Some Studies on Pseudomonas Infection in Experimentally Infected
*Oreochromis niloticus*

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**ABSTRACT**

Bacterial fish diseases are the major problems in aquaculture as it found naturally in the fish environment and under certain stresses condition causes severe economic losses to fish farms. This study was to achieve the aim of this study, the following points were done: 1) Evaluation of the virulence of isolated *Pseudomonas flourescence*. 2) And effect of isolated strain of *Pseudomonas flourescence* on different blood parameters and serum enzymes. Examination the potency of locally prepared bacterin against isolated strain of *Pseudomonas flourescence*. the clinical signs were manifested as, loss of scales from some areas of the body with excessive mucus all over the body. While, the lymphocyte showed a tendency to decreasing from the 1st week to the 4th. While, the monocyte level decreased from the 1st week to the 3rd week. The Phagocytic activity and index increased progressively from the 1st week to the 4th week of the experiment. The results also showed that, the total protein, albumin, globulin and albumin/globulin ratio differ significantly among infected and control group and among different weeks. The antibody titer and RLP the antibody titer in the groups treated with *P. flourescence* only of lower RLP than that of the control groups.

**INTRODUCTION**

Fish diseases are a substantial source of great losses to aquaculturists. Production costs are increased by fish diseases outbreaks because of the investment lost in dead fish, cost of treatment and decreased growth during convalescence. (Floyd, 2003).

The most prevalent diseases affecting fish farms in Egypt were Motile Aeromonads, *Pseudomonas* species, *Streptococcus* spp. and *Staphylococcus* spp. *Vibrio* spp. and *Flexibacter* spp. (Khalil et al., 2001).

**MATERIALS AND METHODS**

**A- Materials :**

*Fish for experimental infection*

A total number of ninety apparently healthy *O. niloticus*, were obtained from private fish farm. Fish were transported alive to the laboratory of Departement of poultry and fish disease, Faculty of Veterinary Medicine, Alexandria University in plastic bags contained water enriched by air (2/3). Average body weight of fish about (50 ± 5 gm).

**Fish pathogens :**

a. Bacteria used for preparation of bacterin and challenge. (*Pseudomonas flourescence*) isolated strains kindly obtained from Poultry and Fish Diseases Dept.

b. *Candida albicans* used for phagocytes was kindly provided by Poultry and Fish Diseases Dep. Fac. of Vet. Med. Alex. Univ.

**B- Methods :**
1. Clinical and Postmortem examinations

The collected fish were examined clinically according to the method described by McVicar (1982).

A. Experiment I

2. Chronic experiment

Another same 60 apparently healthy \textit{O. niloticus} were equally divided into 2 groups; each group contains 2 replicates (10 fish / each replicate). First group was inoculated I/M with 0.2 ml of 1/10 LD\textsubscript{50} of tested bacteria. The second group was inoculated I/M with 0.2 ml / fish of normal saline and served as a control group. All infected and control fish were observed daily to record their general health condition, clinical signs and mortalities. Experimental period was 28 days. Postmortem examination was performed on dead fish. The survivors at the end of the observation were sacrificed and examined for postmortem changes and specimens for histopathological studies were collected.

3. Haematological examination

Differential leucocytic count

Blood film was done according the method described by Lucky (1977). The percentage and absolute value for each type of cells were calculated according to Schalm (1986).

Determination of phagocytic activity and phagocytic index

Phagocytic activity was determined according to Kawahara \textit{et al.} (1991).

\[
\text{Phagocytic index (PI)} = \frac{\text{No. of yeast cells phagocytized}}{\text{No. of phagocytic cells}}
\]

WBCs, RBCs counts and Packed cell volume (PCV %)

Were determined according to (Stoskopf, 1993).

Blood hemoglobin

Blood hemoglobin (Hb gm %) was assessed by cyanomtahemoglobin method (Drubkin, 1964).

Haematocrite value

According to (Blaxhall and Daisley, 1973).

Clinico-biochemical analysis

1) Determination of serum total protein

Serum total protein was determination according to Doumas \textit{et al.} (1981) using commercial kits produced by Pasteur Lab.

2) Determination of serum albumin

Serum albumin was determined according to Reinhold (1953) using commercially available kits of Chemroy.

3) Determination of serum globulin

Serum globulin was determined by subtract the total serum albumin from total serum protein according to (Coles, 1974).

Protein fractions determination

Blood serum was used for the determination of the relative concentrations (%) of major protein fractions of fish serum Bossuyt and Sporrow (1998) and Boyanton, and Blick, (2002).

