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RESEARCH ARTICLE **OPEN ACCESS**

Effects of Single and Co-infections of *Proteus Mirabilis* and *Aeromonas Hydrophila* on Baseline Hematological, Serological, and Histological Data in Cultured *Clarias Gariepinus*

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Abstract:

Background and Aim: Significant mortality and production disruptions in fish culture are brought on by diseases and parasites. The purpose of the current study was to collect baseline data on the effects of single and co-infections of *Proteus mirabilis,* and *Aeromonas hydrophila* in *Clarias gariepinus*.

Materials and Methods: One hundred and twenty sub-adults of *C. gariepinus* were divided into control, *P. mirabilis*, *A. hydrophila*, and co-infection groups (*P. mirabilis* X *A. hydrophila*). Standard methods were used to determine hematology, serology, and histology. Standard microbiology methods were used for microbial analysis.

Results: The single *A. hydrophila*-infected group had the highest mortality (60% *versus* 37%) in the co-infected and *P. mirabilis* groups. A marked decrease was observed in the RBC, hemoglobin, and Packed Cell Volume (PCV) of 2.9×10^{12}/L, 32.8 g/L, and 33.5% in the co-infected fish, compared to the control with 3.6×10^{12}/L, 35.8 g/L, and 41.0%, respectively. Alanine transaminase, alkaline phosphatase, and aspartate transaminase levels were significantly lower in the co-infected fish (13.8, 236.0, and 66.3, respectively) compared to the *A. hydrophila*-infected group. Creatinine and urea levels were, however, higher in the co-infected treatment. The kidneys and livers of the *A. hydrophila* and co-infected groups were more severely damaged than those of the *P. mirabilis* and control groups. Vacuolation and necrosis of hepatocytes led to the desquamation of tubular and glomerular epithelial cells in the livers and kidneys of infected fish. Fish infected with *A. hydrophila* had the highest bacterial load count.

Conclusion: It was concluded that an antagonistic association exists between *A. hydrophila* and *P. mirabilis* when they are co-infected.

Keywords: *Aeromonas hydrophila*, *Clarias gariepinus*, Multiple infection, Pathology, Produce, *Proteus mirabilis*.

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1. INTRODUCTION

Food and nutrition insecurity has been a serious subject of concern to Nigerians and Africans in general resulting to several health concerns [1, 2]. Fish play an important role in meeting food and nutrition problems with Nigeria being among the world's highest fish consumers, with a consumption of over 3.2 million metric tons of fish per year [2]. Nigeria's aquaculture output has increased by 12 percent yearly for the last 35 years when compared to 8% globally. From just over 6,000 metric tons in 1980 to about 307,000 metric tons in 2016, Nigeria has become one of Africa's top fish producers, next to Egypt [2]. Nigeria produces the most farmed fish in sub-Saharan Africa. This accounts for 52% of all fish produced on that continent. The Nigerian aquaculture industry is primarily focused on freshwater fish. In 2015, the production of the *Clarias* species accounted for 64% of total aquaculture production [3]. Aside from *Clarias* and *Heterobranchus* spp. (catfish), other fish species are also cultivated in Nigeria, including *Tilapia* spp. (tilapia), *Cyprinus carpio* (common carp) and *Heterotis niloticus* (slap water) [1, 4]. However, this expansion of aquaculture, and specifically fish farming, brings with it the introduction of parasites and diseases. Diseases and parasites are responsible for significant catfish mortality and production disruptions due to missed feeding times (growth) and treatment with chemicals or antibiotics (economic expenses).

