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Studies on the Biochemical and Histological Changes Due to *Pseduomonas aeruginosa* PKB 113 Infection in *Cirrhinus mrigala* Hamilton, 1822

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A B S T R A C T

Cirrhinus mrigala was found to be infected with a new strain of fish pathogenic pseudomonad bacteria, *Pseduomonas aeruginosa* PKB113 and the change in the tissue architecture as well as the biochemical parameters of both blood and vital organs like liver, kidney have been observed. Among the biochemical parameters protein concentration, the activity of important metabolic enzymes like GOT, GPT, ACP, and ALP concentration have been monitored. The Total protein concentrations have been found to be decreased in tissues and serum of infected fishes whereas all the enzyme activity showed increased value in vital organs and serum of infected fishes.

***Key words*:** Bacteria, *Pseudomonas,* Biochemical, Enzymes, Total proteins

Aquaculture is one of the most economically important applied strategies all over the world. Fishes are one of the most beneficial food and nutritional resources to human and other organisms. Disease is a biggest problem in fish culture farms for which they suffer huge economic loss. Bacteria are one of the major factors causing infections to the edible fishes. Bacterial disease outbreaks impose deleterious effects on fish and shellfish production [1]. Among the bacterial pathogen, *Pseudomonas* is very common Gram-negative bacterial organism which causes disease in fresh water fish. *Pseudomonas* is frequently associated with fishes and has been isolated from skin, gills and intestine. They are so widespread and numerous that they may become involve in the disease processes and act as secondary invaders of fish compromised by the pathogens or other factors [2]. The infection due to *Pseudomonas aeruginosa* PKB113 strain is characterized by high mortalities, petechial haemorrhage, and darkness of the skin, detached scales, abdominal ascitis and exophthalmia. Bacterial infection causes harm to host fish by changing host physiology as well as the biochemical parameters of both blood and tissue. The internal organs such as liver and kidney of the infected fishes showed some necrosis. Among the biochemical parameters protein concentration, the activity of important metabolic enzymes like GOT, GPT, ACP, and ALP concentration have been observed and presented here.

MATERIALS AND METHODS

In the present study, one bacterial strain has been isolated from the fresh water edible fish *Cirrhinus mrigala* from the haemorrhagic septicemia regions of their body. Bacteria were also collected from the internal organs like liver and kidney. The bacteria were then cultured in the nutrient broth for overnight and on the next day streaked on the pre -prepared nutrient agar media [3]. The agar plates were incubated at 37 °C for 24 hours for appropriate colony formation. The predominant bacterial colonies from the media were isolated, purified and characterized following standard methods [4]. The bacterial isolates were identified both phenotypically and genotypically on the basis of 16SrRNA sequencing and the bacteria isolates were considered as *Pseudomonas aeruginosa* PKB 113 after deposition in GenBank database under the accession number JX426137.

Inoculation of fish with pure pathogenic strain Pseudomonas aeruginosa PKB113

A single colony from the pure culture was transferred to a liquid agar medium i.e., nutrient broth (Hi Media) or Luria broth, incubated overnight at 37 °C until the turbidity of the 0.5 McFarland standards. This resulted in a suspension containing approximately $3X10^8$ cfu/ml although the maximum density depends upon the species of the bacteria and the medium where they are growing. The stock culture having the density of 10^8 cfu/ml is serially diluted up to 10^3cfu/ml in small tubes or vials. Each of these dilutions serves as inoculums. The density

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required to develop symptoms and minimum lethal concentration was determined by injecting the inoculums to different groups of fishes.

