Discussion

Epizootic ulcerative syndrome is at present known to be one of the most common and most dreaded disease of fresh and brackish water fishes in India. Severe losses have been reported from all types of water bodies. Considerable amount of research activity is currently focused on the identification of major causative agents and an effective method for controlling this disease. However, there is a lack of basic knowledge on the pathogenesis of disease agents on the host. In the present study an attempt has been made to study the exact role of pathogenic bacteria which have been isolated from ulcerative lesions of diseased fishes of North Bengal in causing ulcers in air breathing fishes which are economically important in this region along with a study on the bacteria isolated from EUS affected fishes of the district of Midnapur in South Bengal and a study on the effect of chemotherapeutic agents on the bacterial isolates. It is likely that the results of these studies will broaden the scientific base upon which total control of this dreadful disease can be established.

Two virulent strains of *Pseudomonas* sp. and one virulent strain of *Aeromonas caviae* which were isolated from ulcerative air breathing fishes from North Bengal in 1988 were reported earlier to be pathogenic to *Anabas testudineus* (Pal and Pradhan, 1990) and *Channa punctatus* (Pradhan and Pal, 1990) but the pathogenicity was not measured and there were no reports on their LD_{50} values in these fishes.

In the present experiment (section 4.1.1), the serially diluted dose of different bacterial suspensions produced consistent trends of mortality. Isolation and identification of injected bacterial strains from external lesion, liver, and kidney of freshly dead fishes clearly indicate that the cause of death was associated with these bacterial strains. Various workers have observed changes in the liver and kidney of fishes infected by pathogenic *Pseudomonas* and *Aeromonas* strains (Takahashi, 1984b; Miyazaki and Kaige, 1985; Okpokwasili and Okpokwasili, 1994; Pal and Pradhan, 1995). Prasad *et al* (1995) could re-isolate the pathogenic strain of *Aeromonas hydrophila* from liver, kidney and spleen of fish experimentally infected by the bacteria.

The LD_{50} values of the two Pseudomonads (R1 and R2) and the Aeromonad (R3) in *H. fossilis* were found to be 9.97×10^5 c.f.u., 4.45×10^5 c.f.u.

and 6.29 $x10^5$ c.f.u. respectively. Descriptions of the degree of virulence by previous workers (Mittal *et al*, 1980 ; Santos *et al*, 1996) suggested that strains of *Aeromonas* sp. showing LD_{50} $<10^6$ could be classified as virulent, those with LD_{50} 10^6 to 10^8 as weakly virulent and those with LD_{50} > 10^8 as nonvirulent. Accordingly R1, R2 and R3 could be denoted as virulent. There was no significant difference between the virulence of R2 and R3. The virulence of R1 was slightly low as suggested by its slightly higher LD_{50} value.

Intramuscular injections of R1, R2 and R3 were given to three economically important species of air breathing fishes (*Anabas testudineus, Channa punctatus* and *Heteropneustes fossilis*) in pure and in mixed form (section 4.1.1.2). Mortality data (Tables 3, 4 and 5) 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.

Aeromonads and Pseudomonads were found to experimentally induce EUS like skin lesion when injected intramuscularly to healthy snakehead (*Ophiocephalus striatus*) and walking catfish (*Clarias batrachus*) using at least 10^6 c.f.u./ml (Lio-Po *et al*, 1992 ; Leano *et al*, 1995). Karunasagar *et al* (1995) isolated a number of *Aeromonas* strains from external ulcer and internal organs of EUS positive fish and found that these bacteria had very low LD_{50} values ranging from 10^3 to 10^5 in catfish fingerlings and 10^4 to 10^6 in mouse.

Prasad *et al* (1995) observed that *Cirrhinus mrigala* intraperitoneally injected by virulent *Aeromonas hydrophila* strain isolated from EUS affected *Mastocembelus armatus* exhibited symptoms of EUS including lethargic behaviour and haemorrhages on the general surface of the body. *C. mrigala* showed 100%, 90%, 60% and 30% mortality in 2, 3, 4 and 5 days after intraperitoneal injection with 0.1 ml of viable bacterial suspension containing 2.0 x 10^3, 1.6 x 10^3, 1.2 x 10^3 and 0.8 x 10^3 c.f.u. respectively.

Sahu *et al* (1996) found that experimentally *infected Aeromonas hydrophila* could produce lesion in the catfish, *Clarias batrachus* on 30th day and in Rohu (*Labeo rohita*) on the 10th day. However, the infection was limited

to ulcer in the skin and muscle only. Histopathological changes in the internal organs were not as remarkable as it was in the skin and muscle.

Besides *Aeromonas* sp. and *Pseudomonas* sp., various other types of bacteria were also found to be associated with epizootic ulcerative syndrome. Jhingran and Das (1990) observed that *Micrococcus* sp. isolated from lesions and haematopoetic tissues of EUS affected fishes could induce ulcers within 72 hours both through inoculation and when kept in association with the bacteria. Kar *et al* (1990) found *E. coli* and *Pseudomonas aeruginosa* to be associated with EUS affected fishes.

Ali and Tamuli (1991) isolated three types of bacteria from ulcers of four species of affected fishes and reinfection studies showed that *Aeromonas* sp. induced only mild infections, *Vibrio* sp. induced similar disease symptoms and *Micrococcus* sp. failed to induce any disease symptom.

Mukherjee *et al* (1995) reported constant isolation of chemoautotrophic nocardioform (CAN) bacteria from EUS affected fishes which bears close relationship to the human/rat leprosy bacillus and demonstrated its pathogenicity in mouse model.

Qureshi *et al* (1995b) isolated nine types of bacteria from EUS affected fishes out of which Pseudomonads and Aeromonads were found to be highly pathogenic while Micrococcans and Cytophagans were less pathogenic.

These previous findings show that there is little similarity in the type of bacteria isolated from EUS positive fishes though many of these bacteria are virulent and are able to induce EUS like lesion when injected to healthy fishes. R1, R2 and R3 also belong to different genera and species though they are almost equally virulent. A possible explanation for this discrepancy in bacterial types may be that EUS is not caused by any single bacterium. It seems to be a complex disease caused by mixed infection.

A study on the pathogenicity of these bacteria in three economically important fishes in pure as well as mixed form was undertaken (Fig. 18). When compared among the three fish species *Channa punctatus, Heteropneustes fossilis* and *Anabas testudineus*, the obtained data indicated that the mortality rates in *Anabas testudineus* inoculated by all the three bacteria R1, R2 and R3

and mixed culture were slightly lower than *Channa punctatus* and *Heteropneustes fossilis* although the differences were not statistically significant (Fig. 17).

The range of incidences of EUS recorded from the different species of naturally infected fish shows that *Channa punctatus* is one of the most susceptible fishes. The incidence percentage ranges from 20% to 100%. The other two fish species are also highly susceptible in natural waters. The incidence percentage ranges from 10% to 20% in *Heteropneustes fossilis* and 10% to 55% in *Anabas testudineus* (Das and Das, 1993). The relative susceptibilities of various other fish species to several bacterial pathogens have been investigated by several workers (Plumb and Sanchez, 1983; Plumb and Hilge, 1987; Whittington and Cullis, 1988; Sakai *et al*, 1989; Soltani *et al,* 1994).

In natural infections, though most of the pond cultured fishes are susceptible, some like the Nile tilapia (*Oreochromis niloticus*) is found to be resistant to EUS (Ahmed and Rab, 1995). Thus, a comparative susceptibility data among all types of pond cultured fishes is necessary because this will provide vital clues in fish farm management. Choice of fish species other than which are highly susceptible to the disease will minimize the losses and segregating fishes which are more susceptible can lower the risk of disease outbreak.

