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Research Article

STUDIES ON HISTOPATHOLOGICAL AND HISTOCHEMICAL CHANGES IN THE LIVER OF *CHANNA STRIATUS* AND *CHANNA PUNCTATUS* INFECTED WITH BACTERIA INDUCED EPIZOOTIC ULCERATIVE SYNDROME

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ABSTRACT

The hazardous affect of the different bacterial infection induced epizootic ulcerative syndrome (EUS) the present study focus on the histopathology and histochemistry of liver of the freshwater edible fishes of *Channa striatus* and *Channa punctatus* are infected with *Aeromonas hydrophila, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida* was investigated. The results showed that the histopathological changes induced in the liver are mainly represented bycytoplasmic vacuolization of the hepatocytes, blood vessel congestion and inflammatory condition and coagulated necrosis. The histochemical observation revealed a marked reduction in the infected fishes the glycogen content and total a protein content of the liver cells was compared with the control fish.

INTRODUCTION

The history of freshwater aquaculture, particularly fish farming has been characterized by a great deal of health problems which have often led to drastic declines in production and major economic loss. The rapid global spread of bacterial, fungal, protozoan, trematode parasites, poor oxygen, heavy native fish species and environmental pollution diseases of fish has emerged as one of the most important issues in aquaculture. Infectious diseases have had major impact on freshwater fish farming in India. Therefore, the target organs in which the bacteria reside and cause degenerative changes need to be studied. An important epidemiological tool used for disease diagnosis is histopathological studies.

The liver is a metabolic hub of activities performing most of the catabolic, anabolic, and detoxifying processes besides recycling of nutrients and maintaining mineral balance, and thence occupies a central position within the life of hepatopancreas having fishes (Ajit Hund *et al*., 2014). Fish liver is a very interesting model for the study of interactions between environmental factors and hepatic structures and function (Brusle and Anadon 1996; Velkova and Kostoski, 2005). Fish liver is important, especially in the field of problems induced by aquaculture conditions, aquatic pollution and diseases (Gochfeld 2003; Bertolucci *et al*., 2008; Millan *et al*., 2011).

The liver is one of the digestive glands that composed of parenchyma cells and lattice fibers. It plays a prominent role in metabolism and acts as a storage center for many substances. Fish liver with a higher phylogenic status had structures identical to the mammalian arraignment, which possessed higher metabolic functions. The fish liver is a relatively large organ. In wild fish, it is usually reddish brown in carnivores and lighter brown in herbivores, but at certain times of year it may be yellow or even off white. In farmed fish, it can be lighter in color than in an equivalent wild specimen but this is diet dependent. According to (Rohit Kumar *et al*., 2016) the liver was seen to be yellowish brown having haemorrhagic symptoms on the surface after 6 h. The liver was pale, and greyish white foci were observed at 12 and 24 h p.i. The degenerative changes in the liver with focal necrosis of hepatocytes were observed histologically at 48 h p.i.

Histopathology is able to evaluate the early effects and the responses to diseases. Environmental factors and poor water quality resulting from increased pollution due to effluents discharge and pathogen transfer appear to be as important underlying cause of epizootics. EUS is one of the most destructive diseases of freshwater as well as brackish water fish species in the Asian Pacific region (Muthu Ramakrishnan *et al*., 2015). Histopathological study of the Liver of the control *C. striatus* and *C. punctatus* showed that the polyhedral hepatic cells are arranged in chains of which some of them are organized into a group of 4-6 cells, bound by a very thin

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membrane. These hepatic cells containing centrally placed nucleus show prominent nucleoli, granular cytoplasm, but certain cells have peripheral cytoplasm.