Challenge trials were conducted using healthy fishes obtained from pond where no symptom of being diseased have been observed. Three experimental groups and a control group have been set up; twenty fishes were used in each group. Fishes were kept in glass aquaria with aeration, and water was maintained at a daily exchange rate of 5–10%. Water temperature was maintained at 22 – 26°C. The average body weight of the fishes was 10gm and the average body length was 8cm. All fishes were fed with artificial diet meal twice a day. Infectivity trials were performed by intraperitonial injection with four doses (4.2×10^6, 3.7×10^5, 3.0×10^4, 2.5×10^3) cfu/ml of purified bacteria to the three different experimental groups. Each animal received 200µl of the innoculums. The bacterial inoculation brought out the disease symptoms in host fishes. The 4.2×10^6 cfu/ml was LD100 or lethal dose, 3.7×10^5 cfu/ml dose proved to be the LD_{50} dose, 3.0×10^4 cfu/ml was a sub lethal dose, 2.5×10^3 cfu/ml of isolate did not give any significant variation and below doses did not develop any diseased condition. The fishes exposed to 4.2×10^6 cfu/ml died within twelve hours. The group of fishes injected with 3.7×10^5 cfu/ml and 3.0×10^4 cfu/ml from challenge trials were examined for various biochemical and histological changes that has occurred as a result of the bacterial infection.

Histopathology of the affected organs

Internal organs namely liver, kidney were aseptically dissected out from both normal and infected animal. The tissues were fixed in 10% formaldehyde. The fixed tissues are then processed in the series of alcohol gradation and stained by haematoxylin and eosin following the method described by Bell and Lightner [5].

Collection of blood

The challenged fishes showing the external symptoms of infection were separated and blood samples were collected from the caudal vein by plastic syringe. The clot was stored at –20 °C overnight. The clot was then spun down at 5000 rpm for ten minutes. The serum collected by aspiration was stored in sterile microcentrifuge tubes at –20 °C for further use.

Homogenization and centrifugation of tissue sample

The same infected fishes were then sacrificed and liver, kidney were removed aseptically, rinsed and homogenized in PBS buffer. 50 mg of tissues were homogenized in 2ml of phosphate buffer saline (PBS). The homogenized tissues were spun in refrigerated centrifuge (REMI C 24 Model, India) at 5000 g for fifteen minutes at 4 °C. After centrifugation the supernatant were used directly as aliquots or else were stored at -80 °C till they were used within the next four days (generally within a day for enzymatic studies).

For the quantitative estimation of total protein, the method of Lowry *et al*. [6] have been followed. Glutamic Oxaloacetic Transaminase (GOT) or Aspartate Aminotransferase (ASAT) and Glutamate Pyruvate Transferase (GPT) or Alanine Aminotransferase (ALAT) are the enzymes associated with liver parenchymal cells which have been commonly measured clinically as a part of diagnostic liver function test, to determine health of the liver. The method of Brent and Bergmeyer [7] was followed to analysis of Glutamic Oxaloacetic Transaminase (GOT) and and Glutamate Pyruvate Transferase (GPT).

RESULTS AND DISCUSSION

Biochemical changes due to Pseudomonas aeruginosa PKB113 infection

Changes in liver

In the present study the fishes inoculated with *Pseudomonas aeruginosa* PKB113 revealed significant changes in the total protein, and enzyme concentration in the infected liver in comparison to uninfected liver. The average total protein concentration of liver tissue was 15.65±0.63 mg/ml in normal condition, while, in infected condition the concentration was significantly decreased to 12.09±0.88 mg/ml (paired T- Test, $P < 0.05$). It was observed that the concentration of the four metabolically important enzymes GOT, GPT, ACP and AKP have found to be increased significantly (paired T-Test, $P < 0.05$) in infected fishes than the uninfected one. The concentration of GOT and GPT in liver tissue of infected fishes were 1.36 ± 0.31 nM/min/mg protein and 1.34 ± 0.28 nM/min/mg protein respectively. These values were significantly higher than that of the normal level which is 0.94 ± 0.13 nM/min/mg protein and 1.06 ± 0.21 nM/min/mg protein respectively. In case of ACP and ALP enzyme, the concentration were found to be 0.82 ± 0.10 nM/min/mg protein and 0.83 ± 0.09 nM/min/mg protein respectively in the infected group. But in uninfected fishes they were observed as 0.53 ± 0.12 nM/min/mg protein and 0.81 ± 0.13 nM/min/mg protein respectively. The concentration of both ACP and ALP enzyme were increased in infected fishes. The overall comparison of these biochemical parameters are summarized in the following figures (Fig 1).

