ROLE OF NIGELLA SATIVA IN DECREASING MORTALITIES IN NILE TILAPIA CAUSED BY PSEUDOMONAS SEPTICEMIA

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ABSTRACT

This work was carried out to investigate Pseudomonal septicemia in Nile tilapia, *Oreochromis niloticus*, at Assiut governorate and to evaluate the effects of oral administration of black cumin *Nigella sativa* on resistance against *Pseudomonas aeraginosa* infection. A total of 100 Nile tilapia were collected from River Nile and El-Ibrahemia canal, Assiut governorate, and were subjected to clinical and bacteriological examination. Pseudomonase like isolates were detected from 29 out of 100 collected and examined fish samples (The incidence of pseudomonase infection was 29%). Biochemically, the collected isolates were identified as *P. aureginosa* and *P. putida* and other unidentified pseudomonase species. The organisms were mainly isolated from liver, spleen and kidney. The pathogenicity of the isolated *P. aureginosa* from Nile tilapia was confirmed by an experimental pathogenicity challenge. A total of 90 Nile tilapia were divided into two equal groups with three replicates to investigate the effect of dietary supplementation of black cumin on diseased resistance throughout the experimental challenge. Fish of the first group were fed on basic diet, while fish of the second group fed on basic diet with 3% black cumin /kg diet (3 g. black cumin /100 g diet) for 30 successive days. Experimental challenge was done by immersion where the infected fish showed typical signs of pseudomonal septicemia; redness all over the body, ulceration, scales detachment, darkening of body and congestion of all internal organs. Cumulative mortalities of fish challenged were significantly less in groups fed on black cumin diet (13.33%) than those fed on the basic diets (53.33%). Moreover, lesions and symptoms were less or sporadically seen in group treated with black cumin. These results showed black cumin improve the fish resistance to disease.

Key words: *Pseudomonal septicemia, Nile tilapia, black cumin*

INTRODUCTION

Bacterial pathogens are the causative agents of most serious disease problems in both wild and cultured fish causing mortalities and severe economic losses (Roberts, 2001). Pseudomonas infection has been incriminated as an important bacterial infection among fish and appear to be stress related disease of freshwater fish especially under culture conditions (Kitao *et al.,* 1993)

Pseudomonades are opportunistic Gram negative pathogens, naturally occur in aquatic environment and as a part of normal gut flora of healthy fish, it causes outbreak when the optimum environmental conditions change (Angelini and Seigneur 1988). The genus Pseudomonas contains five species which have been described as etiological agents of diseases in fish in Egypt. *Pseudomonas fluorescens,P. angulliseptica, P. aeruginosa* and *P. putida* were identified in various species of fish as causative agents of pseudomonas septicemia (Sakar and Azza 2008 and EL-Nagar 2010), which characterized by fin rot, petechial hemorrhage, darkness of the skin, detached scales, abdominal ascitis and exophthalmia (Khalil *et al.,* 2010).

Using natural feed additive is becoming useful for fish feeding rather than classic chemical feed additives due to the cumulative effects of the chemical components induced deterrent effects on human health (El-Dakar *et al*., 2008). Black cumin, *Nigella sativa*, an annual herb that belongs to the botanical family of *Ranuculaceae*, showed antibacterial, fungicidal effects (Akgul, 1989). Black cumin have been used as enhancer for performance, growth and immune system of some fish species (Abdel-Ghaffar *et al.,* 2003; Diab *et al.,* 2008)

The aim of the present study was to investigate the incidence of pseudomonas species in Nile tilapia at Assiut governorate, as well as the pathogencity of the

isolated bacteria to Nile tilapia. This study was also performed to evaluate the effect of dietary supplementation of black cumin on diseases resistance of Nile tilapia challenged with pseudomonas.

MATERIALS and METHODS

Clinical and Postmortem Examination of Naturally Infected Fish:
A total of 100 alive Nile tilapia, *Oreochromis niloticus*, and weighing 100-350 g with total length of 14-26 cm were collected from El-Ibrahemia canal and River Nile from November 2012 to April 2013. Fish were transported immediately to the Aquatic Animals Wet Lab., Veterinary Hospital Clinic, Faculty of Veterinary Medicine, Assiut University. Fish were subjected to clinical and bacteriological examination (Plumb and Bowser, 1982), observed signs were recorded and detected lesions were reported.

