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ORIGINAL ARTICLE

CHANGES ASSOCIATED WITH *PSEUDOMONAS* INFECTION IN CULTURED *OREOCHROMIS SPECIES* AND ITS RELATIONS TO ECONOMIC LOSSES OF FISH PRODUCTION FARMS

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ABSTRACT: This study aimed to throw the light on the effect pseudomonas infection in the freshwater fish under Egyptian condition through study the clinical signs and post mortem (P.M) lesions as well as its biochemical changes also study the economic losses resulted from dead fish attributed to the incidences of pseudomonas infection. This study was carried-out on 350 of O. niloticus (mean weight 80±5g, mean length 20±2cm) and 350 of O. galilas (mean weight 70±5g, mean length 18±1cm) were collected from commercial fish farms in Behera Province. The fish stocks received regular health checks and differentiate to both species and enteric red mouth (ERM) had not been found. Fish were acclimated for one week in the laboratory of the Dept. of Avian and Aquatic Animal Medicine of Alexandria University before any experimentation was started. This study, showed that pseudomonas infection in freshwater fish causes high mortality, with severe changes in blood parameters as RBCs and WBCs as well as the serum enzymes as GOT, GPT and alkaline phosphates, also the changes extended to include the total serum proteins, albumin, globulin and albumin globulin ratio, with severe histological changes in liver, kidney, spleen and gills. The results also showed severe economic losses due to high mortality in O.niloticus and O. galilus and reached to 57.75, 14.40 and 43.20 LE/100 fish for control fish, infected; vaccine treated fish and starved of O. niloticus fish, respectively. While, in O.galilus the losses were 135, 15 and 37.50 LE/100 fish for the same groups respectively, the results also cleared that, the best method for decreasing the economic losses of pseudomonas infection is the vaccination of the fish against Pseudomonas infection.

INTRODUCTION

Freshwater aquaculture is one of the major cash crops in Egypt and developing countries. Egyptian freshwater aquacultures are dominated by the fish species like *O. niloiticus and O.* galilas. Owning to increase in aquacultural industries, more environmental friendly strategies for controlling fish infections are urgently needed to make the aquaculture industry more sustainable (Khairnar *et al.*, 2013). Cultured fish and shelfish are constantly threatened by microbial infections because of various stress conditions resulting into occurrence of infectious diseases (Jang *et al.*, 2014).

Aquaculture industries are rapidly progressing to fulfill food requirement and provide employment opportunities for growing population. One of the major threatening problems faced by the aquaculture industries is the development of bacterial diseases that causes severe financial loss (Thomas *et al.*, 2014).

Fish diseases caused by Pseudomonas aeruginosa and fluorescence, a known pathogenic organism, is responsible for considerable economic losses in the commercial cultivation of Oreochromis niloticus (Mishra *et al.*, 2014; Seo *et al.*, 2014).

Under stress conditions like, the physical stagnation of the stream, high levels of ammonia, phenol and polycyclic aromatic hydrocarbons (PAHs) and low levels of dissolved oxygen (DO) were all incriminated as the initial stimulus behind biological invasion of pathogenic bacteria (Pseudomonas fluorescence) (Eissa *et al.*, 2013; Khairnar *et al.*, 2013). Pseudomonas spp especially poseudomonas fluorescence causes severe spoilage to the fish with elevation of serum enzymes (Sallam, 2007).

The main clinical signs caused by Pseudomonas fluorescens were white nodules in the spleen and abscesses in swim-bladder of tilapia (Miyazaki *et al.*, 1984), darkness, ascitis, peteicheal

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haemorrhages, congestion in kidneys, liver, ovaries and spleen (Badran, 1993), the disease is indistinguishable from Motile Aerommonas septicemia (Roberts, 1989).
The Pseudomonas anguilliseptica is the etiological agent of Sekiten-byo or red-spot disease of Japanese eels (Woo and Bruno, 1999), while Pseudomonas putida has been mentioned as a fish pathogen in Japan (Murogao, 1990).
The infection with pseudomonas in fish causes decrease in the lymphocytes, monocytes, eosinophils and neutrophils, also there is adecrease in the level of phagocytic activity and its index, there is a decrease in the level of WBCs, RBcs, Hb % and PCV %, there is also decreasing of the level of serum proteins (Albumin, globulin and total protein) (Austin and Austin, 1987).
The pseudomonas infection caused increasing of cholesterol, glucose, alkaline phosphatase, GPT and GOT level (Austin and Austin, 1993; Badran, 1993).
The infection and the severity of the symptoms differ according to the period of infection and its length as the clinical signs become clear in the first period then it regress and returned to its normal level at the last period of infection, also the fish infection differ according to the fish species as the monosex fish have the ability to resist the infection and overcome on it than that of O. niloticus and carp fish (Badran, 1993).
Vaccination of O.niloticus against pseudomonas flurescence infection causes improvement of feed intake, body weight gain, food conversion, serum protein level, RBCs and WBCs than the infected and non-vaccinated fish. It is recommended to use Pseudomonas species as probiotic in vitro only as it as Ps. species antagonized P. fluoresce with inhibition zone of 9 cm diameter (Aly *et al.*, 2008; Abd El-Rhman *et al.*, 2009).
This study aimed to throw the light on the effect pseudomonas fluorescence on the freshwater fish under Egyptian condition and its symptoms through study the clinical signs and PM lesions as well as its biochemical changes also study the economic losses resulted from dead fish attributed to the incidences of pseudomonas infection.

