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Studies on *Aeromonas hydrophila* in Cultured *Oreochromis niloticus* at Kafr El Sheikh Governorate, Egypt with Reference to Histopathological Alterations in Some Vital Organs

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Abstract: The present study was carried out to isolate *Aeromonas sp* from cultured *Oreochromis niloticus* (*O. niloticus*) that identified by Biochemical tests (API 20 E) and PCR. A total of 10 *Aeromonas hydrophila* strains were isolated from 40 cultured *O. niloticus* collected randomly from the ponds of private fish farm in Kafr El Sheikh Governorate, Egypt with a prevalence of 25%. The clinical picture of the collected fish exhibited loss of escape reflex; skin darkness; bilateral exophthalmia and ulcers varied in their degrees, congestion, hemorrhage and enlargement in internal organs were appeared in postmortem examination. The complete identification was achieved by traditional biochemical tests and API 20 E system as both of them showed the same results. The isolated *A. hydrophila* were screened for presence of aerolysin gene and the results showed the amplification of the concerned gene at molecular size 309bp in two strains. Histopathological examination revealed necrosis of most internal organs, inflammatory reaction, associated with hemosiderosis.

Key words: *Aeromonas hydrophila* · *Oreochromis niloticus* · Aerolysin Gene · Histopathological

INTRODUCTION

Bacterial diseases in cultured fish are considered the main problem to aquaculture system in Egypt. Fish farms have been facing great problems due to bacterial fish diseases that cause severe damage and mortality in Egypt [1]. *Aeromonas* species are responsible for wide range of human diseases that vary in severity from a self-limiting gastroenteritis to potentially fatal septicemia and traveler's diarrhea, in addition to extra intestinal symptoms such as meningitis, endocarditis and osteomyelitis with a high mortality rate in immune-compromised person [2]. *Aeromonas hydrophila* is an opportunistic pathogen in fish and humans. The bacterium is widely distributed in aquaculture and it has been shown to inflict diseases in warm water fish including tilapia [3]. Motile *Aeromonas* septicemia (MAS) is a more dramatic bacterial disease affecting various species of fish and shellfish, feral as well as farmed in both fresh and seawater and cause a serious problem for the fish farming industry in Egypt as well as in other countries [4, 5]. *A. hydrophila* has ability to grow at refrigerated temperature so it considered as food borne pathogen of emerging importance [6]. There are many extracellular proteins were produced by *A. hydrophila* which associated with pathogenicity and environmental adaptability. The main virulence factors that have effect on pathogenicity of *Aeromonas* species are enterotoxins, aerolysin and hemolysin in addition to other factors such as adhesin and mucinase production [7]. Aerolysin gene is recorded to be the putative virulence gene produced by some strains of *A. hydrophila,* which is an extracellular, soluble, hydrophilic protein exhibiting both hemolytic and cytolytic properties [8, 9]. Aerolysin is a cytolytic pore-forming toxin (PFT) which creates unregulated pores in the membrane of targeted cells. The mature toxin binds

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to eukaryotic cells and aggregates to form holes (Approximately 3 nm in diameter) leading to the destruction of the membrane permeability barrier and osmotic lysis [10]. Our study aimed to isolate and identify *A. hydrophila* from tilapia fish farms in Kafr El Shiek Governorate, in addition to target its most virulence gene (aerolysin gene). The importance of virulence genes study is to detect the potential pathogenicity of the organism and subsequent possible targets for prevention of infection.

MATERIALS AND METHODS

A total number of 40 cultured *O.niloticus* were collected randomly from the ponds of a private fish farm in Kafr El Sheikh Governorate, Egypt. The Body weights of fish were ranged from 150±10 g. The collected fish were examined clinically with paying an attention to the behaviors in the ponds, changes in color and respiratory manifestations with a special care to the external lesions according to the methods described by Noga [5]. The fish samples were kept in tanks partially filled with the same water of the pond then transported to the Lab. In the Lab, each fish was rinsed with de-ionized water and the surface of the fish was decontaminated by dipping it in ethyl alcohol and lightly flamed. The samples were taken according to Bullar [11]. Swabs from the different organs of each fish were inoculated on Tryptic Soya broth then incubated at 28°C for 24 hours according to Austin and Austin [12].

Isolation and Identification of *A. hydrophila*: A loop full from each broth tube was streaked onto the following media; MacConkey's agar, Rimler and Schotts (R-S agar) (Oxoid) then incubated at 28ºC for 24 hours. Purified isolates were used as stocks for further morphological and biochemical identifications.

