Discussion

Fish have long been an automatic and rich source of nutrients and growth accelerating ingredients from the onset of human civilization. But their survivability has largely been affected by innumerable diseases caused by pathogens of diverse origin. A wide range of pathogenic bacteria have so far been recovered from fish suffering from various ailments and among them, different strains of *Aeromonas* are quite common. Although many strains of *Aeromonas* are regarded as opportunistic pathogens, others are clearly treated as primary pathogens on their own right. *Aeromonas* bacteria are known to cause several fish diseases including red spot disease of European eel, *Anguilla anguilla* (Schäperclaus, 1934), exopthalamus and subcutaneous ulceration of cultured ayu, *Plecoglossus altivelis* (Jo and Onishi, 1980), tail rot, fin rot and haemorrahagic septicemias (Miyazaki and Kaige, 1985), brown patch diseases of Tilapia (Okpokwasili and Okpokwasili, 1994) and dropsy (Edun, 2007). Representatives of *Aeromonas* sp. have been isolated most frequently from ulcers and various internal organs of EUS affected fish in different regions of EUS outbreak. Reinoculation studies in healthy fish suggest that *Aeromonas* bacteria must have a crucial role in the fish mortalities caused by EUS (Pal and Pradhan, 1990; Chattopadhyay *et al.*, 1990; Karunsagar *et al.*, 1995; Yambot 1998; Chandrakanthi *et al.*, 2000; Rahaman *et al.*, 2002).

In the present study, twenty bacterial isolates were collected from the ulcers of four different EUS affected fish using *Aeromonas* isolation medium supplemented with *Aeromonas* selective supplement (HiMedia Laboratories, Mumbai, India) by pour plate method. The morphological features and biochemical profiles of these isolates suggested that they were motile, non-spore forming, glucose fermenting, Gram-negative bacilli. They were straight rods, grown in agar with 0% but not in 6% NaCl and reduced nitrate to nitrite (Tables 3, 4, 5 and 6). Thus, they all belonged to the genus *Aeromonas* (Popoff, 1984). Of the twenty *Aeromonas* isolates, seventeen (M6, M7, M8, M9, Ct1, Ct2, Ct3, Ct4, Ct5, Ch3, Ch4, Ch5, P1, P2, P3, P4 and P5) of them were positive to esculin hydrolysis test, sensitive to antibiotic cephalothin, produced gas from glucose and acid from arabinose (Carnanhan *et al.*, 1991). These bacteria were positive to lysine decarboxylase and arginine dehydrolase test but negative to ornithine decarboxylase test, produced acid from mannitol and sucrose and gave a positive to VP test (Abbott *et al.*, 1992).Thus, these bacteria were regarded as *Aeromonas hydrophila*. The isolate Ch1 was

negative to esculin hydrolysis test, positive to indole test, produced acid from sucrose and gave a positive VP test (Carnanhan *et al.*, 1991). It was tested positive to arginine dihydrolase, lysine decarboxylase and negative to ornithine decarboxylase, produced acid from arabinose and mannitol (Abbott *et al.*, 1992). Therefore, it belonged to *Aeromonas veronii* biovar sobria. The isolate M10 was tested positive to esculin hydrolysis but could not produce gas from glucose (Carnanhan *et al.*, 1991). It was tested positive to arginine dihydrolase, negative to lysine decarboxylase and ornithine decarboxylase, gave a negative VP test and produced acid from arabinose, mannitol and sucrose (Abbott *et al.*, 1992). Thus it was identified as *Aeromonas caviae*. The isolate Ch2 could not follow the identification schemes mentioned in the materials and methods and therefore, it was only stated as *Aeromonas* sp. A further detailed study is awaited in order to decide its taxonomic status and nomenclature.