Histochemical techniques help to analyze not only the localization of glycogen total protein but also molecular changes at cellular level. The main advantage of histochemistry lies in the analysis of biological phenomena in the "particular cells". The altered state of cell constituent can be studied by using histochemical techniques. The histochemical tests reveal the localization of chemical product of cellular activity. The present investigation deals with the histochemical nature of the liver of *Channa striatus* and *Channa punctatus*in order to discuss, the contrast among control and the effect of bacterially infected fishes. The bacterial diseases create a metabolic crisis and impairment in fish liver. The depletion in the level of protein, lipid and glycogen, points towards exhaustion of cellenergy to meet high demand of fish in a stressful condition. Hence, in present study histochemical tests were used for localization of chemical products of cellular activities by the intensity of staining reactions. It is used for comparing the glycogen content and total protein present in the liver cells of the control and infected fish, *Channa striatus* and *Channa punctatus.*

MATERIAL AND METHODS

The microbial infected *Channa striatus* and *Channa punctatus* showing external symptoms were collected from Dharmasagar and Bandham lakes at Warangal district, Telangana, India. The specimens (fishes) were collected alive by using the fishing net and are brought immediately to the laboratory for further clinical examination plastic containers with oxygen filled water. The specimens were deeply anaesthetized by immersion into 5 ml/L aqueous solution of ethylene glycol monophenylether. After dissection of fishes, sample tissue of liver was carefully removed and small pieces were preserved in fixatives like Bouin's, and Carnoy standard fixatives as per protocols. The fixed samples were dehydrated in ascending series of ethanol, cleared in methyl benzoate and embedded in paraffin wax. Sections of 3-4 microns thickness were cut, mounted and stained with different stains according to the target of the investigation. For histopathological investigations, Bouin's fixed sections were stained with Azan and Aniline Blue. For histochemical investigations, materials fixed in Carnoy's fluid were stained with Periodic acid Schiff's (PAS) technique (Hotchkiss, 1948). For a demonstration of polysaccharides (liver glycogen). Total proteins were demonstrated by the mercury Bromophenol Blue method (Mandel *et al*., 1980).

The Microbial investigation was carried out in infected liver organ parts were separated from *Channa striatus* and *Channa punctatus*. The bacterial microorganisms such as *Aeromonas hydrophila, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida* were determined in above the organ.

Processing For Histopathological Investigations

Sample of liver tissue was taken out of fixative.

***Hydration*:** Washing of these tissues was done with saline water/solutions as per procedures adopted.

***Dehydration*:** The removal of water or dehydration was done by' transferring tissue into the 70% alcohol of sufficient quantity and then graded alcohol upto 90% for one change and two changes of absolute alcohol.

***Clearing*:** The clearing of tissues was done by subjecting to a clearing agent *i.e.* xylene.

***Embedding and Impregnating*:** The impregnation agent used was paraffin wax, as it facilitated easy penetration into the tissue without causing structural damage and without much shrinkage or crystal formation.

***Trimming*:** The embedded block containing tissue was further trimmed such that only one tissue is subject for cutting into sections.

***Mounting of the Block*:** An iron blocks of 30 mm/was used to hold the block at the correct angle and in position for cutting.

***Section cutting*:** The block containing tissue was cut at 5µmthickness with the help of a microtome.

***Floating out and Mounting of sections*:** The cutting sections were allowed to float in a water bath so as to avoid wrinkles. Further it is also seen that the cut sections were adhered to the slides firmly and in a right position. The slide was given an egg albumin coating before affixing the sections over it.

***Staining the slides with Azan and mounting*:** The stained sections were observed under light microscope andmicrophotographs were taken for pathological observations.

The following various histochemical methods have been employed to elucidating the chemical nature of viz., the presence of glycogen and total proteins in the tissue in liver of *Channa striatus* and *Channa punctatus*. The procedure as outlined in Pearse (1968) for the different histochemical tests was adopted in the present study. However, the techniques mentioned in the following books were also referred (Gomori, 1952; McManus and Mowry 1960; Lillie, 1965; Humason, 1967; Bancroft, 1975; Carleton and Drury 1957; 1974; Bancroft and Steyans, 1977; Kiernan, 1999; Shyamasundari and Hanumantha Rao, 2007).

Glycogen Content: The conventional method for detecting polysaccharides by the periodic acid/ Schiff (PAS) method. As varieties of substances were known to give a positive reaction with PAS technique, suitable controls were employed to determine the actual compound responsible for the positive reaction. It was performed without prior oxidations with periodic acid to know whether the reaction was due to performed aldehydes, To detect glycogen, sections were subjected to PAS/ light green technique in conjunction with diastase digestion. Best's caramine method was also used to determine the presence of glycogen. The PAS reaction was conducted after acetylation (24 hours at room temperature in 16 ml of acetic acid and 24 ml of pyridine) and subsequent deacetylation (45 minutes in 0.1 N potassium hydroxide at room temperature or with 20% ammonia in 70% alcohol for 24 hours) to establish the presence of 1: 2 glycol groups.

