Haemato- histopathological effect of experimental infection of *Aeromonas hydrophila* isolates on *Oreochromis niloticus*

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Abstract

Motile Aeromonas Septicemia caused by *Aeromonas hydrophila* is an important bacteria disease of Nile tilapia (*Oreochromis niloticus*). The study was aimed to evaluate the effect of the infection on the blood parameters and the architectural integrity of the tissues of the fish. Experimental *A. hydrophila* infection of *O. niloticus* juveniles was carried out using isolates from infected fishes of the Kainji lake area. Following intraperitoneal injection, the effect of the bacteria on the gross, hematological and histological integrity of the fish was analyzed. The bacteria isolate induced mortality and morbidity on the fish. There were significantly ($p<0.05$) lower red blood cell and higher total white blood cell counts in experimentally infected fishes than the control. Lesions observed following *A. hydrophila* infection included dilation of the sinusoids and vacuolation of hepatocytes of the liver, interstitial necrosis with infiltration of lymphocytes and macrophages in the kidneys. Macrophage hyperplasia and lymphoid depletion in the spleen, fusion of adjacent secondary lamellae, epithelial necrosis and desquamation in the

gills were recorded in the infected fishes too. The study demonstrates that *A. hydrophila* induces both hematological and histopathological alterations in the tissues of infected *O. niloticus*.

Key words: *Aeromonas hydrophila*, *Oreochromis niloticus*, Haematology, Histopathology, Kainji Lake

1. Introduction

Aeromonas hydrophila is a ubiquitous, Gram negative, motile, rod-shaped, oxidase positive, catalase positive, glucose fermenting bacterium which can be commonly isolated from freshwater ponds (Li, Zhu, Ringø, & Yang, 2020). Diseases caused by *A. hydrophila* have been observed in some freshwater fish species such as tilapia (Zaher *et al*., 2021), African catfish (*Clarias gariepinus*) (Kusdarwati, Kurniawan, & Prayogi, 2017) and other fish species where it causes a systemic disease known as "Haemorrhagic septicaemia", "Motile Aeromonas septicaemia". This disease is characterized by ulcers of various degrees on the skin, abscesses and haemorrhages in the internal organs of the peritoneal cavity (Alavinezhad, Kazempoor, Ghorbanzadeh, & Gharekhani, 2021). The organism haemolyses red blood cells and hydrolyses esculin. *Aeromonas hydrophila* is very toxic to many organisms (Rey, Verjain, Ferguson, & Iregui, 2009). The infection spread in the system of the susceptible host by way of the blood circulatory system and on getting to any organ causes extensive destruction of the tissues with the help of the aerolysin cytotoxic enterotoxin it produces (Singh, Rathore, Kapoor, Mishra, & Lakra, 2008) and these have been attributed to the pathogenicity of bacterium (Abd-El-Malek, 2017). Histopathological manifestations in liver, kidney, pancreas and intestines have been reported in fish infected with *A. hydrophila* with extensive focal necrosis in muscle, liver and

pancreas (Al Yahya et al., 2018). Renal tubular necrosis, depletion of the cells in the tubular interstitium and glomerular necrosis has also been reported (Azad, Rajendran, Rajan, Vijayan, & Santiago, 2001). Scientists also reported severe intussusceptions and wrinkling of the intestinal wall of tilapia hybrid infected with *A. hydrophila* (Rey et al., 2009). Blood participates directly or indirectly in almost all biochemical processes in the body and its composition is usually altered during diseases and malnutrition condition (Bello-Olusoji *et al.*, 2007). Analysis of haematological profile has been employed by fish disease experts in the diagnosis of disease conditions in fish (Hamid, Mohd Daud, Srisapoome, Abu Hassim, & Mohd Yusoff, 2018). In an experimental infection of Hybrid catfish with *A. hydrophila*, (Koeypudsa, Jongjareanjai, Phalitakul, & Punnarak, 2020) established the link between changes in the blood profile and a disease conditions. In stressed fish, resistance to disease decreases, the metabolic processes and assimilation of food are disrupted. These lead to morphological, biochemical and physiological changes in response to the stressful condition and this implies profound disturbances in metabolism and in the functioning of enzymatic, nervous and other systems. The objective of this study was to determine possible effect of *A. hydrophila* infection on the blood parameters of *O. niloticus* and also to assess the changes induced in the histology of the tissues following experimental infection.

