INTRODUCTION

Intensive and semi-intensive inland fish culture with the use of different species has become one of the important expanding practices in India. With the improved intensification of aquaculture, the problem of diseases caused by bacteria is also increasing. Bacterial diseases generally result in heavy mortality of fishes and consequently effect the economy of the fish farmers. The Indian major carps are highly nutritious and most prestigious of all fishes in India constituting nearly 90% of India’s total production. Among the Indian major carps, rohu (Labeo rohita) is most preferred species and constitute about 35% of the Indian major carps production (FAO, 2000). In India, majority of the fishes may be effected by Aeromonas and Pseudomonas groups of bacteria and from the diseased fish, Aeromonas liquefaciens and Aeromonas punctata have been isolated. Aeromonas hydrophila is causing the abdominal dropsy in the major carps (Kanai and Takagi, 1986). The disease caused by A. hydrophila was classified under four categories: acute form, chronic form, fatal septicemia and acute dropsy. In the Indian major carp, rohu, an acute septicemia due to A. hydrophila has been reported (Karunasagar et al., 1986 and 1989; Lakshmanan et al., 1986).

Aeromonas infections are regarded as the most common bacterial infections in fresh water fish. There are several species of Aeromonas which can infect fish. Aeromonas, a genus of facultatively anaerobic, gram-negative, rod shaped bacterium in the family pseudomonadaceae, usually resident in richly organic water and soil, and on fish species. Some strains of Aeromonas cause serious disease out breaks in a population of fish. One among the pathogenic species of Aeromonas is Aeromonas liquefaciens. This pathogenic bacterium may cause hemorrhagic septicemia, fin rot, tail and snout
erosions in cultured fish. Usually mortality rates are low and losses may occur after a period of three weeks or longer after the onset of disease. Fish become more susceptible to the pathogenic bacteria due to stress (poor water quality, over crowing, over feeding) or temperature disturbances. Fish suffering by *Aeromonas* infections show external symptoms like small, pin point haemorphages at the base of the fins or on the skin, distended abdomen and protruding eyes. Internal signs include fluid in the abdomen, swollen liver and spleen and fluid filled distended intestines (http://edis.ifas.uff.edu/fa042,2009). Aeromonads, the gram-negative bacteria are native to aquatic environments (Hazen et al., 1978). The pathogenic bacteria have been reported from fresh water, brackish and estuarine fish and freshwater and marine aquatic environments (Hazen et al., 1978; Kaper et al., 1981; Van der Kooj, 1988). Pathogenic aeromonads have also been reported from diseased cold and warm blooded animals (Mathewsu and Dupont, 1992). *Aeromonas hydrophila* has been isolated from a wide variety of pond water fish worldwide (Larsen and Jensen, 1977; Eurell et al., 1978).

The scientific classification of *Aeromonas liquefacenes* is:

- **Kingdom**: Bacteria
- **Phylum**: Proteobacteria
- **Class**: Delta Proteobacteria
- **Order**: Aeromonadales
- **Family**: Aeromonadaceae
- **Genus**: *Aeromonas*

*Aeromonas* infections are causing septicemia and great economic losses in fish culture worldwide (Holliman, 1993). In many cases, fish may resist the bacterial disease by developing resistance (Ansary et al., 1992; Pettibone et al., 1996; Son et al., 1997;
Haemorrhagic septicaemia is a common bacterial disease caused by *Aeromonas* species (Bullock *et al.*, 1971; Khardori and Fainstein, 1988). The stress-mediated disease, aeromoniasis is also described as a ulcerative disease of fish (Karunasagar *et al.*, 1995). The motile *Aeromonas* group shows great adaptability to different aquatic environments in the world (Mateos *et al.*, 1993). Aeromoniasis had been noticed in various fresh water and marine fish, reptiles, bovine animals and humans (Bullock *et al.*, 1971; Khardori and Fainstein, 1988). Several *A. hydrophila* strains had shown multiple antibiotic resistance in fresh water farms (Pettibone *et al.*, 1996 and Son *et al.*, 1997). However, little attention has been paid on the bacterial ecology of farmed fish (De Paola *et al.*, 1995). *Aeromonas* associated gastroenteritis was reported in children under two years of age as watery diarrhea of short duration in Western Australia (Gracey *et al.*, 1982).