Morphological Characterization: Bacterial films were prepared from each suspected purified isolate and stained with Gram's stain [13] then examined under the bright field microscope with the oil immersion lens.

Biochemical Identification

Traditional Methods: The separate colonies were subjected to biochemical identification by the following tests: oxidase "Biomerieux", triple sugar iron agar, indol, Voges Proskauer, urea utilization, Simmon`s citrate agar and methyle red according to Collee *et al*. [14].

B-API 20 E Kits: API 20E kit BioMerieux [15] biochemical profiling test was performed according to manufacturer's instructions. Finally, isolates were stabbed into tubes containing semi-solid nutrient agar medium and then incubated at 37°C for 24 hrs. The incubated tubes were examined for detecting motility of inoculated isolates then preserved in the refrigerator at 4°C.

Genomic DNA Extraction and PCR Amplification: DNA of *A. hydrophila* was extracted by using QIA amp mini kit, Qiagen, specific oligonucleotide primer for the aerolysin toxin gene was used according to Wang *et al*. [16] as illustrated in Table (1).

DNA Amplification and PCR Running: The PCR mixture consisted of 20 µl reaction. The PCR amplification included an initial denaturation of at 95°C for 5 min followed by 50 cycles of denaturation at 95°C for 0.5 min, annealing of the primers at 59°C for 0.5 min and extension of at 72°C for 0.5 min. A final extension at 72°C for 7 min was used in the thermocycler (Biometra, Germany). Volumes of each (10 µl) PCR product were subjected to electrophoresis in a 1.5% (w/v) agarose gel according to Casiano and Choresca [17].

Histopathological Examination: Tissue specimens from intestine, gills, liver, spleen and kidney of naturally infected fish were collected for histopathological examination. The tissue specimens were fixed in 10% neutral buffered formalin for 24h. The fixed tissue were rinsed in tap water, dehydrated through graded series of alcohols, cleared in xylene and embedded in paraffin wax [18]. 5 µm thick sections were cut and stained with hematoxylin and eosin (H and E) and then examined by light microscopy.

Table 1: The primer sequence and amplified product size of Aerolysin gene

Target gene	Primer sequence	Amplified product size	Reference
Aerolysin gene	F.CAAGAACAAGTTCAAGTGGCCA	309 bp	Wang *et al*. [16]
	R. ACGAAGGTGTGGTTCCAGT		

RESULTS

Clinical and Post Mortem Findings: The clinical examination of diseased fish exhibited: loss of equilibrium, ascitis, skin darkness, exophthalmia and ulcers varied in their degrees. Congestion and enlargement in internal organs were appeared in postmortem examination (Figure 1).

Bacteriological Isolation and Identification: A total of 10 *A. hydrophila* strains were completely identified with an incidence of isolation 25% by culturing on R-S and MacConky agar media. The strains gave smooth, yellow and non- lactose fermenter colonies on the previous media. All strains were Gram negative, motile rods. Traditional biochemical identification showed the same results as obtained by the API 20 E system.

Results of PCR for the Detection of Aerolysin Gene: A total of 2 *A. hydrophila* strains out of the tested 10 isolates carried aerolysin gene at the expected product size (309 bp) as showed in photo (1).

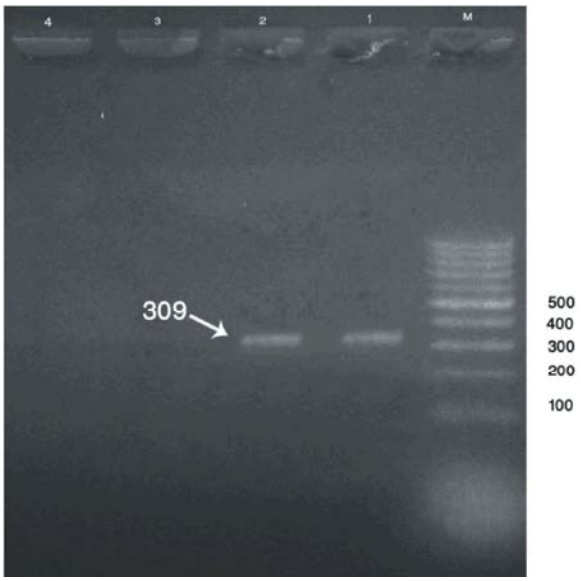

Photo 1: Amplification of 309 bp by PCR for detection of aerolysin gene.
Lane M: 100 bp Ladder, lanes 1and 2 showing positive samples while lanes 3and 4 shows negative result

Histopathological Findings

Gills: Gills showed hyperplasia and fusion of gill lamellae (Fig. 2a), dilatation with congestion of central venous sinus (Fig. 2b), lamellar telangiectasia (Fig. 2c) and branchitis characterized by congestion of the branchial blood vessels with intense leukocytic infiltration (Fig. 2d).

