Role of Some Biological Pollutants in Relation to Disease Occurrence in *Oreochromis Niloticus*

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Abstract
The current study was performed to investigate the role of biological (bacterial) pollutants in disease occurance in *Oreochromis niloticus* obtained from the western lagoon of Lake Temsah, Ismailia. The naturally infected *O.niloticus* showed no pathognomonic signs or lesions, except some fish revealed haemorrahage, tail rot with enlarged and different grades of congestion in the internal organs. There were different isolated bacteria as identified *Pseudomonas aurginosa, Proteus vulgaris, Enterobacter cloacae,E.coli* and salmonella sp. They were identified using ViteK2 system. *Ps. aurginosa* was confirmed by using PCR. Experimental infection was carried out and reisolation of *Ps. aurginosa* was tried.

Introduction:

Fish and seafood are considered important sources of high quality protein, minerals and essential polyunsaturated fatty acids ***(kris-Etherton et al., 2003 and Guerin et al., 2011).*** In Ismailia as a coastal city,where are people basically depend on fish as a main source of animal protein ; most caught from Suez Canal and lake Temsah ***(Abdel Fattah,1992).*** Lake Temsah, the main site of the study, besides being one of the well-known wetlands in Suez Canal region and a main tourist attraction , it is one of the main sites in Egypt where vast numbers of migratory birds are passing through ,especially during winter on their way from Europe to Africa ***(Varo et al., 2002).*** Faecal indicator bacteria such as total coliforms, faecal coliforms, and faecal streptococci were utilized worldwide to measure health hazards in aquatic systems ***(Sanders et al., 2005).*** The present study was planned to investigate the role of some biological pollutants in western lagoon of Lake Temsah in the occurrence of diseases in *Oreochromis niloticus*.

Material and Methods

Fish

A total of 120 fish *Oreochromis niloticus* were randomly collected alive from western lagoon of Lake Temsah with an average body weight (257±10g) and transferred

under complete aseptic conditions in ice box to the laboratory of Department of Fish Diseases and Management, Fac. of Veterinary Medicine,Suez Canal University.

A total number of 26 apparently healthy *O.niloticus* were collected alive used for experimental infection.

Clinical examination;

All live and freshly dead fish samples were clinically examined according to the methods described by ***Amlacher (1970).*** The postmortem examination was performed on all freshly dead and moribund fishes according to ***Conroy and Herman (1981).***

Bacteriological examination;

Isolation of bacteria were done on different media(Nutrient agar slant, MacConkey agar, Brilliant green medium, Sallmonela Shigella agar (SS agar) ,XLd medium ,EMB medium, pseudomonas F agar) according to ***Austin and Austin (2007).***

Isolation;

Under complete aseptic conditions, fish specimens (skin, gills and internal organs as liver, kidneys, intestines and spleen lesions) were inoculated over nutrient agar and incubated at 37^0C for 24 hrs. Re-inoculation of cultured bacteria occurred until separated colonies appeared. The suspected purified colonies were picked up for further identification according to ***Austin and Austin (2007).***

Identification:

Bacterial isolates were identified using colony characters and Gram – stain ***Mackie and McCartney (1989)*** for microscopical examination to reveal reaction, bacterial shape and cell arrangement.

The identification of isolated bacteria was applied using the classical and Vitek2 system according to ***Pincus (2009).***

The detection of haemolysin (Asal) gene of pathogenic *Pseudomonas aeroginosa* isolates was adopted using PCR according to ***Touihri et al. (2009)***.

Primer sequences.

Cycling conditions of PCR.

Experimental infection:

The bacteria was inoculated into nutrient agar and incubated at 37^0C for 24 hrs. Bacterial colonies were collected in sterile saline (0.85% NaCl). According to ***Iqbal et al., (1999)***, each ml of saline contained 1.2 x 10^8 cells /ml (LD50 by using McFarland standard tube.

This experiment was done by using 26 apparently healthy *Oreochromis niloticus* with an average body weight 100$\pm$10 g. The fish were divided into two equal groups (1 and 2). Each group contained 13 fish and kept in the fully prepared glass aquaria which supplied with dechlorinated tap water and conducted with electric air pumping to obtain continuous aeration. The tested fish were fed on commercial pelleted feed containing 30% crude protein and kept in this condition for one week before injection for

acclimation. The first group was served as control and second group was experimentally infected fish by intra-peritoneal route (I/P) with 0.1 ml of saline containing 1.2 x 10^8 cells /ml (LD50) of 24 hrs bacterial cultures. The experimentally infected fish were monitored and observed daily for any clinical signs and mortalities were recorded. The postmortem examination was done to detect the internal lesions and bacterial reisolation was tried.