Changes in kidney

Pseudomonas aeruginosa PKB113 infection resulted marked deviation in both total protein and enzyme concentration in the kidney of the infected fishes from that of the normal. The total protein concentration of kidney tissue in normal condition was found to be 10.45 ± 0.77 mg/ml. But in infected fishes the total protein concentration was 8.91 ± 0.65 mg/ml. The decrease in protein concentration in the infected group was significant (paired T- Test, $P < 0.01$). The concentration of GOT and GPT in kidney tissue of infected fishes were 1.19 ± 0.29 nM/min/mg protein and 1.19 ± 0.28 nM/min/mg protein respectively. These values were significantly higher than that of the normal level which is 0.95 ± 0.25 nM/min/mg protein and 1.08 ± 0.20 nM/min/mg protein respectively. In case of ACP and ALP, the enzyme concentration were found to be 0.48 ± 0.05 nM/min/mg protein and 0.71 ± 0.16 nM/min/mg protein respectively in the infected group. But in uninfected fishes they were found to be 0.37 ± 0.07 nM/min/mg protein and 0.54 ± 0.14 nM/min/mg protein respectively. The concentration of both ACP and ALP enzyme were increased in infected fishes. The overall comparison of these biochemical parameters have been summarized in the following figures (Fig-2).

Changes in serum

The present study depicted that a profound change in different serum biochemical parameters of fishes have been found due to innoculation of pure strain of *Pseduomonas aeruginosa* PKB113. The total protein level in serum of the infected fishes was found to be 28.37 ± 1.34 mg/ml where as it was 30.38 ± 1.29 mg/ml in uninfected group. The average protein concentration of the serum has significantly decreased (paired T- Test, $P < 0.01$) in the infected fishes than the normal ones. On the other hand, the other vital enzyme concentrations namely, SGOT, SGPT, ACP, ALP were found to be increased significantly (paired T- Test, $P < 0.01$) in the infected tissue.

The average concentrations of SGOT and SGPT enzymes were observed 3.09 ± 0.24 nM/ min/mg protein and 3.19 ± 0.28 nM/ min/mg protein respectively in infected group. In normal fishes the concentrations of SGOT and SGPT were found to be 2.61 ± 0.40 nM/min/mg protein 2.69 ± 0.43 nM/min/mg protein respectively. The activity of SGOT and SGPT enzymes were increased in infected fishes in comparison to uninfected fishes.

The average concentrations of ACP and ALP enzymes in normal fishes were 0.75 ± 0.11 nM/min/mg protein and 0.83 ± 0.11 nM/min/mg protein respectively. Whereas in infected fishes their concentrations were increased to 0.93 ± 0.10 nM/min/mg protein and 0.93 ± 0.08 nM/min/mg protein respectively. The comparison of these blood parameters are summarized in the following figures (Fig 3).

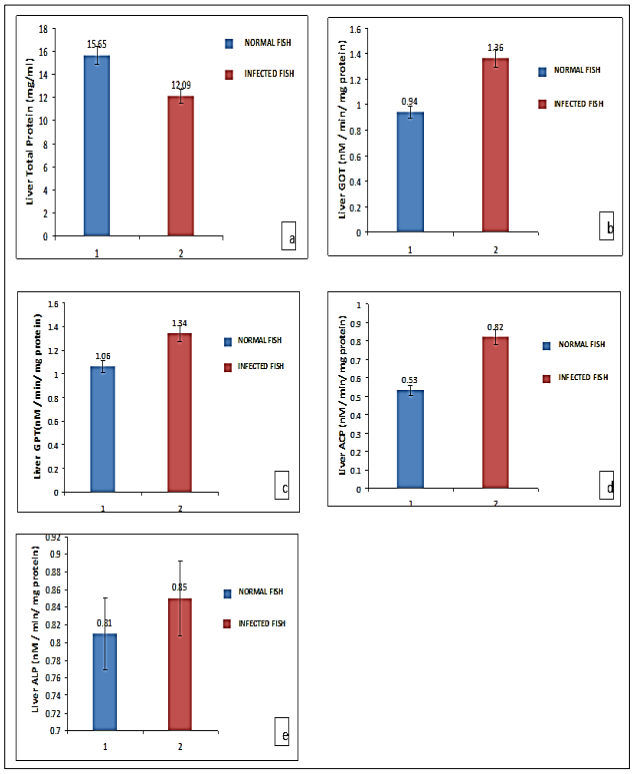


Fig 1 Changes in biochemical parameters of liver in normal non-innoculated fishes and inoculated fishes

