한국어병학회지 제30권 제1호 (2017)
J. Fish Pathol., 30(1) : 11~24

www.ksfp.org
pISSN 1226-0819, eISSN 2233-5412
http://dx.doi.org/10.7847/jfp.2017.30.1.011

# An outbreak of Motile *Aeromonas* Septicemia in cultured Nile tilapia, *Oreochromis niloticus* with reference to hematological, biochemical and histopathological alterations

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The current investigation dealing with the causative agent of mass mortalities in cultured *Oreochromis niloticus*. The diseased fish showed external hemorrhage, unilateral and bilateral eye opacity, ended by blindness and fish death. The postmortem lesions revealed congested friable kidney and spleen, and liver has yellow nodules. Obtained isolates were identified as *Aeromonas hydrophila* (the causative agent of Motile *Aeromonas* Septicemia) and found to be highly pathogenic as they contained hemolysin virulence gene causing mortality reached to 100 and 70% in intraperitoneal and intramuscular infection. The prevalence of MAS was 80% among the surveyed *O. niloticus*. Blood and serum were collected from naturally diseased, intraperitoneal and intramuscular injected *O. niloticus* for hematological and biochemical examination. Similarly, gills, musculature, kidney, liver and spleen were collected for histopathological evaluation, and micropathomorphological analysis of spleen was done. Macrocytic hypochromic anemia was recorded in the intraperitoneal infection. Serum protein, albumin and globulin were decrease only in naturally diseased fish. Leucocytosis with heterophilia and lymphocytosis were observed in naturally diseased and intraperitoneal infected fish. There were severe degenerative changes and hemorrhagic necrosis in the examined tissues which were more obvious in intraperitoneal than intramuscular infection. Activation and proliferation of melanocytes macrophages centers with severe hemosiderosis were recorded in spleen of naturally diseased and experimentally infected fish.

**Key words:** *Oreochromis niloticus; Aeromonas hydrophila,* Motile *Aeromonas* Septicemia; Leucocytosis; Melanocytes macrophages centers.

Bacterial pathogens are the most serious disease problem in tilapia production causing 80% of fish mortalities (Clark et al., 2000; Shoemaker et al., 2000). Motile *Aeromonas* Septicemia (MAS) is a serious problem for fish farming industry in Egypt as well as in other countries and affects a great variety of freshwater fish (Woo and Bruno, 2003). The causative agent of MAS, *Aeromonas hydrophila* (*A. hydrophila*) is an opportunistic pathogen of humans causing diarrhea, septicemia and meningitis (Inglis et al., 1993). Moreover, external clinical signs of the affected fish make them unmarketable. The clinical picture of MAS disease includes acute, chronic, and latent form. The acute form is characterized by a rapidly fatal septicemia with little clinical signs of the disease with unilateral/bilateral opaqueness accompanied by exophthalmia and bursting of the orbit (Yambot and Inglis, 1994). Additionally, Liver and

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kidneys are the most predominant affected organs during acute septicaemia (Laith and Najiah, 2013). Chronic form is often ulcerous and characterized by ulceration, inflammation, and skin lesions associated with focal hemorrhages (Cipriano et al., 1984).

Moreover, studying hematological variables may allow the magnitude of the pathogen's influence on the host health (Figueredo et al., 2014) to be estimated. In this concern, it can be stated that bacterial diseases cause significant haematological alterations in fish leading to immunological variables (Clauss and Arnold, 2008). Therefore, the gap between behavioral alterations and hematological, biochemical changes, as well as histopathological changes induced by bacterial diseases will be targeted in to predict economic impacts of them on the aquaculture.

The pathological picture of the disease is characterized by diffuse degenerative changes and necrosis along the entire internal organs associated with the presence of melanin-containing macrophages in the blood (Ventura and Grizzle, 1988). Melanomacrophage centers (MMCs) are defined as clusters of cells containing pigments, which contain variable quantities of macrophage aggregates, in both spleen and kidneys (Manrique et al., 2014). After phagocytic activity, macrophages are packed into activated large aggregates on heterogenous material including cell particles, melanin pigments and hemosiderin granules (Agius and Roberts, 2003). Accordingly, MMCs play an important role in fish response against different pathogens (Roberts, 2001).

Thus, the aim of this study was to identify the disease responsible for the mortality of cultured *Oreochromis niloticus* (*O. niloticus*) in Abo-Saleh fish hatchery, Beni-Suef, Egypt. In addition, hematological, immunological and histopathological changes, as well as micropathomorphological changes of splenic MMCs induced by this disease in O. *niloticus* were performed.

## Material and methods

### Fish

**Surveyed fish for identifying the disease and for hematological, biochemical and histopathological studies**

A total of 400 *O. niloticus* with average body weight (75± 5 g) were randomly collected alive from Abo-Saleh fish hatchery, Beni-Suef, Egypt, during summer season at water temperature 26 ± 2ºC. They were brought back to wet laboratory of Fish Department, Faculty of Veterinary Medicine, Beni-Suef University, Egypt. They were kept in four fiberglass tanks each of 400 L capacity supplied with chlorine free tap water and continuous aeration. They were subjected to clinical and postmortem examinations as described by Lucky, (1977).

