7. ULCERATIVE DISEASE IN INDIAN MAJOR CARPS AND ISOLATION OF BACTERIA FROM ULCERS TISSUE OF *Cirrhinus mrigala.*

Materials and Methods

Infected major carps such as *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala* were collected from different affected areas of West Dinajpur, Jalpaiguri, Cooch Behar and Darjeeling districts for investigation. Behaviours of the infected fishes were observed in a pond one kilometer from the laboratory. Bacteria were isolated from ulcer tissues of infected *C. mrigala.* A portion of the ulcer tissues was incubated in sterilized bacterial nutrient medium and nutrient medium supplemented with 0.1% glucose, after surface sterilization in 0.1% mercuric chloride, at 30°C for 24 hrs. Bacteria were isolated by the pour-plate method. The isolated bacteria were maintained routinely on nutrient agar slants. Bacteria were cultured in nutrient broth when required. Microbiological studies of the isolated bacteria were done by following methods (Cowan and Steel, 1965).

The morphological characteristics of isolates includes the shape and size of the cells. The colour, texture and shape of the colonies on agar media were also noted. Presence of spores and capsules were noted after appropriate staining (Cowan and Steel, 1965).

The bacteria were grown 24 hrs. in nutrient broth at different temperature e.g. 25°, 30°, 37°, 42°C and optical density was measured at 630 nm in a spectrophotometer to determine the growth at different temperature. All the bacteria were stained for Gram reaction and tested for i) catalase activity, ii) oxidation or fermentation of glucose (O-F test), iii) acid and gas production in media containing different carbohydrates, (iv) nitrate and nitrite reduction, (v) production of indole, (vi) hydrolysis of gelatin, (vii) Oxidase activity, (viii) levan formation, (ix) Voges Proskauer test, (x) pigment production and (xi) utilization of amino acids, and L-arabinose and (xii) arginine dehydrolase.

Observation and Results

Affected fishes, C. catla, L. rohita and C.mrigala showed infection of different stages of development. In some fishes infections were at the primary stage, i.e. haemorrhagic red spots on the body surface were found (Fig. 111). Some fishes showed superficial ulcers on their bodies. A few fishes showed deep haemorrhagic ulcers (Fig. 112). In advanced stage ulcers were deep and necrotic. Fins were also affected in a few cases. Only one fish (C. mrigala) showed skin lesion with accumulation of fluid in the intestine. In the affected pond (Fig. 113)(1 km. from laboratory) some infected fishes showed abnormal swimming behaviour and occasional jumping out of water.

Stained smear preparations of Giemsa and Carbol fuchsin showed presence of bacteria only. Three rods (R_4, R_5 and R_6) and one sphere (C_1) were isolated from the nutrient broth supplimented with 1% glucose incubated with a portion of ulcer tissues. Morphological and physiological characteristics of isolated bacteria were given in Table-12.

Plate XXXII

Fig. 111. Showing haemorrhagic spots on the body of infected carps.

Fig. 112. Showing deep haemorrhagic ulcer in *cirrhinus mrigala.*


11b


11c

Plate XXXIII

Fig. 113. Showing affected pond


113

Table 12: Morphological and physiological characteristics of four bacteria isolated from ulcer of C.mrigala

Parameter	R_4	R_5	R_6	C_1
Shape	Rod	Rod	Rod	Sphere
Occurence	Single, in pair or in chain	Single, in pairs or in chain	Single, in pairs or in chain.	Single, in pairs, in tetrad, in irregular cluster or in short chain.
Size	2.7-3.0 x 0.72-0.75 µm	1.5-2.2 x 0.68-0.72 µm	1.6-2.2 x 0.72-0.8 µm.	0.9-1.5 µm.
Spore	-	-	-	-
Gram stain	-	-	-	+
Agar colonies	Circulars, smooth, slight convex.	Circular, smooth, slight, convex.	Circular, smooth, slight, convex.	Small, smooth convex.
Catalase activity	+	+	+	+
Oxidase activity	+	+	+	-
Motility	+	+	+	-
Broth	Turbid	Turbid with pellicle and sediments.	Not dense turbid and sediments	First turbid clear with sediments.

Contd.....

