Summary

The present investigation dealt with "Studies on R-plasmid in bacteria isolated from Epizootic Ulcerative Syndrome (EUS) affected fish" consisting of 1) Isolation of *Aeromonas* bacteria from ulcers of EUS affected fish *Cirrihinus mrigala, Catla catla, Channa striata* and *Puntius* sp. from the Darjeeling and Jalpaiguri districts of West Bengal, 2) Biochemical characterization and identification of isolated bacteria, 3) Pathogenicity test of the isolated bacteria on healthy fish, *Channa punctatus*, 4) Detection of some extra cellular virulence factors in the isolated bacteria, 5) Cytotoxic effect of the bacterial isolates on head kidney cells of healthy fish, *Channa punctatus*, 6) *In vitro* antibiotic sensitivity assay of twenty bacterial isolates from EUS affected fish, 7) Isolation of bacterial genomic DNA and plasmids from isolated bacteria, 8) Horizontal transfer of plasmids from isolated bacteria to susceptible species and 9) Detection of both cytolytic enterotoxin and aerolysin genes responsible for pathogenicity in the genomic DNA and plasmids of bacteria isolated from affected fish.

Twenty bacterial isolates, five from each fish species were isolated from the ulcers of affected fish using *Aeromonas* isolation medium supplemented with *Aeromonas* selective supplement (HiMedia Laboratories, Mumbai, India) by pour plate method during winter months from different affected ponds in various locations of the Darjeeling and Jalpaiguri districts of West Bengal. Among the isolates of C. *mrigala* four were *A. hydrophila* and one belonged to *A. caviae.* All the five bacteria isolated from ulcers of *C. catla* were identified as *A. hydrophila.* Among the isolates of *C. striata*, three were *A. hydrophila*, one belonged to *Aeromonas veronii* biovar sobria and the remaining one was stated as *Aeromonas* sp.as because it did not fulfill the known characteristics of the present procedure of identification. All isolates from the ulcers of *Puntius* sp. were identified as *A. hydrophila.*

All the isolates were tested for their ability to induce ulcers in healthy fish *C. punctatus* fish weighing of 40-50 g by intramuscular application of 0.5 mL of bacterial cell suspension (1×10^7 c.f.u /mL) per 100 g of fish body weight in 0.85% NaCl. Of the twenty bacterial isolates, nineteen were found to be pathogenic after intramuscular administration of these isolates to the healthy fish. Initially red patches appeared at the site of injection, it swelled gradually and after 72-96 hrs, the skin and underlying muscle layer eroded and it developed into ulcer. One isolate, Ch2 (*Aeromonas* sp. from *C. striata*,) could not induce any ulcer at the site of injection in healthy fish.

Studies were conducted on the virulence associated characters of all the isolated bacteria and it was found that nineteen of them exhibited proteolytic, lipolytic, haemolytic and amylolytic activities. Lecithinase was secreted by all the nineteen isolates and all of them were able to hydrolyze gelatin. Only the non pathogenic isolate, Ch2 could not show any positive test for virulence factors.

Cytotoxic effect on head kidney cells of healthy fish was assayed by using cell free culture filtrates of all the twenty bacterial isolates from diseased fish. It was observed that single dilution of culture filtrates of 95% isolates showed more than 50% *in vitro* cell death. Double (1/2) dilution of cell free culture filtrates of 70% bacteria showed more than 50% cell death whereas 1/4th dilution of culture filtrates from 45% bacteria showed more than 50% cell death. Crude culture filtrate of non pathogenic isolate Ch2 showed only 24.9% head kidney cell death compared to the 12% cell death in control.

The antibiotic sensitivity of all bacteria isolated from the ulcers of EUS affected fish were measured by disc diffusion method in Muller Hinton agar plates and results showed that all the bacterial isolates (100%) were resistant to ampicillin. Nineteen isolates (95%) showed resistance to erythromycin and novobiocin whereas seventeen (85%) and twelve (60%) isolates were found to be resistant to sulphadiazine and rifampicin respectively. Resistance to kanamycin was showed by five of the twenty isolates (20%) while three isolates (15%) were resistant to chloramphenicol and gentamycin. Tetracycline resistance was showed by two isolates (10%) while streptomycin and nalidixic acid was found to be resistant to only one isolates (5%).

Bacterial genomic DNA and plasmids were extracted from all bacterial isolates obtained from the affected fish in the present study. Analysis of plasmid profiles reaveled that all isolates contained plasmids, ranging in sizes from 2.0 to 63 kb, with a 23 kb plasmid in common. All isolates from *C. mrigala* harbored a 23 kb plasmid. Among the isolates of *C. catla,* Ct1 and Ct3 harbored 64 kb and 23 kb plasmids and Ct2, Ct4 and Ct5 carried 56 kb and 23 kb plasmids. Among the isolates of *C. striata,* Ch3 bacteria had three plasmids measuring 63 kb, 23 kb and 2.6 kb in length. Ch1 contained two plasmids of 23 kb and 2 kb whereas Ch4, Ch5 and non pathogenic isolate Ch2 had a single plasmid of 23 kb. All isolates from *Puntius* sp. harboured a single plasmid of 23 kb.

Of the twenty bacterial isolates, plasmids from sixteen were transferred to the nalidixic acid resistant *E. coli* DH5α strain through bacterial transformation and thirteen were transferred to E. *coli* DH5α strain through broth conjugation. Ampicillin (Am) erythromycin (E) and chloramphenicol (Cm) antibiotic resistance determinants in the bacterial isolates were found to be transferable to DH5α recipient with the transfer of plasmids. Frequencies of conjugal transfer ranging from 1.9×10^{-6} to 4.2×10^{-5} transconjugants per recipient cell were detected in the mating mixture. Donor isolates and transconjugants showed same plasmid bands. Plasmids from four isolates (M7, Ct1, Ch3 and P2) could not be transferred to the recipient strains through bacterial transformation. Plasmids from six bacterial isolates (M6, M10, Ct2, Ch4, Ch5 and P3) could not be transferred to the recipient strains through broth conjugation and the bacterial isolate, Ch3 could not take part in the conjugation experiment as because it was resistant to nalidixic acid.

Detection of both cytolytic enterotoxin and extracellular aerolysin genes in bacterial genomic DNA as well as in plasmids was carried out by amplification of DNA using combination of specific primers. Out of twenty bacterial isolates, nineteen were found to be positive to PCR test for the cytolytic enterotoxin gene and the extracellular aerolysin gene using the bacterial genomic DNA as template. DNA from non pathogenic isolate, Ch2 (*Aeromonas* sp. isolated from *C. striata*) gave negative result to PCR. All the twenty isolates were tested negative in the PCR when the plasmids obtained from all the bacteria were used as template