CONTRIBUTION ON PSEUDOMONAS SEPTICEMIA CAUSED BY *PSEUDOMONAS ANGUILLISEPTICA* IN CULTURED *OREOCHROMIS NILOTICUS*

SALEH F. M. SAKR AND AZZA M.M. ABD EL-RHMAN

Fish Diseases Dept., Central Lab for Aquaculture Research (El-Abbassa), Agriculture Research Center, Egypt.

Abstract

Pseudomonas anguilliseptica (*Ps. anguilliseptica*) was isolated from naturally infected *Oreochromis niloticus* (*O. niloticus*), showing septicemic picture. The prevelance of *Ps. anguilliseptia* was 62.5 % to the other isolated *Pseudomonas* species. The clinical signs noticed were inappetence, dark skin, petechial hemorrhage on the body and at the base of fins, easily detached scales, eroded and erected fins. Some fish showed slight abdominal distension, exophthalmia and pale gills. The post mortem changes were manifested by congestion in the internal organs with enlarged kidneys and spleen. Experimental infection of *O. niloticus* with the isolated *Ps. anguilliseptica* intraperitoneally (I/P) gave more or less the same changes recorded in naturally infected fish. *Ps. anguilliseptica* displayed in vitro sensitivity to Ciprofloxacin, Erythromycin, Gentamycin, Oxytetracycline, Streptomycin and Trimethoprim and sulphamethoxazole. Also it is sensitive to methanolic extract of *Anabaena wisconsinense* and *Oscillatoria curviceps.* Ciprofloxacin and methanolic extract of *Anabaena wisconsinense* were highly effective in the experimental treatment of pseudomonas septicemia at a dose of 10 mg / k. b. wt. I/P. Results of vaccination of *O. niloticus* with formalin-killed bacterial cells of *Ps. anguilliseptica* provided a good protection on challenge by *Ps. anguilliseptica* under laboratory conditions. The histopathological changes used as a tool for evaluation of potency of the vaccine where, the results revealed sever pathological changes manifested in sloughing of most superficial layer of the epidermis, congestion and leucocytic infiltration of the dermis. Sever hyperplasia of gill filaments as well as congestion of branchial blood vessels. Congestion of hepatic and portal blood vessel. Vacuolation and hydropic degeneration of liver and renal tubules were noticed, also hyperplasia of MMCs in the spleen was encountered. In comparison with immunized fish, no or slight histopathological changes were recorded.

Keywords: *Pseudomonas anguilliseptica*, *O. niloticus*, Antibiotcs, Algal extract, Vaccine, Histopathology.

INTRODUCTION

Aquaculture is playing an important role in boosting global fish production and in meeting the rising demand for animal protein. Nile-tilapia production is considered to be the fastest growing sector of the Egyptian fish farming industry has spread to several countries all over the world. Bacterial fish diseases constitute some of the

major challenges facing sustainable aquaculture production. Diseased fishes were the vehicles for human infection and deaths by septicemia (Veenstra *et al.*, 1992 and Woo and Bruno, 1999).

Pseudomonas anguilliseptica is an opportunistic pathogen for variety of fish species cultured in marine and brackish waters worldwide (Daly, 1999). *Ps. anguilliseptica* was originally described as the bacterial causative agent of "Sekiten-byo" of red spot disease of pond-cultured Japanese eel *Anguilla japonica* (Wakabayashi and Egusa 1972). In Finland, *Ps. anguilliseptica* was identified as the causative agent of sever disease out-breaks in several species of farmed salmonid fish (Wiklund and Dalsgaard 1987). Aquaculture health management is vital to successful industry. The lack of effective diseases prevention and control are the chief limiting factors of the realization of highly stable tilapia production. Preventive treatment by vaccination in combination with improved general management techniques is an ideal method for the pseudomonas septicemia control (Austin and Austin 1993, Woo and Bruno, 1999, Esteve-Gassent *et al.*, 2004a, Esteve-Gassent *et al.*, 2004b and Vervarcke *et al.*, 2005). A renewed interest in development of fish vaccine occurred in the early 1970s, and vaccination of cultured aquatic animal has become a viable fish health management tool for some diseases.

Therefore, the objectives of the current investigation were to through a beam of light on pseudomonas septicemia as a serious emerging bacterial disease caused by *Ps. anguilliseptica* among the cultured *Oreochromis niloticus*. Also, monitoring the effect of vaccination through the histological evaluation.

MATERIALS AND METHODS

1- Bacterial examination

Eighty (80) naturally infected *Oreochromis niloticus* of different sizes were collected from a private fish farm during winter season and transferred alive to the laboratory. The clinical signs and post-mortem findings were recorded. Bacteriological examination was carried out on samples taken from gills and the internal organs (liver, kidney, spleen, gonads, stomach and intestine) of collected fish. Bacterial swabs were cultured on Tryptic Soya Agar (TSA) and incubated at 25ºC for 24-48 hours. Pure bacterial isolates were identified using phenotypic and biochemical tests according to Austin and Austin (1993). The type strain of the species *Pseudomonas anguilliseptica* NCIMB 1949^T was also included in all of the identification tests as a reference strain. The prevalence of *Ps. angilliseptica* to other isolates and the organs was recorded.

2- Sensitivity test

2.I- Antibiograms:

Sensitivity of *Ps. angilliseptica* to different antibiograms (Ampicllin, Ciprofloxacin, Colistin, Erythromycine, Gentamycin, Kanamycin, Oxytetracycline, Pencillin, Streptomycin and Trimethoprim + sulphamethoxazol) were estimated according to Ericsson and Sherris (1971).

2.II- Sensitivity to algal extract:

Sensitivity of *Ps. angilliseptica* to methanolic extracts of *Anabaena wisconsinense* and *Oscillatoria curviceps* collected from the earthen ponds in Abbassa Fish Farm (biological control) was examined as paper disk assay according to (Bauer *et al.*, 1966). After cultivation of algae in carboys and harvesting, the algal were extracted by methanol 1: 15 (algal powder: volume of methanol) using a Soxhlet Extractor at 55-60°C all samples were refluxed until saturation (24 hours). The respective extracts were dried in an oven at 50°C or rot vapor according to (José-Vitor *et al.*, 2002). Sterilized paper discs impregnated with 30 ml of the extracted materials of *A. wisconsinense* and *O. curviceps* and air dried. The paper discs were placed over the agar surface after inoculation the plates with 0.1 ml of fresh bacterial suspension. Also an impregnated sterilized paper disc in the methanol alone was put in the surface of agar as a control. The plates were incubated at 25°C for 24 hrs. to be examined for inhibition zones.