Results

Clinical signs:

Some fish revealed no pathognomic clinical abnormalities, some fish showed slimy. Others showed clinically one or more from the followings hemorrhages all over the body surface , base of dorsal , pectoral and caudal fins and redness around eyes . Ulcers were observed on the skin varied from shallow to deep with detachment of the scales. In most cases, the gills were congested and eroded with abdominal distention and inflamed vent (photo1).

Postmortem examination:

The freshly dead fish revealed enlarged pale liver and haemorrhagic or detached liver, enlarged full distended gall bladder and enlarged congested spleen. Also, in some cases showed enlargement and variable degrees of congestion in liver, congested gills and kidneys (photo2,3).

A- Clinical signs and Postmortem lesions of experimentally infected fish

The experimentally infected fish (*O. niloticus*) suffered from exophthalmia, scale detachment, ulceration of the skin, hemorrhages distributed all over the body surfaces and base of fins. The postmortem examination revealed congested and enlargement of all internal organs as liver, spleen, kidneys and gall bladder (photo 4and 5).

Results of Polymerase Chain Reaction of isolated *P. aeruginosa*:

Figure (1) gave PCR product with specific band at 530 base pair which confirmed the presence of *P. aeruginosa* in 5 isolates by using 16S rDNA species-specific *P. aeruginosa* primer.

Photo (1): *Showing distended abdomen O.niloticus infected with seudomonas aeriginosa fish.*

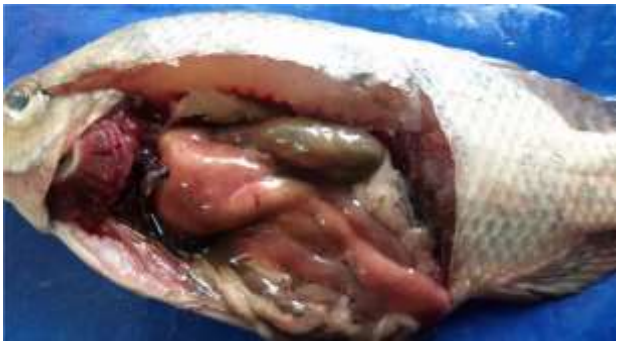

Photo (2): *Showing pale enlarged liver with ascetic fluid and congested gills in O.niloticus.*

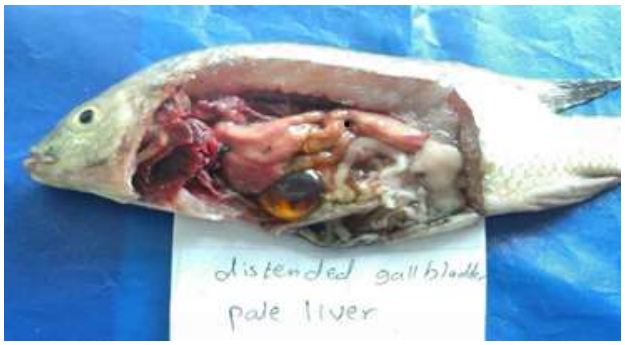

Photo (3): *Showing enlarged pale liver with distended gall bladder and congested gills.*

Bacteriological examination

Biochemical characters according to Vitek system:

Table (1): *Showing the Vitek 2 system results for Pseudomonas aeruginosa*

Biochemical Details																	
2	APPA	+	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H_2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATK	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	+	61	IMLTa	+	62	ELLM	-	64	ILATa	+			

Table (2): *Prevelence of bacterial infection in O.niloticus at western lagoon of Lake Temsah in different seasons.*

Season	No. of examined fish	No. of infected fish	%
Winter	30	8	26.66
Spring	30	18	60.00
Summer	30	22	73.33
Autumn	30	14	46.66
Total	120	62	51.66

Table (3): *Prevalence of microorganisms isolated from O.niloticus in different seasons:*

Micro organism	Autumn		Winter		Spring		Summer	
	No. of infected fish	Infected fish %	No. of infected fish	Infected fish %	No. of infected fish	Infected fish %	No. of infected fish	Infected fish %
Pseudomonas aeruginosa	**3**	**10**	**2**	**6.6**	**2**	**6.6**	**2**	**6.6**
E-coli	**9**	**30**	**3**	**10**	**3**	**10**	**3**	**10**
Enterobacter coloacae	**1**	**3.3**	**1**	**3.3**	**1**	**3.3**	**5**	**16.6**
Proteus vulgaris	**2**	**6.6**	**1**	**3.3**	**3**	**10**	**6**	**20**
Salmonella sp.	**-**	**-**	**1**	**3.3**	**9**	**30**	**6**	**20**
total	**15**		**8**		**18**		**22**	

