PATHOGENICITY OF *Pseudomonas aeruginosa* IN RECIPROCAL HYBRIDS OF *Clarias gariepinus* AND *Heterobranchus bidorsalis*

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ABSTRACT

Farming of hybrids of two African Clariid catfish species, "Heteroclarias" is becoming a focus of attention in many aquatic farms in Nigeria. Pseudomonas sp. is one of the major pathogens responsible for disease in aquaculture. A total of 150 Heteroclarias juveniles were orally inoculated with different concentrations of Pseudomonas aeruginosa ranging from 3 x 10^1 to 3 x10^4 CFU/100µl in 4 groups (A to D) replicated thrice, for 14 days with the aim of evaluating its pathogenicity. The control group (E) also with 10 fish in 3 replicates were given normal saline orally. Clinical signs, mortality, histopathology and haematology were observed following experimental infection. Haematological parameters observed were statistically analysed using SPSS 20. Clinical signs observed in group A to C were discolouration, sloughing and patches of haemorrhage on their skin, Frequency of mortality recorded in group A to E were 20%, 10%, 10%, 6.6% and 0% respectively. Histopathological changes observed were vacuolar degeneration of renal tubular epithelium, diffuse hepatocellular vacuolar degeneration, and hyperplasia, fusion of secondary lamellae, with haemorrhage in the gills. Significant difference in haemoglobin concentration parameter was observed, translating to reduction in oxygen carrying capacity of the infected fish. Heteroclarias were susceptible to Pseudomonas aeruginosa, with resultant significant mortality.

Keywords: Bacteria; Lesion; Pathogenic; Stress.

INTRODUCTION

Hybrids among African catfishes; *Clarias gariepinus*, *Heterobranchus bidorsalis* and/or *Heterobranchus longifilis* has been reported by Ataguba *et al.* (2009) and Nlewadim *et al.* (2004). In Nigeria, *Clarias gariepinus* captures the interest of commercials fisheries because of its taste, hardy nature and tolerance to poor water conditions. Also, the potential of *Heterobranchus bidorsalis* as a good aquaculture species has been described by Teugels (1990). However, *Clarias gariepinus* possess qualities considered better than those of *Heterobranchus* spp., and these include high fecundity, earlier sexual maturity, faster growth rate and more adaptability, while *Heterobranchus* spp. grow bigger than *Clarias gariepinus* reaching about 14kg (Solomon, 2013; Idodo–Umeh, 2003). The differences in the qualities of these catfishes, prompted scientists to harness the qualities of the species by cross breeding to produce a hybrid (literarily called "Heteroclarias" for crosses between *Heterobranchus* female and male Clarias while "Clariobranchus" is reciprocal cross of the latter) that combines hardy and faster growth traits of the two species (Solomon, 2013).

One of the key problems in fish farm industry is diseases (Abdullahi *et al.*, 2013). Studies have revealed globally that bacteria are accountable for high mortality in fish hatcheries and culture (Iglewski, 1996). Effects cause of diseases in fish industry causes economic losses, due to factors like fish mortality, treatment expenses, delay or loss of the opportunity to sell the fish and infection with zoonotic diseases by the handler and final consumer of the affected fish (Abdullahi *et al.*, 2013). Studies by Ikpi and Offem (2008) and Ugwuzor *et al.* (1990), have shown that *Pseudomonas* sp is one of the common fish bacteria responsible for diseases in fish farms in Nigeria. *Pseudomonas* infection is one of the most common bacterial infections among fish (Oh *et al.*, 2019). It is an opportunistic pathogen to plants, animals and humans and appears to be a stress related disease of freshwater fish especially under culture conditions (Iglewski, 1996).

Pseudomonas aeruginosa is a gram-negative, rod-shaped, anaerobic, and polar-flagellate bacterium with unipolar motility belonging to the family Pseudomonadaceae. This species is a highly adaptable pathogen, capable of surviving in a variety of environment (Abdullahi *et al.*, 2013; Iglewski, 1996). *Pseudomonas aeruginosa* is capable of growing in conditions of extremely low nutrient content (Palleroni, 1984). The species was found to survive and proliferate in water for up to 100 days or longer (Warburton *et al.*, 1994). *Pseudomonas aeruginosa* has been isolated from skin, gills and stomach content of cultured *Clarias gariepinus* fingerlings in Nigeria (Oni *et al.*, 2013).

