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Research Article

HISTOPATHOLOGY OF ROHU *LABEO ROHITA* (HAMILTON) AFTER INFECTING WITH *AEROMONAS HYDROPHILA*

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ABSTRACT

Rohu fishes grown with biofloc for 120 days have been examined for the histopathological changes occurred in the intestine and kidney after infecting with *Aeromonas hydrohila* with 0.5 ml, 1.0 ml and 1.5 ml where 1.5 X 10^5 bacteria per ml. There was drastic changes observed in control compared with treatments and within the treatments 1.5 ml dose has shown massive destruction in the tissues compared with 0.5 and 1.0 ml. 0.5 ml have shown least changes in external as well as internal parts of kidney and intestine. In the present study histological alterations were documented for different tissues intestine and kidney of *A. hydrophila* infected rohu fish *Labeo rohita* fingerlings, fed with probiotic supplemented diets with rice bran and ground nut oil cake in biofloc with added ammonium chloride when NH_3 is reduced. Histological observations on intestine and kidney of control and biofloc fed fingerlings showed abnormal architecture of primary and secondary lamellae. Blood (0.5 ml) removed from the fishes to observe the blood cells after infecting with *A.hydrohila.* Heamoglobin and RBC were decreased. Neutrophils, WBC and lymphocytes, monocytes count has been increased.

Keywords: *Aeromonas hydrohila,* WBC, *Labeo rohita,* Neutrophils.

INTRODUCTION

Histopathology is concerned with the study of alteration of tissue structure and function that occur in disease and the correlation of these changes with the clinical sign and symptoms. Histopathology studies are the most important tool for disease diagnosis. Because of their low insistence, frequent application of these pesticides are life form skillful for the have power over of pests in agricultural fields and thereby large quantities find their way into water bodies(Tamizhazhagan *et al.*, 2017). It confirms the involvement of etiologies in the organ and tissues of diseased fish. It also describes the extent of damage caused in various organ systems. Histology is the microscopic study of plant and animal tissues. Although all organisms are comprised of at least one cell, we will be focusing on observing cells and tissues of the human body. All organisms are composed of cells. Humanoid body cells are grouped by their similarities in structure and function into tissues (Tamizhazhagan & Pugazhendy, 2017). Histological technique deals with the preparation of tissue for the microscopic examination. Histopathological changes in fish organs have been increasingly (Tamizhazhagan *et al*., 2016). Histopathological studies reveal the impact of

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toxicants on fish as it provides direct translation of toxic xenobiotics effects on vital anatomical functions. Histological analysis appears to be a very sensitive parameter and is crucial in determining cellular changes that may occur in target organs such as the gills, muscle, liver and kidney (Dutta, 1996).

MATERIALS AND METHODS

Sample collection

Rohu fish (30+30 numbers) cultured in biofloc for 120 days were collected and kept in separate aquaria tanks (6) 10 fishes in each aquarium for control and treatments. After acclimatizing in aquaria they have been infected with *A.hydrophila* with the concentrations of 0.5 ml, 1 ml and 1.5 ml and the bacterial count in each ml was 1.5X105 cells per ml of culture media.

Important steps involved in histopathology

Fixation, Dehydration, Clearing, Embedding, Sectioning, Staining (Tamizhazhagan & Pugazhendy, 2017). Samples should be collected from freshly dead fishes, not frozen or extremely dead and sample volume should not exceed $1/10^{th}$ of the fixative. The samples should be placed in an appropriate fixative.

Fixation

The process of fixation is done to preserve cells and tissue constituents in a life like state possibly. Cells and tissues are fixed in a physical and partly also in a chemical state, so that they will withstand subsequent treatment with various reagents with minimum loss of architecture. This is achieved by exposing the tissue to chemical compounds, called as fixatives, which coagulate tissue proteins and constituents, a necessary event to prevent their loss or diffusion during tissue processing. The amount of fixative should be approximately 10-20 times the volume of the specimen on all sides. In the present histology studies neutral buffer formalin is used as common fixative. Formalin is available as 37 or 40% w/w solution of formaldehyde gas in water. 10% of the solution in normal saline has been used to remain the tissue in it for prolonged period without distortion. It is compatible with most special stains also which is cheapest and most popular.