MATERIALS AND METHODS

Fish

350 of O. niloticus (mean weight 80±5g, mean length 20±2cm) and 350 of O.galilas (mean weight 70±5g, mean length 18±1cm) were collected from commercial fish farms in Behera Province. The fish stocks received regular health checks and differentiate to both species and enteric red mouth (ERM) had not been found. Fish were acclimated for one week in the laboratory of the Department of Avian and Aquatic Animal Medicine of Alexandria University before any experimentation was started.

Bacteria

Pseudomonas were enumerated on Pseudomonas Agar Base (CM 559; Oxoid) supplemented with cetrimide, fucidin, and cephaloridine (CFC) supplements (SR 103; Oxoid, Basingstoke, Hampshire, UK) providing a selective isolation medium for Pseudomonas spp. Colonies were counted after 2-days incubation at 25°C (Sallam, 2007).

Bacterin Preparation

A virulent strain of Pseudomonas fluoresce was inactivated by formalin according to (Sakai *et al.*, 1984). The inactivated strain was tested for safety and sterility according to (Anderson *et al.*, 1970). The formalin inactivated Pseudomonas fluoresce preparation was mixed with an equal volume of sterile saline (Badran, 1990). The bacterial number was adjusted at 1.0 107 CFU/ml formalin inactivated bacterial suspension and it was injected 1ml intraperitoneally (IP) into fish.

Bacteriological examination

The bacterial isolates recovered from the kidney, liver and intestine of diseased fish during June July 2013 when temperature of water reached to 32°C, Ammonia unionized NH3 0.2 mg/L and 2ppm of dissolved oxygen. Nutrient agar, MacConkey agar and 5% sheep blood agar were used for primary isolation. Smears of colony from liver, heart, kidney and spleen were stained by Gram stain. The *Pseudomonas Spp.* was Gram negative bacilli. Smears from spleen and blood were stained by Giemsa stain. Using API 20 E System, identification of the isolates was carried out according to Bergey`s Manual of Determinative Bacteriology. The antibiogram of the isolates was conducted on sensitivitynutrient agar medium according to the method mentioned by Lanyi.

Experimental design
The fishes were held at 32ºC and divided into equal six groups (each 30 fish):
Group 1: Infected (IP) with 0.1 ml viable Pseudomonas fluoresce (1.0 107 bacteria/ml) in saline (O. niloticus).
Group 2: The same procedure carried out as in the first group but in O. galilas.
Group 3: (non-treated group) O. niloticus was IP injected with 0.1 ml formalin killed Pseudomonas fluoresce (1.0 107 bacteria/ml).
Group 4: The same procedure carried out as in the third group but in O. galilas.
Group 5: O. niloticus starved for 35 days.
Group 6: O. galilas starved for 35 days.
To ensure the adequacy of the formalin treatment before administration of samples, formalin killed bacteria were streaked onto Pseudomonas Agar Base media.

Examination procedures
Groups 1 and 2 were sampled before any treatment and labeled as day 0. Groups 1, 2, 3 and 4 were then sampled at 7, 14 and 21 days post-injection. Infected fish (group 5 and 6) were gradually stopped feeding and the influence of starvation on each parameter was assessed by the inclusion of groups 5 and 6 which were sampled at 14 and 21 days post-starvation. Between two and five random fish were examined in groups and for each parameter at each sampling time.

