Branchiomyosis In Nile tilapia *(Oreochromis niloticus)* in Behiera Governorate With Trials For Treatment

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ABSTRACT

A total number of 100 Nile tilapia (*Oreochromis niloticus*) were collected from Edco private fish farms in Behiera governorate during the period between July and September 2013 and screened for branchiomyces infection. The infected fish were suffering from respiratory distress (resulted from gill tissue damage); gasping air from the water surface, rapid movement of operculum and massive mortality, which resulted in the loss of 90% of the collected fish. Squash preparations from the infected gill tissue revealed brown, broad, branched and non-septated hyphae. On Sabouraud's dextrose agar (SDA) media with 10% duck decoction showed bright white colonies after 2 days which reached its maximum growth 8 days post inoculation. Microscopical examination of stained growth with Lactophenol cotton blue, branched hyphae at their tips were identified which were characteristic for *Branchiomyces* sp. The causal pathogen was identified as *Branchiomyces demigrans*, in which the diameter of spores and non-septated hyphae were 4-10 µm and 16-24 µm respectively. The fungus was confirmed using polymerase chain reaction (PCR).Experimental infection and reisolation of fungus revealed the same findings of natural infection. Clotrimazole was more effective than clove oil, while using both of them revealed higher lysozyme activity and phagocytic activity. Histopathological examination from naturally and experimentally infected fish gills revealed non-septated hyphae and spores were embedded inbetween affected gill tissues, which confirmed that the isolated organism was *Branchiomyces demigrans.*

INTRODUCTION

Oreochromis niloticus is a highly valued commercial freshwater fish and gain more popularity allover the world. Fish diseases negatively affect production and reproduction. Branchiomycosis also known as gill rot, is an acute, localized fungal disease of the gill affecting a wide variety of freshwater fish. It has fairly broad wide geographical range with cases reported in United States, Japan, Endochina, Israel and Europe, where it is considered to be a major problem in commercial fish production *(1)*. Two species were recognized, *B. sanguinis* the causative agent of carp branchiomycosis and *B. demigrans* causing gill infections in tench and pike. Growth of the former species is confined to the vascular system while the latter expands to extra vascular tissues *(2)*. The diameter of the hyphae of *Branchiomyces demigrans* usually is 13–14 µm and may be up to 22–28 µm and the diameter of the spore is 12–17 µm *(3)*. The disease occurs most frequently in the warm climatic regions. The rise, and course of the disease depend on factors that underline them; water temperature is one of the factors that play the most important part with a high load of organic matter, ponds fertilized by organic manure, and high levels of unionized ammonia in the water *(4 ,5)*.

Branchiomycosis causes a respiratory insult.The fish with subacute to acute infections most often present with respiratory distress, lethargy, mortality may reach as high as 50% and morbidity may reach as high as 100% because the disease has an acute onset and rapid progression *(1)*.

Diagnosis of Branchiomycosis was based on macroscopic observation of the marbling appearance with necrotic areas on the localized damage gills, on gill wet mount from the affected gills, fungal hyphae appeared brown, branching and non septated, Hyperplasia and areas of massive necrosis resulting from thrombosis of vessels by fungal hyphae with talengiectasis in histological sections from affected gills and in vitro culture of the *Branchiomyces* sp. on Sabouraud's dextrose agar media with 10% duck decoction and pH of media adjusted at 5.8 also aid in confirmatory diagnosis ***(1,5)***.

Ketoconazole was tested using disc diffusion method against *Branchiomyces demigrans* which showed an inhibition zone of 16-25mm diameter ***(6)***.

Several chemical studies have been suggested for the treatment of Branchiomycosis. These include malachite green, formaline and cupper sulfate. However, these treatment protocols and their efficacies are not well established ***(1)***.

The aim of present study includes: Isolation and identification of the causative agent of Branchiomycosis from naturally cultured *O.niloticus* in Behiera and trials for treatment from experimentally infected *O.niloticus* using antifungal drug (clotrimazole) and plant extract (clove oil).