3- Pathogenicity of isolated *Pseudomonas anguilliseptica*:

A randomly selected two hundred and forty (240) apparently healthy *O. niloticus* (average body weight of 50 ± 5 g) were maintained in 24 glass aquaria (70 × 80 × 50 cm). They were acclimatized in the aquaria for two weeks and fed on the basal diet twice a day. The aquaria were supplied with well-aerated de-chlorinated tap water and water temperature was maintained at 20°C. Siphoning of fecal matters and changing of one third of water was carried out daily. The fish were divided into 8 equal groups (each in a three replicates). Three isolates of *Ps. anguilliseptica* formerly isolated from the naturally morbid fish (from different organs) , was suspended in saline 0.85% to give a final bacterial count of 10^7 cells/ml. Fish groups 1, 2 & 3 were inoculated I/M (intra-muscular) 0.2 ml of the three prepared bacterial suspensions. Fish groups 4, 5 & 6 were inoculated I/P (intra-peritoneal) 0.2 ml of the same bacterial suspensions. The seventh and eight groups of fish were inculcated IM and IP with 0.2 ml of sterile saline as control groups respectively. All groups of fish were observed for 14 days and the mortality rate recorded. Moribund fish were subjected to laboratory examination and bacterial re-isolation.

4- Disease control

4.I- Efficacy of Ciprofloxacin and algae extract for treatment of infected *O. niloticus* with *Ps. anguilliseptica*

4.I.a- Ciprofloxacin

Ciprofloxacin (Amyria Pharm. Ind. Co., Egypt) was chosen according to the result of in-vitro sensitivity test. Forty *O. niloticus* were allotted into four equal groups. The first group was injected I/P with 0.2 ml of 10^7/ml *Ps. anguilliseptica* and kept for 18 hr. prior to Ciprofloxacin administration once intraperitoneal at a dose of 10 mg / Kg B. wt. (Nau *et al.*, 1995). The second group was kept as infected and non-treated. The third group was left as non-infected and treated. The fourth group was maintained as non-infected and non-treated. All groups were observed for 21 days post-infection.

4.I.b- Alga extract

Algae extract was chosen according to the result of in-vitro sensitivity test. Eighty *O. niloticus* were allotted into four equal groups. The first group was injected I/P with 0.2 ml of 10^7/ml *Ps. anguilliseptica* and kept for 18 hr. prior to algae extract once intraperitoneal at a dose of 10 mg / Kg b. wt. (Nau *et al.*, 1995). The second group was kept as infected by *Ps. anguilliseptica* and non-treated. The third group was left as non-infected and treated by algal extract. The fourth group was maintained as non-infected by *Ps. anguilliseptica* and non-treated. All groups were observed for 21 days post-infection.

4.II- Vaccination

4.II.a- Preparation of the bacterin

A formalin-killed vaccine was prepared as described by Yin *et al.* (1996). The selected isolate of *Ps. anguilliseptica* was grown in Tryptic Soya Broth (TSB) for 48 h. at 25ºC. Bacterial cells were killed by addition of formalin in a concentration of 0.2% and incubated at 25ºC overnight. Bacterial cells were collected by centrifugation at 2000 × g for 15 min at 4 ºC and washed three times in Phosphate Buffer Saline (PBS) at a final concentration of 1×10^7 cells ml^{-1}

4.II.b- Vaccination experiment

Ninety (90) *O. niloticus* (average body weight of 50 ± 5 g) were divided into two groups. The fish in the first group (60 fish) were injected I/P with 0.2 ml of the formalin-killed bactrin which prepared previously. The second group (30 fish) included the non-vaccinated fish. All fish groups were maintained at 20ºC in freshwater aquaria with aeration. The vaccinated and non-vaccinated fish were challenged at 2, 4, 6 weeks by I/P injection of 0.2 ml of 10^7 live bacteria of *Ps. anguilliseptica*. The mortalities were recorded daily for 2 weeks period and all dead fish were examined

quickly to confirm the re-isolation of the inoculated bacteria from internal organs. Protection was evaluated by determining the relative percent of survival (RPS) in each group using the formula:

RPS = [1 – (% mortality in vaccine fish / % mortality in control fish] × 100

5- Histopathology

Histopatological studies were carried out on tissues from naturally infected, challenged and vaccinated *O. niloticus*. Samples of fins, gills, liver, kidney, and spleen were fixed in 10% buffered formalin, dehydrated in ascending grades of ethanol and cleared in xylene. Fins were decalcified by 10% EDTA. Tissues were then embedded in paraffin and processed routinely for light microscopy. They were sectioned at 5 µm thickness and stained with haematoxylin and eosin (H & E).

RESULTS

Clinical signs and postmortem lesions

The naturally infected fish showed in-appetence, dark skin, easily detached scales, petechial hemorrhage on different parts of the body and at the base of fins with eroded and erected fins. Some cases showed slight abdominal distension, exophthalmia and pale gills. The post mortem changes were manifested by petecial hemorrahges in the internal organs with enlarged kidneys and spleen in some cases.

Isolation and identification of the pathogen

Pure cultures of the bacterium isolated from the gills, intestine and liver of naturally infected fish were identified morphometrically (table 1) together with the characteristics of the type strain NCIMB 1949^T. On the basis of morphological, physiological and biochemical characteristics it was identified as *Ps. anguilliseptica*. Also other bacteria were isolated from the clinically infected fish other than *Ps. anguilliseptia* were identified as *Ps. putida* and other Ps. species. The prevalence of *Ps. anguilliseptica* to other Ps. sp. which isolated from the same samples was 62.5%, *Ps. putida* 12.5% and Ps. sp. 25.5%. The prevalence of *Ps. anguilliseptica* to fish organs was 37.5. 37.5 and 25% from gills, intestine and liver respectively.

Drug sensitivity

Ps. anguilliseptica was sensitive to Ciprofloxacin, Erythromycin, Gentamycin, Oxytetracycline, Streptomycin and Trimethoprim & sulphamethoxazole. On the other hand, it was resistant to Ampicllin, Colistin, Kanamycin and Penicillin (table, 2).

