

# *Summary*

The present study dealt with "Studies on the role of bacteria isolated from ulcers of fishes in causing ulcer in experimental fish" consisting of, 1) Studies on the pathogenicity of the three bacteria, R1, R2 (fluorescent Pseudomonads) and R3 (*Aeromonas caviae*) which were isolated from ulcers of fishes affected with epizootic ulcerative syndrome, 2) Histopathology of experimentally infected fish *Heteropneustes fossilis* after intramuscular injection with R1, R2 and R3, 3) Effect of the three pathogenic bacteria, R1, R2 and R3 on the morphology of erythrocytes, total erythrocyte count (TEC) and haemoglobin (Hb) content of *H. fossilis*, 4) Studies on some virulence associated characters of R1, R2 and R3, 5) Detection and partial purification of extracellular virulence factors of R1, R2 and R3, 6) Observations on the external pathological condition of the epizootic ulcerative syndrome affected fishes, 7) Histopathological studies of the naturally infected EUS positive fishes, 8) Isolation of bacteria from the ulcerative lesion of naturally affected fishes, *Channa punctatus*, *Mystus* sp. and *Puntius* sp. from the South Bengal region and their characterization 9) Isolation of fungus from the fish, *Clarias batrachus* from the North Bengal region, 10) Pathogenicity testing of the isolated bacteria in *C. punctatus* and *H. fossilis* and a comparison with the pathogenicity of R1, R2 and R3, 11) *In vitro* antibiotic susceptibilities of nineteen bacterial isolates from EUS affected fish 12) Antibiotic treatment of ulcers of the fish *Channa punctatus* caused by experimental induction of the bacteria, R1, R2 and R3.

The ulcerative fish disease in an epizootic form occurred for the first time in Australia in 1972 with reccurrences in subsequent years. From the beginning of the last decade, severe disease outbreaks of a similar nature causing mass mortalities were reported from the countries of the Asia Pacific region one after another. Since the first appearance of the epizootic ulcerative syndrome in India, in May, 1988, in various north eastern states, it was distinct by its destructive nature and capacity of affecting a wide variety of fish species in both wild and cultured waters. It spread alarmingly and by the next two years it spread all over West Bengal, and also affected the adjoining states. By 1993 the disease spread all over India barring a few states.

The experiments conducted during the investigation has been presented in two broad sections. The first section (section 4.1) deals with studies on the involvement of three pathogenic bacteria R1, R2 (fluorescent Pseudomonads) and R3 (*Aeromonas caviae*) in the ulcerative disease of fishes. These bacteria were isolated from ulcers of epizootic ulcerative syndrome affected air breathing fishes from North Bengal region in 1988. The second section (section 4.2) deals with studies on fishes affected by epizootic ulcerative syndrome (EUS) collected from natural sources and isolation of microorganisms present in the lesion of EUS affected fishes from South Bengal. This section also includes a study on the treatment of ulcers of fishes experimentally infected with R1, R2 and R3 in pure and mixed form by using the antibiotic oxytetracycline.

Pathogenicity of R1, R2 and R3 were tested on *Heteropneustes fossilis*. The LD<sub>50</sub> values of R1, R2 and R3 were found to be 9.97x10<sup>5</sup> cfu, 4.45 x 10<sup>5</sup> cfu and 6.29 x 10<sup>5</sup> cfu respectively. The values showed that all the three bacteria were virulent. The virulence of R2 and R3 were almost similar and the virulence of R1 was slightly low as suggested by its slightly higher LD<sub>50</sub> value. R1, R2 and R3 were intramuscularly injected to three economically important species of air breathing fishes (*Anabas testudineus*, *Channa punctatus* and *Heteropneustes fossilis*) in pure and in mixed form. Results showed that the mixed bacterial suspension was more virulent than the pure bacterial suspensions. Severe ulcers were induced at the injection site in fishes treated with a mixed suspension while moderate ulcers were induced in fishes injected with pure bacterial suspensions of R1, R2 and R3. All the three species were equally susceptible to R1, R2 and R3 when injected intramuscularly either in pure or in mixed form.

Histopathological studies were conducted on *H. fossilis* after intramuscular injection given to healthy fish with pure and mixed bacterial suspension of R1, R2 and R3. Results showed that, there was loss of epidermis, muscle necrosis, granulomatous response and blood capillary infiltration in the dermal and subdermal layers at the site of injection. Liver showed vacuolation of hepatocytes, necrosis and infiltration of blood capillaries. In the kidney,

vacuolation, tubular degeneration and haemorrhage were the major changes. Haemosiderin laden macrophages were detected in the kidney of all infected fishes. The degree of pathological changes were comparatively less in the sections from fishes injected with pure bacterial suspensions than the sections from fishes injected with mixed bacteria. Presence of bacteria was observed in all tissue sections from affected fishes.