**Experimental injection fish**

**Fish for pathogencity**

A total of 80 apparently healthy *O. niloticus* with average body weight 80 ± 10g were collected from Abo-Saleh fish hatchery, Beni-Suef, Egypt, during summer for monitoring the pathogenicity of the disease causative agent. The identified isolate was inoculated onto tryptone soya agar at 30 °C for 18 hours. The pure culture was suspended into a sterile saline and adjusted to 1.5x $10^8$CFU/ml (McFarland 1). Fifteen days after acclimation, the fish were randomly divided into four equal groups (20 fish/ group). Fish in first and second groups were injected intraperitoneal (IP) and intramuscular (IM), respectively with 0.3 ml of $1.5\times10^8$ CFU/ml. Fish in the third and fourth groups was kept as control and injected IP and IM respectively with 0.3 ml of sterile physiological saline. All fish groups were kept under daily close observation for two weeks and mortalities were recorded. All freshly dead fish were submitted to bacteriological isolation and identification of the disease causative agent to verify the specificity of mortality.

**Fish for hematological; biochemical and histopatholoical studies**

A total of 120 apparently healthy *O. niloticus* (mean weight 60 ± 10g) were randomly divided into four equal groups. Fish in the first group were injected IP with sterile saline, while that in the second group were injected IP with 300 µl of the identified causative agent of the disease ($1.5\times10^8$ CFU/ml). The third and fourth groups were injected IM with sterile saline and identified isolate, respectively. Fish were anesthetized by MS 222, blood and serum samples were collected after $7^{th}$ and $14^{th}$ days post-inoculation. Tissue samples from gills, musculature, kidney, liver, and spleen were taken for histopathological examination.

### Hematological analysis

Blood samples were collected from the caudal vein, into a tube with a syringe moistened with heparin as an anticoagulant. The erythrocyte count (RBC) and total leukocyte count (WBC) were determined using a Neubauer counting chamber. The packed cell volume (PCV) value was determined by the microhaematocrit method. Haemoglobin concentration (Hb) was estimated by the cyanomethaemoglobin method. The haematological indices, mean corpuscular volume (MCV), and mean corpuscular haemoglobin concentration (MCHC) were calculated according to (Dacie and Lewis, 1966). Differential leukocyte counts were performed on routinely prepared Geimsa-stained blood films using the cross-sectional technique (Jain 1993).

### Biochemical analysis

Blood samples were collected from the caudal vein by using a syringe without anticoagulant for serum separation. The collected serum were stored at -20 °C for estimation of total protein using the method of Bradford (1976); albumin concentration (Doumas et al. 1971). Globulin was calculated by subtracting albumin from total protein values. Albumin-globulin ratio was calculated by dividing albumin by globulin values. Lysozyme activity was measured by agarose gel cell lysis assay according to Schltz (1987).

### Histopathology

The collected samples were fixed in 10% buffered formalin at room temperature. Samples were trimmed for a size of one cubic centimeter. Sections underwent routine histological procedures of dehydration, clearing and paraffin embedding. Sections of 4-6 µm thickness were stained with hematoxylin and eosin (Howard et al. 2004) and Prussian blue according to Perl's method (Howard et al. 2004) to identify the biological iron.

### Micropathomorphological analysis

Based on the quantification of melanomacrophage centers (numbers and areas), and hemosiderosis (area percentages and intensities), morphometric analysis of spleen, was performed with an optical microscope, and images were captured using a digital camera (Leica, DM2500 M), with a freeware version of Image-J v1.45s downloaded from the NIH website (http://rsb.info.nih.gov/ij) (Manrique et al. 2014) as following:

a- Calculation of melanomacrophage centers (MMC) numbers; ten 100 x magnification fields were randomly selected on each slide. The number of MMCs/field was counted.

b- Calculation of MMC center areas; 25-30 at 400x magnification fields were randomly selected on each slide. The areas were measured in $\mu m^2$.

c- Area percentages and intensities of hemosiderosis; 25-30 at 400 x magnification fields were randomly selected on each slide for image analysis, in addition to a freeware version of Image-J v1.45s, the threshold Color plug in was downloaded from the NIH website (http://rsb.info.nih.gov/ij) to measure the area percentages and intensities of hemosiderosis by examining the slides stained with Prussian blue according to Perl's method (Howard et al. 2004).

### Bacteriological isolation

Samples were taken under complete aseptic condition from eyes, ulcerative skin areas, kidney, liver, and spleen and inoculated onto *Aeromonas* agar media (LAB) and incubated at 25°C for 24 hours.

### Bacteriological identification

**Morphological examination**

Smears from the suspected colonies were prepared, stained with Gram's stain and microscopically examined (Kreig and Holt, 1984).

**Biochemical identification**

The separate colonies were subjected to biochemical identification according to Collee et al., (1996) and Austin and Austin, (1999) by using cytochrome oxidase test "Biomerieux, France", detection of motility, $H_2S$ production and indole tests by using SIM medium (Oxide), urease test, citrate utilization test, catalase test, methyl red, voges proskauer and fermentation of esculin, maltose, fructose, xylose, lactose, sucrose and mannitol.

**API 20 E strips**

Biochemical characteristic of the eleven pure isolates were determined using the API 20E strips system (BioMérieux, France) according to the manufacturer's instructions. Single well-isolated colony was inoculated onto trypticase soya agar at 25ºC for 18-24 hours (young culture) to make bacterial suspension (Quinn et al., 2002).