***Total Proteins*:** The presence of basic proteins in the tissues was demonstrated by using Mercuric Bromophenol blue. The method followed was that of Mazia *et al*., (1953). Bromophenol blue is an anionic dye which binds with the actions of proteins in the presence of salts like mercuric chloride. It binds with both anionic and cationic groups of proteins and imparts blue color. This test was confirmed simultaneously by control slides in vans like's reagents.

RESULTS

In the present study pathological changes were observed in the liver of *Channa striatus* and *Channa punctatus* due to the infection of *Aeromonas hydrophila, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida*. Indeed in many cases their presence is responsible for some of the variation in inflammatory response. EUS affected fish have shown considerable changes that were brought about by the infectious agents. Nevertheless, the overriding common finding was the bacteria associated necrosis and it is the consequences of these lesions that should probably direct investigations into the cause of health in these fishes.

Clinical and physical signs of the EUS diseased fish

The present study reveals that the following gross observations in the infected fish are dullness, loss of balance, loss of appetite, sluggish movements, swimming near the surface water, lethargic, erratic and spiral swimming. Rough and dark pigmented skin haemorrhage at the base of the fins and sometimes ulcers on the skin, unilateral or bilateral exophthalmia. The protrusion of the eyeball so that the eyelids will not cover it, in consequence of disease, the opaqueness of the eyes with haemorrhages and abdominal distention.

Histopathological Results

Bacterial, environmental pollution, and lower oxygen cause EUS infection which had induced discrete pathological changes in the liver tissue. Histopathological examination of liver of *C. striatus* and *C. punctatus*. The infectious liver had shown formation of numerous tiny spaces between the hepatic cords, complete destruction of connective tissues at some places, clear atrophy in the hepatic cell nucleus and homogeneity in hepatic mass. The organ is also most affected by *Aeromonas,* the most common cause of the cellular degeneration observed in the liver.The vascular dilation; intravascular hemolytic and thrombosis formation observed in the blood vessels with subsequent stasis of blood may also be responsible for the cellular degeneration and necrosis in the liver (Premaraju, 2011). Dilation and thrombosis formation central veins, dilation and congestion in blood sinusoids and intravascular haemolysis in hepatic blood vessels and hepatoportal blood vessels were also observed. Moreover, focal areas of coagulative necrosis and fibrosis were clearly seen. In the present study, the main alterations were found in the EUS infected *C. striatus* and *C. punctatus* liver shows the irregular shape of nuclei, nuclear hypertrophy, nuclear vacuolation and the presence of eosiniophilic granules in their cytoplasm. Bile stagnation is identified as brownish yellow granules in the cytoplasm. The cytoplasmic and nuclear degenerated cells are common. *C. striatus* (Fig 1&2) and *C. punctatus* (Fig 3&4).