Dehydration

Dehydration is the process of removal of fixative and water from the tissue and replacing it with dehydrating fluid. Tissues are dehydrated by using by using gradient concentration of alcohol like 50%, 70%, 90% and 100%. The duration for the tissues were kept in each strength of alcohol depends upon the size of the tissue, fixative used and types of tissue. Here the concentration of ethyl alcohol was used is 50% because intestine and kidney are smooth and delicate parts which may get shrinkage by increased alcohol concentration.

Clearing

Clearing is a process of replacement of hydrating fluid that totally miscible with both the dehydrating fluid and the embedding medium. During the dehydration water in tissue has been replaced by alcohol. After that alcohol was replaced by paraffin wax. As paraffin wax is not alcohol soluble, we replaced alcohol with xylene in which wax is soluble. Small piece of tissues were cleaned in 0.5 – 1 hour.

Embedding

Embedding is the process of replacement of clearing agent with the embedding medium. Impregnation with wax was allowed to occur at melting point temperature of paraffin wax (55 60^0 C). Volume of wax was about 25-30 times the volume of tissues was used. The duration of impregnation was around 4 hours because in this experiment only intestine and kidney were used. The section has been taken 3-4 microns thickness and the tissues have been oriented and placed in a mould with labels. After that fresh melted wax was poured in it and allowed to settle and solidify. Once the block has been cooled they were immersed in cold water to cool rapidly. The wax block has provided the external support during the sectioning.

Sectioning

This is a process where the wax hardened the tissues and was cut into very thin sections 3-5 microns by using microtome.

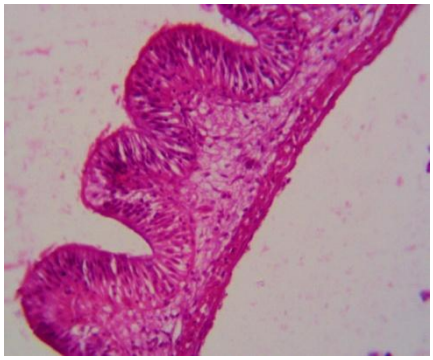
Staining

Staining is a process which gives colour to the sections of tissues by using special dyes in order to enhance contrast in microscope image. Eosine was used as dye and the slides stained manually.

RESULTS AND DISCUSSION

The inoculation of *A. hydrophila* has been done through injection near the caudal vein by using No. 24 gauge syringe. Ten numbers of fish were infected with *A. hydrophila* including control with three doses *i.e* 0.5 ml, 1.0 ml and 1.5 ml. The symptoms were found from the day three very clearly. Inflammation, reddening of the body and abdomen, erosion of caudal and dorsal fins bulged abdomen, hemorrhages on the body etc. Internal parts were lost their normal colour due to necrosis. Hemoglobin and RBC increased and nutrophils have been decreased in the all treatments compared to control and WBC and lymphocytes, monocytes count has been increased when compared with control and the same has been shown in the results of Dharmakar (2017). The fish presented necrosis in the site of *A. hydrophila* inoculation tissue more and hemorrhage and necrosis of kidney and intestine were observed (Schlotfeldt *et al.*, 1995) when they were infected by the bacteria. The fishes started dying after 3^{rd} day but showed symptoms after 36 hours. Control animals died in all the three groups but only 3 died in treated groups. The histopathological changes were observed in only dead

fishes compared with negative control but blood was taken from live animals after injecting *Aeromonas hydrophila* and showing characters of infection.



