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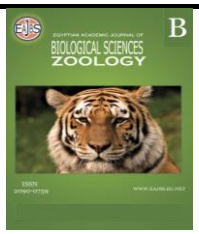

Morphological Examination and Histopathological Investigation of Gills In Common Carp, Experimentally Infected With *Arenomas hydrophila*

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ABSTRACT

Infectious diseases are the main reason for economic harm in the aquaculture industry, which is depressingly impacted by numerous pathogenic organisms. *Arenomas hydrophila (A. hydrophila)* recognized as an opportunistic pathogen. Forty fish were used in the present study, which was divided into two groups. One serves as control group (n=20) and the other group was experimentally infected with a strain of *A. hydrophila.* The clinical response was observed every day for 7 days. Then at the end of the experimental period, the fish dissected and the gills were removed for histological investigation. External signs of infection revealed many lesions such as hemorrhage in fins as well as a caudal peduncle. Also, reddish/gray ulcers with necrosis extending to the muscle were observed. Histopathologically, gills of *Cyprinus carpio* exhibited a varying degree of histopathological changes. Epithelial changes such as lifting and rupture of the lamellar epithelium, lamellar fusion.

INTRODUCTION

The global aquaculture industry currently accounts for over 45% of all seafood consumed. That figure has been projected to increase to 75% over the next 20 years (**Olsen *et al.*, 2012**). Infectious diseases are the main cause of economic losses in the aquaculture industry, which is negatively impacted by various pathogenic organisms (Lafferty *et al.*, 2015). *Arenomas hydrophila* is a facultative anaerobic Gram-negative enterobacterium of the family Aeromonadaceae, *A. hydrophila* has gained public health recognition as an opportunistic pathogen. Diseases caused by *A. hydrophila* (hemorrhagic septicemia, fin-tail rot, and epizootic ulcerative syndrome) have a major impact in aquacultures (Shar, 2010; Austin, *et al*., 2012)

Aeromonas hydrophila is the causative agent of motile aeromonad septicaemia, found in a wide variety of freshwater fish species (Cipriano *et al.*, 1984). Motile aeromonad septicaemia outbreaks are common all over the world (Kozinska and Guz, 2004). Outbreaks of motile aeromonad septicaemia usually occur only when the fish are immunocompromised by stresses such as overcrowding or concurrent disease (Stevenson, 1988). *A. hydrophila* produces several virulence determinants, including cytotoxins and enterotoxins (Yadav *et al*., 1992) and a repertoire of enzymes that

digest cellular components, mostly proteases and haemolysins (Leung and Stevenson, 1988).

Invasion of cells by bacterial pathogens involves adherence of the organism to the host cell, followed by bacterial internalization into a membrane-bound vacuole inside the host cell (Finlay and Falkow, 1989). Once inside the cell, these bacteria either remain within membrane-bound inclusions, as does *Salmonella typhimurium*, which multiplies efficiently within vacuoles (Garcia-del Portillo *et al*., 1993), or escape into the cytoplasm, as does *Shigella flexneri*, which encodes enzymes to lyse the vacuolar membrane (Hale and Boventre, 1979).

Although motile aeromonads appropriately receive much notoriety as pathogens of fish, it is important to note that these bacteria also compose part of the normal intestinal microflora of healthy fish (Trust *et al*. 1980). Therefore, the presence of these bacteria, by itself, is not indicative of disease and, consequently, stress is often considered a contributing factor in outbreaks of disease caused by these bacteria. Such stressors are most commonly associated with environmental and physiological parameters that adversely affect fish under intensive culture (Yambot and Inglis, 1994).

The present study was designed to Isolate and identify *Aeromonas hydrophila*, from *Cyprinus carpio.* Investigating of experimental infection of *Aeromonas hydrophila* and determination of antibody titre and challenge infection as well as morphological and histopathological investigation of fish.

MATERIALS AND METHODS

Fish:

A total number of 40 *Cyprinus carpio* weight 25±5 g were collected alive from Fish Research Center, Suez Canal University.

Fish were transported to the Wet–Lab of Fish, Resources and Aquaculture Department, Faculty of Environmental Agricultural Sciences, Suez Canal University, El-Arish, North Sinai, Egypt. The fishes were kept into polyethylene bags filled with their natural freshwater pressed with enough amount of oxygen. Water temperature was gradually decreased by adding crushed ice outside the bags. After arrival, all fish were held for three days in rearing fiberglass tanks (600 L- capacity) to diminish stress during transportation, and to be adapted by adding gradually amounts of brackish water until they were transferred to the experimental aquaria.