Parameter	R_4	R_5	R_6	C_1
Growth at				
25°C	m	m	m	++
30°C	++	++	++	++
37°C	++	++	++	++
42°C	++	m	m	-
O-F test	F	O	O	O
Acid from carbohydrates:				
L-arabinose	+	+	+	-
D-glucose	+	+	+	+
D-Fructose	+	+	+	+
Sucrose	+	+	-	+
D-Lactose	+	-	-	+
Adonitol	-	-	-	-
D-sorbitol	-	+	+	+
Manitol	+	+	+	+
meso-Inositol	-	+	-	-
Utilization of chemicals as sole carbon source				
β-Alanine	-	+	-	-
L-arginine	+	+	+	-
L-aspartic acid	-	-	-	-
L-Valin	-	+	+	-
L-arabinose	-	-	-	-

Contd.....

Parameter	R_4	R_5	R_6	C_1
Gas from:				
Glycerol	+	-	-	-
Glucose	+	-	-	-
Nitrate reduction	+	-	+	+
Nitrite reduction	+	-	+	+
Indole production	+	-	-	-
Arginine dihydrolase.	+	+	+	-
Hydrolysis of gelatin	+	+	+	+
Levan formation	-	+	-	-
V-P test	-	-	-	-
Pigment	-	Yellowish green (in medium 'B' of King et al.)	Yellowish green(in medium'B' of King. et al)	Yellow

+, positive; +, good; m, moderate;

-,negative, O, oxidative; F, fermentative.

Three rod shaped bacteria R_4, R_5 and R_6 were straight, measuring about 0.72 - 0.75 by 2.7 - 3.0 µm, 0.68 - 0.72 by 1.5 - 2.2 µm and 0.72 - 0.80 by 1.6 - 2.2 µm, respectively (Fig. 114-116) found singly, in pairs or in short chains and motile. The cells were Gram negative and spores were absent. Colonies on agar plates were circular, smooth and convex. In nutrient broth, cultures of R_4 bacteria were turbid. Cultures of R_5 and R_6 bacteria in nutrient broth showed turbidity with pellicle and sediments. All three rod shaped bacteria showed good growth at 30°C and 37°C. At 25°C they showed moderate growth. R_4 also showed good growth at 42°C where as R_5 and R_6 showed moderate growth at 42°C.

C_1 bacterium was sphere shaped, (measuring about 0.9 - 1.5 µm in diameter), found singly, in pairs, in tetrads, in irregular cluster and in short chains. Cells of C_1 bacterium were Gram positive and no spores were found. Colonies, on agar plates, showed small, smooth and convex. Cultures in nutrient broth were first turbid then became clear with sediments. This bacterium showed good growth at 25°, 30° and 37°C but there was no growth at 42°C.