Normal intestine

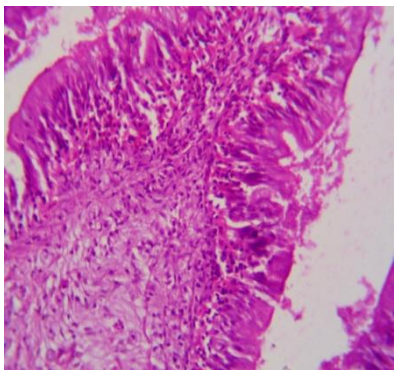


Figure 1. Mild to moderate infiltration of lymphocytes, macrophages, plasma cells and fibroblasts (a)

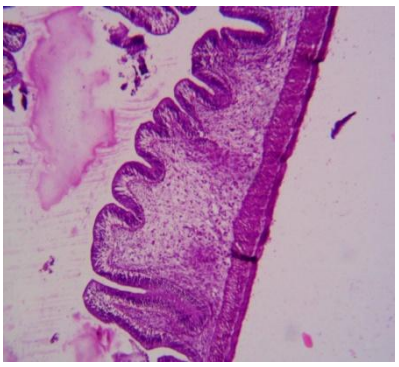


Figure 1. Fusion of intestine villi (b)

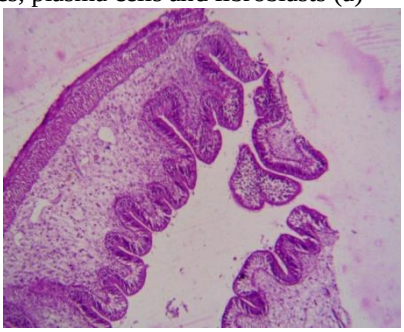


Figure 2. Mild to moderate infiltration of MNC and fibroblasts in sub mucosa and mucosa, desquamated villus epithelial lining and mild increase in number of goblets (a)

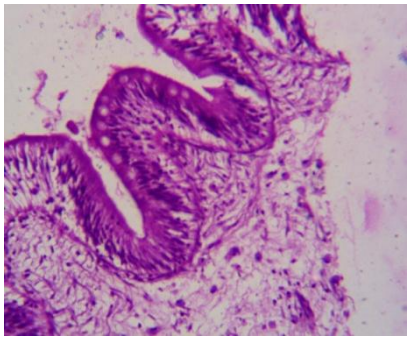


Figure 2. Mild infiltration of inflammatory cells and increased number of goblet cells in villus epithelial lining (b)

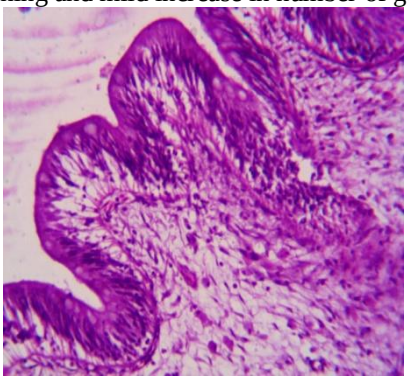


Figure 3. Mild infiltration of MNC, few PMN cells and increased number of goblet cells in villus epithelial lining (a)

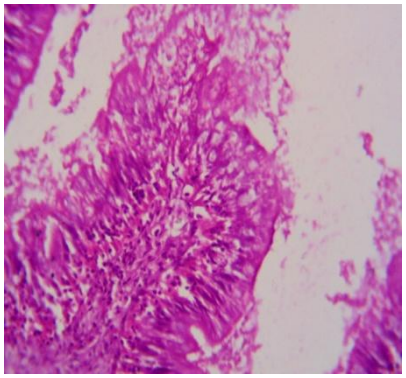


Figure 3. Moderate infiltration of MNC, fibroblasts, loss of ciliated epithelium on tip of the villi, and few number of goblet cells are observed (b)