Globally *Aeromonas* sp. is one of the most common bacteria associated with fish diseases. Although many strains are regarded as opportunistic pathogens, others are clearly primary pathogens in their own right (Trust, 1986). The fish diseases which involve *Aeromonas hydrophila* includes motile Aeromonad septicemia (AFS, 1975), red spot disease of European eel, *Anguilla anguilla* (Schäperclaus, 1934), red disease of Japanese eel, *A. japonica* (Hoshina, 1962) and red disease of carp, *Cyprinus carpio* (Egusa, 1978). Jo and Onishi (1980) isolated *A. hydrophila* from all diseased cultured ayu, *Plecoglossus altivelis* characterized by exopthalamus and subcutaneous ulceration. Rahim *et al* (1985) isolated *A. hydrophila* from the wounds of five species of fishes in Bangladesh. Okpokwasili and Okpokwasili (1994) found that *Pseudomonas* spp. and *A. hydrophila* isolated from brown patch disease of tilapia were more virulent to tilapia fingerlings when infected by a mixed culture

than *A. hydrophila* or *Pseudomonas* spp. alone. Esteve *et al* (1993) isolated *Aeromonas hydrophila* and *Aeromonas jandaei* from diseased European eel (*Anguilla anguilla*) from an eel farm in Spain which caused ulcerous disease by intraperitoneal injection (LD_{50} dose : $10^{5.4}$ to $10^{7.5}$ c.f.u. / fish) and also by bath exposure to 10^7 to 10^8 c.f.u./ml in healthy eels.

Deep seated muscle lesions (furuncles) are caused by *Aeromonas salmonicida* principally in salmonids although other fish species may be affected (McCarthy, 1977; Patterson *et al*, 1980; Boomker *et al*, 1984; Ostland *et al*, 1987; Wichardt *et al*, 1989; Rintamaki and Valtonen, 1991 and Wiklund *et al*, 1994). The disease known as furunculosis has affected various species of fishes in fish farms worldwide including wild fish stocks (Wiklund and Bylund, 1993). *Aeromonas salmonicida* subsp. *salmonicida* has been isolated as the etiological agent of furunculosis in salmonids and has often been referred to as the typical strain (Austin and Austin, 1993). The atypical strains belonging to other *A. salmonicida* subspecies have also been isolated which causes ulcerative diseases in a variety of both marine and freshwater fish (Nakatsugawa, 1994). Gudmundsdottir (1996) analyzed extracellular products of 32 strains of *A. salmonicida* from various fish species and geographical locations and found that all typical strains belonged to a single group while the atypical strains were more diverse.

The goldfish ulcer disease (GUD) is caused by an atypical strain of *Aeromonas salmonicida* which is responsible for loss of both wild and cultured goldfish (Trust *et al*, 1980; Whittington *et al*, 1987).

Aeromonas salmonicida has been reported to be isolated from head ulcer of eel, *Anguilla japonica* characterized by ulcerative lesion on the head (Ohtsuka *et al*, 1984) and carp erythrodermatitis characterized by cutaneous ulcerative lesion (Csaba *et al*, 1984).

Aeromonas liquifaciens, another pathogenic strain of *Aeromonas* sp. was isolated from scale protrusion disease in carp by Kusuda and Takahashi (1970), which affected fish farms of Japan. Reinfection studies in carp and gold fish showed that percentage mortality of carp was higher than that of gold fish. *Aeromonas punctata* has been regarded as the etiological agent of infectious

dropsy in carps by Schäperclaus (1965) and he thought that primary infections resulting in disease may be induced by *Pseudomonas fluorescens*. Later he indicated that 3 different microorganism, *A. punctata*, *P. fluorescens* and a virus may be involved in this disease which manifest itself in several forms (Schäperclaus, 1969).

Different species of *Pseudomonas* has been reported to be the causative agent of various fish diseases throughout the world. *Pseudomonas anguilliseptica* was identified as the etiological agent of red spot disease in Japan characterized by petechial haemorrhage in the mouth, opercula and ventral portion of the body of the fish (Wakabayashi and Egusa, 1972; Muroga *et al*, 1973; Jo *et al*, 1975; Nakai *et al*, 1985). *P. anguilliseptica* was also isolated from red spot disease of pond cultured eel, *A. japonica* in Taiwan (Kuo and Kou, 1978), from *A. anguilla* in Scotland (Nakai and Muroga, 1982; Stewart *et al*, 1983) and from salmonid fish in Finland (Wiklund and Bylund, 1990). Muroga and Nakajima (1981) reported artificial induction of red spot disease in *Anguilla japonica* with *P. anguilliseptica* and was able to induce the same clinical signs as those observed during disease outbreak by reinfection tests.

Pseudomonas fluorescens has been reported to cause haemorrhagic septicemia in European eel, *Anguilla vulgaris* (Andre *et al*, 1970), pond cultured tilapia, *Sarotherodon niloticus* (Miyashita, 1984; Miyazaki *et al*, 1984), yellowtail *Seriola quinqueradiata* (Kusuda, 1980) and cyprinid fishes (Bullock and Mc Laughlin, 1970; Shiose *et al*, 1972). Sakai *et al* (1989) isolated *P. fluorescens* from diseased rainbow trout *Onchorhinchus mykiss* in Japan and found the bacteria to be pathogenic to rainbow trout and tilapia (*S. niloticus*). Saeed *et al* (1987) isolated *P. putrefaciens* from diseased rabbit fish, *Siganus rivulatus* in Red sea. Pal and Pal (1986b) reported induction of ulcer in *A. testudineus* by mixed culture of two bacteria, one fluorescent Pseudomonad and another coccus, *Micrococcus varians*.

Several other workers (Boonyaratpalin,1989; Subhashinghe *et al*, 1990; Chattopadhyay *et al*, 1990; McGaray *et al*, 1991; Torres *et al* 1993; Cartwright *et al* 1994.) also reported the association of mainly *Aeromonas* and occassionally

Pseudomonas with epizootic ulcerative syndrome. Among Aeromonads, Karunasagar *et al* (1989) and McGaray *et al* (1991) had recovered *Aeromonas hydrophila* and *Aeromonas sobria* more often than other bacteria. Pal and Pradhan (1990) on the other hand found *Aeromonas caviae* and two other fluourescent Pseudomonads to be involved in EUS. This tends to indicate that *Aeromonas* sp. and *Pseudomonas* sp. are highly opportunistic pathogens which invade the fish once the skin barrier is breached. This however does not eliminate the fact that these bacteria are primary pathogens. In order to arrive at a conclusion on this aspect, a detailed study on the role of these bacteria in causing EUS is necessary.

In the present study, histological observations reveal severe hitopathological changes of the liver and kidney and at the injection site of *H. fossilis* experimentally infected by the two pathogenic Pseudomonads (R1 and R2) and the Aeromonad (R3) (section 4.1.2). A nectrotic granulomatous response in the dermis and cloudy degeneration of the muscle fibres was observed. The liver showed vacuolation, necrosis and infiltration of blood capillaries in some regions. The kidney showed hemorrhages and necrotic changes in certain hematopoietic regions along with tubular necrosis, tubular degeneration and haemosiderin accumulation. Bacteria were found to be present in the tissue sections from liver, kidney and from the ulcer region of inoculated fishes. Presence of bacteria were also detected in the histological sections of liver, kidney and ulcer tissues of the naturally infected fishes. The changes resembled the observations by previous workers who investigated histological changes in fishes experimentally induced by pathogenic strains of Aeromonad and Pseudomonad (Kumar *et al*, 1991; Pradhan, 1992; Pal and Pradhan, 1995; Prasad *et al*, 1995; Qureshi *et al*, 1995b).