Algal extract sensitivity

Ps. anguilliseptica was sensitive to methanolic extract of *Anabaena wisconsinense* and *Oscillatoria curviceps* and had inhibition zone 50 and 16 mm in

diameter respectively, while control (methanol alone) had inhibition zone 10 mm in diameter

Table 1. Morphometrical and biochemical characters of *Ps. anguilliseptica* isolated from naturally infected cultured *O. niloticus.*

Item	Isolate character	NCIMB 1949^T	Item	Isolate character	NCIMB 1949^T
Gram-stain	-ve	-ve	Lactose	+	-
Shape	Bacilli	Bacilli	Galactose	+	+
Arrangement	Single	Single	Maltose	+	+
Oxidase	+	+	Xylose	+	+
Catalase	+	+	Manitol	+	+
O/F	-	-	Sorbitol	-	-
Motility	+	+	L-Tyrosine	+	+
V.P.	+	+	Tween 80	+	+
M.R.	+	+	Nitrate	+	+
H_2S	-	-	Arginine hyd.	+	+
Citrate	+	+	Dec. of Lycin	-	-
Gelatin	+	+	Ornithine	-	-
Acid from: glucose	-	-	Growth on Nacl 0.0%	+	+
Sucrose	-	-	Growth on Nacl 3%	-	+
Glycero	-	-	Growth on Nacl 5%	-	-
Salicin	-	-	Growth at 5°C	+	+
Arabinose	+	+	Growth at 37°C	-	-
Fructose	+	+	Indol	-	-

Table 2. Sensitivity of *the isolated Ps. anguilliseptia* to different antibiograms.

Antibiotic againt	symbol	Concentration (mcg)	Susceptible zoon (mm)	inhibition zoon (mm)	Sensitivity reaction
Ampicllin	AM	10	≥30	10	R
Ciprofloxacin	CIP	5	≥21	32	S
Gentamycin	GM	10	≥15	20	S
Kanamycin	K	30	≥18	18	R
Oxytetracycline	OT	30	≥19	22	S
Streptomycin	S	10	≥15	22	S
Trimethoprim + sulphamethoxazol	Sxt	1.25 / 23.75	≥16	26	S
Penicillin	P	10 u	≥29	0.0	R
Collistin	CT	10 mcg	≥11	8	R
Erythromycine	E	15	≥18	20	S

Pathogenicity of *Ps. anguilliseptica*:

The three isolates of *Ps. anguilliseptica* recovered from the naturally diseased fish were pathogenic to *O. niloticus.* The mortality rate was illustrated in table (3). The clinical signs of experimental infected fish were similar to those noticed in

thenaturally infected fish. Bacterial re-isolation from experimentally moribund and freshly dead fish revealed the isolation of *Ps. anguilliseptica* in pure culture as a single infection.

Table 3. Mortality of *O. niloticus* experimentally inoculated with 0.2 ml of 10^7 cells/ml *Ps. anguilliseptica*.

group	Number of fish	*Origen of Ps. anguilliseptica*	*Route of injection*	Mortality %*
1	30	Gills	I/P	100
2	30	Intestine	I/P	100
3	30	Liver	I/P	75
4	30	Gills	I/M	75
5	30	Intestine	I/M	75
6	30	Live	I/M	60
7	30	Sterile saline	I/P	0.0
8	30	Sterile saline	I/M	0.0

I/P- intra-peritoneal, I/M – intra-muscular. Each group contained three replicates of ten fish each. * Mean of mortality percent between each group.

Efficacy of Ciprofloxacin in the treatment of Pseudomonas septicemia:

With regard to the laboratory trial for efficacy of Ciprofloxacin in the treatment of Pseudomonas septicemia, Ciprofloxacin decreased mortalities of *O. niloticus* from 30% during *Ps. anguilliseptica* infection to 14.3% at 72 hours post Ciprofloxacin injection as shown in table (4). Clinical signs started to relive after 12 hrs of treatment. No bacteria were isolated from any fish that survived to the end of trial. On the contrary, *Ps. anguilliseptica* was isolated from dead fish. Neither mortalities nor *Ps. anguilliseptica* were detected in the non-infected and treated group and non-infected and non-treated one. While the mortality rate of non-treated and infected group was 80% throughout the experiment.

Table 4. Mortality percent in *O. niloticus* with Pseudomonas septicemia after treatment with Ciprofloxacin

Fish group	No. of fish	No. and mortality rate	
		Before treatment %	After treatment %
Treated &infected	10	30	14.3
Non treated &infected	10	80%	
Treated & noninfected	10	0	0
Non trated & non infected	10	0	0

Efficacy of algal extract in the treatment of pseudomonas septicemia

According to the in-vitro activity of the results of algal extracts, we chose methanolic *A. wisconsinense* extract in the treatment of *O. niloticus* infected by *Ps. anguilliseptica*. From table (5) methanolic *A. wisconsinense* extract decreased the mortalities of *O. niloticus* from 48% during infection to 19% at 72 hours post treatment. No bacteria were isolated from any fish that survived to the end of the trial. On the contrary, *Ps. anguilliseptica* was isolated from dead fish. Neither mortalities nor *Ps. anguilliseptica* were detected in the non-infected and treated group. While the mortality rate of non-treated and infected group was 80% throughout the experiment.

Table 5. Mortality percent in *O. niloticus* with Pseudomonas septicemia after treatment with methanolic *A. wisconsinense* extract

parameters		Treated &infected	Non treated &infected	Treated & non infected	Non treated & non infected
No. of examined fish		20	20	20	20
Mortality %	before treatment	80%			
	after treatment	19%	48%	0.0	0.0

Formalin killed vaccine

Results indicated that formalin killed vaccine of *Ps. anguilliseptica* conferred a good protection at controlling mortalities when applied by intraperitoneal route (table 6). The r.p.s. were 90, 100 and 100 % on challenge at 2, 4 and 6 weeks post-vaccination respectively. While 100 % mortalities were recorded at each time for non-vaccinated group.

Table 6. The relative percentage of survival of the intraperitoneally vaccinated *O. niloticus* after challenge by 0.2 ml of 10^7 cells/ml *Ps. anguilliseptica*

Fish group	Vaccinated fish			Non vaccinated fish		
Time/week	2	4	6	2	4	6
No. of vaccinated fish	20	20	20	10	10	10
R.P.S.	90	100	100	-	-	-

Histopathological findings in naturally infected fish:

Fins

The free portions of the eroded fins displayed desquamation of the most superficial layer of the epidermis and increased number of alarm substance cells (Fig 1). The basement membrane was frietly hyalinized and /or showed disorganized basal cells. Focal aggregation of melanin carrying cells (MMC), especially under the basement membrane at the area of necrosis, were encountered (Fig 2). Sometimes congested blood capillaries were surrounded by MMCs and round cells were detected (Fig 3).

