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Impairment of Bioenergetic Function and Loss Ions Regulation in *Cyprinus carpio* Experimentally Infected with *Providencia alcalifaciens*: Biochemical and Histopathology Study

Ameer Hameed Almohammadi[1], Shahbaa Khalil AL-Taee[1*] and Balsam Y. Rasheed[2]
[1]Department of Pathology and Poultry Diseases, College of Veterinary Medicine, University of Mosul, Mosul, Iraq
[2]Department of Microbiology, College of Veterinary Medicine, University of Mosul, Mosul, Iraq
[*]**Corresponding Author: shahbaa_khal@uomosul.edu.iq**

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ABSTRACT

It is a scientific fact that bacteria cause disturbance in energy metabolism. Studies about the *Providencia* spp. in carp fish are rare. Therefore, the study aimed to molecularly reisolate and investigate Providencia's effect on enzymes of the phosphorylation network as creatine kinase (CK), ionic balance in the serum and semi-quantitative and histochemistry analysis of the gill tissue. Thirty fish were divided into three groups: control group, second and third fish were intraperitoneal injected with 108 ×12 and 108×6 CFU, respectively. The 16S rRNA gene sequence was related to *Providencia alcalifaciens*. The statistical analysis showed a significant decline ($P \leq 0.05$) of the CK and a loss of ionic balance (Na^+, K^+, Cl^-, and Ca^{++}) in the two study groups compared to the control group. The semi-quantitative analysis of the gills arch and filaments showed pathological alteration occupied score (0-3), moreover the histological alteration index (HAI) was highly significant ($P \leq 0.01$) in both groups from 216 and 136. The histochemistry analysis of the mucocytes showed positive reactivity for Alcian Blue stains for acid mucus secretion. Additionally, mucocytes were highly significantly distributed ($P \leq 0.05$) at the apex of filaments. In conclusion, the decrease in the concentration of CK is an indicator of the effect of *Providencia* on energy metabolism as well as the disturbance of the ionic balance. This leads to pathological lesions in the gills. Moreover, the semi-quantitative and statistical analysis of histological lesions and histochemistry analysis are considered evidence of the significance and severity of the lesions.

INTRODUCTION

Fish culture is an essential source of aquatic nutrition and food chain and meaningful livelihoods for millions of people around the world. Fish and their products represent a significant source of proteins, vitamins, and other micronutrients, which is important for human health **(Gobi *et al.*, 2016; Al-Jumaa *et al.*, 2024)**. However, fish rearing in an intensive culture may be exposed to stress factors such as improper handling, high stock density, extensive use of chemotherapeutic agents, antibiotics and

insufficient dissolved oxygen concentration. All these factors lead to immunosuppression and increase susceptibility to bacteria, parasites, fugal and viral pathogens **(Ramesh & Souissi, 2018; Fitriadi *et al.*, 2023)**. Several bacterial pathogens are responsible for morbidity and disease outbreaks in different fish species, including *Aeromonas septicemia*, Streptococcosis, vibriosis, and Columnaris **(Declercq *et al.*, 2013; Luo *et al.*, 2017; Ji *et al.*, 2020; Mohammad *et al.*, 2021; Qosimah *et al.*, 2023)**.

The genus *Providencia*, related to Enterobacteriaceae, is a Gram-negative bacterium bacillus. It includes urease-producing bacteria responsible for a wide range of domestic animals, reptiles and humans in addition to insect infections. Furthermore, it has been identified as a pathogen of aquatic animals, commonly isolated from fish organs **(Abdallah & Balshi, 2018)**. It has been isolated from the muscle and skin of *Labeo rohita*, *Lagocephalus sceleratus*, and the Nile tilapia (*Oreochromis niloticus*), displaying symptoms such as ulcers and fin rot at the pelvic and tail fin bases, abdominal lesions around the head, orbital hemorrhage with exophthalmia and redness at the abdominal and anal region, darkening coloration of the skin, complete loss of the reflexes and responses to the external stimuli, with respiratory disturbances, ascites and lepidarthosis **(Faisal *et al.*, 1987; Ramkumar *et al.*, 2014; Tu *et al.*, 2014)**. Moreover, this pathogen causes septicemia in crocodile **(Benedict & Shilton, 2016)** and causes red legs in shrimp **(Cao *et al.*, 2022)**.

Gill diseases have a direct effect on the status of fish health since they play a vital physiological role in maintaining ions, and acid-based balance. Additionally, they are the main branchial tissue which are responsible for fish respiration **(Hussein *et al.*, 2024)**. Furthermore, it is considered an effective biological monitoring tool for their unique features, such as the great surface area and direct interaction with aquatic environment (**Sweidan *et al.*, 2015; Al-Taee & Alhamdani, 2022)**. Despite the importance of gill function, studies on *Providencia* infected branchial tissue remain limited, there is an uncertainty about whether the *Providencia* sp. is considered a primary pathogen to the gills **(Austin & Austin, 2007; Ramesh & Souissi, 2018)**.

