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Antimicrobial resistance and pathological impacts of Proteus mirabilis Infection in African Catfish (Clarias gariepinus) Juveniles

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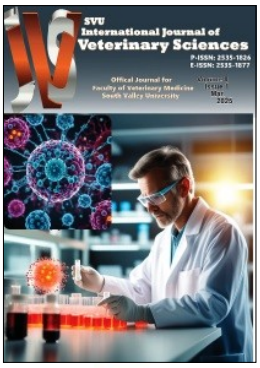

ABSTRACT

Bacteria are the main cause of diseases in freshwater fish. This study evaluates the antibiogram profile of Proteus mirabilis and its impact on hematology, serum chemistry, and histopathology in African catfish. The agar disc diffusion method was used for the antibiogram profile of P. mirabilis. One hundred and sixty Clarias gariepinus juveniles were acclimatized and divided into two groups with four replicates (20 fish per replicate; infected and control group). The fish were infected with P. mirabilis isolates at 8.5 x 108 CFU/ml by immersion. The control group was not infected. The control and infected groups were observed for 14 days, and mortalities, clinical signs, skin, and gross lesions were recorded. Hematology, histopathology, and blood chemistry were studied. Proteus mirabilis isolates showed multiple drug-resistant characteristics, with a MAR index of 0.60. The mortality rate was 0% and 68.8% in control and infected fish, respectively. Anemia and lymphocytosis were observed in the infected group. Increased serum globulin, albumin, and total protein were also reported. Moreover, hepatic and renal damage due to high levels of ALT, AST, and creatinine were observed in the infected group. The histopathological changes in the skin and kidney section of infected groups showed focal area degeneration in the epidermis, generalized vacuolation of hepatocytes, loose connective tissues in the dermis, renal thrombosis, tubular degeneration, and necrosis of infected fish kidney. Proteus mirabilis was pathogenic and caused organ damage in infected African catfish. Effective biosecurity measures and antimicrobial susceptibility testing before treatment are recommended.

Keywords: *Antimicrobial resistance, Bacterial isolates, Biosecurity, Pathology, Proteus mirabilis*

INTRODUCTION

African catfish (Clarias gariepinus) is one of the most cultured fish species in Nigeria as a result of its economic importance, abilities, and capabilities such as; high fertility rate, level of growth rate, proliferation, disease resistance, and environmental tolerability (Tiamiyu et al., 2019). Moreover, a highly profitable index relating to customers' acceptability of Clarias gariepinus leads to increased fish rearing in every part of Nigeria (Adewumi & Olaleye 2011). Meanwhile, disease outbreaks due to bacterial infection pose serious economic devastation to the rapid growth of aquaculture in Nigeria (Remilekun et al., 2021). The morbidity and mortality caused by bacterial infection is usually high due to environmental and biological. The causes of bacterial infection in farms include lack of biosecurity measures, stocking density, poor water quality, contaminated feed, and lack of prompt

diagnosis and treatment of infected fish (Remilekun et al., 2021).

Proteus mirabilis is a gram-negative bacterium that belongs to the family of Enterobacteriaceae and can also be referred to as facultative anaerobes. The bacteria are environmental, though opportunistic in occurrence (Zhai et al., 2023). The bacteria may exist in wastewater, soil, and intestinal tracts of the animal. The incidence of P. mirabilis was earlier reported in Tuna fish, ornamental fish; African catfish, Indian carp, and Nile Tilapia, (EL-Deen, 2013; Kumar et al., 2015; Pattanayak et al., 2018; Qasim, 2020; Anifowose et al., 2024a). Moreover, the antimicrobial resistance profile and pathogenicity of P. mirabilis isolates in African catfish were scarcely reported. Antibiotic resistance has become a menace facing aquaculture worldwide (Preena et al., 20220). The antimicrobial resistance profile of P. mirabilis is important for controlling bacterial diseases, providing information and knowledge on the selection of antimicrobial agents, and assessing the level of resistance

Most nations in which Clarias gariepinus is farmed are mainly developing nations where regulation on the antibacterial agents' usage is not functioning and an influence that might contribute to the increase of antibacterial resistance (AR) (Cabello et al., 2013). This practice is in contrast to what obtains in advanced countries where limited controlled antibacterial agents are acceptable for treatment. Moreover, the warm steamy climate of developing countries contributed to the spread and increase of antimicrobial raises. A lot of antibacterial drugs used in human medication are also employed in Clarias gariepinus aquaculture (Laxminarayan et al., 2013).