MATERIALS AND METHODS

Samples

One hundered *O. niloticus* were obtained from Edco private fish farms in Behiera governorate and examined during the period between July and September 2013. Fish ponds were rich with organic matter. The fish were transferred alive to the Laboratory of Fish Diseases and Management Department, Faculty of Veterinary Medicine, Zagazig University.

Infected fish

A total of 320 apparently healthy *O. niloticus* were collected from Abbassa private fish farms for experimental infection and treatment trials.

Clinical examination

Clinical examination of fish was carried out ***(7)***.

Gross examination

Gross examination was carried out for presence of external lesion.

Mycological examination

Squash preparation

Squash preparations were done from the infected gills and examined microscopically.

Mycotic culture

Pieces from the infected gill tissue were inoculated onto Sabouraud's dextrose agar (SDA) media with chloramphenicol and supplemented by 10% duck decoction, 10% Gelatin and 0.1% Citric acid ***(1)***, incubated at room temperature 25-30°C and the culture was examined daily for 9days. The identification of fungus was done through staining preparation from positive culture growth on SDA media using Lactophenol cotton blue stain (LPCB) and through Measuring of hyphae and spores diameters using light microscope and high power magnification lens, (X 400).

Molecular detection by PCR

PCR tests for detection of DNA in samples (a pure isolate of *Branchiomyces* sp. on SDA with 10% duck decoction) was performed using two universal primers to ensure that *Branchiomyces* sp. is related to fungi. The universal primers used for fungal amplification were ITS1 (5′TCC GTA GGT GAA CCT GCG G 3′), which hybridized at the end of 18S rDNA, and ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3′), which hybridized at the beginning of 28S rDNA. ***(8-10)***.

Histopathological examination

Tissue specimens were taken from the suspected lesions from gills of naturally infected fish and fixed in 10% Phosphate buffered formalin, embedded in paraffin, sectioned and stained with Haematoxylin and Eosin stains (H&E) ***(11)***. and PAS reaction ***(12)***.

Antifungal Sensitivity test

The antifungal susceptibility testing for Amphotericin B, Fluconazole, Itraconazole, Nystatin, Clotrimazole and Ketoconazole was based on Clinical Laboratory Standards Institute disc diffusion method, Mueller-Hinton agar plate supplemented with 0.2% glucose and 0.5µg/ml methylene blue dye was inoculated with *Branchiomyces* sp. suspension, its concentration compared with 0.5 McFarland standard ***(13)***. The methods of test were applied ***(14,15)*** and the plates were incubated at 25◦C for 48 hours prior to determination of results.

Experimental infection

Preparation of the inoculum (the infective dose)

1-Culture of the *Branchiomyces* sp. was done on SDA medium with chloramphenicol and supplemented by 10% duck decoction, 10% gelatin, 0.1% Citric acid, pH of the media adjusted at 5.8 and the colony growth appeared after 7 days, then dissolve three colony growth in three liter of distilled autoclaved water and do frequent stirring using large sterilized swab, then add the three liter to 27 liter of clean water to become a total of 30 liter *Branchiomyces* sp. suspension in the water.

2-A synthetic corticosteroid, triamcinolone acetonide suspension (Kenacort-A, Galaxo smithkline company),will be used as immunosuppressors, at a dose of 20mg for each Kg body weight ***(16)***.

Performance of experiment

A total of 80 apparently healthy *O.niloticus* with an average body weight 30 ± 5 g were divided into 4 equal groups (group1 &2 as a control and group 3&4 infected) in a well aerated glass aquaria, each aquarium (80cm x 40cm x 20cm) supplied with dechlorinated 30 liter tap water and fed on basal ration twice daily at the rate of 3 % of the fish biomass. The water temperature was 20±1°C, pH was 7 ± 0.2 and the dissolved oxygen was 5-6 mg/L.

Control groups

The fish in the group 1 not subjected to any stress factors. The fish in the group 2 was injected with a synthetic cortisone (20mg\kg body weight).