Blood analysis
Anaesthesia using commercial clove oil 10% was generally complete within 10 min at a concentration of 20 mg/L. Blood was collected from caudal vessels of anesthetized fish and expressed into a 1 ml blood EDTA collection tube (Teklab Ltd.). The following were determined in all groups at each sampling time: fish weight and length, erythrocyte count, differential leukocyte count, hemoglobin and haematocrit (Blaxhall and Daisley, 1973). A 1 in 50 dilution of a non-coagulated blood sample containing 0.98ml of modified Dacie's fluid (Blaxhall and Daisley, 1973) was used to determine the erythrocyte and lymphocyte counts. Duplicate blood films were air dried, one was stained with leishman/Geimsa and a differential leukocytes count carried out on 100 blood cells from each smear.
Lymphocytes were divided into two groups, based on the length of the long axis. Large lymphocytes were recorded when the long axis was >9µm (Blaxhall and Daisley, 1973). The second was examined unstained to measure the longitudinal diameter of erythrocytes from the tilapia group using a calculated micrometer. The results were expressed as the mean diameter (µm) and as the frequency of the length of their long axis and were based on 100-300 observations (25 per fish) at each sampling time. Mean erythrocytes thickness was calculated using the formulae described by (Baker *et al.*, 1966). Serum samples were collected from all treated groups to determine s.AST, s.ALT and alkaline phosphatase which estimated according to (Reinhold, 1953) and serum proteins estimated according to (Reitman and Frankle, 1957). Selected tissues were routinely processed and stained with hematoxylin and eosin (H & E) (Culling, 1974).

Economic losses
The economic losses of the fish due to infection with bacteria were determined from dead fish, weight of dead fish and the losses in return due to dead fish/100 fish according to the following equations (Atallah and El-Banna, 2005; Saad *et al.*, 2007):

a- Weight of dead fish = Number of dead fish X Average weight of the fish (gm).
b- Losses in returns (L.E) = Weight of the fish (Kg) X Price of Kg fish (L.E).

Statistical analysis
The statistical analysis was made using One Way Analysis of Variance (ANOVA) and Duncan's test to study the significant differences among the means of the different groups according to SAS, (2004).

RESULTS

Clinical signs and PM lesions

externally, scalloss, tail rot, skin discoloration, scale loss, tail erosion, erythema at the base of fins and some fish showed slight abdominal distension and exophthalmia as well as bilateral blindness of eye. Moreover, fish showed hemorrhagic patches all over the body particularly around the base of the fins sometimes darkening of the skin. Necrotic (frayed) fins, hemorrhaged scale pockets and pale pockets and pale gills indicative anaemia. as well as oedematous musculature were also recorded Internally, organs are friable and have a generalized hyperemic appearance; the kidney and spleen are swollen; and the liver is often mottled with hemorrhage increased with light areas. The abdomen was enlarged with ascitis. The body cavity contains a clear fluid but more often the fluid is bloody and cloudy. The intestine is flaccid, hyperemic, contains yellowish mucous, and is void of food. Blood, heart, kidney and spleen smears stained with Giemsa stain showed numerous number of capsulated rod shaped organism (Figure 1 and 2).

The first deathsfrom Pseudomonas florescence occurred at day 6 post infections in the *O.niloticus* and day 11 post-infection in the *O. galilas*. Cumulative mortalities of non- vaccinated (infected) fish reached to 70% and 85% respectively in 21 days. No deaths occurred in the vaccinated or starved fish. The present results verified the success of experimental infection by an injection of 0.1 ml suspension ($1.0*10^7$ CFU/ml) of Pseudomonas fluorescence /fish IP in *Tilapia* species.

Figure 1: O. niloticus infected with P.fluoresce showing scale loss and tail rot.

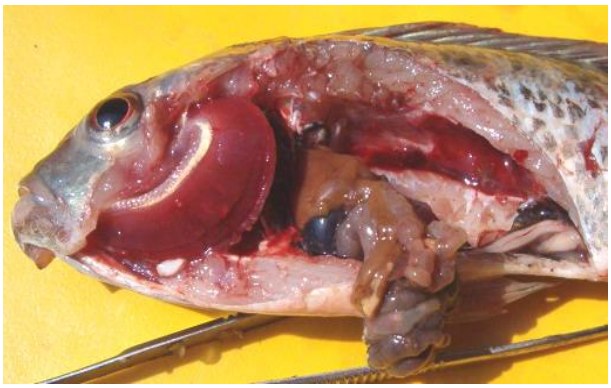

Figure 2: O.niloticus infected with P.fluoresce showing congestion of all internal organs.

Erythrogram

A rapid decline was recorded in the number of circulating erythrocytesin experimentally infected *O. niloticus* $9.50\pm0.4*10^6/mm^3$ to $3.2\pm0.3*10^5/mm^3$ (Table 1) more than who recorded in *O. galilas*. The mean erythrocyte count from the infected *O. niloticus* was significantly increased by 7 days post-infection and in *O. galilas* decreased by 14 days post-infection ($p<0.05$) (Tables 2, 3) A significant difference from the infected, vaccinated and starved groups was first noted at day 14 ($p<0.05$). Between 7 and 21 days post-infection the mean erythrocyte diameter in infected *O. niloticus* decreased significantly from 16.8 um to 13.90µm, while in case of *O. galilas* decreased from 19.10 µm to 15.7µm (Table 1, 2). These were no significant changes occurred in erythrocyte diameter in both *O. niloticus* and *O. galilas* in vaccinated and starved groups. A concurrent increase in the mean erythrocyte thickness was recorded in the infected *O. niloticus* measuring