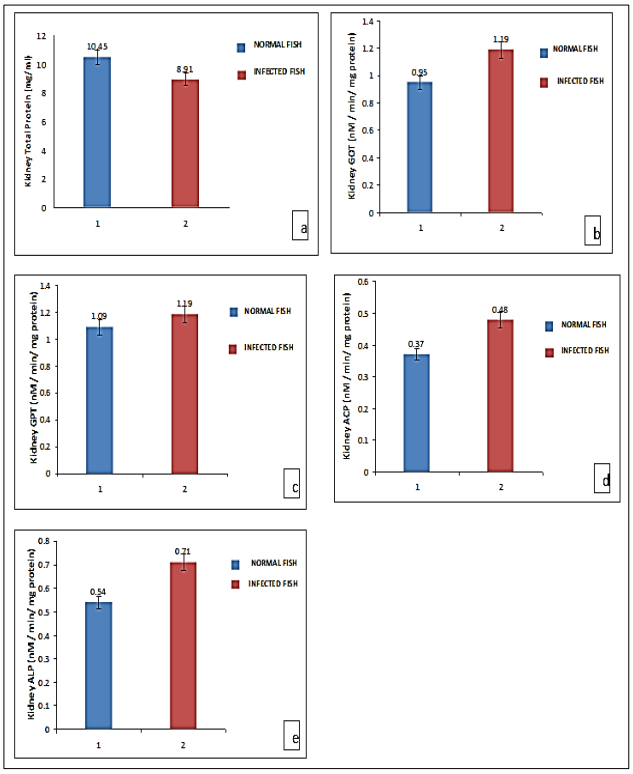


Fig 2 Changes in biochemical parameters of kidney in normal non-innoculated fishes and inoculated fishes

