±2 cm. The diseased fish were susbected to clinical, post mortem and mycological examination according to **(*Chauhan, 2012),*** the isolation of fungal isolates samples were done according to ***(Iqbal et al., 2012)*** and a specific methodology for isolation of Saprolegnia was described by ***(Willoughby and Pickering, 1977).*** Both methods used SDA media with chloramphenicol (SDA, Difcolab., USA). for Identification,The isolates were subjected to Lacto phenol cotton blue (LCB) stain. Following the protocol of ***(Thomas et al.1991; Pelczar et al.2008).***

Identification of suspected Saprolegnia isolates by PCR technique: that was done according to ***Prabha et al. 2013.***

- DNA extraction:

-The 750 bp of the internal transcribed spacer (ITS) gene amplified by PCR using two (ITS) gene primers:

5`-TCCGTAGGTGAACCTGCGG -3` and5`-TCCTCCGCTTATTGATA TGC-3` (ITS4).

- The PCR product was electrophoresed on 1% agarose gel and observed via ultraviolet trans-illumination dideoxynucleotides termination method.

In vitro trials of treatment:

-Sensitivity test of Saprolegnia to ethanolic extract of *Ruta graveolens* by wells method was done according to **(*Caruan et al., 2012 and Hashemi Karouei et al., 2012).*** -iodine solution was used in sensitivity test by the same previous method and the results of inhibitory zone were recorded.

Results:

Clinically the infected fish showed focal white to brownish cotton-like patches on the surface of skin of infected fish. (As shown in fig.1, 2) and degeneration of caudal and dorsal fins (as shown in fig.3). Also presence of dry depigmentation patches on head of infected fish (as shown in fig.4) and when infection reach eye lead to eye opacity and blindness (as in fig. 5).

The mycological examination showed: Macroscopically growth of mold colonies as cysts of whitish cottony long hairs that quickly shifted to gray then black (as in fig.6). Microscopically the fungal isolate showed branched non septated hyphae together with masses of sporangia which not contain sporangiospore (as in fig. 6).

Suspected Saprolegnia isolates grew on hemps seeds plates zoospore are found and discharged from sporangium (as in fig.6) and other Saprolegnia mycelia showed broad, thick hyphae and have septa which are only produced in sexual production **(*Dieguez-Uribeondo et al., 2007*).**

The 750 bp lenghth of rDNA extracted from 5 isolates were amplified using the targeting

primers. The ITS region sequence of *Saprolegnia species* were submitted to the GenBank database. The similarity between ITS region of Saprolegnia isolates and those of Saprolegnia strains in GenBank database were 99% (Fig.7) that confirming the initial identification.

In vitro trial of treatment showed that:*Saprolegnia spp.*was senstive to the ethanolic extract of *Ruta graveolens* as represented by observation of an inhibiting halo of growth (as in fig.8,9).

The effect of Iodine soltion on *Saprolegnia spp*.growth:

In vitro: betadine inhibited Saprolegnial growth by using both well diffusion and disc diffusion method.

Field trials: betadine used in field trials showed good protective effect against Saprolegial infection in Nile tilapia.

Fig.:1-5

1- Depigment area of skin with Hemorrhagic margins, 2-Lifted scales & ulcerated skin, exposing underlying musculature,

3-Degeneration of dorsal and Caudal fins& hemorrhage on skin, 4-Dry, depigmentation patches on head, 5- Eye opacity, hemorrhage on head, *Nile tilapia.*