There is a relationship between the environment, the host, the pathogen, and the control of morbidity or mortality of fish diseases (Anifowose et al., 2022; Aadil et al., 2023). The clinical features of infected fish may include septicemia, erosion, skin ulceration, and tail and fin rot (Avani et al., 2023). The pathogenesis and virulence of pathogenic agents may divulge as a result of fish species, prompt response to infection, and immune components of infected fish. In addition, hematology and histopathology investigation may help in the control and management of fish diseases

Hematological investigation may reveal the health, and physiological status of the fish, and its profile provides useful information for disease diagnosis in fish (Havixbeck et al., 2015; Fazio, 2019). Extensive information on disease status, level of stress, the oxygen transport capacity of fish, and the response of certain organs of fish to diseases may be provided by haematological and biochemical studies (Uiuiu et al., 2021).

Moreover, pathogenic agents usually cause damage and pathological effects to the fish host tissues and organs. The pathological investigation is important, as an essential indicator pointing to the exposure of fish to stressful and diseased or contaminated environments (Tiamiyu et al., 2020). The histopathological analysis of infected fish organs and tissues showed modifications at the cellular level and perceived acute and chronic alterations of infected fish organs and tissues. The identification of definite altered cells and organelles of infected fish is provided by histopathological examination (Salamat and Zarie, 2014). The scarce information on haematology, serum biochemistry, and histopathology of infected fish with P. mirabilis imposes a gap in the control of the infection. Therefore, this study aims to assess antimicrobial resistance, haematology, serum biochemistry, and histopathology of experimentally infected fish with P. mirabilis .

MATERIALS AND METHODS

Experimental Infection Trial

African catfish juveniles (n=160) were collected from reputable fish farms. The average weight and length of the fish were; 25.0 ± 0.69 g and 13.1 ± 0.4 cm. The fish were divided into two groups (n=20 each, infected and control) with four replicates. Furthermore, a suspension of 1 ml of TSA broth culture of P. mirabilis based on McFarland 0.5 was 1.7 x 108 CFU. Fish were infected by immersion in one liter of water containing 8.5 x 108 CFU. The control groups were not infected. The fish were fed three percent body weight once daily starting from 96 hours post-infection. Water contamination was prevented by changing 50% of the tank water daily. Fish tank water Ammonia, nitrite, dissolved oxygen, pH, and temperature were observed and assessed daily. The fish were examined for skin lesions, clinical symptoms, and mortalities for 14 days

(Anifowose et al., 2024a).

Antimicrobial Susceptibility Testing

The antibiotic susceptibility was carried out using 20 isolates of *P. mirabilis*. The method used was agar-disc diffusion and CLSI standards Mo2 was carefully followed (CLSI, 2020).

The resistance and sensitivity of *P. mirabilis* isolates to antibiotics was carried using 15 antibiotics belonging to 9 different classes: Furaltadone 30 µg (F), Macrolide (Azithromycin 15 µg), Polymyxin (Colistin sulfate 10 µg (CS)), fluoroquinolone (Enrofloxacin 5 µg (ENR), ciprofloxacin 5 µg (CIP), Metronidazole 5 µg (M), aminoglycoside (gentamicin 10 µg (G), streptomycin 10 µg (S),), chloramphenicol 30 µg (CH), tetracycline (doxycycline 30 µg (D), tetracycline 30 µg (T), cephalosporin (third-generation) ceftriaxone 30 µg (CEF), penicillin; amoxicillin 25 µg (AM), ampicillin 10 µg (AP), and amoxicillin-clavulanic acid 30 µg (AC).

Preparation of Antibiotic Disk

Each antibiotic was infused into sterile Whatman filter paper disks based on the CLSI Mo2 disk preparation guide. A maximum of six 6-mm disks were placed on a bacterial isolate streaked sterile Mueller-Hinton agar plate (CLSI, 2020).

Preparation of Inoculums

The suspension of *P. mirabilis* was made from a pure overnight culture of the isolates on Tryptic Soy agar (HiMedia®). The density was adjusted to McFarland 0.5 turbidity standard using normal saline. Then, the suspended P. mirabilis colonies were streaked over Muller-Hinton agar (HiMedia®). The *Staphylococcus aureus* strain was used for quality control (CLSI, 2020).