The nature of histopathological changes has some similarities with the observations reported by various authors on histopathological changes caused by bacteria in infected fishes. Hepatic edema and liver congestion, glomerulities and atrophy of hematopoietic tissues in the kidney was observed by Miyazaki and Egusa (1977) in Japanese eel (*Anguilla japonica*) affected with red spot disease. Miyazaki (1980) observed degeneration of hepatic cells, necrosis and degeneration of epithelia of renal tubules and atrophy of renal hematopoietic

tissue, in *Anguilla japonica* infected by *Aeromonas hydrophila*. Miyazaki *et al* (1984) reported liver and kidney tissue necrosis in tilapia, *Sarotherodon niloticus* infected with *Pseudomonas fluorescens*. Later Miyazaki and Kaige (1985) found that on experimental induction of motile Aeromonad disease in crucian carps produced similar pathological signs as in the natural infections. The major observations were hemosiderosis in the liver, spleen and kidney and deposition of hematoidin crystals in spleen, kidney and blood vessels of diseased fishes. Regressive changes such as necrosis of the kidney and spleen tissues were seen after inoculation of healthy carps with pathogenic *Aeromonas* (Takahashi, 1984b). Chien and Chieh (1994) observed necrosis in the muscle, septic haemorrhage, spleen damage, fatty liver and renal hematopoietic tissue atrophy in eels after artificial infection by *A. hydrophila*. They suggested that the pathological changes were due to the proteolytic, haemolytic and cytotoxic attacks of liver and hematopoietic system by the *A. hydrophila* toxin.

During the present study, the histopathological changes observed in the ulcers indicate that bacterial multiplication takes place in the muscle. The haemorrhage, cellular degeneration and tissue necrosis in the skin, liver and kidney appear to be due to extracellular toxic substances secreted by these bacteria. Histological studies indicate toxemic changes that may be induced by extracellular bacterial toxins like haemolysin and proteases. The bacteria may enter through the injured skin and multiply in the adjoining muscle causing ulceration. From the muscle, the bacteria are disseminated to different organs such as liver and kidney through blood causing different pathological changes in these organs.

Aeromonas spp. is reported to produce a wide range of extracellular products including haemolysin, proteases, and cytotoxins. The major extracellular proteases produced by *Aeromonas hydrophila* which are also produced by some strains of *A. sobria* and *A. caviae* are a thermostable metalloprotease (TSMP) and a thermolabile serine protease (TLPS). Besides these there is another distinctly different protease produced only by *A. caviae* (Leung and Stevenson, 1988a). Extracellular toxic substances are also produced by *Pseudomonas aeruginosa* (Morihara, 1963; Snell *et al*, 1978;

Callahan III, 1974; Stapleton *et al*, 1984) and *Pseudomonas fluorescens* (Bullock and McLaughlin, 1970; Margesin and Schinner, 1992). These toxic substances produced necrosis, ulceration and haemorrhages (Diener *et al*, 1973; Kawaharajo *et al*, 1975a,b; Gray and Kreger, 1979). These previous findings and the histopathological effects produced by R1, R2 and R3 tend to indicate that R1, R2 and R3 may cause haemorrhage and considerable tissue damage due to the action of extracellular toxins.

A study on the virulence characteristics of these pathogenic bacteria was therefore necessary in order to explain the histopathological observations of experimentally and naturally infected fishes. It was also necessary to study the erythrocyte morphology, total erythrocyte count (TEC) and haemoglobin content of diseased fishes because haemorrhages were frequently noticed in the tissue sections of all artificially infected and also of the naturally infected fishes.

Experimental infection of *H. fossilis* with R1, R2 and R3 and the subsequent development and progress of ulcerative disease caused a decline in the Hb content and TEC of the diseased fishes (Figs. 21 and 22, section 4.1.3). Significant decrease ($P<0.01$) was observed after 48 hours of injection with the mixed bacterial suspension and after 72 hours of injection with R1, R2 and R3. This revealed anaemic condition in fishes. Disintegration of erythrocytes were observed in the blood smear preparations of all diseased fishes after 72 hours of injection. Reddish purple nuclei of disintegrated erythrocytes (nuclear shadow) were present in large numbers in the blood smear of fishes treated with mixed bacterial suspension. Presence of rod shaped bacteria in the blood of experimentally infected fishes indicated that after proliferation in the muscles the bacteria disseminated to other principal organs through blood.

Pradhan and Pal (1992) observed significant decrease in haemoglobin content and erythrocyte count in *Channa punctatus* after 24 and 48 hour of inoculation respectively with pure and mixed culture of the three pathogenic bacteria R1, R2 and R3. The nonpathogenic coccus (C) did not induce any change in Hb content or TEC. Das and Das (1993) observed that there was a decline in counts of erythrocytes (RBC) followed by a drop in haemoglobin

content and hematocrit values which indicated anaemic condition in the naturally infected *Cirrhinus mrigala*. Prasad and Qureshi (1995) reported remarkable changes in the number of RBCs and haemoglobin concentration in fishes (*Clarias batrachus*) affected with epizootic ulcerative syndrome. Pathiratne and Rajapakshe (1995) observed low TEC and haemoglobin content in EUS positive fish *Etroplus suratensis* and suggested that the anaemic condition resulted from loss of blood and destruction of erythrocytes.

Decrease in number of erythrocytes and haemoglobin concentration was also noted in case of other diseases caused by *Pseudomonas* sp. and *Aeromonas* sp. Takahashi and Kusuda (1979) observed that the erythrocyte count in induced scale protrusion disease by *Aeromonas liquefaciens* in coloured carp was decreased with lapse of time. They concluded that the cause of decrease in the number of erythrocytes was due to enormous disintegration of erythrocytes by the infection. Barham *et al* (1980) found that bacterial infection caused significant reduction in total erythrocyte count and haemoglobin concentration in rainbow trout, *Salmo gairdneri* Richardson. Takahashi (1984a,b) reported marked decrease in TEC and haemoglobin levels in experimentally infected carp treated with *A. hydrophila* and suggested that the erythrocytes were destroyed by haemolysin produced by *Aeromonas* entering the blood and there was a decrease in the haematopoetic function of kidney and spleen due to necrosis in these organs.

In this study, it was found that in experimentally infected fishes there were large number of disintegrated erythrocytes along with rod shaped bacteria. This tends to indicate that bacterial infection had caused bacteria to enter the blood and destroy the erythrocytes by their haemolytic toxins which was reflected in lowering of TEC and Hb content values. Haemopoiesis also seems to be affected because severe necrosis was observed in some areas of the haemopoietic region of the kidney. In the later stages of the disease, severe haemorrhagic ulcers also contribute to the loss of red blood cells.