Bacteriological Studies:

Preparation and Sterilization of Bacterial Culture Media:

For isolation and identification of *Aeromonas hydrophila* certain specific culture media; Rimelar-Shotts agar (R-S) medium, Trypticase soy agar (TSA) and Trypticase soy broth (TSB) were used.

Sampling of Bacteria from Different Organs of the Body:

The fish used for bacterial sampling were killed by taking out of the water without administering any drug. The bacterial samples were obtained from the liver, spleen, and kidney. An alcohol-soaked cotton swab was rubbed on the side of the body. Then with sterilized scalpel and forceps, the body was cut. The abdominal cavity was further opened for exposing the internal organs. In order to obtain the sample from these organs, the external surface of these organs was sterilized by a red hot scalpel and then a portion of the organ was removed with the help of an inoculating loop and placed in TSB. All culture tubes inoculated with the bacterial samples from different organs were incubated at 28º C for 24 hours. These tubes were then used for further streaking to obtain a pure culture. For this purpose, R-S media and TSA were used.

In order to facilitate the recovery of motile aeromonads upon primary isolation, Shotts and Rimler (1973) designed a differential medium for selective isolation of motile aeromonads. The isolated colonies were picked up and streaked onto the surface of tryptic soy agar (TSA). The isolated bacteria were identified by culture morphology, Gram-stain and biochemically according to (Bullock *et al*., 1971, Popoff, 1984, Palumbo *et. al*. 1985 and Bisgaard *et al*., 1995). The colonies that showed a typical reaction in TSI (Trible Sugar Iron) and positive for cytochrome oxidase test, oxidation and fermentation reaction of glucose and catalase test were confirmed as *A.hydrophila.*

Identification of Isolated Bacteria:

The isolated bacteria were used to study the colony, morphological, physiological and biochemical characteristics for identifying the bacteria, the tests performed were: Gram's staining, motility test, oxidation and fermentation of glucose (O/F), lactose fermentation, oxidase test, hydrogen sulphide production in TSI agar.

Colony Characteristics:

The colony characters such as shape, size, surface, edges, elevation, and color were recorded.

Morphological Characteristics*:*

Under the morphological characteristics, Gram's staining was carried out. The motility, shape was also studied.

Gram's Staining:

Gram's staining indicates the nature of the cell wall, shape and arrangement of the cells.

Motility:

The motility was determined by using a semisolid TSB. The medium was sterilized and dispensed in a test tube. On solidification, the test bacteria was stabbed deep into the center of the tube and incubated for 24 hours at 37º C. The dispersion from the stabbed line indicated the motility and no diffusion in the medium showed non-motile behavior of the bacteria.

Oxygen Requirement:

The status of bacteria regarding their oxygen requirements was carried out along with the motility test. If the stabbed bacteria grow on the upper layer of the medium, it would be aerobic; when it grows in the lower portion of the agar stab tube, it would be anaerobic and grew throughout the medium, the bacteria would be facultative anaerobic.

Physiological and Biochemical Tests:

Carbohydrate Fermentation*:*

The carbohydrate fermentation test was performed in the phenol red carbohydrate medium. The medium was dispensed in cotton-plugged test tubes having an inverted Durham's tube and was sterilized. To these tubes, a particular carbohydrate was added at the rate of 5g/liter or a one percent solution be used. The test bacteria were then inoculated in each tube and incubated for 24 hours at 37º C. the control was also run along with the experimental ones.

Some bacteria can ferment carbohydrates while others can also produce gas from the same carbohydrate. The results of the tests were interpreted as:

a) Yellow color in the culture tube shoes the fermentation of the specific carbohydrate by the inoculated bacteria.
b) If no change in color observed, it indicates –ve results
c) If the Durham's tube is filled with gas, the bacteria can produce gas from that carbohydrate.

Catalase Test*:*

The catalase test was performed simply by dipping the capillary tube in 3% hydrogen peroxide solution and then placing it on a colony of the test bacteria. The formation of bubbles in the capillary tube showed +ve catalase test.

Oxidase Test:

For the oxidase test, a drop of freshly prepared 1% aqueous solution of Tetramethyl paraphenylene diamine hydroxide was placed on a glass slide. A colony of the test bacteria was smeared on it with the help of the inoculating loop. After 20-30 minutes. The appearance of purple or black color indicated the bacteria as oxidase +ve while no change in color indicated a –ve test.