Inoculation of agar plates

Antibiotic disks were selected with sterile forceps and placed on a contact surface with a Mueller-Hinton agar plate. Disks made contact with the medium and incubation of plates was carried out at 37^0C for 18 hours. The zone of inhibition was measured with a ruler in millimeters. The *P. mirabilis* was interpreted as resistant or sensitive to the antibiotics based on the zone of inhibitions measurement using the CLSI guide (CLSI, 2020).

Haematology and Serum Biochemistry

At the end of the experiment, blood samples (n=4) were collected for hematology and serum biochemistry. Fish were euthanized by transecting the spinal cord behind the skull for post-mortem examination. After fourteen days of the infection trial, fish were randomly sampled in each tank for blood collection. Four fish (n=4) were selected per tank. Blood was collected from a caudal vein of four sampled fish using a 1-mL sterile hypodermal syringe affixed with a 24-gauge needle. The collected blood was placed in either non-heparinized or heparinized Eppendorf tubes. The blood samples were transported to the Clinical Pathology Laboratory of the Department Veterinary Pathology, University of Ibadan. Hematology and serum chemistry was carried out according to the method described by Adeshina *et al*. (2020).

Histopathological Examination

Tissue specimens were obtained from the skin, liver, and kidney (based on post-mortem lesions) for histopathological examination. Four fish each (n=4) were sampled from each infected and control group. Tissue specimens from the skin, liver, and kidney were fixed with 10% neutral buffered formalin, dehydration, infiltration, embedment in paraffin, and stained with hematoxylin and Eosin according to Robers, (2004).

STATISTICAL ANALYSIS

Statistical software SPSS version 23 was employed for statistical analysis of data. The Antibiogram profile was subjected to a Chi-square test. Mortality rate, hematology, and serum biochemistry were subjected to Independent T-test. Differences were considered significant at $p < 0.05$ for all the datasets. Significant levels were determined using F-tests and P-values

RESULTS

The antibiogram profile showed that the tested strains were resistant to chloramphenicol (100%), tetracycline (90%), amoxicillin-clavulanic acid (85%), doxycycline (80%), ampicillin (70%), gentamicin (45%), and colistin sulfate (35%). Moreover, the

Table 1: Frequency of antimicrobial susceptibility for Proteus mirabilis

Antimicrobial Class	Antimicrobial agent	Disk Potency	Sensitive, n* (%)	Intermediate,n (%)	Resistance, n (%)
Aminoglycoside	Streptomycin	10 µg	12 (60.00)	1 (25.00)	7 (15.00)
Aminoglycoside	Gentamicin	10 µg	9 (45.00)	2 (10.00)	9 (45.00)
Cephalosporin (3rd generation)	Ceftriaxone	30 µg	18 (90.00)	2 (10.00)	0 (0.00)
Penicillins	Ampicillin	10 µg	3 (15.0)	3 (15.0)	14 (70.0)
Penicillins	Amoxicillin clavulanic acid	30 µg	2 (10.0)	1 (5.0)	17 (85.0)
Fluoroquinolones	Enrofloxacin	5 µg	16 (80.0)	1 (5.0)	3 (15.0)
Fluoroquinolones	Ciprofloxacin	5 µg	14 (70.0)	2 (10.0)	4 (20.0)
Tetracyclines	Doxycycline	30 µg	0 (0.0)	4 (20.0)	16 (80.0)
Tetracyclines	Tetracycline	30 µg	0 (0.0)	2 (10.0)	18 (90.0)
Phenicols	Chloramphenicol	30 µg	0 (0.0)	0 (0.0)	20 (100.0)
Polymyxin	Colistin sulpate	10 µg	10 (50.0)	3 (15.0)	7 (35.0)
Chi-square			261.75	58.7	142.19
p values			0.001	0.001	0.001

**Number of isolates n = 20; p < 0.05, MAR index = 0.60*

Figure 1: Skin ulceration of Clarias gariepinus

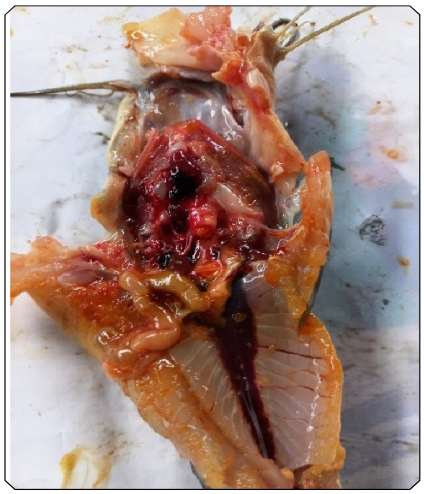

Figure 2: Congestion of posterior kidney of Clarias gariepinus infected with Proteus mirabilis (white arrow)