Gills

The gills showed congestion of the branchial blood capillaries, especially at the base of gill filament (Fig 4). Sever hyperplasia of epithelial cells covering the secondary lamellae were seen (Fig 5). Other filaments showed sever edema (Fig 6). Few round cells together with a considerable number of esinophilic granular cell (EGC) were detected at the base of gill filament. The gill arch showed sever congestion, edema and hemorrhage together with sever dilatation of lymph vessels and numerous EGC (Fig 7).

Liver

The hepatic portal veins and the blood sinusoids were highly congested (Fig 8), moreover vaculation and edema of blood vessels were seen. The hepatocytes showed vacuolar and hydropic degeneration (Fig 9). The bile ducts revealed mild perductal fibrosis and newly formed bile ductules (Fig 10). Some bile ducts showed hyperplasia of its epithelial lining (Fig 11), other bile ducts were surrounded by fibroblasts, EGC and round cells (Fig 12). The pancreatic acini showed partial to sever inactivation with lack of the zymogenic granules, necrotic changes and sever infiltration by MMC and EGCs.

Kidneys

The renal tubules showed vacuolar and hydropic degeneration, other tubules showed separation of the renal epithelium from its basement membrane (Fig 13). Minute focal coagulative necroses were detected. Focal hemorrhage and interstitial edema were encountered in the renal parenchyma (Fig 14). The archinephric duct showed vacuolation and hyperplastic epithelial lining (Fig 15). The renal parenchyma was infiltrated by MMCs. Some gromerli appear contracted with edema in the bowman's capsule (Fig 16). The kidney showed sub-capsular hemorrhage (Fig 16). Alternative area of activation and depletion of hemopiotic elements were noticed.

Spleen

Proliferation and increased number of MMCs which enchroached upon the spleenic tissue was evidently seen (Fig 17). Other cases showed depletion of hemopiotic element specially under the capsule (Fig 18). The splenic ellipsoid showed hyalinized wall. Excessive golden yellow or brown pigments were detected in the splenic parenchyma. The clinical, postmortem and histopathological results of experimentally infected fish showed nearly the same results of natural one.

Comparing the histopathological lesions in vaccinated and challenged with virulent *Ps. anguilliseptica* with those of naturally infected one, the results are to be seen in table (7).

Table 7. Histopathological comparison between vaccinated and non vaccinated fish challenged with 0.2 ml of 10^7 cells per ml of *Ps. anguilliseptica*

organ	Immunized fish	Non immunized fish
Fins	- Slight increase of alarm and mucus secreating cells.	- Focal sloughing of the epidermis and increased number of alarm substance cells. - Edema, congestive and leukocytic infiltration in dermis.
Gills	- Slight hyperplasia of epithelial covering of secondary lamella. - Slight congestion of the branchial blood vessel.	Sever hyperplasia of epithelial cells covering the secondary lamellae. Congestion in the branchial blood vessels. Edema, hemorrhage and aggregation of EGC in gill arch.
Liver	- Some hepatocyts showed cloudy swelling and vacuolation. - Few RBCs were seen in the central vein.	Congestion of hepatoportal vein and hepatic sinusoid. Hyperplasia of epithelial lining of bile duct. Inactivation of pancreatic acini which infiltrated by leucocyts.
Kidney	- Hydropic degenerated of some renal tubules. - Slight congestion of some pertubular blood vessels.	Sever hydrpic degeneration of the renal tubules. Variable degree of activation and depletion of the hemopiotic elements. Congestion and focal hemorrhages of the pertubular blood vessels. Some glomurli were appeared contracted with edema in Bowman's capsule.
Spleen	- Hyperplasia of melanomacrophage center	Increase the number and size of melanomacrophage center which appear dark brown in color. Focal areas of depletsion of hemopiotic elements.

Photos showing histopathological changes of *Oreochromis niloticus* infected with *Pseudomonas anguilliseptica*.

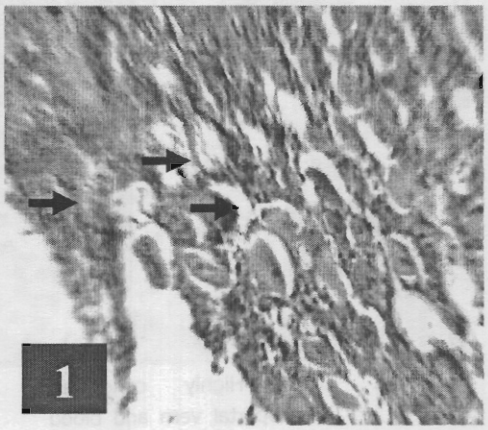

Fig. 1. Fin, showing desqumation of the most superficial layer of the epidermis and increased number of alarm substance cells, H & E., × 150.

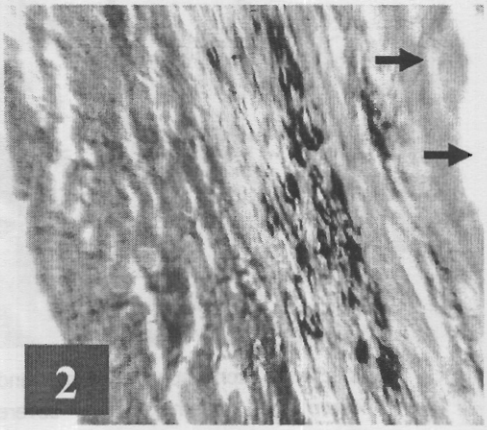

Fig. 2. Fin, Focal aggregations of melanomacrophage cells (MMC) under the basement membrane. H&E., × 150.

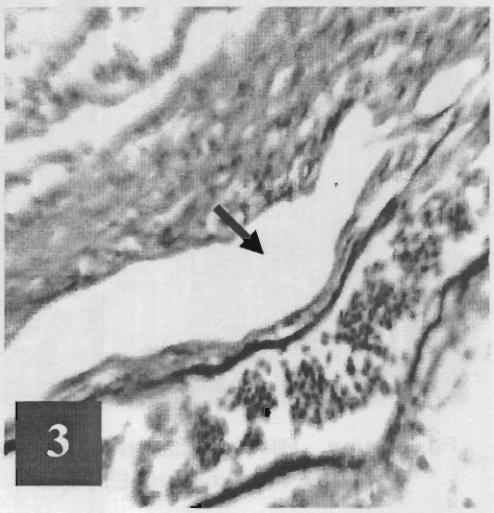

Fig. 3. Fin, Congested blood capillary was surrounded by MMC as round cells H & E., x 300 × 300.

