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Investigation on the Bacterial Haemorrhagic Septicemia Disease of *Cyprinus carpio* and *Channa striatus*

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Abstract

Bacterial Hemorrhagic Septicemia (BHS) was found affecting *Cyprinus carpio* and *Channa striatus*. Bacteriological examination and artificial infection trials were carried out on these fishes. Hematological examination showed substantial decline in the number of erythrocytes and lymphocytes, increase in the number of granulocytes and significant loss in the amount of hemoglobin. Histopathological examination suggested that the affected part of the skin had got completely destroyed. The peripheral area of the liver showed loosening of tissue and cell distension. There was complete shrinkage in all the components of kidney and glomeruli appeared smaller in size than healthy ones. Artificially infected specimens of *C. carpio* and *C. striatus* were treated with 20 ppm and 30 ppm chloramphenicol solution and oxytetracycline @ 50 and 60 mg/kg of feed for 20 and 30 days respectively. The results of this work indicated that *Aeromonas hydrophila* and *Pseudomonas fluorescens* were the etiological agents for this disease.

Keywords: Bacteria; Disease; C*yprinus carpio; Channa striatus*

Introduction

The term 'hemorrhagic septicemia' was first introduced by Snieszko in the year, 1933. This disease is known by various synonyms such as red-mouth disease, infectious dropsy, rubella disease, red-pest and freshwater eel disease. In India, it is known either by the name of infectious dropsy or bacterial hemorrhagic septicemia [1].

Bacterial hemorrhagic septicemia has been described by many investigators [2-7]. Definitive diagnosis of bacterial hemorrhagic septicemia can be made on the basis of external signs and the isolation and identification of the etiological agents [8]. Although motile *Aeromonas* species are typically recognized as opportunistic pathogens or secondary invaders, cases have been reported of *A. hydrophila* acting as a primary fish pathogen [9-13].

P. fluorescens was originally described as the causative agent of bacterial hemorrhagic septicemia disease of pond-cultured fish [14,15]. It is considered as a primary pathogen of freshwater fish and opportunistic pathogen for different fish species cultured in marine and brackish waters worldwide [16,17]. Isolation and characterization of *P. fluorescens* from gills of silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), African magur (*Clarias gariepinus*) and Nile tilapia (*Oreochromis niloticus*) has also been reported by some workers [18,19].

The present work was, therefore, undertaken to study the etiology, symptoms, causes and treatment of bacterial hemorrhagic septicemia in *Cyprinus carpio* and *Channa striatus.*

Materials and Methods

Diseased fish brought to the laboratory were examined grossly and photographed for characterization of lesions. For the purpose of bacteriological examination, the methodologies applied by [5,20-22] were followed. Samples for bacteriological examination were collected by inserting sterile inoculating loop into the lesions or tissues. All bacterial isolates were examined for gram reaction. Characterization of pure cultured organisms was done by applying morphological and biochemical tests. Artificial infection trials were conducted on the fingerlings of *C. carpio* and *C. striatus*, averaging 12.4 ± 0.28 g in weight and 8.17 ± 0.13 cm in length kept in aquaria (90×45×45cm), washed with KMnO4 and filled with 100 liters of freshwater. In each experiment, ten fishes were used which were acclimatized in laboratory conditions for one week prior to the experiment and fed with dry pelleted diet.

The hematological parameters of diseased fishes were compared with those of apparently healthy specimens with no visual disease symptoms. For this purpose, the blood samples were taken from the caudal peduncle and heart with the help of 3 ml syringe. R.B.Cs. and W.B.Cs. were counted with the help of hemocytometer. Blood smears were prepared and stained with Leishman's stain for the differential count of W.B.Cs. Hemoglobin concentration was measured by using hemoglobin meter. The data were analyzed statistically by using student 't' test. For histological examination, the infected tissue was taken out and preserved in aqueous Bouin's fluid for 48-72 hours. The tissue was then processed routinely and prepared into paraffin blocks. The blocks of the tissue were cut at 6µm thickness and stained with Delafield's Haematoxylin and Eosin (H-E). Sections were studied under microscope on different magnifications and photographed.

After making accurate diagnosis, the affected fishes were either bathed/injected or fed with different concentrations of antibiotic drugs and other commonly used chemicals following the methods [23]. Chloramphenicol and Potassium permanganate ($KMnO_4$) were applied for treatment. Percentage of mortality was based on the number of fishes dead after the treatment of bacterial suspension in each set of experiments. Abbot's (1925) formula was applied to correct the percentage of mortality.