### Detection of hemolysin gene

The hemolysin gene forward primer was 5′-GCCG AGCGCCCAGAAGGTGAGTT-3′ and the reverse primer was 5′-GAGCGGCTGGATGCGGTTGT-3′. The PCR mixture consisted of 20 μL reaction. The PCR amplification included an initial denaturation of at 95ºC for 5 min followed by 50 cycles of denaturation at 95ºC for 0.5 min, annealing of the primers at 59ºC for 0.5 min and extension of at 72ºC for 0.5 min. A final extension at 72ºC for 7 min was used in the thermocycler. Volumes of each (10 μL) PCR product were subjected to electrophoresis in a 1.5% (w/v) agarose gel (Casiano et al., 2010).

### Statistical analysis

Statistical analyses were done using Advanced Models 16.0 software (SPSS, Tokyo, Japan). Hematological and biochemical data was analyzed using independent t-test. Morphometric analysis of the splenic MMCs and hemosiderosis was done by using one way ANOVA followed by post hoc test (Tukey test) and t-test. $P<0.05$ was considered as statistically significant.

## Results

### Clinical signs and postmortem lesions

**Surveyed fish**

In the acute and sub-acute form of MAS disease fish showed gross signs of the disease as skin redness; dark coloration; loss of scales at the dorsal musculature and ventral aspects; enlarged inflamed vent; congested gills (Fig. 1.a); and unilateral eye opacity (Fig. 1.b). In the chronic form, fish manifested a severe loss of scales and external hemorrhage (Fig. 1.c and1.d). In addition, unilateral eye opacity was observed that progressed into bilateral one ended by blindness and death.

The postmortem lesions revealed dark abdominal exudates with offensive odor, congested friable kidney and spleen, the liver become pale with characteristic yellow nodules (Fig. 1.e). The intestine devoid from food and contains a yellowish-mucoid fluid.

The prevalence of MAS was 80% among the surveyed *O. niloticus* where 320 *O. niloticus* out of 400 fish were naturally infected with MAS.

**Pathogenicity test**

Experimentally infected *O. niloticus* showed clinical signs and postmortem lesions similar to those observed in naturally infected fish. The IP inoculated

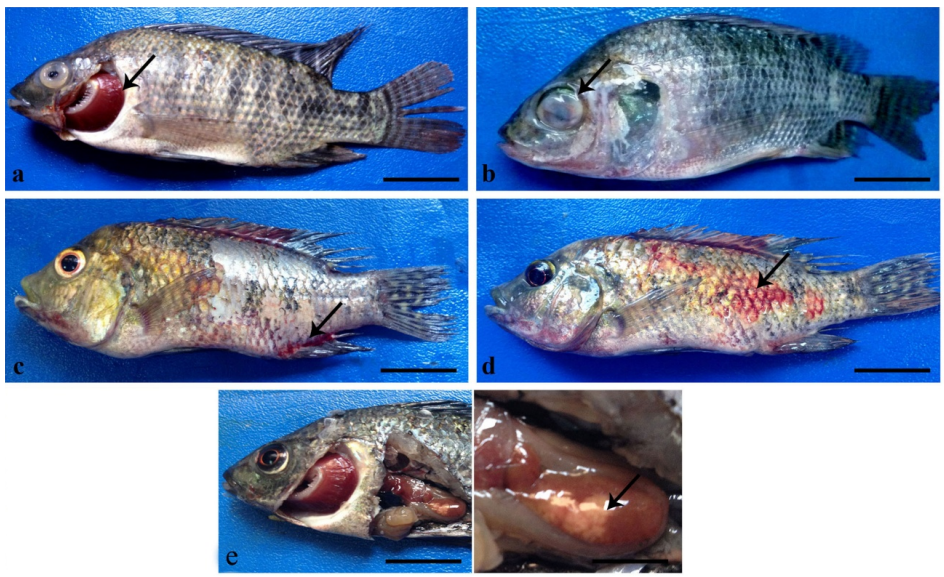


Fig. 1. Clinical signs and postmortem lesions of motile *Aeromonas* septicemia in naturally infected *O. niloticus*. a. congestion of gills (Scale bar 1 cm). b. unilateral eye obacity (Scale bar 1 cm). c. severe loss of scales and hemorrhage at anal fin (Scale bar 1 cm). d. severe hemorrhage at skin and dorsal fin (Scale bar 1 cm). e. yellow nodules in liver (Scale bar 1 cm).

fish exhibited more severe clinical picture and higher mortality rate (100%) than intramuscular injected one (70%). In the control group, fish showed neither clinical signs nor mortalities.

## Identification

### Biochemical identification

Eleven strains of *A. hydrophila* were identified. They appeared Gram negative, short motile rods, cytochrome oxidase positive, positive to $H_2S$, indole, and citrate utilization. Moreover, they fermented maltose, xylose, lactose, sucrose, fructose, mannitol and esculin.

### API 20 E.

The identification of MAS causative agent *(A. hydrophila)* was confirmed by the API 20E biochemical system. All isolates were positive for cytochrome oxidase, indole and $H_2S$, failed in urease and tryptophane deaminase but positive in arginine dihydrolase, ornithine decarboxylase, ortho-nitrophenyl-b-D-galactopyranoside, lysine decarboxylase, voges-proskauer and gelatin hydrolysis test. All isolates were able to ferment glucose, mannitol, Sucrose, Arabinose and Amygdaline, but they were non-fermentable to Inositol, sorbitol, Rhamnose and Melibiose.