Infected groups

The fish in the group 3 subjected to *Branchiomyces* sp. suspension in the water. While, the fish in the group 4 was injected with a synthetic cortisone (20mg\kg body weight) and subjected to *Branchiomyces* sp. suspension in the water.

Treatment trials design

A total number of 240 *O.niloticus* with average body weight 30 ± 5g were divided into 8 equal groups, each group had 3 replicate (10 fish per replicate).The experiment design was showed in table (1). Clinical signs and mortalities were recorded for one week during the experimental period. Two types of blood samples were collected at the end of the experimental period from the caudal blood vessels from each group ***(17)***, one sample with addition of heparin for measuring the phagocytic activity and the other sample used for serum separation for measuring the lysozyme activity.

Table 1.Treatment of *O.niloticus* experimentally infected with *Branchiomyces* sp.

Types of treatment	Infected groups	Control groups, treated, non-infected)
	Fish were subjected to *Branchiomyces* sp. suspension in the water. Fish were subdivided into three groups, each group received type of treatment.	Three groups, each group received one type of treatment
1- Clotrimazole	Used as a bath with a dose of 0.5 µg/ml, ***(18)***, water was exchanged daily with percentage of 20% and the drug was added for each aquarium with dose of 0.1 µg/ml to compensate the losted drug.	
2-Clove oil	Clove oil dissolved firstly in ethanol 70% (1:9 ratio) and used as a bath with dose of 4.3 µ/ L, ***(19)***, water was exchanged daily with percentage of 20% and the oil was added for each aquarium with dose of 0.26mg to compensate the losted oil.	
3-Both Clotrimazole and clove oil	Both treatment were used with the same doses as previously described	
4-Control (Non-treated)	Control (+ve), non treated and infected through bath route as mentioned above	Control(-ve), non treated, non-infected

Determination of immunological parameters

Assay procedure for Lysozyme determination

The lysozyme activity was measured using the turbidity assay ***(20)***.

Assay procedure for Phagocytic activity

To measure the phagocytic capacity, the white blood cells were separated from blood of the tested fish in the different experimental groups. Heat-inactivated *Candida albicans (C. albicans)* was used to determine the phagocytic capacity of the phagocytic cells in each experimental group ***(21)***. phagocytic activity (PA) was determined ***(22)***.

N.B. phagocytic activity (PA) =Number of phagocytic cells with engulfed yeast / total number of phagocytic cells x 100.

Histopathological examination

Tissue specimens were taken from gills of treated groups of experimentally infected fish and fixed in 10% Phosphate buffered formalin, embedded in paraffin, sectioned and stained with Haematoxylin and Eosin stains (H&E) ***(11)***.

RESULTS

Mortality rate of *Branchiomycosis* was 90% in examined fish. The fish suffered from weakness, lethargy and respiratory distress (resulted from gill tissue damage) which represented by swimming vertical position to gasp the air, surfacing, accumulating at the water inlet with rapid operculur movement and finally die with open mouth. Gills appeared congested in acute condition (Fig.1A) and white in chronic condition when the disease progressed as a result of necrosis of gill tissue (Fig.1B).

Microscopical examination of squash preparations from the gills revealed brown, broad, branched and non-septated hyphae which contained numerous spores which may indicate the infection with Branchiomyces sp.(Fig.2).

On SDA media which supplemented with 10 % Duck decoction ,10% Gelatin,0.1 Citric acid and Chloramphenicol, primary growth of *Branchiomyces* sp. was observed after 2 days which was characterized by creamy, pasty and convex colonies in form of small pellicles (Fig.3A).Pieces from colonies stained with lactophenol cotton blue (LPCB) revealed broad, non-septated and branched hyphae at their tips (Fig.3B).Typical full growth of

colonies on culture were completed 8 days post inoculation and the shape of the colony modified to be bright white in colour, like cotton and firmly attached to the media (Fig.4A) and microscopic identification of the culture after staining with lactophenol cotton blue (LPCB) revealed an increase in thickness and more branching of the hyphae and filled with numerous spores .This branching was the key diagnostic feature to *Branchiomyces* sp. (Fig.4B).