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Results and Discussion

Bacterial hemorrhagic septicemia was found to affect *C. carpio* and *C. striatus*. The diseased specimens ranged between 16 - 20cm in total length and 550-625g in weight in case of *C. carpio* and 13 - 16cm in total length and 350-425g in case of weight in *C. striatus*. As observed in the present case, Kabata (1985) has also reported the occurrence of BHS in *C. carpio* in Indonesia and West Java. Kumar et al. [24] and Kumar and Dey [25] have reported that all the Asiatic major carps are prone to such infection, but silver carp and catla have been found to be relatively more susceptible.

The diseased fish displayed abnormal behavior, swam slowly, remained lethargic, preferred to stay at the bottom of aquarium and refused to feed. They exhibited the symptoms such as the swollen vent filled with reddish fluid, descaling, swelling and softening of the spleen and kidney and the presence of thick yellowish fluid in the intestine. Skin lesions, with hemorrhages, reaching deep into the muscles, appeared on the general surface of the body (Figure 1). Similar symptoms of BHS have also been reported by [2,3,8,20,26,27].

The bacteriological examination of the diseased fishes indicated that mainly two pathogenic bacteria, *Aeromonas hydrophila* and *Pseudomonas fluorescens* have been associated with this disease. After 24-48 hours of incubation at 28 °C, numerous small colonies appeared which were subsequently isolated in pure form on TSA. *A. hydrophila* was gram-negative rod, motile, oxidase-positive, and catalase-positive and glucose fermenting bacteria while *Pseudomonas fluorescens* was gram-negative rod, motile, oxidase-positive, catalase-positive and glucose oxidizing bacteria. Other biochemical characteristics of these bacterial species are given in Table 1. The characteristics exhibited by these bacteria conform to those as given by [20,22,28].

Experimental infection trials were carried out by keeping healthy specimens of *C. carpio* and *C. striatus* in water at 28°C inoculated with bacterial suspension of *A. hydrophila* and at the dilution of 0.5 and 0.05 ml/l which got infected and died in twelve to twenty days showing acute symptoms of BHS i.e., the external hemorrhages (Tables 2 and 3). Snieszko [29] suggested that *A. hydrophila* is abundant in water receiving sewage. Richards and Roberts [30] have also forwarded the similar views. They pointed out that *A. hydrophila* and *P. fluorescens* are ubiquitious in the aquatic environment and frequently implicated in the aetiology of BHS. *A. hydrophila* has been recorded as a pathogen from a wide variety of freshwater fish species and occasionally from marine fish [31]. In the present study, as observed in experimental infection trials, it is confirmed that *A. hydrophila* and *P. fluorescens* are the etiological agents and can produce the disease in fish.

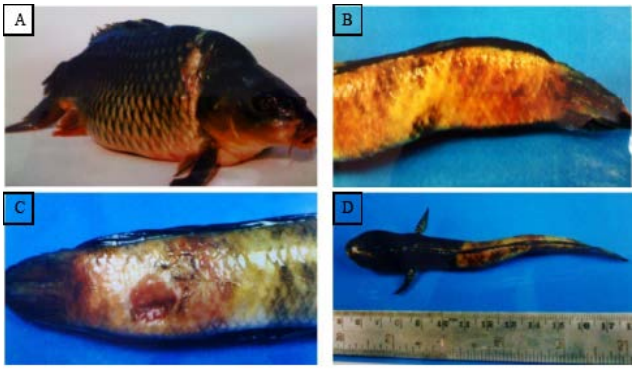

Figure 1: External symptoms of BHS. A. Showing descaling and erection of scales on the lateral side of diseased *Cyprinus carpio*. B. Showing hemorrhages on the body of diseased *Channa striatus*. C. Showing skin lesions reaching deep into the muscles on the general body surface of *Channa striatus*. D. Showing discolored body surface of the diseased *Channa striatus*.

S.No.	Tests	Aeromonas hydrophila	Pseudomonas fluorescens
1.	Colony morphology	creamy yellow	yellow green
2.	Gram staining	- rods	- rods
3.	Motility	+	+
4.	Oxidase	+	+
5.	Catalase	+	-
6.	Oxidation/Fermentation	F	O
7.	Nitrite	+	+
8.	Nitrate	+	+
9.	4% NaCl	-	-
10.	40% Bile	-	-
11.	Methyl red	-	-
12.	Voges-Proskauer	+	-
13.	Arginine dihydrolase	+	+
14.	Lysine	-	+
15.	Ornithine decarboxylase	-	-
16.	Indole	+	-
17.	Citrate	+	+
18.	Triple sugar iron	-	-
19.	Adonitol	-	+
20.	Arabinose	-	+
21.	Dextrose	+	+
22.	Fructose	+	+
23.	Galactose	+	+
24.	Inositol	-	+
25.	Lactose	-	-
26.	Maltose	+	+
27.	Mannitol	+	+
28.	Mannose	+	+
29.	Raffinose	-	-
30.	Rhamnose	-	+
31.	Salicin	-	+
32.	Sucrose	-	+
33.	Trehlose	+	+
34.	Xylose	+	+
35.	H_2S	+	-
+ = Positive,		O = Oxidative,	