2. Materials and methods

2.1. Experimental Animal

Post fingerlings (juveniles) of *Oreochromis niloticus* were obtained from the hatchery complex of the National Institute for Freshwater Fisheries Research (NIFFR), New Bussa, Nigeria for the study. The fish were acclimatized for two weeks prior to the commencement of the study. The weights and the lengths of the experimental fish were taken with weighing balance and

measuring boards respectively prior to stocking and the average weight of 41.8±0.98g (37.8 – 56.2) and standard length of 14.4±0.68cm (12.6 – 16.0) was obtained. All the experimental fish were certified as showing no clinical presentation of any bacterial infection before stocking. A total of nine experimental indoor glass aquarium systems of 40.5L (30cmX30cmX45cm) containing air stones were used for the experiment. The glass aquaria were covered with a net of mesh size 3mm to protect the fish from jumping out and from predators. Weekly monitoring of water quality parameters such as pH, temperature and dissolved oxygen were carried out using pocket-sized pH meter manufactured by Hanna instruments and portable dissolved oxygen (DO) meter JPB – 607 A respectively. The water quality parameters recorded such as DO (5.04 ± 0.62); Temperature (25.44 ± 1.38^{0}C) and pH (6.08 ± 0.12) were within the tolerable limits for fish culture (Timmons and Losordo, 1994).

2.2. Bacterial strain

Characterized *Aeromonas hydrophila* isolates used for the study were isolated from diseased catfishes (*Clarias gariepinus*). Isolation and identification of the bacteria was carried out following the standard morphological (Holt, 1982) and biochemical assays (Cheesebrough, 2002). The isolates were also subjected to polymerase chain reaction (PCR) and molecular studies to confirm the organism (Imron *et al*., 2020). Prior to use, the bacteria colonies were homogenized in phosphate buffered saline and turbidity adjusted to correspond to 0.5 McFarland's turbidity standard (equivalent to 1 x 10^8 colony forming units/ml). The pathogenicity (LD_{50}) of the isolate was previously determined to be 10^8 CFU/ml and therefore was considered as the suitable dose.

2.3. Experimental Design

The experimental design was that of three treatment group (A, B and C) each with three replicates. Each aquarium represents a replicate and the stocking density were 40 fish per replicate making a total of 120 fish per treatment group. Fishes in group A were injected intra-peritoneally (ip) using 1ml tuberculin syringe and a 26 gauge needle with 0.2ml of a 0.5% saline suspension containing approximately 10^8 cfu of *Aeromonas hydrophila.* Group B were infected by immersing them in appropriate tank containing 1ml/ liter of *A. hydrophila* inoculums while fishes in group C were injected with phosphate buffered saline (PBS) only to serve as control. The experiment lasted for 5 weeks (35 days) and the infected fish were monitored daily for the manifestation of clinical signs of the disease and all mortalities removed and recorded as they occurred. Infection was confirmed by re-isolating the bacteria from the intestine of dead fishes.

At the end of the experimental period, sixty fish samples were randomly selected from the surviving fish from each of the treatment groups and the weights and total lengths measured. The analysis of the blood of *O. niloticus* experimentally infected with *A. hydrophila* isolates and the control were carried out. At the end of the experimental period, blood samples were collected from the caudal vessels of the surviving fish with the aid of 3ml disposable plastic syringes (containing EDTA) and a 21 gauge disposable hypodermic needle for evaluation of the parameters. Blood were collected from sixty of the surviving fish from each experimental group. The universal bottles treated with ethylene diamine tetra acetic acid (EDTA) were used for the blood collection for hematological determination. The collected blood was well mixed with the anticoagulant. The hematological parameters assessed include red blood cell count (RBC), white blood cell count (WBC), Hemoglobin and Haematocrit (PCV). Derived Erythrocytes indices

such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were also calculated.

At the end the experimental period, 20 fish samples from the infected and control groups were selected, killed by over dosing with anesthetic agent (clove oil), the abdomen dissected and then fixed in 10% buffered formalin with two changes at 24 hour intervals. Some pieces of tissues were also dissected out from the liver, kidney, gill and spleen of each specimen and prepared histologically according to the method adopted by Culling (1963).

Statistical analysis

Results are presented in tables and as mean with standard deviation. All data collected were subjected to single factor analysis of variance (ANOVA). Variant means were separated using Bonferroni (Dunn) t test. Mean differences with $p<0.05$ were considered statistically significant.