Disease issues are a serious menace in the fish farming industry. This menace is a limitation to sustainable aquaculture production and product trade [5]. In nature, co-infections are common. They occur when two or more pathogens infect the same host or when one pathogen causes a secondary infection. Mixed infections can make pinpointing the actual cause of death extremely difficult, further complicating treatment. Despite the fact that co-infections are common in an ordinary fish farming enterprise, there is little knowledge of them. Co-infections involving bacterial pathogens, viruses, and parasites can occur in pond habitats, resulting in various clinical symptoms and therapeutic problems [6]. Recently, a high prevalence of multi-drug resistant and virulent *Aeromonas hydrophila* in Nile tilapia was reported in Egypt [7]. The prevalence of the bacteria has also been reported in freshwater fish [8], indicating an increased risk of diseases, such as gastroenteritis, septicemia and necrotizing fasciitis caused by *A. hydrophila* and raising concerns about its importance for public health. Similarly, *Proteus mirabilis* infection (histamine poisoning or Scombroid poisoning) had a high prevalence and mortality in Ugandan carp farms [9].

The goal of the study was to look into the effects of single and co-infections of *A. hydrophila* and *P. mirabilis* in cultured *C. gariepinus*.

2. MATERIALS AND METHODS

2.1. Study Area

The study was carried out in the Fisheries House of the Landmark University Teaching and Research Farm, Omu-Aran, Kwara State, Nigeria.

2.2. Collection of Experimental Fish

A total of 120 sub-adult *C. gariepinus* were purchased from a commercial fish farm in Ilorin, with each weighing 100±0.5 g on average. Experimental fish were transported to the Landmark University Teaching and Research Farm in an oxygenated tank. They were kept in a 1500-liter plastic tank to adjust to their new environment for two weeks. The fish were fed twice daily at 5% of their body weight throughout the acclimatization period. Temperature, dissolved oxygen, and pH were monitored using the Consort C6020 all through the study period.

2.3. Bacteria Strains

The bacterial strains used in this study were *P. mirabilis* and *A. hydrophila*. The *P. mirabilis* (isolated from human feces) was obtained from the Microbiology Laboratory of the University of Ilorin Teaching Hospital in Kwara State, Nigeria. On the other hand, the *A. hydrophila* (isolated from cattle wounds) was obtained from the National Veterinary Research Institute in Vom, Plateau State, Nigeria. *P. mirabilis* was sub-cultured on blood agar and MacConkey agar before use. The observance of growth in successive waves that form a thin, filmy layer of concentric circles on blood agar and growth that lacked swarming but formed smooth, pale, or colorless colonies on MacConkey agar validated *Proteus*. In the case of *A. hydrophila*, sub-culturing was done on blood agar, MacConkey agar, and thiosulfate citrate bile salt-sucrose (TCBS) agar. On blood agar, colonies were observed to show a grayish color due to beta-hemolysis, which turned dark green after 72 h.

2.4. Experimental Design

The study adopted a complete randomized design. After acclimation, experimental fish were placed randomly into 50-liter plastic aquarium tanks at a 10 fish-per-bowl ratio. They were divided into four groups: control (Trt. 1), *P. mirabilis* (Trt. 2), co-infection (Trt. 3), and *A. hydrophila* (Trt. 4). All groups were replicated thrice.

2.5. Haematology

Using a 2 mL needle and syringe, 0.5 mL of blood was drawn from the experimental fish's vertebral column. Twelve fish, one from each replicate, were used. Blood collection was reported elsewhere [10]. Cold water anesthesia was adopted as described in this study [11]. The water temperature was gradually reduced until it reached 17 °C, and fish movement was gradually halted. The period between fish removal from the culture environment and the completion of blood sampling was less than 3 minutes. The blood sample was estimated as follows:

- Packed cell volume (PCV) was calculated using the Hawksleymicrohematocrit centrifuge (Hematospin 1400, England). It was read using the microhematocrit reader.
- Franco's (1984) spectrophotometric method was used to determine hemoglobin (HB). The Cypress

diagnostic kit (Cypress Diagnostics, Hulshout, Belgium) was used.

• Red blood cells (RBC) and white blood cells (WBC) were viewed under the Olympus Microscope CH. The cells were estimated using the Hawksleyhemocytometer (Hawksley, England).

• A drop of blood was smeared on a microscope glass slide and viewed through an Olympus Microscope CH to calculate the WBC differential.