Therefore, our hypothesis was that there is a possibility of *Providencia* infecting the gill of the carp fish, causing histological pathological alteration that led to a disturbance of the vital ions balance. Moreover, this study aimed to investigate the bacteria's influence on ATP-dependent enzymes, particularly the creatine enzyme activity.

MATERIALS AND METHODS

Ethical approval

The methodology used in the study was approved by the Institutional Animal Care and Use Committee UM.VET.2024.091 University of Mosul College of Veterinary Medicine.

Inoculum confirmation, preparation, and infection

The strain *Providencia* was previously obtained from farmed infected carp (naturally) and was isolated and reisolated from experimentally infected fish by

conventional bacterial methods (seeding in MacConkey agar and sheep's blood agar) and by Gram staining protocol (-ve bacilli). The identification of these Gram-negative bacilli was carried out using Vitek 2® (BioMérieux, Marcy-l'Étoile, France), using a GN colorimetric identification card (64 parameters), which presented an excellent confidence level (96 to 99% probability). The isolate pathogens were preserved in skimmed milk and glycerol at a temperature of −80°C before being used for pathogenicity purposes. The *Providencia alcalifaciens* suspension was washed in NaCl 0.9% as sterile saline, and the turbidity was measured at OD_{600}.

Experimental design

A total of 30 common carp (*Cyprinus carpio*) **(Al-Sabaawy *et al.*, 2024)** with an average body weight of 250 ±10g were used to evaluate the histopathological effects of *Providencia* bacteria in gills, ion balance in the serum, and creatine kinase activities in the serum.

The carp fish were kept randomly into two groups, control group uninfected (U) and infected group(I). Fish in (I) group was injected intraperitoneally with 1ml of *Providencia alcalifaciens* suspension containing $10^8 \times 12$ and $10^8 \times 6$ CFU, according to the procedure reported by **Souza *et al.* (2019)** for the Nile tilapia *Oreochromis niloticus*. The uninfected groups were kept in freshwater without any treatment. The fish were evaluated on day seven post-infection (IP), in which serum collection for biochemical parameters (Creatinine kinase and ions balance as Na^+, K^+, Cl^-, and Ca^{++}) and gills were dissected for molecular re-isolation and histopathological analysis.

Molecular identification of reisolate

The DNA of bacteria was mainly extracted directly from the tissue of infected carp fish, according to **Shima *et al.* (2012)** and the manual's guidance of PCR SuperMix – Invitrogen, then the DNA extracted were stored at −20°C until examination. 16S rRNA gene of the bacterial isolates were amplified by universal primers Psp16s-F1 (5/-ACCGCATAATCTCTT AGG-3/) and Psp16s-R2 (5/-CTACACATGGAATT CTAC-3/). The PCR reactions were carried out in a total reaction volume of 50µL containing Taq DNA polymerase (5 units), 400mM each dNTPs, 1.5mM 168 MgCl2, and 20ng template DNA. The PCR reaction was carried out under the conditions, as shown in Table (1).

Table 1. The amplification program

Number of cycles	Temperature °C	Time	Reaction
1	94	5min	Initial denaturation
30	94	30s	Denaturation
1	50	30s	Annealing
1	72	30s	Extension

The products of PCR were visualized by using 0.2µL ethidium bromide in TBE buffer with agarose gel electrophoresis, 2% (Biometra, Germany), DNA bands were analyzed with a UV transilluminator.

Histopathological analysis

Following the collection of infected gills, they were fixed for 24 hours in 10% natural formalin buffered phosphate, dehydrated in ethanol at progressively higher concentrations, immersed in xylene, and then embedded in paraffin wax at 50 degrees Celsius. A microtome was used to cut a block of paraffin into slices that were 5 microns thick. Tissue samples were fixed on slides and then stained using routine and histochemical stain (Alcian blue pH2.5), **(Santos *et al.*, 2011; Al-Taee *et al.*, 2024)**.

Semi-quantities score system

Quantification of histological gills index

According to **Flores-Lopes and Thomaz (2011)** and **Nascimento *et al.* (2012)**, the histological lesions were scored and graded, as shown in Table (2).

