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Induction of Saprolegniosis in *Oreochromis niloticus* with Special Reference to its Biological Control

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Abstract: A method was developed to induce experimental saprolegniasis in tilapia (*Oreochromis niloticus*) exposed to physical stress through, descaling and descaling with wounding in addition of sudden and graduall drop of water temperature. Fish which descaled and wounded were mostly affected with saprolegniasis than the other groups. Thus combination of descaling with abrasion and sudden drop of water temperature were more effective in inducing saprolegniasis in *O. niloticus*. Also, the present study used some biological treatment of natural saprolegniasis infected *O. niloticus* using non pathogenic intestinal aeromonas strain either *in vitro* (plate) and *in vivo* (treatment tank) as a bath of aeromonas suspension 2 times for 3 days.

Key words: Saprolgniasis · Tilapia *O. niloticus* · temperature · biological treatment

INTRODUCTION

Saprolegniasis is a worldwide serious mycotic winter freshwater disease often affects wild and cultured fishes. Its emergence is correlated to stress factors such as abrasions, cutenous wounds sexual maturity, poor water quality, crowdness, malnutrition, handling and bacterial and/or parasitic infections [1, 2]. Several authors have carried out experimental infections with various spp of saprolegnia using some predisposing factors to increase the susceptibility of fish to infection using cutenous scarification [3], modification of water temperature [4, 5] and combination of scarification and drop of water temperature [3]. Saprolegniosis usually starts as a cotton wool-like white to dark grayish or brownish growth on the head region and dorsal fin then spread allover the body in the form of focal patches [6, 7].

Saprolegniosis causes enormous economic losses in intensive fish farming [8, 9]. Treatment of saprolegniosis using anti fungal agents are vital for the maintenance of healthy fishes and their eggs [10-11]. Although, the disadvantages of using chemical fungicides (malachite green and formalin) reprsented as low withdrawal affinity and high carcinogenic activity on human and fish, yet, they used by many veterinarians for the control of saprolegniasis. Biological control of saprolegniosis has received little attention in Egypt, therefore, the present study was designed to investigate potential biological agent for biocontrol of saprolegniosis in *Oreochromis niloticus* by the using of intestinal non pathogenic aeromonas strain and to confirm the hypothesis that it could be used in treatment of saprolegniosis in the field.

MATERIALS AND METHODS

Fish:

A. Natural infected fish: Twenty natural infected *O. niloticus* fish with saprolegniasis were obtained from private fish farm.

B. Experimental Fish: Apparantly healthy alive sixty *O. niloticus* fish of body weight of 80±10g were brought from private cement fish farm for experimental induction of saprolgniosis. Fish transported in plastic tanks aerated with battery air pumps to the Hydrobiolgy Separtment, National Research Center. Fish were subdivided into 6 groups of ten fish each in 6 glass aquaria of 50 x 50 x 100 cm dimensions, supplied by the natural water from the farm, fishes were fed with commercial feed pellets twice daily.

Induction of saprolegniosis: Fishes were acclimated at water temperature of 22±1°C using thermostatically adjusted heater for 7days. The first three groups (1,2,3) were descaled only while the other groups (4,5,6) were descaled and wounded on the sides and peduncle of the tail using sharp scalple. First and fourth groups were

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subjected to sharp drop of water temperture (5°C±1°C) within 5 hrs using ice pieces placed around the aquaria from outside to avoid direct contact of fish with ice. The 2nd and 5th groups were subjected to graduall drop of water temperature to 5±1°C within 10 days (the time of the experiment). The 3rd and 6th groups subjected to temperature of 22°C ± 1 during the time of the experiment (control). Fish groups were observed for behavioral, clinical signs of infection and morbidity /mortality rate. Spores of saprolegnia were placed in each tank with each group of fish [12, 13]. The spores were counted according to [4, 14] to determine the mean number of spores / ml of holding water.

Identification of the involved saprolegnia: Wet mount preparations of fungal skin lesions were microscopically examined according to [15]. Materials from fungal skin lesions of naturally infected fish were cultured on Sabaroud's dextrose agar (SDA, Difco), with adding of chloramphenicol at the rate of 25 mg/L. Plates were incubated at 22°C (temperature resembled to that of the experimental aquaria) and periodically examined and reisolation and cultivation of saprolegnia sp. on plates of Sabaroud's dextrose agar enriched with crushed hempseed for flourishing saprolegnian hyphae. (Fig. 3). Identification of recovered saprolegnia spp. was carried out based on cultural morphological and microscopic characteristics recorded by [16].