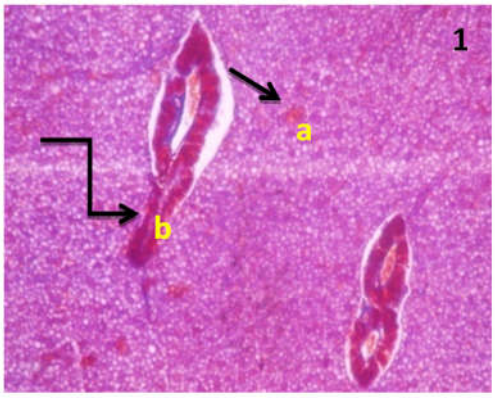

Fig. 1

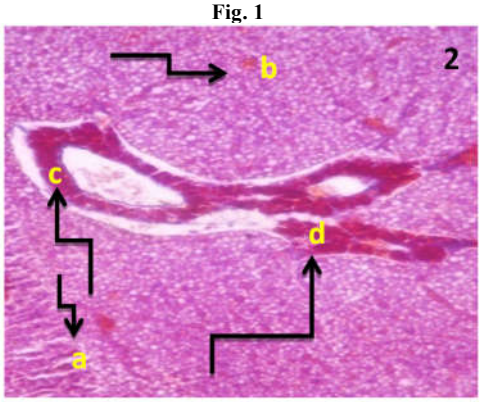

Fig. 2

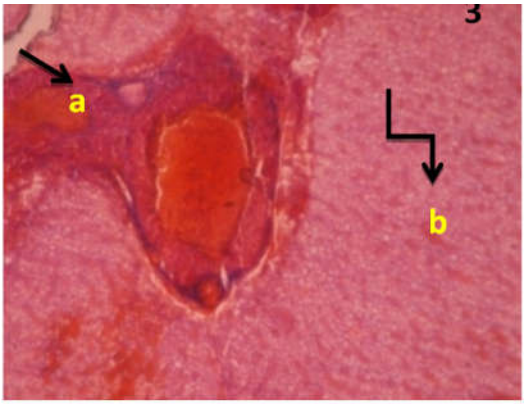

Fig.3

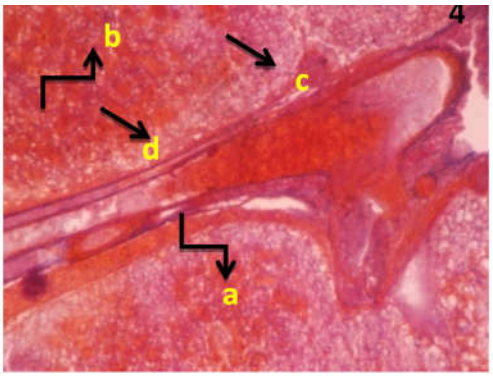

Fig. 4

Fig. 1 Section of Control Liver of *Channa straitus* (Azan); **a)** Hepatocyte, **b)** Exocrine pancreatic tissue. Fig. 2 Section of Infected Liver of *Channa straitus* (Azan); **a)** Coagulated necrosis, **b)** Congested sinusoids, **c)** Vacuoles, **d)** Hepatopancreas. Fig. 3 Section of Control Liver of *Channa punctatus* (Azan); **a)** Hepatocyte, **b)** Exocrine pancreatic tissue. Fig. 4 Section of Infected Liver of *Channa punctatus* (Azan); **a)** Coagulated necrosis, **b)** Congested sinusoids, **c)** Vacuoles, **d)** Hepatopancreas.

HISTOCHEMICAL RESULTS

***Glycogen Content (PAS) Reaction*:** In the control liver of cells or hepatocytes when subjected to PAS technique reacts moderately and have been observed a considerable amount of polysaccharide material in the cytoplasm as shown by the PAS technique. In the infected liver, a considerable depletion of polysaccharides was observed in hepatocyte cytoplasm. The colour of the PAS was not abolished after acetylation. The pancreatic tissue had shown moderate positivity with Schiff's, without prior oxidation and periodic acid. The infected pancreatic tissue presented deeply stained nuclei. *C. stratus* (Figl&2) and *C. punctatus* (Fig 3&4).