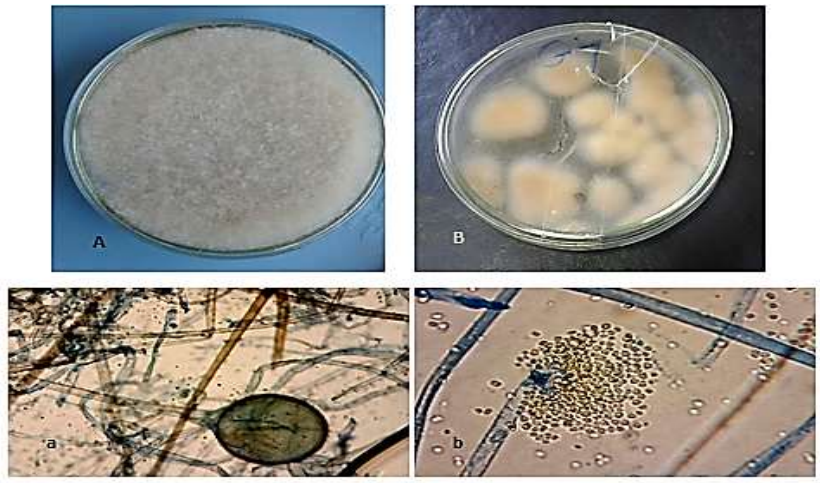

Fig.6: A, an extensive and dense mycelium growth whit in color on SDA. a, long branching hyphae with sporangia free from zoospores.
B, Saprolenial growth on SDA with hemp seeds. b. long branching hyphae, presence of zoospores, *Saprolegnia spp.,* Macro and micro morphological character.

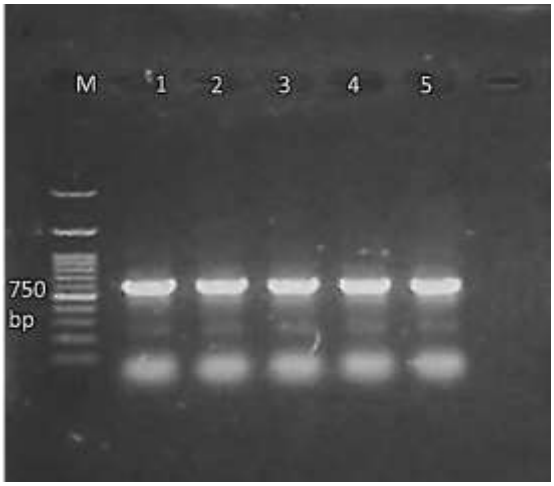

Fig.(7):Gel electrrophoresis of PCR showing 750bp band amplified from 5 Saprolegnial isolates from Nile tilapia*(O.niloticus).*

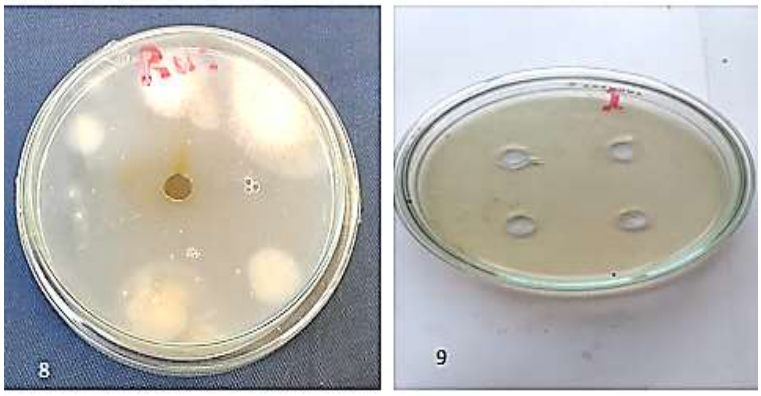

Fig.: (8-9) Effect of *Ruta graveolens* and Iodine on Saprolegnia growth (well diffusion method).

Discussion:

The current study supported the findings of ***(Osman et al., 2010)*** who reported that Saprolegnial infection in fish showed cotton wool like white to dark gray mycelial growth on head, dorsal fin and then spread all over the body of the fish in the form of focal patches and similar to findings of ***(Khoo, 2000)*** who recorded that the presence of cotton white growth of *Saprolegina spp.* on skin of fish when it present in water, but when it out of water the cottony appearance quietly disappear because the mycelia collapsed into a slimy mass.

