

## Treatment Trails of Saprolegniosis in *Oreochromis Niloticus*

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### ABSTRACT

#### Key words:

*Oreochromis niloticus*,  
*Saprolegnia*,  
H<sub>2</sub>O<sub>2</sub>, KMnO<sub>4</sub>  
and *A. hydrophila*

This study was performed to compare between different methods of *Saprolegnia* treatment hydrogen peroxide H<sub>2</sub>O<sub>2</sub>, potassium permanganate KMnO<sub>4</sub> and biological treatment non pathogenic bacterial strain *Aeromonas hydrophila* (NPAH). 120 *Oreochromis niloticus* were randomly collected and investigated for Saprolegnia fungal infection and infection rate were 10%, 5% and 15% in tolompate village, Elhamool and om-sin village respectively. Obtained *Saprolegnia parasitica* (*S. parasitica*) was examined by Polymerase chain reaction (PCR of ITS gene, 750 bp). Results obtained revealed that Kmno4 had the highest effective treatment in *S. parasitica* infection compared with biological and control treatment. Also, biological treatment with *A. hydrophila* showed hazard of bacterial infection. So, KMnO<sub>4</sub> recommended in case of *S. parasitica* infection as treatment of choice followed by H<sub>2</sub>O<sub>2</sub> and a biological treatment with *A. hydrophila* is not recommended.

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## 1. INTRODUCTION

Aquaculture activities contributed 63% of total national fish production. The production, progressively become more subjected to many problems related to artificial feeding, water quality, management, pathogens and low immunity. Mycotic infections of freshwater fish often affect wild and farmed fish in freshwater environments (Pickering and Wiloughby, 1982). One of the most destructive infections for fish culture is *Saprolegnia* spp., which is widespread in freshwater habitats around the world and responsible for significant contaminations involving living and dead fish as well as incubating fish eggs (Noga, 1993). Control of *saprolegniasis* had turned to a problem since the malachite green treatment was banned worldwide (Noga, 2010). (Whisler 1997) claimed that stressors such as improper water temperature, water quality, handling, or crowding are frequently accompanied with outbreaks of *saprolegniosis*. Ke *et al.* (2009) mentioned that usage of PCR coupled with partial sequencing of inter transcribed spacer (ITS) gene are the most currently used approaches to differentiate *S. parasitica* from other *Saprolegnia* spp.

So this study was aimed to compare between different treatments methods of *Saprolegniosis* in *O. niloticus* fish.

## 2. MATERIAL AND METHODS

### 2.1. Additives and methods of application:-

**2.1.1.** Hydrogen peroxide (3%) H<sub>2</sub>O<sub>2</sub> Nasr. Co., Egypt. 14 ml/l a bath last for 10-12 minute (Noga, 2010) repeated day after day 5 times.

**2.1.2.** Potassium permanganate KMnO<sub>4</sub> Nasr. Co., Egypt. 100 mg/l a bath last for 5 minute (Noga 2010) repeated day after day 5 times.

**2.1.3.** Isolation of Non-Pathogenic *A. hydrophila* (NPAH) in fish diseases department, Animal health research institute from the gut of healthy *O. niloticus*. 20 *O. niloticus* apparently healthy were injected I/P with 0.2mlx10<sup>7</sup> CFU (colony forming unit) for determination of its pathogenicity and fish were kept under supervision for 14 days and no clinical signs were recorded. A bath with NPAH 104 cell /ml last for 1 repeated day after day 5 times.

### 2.2. Tested fish:-

**2.2.1.** Survive fish: 120 *O. niloticus* were randomly collected from 3 freshwater fish farms (40 fish / farm).

**2.2.2.** Experimental fish: A total of 150 apparently healthy *O. niloticus* fish were collected from private fish farms Kafr El-Sheikh Governorate and previously acclimated in indoor tanks in full glass aquaria measuring (80 X 40 X 40 cm) and maintained in aerated de-chlorinated fresh water at

27 ± 2 °C for 14 days. They seemed healthy and had a uniform size and weight with average body weight 115±4.5 gram.