Hyphal diameters of isolated *Branchiomyces* sp. were ranging from 16-24 µm and spores diameters were ranging from 4-10 µm.

The PCR test for *Branchiomyces* sp. was very sensitive for the detection of infection. There is no doubt that the infective agent of Branchiomycosis is related to fungi (Fig.5).

Histopathological alterations of gills infected with Branchiomycosis revealed non-septated, thick hyphae and aplanospores in between gill fillament, sloughing of the secondary lamellae, round cells infiltration and telangictiasis(H&E)(Fig.6A). PAS reaction revealed localization of the spores in between gill fillament, which confirmed that the isolated organism was *Branchiomyces demigrans* (Fig.6B).

Antifungal drugs sensitivity testing showed that, Clotrimazole is the highest inhibition zone (Table 2) (Fig.7).

Table 2. Antifungal sensitivity testing of *B. demigrans*.

Antifungal discs	Disc content	Diameter of inhibition zone (mm)	Sensitivity of fungi
Amphotericin B	100 unit	5mm	sensitive
Fluconazole	10µg/ml	-	resistant
Itraconazole	10µg/ml	-	resistant
Nystatin	100 unit	14mm	sensitive
Clotrimazole	10µg/ml	18mm	sensitive
Ketoconazole	10µg/ml	17mm	sensitive

Experimentally infected *O.niloticus* nearly showed similar clinical signs. Reisolation of the fungus on SDA media with 10% duck decoction revealed positive results for *B.demigrans* (Fig.8).The mortality percentage was 80% in the group which subjected to 30 liter *B.demigrans* suspension in water and injected with a synthetic cortisone and was 60% in the group which subjected to 30 liter *B.demigrans* suspension in water. While the mortality percentage was zero in the control groups (Table3).

Table 3. Mortality rate in experimentally infected *O. niloticus* with *B.demigrans*

Group No.	No. of fish	Mode of infection	Mortalities within							No of dead fish	Mortality rate %
Control groups			48hr	72hr	96hr	5days	7days	10days	14days		
1	20	Not subjected to any stressors	0	0	0	0	0	0	0	0	0
2*	20	Injected with cortisone at dose 20mg/kg body weight.	0	0	0	0	0	0	0	0	0
Infected groups											
3	20	Bath challenges with 30 liter *B.demigrans* suspension in the water	1	2	3	2	2	1	1	12	60%
4*	20	Bath challenges with 30 liter *B.demigrans* suspension in the water	4	3	3	2	4	0	0	16	80%

N.B. (*):Fish groups injected with cortisone at dose of 20mg/kg body weight.

The duration of treatment for one week revealed an improvement in the health state of the experimentally infected fish which indicated by gradual disappearance in the clinical signs of the disease and few mortality rates were recorded compared to infected &non-treated group (Table 4).

Table 4. Treatment of experimentally infected *O.niloticus* with *B.demigrans*

Group No=30	Treatment			Dead fish	
	Clove oil	Clotrimazole	Both treatments	No	%
Control (-)	Neither treated nor infected			0	0
Non-infected	+			0	0
Non-infected		+		0	0
Non-infected			+	0	0
Infected through bath route (3 groups, each group received type of treatment)	+			3	10%
		+		2	6.7%
			+	1	3.3%
	Non treated			15	50%

Table 5 showed the lysozyme activity between the infected groups and non-infected treated groups and effect of type of treatment on both groups.

Table 5. Lysozyme activity after treatment

Route / Treat	Infected with *B.demigrans*	Non-Infected & Treated
Clove Oil	0.230 ± 0.012^{Aa}	0.493 ± 0.036^{Ba}
Clotrimazole	0.362 ± 0.016^{Ab}	0.552 ± 0.021^{Bb}
Both	0.451 ± 0.023^{Ac}	0.645 ± 0.066^{Bc}
Infected & Non treated	0.187 ± 0.046^{Ad}	

Table 6 showed the phagocytic activity between the infected groups and non-infected treated groups and effect of type of treatment on both groups (Fig.9).