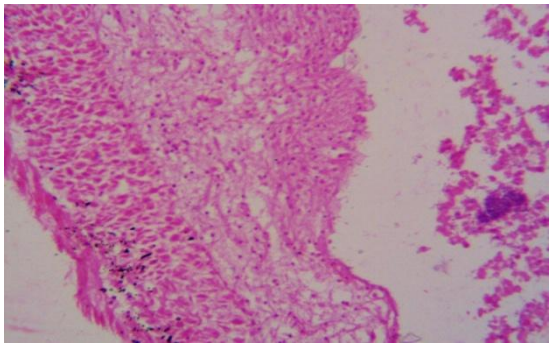
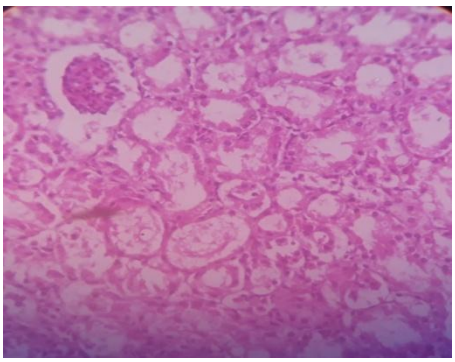


Figure 3. Mild infiltration of mononuclear cells in sub mucosa and mucosa with high bacteria (c)

Note: Figure 1: – 0.5 ml, Figure 2 – 1.0 ml, and Figure 3 – 1.5 ml of *Aeromonas hydrophila* medium which has the bacterial number of $1.5X10^5$.

According to (Abdelhamed *et al*., 2017) intestine of control group exhibited normal intestinal layers including mucosa, sub mucosa, muscularis, and serosa. At 5 HPC, approximately 75% of the surface of entire mucosal layer was necrotic in two fish. At 24 and 48 HPC, extensive necrosis of the surface of entire mucosa with an accumulation of necrotic enterocytes and homogenous substance within the lumen in six fish. Multifocal clumps of monomorphic rod bacteria were detected in the intetstin al lumen.In the present experiment mild to moderate infiltr ation of lymphocytes, and Fusion of intestine villi (Figure 1) Mild to moderate infiltration of MNC and fibroblasts in sub mucosa and mucosa, desquamated villus epithelial lining and mild increase in number of goblets, (Figure 2a) Mild infiltration of inflammatory cells and increased number of goblet cells in villus epithelial lining(Figure 2b) and Mild infiltration of MNC, few PMN cells and increased number of goblet cells in villus epithelial lining (a), Moderate infiltration of MNC, fibroblasts, loss of ciliated epithelium on tip of the villi, and few number of goblet cells are observed (b) and Mild infiltration of mononuclear cells in sub mucosa and mucosa with high bacteria (c)



Normal kidney

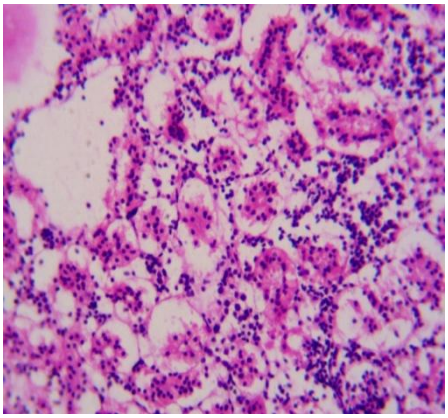


Figure 4. Severe infiltration of inflammatory cells with peri tubular and glomerular spaces (a)

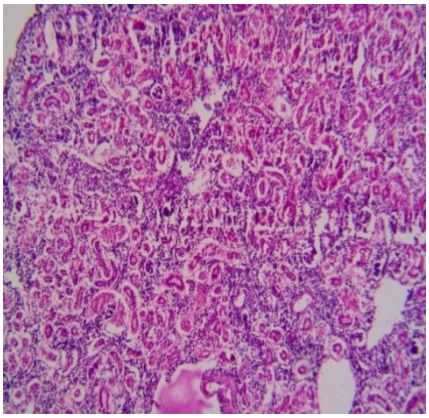


Figure 4. Severe inflammation with hemorrhage and tubular necrosis (b)

