IMMUNE RESPONSE OF *OREOCHROMIS NILOTICUS* AGAINST *STREPTOCOCCUS INIAE*

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ABSTRACT

Skin darkening, gasping, exophthalmia, eye opacity, scale loss, hemmorrhage at the base of fins, fin rot, vertical position with the head down or up and protruded haemorrhagic vent were noticed on Oreochromis niloticus (Nile tilpia) experimentaly infected with Streptococcus iniae. The mortality rate was 31.66 % in the infected. Leucogram of O. niloticus experimentally infected with streptococcus iniae revealed leucocytosis associated with heterophilia and lymphocytopenia at the 3rd day.

Leucocytopenia accompanied with heteropenia, lymphocytosis and monocytosis were noticed at the end of the 1st week till the end of the 4th week of experiment whlie heterophils and lymphocytes came back to normal at the last (5th) week of infection. The total proteins were significantly increased at the 3rd day then significantly decreased at the 1st week of infection, till the end of the experiment at period. The globulins were significantly increased at the 1st week of infection, till the end of the experiment. AGPT demonstrated precipitating antibodies in serum samples of experimentally infected fish at the end of the 2nd week till the end of the experiment. ELISA was more rapid and sensitive than AGPT and showed that the antibody titers agianst Streptococcus iniae increased significantly from the end of the 1st week, and reached its peak at the end of the 3rd week and stay there till the end of the experiment.

INTRODUCTION

Fishes are continously exposed to a wide range of micro-organisms present in the environment ***(Ringoe and Gatesoupe, 1998)*** and the bacterial diseases are responsible for extreme mortality in both wild and cultured fish. Cichlids mainly *Oreochromis niloticus* is greatly cultured for commercial production of food fish. Streptococcal infection is one of the bacterial diseases which reduced the stocks of tilapia and have severe economic consequences on fisheries in many areas of the world **(*Eldar et al., 1994).*** It has been found that there is a great incidence of streptococcal infections in tilapias than other cultured fish ***(Chang and Plumb, 1996).***

Streptococcosis in fish is caused by different species of streptococci; the most important species is *Streptococcus iniae* which firstly isolated by ***Pier and Madin (1976)*** from a diseased Amazon freshwater dolphin and ***Kitao et al. (1981)*** isolated *S. iniae* for the first time from tilapia. *Streptococcus iniae* has a zoonotic importance displayed in humaus as a localized cellulitis, infective endocarditis, meningitis and septic arthritis; tilapia was the most common fish associated with these cases ***(Weinstein et al., 1997; Getchell, 1998 and Susanna et al., 2003).***

Pulido et al. (1999), Shoemaker et al. (2000) and ***Yanong*** and ***Floyd (2002)*** described the clinical signs of fish *Streptococcosis* in the form of loss of orientation, exophthalmia, corneal opacity, haemorrhages in or around the eye, base of fins, vent, over the heart or else where on the body, ulcerations and fluid accumulation in the peritoneal cavity were also seen. More added, nervous manifestations with erratic swimming, pale liver and reddish intestinal contents were recorded in many infected fish.

The physiological responses of the fish immune system to an infection are based on a complex, stepwise activation and proliferation, especially of the specific immune mechanism after first contact to the microorganisms ***(Köllner et al., 2002)***.

Detection of specific antibodies in the serum of fish is recognised as a useful indicator of previous exposure to pathogens and this type of serology is often used when rapid tests to identify the pathogen have not yet been developed ***Delamare et al. (2006)***.

The present study was designed to investigat the leucogram, proteins profile and immune response (antibody titer) of *Oreochromis niloticus* fish against *Streptococcus iniae* by Agar Gel Precipitation Test (AGPT) and Enzyme Linked Immunosorbent Assay (ELISA).