Table 6. Phagocytic activity after treatment

Route / Treatment	Infected with *B.demigrans*	Non-Infected & treated
Clove Oil	30 ± 1.15^{Aa}	50 ± 4.70^{Ba}
Clotrimazole	50 ± 1.33^{Bb}	83.3 ± 2.19^{Cb}
Both	55.6 ± 2.54^{Bc}	93.7 ± 2.29^{Cc}
Infected & not treated	5 ± 2.89^{Bd}	

Means carrying different superscripts **(capital letters) within the same raw** and those carrying Means carrying different superscripts **(small letters) within the same column** are significantly different at **($P < 0.05$)** based on **Duncan's multiple range test (DMRT).**

Fig.1. A. *O.niloticus* infected with *Branchiomyces* sp. showing congested gills in acute condition. B. white-coloured gill tissue in chronic condition.

Fig. 2: Squash preparations from the gills affected with *Branchiomyces* sp. revealing brown, broad, branched and non-septated hyphae which contained numerous spores.

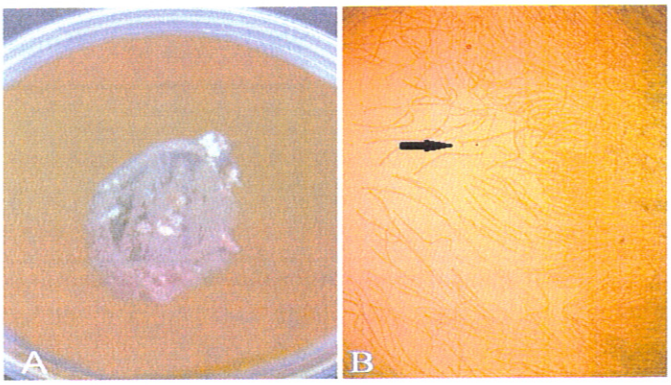

Fig.3. A. *Branchiomyces* sp. culture growth at 3rd day on SDA media with 10% duck decoction, the colonies were creamy, pasty and convex in form of small pellicles. B. microscopiclly, through a wet mount preparation, the hyphae were broad, non-septated, intermingled with each other and branched at their tips.

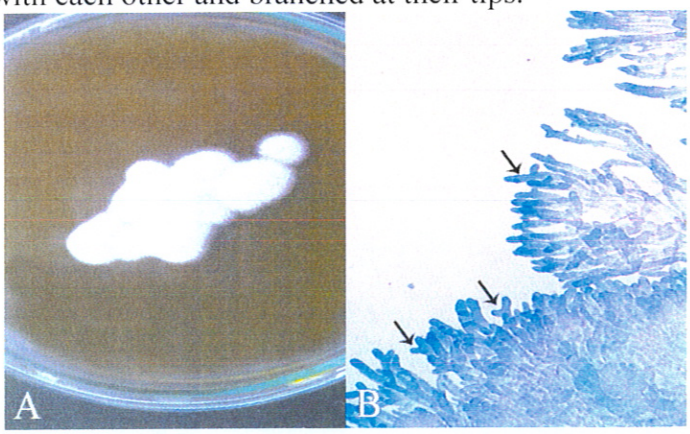

Fig.4. A. *Branchiomyces* sp. culture growth at 9days on SDA media with 10% duck decoction, the colonies were bright white in colour, like cotton and firmly attached to the media. B. Microscopic identification of the culture after staining portion of colony with (LPCB) revealing an increase in thickness, more branching of the hyphae and filled with spores.

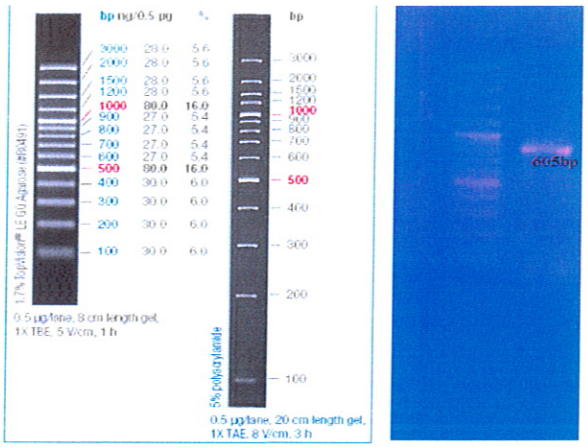

Fig.5. The PCR product of *Branchiomyces* sp. gene give band at 605bp.