All parameters were measured at the beginning and every two weeks after, as described in this study [12]. The entire experiment lasted for 12 weeks.

2.6. Serum Chemistry Assay

For serum chemistry, 1 ml of whole blood was collected in plain bottles. The blood was centrifuged at 1100 rpm for 5 minutes using a centrifuge machine (Model 800-B, China). Alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), creatinine, and blood urea nitrogen (BUN) were measured [13].

2.7. Histology

The paraffin technique was adopted to complete this operation. The liver and kidney of experimental fish were collected and prepared for histology [14]. After preparation, sections were properly dried before being stained with hematoxylin and eosin (H&E) and mounted using Histomount [15].

2.8. Challenge Experiment

Before the Challenge experiment, experimental fish were immobilized by lowering the water temperature [11]. Fish in treatment 1 were injected with 0.1 mL of 1×10^7cfu/fish of *P. mirabilis*; fish in treatment 2 were injected with 0.1 mL of 1×10^7 and 2.5×10^3cfu/fish of both *P. mirabilis* and *A. hydrophila*; and fish in treatment 3 were infected with *A. hydrophila* at a dosage of 2.5×10^3cfu/fish [16]. The pathogens were introduced to the fish intraperitoneally, allowed to stand for 5 minutes to observe its physiological stability, and then released into the controlled environment. Experimental fish were observed daily for 21 days for signs of abnormality. Mortalities were recorded.

2.9. Culture and Isolation of Bacteria from Experimental Fish

One gram of gills, stomachs, and intestines were collected from *C. gariepinus* and suspended in 0.85 NaCl (w/v) normal saline solution. It was diluted serially for bacterial isolation. Following serial dilution, 0.1 mL from each of the diluents 10-1 to 10-9 was cultured on nutrient agar (NA) (M001-500g, Hi Media, India) and thiosulfate citrate bile salts-sucrose (TCBS) agar (Rapid Labs Ltd., Colchester, ESSEX, U.K.) Petri dishes and incubated at 37 °C for 24 hours. Colonies from the NA and TCBS mediums were observed on the dish, which was counted using a colony counter (Thomas Scientific: MFG050) meticulously to determine the microbial load.

2.10. Statistical Analysis

A test for the significance of means was carried out using the one-way analysis of variance (ANOVA) test. Multiple comparisons were done using the Duncan multiple range tests. The computer's Statistical Analytical System (SAS) application, version 9.4, was used for each analysis.

3. RESULTS

3.1. Mortality and Water Quality Parameters

As shown in Table **1**, the pH of the water during the experiment was lower than the recommended range (6.5-9.0) for *C. gariepinus* culture, while the dissolved oxygen level was within the normal range. With respect to mortality, the highest value (60%) was reported in the *A. hydrophila*-infected group (Table **2**).

3.2. Clinical Signs in Infected Fish

Fig. (**1**) shows the clinical signs of infection. *A. hydrophyla,* single infections, and co-infected fish showed distinct signs.

Table 1. Water parameters measured during the experiment.

Parameters	*Proteus mirabilis*	Co-infection	*Aeromonas hydrophila*	Control	Standard Deviation
pH	5.63	5.67	5.60	5.68	0.24
Dissolved Oxygen (mg/l)	6.11	6.24	6.12	6.16	0.22
Temperature (°C)	24.78	24.67	24.89	24.56	0.61

Table 2. Mortality recorded in experimental fish from all treatments.

Treatment	Number of Fish Stocked	Mortality	% Mortality
Proteus mirabilis	30	11	37
Co-infection	30	11	37
Aeromonas hydrophila	30	18	60
Control	30	2	7

Table 3. Blood parameter analysis of experimental fish.