Isolation and Identification of *Pseudomonas spp.* From Fish:
Samples from internal organs of the examined fish were streaked onto bile salt brilliant green agar (Lab M), Pseudomonas p. agar medium plates ((Lab M) and brain heart infusion agar (Lab M), then incubated at 28ºC for 24hr. Bacterial colonies were identified according to colony morphology, bacterial staining character, and biochemical character (Palleroni,1984 and Buller, 2008).

Experimental Fish:
Apparently healthy Nile tilapia with an average body weight of 100 ± 5 g were obtained from a private fish farm in waladya area at Assiut Governorate and transported to the Aquatic Animals Wet Lab., Veterinary Clinical Hospital, Faculty of Veterinary Medicine, Assiut University where they kept in well prepared aquaria. Random samples were used to check whether they are Pseudomonal septicemia free. Fish were acclimated for 2 weeks according to the protocol of maintaining bioassay fish as was previously described by Ellsaesser and Clem (1986) and received commercial food.

Experimental challenge:
Bacterial strain:
Bacterial strains were kept in BHI broth with 15% glycerol (El-Gomhurrhia, Cairo, Egypt) at -20ºC. *Pseudomonas aeraginosa* strain was passed three times in Nile tilapia through intraperitoneal injection before using for experimental challenge.

Bacterial challenge suspension and counts:
Colony forming units (cfu) counts in bacterial suspensions were determined using spectrophometery optical density values at wavelength of 600 nm and standard-plate-count method with ten-fold serial dilution (Elkamel and Thune, 2003).

Experimental challenge:
Acclimated Nile tilapia were divided into three groups with 15 fish each. The first group was infected through immersion in 1 X 10^7 cfu/ml, suspension of *p. aeraginosa* for 30 minutes in 30L, while the second group was subjected to sterile BHI broth for the same duration and the other group remained unchallenged. The whole experiment was repeated three times. Re-isolation and identification of the inoculated organism from freshly dead and moribund fish were carried out as described above.

Diets and feed additives:
Two different diets with or without additives, representing two diet variants, were formulated to be used for feeding of fish. A basic diet (control) was formulated of grounded yellow corn (34.9%), soya bean meal (28.6%), fish meal (17.0%), wheat bran (9.3%), vegetable oils (6.5%), ground lime stone (0.70%), bone meal (0.30%), mineral mixture (1.7%) and vitamin mixture (1.0%). The other experimental diet was formulated as a Nigella diet (3 g. black cumin, *N. sativa* /100 g. of basic diets).

Experimental design:
Acclimated Nile tilapia were allotted into two replicates, one replicate received the nigella diet, while the other replicate received the basic diet. Each replicates were subdivided in to three groups (15 fish each). Each replicate was fed twice daily for 30 successive days.

The two challenge groups of each replicates were challenged through immersion in 1 X 10^7cfu/ml of *Pseudomonas aeraginosa* for 30 min in a volume of 30 L. While challenged control groups of each replicates was subjected to sterile BHI broth for the same duration (30 min), while the other group remained unchallenged. Mortalities and clinical signs were recorded daily for 21 days. Re-isolation and identification of bacteria was done from freshly dead fish as mentioned above. The whole experiment was repeated three times.

RESULTS

Clinical and postmortem examination:
Clinical examination of naturally infected fish revealed the presence of septicemia signs on some fish represented by skin darkness and scales detachment in 6 examined fish. Fish exhibited congestion and petechiae on the body surface, especially on the ventral part of abdomen and fins in 22 fish. The postmortem examination revealed congestion of the spleen, kidney and liver in 18 fish. In 17cases, showed enlarged gall bladder and distended with bile. The remaining fish appeared to be clinically healthy.