Table 2: Mortality rate of Clarias gariepinus juveniles infected with Proteus mirabilis

Tank	Control Fish	Dead Fish
Tank 1	0^a	14^b
Tank 2	0^a	13^b
Tank 3	0^a	16^b
Tank 4	0^a	12^b
Total	0^a	55^b
	0.0^a	68.8^b

Significance (α < 0.05) based on independent T-test indicates different superscripts along rows

recovered isolates were sensitive to ceftriaxone (90%), enrofloxacin (80%), ciprofloxacin (70%), streptomycin (60%), colistin sulfate (50%), and gentamicin (45%). The P. mirabilis isolates indicated a statistical significance in their susceptibility patterns to different tested antimicrobial classes ($p<0.05$)

The tested P.mirabilis isolates showed a Multiple Antibiotic Resistance (MAR) index of 0.60 (Table 1). The mortality rate observed was 68.8% and 0% in infected and control groups, respectively (Table 2). Exophthalmia and skin abrasion were observed in

Table 3: Haematology of infected Clarias gariepinus juvenile (n=32) with Proteus mirabilis isolate

Parameters	Control (Mean±S.E.M)	Infected (Mean±S.E.M)
PCV (%)	26.00±0.41[a]	17.83±0.13[b]
Hb (dL)	8.29±0.40[a]	4.97±0.20[b]
RBC (x10^6/µL)	2.83±0.20[a]	1.33±0.02[b]
WBC (x10^3/µL)	13.42±0.07[a]	15.38±0.11[b]
Lymphocytes (%)	66.38±0.98[a]	68.78±0.22[b]
Heterophils (%)	24.71±0.81[a]	26.89±0.31[b]
Monocytes (%)	2.44±0.18[a]	2.00±0.24[a]
Eosinophils (%)	2.67±0.14[a]	1.27±0.29[b]
Basophils (%)	0.98±0.02[a]	0.11±0.11[b]
Platelets (x10^5/µL)	2.82±0.01[a]	0.95±0.04[b]

Significance (α < 0.05) based on independent T-test indicates different superscript along rows S.E.M – Standard Error of Mean

Table 4: Serum biochemistry of infected *Clarias gariepinus* juvenile (n=32) with *Proteus mirabilis* isolate

Parameters	Control (Mean±S.E.M)	Infected (Mean±S.E.M)
Total Protein (%)	6.00±0.10[a]	7.98±0.35[b]
Albumin (dL)	1.50±0.05[a]	2.90±0.28[b]
Globulin (dL)	4.50±0.05[a]	5.20±0.37[b]
AST (µL)	176.10±3.42[a]	213.56±5.32[b]
ALT (µL)	49.23±0.29[a]	82.67±6.57[b]
Creatinine (mg/dL)	0.48±0.01[a]	1.37±0.18[b]

Significance (α < 0.05) based on independent T-test indicates different superscripts along rows S.E.M – Standard Error of Mean

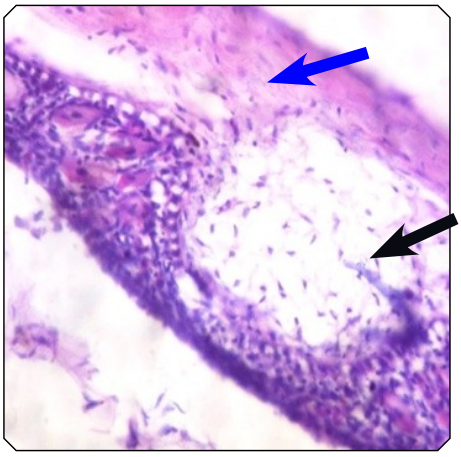

Figure 3: Photomicrograph of an infected Clarias gariepinus skin section stained by Haematoxylin and Eosin (x400), showing epidermal layers with focal area degeneration (black arrow). The dermal layer (blue arrow) shows loose connective tissues and fat(blue arrow)

