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Original Article

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 bacterial disease of Nile tilapia (*Oreochromis niloticus*). This study aimed to evaluate the effects of infection on the blood parameters and on architectural integrity of tissues of the fish. Experimental *A. hydrophila* infection of *O. niloticus* juveniles was carried out using isolates from infected fish in the Kainji lake area. Following intraperitoneal injection, the effects of the bacteria on gross, hematological and histological integrity of the fish were analyzed. The bacterial isolate induced mortality and morbidity in the fish. There were significantly ($p<0.05$) lower red blood cell and higher total white blood cell counts in experimentally infected fish than in the control. Lesions observed following *A. hydrophila* infection included dilation of the sinusoids and vacuolation of hepatocytes of the liver, and 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 fish, too. The study demonstrates that *A. hydrophila* induces both hematological and histopathological alterations in the tissues of infected *O. niloticus*.

Keywords: *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, in which it causes a systemic disease known as "Haemorrhagic septicaemia", or "Motile Aeromonas septicaemia". This disease is characterized by ulcers of various degrees on the skin, and abscesses and haemorrhage 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 spreads in the system of a susceptible host by the blood circulation and on getting to any organ it 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 have also been reported (Azad, Rajendran, Rajan, Vijayan, & Santiago, 2001). Scientists have also reported severe

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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 or by malnutrition (Bello-Olusoji *et al.*, 2007). Analysis of the 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, and the metabolic processes and assimilation of food are disrupted. These lead to morphological, biochemical and physiological changes in response to the stressful conditions, 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 effects 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 animals

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 was 41.8±0.98 g (37.8 – 56.2) and length was 14.4±0.68 cm (12.6 – 16.0). 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 40.5 L (30 cm X 30 cm X 45 cm) containing air stones were used for the experiment. The glass aquaria were covered with a net of mesh size 3 mm to prevent the fish from jumping out and to protect them from predators. Weekly monitoring the water quality parameters pH, temperature, and dissolved oxygen, was carried out using a pocket-sized pH meter manufactured by Hanna instruments and a portable dissolved oxygen (DO) meter JPB – 607 A. The water quality parameters recorded had the ranges DO 5.04 ± 0.62; temperature 25.44 ± 1.38 ^{0}C; and pH 6.08 ± 0.12, and all were within tolerable limits for fish culture (Timmons and Losordo, 1994).

2.2 Bacterial strain

Characterized *Aeromonas hydrophila* isolates used in 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 bacterial colonies were homogenized in phosphate buffered saline and turbidity was 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 this was considered a suitable dose.

2.3 Experimental design

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

At the end of the experimental period, sixty fish were randomly sampled from the survivors of each treatment group, and the weights and total lengths were measured. The analysis of blood samples of *O. niloticus* experimentally infected with *A. hydrophila* isolates and of the control group 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 3 ml disposable plastic syringes (containing EDTA) and a 21-gauge disposable hypodermic needle for evaluation of the parameters. Blood samples were collected from sixty of the surviving fish in each experimental group. 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 were 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 of the experimental period, 20 fish samples from the infected and control groups were selected, killed by overdosing with an anesthetic agent (clove oil), the abdomen was dissected, and then fixed in 10 % buffered formalin with two changes at 24-hour intervals. Some tissue samples were also dissected from liver, kidney, gills, and spleen of each specimen, and prepared histologically according to the method adopted by Culling *(*1963*).*

2.4 Statistical analysis

Results are presented in tables as means with standard deviations. 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 it remained in the infected fish. 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 aquarium's 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 had died among group A that was infected by intraperitoneal injection, while 7 were recorded among group B infected by immersion, and 5 deaths were also observed among 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 group. By day 35, the terminal day of the experiment, mortality was still observed but had reduced drastically. A total of 15 fish died among the control group representing 12.5 %, while in group A infected by intraperitoneal injection 69 deaths (57.5 %) were recorded, and 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.

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, between group A infected by intraperitoneal injection and group B infected by immersion in the bacterial inoculum. However, there were significantly ($p<0.05$) higher mean body weight, mean total length, and specific growth rate in the control group, when compared with those of the infected groups (A and B) as shown in Table 1.

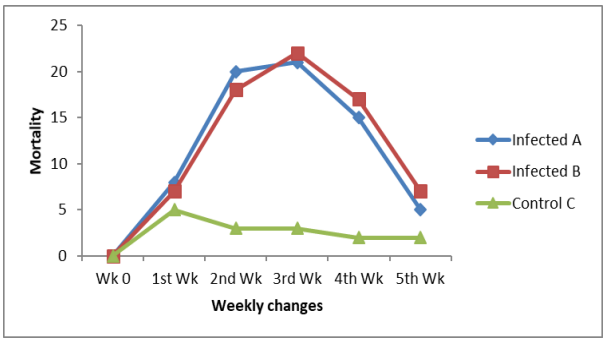

Figure 1. Mortality recorded among *Oreochromis niloticus* infected with *Aeromonas hydrophila* and the control.

3.3 Haematological changes

The mean values for the haematological parameters of *O. niloticus*, infected or control, are shown in Table 2. There was a significant decrease ($p<0.05$) in mean RBC count in the infected groups (A and B) when compared with that of the control, while there was a 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 between group A infected by intraperitoneal injection and group B infected by immersion into the bacterial inoculum.