During the present study, it was observed that all the three bacteria (R1, R2 and R3) exhibited extracellular proteolytic, haemolytic and lipolytic activity (Table 8, section 4.1.4). All the bacteria secreted β haemolysin as indicated by clear zones around bacterial colonies. Lecithinase was also secreted by all the

bacteria. However all the bacteria showed negative Voges Proskaur (VP) test. Karunasagar *et al* (1995) found that majority of the *Aeromonas* spp. isolated from internal organs of EUS affected fishes produced haemolysin and all of them were able to hydrolyse gelatin. Majority of the isolates, but not all showed a positive VP test. Motile *Aeromonas* sp. from fish which produces neither gas nor acetoin from glucose (negative VP test) had been considered as non or low virulent organisms (Eddy, 1960; Kou, 1973). In the present study the pathogenic Aeromonad (R3) did not show positive reaction for the VP test. The Pseudomonads and also *A. caviae* in general does not produce acetoin from glucose (Popoff, 1984) but has been reported to be pathogenic (Santos *et al*, 1988, 1996). Lack of relationship between VP positivity and pathogenicity was also reported by De Figueirdo and Plumb (1977) who studied the biochemical properties of nine *A. hydrophila* isolates of varying pathogenicity. Torres *et al* (1990) also did not find any correlation between the biochemical characteristics of virulent and nonvirulent strains of *Aeromonas* spp. isolated from healthy and diseased fishes affected by the epizootic ulcerative syndrome. Wakabayashi *et al* (1981) found that almost all strains of *Aeromonas* studied by him were able to produce haemolysin, protease, lipase and lecithinase and showed positive VP test. He found no clear difference in biochemical properties between the virulent and avirulent strains. Santos *et al* (1988) reported that more than 96% of the 59 *Aeromonas* strains tested (both avirulent and virulent) were proteolytic and amylolytic. Regardless of the species considered, a large number of isolates (81.3%) also produced lipase. He also did not observe any particular virulence determinant which could be considered to be an absolute criteria of pathogenicity in environmental motile *Aeromonas* sp. Chabot and Thune (1991) purified three different proteases from the extracellular products of *A. hydrophila*, but found no correlation between quantitative protease production and virulence by comparative studies involving some other pathogenic and non pathogenic strains of *A. hydrophila*. Apart from producing extracellular toxic enzymes, other virulence determinants like siderophore production (Crosa, 1984; Gierer *et al*, 1992), presence of S-layer (Dooley and Trust, 1988), serum

resistance (Mittal *et al*, 1980; Leung and Stevenson, 1988b; Leung *et al*, 1995), agglutination in Acriflavin (Mittal *et al*, 1980), autoagglutination (Mittal *et al*, 1980) and cytotoxicity (Leung *et al*, 1996; Shome *et al*, 1996; Santos *et al*, 1996) were also reported to be involved in the pathogenicity of *Aeromonas* and *Pseudomonas* strains in fish.

From the study of virulence characters of R1, R2 and R3, it is clear that these pathogenic bacteria secrete a number of lytic enzymes which may have important roles in manifestation of the symptoms of the ulcerative disease of fishes.

Though there is no clear correlation between extracellular enzyme production and virulence of the fish pathogenic bacteria, various workers have isolated and characterized proteases (Thune *et al*, 1986; Kanai and Wakabayashi, 1984; Leung and Stevenson, 1988a,b) and haemolysins (Allan and Stevenson, 1981; Kanai and Takagi, 1986) which they found to be involved in the virulence of *Aeromonas* spp. *Pseudomonas* spp. are also reported to produce protease (Morihara, 1963; Morihara and Tsuzuki, 1977; Snell *et al*, 1978) and haemolysin (Berk, 1964; Altenbern, 1966; Vasil *et al*, 1982). In other fish pathogenic bacteria such as *Aeromonas salmonicida* (Nielson *et al*, 1994) and *Vibrio anguillarum* (Inamura *et al*, 1985) extracellular proteases and haemolysins have been reported to be associated with disease production.

In the present study, the culture filtrates of R1, R2 and R3 were capable of inducing ulcers in healthy *A. testudineus* (section 4.1.5.1). This showed that the virulence factors of these bacteria are constituents of the extracellular products. The culture filtrate initially produced the typical reddish swelling at the site of injection but the ulcer remained superficial to moderate and did not progress to the severe stage. A higher dose of culture filtrate may be required to produce more acute ulcers which was not attempted in this study.

The extracellular virulence factors of R1, R2 and R3 were separated out from the crude culture filtrate by filtration through a short sephadex G-200 column (section 4.1.5.2, Fig. 25, 26 and 27). Two peaks were obtained in all cases of which the pooled fractions of the first peak was non toxic while that of the second peak was found to be toxic when injected to healthy *Anabas*

testudineus. The toxic fraction showed both haemolytic and proteolytic activity. The recovery of proteolytic activity was about 35% to 39%. The haemolytic activity was mostly lost during purification and only 12 to 15% of the activity could be recovered.

Among the three pathogenic isolates, R3 was of particular interest because firstly, *Aeromonas caviae* was not as frequently isolated as *A. hydrophila or A. sobria* and secondly, there was no other report of isolation of such highly virulent strain of *Aeromonas caviae* from EUS affected fishes which was capable of inducing ulcer in healthy fishes. Almost all studies on motile *Aeromonas* spp. was found to be concentrated on *A. hydrophila* since this was the most well known fish pathogen around the world. Occasionally some strains of *A. sobria* had also been included mainly in comparative studies among motile *Aeromonas* strains but a reference to *A. caviae* was found to be very rare (Santos *et al*, 1996). The bacteria which was mostly reported to be involved in EUS also was *A. hydrophila*. Karunasagar *et al* (1995) however, reported association of pathogenic *A. sobria* along with *A. hydrophila* in the EUS affected fishes. Subasinghe *et al* (1990) reported occasional occurrence of *Pseudomonas fluorescens* in the haemorrhagic lesions of EUS affected fishes while *A. hydrophila* was consistently isolated. Though there are many reports of isolation of pathogenic bacteria from EUS affected fishes, very little experimental work has been done so far (Yadav *et al*, 1992, Sahu *et al*, 1996) on the factors that may be involved in the pathogenicity of the virulent bacteria such as haemolysins and proteases although such works have been reported with pathogenic isolates from other disease. (Inamura *et al*, 1985; Nielson *et al*, 1994).

Among the motile Aeromonads, the extracellular haemolysins from *Aeromonas hydrophila* has been studied extensively. Wretlind *et al* (1973) observed that the haemolytic activity of *A. hydrophila* was synthesized during the late logarithmic phase of growth and released into the medium by lysis. Bernheimer and Avigad (1974) purified an extracellular haemolytic toxin of *A. hydrophila* by salt fractionation, dialysis and gel filtration with a yield of 24% of the starting activity. A study on the properties of this toxin showed that it

considerably resembled the exotoxin of *Pseudomonas aeruginosa* and the authors suggested that they may be members of a new class of exotoxins of the gram negative bacteria.

Allan and Stevenson (1981) purified protease and haemolysin of *A. hydrophila* from the extracellular products collected on the cellulose dialysis membrane discs placed on the agar cultures in petridishes. He reported a 70% recovery of protease activity by cold acetone precipitation compared to a 7% recovery by 30 to 50% ammonium sulphate precipitation. He separated the haemolysin and protease by chromatography on sephadex G-200 column and observed close correlation between the haemolytic activity and toxicity to fish. The protease deficient mutants were on the other hand capable of producing pathological changes and death in fish which implicated haemolysin as the lethal factor. He further observed that some common pathogenic mechanism was involved in both motile Aeromonad disease and furunculosis caused by *A. salmonicida.* Ljungh *et al* (1981) observed that both α and β type haemolysins are produced by *Aeromonas hydrophila in vitro.*

Kanai and Wakabayashi (1984) demonstrated that purified protease preparation from extracellular products of *A. hydrophila* possessed lethal effect to carp. Injecting the enzyme intramuscularly caused local haemorrhage and necrosis in carp and guinea pig. He also reported similarities in his observation with that observed in case of *A. salmonicida* and *P. aeruginosa.* In his purification procedure, he recovered 82% of the enzyme activity after 100% ammonium sulphate precipitation of culture filtrate and after several other purification steps the final yield was 35%.

Thune *et al* (1986) described a single step procedure for the purification of B-haemolysin from *A. hydrophila* which recovered 94% of the haemolytic activity with a six fold increase in specific activity. He concentrated extracellular products of culture supernatant by ultrafiltration and purified the B-haemolysin by chromatofocusing technique. He however suggested that proteases rather than the haemolysin, despite its toxicity, may be the significant virulence factor in *A. hydrophila* infection in channel catfish.