B. Experiment II

Thirty \textit{O. niloticus} were equally divided into three groups.

Antibody titration against \textit{Pseudomonas flourecence} bacterin:

For determination of antibody titration the design of the experiment as in the following Table:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. Of fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected fish+ bacterin</td>
<td>10</td>
</tr>
<tr>
<td>Control +ve with bacterin</td>
<td>10</td>
</tr>
<tr>
<td>Control –ve</td>
<td>10</td>
</tr>
</tbody>
</table>
Evaluation of potency of prepared vaccine against Pseudomonas fluorescens

**Bacterin preparation**

*Pseudomonas fluorescens* isolate was used in the bacterin preparation according to the method described by Sakai et al. (1984) and Badran (1990). The preparation of bacterin for injection was carried out according to the method of Badran (1990). The formalin inactivated bacterin cells were mixed with an equal volume of 0.85% sterile saline. Bacterial number was adjusted to MacFerland's No. 2 (approximately 6 x 10^8 cells/ml).

**Antibody titration against Pseudomonas fluorescens bacterin**

Detection of immune response to *Pseudomonas fluorescens* was evaluated by microagglutination (MA) test according to the method described by Badran (1990).

**Challenge test**

\[
RLP = 1 - \frac{\% \text{ mortality of vaccinated fish}}{\% \text{ mortality of control}} \times 100
\]

According to Newman and Majnarich (1982).

**4) Statistical analysis**

The data of hematological and biochemical examinations of exposed fish were statistically analyzed using t-test, Duncan-test after ANOVA and simple correlation according to (SAS, 1987).

**RESULTS**

1. **Results of Clinical and postmortem lesions in experimentally infected fish**

The experimentally infected fish species (*O. niloticus*) with the strains of *Pseudomonas fluorescens* showed the following clinical signs: paleness coloration of the body, loss of eye, ascitis, scale loss and exophthalmia, fin and tail rot, darkness coloration of the body and tail rot.

2. **Post mortem findings for experimentally infected fish**

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 cavity contain a clear fluid but more often the fluid is bloody and cloudy. congestion of all internal organs especially gills, liver and kidneys with necrosis of liver and kidneys (Fig. 1, 2 and 3).

3. **Effect of Pseudomonas fluorescens infection on differential leucocytic counts, Phagocytic activity and Phagocytic index, T.WBCs and T.RBCs.**

Table (1) indicated the significant effect of *Pseudomonas fluorescens* infection (P < 0.01) on differential leucocytic counts in *O. niloticus* fish at different weeks of infection.

The lymphocyte showed a tendency to decreasing from the 1st week to the 4th of the experiment than that of the control group. While, the monocyte level decreased from the 1st week to the 3rd week of the experiment but at the 4th week of the experiment it increased and returned to its normal level.

**Fig. (1) : O. niloticus infected with P. Flourescence at 2nd week showing unilateral exophthalmia.**
The results in Table (1) indicated the significant effect of *Pseudomonas fluorescens* infection (P < 0.01) on Phagocytic activity and Phagocytic index in *O. niloticus* fish at different weeks of infection.

The Phagocytic activity increased progressively from the 1<sup>st</sup> week to the 4<sup>th</sup> week of the experiment in infected fish and the maximum level of Phagocytic activity observed at the 4<sup>th</sup> week of the experiment but in the infected group lower than that of the control group.

The Phagocytic index, also increased progressively from the 1<sup>st</sup> week to the 4<sup>th</sup> week of the experiment but in control group higher than that of infected group.

The results in Table (2) indicated the significant effect of *Pseudomonas fluorescens* infection (P < 0.01) on T.WBCs and T.RBCs in *O. niloticus* fish at different weeks of infection.

### Table (1) : Effect of *Pseudomonas* infection on differential leucocytic counts, Phagocytic activity, phagocytic index, T. WBCs and T. RBCs counts at different weeks of experiment.