MATERIAL AND METHODS

Fish:

Two- hundred and sixty apparently healthy *O. niloticus* fish were randomly obtained from earthen pond fish farm at Beni-suef governorate, Egypt, and transported alive to the wet lab. of Fish Dept., Faculty of Vetrinary Medicine, Beni-suef University, The collected fish were about 65-90 gs in weight, the fish were acclimated for one week before use, and fed on a commercial ration of 25% protein content.

Clinical examination:

The fish were examined clinically for the presence of abnormal behaviors, skin lesions and fin erosions.

Streptococcus iniae strain:

Micro-organisms used for experimental infection were isolated from diseased tilapia fish and identified in Bacteria, mycotic and immunity Dept., Fac. Vet. Med., Beni-suef University.

Experiment - I

Sixty apparently healthy *O. niloticus* fish were divided into two groups, the first group (30 fish) was inoculated intra-peritoneally (I. P.)with 0.2 ml of $1x10^7$ CFU/ml $fish^{-1}$ of *streptococcus iniae* ***(Perera et al., 1997),*** the second group (30 fish) was inoculated intra-peritoneally with 0.2 ml of sterile saline and kept as a control. This experiment was desgined to study the clinical sings, mortality rate, post mortem lesions.

Experimente - II

Two- hundred apparently healthy *O. niloticus* fish were divided into two groups, the first group (120 fish) was inoculated intra-peritoneally with 0.2 ml of 1×10^7 CFU/ml $fish^{-1}$ of *streptococcus iniae***,** the second group (80 fish) was inoculated intra-peritoneally with 0.2 ml of sterile saline and kept as a control. This experiment was desgined to study the immune response and leucogram of *O. niloticus* fish against *streptococcus iniae*.

Collection of blood sample:

Two blood samples were collected from the caudal blood vessels of 10 investigated and another 10 control fish according to ***Lucky (1977)*** at the 3^{rd} day and at the end of the 1^{st}, 2^{nd}, 3^{rd}, 4^{th} & 5^{th} week post inculation. The first blood sample was collected by a syringe flushed with heparinized saline for hematological studies. The second sample was collected without anticoagulant in a centrifuge tube for serum separation.

Hematological studies:

Total leucocytic count was carried out according to ***Blaxhall*** and ***Daisley (1973)*** and differential leucocytic count was performed on the stained blood smears. ***(Jain, 2000).***

Determination of serum total proteins and albumin:

Total proteins were determined according to ***Peters (1968)*** and albumin was determined colorimetrically according to ***Doumas*** and ***Biggs (1972)***.

Preparation of *S. iniae* antigen:

The pure *S. iniae* in tryptone soy broth was incubated in formalin 3% for 4 hours and then centrifuged and washed three times (the supernatant was discarded in each time) to obtain killed *S. Iniae*.

Preparation of fish immunoglobulin:

Added to a volume of fish serum in adropwise manner, an equal amount of saturated ammonium sulphate with gentle shacking, then centrifuged at 3000g x for 15 minutes for globulin sedimentation. The supernatant was discarded and the sediment in redissolved a minimal amount of PBS and dialyzed against 100 times volume of HCl, that has been repeated later step three times, the suspended material was centrifuged and the supernatant fish immunoglobulin was stored at -20ºC ***(Roberts, 1989).***

Preparation of anti-fish immunoglobulin:

Four adult ballady chickens were used for the preparation of hyper immune serum agaisnt fish immuno-globulins (chicken antifish immuno-globulins). The birds were subdivided into two equal groups, the first group received 5 doses of previously prepared fish immuno-globulins S/C, the first dose was 1 ml of fish immuno-globulins and 1 ml of complete Freund's adjuvant. One month later, the birds received four poster doses IM; each of 1 ml fish immuno-globulins and 1 ml of

incomplete Freund's adjuvant with two weeks intervals. The second chicken group was kept as control and similarly inoculated with saline. Two weeks from the last dose, they were sloughtered and their blood was collected, clotted and centrifuged for serum (containing antifish immuno-globulins) collection ***(Nakane** and **Kawaoi, 1974).***

Agar Gel Precipitation Test (AGPT):