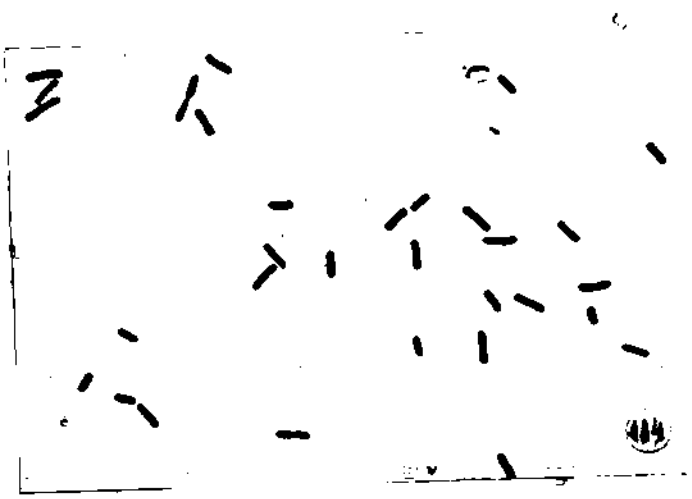
Plate XXXIV

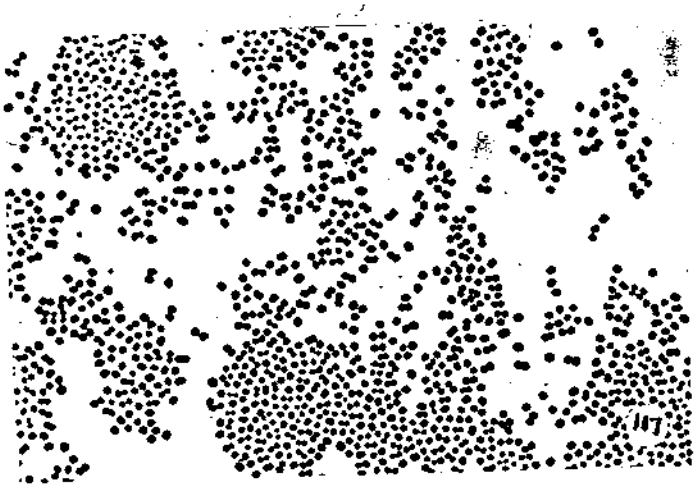
Fig. 114. Showing rod shaped bacteria, R_4 (Aeromonas sp.) phase contrast photomicrograph. X 800.

Fig. 115. Showing rod shaped bacteria, R_5 (Pseudomonad) phase contrast photomicrograph. X 800.

Fig. 116. Showing rod shaped bacteria R_6 (Pseudomonad) Phase constrast photomicrograph. X 800.

Fig. 117. Showing the sphere shaped bacteria, C_1 (Micrococcus varians) phase contrast photomicrograph. X 800.





L-arabinose, D-glucose, fructose, sucrose, lactose, adonitol, sorbitol, meso-inositol and mannitol were used to test for acid and gas production.

R_4 bacteria produced acid from L-arabinose, D-glucose, D-fructose, sucrose, D-lactose and mannitol but no acid was produced from adonitol, D-sorbitol and meso-inositol. R_5 bacteria produced acid from L-arabinose, D-glucose, D-fructose, sucrose, D-sorbitol, meso-inositol and mannitol. R_5 bacterium produced no acid from D-lactose and adonitol, R_6 bacterium produced acid from L-arabinose, D-glucose, D-fructose, D-sorbitol, mannitol but no acid was produced from sucrose, D-lactose, adonitol and meso-inositol. R_4 bacteria produced gas from glycerol and glucose. C_1 bacterium produced acid from D-glucose, D-fructose, sucrose, D-lactose, D-sorbitol and mannitol, and no acid was produced from L-arabinose, adonitol and meso-inositol.

O-F test showed that two rod shaped bacteria (R_5 and R_6) and one sphere shaped bacteria(C_1) utilized glucose by oxidation as because only the unsealed tube turns yellow. Another rod shaped bacteria (R_4) utilized glucose by fermentation as both the tube turned yellow.

After addition of nitrite reagent red colour developed in the culture tubes containing nitrate broth inoculated with R_4, R_6 and C_1 bacteria. This indicated that R_4, R_6 and C_1 bacteria could reduce nitrate to nitrite, on the other hand no red colour was developed in the culture tubes containing nitrate broth inoculated with rod shaped bacteria R_5 after addition of nitrite reagents which indicated absence of nitrite in the culture tubes. Addition of zink dust in the same tube developed red colour which showed presence of nitrate in the culture tubes. So it indicated that the R_5 bacteria could not reduce nitrate.

Nitrite broth inoculated with R_4, R_6 and C_1 bacteria, developed no red colour after addition of nitrite reagent which indicated absence of nitrite in the culture tubes which were reduced by these bacteria. On the other hand, nitrite broth inoculated with R_5 bacteria developed red colour after addition of nitrite reagent indicated the presence of nitrite in the culture broth which was not reduced by R_5 bacterium.

R_5, R_6 and C_1 bacteria did not produce indole. R_4 bacteria showed positive test for indole production. The tryptone broth culture of R_4 bacterium developed pink colour after addition of Kovac's reagent.

Gelatin were hydrolysed by all three rod shaped bacteria (R_4, R_5 and R_6) and sphere shaped bacterium (C_1) as clear zones were developed both on culture plates and on slants after addition of acid mercuric chloride solution.

All bacteria three rods (R_4, R_5, and R_6) and one coccus (C_1) showed positive test for catalase activity as bubbles of gas came out after pouring down of hydrogen peroxide (H_2O_2) over the culture slants.