All the bacterial isolates were examined for their ability to induce ulcers when administered intramuscularly to healthy *Channa punctatus* fish. It was observed that all isolates apart from Ch2 (*Aeromonas* sp. from *C. striata*) showed manifestation of EUS like lesions at the site of injection of healthy fish within 24-72 hrs (Fig. 13, 14 and 15). *Aeromonas* bacteria were reported to induce EUS like lesions when injected intramuscularly to healthy snakehead (*Ophiocephalus striatus*) and walking catfish (*Clarias batrachus*) using the bacterial suspension of 10^6 c.f.u/mL (Lio-Po *et al.*, 1992; Leano *et al.*,1995). Saha and Pal (2000) suggested that *Aeromonas* and *Pseudomonas* bacteria could induce ulcer when 0.5 mL of each bacterial cell suspension was injected intramuscularly to healthy *C. puntatus* fish per 100 g of fish body weight containing 1×10^7 bacterial cells/mL. Chandrakanthi *et al.* (2000) showed that *A. hydrophila* suspension manifested ulcers in healthy EUS susceptible *Etroplus suratensis* fish when 0.05 mL of bacterial culture (5×10^6 c.f.u/mL) was injected intramuscularly per 19-27 g of fish body weight. In the present study, a volume of 0.5 mL of bacterial suspension containing 1×10^7 c.f.u/mL per 100 g of fish body was injected to healthy *C. puntatus* fish to induce ulcers and it was in agreement with the findings of earlier investigations.

Aeromonas bacteria were reported to secrete a wide range of biologically active extracellular products (ECP), such as glycophospholipid cholesterol acyltransferase (GCAT) (Buckley *et al.*, 1982), haemolysin (Asao *et al.*, 1984), pore forming areolysin

(Howard *et al.*, 1986), cytolytic enterotoxin (Chopra *et al*, 1993), amylase (Chang *et al.*, 1993) lipase (Pemberton *et al.*, 1997), several proteases (Coleman, 1992; Pemberton *et al.*, 1997) and cytotonic enterotoxin (Chopra, 2006). These virulence factors are useful to distinguish between potentially pathogenic and non-pathogenic strains of *Aeromonas* bacteria (Zhang *et al.*, 2000), though further evidences are required to establish the exact correlation between extracellular enzymes and virulence of fish pathogenic *Aeromonas* bacteria. The primary function of aerolysin or other enterotoxins is likely to kill target cells, whereas other proteins are enzymes whose main purposes are perhaps to produce utilizable nutrients from the host macromolecules (Howard *et al.*, 1996). In order to ascertain the correlation between pathogenicity and the role of extracellular products released by different species of *Aeromonas*, a number of biochemical tests (section 4.2.2) were conducted in the present investigation. It was observed that out of twenty isolates, nineteen were found to exhibit extracellular amylolytic, proteolytic, haemolytic and lipolytic activities. Only one isolate (Ch2, *Aeromonas* sp. from *C. striata*), which was non pathogenic to healthy fish could not show any lytic activities (Table 8). Karunsagar *et al.* (1995) suggested that most of the *Aeromonas* bacteria isolated from internal organs of EUS affected fish produced haemolysin and all of them were able to hydrolyse gelatin. Saha (1998) investigated the involvement of virulence factors in two pathogenic fluroscent pseudomonads and one aeromonad isolated from the ulcers of EUS affected fish and found that all of them were able to secrete extracellular lytic enzymes such as, protease, lipase, gelatinase and haemolysin. Presence of extracellular haemolysin and lipase in *A. hydriphila* isolated from Ulcerative Disease Syndrome affected fish was also detected by Majumdar *et al.* (2006).

From the study of extracellular products of bacteria isolated from different EUS affected fish, it is clear that the *Aeromonas* bacteria secrete a variety of extracellular lytic enzymes which may have crucial roles in manifestation of the signs of the ulcerative disease of fish. Toxins produced by *Aeromonas* bacterial strain are reported to have cytotoxic and enterotoxic activities. Aerolysin, a toxin molecule obtained from a fish isolate of *A. hydrophila,* can penetrate the lipid bilayers of cell membranes of eukaryotic cells (Howard and Buckley, 1982) and manifest haemolytic and cytotoxic activities (Janda *et al.*, 1985; Chakraborty *et al.*, 1987). Chopra *et al.*, (1993) have isolated a cytolytic enterotoxin from

A. hydrophila which can destroy rabbit blood cells and lyse Chinease hamster ovary cells. In the present investigation, cytotoxic effect on head kidney cells obtained from healthy fish was conducted by serial double dilution of cell free cultures of bacteria isolated from the EUS affected fish (section 4.2.3). It was observed that out of twenty bacteria crude culture filtrates from nineteen isolates showed more than 50% *in vitro* cell death whereas 1/2 dilution of fourteen bacterial filtrates caused more than 50% cell death. Culture filtrates having 1/4th dilution from nine isolates showed more than 50% cell death. Crude culture filtrate from non pathogenic isolate Ch2 showed only 24.9% cell death in compared to the 12% cell death in control. It was clear from the above experiment that the higher doses of culture filtrates had more lytic effect on the *in vitro* fish cell culture whereas the culture filtrates from non pathogenic bacteria Ch2, could not have any significant affect on head kidney cells.