Experimental infection with *A. hydrophila* resulted in an acute septicemic disease in *Mugil cephalus* (Soliman *et al.*, 1989). Fish like carps can ingest attached bacteria (Rahmatullah and Beveridge, 1993). Fish culture is an age-old practice in India and stand second in the world culture fish production (Chakrabarty and Chattopadhyay, 1998). One of the major bacterial fish pathogen in Indian aquaculture is *A. hydrophila* causing diseases such as haemorrhagic septicemia, infectious dropsy, tropical ulcerative disease and fin rot bringing heavy mortality in aquafarms (Kumar and Dey, 1988; Rath, 1993; Karunasagar *et al.*, 1997). A new viral disease, viremaia associated anaakibyo was found in combination with *A. hydrophila* infection in carp, *Cyprinus carpio* in Japan (Miyazaki *et al.*, 2000). *Aeromonas* species may cause severe out breaks among carp species in different fish hatcheries in Egypt (Ahmed and Shoreit, 2001). Bacterial haemorrhagic
ascites, a serious disease of ayu Plecoglossus altivelis is responsible for significant losses in Japan; the causative agent has been identified as Pseudomonas plecoglossicida (Nishimori et al., 2000). Pathogenic bacteria Pseudomonas species were experimentally used by various methods like immersion (Sukenda and Wakabayashi, 2000), intraperitoneal infection (Wakabayashi et al., 1996), intramuscular injection and oral administration (Park and Nakai, 2003) to induce disease. A. hydrophila was detected from the gill of carp species and cat fish suffering from Aeromonas septicemia (Gamal et al., 2002).

Aeromonas bacteria are the most common and trouble some pond fish pathogens widely distributed throughout the world (Ho et al., 1990). A. hydrophila was found in the vast majority of fish pathogens causing mortalities in aquaculture industry (Austin and Austin, 1993; Angkra et al., 1995; Das and Mukherjee, 1997). Aeromonas bacteria may cause haemorrhagic septicemia, epizootic ulcerative syndrome (EUS) and abdominal dropsy to fishes (Freriches, 1989; Das and Mukherjee, 1998). It has been well known that A. hydrophila is often associated with epizootic ulcerative syndrome in South East Asian countries (Roberts et al., 1986) and India (Karunsagar et al., 1986; Pal, 1996; Das and Mukherjee, 1997, 1998; Nayak et al., 1999). A. hydrophila infection may bring scale sac edema, ascities, exophthalmus and ulcers in diseased carps (Miyazaki et al., 2001). Aeromoniasis in Indian major carps poses one of the major threats in aquaculture. A wide genetic variation in resistance has been noted in L. rohita (Ham.) for A. hydrophila infection (Sahoo et al., 2004). Juveniles of Labeo rohita infected with A. hydrophila show degenerated hepatocytes, edema and leucocytic infiltration in parenchymatous tissues and extensive haemorrhages in the kidney (Gupta et al., 2008).
Occurrence of skin lesions with haemorrhages due to *A. hydrophila* and the effective antibiotic treatment was reported in a carp (*Cyprinus carpio*) hatchery farm in Turkey (Adanir and Turutoglu, 2007). In Turkey, *A. hydrophila* had been reported in Eel (*Auguilla anguilla*), grass carp (*Ctenopharyngodon idella*) and mirror carp (*Cyprinus carpio*) (Swan and White, 1989; Uzbilek and Yildiz, 2002; Guz and Kozinska, 2004; Yildiz et al., 2005).

Members of the genus *Aeromonas* are facultatively anaerobic and gram negative bacteria. Their natural habitat is the aquatic environment and some are pathogenic for animals and humans (Carnahen and Joseph, 2005). *A. liquefaciens* is the causative agent of the disease known as haemorrhagic septicemia. Species of *Aeromonas* are generally found in the gastrointestinal tract of fish and are considered as opportunistic pathogens (Swann and White, 1989; Yildiz et al., 2005). *Aeromonas* species are always capable of producing disease outbreak if fish are weakened by bad managerial practices.