## PCR findings

Two strains were positive for hemolysin gene. PCR amplification revealed the presence of *A. hydrophila* extracellular hemolysin gene (130 bp) (Fig. 2).

## Hematological and biochemical analysis

There was no significant change in RBCs count, PCV %, Hb concentration, MCV, and MCHC values between the control and natural infected group. Total leucocytic, heterophil and lymphocyte count were significantly higher in natural infected group than the control -. There was a significant decrease in total protein; albumin and globulin values in the natural infected group compared with the control group. The albumin/globulin ratio showed significant decrease

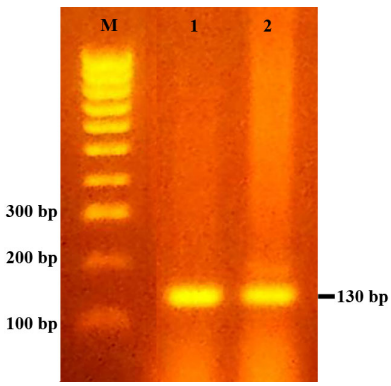


Fig. 2. PCR amplification products profile of *A. hydrophila* extracellular hemolysin gene (130 bp). M: molecular weight marker using 100 bp ladder, Lane1 and 2: positive.

(Table 1).

On day 7 post-challenge, the IP injected fish showed a significant decrease in the mean values of RBCs, PCV, Hb and MCHC compared to the control group. The MCV value, total leucocytes, heterophils and lymphocytes counts were significantly elevated in the IP group as compared to the control. Whereas, in the IM infected group, there was no significant changes in the measured hematological parameters except the total leucocytic and lymphocytic counts as they showed significant increase compared to control group. There was no significant impact of *A. hydrophila* injection on total protein, albumin, and globulin

Table 1. Hematological and biochemical parameters of naturally and experimentally infected *O. niloticus* by Motile *Aeromonas* Septicemia causative agent (*A. hydrophila*), (mean ± SE)

| Parameter | Survey | | Experimental study | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 7th day | | | | 14th day | |
| | Control | Infected | IP | | IM | | IM | |
| | | | Control | Infected | Control | Infected | Control | Infected |
| RBCs ($10^6$) | 3.70 ±0.16 | 3.1 ±0.08 | 4.47 ±0.08 | 3.06 ±0.03* | 4.44 ±0.48 | 4.6 ±0.3 | 3.7 ±0.48 | 3.6 ±0.63 |
| PCV% | 25.67 ±1.20 | 22.60 ±0.33 | 27.00 ±1.0 | 22.00 ±0.58* | 26.7 ±0.72 | 29.66 ±1.5 | 23.75 ±1.5 | 23.25 ±2.7 |
| Hb (g/dl) | 5.94 ±0.73 | 4.98 ±0.50 | 6.77 ±0.45 | 4.77 ±0.16* | 6.82 ±0.41 | 6.66 ±0.55 | 5.37 ±0.49 | 5.06 ±0.91 |
| MCV (fL) | 69.90 ±0.96 | 72.18 ±0.59 | 60.50 ±0.1 | 72.00 ±0.52* | 60.40 ±5.12 | 64.50 ±1.09 | 64.73 ±5.9 | 64.69 ±4.6 |
| MCHC (%) | 23.00 ±1.8 | 22.07 ±2.5 | 25.03 ±1.8 | 21.68 ±0.71* | 25.55 ±1.17 | 22.44 ±0.94 | 22.58 ±0.8 | 21.67 ±1.7 |
| WBCS ($10^3$) | 27.32 ±0.45 | 35.33 ±1.00* | 25.33 ±0.5 | 30.00 ±0.91* | 24.75 ±0.59 | 29.53 ±0.35* | 25.00 ±4.8 | 27.00 ±4.2 |
| Hetero ($10^3$) | 6.19 ±1.50 | 7.41 ±0.88* | 5.32 ±1.7 | 7.12 ±0.80* | 5.21 ±0.55 | 5.74 ±0.45 | 5.19 ±3.8 | 5.06 ±2.0 |
| Lympho ($10^3$) | 20.49 ±1.15 | 27.58 ±0.88* | 19.24 ±0.65 | 22.20 ±0.23* | 18.76 ±0.33 | 22.82 ±0.28* | 19.25 ±2.9 | 21.33 ±1.8 |
| Mono ($10^3$) | 0.64 ±0.33 | 0.34 ±0.57 | 0.60 ±0.57 | 0.64 ±0.50 | 0.78 ±0.04 | 1.02 ±0.20 | 0.56 ±1.5 | 0.62 ±0.5 |
| T.p (g/dl) | 3.25 ±0.08 | 2.46 ±0.06* | 3.28 ±0.09 | 4.37 ±0.56 | 3.28 ±0.26 | 3.30 ±0.16 | 3.77 ±0.94 | 3.66 ±0.80 |
| Albumin | 1.25 ±0.05 | 0.68 ±0.05* | 1.28 ±0.04 | 1.23 ±0.11 | 1.21 ±0.02 | 0.98 ±0.5 | 1.24 ±0.11 | 1.01 ±0.10 |
| Globulin | 2.05 ±0.01 | 1.78 ±0.01* | 2.09 ±0.06 | 3.14 ±0.45 | 2.08 ±0.05 | 2.33 ±0.10 | 2.52 ±0.74 | 2.62 ±0.73 |
| A/G ratio | 0.61 ±0.01 | 0.38 ±0.02* | 0.61 ±0.02 | 0.39 ±0.21 | 0.58 ±0.01 | 0.42 ±0.11 | 0.49 0.20± | 0.39 ±0.22 |
| Lysozyme (ug/ml) | 236.7 ± 23.6 | 233.02 ±28.2 | 259.1 ±21.2 | 268.06 ±19.0 | 231.4 ±19.5 | 207.3 ± 25.4 | 229.6 11.5± | 200.2 ±13.4 |