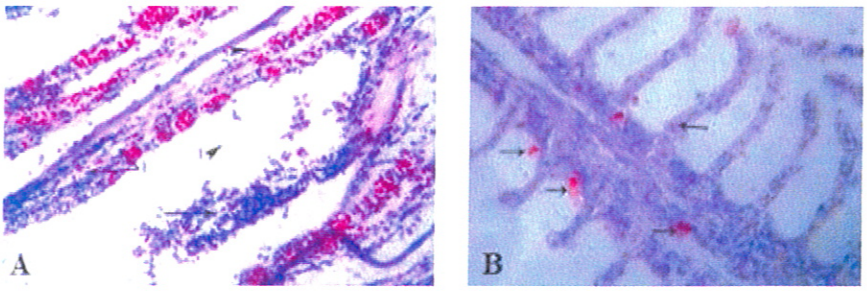

Fig.6. (A) Gills of naturally infected *O.niloticus* showing hyphae (arrow1) and spores (arrow2) of *Branchiomyces* sp. beside sloughing of secondary lamellae (arrowhead 1), round cells infiltration and telangictiasis (arrowhead2). H&E. (Bar 100 µm). (B)Gills of naturally infected *O.niloticus* showing few bright red spores (arrows)in between the gill fillaments, PAS reaction x 400.

Fig.7. Sensitivity test of *B.demigrans* to tested antifungal discs (Amphotericin B, Fluconazole, Itraconazole, Nystatin, Clotrimazole and Ketoconazole) using disc diffusion method.

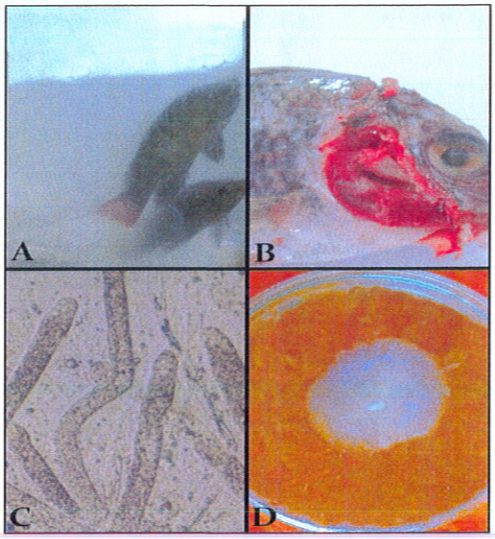

Fig. 8. (A)*O.niloticus* experimentally infected with *B.demigrans* showing signs of asphaxia, fish try to gasp air from water surface.(B)Gill tissue showing areas of necrosis.(C) Squash preparation from the affected gills revealing brown, broad, branched and non-septated hyphae which contained numerous spores.(D)Reisolation of *B.demigrans* on SDA media revealing white colony growth.