Nieto and Ellis (1986) detected the presence of at least 4 to 5 heat stable proteases of *A. hydrophila* by isoelectric focusing and suggested that significant differences in the character of extracellular products and extracellular proteases exist between different isolates of *A. hydrophila*.

Kanai and Takagi (1986) partially purified the toxic substance of *A. hydrophila* produced *in vivo* from necrotized muscle extracts by ammonium sulphate precipitation, ion exchange chromatography and gel filtration. Crude toxins from culture supernatants and sonicated cell extracts produced *in vitro* were also studied. Results showed that haemolysin was implicated in the toxicity and the mode of haemolysis was α-type.

Leung and Stevenson (1988a) purified extracellular proteases of *A. hydrophila* by ion exchange chromatography and recovered 10% of the original proteolytic activity with a purification of 26 fold. He also studied the characteristics and distribution of the proteases of *A. hydrophila* and compared them serologically with other Aeromonads. He observed that the major extracellular proteases of *A. hydrophila* NRC 505 and ATCC 7966 were a thermostable metalloprotease (TSMP) and a thermolabile serine protease (TLSP) respectively which were antigenically distinct. Serological reactions between the antisera against the two purified proteases and different *A. hydrophila* strains and also 29 other Aeromonads of which 9 were *A. caviae*, showed that six strains of *A. caviae* did not exhibit extracellular protease activity. One strain however, produced both enzymes. Two other strains did not react with either antisera, but produced proteolytic activity in ECP that was moderately EDTA resistant, PMSF resistant and heat stable. He suggested this to be a third protease enzyme which is highly significant in strains of *A. caviae*. In another study (Leung and Stevenson, 1988b), the authors used Tn5 mutagenesis in order to produce protease deficient strains of *A. hydrophila* which lost its pathogenic properties after mutation. The mutants were not deficient in haemolysin and its surface characters were retained. They also observed that some characters of this protease deficient strain were similar to Tn5 induced protease deficient strains of *P. aeruginosa*.

Santos *et al* (1988) observed that none of the virulence determinants studied could be considered as absolute criterion of pathogenicity in motile *Aeromonas* spp. since some pathogens lack the virulence properties which by contrast were found in non pathogenic strains. He suggested that haemolysins and proteases could be a part of several factors determining pathogenicity but were not required for virulence in all *Aeromonas* sp. However, it was clear that all the virulent strains produced an extracellular heat labile substance which was lethal for fish.

Pseudomonas sp. is also known to produce a variety of extracellular substance which may contribute substantially to the virulence of the bacteria. Morihara (1963) purified and crystallized an extracellular protease from *P. aeruginosa* by DEAE cellulose column chromatography. Berk (1964) partially purified a haemolytic toxin from the extracellular substance of *P. aeruginosa.*

Callahan (1974) purified a heat labile exotoxin from *P. aeruginosa* by a series of processes including membrane ultrafiltration, hydroxylapatite chromatography, ion exchange cellulose chromatography and gel filtration. He recovered 48% of the exotoxin with a 40 fold increase in specific activity. Various other workers have also purified similar toxins from *Pseudomonas* sp. (Liu *et al*, 1973; Jensen *et al*, 1980).

In the present study, the partially purified lethal factor possessed haemolytic and proteolytic activity. While the haemolysin may cause lysis of fish erythrocytes leading to a lowering in the total erythrocyte count and anaemia, the protease may support *in vivo* proliferation by making available nutrients in the form of amino acids by digestion of tissues in the host environment. Protease and other lytic enzymes may be responsible for the tissue damages that were observed in the skin, liver and kidney. The haemolysin and protease might play a role in the virulence, since the haemolysin and protease produced by all the three bacteria possessed similar specific activities which may be reflected in their similar LD_{50} values. The severe effect in mixed infections may be due to the cumulative effect of the three bacteria.

However, though the toxic principle which was responsible for the pathogenicity of the bacteria in fishes was detected in the culture filtrates, it is essential to note that this toxicity has been produced when grown *in vitro* and

not *in vivo*. There is a possibility that some factors may be produced *in vivo* but may not be produced under *in vitro* conditions. Kanai and Takagi (1986) partially purified the toxic substance of *A. hydrophila* produced *in vivo* from necrotized muscle extracts. Crude toxins from culture supernatants and sonicated cell extracts produced *in vitro* were also studied. Results showed that *in vivo* haemolysis was of α type and β type haemolysin was produced *in vitro*. Ellis *et al* (1988) observed that in case of *A. salmonicida*, high virulence of a strain depended on its ability to produce protease positive variants *in vivo*. Ellis (1991) pointed out that based on the factors produced *in vitro*, a picture of the virulence factors of the pathogen may be obtained but a complete understanding of the pathogenicity may not be possible.

Thus it may be concluded that the presence of protease and haemolysin in the toxic fraction of the culture filtrate can be related with the development of the typical ulcerative lesion and pathological changes in the blood and internal organs like liver and kidney associated with epizootic ulcerative syndrome, and may cause speedy mortality. A study on the toxins secreted *in vivo* is necessary to conclusively comment on the exact role of the bacteria in causing mortality in fishes.

During the present study, 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. Histopathological observation of tissues from naturally infected fish, *H. fossilis* showed loss of epidermis, severe granulomatous changes, blood capillary infiltration and muscle necrosis in the sections from the skin ulcers of affected fishes. 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. Presence of bacteria was detected in all the histological sections of liver, kidney and ulcer tissues. No structures like fungal mycelia was detected in the sections of ulcers barring only one fish (*Clarias batrachus*) from where the fungus was isolated. Blood smear preparation showed erythrocyte disintegration and presence of bacteria.

The nature of histopathological changes has some similarities with the observations reported by some other workers. Kumar *et al* (1991) found that there was complete loss of the epidermis of the skin at the ulcerated areas where dermis and hypodermis showed characteristic cyst like or nodule like granulomatous formations in abundant numbers. Granuloma formations were also seen in the muscle layers of the skin which were degenerated and necrosed in many areas. Kidney haematopoietic tissue showed proliferation of macrophage cells especially indicating inflammatory reactions. In the liver, most of the sinusoidal spaces and blood vessels were congested (Hyperaemic) and wandering lymphocytes were plenty in the liver parenchyma. Das *et al* (1990) however did not find any significant changes in the liver except vacuolization in certain cases. Prasad and Qureshi (1995) observed that the epidermis of the skin of infected *Clarias batrachus* were completely destroyed and wherever it existed it showed spongiosis. The dermis lost its original compactness due to necrosis. The hypodermis appeared in the form of a network of thin fibrils.

The histopathological observations in the present study however differed with some other workers who found fungal hyphae to be associated with the severe necrosis of the dermis and epidermis (Mohan and Shankar, 1995). Histopathological observations by Cruz-Lacierda and Shariff (1996) of EUS positive snakehead *Ophiocephalus striatus* showed that all stages of the disease exhibited chronic, necrotic and mycotic granulomatous response. They reported association of a highly invasive, broad (upto 24.6 μm in diameter), branching aseptate fungal hyphae with all stages. The internal organs of fish with advanced stages of EUS also exhibited mycotic granulomatous response. Viswanath *et al* (1997) observed the presence of fungal hyphae in the sections from ulcers of EUS positive fishes stained by Grocott's methanamine silver staining (GMS) technique. The ulcers in the advanced stage showed mycotic granulomatous response and massive necrotic changes associated with fungal invasion in the integument and skeletal musculature.