It was performed according to ***Beard (1982),*** the media consisted of Agarose 1.5 g, Glycine 1.5 g, Na cl 8 g and Dist. water 100 ml, the mixture was boiled in water bath for agarose dissolving then left to cool to 45ºC, poured in petri dishes, seven wells of 5 mm in diameter were cut in the prepared media, one well central and 6 wells at 3-4mm peripheral to the central well. The central well was filled with the preparred *streptococcus iniae* antigen, one peripheral well was filled with negative control serum, another well was filled with positive control serum, the last 4 wells were filled with the serum collected from *streptococcus iniae* experimentally infected fish. The dish was incubated at room temperature in humid chamber and examined at 24, 48, 72hrs for lines of precipitation.

ELISA standardization:

According to ***Klesius and Johnson, (1991)***, the ELISA assays were performed in 96 wells, flat bottom microtiter plates (Dynatech, USA). The inner wells of the plate were coated overnight at 4˚ C with 100 µl of prepared bacterial antigen dilution. Vertically in the plate, double fold serial dilution steps were prepared with the coating buffer (carbonate bicarbonate buffer). Using 100 µl of blocking solution for 1 hour blocked the non-binding sites in coated wells of the microtiter plate. Into the wells 100 µl of positive and negative fish serum prediluted 1: 100 in

diluting buffer were pipetted and incubated for 1 hour at room temperature then washed three times using PBS (10 minutes for each washing), each two adjacent wells of the plate is filled vertically with 100 µl of prepared chicken antifish serum prediluted 1: 100, 1: 200 and 1: 300 in diluting buffer respectively and incubated for 1 hour at room temperature, then washed three times using PBS. The washed wells of the plate were received 100 µl of antichicken peroxidase prediluted 1: 500 (standardized) in diluting buffer and incubated for 1 hour at room temperature, then washed three times using PBS. Finally the wells of the plate were filled with 100 µl of freshly prepared substrate working solution, and incubated for 15 - 20 minutes in a dark place. Finally, 50 µl of stop solution was added to stop the reaction and the plates were read using ELISA reader Dynatch MR 700 at a wavelength of 490 nm.

Optimal antigen and chicken antifish serum dilution were determined to be used in determining the antibody titer in collected samples and they were 1:16 & 1:200 respectively.

Enzyme Linked Immunosorbent Assay (ELISA)

It was conducted in 96 wells, flat bottom microtiter plate (Dynatech, USA), The wells of the plate were filled with 100 µl of *streptococcus iniae* suspension diluted 1/16 in carbonate bicarbonate buffer and coated overnight at 4ºC. The non binding sites of the plate wells were blocked using 100 µl of blocking buffer for 1 hour, the blocking buffer was removed from the wells and the wells were filled with 100 µl of tested, positive and negative control fish sera prediluted 1:100 in diluting buffer and incubate at room temperature for 1 hour then the plate was washed three times with washing buffer. The wells of the plate were filled with 100 µl of chicken antifish serum prediluted 1:200 and incubated at room temperature for one hour then the plate was

washed three times with washing buffer. Then the wells were filled with 100 µl of antichicken peroxidase prediluted 1:500 and incubated at room temperature for one hour then the plate was washed three times with washing buffer. Finally, the washed wells of the plate were filled with 100 µl of freshly prepared substrate working solution and incubated at room temperature for 15-20 minutes in dark place then 50 µl of stopping solution was added and the plate was read using ELISA reader Dyantech MR 700 at wave length 490nm for determining the optical densities of the tested samples.

Statistical analysis:

The obtained data of hemogram, total proteins, albumin and globulin were statistically analyzed for the mean and the standard error of the mean according to Excel Microsoft.

RESULTS

O. niloticus experimentally infected with *streptococcus iniae* showed behavioral changes as sluggish movement, loss of escape reflex at the 2nd day of experiment, few fish had a decreased appetite; but most fish feed normally during the experiment period. Gasping. Exophthalmia, eye opacity **Photo (1),** skin darkness, scaleloss areas, hemmorrhage at the base of fins and fin rot were recorded **Photo (2)**. Immediatly before death some fish lied in a vertical position with the head down or up **Photo (3)**.