A lot of work has been carried out on growth performance of different hybrids of catfish (Solomon, 2013; Owodeinde *et al*., 2017). Therefore, this study is aimed at conducting the pathogenicity of *Pseudomonas aeruginosa* on juvenile heteroclarias in order to check their tolerance to pathogenic organisms.

Study area

This experiment was conducted at Oyo State College of Agriculture and Technology, Igboora located within Ibarapa Central Local Government (ICLG) Area of Oyo State. Igboora is located Latitude 70 26' 1.79" N and Longitude 30 17' 16.37" E. and 140 meter above the sea level.

MATERIALS AND METHODS

A total of 150 juvenile heteroclarias fish about 57-70g were purchased from a reputable commercial fish farm. The fish were acclimatized for 14 days before the commencement of the experiment, after which they were allotted into five treatment groups (A to E) with three replicate each having 10 fish. *Pseudomonas aeruginosa* was isolated on nutrient agar, MacKonkey agar and TCBS agar from the liver and skin of *Clarias gariepinus* that was aseptically dissected, and further characterised biochemically. *P. aeruginosa* for inoculation was prepared using serial dilution technique and harvested in normal saline. Treatment groups A to D were inoculated orally with different concentrations of *P. aeruginosa* (i.e. 3×10^1, 3×10^2, 3×10^3, 3×10^4) CFU/100µl respectively, while treatment E was inoculated with normal saline via oral administration. The fish were observed for 14 days during which the fish were observed for mortality, clinical signs and lesions of diseases. Thereafter, blood samples were collected from the fish through the heart using 21gauge needle and 2ml syringe. The blood samples were stored in heparinized bottles and preserved in a cold chain compartment. Two fish were sacrificed from each replicate per treatment group. Thereafter, tissue samples were collected from the skin, gills, gut, liver and kidney, and stored in universal bottles containing 10% formalin. The tissue samples and the blood samples were transported to the laboratory for analysis. Haematological parameters observed in this study were statistically analysed by general linear model using Statistical Package for the Social Science (SPSS, 20). To obtain the differences among the treatment means, data collected were subjected to Duncan Multiple Range Test.

$Yij = \mu + Ti + Єij$

Where:

Yij= Trait of interest

µ =Population mean

Tij= Fixed effect i^{th} *Pseudomonas aeruginosa* (i = 0, 10^1,10^2,10^3,10^4)

Єij = Random/residual error

RESULTS

COLONY AND MORPHOLOGY OF *Pseudomonas aeruginosa*

Colony of *Pseudomonas aeruginosa* used was green on nutrient agar, colourless on MacConkey and bluish on TCBS (Thiosulphate Citrate Bile Salts Sucrose agar). The Gram reaction result was Gram negative, rod shaped morphology when viewed under light microscope (x100).

Clinical observations

Treatment groups A to C exhibited slight skin discolouration, slight sloughing on their skin and patches of haemorrhage on their Skin. Mortality observed was recorded in treatment A to D, the occurrence was 20%, 10%, 10%, and 6.6% respectively.

Post-mortem and histopathological observations

After two weeks of the experiment, it was terminated and the tissue (blood) and organs (gills, intestine, kidney, liver) were harvested.

At post mortem examination, much was not seen in the kidney. However, on histology, there was marked histopathological modifications on some of the organs and tissues as a result of the invasive actions of the bacteria pathogen on them; vacuolar degeneration of renal tubular epithelium was observed. There was no appreciable lesion seen in the gut and the skin throughout all the treatment groups of this experiment. The gills of the fish subjected to infection revealed severe hyperplasia and fusion of secondary lamellae, while some had severe haemorrhage (shown in figure 1c, arrow pointing to haemorrhagic area), the liver had diffuse hepatocellular vacuolar degeneration (shown in figure 1b), figure 1a shows arrow pointing to area of vacuolar degeneration of renal tubular epithelium in the kidney.

Haematological parameters of Heteroclarias exposed to *P. aeruginosa*

There was no significant difference ($p \geq 0.05$) in most of the haematological parameters measured across the treatment groups except haemoglobin (Table 1). The haemoglobin parameter of hybrid catfish in treatment group B was significantly higher ($p \leq 0.05$) than that of the other treatment groups. Treatment A was significantly lower ($p \leq 0.05$) than treatment B, but significantly higher ($p < 0.05$) than treatment group D and E. Treatment group A and C, D and E are not significantly different.