It has been observed that the nature of ulcers in fishes collected from natural sources is comparable to that induced by a mixed culture of the three pathogenic bacteria R1, R2 and R3. None of these bacteria individually could produce ulcers of such severity. This was confirmed by histopathological examinations of the ulcerative region along with internal organs like liver and kidney of the experimentally and naturally infected *H. fossilis* fishes. Similarities were also noted in blood smear preparations from naturally and experimentally infected fishes. The manifestation of similar pathological signs in the ulcer, liver and kidney of naturally infected fishes and fishes experimentally infected with a mixed culture of the three bacteria R1, R2 and R3 implies that this ulcerative disease has a complex etiology involving more than one etiological agent which act in concert to bring about heavy losses in an EUS outbreak.

Among the bacteria that were isolated in this study (section 4.2.3) from the external lesion of EUS positive fishes of South Bengal, the morphological features and biochemical profile of P01, P02, P03, P04, P05 and P06 reveal that these bacteria were gram negative rods, motile, catalase positive, oxidase positive non spore forming, attacked glucose oxidatively and except P01, all produced a yellow-green pigment which diffused into the medium when grown in medium B of King. (Tables 13-19). Thus they belonged to the genus *Pseudomonas* (Stanier *et al*, 1966; Palleroni, 1984). P01 satisfied most of the characteristics of *Pseudomonas fluorescens* but it produced a reddish brown pigment instead of the usual yellow-green pigment and was capable of growing at 42^{0}C. So it was not regarded as *P. fluorescens*. Some strains of *Pseudomonas aeruginosa* produces a dark red (Palleroni, 1984) or brown (Cowan and Steel, 1993) pigment and this bacteria is also capable of growing at 42^{0}C. However, P01 differed from *Pseudomonas aeruginosa* in that it was capable of producing acid from sucrose, sorbitol and m-inositol. Ajellow and Hoadley (1976) have reported a fluorescent Pseudomonad capable of growing at 41^{0}C but distinct from *P. aeruginosa*. Pal and Pal (1986a) reported isolation of a fluorescent Pseudomonad from epithelial carcinoma of *Anabas testudineus* which had similarities with *P. fluorescens* but was capable of growing at 42^{0}C. The biochemical tests that were done did not give any clue as to which species

P01 may belong. Thus a further detailed study is awaited in order to decide its taxonomic status and nomenclature.

Pigment production, inability to grow at 41^{0}C and acid production from sucrose indicate that P02, P03, P04 and P06 resembled *P. fluorescens*. Denitrification ability, inability to produce levan from sucrose and acid from sorbitol and m-inositol suggested that P02 belonged to biovar III. However, this strain differed from biovar III in that it was able to produce acid from sucrose.

P03 and P04 could not reduce nitrate and was unable to produce acid from adonitol. The overall biochemical characters were similar to *P. fluorescens* biovar V. All reactions of P03 and P04 were similar except that P03 could not produce acid from m-inositol.

P05 like P01 was also capable of growing at 42^{0}C but unlike P01, it was unable to produce acid from sucrose, sorbitol, m-inositol and adonitol. Thus it resembled *P. aeruginosa* (Palleroni, 1984) but it did not produce pyocyanin in King's A medium. The biochemical characteristics of P05 resembled R2 but it was found to be non pathogenic.

The biochemical profile of P06 indicated that it had similarities with biovar II and IV of *P. fluorescens* but by the tests that were done, it was not clear that among the two biovars, exactly to which it belonged. Thus a more detailed characterization is required in order to specify its exact taxonomic status.

Isolates A01, A02, A03, A04, A05 and A06 were gram negative straight rods, motile, catalase positive oxidase positive, indole positive, reduced nitrate to nitrite and attacked glucose by fermentation. Thus they belong to the genus *Aeromonas* (Popoff, 1984). They were not considered to belong to the genus *Vibrio* because all the isolates produced gas from glucose. Species of *Vibrio* except *V. fluvialis* biotype II and *V. gazogenes* do not produce gas from glucose (Baumann *et al*, 1984) *V. fluvialis* biotype II are curved rods and gives a negative indole test, while *V. gazogenes* produces a red pigment and gives a negative oxidase test. Morphologically they were distinct from *Aeromonas salmonicida* in that all of them were motile straight rods mostly in singles and some in pairs and no chains or clumps of coccobacilli as shown by *A.*

salmonicida were observed. Moreover, all the isolates were capable of growing at 37°C while none of the strains of *A. salmonicida* can grow at this temperature.

Among the *Aeromonas* strains isolated, A01, A03, A04 and A05 phenotypically resembled *Aeromonas hydrophila*, since it produced gas from glucose, H_2S from cystein, gave a positive VP test and produced acid from L-arabinose (Popoff, 1984). A02 showed a negative VP reaction and also did not produce acid from L-arabinose. However, it was capable of producing gas from glucose and H_2S from cystein. Hence A02 phenotypically resembled *A. sobria*. A06 also phenotypically resembled *A. sobria*, but it differed from A02 in that it showed a positive VP reaction.

The isolate V01 was gram negative straight rods, motile, oxidase positive, catalase positive, did not produce gas from glucose and attacked glucose by fermentation. The overall biochemical profile showed that the bacteria belonged to the genus *Vibrio*. It was not considered to belong to the motile *Aeromonas* group because it gave a negative indole test and also did not produce arginine hydrolase in Thornley's media.

The morphological features and biochemical tests showed that the isolate M01 belonged to the genus *Moraxella* (Bøvre, 1984a). The bacteria were gram negative short rods, non motile, occurring mostly in pairs and some in short chains. The isolate was both oxidase and catalase positive which distinguished it from *Acinetobacter* and *Kingella*, which are two other genus of the family Neisseriaceae (Bøvre, 1984b). The genus *Neisseria* is also catalase and oxidase positive but morphologically, they are coccoid (Vedros, 1984).

Morphological and biochemical tests show that the isolate B01 belongs to the genus *Bacillus*. The bacteria were motile gram positive rods, catalase positive, oxidase positive, produced endospores and attacked glucose by oxidation. The spore was in the central position of the bacterium and the sporangium was not swollen. It differed from other endospore forming bacteria belonging to other genera in that it was both catalase and oxidase positive. (Claus and Berkeley, 1986). Differentiation between species in the three genera, *Bacillus, Moraxella* and *Vibrio* was not attempted.

The morphological and biochemical characteristics of the isolate C01 showed that the bacteria was coccoid, gram positive, non motile, catalase positive, oxidase positive and attacked glucose by oxidation (Kocur, 1986a). Thus it belonged to the genus *Micrococcus*. It differed from the genus *Staphylococcus* in that unlike *Staphylococcus* which ferments glucose anaerobically, the isolate C01 attacked glucose oxidatively. C01 was not considered to belong to the genus *Planococcus*, which is another member of the family Micrococcaceae because unlike *Planococcus* which was motile with one or two flagella, C01 was non motile (Kocur, 1986b). Since C01 produces a yellow pigment and was able to grow on Simmons citrate agar, it was similar to *Micrococcus varians*.

Among other microorganisms, a fungus was observed in the lesion of *C. batrachus* (section 4.2.4). Morphologically it was aseptate and moderately wide with branchings. However, in culture, it became thin and delicate and its overall maintenance was very difficult. Various workers (Roberts *et al*, 1993; Willoughby *et al*, 1995) have emphasized the association of a highly invasive oomycetic fungus in the lesions of epizootic ulcerative syndrome affected fishes. It seems likely that the fungus isolated during this study was almost similar to that described by Roberts *et al* (1993) and Willoughby *et al* (1995). It's growth temperature also matched that of the *Aphanomyces invaderis* observed by Roberts *et al* (1993). The fungus died if kept at 32^{0}C for a long period.