Table 2. Semi quantities and catalog histological lesions with sequalae of gills in *Cyprinus carpio*

Score	Grade	Catalog lesions	Sequalae
0	No alterations	Normal appearance	
1	Slight alterations	Edema Infiltration of leukocyte Hyperplasia of lamellar epithelium Secondary lamellar fusion Blood vessels dilatation and congestion	Minor alteration without tissue damage and functions (revisable alteration)
2	Moderate alterations	Epithelial lifting	Alterations are more severe lead deleterious gills tissues associated with functioning
3	Severe alterations	Aneurysms and lamellar necrosis	Irreversible alteration with loss normal functioning

The histopathologic alterations index (HAI) depended on the classes' severity of the lesions and progressive gills tissue damage **(Poleksic & Mitrovic-Tutundzic, 1994; Nascimento *et al.*, 2012)**. The HAI was calculated according to the following formula: HAI = (1 × SI) + (10 × SII) + (100 × SIII), where I, II and III represent the number of alteration stages 1, 2 and 3; while S corresponds to the sum of the number of pathological lesions at each particular stage, the classes catalogue of HAI values determined in Table (3).

Table 3. Classes catalog, indexes, and description of the histopathologic alterations index

Classes catalog	Lesions indexes	Attribute
Class I	0 -10	Normal organ functioning
Class II	11 -20	Slight alteration in the organ
Class III	21 -50	Moderate alteration in the organ
Class IV	51 – 100	Severe lesions
Class V	≥ 100	Irreparable organ lesions

Quantification and histochemistry analysis of mucus cells

The number of mucous cells/ mm^2 of gills tissue per fish was determined by investigating three gills filament regions (apex, middle and base) at 3 sections, each having 4 filaments. These 3 sections were separated by at least four intermission sections. The examination region involved each side (left and right) of the filament including ten adjoining lamellar and interlamellar spaces. In total, 36 measurements were taken per individual. Histological alterations were evaluated under a light microscope at power magnification 40X **(Ledy *et al.*, 2003)**.

Statistical analysis

The data of this experiment were analyzed using a completely randomized design (CRD), one-way ANOVA, and the Chi-square test. Semi-quantitative analysis of mucosal cells was analyzed using two approaches. To determine significance, Duncan's test was used up to the probability level of $P \leq 0.01$ and $P \leq 0.05$ **(SAS Institute, 2014)**.

RESULTS

Molecular identification of reisolating bacteria

PCR analysis of the extracted DNA from experimentally infected fish was amplified to identify *Providencia* bacteria molecularly using universal primers. The amplification PCR products showed the target identified 515 bp DNA fragment (Fig. 1), which indicates the *Providencia* bacteria. All 20 samples from the experimental groups were analyzed and presented positive results. The 16S rRNA gene sequence of isolate *Providencia alcalifaciens* was deposited in GenBank with accession No. PV101577 (Bacterium strain *Providencia alcalifaciens* 2 16S ribosomal RNA gene, p-Nucleotide-NCBI) and showed a similarity of 100% with other *P. alcalifaciens* strains (PP858093.1 Nigeria strain and JX827242.1 China strain) in the GenBank database.

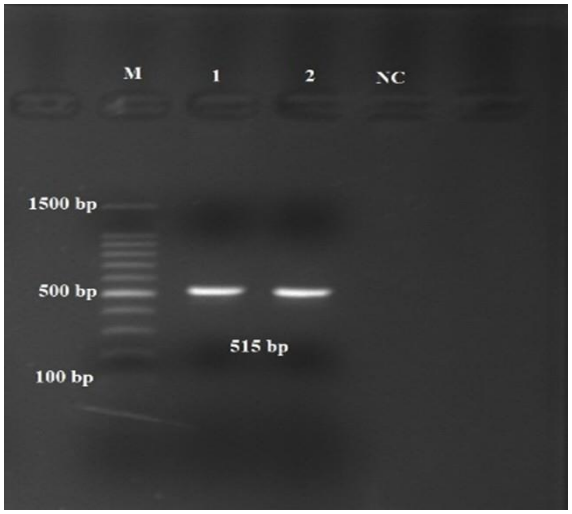

Fig. 1. *Providence* spp. from fish, M: 100 bp marker, NC: Negative control, 1 and 2: natural isolated *Providencia* spp.

Biochemical analysis

Creatine kinase activity in fish serum significantly decreased in both groups inoculated with $10^8 \times 12$ and $10^8 \times 6$ CFU, resulting in the reduction of 85.1 and 78.5% on day 7 IP. In the second treated group, CK levels decreased significantly ($P \leq 0.05$) reaching 6.5mmol/ L in second treated group in contrast to control group (8.28) (Fig. 2).