### 2.3. Isolation and identification of *Saprolegnia* spp:-

Fish naturally infected with *Saprolegnia* were brought to Animal health research institute Kafr El-Sheikh branch and cultivated on Sabouraud's dextrose agar (SDA) (Adwic SCG) was used for isolation of fungus and was prepared by dissolving 65 gm /liter of distilled water by gentle heating and sterilized in autoclave at 121 °C for 15 minute and chloramphenicol was added by 50 mg/ml (Cruickshank *et al.*, 1975). **Identification** of isolates using Polymerase Chain Reaction PCR

a- DNA extraction: The protocol of DNA extraction was adopted from (Moller *et al.* 1992).

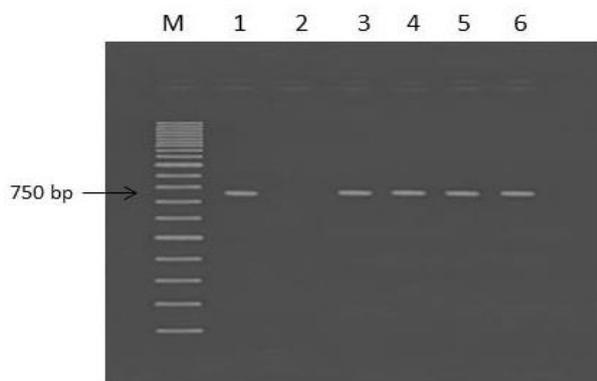
b- Detection of ITS gene by PCR: The 750 bp of the internal transcribed spacer (ITS) gene was amplified by PCR using two ITS gene primers:

50-TCCGTAGGTGAACCTGCGG-30 (ITS1) and 50-TCCTCCGCTTATTGATATGC-30 (ITS4)

using a PCR gradient thermal cycler (TC-3000G, Bibby Scientific Ltd., Staffordshire, United Kingdom) (Cao *et al.*, 2012). The PCR product was subject to electrophoresis on 1% agarose gel and specific bands were detected under the ultraviolet (UV) transilluminator.

**2.4. Experimental design:-** isolation, identification and infection rate of *Saprolegnia* in three freshwater fish farms. Also, performed experiment of artificial infection by *Saprolegnia* isolated from field, trails of treatment, detection of survival rate after three week of stating treatment and antioxidants levels.

**5-Induction of experimental *Saprolegniosis*:-** The challenge infection was done by immersing a manual wounded *O. niloticus* in a zoospore suspension of *S. parasitica* (4x10<sup>6</sup> zoospore/L) for 10 min. according to (Willoughby, 1994).



**2.5. Survival rate (SR %):-** SR%= (No. of fish at end / No. of fish at the start) ×100. Where calculated after three week of starting treatment.

**2.6. Blood&serum analyses:-** Red blood cell (RBCs) and White blood cell (WBCs) counts were counted by haemocytometer according to Stoskopf (1993). Blood film was prepared according to the method described by Lucky (1977). Differential leukocytic count was calculated according to Schalm (1986). Blood hemoglobin (Hb) was assessed by cyanometahemoglobin method (Drubkin, 1964). The activity of the liver enzymes, aspartate amino transaminase (AST) and alanine amino transaminase (ALT) were determined according to (Reitman and Frankel, 1957) by using kits reagent supplied by Diamond Diagnostic Co. Glutathion peroxidase (GPx) activity was assayed by the method of (Mohandas *et al.* 1984). Nitric Oxide (NO) It was measured using the method described by (Rajaraman *et al.*, 1998).

**2.7. Statistical analysis:-** Statistical analysis was performed using the analysis of variance (ANOVA). Duncan's Multiple Range Duncan (1955) was used to determine differences among treatments mean at significance level of 0.05. All statistics were run on the computer using the SPSS program (SPSS, 2004).

## 3. RESULTS

### 3.1. Infection rate in fish farms

Examined one hundred and forty randomly collected *O. niloticus* in three farms in late autumn had revealed infection with *Saprolegnia* in rate of 10%, 5% and 15% in tolongpate village, Elhamool and om-sin village. Laboratory examination using PCR technique presented in photograph (1) indicated that *Saprolegnia* strain was *S. parasitica*.