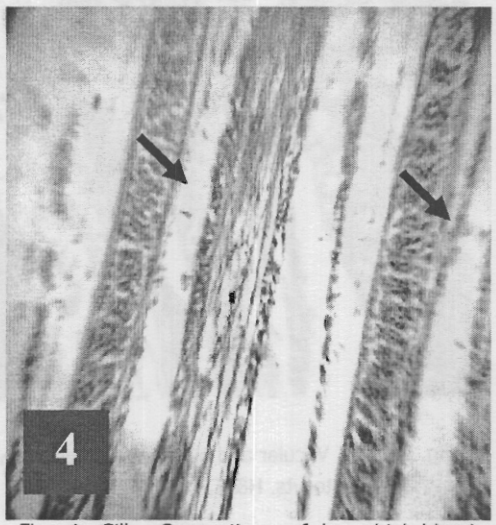

Fig. 4. Gills, Congestions of branchial blood vessels H & E., × 300.

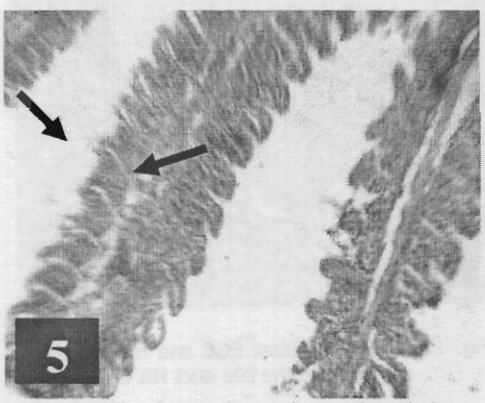

Fig. 5. Gills, Severe hyperplasia of epithelial cells cover the secondary lamellae H& E., × 300.

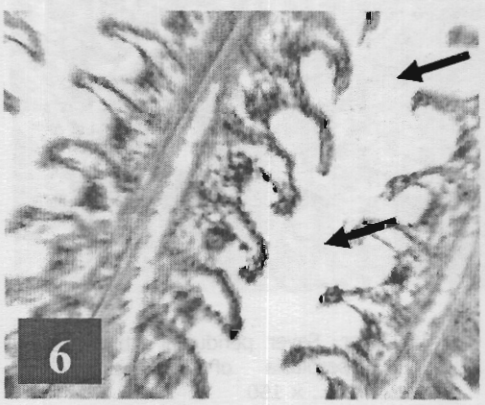

Fig. 6. Gills, Oedema of gill lamellae H& E., × 300.

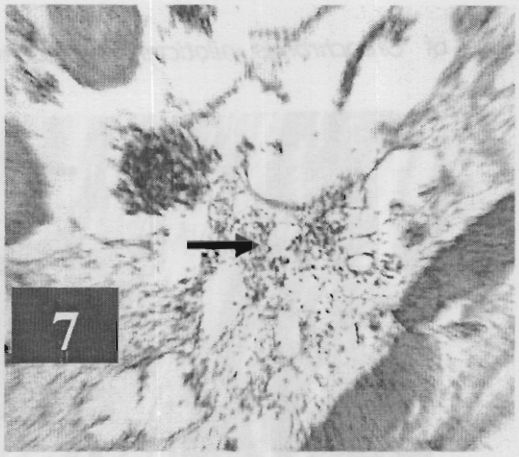

Fig. 7. Gills, Severe congestion, oedema and hemorrhage together with severe dilatation of lymph vessels and

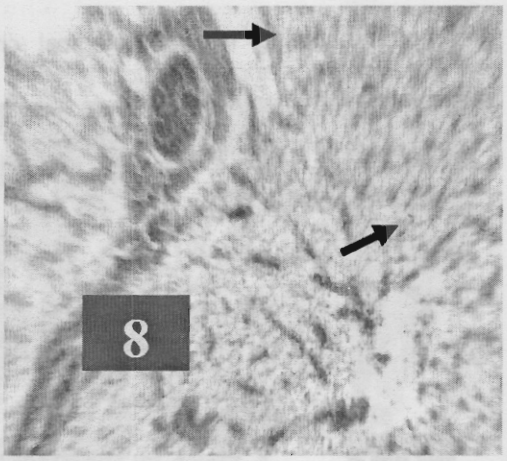

Fig. 8. Liver, Highly congested hepatoportal vern and blood sinusoids H& E., × 150

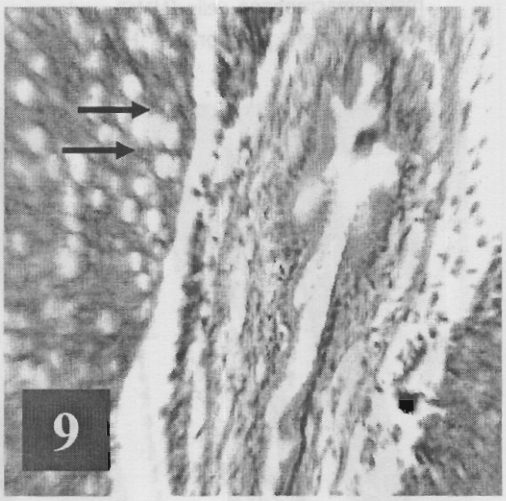

Fig. 9. Liver, Vacular and hydropic degeneration of hepatocyts. H& E., × 300

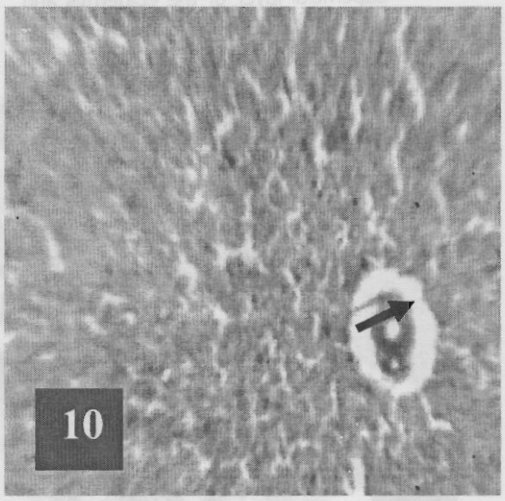

Fig. 10. Liver, Mild periductal fibrosis and newly formed bile ductules H& E.,× 150.