Liver and Kidney: Liver showed marked dilatation with congestion of hepatoportal blood vessel and sinusoids with diffuse vacuolar degeneration of hepatocytes (Fig. 3a), liver hemorrhage (Fig. 3b) and liver cell necrosis with pyknotic nuclei associated with loss of structural integrity (Fig. 3c).

Kidney showed congestion of interstitial blood vessels and renal glomerular tuft (Fig. 3d), focal interstitial hemorrhage with necrobiotic changes of the surrounding renal tubular epithelium characterized by karryopyknosis and chromatolysis (Fig. 3e) and focal interstitial infiltration of mononuclear cells with vacuolar and hyaline droplet degeneration of the surrounding renal tubular epithelium (Fig. 3f).

Spleen: Spleen showed subcapsular necrosis (Fig. 4a) and splenic hemorrhage with aggregation of hemosiderin-laden macrophages (Fig. 4b).

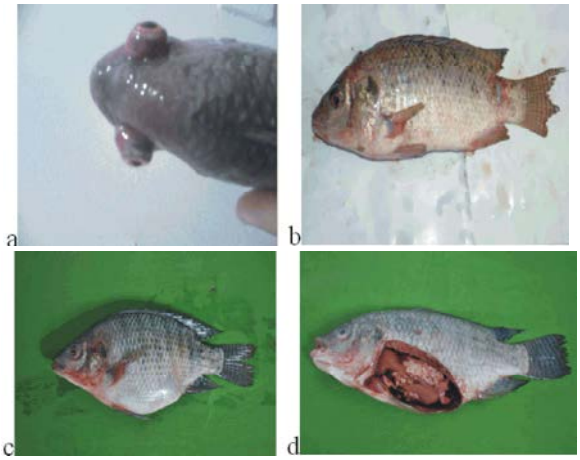

Fig. 1: In *Oreochromis niloticus* fish (a) bilateral showing exophthalmia, (b) ulcer on skin and erosion on fins and tail, (c) severe abdominal ascites and (d) congestion and hemorrhages in all internal organs

Intestine: Intestine showed necrosis of intestinal mucosa especially the apical portion of intestinal villi (Fig. 5a) with marked congestion of the sub mucosal blood vessels associated with edema and inflammatory cell infiltration (Fig. 5b). The inflammatory cellular infiltrates consisted mainly of mononuclear cells and eosinophilic granular cells (EGCs) (Fig. 5c) that were degranulated (Fig. 5d). The lumens of intestinal glands were filled with necrotic materials.

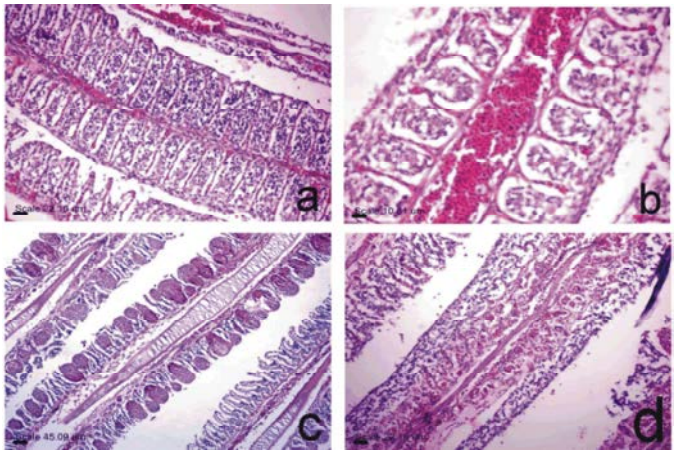

Fig. 2: Light micrograph of gills of *O. niloticus* showing (a) hyperplasia and fusion of gill lamellae, (b) dilatation with congestion of the central venous sinus, (c) lamellar telangiectasia, (d) branchitis.
Scale bars: 22.16 µm (a), 10.81µm (b), 45.09 µm (c) and 22.16 µm (d). H and E stain.

