Studies on Some Septicemic Bacterial Diseases Affecting *Oreochromis Niloticus* in Earthen Fish Farm

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Abstract
This study was carried out on 150 *Oreochromis niloticus* at Dakhalia governorate within winter and summer seasons of the year 2015. The clinicohistopathological picture and biochemical investigations of the diseased fish were recorded. Sixty fish were infected with various types of bacterial species, of which, (49) fish were infected with Gram-negative bacteria while (11) fish were infected with Gram-positive one. The most common bacterial infection were *Aeromonas hydrophila* (33%), *Pseudomonas fluorescens* (20.5%), *Streptococcus agalactiae* (17.8 %), *Aeromonas sobria* (12.3 %), *Vibrio anguillarum* (9.5%) and *Vibrio alginolyticus* (6.8%). The highest total prevalence of bacterial infections was recorded in summer season (39%) compared to 21 % in winter. Isolates varied in their antibiotic sensitivity pattern. Inflammatory changes associated with these bacterial diseases were obvious in histopathological sections. Disease conditions were affected by various environmental stressors especially temperature, pH, salinity, ammonia and some heavy metals.
Keywords: *Oreochromis niloticus,* Bacterial diseases, Diagnosis, Pathology, water quality.

Introduction

The most healthiest and cheapest source of protein is obtained from aquaculture ***(Nijdam et al, 2012).*** So, we now trend to increase production of aquatic animal food products more than others ***(FAO, 2012).***

Tilapia is the most well known cultured species in all over the world , as it occupies the second prevailing species after carp ***(FAO, 2012)***. Egypt is ranked 2nd one in production of Tilapia and the 7 th of top ten countries in the total production of fish ***(El Zokm et al, 2012)***.

Diseases are considered one of the hazard factors that increase the incidence the Epidemiological aquaculture ***(Umesh et al, 2008)***. Any disease usually originates from synergism of the pathogen, host and environment as temperature,pH,

nitrogenous waste products, salinity and some heavy metals ***(Madhun et al, 2015).***

Bacterial diseases are considered one of the important diseases affecting fish mainly the septicemic bacterial diseases which caused great loss and mortality ***(Noor El-Deen et al, 2010)*** .The bacterial fish pathogens comprise natural inhabitants of the aquatic environment ***(El-Refaey, 2013)***.The current study was investigated the most predominant bacterial diseases in tilapia fish in earthern pond at Dakahlia province.

Materials and Methods:

Fish:

The study was carried out on 150 freshly captured *Oreochromis niloticus* of different body weight ranged from (175 to 200 g) collected from earthen ponds in private fish farms in Dakahlia governorate in Egypt showing signs of septicemia during the period of December 2014 to July 2015 (winter and summer seasons). Seventy five fish were collected per season.

Bacteriological examination

Sampling and processing

Samples were retrieved from liver, kidney, spleen, gills under fully sterile conditions. Loopfuls were cultured into tryptic soy agar, Aeromoas medium base supplemented with ampicilin, thiosulphate citrate bile salt sucrose agar (TCBS) and Blood agar medium. The inoculated plates were incubated at 25 °C for 18-48 hours. Representative numbers of the different colonial types detected on the media were collected from plates and streaked on TSA for purity and identification.

Identification of isolates

Identification of the obtained bacterial isolates was carried out by performing biochemical and morphological characters using traditional , API 20 E and API strept kits according to ***(Buller, 2004).***

Water quality examination

In summer, water samples were obtained from various locations from Dakahlia fish farm (2times) in sterile plastic bottles and stored according to methods adopted from ***(APHA, 2000).*** Then Physico-chemically (Temperature, pH and salinity), unionized ammonia (NH3) and heavy metals (iron and copper) analysis were measured using Thermometer, Symphony VMR PH meter, Martin conductivity meter, Cintza 101 double beam spectrophotometer and Atomic absorption spectrometer respectively.