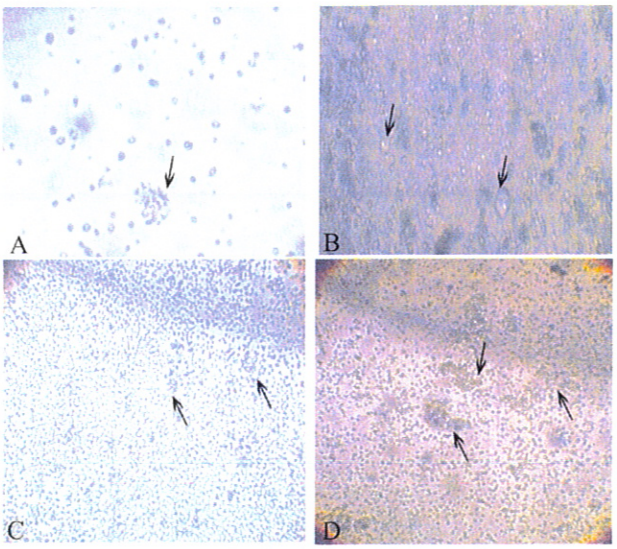

Fig. 9. Effect of different types of treatment on cells phagocytic activity after 5 days of experimental infection (A) Control group (untreated cells).(B) Cells exposed to clove oil.(C) Cells exposed to clotrimazole. (D) Cells exposed to both clove oil and clotrimazole.

Effect of treatment was evaluated through histological sections from treated groups which revealed the best regeneration of the gill fillament in the group which treated with clove oil and Clotrimazole together followed by group which treated with Clotrimazole then group which treated with clove oil(Fig.10).

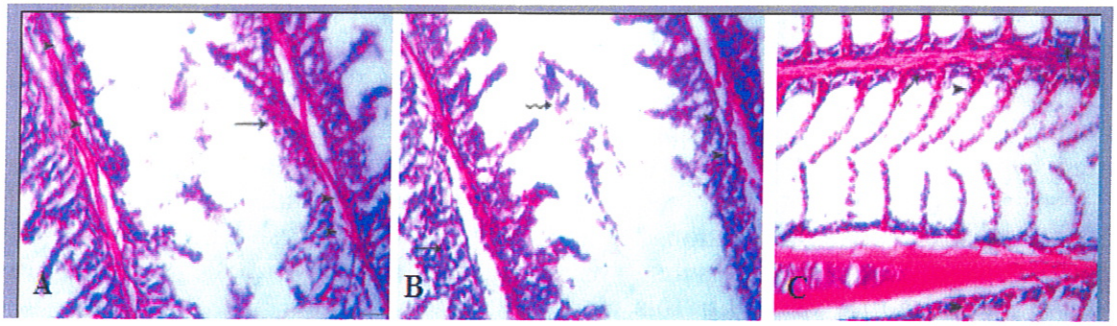

Fig.10. (A) Gills treated with Clove oil showing sloughing of the secondary lamellae with numerous spores and hyphae (arrows) and focal regenerative attempts were irregularly encountered. HE x 50 µm. (B) Gills treated with Clotrimazole showed slight desquamation in the covering epithelium (irregular arrow) with few hyphae (arrowheads) and regeneration of the secondary lamellae. HE x 50 µm.(C) Gills treated with Clove oil and Clotrimazole showed complete regeneration in the gill fillaments with slight congestion (arrowheads) and round cells infiltrations (arrow). HE x 50 µm.

DISCUSSION

Initial assessment of the mass mortalities which had occurred among adult tilapia were very complicated if multifactorial hypothesis was considered. However, by progress of diagnostic investigations through the entire event of mass kills during summer season, visual detection of signs of asphyxia (fish accumulated at water surface and gasping of air and finally died with open mouth) shortly before death, shape of gills (congested in acute cases and white in chronic cases) and histopathological sections from infected gills were primary suggestive of a Branchiomyces infection. Massive mortalities may be attributed to elevated water temperature, higher organic matter in the pond, low oxygen water content and lower pH water. Squash preparations from the affected gills revealed brown, broad, branched and non-septated hyphae and contained numerous spores, similar results were previously obtained ***(1,5, 23, 24).*** Cultivation of *Branchiomyces* sp. on SDA media with 10% duck decoction and antibiotic revealed bright white colonies, like cotton and firmly attached to the media, the results were in agreement with the results previously recorded ***(5).*** Slides stained with lactophenol cotton blue revealed broad, non-septated hyphae, intermingled with each other, contained numerous spores and branched at their tips, this branching was the key diagnostic feature to genus branchiomyces, the results were previously obtained ***(23)***.