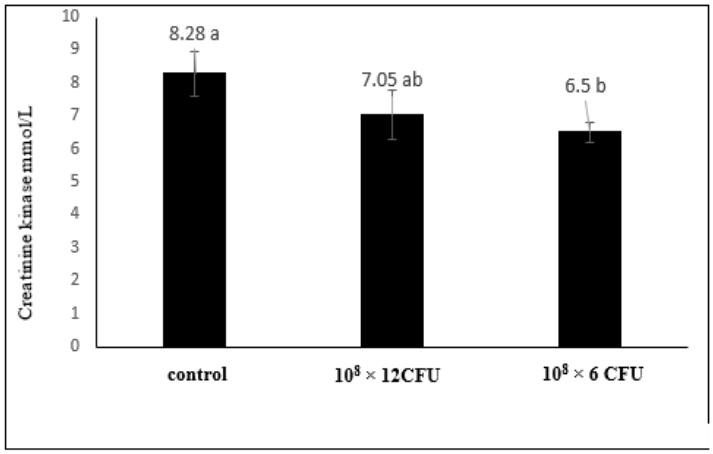

Fig. 2. Diagram of creatine kinase activity (mmol/L) in serum of fish treated with different concentrations of Prov. and variable way to exposure

Ions balance

The concentrations of Na^+ and Ca^{+2} ions in *Cyprinus carpio* blood serum significantly declined ($P \leq 0.05$) in both injectable groups compared to control groups. Moreover, there were significant reductions in K+ and Cl- ions in serum of fish

inoculated with bacterium suspension of 10^8 ×12 CFU, reaching 2.41 and 84.00%, compared to the control group (1.83 and 102.25%, respectively) (Table 4).

Table 4. Ion homeostasis in blood serum of treated *Cyprinus carpio* to variable concentration of *Providencia alcalifaciens*

Groups	Na^+ mmol/L	K^+ mmol/L	Cl^- mmol/L	Ca^{++} mg/dl
Control	126.25±5.56 a	1.83±0.21 c	102.25±3.77 a	9.36±0.34 a
IP: 10^8 ×12 CFU	113.75±4.99 b	2.41±0.21 b	84.00±3.65 b	7.01±0.44 c
IP: 10^8 ×6 CFU	105.25±9.39 b	3.11±0.37 a	103.50±2.52 a	7.72±0.42 b

The different litter refers to significant difference between groups at $P \leq 0.05$.

Histopathological analysis

To evaluate the histopathological alteration of carp's osmoregulation organ caused by IP infection Prov. at bacterial solution 10^8 × 12 CFU, sections of gills arch were examined. These sections included primary gills filament, secondary gill lamellae, adipose tissue, and, externally, the gills racker (Fig. 3-A).

The microscopic examination showed alteration in brachial arch represented by hyperplasia of epithelial cells with severe infiltration of mucocytes (Fig. 3-B). Additionally, leukocytes infiltration was observed in the elastin connective tissue and proliferation of fibroblast at the subepithelial tissue of the brachial gills tissue (Fig. 3-C). Hemorrhage, necrotic gill arch tissue, and damage to the epithelial cells of the convex arch surface were also noted (Fig. 3-D). These lesions were less severe in fish inoculated with 10^8 × 6 CFU.

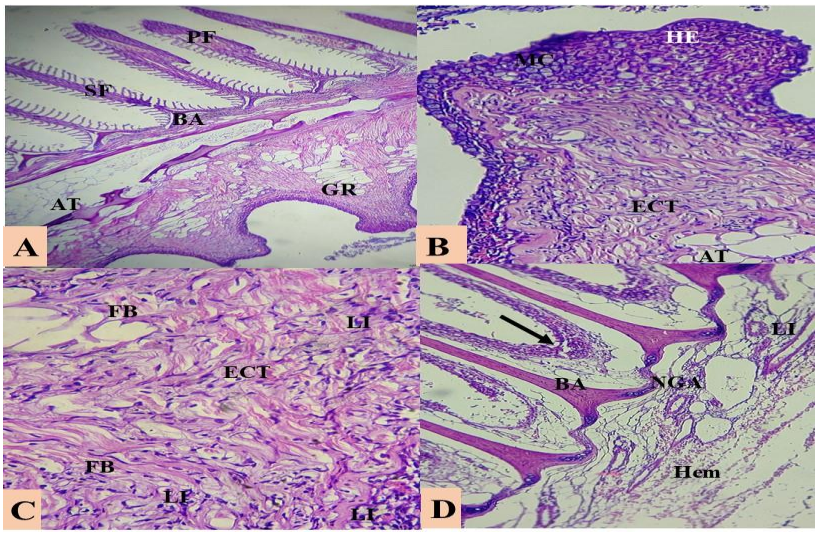

Fig. 3. Microscopic examination of gills arch of fish inoculated with 10^8 × 12 CFU on day 7 IP, its structure was primary gills filaments (PF), secondary gill filaments (SF), adipose tissue (AT) with gills racker (GR) **(A)** 40X. There was alteration in brachial arch represented by hyperplasia of epithelial cells (HE) with sever infiltration of mucocytes (MC) **(B)**, 100X, leukocytes

infiltration in the (LI) elastin connective tissue (ECT) and proliferation of fibroblast (FB) at the subepithelial tissue of the brachial gills tissue **(C)**, 400X, hemorrhage (Hem), necrosis of both gills arch (NGA) and at the epithelial cells of the arch convex surface (black arrow) **(D)**, 40X, H&E