Streptococcus iniae cuased death for 38 out of 120 experimentally infected *O. niloticus* fish during the five weeks of observation, thus the commulative mortality rate reached (31.66 %) comparing to 3.75% mortality rate of the control group.

Post-mortem examination of the internal organs of the dead fish showed congested liver, congested and enlarged spleen and accumullation of fliuds in the abdominal cavity. The stomach and intestine were filled with food in most examined fish while as, in some few were deviod of food and contian mucoid yellowish fliuds**.**

Streptococcus iniae was reisolated from the liver and kidney of experimentally infected fish and reidentified.

Leucogram of *streptococcus iniae* infected *O.niloticus* fish revealed leucocytosis associated with heterocytophilia and lymphocytopenia at the 3rd day. The total leucocytic count significantly decreased accompanied with heterocytopenia, lymphocytosis and monocytosis from the 1st week till the end of the 4th week of experiment. In the last week of infection, heterophil and lymphocyte came back to normal accompanied with monocytosis.

Total proteins significantly increased at the 3rd day post-infection then significantly decreased at the 1st week of infection till the end of the experiment. Globulins were significantly increased at the 1st week of infection till the end of the experiment.

Agar gel precipitation test, detected the precipitating antibodies in 16.7, 33.33, 50 & 50 % of the serum samples of experimentally infected fish at the 2nd, 3rd, 4th and 5th week of the experiment respectively however, no precipitating antibodies could be detected in serum samples of infected fish at the 3rd day and 1st week or control fish along the experiment.

ELISA optical density revealed that the *Streptococcus iniae* antibody titer began to increase significantly from the end of the 1st week, reach its peak at the end of the 3rd week and persisted at the peak till the end of the experiment.

Table (1): Showed total and differential leucocytic count of control and *Streptococcus iniae* experimentally infected *O. niloticus*.

Parameter		Total WBCs (x 10^3/µl)	Differential leucocytic count (%)		
Time	Fish group		Heterophil	Lymphocyte	Monocyte
3rd day	Control	4.68±0.08	19.20 ±0.42	80.21±0.42	0.58±0.18
	Infected	6.66** ±0.01	36.50** ±0.42	62.89**±0.37	0.61±0.20
1st W	Control	4.75 ±0.07	18.10±0.31	81.32±0.20	0.57±0.21
	Infected	3.62** ±0.05	15.66*±0.36	83.24*±0.20	1.10*±0.23
2ndW	Control	4.53 ±0.04	18.18±0.43	81.22±0.31	0.59±0.20
	Infected	3.80** ±0.07	15.40*±0.54	83.30*±0.59	1.30*±0.20
3rdW	Control	4.68 ±0.07	18.30±0.35	81.18±0.24	0.51±0.20
	Infected	3.70** ±0.08	15.84*±0.50	83.27*±0.56	0.87*±0.18
4th W	Control	4.26 ±0.06	19.79±0.31	79.61±0.14	0.59±0.21
	Infected	3.32** ±0.05	16.26*±0.40	82.79*±0.42	0.94*±0.21
5th W	Control	4.20 ±0.07	18.47±0.52	80.95±0.50	0.56±0.18
	Infected	3.80** ±0.06	18.07±0.43	81.00±0.60	0.93*±0.20

*Significantly different from control, p<0.05 ** Significantly different from control, p<0.01

Table (2): showed the total proteins, Albumin and Globulin values of *O. niloticus* fish experimentally infected with *streptococcus iniae*.