**Photograph(1):** Agarose gel electrophoresis of PCR of ITS gene (750 bp) specific for demonstration and characterization of *Saprolegnia* species.

**Lane M:** 100 bp ladder as molecular size DNA marker.

**Lane 1:** Control positive *E.cloacae* for ITS gene.

**Lane 2:** Control negative. Lanes 3, 4, 5 & 6: Positive *saprolegnia* species for nr gene.

### 3.2. Survival rate in experimental fish

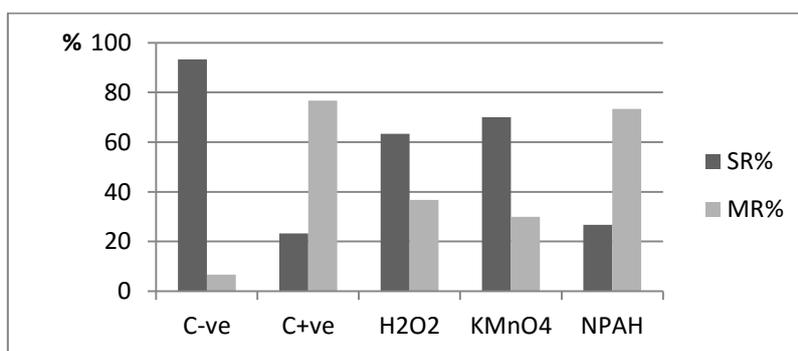
Data presented in table (1) showing survival and mortality rate of experimental *O. niloticus*. Infection procedure last till appearance of cotton like patches on *O. niloticus* skin. After three weeks of starting treatment it was obvious from table (1) and fig (1) that KMnO4 had the highest SR% 70% when compared with infected groups followed by H<sub>2</sub>O<sub>2</sub> then NPAH had recorded the lowest value 26.7% close to control +ve indicating that NPAH had no significant *saprolegniosis* treatment.

### 3.3. Antioxidant analyses

**Table 1 Survival rate of experimental *O. niloticus*.**

Item	Control -ve	Control +ve	H <sub>2</sub> O <sub>2</sub>	KMnO <sub>4</sub>	NPAH
NO	30	30	30	30	30
SR	28	7	19	21	8
SR%	93.33	23.33	63.33	70	26.7
MR%	6.67	76.67	36.67	30	73.3

NO=number of fish, SR =survival rate, MR= mortality and NPAH = Non Pathogenic *A. hydrophila*.

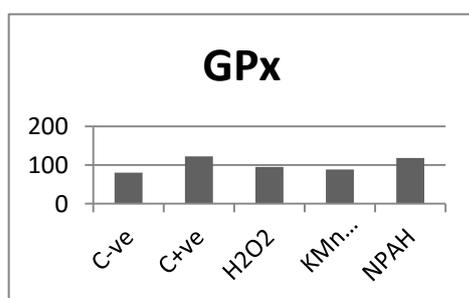


**Fig(1):** percentages of survival and mortality rate of *O. niloticus*.

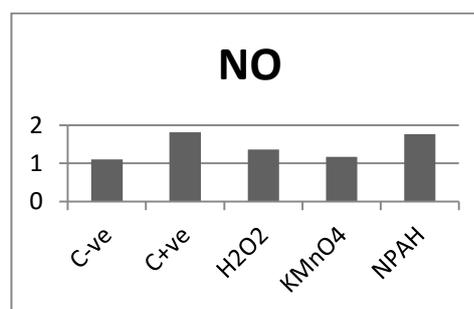
**Table 2 Antioxidants analyses in experimentally infected *O. niloticus* (mean)±SE.**

Item	Control -ve	Control +ve	H <sub>2</sub> O <sub>2</sub>	KMnO <sub>4</sub>	NPAH
GPx	80 <sup>c</sup>	122 <sup>a</sup>	95.3 <sup>b</sup>	88.3 <sup>b</sup>	118 <sup>a</sup>
μU.ml <sup>-1</sup>	±4	±1.2	±2.7	±2.7	±1.2
NO	1.1 <sup>c</sup>	1.81 <sup>a</sup>	1.36 <sup>b</sup>	1.17 <sup>c</sup>	1.76 <sup>a</sup>
μ.mol <sup>-1</sup>	±0.06	±0.02	±0.02	±0.07	±0.06

Different values letter within the same raw are significantly different at P≤0.05.