Histopathological studies

Tissue specimens used for histopathological techniques were obtained from liver, spleen, kidney, gills, intestines and gonads of infected *O. niloticus*. Samples were conserved in 10 % buffered formalin, dehydrated by ethanol solution, then fixed with paraffin, and cut at 5 µm thick. Tissue sections were routinely processed

and stained with Hematoxylin and Eosin (H & E) and finally examined under light microscopy following the criteria reported in ***Bancroft (1996).***

Results

Naturally infected *O. niloticus* showed: hemorrhagic patches extensively distributed on the external body surface, scales detachment, erosions, fin and tail rot. The gills were congested with prescence of excessive amount of mucus. Some cases showed abdominal distention, ascites exophthalmia (fig1A) .On the other hand, postmortem findings revealed paleness of liver, enlargement of gall bladder and congestion of kidney (fig1B).

Isolation and identification of retrieved bacterial isolates

The phenotypic characteristics of obtained loopfuls from different fish organs showed a total number of seventy three bacterial isolates retrieved from sixty infected fish. Most of obtained isolates were Gram-negative 82.2 % represented as; *A. hydrophila* 33% followed by *Ps. fluorescens* 20.5 %, *A. sobria 12.3* %, *V. anguillarum* 9.5%, *V. alginolyticus* 6.8%.On the other hand *Streptococcus agalactiae* was the incriminated Gram-positive bacterial pathogens involved in this investigation 17.8% .Full phenotypic and biochemical characteristics of retrieved isolates are reported in **(tables 1, 2)** and the prevalence of the different bacterial species is summarized in **(table 3)**.

Antibiogram sensitivity testing

Isolates differed in their sensitivity pattern according to different antimicrobial agents tested (table4). The most effective antimicrobial agent tested has been established to be Sulphamethoxazole + Trimethoprim complex 66 %. On the other hand, the highest resistances displayed by these bacterial isolates were noticed against novobiocin59 % and tetracycline 46.5%.

Histopathological lesions

Inflammatory and necrotic alterations were obvious, also some bacterial colonies were observed in histological sections (Fig. 2). Degeneration, hemorrhagic necrotic changes were commonly observed in haemopoeitic tissues, liver and kidney. On other hand, gills showed congestion, necrosis and desquamation of epithelial lining of primary gill lamellae and also fusion of secondary gill lamellae.

Water quality parameters

Disease conditions reported in this study were appeared to be related to stressful environmental condition expressed by unseemly water quality parameters in examined fish farms **(Table 5).** Investigated fish farms at summer season showed slightly alkaline waters as the pH values measured about 8.9 and salinity recorded 2 ‰ .

Results also demonstrated presence of unfavorable values of some heavy metals such as iron which

ranged about 2.95 ppm with no presence of copper in summer season. On other hand, levels of some nitrogenous waste products as NH3 was 1mg/l which above the permissible level.

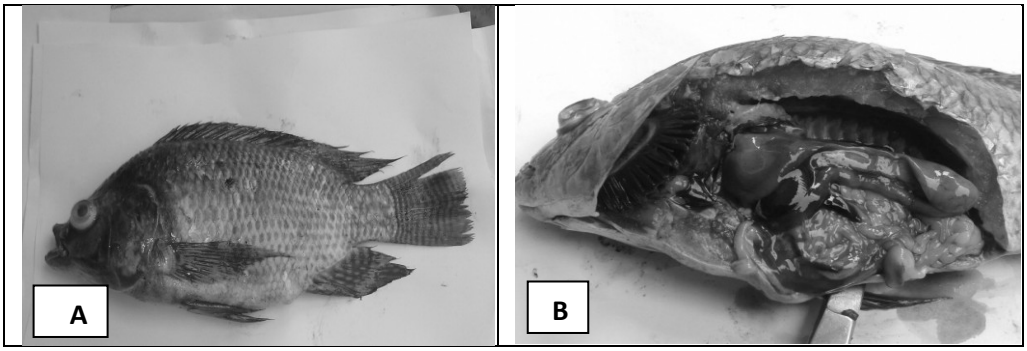

Fig1: ***(A)****Naturally infected tilapia showing haemorrhages on the external body surface and exopthalmia.*
(B)*Naturally infected tilapia showing paleness of liver,enlargement of gall bladder and congestion of kidney.*

Table1: *biochemical characteristics of Gram- negative bacterial isolates retrieved from naturally infected O. niloticus obtained from API20E.*