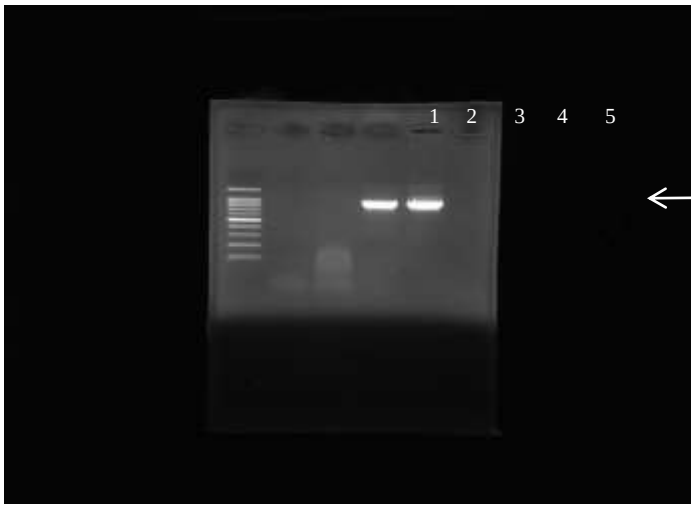

Photo (4): *Detection and identification of P. aeruginosa 16S rRNA by amplification of fragments in the multiplex PCR assays.*

Table (4): *Mortality rates in experimentally infected O. niloticus with Pseudomonas aeruginosa*

Fish	Group No.	Fish No.	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	Total	%
Oreochromi niloticus	1:control	13	0	0	0	0	0	0	0	0	0
	2: (I/P)	13	0	0	2	3	5	2	0	12	92

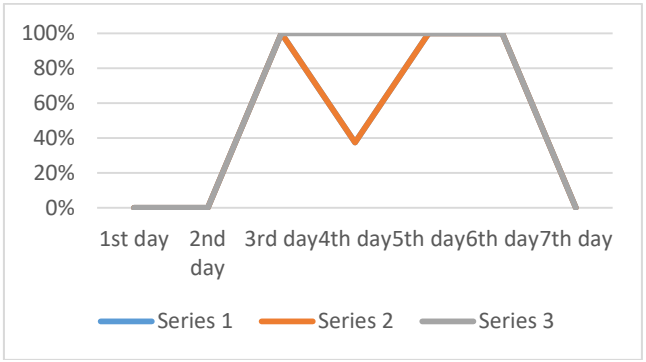

Figure (1): *Mortality rates in experimentally infected O. niloticus with Pseudomonas aeruginosa*

Photo (5):- *O.niloticus showing hemorrhages at the dorsal area and fins*

Photo (6): *O. niloticus showing enlarged pale liver*

Discussion

Environmental pollution is a worldwide problem. The progress of industry has led to increased emission of pollutants into ecosystem. Environmental pollution can cause poisoning, diseases and even death to fish. The absorption and accumulation of different pollutants vary among different biological systems ***(Zeitoun and Mehana, 2014).***

In the present study , isolation and identification of the bacteria was done by traditional methods, Vitek 2 compact system and the confirmation of the isolates was done by using PCR for detection of 16S rRNA and hemolysin gene (asal).

In this study, the most observed clinical signs of infected *O. niloticus* by different isolated bacteria were hemorrhages all over the body surface , base of dorsal ,pectoral and caudal fins plate and redness around eyes. Ulcers were observed on the skin varied from shallow to deep and detachment of the scales.The present results were nearly similar to that recorded by ***Azza et al., (2002) Altinok et al., (2006); Austin and Austin, (2007); Eissa et al., (2010); Janga et al., (2014); Enany et al., (2016)***

Regarding the postmortem lesions of freshly dead fish revealed enlarged pale liver and haemorrhagic or detached liver, enlarged full distended gall bladder and enlarged congested spleen. Also, in some cases showed enlargement and variable degrees of congestion in liver, congested gills and kidneys, haemorrhage on internal organs and bloody intestine and white patches on intestine. These results are nearly similar to that recorded by ***Blanco et al. (2002); Olurin et al .,(2006) : Altinok et al. (2007); Saleh and Azza (2002); Azza et al., (2012) and Enany et al. (2016)*** who recorded that the internal organs of naturally infected fish appeared pale , anaemic with enlargement and congestion of spleen , liver with distended gallbladder while intestinal wall was congested with the presence of ulcers and protruded of anus.