Furthermore, the deleterious effects of inoculum Prov. 10^8 × 12 CFU on day 7 IP, represented by and circulatory disturbances characterized by edema at the apex of primary gill filament, vasodilatation, (Fig. 4-A). Additionally, sloughing of the epithelial cells lining the secondary gill lamellae was observed, giving them a drumstick-like appearance. This was accompanied by leukocyte infiltration (Fig. 4-B), loss of structural alignment and the presence of aneurysms (Fig. 4-C), as well as vacuolar degeneration in both pillar cells and chloride cells, the latter appearing enlarged (Fig. 4-D).

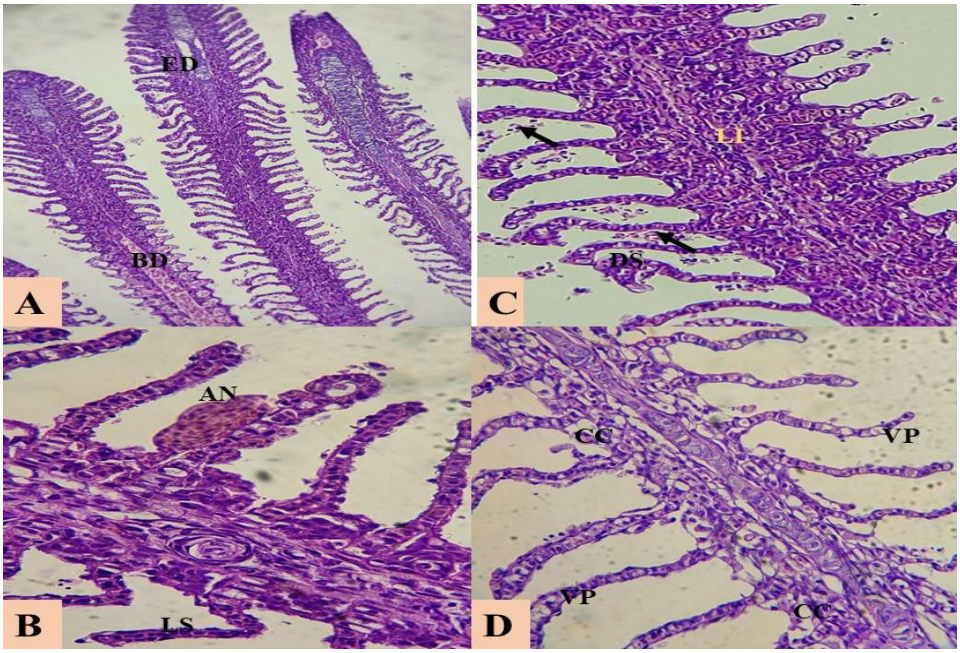

Fig. 4. Microscopic examination of gills arch of fish inoculated with 10^2 × 12 CFU on day 7 IP, show blood vessels dilatation (BD), edema (ED) **(A),** 40X, curling appearance of secondary (loss its straight -LS) with aneurism (AN) **(B)**, 400X, sloughing of the epithelial cells lining secondary gills lamellae that have been drum steak appearance (DS), infiltration of leukocytes (LI) **(C)**, 100X, vacuolar degeneration of pillar cells (VP) and chloride cell (CC) which appear enlargement **(D)**, 100X, H&E

The histological lesions in the gills of fish of inoculum Prov. 10^8 × 6 CFU on day 7 IP are represented by an increase in the cellularity activity with circulatory disturbances as hyperplasia of mucus cells and edema (Fig. 5-A). A loss of the normal secondary gills architecture which has curling appearance was observed. Additionally, the infiltration of inflammatory cells leads to adhesion at the apex of secondary gills filaments with severe congestion of capillary (Fig. 5-B). Additional cellular injuries observed in this study included vacuolar degeneration of pillar cells, chloride cells, and undifferentiated cells. These changes resulted in hypertrophy of the epithelial cells lining the secondary gill

filaments, along with noted hyperplasia. A marked decline in the interlamellar space was also evident (Fig. 5-C, D). These adaptive responses led to an adhesion between the secondary gill filaments at their apices, accompanied by epithelial sloughing and ruptured blood vessels (Fig. 5-E, F), as well as shortening and distortion of the normal structure of the secondary gill filaments.