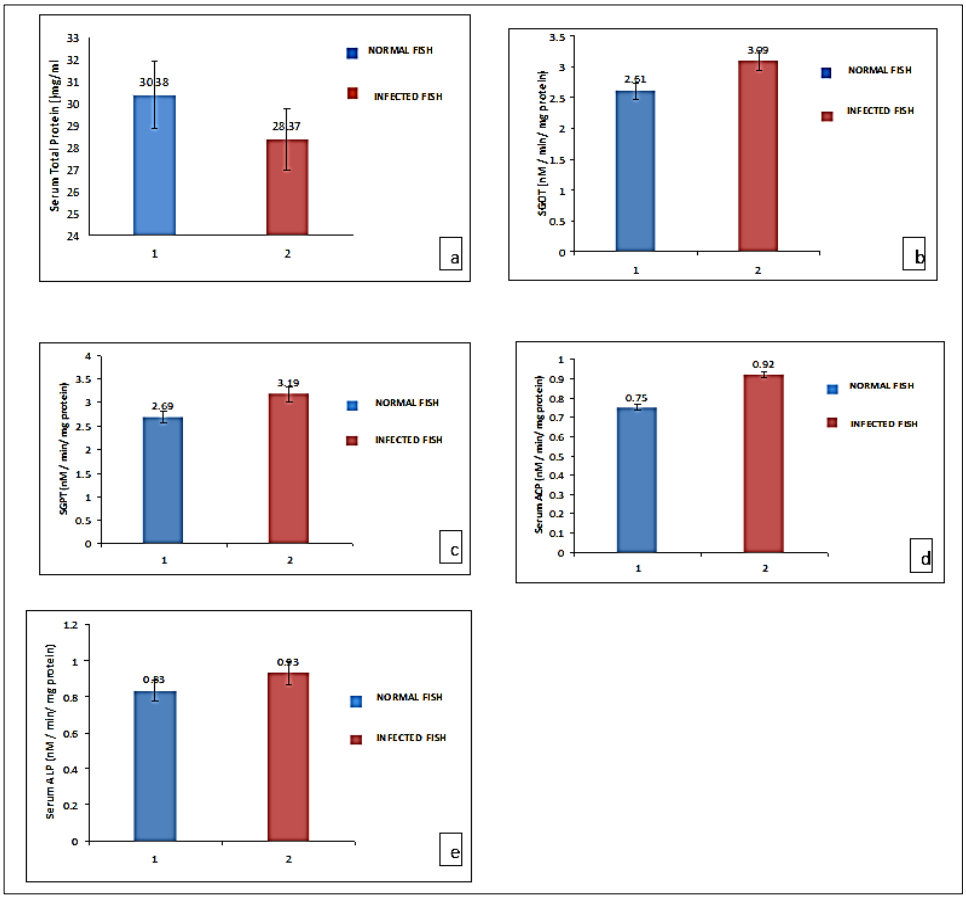


Fig 3 Changes in biochemical parameters of blood serum in normal non-innoculated fishes and inoculated fishes

The result showing concentration of different biochemical parameters has been presented in the following table (Table 1).

Histological changes due to Pseudomonas aeruginosa PKB113 infection

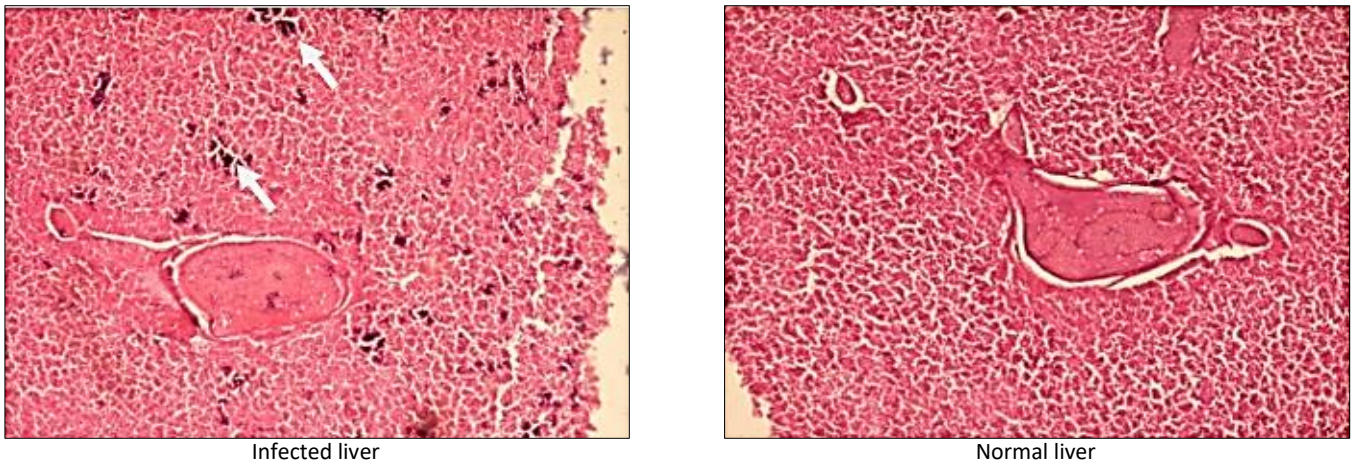
Histopathological changes reveal gross clinical and pathological alterations due to bacterial invasion in the tissues studied from infected fishes [8]. In the present study, the most prominent pathological changes have been observed in the vital organs like liver and kidney.

Table 1 Concentration of different biochemical parameters in tissues and serum

Sample	Concentration of biochemical parameters									
	Total protein (mg/ml)		GOT (nMmin/mg protein)		GPT (nMmin/mg protein)		ACP (nMmin/mg protein)		ALP (nMmin/mg protein)	
Groups	Normal	Infected	Normal	Infected	Normal	Infected	Normal	Infected	Normal	Infected
Liver	15.65±0.63	12.09±0.88	0.94±0.13	1.36±0.31	1.06±0.21	1.34±0.28	0.53±0.12	0.82±0.10	0.81±0.13	0.83±0.09
Kidney	10.45±0.77	8.91±0.65	0.95±0.25	1.19±0.29	1.08±0.20	1.19±0.28	0.37±0.07	0.48±0.05	0.54±0.14	0.71±0.16
Serum	30.38±1.29	28.37±0.34	0.61±0.40	3.09±0.24	2.69±0.24	3.19±0.28	0.75±0.11	0.93±0.10	0.83±0.11	0.93±0.08