Table 1: Origin, colony and morphology of *Pseudomonas aeruginosa* isolate inoculated

Sample number and name	F2P3 FISH 1 (Liver)	F3P1 FISH 1 (Skin)
Bacteriological Test	Result	
Growth on MacConkey agar	Colourless	Colourless
Growth on Nutrient agar	Green	Green
Growth on TCBS	Blue	Blue
Gram	Negative	Negative
Morphology	Rod	Rod
Organism	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*

Key: F – Farm, P – Pond

Table 2: Frequency of occurrence of mortality of fish inoculated

TG	DPT (CFU/100µl)	NIF	Mortality	FOM (%)
A	3×10^1	30	6	20
B	3×10^2	30	3	10
C	3×10^3	30	3	10
D	3×10^4	30	1	6.6
E	Control (NS)	30	0	0

Key: FOM (%) - Frequency of occurrence of mortality,
DPT - Dose per treatment (CFU – Colony forming unit),
NS – Normal Saline, NIFB. – Number of inoculated fish, TG – Treatment Group

Table 3: Haematological parameters of Heteroclarias infected with *P. aeruginosa* and the controlled treatment groups

Parameters	A	B	C	D	E	SEM	PVALUE
PCV (%)	22.67	24.75	21.00	21.33	17.50	0.97	0.12
HBC (g dL^{-1})	7.30^{ab}	8.13^{a}	6.73^{ab}	5.80^{b}	5.73^{b}	0.33	0.05
RBC (cells × 10^6 mm^{-3})	2.02	2.40	1.44	1.37	1.34	0.19	0.24
NØ	15550	15275	12833	14667	13263	577	0.53
PLT	145.67	149.50	139.33	141.00	167.25	8.86	0.86
LYM	59.33	62.00	56.67	54.33	57.53	1.54	0.48
HET	33.67	30.25	37.00	37.33	38.50	1.62	0.47
MONØ	2.67	2.75	3.67	2.67	3.00	0.20	0.57
ESIØ	4.00	4.75	2.67	5.33	3.75	0.34	0.15
BAØ	0.33	0.25	0.00	0.33	0.00	0.10	0.70

[ab]Means in the same row with different superscripts differed significantly (P≤0.05)
SEM- standard error of means, PCV - packed cell volume, HBC - haemoglobin concentration, RBC - red blood cell count, NØ neutrophils count, PLT - platelet count, LYM - lymphocytes, HET - heterophil count, MONØ - monocytes count, ESIØ - eosinophils count, BAØ - basophils count

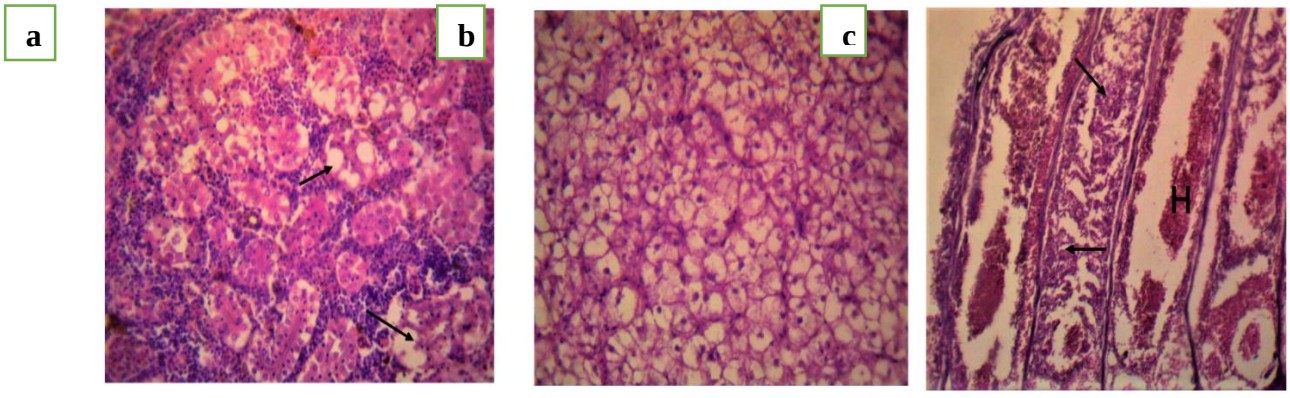

Figure 1: Histopathological changes of organs of Heteroclarias juveniles subjected to *Pseudomonas aeruginosa*
Slide a. Kidney: Vacuolar degeneration of renal tubular epithelium (arrows) (H&E x150)
Slide b. Liver: Diffuse hepatocellular vacuolar degeneration (H&E x 400)
Slide c. Gills: Diffuse hyperplasia and fusion of secondary lamellae (arrows) with severe heamorrhage (H&E x150)