**Fig(2):** GPx level in different treatment.



**Fig(3):** NO level in different treatment.

**Table 3 Blood analyses and Liver enzymes in experimentally infected *O. niloticus* (mean±SE).**

Item	Control -ve	Control +ve	H <sub>2</sub> O <sub>2</sub>	KMnO <sub>4</sub>	NPAH
RBCs X10 <sup>6</sup>	1.85 <sup>a</sup> ±0.3	1.54 <sup>c</sup> ±0.3	1.69 <sup>b</sup> ±0.00	1.83 <sup>a</sup> ±0.2	1.58 <sup>c</sup> ±0.01
WBCs 10x3	6.4 <sup>a</sup> ±0.06	5.6 <sup>b</sup> ±0.1	6 <sup>ab</sup> ±0.2	6.4 <sup>a</sup> ±0.06	5.6 <sup>b</sup> ±0.27
Hb g/dl	11.18 <sup>a</sup> ±0.17	9.34 <sup>c</sup> ±0.18	10.3 <sup>b</sup> ±0.04	11.1 <sup>a</sup> ±0.14	9.6 <sup>c</sup> ±0.9
ALT Ug/g	11.3 <sup>a</sup> ±0.06	11.8 <sup>a</sup> ±0.09	11.5 <sup>a</sup> ±0.15	11.3 <sup>a</sup> ±0.3	11.8 <sup>a</sup> ±0.09
AST Ug/g	21.4 <sup>c</sup> ±0.7	25.6 <sup>a</sup> ±1.2	23.8 <sup>ab</sup> ±0.4	22.9 <sup>bc</sup> ±0.4	25.3 <sup>a</sup> ±0.4

Different values letter within the same raw are significantly different at P≤0.05.

#### 4. 3.4. Heamtological investigation

5. *O. niloticus* RBCs, WBCs and Hb (table, 3) had been investigated in order to evaluate the impact of *Saprolegnia* infection on the health status. From data obtained it was obvious that infected groups had significantly impacted as the lowest values of RBCs, WBCs and Hb had recorded in control +ve 1.54, 5.6 and 9.34 and enhanced by treatment with KMnO<sub>4</sub> 1.83, 6.4 and 11.1 as compared with control –ve 1.85, 6.4 and 11.18 respectively.

#### 6. 3.5. Liver enzyme

7. It was clear from liver enzyme level presented in table (3) that ALT hadn't effect while AST had raised significantly with infection.

### 8. DISCUSSION

Our results concerning infection rate in freshwater fish farms agreed with those obtained by (Van West 2006 and Phillips *et al.* 2008) as they stated that *S. parasitica* considered as one of the most important mycotic infections that can cause tremendous economic loss in cultured ecosystems. Also, Ruthig (2009) mentioned that *S. diclina* and *S. parasitica* occurred mostly in areas with a temperate climate. Also, Van den berg *et al.* (2013) mentioned impaired osmoregulation is caused by haemodilution resulting from wounds over a large surface area. Also, they added that *Saprolegnia* in nature is not thought to pose a large threat to populations of fish as it mostly infects wounded or otherwise immune compromised animals.

Concerning evaluation of treatment trails efficacy in mortalities reduction, SR% and MR%

recorded (table.1 and fig.2) was agreed with Hussein and Hatai (2002) as they recorded a cumulative mortalities of 100% in all salmonid species exposed to concentrations 2 × 10<sup>5</sup> spore/L of *S. parasitica* NJM 9868. Also, Zahran and Risha (2013) stated that challenged *O. niloticus* with *S. ferax* zoospores infection showed mortality rate in KMnO<sub>4</sub>, FCA (Freund's complete adjuvant), and control positive group was 25, 27.5 and 30% respectively also, they noticed that the mortality rate in control positive group was continued to increase peaked upto 92.5% after 3 weeks.