Time	Fish Group	Total proteins	Albumin	Globulin
3rd day	Control	4.72 ± 0.06	3.23 ± 0.04	1.48 ± 0.05
	Infected	5.28 ± 0.09**	3.6 ± 0.04**	1,7 ± 0.05*
1st Week	Control	4.56 ± 0.06	3.13 ± 0.09	1.51 ± 0.05
	Infected	3.78 ± 0.05**	1.92 ± 0.05**	1.9 ± 0.05**
2nd Week	Control	4.65 ± 0.05	3.11 ± 0.04	1.43 ± 0.09
	Infected	3.76 ± 0.05**	1.92 ± 0.04**	1.84 ± 0.04**
3rd Week	Control	4.67 ± 0.06	3.21 ± 0.02	1.49 ± 0.04
	Infected	3.99 ± 0.05**	1.90 ± 0.04**	2.05 ± 0.07**
4th Week	Control	4.53 ± 0.07	3.06 ± 0.05	1.51 ± 0.08
	Infected	3.73 ± 0.06**	1.71 ± 0.03**	2.01 ± 0.05**
5th Week	Control	4.56 ± 0.05	3.15 ± 0.04	1.52 ± 0.09
	Infected	3.96 ± 0.06**	2.01 ± 0.04**	1.93 ± 0.05**

* Significantly different from control, p<0.05 ** Significantly different from control, p<0.01

Table (3): Results of agar gel precipitation test in *O. niloticus* fish experimentally infected with *streptococcus iniae*.

Time	Fish group	Number of examined samples	Number and (%) of positive samples
3rd day	Control	6	0 (0 %)
	Infected	6	0 (0 %)
1st W	Control	6	0 (0 %)
	Infected	6	0 (0 %)
2ndW	Control	6	0 (0 %)
	Infected	6	1 (16.7 %)
3rdW	Control	6	0 (0 %)
	Infected	6	2 (33.3 3%)
4th W	Control	6	0 (0 %)
	Infected	6	3 (50 %)
5th W	Control	6	0 (0 %)
	Infected	6	3 (50 %)

Table (4): Showed mean values and S.E. of ELISA optical density in *streptococcus iniae* experimentally infected *O. niloticus*.

Time / Fish group	3rd day	1st W	2nd W	3rd W	4th W	5th W
Control	0.187 ± 0.04	0.194 ± 0.01	0.189 ± 0.003	0.189 ± 0.01	0.178 ± 0.004	0.191 ± 0.02
Infected	0.197 ± 0.02	0.501** ± 0.2	0.714** ± 0.01	0.810** ± 0.03	0.796** ± 0.02	0.788** ± 0.01

** Significantly different from control, $p<0.01$

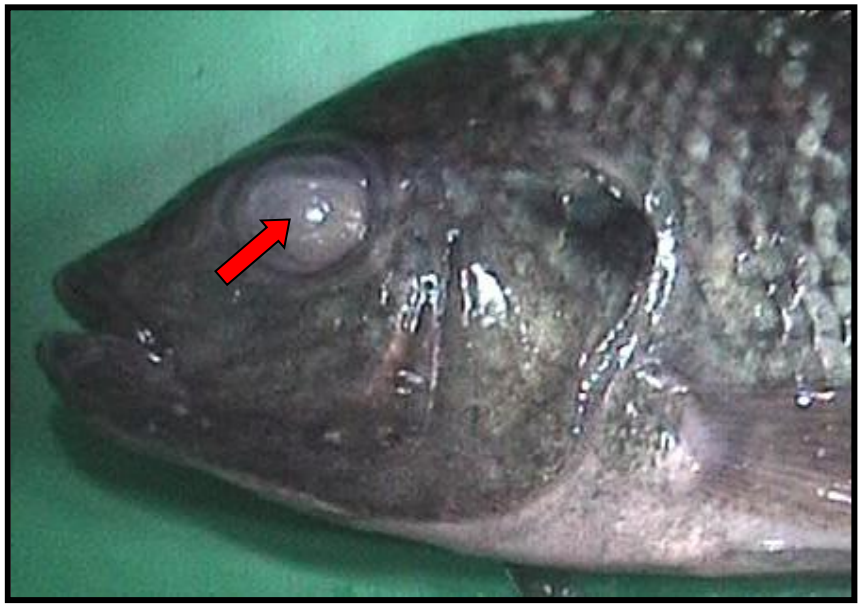

Photo (1): *O. niloticus* showed eye opacity and skin darkening.