Bacterial isolation and identification:
Bacteriological examination resulted in isolation of 34 isolates suspected to be *Pseudomonas aureginosa*

(n=18), *Pseudomonas putida* (n=6) and unidentified *Pseudomonas spp*.(10) according to Morpho-biochemical test. Bacterial colonies grown on BHI agar were circulated, convex, entire edge, glistening, creamy color and 1-2 mm in diameter. On pseudomonas p agar colonies were greenish white colonies, while on bile salt brilliant green agar, bacterial colonies were whitish, convex and 1-2 mm in diameter. Results of the biochemical characters and enzyme activities of suspected isolates are shown in Table (1).

Results also revealed that the organism could be mainly isolated from spleen, liver, kidney. Pseudomonas existence ratios in different fish organs was 12in spleen, 10 from liver and 12 from kidney.

Experimental challenge was done by immersion in 1 X 10^7 CFU/ml viable cells of *P. aerugenosa* in two groups of fish. The infected fish in two groups of fish display the same clinical signs and postmortem lesions but in different percentage (level).The challenged fish exhibited signs of ulceration on the body and fin rot (in four fish in group 1 and 2 fish in group two). Petechial haemorhage on different part of the body surface especially on the ventral part of abdomen, fin and gill cover were recorded in six fish in group one, however 4 in group two.Dark pigmentation were observed in three fish in group one. Internally there were congestion of all internal organs in eight fish in group one and in two fish in group two. Gall bladder was enlarged and distended by bile. Intestine was filled with bloody serous fluid in two fish of group one (Fig 1).

Mortality rate after bacterial infection showed a significant decrease in *Nigella sativa* treatment($p<0.05$) in a way that mortality rate in *Nigella sativa* treatment group, basic diets group and in control group were 13.33%, 53.33% and 0% respectively.

Table 1: Cultural and biochemical characters of the isolated bacteria (n=34).

characters	*P.aeraginosa*	*P.putida*	Un-identify strains
No. of isolates	18	6	10
Gram stains	G-ve	G-ve	G-ve
Motility	+	+	+
Oxidase test	+	+	+
Indole	-	-	-
O/F test	-/-	-/-	-/-
Catalase	+	+	+
H2S production	-	-	4/10
Urease	+	-	2/10
V.P.	-	-	-
M.R.	-	-	5/10
Gelatin liquification	+	-	4/10
Growth on 5%Nacl	+	+	7/10
mannitol	+ acid only	-	
Sucrose	-	-	
Lactose	-	-	
Maltose	-	-	

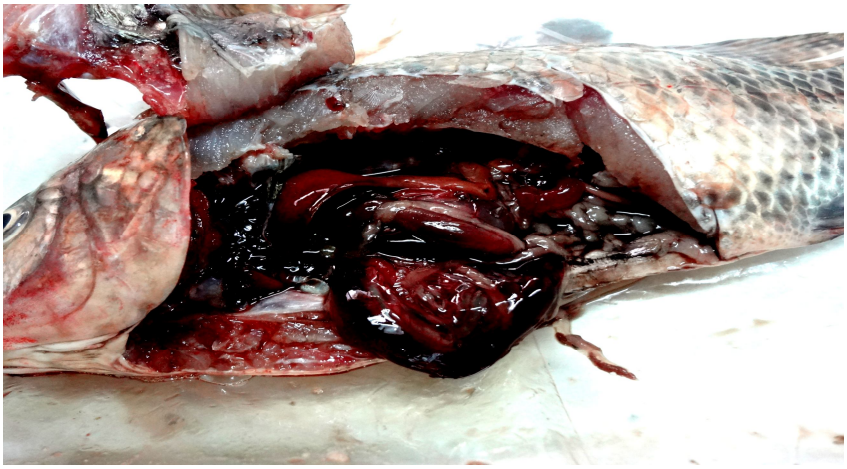

Fig. 1: Nile tilapia, experimentaly infected with *Pseudomonase aeraginosa* showing congested liver, kidney and bloody fluids filling the intestine

DISCUSSION

Pseudomonas septicemia is one of the important pathogenic bacteria affecting fish farm in Egypt (Khalil *et al.,* 2010). The present study was done to assess and characterize the pseudomonas infection in Nile tilapia, *O. niloticus* in Assiut governorate and to investigate the effect of dietary supplementation of black cumin*, Nigella sativa* on the diseases resistance of cultured Nile tilapia.