3.5µm at day 0 and 4.6µm at day 14 (P<0.01). No changes occurred in the vaccinated and starved groups in both *O. niloticus* and *O. galilas*. Hemoglobin levels decreased in infected and starved fish than those of vaccinated fish in both infected species (P< 0.001) (Table 1, 2). Significant changes were found among infected, vaccinated or starved fish. A progressive decline in the mean hematocrit levels was recorded in the infected *O.niloticus* more than infected *O. galilas* with significant differences from the vaccinated and starved group occurring from 14 days post-infection (p<0.05) (Table 1, 2). Overall the decrease was highly significant in both infected groups (p<0.01). These values parallel the decrease in circulating erythrocytes of the infected groups.

Table 1: Erythrogram of *Pseudomonas fluorescence* in infected, vaccinated and starved *Oreochromis niloticus*

Groups	Day	Erythrocyte count 10^6/mm^3	Erythrocyte diameter (µm)	Calculated erythrocyte Thickness (µm)	Haemoglobin (g/100 ml)	Haematocrit (%)
		Mean±SE	Mean±SE	µm	Mean±SE	Mean±SE
Infected	0	8.2±0.4E	15.7±0.1C	3.5	7.9±0.4B	26±1.4E
	7	9.5±0.4D	16.8±0.1B	3.9	3.9±0.3C	24±1.2G
	14	9.0±0.3D	15.9±0.1C	4.6	3.7±0.4C	22±1.1F
	21	3.2±0.3F	13.9±0.1D	2.9	3.5±0.4C	19±0.9H
Vaccinated	7	10.8±0.6B	17.8±0.1A	2.6	7.9±0.3B	29±0.9C
	14	11.4±0.5A	15.6±0.1C	1.9	9.6±0.3A	32±1.3B
	21	11.2±0.2A	15.9±0.1C	2.2	9.5±0.2A	33±1.2A
Starved	14	9.5±0.2C	15.7±0.1C	2.4	7.5±0.2B	28±0.8D
	21	9.1±0.2C	16.8±0.1B	1.9	7.6±0.3B	26±1.2E

Means within the same column carrying different letters are significantly different at (P < 0.01).

Table 2: Erythrogram of P. fluorescence infected, vaccinated and starved O. galilus.

Groups	Day	Erythrocyte count 10^6/mm^3	Erythrocyte diameter (µm)	Calculated erythrocyte thickness (µm)	Haemoglobin (g/100 ml)	Haematocrit (%)
		Mean±SE	Mean±SE	µm	Mean±SE	Mean±SE
Infected	0	12.9±0.9A	19.1±0.2A	6.9	9.9±0.9C	42±1.2A
	7	9.4±0.6D	18.3±0.2B	5.6	11.8±0.7A	39±2.1B
	14	9.7±0.4D	17.8±0.2D	4.8	8.3±0.7D	32±1.4E
	21	6.7±0.4G	15.6±0.2G	6.5	5.4±0.8E	29±1.5F
Vaccinated	7	9.6±0.6D	15.8±0.2G	2.8	9.3±0.6C	38±2.5C
	14	11.7±0.7B	15.7±0.2	3.3	10.7±0.8B	37±1.7D
	21	8.9±0.6E	16.8±0.2EF	2.9	9.7±0.7C	39±1.8B
Starved	14	7.8±0.2F	16.5±0.2E	2.9	9.9±0.4C	37±1.6D
	21	10.80±0.3C	18.3±0.2C	3.9	8.9±0.4D	32±1.7E

Means within the same column carrying different letters are significantly different at (P < 0.01).

Significant (P<0.01) changes were recorded in the vaccinated or starved fish. An decrease in monocytes occurred at 0 and 14 days post- infection in the infected fish of *O. niloticus*, while this occurred at 0 and 7 days post-infection in the *O. galilas* (P<0.05), but no change was noted in the starved groups (Table 3 & 4).

The monocytes significantly increase throughout the period of vaccination in both fish species. Overall, there was no significant change in the small or large lymphocyte counts from the infected or starved of both fish species (Table 3, 4).

In vaccinated both fish species, there was slight increase of small lymphocytes all-over the period of vaccination, while large lymphocytes no changes reported. A significant (P<0.01) slight decrease in neutrophils occurred in both species during 0 day of infection and 7, 14 and 21 days post-vaccination. Thromobocytes were very variable between groups in both fish species. A significant difference was only recorded in the infected *O. niloticus* and *O. galilas* at day 7 (P<0.01).

Table 3: Erythrogram of P. florescence infected, vaccinated and starved O. niloticus.