Though this was only a single case where *Aphanomyces* sp. was observed, it is still possible that this fungus is associated with EUS. This is because, the time of isolation of this fungus was during early January which is the coldest period of the year in the northern part of West Bengal (North Bengal) when temperature normally varies between 8^{0} to 20^{0}C. The isolation of bacteria from fishes in South Bengal was during early December when the temperature just begins to fall and at this time it normally varies between 16^{0}C to 32^{0}C in this region. Moreover, the summer temperature in some regions of South Bengal including the area from which the diseased fishes were collected may reach as high as 41^{0}C when there is little possibility of this fungus to survive within the fish body (Chinabut, 1995). But in North Bengal, the summer temperature is

usually around 30 to 35^{0}C and it very rarely goes above 35^{0}C. Thus North Bengal is a better habitat for the fungus to survive and this may explain its presence only in fishes of North Bengal. However, this is only a preliminary indication of the association of fungal elements with EUS and a more detailed study on a wider geographical expansion with more fishes remains to be done. However, the non association of fungus in fishes from warmer climates indicates that A*phanomyces* sp. is also a secondary pathogen and not the initiating factor behind the outbreak of the disease. Srivastava (1979) reported *Aphanomyces* infection in *C. batrachus* in the state of Uttar Pradesh, India, in 1974, long before the outbreak of EUS in this country. Qureshi *et al* (1995a) isolated seven species of fungi belonging to the genera *Saprolegnia*, *Achlya* and *Aphanomyces* from the lesions of EUS affected fishes. Chinabut *et al* (1995) observed that since the epizootic ulcerative syndrome related *Aphanomyces* sp. is pathogenic at low temperatures, this can only explain the lack of infection other than the winter season but does not resolve the problem of persistence of infection from year to year. The hyphae and the spores of the fungus do not survive in water temperatures over 31^{0}C for any length of time which rules out the possibility of carrier fish holding the infection over the period.

In tropical Asia, especially in countries like India, water temperature regularly reach upto 33^0 to 36^{0}C for some time each year. So it is very much unlikely that this delicate strain of *Aphanomyces* sp. can at all survive in tropical Indian waters and therefore cannot be thought as the cause of the ulcerative disease outbreaks in India.

During the present study, *Pseudomonas* and *Aeromonas* were the most predominant bacteria isolated from the diseased fishes. Bacteria belonging to other genera were also present but they seem to be unrelated to the disease. Only strains of *Pseudomonas* sp. and *Aeromonas* sp. were found to be pathogenic. Qureshi *et al*, (1995b) also found *Aeromonas* sp. and *Pseudomonas* sp. to be pathogenic among nine types of bacteria isolated from EUS positive fishes. In the present study, four of the six strains of *Aeromonas* isolated resembled *A. hydrophila* and two strains resembled *A. sobria*. Two strains resembling *A. sobria* were found to induce ulcers in healthy *C. punctatus* and *H. fossilis*. Two strains of *A. hydrophila* ere also found to induce ulcers.

Among the Pseudomonads, the two strains which were found to be pathogenic resembled *P. fluorescens*.

There is some difference in the types of bacteria isolated from different fish species. While among the six isolates from *C. punctatus*, four were Pseudomonads and one Aeromonad; one Pseudomonad and three Aeromonads were isolated from ulcers of *Puntius* sp. Among the bacteria present in the ulcers of *Mystus* sp., two were identified as *Aeromonas* sp. and one was identified as *Pseudomonas* sp. While Pseudomonads and Aeromonads were consistently isolated from all fishes, there were variations in other types of bacteria that were present at the ulcer site. Beside Pseudomonads and Aeromonads, *Bacillus* sp. was the only other bacteria that was isolated from *C. punctatus* while *Micrococcus* sp. was the only other bacteria that was isolated from *Puntius* sp. In *Mystus* sp. one strain of *Moraxella* sp. and one strain of *Vibrio* sp. were isolated from the skin lesion besides *Aeromonas* and *Pseudomonas*. This wide difference may be explained by the fact that barring *Pseudomonas* and *Aeromonas*, the other bacteria reflects the microflora of the environment and bears no relationship with the disease. These explanation is supported by the non pathogenicity of these bacteria. Other workers (Shewan, 1961; Campbell and Buswell, 1983) have also reported that fish microflora is affected by the environment.

One pathogenic Pseudomonad and one pathogenic Aeromonad each were isolated from *C. punctatus* and *Mystus* sp. These bacteria, however, differed phenotypically from each other. While A01, isolated from *C. punctatus* resembled *A. hydrophila*, A07 isolated from *Mystus sp.* resembled *Aeromonas sobria*. These findings were similar to those observed by Karunasagar *et al* (1995) who isolated both *A. hydrophila* and *A. sobria* from internal organs and external lesions of EUS affected fishes. They found *A. sobria* to be the more common species both in ulcers and internal organs of the diseased fishes. They also found *A. hydrophila* and *A. sobria* to be present in the internal organs of fishes during the assymptomatic stage of the disease. However, due to lack of homogeneity in the phenotypic characters and LD_{50} values among these isolates, they suggested that the role of *Aeromonas* sp. in the outbreak may be

secondary in nature. However, these results differ from Torres *et al* (1993) who found *A. hydrophila* to be the only pathogenic (both highly virulent and weakly virulent) bacteria. Subasinghe *et al* (1990) and Llobrera and Gacutan (1987) also found *A. hydrophila* to be consistently associated with the ulcers.

Among the Pseudomonads, though both P02 isolated from *Channa punctatus* and *Mystus* sp. resembled *P. fluorescens*, they differed in their biochemical characters. While P02 resembled biovar III of *P. fluorescens*, P06 was similar to biovar II and IV . On the other hand, no pathogenic *Pseudomonas* was isolated from ulcers of *Puntius* sp. Both the pathogenic bacteria belonged to *Aeromonas* sp. among which A02 resembled *A. sobria* and A03 resembled *A. hydrophila*. Thus the inconsistency in the bacterial isolates from different fish species is quite distinct. This tends to suggest that these are opportunistic pathogens which have invaded the fish secondarily. Another notable feature is that *A. caviae* (R3) which was isolated by Pal and Pradhan (1990) from EUS affected fishes of North Bengal and was thought to play an initiating role in the disease (Pradhan, 1992) was not at all detected in diseased fishes from South Bengal. Moreover, the virulence of all the present isolates were much less than R1, R2 and R3.

The complex nature of epizootic ulcerative syndrome which involves multiple pathogens makes it difficult to control the disease by the development of suitable vaccines. Application of antibiotics provides an easy alternate to control the spread of the disease effectively.

The results of drug disc diffusion study of the pathogenic bacteria i.e., R1, R2 and R3 responsible for inducing ulcers in fishes along with other pathogenic Pseudomonad and Aeromonad isolates showed that all these bacteria were highly susceptible to the antibiotic oxytetracycline (section 4.2.6, Tables 22-25). Moreover, 94.74% of all the bacteria tested were found to be sensitive to oxytetracycline (Table 27). The low MIC values (3·2 μg/ml for R1 and R2 and 6·4 μg/ml for R3) also confirmed that oxytetracycline would be suitable for the treatment of ulcers induced by R1, R2 and R3. This was an important finding considering that oxytetracycline resistant strains of *Aeromonas* and *Pseudomonas* were reported from different countries around the world. It

was reported from Scotland (Inglis *et al*, 1991), France, UK, Ireland (Hedges *et al*, 1985), Thailand (Aoki *et al*, 1990), Nigeria (Okpokwasili and Okpokwasili, 1994), China (Bai, 1995), Japan (Aoki, 1977), USA (Shotts *et al*, 1976) and recently and most alarmingly from adjoining Bangladesh (Chowdhury and Inglis, 1995).

