VOLUME 11 ISSUE 1 2025

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 11, No. 1, 843-850 (2025)

International Journal of Zoological Investigations
Contents available at Journals Home Page: www.ijzi.net
Editor-in-Chief: Prof. Ajai Kumar Srivastav
Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

Haematological and Histological Changes Associated with Artificial Infection of *Aeromonas jandaei* in *Anabas testudineus*

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Received: 8th August, 2024; **Accepted:** 25th October, 2024; **Published online:** 30th June, 2025

https://doi.org/10.33745/ijzi.2025.v11i01.088

Abstract: The present study is an attempt, to observe and analyse the onset of clinical symptoms, certain haematological parameters and histological alterations in climbing perch, *Anabas testudineus* following artificial infection with *Aeromonas jandaei*. Clinical signs noticed in the experimental organisms include: loss of appetite, erratic swimming behaviour, haemorrhages and ulceration. Fishes were sampled at 24, 48, 72 and 96 h interval post infection. Estimation of total RBC and haemoglobin (Hb) till 96 h of post infections showed significant reduction and WBC counts were higher in infected groups when compared to the control group. The histopathology revealed marked degenerative changes in kidney and liver tissues. The present analyses clearly revealed the pathogenicity of *Aeromonas jandaei*, to *Anabas testudineus,* and this might be helpful in the identification and the control of such infections in cultured fishes.

Keywords: *Anabas testudineus, Aeromonas jandaei*, Haemorrhage, Haematology, RBC, WBC, Haemoglobin

Citation: Mazumder Abhishek and Dey Abhinit: Haematological and histological changes associated with artificial infection of *Aeromonas jandaei* in *Anabas testudineus.* Intern. J. Zool. Invest. 11(1): 843-850, 2025.

https://doi.org/10.33745/ijzi.2025.v11i01.088

Introduction

Anabas testudineus, commonly known as "Koi" is a freshwater fish with wide geographical distribution. This fish species is cultured in several countries due to its ability to sustain adverse environmental condition, and proves to be good for fish culture system. It is one of the important freshwater food fish with high demand. *A. testudineus* is prone to bacterial infection, and one of the potential candidate species of *Aeromonas* infection (Chhanda *et al*., 2019), leading to high mortality and economic loss. The bacterium of genus *Aeromonas* is ubiquitous inhabitant of freshwater ecosystem and has been reported to cause infections in fish as well as in humans (Wu *et al*., 2007). In freshwater fishes, *Aeromonas* spp. are mostly the causative agents for bacterial infection which causes local inflammations, septicaemia, necrosis, muscle and soft tissues in

fishes (Austin and Austin, 2007; Skwor *et al*., 2014). *Aeromonas jandaei,* a gram-negative, rod shaped bacterium associated with haemorrhages and ulceration has proven to be virulent in *Anabas testudineus* (Mazumder *et al*., 2020).

Blood, a specialised body fluid in animals, plays an important role in the immune system of the organism. Haematological parameters are vital indicators used for fish health monitoring (Hesser, 1960). Haematological parameters (RBC, WBC, Hb etc.) and analysis of serum constituents helps in detection, diagnosis of metabolic disturbances, diseases in fishes and assessing their immune system (Ballarin *et al*., 2004; Fazio *et al*., 2015a, b).

Histopathology is one of the most important techniques for monitoring of diseases in fishes (Islam *et al*., 2008). The natural infection caused by *A. jandaei* showed clinical symptoms such as reddish lesions with haemorrhages on pelvic fin and belly region of *Pangasianodon hypohthalmus*; the histopathology of same revealed marked alteration of hyperplasia of primary and secondary lamellae of gill tissue, necrosis with pyknotic nuclei in hepatic tissues and tubular necrosis in kidney (Kumar *et al*., 2014). *A. jandaei* have been observed in manifestation of histological alterations in internal organs – kidney, liver, gills, spleens (Dong *et al*., 2017).

Since there is no report on haematological and histological alterations caused by *A. jandaei* on *Anabas testudineus*, the present study is a pioneering work on clinical findings with pathological changes in haematological parameters and histoarchitechture of kidney and liver tissues from moribund fishes.