Mean while, Rasowo *et al.* (2007) had investigated the efficacy of formaldehyde, sodium chloride, KMnO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub> in control of *saprolegniosis* in *C. gariepinus* eggs and recorded the highest hatchability performance when eggs was treated with 2 ppm KMnO<sub>4</sub> for 30 min (96.7%). Also, Kitancharoen (1997) found that treatment of ova and fish with H<sub>2</sub>O<sub>2</sub> 250-500 mg.L<sup>-1</sup> for 15 min on alternate days had control *saprolegniosis* with no chemical residues.

Failure of biological treatment could be due to differences between in vivo and in vitro conditions these in the same trend with Abouelatta (2008) who had isolated Gram negative bacteria and identified as *Aeromonas* species and *Pseudomonas* species from fish suffered *saprolegniosis*.

NO is an important regulating signaling molecule, it produced endogenously from L-arginine and molecular oxygen by the enzyme nitric oxide synthesis (Bogdan, 2001). Data concerning antioxidant it was significantly increased the antioxidant level in response to *S. parasitica* infection these findings agreed along with Zahran

and Risha (2013) results as they found that NO level was significantly increased in the infected group compared to the control one. As Ignarro (2000) mentioned that high NO level was accompanied with stress conditions (infectious diseases and toxicity).

A similar response was observed in rainbow trout inoculated with a virulent strain of *Renibacterium salmoninarum* compared with avirulent strains (Camposperez *et al.* 2000). Mean while, Storey (1996) mentioned that the damage produced by infection was compensated with antioxidant enzymes released namely GSH and glutathione peroxidase (GPX).

In table (3) RBCs, WBCs and Hb had low values in control +ve and treated groups compared with control -ve mean while (Abouelatta, 2008) blood parameters *O. niloticus* experimentally infected with *saprolegniosis* showed that no significance difference between control healthy non treated and the healthy treated fish, except glucose which is higher in healthy treated than non treated one due to H<sub>2</sub>O<sub>2</sub> affect on cortisol level in the blood. While, Musa and Omeregie (1995) observed that the reduction of haemoglobin, PCV, RBCs and iron level in *O. niloticus* was resulted of loss appetite or the direct of catabolic effect of cortisol. In the same trend, Fawzi *et al.* (2008) significant decrease in RBCs, Hb and PCV was observed in *O. niloticus*, after 7 days of *saprolegnia* post- infection and 10 days of post-treatment; while MCV and a reticulocytes showed a high significant decrease at the same period of sampling in comparison with control.

Data concerning liver enzyme indicated that *S. parasitica* had insignificant impact ALT while increased level of AST maybe dueto skeletal muscle damage. In agreement Zahran and Risha (2013) noticed that *Saprolegniosis* had no significant effect in ALT levels at any time of experiment. However, AST had a significant rise in the infected group at 1<sup>st</sup> week which declined by 2<sup>nd</sup> week and 3<sup>rd</sup> week in KMnO<sub>4</sub> group to be within the control group level. Mean while, Fawzi *et al.* (2008) reported that marked elevations noticed in the activity of (AST) and (ALT) was due to the liver is the primary organ of detoxification therefore, the significant increase in liver enzymes couldbe explained by the presence of the *S. parasitica* or its toxins in liver. Tripathi *et al.* (2003) mentioned that sorbitol dehydrogenase and ALT presented in low concentrations in skeletal muscle and may be a better indicators of

hepatocellular damage and this indicate that there were no notable damage to other internal organ. AST had showed higher level in treated healthy fish than healthy non treated (Abouelatta, 2008).

So it was concluded that, KMnO<sub>4</sub> is the best treatment for *Sproplegniosis* followed by H<sub>2</sub>O<sub>2</sub> while biological treatment with Non-pathogenic *A. hydrophila* showed no significant improvements. It is recommended usages of KMnO<sub>4</sub> in *Saprolegnia* treatment.

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