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STUDIES OF HEMATOLOGY AND HISTOLOGY IN *LABEO ROHITA* INFECTED WITH CUTANEOUS COLUMNARIS DISEASE

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INTRODUCTION

Bacterial disease is extremely common in freshwater fishes caused by primary or obligate pathogens. Most bacterial infections are caused by gram negative and systematic or ulcer forming bacteria seriously through genera *Aeromonas*, *Vibrio*, *Edwardsiella*, *Pseudomonas*, *Flavobacterium* and others. Columnaris disease is also commonly known as saddle back disease, cotton wool disease, cotton mouth disease and fin rot. Columnaris disease has been reported worldwide in most species of freshwater fishes with rare infections reported in marine fishes (Tripathi et *al*., 2003). The etiologic agent of columnaris disease is a long thin, gram-negative, gliding rod that has recently been reclassified as *Flavobacterium columnare* (Bernardet et *al*., 1996). Natural infections with F. Columnare may occur frequently at > 20^0C water temperature. Such disease ontbreaks are associated with high mortality that may reach 100%. Columnaris disease has also been reported in coldwater fish at normal environmental temperatures ranging from 6 to 12^0C (Tripathi et *al*., 2003).

The pathogenesis of columnaris disease is not well understood. In addition, most of these studies focused on gill lesions, Skin. This study was conducted to detect skin infections, to assess the bacteriostatic effects of cutaneous mucus and to evaluate hematologic and biochemical changes during disease.

MATERIALS AND METHODS

One hundred clinically healthy rohu (*Labeo rohita*), with a mean length of 12-18 cm and an average weight of 150 gram were obtained from Kushinagar hatchery plant. These fish were maintained in stocking tanks with a flow-through water system. The photoperiod of 12 hr of light was provided, and the fish were fed a commercial feed once daily. In addition, the fishes were observed twice daily for clinical signs of disease or mortality during the study period.

Twenty clinically healthy *rohu* were chosen at random and assigned to infected or control group. The fish were anesthetized in aerated water. Anesthetized fish were quickly laid on flat surface and their right side was wiped gently Kim knife containing a 10-fold dilution of detergent. The fish in the infected group were immersed for 1 hr in a bacterial culture of *F.columnare* (106−107 CFu/Me of inoculam) with 0.35% Saline of 2 gallon volume. The control group was immersed in aerated 0.35% saline containing a similar volume.

Tissue imprint preparations were made from skin and gill lesions. These specimens were air-dried, stained with wright-Leishman stain and examined for presence of characteristic long, thin bacill suggestive of *F. Columnare*.

Fifteen healthy acclimatized *rohu* were randomly divided into an infected group (x=10) and

a control group (x=5). The fish were anesthetized and blood specimens collected from the caudal vein for baseline hematology and biochemical heparin anticoagulant. Smears of heparinized blood were prepared for leukocyte differential counts, and the remaining blood specimens were refrigerated immediately. The determinations included PCV, total and differential leukocyte counts, erythrocyte counts with calculation of erythrocyte indices, and morphologic examination of leukocytes, erythrocytes of erythrocyte indices, and morphologic examination of leukocytes, erythrocytes and thrombocytes. For biochemical parameters, blood was collected without anticoagulant, placed in sterile glass tubes and allowed to clot for 15 min at room temperature. The serum was separated by centrifugation and analyzed. During the course of the experiment any mortality was recorded and dead fish were discarded.

Data of various hematologic and biochemical parameters were compared in the same group of fish before and after infection with *F. Columnare* to minimize the effect of individual variations. Means of all parameters were analyzed and comparison were made by t-test at a significant level of P=0.05. Similar comparisons were made in the uninfected control group to exclude the effect of handling.

RESULTS AND OBSERVATIONS

In 3 separate trials, the mortality rate ranged from 80% to 100% in the infected group as compared with 0% to 20% in the uninfected control groups. Initial infection showed cotton-wool like bacterial growth on the skin from right side of the fish where mucus had been removed with detergent, but the contra-lateral side of the fish remained normal (Fig. 1a and 1b). These fish also had clinical signs of disease, including lethargy, depression, and anorexia. Deep skin ulcers were ultimately observed in the *rohu* from the infected group. Neither clinical signs nor skin lesions were observed in the uninfected control group.

Fig. 1a and 1b. Control and infected fish with bacterial treatment.

Wright-Leishman stained cytology imprints of cutaneous lesion contained a homogenous population of slender, elongate bacilli (Fig. 2).