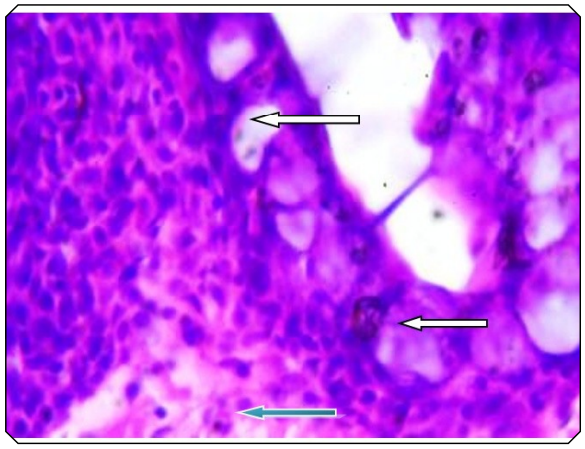

Figure 4: Photomicrographs of a control Clarias gariepinus skin section stained by Haematoxylin and Eosin (x400) showing epidermal layers with normal epithelial cells in layers. Mucous goblet cells (white arrow). The dermal layer (blue arrow).

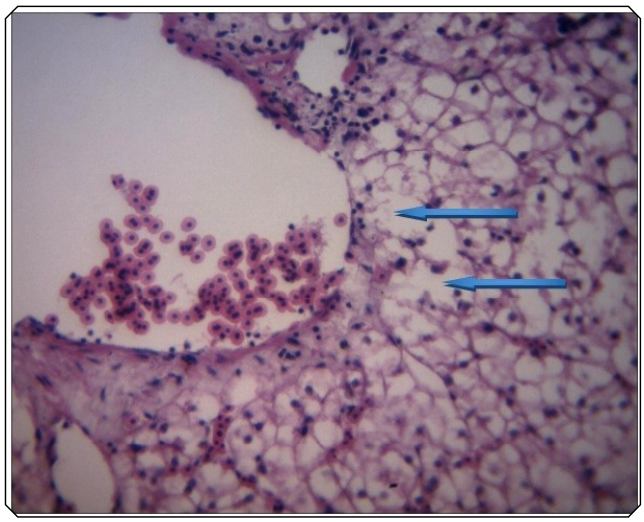

Figure 5*: Photomicrograph of an infected* *Clarias gariepinus* ***liver section stained by Haematoxylin and Eosin (x400), showing generalized vacuolation of hepatocytes (blue arrow)***

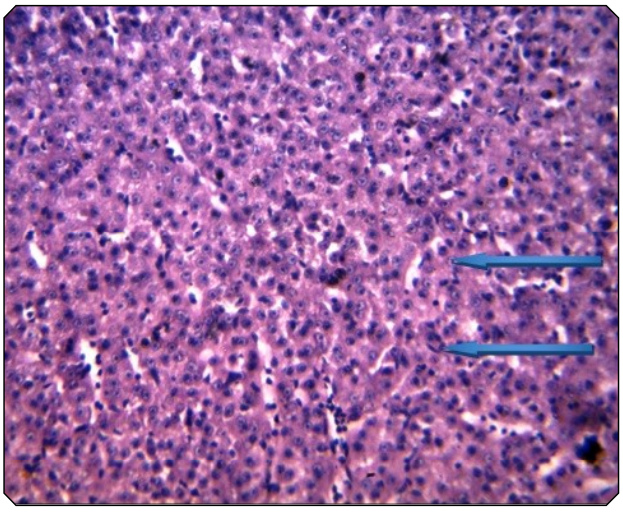

Figure 6*: Photomicrographs of a control* *Clarias gariepinus* ***liver section stained by Haematoxylin and Eosin (x400) showing normal hepatocytes (blue arrow).***

infected groups. Additionally, skin ulceration and congestion of the posterior kidney were observed in infected groups (Figures 1 and 2). Histopathological analysis revealed cellular modifications and acute, and chronic tissue alterations in infected fish organs. The histopathological changes observed included focal area degeneration in the epidermis, loose connective tissues in the dermis, generalized vacuolation of hepatocytes, renal thrombosis, tubular degeneration, and necrosis of infected fish kidneys (Figures 3, 5, and 7). Significant (α0.05) anemia and lymphocytosis due to decreased hemoglobin (Hb), packed cell volume