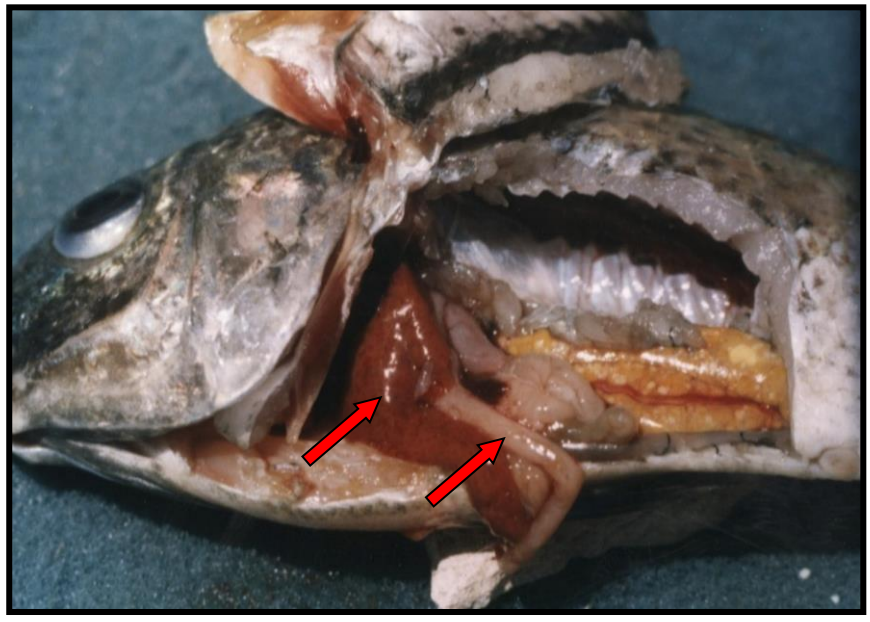

Photo (2): *O. niloticus* showed congested liver and the GIT was devoided of food