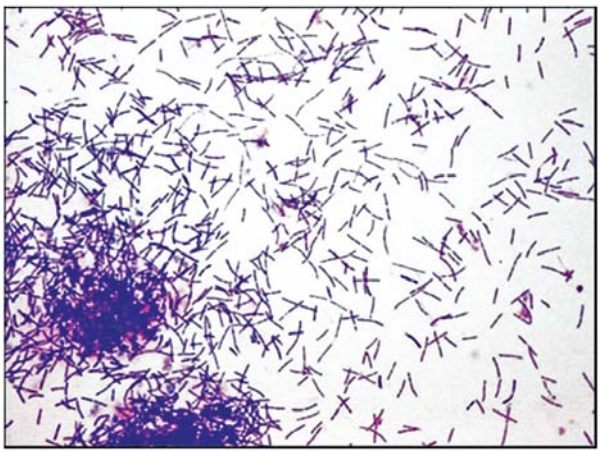

Fig. 2. Cytological preparation stained with Wright-Leishman reagent of Flavobacterium columnare (0.5-10μm) from cutaneous ulcer of infected fish.

Histopathologic study indicated that *F. Columnaris* infection in *rohu* was primarily

associated with Skin and finuleers; gill necrosis was rarely observed. Large numbers of *F. Columnare* were observed in the Skin ulcers and attached to the exposed layers of the Skin and dermis. The cutaneous ulcers extended to the deep dermis and underlying skeletal muscle in Occasional cases. Necrosis of Skin and muscle was accompanied by infiltrates of neutrophils (Fig. 3) Bacteria usually were not observed associated with fin lesions and were probably lost during tissue processing. Neither bacilli nor microscopic lesions were observed in internal organs including liver, spleen and anterior Kidney.

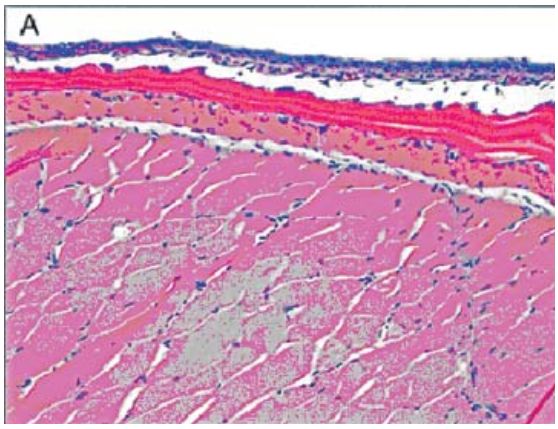

Fig. 3A. Normal skin showing the epidermis, scale, dermis and underlying skeletal muscle.

Pre-and post infection values of biochemical parameters are presented in Table 1. Marked hyponatremia and hypochloridemia were observed, but minor decline was observed in calcium and magnesium concentration. The increase in anion gap was minimal. Mild decreases were observed in total serum protein and albumin like protein concentrations. A significant hyperglycemia was observed after infection with *F. Columnnare*. Alkaline phosphatase (ALP), aspartate aminotransferase (AST), Lactate dehydrogenase (LDH) and creative kinase (CK) activities were significantly increased. In contrast, insignificant changes were observed in alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) content. Significant changes were not observed in the uninfected control group (Table 2).

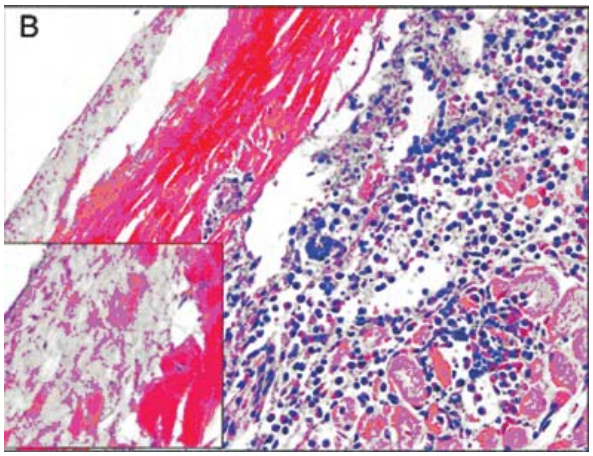

Fig. 3B. Infected skin with bacteria has epidermal ulceration and necrosis with scale loss and a severe dermal infiltrate of neutrophils.

Table-1. Change in hematology parameters in *Labeo rohita* to control and post-infection.