Red blood cells, (RBCs); -Packed cell volume, (PCV); Hemoglobin, (Hb); mean corpuscular volume, (MCV); mean corpuscular hemoglobin concentration (MCHC); White blood cells, (WBCs).
*Significantly different in comparison with control group. ($P< 0.05$)

values as compared to healthy fish.

On day 14 post-challenge, no significant differences in the hematological parameters, total protein, albumin and globulin values were observed between the healthy and IM infected fish (Table 1). There was no significant change in lysozyme activity between all groups.

### Histopathology

Both naturally and experimentally infected groups with *A. hydrophila* revealed similar microscopic picture. However, fish inoculated intramuscularly showed relatively milder lesions than those inoculated intraperitoneally.

**Gills** showed hyperplasia and fusion of gill lamellae, dilatation with congestion of central venous sinus and branchitis which characterized by congestion of the branchial blood vessels associated with intense leukocytic infiltration (Fig. 3a).

**Liver** showed passive hyperemia of central veins hepatoportal, blood vessels and sinusoids with severe diffuse vacuolar degeneration of hepatocytes especially in naturally infected group. Liver hemorrhage and aggregations of inflammatory cells were in all groups with severe degree in intraperitoneally infected one. Hepatocytes necrosis with pyknotic nuclei associated with loss of structural integrity and pancreatic cells degeneration and necrosis were predominant. In some cases, infiltration of pancreatic acini by melanomacrophages could be detected (Fig. 3b).

**Spleen** showed subcapsular necrosis (Fig. 3c) and splenic hemorrhage with activation of melanocytes macrophages centers (MMCs), Deposition of haemosiderin pigments in the melano-macrophage centres of infected fish could be easily detected by Perl's Prussia stain either in naturally infected or experimentally infected groups (Fig. 3d).

**Kidney:** Renal lesions could be detected in mild to moderate forms in all groups. Kidney showed congestion of interstitial blood vessels and glomeruli, focal interstitial hemorrhage and interstitial infiltration of mononuclear cells with vacuolar and hyaline droplets degeneration of renal tubular epithelium (Fig. 3e).

**Musculature** exhibited mild edema and severe focal hyaline degeneration with focal mononuclear cells infiltration (Fig. 3f).

### Micropathomorphological examination

MMCs number, MMCs areas, hemosideriosis areas percentage and hemosiderosis intensities in the spleen of both naturally and experimentally infected groups were illustrated in (Table 2). There was a variation in their shape; the largest of them were sometimes spherical. There was no specific pattern in the distribution of MMCs over the organ, but some of them adjacent to the wall of blood vessels. Staining according to Pearls revealed the presence of hemosiderosis in all groups, but the severe form was found in infected groups with *A. hydrophila* either in the natural or experimental infection.

Comparison of MMCs numbers (Fig. 4a), MMCs areas (Fig. 4b), hemosiderosis area percentages (Fig. 4c) and hemosiderosis intensities (Fig. 5d) between naturally infected and non-infected fish were carried out using t-test, and the results revealed high statistical differences between them ($P<0.001$).

Statistical analysis of possible pairwise comparisons of values for MMCs number, MMCs areas hemosiderosis area percentages and hemosiderosis intensities in experimentally infected fish (IP and IM) and control non-infected groups using ANOVA and post hoc test showed:

A- Significant differences in all of them between IP infected group and control non-infected one ($P<0.001$).

B- Significant differences in MMCs areas (Fig. 4f), hemosiderosis area percentages (Fig. 4g) and hemosiderosis intensities (Fig. 4h) between IM infected group and control non-infected one ($P<0.001$). While the MMCs number (Fig. 4e) showed lower significant level between the same groups ($P<0.01$).