Parameters	Sample Before Research Commencement	Control	*Proteus mirabilis*	*Aeromonas hydrophila* & *Proteus mirabilis*	*Aeromonas hydrophila*	Standard Error
Red Blood Cells ($\times10^{12}$/L)	2.20^{b}	3.60^{a}	3.20^{a}	2.90^{ab}	3.00^{ab}	0.17
White blood cells ($\times10^{9}$/L)	9.97^{b}	10.87^{ab}	11.17^{a}	10.6^{ab}	10.00^{b}	0.03
Haemoglobin (g/l)	26.50^{b}	35.77^{a}	34.00^{a}	32.80^{a}	32.80^{a}	1.00
Packed Cell Volume (%)	32.50	41.00	36.00	33.50	34.00	1.50
Mean Corpuscular Volume (fl)	14.97^{a}	11.37^{b}	11.20^{b}	11.60^{b}	11.23^{b}	0.02
Mean Corpuscular Haemoglobin (pg)	12.07^{a}	10.00^{b}	10.70^{ab}	11.57^{ab}	11.23^{ab}	0.28
Mean Corpuscular Haemoglobin Concentration (g/dl)	12.90^{a}	8.77^{b}	9.67^{b}	10.00^{b}	10.07^{b}	0.44
Lymphocyte (%)	37.50	34.50	32.50	35.50	35.50	0.87
Neutrophil (%)	62.00	64.00	65.00	63.50	64.00	0.69
Eosinophil (%)	0.50	1.50	1.50	1.00	0.50	0.21
Basophil (%)	0.00^{b}	0.00^{b}	1.00^{a}	0.00^{b}	0.00^{b}	0.14

Note: Means with the same superscript running down the rows do not vary substantially at the 95% confidence level.

Table 4. Serum parameters analysis of experimental fish.

Parameters	Sample Before Research Commencement	Control	*Proteus mirabilis*	*Aeromonas hydrophila* & *Proteus mirabilis*	*Aeromonas hydrophila*	Standard Error
Alanine aminotranseferase	14.06^{bc}	14.10^{bc}	15.67^{a}	13.77^{c}	14.57^{b}	0.27
Alkaline phosphate	241.00^{bc}	253.00^{ab}	246.00^{bc}	236.00^{c}	260.00^{a}	2.70
Aspartate aminotranseferase	67.17^{ab}	69.47^{ab}	69.10^{ab}	66.30^{b}	70.10^{a}	1.74
Creatinine	22.50^{c}	25.67^{bc}	29.00^{ab}	31.10^{a}	25.07^{bc}	2.65
Urea	0.80^{b}	0.80^{b}	0.80^{b}	0.90^{a}	0.80^{b}	0.02

Note: Means with same superscript along the rows are not significantly different at 95% confidence limit.

Fig. (1). a) Haemorrhage observed in *Aeromonas hydrophila* single infection. **b**) Erosive skin lesion observed in *Aeromonas hydrophila* co-infected with *Proteus mirabilis*.

3.3. Haematology

There was no significant variation in Packed Cell Volume (PCV), Hb, and RBC levels observed between the infected and uninfected control groups. RBC, Hb, and PCV levels were markedly lower in fish sampled before infection. RBC indices Mean Corpuscular Haemoglobin Concentration (MCHC), Mean Corpuscular Haemoglobin (MCH), and Mean Corpuscular Volume (MCV) showed a similar trend, with MCV and MCH higher in fish sampled before infection (Table **3**). The total WBC in fish infected with a single infection of *P. mirabilis* increased, but this was not significantly different from the control and co-infected fish groups ($p < 0.05$).

Neutrophil and lymphocyte counts showed no significant difference ($p < 0.05$) between and among the infected and uninfected control groups.

3.4. Serum Chemistry Analysis

The level of ALT in the *P. mirabilis* and co-infected groups was significantly higher and lower than that in the control and *A. hydrophila* groups, respectively. There was no significant difference ($P< 0.05$) between the co-infected and the uninfected control groups. Similarly, low values were obtained for AST and ALP, while creatinine and urea were observed to be higher in the co-infected group (Table **4**).