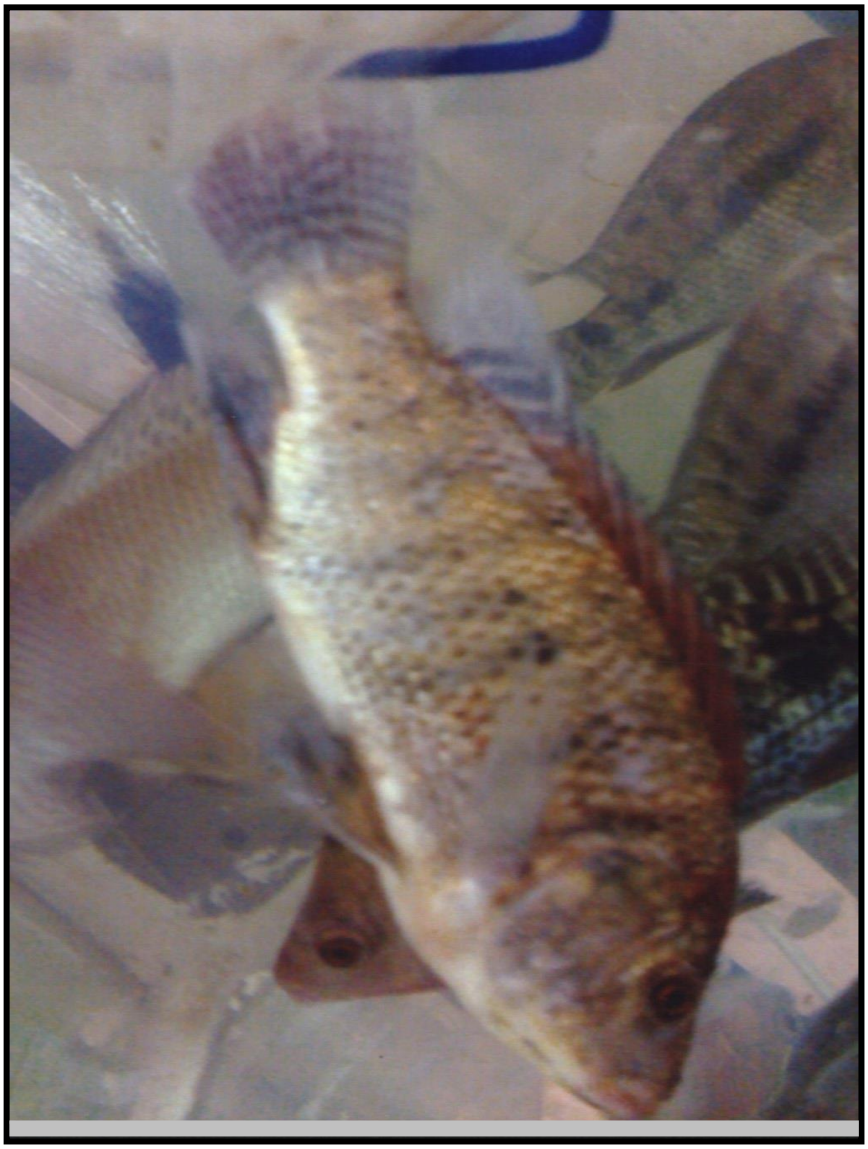

Photo (3): *O. niloticus* fish lied in a vertical position with the head dowen.

DISCUSSION

Streptococci have increasing prominence as agents of fish disease with particularly severe losses reported from many aquacultures ***(Kitao et al., 1981; Humphrey et al., 1987 and Carson et al., 1993)*** and the ability to measure immune response to *S. iniae* can be used to monitor disease outbreaks ***Shelby et al. (2004).***

Streptococcus iniae infected *O. niloticus* fish showed sluggish movement, loss of escape reflex, in addition gasping, exophthalmia, eye opacity, skin darkness, scaleloss, hemmorrhages at the base of fins and on the operculum as well as fin rote were also noticed. Immediatly before death, the fish lied in a vertical position with the head down or upward. The PM lesions revealed liver congestion, congestion and enlargment of spleen and accumullation of fliuds in the abdominal cavity. Similar clinical signs were recorded by ***Perera et al. (1994)***, ***Chang and Plumb (1996a), Pulido et al. (1999), Shoemaker et al. (2000) and Yanong and Floyd (2002),*** these signs may be attributed to the production of bacterial toxins that may lead to septicemic signs or fish death ***(Wedemeyer and Goodyear, 1984).***

The commulative mortality rate in *O. niloticus* experimentally infected with *S. iniae* was 31.66 %, nearly similar mortality rates were recorded by ***Shoemaker et al. (2000) Evans et al. (2000)***, while ***Ferguson et al. (1994), Perera et al. (1997) and Mcnulty et al. (2003)*** recorded 90%:100% mortality rate which may attributed to the differences in doses, route of infection, fish species and temperature.

At the 3rd day, the streptococcus infected fish showed leucocytosis which may be considered as an inflammatory response against injected antigen ***(Iwama et al., 1986 and Stoskopf, 1993),*** the temporary lymphocytopenia may be attributed to the immuno-suppressive effect of stress due to catching and injection ***(Ellsaesser and Clem, 1987 and Bly et al., 1990).*** The total leucocytic count significantly decreased accompanied with heterocytopenia, lymphocytosis and monocytosis at the end of the 1st week till the end of the 4th week. The leucocytopenia may be attributed to the depletion of the leucocytes from the blood to the tissues, also and the monocytosis may be due to enhanced phagocytic activity of monocytes ***(Jain, 2000).***