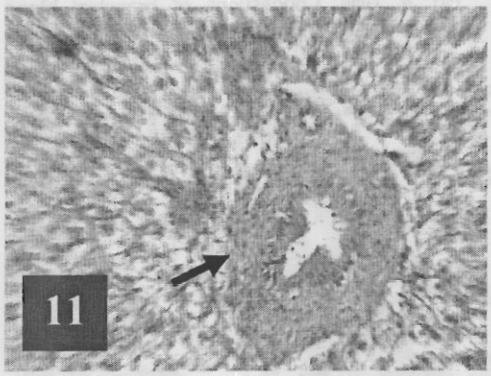

Fig. 11. Liver, Sever periductal Fibrosis and hyperplasia of its epithelial lining H& E., × 150

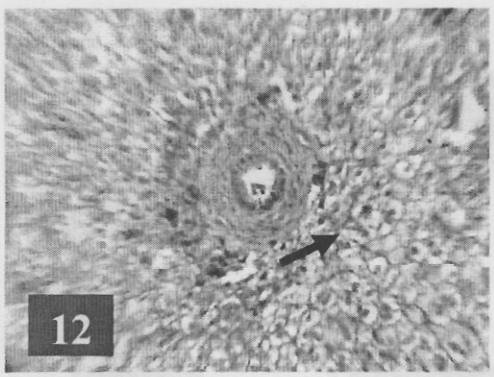

Fig. 12. Liver, Fibroblast EGC and round cells, surrounded the bile duct H& E., ×150

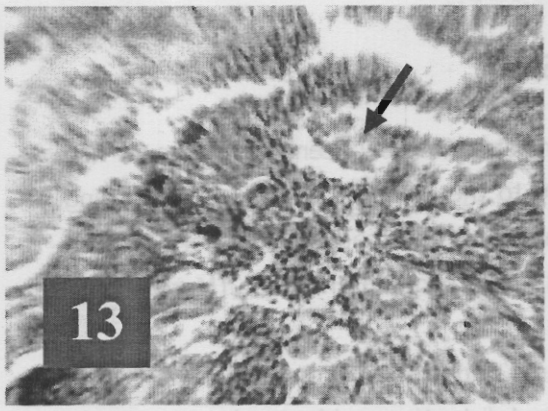

Fig. 13. Kidney, Separation of the renal epithelia from its basement membrane in some renal tubule. H& E., × 300

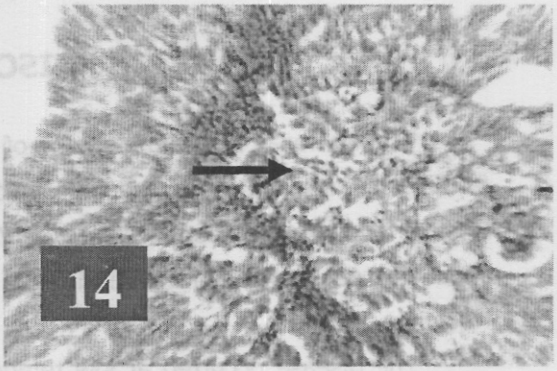

Fig. 14. Kidney, Focal hemorrhage and interstitial oedema in the renal parenchyma H& E., × 150.

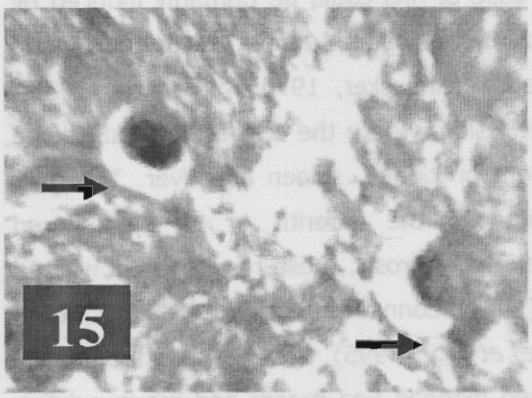

Fig. 15. Kidney, Some glomeruli appear contracted with oedema in Bowmanis capsule H& E., × 300.

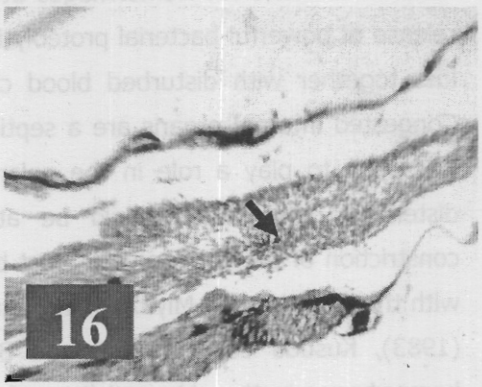

Fig. 16. Kidney, Sub- capsular hemorrhage. H& E., × 150

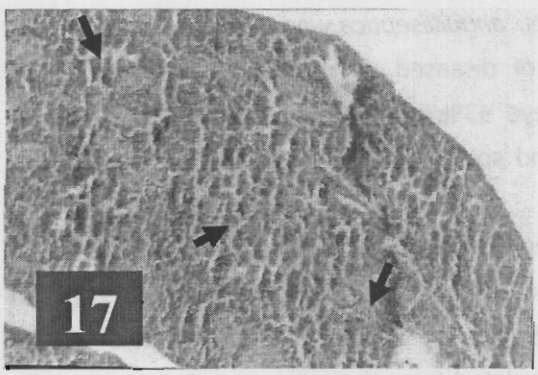

Fig. 17. Spleen, Proliferation and increased number of MMCs: H& E., × 150.

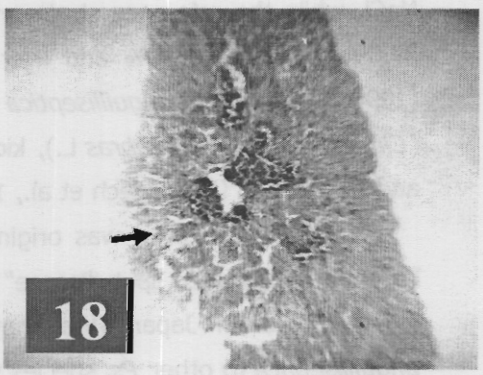

Fig. 18. Spleen, Depletion of hemopoiehtic elements especially under the splenic capsule H& E., × 150.

DISCUSSION

Pseudomonas septicemia is one of the most serious septicemic diseases for fish farming industry caused by bacteria belonging to the genus *pseudomonades* leading to sever economic losses. *Ps. anguilliseptica* infections have caused high mortalities on Japanese eel farms since the disease was first recorded in 1971 (Muroga and Nakajima 1981).