Biochemical test	*A. hydrophila*	*A. sobria*	*Ps. fluorescens*	*V. alginolyticus*	*V. anguillarum*
B –Galactosidase production (OPNG)	+	+	-	-	+
Arginine dihydrolase production (ADH)	+	+	+	-	+
Lysine decarboxylase production (LDC)	+	+	-	+	-
Ornithine decarboxylase production (ODC)	-	-	-	-	-
Citrate utilization (CIT)	V	V	V	+	+
H2S production (H2S)	+	+	-	-	-
Urease production (URE)	-	-	-	V	-
Tryptophane deaminase production (TDA)	-	-	-	V	-
Indole production (IND)	+	+	-	+	-
Acetoin production(VP)	+	+	+	-	-
Gelatinase production (GEL)	+	+	-	V	+
Acid from glucose(GLU)	+	+	-	+	+
Acid from manitol(MAN)	+	+	-	+	+
Acid from inositol(INO)	-	-	-	-	+
Acid from Sorbitol(SOR)	V	-	-	V	+
Acid from rhamnose(RHA)	-	-	-	-	-
Acid from sucrose(SAC)	+	+	-	+	+
Acid from melibiose (MEL)	-	V	V	-	-
Acid from amygdalin (AMY)	+	V	-	-	-
Acid from arabinose (ARA)	V	V	-	-	+

(+) Positive, (-) Negative, (V) Variable

Table2: Phenotypic characteristics of *S. agalactiae* obtained from naturally infected *O. niloticus* fish using API strept.

Bacterial isolates	Winter	%	summer	%	Total isolate	%
A.hydrophila	6	30	18	34	24	33
A.sobria	1	5	8	15	9	12.3
Ps.flurorescens	11	55	4	7.5	15	20.5
V.anguillarum	2	10	5	9.4	7	9.5
V.alginolyticus	0	0	5	9.4	5	6.8
S.agalactia	0	0	13	24.5	13	17.8
Total	20	100%	53	100%	73	100%

Table3: *Prevalence of bacterial isolates obtained from infected fish*

Colony characters	Onto marine and TSAS agar	Small pin point, whitish, rounded and slightly raised
	Onto Blood agar	β Haemolysis
Microscopical Examination	Gram- stain and cell form	Gram positive cocci arranged in short chains
Biochemical characteristics obtained from API 20 Strep	Voges-Proskauer (VP)	+
	Hippurate (hip)	+
	Aesculin (ESC)	-
	pyrrolidonyl arylamidase (PYRA)	-
	α galactosidase (α-GAl)	-
	β glucuronidase (β GUR)	Variable
	β galactosidase (β GAL)	-
	Alkaline phosphatase (PAL)	+
	leucine arylamidase (LAP)	+
	Arginine dihydrolase (ADH)	+
	Ribose (RIB)	+
	Arabinose (ARA)	-
	Mannitol (MAN)	-
	Sorbitol (SOR)	-
	Lactose (LAC)	Variable
	Trehalose (TRE)	Variable
	Inulin (INU)	-
	Raffinose (RAF)	-
	Amygdalin (AMD)	Variable
	Glycogen (GLYG)	-

Table 4: Antibiogram of obtained bacterial isolates

	Conc	*A.hydrophila*		*A.sobria*		*Ps. fluorescens*		*V.anguillarum*		*V.alginolyticus*		*S.agalactia*		total	
		N:24	%	N:9	%	N:15	%	N:7	%	N:5	%	N:13	%	N:73	%
Tetracycline	30 µg				44		93						69		46.5
R		13	12.5	4	22	14	70	4	57	2	40	9	31	34	15
I		20	83	2	33	1	0	2	28.5	3	60	4	0	28	38
S				3		-						-			
Novobiocin	30 µg				67		67						46		
R		18	75	6	73	10	20	3	43	-4	0	6	31	43	59
I		4	17	3	30	3	13	3	43	1	80	4	23	21	29
S		2	8	-		2		1	14		20	3		9	12
Streptomycin (S)	10 µg				55		47						02		
R		5	21	5	22	7	13	2	28.5	3	60	-	37	22	30
I		4	17	2	22	2	40	1	14	2	40	3	7	14	19
S		15	62.5	2	22	6		4	57	-	0	10	7	37	51
Sulphamethoxazole +Trimethoprim (SXT)	25 µg		0		03		27						01		
R		-	0	-	36	4	33	1	14	4	80	-	58	9	12
I		-	10	3	66	5	40	5	71	1	20	2	5	16	22
S		24	0	6		6		1	14	-	0	11		48	66
Amoxicillin AML	10 µg				44		20						23		
R		12	50	4	11	3	47	-	0	4	80	3	23	26	36
I		10	42	1	14	7	33	6	86	1	20	3	54	28	38
S		2	8	4	44	5		1	14	-	0	7		19	26
Doxycycline (DO)	30 µg				78		60						15		
R		10	42	7	81	9	02	2	28.5	3	60	2	56	33	45
I		3	12	1	11	4	27	2	28.5	-	0	9	91	19	26
S		11	46	1	11	2	13	3	43	2	40	2	5	21	29
Kanamycin (K)	30 µg				11		27						37		
R		7	29	1	55	4	20	2	28.5	1	20	5	31	20	27
I		6	25	5	33	3	53	5	71	1	20	4	13	24	33
S		11	46	3		8		-	0	3	60	4	1	29	40