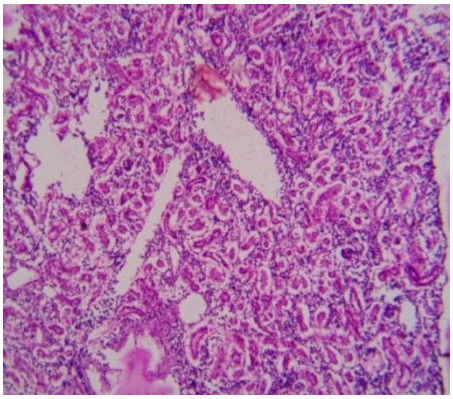


Figure 5. Cystic spaces, severe infiltration, atrophy of glomeruli, increased peri tubular and glomerular spaces, degenerated and necrotic tubules (a)

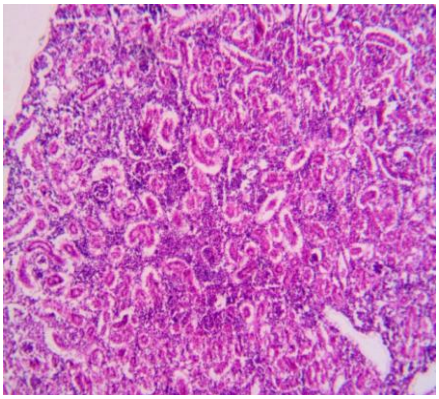
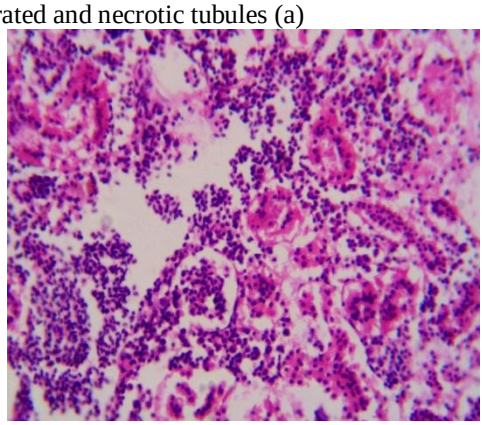


Figure 5. Severe inflammation with hemorrhage and tubular necrosis (b)



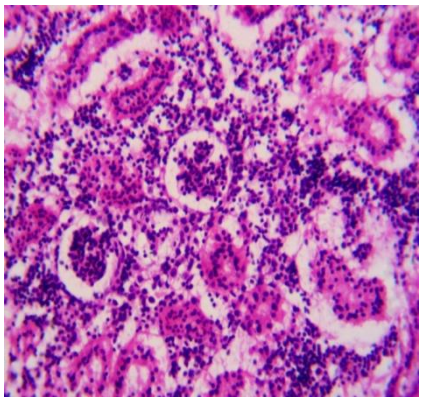


Figure 6. Severe infiltration of inflammatory cells increased peri tubular and glomerular spaces(a)
Note: Figure1 is 0.5 ml, Figure2 is 1.0 ml Figure 3 is 1.5ml of *Aeromonas hydrophila*

In the three treatments of intestine (0.5 ml, 1.0 ml and 1.5 m. *A. hydrophila*) injected fishes have been dissected and observed under microscope. In the first dose (0.5 ml.) the intestine of the fishes observed were mild to moderate infiltration of lymphocytes, macrophage, plasma cells and fibroblasts were observed as shown in the Figure 1a and fusion of intestinal villi were observed which has presented in the Plate 1b compared to the control. In the kidney (Figure 1a) severe infiltration of inflammatory cells with peri tubular and glomerular spaces were observed and (Plate 1b) inflammation with hemorrhage and tubular necrosis were observed.