Table 1. Mean morphometric parameters and specific growth rate of *O. niloticus* infected with *A. hydrophila* over a 35-day 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] Different superscripts within a column indicate significant differences ($p<0.05$)

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

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

[a,b] Different superscripts within a row indicate significant differences ($p<0.05$)

3.4 Histological observations

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

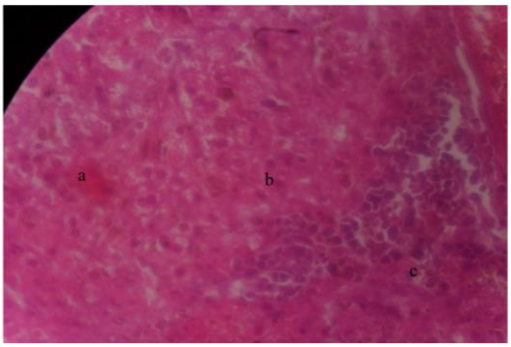

Figure 2. Histopathological changes observed in the liver tissues of fish infected with *A. hydrophila*: (a) dilation of sinusoid, (b) vacuolation of hepatocytes, and (c) hepatic degeneration (H&E, x100)

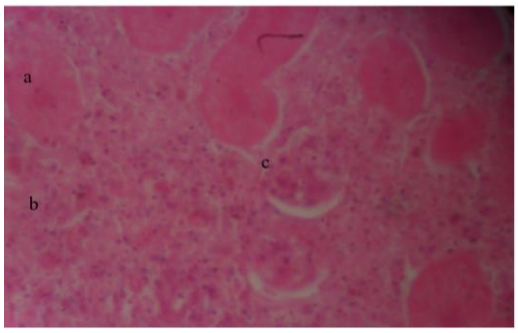

Figure 3. Histopathological changes observed in the kidney tissues of fish infected with *A. hydrophila* include (a) interstitial congestion, (b) interstitial necrosis, and (c) infiltration of lymphocytes and macrophages (H&E, x100)

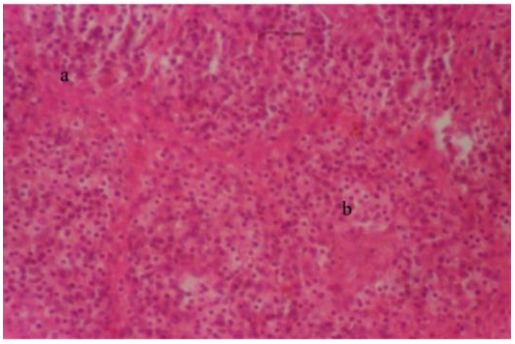

Figure 4. The histopathological changes observed in the spleen tissues of fish infected with *A. hydrophila* include (a) macrophage hyperplasia, and (b) lymphoid depletion (H&E, x100)

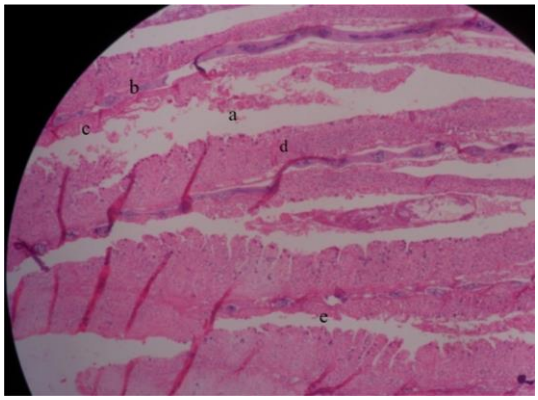

Figure 5. The histopathological changes observed in the gill tissues of fish infected with *A. hydrophila* include (a) fusion of adjacent secondary lamella, (b) epithelial necrosis, (c) desquamation, (d) bacterial plugs blocking the primary lamella, and (e) hyperplastic epithelium of the secondary lamella (H&E, x100)

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 infections. Reddening, swelling and necrotic ulceration first on the site of injection of the bacterium were noticed in this study, similar to the observations 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 % when infected through immersion in the bacterial inoculum, against 12.5 % in the control treatment. The mortality recorded among the control group may be due to trauma from the injection with phosphate buffered saline, since the mortality in this group was at its highest (5 fish) on the first week, but 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 and that infected by immersion in the bacterial inoculum indicates 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 have 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 attributed to 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) both of which studies reported adverse effects of *A. hydrophila* on 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 pathogen 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 show significantly ($p<0.05$) lowered RBC among the infected groups compared with the control. There were higher levels of white blood cells among the infected groups than in their control counterparts. The differences 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 cases were not statistically significant ($p>0.05$). In the present study, the lowered erythrocyte with normal MCHC demonstrates normochromic anemia, which most probably is non-regenerative as is common in bacterial infections (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, as according to Youssef (2019) pathogenic strains of the bacteria are capable of exhibiting high haemolytic activity in an infection. Haghparast, Alishahi, Ghorbanpour, and 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 unfavorable 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.

The histological studies revealed changes in the architecture of the internal organs resulting from 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 dilation of the sinusoid, vacuolation of hepatocytes, multifocal perivascular coagulative necrosis, and massive hepatic degeneration. The extent of damage to the liver of the infected fish was 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 time 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. Effects of *A. hydrophila* on the tissue architecture have been reported in other animals, and 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, and Chiayvareesajja (2018), necrosis and degeneration were 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 indicate 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, and 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 spleen of the infected fish indicate adverse effects on the haematopoietic system of the fish. According to Dalmo, Ingebrigtsen, and Bogward (1997) and Manca, Glomski, and 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, and 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 in 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 changes observed in the gill architecture of infected fish. Further investigation into hematological, histopathological and immunological changes in diseased and antimicrobial treated tilapia (*Oreochromis niloticus*) is recommended

5. Conclusions

The study has demonstrated that *Aeromonas hydrophila* is pathogenic to Oreochromis niloticus since it induced both haematological and histological tissue changes in the infected fish, which signify the invasive nature of the bacteria. These clinical features represent potentially disruptive impacts on survival and overall production the fish when cultured.

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