Studies were conducted on the progressive changes in erythrocyte morphology, total erythrocyte count (TEC) and haemoglobin (Hb) content of *H. fossilis* after intraperitoneal injection with pure and mixed bacterial suspension of R1, R2 and R3. Disintegration of erythrocytes and presence of bacteria were noted in the blood smears of all infected fishes. Significant lowering of TEC and Hb content in all experimentally infected fishes revealed anaemic condition. Mixed bacteria infected fishes showed a greater decline in TEC and Hb content than pure bacteria injected fishes.

Studies were conducted on the virulence associated characters of the three pathogenic bacterial isolates R1, R2 and R3. It was observed that all the bacteria exhibited proteolytic, lipolytic and haemolytic activity. Lecithinase was secreted by all the bacteria and all were able to hydrolyze gelatin. However, all the bacteria showed negative VP test.

Cell free culture filtrates containing the extracellular products of the pathogenic isolates R1, R2 and R3 were injected to healthy *Anabas testudineus*. Local haemorrhage was observed in the experimental fishes indicating that all the bacteria secrete extracellular toxic substances which may be responsible for the pathogenicity of these bacteria. The extracellular virulence factors were partially purified by gel filtration in a sephadex G-200 column. Two major peaks were eluted from culture filtrates of all bacteria and in all cases, the second peak was found to be toxic. The second peak also showed haemolytic and proteolytic activity.

EUS affected fishes collected from natural sources were brought to the laboratory for further observations. The ulcers were grouped into three major types : superficial, moderate and severe. A description of the three types of ulcers has been presented.

Histopathological observation of the sections through the ulcers of naturally infected fishes (*H. fossilis*) showed loss of epidermis, severe granulomatous changes, blood capillary infiltration and muscle necrosis. Liver showed severe vacuolation of the hepatocytes and infiltration of blood capillaries. Sections from kidney showed presence of haemosiderin laden macrophages along with tubular vacuolation and necrosis in some regions. Blood smear preparation showed erythrocyte disintegration and presence of bacteria in the naturally affected fish, *H. fossilis*. Tissue sections from naturally affected fishes and experimentally inoculated fishes (*H. fossilis*) with a mixed bacterial suspension of R1, R2 and R3 showed notable similarities. Similarities were also noted in blood smear preparations from naturally and experimentally infected fishes.

Bacteria were isolated from the lesions of naturally infected *Channa punctatus*, *Mystus* sp. and *Puntius* sp. collected from South Bengal and they were characterized by various biochemical tests. Among the isolates of *C. punctatus*, four belonged to the genus *Pseudomonas* and among the rest two, one belonged to the genus *Bacillus* and one belonged to the genus *Aeromonas*. Among the isolates of *Mystus* sp. three belonged to the genus *Aeromonas*, one was found to be *Moraxella* sp. and the remaining one belonged to the genus *Pseudomonas*. Among the isolates of *Puntius* sp. one belonged to the genus *Micrococcus*, another one belong to the genus *Pseudomonas* and the rest were identified as motile *Aeromonas* sp. Fungus or any other microorganism could not be detected in the ulcers of fishes from South Bengal.

Fungus was detected in the smear preparation of only one fish *C. batrachus* obtained from North Bengal. The fungus was isolated and its morphological features were examined after staining with cotton blue. the fungus from the fish tissue was aseptate and moderately wide with branchings. The spores were arranged in a single row inside the zoosporangia. The zoospores were circular and were encysted. It showed similarities with *Aphanomyces* sp.

Pathogenicity studies with the new bacterial isolates showed that only some strains of bacteria belonging to the genus *Aeromonas* and *Pseudomonas*

were virulent. Among the Aeromonads, A01, A02, A03 and A06 were found to be pathogenic. Among the Pseudomonads, P02 and P06 were found to be pathogenic. Isolates belonging to the other genera such as *Micrococcus* sp., *Moraxella* sp., *Bacillus* sp. and *Vibrio* sp. were found to be non pathogenic. All these pathogenic isolates were however, much less virulent in comparison to R1, R2 and R3.

The antimicrobial susceptibility of all bacteria isolated during the present study from skin ulcers of diseased fishes and R1, R2 and R3 were measured by the drug disc diffusion method in Mueller Hinton agar plates. Results showed that more than 50% of the bacteria were resistant to penicillin, ampicillin and erythromycin. More than 90% of the bacteria were sensitive towards chloramphenicol, kanamycin, nalidixic acid, norfloxacin, streptomycin and oxytetracycline.

Considering the susceptibility of R1, R2 and R3 to oxytetracycline, this antibiotic was chosen for treating the ulcers induced by these bacteria. This finding was further confirmed by the MIC values which clearly showed that oxytetracycline would be suitable for the treatment. In the present study, the MIC values of oxytetracycline for R1 and R2 were 3.2 µg/ml and for R3, the value was 6.4 µg /ml. Studies were conducted on the bath treatment of fishes experimentally infected with R1, R2 and R3 with the antibiotic oxytetracycline. The results showed that among the doses of oxytetracycline that were tried, some were capable of lowering the mortality rates of the experimentally infected fishes. However, for exact formulation of the drug, field trials are awaited.