Hydrogen Sulphide Production in TSI Agar*:*

This test is specially designed for the detection of hydrogen sulphide production along with the fermentation of glucose, lactose, and sucrose. The TSI agar was prepared by dissolving Peptone (Difco) 2g, Sodium chloride (Merck) 5g, Glucose (Merck) 0.1g, Lactose (Merck) 1g, Sucrose (Merck) 1g, Ferrous ammonium sulphate (China) 0.02g, Sodium thiosulphate (China) 0.02g, Agar (Difco) 1.3g and Phenol red 0.002g in 100 ml distilled water.

The sterilized medium was dispensed in cotton plugged tubes and was allowed to solidify to form a slant to these tubes the bacteria to be tested was streaked on a slant and incubated for 24 hours at 37ºC.

Reading Results: H2S will react with the iron and produce a black precipitate. A positive result has a black precipitate present and a negative result has no black precipitate.

Experimental Design:

Forty fishes were divided into two groups as follows:

Group (1): doesn't receive bacterial challenge

Group (2): *Aeromonas hydrophila* infected group, where the fish injected intraperitoneally 5×10^8 CFU of bacteria After challenge fishes were observed for 7 days

Necropsy and Histopathological Examination:

Fish were examined for external lesions during the experimental period. After the end of the experiment period; the fish were dissected; gill was removed then tissue samples were fixed in Bouin's Fixative and routinely embedded in paraffin wax. Serial sections of 5 μm were stained with hematoxylin and eosin and examined by microscopy (**Drury and Wallington, 1980**).

RESULTS AND DISCUSSION

Isolation and Identification of *Aeromonas* Strains.

All the cultures were maintained at 37°C and readings were recorded after 24 hours on RS media. These colonies were rounded, 2-3 mm in diameter, and yellow to orange in color. On TSA the colonies were about 2 mm in diameter, white to pale pink, round and covex colonies, butyrous inconsistency and the edges were entire. On TSA slant the growth was spreading, abundant and creamy-white with a smooth, glistening raised surface. In TSB the growth appeared dense, uniformly turbid with a surface pellicle after 24 hours. On the basis of morphological, physiological and conventional biochemical tests, the *Aeromonas* strains isolated from diseased and healthy *Cyprinus carpio* were identified. The identified strain was Gram-negative, rod-shaped, oxidase-positive, fermentative and motile were identified at the species level with biochemical tests.

The isolated strains were Gram-negative, rod and motile. Complete identification at the species level with biochemical tests as described in Table (1).

Table (1): Biochemical tests for identification of *Areomonas sp.*

Characteristic	*A. hydrophila*	*A. sobria*	*A. caviae*
Gram stain	Gram Negative	Gram Negative	Gram Negative
Motility	motile	motile	motile
Growth Triple sugar iron (TSI) agar	acid production	acid production	Negative gas production.
H_2S production	H_2S Positive	H_2S Positive	Negative gas production.
O-F Test (growth on glucose fermentation broth)	acid production	acid production	Negative gas production.
Catalase	Negative	positive	positive
Oxidase	positive	positive	positive

Experimental Pathogenicity of *Aeromonas hydrophila:*

Experimental infection of fish with *Aeromonas hydrophila* by injection method leads to the appearance of pathological changes and mortalities among challenged fish. Clinical pictures of the experimentally infected fish were nearly similar but varied in the severity of the developed lesions. They included poor appetite, loss of equilibrium with the erratic movement of some fish, swimming with head down abdominal distension and finally loss of all reflexes just prior to death. Hemorrhage of all fins, caudal peduncle and ventral abdominal wall with hemorrhagic and protruded anal opening was seen. Some animals had a distended abdomen with serosanguinous fluid. Also reddish/gray ulcers with necrosis extending to the muscle (Fig. 1). Moreover, *Aeromonas hydrophila* infection revealed an extensive amount of mucus with hemorrhagic lesions on the skin at the base of fins. Ulceration on the gill cover and slightly protruded reddish vent, skin darkness and tail rot were also noticed.

Histopathological Studies:

Control non-infected Common carp gills are comprised of four-gill arches on both sides and extending from floor to roof of the buccal cavity. Two holobranchs (rows of filaments called primary lamellae) project from the posterior edge of each gill arch. The gill arch is a curved osseous structure from which radiate the bony supports of the primary lamellae. The primary lamella is covered by a mucoid epidermis (Fig 2). The gills of cyp*rinus carpio* experimentally infected with bacteria revealed necrosis. Either scattered or local foci of foam cells were seen between primary lamellae (Fig.3). The most marked lesions detected in gill tissue of *Cyprinus carpio* treated with experimentally infected with *Aeromonas hydrophilla* was a fusion of secondary lamellae associated with lymphatic infiltration and hyperplasia of chloride cells and congestion in secondary lamellae (Fig.4).