Results revealed that fish naturally infected with *Pseudomonas spp*. showed signs of infection were nearly similar to those reported by (Khalil *et al.,* 2010, EL-hady and Samy 2011).

Bacteriological analysis of naturally infected fish resulted in the isolation of two different *Pseudomonas spp*. including *P.aeraginosa* and *P.putida*. The isolates showed phenotypic and biochemical characteristics of the isolated *P. aureginosa* and *P.putida* were parallel to previous studies which identified the same organisms from other fish species (Austin and Austin, 2007 and Buller 2008).

In this study, the incidence of *Pseudomonas spp*. in the examined *O. niloticus* was 29%. The result supports previous studies of the examined *O. niloticus* 25.5% (Saleh *et al.,* 2008) and 30.83% (Eissa *et al.,* 2010). These results are not in agreement with those reported by EL-hady and Samy (2011) who isolated *Pseudomonase spp.* From *O. niloticus* with percentage of 55.3%.

Regarding the samples of bacterial isolates among various organs of fish, it was revealed that the isolation from liver, spleen and Kidney approximately had the same rates. This result agreed with (El-Refaey, 2013). This may be due to most of bacterial infections affect haemobiotic system mainly liver, kidney and spleen.

Experimental infection was successfully done by immersion. The result of the current study demonstrated that clinical picture of pseudomonas septicemia characterized by signs of dark pigmentation, petechial hemorrhage on different parts of the body surface, ulceration, especially at dorsum part and at the base of fins with eroded fin (fin and tail rot). It may be due to the toxic proteases produced by this organism, thus serving to destroy the body tissues. Hemorrhages at the base of fins could be primarily induced by release of powerful bacterial proteolytic enzymes which lead to electrolyte and protein loss together with disturbed blood circulation (Amlacker, 1970 and Mortia, 1975). Congested internal organs are a septicemic lesion, where the congestion and edema was seen to play a role in the enlargement of kidney, spleen and liver. The over distended gall bladder could be attributed to the enteritis or to encountered constriction of the common bile duct by peri-duct fibrosis, these results are conceited with those noticed by Eissa *et al.* (2010); Kalil *et al.* (2010).

The pathogenicity of *Pseudomonas spp*. For experimentally infected Nile tilapia may be attributed to the production of extracellular enzymes and total toxins (as protease, haemolysins, enterotoxins, enterotoxins cytotoxins and others) (El-Attar and Mostaf 1996) Abou El-Geit *et al.,* 2013).

The challenge infection revealed cumulative mortalities of Nile tilapia were significantly less in fish fed on nigella diets (13.33 %) than those of fish fed on the basic diets (53.33%). Moreover, lesions and symptoms were minimized or sporadically seen in group treated with black cumin. In this study, stimulation of the immune system of Nile tilapia as a result of feeding of black cumin diets have positively impacted the resistance of fish to *P. aeraginosa* infection as was indicated by the significantly lower mortality rates of fish challenged with virulent *P. aeraginosa*. It was reported that *Ocimum sanctum* enhanced the disease resistance in *Oreochromis mossambicus* against *A. hydrophila* infections (Logambal and Michael 2000). Furthermore, Nile tilapia fed with probiotics and challenged with *A. hydrophila* showed significant decrease in mortalities (Ali *et al.,* 2010). Moreover Yılma *et al.* (2012) reported that cumulative mortality was 60% in fish fed the 0% control diet and challenged with *Streptococcus iniae*. However, in fish fed the 2.0% supplemented diets with black cumin, mortality was only 37.50%. These results are in fair agreement with the administration of herbal supplemented diets showing resistance against streptococcal disease in tilapia fed *Rosmarinus officinalis* (Abutbul *et al.*, 2004; Zilberg *et al.,* 2010).