<table>
<thead>
<tr>
<th>Week</th>
<th>Group</th>
<th>Lymph</th>
<th>Monocyte</th>
<th>PA</th>
<th>PI</th>
<th>T. WBCs</th>
<th>T. RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; week</td>
<td>Infected</td>
<td>46±3.40</td>
<td>12±2.20</td>
<td>16±3.55</td>
<td>1.4±0.04</td>
<td>25±5.22</td>
<td>1.3±0.55</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>44±4.40</td>
<td>10±1.20</td>
<td>19±4.55</td>
<td>1.6±0.05</td>
<td>27±3.77</td>
<td>1.4±0.33</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; week</td>
<td>Infected</td>
<td>45±4.40</td>
<td>10±1.20</td>
<td>18±3.77</td>
<td>1.5±0.06</td>
<td>26±5.44</td>
<td>1.5±0.33</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>47±4.30</td>
<td>15±3.50</td>
<td>19±4.31</td>
<td>1.7±0.07</td>
<td>28±5.22</td>
<td>1.4±0.44</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; week</td>
<td>Infected</td>
<td>45±4.50</td>
<td>10±1.3</td>
<td>19±3.56</td>
<td>1.6±0.06</td>
<td>26±6.22</td>
<td>1.6±0.55</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>48±6.60</td>
<td>13±1.20</td>
<td>21±4.53</td>
<td>1.7±0.07</td>
<td>26±5.33</td>
<td>1.7±0.52</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt; week</td>
<td>Infected</td>
<td>44±4.33</td>
<td>12±1.20</td>
<td>20±5.22</td>
<td>1.5±0.05</td>
<td>28±4.88</td>
<td>1.6±0.52</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>48±4.22</td>
<td>14±1.22</td>
<td>20±5.22</td>
<td>1.4±0.04</td>
<td>29±4.55</td>
<td>1.8±0.43</td>
</tr>
</tbody>
</table>

*Means within the same column of different letters are significantly different at (P < 0.01).*
The T.WBCs increased progressively from the 1st week to the 4th week of the experiment in infected fish and the maximum level of T.WBCs observed in the infected fish at 4th week of the experiment, but its level lower than that of the control groups.

While, the T.RBCs also increased progressively from the 1st week to the 4th week of the experiment, but its level lower than that of the control groups.

4. Total protein, albumin, globulin and albumin / globulin ratio level in different fish groups at different periods:

The results in Table (2) showed that, the total protein, albumin, globulin and albumin/globulin ratio differ significantly among infected and control group and among different weeks.

The total protein level increased progressively from the first week to the last week and the level of total protein in infected group lower than that of control group. While the albumin level showed increasing then decreasing level of albumin and in the 4th week it increased and the albumin level in infected groups lower than that of the control groups. While, the globulin level not show any significant difference among the control and infected groups but its level in the 1st weeks lower than that of the last weeks.

The albumin/globulin ratio showed that, its level decreased gradually from the first week to the last week but in the last week it return to its normal level. But, the albumin globulin ratio level in infected group lower than that of the control group all over the period of the experiment.

5. Effect of P. Fluorescence infection on serum T-globulin level (α-globulin, β-globulin and γ-globulin) at different weeks of experiment

Fig. (4, 5, 6 and 7) indicated the significant effect on total serum globulin fractions (α - globulin, β -globulin and γ-globulin) levels among different treatment groups at different weeks of the experiment.

| Table (2) : Total protein, Albumin, globulin and albumin/globulin ratio level in different fish groups at different periods. |
|---|---|---|---|---|---|---|
| Time | Group | Number | Total protein | Albumin | Globulin | Albumin/globulin ratio |
| | | | Mean | Mean | Mean | Mean |
| | | | Std. Error | Std. Error | Std. Error | Std. Error |
| 1st week | Infected | 3.00 | 4.30±1.30 | 2.40±0.45 | 1.90±0.74 | 1.27±0.20 |
| | Control | 3.00 | 4.60±1.60 | 2.70±0.70 | 1.90±0.74 | 1.42±0.40 |
| 2nd Week | Infected | 3.00 | 4.40±1.30 | 2.60±0.65 | 1.80±0.45 | 1.45±0.42 |
| | Control | 3.00 | 4.50±1.44 | 2.70±0.74 | 1.80±0.43 | 1.5±0.50 |
| 3rd Week | Infected | 3.00 | 4.70±1.45 | 2.40±0.74 | 2.30±0.44 | 1.04±0.30 |
| | Control | 3.00 | 4.90±1.49 | 2.60±0.61 | 2.30±0.43 | 1.14±0.25 |
| 4th Week | Infected | 3.00 | 4.60±1.47 | 2.50±0.51 | 2.10±0.31 | 1.20±0.22 |
| | Control | 3.00 | 5.10±1.52 | 2.90±0.71 | 2.20±0.20 | 1.31±0.23 |

Means within the same column of different litters are significantly different at (P < 0.05)
The serum globulin level increased in the 3rd and 4th of the experiment and the level of serum globulin increased in control group than infected one all over the period of the experiment.