Groups	Day	Monocytes 10^3/mm^3	Small lymphocytes 10^4/mm^3	Large lymphocytes 10^4/mm^3	Neutrophils 10^3/mm^3	Thrombocytes 10^3/mm^3
		Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Infected	0	5.13±1.2C	5.74±0.2B	1.54±0.21D	3.75±0.4A	2.85±2.8A
	7	4.68±0.9D	3.83±0.3D	1.92±0.21C	1.44±0.3C	2.90±0.6A
	14	5.23±1.3E	2.91±0.3E	2.45±0.21A	1.30±0.2C	1.99±0.6B
	21	3.12±0.6F	3.34±0.4D	1.22±0.31C	1.18±0.2C	0.79±0.4D
Vaccinated	7	5.76±0.10C	4.38±0.8C	1.15±0.21C	2.68±0.2B	0.40±0.7D
	14	7.89±0.21B	5.04±0.3B	2.39±0.21B	3.71±0.2A	1.28±0.5C
	21	8.62±1.41A	6.67±1.5A	1.83±0.21C	2.29±0.2B	0.84±0.6D
Starved	14	2.43±0.40G	3.95±0.3D	1.91±0.31C	2.28±0.2B	1.69±0.4B
	21	3.71±0.50F	2.58±0.4F	2.19±0.41B	1.18±0.2C	0.88±0.5D

Means within the same column carrying different letters are significantly different at (P < 0.01).

Table 4: Leukogram of *pseudomonas florescence infected*, vaccinated and starved of *Oreochromis galilas*.

Groups	Day	Monocytes $10^3/mm^3$	Small lymphocytes $10^4/mm^3$	Large lymphocytes $10^4/mm^3$	Neutrophils $10^3/mm^3$	Thrombocytes $10^3/mm^3$
		Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Infected	0	3.28±1.3D	2.14±0.2D	3.24±0.2A	1.99±0.3B	1.97±0.4B
	7	2.19±0.80F	3.07±0.2C	1.11±0.2C	2.24±0.2A	1.64±0.6B
	14	1.94±0.6G	1.88±0.3E	2.69±0.3B	0.99±0.2C	0.38±0.4C
	21	2.89±0.8E	2.78±0.4D	1.56±0.2C	1.79±0.2B	1.23±0.3B
Vaccinated	7	5.72±0.5B	3.89±0.4C	2.27±0.3B	2.09±0.2AB	1.24±0.5B
	14	5.61±0.3B	5.15±0.4A	2.79±0.3B	2.99±0.2A	2.37±0.5A
	21	6.85±0.3A	4.74±0.4B	2.58±0.3B	2.37±0.2A	1.78±0.5B
Starved	14	4.31±0.3C	3.76±0.3C	1.19±0.3C	0.80±0.2C	0.89±0.6C
	21	3.89±0.3D	2.66±0.3D	2.33±0.2B	0.66±0.2C	1.59±0.2B

Means within the same column carrying different letters are significantly different at ($P < 0.01$).

AST, ALT and alkaline phosphatase levels:

The results in (Table, 5 and 6) indicated that the level of AST and ALT without any differences in *O. niloticus* fish and *O. galilas* and also, the results indicated that, the AST and ALT levels increased in infected fish, than the starved fish and the lowest level in vaccinated fish.

Protein levels

Table (5 and 6) explain that, the level of serum protein increased in *O. galilas* than that of *O. niloticus* and the level of serum albumin, globulin and total proteins is higher in vaccinated than that of starved fish, and the lowest value in infected fish.

Histopathological findings

Gills: Focal necrosis invaded primary lamellae of gill filaments with mononuclear inflammatory cells (Figure 3).

Liver: The hepatocytes membrane started as small circular white patches on the serosa which later appeared filamentous and infiltrated by an inflammatory reaction (Fig. 4).

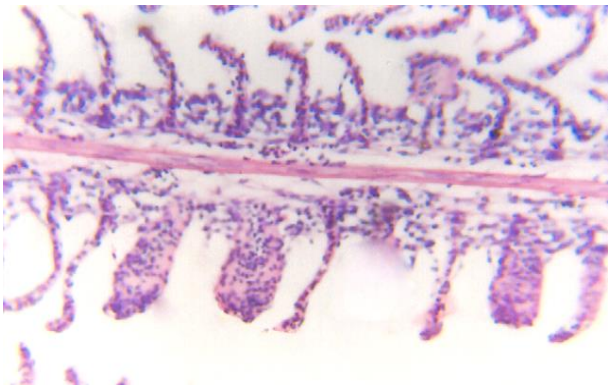

Figure 3: Gill of O. niloticus infected with P.fluoresce showing focal lamellar necrosis that invaded with mononuclear inflammatory cells (H&E x250).