In the second dose (1.0 ml.) mild to moderate infiltration of MNC and fibroblasts in sub mucosa and mucosa, desquamated villus epithelial lining and mild increase in number of goblets cells were observed (Figure 1a) and infiltration of inflammatory cells and increased number of goblet cells in villus epithelial lining was observed as shown in the (Figure 1b) compared to the control. Cystic spaces, atrophy of glomeruli, increased peri tubular and glomerular spaces, degenerated and necrotic tubules were observed in the kidney (Figure 3a) and severe inflammation with hemorrhage and tubular necrosis has been noticed (Figure3b). Gobinath & Ramanibai, (2014) have shown that *Vibrio cholerae* after 30 days of feeding with experimental diets showed prominent tubular epithelium, glomerulus with Bowman's capsule and inter-renal cells . Whereas fingerlings with bacterial treatment showed invading lymphocytes, hyperplasia of interregnal cells, fibrosis, dark granule accumulation in tubular cells, vacuolation of kidney tubules, necrosis of epithelial lining and depletion of lymphoid cells in kidney. However the pretreated groups 3 probiotic did not show any marked deformities in the kidney. Histological kidney tubules, observation on kidney showed the normal architecture as seen in fingerlings fed with control diet. In the present experiment Figure 1 shows that Severe infiltration of inflammatory cells with peri tubular and glomerular spaces(a) and severe inflammation with hemorrhage and tubular necrosis (b), Figure 2 shows Cystic spaces, severe infiltration, atrophy of glomeruli, increased peri tubular and glomerular spaces, degenerated and necrotic tubules (a) and Severe inflammation with hemorrhage and tubular necrosis (b) and Figure 3 shows severe infiltration of inflammatory cells increased peri tubular and glomerular spaces(a).

According to Abdelhamed *et al*. (2017) intestine of control group exhibited normal intestinal layers including mucosa, sub mucosa, muscularis, and serosa. At 5 HPC, approximately 75% of the surface of entire mucosal layer was a necrotic in two fish. At 24 and 48 HPC, extensive necrosis of the surface of entire mucosa with an accumulation of necrotic enterocytes and homogenous substance was observed within the lumen in six fish. Multifocal

clumps of monomorphic rod bacteria were detected in the i ntestinal lumen. In the present experiment, it is observed that mild to moderate infiltration of lymphocytes and fusion of intestine villi (Plate 1 a and b). Mild to moderate infiltration of MNC and fibroblasts in sub mucosa and mucosa, desquamated villus epithelial lining and mild increase in number of goblets were observed (Figure 2a). Mild infiltration of inflammatory cells and increased number of goblet cells in villus epithelial lining (Figure 40b), and Mild infiltration of MNC, few PMN cells and increased number of goblet cells in villus epithelial lining (3 a). Moderate infiltration of MNC, fibroblasts, loss of ciliated epithelium on tip of the villi, and few number of goblet cells were observed (3 b) and Mild infiltration of mononuclear cells in sub mucosa and mucosa with high bacteria were also detected. (Tiwari & Pandey, 2014) indicated that *Flexibacter columnare* infection in rohu was primarily associated with skin and finuleers and gill necrosis was rarely observed. Large number of *F. columnare* were observed in the skin ulcers and attached to the exposed layers of dermis. The cutaneous ulcers extended to the deep dermis and underlying skeletal musclein occasional cases. Necrosis of skin and muscle was accompanied by infiltrates of neutrophils. In the internal organs like kidney, liver and spleen neither *Bacilli* nor microscopic lesions were observed. In the present study, skin hemorrhages, fin necrosis, increased number of goblet cells, mild to moderate infiltration of poly nuclear and mononuclear cells, lymphocytes, macrophages and fusion of intestinal villi have been observed in the intestine including heavy bacteria in the infected fish.

CONCLUSION

The fishes dead with *A. hydrophila* have shown almost all the characters of the bacteria compared with control. The bacteria were observed in the intestine and kidney confirmed by histopathology. The bacteria injected to the rohu were pure strain and has been brought from CIFT Kochi. After that it has been cultured by using standard methods and inoculated to the rohu grown in biofloc and finally the results have been shown.

ACKNOWLEDGMENT

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