Materials and Methods

Experimental animals

Healthy adult *A. testudineus* (n = 30), weighing 20 – 45 g were collected from a commercial koi farm and then maintained in glass aquarium having a capacity of 30 L to acclimatise for 15 days prior to bacterial infection. The aquarium water was monitored and renewed with 50% fresh water daily.

Bacterial strain

Pure culture of *A. jandaei* (isolate AM-05) isolated from naturally infected *A. testudineus* was inoculated into Brain Heart Infusion (BHI) broth and was incubated for overnight at 30°C. After incubation, the culture was aseptically transferred to 2 ml eppendorf tube and centrifuged at 10000 rpm for 5 min in a cooling centrifuge. The supernatant was discarded and the bacterial pellet was washed in phosphate buffered saline (PBS pH 7.4). The number of bacterial colony in the suspension was quantified by using spread plate method in order to obtain an accurate estimate of the number of bacteria. The bacterial isolate was identified using both biochemical and molecular method (GenBank accession number MN204041).

Experimental infection

Two sets of experimental groups were arranged, each group comprising of ten individuals of fishes. One group of fish were employed as control and other group of fishes were intra-peritoneally injected 100 µl of bacterial suspension with 4.2 x 10^4 cfu/fish of *A. jandaei* (bacterial dose concentration were selected on the basis of 50% mortality of reinfection experiment) isolates. Changes in the haemoglobin content, total erythrocyte and leucocyte counts were determined at different time intervals of 24, 48 72 and 96 h post infection.

Morphological and behavioural examination

The fish were observed for colour change of body and other organs as well as behavioural aspects after infection with *A. jandaei.*

Sampling and haematology

For blood sampling, fishes were initially anesthetized for 3–4 min using 50 ppm clove oil. The fishes were wiped and cleaned properly, in order to avoid mucus mixing into the blood. Blood samples were collected from the caudal vein using 2 ml syringe with 22 gauge needle and stored in EDTA (Ethylene diamine tetra acetic acid 2.5%) containing tubes for hematologic tests. Red blood cells (RBC) and white blood cells (WBC) were

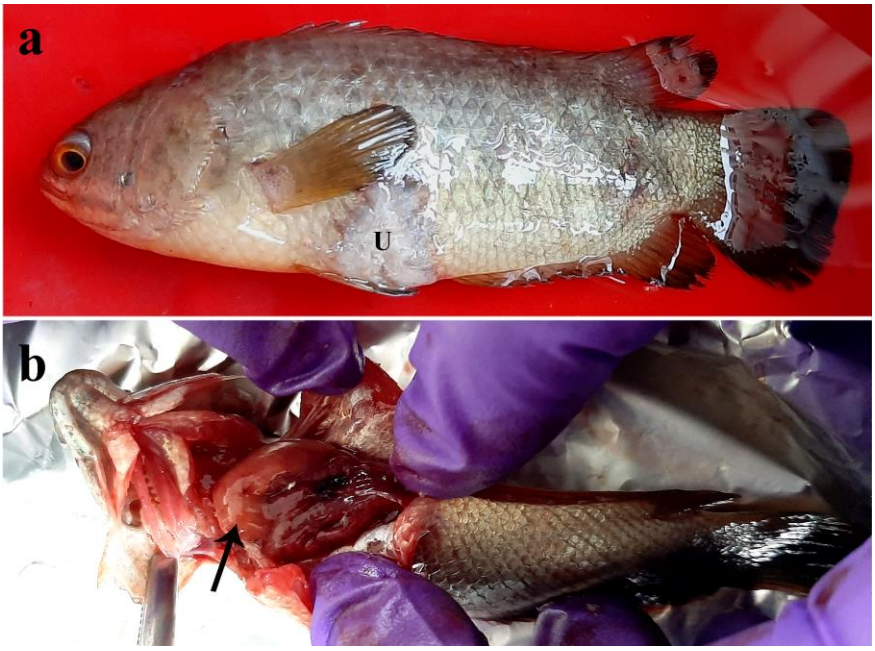

Fig. 1: Bacterial infections in experimentally-challenged *Anabas testudineus* with *A. jandaei*: (a & b) Clinical signs and gross pathology at 48 h of post infection. "U" represents ulceration; Black arrow mark represents change in liver colour.

counted using Dacie's solution as diluting fluid (Blaxhall and Daisley, 1973). Hb concentration was measured by cyano-methemoglobin method with spectrophotometer at 540 nm absorbance (spectrophotometric method).