C- High significant differences in MMCs numbers

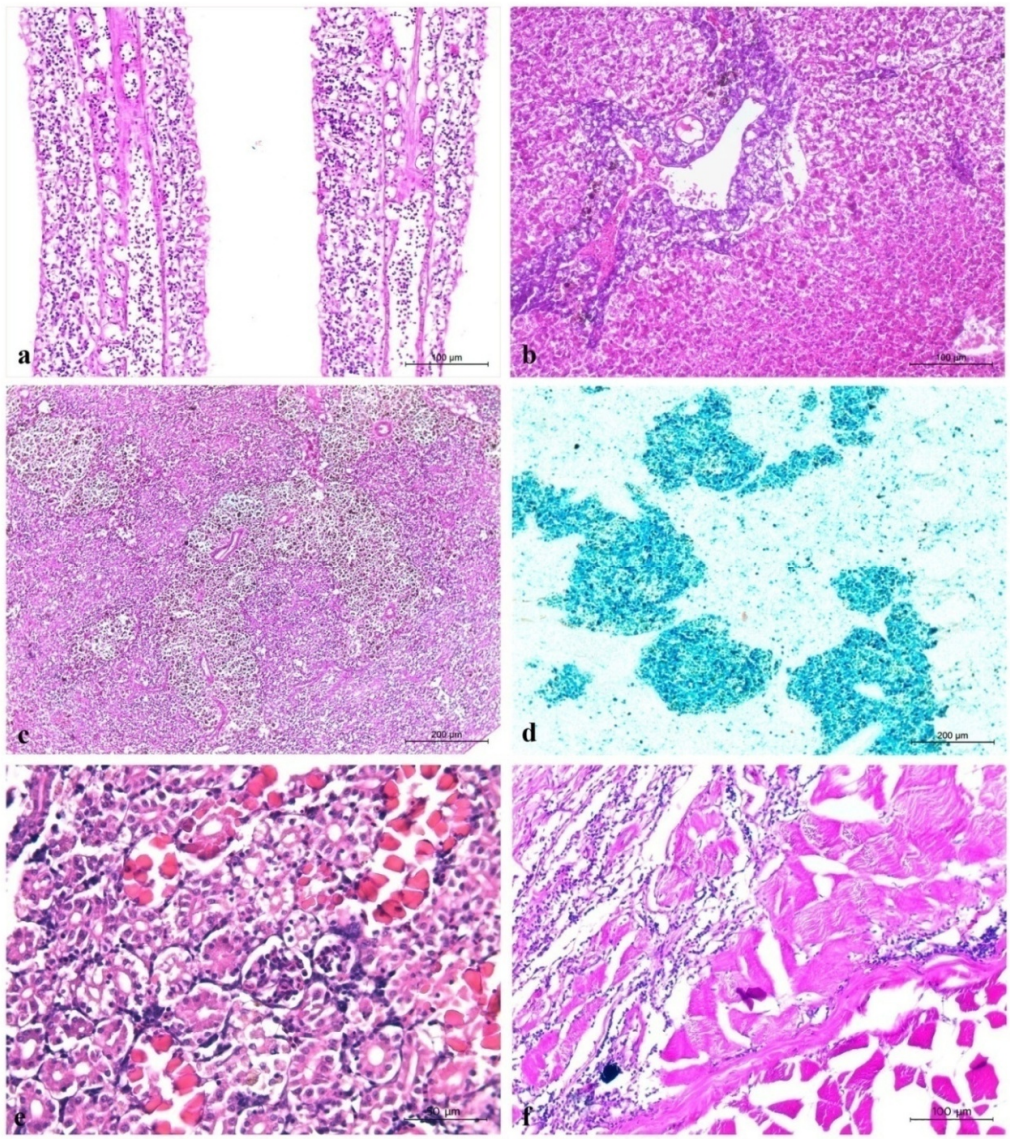


Fig. 3. (a) Light micrograph of *O. niloticus* gills, naturally infected with *A. hydrophila* showing; fusion of secondary gill lamellae, dilatation with congestion of the central venous sinus and branchitis. H and E stain. (b): Light micrograph of *O. niloticus* liver, naturally infected with *A. hydrophila*, showing, severe degenerative changes and necrosis of hepatocytes acinar cells of pancreas and loss of zymogen granules in addition to presence of melanomacrophages cells. H and E stain. (c): Light micrograph of *O. niloticus* spleen naturally infected with *A. hydrophila* of *O. niloticus* showing activation of melanomacrophage. H&E, Prussian blue stain. (d) special stain with Perl's Prussian blue. (e): Light micrograph of *O. niloticus* kidney of intra muscular infected group with *A. hydrophila*, showing, severe hyaline e droplets degeneration, severe degenerative changes and coagulative necrosis of renal tubules with pyknotic neuclei. H&E stain. (f): Light micrograph of musculature of *O. niloticus* naturally infected with *A. hydrophila* showing severe necrosis with haylanized appearance and leuckocytic infiltration in the interstitial tissue. H&E stain.