This result agreed and explained by (Elkamel and Mosaad 2012) who reported that dietary supplementation of black cumin enhanced the overall immune response of Nile tilapia as was indicated by the significant increase of the WBC numbers, (White blood cells (WBCs) of fish play a crucial role in the cellular immunity and resistance to infectious diseases (Whyte, 2007), globulin proteins and the phagocytic activities of fish phagocytes. This modulation of the fish immunity has greatly enhanced the resistance of challenged fish to *A. hydrophila* as was indicated by the significant decrease in mortalities in fish received the nigella diets. Al-Dubakel *et al.* (2012**)** reported that black cumin enhance T cell immunity and production of cytokines (Haq *et al.* 1995), natural killer cell and compliment (Mahdi, 1993). It also inhibit some microbe and has anti-helmintic activity against nematodes and cestodes (Agarwal *et al*. 1997). Black cumin extract has positive effect on leukocytes (Mona *et al*., 2002). Diab *et al.* (2008) argued that black cumin could increase the survival rate and the resistance of fish to some infectious diseases. Black cumin seed could be recommended to be used for farmed fish to decrease

mortalities caused by pathogenic microorganisms (Dorucu1 *et al.,* 2009).

On conclusion, *P. aerugenosa* can be considered as accountable fish infection under culture condition. The use of 3% black seeds for 30 days could increase the survival rate and the resistance of fish to some infectious diseases.

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دور الحبة السوداء فى تقليل الوفيات فى السيدوموناس الدموية فى اسماك البلطى النيلى

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أجريت هذة الدراسة بهدف دراسة مرض السيدوموناسى فى اسماك البلطى النيلى واجريت أيضا لتقييم (للاستدلال)على مدى تاثير تناول حبة البركة على مدى مقاومة أسماك البلطى النيلى للعدوى البكتيرية بميكروب *pseudomonas aeraginosa*. تم جمع عدد ١٠٠ سمكة بلطى نيلى من نهر النيل والترعة الإبراهيمية بمحافظة اسيوط كما تم فحصها أكلينيكيا وبكتيرولوجيا .وقد تم تسجيل الأعراض الظاهرية والتشريحية لها. وقد تم عزل ٣٤ من عترات السيدوموناس من الأعضاء الداخلية ل ٢٩ سمكة من أجمالى ١٠٠ سمكة تم فحصها (٢٩%) وقد تم تصنيف العترات المعزولة بيوكيميائياعلى أنها سيدوموناس أيروجينوزا ، سيدوموناس بيوتيدا ومجموعة مجهولة (غير مصنفة) من السيدوموناس. تمت العدوى الصناعية بنجاح لتقصى قدرة بكتيريا السودوموناس أيروجينوزا المعزولة من سمك البلطى على الامراض. خصصت عدد ٩٠ سمكة من أسماك البلطى النيلى ، قسمت الى مجموعتين لتقييم تاثير الحبة السوداء على مدى مقاومة هذة الأسماك للامراض طوال الفترة العلاجية (٢١ يوم). الأسماك فى المجموعة الأولى تم تغذيتها غلى علف خالى من أى اضافات ، بينما الأسماك فى المجموعة الثانية تم تغذيتها على علف مضاف الية الحبة السوداء مجروشة بجرعة ٣ جرام حبة سوداء /١٠٠ جرام علف.تم اطعام هذة الاعلاف لكل مجموعة من الأسماك على مدى ٣٠ يوم متتالية. وقد تم العدوى التجريبية بنجاح عن طريق الغمر. أتصفت الأسماك المصابة بالأعراض العامة للسيدوموناس الدموية ومنها أحتقان وأنزفة على سطح الجسم ، تقرحات ، فقدان القشور، اسوداد لون الجسم وأحتقان الأعضاء الداخلية. وجد أن معدل الوفيات (النفوق) فى مجموعة الاسماك التى غذيت على العليقة التى تحتوى على حبة السوداء (١٣.٣٣%) أقل من مجموعة الأسماك التى غذيت على العليقة الضابطة (٥٣.٣٣) خلال فترة التجربة، كما قلت نسبة ظهور الأعراض والآفات المرضية المصاحبة فى مجموعة الأسماك التى غذيت على عليقة التى تحتوى على حبة البركة. وأظهرت النتائج أن الحبة السوداء تحسين مقاومة الأسماك للأمراض.