3.5. Histopathology of the Kidney and Liver of Experimental Fish

The kidneys of experimental fish showed varying stages of degeneration. Kidney sections showed degenerations ranging from vacuolation and necrosis of hepatocytes to desquamation of tubular and glomerular epithelial cells. Experimental fish liver before infection

showed vacuolation and necrosis of hepatocytes on a section of the liver. The kidney of a fish before infection revealed necrosis of tubular epithelial cells and infiltration of inflammatory cells. The kidney and liver of co-infected fish showed greater damage than the single-infected and uninfected (control) groups (Figs. **2** and **3**).

3.6. Microbial Isolation, Count, and Identification

Bacteria isolated from control and freshly moribund fish and counted were shown in Table **5**. The bacterial load in the experimental fish intestine showed the highest bacterial load in the *A. hydrophila*-infected fish.

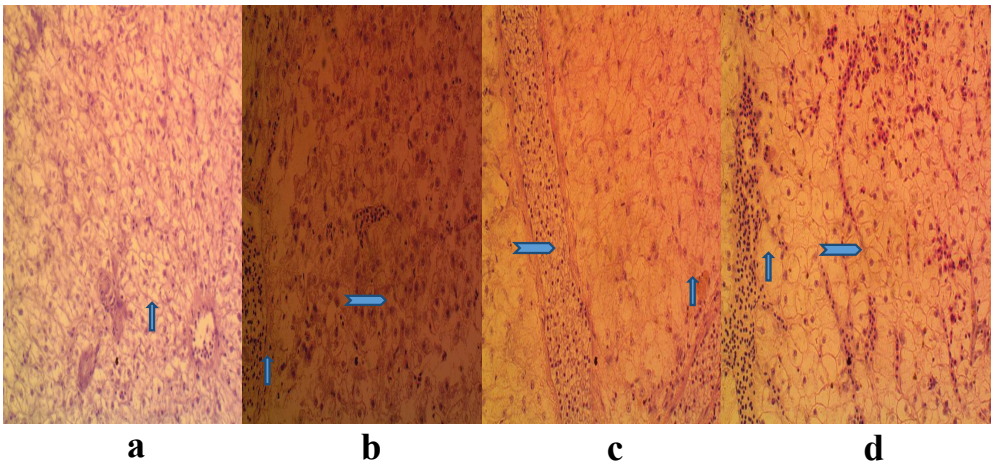

Fig. (2). Liver of experimental fish.
(**a**). Section of an experimental fish liver before infection, showing vacuolation and necrosis of hepatocytes (arrow). (**b**). Section of the fish liver infected with *Proteus mirabilis* showing congestion of blood vessels (arrow) and vacuolar degeneration and necrosis of hepatocytes (arrow head). (**c**). Section of fish liver infected with *Proteus mirabilis* and *Aeromonas hydrophila* showing severe vacuolar degeneration/fatty change and necrosis of hepatocytes (arrow) as well as the presence of fat globules in blood vessels (arrowhead). (**d**). Section of the fish liver infected with *Aeromonas hydrophila* showing congestion of blood vessels and sinusoids (arrow), and severe vacuolar degeneration/fatty change and necrosis of hepatocytes (arrowhead) (x400; Hematoxylin and Eosin).