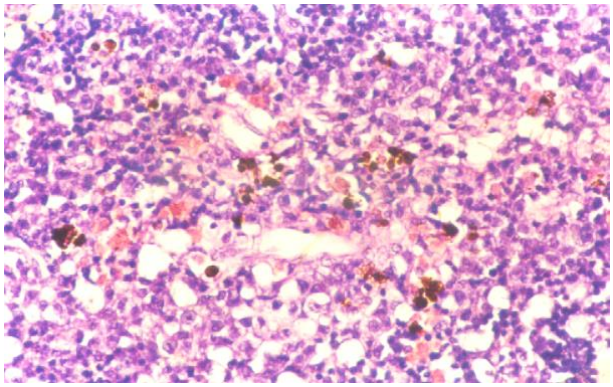

Figure 4: Hepatopancreas of O. niloticus infected with P. fluoresce showing individualization of pancreatic acini surrounded by individual atrophied hepatic cells contain minute vacuoles (H&E x250).

Table 5: Effect of different treatment groups on serum GPT, GOT, Alkaline phosphatase and serum proteins (albumin, globulin, total protein and albumin/globulin ratio) among different weeks in O. niloticus.

Groups	Day	N	GPT	GOT	ALP	Albumin	Globulin	Total protein	Albumin/globulin
			Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Infected	0	3	61.33±0.33E	78.33±1.20A	13.00±0.58A	2.47±0.22BB	0.93±0.38D	3.40±0.17D	2.65±0.24C
	7	3	70.42±0.33C	73.67±1.20C	12.00±0.58B	3.40±0.17A	0.43±0.28F	3.83±0.15C	7.90±1.84A
	14	3	69.33±0.88C	69.33±0.33D	12.33±1.20B	2.87±0.24B	1.30±0.26B	4.17±0.03B	2.20±0.64D
	21	3	70.00±1.00C	73.33±1.33	11.67±0.33C	2.00±0.06C	1.23±0.07C	3.23±0.03E	1.62±0.14F
Vaccinated	7	3	61.67±0.88	70.33±0.88D	11.00±0.58C	3.60±0.15A	2.00±0.12A	5.60±0.26A	1.80±0.04E
	14	3	64.00±0.58	71.67±0.67D	10.00±0.58D	3.20±0.06A	1.20±0.15B	4.40±0.12B	2.66±0.44C
	21	3	65.33±0.33D	74.67±0.88B	11.00±0.58C	2.23±0.09B	1.43±0.27A	3.67±0.30D	1.55±0.38F
Starved	14	3	73.69±1.53B	71.33±0.88	10.67±0.88D	3.03±0.09A	0.73±0.27C	3.77±0.34CD	4.15±0.80B
	21	3	74.67±1.33B	75.33±0.88B	10.00±0.58D	2.40±0.17B	1.17±0.15B	3.57±0.03E	2.05±0.44E

Means within the same column carrying different letters are significantly different at ($P < 0.01$).

Table 6: Effect of different treatment groups on serum GPT, GOT, Alkaline phosphatase and serum proteins (albumin, globulin, total protein and albumin / globulin ratio) among different weeks in O. O.galilus.

Groups	Day	N	GPT	GOT	ALP	Albumin	Globulin	Total protein	Albumin/globulin
			Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Infected	0	3	70.67±0.67F	76.33±0.33A	13.33±0.88A	2.83±0.47A	1.37±0.41BC	4.20±0.20B	2.06±0.10B
	7	3	72.33±1.45D	75.67±0.88A	13.00±0.58A	2.90±0.60A	0.67±0.12D	3.57±0.48D	4.32±0.58A
	14	3	73.00±0.58D	71.00±1.00C	12.33±0.33B	2.30±0.21BC	1.50±0.15B	3.80±0.36C	1.53±0.02E
	21	3	67.67±1.20G	73.67±0.67B	12.67±0.33B	2.53±0.09B	1.67±0.17A	4.20±0.12B	1.51±0.18E
Vaccinated	7	3	71.00±2.08E	72.00±0.58B	9.00±1.15E	2.13±0.12C	1.33±0.07C	3.47±0.19C	1.60±0.02D
	14	3	72.33±0.33D	71.67±0.67C	10.00±0.58D	2.57±0.32BC	2.77±0.20A	5.33±0.15A	0.92±0.19E
	21	3	73.67±0.33D	72.00±0.58B	9.67±0.88E	3.20±0.11AB	1.66±0.14C	4.87±0.24C	1.92±0.12B
Starved	14	3	75.67±0.88C	75.67±0.67A	10.33±0.33D	2.20±0.06C	2.73±0.46A	4.93±0.41B	0.80±0.18F
	21	3	76.00±0.58B	74.00±0.58A	12.67±1.76B	2.20±0.25C	1.70±0.21C	3.90±0.15E	1.29±0.30C

For each week means within the same column carrying different letters are significantly different at ($P < 0.01$).