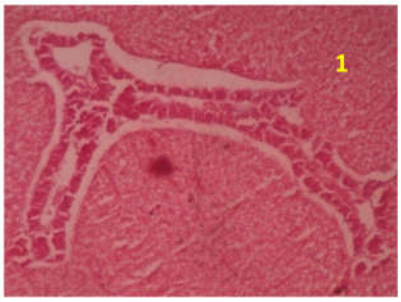

Fig. 1

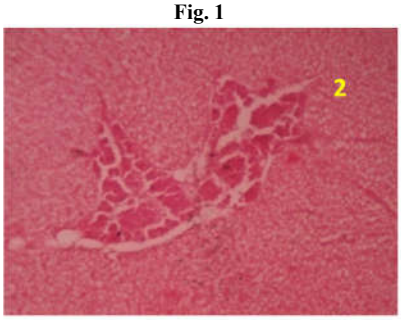

Fig. 2

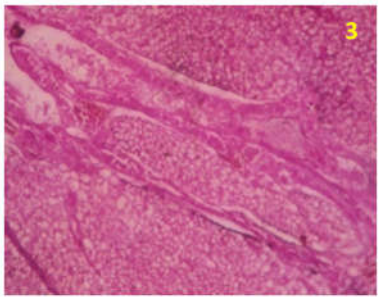

Fig. 3

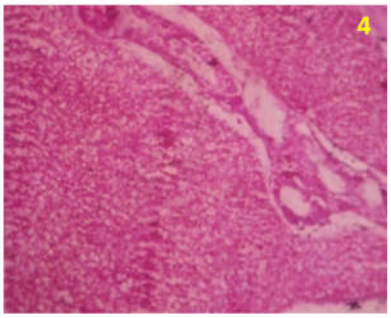

Fig. 4

Fig1 T.S of Contral *C. striatus* liver tissue displaying polysaccharides (PAS). Fig2:T.S of Infected *C. striatus* liver tissue displaying absence of glycogen (PAS).Fig3:T.S of Control *C. punctatus* liver tissue displaying polysaccharides (PAS).Fig4:T.S of Infected *C. punctatus* liver tissue displaying absence of glycogen (PAS).

Total proteins (Mercuric Bromophenol Blue)

The normal liver hepatocytes and pancreatic tissue were demonstrated intense positivity of Bromophenol blue. This exhibited the presence of a basic and high concentration of total protein in the tissues. The hepatocyte cytoplasm has presented a positive reaction that appeared as fine granulation thoroughly distributed. While the infected hepatocytes and pancreatic tissue was moderately positive.

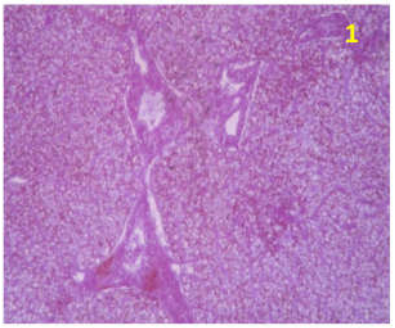

Fig.1

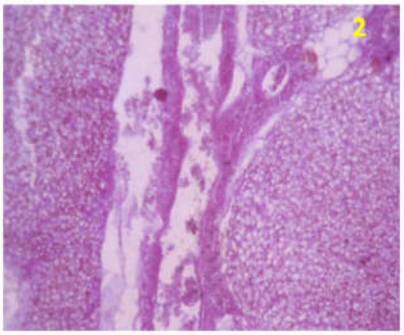

Fig.2

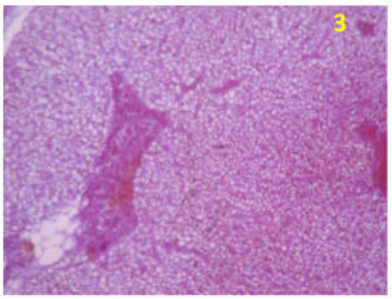

Fig.3

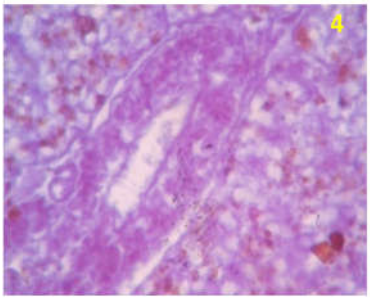

Fig.4

Fig1 T.S of Control *C. striatus* liver tissue displaying the total basic protein substances (Bromophenol Blue). Fig2: T.S of Infected *C. striatus* liver tissue showing the absence of basic proteins (Bromophenol Blue). Fig3:T.S of Control *C. punctatus* liver tissue displaying the total basic protein substances (Bromophenol Blue). Fig4: T.S of Infected *C. punctatus* liver tissue showing the absence of basic proteins (Bromophenol Blue).

Total protein was found to exhibit a noticeable decrease in cytoplasm and nucleus of the hepatocytes and pancreatic tissue. The histochemical procedures followed in the present study have clearly indicated that in the two species of fishes. The liver contains a fairly good amount of proteinaceous material. This was confirmed; when the sections of liver were subjected to other specific stains for the detection various proteins.*C. striatus* (Figl&2) and *C. punctatus* (Fig 3&4).