+ = Positive, - = Negative, O = Oxidative, F = Fermentative

Table 1: Showing physical and biochemical characteristics of A. hydrophila and P. fluorescens isolated from C. carpio and C. striatus suffering from BHS.

	Aeromas hydrophila		*Pseudomonas fluorescens*	
	First trial	Second trial	First trial	Second trial
Number of fish used	10	5	10	5
Mean weight (g)	35	35	35	35
Number of days	20	20	20	20
Mortality (%)	62.5	25	50	-
Symptoms observed	Haemorrhages	Abnormal behaviour	Haemorrhages	-

Table 2: Artificial infection produced in C. carpio with the bacterial strain isolated from BHS.

In the healthy specimens of *C. carpio*, the number of erythrocytes was found to be $1.6\times10^6/mm^3$. The number of granulocytes was 60 - $90\times10^3/mm^3$. Hemoglobin content was 10.0 g /100 ml. The percentage

	Aeromas hydrophila		*Pseudomonas fluorescens*	
	First trial	**Second trial**	**First trial**	**Second trial**
Number of fish used	10	5	10	5
Mean weight (g)	50	50	50	50
Culture dilution (ml/l)	0.5	0.05	0.5	0.05
Number of days	20	20	20	20
Mortality (%)	50	-	50	-
Symptoms observed	Haemorrhages	-	Haemorrhages	-

Table 3: Artificial infection produced in C. striatus with the bacterial strains isolated from BHS.

of lymphocytes and monocytes was 80 and 20, respectively. There was no trace of degenerated granulocytes in the blood of healthy fish. In case of diseased specimens, the number of erythrocytes varied from 0.55-0.92×10^6/mm^3 and that of granulocytes varied from 91-130×10^3/mm^3. Hemoglobin content was 3.8-6.2 g/100 ml. The percentage of lymphocytes and monocytes was 39 and 14-45, respectively. The percentage of degenerated granulocytes was observed to be 0-30 (Table 4). In the healthy specimens of *C. striatus*, the average number of erythrocytes was found to be 2.16×10^6/mm^3 and the number of granulocytes varied from 180-195×10^3/mm^3. Hemoglobin content was 13.2 g /100 ml. The percentage of lymphocytes and monocytes was 60 and 38, respectively. There was no trace of degenerated granulocytes in the blood of healthy fish. In case of diseased specimens, the number of erythrocytes varied from 1.22-1.46×10^6/mm^3 and the number of granulocytes varied from 270-310×10^3/mm^3. Hemoglobin content was 6.2-8.1 g /100 ml. The percentage of lymphocytes and monocytes was 28 and 40 - 70, respectively. The percentage of degenerated granulocytes was observed to be 0-40 (Table 5). Similar findings have been reported by [32-34]. Increase in the number of granulocytes is reported by Iwami et al. [35]. Though, the real cause of the patho-physiological changes in the erythrocytes is not clearly understood but it seems that the toxins released by the pathogenic bacteria may be the cause of destruction of erythrocytes in the diseased fish [33]. The same view is conceded in the present study also. The increase in the number of granulocytes in the infected fish may be due to the increase in the tissue damage by the pathogens and other stress factors [35]. No trace of degenerated granulocytes has been found in the normal fish which means that the granulocytes get degenerated only when they come in contact with the toxins produced by the bacteria or any other factors. The decline in lymphocytes percentage may also be due to the presence of bacteria or other stressful conditions.

Histopathological examination showed that affected part of the skin had got completely destroyed. There was no distinction between the different layers of skin (Figure 2) unlike the normal skin. The central part of the liver showed the same picture as in normal liver but the peripheral area of the liver showed loosening of its tissue and the distension of its cells. Most of the distended cells have become empty and have lost their nuclei (Figure 3). Kidney of a normal fish is a compact structure which is mainly composed of lymphoid tissue containing large number of uriniferous tubules, glomeruli and blood capillaries. In this case, there was complete shrinkage in all the components of kidney. The internal spaces of the uriniferous tubules have reduced to a greater extent due to the shrinkage. Glomeruli appeared smaller in size in comparision to those of healthy ones (Figure 4). Similar results have been reported for BHS caused by *A. hydrophila* and *P. fluorescens* by many workers [2,20,22,24,25].