Economic analysis

The economic results cleared that, the pseudomonas infection in the fish causes severe economic losses and the losses in O. niloticus higher than that of the O. galilus. While, the results of economic losses for O.niloticus due to infection with pseudomonas were 57.75, 14.40 and 43.20 LE/100 fish for control fish, infected, vaccinated and starved O. niloticus fish, respectively. While, in O.galilus the losses were 135, 15 and 37.50 LE/100 fish (Table, 7).

Table 7: Effect of different treatment groups on serum glucose and serum proteins (albumin, globulin, total protein and albumin / globulin ratio) among different weeks

Spp.	Groups	Day	N	Mortality number	Weight of dead fish (Kg)**	Return losses	Return losses /100
O. niloticus	Infected	0	100*	30	5.25	57.75	57.75
		7	100	20	4	48	192
		14		20	4	48	
		21		10	2	24	
	Vaccinated	7	100	2	0.40	4.80	14.40
		14		1	0.20	2.40	
		21		-	0	0	
	Starved						43.20
		14	100	4	0.80	9.60	
		21		8	1.60	19.20	
O. galilus	Infected	0	100	22	3.30	33	135
		7		21	3.15	31.50	
		14		12	1.80	18	
		21		35	5.25	52.50	
	Vaccinated	7	100	3	0.45	4.50	15
		14		2	0.30	3.0	
		21		-	0	0	
	Starved						37.50
		14	100	6	0.90	9.0	
		21		10	1.50	15	

For each week means within the same column carrying different letters are significantly different at ($P < 0.01$).
* 50 O.niloticus +50 O.galilus; **Average weight of O. niloticus dead fish = 200 gm, while the, average weight of O. niloticus dead fish =150gm. Price of Kg O. nilioticus = 12 LE; Price of Kg O.galilus = 10 LE.

DISCUSSION

The clinical signs of experimentally infected O. niloticus and O. galilas after injection of 0.1 ml viable Pseudomonas flourscence from suspension of 1.0 10^7 CUF/ml.

Internally, organs are friable and have a generalized hyperemic appearance ; the kidney and spleen

are swollen ;and the liver is often mottled with hemorrhage increased with light areas. The enlarged abdomen with ascitis. The body cavitycontain a clear fluid but more often the fluid is bloody and cloudy. The intestine is flaccid, hyperemic, contains yellowish mucous, and is void of food. These signs were nearly similar to the result recorded by (Stoskopf, 1993; Saad *et al.*, 2007; Khairnar *et al.*, 2013). The clinical signs and P.M. lesions may be attributed to the role of plasmids in virulence which differs in number according to pathogencity (Austin and Austin, 2007). Further work is necessary, however, to resolve the precise role of this large plasmid in the pathogenicity of P. flourscence. The experimental infection of O. niloticus and O. galilas with P. flourscence and the subsequent progression of septicemia resulted in a significant decline in red cell count, haematocrit and hemoglobin with an associated generalized bacteraemia occurs in the principal organs, with slight enlargement of the kidney and spleen (splenomegaly), which supports previous findings in both experimentally and naturally infected O. niloticus and O. galilas (Hester, 1973; Newman and Majnarich, 1982; El-Gamal, 2005; Jang *et al.*, 2014). In addition, this study showed a significant increase in monocytes, small lymphocytes, large lymphocytes and neutrophils in vaccinated groups (Bruno and Munro, 1986; Thomas *et al.*, 2014).

It was observed that formalin-killed Pseudomonas flourscence caused a minor but significant change in the mean red cell diameter, is suggestive that a bacterial toxin resistant to formalin is active (Bruno and Munro, 1986). The decline in circulating red cell levels in infected fish was accompanied by a decline in the mean cell diameter and a corresponding increase in the calculated cell thickness, changes which were among the earliest detectable pathophysiological effects. The 14-day frequency profile of red cell diameters showed this loss appears to have been principally of mature erythrocytes, which were replaced by immature forms identified as reticlouytes. It was concluded that during the early part of infection an increased release of immature forms occurs in response to those lost from the circulation. At a later stage, release of even less mature forms occurs, particularly erythroblasts. The early and continued appearance of immature erythroblasts in the circulation is a reflection of the pathophysiological changes which occur as the infected fish attempts to maintain homeostasis (Hunn, 1964; Härdig, 1978; Mishra *et al.*, 2014). In vaccinated fish with P.Flourscence vaccine showed increase the cells of monocytes, lymphocytes and neutrophils which responsible to phagocytes of microorganisms so all changes of haematological assessment not occurred and mortalities. So late similar results was obtained by El-Gamal, (2005) and Saad *et al.*, (2007) who found that the protection against Yersiniasis was increased by use vaccine of bacterin of Y. ruckeri in monosex O. niloticus and Marone Labrax respectively. In the later stage of disease additional erythroblasts would be lost from the infected fish through areas of necrosis, particularly around the vent region and the petechial hemorrhages, and this may also contribute to red cell loss. Also bacterial toxin induced direct damage of red cell membrane (Aldrin *et al.*, 1978; Bruno, 1984), which may have been sufficient to cause instability to the red cell membranes. Infection with P. florescence resulted in a lymphocytopenia and monocytosis. However, the response by individual cell types was not synchronous and therefore does not necessarily reflect what is happening to the cell lymphocyte and monocyte population. This may be due to increase number of monocytes or decrease number of lymphocytes according to degree of infection which reflection on the antibody production (Saad *et al.*, 2007; Mishra *et al.*, 2014). Early in the infection both neutrophils and thrombocytes increased, although they returned to the starved group levels within a few days. Changes of this cell type are considered part of the non-specific aspect of the stress syndrome (Pickering *et al.*, 1982; Stoskopf, 1993). Sites of infection often contained manysmall lymphocytes as well as monocytes and tissue macrophages phagocytosing P.flourscence. Macrophage stimulation and responsiveness is also non-specific, although activated by products of the specific immune system, such as lymphocytes which may perform as T- cell equivalents or as natural killer cells (Ellis *et al.*, 1981; Safinaz, 2001).