Histological analysis

Kidney and liver tissues from *Anabas* 48 and 96 h after artificial infections were fixed in 10% neutral buffer formalin (NBF) for histological studies. Tissues were then subjected to dehydration through a series of increasing alcohol grades, cleared with xylene and embedded into paraffin following standard method. The paraffin-embedded tissues were sectioned at 5 µm thickness using a microtome and stained with haematoxylin and eosin (Roberts, 2012). Pathological changes manifested in the tissue sections were examined under a Leica DM 3000 microscope at 40 × magnification.

Statistical analysis

The data obtained from haematological tests were statistically analysed using ANOVA and Duncan multiple range test in order to test the significance among groups using Statistical Package for Social Sciences (SPSS) (version 16).

Results and Discussion

Haemorrhagic septicaemia, ulceration in skin, fins and tail-rot were the clinical signs observed in post artificial infection (Figs. 1 a, 2 a). The gross pathology revealed change in colour of liver (Figs. 1b, 2b). The haematological parameters showed significant reduction in the mean values of RBC and Hb. WBC count elevated in comparison to normal healthy fishes (Table 1). The values were significant ($p<0.05$) than those of the controls at 24, 48, 72 and 96 h. RBC counts decreased from control value of 1.71±0.03 to 1.40±0.01 for *A. jandaei* infected fishes. Hb of infected fishes dipped to 7.22±0.10 in contrast to 9.65±0.12 for control fishes at 96 h. WBC increased in number from 15.0±7.6 to 22.5±9.4. Histopathology of liver and kidney tissues revealed marked pathological changes. Histo-architechtural abnormalities in kidney includes glomerular atrophy and haemo-cyte aggregation at 48 h experimental infectivity and in 96 h of kidney showed glomerular