3. Results

3.1. Clinical signs of infection

About two to three hours post injection with the bacterium, there were reddening and swelling at the site of injection in both the control and the fish inoculated with the isolate but by the second day the swelling noticed on the control group of fish started to disappear while those of the infected remained. Also on that second day, it was noticed that some of the infected fish were off feed and clustered at the blind end of the aquaria bottom. By the third day, loss of scales at the site of injection was observed in tilapia injected with the bacterium. At the end of the first week, 8 fish has died among the group A infected by intraperitoneal injection while 7 was recorded among the group B infected by immersion, 5 deaths were also observed among the group C

(control). Whereas the mortality continued and rose gradually to a peak on the 3rd week among the infected groups (A and B), it reduced among the control. By the day 35 which was the terminal day for the experiment, mortality was still observed but reduced drastically. A total of 15 fish died among the control group representing 12.5% while group A infected by intraperitoneal injection recorded 69 deaths (57.5%) while the group B infected with the bacterium by immersion recorded 71 deaths (59.2%). There were no discernible differences in the pattern of mortality curve observed among the two infected groups as shown in figure 1. Respiratory distress was evident before death in most of the dead fish.

FIGURE 1

3.2. Morphometric characteristics and specific growth rate of *O. niloticus* infected with *A. hydrophila* and the uninfected control.

At the end of the experimental period of 35 days, there were no significant differences ($p>0.05$) in the mean body weights, mean total lengths and specific growth rates of the group A infected by intraperitoneal injection and those of group B infected by immersion in the bacteria inoculums. Whereas there were significantly ($p<0.05$) higher mean body weight, mean total length and specific growth rates obtained among the control group compared with those of the infected groups (A and B) as shown in Table 1.

TABLE 1

3.3. Haematological Changes

The mean values for the haematological parameters of *O. niloticus*, infected and the control are shown in Tables 2. There were significant decrease ($p<0.05$) in mean RBC counts of the infected groups (A and B) when compared with that of the control while there were significant increase ($p<0.05$) in the WBC and the MCH of the infected fish. However, there were no significant

differences ($p>0.05$) in the haemoglobin, PCV, MCV and MCHC of the infected and the control fish as shown in Table 2. It was observed that there were no significant differences in the hematological parameters of the group A infected by intraperitoneal injection and those of group B infected by immersion into the bacteria inoculums.

TABLE 2

3.4. Histological observations

The photomicrographs of the fish tissues of the experimentally *A. hydrophila* infection shows varied effects of the bacterium on the tissue architecture. Figures 2, 3, 4 and 5 shows various histopathological changes induced on the tissues of *O. niloticus* when infected with *A. hydrophila*. The pathological lesions were similar among the group infected either by intraperitoneal injection of the bacterium or by immersion of the fish on the bacteria inoculums. However, there were no histological lesions on the control group injected with phosphate buffered saline only.

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

4. Discussion

Aeromonas hydrophila is an important disease causing pathogen in fish (Adeshina, Jenyo-Oni, Emikpe, Ajani, & Abdel-Tawwab, 2019) and it manifests varied pathology in both natural and experimental infection. Reddening, swelling and necrotic ulceration first on the site of injection

of the bacterium noticed in this study are similar to the observation of Azad et al. (2001). They attributed reddening and pronounced necrotic ulceration observed to haemolysin and protease activity of *A. hydrophila*. De Figueiredo and Plumb (1977) were of the opinion that *A. hydrophila* isolated from diseased fish are more virulent than the water borne pathogen. At the end of the experimental period of 35 days, 57.5% mortality was recorded in *O. niloticus* infected with the bacteria through intraperitoneal injection and 59.2% infected through immersion in the bacteria inoculums against 12.5% obtained in the control. The mortality recorded among the control group may be due to the trauma of the injection with the phosphate buffered saline, this is because the mortality in this group was highest (5 fish) on the first week which then regressed thereafter until the end of the experimental period. The insignificant ($p>0.05$) differences in mortality rate observed among the group infected by intraperitoneal injection when compared to the group infected by immersion in the bacteria inoculums indicate that the route of infection does not affect the infectivity of the bacteria. This finding is not out of place owing to the fact that several experimental infections employing either immersion or intraperitoneal injection methods indicated successful infections with *A. hydrophila* (Anyanwu, Chah, & Shoyinka, 2015; Ibrahim, Eleiwa, Galal, El-Ekiaby, Abd El Rahman, 2020; Pauzi *et al*., 2020). However, the significantly ($p<0.05$) higher mortality rate recorded among the infected groups compared to the control shows the pathogenicity and virulence of *A. hydrophila* to *O. niloticus*. The significantly ($p<0.05$) reduced mean body weights, mean total lengths and specific growth rates recorded among the infected groups when compared to the control may be due to reduced food intake and the disturbances in the general wellbeing occasioned by the disease condition induced by the infection. This is in agreement with Adeshina *et al*. (2019) and Neamat-Allah, El-Murr, & Abd