Changes in liver

The hepatomegaly of liver was not pronounced although slight sinusoidal congestion and areas of vasodilatation were observed. Bacterial colonies were found in vessels, mainly in the sinusoidal lumen and were usually associated with hepatocellular degeneration. The main alterations observed in the liver were: irregular-shaped nuclei, nuclear hypertrophy, nuclear vacuolation and the presence of eosinophilic granules in the cytoplasm. Liver diffuse necrosis and individualization of hepatocytes were found in the infected condition. In case of normal uninfected liver tissue, the regular-shaped hepatocytes with large centrally located nuclei and some lipid vacuoles in the cytoplasm were found. Cytoplasmic and nuclear degeneration have also been found (Fig 4).

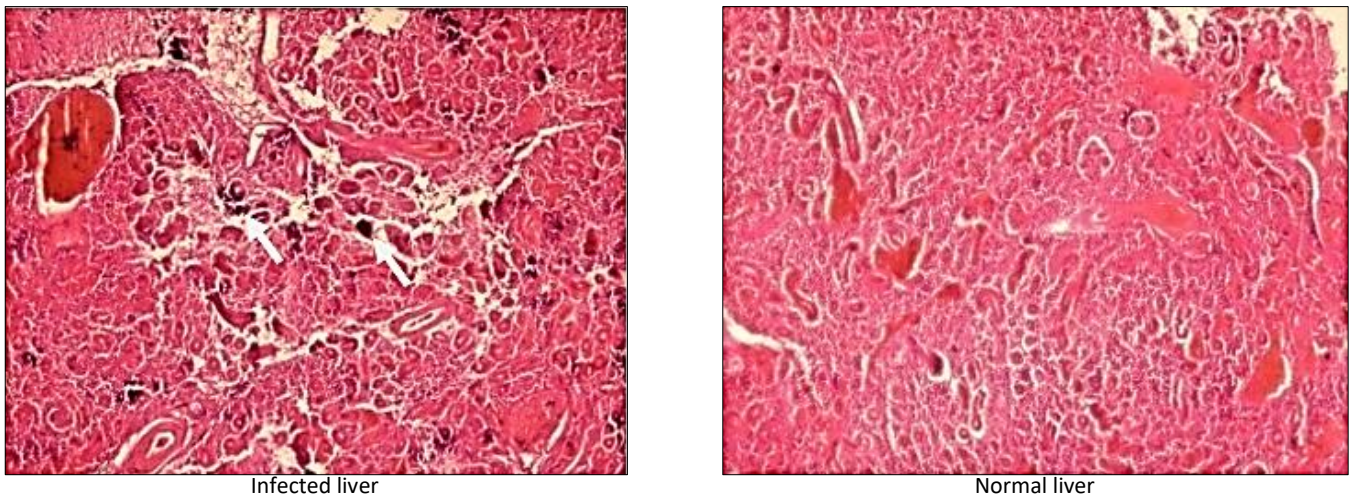


Infected liver Normal liver

Fig 4 Histological section of liver of Infected and Normal fishes (Arrow showing bacterial colony)

Changes in kidney

In the kidney, the changes appeared as vasodilation of the sinusoids and slight inflammatory response underlying the kidney capsule tissue. Oedema and focal necrosis were present in the interstitial kidney tissue. Bacterial infection caused degeneration of glomeruli and hypertrophy of the epithelial cells of the renal tubules resulting in the blockage of the tubular lumen. The change also attributed with glomerular expansion, leading to the reduction of Bowman's space. But in normal condition the glomerulus is evenly distributed with proper Bowman's space (Fig 5). The findings are co-related with the findings of Roy and Bhattacharya [9] with other bacterial infection.