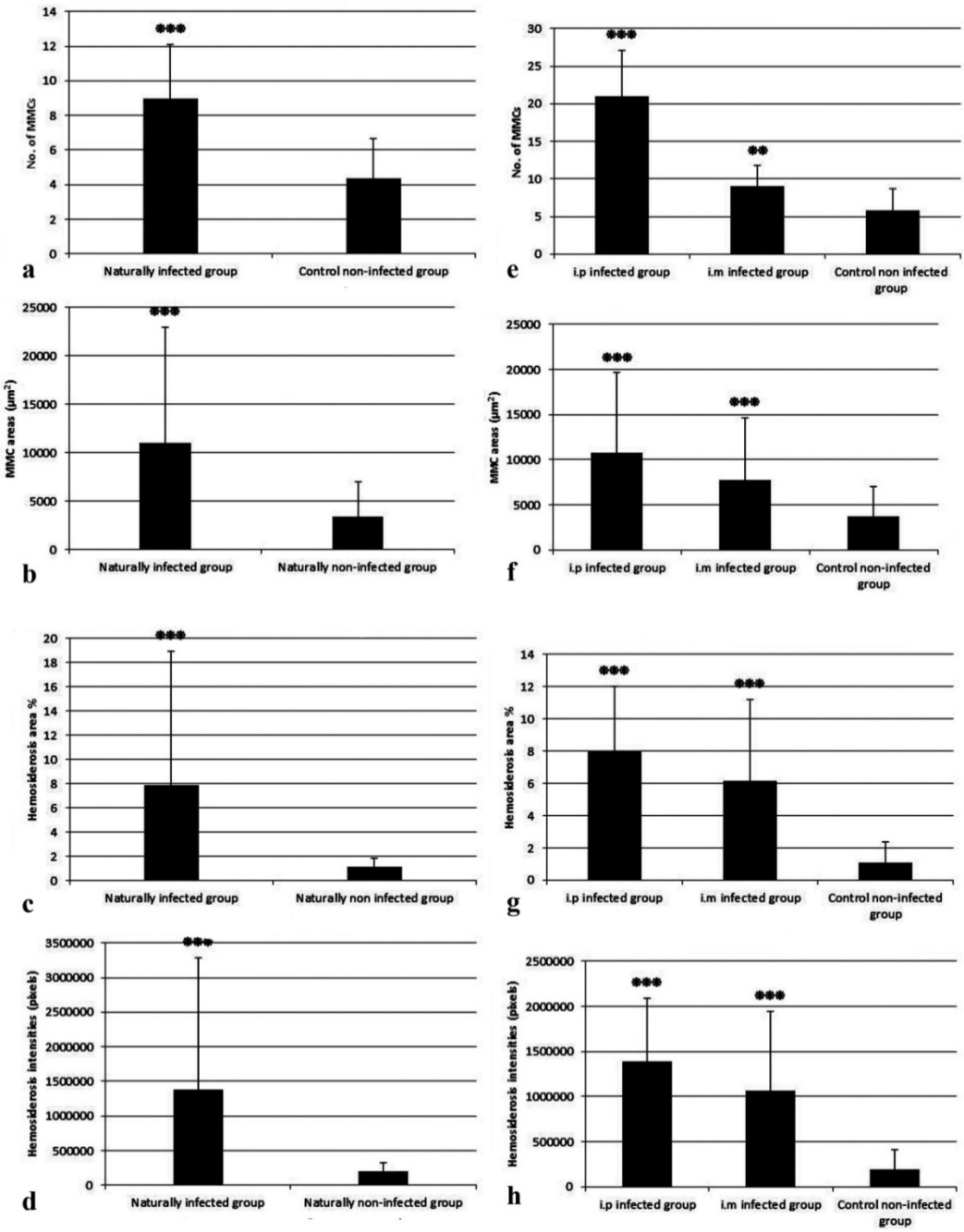


Fig. 4. (a,b,c,d): Morphometric analysis of the spleen in naturally infected and non-infected groups showed statistical significant difference between two groups in: (a) MMCs numbers. (b) MMCs areas. (c) Hemosiderosis area percentages and (d) Hemosiderosis intensities. (t test, $P<0.001$).(e,f,g.h): Morphometric analysis of the spleen in experimentally infected and control non-infected groups showed statistical significant difference among groups in: (e) MMCs numbers. (f) MMCs areas. (g) Hemosiderosis area percentages and (h) Hemosiderosis intensities. (One way ANOVA, $P<0.001$).

Table 2. Biometric analysis of melanomacrophage centers (MMCs) and hemosiderosis of spleen (± standard error) of *O. niloticus* infected Motile *Aeromonas* Septicemia causative agent (*A. hydrophila*) in naturally and experimentally infected groups

| Parameter | Natural group | | Experimental group | | |
|---|---|---|---|---|---|
| | Infected | Control Non-infected | IP | IM | Control non-infected |
| MMCs | | | | | |
| Number | 8.95±3.16*** | 4.37±2.29 | 20.98± 6.10***,■ | 9±2.75** | 5.85±2.84 |
| Area ($\mu m^2$) | 10967.95± 1957.2*** | 3438.7±3558.8 | 10785.7±8896.9***,▼ | 7790.6±6802.4*** | 3775.6±3210.0 |
| Hemosiderosis | | | | | |
| Area % | 7.92±11.00*** | 1.11±0.74 | 8.03±3.98***,▼ | 6.19±5.03*** | 1.11±1.27 |
| Intensity (pixels) | 1373895±1908504*** | 191757±128553 | 1393597±69073***,▼ | 1068834±874769*** | 191921±220772 |

***$P$<0.001(in comparison with corresponding control non-infected group).**$P$<0.01 (in comparison with corresponding control non-infected group).■ $P$<0.001 (in comparison with IM infected group).▼$P$<0.01 (in comparison with IM infected group)

between intraperitoneal and intramuscular infected groups ($P$<0.001), and a significant difference at level of ($P$<0.01) between them in MMCs area, hemosiderosis area percentages and hemosiderosis intensities.

## Discussion

Motile *Aeromonas* Septicemis is a serious disease for aquaculture industry in Egypt, causing heavy economic losses. Severity of disease is influenced by number of factors, the most important is bacterial virulence (Rocco 2001).

The observed clinical signs of acute, sub-acute and chronic forms of MAS disease were similar to those reported by Rocco (2001); Basheer et al. (2013); Noor El Deen et al. (2014) and Ngoc et al. (2015). In the present study, the prevalence of MAS was 80% (320/400) among the surveyed *O. niloticus*, these findings were agreed with Omeje and Chukwu, (2012) who noticed that the highest prevalence of MAS was among *O. niloticus* than other fish species. On contrast, Mai et al., (2008) and Noor El Deen et al. (2014) recorded prevalence of 10% and 25 % among cultured *O. niloticus*, respectively.