Aeromoniasis is a serious disease causing heavy damage in pond and aquarium culture. The outbreaks of aeromoniasis have a major impact in aquaculture (Austin and Austin, 1993). Aeromonads are opportunistic pathogens infecting a wide variety of hosts (Huizinga et al., 1979; Yadav and Verma, 1998; Soltani et al., 1998). The pathogenesis of aeromoniasis, caused by *A. hydrophila* has been reported in the common carp (*Cyprinus carpio*) (Vivas et al., 2004), Channel cat fish (*Ictalurus punctatus*) (Galindo et al., 2004), Cultured eel (*Auguilla japonica*) (Hoshina, 1962), large mouth bass (*Micropterus salmonides*) and several other fish species (Miyazaki et al., 2001). Lesions, exophthalmus and cutaneous haemorrhages in the tail and anus were found in cultured ayu...
(Plecoglossus ultivelis) during *A. hydrophila* infection (Lipsitch, 2001; Jain and Wu, 2004). *A. hydrophila*, which is widely distributed in aquatic environment causes ulcers in fish (Stevenson, 1988; Torres *et al*., 1993; Gonzalez *et al*., 2002). The development of aeromoniasis by *A. hydrophila* in fish is due to the virulent factors (expressed by this bacterium) like haemolysins, proteases, cholinesterases, enterotoxins, endotoxins and adhesions (Torres *et al*., 1993; Gonzalez *et al*., 2002).

*A. hydrophila* is the causative agent of haemorrhagic septicemia or ulcer disease or red sore disease and is generally found in the gastrointestinal tract (Swann and White, 1989; Guz and Kozinska, 2004; Yildiz *et al*., 2005). Abnormal conditions of the pond environment like stress, over crowding, temperature fluctuations, poor water quality, high nitrite and carbondioxide levels and mishandling of fish are found to be associated with disease outbreaks (Dixon and Issvoran, 1993; Aoki, 1999; Cipriano, 2001; Lakshmanaperumalswamy *et al*., 2005; Yildiz *et al*., 2005). *A. hydrophila* has been reported in eels (*Anguilla anguilla*) and grass carp (*Ctenopharyngodon idella*) in Turkey (Uzbilek and Yildiz, 2002; Yildiz *et al*., 2005; Yucel *et al*., 2005). Adanir and Turutoglu (2007) isolated *A. hydrophila* from the skin, kidney, heart and liver of the carp (*Cyprinus carpio*) (in a hatchery form).

Liver and kidneys are the larger organs in an acute septicemia. The liver may become pale or have a greenish colouration while the kidney may become swollen and friable tissue damage occurs and these organs loose their integrity (Huizinga *et al*., 1979; Ventura and Grizzle, 1988 and Fuentes and Perez, 1998). Histopathologically, fish may exhibit haemorrhages on the gill, ulcers in the dermis and tissue damage in liver and kidney. Heart and spleen are not damaged. Bach *et al*. (1978) noted marked pathological
changes in the spleen of fish infected intramuscularly with varied doses of virulent *A. hydrophila*. Whereas fish received oral infection showed little or no spleenic involvement. In chronic aeromonial infections, both the dermis and epidermis are eroded and the underlying musculature becomes severely necrotic (Huizinga *et al*., 1979).

Fish are considered as the excellent experimental models for doing research on toxicology, infectious diseases etc., because the contaminants exert their impact on aquatic systems or organisms (Leblond and Hontela, 1999; Lacroix and Hontela, 2001; Law, 2003; Raisuddin and Lee, 2008; Parikh *et al*., 2010). Histopathological studies are recognized as biomarkers in the evaluation of the health of fish exposed to contaminants/pathogenic microbes/metazoan parasites, both in the laboratory (Wester and Canton, 1991; Thophon *et al*., 2003; Parikh *et al*., 2010) and field studies (Hinton and Lauren, 1993; Schwaiger *et al*., 1997; Teh *et al*., 1997). The histopathological studies on various target organs like gill, kidney and liver are useful to assess the damage to fish and animal health (Hinton and Lauren, 1993; Gernhofer *et al*., 2001). In all teleost species, both in normal and diseased fish, only the spleenic centres are normally involved in handling antigen (Agius, 1979). Despite the morphological similarity of the melano-macrophage centres of the kidney, spleen and liver, there are important functional differences between the centers of these three organs; when they were spleenectomized, that role was taken over by the kidney (Agius, 1981 and 1985). Thus, in the present investigation both the kidney and spleen were selected for histopathological studies.