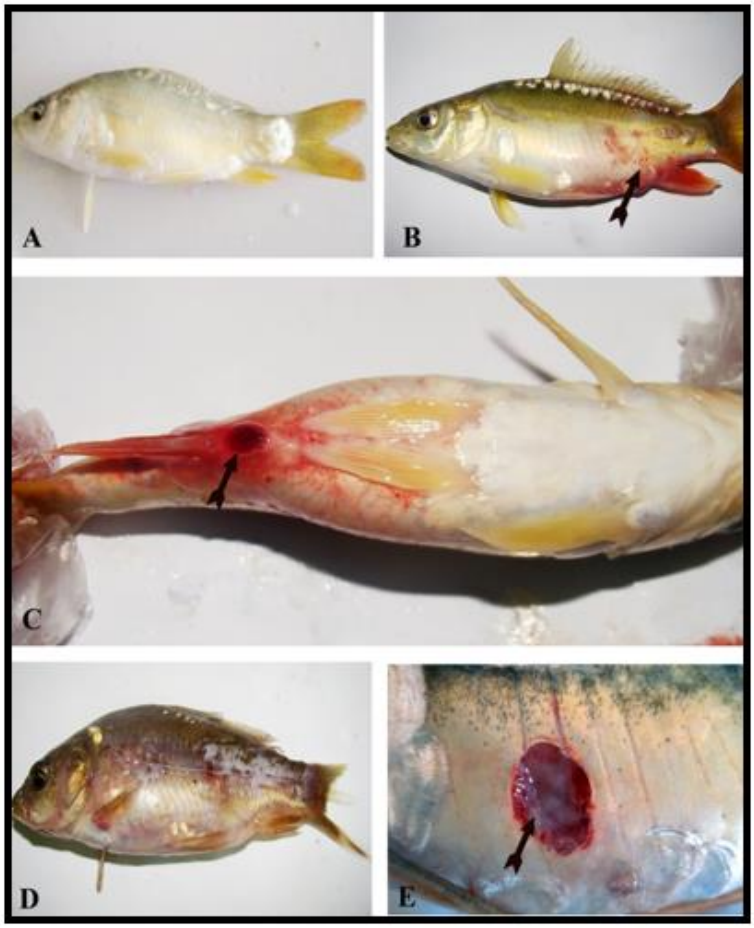

Fig (1). Clinical signs and post mortum lesions after challenge with *Aeromonas hydrophila.* **(A)** Control, **(B)** Hemorrhage, **(C)** Distended abdomen and protruded anal opening, **(D)** Tail and fin rot, **(E)** Dermal ulceration.

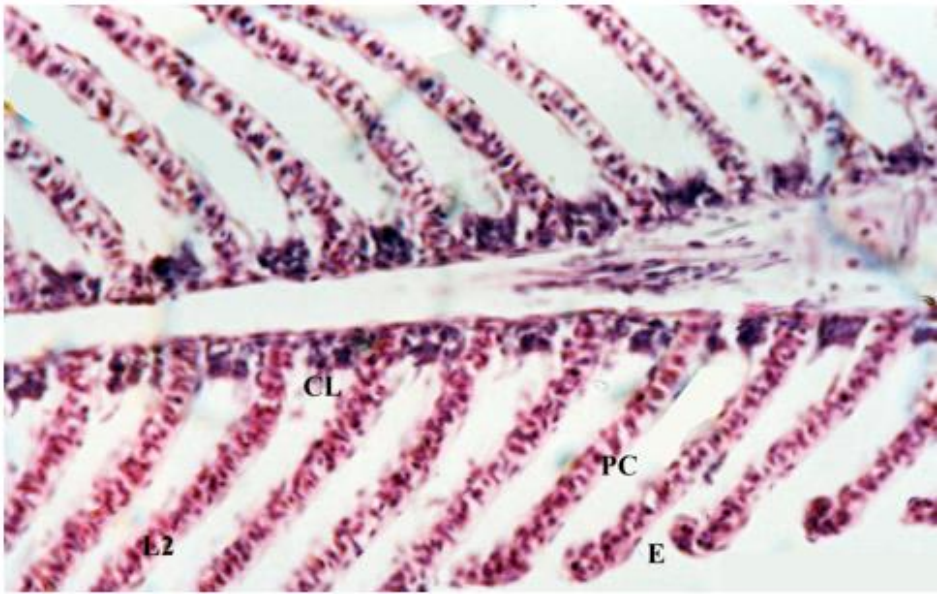

Fig (2): Section of gills of control *Cyprinus carpio*, showing secondary lamellae (L2) originate at the superior and inferior surface of primary lamellae. Besides, the thin epithelial (E) covering secondary lamellae supported by pillar cells (PC), and chloride cells (CL) located between secondary lamellae (L2). (200 X).