Therapy was carried out on artificially infected specimens of *C. carpio* and *C. striatus*, which were kept in 20 ppm and 30 ppm chloramphenicol solution for a period of twenty days and by feeding oxytetracycline @ 50 and 60 mg/kg of feed for thirty days, respectively. These fishes were also treated with intra-peritoneal injections of chloramphenicol @ 10 and 15 mg/kg body weight of the fish at weekly intervals. Moore et al., [23] have recommended the treatment of bacterial fish diseases with the help of antimicrobial drugs and the chemicals of general use. It was observed that no mortality occurred during the first eight days period in any of the experimental media i.e., control and the water with 20 ppm and 30 ppm chloramphenicol. After eight days, 55% mortality was observed in control group and 15% in that kept in 20 ppm chloramphenicol solution in *C. carpio* and 60 % mortality in control group and 20% in 20 ppm chloramphenicol solution in *C. striatus*. After fifteen days, 100% mortality was recorded in control group and

S.No.	Parameters	Units	Healthy	Diseased
1.	Erythrocytes	$10^6/mm^3$	1.6	0.55-0.92
2.	Granulocytes	$10^3/mm^3$	60-90	91-130
3.	Haemoglobin	g /100ml	10.0	3.8-6.2
4.	Differential count of Leucocytes (%)			
I	Lymphocytes	$10^3/mm^3$	80	39
ii	Normal granulocytes	$10^3/mm^3$	12	14-45
iii	Degenerated granulocytes	$10^3/mm^3$	-	0-30

Table 4: Haematological parameters of healthy and diseased Cyprinus carpio suffering from BHS.

S.No.	Parameters	Units	Healthy	Diseased
2.	Granulocytes	$10^3/mm^3$	180-195	270-310
3.	Haemoglobin	g /100ml	13.2	6.2-8.1
4.	Differential count of Leucocytes (%)			
I	Lymphocytes	$10^3/mm^3$	60	28
ii	Normal granulocytes	$10^3/mm^3$	38	40-70
iii	Degenerated granulocytes	$10^3/mm^3$	-	0-40

Table 5: Haematological parameters of healthy and diseased Channa striatus suffering from BHS.

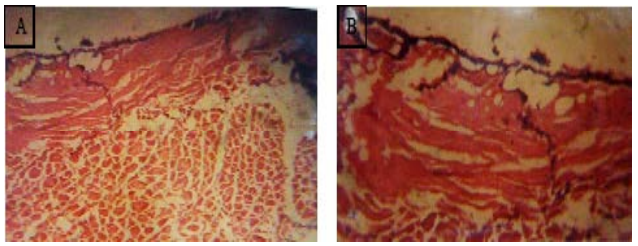

Figure 2: Histopathology of Skin. A. Necrotic changes in the skin of BHS affected fish at 40X. B. Necrotic changes in the skin of BHS affected fish at 100X.

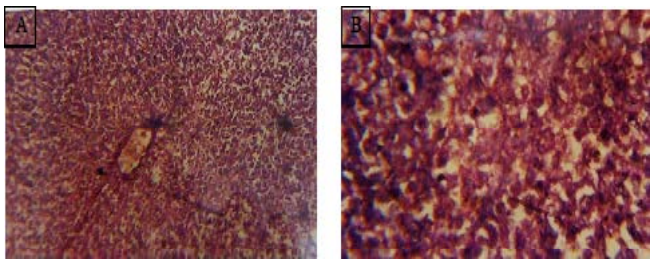

Figure 3: Histopathology of Liver. A. Loosening of hepatic cells and loss of nuclei and cytoplasm in BHS affected fish at 100X. B. Loosening of hepatic cells and loss of nuclei and cytoplasm in BHS affected fish at 400X.

15% in those kept in 30 ppm solution but no mortality was recorded this time in fishes kept in 20 ppm solution in *C. carpio*. 100% mortality in control group and 20% in 30 ppm solution but no mortality were recorded this time in fishes kept in 20 ppm solution in *C. striatus*. These fishes survived and got completely cured within thirty days period. Similarly, it was noticed that 15% mortality occurred after eight days in *C. carpio* which were fed with the feed containing oxytetracycline @ 50 mg/kg of feed and 20% mortality occurred after eight days in *C. striatus* which were fed with the food containing oxytetracycline @ 50 mg/kg of feed. No mortality was recorded in those which were fed with the food containing oxytetracycline @ 60 mg/kg of feed. Here again, 100% mortality was recorded in control group within fifteen to seventeen days. The fishes under treatment got cured within thirty days. In the injected groups, 100% mortality took place in the control group within fifteen to seventeen days while no mortality was recorded in the fishes, injected with chloramphenicol @ 10 and 15 mg/kg body weight for fifteen days. Later on, 15% mortality was noticed in *C. carpio* injected with chloramphenicol @ 15 mg/kg body weight and 20% mortality was noticed in the *C. striatus* injected with chloramphenicol @ 15 mg/kg body weight. Thereafter, no mortality was noticed in treated fishes. The remaining fishes got cured within 30 days (Tables 6 and 7).