Plasmids are self replicating extra chromosomal DNA varying in sizes from 1 to more than 200 kb and mainly encode proteins for antimicrobial resistance, toxin, adhesion, metabolic enzyme and bacteriocin (Mayer, 1988). Studies by different workers reported the presence of plasmids in different motile and non-motile *Aeromonas* bacteria with sizes ranging from 2 to 150 kb (Schmidt *et al.*, 2001; Nawaz *et al.*, 2006) which confer antibiotic resistance (Son *et al.*, 1997) and play a role in virulence (Mazumdar *et al.*, 2006). In the present investigation, plasmid profiles of all the bacterial isolates from EUS affected fish were examined and analysis of plasmid profiles revealed that all isolates contained plasmids, ranging in sizes from 2.0 to 63 kb, with a 23 kb plasmid in common (Table 13).

It was observed that the use of antibiotics is an effective and easy method to prevent *Aeromonas* infections in aquaculture. Several workers reported and recommended the application of antibiotics for the treatment of EUS affected fish (Jhingran, 1990; Pradhan and Pal, 1993; Das 1997; Saha and Pal, 2002). Jhigran (1990) observed total recovery of EUS after using nalidixic acid and erythromycin at the rate of 50 mg per kg fish body weight per day in a formulated micro capsulated feed containing 30% protein and fortified with vitamin A and C together with chloramphenicol bath at the rate of 15 ppm. Pradhan and Pal (1993) studied the antibiotic susceptibility of bacteria isolated from EUS infected fish and suggested that bactrim and streptomycin could be effective in controlling the disease. Das (1997) reported that either erythromycin or oxytetracycline at 60 to 100 mg

per kg of feed for 7 days could cure the ulcers of EUS affected fish. Saha and Pal (2002) recommended that the oxytetracycline could be useful to treat the EUS affected fish.

Other *Aeromonas hydrophila* infections like "Motile Aeromonas Septicemia" (MAS) or "Hemorrhagic Septicemia" could be treated by the application of antibiotics Terramycin®, an oxyteracyline using 2.5-3.5 g/100 lb of fish per day for 10 days in feed and Remet-30®, a potentiated sulfonamide using 50 mg/kg of fish per day for 5 days. (CES, 1989).

Several investigators have opined that excessive use of antibiotics and other chemotherapeutics in fish farm either directly or as dietary supplement have led to increase in a number of drug resistant bacteria (Huys *et al.*, 2000; Miranda *et al.*, 2003; Nawaz *et al.*, 2006). Antibiotics used in the aquaculture remain in the bottom soil or sometimes wash away to distant places by water body and may exert selective pressure on microorganisms leading them to become resistant to antibiotics (Krush and Sørum, 1994; Kim *et al.*, 2004; Balban *et al.*, 2004). Numerous studies indicate that the bacteria flora in the environment surrounding aquaculture sites contain an increased number of antibiotic resistant bacteria (Huys *et al.*, 2000; Sørum, 2000; Miranda and Zemelman, 2002) and these bacteria harbour new and previously uncharacterized plasmids encoding antibiotic resistant determinants (Poirel *et al.*, 2005; Sorum, 2006) These resistance plasmids or R-plasmids harbouring antibiotic resistance determinants can be transmitted to a wide range of bacterial species including bacteria pathogenic to human. (Krush and Sørum, 1994; Sørum, 2006).

In the present study, antibiotic sensitivity test of all the twenty bacterial isolates was carried out (section 4.3) and it was observed that all of them were resistant to ampicillin and majority of them were resistant to erythromycin, sulphadiazine, novobiocin and rifampicin (Table 10). *Aeromonas* bacteria have been found to be resistant to ampicillin, sulphadiazine and novobiocin by several other workers (Torenzo *et al.*, 1984; Thampuran *et al.*, Son *et al.*, 1997; Roy *et al.*, 2003; Hatha *et al.*, 2005), but they did not detect high resistance to erythromycin. Tetracycline was recommended as a therapeutic agent for the treatment of EUS by different authors (Das, 1997; Saha and Pal; 2002), but 10 % of the bacteria isolated in the present investigation were resistant to tetracycline.