DISCUSSION

The organ most associated with the detoxification and biotransformation process is the liver, due to its function, position and blood supply. These results coordinated with many investigators. (Nakazawa *et al*., 1985), also reported that polymorphonuclear lecuocyte infiltration was a primary response to *Aeromonas hydrophila*. The polymorpho nulear leucocytes (PMN) infiltrated into the inflammatory area. It was indicated by large ulcerative lesions on the flank and these may cause the death of some of the infected fish. The hepatic cells damaging processes caused by bacterial activities. It is also one of the organs most affected by *Aeromonas* (Chinabut *et al*., 1999). The therapeutic sites were distinguished grossly as dark concave scars. The infected liver showed vacuolar degeneration in the hepatocytes, focal areas of necrosis, thrombosis formation in central veins, dilation, and congestion in blood sinusoids and fibrosis. The vascular dilation, intravascular haemolysis, and thrombosis formation observed in the blood vessels with subsequent Stasis of blood may be also responsible for the cellular degeneration and necrosis in the liver these reports are similar to that of (Mohamed, 2001; Butchiram *et al*., 2009 and Fatma, 2009).The major histopathological changes in the present study included massive hepatic atrophy, haemorrhages, necrotic hepatocytes, focal necrosis and atrophy of hepatic sinusoids. *A. hydrophila* bacteria were distributed all over the hepatic tissue (Kumar *et al*., 1991). Hepatocytes were severely necrotic and many empty spaces were created as a result of cell missing Taveekijakaran *et al*., 1996 observed focal atrophied hepatocytes with dilated sinusoids, multifocal necrosis, perivasculitis and pericholangitis in *Amago salmon Oncorhynchus rhodurus*.

Histochemically rich amounts of glycogen and fat were found in the liver tissue. This is in agreement with the observations of Scarpelli *et al*., (1963) and Weis (1972)1:2 glycol and protein ratio was observed in this *Channa striatus* and *Channa punctatus*, the liver and pancreas mainly serves to store glycogen. The vacuoles in the cytoplasm of the hepatocytes can contain lipids and glycogen, which are related to the normal metabolic function of the liver similar observations were investigated (Seyrafi *et al*., 2011) who reported the appearance of vacuolar structures in the hepatic cells, probably due to the presence of lipids and with PAS reactions, large glycogen deposits were identified throughout the cellular level. Depletion of the glycogen in the hepatocytes is usually found in infected animals (Hinton and Lauren, 1990; Wilhelm Filho *et al*., 2001). Because the glycogen acts as a reserve of glucose to supply the higher energetic demand occur in such situations (Panepucci *et al*., 2001).

In the present study hepatic total protein contents significantly decreased in infected fish shown by BPB method. These results agree with those of Tripathi and Verma (2004) who reported that exposing fish *Clarias batrachus* to fenvalerate induced a highly significant decrease of protein contents of the liver, brain and skeletal muscle. (Reddy *et al*., 1991) found that total, structural and soluble proteins were decreased, whereas the free amino acids and the activities of protease, aspartate aminotransferase, and alanine aminotransferase significantly increased infenvalerate exposed fish *Cyprinuscarpio*. The results reveal that the bacteria *Aeromonas hydrophila, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida sp* infection induced *C. striatus* and *C. punctatus* fishes have been shown drastic reduction in the proteins and glycogen contents in the liver tissue.

CONCLUSION

The present study will conclude, according to our review of bacterial pathogens of fish have highlighted current knowledge gaps that need to be rapidly filled if future epizootics are to be prevented. This would allow a simulation of the true extent of the ecological risk and provide elements for a better environmental monitoring and understanding of these types of pathogens. Finally, the present study concludes the bacterial infections have produced rapid degeneration, hypertrophy, necrosis and vacuolation of hepatocytes with splitting off the liver tissue. Severe effects were observed when in the seasonal infection. This includes loss of characteristic hexongonal shape of hepatic cells, rupture of nuclear and cell membranes, the formation of binucleate hepatocytes, a higher degree of atrophy at the centre and disorganization of hepatic chords. The extent of damage to the liver depended on the seasonal variations leads to EUS condition.

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