Table 5: *Mean water quality measures in examined fish farm*

Parameters	Summer season
Salinity (‰)	2 ‰
Temperature (°C)	31.5
pH	8.9
NH3(mg/l)	1
Iron (ppm)	2.95
Cu(ppm)	0

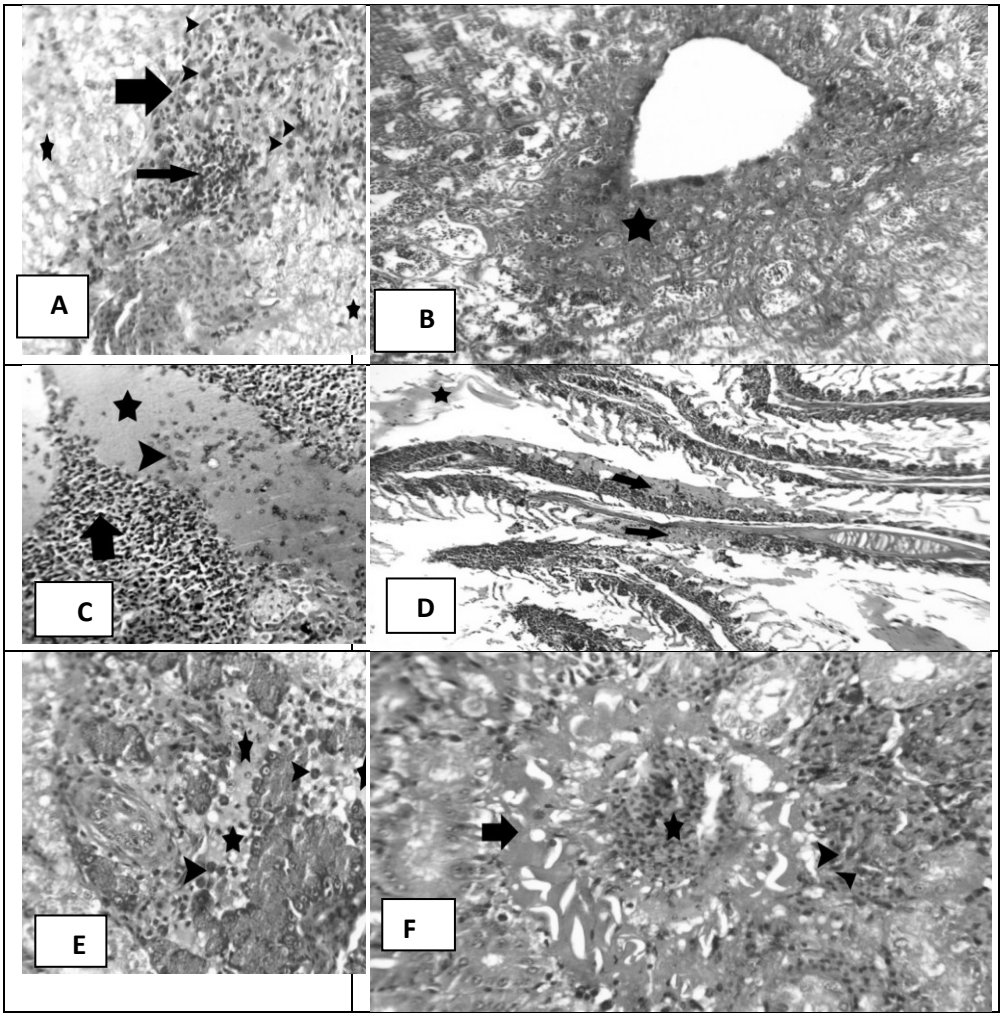

Fig3.A:*Liver infected with A.hydrophila shows hydropic degeneration in hepatocytes (asterisks), perivascular fibrosis with mononuclear cells(thin arrow) and eosinophilic granulocytes infiltration(arrowheads)(H&E, x200).*
B:*Testis infected with A.sobria shows perivascular necrosis and fibrosis(asterisk)(H&E, x100).*
C: *Spleen infected with Ps. fluorescence shows bacterial colonies(arrowhead) inside area of necrosis (asterisk) surrounded by leukocytes infiltration(thick arrow)(H&E, x200).*
D: *Gills infected with V.anguillarum shows necrosis(arrows) and desquamation of epithelial lining primary gill lamellae(asterisk)(H&E, x100).*

E: *Liver infected with V.alginolyticus shows focal necrosis(asterisks)and fibrosis in hepatopancreas with few eosinophilic granulocytes infiltration (arrowheads) (H&E, x200).*
F: *Caudal kidneys infected with S.agalactia shows congested renal blood vessel (asterisk) with perivascular fibrosis (arrow) and basophilic bacterial colonies trapped inside glomerulus (arrow heads) (H&E, x200).*

Discussion

Bacterial disease is the most critical sources of disease problems in all types of fish. Specific bacterial pathogens in every type of fish either freshwater or marine are responsible for severe mortalities (**Moustafa *et al*., 2015; Elgendy *et al*., 2015**).Freshwater aquaculture is considered the main investment in Egypt and other countries **(Saad *et al*., 2014)**.Tilapias (*Oreochromis* sp.) are one of the most important cultured fish in freshwater aquaculture industry **(Bostock *et al*., 2010).**