The enzymatic examination revealed the significant increase of AST and ALT enzymes in case of infected fish, followed by starved fish and the lowest level in case of vaccinated fish. This may be attributed to, the P. flourscence infection were mainly causes anticonvulsant activity and also due to increase in alkaline phosphatase activity which leads to hepatocytic destruction and unbalanced metabolism (Gupta and Chatterjee, 1980). Also, increase in serum transaminases (ALT and AST), may reflect the myocardial and hepatic damage leading to extensive liberation of the enzymes into blood circulation (Verma *et al.*, 1975; Fuchs *et al.*, 1986). Moreover, serum ALT and AST activities are considered as a sensitive indicator to evaluate hepatocellular and myocardial damage (Abd-Allah *et al.*, 1991; Raa, 1994; Mishra *et al.*, 2014). The results indicated that albumin, globulin, total protein levels decreased progressively from the 1st period to the last period and the level of serum proteins in O. niloticus was slightly higher than that of O. galilas. Hypoalbuminemia, hypoglobulinemia and hypoproteinemia which observed may be attributed to stress

condition (bacterial infection) causing liver damage that causing decrease in serum protein concentration. The present study showed that there was a decrease in serum globulin in bacterial infection and this decrease commonly in the last stages of experiment which may be attributed to lymphopenia and this due to liver damage where all plasma protein synthesis usually occurs in liver except gamma globulins which produced by lymphocytes (Coles, 1986).

Moreover, the decrease in protein and globulin can explain the drastic effect of bacterial infection on immune response of infected fish with subsequently increased the drastic damage effects of bacterial diseases. Also, the liver disorder is usually, accompanied by hypoalbuminemia. Both hypoglobulinemia and hypoalbuminemia confirmed the recorded hypoproteinemia, which was associated with liver damage. The causes of decreased serum total protein may be occurred after vascular leaking due to increasing permeability after histamine release (Green, 1978; Ellis, 1981), liver damage and anorexia, non-specific proteolysis (Ellis, 1981; Mishra *et al.*, 2014).

From this study, we concluded that the routine hematological methods for examining fish blood should be used to assess the development of P. flourscence vaccine by its influence on the blood components. Although physiological normal values are wider in fish than those in human medicine as several significant changes were recorded before evidence of clinical diseases. This indicates the immune status of fish and diseases resistance.

The economic analysis revealed that, the pseudomonas infection causes severe economic losses to the fish industry analysis

The economic results cleared that, the pseudomonas infection in the fish causes severe economic losses and the losses in O. niloticus higher than that of the O. galilus. While, the results of economic losses for O.niloticus due to infection with pseudomonas were 57.75, 14.40 and 43.20 LE/100 fish for starved fish, infected, vaccinated and starved O. niloticus fish, respectively. While, in O.galilus the losses were 135, 15 and 37.50 LE/100 fish.

From this study, we can concluded that pseudomonas infection in freshwater fish causes high mortality, with severe changes in blood parameters as RBCs and WBCs as well as the serum enzymes as GOT, GPT and alkaline phosphates, also the changes extended to include the total serum proteins, albumin, globulin and albumin globulin ratio, with severe histological changes in liver, kidney, spleen and gills. The results also showed severe economic losses due to high mortality in O.niloticus and O. galilus and reached to 57.75, 14.40 and 43.20 LE/100 fish for starved fish, infected, vaccinated and starved of O. niloticus fish, respectively. While, in O.galilus the losses were 135, 15 and 37.50 LE/100 fish for the same groups respectively, the results also cleared that, the best method for decreasing the economic losses of pseudomonas infection is the vaccination of the fish against Pseudomonas infection.

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