The total proteins were significantly increased at the 3rd day post-infection with *S. iniae* and this may be attributed to the inflammatory response or destruction of body proteins due to infection. At the 1st week to the end of the experiment, the total proteins were significantly decreased and this may be attributed to the hepatic degradiation due to the proteolytic enzymes released from the destructed endothelial cells due to bacterial infection ***(Stoskopf, 1993)*** or due to loss or dilution of serum proteins as a result of increased capillary permiability due to infection ***(Hargens et al., 1974).***

Globulins was significantly increased at the 1st week post-infection and reach its peak at the 3rd week and persisted there till the end of the experiment, similar results were reported by ***Abd El-Rahman (2002)*** and this result improve the humral immune response of *O. niloticus* fish against *S. iniae* and confirmed by the results of ELISA test and the lymphocytosis of the leucogram.

AGPT demonstrated precipitating antibodies in 16.7 % of the experimentally infected fish at the 2nd week and 50% at the end of the experiment while as, can not detect this antibodies at the 1st week; this explains that AGPT has a low sensitivity to detect the fish antibodies against *S. iniae*.

On the other hand, the readings of ELISA optical density revealed that *Streptococcus iniae* antibody titer begin to increase significantly from the 1st week, and reached its peak at the 3rd week that persists till the end of the experiment. ***Shelby et al. (2001, 2002 & 2004)*** and ***Delamare et al. (2006)*** succefully used enzyme linked immunosorbent assay (ELISA) to determine the antibody titer of *Oreochromis niloticus* against *S. iniae*, ***Shelby et al. (2001)*** found that the antibody response of the experimentally infected tilapia was significantly different from the non-infected group; and the ELISA results were highly correlated with the agglutination test results for tilapia antibody against *S. iniae*. ***Delamare et al. (2006)*** detected a strong specific antibody response in the serum at 21 days following intraperitoneal injection of *Lates calcarifer* fish with *streptococcus iniae*.

In conclusion**,** evaluation of humral immune response of *S. iniae* infected fish revealed formation of antibodies in the survived infected *O. niloticus* fish from the 1st week of infection and reached its peak at the 3rd week of infection. ELISA test is more sensitive and rapid than AGPT in the detection of antibodies against *S. iniae* in the serum of infected fish and could be used in diagnosis of infections.

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الاستجابة المناعية لأسماك البلطي النيلى ضد الاستربتوكوكس إنياي

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قتامة لون الجلد وجحوظ العينين و عتامة على العين ونزيف عند قواعد الزعانف وتساقط القشور مع بروز فتحة الشرج كانت أهم الأعراض على اسماك البلطي النيلى المصابة تجريبياً بميكروب ستربتوكوكس إنياي, حيث سببت هذه الإصابة نفوق بنسبة 31.66 % . وبدراسة صورة الدم الخلوية لهذه الأسماك, تبين أنه عند اليوم الثالث من العدوى ارتفع عدد كرات الدم البيضاء ارتفاعا معنويا مصحوبا بنقص خلايا الليمفوسا يت وزيادة خلايا المونوسا يت والهيتيروفيل. وبحلول الأسبوع الأول وحتى نهاية الأسبوع الرابع انخفض العدد الكلى لخلايا الدم البيضاء مصحوبا بزيادة في عدد خلايا الليمفوسايت والمونوسايت بينما انخفضت خلايا الهيتيروفيل، كما ارتفع البروتين الكلى ارتفاعا معنويا عند اليوم الثالث من العدوى وعاد لينخفض معنويا من الأسبوع الأول حتى نهاية التجربة بينما ارتفع الجلوبيولين ارتفاعا معنويا من الأسبوع الأول حتى نهاية التجربة. وباستخدام اختبار الإليزا تبين وجود الأجسام المضادة للا ستربتوكوكس إنياي بصورة معنوية فى دم الأسماك المصابة بداية من الأسبوع الأول من العدوى ووصلت إلى قمتها عند الأسبوع الثالث وحتى نهاية التجربة.

أظهرت النتائج أن اختبار الاليزا كان أكثر حساسية وسرعة من اختبار ترسيب الاجارز