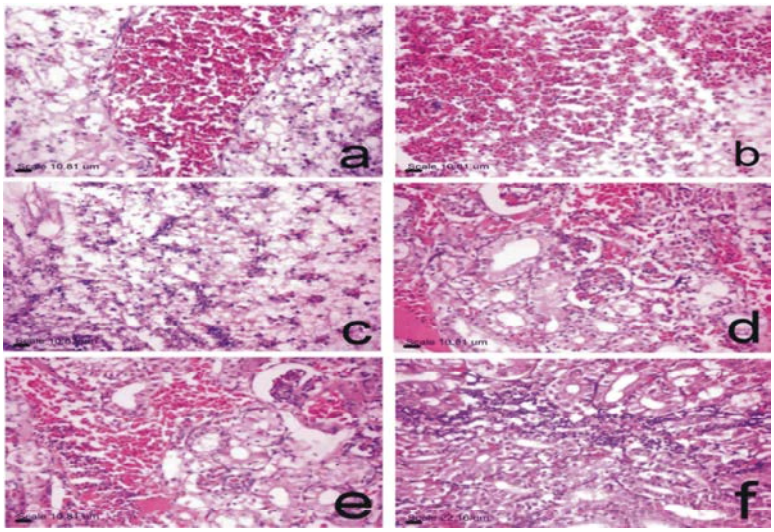

Fig. 3: Light micrograph of, liver (a,b,c) of *O. niloticus*, showing (a) dilatation with congestion of hepatoportal blood vessel and sinusoids, (b) liver hemorrhage and (c) liver cell necrosis. kidney (d,e,f) of *O. niloticus*, showing (d) congestion of interstitial blood vessels and renal glomerular tuft (e) focal interstitial hemorrhage with necrobiotic changes of the surrounding renal tubular epithelium (f) focal interstitial infiltration of mononuclear cells with degeneration of the surrounding renal tubular epithelium.
Scale bars: 10.81µm (a,b,c,d,e) and 22.16 µm (f), H and E stain.

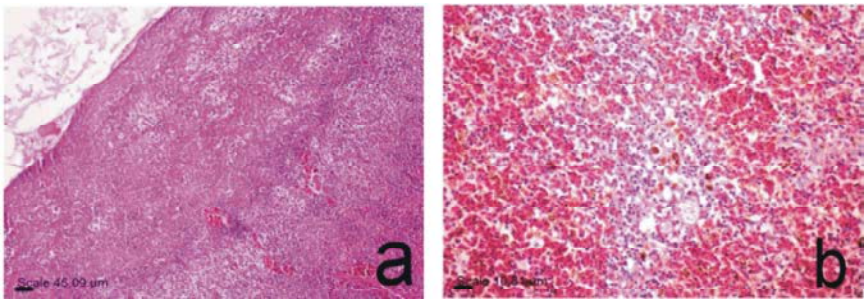

Fig. 4: Light micrograph of spleen of *O. niloticus* showing (a) subcapsular necrosis and (b) splenic hemorrhage.
Scale bars: 45.09 µm (a) and 10.81 µm (b), H and E stain.

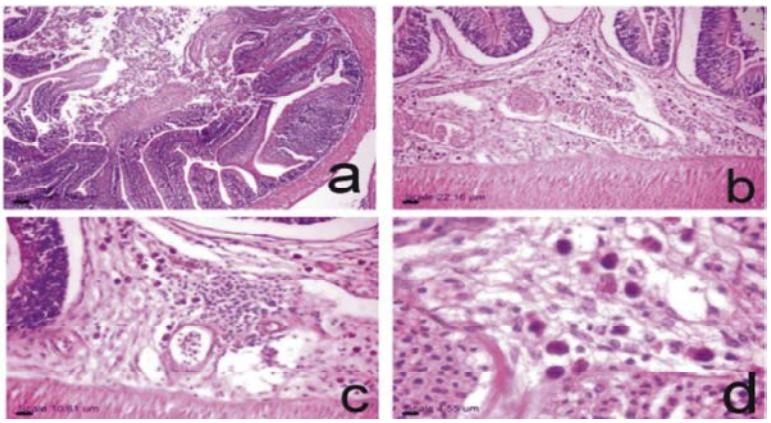

Fig. 5: Light micrograph of intestine of *O. niloticus* showing (a) necrosis of intestinal mucosa (b) congestion of the sub mucosal blood vessels associated with edema and inflammatory cell infiltration (c) inflammatory edema associated with aggregation of mononuclear and eosinophilic granular cells. (d) degranulated eosinophilic granular cells. Scale bars: 45.09 µm (a) and 22.16 µm (b), 10.81 µm (c) and 4.55 µm. H and E stain