The disease occurred at low temperatures during the winter. The main clinical signs in the diseased *O. niloticus* were abdominal distention and hemorrhagic petechia in skin and internal organs as described from European eel (Wakabayashi & Egusa 1972, Doménech *et al.*, 1997, Wiklund and Bylund 1990 and Haenen and Davidse, 2001). Sluggish movement was probably the result of eroded and hemorrhagic fins in addition to anorexia. 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 Miyazaki and Egusa (1977), Lönnström et al. (1994), Ellis *et al.*, (1983), Kusuda *et al.* (1995) and Berthe *et al.*, (1995) who described petechial hemorrhages in the skin and liver of farmed gilthead Sea breem and moribund fish exhibited abdominal distension.

The isolated bacteria were identified as *Ps. anguilliseptica* which had almost the same biochemical tests of the reference strain NCIMB 1949^T except the growth on medium contained 3% NaCl. Our isolates did not grow on lab media containing 3% NaCl, while the reference strain grows. *Ps. anguilliseptica* was isolated from the gills 37.5%, intestine 37.5% and liver 25% of diseased *O. niloticus*. Lönnström *et al.* (1994) isolated *Ps. anguilliseptica* from eye 43% and kidney 30% of Baltic herring (*Clupea harengus membras* L.), kidney and spleen (Berthe *et al.,* 1995) liver, kidney and ascitic fluid (Doménech et al., 1997).

Ps. anguilliseptica was originally described in 1972 as the aetiolgical agent of "Sekiten-bio" or "red spot disease" which caused massive mortalities in pond cultured Japanese eel in Japan (Wakabayashi & Egusa 1972**).** The prevaelance of *Ps. anguilliseptica* to other *Ps.* species which isolated from the same samples was 62.5%, *Ps. putida* 12.5% and *Ps.* species 25.5%. Among Pseudomonas species recovered from diseased fish (*Ps. chlororophis, Ps. anguilliseptica, Ps. fluorescens, Ps. putida and Ps. plecoglossicida), Ps. anguilliseptica* is considered the most significant pathogen for cultured fish (Austin and Austin 1999).

Ps. anguilliseptica has been isolated in different countries from a variety of cultured and wild fish species such as European eel (*Anguilla anguilla*), black seabreem (*Acanthopagrus sehlegeli*), ayu (*Plecoglossus altivelis*), Atlantic salmon (*Salmon salar*) sea trout (*Sparus trutta*), rainbow trout (*Onchorhynchus mykiss*), whitefish (*Oregonus* sp.), Baltic herring (*Clupea harengus membras*), striped jack (*Pseudocaranx dentrx*), orange-spottod grouper (*Epinephelus coioides*), Nile tilapia (*O. nilotcus*), gilthead seabream (*Sparus aurata*) and turbot (*Scophthalmus maximus*), black spot seabream (*Pagellus bogaraveo*) and cod (*Gadus morhua*) (El-Attar and Moustafa, 1996, Austin and Austin, 1999, Daly, 1999, Al-Marzouk 1999, López-Romalde *et al.*, 2003, Romaled *et al.*, 2005 and Balboa *et al.,* 2007).

Ps. anguilliseptica was sensitive to Ciprofloxacin, Erythromycin, Gentamycin, Oxytetracycline, Streptomycin and Trimethoprim & sulphamethoxazole. Sensitivity of *Ps. Anguilliseptica* to antibiotics varies as indicated by others (El-Attar and Moustafa, 1996, Doménech *et al.*, 1997, Haenen and Davidse, 2001 and López-Romalde *et al.*, 2003).

The pathogenicity of *Ps. anguilliseptica* which isolated from gills and intestine to *O. niloticus* was high pathogen than the strain which isolated from liver. The pathogenicity of *Ps. anguilliseptica* varies according to fish species, temperature, type of water, size and age of the same fish species (Lönnström *et al.*, 1994, Berthe *et al.*, 1995 and Haenen and Davidse, 2001). They used nearly the same dose of *Ps. anguilliseptica* (10^7 cells per ml of saline). Also *Ps. anguilliseptica* was sensitive to methanolic extract of *Anabaena wisconsinense* and *Oscillatoria curviceps* but no report about the use of algal extract.

Successful control of bacterial diseases of cultured fish is a major management and economic problems for the aquaculture industry (Plumb 1999). Many antibiotics have proved to be very useful in controlling pseudomonas septicemia (Austin and Austin, 1993 and Woo and Bruno, 1999). Ciprofloxacin was found to be the drug of choice both in-vitro and in-vivo for the treatment of infected fish under laboratory conditions. Ciprofloxacin revealed a significant reduction in mortalities compared to that of the infected and non-medicated group. Doménech *et al.* (1997) used oxolinic acid and oxytetracyline orally by feed in some of the affected fish farms and recorded that these antibiotics was difficult to evaluate because they were effective only when the water temperature was above 17-18°C.

Recently, probiotic and prebiotic are receiving attention as candidates to improve the fish health and the prevention of bacterial diseases for environment friendly aquaculture. The methanolic *A. wisconsinense* extract decreased the mortalities of *O. niloticus* from 48% during infection to 19% at 72 hours post

treatment as nearly which recorded by Abd El-Rhman *et al.* (2008). Austin *et al.* (1992) indicated that extracts derived from *Tetraselmis suecica* were observed to inhibit Gram-negative bacteria in-vitro, and when used as a food supplement, the algal cells inhibited laboratory –induced infections in Atlantic salmon.

Immunization has played an important role as good fish farming practices in the control of infectious diseases (Vervarcke *et al.*, 2005). The most efficacious route of vaccination is intraperitoneal injection. The vaccination of *O. niloticus* with formalin killed vaccine of virulent strains of *Ps. anguilliseptica* appeared to be more effective in the prevention of Pseudomonas septicemia among cultured *O. niloticus* as mentioned by Nakai *et al.* (1982) and Romaled *et al.* (2005) who recorded that a bacterin against *Ps. anguilliseptica* gave protection for 12 weeks when tested in an experimental challenge trial in turbot Also they still have efforts to the development of vaccine which proved to be effective in experimental trials in gilthead seabream and trubot.