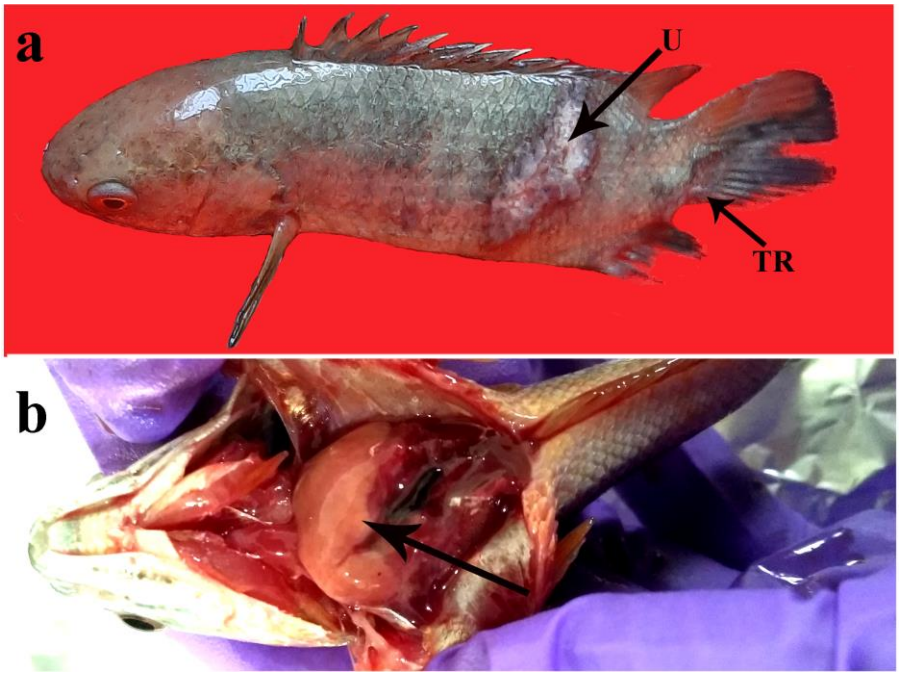

Fig. 2: Bacterial infections in experimentally-challenged *Anabas testudineus* with *A. jandaei*: (a & b) Clinical signs and gross pathology at 96 hours of post infection. "U" represents ulceration; "TR" represents Tail-rot; Black arrow mark represents change in liver colour.

Table 1: Blood parameters of climbing perch (*Anabas testudineus*) exposed to *A. jandaei*

Hours post infection	**Mean RBC/WBC counts ($10^6/10^5$ cells/mm) Control group**	**Mean RBC/WBC counts ($10^6/10^5$ cells/mm) Infected with AM-05 (*A. jandaei*)**	**Mean Hb (g/dl) Control group**	**Mean Hb (g/dl) Infected with AM-05 (*A. jandaei*)**
24 h	1.71±0.03/15.0±7.6	1.54±0.03/15.91±5.6	9.65±0.12	9.14±0.23
48 h	1.72±0.11/15.02±7.2	1.50±0.04/20.25±6.2	9.63±0.16	8.72±0.06
72 h	1.73±0.02/16.04±8.6	1.46±0.05/23.01±4.7	9.60±0. 20	7.43±0.05
96 h	1.67±0.05/14.97±9.5	1.40±0.01/22.5±9.4	9.62±0.05	7.22±0.10

Values are presented in means ± SE.

shrinkage, haemocyte aggregation and necrosis of the tissue (Figs. 3a, b). Haemorrhagic lesion and vacuolar degeneration were detected in 48 h of post infection and at 96 h infection revealed increased haemorrhages, vacuolation and necrosis of hepatic tissue (Figs. 4 a, b).

Species of *Aeromonas* are considered to be opportunistic pathogens, responsible for causing diseases in many fish species or act as secondary invaders in fishes suffering under stress conditions (Roberts, 2012). The clinico-pathological symptoms such as - abnormal swimming, dark bodies, haemorrhagic fin and abdomen, observed in the present investigation is in agreement with earlier report of *A. jandaei* infection in *Oreochromis niloticus* (Dong *et al*., 2017). Many authors have reported haemato-