Treatment through injection proved to be more effective than bath treatment. When the injection of chloramphenicol was administered to infected fishes, at the rate of 10 mg/kg body weight, they got cured within fifteen to twenty days. Oral adminstration of oxytetracycline at the rate of 50 mg/kg of feed has also given satisfactory results. In a series of reports, Schaperclaus [36-38] described the results of his approach to the prophylactic chemotherapy of BHS by means of oral, anal and intra-peritoneal introduction of antibiotics to the carp, *C. carpio*, at the time of spring transfer from winter to production ponds. Transient protection against BHS in carps through intra-peritoneal injections has also been advocated by Bullock and Snieszko [20] of the antibiotics tested by Schaperclaus [38], the most satisfactory results were obtained with chloramphenicol. Reduction in the rate of mortality from 80 - 90% was obtained by a single intra-peritoneal injection of chloramphenicol given at the rate of 20-50 mg/kg body weight of fish. Streptomycin injection given at the rate of 50-200 mg/kg body weight of fish was reported to be more effective against this disease by schaperclaus [36,37]. European fish pathologists have also been employing chloramphenicol at the rate of 3 and 5 mg/kg body weight of fish, in a single intra-peritoneal injection to protect winter stressed carp (C. carpio) from mortality due to infectious dropsy [39]. Oxytetracycline is probably the most widely used antibiotic in fish therapy and the general recommended dosage is the same as that of chloramphenicol i.e., 50-75 mg/kg body weight of fish [40]. Similar observations have also been made by [1,41]. Oxytetracycline has been approved for use with pond fishes, channel catfish and salmonids at a daily rate of 5-75 mg/kg of feed to fish for ten days [42,43].

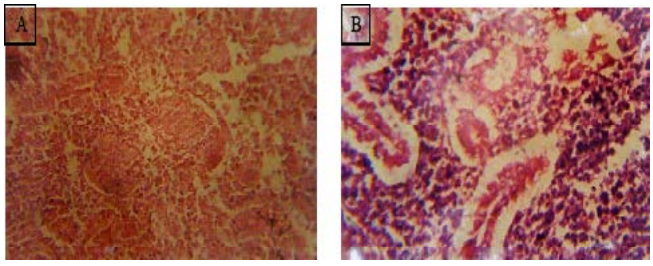

Figure 4: Histopathology of Kidney. A. Shrinkage and necrotization in kidney of BHS affected fish at 280X. B. Shrinkage and necrotization in kidney of BHS affected fish at 400X.

		Percentage of mortality in lapsed days		
		1 to 8 days	9 to 15 days	16 to 30 days
Control		-	55	100
Chloramphenicol ppm	20	-	15	-
(Bath)	30 ppm	-	-	15
Chloramphenicol	10 mg	-	-	-
(Injection)	15 mg	-	-	15
Oxytetracycline	50 mg	-	15	-
(Oral with feed)	60 mg	-	-	-

Table 6: Effect of drugs on *Cyprinus carpio* artificially infected with BHS.

		Percentage of mortality in lapsed days		
		1 to 8 days	9 to 15 days	16 to 30 days
Control		-	60	100
Chloramphenicol	20	-	20	-
ppm				
(Bath)		-	-	20
	30 ppm			
Chloramphenicol	10 mg	-	-	-
(Injection)				
	15 mg	-	-	20
Oxytetracycline	50 mg	-	20	-
(Oral with feed)				
	60 mg	-	-	-

Table 7: Effect of drugs on Channa striatus artificially infected with BHS.

Conclusion

The present work shows that bacteria are the etiological agents of the diseases discussed above but stress also plays a vital part in the outbreak of diseases. We should take extra care of the stress factors associated with the fish as these usually trigger the bacteria to invade these fish species. So steps should be taken to minimize the stress factor for fish to be healthy and not to succumb to secondary invasion by opportunist pathogens. Although, we have used chloramphenicol in our study but it should be borne in mind to use it as minimally as possible due to its carcinogenic nature.

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