This work revealed that during the colder winter months the most common isolated mold pathogen identified as *Saprolegnia spp.* Which infect Nile tilapia (*Orechromous niloticus*) leading to sever mortality of all ages including market size. These results is agree with ***(Fregeneda-crandes et al., 2007)*** who recorded that the sharp decrease in water temperature enhances the quick proliferation of Saprolegnia free swimming zoospores with consequent attachment to skin/eggs of fish and also in tune with ***(Pelczar et al., 2008)*** who reported that *Saprolegnia spp.* is ubiquitous in fresh water ecosystems and is the main genus of water molds responsible for significant fungal infections of fresh water fish and eggs.

Determination of *Saprolegnia spp.* is complex and sometimes confusing. However, several typical morphological features involving asexual and sexual reproductive organs serve for classic *Saprolegnia spp.* identification ***(Stueland et al., 2005).*** *Saprolegnia spp.* is usually difficult or even impossible to identify by traditional morphological criteria alone. So in this study, the molecular sequence

analysis are strongly indictable for *Saprolegnia spp*.

In this study, DNA isolation procedure developed is based on the sodium dodecyl sulphate/phenol method, without addition β-mercaptoethanol and proteinase k; instead it uses phenol/chloroform extraction. This protocol resulted in good quality DNA; it is an easy and rapid protocol for the isolation of good quality DNA from fungi such as Saprolegnia. This is agreeing with **(*Prabha et al., 2013).*** Other reports have described procedures for the extraction and purification of fungal DNA. Many of these are modifications of the CTAB method originally developed for plant tissue extraction **(*Petrisko et al., 2008).***

In the current study, the results of application of ethanolic extract of *Ruta Graveolens* revealed that it was an effective candidate substance for inhibition of Saprolegnia species growth, these results was in agrees with **(*Hashmi et al., 2012)*** who revealed that the ethanolic extract of *Ruta Graveolens* root was an effective antifungal against Saprolenia species growth. Also it was in tune with **(*Meepagala et al., 2005)*** who mentioned that *Ruta Graveolens* extract contains antifungal and phytotoxic component. And also in tune with **(*Oliva et al., 2003)*** who reported that the ethyl acetate extracts of leaves of *Ruta Graveolens* had antifungal effect.

Also this work supports the assumption of the Iodophors has high efficacy/safety and widely used as disinfectant in both fish brood stocks and eggs at in modern fish farms/hatchery facilities ***(Eissa et al., 2013).***Betadine antifungal disinfectant effect was confirmed by the failure to re-isolate of mold back from the treated eggs.

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الملخص العربى
كفاءة تفاعل انزيم البلمرة المتسلسل فى تشخيص السابروليجنيوزس فى اسماك البلطى النيلى

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تمت هذة الدراسة على100 سمكة من اسماك البلطى النيللى ذات اوزان مختلفة تم تجميعها من مزارع المعمل المركزى لبحوث الثروة السمكية بالعباسة ابو حماد شرقية خلال الفترة من اواخر نوفمبر2014 الى مارس2015. ولقد كانت هذة الاسماك مصابة وتعانى من مشاكل تنفسية واضطرابات فى الحركة والتوازن. كما انها كانت تعانى من وجود تجمعات قطنية بيضاء ورمادية من الفطر على جلد الراس والزعانف الظهرية والخلفيةواحيانا وجود عتام فى احدى العينين. وعند فتح هذة الاسماك لوحظ ان الامعاء خالية تماما من الطعام مع وجود احتقان وتضخم فى كل من الكبد والكلية والطحال وقد تم اخذ عينات وتعرضت الى الفحص الفطرى والتى اعطت نتائج مبدئية ان هذا الفطر هو فطرالسبرولجنيا ثم تم استخدام تفاعل انزيم البلمرة المتسلسل فى تاكيد هذا التشخيص. وايضا تم استخدام بعض الوسائل العلاجية باستخدام مستخلص نبات السذاب شديد الرائحة واليود و تاثير استخدام كل منهما على السبرولجنيا فى المعمل.