DISCUSSION

A number of aquatic animals like fish, frogs and soft-shelled turtles were recorded to be susceptible to *Pseudomonas* spp. in earlier studies (Somsiri and Soontornvit, 2002). Relatively few disease outbreaks occur even though the bacteria are present in the water; thus, these pseudomonads are probably opportunists which produce disease when fish are stressed or injured (Bullock, 1965).

The experiment conducted on Heteroclarias revealed that fish in treatment A to C exhibited skin discoloration and slight sloughing all over their skin which was not observed in the control experiment. This is similar to what was recorded in experimental exposure of *Clarias gariepinus* to *Pseudomonas aeruginosa* by Amrevuawho *et al.* (2014). The superficial clinical signs limited to the skin were discoloration of the skin, and ulceration, noticed toward the tail and fin. Although, heamorrhages were not observed and there was no fin rot. This may be due to period of the Heteroclarias exposure to the infection (14 days whereas *C. gariepinus* were exposed for 21 days) or it may be their high threshold to withstand infection. Similar experiment conducted by Magdy *et al.* (2014) observed similar superficial lesions of haemorrhage, tail and fin rot, and skin ulceration at infection exposure period of 7 days. The variance in duration and similar lesion observed post experimental infection suggests that the concentration of the organism inoculated or the strain of the organism inoculated could be the reason for lesion observed. The mortality observed in this study ranges from 6.6% to 20% at 3×10^1 to 3×10^4 CFU/fish/100µl concentration of *Pseudomonas aeruginosa* that was inoculated. This report is similar to Hossain *et al*. (2006) and Magdy *et al.* (2014) who studied the infection of *P. aeruginosa* in *Oreochromis niloticus* and *Clarias gariepinus* reported mortality range between 20% to 90% and 40% respectively, but contrary to Amrevuawho *et al.* (2014) who conducted similar studies in *Clarias gariepinus* although claimed to record some mortalities but was not reported. Oh *et al.* (2019) who also reported 15% mortality when he challenged rainbow trout with *Pseudomonas tructae.*

Histopathology revealed vacuolar degeneration of renal tubular epithelium this is in line with what was observed by Magdy *et al.* (2014) who subjected *C. gariepinus* to similar experimental infection conditions. He reported vacuolar degeneration with necrotic changes in the tubular epithelium, sloughing off of the epithelial cells, hypertrophy of the epithelial cells of the renal tubules with reduction in tubular lumens and contraction of glomeruli. Significant tubular degeneration (with cellular debris surrounding the tubules) and hyaline droplet accumulation in tubular epithelium was observed in the infected posterior kidneys by Oh *et al.* (2019) who experimentally challenged rainbow trout with *Pseudomonas tructae*. Damaged kidney indicates attack from bacterial toxins. *Pseudomonas aeruginosa* produces quite a number of toxins which are responsible for the above mentioned lesions in the kidney (of which the liver is not exempted) (Magdy *et al.* 2014; Suprapto, 2005).

In the liver, diffuse hepatocellular vacuolar degeneration was noticed, this is close to what Magdy *et al.* (2014) reported. However, Amrevuawho *et al.* (2014) reported a few foci of large cytoplasmic vacuolation in the hepatocyte cells. The sinusoids were also moderately congested in *C. gariepinus*. Also, Oh *et al.* (2019) observed vacuolation of hepatic cells, few necrotizing areas and infiltration of macrophages and lymphocytes in the liver of rainbow trout infected with *Pseudomonas tructae*. The effect of *P. aeruginosa* on the liver acknowledges its invasive nature, resulting in failure to detoxify foreign bodies leading to liver dysfunction.