DISCUSSION

Tilapia (*O. niloticus*) fish is characterized by fast-growing massive production which led to inappropriate farm management systems e.g., increasing stocking density, fluctuating temperature and poor aeration so susceptibility to pathogenic infections was enhanced and farmed tilapia has been challenged with several diseases outbreaks mainly motile aeromonas septicemia in Egyptas these were recommended by El-Sayed [19]. The present study showed that the main clinical signs of infected fish were aggregated on the water surface, accumulated at water inlet of pond. Others appeared dull with loss of escape reflex, haemorrhage, skin ulcers, sloughed tail and bilateral exophthalmia. These results may be attributed to bacterial toxins. These results were recorded by Fang [20] who mentioned that the sluggish movement associated with *A. hydrophila* infection was probably the result of frayed and sloughed tail, beside hemorrhagic edematous and ulcerated fins, in addition to anorexia which affected the vital activities of the diseased fish. The common gross pictures observed in the diseased fish were abdominal ascites, congestion and enlargement in the internal organs. These results may be attributed to septsemic reaction of MAS. These results were as the findings by Shoemaker *et al*. [21]. The characteristic colonies of *A. hydrophila* were indicated by yellow colonies due to maltose fermentation on RS Media, these results agreed with that findings by Hazen *et al*. [22] who stated that RS Media was 94% efficient for isolation of *A. hydrophila.* According to the results of morphological, biochemical and API 20 E test, a number of 10 strains were confirmed to be *A. hydrophila*, these results agreed with that recorded by Cantas *et al*. [23]. The primary toxin haemolysins produced by some *Aeromonas* species is termed 'aerolysin', a heat-labile - haemolysin and it possesses both hemolytic and enterotoxic activity which expressed by many strains of *A. hydrophila* Yu *et al*. [24]. Ullmann *et al*. [25] used PCR method for diagnostic purposes of cytotoxin-encoding genes of aerolysin (*aer* A) and haemolysin (*hly* A). Buckley [26] explained the mechanism of action of aerolysin gene, he revealed that *A. hydrophilia* exported autolysins as a protein which was activated by protolysis, the activated autolysin blinded to the eukaryotic cell receptor glycophorin and oligomerizes, forming holes in the erythrocytes membrane so called hole forming toxin aerolysin. In this study, 2 strains of *A. hydrophila* carried aerolysin (*aer* A) gene at molecular size 309bp, This result was in harmony with Casiano *et al*. [17] who detected aerolysin (*aer* A) gene of *A. hydrophila* which isolated from Moribund albino catfish and *Clarias* sp., at molecular size 309bp.

In this study, *O. niloticus* exhibited histopathological lesions in nearly all examined organs including, gills, liver, kidney, spleen and intestine. The histopathological alterations demonstrated in this study, were similar to those observed in channel catfish [27], crucian carp [28], rainbow trout [29] and gold fish [30] infected with *A. hydrophila*. In the present study, the renal lesions were glomerular congestion, tubular necrosis and interstitial hemorrhage. The same histopathological alterations were previously recorded by Canden [29], Harikrishnan *et al*. [30], Ventura and Grizzle [31], Angka [32], Carraschi *et al*. [33] and Yardimci & Aydin [34].

Gills lesions were showed hyperplasia and congestion of the branchial blood vessels with intensive leukocytic infilteration. These results could be attributed to the bacterial toxin expressed by MAS. Liver and splenic lesions were necrotic changes with loss of structural integrity and hemorrhage associated with hemosiderosis. Affinity of *A. hydrophila* to the liver was previously reported by Miyazaki *et al*. [35] and Grizzle and Kirya [36]. Cipriano, [37] reported that the liver and kidneys are target organs of an acute septicemia. These organs are attacked by bacterial toxins and lose their structural integrity Huizinga *et al.* [38]. Among these bacterial toxins are the extracellular products including aerolysins, α and β-haemolysins [39, 40], enterotoxins, proteases, haemagglutinins and adhesions [41-43]. These extracellular products induced more severe tissue changes [44] and contribute to the development of disease in fish [45]. One of the most characteristic features demonstrated in this study was hemosiderosis. Miyazaki and Kaige [28] attributed the lesions to the β-haemolysin secreted by the bacterium that cause haemolysis inside the fish body followed by deposition of haemosiderin. Moreover, β-haemolysin (Aerolysin) has proteolytic activities lethal to fish [46, 47]. The intestinal lesions demonstrated in this study was greatly similar to those reported by Roberts *et al*. [48] and these lesions could be attributed to the different bacterial toxins expressed by *A. hydrophila.*

CONCLUSION

- PCR is an accurate and rapid technique for detection of aerolysin gene in naturally infected *O. niloticus* with MAS which is considered one of the most virulent factors of *Aeromonas hydrophila*.
- The postmortem and histopathological findings reflect the action of aerolysin gene produced by *Aeromonas hydrophila*.

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