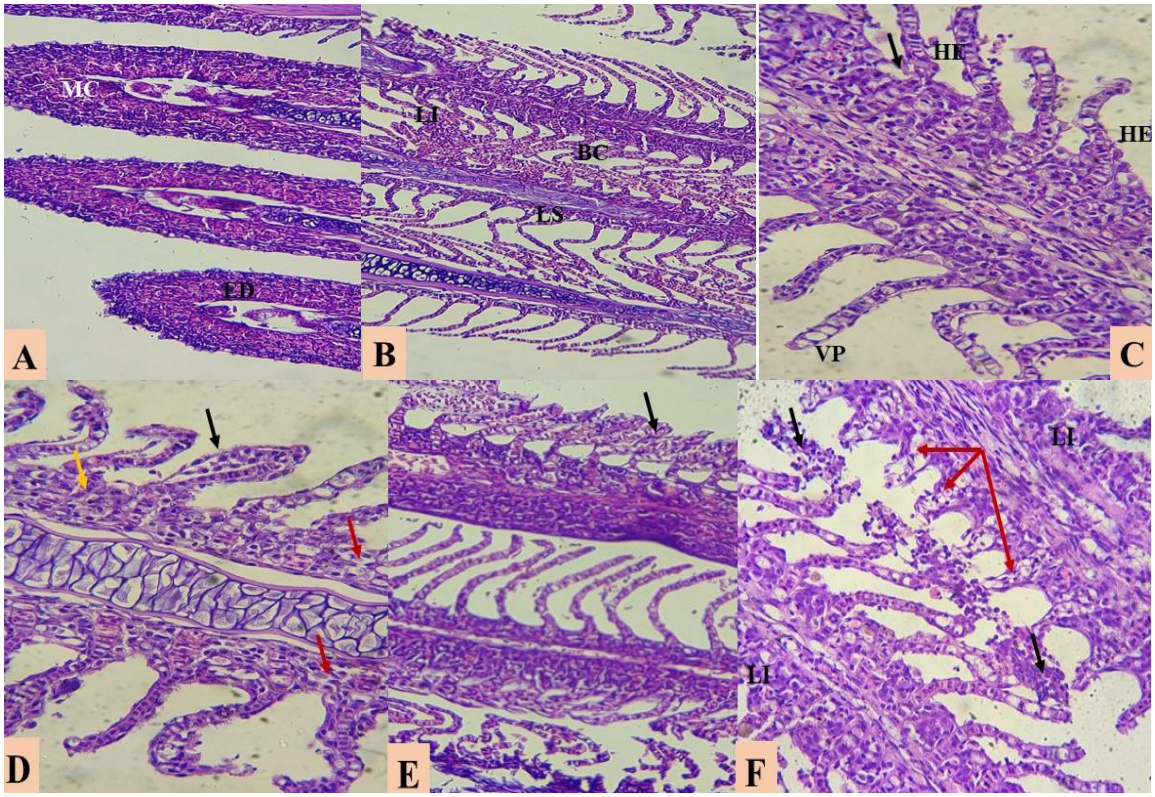

Fig. 5. Microscopic examination of gill arches from fish inoculated with 1.08 × 10^6 CFU on day 7 post-infection (IP). **(A)** Edema (ED) and hyperplasia of mucous cells (MC) observed at 40× magnification. **(B)** Curling appearance and loss of structural alignment of secondary gill lamellae (LS), along with blood vessel congestion (BC) and leukocyte infiltration (LI), ×40. **(C)** Hypertrophy of epithelial cells lining the secondary gill filaments (HE), leukocyte infiltration (LI), and vacuolation of pillar cells (VP), 100×. **(D)** Chloride cells (red arrow), undifferentiated cells (yellow arrow), and epithelial hyperplasia (black arrow), 400×. **(E)** Adhesion between secondary gill filaments and ruptured capillary vessels (black arrow), ×100. **(F)** Sloughing of gill epithelium (black arrow) and shortening of secondary gill filaments (red arrow), 400×. Stained with Hematoxylin and Eosin (H&E)

The histological analysis demonstrates irreversible gills damage; all gills obtained from fish inoculated with 10^8 × 12 and 10^8 × 6 CFU showed HAI equal to 216 and 136, which are highly significant in comparison to the control group (20). Based on the quantification and frequency of histological alteration (Table 3), score 3 with severe alterations were more common in fish inoculated with 10^8 × 12 CFU, whereas score 1

and 2, with slight and moderate alterations, were often investigated in fish inoculated with 108 × 6 CFU, (Table 5).