Histopathological observations in diseased fish are useful to understand the causes of their mortality (Iwalokun *et al*., 2001). Histopathological reactions in cat fish, *Clarius butrachus* and *Salmo gairdneri* experimentally infected with *A. hydrophila* have been
documented by Candan (1990). In large mouth bass (M. salmonoides), A. hydrophila infection caused necrosis in liver, kidney and heart (Huizinga et al., 1979). The channel cat fish, Ictalurus punctatus showed large number of lesions during natural and artificial infections of A. hydrophila (Ventura and Grizzle, 1988). Bach et al., (1978) found marked destruction in splenic tissues of channel cat fish infected with A. hydrophila. Candan (1990) observed fragmentation of renal tissues and cellular infiltration in rainbow trout infected with A. hydrophila. Cyprinus carpio infected with viraemia developed skin ulcers (Moore et al., 1998). I. punctatus infected with A. hydrophila showed haemorrhagic septicemia and marked histopathological changes in gill – sloughing of respiratory epithelium (Miyazaki, 1985). Histopathological changes in A. hydrophila infected liver, kidney, pancreas and intestine were characterised by necrosis and haemorrhages in different fishes – Clarius bacteriachus, S. gairdneri and I. punctatus (Ventura and Grizzle, 1988; Candon, 1990). Ramasamy et al., 2009) injected goldfish (Carassius auratus) with A. hydrophila. The pathogen was injected intramuscularly into the experimental fish. Sloughing of scales at the site of infection with muscular haemorrhagic protuberance was recorded in test fish. Also, test fish showed ulcerative dermatitis linked with focal haemorrhaging, oedema and dermal necrosis exposing the underlying muscle. Muscle, gill, liver and heart were affected by the disease. Pathogenicity of the disease progressed in muscle, gill, liver and finally to the heart. After day 24 of infection, diseased fish exhibited fragmentation of muscle fibers with necrosis. In the early stages of infection, there were no external skin ulcers in diseased fish. Gill, liver and pancreas showed marked damage in channel cat fish infected with A. hydrophila complex (Grizzle and Kiryu, 1993).
In a preliminary study, the potential correlation of natural and acquired immune response with aeromoniasis was investigated in *L. rohita* under laboratory conditions. Bactericidal activity plays an important role in defense mechanism during aeromoniasis (Pramoda et al., 2004). Aqueous root extract of *Achyranthes aspera* was incorporated in the diet and fed to test groups of *L. rohita* (Rao et al., 2004), *Catla catla* (Rao and Chakrabarti, 2005) and *Cyprinus carpio* (Vasudeva Rao and Chakrabarti, 2005) and immunized with chicken RBC after 4 weeks. Antigen specific antibody, total serum globulin, DNA/RNA ratio of spleen and protein levels were found higher in all the test groups of fish than the control group on day 7 and 14. Pramoda et al. (2004) reported that the experimental infection of *L. rohita* through the intraperitoneal route of *A. hydrophila* produced mortality ranging from 0 to 100%. Significant positive correlation between serum bactericidal activity and aeromoniasis was observed.