Isolation of saprolagnia spores: In test tubes containing sterilzed distilled water, one sterilize pierced hemp seeds in each tube with the cotton wool like hyphae was placed and incubated for 24 hrs at room temperature then the water was centrifuged at 3.000 rpm/for 10 mint to settled down the spores and discard the supernatant. Spores were counted on the haemocytometer and used later in induction of saprolegniasis.

Preparation of Non Pathogenic Aeromonas Strain (NPAS): Under complete aseptic condition intestinal swabs were taken from apparently healthy *O niloticus* and cultured in tryptone soy broth (TSB CM_{129}Oxid) and incubated for 24 hrs at 27°C. Subcultured of these samplas onto TSA were carried out for examination of their growth and colony character. Microscopical examination of such bacteria showed gram negative short bacilli. Confirmatory biochemical identification of these bacteria was done. Aeromanoas colonies were taken from the plates and subcultured into TSB for 24 hrs at 27°C [17].

Exprimental Checking the virulence of NPAS on healthy *O. niloticus*: Alive healthy 15 *O.niloticus* fish were injected I/P with 0.2 ml of $1x10^7$ cells/ ml (NPAS)/fish for determination of the pathogensity of the baterial strain to the fish and observed for 14 days for recording the clinical signs and any abnormality on the fish. Also the PM lasions were not detected.

Prepration of fungal material and inoculating technique (*in vitro*): For testing (NPAS) *in vitro*, hyphal tips obtained from a culture of saprolegnia grown on sabroud's dextrose agar at 25°C were inoculated onto the prepared (NPAS) plates. In the first half of the plate hyphal tips were inoculated onto the area containing (NPAS) while inoculation in the second half of the plate served as a control to observe the saprolegnian hyphae gowth. This for confirmatory testing of the antagonistic activity of (NPAS) to saprolegnia *in vitro* (Fig. 5).

Preparation of NPAS bath for controlling of saprolegnosis (*in vivo*): Twenty natural infected fish with saprolegnosis were subjected for treatment using 4 tanks provided with The prepared (NPAS) which grown in Tryptone Soy Broth (TSB) overnight and diluted in the tank of water to give approximately 10^6-10^8 cells/ml in 10 L of dechlorinated water (provided with air pumps) The suspension was added to the tanks, which contained natural infected fish with saprolegnosis, Fish were observed for behaviour and clinical signs of saprolegnosis. Tankwater was partialy replaced by 2.5 L from each tank daily with addition of (NPAS) at conc. 10^3- 10^4 cell / mL (for presrvation the conc. of NPAS in the water of the treatment tank).

RESULTS

Saprolegniosis is an acute infection affecting *O. niloticus* the natural infected fish revealed focal greyish white patches on the head regions as well as skin, fins and occasionally gills. In advanced stages of infection, saprolegniasis spread out to cover the whole body (Fig. 1).

Regarding to the experimental induction of saprolegniasis, results showed that in the 1st group(subjected to sudden drop of water temperature), 30% of the fish were infected with soprolegniasis (Table 1 and Fig. 2). In the 2nd group (subjected to gradual drop of water temperature), 10% of the fish were infected on the other hand the 4th group (subjected to sudden drop of water temperature), 70% of the fish were infected,