Until now, several studies have reported the horizontal transfer of R plasmids coding multiple antibiotic resistances in fish pathogenic bacteria including *Aeromonas* (Toranzo *et*

al., 1984; Son *et al.*, 1997) but very little information is available on plasmid profiles of fish pathogenic bacteria in India. In order to ascertain whether the antibiotic resistance determinant resides in the plasmid and whether these determinants are transferable with the transfer of plasmids, further experiments were carried out (Section 4.5 and 4.6). Plasmids in different sizes (63, 54, 23, 2.6 and 2 kb) were present in all isolated bacteria of which plasmids from sixteen isolates including non pathogenic bacteria (Ch2) were transferable through bacterial transformation (Table 12) and thirteen were transferable through broth conjugation (Table 13). This was associated with the transfer of ampicillin, erythromycin and chloramphenicol resistance determinants and these resistance markers were expressed in *E. coli* recipient strain. Transfer of ampicillin and erythromycin resistance determinants in motile *Aeromonas* has been reported by other workers (Chaudhury *et al.*, 1996; Son *et al.*, 1997) and their sizes varied from as low as 6.2 kb to >150 kb. Erythromycin resistance was found in 95% of the isolated bacteria and among them, 68% were transferable through broth conjugation (Table 13). During the first outbreak of EUS in India and during subsequent recurrences, erythromycin was included in almost all prescribed formulations for treatment of affected fish (Jhingran, 1990; Das, 1997). This may in part be responsible for excessive erythromycin resistance (95%) in the present isolates.

Schmidt *et al.* (2001) reported the presence of transferable plasmids associated with both streptomycin and sulphadiazine resistance determinants. In contrast, although our isolates showed high resistance towards sulphadiazine (85%), the resistance determinant was not transmissible. The resistance factor may be encoded in the genomic DNA as also novobiocin, gentamycin, kanamycin and rifampicin. Toranzo *et al.* (1984) reported the horizontal transfer of an 80 kb plasmid associated with streptomycin resistance in *Aeromonas* while only one of our isolates (Table 9) was resistant to streptomycin which was not transmissible.

A notable finding during the study was 15% resistance of the isolated strains to chloramphenicol, though chloramphenicol resistance is seldom observed in motile *Aeromonas* (Koehler and Ashdown, 1993; Sørum *et al.*, 2003). Earlier experiments from our laboratory (Pradhan and Pal, 1993; Saha and Pal, 2002) showed that all the bacterial strains isolated from EUS affected fish in this region were sensitive to chloramphenicol. According to a report by Jhingran (1990), chloramphenicol (15ppm) was found to be

effective in treating EUS affected fish. Although direct use of chloramphenicol in fish farms for treating EUS is not recorded, resistance to this antibiotic is increasingly being detected (Vivekanandhan *et al.*, 2002; Hatha *et al.*, 2005) which suggests that farmers may have used it for such treatment. The recent detection of chloramphenicol residues in aquaculture products from India has raised great concern globally leading to a ban on the fish products from some Indian suppliers (Hatha *et al.*, 2005). Thus, there is a strong probability of the widespread use of this antibiotic in aquaculture.

In the present study, mating experiments was carried out at 25°C, since the highest frequencies of gene transfer were earlier reported within 20 to 25°C, which is quite normal in the tropical aquatic systems (Altherr and Kasweck, 1982; Son *et al.*, 1997). A frequency range in between 1.9×10^{-6} to 4.2×10^{-5} was found in the present experiment which may occur in natural system (Table 13). Other authors reported transfer of antibiotic resistance determinants in *Aeromonas* at frequencies that ranged from as low as 1.0×10^{-7} (Toranzo *et al.*, 1984) to as high as 4.3×10^{-3} (Son *et al.*, 1997). In this study, although a 23 kb plasmid was present in all isolates; it was not conjugable from seven strains. This might have been successfully transferred if the conditions of mating or mating procedures were different (Altherr and Kasweck, 1982; Son *et al.*, 1997; Sørum *et al.*, 2003).

It has been reported that a single high molecular weight plasmid can be responsible for multiple drug resistance in different species of *Aeromonas* (Roy *et al.*, 2003; Sorum *et al.*, 2003). Therefore, isolation of 23 kb plasmid from all the bacterial isolates from different EUS affected fish and its horizontal transfer to *E. col* DH5α strains in every successful single step conjugation indicated that it was likely to be a stable plasmid and conferred resistance to different *Aeromonas* isolates.