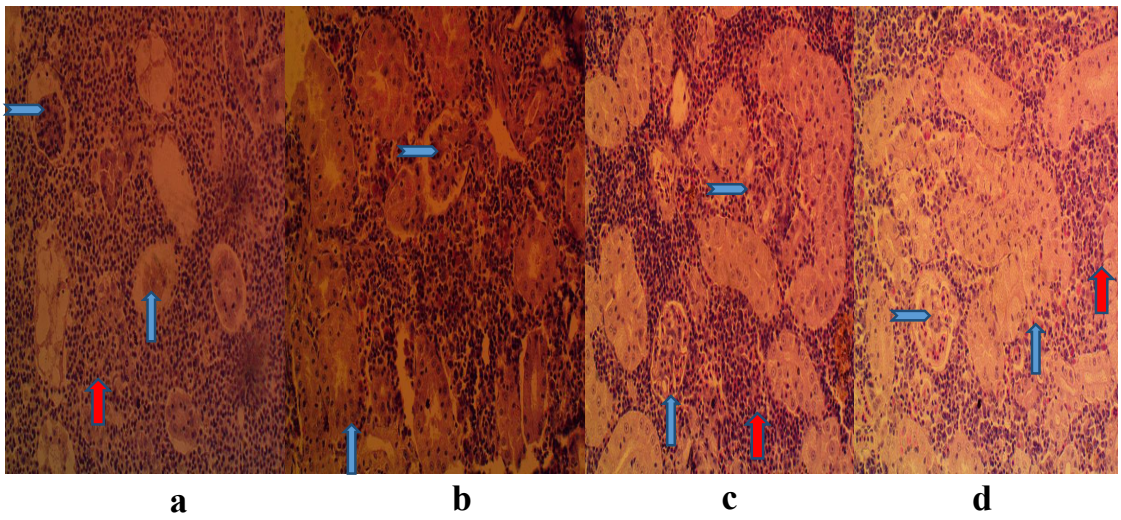

Fig. (3). Kidney of experimental fish.
(**a**). Fish kidney infected with *Proteus mirabilis*. Section of the kidney showing severe necrosis and desquamation of tubular (blue arrow) and glomerular (arrowhead) epithelial cells and infiltration of inflammatory cells in the interstitium (red arrow) (**b**). Fish kidney co-infected with *Proteus mirabilis* and *Aeromonas hydrophila*. Section of the kidney showing severe necrosis and desquamation of tubular (blue arrow) and glomerular (arrowhead) epithelial cells and infiltration of inflammatory cells with hemosiderosis in the interstitium (red arrow). (**c**). Fish kidney infected with *Aeromonas hydrophila*: section of the kidney showing severe necrosis and desquamation of tubular (blue arrow) and glomerular (arrowhead) epithelial cells, hemorrhages, and infiltration of inflammatory cells in the interstitium (red arrow). (**d**). Kidney of fish in the control group showing necrosis and desquamation of tubular (blue arrow) and glomerular (arrow head) epithelial cells, hemorrhages, and infiltration of inflammatory cells in the interstitium (red arrow) (x400; Hematoxylin and Eosin).

Table 5. Microbial count of the intestine of experimental fish.

Treatment	Microbial Load (cfu/g)
Before infection	25.3×10^4
Control	24.7×10^5
Aeromonas hydrophila	2.7×10^8
Proteus mirabilis	2.3×10^4
Aeromonas hydrophila X *Proteus mirabilis*	4.3×10^5

4. DISCUSSION

The higher mortality recorded in the *A. hydrophila* group compared to the *P. mirabilis* and co-infected groups confirms the pathogenicity of the organism. Previous authors [17-24] have reported similar findings. The pathogenic organism *P. mirabilis* has also caused scombroid poisoning and high mortality in carp farms [9]. However, *A. hydrophila* causes more harm because of its hemolysin virulence factor, which affects both people and animals [25, 26]. Possibly contributing to this is the fact that *A. hydrophila* is a water-borne microbe. This result did not corroborate the findings of [16], who reported lower mortality in the *A. hydrophila*-infected group compared to the co-infected group (*Edwardsiella ictaluri* and *A. hydrophila*). The reason for these differences may be a result of the synergistic association of *E. ictaluri* and *A. hydrophyla*. Machimbirike *et al.* [27] also reported that *E. ictaluri*-infected fish showed a high mortality rate.

Additionally, the fact that both organisms are water-borne diseases could explain this. The lower mortality reported for the co-infection group could be due to the antagonistic relationship between *A. hydrophila* and *P. mirabilis* organisms [28, 29]. This was, however, not the case in the study of Grayson *et al.* [30] and Loch *et al.* [31], who reported a synergistic relationship between *Renibacterium salmoninarum* and *A. hydrophila* from the wild. This could be due to the differences in the organisms co-infected with *A. hydrophila*.