Regarding the highest prevalence of *Pseudomonas arigunosa* it was showing the highest in winter followed by spring , summer and autumn 10 ,6.6 , 6.6 and 6.6% respectively. These results were nearly similar to that recorded by ***Arafat Goja (2013)*** who reported that the the percentage of pseudomonas isolates from fish skin as 15.3% and 11.3%.

In our study, clinically infected fish were externally examined and showed presence of irregular hemorrhages on body surface, scales detachment and congested gills. These findings were agreed with ***Austin and Austin (2007):Eissa et al ., (2010) : Eissa et al., (2017)*** who mentioned that *Pseudomonas aeriginosa* cause petechial hemorrhage , darkness of

the skin , detached scales and abdominal ascites .

Using the classical and ViteK2 system, all the isolates of *Pseudomonas aeruginosa* produced pale colonies on Macconkey agar due to they were non lactose fermenter. Also, on nutrient agar they produced blue green colonies due to production of pyoverdin and pyocynin pigments. Besides, they produced green colonies on Pseudomonas F agar and Pseudomonas agar base. These results were in agreement with ***Altinok et al. (2007); Abro et al. (2009); Hanna et al. (2014).***

The Polymerase Chain Reaction (PCR) was highly sensitive specific and rapid method which improved the detection of *P. aeruginosa* especially when using species-specific primer ***(Buller, 2004; Xu et al., 2004)***

Selective amplification of pseudomonas16S rRNA gene by PCR has been used to detect and differentiate Pseudomonas species**.** It was also used for genus or species level identification of *P. aeruginosa* ***(Drancourtn et al., 2000; Porteous et al., 2002).***

The experimentally infected fish *O. niloticus* were suffered from exophthalmia, scales detachment, ulceration of the skin, hemorrhages distributed all over the body surfaces and base of fins. In addition to the postmortem examination revealed congested and enlargement of all internal organs as liver, spleen, kidney and gall bladder. These findings were in agreement with ***Azza et al. (2002); Eissa et al. (2010); Khalil et al. (2013).***

The mortality rate in experimentally infected *O. niloticus* with *Pseudomonas aeruginosa* by intra-peritoneal route were 92% of the total number of fishes used in the experiment. On the other hand ***Mona et al. (2017)*** who found that the mortality rates in experimentally infected *O. niloticus* with *Pseudomonas aeruginosa* by intra-peritoneal route were (80 and 50%) respectively of the total number of fishes used in the experiment. These results nearly agreed with ***Enany et al. (2016)*** who found that mortality rate in experimentally infected *Oreochromis niloticus* was 70% after 7 days. However, these findings disagreed with ***Austin and Stobie (1992)*** who found that pseudomonads cause 100% mortalities within 7 days by I.P. or I.M. routes into rainbow trout. In addition, ***Hossain et al. (2006)*** reported that *Pseudomonas aeruginosa* produced 30% mortality in *O. niloticus*.

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الملخص العربي

تم في هذة الدراسة عزل وتصنيف البكتريا الموجودة في مياة بحيرة التمساح وفي اسماك البلطي باستخدام الطرق التقليدية والحديثة مثل جهاز الفيتيك 2 وتفاعل البوليميريز المتسلسل والتأكد من التصنيف عن طريق تحديد جين 16sRNA.

وقد تم رصد العلامات الأكلينيكية للأسماك المصابة وهي انتشار بقع نزيفية على جميع انحاء الجسم والزعانف ووجود تساقط للقشور وتقرحات للجلد مع وجود استسقاء بالبطن واحتقان الخياشيم.كما تمثلت الصفة التشريحية للأسماك في صورة شحوب واحيانا احتقان في جميع الأعضاء الداخلية مثل (الكبد ,الطحال,الكلى,المرارة والأمعاء).البكتيريا المعزولة تم التعرف عليها بالطرق التقليدية وبجهاز الفيتيك2.فقد تم التعرف على وجود ميكروبات السيدوموناس , اي كولاي والانتيروباكتر باستخدام جهاز الفيتيك 2.كما تم الكشف على وجود جين الهيموليسين وجين 16س باستخدام تفاعل البوليميريز المتسلسل في خمسة عترات مختارة للسيدوموناس ايريجنوزا.كما تم اجراء عدوى تجريبية للأسماك البلطي معمليا ببكتريا السيدوموناس ايريجنوزا المعزولة من الأسماك المصابة طبيعياً وتم عزلها مرة اخرى مع رصد العلامات والصفة التشريحية.