The α-globulin level increased in infected group than that of the control group. While, the β-globulin level decreased in infected group than that of the control group. The level of γ-globulin level decreased in infected group than that of the control group. In general our results cleared that, infected groups of lower serum globulins level than control group.

6. Antibody titer (logarithmic value) of Pseudomonas fluorescens in different fish groups at different periods:

Table (3) showed that, the antibody titer level differ significantly (P < 0.01) among infected and control groups and among different weeks of the experiment.

The antibody titer level in control groups higher than the infected groups and its level in in the 1st and 4th week at the same level while at 2nd and 3rd weeks it of higher value.
Fig. (7) : Serum Protein fractionation of *P. Flourescense* infected fish at 4th week

**Table (3) : Antibody titer (logarithmic value) of Pseudomonas fluorescens in different groups at different weeks.**

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Number</th>
<th>Antibody titer (logarithmic value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean Std. Error</td>
</tr>
<tr>
<td>1st week</td>
<td>Infected</td>
<td>3.00</td>
<td>A 0.477±0.07</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>3.00</td>
<td>B 0.60±0.04</td>
</tr>
<tr>
<td>2nd Week</td>
<td>Infected</td>
<td>3.00</td>
<td>B 0.60±0.05</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>3.00</td>
<td>B 0.60±0.01</td>
</tr>
<tr>
<td>3rd Week</td>
<td>Infected</td>
<td>3.00</td>
<td>A 0.477±0.07</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>3.00</td>
<td>C 0.69±0.06</td>
</tr>
<tr>
<td>4th Week</td>
<td>Infected</td>
<td>3.00</td>
<td>A 0.477±0.07</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>3.00</td>
<td>B 0.60±0.03</td>
</tr>
</tbody>
</table>

**Means within the same column of different litters are significantly different at (P < 0.05)**
Table (4) : Mortality % and RLP in different fish groups at different periods.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mortality</th>
<th>RLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected + Vaccinated</td>
<td>6</td>
<td>1 – 6/8 = 2/8 = 25 % C</td>
</tr>
<tr>
<td>Control +ve</td>
<td>5</td>
<td>1 – 5/8 = 3/8 = 37.5 % B</td>
</tr>
<tr>
<td>Control –ve</td>
<td>8</td>
<td>0 A</td>
</tr>
</tbody>
</table>

*Means within the same column of different litters are significantly different at (P < 0.05)*

7. Relative level of protection against *Pseudomonas fluorescens* in different fish groups at different periods:

Table (4) showed that, the relative level of protection differed significantly (P < 0.01), the higher level of protection observed in infected + vaccinated group, followed by control +ve group and finally the control –ve group.

DISCUSSION

Fish diseases due to bacterial infections are the major problems in aquaculture as it found naturally in the fish environment and under certain stress condition causes severe economic losses to fish farms (Olsson et al., 1998).

In experimentally infection with *P. Flourescence* the clinical signs were manifested as, loss of scales from some areas of the body with excessive mucus all over the body surface and petechial haemorrhages over the body.

The clinical signs & P.M. lesions mainly due to septicemia effect of *P. Flourescence* infection and its endotoxin which affecting body of fish, these observation were partially similar to those reported by (Hicks, 2008).

El-saka (2006) reported that there was a high mortality percentage among the experimentally infected fish under different stress conditions. These conditions were overcrowdingness, external parasitism and transportation with mortality rates of 83%, 70% and 60% in *O. niloticus*, respectively, and all of these stress conditions facilitated the infection with *P. Flourescence*.

The present results cleared that the Effect of *Pseudomonas fluorescens* infection on differential leucocytic counts:-

The lymphocyte showed a tendency to decreasing from the 1st week to the 4th week of the experiment than that of the control group. While, the monocyte level decreased from the 1st week to the 3rd week of the experiment but at the 4th week of the experiment it increased and returned to its normal level.

Dovale et al. (2002) reported that neutrophils and macrophages are important phagocytic cells depending on the opportunity to encounter the invading agent, macrophages from peritoneal exudates showed a greater capacity for engulfing bacteria Esteban et al. (1977).

In the present work injection of *P. Flourescence* increased the neutrophils count at the first week and significant decrease in its count in the second week.