The gills are important organ for respiration, regulation of osmotic and ion balance and excretion of nitrogenous waste (Magdy *et al.,* 2014; Wani *et al*., 2011; Hassan *et al.,* 2010); because the gills keep in contact with external environment, it is considered the primary target of the bacteria infection (Wani *et al*., 2011). In this experiment, diffuse hyperplasia and fusion of secondary lamellae with severe heamorrhage was seen in the gills. This contradicts the report given by Amrevuawho *et al.* (2014), that observed marked loss and sloughing off of the gill lamellar epithelium whereas, fusion of the secondary lamellae due to hypertrophy and hyperplasia of the epithelial cells with epithelial lifting and necrosis, oedema and desquamation of lamellar epithelium was observed in the study conducted by Magdy *et al.* (2014). Hassan *et al.* (2010) reported necrotic changes in gills lamellae plus rupture of lamellar epithelium, lamellar fusion, hypertrophy and hyperplasia of epithelial cells of gill structure of *C. gariepinus*, infected with bacterial diseases. These gills lesions led to hypoxia, because surface area available for oxygen exchange has been reduced, oxygen distance between water and blood has increased and toxic waste has accumulated within body and finally, death.

The gut and the skin did not present any lesion on histology which contradict the scholarly report presented by Hassan *et al.* (2010) and Magdy *et al.* (2014). Report from Hassan *et al.,* (2010) on histology of skin of diseased fish were massive thinning of epidermis, sloughing of epithelial cells, contents of club cells at the surface of the epidermis were squeezed out, extensive vacuolation of club cells especially around their nuclei and decrease in density of mucous cells. Magdy *et al.*, (2014), described the skin lesion on histology as vacuolar

degeneration and necrosis in the epithelial cells with mononuclear inflammatory cells infiltrating in between the epidermal cells. The goblet cells were activated and the oedematous fluid accumulation of the sub-epidermal led to splitting of the sub-epidermal connective tissue.

The stomach of fish exposed to *P. aeruginosa* in this study did not reveal any change. This contradicts Amrevuawho *et al.* (2014) and Magdy *et al.* 2014 studies where the rugae were shortened; the submucosa glands were reduced in numbers, the surface epithelial cells appear to be proliferating rapidly, immature (hyperplastic) hypertrophy in the columnar epithelium of the intestinal villi, crypts with increased secretory activity, vacuolated clear cytoplasm and infiltration of inflammatory cells where observed in the stomach and intestine of *C. gariepinus* challenged with *P. aeruginosa*.

Haematological parameters are useful indicators for evaluations of physiological state of animals (Khan & Zafar, 2005) and fish is not excluded (Thrall, 2004). Parameters that are related to the blood and blood forming organs are used in the diagnosis of many diseases, and in investigation of the extent of damages done in the blood forming organs (Bamishaiye *et al.*, 2009; Togun *et al.*, 2007). Haematological parameters have been used to assess the effects of pathogenic bacteria in African catfish, *C. gariepinus* (Ezeri, 2001). In this study, *Pseudomonas aeruginosa* has no effect on most haematological parameters of Heteroclarias, only with the exception of haemoglobin, where treatment group B is significantly higher ($P \leq 0.05$) than other treatment groups. This finding disagrees with Amrevuawho *et al.* (2014), who reported significant reduction in the mean values of packed cell volume, haemoglobin, red blood cell and lymphocyte count of *C. gariepinus* after three weeks of exposure to *P. aeruginosa* and significant increase in the mean values obtained for white blood cell and neutrophil of infected fish. Haemoglobin is the iron containing protein found in the red blood cells whose function is to assists in the transportation of oxygen. RBC count, PCV and haemoglobin concentrations (HBC) are used as indicator for oxygen transportation in all animals including fish. In this study, the value of RBC and PCV were not affected, but the HBC value was higher in the infected fish. This indicates that oxygen carrying capacity of the fish was not impaired during the study.

CONCLUSION

The invasive nature of *P. aeruginosa* was pronounced in the internal organs, while the effect was minimal on blood parameters and external features (symptoms) when compared to other similar experiments reported by other scholars. Heteroclarias might not have shown any external symptoms of infection but the vital organs were affected. All the examined organs are vital to the survival of fish, injury to anyone can lead to death not to mention injury to all. It was also observed that the extent of lesions seen in this experimentation were in diverse degrees which is irrespective of the concentration of the inoculated organism but conclusion cannot be drawn whether it is time dependent. Hence, there is need for more studies to be conducted on the effect of bacteria pathogens on the newly trending aquaculture farming of hybrids of the two African Clariid catfish "Heteroclarias". This will help aquaculture farmers to take necessary precautions especially on biosecurity measures to prevent outbreaks of diseases and invariably prevent farmers from any economic loss.

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