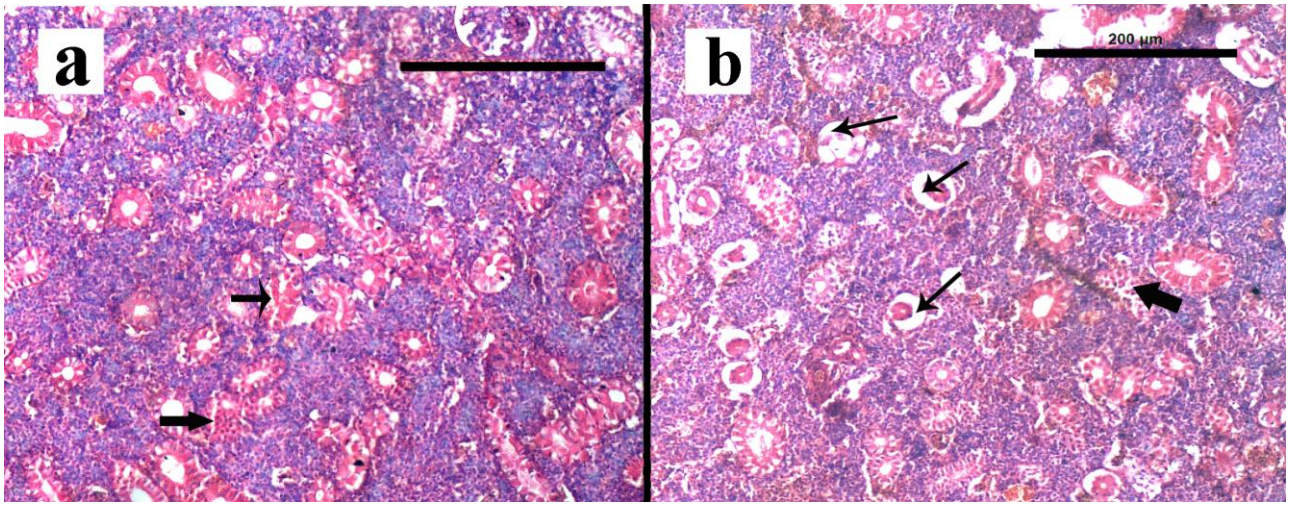

Fig. 3: Histological alterations in experimentally-challenged *A. testudineus*: T. S. of kidney infected with *Aeromonas jandaei* (a) at 48 h of post infection [Thin arrow represents glomerular atrophy & Thick arrow represents haemocyte agregation]; (b) at 96 h of post infection [Thin arrow represents glomerular shrinkage & Thick arrow represents haemocyte agregation].

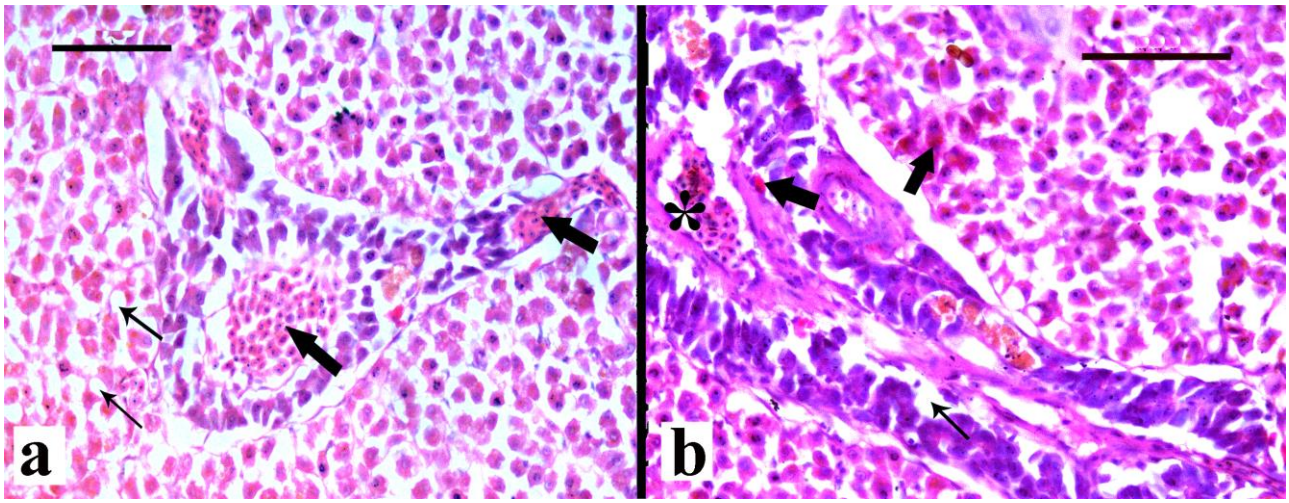

Fig. 4: Histological alterations in experimentally-challenged *A. testudineus*: T. S. of liver infected with *Aeromonas jandaei* (a) at 48 h of post infection [Thin arrow represents vacuolations & Thick arrow represents haemorrhages]; (b) at 96 h of post infection [Star represents necrosis; thin arrow represents vacuolation & Thick arrow represents haemorrhages].