Rao et al. (2004) fed *L. rohita* with a diet containing root extract (0.5%) of *Achyranthes aspera* as an ingredient (experimental) and a normal diet without the root extract (controls). After 4 weeks of feeding, fish were sensitized with chicken RBC. It was found that the antibody response, total serum globulin, RNA/DNA ratio of spleen and proteins increased on day 4 and 14 in experimental fish. Rao and Chakrabarti (2005) and Vasudeva Rao and Chakrabarti (2005) reported similar observations in *Catla catla* and *Cyprinus carpio* fed with a diet containing the seed extract of *A. aspera*. Vasudeva Rao et al. (2005) tested the efficacy of seed extract of *A. aspera* on *L. rohita*. Fish were fed with varied diets (containing 0.01%, 0.1% and 0.5% seed extract) for 2 weeks. After 2 weeks of feeding, fish were sensitized with heat killed *A. hydrophila* and after a further 2 weeks, the rohu were infected with pathogenic *A. hydrophila*; seven days after infection,
blood was collected and analyzed for some biochemical constituents. Superoxide anion production, serum bactericidal activity, lysozyme, ALP, serum proteins and albumin: globulin (A/G) ratio were enhanced in *Achryanthes* treated groups compared to the control group. Prevalence of pseudomonas infection was reported in Qaroun and El Rayan lakes in Egypt (Eissa *et al.*, 2010). The clinical picture of pseudomonas septicemia characterised by irregular hemorrhage all over the body surface and detachment of scales in Tilapia (*Oreochromis niloticus*). The toxic substances produced by *A. hydrophila* caused nephrosis, hepatocyte degeneration and cardiac hemorrhage in *Carrassius auratus* and *Cyprinus carpio* (Citarasu *et al.*, 2011). Major histopathologic findings of aeromoniais were observed in liver, kidney and heart tissue of *Nile tilapia* (*Oreochromis niloticus*) (Yardimci and Aydin, 2011).

*Labeo rohita* is one of the most important Indian major carp species used in small scale and intensive fish farming. Very little information is available on aeromoniais in Indian major carps. The clinical aspects like biochemical characteristics, total protein banding and histopathological observations from various organs of healthy and/or diseased fish have not been described clearly. Hence, a new vista has been opened to study the alteration in the level of total proteins and DNA from the muscle, gill, head kidney and brain, and total protein extraction and banding from muscle and head kidney of experimentally infected fish with varied doses of *A. liquefaciens*. Histopathological observations were extended to muscle, gill, head kidney and spleen of diseased and control fish. Total protein extraction and banding from muscle (on day 1 and 4) and head kidney (on day 1 and 4) and histopathology of muscle (day 3), gill (day 1), head kidney (day 9) and spleen (day 9) was made on few specific days of infection period from
various experimental and control groups of fish. The protein band alteration and/or the pathological changes in various organs may be a reaction to pathogens or an adaptive response to prevent the entry of pathogens through the muscle and gill. The response of lymphoid organs (head kidney and spleen) to infection/antigen may be somewhat delayed. Also, very little attention has been paid towards the histopathological changes of muscle on day 3, gill on day 1, head kidney and spleen on day 9 in rohu during aeromoniasis. Hence, in the present study, the above specific days have been selected to study the SDS-PAGE analysis of protein and histopathology.

The present work was designed to determine the following:

A. Level of total proteins and DNA from the muscle, gill, head kidney and brain of fish infected with a single dose of $10^{-2}$ CPU/fish of *Aeromonas liquefaciens*.

B. Level of total proteins and DNA from the muscle, gill, head kidney and brain of fish infected with a single dose of $10^{-4}$ CPU/fish of *Aeromonas liquefaciens*.

C. Level of total proteins and DNA from the muscle, gill, head kidney and brain of fish infected with a single dose of $10^{-5}$ CPU/fish of *Aeromonas liquefaciens*.

D. Level of total proteins and DNA from the muscle, gill, head kidney and brain of fish infected with a single dose of $10^{-6}$ CPU/fish of *Aeromonas liquefaciens*.

E. Level of total proteins and DNA from the muscle, gill, head kidney and brain of fish infected with repeated doses of $10^{-2}$ CPU/fish and $10^{-2}$ CPU/fish at 5 days interval.

F. Level of total proteins and DNA from the muscle, gill, head kidney and brain of fish infected with repeated doses of $10^{-3}$ CPU/fish and $10^{-3}$ CPU/fish at 5 days interval.

G. Level of total proteins and DNA from the muscle, gill, head kidney and brain of uninfected fish (controls).