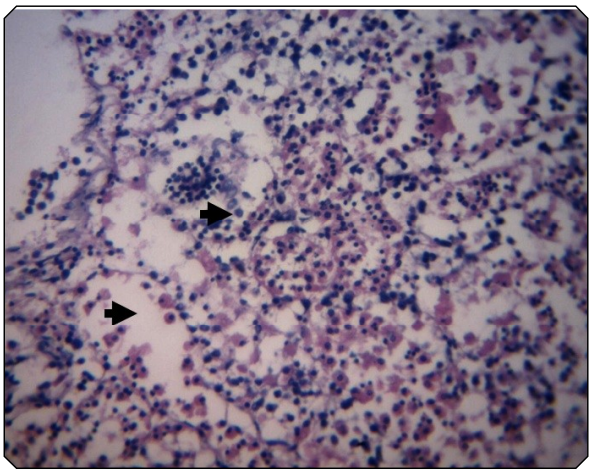

Figure 7*: Photomicrograph of an infected* *Clarias gariepinus* ***kidney section stained by Haematoxylin and Eosin (x400), showing renal thrombosis, tubular degeneration, and necrosis (arrowheads).***

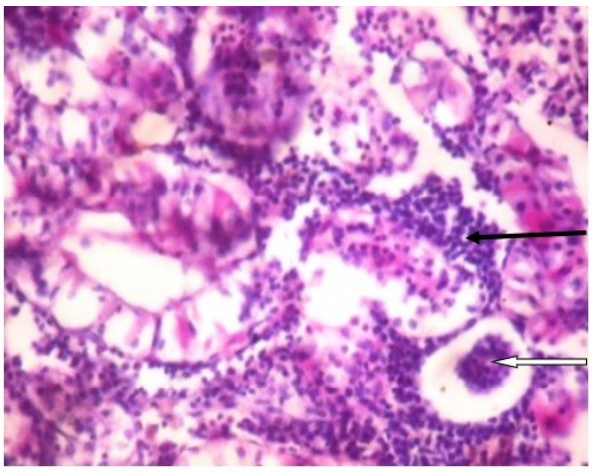

Figure 8: Photomicrograph of a control Clarias gariepinus kidney section stained by Haematoxylin and Eosin (x400), showing renal cortex with normal glomeruli (white arrow), and the interstitial spaces show haematopoietic elements (slender arrow)

(PCV), red blood cell (RBC), increased lymphocytes, and white blood cell (WBC) counts of infected fish were detected in comparison to control fish. A significant (α0.05) increased heterophil count was detected in the infected group compared to the control group. Significant (α0.05) increased monocyte, eosinophil, basophil, and platelet counts were detected in the control group compared to the infected group (Table 3).

Moreover, infected fish exhibited significantly ($\alpha < 0.05$) higher serum protein, albumin, and globulin levels than controls. Significant ($\alpha < 0.05$) increased serum Alanine aminotransferase(ALT), aspartate aminotransferase(AST), and creatinine were detected

in infected fish in comparison to control fish (Table 4). The histopathological sections of control groups showed epidermal layers with normal epithelial cells in layers, and few secretory cells in the epidermis including mucous goblet cells. the dermal layer. moreover, the renal cortex with normal glomeruli and the interstitial spaces show hematopoietic elements (Figures 4 and 6).

DISCUSSION

Antimicrobial resistance patterns of the isolated *P. mirabilis* showed notable resistance to phenicols, tetracyclines, penicillins, aminoglycosides, and polymyxin. The findings in this study agreed with Chauhan *et al*., (2015). The indiscriminate use of antibiotics in fish farms and the capacity of *P. mirabilis* to acquire antibiotic-resistance genes from other resistant bacteria may be responsible for antibiotic resistance(Hess *et al*., 2020). The implication of these findings is an indication of rampant antimicrobial resistance usage in the study area (Wamala *et al*., 2018).

The mortality rate in the infected group (68.8%) was lower than the 100% mortality observed by Zhai *et al*., (2021) in yellow catfish and zebrafish experimentally infected with P. mirabilis. Yellow catfish were infected by immersion and the bacterial dose may be responsible for the higher mortality rate reported than the findings of this study (Remilekun *et al*., 2021). This implies that *P. mirabilis* was pathogenic and was responsible for the high mortality observed in experimental infection.

The current study showed a disparity in Neutrophils, PCV, Hb, red blood cells, white blood cells, heterophils, lymphocytes, and platelets among experimentally infected and control groups. This indicated the influence of the bacterial infection on the hematological parameters of infected African catfish.