Infected liver Normal liver

Fig 5 Histological section of kidney of infected and normal fishes stained (Arrow showing bacterial colony)

The result of the present study demonstrated that *Pseudomonas* causes septicemia characterized by irregular hemorrhages all over the body surface, especially at the ventral part of the abdomen and ulceration on skin. The present study showed depletion in total protein concentration in liver, kidney and serum due to the infection of *Pseudomonas aeruginosa* PKB113 strain. Total proteinaemia is a significant parameter of homeostasis as it acts as physiological and biochemical parameter of nitrogen metabolism [10]. The consistent decrease of the total protein in the tissues and serum in infected fishes revealed the occurance of proteolysis in tissues as a result of degeneration [11]. The above proteolysis may be due to the action of the extra-cellular secretion of the proteases by the bacterial strain. Cardwell and Smith [12] also stated that the dramatic reduction in Total Plasma Dissolved Solids (TDS) revealed extensive diminutions in plasma proteins as because TDS and plasma protein concentrations are highly correlated. The present study about total protein concentration in diseased condition in fishes also co-related with the findings of other researchers who worked on other bacterial diseases of fish [13-15].

Both tissue and plasma enzyme levels depend on the rate of release of enzymes from damaged cells, which in turn depend on the rate of damage of the cells. According to Bell [16], measurement of enzymatic activity is one of the most useful clinico-biological methods for diagnosis of certain diseases. Glutamic Oxaloacetic Transaminase (GOT) or Aspartate aminotransferase and Glutamic Pyruvic Transaminase (GPT) or Alanine Transaminase present in low concentrations in the liver, kidney and serum [17]. Liver is the primary site of detoxification therefore, a significant increase in liver enzymes in the plasma of infected fishes suggests the presence of bacterial toxins in liver. At the same time the structure and integrity of different cellular organelles like endoplasmic reticulum and membrane transport system of internal organs become damaged and disrupted due to stress, allowing the enzymes to leak out of the cell [18]. This explains for the increase activity of metabolic enzymes in liver, kidney and serum due to the stress condition occurs by the infection with *Pseudomonas aeruginosa* PKB113 bacteria strain. According to Rehulka and Minarik [19] the increased level of GPT signals liver cell insufficiency and the increased level of GOT signs as serious damage to liver with release of mitochondrial GOT. Thus, significant changes in the activities of aminotransferase enzyme in blood plasma indicate tissue impairment caused by stress which was co-related with the work of Svoboda [20] with other fish pathogenic bacteria. Aminotransferases play a vital role in carbohydrate-protein metabolism in fish [21]. These enzymes occupy a central position in the amino acid metabolism as they help in retaining the amino groups during degradation of amino acid and also involved in the biochemical regulation of intracellular amino acid pool. They help in providing necessary intermediates for gluconeogenesis. Thus, increased activities of both aminotransferases indicate amplified transamination processes which in turn accounts for increased input of amino acids into the TCA cycle in order to cope up with the energy crisis due to bacterial infection.

Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP) also act as the indicators that can be considered as significant manifestations of pathological processes [19]. The activity of these enzymes can act as stress indicator. ALP is a brush border enzyme that splits phosphate esters at an alkaline pH and mediates membrane transports [22]. The increase in the activity of ALP enzyme indicated the increase rate of transphosphorylation due to the presence of pathogenic bacteria. ACP is a lysosomal enzyme that takes part in the autolysis of cells after its death [23]. Increase in the activity of ACP indicated increase in the lysozyme activity which could be correlated with the enhanced leucocytic response and the production of reactive oxygen species. This finding was co-related with the work of Harikrishnan *et al*. [24] who worked with fish pathogenic *Aeromonas* bacteria. The increase in the level of all the biochemical parameters indicated about the stress condition only due to the infection caused by *Pseudomonas aeruginosa* PKB113 strain which was found to be highly fish pathogenic. This causes depletion in fish production in Indian sub-continent each year.

CONCLUSION

This study revealed that *Pseudomonas aeruginosa* PKB113 is an opportunistic fish pathogen which create stress condition in fishes. This condition was clearly recognized by histological studies of major organs like liver and kidney. Increase in the amount of some stress enzymes and decrease in the amount of total proteins in this study also support the occurrence of bacterial infection in fish.

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Conflict of Interest

Both the authors don't have any conflict of interest from this research work.

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