Table 5. Semi-quantities analysis of histological alteration index (HAI)

Groups	Freq	%	Chi^2 value
Control	20	5.38	156.64 **
IP: 10^8 ×12 CFU	216	58.06	
IP: 10^8 ×6 CFU	136	36.66	
Total	372	100%	

** refers to high significant difference at $P < 0.01$ between groups according to Chi squared test.

Quantification and histochemistry of mucus cells

Mucous cells in both experimental groups (10^8 × 12 and 10^8 × 6) CFU was dependent as principle of both primary filaments and secondary lamellar. The histochemistry analysis revealed a positive AB staining reactivity of acid mucus – glycoconjugates and uniform distribution at the apex and middle region of gills filaments (Fig. 6A, B, and C). Additionally, the statistical analysis showed a highly significant ($P \leq$ 0.05) counting of mucus cells in both groups in contrast to control group. These cells significantly occupy the apex of the gill filaments of fish in the second group (Table 6).

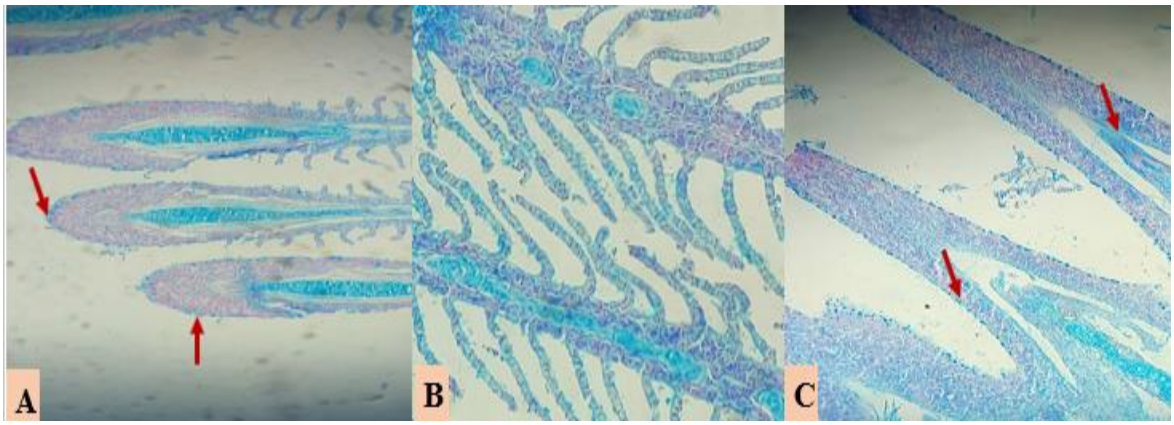

Fig. 6. Histology and histochemistry in gills of *C. carpio* inoculated *P. alcalifaciens* at 10^8 × 12 and 10^8 × 6 CFU on day 7 I, **(A)** Mucus cells (MC) at the apex 100X and **(B)** in the middle, 400X, uniform distribution of acid mucus cells secretion **(C)**, Positive stained for AB, 100X

Table 6. Semi-quantities analysis of mucus cells/mm^2 distribution in the three regions in gills filaments

Groups	Apex	Middle	Base
Control	10.00±1.00 d	6.11±1.05 e	2.33±0.25 f
10^8 × 12 CFU	29.00±2.83 b	16.67±0.87 c	3.78±0.83 ef
10^8 × 6 CFU	59.78±6.92 a	26.78±3.07 b	28.56±1.67 b

The different letters refer to significant differences between groups at $P \leq 0.05$.

DISCUSSION

In general, outbreaks of bacterial pathogens are mainly responsible for several diseases and high mortality in both wild environment and farm-cultured fish. The microorganisms vary from a main pathogen to that of an opportunistic, making their fish diseased by commencing pathogenesis and infection expansion. Currently observed ulcerative and hemorrhagic septicemia diseases breakout are major diseases in the carp fish **(Mastan & Ahmed, 2013)**.

The reports on *Providencia* spp. associated with fish-sources are rare. To date, only one study investigated *Providencia stuartii* as a causative agent of infection and mortality, isolated from *L. rohita* **(Ramkumar *et al.*, 2014)**. This current study has successfully molecularly identified *P. alcalifaciens* from experimentally diseased carp fish. The nearly complete 16S rRNA genome sequence (515bp) confirmed the presence of *P. alcalifaciens* and its infection in fish through intraperitoneal injection. Moreover, the molecular analysis showed that our strain is closely related to *P. alcalifaciens* with a 100% similarity in 16S rRNA sequence analysis.

This study is the one of the early investigations of *P.alcalifaciens* pathophysiology in *C. carpio*. In the current study, creatine kinase (CK) activity in fish serum was significantly inhibited on day 7 post-infection. These findings are consistent with the results reported by **Baldissera *et al.* (2019)**. CK is a key enzyme in the phosphoryl transfer pathway, playing a vital role in maintaining cellular energy balance through the reversible conversion of creatine (Cr) to phosphocreatine (PCr). It is especially important in organs with high and fluctuating energy demands, such as the gills, muscle, and brain **(Schlattner *et al.*, 2006)**.