logical changes in different cultured fish species to identify variable parameters and determine the fish health status due to bacterial infections. In this study changes in RBC, WBC and Hb were reported in *A. testudineus* after administration of bacterial isolate, *A. jandaei*. The present results corroborate with the previous report of Rehulka (2002) and Milud *et al*. (2015) on haematological response, in infectious diseases. The significant decrease in erythrocyte and haemoglobin content is possibly due to hypochromic microcytic anaemia caused by the bacterial infections (De Chavez and Encinares, 2018). Ishikawa (1998) reported that in unhealthy fish there was severe infection of haematopoiesis and decrease in erythrocyte volume. Moreover, from biochemical evident *A. jandaei* was hemolysin positive. Therefore, hemolysin released by *A. jandaei* could cause erythrocyte lysis and play important roles in pathogenesis, in addition to different enterotoxins

released by the bacterium. The significant decrease in total erythrocyte count in *A. jandaei* infected fish compared to the control fishes, in the present study could be attributed to the severe haemolytic activity of aerolysin protein. Significant reduction in Hb content in infected fishes may be due to the destruction of the haematopoietic organs located in spleen and pronephros (Milud *et al*., 2015). Decrease in haemoglobin content also indicates that the oxygen carrying capacity of the fish was impaired by the bacteria which may correspond to anaemic hypoxia. The increase in WBC count in present study validate to the results obtained by Pathiratne and Rajapakshe (1998), who have reported that more production of leucocyte in bacterial injected fish is due to the non-specific (cellular) defense system which is stimulated during the first stage of disease manifestation of fish defense mechanisms. The findings of blood parameters are supported by the explanation of previous workers – leukopenia with lymphopenia, neutrophilia and occasional monocystosis were frequently caused by gram-negative bacterial disease of fish (Noga, 2000). Therefore, it can be inferred that the bacterial infections are major causes of changes in haematological parameters.

Quite significant and systematic pathological alterations were seen in the internal organs – kidney and liver of *A. testudineus* experimentally challenged with *Aeromonas jandaei*. Kidneys and liver play important role in fish physiology and are very sensitive to *Aeromonas* disease attacks. Fish kidneys exposed to *A. jandaei* revealed severe damage and show glomerular atrophy, haemocyte aggregation. Similar observations were also made by Abraham *et al*. (2015). These alterations might be due to the enzymatic degradation caused by the bacterial infection as reported by Burr *et al*. (2005). Bacterial toxins causes glomerular shrinkage and necrosis of cells which triggers loss in the structural integrity of kidneys. This finding corroborates with the results of Belgin *et al*. (2010). The important pathological symptoms detected in koi liver affected by *A. jandaei* revealed damaged hepatocytes having vacuoles, haemorrhages and necrosis. Similar liver pathology in *A. testudineus* was previously reported by Kulsam (2005) which validates the present findings. The current findings are in concurrent with the previous results reported by Afifi *et al*. (2000) who described that extracellular products and toxin released by *A. hydrophila* caused severe necrosis in liver. Hassan *et al*. (2017) stated that haemorrhages, necrosis and vacuolar degeneration are characteristics for much non-proliferative bacterial disease in teleost and these results are attributed to the effect of combined virulence factors of the representative bacterial isolate and sometimes it is species specific also. Thus, from above discussion it is evident that bacterial toxins released by bacteria interrupts the normal functioning of the organs which proves to be fatal for fish physiology.

Conclusion

The present investigation observed abnormalities in haematological parameters and tissue architecture caused by bacterial exposure. Therefore, on the basis of above-mentioned alterations, it is interpreted that severe imbalance in physiological process might have occurred due to bacterial infection. The results of this study may provide guidance to the clinical and pathological signs of disease outbreak of *A. jandaei* and therefore, better management practices in aquaculture are needed to prevent the outbreak of this emerging disease and its control.

Ethical Statement

Ethical protocol followed in the present study was approved by the Institutional Animal Ethical Committee (IAEC) of Gauhati University, Assam (India), under protocol number IAEC/Per/2018/PP-IAEC/2018-039 in accordance with guidelines for care and use of laboratory animals.

Author Contributions

Each author has equally collaborated in designing the research, conducting thorough analysis, drafting the manuscript, and refining its content. All authors have read and agreed to the published

version of the manuscript.

Acknowledgements

Mazumder is grateful to the University Grants Commission, New Delhi (under UGC-BSR scheme) for fellowship grant.

Conflict of Interest

The authors declare no conflicts of interest.

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