El-Hakim (2019) which both studies reported adverse effect of *A. hydrophila* on the growth and survival rates of *C. gariepinus.*

Results of the haematological examination of the infected *O. niloticus* and that of the control indicate the possibility of the organism inducing different changes in the haematological parameters of the fish species. The changes induced by *A. hydrophila* isolates among the infected fish as observed in the study shows significantly ($p<0.05$) lowered RBC among the infected group compared with the control. There were higher values of white blood cells among the infected group than their control counterparts. The difference in WBC and MCH were also statistically significant ($p<0.05$). The changes induced by the bacterial isolates on the haemoglobin concentration, MCV, MCHC and the packed cell volume of the infected and the control were not statistically significant ($p>0.05$). In the present study, the lowered erythrocyte with normal MCHC demonstrates normochromic anaemia which most probably is non-regenerative which is common in bacteria infection (Tiwari & Pandey 2014). It has been shown that *A. hydrophila* and other pathogenic infections alter blood values. Raji *et al*. (2019) observed similar changes in the haematological profile of *C. gariepinus* infected with *A. hydrophila* and they posited that the reduced RBC counts were an indication that the infection affected the haemopoiesis system severely. The decrease in the erythrocyte may also be due to the haemolytic activity of the *A. hydrophila* organism, according to Youssef (2019) pathogenic strains of the bacteria are capable of exhibiting high haemolytic activity in an infection. Haghparast, Alishahi, Ghorbanpour, & Shahriari (2020) and Elayaraja *et al*. (2020) observed that intra-specific changes in blood parameters occur as a result of stress caused by diseases, malnutrition and unfavourable environmental factors. As an aquatic organism, the circulatory system of a fish is in close association with its environment and is sensitive to external stimuli

and this is reflected on the homeostasis of the fish. Systemic response to such stimuli leads to changes in blood parameters. The pattern of changes in this study was similar to that observed in other bacterial infections. Barham, Smit, Schoonbee (1980) reported a decreased RBC in bacterial infection of rainbow trout (*Salmo gairdneri*). Ogbulie and Okpokwasili (1999) also reported similar changes in blood parameters of *Clarias gariepinus* and *Heterobranchus bidorsalis* infected with bacteria organisms.

The histological studies revealed changes in the architecture of the internal organs resulting from the invasive activities of the bacteria leading to structural collapse and erosion of the tissues of infected fish. Normal architecture of the liver was disrupted leading to the dilation of sinusoid, vacuolation of hepatocytes, multifocal perivascular coagulative necrosis and massive hepatic degeneration. The extent of damage to the liver of the infected fish is obvious and suggests that the bacteria may have a specific hepatotoxic effect. Virulence and toxigenicity are associated since virulent strains produce toxins whereas avirulent strains most of the times do not. There have been few reports of changed liver function after exposure of fish to *A. hydrophila* organism. In some cases, liver dysfunction was identified (Aydin and Ciltas, 2004) whereas (Ventura and Grizzle, 1988) found no histopathological changes in channel catfish (*Ictalurus punctatus*) after exposure to the bacteria. The effects of *A. hydrophila* on the tissue architecture has been reported in other animals, Ocholi and Spencer (1989) reported patchy areas of focal necrosis in the lung, liver and kidneys of Caracal lynx (*Fellis caracal*) infected with the bacteria. According to Juntarut, Kaewnopparat, Faroongsarng, & Chiayvareesajja (2018), necrosis and degeneration observed histologically in *A. hydrophila* infection are as a result of protease, haemolysin and leukocidin production by the bacteria. The proteases and haemolysins digest tissues and destroy erythrocytes while leukocidin is an exotoxin that kills inflammatory cells. The marked interstitial