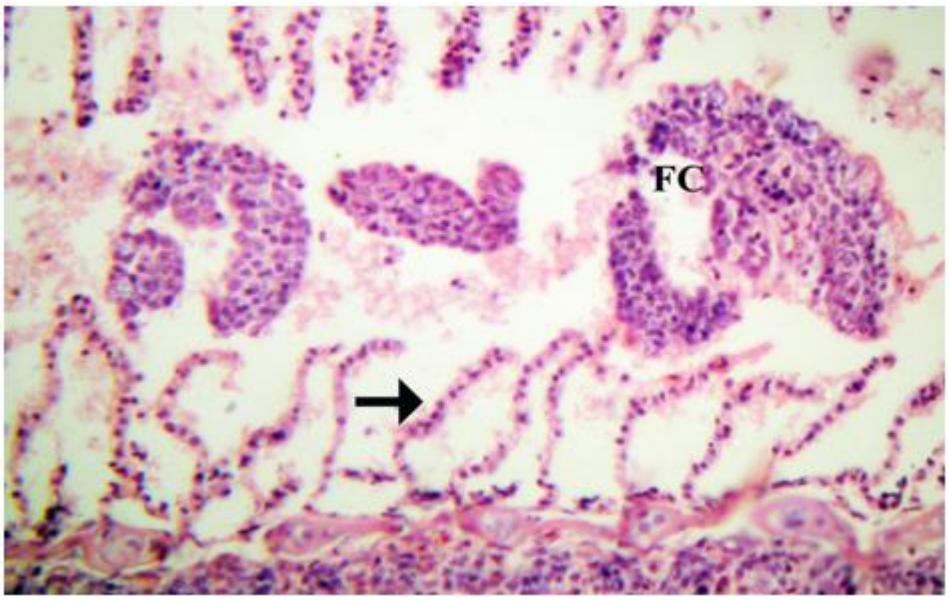

Fig (3): Section of gills of *Cyprinus carpio* experimentally infected with *Aeromonas hydrophila*, revealed degenerative changes mainly necrosis (arrow). Besides Either scattered or local foci of foam cell (FC) reactions were seen between primary lamellae. (200 X)

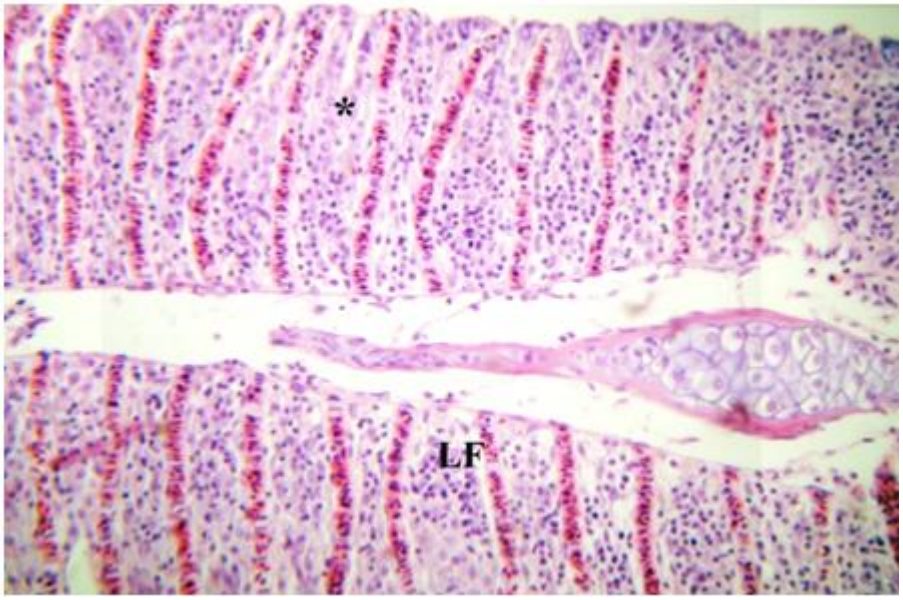

Fig (4): Section of gills of *Cyprinus carpio* experimentally infected with *Aeromonas hydrophila*, displayed epithelia necrosis associated lymphatic infiltration (LF) and Fusion of secondary lamellae (*). (200 X)