The present study confirmed that tilapia are susceptible to many bacterial pathogens which able to cause diseases. In agreement with other studies relating to freshwater bacterial diseases ***(Akinbowale et al, 2006; Al-Harbi and Uddin, 2010)*** most of retrieved bacterial isolates were Gram-negative 82.2%. On the other hand gram-positive bacteria were about 17.8%. Results of bacteriological examination established that *A.hydrophila 33% ,Ps. fluorescens 20.5 %, S.agalactia 17.8% ,A.sobria 12.3 %, V.anguillarum 9.5% and V.alginolyticus 6.8%.*

Vibrio spp, Ps. fluorescens, S.agalactia and Aeromonas spp were the most common isolated bacteria from this study. These results are similar to those reported by ***(Tatsuro et al, 2004; Abdel hamid et al, 2013; Elgendy et al, 2013).***

A.hydrophila were the most prevalent bacterial pathogens representing 33% in this study which in accordance to those reported by ***Al-Laham,et.al (2014) ; Jovanović et al (2015).*** *A. hydrophila* recorded 39 % in summer,but 21% in winter season..Also, *A.sobria* was reported in summer (15%) while winter season (5 %). This result can be explained as the maximized activity of cytotoxins, hemolysin and enterotoxins above 22 ℃ increase the incidence of disease in summer ***(Krovacek et al, 1991).***

Pseudomonads septicemic infections are widly spread.The high frequency of *Ps. fluorescens* infection was recorded in winter 55%**,**but in summer 7.5 %. This can be explained as proteinases and lipases of *P. fluorescens* have ability to produce at 7 C which trigger incidence of disease in winter season ***(Wang and Jayarao, 2001)***.

Vibrosis has great importance in marine water fish but also affect

freshwater fish ***(Dyer and Oliver, 2008; Elgendy et al, 2015)***.Seasonally, the highest prevalence of *V. anguillarum* infection was recorded during the winter and summer 10% and 9.4% respectively. On the other hand the highest prevalence of *V. alginolyticus* infection was recorded in summer 9.4% with no presence in winter**.** This explained as *V. alginolyticus* may lose its ability to cause disease in cold season ***(Yan et al, 2007)***.