Microscopically the fines showed sloughing of most superficial layer epidermis, hyperplasia of alarm substance and mucous ells, congested and hemorrhagic dermis with hyalinization of epidermal basement membrane. These finding may be attributed to the intracellular enzymes which had hemolytic and proteolytic activities (Austin and Austin 1987). Such enzymes cause irritation and subsequent hyperplasia of alarm substance and mucus secreting cells as defense mechanisms.

The gills showed congestion, edema and hyperplasia of secondary lamellae together with round cells infiltrations and considerable number of esinophilic granular cells. (Wakabayashi and Iwado 1985) suggested that the bacteria produce an extracellular hyperplasia inducing factor which can reproduce typical BGD (Bacterial Gill Disease) lesions. Edema and hemorrhage in addition to leukocytic and EGC infiltration may be attributed to process of acute inflammation which was initiated by the action of the released vasoactive amines on the microcirculation of the area and the release of cell break down products (Roborts 1987).

The liver showed congestion, hydropic and vacuolar degeneration and periductul fibrosis. Also the kidneys showed congestion and focal hemorrhage. Hydropic degeneration and contracted glomeruli. While spleen showed proliferation of MMC and depletion of hemepiotic elements. These results were nearly similar to that obtained by Miyazaki and Egusa (1977) who showed that the histopathological studies of red spot disease in Japanese ells appeared initially in the dermis, subcutaneous adipose tissue, interstitial musculature tissue, vasocular walls, bulbous arteriosis and heart. Bacteria multiplied in the vascular wall, producing inflammation with serous exudation and cellular infiltration. Many small hemorrhages occurred in the dermal connective tissue, there was congestive edema of visceral organs and fatty degeneration of liver

hepatic cell, serous exudation at cellular proliferation of the spleen, glomerulitis and atrophy of hematopiotic tissue. Simillar results obtained by Miyashita (1984) and Duremdez and Lio-Po (1985) by *Ps. fluorescens* in tilapia.

According to Austin and Austin (1993) and Plumb (1999), proper management is essential to success of aquaculture operations, while the inadequate management is the principle factor in triggering disease outbreaks.

The control of bacterial diseases of cultured tilapia revolves around good aquaculture management, use of chemotherapy, use of probiotic materials and vaccination when indicated. Also we concluded that the histopathological investigations may be used as a tool for evaluation vaccine efficacy.

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اسهامة على مرض التسمم السودوموناسى الذى يسببه بكتيريا السودوموناس انجويللى سيبتيكا فى أسماك البلطى النيلى المستزرعة

صالح فتحي محمد صقر و عزة محمد محمد عبد الرحمن

قسم أمراض الأسماك – المعمل المركزي لبحوث الثروة السمكية (العباسة) – مركز البحوث الزراعية.

تم إجراء هذه الدراسة على بكتيريا السودوموناس انجويللى سيبتيكا أحد المسببات لمرض التسمم السودوموناس في أسماك البلطي النيلي المستزرعة. كانت الأعراض الإكلينيكية على الأسماك المريضة تتمثل في فقدان الشهية، بطء الحركة، العوم قريبا من سطح الماء، تساقط القشور، تآكل بالزعانف، تضخم البطن، جحوظ العينين، أنزفة عند قاعدة الزعانف. وكذلك شحوب في الخياشيم مع وجود كمية كبيرة من المخاط. كما تتميز الصفة التشريحية بوجود احتقان فى الكلى والطحال بينما كان الكبد أصفر اللون إلي الأصفر الشاحب.

و قد تم عزل البكتيريا المسببة لهذا المرض من الكبد والأمعاء والخياشيم و تم تصنيفها سودوموناس انجويللى سيبتيكا طبقا للمراجع العلمية العالمية.

لقد ثبت انه بالعدوى الصناعية وذلك بالحقن البريتونى أو العضلي لهذا الميكروب في أسماك البلطي النيلي السليمة تؤدى إلى نسبة نفوق عالية مع ظهور الأعراض المرضية المماثلة لما هو موجود في الأسماك المصابة طبيعيا.

تبين من اختبار الحساسية أن سودوموناس انجويللى سيبتيكا حساسة لكل من سيبروفلوكساسين، إيرسروميسن، جنتاميسن، أوكسيتيتراسيكلين، ستربتوميسن و سلفاميذوكسازول. وأيضا كان يوجد منطقة مثبطة مع مستخلص الميثانول للطحالب الخضراء المزرقة (سيلاتوريا كرفيسبس و الانابينا وسكونينس).

تم استخدام عدة طرق للسيطرة و الوقاية من هذا المرض في عدة تجارب معملية كما يلى:

١- أوضحت النتائج أن كل من السيبروفلوكساسين و مستخلص الطحالب الخضراء المزرقة لهما كفاءة عالية فى علاج أسماك البلطى النيلى المصابة بميكروب السودوموناس انجويللى سيبتيكا عن طريق الحقن البريتونى بمعدل ١٠مجم/كجم من وزن الاسماك .

٢- كما أتضح من هذه الدراسة أن استخدام لقاح من الخلايا البكتيرية الميتة بعد معالجتها بالفورمالين لميكروب السودوموناس انجويللى سيبتيكا عن طريق الحقن في التجويف البريتونى في أسماك البلطي النيلي قد أعطتها مناعة ضد الإصابة بهذا المرض.

كما تم تقييم اللقاح عن طريق الفحص الهستوباثولوجى لكل من الخياشيم، الزعانف، الكبد، الكلى والطحال وعمل مقارنة بينها وبين العينات المأخوذة من الأسماك المصابة طبيعيا وصناعيا (المجموعة الضابطة). وقد تبين أن هناك فروقا كبيرة في التغيرات الباثولوجية بين الأسماك المحصنة والتي لم يظهر عليها علامات مرضية والأخرى الغير محصنة التي ظهرت عليها علامات مرضية مماثلة لنفس العلامات المرضية والتغيرات الباثولوجية التي كانت في الأسماك المريضة طبيعيا، وكانت هذه التغيرات الباثولوجية هى تمدد في الخلايا الطلائية للخياشيم مع احتقان في الأوعية الدموية مع احتقان وأنزفة وانتشار الخلايا EGC والخلايا الليمفاوية في القوس الخيشومي. وهناك فجوات في خلايا الكبد مع وجود تنكرز وكذلك تليف بالقنوات المرارية وكذلك زيادة في عدد الخلايا الطلائية المبطنة لها. وكذلك يوجد احتقان وأنزفة موضعية في الكلى وانكماش في المرشحات كما يوجد زيادة في مراكز الميلانين واضمحلال في الخلايا المصنعة للدم.