Positive oxidase test was shown by all three rods as a dark purple colour appeared within 10 seconds on the paper impregnated with oxidase reagent. The coccus (C_1) showed negative result for oxidase activity.

Colonies of R_5 bacteria on agar plates and on slants containing 4% sucrose produce slimy secretion. However colonies of R_4, R_6 and C_1 bacteria did not produce any slimy secretion.

All three rods (R_4, R_5 and R_6) and sphere bacteria C_1 showed negative test in Voges-Proskauer reaction.

Yellowish green pigments were produced by two rod shaped bacteria R_5 and R_6 in medium 'B' of King et al.,(1954) and the pigment showed fluorescence under UV light. No pigments were produced on medium 'A' of King et al.(1954). Colonies of C_1 bacteria produced yellow pigment on nutrient agar at room temperature.

R_4 could utilize L-arginine but could not utilize β-alanine, L-aspartic acid and L-valin as a sole carbon source. R_5 utilized β-alanine, L-arginine and L-valin but could not utilize L-aspartic acid. R_6 utilized L-arginine and L-valin but could not utilize β-alanine and L-aspartic acid. C_1 could not utilize any supplied amino acids. None of the bacteria was able to utilize L-arabinose as sole carbon source.

Therefore from the above morphological and physiological characteristics (Table-12) it appeared that bacterium R_4 belonged to the genus Aeromonas; bacteria R_5 and R_6 belonged to the genus Pseudomonas and C_1 belonged to genus Micrococcus.

Discussion

Morphological and physiological characteristics of the R_4 bacterium resembles the characteristics of *Aeromonas hydrophila* (Schubert, 1974). As it produced gas from glucose and glycerol, it was of the subspecies *hydrophila* and as it was Voges-Proskauer reaction negative, it was of the biotype I (Schubert, 1974). Recently Popoff (1984) divided the motile aeromonad into three species, *A. hydrophila*, *A. caviae* and *A. sorbia*. R_4 bacterium satisfied some characteristics of *A. hydrophila* (Popoff, 1984) but it differed from *A. hydrophila* as it did not utilize L-arabinose as sole carbon source and it was Voges-Proskauer reaction negative. It showed some similarities with *A. sorbia* but it differed from *A. sorbia* as it did not utilize L-arginine as sole carbon source. It differed from R_3 bacteria, *A. caviae* (Chapter 1) as it did not produce gas from glucose, glycerol and it was Voges-Proskauer reaction negative. Popoff (1984) mentioned that the classification of motile aeromonads into species is complex. So the bacterium R_4 will be called *Aeromonas* sp.

Results indicated that R_5 and R_6 bacteria were straight, Gram negative, non-spore forming, motile, catalase positive, oxidase positive and produced yellowish green pigment; so they belonged to the genus Pseudomonas (Stanier et al., 1966; Palleroni, 1984). R_5 bacteria satisfied some important characteristics of P. fluorescens, such as production of fluorescent yellowish green pigment in medium B of King et al.,(1954) and levan formation. R_5 also satisfied the two important characteristics of biovar I of P. fluorescens (Palleroni, 1984) for instance, levan formation and denitrification inability. But it differed from biovar I of P.fluorescens as it was capable of growing at 42°C. The R_5 bacterium differed from R_1 bacterium isolated from ulcers of air-breathing fishes (Chapter 1) as it was capable of breaking down L-arabinose into acid and it showed the capicity to grow at 42°C. The R_5 also differed from R_5 (Chapter 1) as it was capable of breaking down sucrose into acid and it did not produce any green pigment in iron deficient medium. Pal and Pal (1986 a) reported isolation of fluorescent pseudomonad from epithelial carsinoma of Anabas which had similarities with P.fluorescens and was capable of growth at 42°C. Ajellow and Hendy (1985) have reported apyocyanogenic fluorescent pseudomonad strains capable of growth at 41°C but distinct from P. aeruginosa.