A significant number of virulence genes have been described among *Aeromonas* bacteria, including genes for aerolysin (Pollard *et al.*, 1990), haemolysin (Hirono *et al.*, 1992), cytolytic enterotoxin (Chopra *et al.*, 1993), protease and haemagglutinin (Thornley *et al.*, 1997). The product of the aerolysin gene secreted by some strains of *A. hydrophila*, makes an attachment with the eukaryotic host cells and aggregates to form hexamers that penetrate the lipid bilayer of cell membranes, resulting the formation of 3-nm channels (Howard and Buckley, 1982). The toxin molecule is activated outside the bacterial cell by the removal of 25 amino acid residues from the carboxy terminus (Howard and Buckley,

1985) and consists of a single polypeptide chain that is synthesized as a 54 kDa prepotoxin with a putative signal peptide at its amino terminus (Chakraborty *et al.*, 1986; Howard and Buckley, 1986).The aerolysin has been reported to be lethal to mice and have haemolytic and cytotoxic activities (Chakraborty *et al.*, 1987).The product of the cytolytic enterotoxin gene can lyse rabbit red blood cells and destroy Chinease hamster ovary cells, cause fluid secretion in rat ileal loops, and are lethal to mice when administered intravenously (Chopra *et al.*, 1993). Kingobe *et al.* (1999) developed a PCR method that detected both cytolytic enterotoxin and aerolysin genes simultaneously in *Aeromonas* sp. by using one pair of primers. Rahaman *et al.* (2002) substantiated the presence of cytolytic enterotoxin and aerolysin genes in *Aeromonas veronii* biovar sobria isolated form EUS affected fish in Bangladesh by using the PCR primer combination strategy of Kingobe *et al.* (1999).

In the present study, the presence of cytolytic enterotoxin and aerolysin genes was investigated in the bacterial genome as well as in plasmids of aeromonads isolated from EUS affected fish by using the PCR method of Kingobe *et al.* (1999) (section 4.7). It has been reported that plasmids are found to be associated with the virulence factors in several motile and non motile strains of *Aeromonas* bacteria. Toranzo *et al.* (1983) reported a plasmid-cured derivative of a wild-type *A. hydrophila* isolate showing a number of differences from the parental bacteria including gelatinase production and alterations in surface properties in addition to tetracycline resistance. Stuber *et al.* (2003) reported the presence of TTSS genes on a large thermolabile plasmid in *A. salmonisida* and at elevated temperatures the bacterial strains lost the plasmid in addition to the loss of its virulence potentials. Majumdar *et al.* (2006) cured a 21 kb plasmid from *A. hydrophila* isolated from a ulcerative disease syndrome (UDS) affected fish and failed to induce UDS in healthy Indian walking catfish, *Clarias batrachus* when administered the plasmid free cured strains intramuscularly. They suggested that the presence of 21 kb plasmid in the *Aeromonas* bacteria probably served as a virulence marker which could play an important role in the regulation of aeromonad UDS.

Our present study revealed that bacterial genomic DNA obtained from nineteen isolates were positive to PCR test (Fig. 32-34) and only bacterial genomic DNA from non pathogenic isolate Ch2 were negative to PCR test. Whereas all plasmids isolated from twenty *Aeromonas* bacteria were found to be negative to PCR test. Therefore, it can be

suggested from the above findings that both the cytolytic enterotoxin and aerolysin genes are located on the bacterial genomic DNA.

The bacterial isolate Ch2 could not induce any ulcers in healthy fish and could not manifest extra cellular amylolytic, proteolytic, lipolytic and haemolytic activities like other pathogenic isolates (Table 7). The cell free culture of this isolate had no significant toxic effect on *in vitro* head kidney cells of healthy fish but it was found to carry plasmids like other pathogenic bacteria isolated in the present investigation (Table 11). This finding suggests that the presence of plasmids is not likely to be associated with the regulation of extracellular lytic activities of the *Aeromonas* bacteria isolated from ulcers of EUS affected fish as suggested by Majumdar *et al.* (2006).

The present investigation indicates that the resistance to common antibiotics used in aquaculture is prevalent among bacteria isolated from the ulcers of EUS affected fish and resistance determinants of some of these antibiotics have been transferred to the bacteria of other origin. EUS poses a serious threat to the fish industry of Asia Pacific region and its prophylaxis is an urgent need to sustain the steady production of fresh water fish. The emergence of antibiotic resistant bacteria and *in vitro* gene transfer suggests that antibiotics should be used more cautiously to treat the EUS or other *Aeromonas* infections in aquaculture.