Hyphal diameters of the isolated *Branchiomyces* sp. were ranging from 16-24 µm and spores diameters were ranging from 4-10 µm. These measurements of fungi were partially in agreement with those previously obtained ***(3).*** These results confirmed that the isolated pathogen was assigned to *B.demigrans.*

The PCR test for *Branchiomyces* sp. was very sensitive for the detection of infection and it was the first trial for detection of *Branchiomyces* sp. infection using the universal primer. There is no doubt that the infective agent of Branchiomycosis is related to fungi. Species identification needs more trials.

Histopathological alterations of gills infected with Branchiomycosis revealed non-septated hyphae and aplanospores in between gill tissues, sloughing of the secondary lamellae, round cells infiltration and telangictiasis (H&E), the results were previously confirmed ***(5, 23, 25)***.In this work, staining with PAS reaction was carried out which confirm that the isolated pathogen is fungus and belonging to *B.demigrans.*

The highest lysozyme activity and phagocytic activity were clearly noticed in fish group which received both treatment. These findings were supported by **Berenji *et al. (2014),(26)*** who mentioned the strong action of clotrimazole which blocks ergostrol synthesis by suppressing fungus demetylase cytochrome enzyme and causes growth stop with effecting on its membrane. Also, **Pinto *et al. (2009),(27)*** recorded that, clove oil is a strong antifungal agent as it caused a considerable reduction in the quantity of ergosterol, a specific fungal cell membrane component. These results were similar to that obtained by **Hoskonen *et al. (2013),(28)*** who mentioned that, clove oil as an effective antifungal agent as it resulted in significantly reduced growth of Saprolegnia (which is related to *Branchiomyces* sp.) hyphae compared with control treatment after exposure to stock solution of clove oil with ethanol.

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الملخص العربى

البرانكوميكوسيس فى اسماك البلطى النيلى فى محافظه البحيره مع محاولات للعلاج

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تم تجميع عدد ١٠٠ سمكه بلطى نيلى من مزارع ادكوالخاصه فى محافظه البحيره اثناء الفتره من يوليوالى سبتمبر ٢٠١٣ لعزل فطر البرانكوميسيس وكانت اهم العلامات المرضيه للاسماك المصابه صعوبه فى التنفس مع سرعه حركه طبقه الخياشيم مع نسبه نفوق عاليه وصلت الى ٩٠%واظهرالفحص المباشر للخياشيم المصابه من خلال وضعها بين شريحتين زجاجيتين وفحصها تحت الميكروسكوب عن وجودالخيوط الفطريه بنيه اللون عديمه الحواجزوبعد الملاحظه اليوميه لمراحل نمو الفطرعلى سبارود اجار اظهرمستعمرات بيضاء بعد يومين والتى وصلت اعلى نمو بعد ثمانيه ايام واظهرالفحص المجهرى لشريحه من فطرالبرانكوميسيس المصبوغه بصبغه الاكتوفينول الزرقاء عن وجودالخيوط الفطريه المتفرعه من اعلاها وهيه المميزه لفطر البرانكوميسيس بالاضافه الى قياس قطرالابواغ والخيوط الفطريه ٤-١٠و ١٦-٢٤ ميكروميترعلى التوالى مما يؤكد على ان الفطرهو برانكوميكوسيس دى ميجرانس وتم استخدام تفاعل البلمره المتسلسل لتشخيص المرض وعمل عدوى تجريبيه وكانت المظاهر الاكلينيكيه للاسماك المصابه تجريبيا مشابه للاسماك المصابه طبيعيا وتم اعاده عزل الفطرمنها وتم عمل محاولات للعلاج باستخدام الكلوتريمازول وهومضاد للفطريات والذى كان اكثر كفائه من زيت القرنفل بينما استخدام كلاهما فى وقت واحد اوضح اعلى معدل من ارتفاع الليزوزيم ونشاط الخلايا الملتهمه وتم فحص التغيرات الباثولوجيه لخياشيم الاسماك المصابه طبيعيا وتجريبيا والذى اظهرالابواغ الفطريه بين انسجه الخياشيم مما يؤكد ان الفطر المعزول هو برانكوميكوسيس دى ميجرانس