congestion, interstitial necrosis with infiltration of lymphocytes and macrophages observed in kidney tissues is an indication that the bacterium has it predilection site in kidneys among other organs of the infected fish. This observation is similar to those of Angka (1990) who opined that the most prominent histopathological changes occurred in the kidney and liver. El-Salam, Ghaly, Baraka, Mahmoud, & El-Makhzangy (2018) while concurring that the liver and kidneys are target organs of *A. hydrophila* infection opined that the liver may become pale and green while the kidneys may become swollen and friable. Ahmed and Shoreit (2001) also reported focal coagulative necrosis in kidneys of *O. niloticus* infected with *A. hydrophila.* The macrophage hyperplasia and lymphoid depletion observed in the spleen of the infected fish indicates an adverse effect on the haematopoietic system of the fish. According to Dalmo, Ingebrigtsen, & Bogward (1997) and Manca, Glomski, & Pica (2019) the thymus, kidney and spleen are the principal lymphomyeloid tissues of teleosts since they unlike mammals lack bone marrow and lymph nodes. Saharia, Pokhrel, Kalita, Hussain, & Islam (2018) also observed histopathological lesions in Indian Major Carp (Labeo rohita) infected with *A. hydrophila*. According to Laith and Najiah (2014), virulent *A. hydrophila* induced severe pathology on the spleen of experimentally infected fish. They opined that phagocytized bacteria usually destroy the endothelial and reticular cells of the splenic ellipsoids. Fusion of adjacent secondary lamella, epithelial necrosis and desquamation are the changes observed on the gill architecture of the infected fish. Further investigation into hematological, histopathological and immunological changes in diseased and antimicrobial treated tilapia (*Oreochromis niloticus*) is recommended

5. Conclusion

The study has demonstrated that *Aeromonas hydrophila* is pathogenic to Oreochromis niloticus since it induced both haematological and histological changes in the tissues of the infected fish

which signify the invasive nature of the bacteria. These clinical features may represent potential disruptive impact on the survival and overall production of the fish culture enterprise.

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Figure

Figures for SJST corrected manuscript

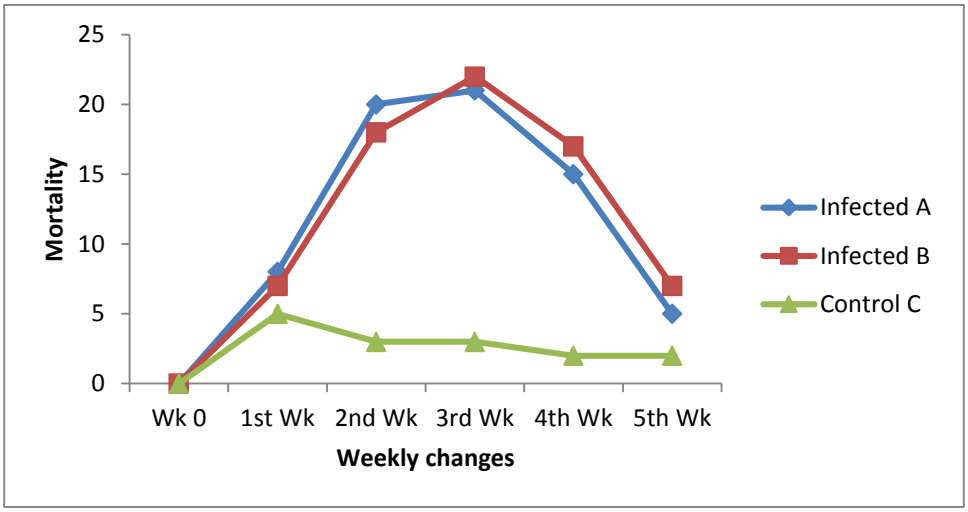

Figure 1: Mortality recorded among *Oreochromis niloticus* infected with *Aeromonas hydrophila* and the control