R_6 bacterium was also a fluorescent pseudomonad as it produced yellowish green pigment in medium B of King et al., (1954). It showed some similarities with Pseudomonas aeruginosa, e.g. no levan formation and growth at 42°C, but it differed from P. aeruginosa as it did not produce any blue pigment in medium A of King et al.,(1954). It differed from R_5 as it reduced both nitrate and nitrite and it did not produce levan from sucrose. R_6 differed from R_1 (Chapter 1) as it reduced both nitrate and nitrite, it was capable of growing at 42°C and did not produce levan from sucrose. R_6 also showed differences with R_2 (Chapter 1) in the following characteristics : it reduced nitrite and it did not produce any green pigment in iron deficient medium.

From the above discussion it became clear that R_5 and R_6 bacteria belonged to the genus Pseudomonas. Both R_5 and R_6 bacteria were fluorescent pseudomonad but differed from each other. They also showed differences with fluorescent pseudomonads (R_1 and R_2) isolated from ulcers of air-breathing fishes (Chapter 1). For their exact taxonomic status and nomenclature further studies are needed.

The spherical bacterium C_1 was Gram positive, occuring singly, in tetrad, in pairs or in short chains, catalase positive, oxidative, oxidase negative and indole negative. So it belongs to genus Micrococcus (Kocur, 1986). It also satisfied the characteristics of species Micrococcus varians (Kocur, 1986) such as reduction of nitrates and nitrites, good growth between 25 - 37° and non pathogenic. The C_1 bacteria also resembles all the characteristics of C bacteria (Chapt.1) isolated from air breathing fishes. Therefore the sphere bacteria C_1 belonged to the species, Micrococcus varians.

The ulcer disease affected not only the air-breathing fishes but also various types of fishes in different affected regions in India (Das, 1988; Jhingran and Das, 1990; Kumar et al., 1991).

Presence of Aeromonas hydrophila in the ulcer of different fishes has been reported by authors of different countries, such as Takahashi (1984), Miyazaki and Jo (1985) in Japan; Rahim et al., (1985) in Bangladesh, Llobrera and Gacutan (1987) in Phillipines. Costa and Wijeyaratne (1981) have reported that the primary cause of the ulcer disease among fresh water and estuarine fishes in Sri Lanka is unknown but A. hydrophila and an unidentified rhabdovirus have been associated with losses in fish in the affected region.

Pseudomonas fluorescens and P. aeruginosa had been reported as fish pathogen by several workers (Andre et al., 1970; Bullock and Mc Laughlin, 1970; Kusuda, 1980; Miyazaki et al., 1984; Okaeme, 1989; Sakai et al.,1989).

Different European countries also reported a haemorrhagic ulcer disease of fishes and it was differently named, such as infectious carp dropsy (Schäperclaus, 1965; Volf and Havelka, 1965; Tomasec and Fijan, 1965); rubella (Goncharov, 1965; Kocylowski, 1965); carp septicemia (Miaczynski, 1965) and viral haemorrhagic septicemia (Bellet, 1965; Ghittino,1965).

There are two opinions regarding the etiology of the disease, bacterial (Schäperclaus, 1965; Volk and Havelka, 1965) and viral (Tomasec and Fijan, 1965: Goncharov, 1965). Ghittino (1965) has described three forms of the disease in trout, acute, chronic and nervous. According to Ghittino (1965) acute form was observed at the begining of the epizootic and was characterised by heavy mortalities and a rapid course. The chronic form followed the initial acute stage characterised by moderate mortalities with prolonged course and sometimes dropsy. Nervous form was the terminal stage of an

epizootic. The affected trout spiraled in the water making circular movements, swimming in a tilted position and darting through or out of water. In France, Bellet (1965) also observed different forms with the nervous form in the final phase of the disease. In this study it was observed that some infected carps show swimming in a tilted position and occasional jumping out of the water. This corresponded the nervous form of trout described by Bellet (1965) and Ghittino (1965). It was observed that majority of the fishes showed acute form of the disease, i.e. skin lesions and no ascitis fluid in the stomach or intestine. Only one fish, Cirrhinus mrigala, with skin lesion showed accumulation of fluid in the intestine. In air-breathing fishes, no such accumulation of fluid was observed (Chapter 1). Goncharov (1965) mentioned that the clinical picture of rubella was variable in the U.S.S.R. and it depended on climatic conditions and species of fish affected. Fijan (1972) concluded that infectious dropsy of carp is a disease complex. So it could be concluded that the Indian major carps showed three forms of the disease, namely, acute, chronic and nervous form.