Inglis *et al* (1991) found that out of 304 *Aeromonas salmonicida* strains that were isolated from fishes suffering from furunculosis, 55% were resistant to oxytetracycline. Okpokwasili and Okpokwasili (1994) observed that about 23 *A. hydrophila* strains isolated from tilapia with brown patch disease showed resistant phenotypes of AmCt x SuTeCz indicating their multiple resistance to ampicillin, colistin sulphate, sulphonamide, tetracycline and cotrimoxazole. The 10 Pseudomonad isolates were resistant only to ampicillin. Aoki (1992) reported an overall increase of drug resistant pathogenic bacteria. Bai (1995) found that out of 55 isolates of pathogenic *A. hydrophila* from catfish, 24% demonstrated high resistance towards oxytetracycline (MIC $\geq$ 250 mg/l). In many cases transferable R plasmids were detected in the drug resistant bacterial strains of *A. hydrophila* (Akashi and Aoki, 1986) and *Pseudomonas fluorescens* (Aoki *et al*, 1977). *Pseudomonas aeruginosa* has also been reported to carry R plasmids (Levy, 1984). The most common type of R plasmids of *A. hydrophila* in Japan had markers resistant to sulphonamide, tetracycline and to chloramphenicol, sulfonamide and streptomycin (Aoki, 1992). During the present study, a particular strain of *Aeromonas* sp. (A04) isolated from the skin ulcer of *Puntius* sp. was found to be resistant to penicillin, ampicillin, erythromycin, gentamycin cotrimoxazole, oxytetracycline and amoxicillin (Table 26). All isolates were resistant to penicillin, ampicillin and erythromycin. In addition some strains were resistant to gentamycin and cotrimoxazole. Among the Pseudomonads, all were resistant to penicillin and ampicillin. Additionally some were resistant to either cotrimoxazole or erythromycin or both. Drug resistance were noted among all other isolates except *Vibrio* sp. (Table 26). However reports of drug resistance among *Vibrio* sp. is quite common. *Vibrio anguillarum* isolated from cultured ayu in Japan were resistant to as many as seven antibiotics (Zhao *et al*, 1992).

Vibrio sp. isolated from diseased specimens of *P. monodon* in Thailand were resistant to oxytetracycline (Chanratchakool *et al*, 1995).

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. Martinsen *et al* (1992) reported a wide range of MIC values of oxytetracycline against different strains of *A. salmonicida*, *V. anguillarum*, *V. salmonicida* and *Y. ruckeri*. The MIC values tested against 44 *A. salmonicida* subsp. *salmonicida* strains ranged from 0.08 to 192.00 μg/ml at 15^{0}C.

Considering the adverse effect of the antibiotic on the fish and the environment, oxytetracycline may be considered to be safer than others. It is one of the few antibiotics approved by the US Food and Drug Administration for use in the treatment of bacterial fish diseases. It is also used in various countries like Japan, Thailand, Indonesia, Norway, Australia, Philippines, Finland, U. K., Canada and Malaysia. Oxytetracycline has a very short half-life in both fresh water and sea water (Choo, 1994) and thus it poses very little danger to the aquatic environment. In the fish body, the antibiotic is rapidly eliminated from the system as soon as its application is withdrawn (Xu and Rodgers, 1994; Somsiri *et al*, 1996). Earlier workers have reported that antibiotics including oxytetracycline are immunosuppressive (Rijkers *et al*, 1980; Grondel and Boesten, 1982). Later, it has been demonstrated that oxytetracycline does not have long term effect on the immune response of fishes (Karunasagar *et al*, 1995; Ali *et al*, 1997). However, long term use of antibiotics may induce resistance in the target bacteria or in other bacteria present in the environment. Plasmid borne resistant determinants can be transferred to other susceptible bacteria which may render them resistant (Klein and Boehm, 1994). Hence it is emphasized that controlled use of antibiotics in the correct dose is very important. A dose lower than that required to kill the bacteria will not only be ineffective but also induce the development of resistance towards the antibiotic.

A higher dose on the other hand may be effective but will have adverse effect on the host and its environment.

During the present study, different concentrations of oxytetracycline were tried for the treatment through bath administration and the fishes were exposed to the antibiotic for different time periods (section 4.2.7). The results clearly showed that among the doses of oxytetracycline that were tried, some were capable of lowering the mortality rates of the infected fishes (Tables 28-31). The mortality rate of fishes exposed to the highest concentration (500 μg/ml) of the antibiotic for the highest time period tried (60 min) was 26.6% in fishes previously inoculated with a mixed bacterial suspension as compared to 80% in the untreated fishes (Table 31 and Figs. 52-67). Results of the present study showed that there was an immediate check in the mortality of fishes especially when higher concentration of the antibiotic was administered for longer periods (Figs. 52-67). Exposure to lower concentrations of the drug for longer periods will require antibiotic in lower quantity and thus reduce the price of application. However, long periods of exposure of the fishes in the antibiotic solution in the treatment tanks will increase the risk of stress on the fishes. Both these factors need to be considered when formulating the exact dose of the antibiotic. The present study gives an idea on the antibiotic sensitivity of R1, R2 and R3 and ensures that the treatment was efficacious at a particular dose. This will help in restricting the unnecessary application of high doses of antibiotics.

According to Jhingran (1990), total recovery was observed in EUS affected fishes where nalidixic acid and erythromycin at the rate of 50 mg per Kg body weight per day in a formulated micro encapsulated feed containing 30% protein and fortified with vitamin A and C was supplied for three days together with antibiotic bath (chloramphenicol @15 ppm). Purkait (1990) reported that antibiotics like terramycin @ 200 mg/ Kg feed had impede the disease within 1-2 weeks in some areas of Chanditala in Hooghly district, West Bengal. Jain (1990) reported that the use of antibiotics like erythromycin @ 200 mg/Kg supplementary feed had been observed to control the ulcerative disease in government farm at Kalyani, West Bengal, India. Mahapatra *et al* (1996) reported that antibiotic tablets (trimethoprim and sulphamethoxazole) were given to the infected fish mixed with the daily supplementary feed @ 100 mg per Kg

feed for 10 days which successfully controlled EUS in fish. Das (1997) reported that in general, antibiotic either erythromycin or oxytetracycline at 60 to 100 mg per Kg of feed for seven days cured the ulcers of the diseased fishes. Besides antibiotics, formulated drugs such as CIFAX developed by CIFA, Bhubaneswar, India, is reported to show encouraging results in controlling EUS. The drug applied at 1 litre per hectare-meter of water area with the notice of the symptoms of EUS in the pond is reported to cure affected fishes within 7 days (Das and Das, 1993). Reports of total recovery of the naturally affected epizootic ulcerative syndrome positive fishes by application of drugs and antibiotics which are primarily antibacterial strongly suggest bacteria to be involved in the development of the disease.

The method of application of antibiotics through the diet for treatment of fish diseases is relatively easier for treatment of large quantities of fish with minor labour cost and has thus become the most popular way of treatment. However, one of the first signs of the disease in fishes is reduction of appetite. Under such conditions, oral administration of antibiotics will create a situation where only the healthy fishes within a population that are still feeding shall be protected by the drug (Samuelson, 1997). Thus medicated feed application is more a prophylactic than a therapeutic measure (Kumar and Dey, 1991). In acute cases of the disease where most of the population is affected, most of the feed often remains untouched and falls to the bottom of the culture tank. If these are not removed, it will have an adverse impact on the environment. Moreover, there will be an wastage of large quantity of expensive drugs. By bath administration, all fishes are exposed to equal doses of the antibiotic.

Thus it may be concluded that the treatment of diseased fishes through bath administration of oxytetracycline will produce effective results. However, an exact formulation of the dose is possible only after field trials in naturally infected fishes which is awaited.