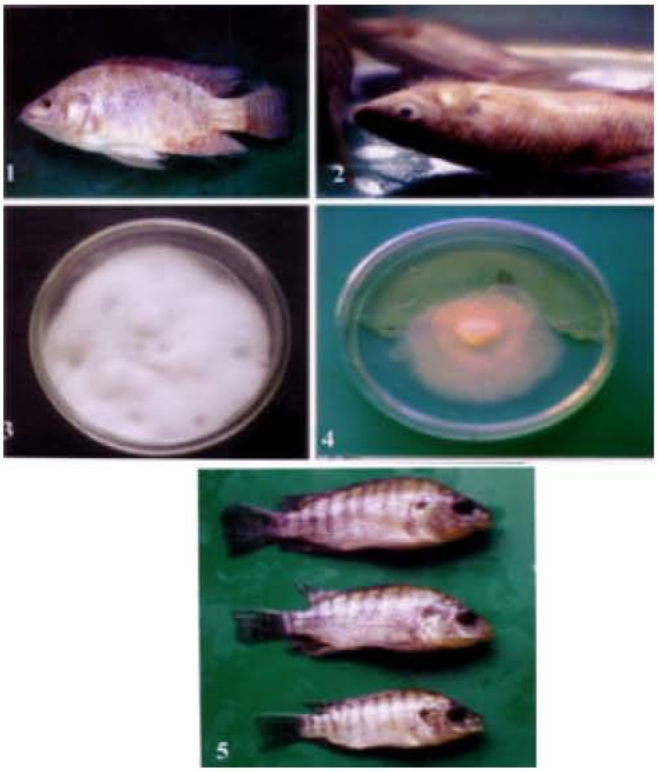

Fig. 1: *Oreochromis niloticus* **natural infected with saprolegniosis**
Fig. 2: *Oreochromis niloticus* **experimentally induced with saprolegniosis**
Fig. 3: Saprolegnia growth on sabaroud's dextrose agar
Fig. 4: The upper half of plate with NPAS while lower have without NPAS showing graoth of sapralegnia hyphae
Fig. 5: Apparently healthy *O. niloticus* **I/P. injected with NPAS after 14 days**

Table 1: Showing the results of exp. inductin of saprolegnia in O.nilotcus in relation tovarious water tamperature

stress	Descaled *O. niloticus*						descaled + wounded *O. niloticus*					
groups	1st group**		2nd group***		3rd group****		4th group**		5th group***		6th group****	
time of exp	no.of inf*	no.of dead	no.of inf	no.of dead	no.of inf	no.of dead	no.of inf	no.of dead	no.of inf	no.of dead	no.of inf	no.of dead
1st day	0	0	0	0	0	0	1	0	1	0	0	0
5th day	1	1	0	0	0	0	3	3	3	1	0	0
10 day	2	0	1	0	0	0	3	3	0	2	0	1
total	30	10	10	0	0	0	70	60	40	30	0	10

* number of infected

** 1st group and 4th group = sudden drop of water temperture (22 ---- 5°C within 5 h)

*** 2nd group and 5th group = gradual1 drop of water temperture (22 ---- 5°C within 10 days)

**** 3rd group and 6th group = room temperture (22±1°C control)

the 5th goup (subjected to gradual drop of water temperature), 40% of fish infected with saprolegnia. The mortality rate was 10% in the 1st group, while it was 60% in the 4th group, 0% in the 3rd group and 30% in the 5th group.

Regarding to checking of the virulence of NPAS on healthy *O.niloticus*, no clinical signs produced nor pathological signs was found on the fish (Fig. 5) following I/P injection of the investigated bacterial strain in apparently healthy fish and observed for 2 weeks,

Regarding the antagonistic action of NPAS on saprolegneasis (*in vitro*). The top half of the plate (Fig. 4) which containg NPAS had not grown the hyphae of saprolegnia while the bottom half lacked NPAS and served as a control to monitor vegetative growth of saprolegnia after 72 hrs incubation at room temperature.

Concerning to treatment of saprolegniasis with NPAS (*in vivo*) the study involved 15 *O. niloticus* naturally infected with saprolegniosis, fish was initially immersed in bath containing NPAS after which normal water of the bath changed (50%) daily. Hyphal masses were observed floating on the water column after overnight exposure to NPAS. The *O. niloticus* appeared to be recovered as judged by absence of saprolegnia gowth although the wound remain unhealed, three days after treatment however the fish began showing clinical signs of saprolegniasis in the inflamed wound at this stage NPAS could not be isolated from the water tank after 3 days another bath was applied using NPAS at the same concentration. Although the wound was free of saprolegnia growth, the wounds began to heal and the fish recovered from the infection.

DISCUSSION

Saprolegniasis is an acute infection affecting *O. niloticus* it is world wide myeotic freshwater disease offects wild and cultured species the clinical signs of soprolegniasis on *O. niloticus* resembled the recorded sings and lesions which were pathognomonic for soprolegniasis [18-23]. Regarding the experimental induction of sapralegniasis, from the results it is cleare that the group of fish which descled only, the rate of infection and the rate of infection and the mortality rate were less than that of the other group which descled and wounded, also water temperature play on important role in susepetability to various infections, especially saprolegnia.