The observed reduction of both mitochondrial and cytosolic CK—particularly in gill tissue—suggests impairment of branchial bioenergetics. This is likely due to an imbalance in the ATP/ADP and PCr/Cr ratios, reflecting disrupted communication between ATP-producing and ATP-consuming sites. Such depletion can severely impact oxidative phosphorylation and energy metabolism **(Baldissera *et al.*, 2017)**.

Furthermore, the pathophysiology of the disease may be influenced by the inhibition of ATP-dependent enzymes, such as Na^+/K^+-ATPase, due to a potential decrease in ATP content caused by disruption in the phosphoryl transfer network **(Baldissera *et al.*, 2017)**. Our findings revealed a significant reduction in the concentrations of sodium, potassium, and chloride ions in the serum of carp—key elements for osmoregulation. This ionic imbalance likely led to the downregulation of Na^+/K^+-ATPase activity, consistent with previous observations by **Baldissera *et al.* (2020)**. According to **Lucu and Towle (2003)**, Na^+/K^+-ATPase plays a critical role in maintaining electrochemical ion gradients across the cell membrane and is essential for energy-driven processes in gill tissue.

Inhibition of this enzyme disrupts normal gill function, as observed in the Nile tilapia experimentally infected with *Providencia alcalifaciens*, where reduced Na^+ extrusion and impaired K^+ uptake was reported **(Souza *et al.*, 2019)**. The prominent histopathological lesions observed in the gills of fish on day 7 post-infection in our study may therefore be attributed to these disruptions in energy-dependent enzyme function and ion regulation. These lesions resemble those caused by *Providencia* spp. in *Labeo rohita* **(Ramkumar *et al.*, 2014)**.

Disruption of CK and Na^+/K^+-ATPase activity leads to imbalances in sodium retention and excessive potassium excretion—early indicators of reactive cellular damage and vacuolar degeneration. Additionally, increased cytosolic calcium influx may activate phospholipase enzymes, resulting in mitochondrial and organelle membrane degradation. This cascade activates further ATPase and protease activity, accelerating ATP depletion and causing damage to the membrane and cytoskeleton, ultimately leading to altered tissue architecture. Activation of phospholipase also promotes arachidonic acid release, a precursor of inflammatory lipid mediators **(Zachary, 2017)**.

Semi-quantitative and histochemical analyses served as vital indicators of lesion severity **(Al-Ali & Al-Sabaawy, 2023; Al-Taee, 2024)**. Positive Alcian Blue (AB) staining indicated the presence of acidic mucous secretions—glycosaminoglycans—which aligns with findings by **Santos *et al*. (2011)**. Mucous cell hyperplasia, particularly at the apex of the gill filaments, was also observed in this study and is consistent with the report by **Carvalho *et al.* (2020)**. Physiologically, the secretion of mucus glycoconjugates serves multiple roles, including lubrication, ion regulation and diffusion, and protection against pathogens **(Whiter & Mittal, 2006)**.

Pathological changes in gill tissue, including hyperplasia, may represent compensatory defensive mechanisms to enhance respiratory surface area in response to infection **(Sollid & Nilsson, 2006)**. The observed blood congestion and aneurysms likely resulted from pillar cell damage, causing fusion of capillaries within the gill lamellae into uniform, blood-filled spaces. This impaired gas and nutrient exchange, and prolonged circulatory disturbances eventually led to necrosis and tissue death **(Martinez *et al.*, 2004; Strzyzewska *et al.*, 2016)**.

From both pathological and statistical perspectives, these lesions are indexed as Histopathological Alteration Index (HAI) and are considered severe and often irreversible **(Nascimento *et al.*, 2012)**. Additionally, the positive response to AB staining supports the presence of acidic glycoconjugates, further confirming the severity and nature of the tissue response.

CONCLUSION

This study suggests that *Providencia alcalifaciens* can infect *Cyprinus carpio* and significantly disrupt enzyme-dependent energy metabolism by impairing creatine kinase (CK) activity, which is crucial for the regeneration, consumption, and utilization of ATP.

Additionally, the infection leads to disturbances in ion homeostasis and results in highly significant histopathological alteration indices (HAI) within the gill filaments.

The quantification of microscopic lesions, supported by histochemical analysis, serves as a vital tool for evaluating and statistically validating the severity of cellular injury and adaptive responses. These alterations not only reflect structural damage but also provide insight into changes in the biological composition and functional distribution of essential elements within the gill architecture.

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