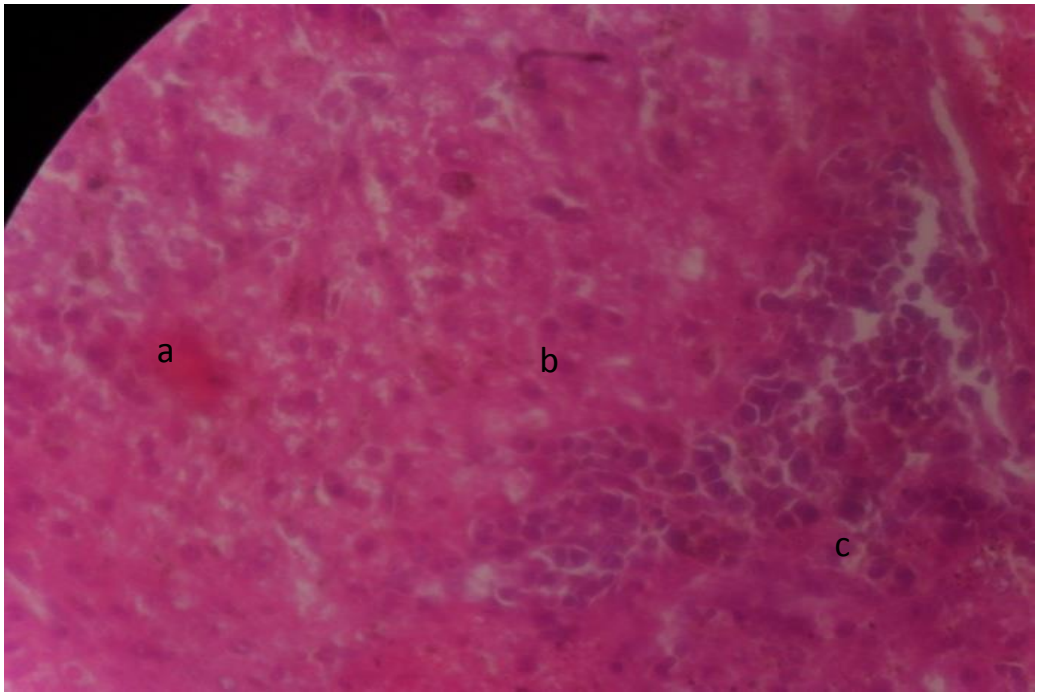

Figure 2: Histopathological changes observed in the liver tissues of fish infected with *A. hydrophila*: (a) Dilation of sinusoid, (b) Vacuolation of hepatocytes and (c) Hepatic degeneration H&E x100

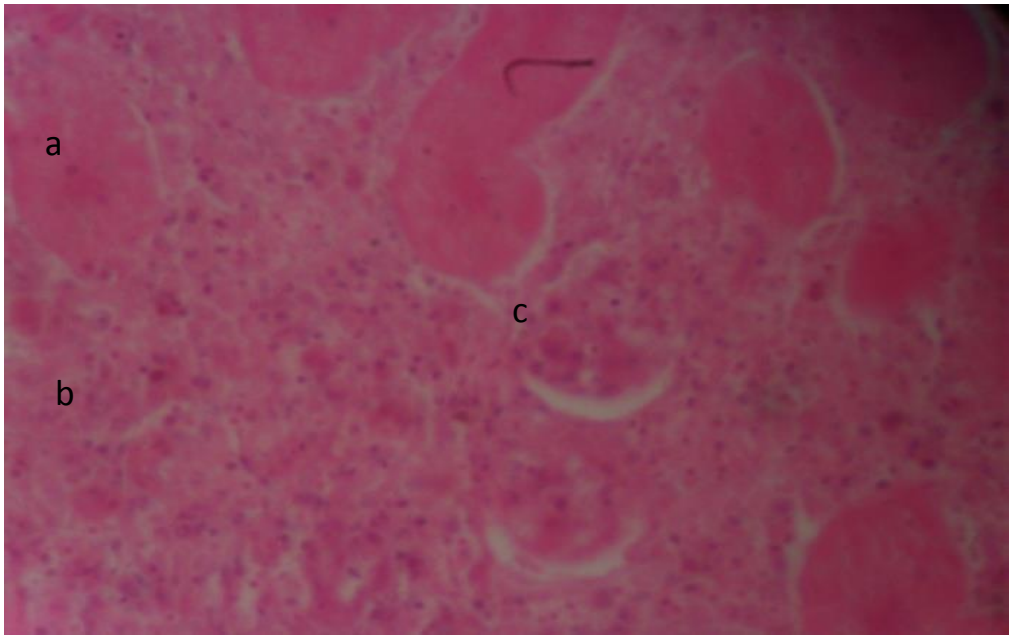

Figure 3: Histopathological changes observed in the Kidney tissues of fish infected with *A. hydrophila* include; (a) interstitial congestion, (b) Interstitial necrosis, (c) Infiltration of lymphocytes and macrophages H&E x100