Several authors induce sapralegniasis in fishes [3]. In rainbow trout [4]. and in catfish but the present study was aimed to investigate, the induction of saprolegniasis in *O. niloticus* using some physical predisposing factors (descaling, wounding and sudden and graduall drop of water temperature) saprolegniasis is disease promoted by physical stressers like, poor water quality, malnutrition, injuries occurred during handling and transportation also crowdness temperature shock, spawning or external porasitism [24, 25].

Scales and skin act as physial barriere against external pathogens, especially mycotic agents. The stressors predisposed fishes to saprolegniasis. In the present investigatio stressors were represented as descaling and/or wounding combined with graduall or sudden drop of water temperature [3]. Also, they demonstrated that, handling, rough surfaces of tanks or cages, over crowsing, parasitic infestation damage skin, fins and gills increasing infection suscepibility causes osmotic stress.

In the present study, the prevalence of saprolegniasis hence mortality rate in the group of fishes predisposed to saprolegniasis by (descaling) were lower than that of the other group (descaled and wounded) this indicate that the importance of the scales and skin as physical barrier this may be owed to disturbance of osmoregultion as infection of saprolegniasis generally occur in the epidermis and dermis and occasionally in the superficial musculature so the destruction of skin can disturbs the fish's osmoregulatory system and cause a lethal dilution of body fluids [12, 26, 27]. Skin of a fish is the envelope for the body and the first line of defense against diseases it also affords protection from and to environmental factors.

Regarding water temperature, fish are cold blooded animals primarily dependant upon water as a medium in which to live. Fish can tolerate wide range of water temperature they can distinguish a rise in temperature from a fall but the physiological mechanism for such discrimination is not known [27, 28]. Temperature stress, particularly cold temperatures can completely halt the activity of immune system eliminating this defense against invading disease organisms [29]. Furthermore, decreasing of water temperature, especially the sudden drop compromise the immune system of the fish, increasing the susceptibility to pathogens with especial reference to mycotic agents. Temperature stress particularly rapid changes severely affect the ability of fishes to release antibodies, giving the invaders the chance to produce and devastate the fish [30].

Regarding the antagonism of NPAS as biological control of saprolegniasis *in vitro* (Fig. 4) and *in vivo*. *In vivo* observations tentatively suggest that NPAS could play a significant role in the management of saprolegnia while the *in vitro* results demonstrated that NPAS was active antagonistic agent against saprolegnia. It can be speculated that the presence of viable NPAS created conditions unfavorable for growth of saprolegnia after initial over night exposure to NPAS. It was clear that the growth of the saprolegnia has been retarded. Hyphal masses were also observed floating in the water after the first and second NPAS baths (3days each). The

observations suggest that in these conditions, the pathogen detaches from the mucus and epidermal layer of the fish and released into the water. The ability of NPAS to inhibit saprolegnia appeared related to its ability to liquefy gelatin of such fungi. However the direct effect of gelatin hydrolase on saprolegnia growth. NPAS is considered as gelatinase positive [31]. Parenthetically another candidate for the inhibitory activity for saprolegnia is cellulase, an enzyme produced by NPAS [15]. The saprolegniacae have cellulose rather than chitin in their cell wall [32, 33]. Using live bacteria for biological control may cause disease in fish. The investigated bacterial strain was non-pathogenic for fish as confirmed by I/p injection of this strain in apparently healthy fish and observed for 2 weeks. No clinical signs produced nor pathological signs were found (Fig. 5). There were reports discussed the *in vitro* inhibition of saprolegnia sp. by a gram negative rod, *Pseudomonas fluorescens* by [8, 10, 34] and they reported that inhibition of saprolegnia by bacteria not related to the secretoray substance but rather the result of competition. Also, [15] showed *in vitro* antifungal activity by a number of Gram negative bacteria inclusive of the genus aeromonas, against pathogenic strains of saprolegnia parasitica. The discovery of existence of both *in vitro* and potential *in vivo* antifungal activity of NPAS increases its suitability as a probiotic and presents a possible approach to the management of saprolegniasis in *O. niloticus*. This is the first report in Egypt about antifungal effect of non pathogenic areomonas strain as biological control of saprolegnia.

In conclusion, *O. nilotcus* were unable to withstand sharp water temperature drop with wounding and descaling. Such factors exclusively were the critical points for induction of fish saprolegniasis. Such idea will enable researchers to carry out further studies to test the efficacy and safety of NPAS as biological antifungal treatments.

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