Also, the current investigation agreed with the result of Siwicki and Dunier (1993). In the present study there was increase in both lymphocytes and monocytes especially with increase of total leucocytic count at the first weeks of infection and this mainly due to the direct effect of stress on the immune response and activation of first line of defense to resist the infection through cellular immune response.
Lymphocyte may perform T-cell equivalent or as natural killer cells (Ellis, 1981)

The T.WBCs increased progressively from the 1st week to the 4th week of the experiment in infected fish and the maximum level of T. WBCs observed in the infected fish at 4th week of the experiment, but its level lower than that of the control groups.

These results nearly agreed with those of (Fumihiko et al., 2008) where they reported that fish fed bacteria of causative agent of disease show neutrophilia and in general there was a leucocytopenia.

Also these results agreed with those of (Fernandez et al., 2003) indicated that the Pseudomonas produces products which causes lysis and destruction of RBCs and reduces its number and its Hb content.

The Phagocytic activity and index increased progressively from the 1st week to the 4th week of the experiment in infected fish and the maximum level of phagocytic activity observed at the 4th week of the experiment but in the infected group lower than that of the control group.

The significant (P< 0.01) decrease of phagocytic activity and phagocytic index of Pseudomonas fluorescence infected fish than the control fish may be attributed to the destructive action of this pathogen on liver, kidney, spleen and other haemopiotic organs (Thampuran et al., 2008), so it causes leucocytopenia and decrease the phagocytic activity and phagocytic index.

This suppression may be mediated directly via the corticosteroid receptors on macrophages or indirectly through the enhanced production of certain factors by the macrophages themselves, which suppress the secretion of other macrophage products (e.g. α-2 macroglobulin) (Pickering et al., 1981 and Thampuran et al., 2008).

The total serum proteins were useful in diagnosis of fish diseases (Mulcahy, 1967). In the present work, significant decrease in albumin, globulin, total protein and albumin/globulin (A/G) ratio were recorded.

The results also showed that, the total protein, albumin, globulin and albumin/globulin ratio differ significantly among infected and control group and among different weeks.

Infection which causes liver damage that causes decreases of serum protein concentration Lee and Marks (2009).

(Dennis et al., 2008) stated that chronic liver disorder is usually accompanied by hypoalbuminaemia. Both hypoglobulinaemia and hypoalbuminaemia confirmed the recorded hypoproteininaemia, which was associated with liver damage.

Naglaa (2004) mentioned that, the mechanisms causing reduction of total serum protein due to bacterial infection was not clear but may be due to the following processes:

- Loss of protein through vascular leaking caused by increased permeability due to histamine release Ellis (1981).
- Impaired synthesis of serum proteins due to liver damage and anorexia in diseased fish. Evenberg et al. (1986).
- The antibody titer and RLP the antibody titer in the groups treated with P. Flourescence only of lower RLP than that of the control groups.

This results attributed to the action of bacterial toxin or whole bacteria and extracellular products on liver, kidney, spleen and other haemopiotic organs as considered as a stress factor on this organs (Fucks et al., 1986 and Gekle et al., 1998), which causes decreases of the relative level of protection of the fish against any stress conditions as well as fish infected with P. Flourescence (El-Gamel, 2005).
REFERENCES


PSEUDOMONAS INFECTION IN EXPERIMENTALLY INFECTED ORECHROMIS NILOTICUS


بعض الدراسات على عدوى السودوموناس في أسماك البلطي النيلي المستزرعة

رياض حسن خليل طلعت سهيل لميس أحمد منتصر

أمراض الأسماك البكتيرية تعتبر من أهم أنواع الأمراض المنتشرة في البيئة المائية التي تصيب الأسماك وتؤثر على الناحية الاقتصادية فتم إجراء هذه الدراسة لتقييم عدوى السودوموناس فلوريسنس ودراسة تأثيرها على مختلف مقاييس الدم والأنزيمات.

تم فحص وتصنيف وعزل بكتيريا السودوموناس فلوريسنس وتسجيل الأعراض المصاحبة لها خارجياً وداخلياً والتي تتلخص في فقدان القشر واحتكانات داخلية وخارجية وتآكل الزعانف واحمرار في مختلف أجزاء الجسم.

أما بالنسبة لقياسات الدم فوجد بها نقصان على طول التجربة المزمنة من الأسبوع الأول إلى الأسبوع الرابع. أما بالنسبة لأنزيمات الدم فوجد بها تغيرات تدريجية سواء بالنقصان أو الزيادة على طول التجربة أما بالنسبة لاختبار الأجسام المضادة واختبار التحدي فكان أعلى في المجموعة الضابطة عنها في المجموعة المصابة ببكتيريا السودوموناس فلوريسنس.