Explicitly, the infected fish showed a significantly reduced PCV, which may imply destruction of components of blood leading to a reduction in host blood performance. Reduction in the infected fish PCV indicates destruction of blood cells, loss of blood, and inability to recompense for blood losses (Adeyemi *et al*., 2014; Achilike and Wusu, 2019). The histopathology sections of the control group skin showed normal epithelial layers. The kidney showed renal thrombosis, tubular degeneration, and necrosis of renal tubules due to systemic infection by the bacteria led to the failure of erythropoiesis. Meanwhile, the heterophilia may be due to systemic bacterial infection (Anifowose *et al*., 2024b).

The higher neutrophils observed in the infected fish may be due to the first leucocytes conscripted to an inflammatory site and can eliminate pathogenic agents through multiple complementary mechanisms (Havixbeck *et al*., 2015). Moreover, neutrophils and macrophages are major components of innate immune cells that protect fish against pathogens (Speirs *et al*., 2024).

The significantly increased white blood cells in infected fish indicated a defense activity through increased phagocytic cell production. White blood cells have been reported to be responsible for antibody production in defense against bacterial infections. The higher significant leukocyte counts showed a high-stress level in an animal (Dauda, 2018). The increased serum level of total protein, albumin, globulin, ALT, AS, and creatinine observed in infected fish indicated a major pathological effect of bacterial infections on biochemical parameters. The significantly high creatinine level indicates damage to the kidney and fundamental health conditions. This was supported by gross lesions and histopathology of the kidney observed in infected fish. Environmental stress may interact with the ALT, AST, creatinine, total protein, albumin, and globulin values in *C. gariepinus* (Dawood *et al*., 2022). A higher level of ALT, AST, and creatinine could indicate damage to fish liver and kidney, and an increase in the level of creatinine and ALT in the blood could indicate liver damage and a range of underlying health conditions. According to Ulas *et al*., (2024), a buildup of creatinine in the blood is a sign of impaired kidney function in the challenged fish. Creatinine values may be different in the same species depending on the diet, season, and presence of environmental stressors (Hore *et al*., 2023). It could be inferred that as much as the control and the challenged fishes

were exposed to similar diets and seasons, the minor increase of creatinine in the challenged could be traced to the effect of the *P. mirabilis* infection.

A significantly higher level of AST was observed in the infected *Clarias gariepinus* juvenile indicating the major effect of the pathogen in this regard. Aminotransferases are intracellular enzymes localized within the cells of fish organs including the liver, heart, gills, kidney, and muscle. According to Anih *et al.*, (2024), Aspartate aminotransferase (AST) is an enzyme found in hepatocytes, myocardial muscles, skeletal muscle, the brain, and the kidneys. The AST is a nonspecific marker of hepatocellular damage. The increased level of this enzyme is usually associated with cellular damage or disrupted cell membranes, allowing the enzyme to leak out of the cells. A significantly high level of AST in the infected fish indicates the possibility of tissue or organ damage leading to a tangible leakage of AST.

CONCLUSION

Proteus mirabilis isolated were multiple drug-resistant and showed resistance to chloramphenicol, tetracycline, amoxicillin-clavulanic acid, doxycycline, ampicillin, gentamicin, and colistin sulfate with an elevated MAR index of 0.60. The bacterial isolates were pathogenic and caused organ damage in *Clarias gariepinus* juveniles. Routine antimicrobial resistance profiling and training for farmers on biosecurity measures is necessary to prevent and curtail the transmission of multi-drug resistant bacteria like *P. mirabilis* isolates. Exploring alternative treatments or vaccine production for *P. mirabilis* in aquaculture is essential to reduce and control bacterial infection.

DECLARATIONS

FUNDING SUPPORT

Financial support was not provided for this study

COMPETING INTEREST

This study has no competing interest to declare

AVAILABILITY OF MATERIAL AND DATA

The data for this study is available on request

CONSENT FOR PUBLICATION

Consent does not apply to this study

CONTRIBUTION OF AUTHORS

All the authors generated the concept note for the study. Anifowse and Obisesan generated data and carried out the fieldwork. Anifowose and Obisesan analyzed data and drafted the manuscript. Adeoye edited the draft of the manuscript. All the authors approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval for this study was obtained from the Animal Care and Use Research Ethics Committee, University of Ibadan (UI-ACUREC; approval number: UI-ACUREC/066-0622/12).

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