Red blood cell parameters and indices observed to be higher in all the treatment groups than the reference range could be attributed to the pathogenicity of the test organisms [32], especially *A. hydrophila*. A similar increase observed in the control group could imply that factors other than infection that were not measured in this study, such as feed, could be to blame for such findings. However, the low level of these parameters observed in the co-infected group as opposed to single infections cannot be scientifically explained as previous research did not cover this scope. This was, however, not the case with the study of Sabri *et al.* [33], who reported a lower value of the red blood cell count in adult *C. gariepinus* infected with *Henneguyosis* sp. The size of the fish and the difference in the challenge organism can be responsible for these differences. The implication of this is that there will be blood thickening, an abnormal increase in RBC production, more concentrated haemoglobin than usual, and macrocytic anemia [34].

Serum parameters could be used to assess the health of fish livers and kidneys. The alanine aminotranseferase, alkaline phosphate, and aspartate aminotranseferase values that were observed to be higher in the infected and uninfected control groups could also be seen in the histology of the liver of the experimental fish, which showed various stages of degeneration even in the fish before the commencement of the study. However, fish infected with a single infection had more effect on the liver enzymes, which could not be said to be true for histology as co-infected fish revealed greater degeneration than single-infected fish. This was not the case with the work of [35].

High levels of serum parameters above the limit indicate chronic liver damage [36]. Creatinine levels in experimental fish exceeding the standard limit, especially in the co-infected group, are interpreted as severe kidney damage [37]. Okoye *et al.* [38] reported the standard range of creatinine for *C. gariepinus* to be between 0 and 3. The level of blood urea observed to be lower than the reference range in this study, especially in the single infected group, could be a result of the failure of the kidney, as depicted in the cell micrograph. Similarly, Ajeniyi *et al.* [39] reported similar results from their research.

In the histology of the liver and kidney, the severe pathological changes observed, especially in the co-infected experimental fish, are consistent with other findings of this study. It shows the pathogenicity of *P. mirabilis* and *A. hydrophila* and/or poor metabolism of fat in the liver, according to Abalaka *et al.* [40]. The research on the co-infection of *Trichodina* sp. and *Aeromonas caviae* on *Lates calcarifer* carried out by Sufardin *et al.* [41] is similar to the findings of this study. Similar work by Ma *et al.* [42], who co-infected *Oncorhynchus mykiss* with infectious hematopoietic necrosis virus and *Flavobacterium psychrophilum*, is consistent with the pathological changes undergone in the livers and kidneys after infection and is corroborated by this study.

The high bacterial load in the *A. hydrophila*-infected group could be attributed to the virulent nature of the organism. Citarasu *et al.* [43] recorded a comparable report. This demonstrates that the test organisms might be to blame for the responses seen throughout the investigation.

CONCLUSION

Fish exposed to a single infection or a co-infection developed a variety of pathological changes in their blood, liver, and kidney, with severe degenerations observed in the co-infection. The study also confirmed the

pathogenicity of *A. hydrophila* over *P. mirabilis*. It also concludes that the co-infections *A. hydrophila* and *P. mirabilis* revealed an antagonistic relationship.

LIST OF ABBREVIATIONS

PCV = Packed Cell Volume

TCBS = thiosulfate citrate bile salt-sucrose

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study adopted methods for handling experimental fish that are in line with international and local regulations guiding the use of animal subjects during experimentation to examine the response of cultured *C. gariepinus* to the co-infection of *P. mirabilis* and *A. hydrophila*, and approval was given by the Landmark University Ethics Committee with approval number LMUIREC/ACSC/003/2021.

HUMAN AND ANIMAL RIGHTS

No humans were used in this research. All the experiments on animals were in accordance with "Guidelines for the Use of Fishes in Research (2004)". https://fisheries.org/docs/ policy_useoffishes.pdf (Accessed July 08, 2022)

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are provided in the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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