DISCUSSION

In this study, isolation and identification of *A.hydrophila* from diseased and healthy fish were established. According to morphological characters, *A.hydrophila* able to grew on RS media after 24 hr. incubation at 37ºC. These colonies were rounded, 2-3 mm in diameter, and yellow to orange in color. This agrees with the findings of Shotts and Rimler (1973) who reported that a characteristic type of colony was obtained when *A. hydrophila* was inoculated on to RS Media and these types of

colonies indicating maltose fermentation. Moreover, our results agree with that reported by Hazen *et al* (1978) who stated that RS Media was 94% efficient for isolation of *A. hydrophila* and Hsu *et al*. (1981) who noted that all 127 strains of *A. hydrophila* tested produced yellow colonies on RS Media, while were. The isolates proved to be a Gram-negative, rod-shaped, facultative anaerobic and motile.

Concerning the biochemical characterization of the isolates, the uniformly positive and uniformly negative results were confirmatory of those reported by other authors including Popoff and Vern (1976), Hus *et al* (1981) and Toranzo *et al* (1986). The biochemical reactions of the isolates showed that typical reaction in TSI, and positive for each of cytochrom oxidase test, oxidation and fermentation reaction in O/F glucose.

The current study revealed some external lesions in common carp (*Cyprinus carpio)* post experimental infection with *A. hydrophila*. Hemorrhage in fins; Distended abdomen as well as protruded anal opening were marked lesions in infected fish. Besides, Tail and fin rot and dermal ulceration were also observed. These results were in agreement with Huizinga *et al*. (1979) who reported that a common infection with *Aeromonas hydrophila* among largemouth bass (*Micropterus salmoides*) results in external lesions varied from pinpoint lesions to chronic ulceration. Focal hemorrhages, edema, and dermal necrosis were detected which resulted in the infiltration of muscle with mononuclear and granulocytic inflammatory cells. Internally, the liver and kidneys showed necrotic foci due to toxins produced by *Aeromonas hydrophila*. Serious pathological changes were not evident in both the spleen and heart. Moreover, Miyazaki (1980) studied the Japanese eel (*Anguilla japonica*) infected with *Aeromonas hydrophila*. The results indicated that the diseased fish displayed a red coloration on their fins, abdomen and tail. Enteritis and hemorrhage in the stomach, liver, dermis, gill epithelia and renal hematopoietic tissue and necrosis of the splenic sheath arteries were reported. Also, Badran and Eissa (1991) found two forms of the motile *Aeromonas Septicaemia* in *Oreochromis niloticus*. The acute form was characterized by peticheal haemorrhages all over the body, congestion of the internal organs (liver, spleen, and kidney) while the chronic form was characterized by abdominal ascitis and exophthalmia, bloody-tinged fluid in the abdominal cavity.

Concerning to the histopathological changes in gills of the experimentally infected common carp by intraperitoneal route with *Aeromons hydrophila* , gills showed odema and epithelial lifting in secondary lamellae. These results confirmed those reported by Miyazaki, (2006) who reported that the histological responses of *Plecoglossus altivelis*, given an intramuscular injection of a formalin-killed bacterin *of Vibrio anguillarum* showed bacterial phagocytosis by infiltrated neutrophils and slight tissue necrosis. Also, gills revealed lymphatic infiltration, hemorrhage, and congestion. On the whole, our results show that infection had an impact on the health of fish that lead to death by affecting one of the most important organs concerning supplying oxygen to the body. Gills lesions represented by leukocytic infiltration of secondary lamellae with mononuclear cells and the presence of local and scattered foci of foam cells as well as the fusion of secondary lamellae. Such findings were met with those reported by Easa and Akeula (1985); Miyazaki and Kaige (1985); Kumar and Day (1998) and Soliman and Hasseib (1990).

In conclusion, experimental infection of *Cyprinus carpio* with *Aeromonas hydrophila* results in pathological changes and mortalities among challenged fish. These pathological changes include some morphological manifestations such as skin ulceration and hemorrhage besides severe histopathological alterations in gill tissue

mainly lamellar fusion which causes a reduction in respiratory surface and in turn causes fish mortality.

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