Biochemical characterization of isolates (particularly $H_2S$ production) were in accordance with those reported by previous literature [Mai et al., (2008); Agniswar et al., (2012); Laith and Najiah, (2013); Muhammad et al., (2014); and Ngoc et al., (2015)].

Extracellular toxins derived from *A. hydrophila* were responsible for the pathogenesis of the organism. PCR amplification of DNA derived from *A. hydrophila* isolates using specific primers for hemolysin gene resulted in the expected PCR products of 130 bp size in lane 1and2, this result was matched with Yogananth et al.,(2009); Casiano et al., (2010); Uma et al., (2010) and Noor El Deen et al., (2014).

In the present study, the pathogenicity test of *A. hydrophila* strain in *O. niloticus* was highly pathogenic in IP and IM injection as the mortality rate reached to 100% and 70%, respectively. Similarly, Das et al., (2014) recorded higher mortality in IP route. Furthermore, Yardimci et al., (2011) observed that IM challenge of *A. hydrophila* in *O. niloticus* induced lesions only in skin and musculature, while the visceral organs such as liver and kidney were affected following the IP inoculation.

In the present study, naturally and IP injected fish

showed leucocytosis accompanied with heterophilia and lymphocytosis suggesting a serious infection process (Ranzani-Paiva et al., 2004). Leucocytosis in infected fish may serve as a protective barrier against pathogenic infection (Talpur et al., 2014). Li et al., (2006) observed the formation of phagolysosomes in trout B cells, revealing the possible contribution of lymphocytes in bacterial killing.

Experimental IP injection resulted in decrease of RBCs, PCV and Hb concentration. These findings were in correspondence with those by Talpur et al., (2014). The reduction in RBC count post challenge correlated with observation of *O. niloticus* after bacterial injection (Ngugi et al., 2015). Based on hematological indices, macrocytic hypochromic anemia was developed in IP injected fish. It could be attributed to the lytic activities of hemolysin, exotoxin produced by *A. hydrophila* on red blood cells (Griffiths et al., 1988). This hypothesis is supported by histopathological finding of hemosiderin in the spleen of infected fish, indicating excessive degradation of erythrocytes. It worthy to mention that, haematopoietic organs (spleen, liver and kidney) of diseased fish showed marked histopathological changes, suggesting affection of the haemopoiesis process. A reduction in serum protein, albumin and globulin levels was detected in naturally infected fish group. These findings were in agreement with those obtained by Aydin et al., (2000) who recorded a significant decrease in serum total protein value in naturally infected fish with *Campylobacter cryaerophila*. Serum proteins were either unchanged or, more usually, lowered in pathological states. Moreover, total serum proteins of diseased fish might be reduced with a high incidence of bacteria in the liver (Barham et al., 1980).

In this study, the histopathology of *O. niloticus* infected with *A. hydrophila* conformed closely to the description in other fish species; including channel catfish (Bach et al., 1978), crucian carp (Miyazaki et al., 1985), rainbow trout (Candan, 1990) and gold fish (Harikrishnan et al., 2010) consisted of septicemia, severe degenerative changes and hemorrhagic necrosis in several internal organs especially liver, kidney, gills, musculature and spleen. These changes were attributed to extracellular products including α and β haemolysins (Hirono and Aoki, 1991), enterotoxins, proteases, haemagglutinins and adhesions (Sha et al., 2002).The experimental infection of *A. hydrophila*, showed more obvious histopathological lesions of intraperitoneal inoculation than those of intramuscular route. These findings were in parallel with Ahmed and El-Ashram, (2002) who stated similar records in cultured tilapias infected with *A. hydrophila*.

In the present study, one of the most characteristic histopathological changes was the activation and proliferation of melanocytes macrophages centers (MMCs) which detected by morphometric analysis of their numbers, areas and hemosiderosis quantifications (area % and intensities) in spleen of infected fish. These changes including the presence of significant difference between naturally infected fish with *A. hydrophila* and non-infected groups. Similarly, a significant difference could be detected between experimentally infected group and control one. A significant increase in hemosiderin in the spleen of infected groups indicated an increased degradation of erythrocytes and the release of iron resulted from damage of cells due to toxins. Hemosiderin in MMCs previously observed in fish with hemolytic anemia and in those living in polluted water (Van der Weiden et al., 1994; Bucke et al., 1992).

Increased number, size and pigment content of MMC, especially hemosiderin, in infected fish have been considered as a sensitive biomarker referred to stressful conditions (Elston et al., 1997), increased environmental contaminants (Nowak and Kingsford, 2003) and in infectious agents (Vogelbein et al., 1987). This elevation in both number and size of MMCs following *A. hydrophila* infection confirmed the existing view of the significant protective role in

the accumulation, retention, and processing of products of catabolic degradation of cellular and tissue structures that can be hazardous for the normal functioning of both tissues and the organism.

## Conclusion

The causative disease of mass mortality in cultured *O. niloticus* was found to be Motile *Aeromonas* Septicemia. Hematological, biochemical and histophological examinations were performed on naturally diseased and experimental infected *O.niloticus*.

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Manuscript Received : May 15, 2016
Revised : May 30, 2017
Accepted : May 31, 2017