Parameter	N	Mean Healthy	Post infection	Chronic infection
PCV%	10	36.84	24.56	21.31
Hb (g/de)	10	9.21	6.73	153.6
MCV	10	185.6	169.3	153.6
MCH (pg)	10	42.23	37.30	33.42
MCHC (g/dl)	10	23.56	23.11	21.64
WBC(10^3/µl)	10	31.4	19.82	16.89
RBC (10^3/µl)	10	2.11	1.72	1.49
Lymphocyte(10^3/µl)	10	22.6	8.87	6.11
Monocyte (10^3/µl)	10	1.62	2.83	3.34
Neutrophil (10^3/µl)	10	6.73	7.55	8.27
Basophil (10^3/µl)	10	1.21	1.34	1.42

Table-2. Various hematologic parameters of a control group of *Labeo rohita* .

Parameter	N	Mean Healthy	Post infection	Chronic infection
PCV%	10	36.84	34.94	21.31
Hb (g/dl)	10	9.01	8.24	4.87
MCHC (g/dl)	10	23.56	24.12	21.64
RBC (10^3/µl)	10	2.11	2.19	1.37
Lymphocyte(10^3/µl)	10	21.6	21.2	6.11
Monocyte (10^3/µl)	10	1.30	1.39	2.97
Neutrophil (10^3/µl)	10	6.73	5.68	8.27
Basophil (10^3/µl)	10	1.44	1.46	1.87

Table-3. Changes observed in biochemical parameters of *Labeo rohita* in control and post-infection with *Flavobacterium columnare* (N=10).

Parameters	Mean		SE	P
	Healthy	Post infection		
Alanine aminotransferase (U/liter)	47.81	76056	14.57	0.05
Albumin (g/dl)	1.20	1.07	0.06	0.04
Alkaline Phosphate(U/liter)	8.11	47.11	13.54	0.01
Aspartate aminotransferase(U/liter)	150.4	96.6	227.9	0.002
Bile Acids (µmole/liter)	21.5	18.7	10.5	0.79
Total Bilirubin (mg/dl)	0.1	0.1	0	
Total Calcium (mg/dl)	10.93	8.43	0.57	0.0002
Chloride (m mol/liter)	114.4	55.7	8.1	0.0001
Cholestral (mg/dl)	237.7	206.4	42.3	0.46
Creatine Kinate (U/liter)	10623	49514	8643	0.0004
Creatinine (mg/dl)	0.14	0.24	0.03	0.002
Globalin (g/dl)	1.49	1.20	0.87	0.002
Glucose (mg/dl)	71.41	225.11	32.6	0.0002
Lactate dehydrogenase (µmole/liter)	376.11	1602.0	383.0	0.005
Phoyphorus (mg/dl)	5.63	6.09	0.74	0.54
Potassium (m mol/liter)	2.23	3.3	0.46	0.01
Sodium (m mol/liter)	140.7	92.3	6.97	0.0001
Sorbitol dehydration (µmole/liter)	0.41	3.0	1.2	0.04
Total protein (g/dl)	2.73	2.30	0.13	0.002
Urea nitrogen (mg/dl)	4	6.06	0.704	0.006

DISCUSSIONS

Bacterial skin infection in rohu induced by *Flavobacterium columnare* was studied using an experimental model of disease. This model was based on the surface mucus layer is par of the innate host resistance of fish to disease and that its removal would promote the establishment of bacterial infection. A previous study demonstrated that flexibacter columnanis infection was not transmitted in healthy Atlantic salmon with infarct skin, but infection did occur after a breach in the Skin surface (Morrison *et al*., 1981). In this study, *F. Columnare* infection performed with cotton wool type bacterial colony attachment to skin on the right side of infected fish where the mucus layer had been removed but these lesions were absent on the contra-lateral side. These primary lesions subsequently developed into extensive ulcers on the right side of the fish.