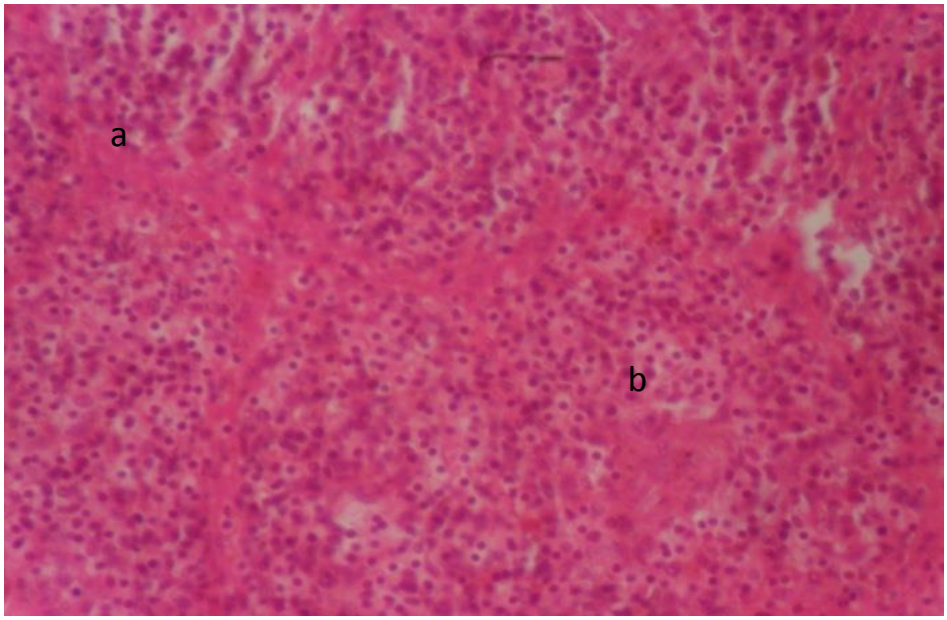

Figure 4: The histopathological changes observed in the tissues of the Spleen of fish infected with *A. hydrophila* include; (a) Macrophage hyperplasia, (b) Lymphoid depletion H&E x100

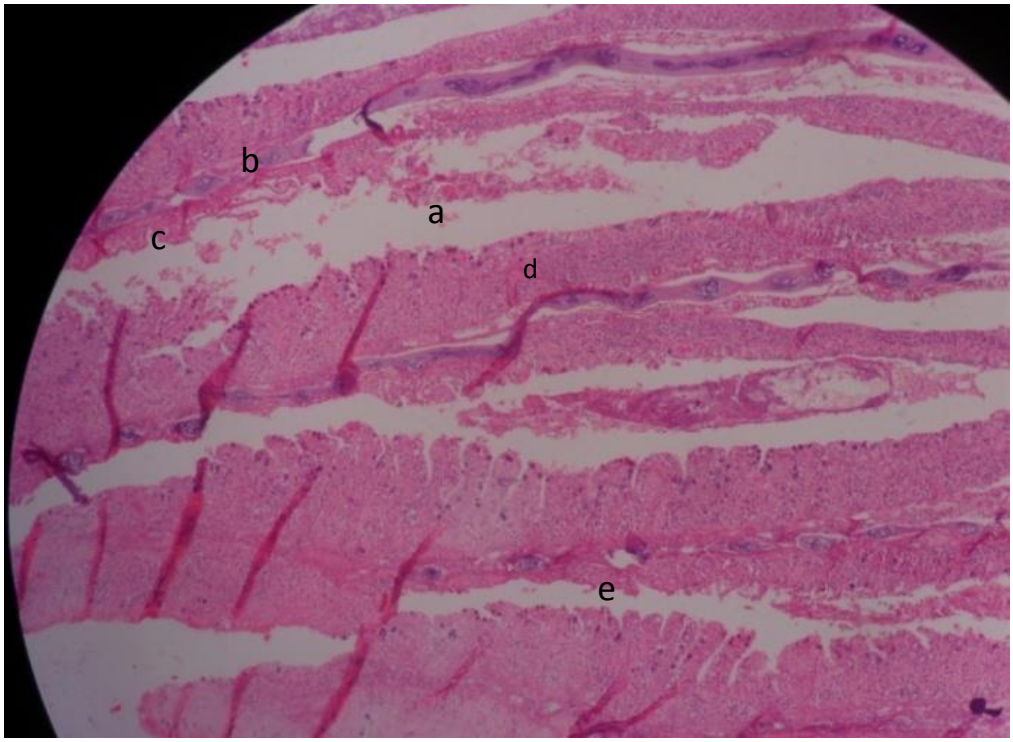

Figure 5: The histopathological changes observed in the gill tissues of fish infected with *A. hydrophila* include; (a) Fusion of adjacent secondary lamella, (b) Epithelial necrosis, (c) Desquamation (d) Bacteria plugs blocking the primary lamella (e) Hyperplastic epithelium of the secondary lamella H&E x100

Tables for SJST corrected Manuscript.