Streptococosis has great economic importance due to high fish mortality ***(Haghighi et al, 2010)***. *S. agalactia* has high incidence in summer about 24% with no prevalence in winter season. This result can be explained that Raising of water temperature (above 26 °C) is optimal for increase the prevalence of streptococosis ***(Mian et al, 2009).***

Prevalence of bacterial isolates was higher in summer 53 % than in winter season 20 %. This may be explained as higher levels of un ionized ammonia was detected in fish farm water during this period

In regards to antibiogram sensitivity testing, freshwater aquaculture is a promising aquaculture sector needing pioneering scientific and technical developments. Excessive using of antibiotics for prophylactic and therapeutic led to increase bacterial resistance ***(Sarter et al, 2007)***. Reservoirs of antibiotic resistance can interact between various environmental conditions and transfer of resistant bacteria from animals to humans ***(Witte, 2000; Vaseeharan et al., 2005)***.

A.hydrophia isolates showed higher resistance to novobiocin 75% and Amoxicillin 50% in compared to other antibiotic tested , On other hand , isolates show intermediate susceptibility to isolates superior susceptibility to Doxycycline 42% .Also, it shows maximum susceptibility in Sulphamethoxazole + Trimethoprim complex 100 % ,tetracyclin82%, Streptomycin 62.5% and Kanamycin42%. Results are nearly in accordance with ***Laith and Najiah (2013) and Al Laham and Al Fadel (2014)***.

A. sobria isolates demonstrated extreme resistance to Doxycycline 75%, novobiocin 67%, Streptomycin 55%, tetracycline 44% were observed in this isolates. Moreover, higher intermediate sensitivity was showed in case of Kanamycin **55.5%.** On other hand, high susceptibility in Sulphamethoxazole + Trimethoprim complex **66%** was characteristic. Results are nearly in accordance with ***Guz and Kozinska (2004) and Çiftci et al (2015)***. In respect to *Ps. fluorescens* **,** revealed resistance were reported to tetracyclinn 93%,novobiocin 67% , Doxycycline60% ,Streptomycin 47%.On contrary, prominent sensitivity was detected in kanamycin 53%and Sulphamethoxazole +Trimethoprim combination 40%,but intermediate resistant to Amoxicillin

47%. Results are nearly in accordance with ***Eissa et al (2010); Foysal et al (*2011**)**.

Regarding *V.anguillarum***,** higher sensitivity was recorded in Streptomycin 57% and Doxycycline *43%.* Furthermore, superior intermediate sensitivity was maximum *in* Amoxicillin86% and both Sulphamethoxazole +Trimethoprim combination and Kanamycin*71%,* but high resistance to tetracycline 57%. Results are nearly in accordance with ***Vaseeharan et al (2004); Jayasree et al (2006).***

In concern to *V. alginolyticus* strains showed reputed sensitivity to both Kanamycin and tetracycline 60%, but maximum intermediate sensitivity to novobiocin 80%. In addition, isolates exploited superior resistance to both Amoxicillin and Sulphamethoxazole +Trimethoprim reach up to 80%, Doxycycline and Streptomycin about 60%. Results are nearly in accordance with ***Li et al (1999); Jayasree et al (2006)***.

Concerning the antibiogram of *S.agalactia* isolates displayed higher sensitivity to Sulphamethoxazole+Trimethoprim8 5%, Streptomycin 77%, Amoxicillin 54%. On other hand, isolates displayed prominent resistance was detected in tetracycline 69%, novobiocin 46%, Kanamycin 37%, on contrary, intermediate sensitivity to Doxycycline 69%. Results are nearly in accordance with ***Al-Marzouk et al (2005) ; Abuseliana et al (2010).***

In concern to water quality parameters reported in this study, in agreement with ***Eissa et al (2013); Moustafa et al (2015)*** unfavorable aquatic environmental condition as low value of water quality parameters increase the prevalence of disease noticed in this study.