The present study showed visible lesions were restricted primarily to the skin and fins; gill involvement was rare. This pattern of lesion may be explained because bacterial infection was established only on the skin and fins where the protective mucus layer was compromised and not on the gills where the mucus layer was undisturbed. A previous study of experimental columnaris disease in salmonids also demonstrated primarily skin disease with inconsistent gill necrosis (Morrison *et al*., 1981). The duration of *F. columnare* infection was approximately 5-7 days until the fish died. In early researches of columnaris disease, the condition was less and had lower mortality when experimentally transmitted (Davis HS, 1922). However, gill necrosis is the major lesion in most natural outbreaks of columnaris disease, and death may occur before cutaneous lesions are evident (Decostere *et al*., 1999). The antibacterial properties of mucus have also been demonstrated previously in carp and other fishes (Ebran *et al*., 1999). Two hydrophobic proteins (27 and 31 KD) have been isolated from the mucus of carp. Both proteins had pore-forming activities correlated with strong antibacterial activity against several grain-negative and gram-positive bacteria (Ebran *et al*., 1999). Spear *et* al. (1992) also observed a damaged mucus coat in association with skin ulcers in fronts infected by *F. columnaris*. Bacteria are frequently observed on the infected skin surface and not in the internal organs. However, organisms may be washed from the surface of some lesions during routine tissue processing.

The hematologic changes in F. Columnaris infected *rohu* included the development of a microcytic, normochromic, non-regenerative anemia. However, wild regeneration was observed in a few blood smears. In *rohu*, microcytosis may reflect an impending regenerative response because erythrocytic precursors in fish are smaller in size than mature erythrocytes as environmental stress may also cause microcytic, normochromic anemia (Graff *et al*., 1999). The WBC count usually indicated a lenkopenia with lymphopenia, mild neutrophils and monocytosis. Hematologic changes are more pronounced in fish with extensive skin ulcers. Leukopenia was lymphocyte as predominant circulating leukocyte (Latimer *et al*., 2003). In fish, leukopenia associated with lymphopenia and neutrophilia is a classical response of stress to leukocyte as in mammals. The exact mechanism of lymphopenia is not clear, but it may be similar to redistribution of lymphocytes induced by corticosteroid level as occur in mammals (Latimer *et al*., 2003). The mild neutrophilia and monocytosis probably occurs in response to tissue demand for these cells as observed in histological sections. Leukopenia with lymphopenia, neutrophilia and occasional monocytosis is frequently observed also in viral and other gram-negative bacterial disease of fish (Noga EJ, 2000).

Biochemical testing indicated hyponatremia and hypochloridemia with minimal decrease in magnesium and calcium concentration. Under stressful conditions, gill perfusion is increased in fish and allows passive diffusion of sodium and chloride ions from gills into the aquatic medium. Also, more water diffuses into the body of fish during stress and subsequently into the tissue fluid.

This process is certain contributor to severity of hyponatremia and hypochloridemia (Graff *et al*., 1999). Finally the loss of sodium and chloride ions probably occurs through a disrupted skin barrier. Decreased total calcium and magnesium content may be associated with proteinemia because a considerable pertions of these ions is bound to serum proteins. Total serum protein and albumin content were moderately decreased might be suggested that plasma protein was lost through skin ulcers or that excess water was being observed by infested fish to resulting in slight hemodilation. Marked hyperglycemia as usually caused by a glycogenolytic effect of catecholamine in acute stress. The corticosteroids maintain this effect in long-term stress by stimulation of hepatic gluconeogenesis or by suppression of glycogen assimilation. Freshwater fish have a hyper osmotic environment inside their body compared with that of aquatic medium. Therefore any damage to the skin barrier allows massive diffusion of water into the body of the fish, resulting in disturbed osmotic regulation and electrolytic homeostasis. The enzymes as ALP, AST, CK and LDH activity were increased markedly, whereas ALT and SDH activities were relatively constant. Previous studies have shown that the increased activity of AST, CK and LDH are associated with venipuncture, which is done through the musculature of caudal peduncle.The enzymes like Sorbitol dehydrogenase and ALT appear to be low content in skeletal muscle and may be better indicators of hepatic cell damage (Tripathi *et al*., 2003). These data generally indicate that there was no substantial damage to live in infected fish.

SUMMARY

The bacterial skin disease was studied to investigate the alteration on hematological and histological levels in freshwater fish, *Labeo rohita*. During infection, lesions were usually restricted to skin and fin with inconsistent gill necrosis.

The bacteria in group were readily detected in skin specimens from infected fish; however the bacterium was occasionally detected in specimens of liver, kidney and spleen. These observations suggest that columnaris disease generally presents as a cutaneous disease and unrelated with systematic infection. Hematologic studies indicated that most infected *rohu* developed microcytic, normochromic, non-regenerative anemia and leukopenia characterized by lymphopenia, mild neutrophilia and monocytosis. Biochemical changes in fish included significant hyperglycemia, hyponatremia and hypochloridemia.

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