Table 1: Mean morphometric parameters and specific growth rate of *O. niloticus* infected with *A. hydrophila* after 35 days culture period

Treatment	**Mean Weight (g)±SE**	**Mean length (cm)±SE**	**Specific growth rate±SE**
A (infected)	48.35±2.56[a]	14.78±0.86[a]	0.92±0.06[a]
B (infected)	49.08±2.88[a]	14.56±0.72[a]	0.91±0.05[a]
C (control)	65.92±2.75[b]	16.98±0.22[b]	1.12±0.08[b]

[a,b] *Columns with different superscripts differ significantly ($p < 0.05$)*

Table 2: Mean values of haematological Parameters of *O. niloticus* infected with *A. hydrophila* and the control (uninfected).

Parameters	Infected A	Infected B	Control
RBC count ($X10^6$/mm)	2.01±0.11 [a]	2.03±0.10[a]	2.15±0.16 [b]
	(1.82-2.16)	(1.80 – 2.18)	(1.90-2.43)
WBC count ($X10^9$/l)	207.39±12.95 [a]	205.23±12.26[a]	191.28±13.96 [b]
	(178.3-226.9)	(180.5 – 220.5)	(165.9-210.2)
Haemoglobin (g/dl)	10.84±1.65 [a]	11.08±1.48[a]	10.06±1.57 [a]
	(8.6 -14.3)	(9.2 – 13.9)	(7.0-12.8)
Haematocrit %	29.52±4.96 [a]	32.01±5.02[a]	31.95±5.03 [a]
	(20.6-37.1)	(22.08 – 41.2)	(24.1-40.6)
MCV (fl)	147.63±25.90 [a]	149.32±31[a]	148.87±20.29 [a]
	(102.8-189.6)	(118.2- 180.0)	(121.5-178.1)
MCH (pg)	54.18±8.37 [a]	52.09±7.28[a]	47.85±8.45 [b]
	(40.0-72.2)	(39.6- 70.5)	(35.4-67.4)
MCHC (g/dl)	38.12±10.15 [a]	35.25±9.8a	32.52±8.48 [a]
	(23.2-55.6)	(21.6-48.7)	(20.5-53.1)

[a,b] Rows with different superscripts differ significantly (P < 0.05)

Response to reviewer's comments Ms. No. SJST-D-22-00255

Keywords: Kainji Lake was added in the list of keywords.

Introduction:

Comment 1: The title is mentioned on hematological parameters. The introduction part has not revealed how important is blood parameter in infected fish.

Response: The importance of blood parameters to infected fish was mentioned in the manuscript. However, additional importance of blood parameters was provided in the corrected copy.

Comment 2: The article does not show the objectives of this investigation

Response: The objectives of the study have been added as indicated.

Materials and Method:

Comment 1: Water quality had not been included in methods

Response: Water quality parameters have been included as indicated

Comment 2: Line 64-65; there are no unit for fish weight and length

Response: The units of weight and length have been included

Comment 3: Line 75; there is no reference for the PCR method, please provide

Response: This have been provided as highlighted

Comment 4: Line 77-78; please give more information, why the author used 10^8 CFU/ml to infect fish

Response: The reason why 10^8 CFU/ml was taken as the infective dose have been provided as highlighted in the corrected manuscript.

Comment 5: Line 90; why the infection was investigated from intestines, not immune-related organs (spleen, kidney).

Response: The organs mostly affected by Aeromonas hydrophila are the intestines, spleen and kidney (Rey, 2009) and any one of the organs can be used for the isolation of the bacteria.

Reference

Rey, A., Verjain, N., Ferguson, H.W. & Iregui, C. (2009): Pathogenesis of Aeromonas hydrophila strain KJ99 infection and its extracellular products in two species of fish. *Veterinary Record*, 164 (16), 493 – 499

Results

Comment 1: Is there any explanation the relationship between blood parameter and histopathological changes?

Response: The issues raised on the results have been addressed

Comment 2: Please describe bacterial results from dead fish during experiment (line 90). They died from Aeromonas infection or other reasons

Response: This was adequately done in figure 1 and lines 137 to 146.

Discussion:

Comment 1: Is there any explanation the relationship between blood parameter and histopathological changes?

Response: The issue raised by the reviewer concerning discussion has been addressed in the manuscript as highlighted.

Comment 2: Please give more information about the further study and advantages in a practical field

Response: This have been provided as highlighted in the corrected manuscript