In respect to the pH values, the farm water were slightly alkaline 7.5 in summer season which increase the prevalence of vibrosis reported in this study as the low ph value increase the mucus secretion which increase adhesion of vibrios ***(Balebona et al, 1995)***. On the other hand, high pH increase the toxicity by non ionised ammonia ***(Ludwig et al, 2007)***.The high pH values was (7.5) in summer due to increasing photosynthetic activity, which decrease levels of Co_2 and also low levels of dissolved oxygen. These findings are supported by ***(Goher, 2002).***

In regards to salinity**,** the high salinity noticed in this study (2 ‰) which also explained the presence of vibrosis in tilapia.This was in agreement to those reported by ***Al-Sunaiher et al (2010); Abou El-Geit et al (2013)***. High level of salinity can be explained by increasing evaporation rate in summer ***(Herbs, 2002).***

Regarding ammonia, the ammonia level reported in this investigation ranged 1.1ppm which was higher than permissible limit (0.02mg/l). This was in agreement to those

reported by ***Sachidan and Yajurvedi (2006)***; ***Moustafa et al (2015)***. Increasing level of ammonia triggers the invasion of bacteria especially immumo-suppression fish ***(Moustafa et al, 2015)***.While, prolonged low levels of ammonia irritates skin and gills facilitating parasitic infestations and opportunistic bacterial infections ***(Goher, 2002)***.

In regard to heavy metals reported in this study, iron ranged 2.95 mg/l which above the permissible limits (0.3 mg/l) according to ***Anzec (2000)*** with no presence of copper in summer season. This was in agreement in case of levels of iron but not in case of copper levels to those reported by ***Karadede and Ünlü (2007); Moustafa et al (2015).*** The high level of iron increases the incidence of some bacterial disease as vibrios since the virulence of vibro spp. enhance with higher iron ***(Muiño et al, 2001).***

In summary the most important bacterial pathogens causing diseases in *Orechromis niloticus* in the studied farm were *A. hydrophila*, *A. sobria, Ps. fluorescens*, *V.anguillarum*, *V. alginolyticus* and S.*agalactia*. Septicemic bacterial infections were related to the noticed unfavorable aquatic environmental stressors. The role of heavy metals including iron in exacerbating bacterial fish diseases is evident. Polluted water weakens the fish host defenses allowing increased opportunities for bacterial infections to affect fish populations**.**

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الملخص العربي

دراسات عن بعض امراض التسمم الدموي التي تصيب اسماك البلطي النيلي في مزارع الاسماك الترابيه

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أجريت هذه الدراسة على عدد١٥٠ سمكة من أسماك البلطي النيلي تم تجميعها مباشرة من مزارع الاسماك الترابيه بمحافظة الدقهليه حيث تم تجميع ٧٥ سمكه من اسماك البلطي خلال فصلي الشتاء والصيف لسنه ٢٠١٥. قد تم تسجيل كل من الأعراض الاكلينيكية والباثولوجيه للاسماك المريضه. كشفت نتائج الفحص البكتريولوجي عن عدد ٦٠ سمكة مصابة اصابه طبيعيه بمختلف العترات البكترية (٤٩ سمكه) وكانت معظم البكتريا المعزوله سالبة الجرام حوالي٨٢,٢ % في حين البكتيريا إيجابية الجرام تمثل نحو ١٨,٩١٪.كان ميكروب الايروموناس هيدروفيليا الاكثر انتشارا ٣٣%تلاها ميكروب السيدوموناس فلوريسينس ٢٠%يليها بالاستريبتوكوكس اجالكتيا١٧%، الايروموناس سوبريا١٢ %،%، الفيبرو انجيولارم٩,٥% بينما كان معدل اصابه بفيبرو الجينوليتيكس٦,٨% فقط. كشفت الدراسه ان أعلى معدل انتشار العدوى البكتيرية في موسم الصيف من فصل الشتاء ، ٣٩٪ من اسمك البلطي مصاب في الصيف ولكن ٢١٪ فقط مصاب في فصل الشتاء. وتختلف العترات المعزوله في حساسيتها لمختلف المضادات الحيويه .التغيرات الهيستوباثولوجي كانت واضحه جدامع الامراض البكتيريه.الامراض تتاثر بالتغيرات البيئيه مثل الحراره، درجة الحموضة،الملوحه،الامونيا،وبعض العناصر الثقيلة.