INCIDENCE OF INFECTIOUS DISEASES IN FARMED CATFISH IN WEST BENGAL

A Thesis

Submitted to the

West Bengal University of Animal and Fishery Sciences

In partial fulfillment of the requirements for the Degree of

Master of Fishery Science

In

Aquatic Animal Health

By

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2014

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CERTIFICATE

This is to certify that the work recorded in the thesis entitled **"Incidence of infectious diseases in farmed catfish in West Bengal"** submitted by **Pradipta Paul** in partial fulfillment of requirement for the degree of **Master of Fishery Science (Aquatic Animal Health)** in the Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, is the faithful and bonafide research work carried out under my supervision and guidance. The results of the investigation reported in this thesis have not so far been submitted for any other degree or diploma. The assistance and help received during the course of investigation have been duly acknowledged.

(Prof. T. J. Abraham)
Chairman
Advisory Committee

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APPROVAL SHEET

Approval of the examiners for the award of the degree of Master of Fishery Science (Aquatic Animal Health)

We, the undersigned have been satisfied with the performance of **Pradipta Paul** in the viva–voce examination, conducted today, the 11th December, 2014, recommended that the thesis be accepted for the award of the degree.

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Acknowledgement

It is my proud privilege to acknowledge indebtedness to my beloved teacher Prof. T. J. Abraham, Professor (Fishery Microbiology), Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, and Chairman of the Advisory Committee for his benevolent guidance and keen interest to carry out this work. I wish to express my deepest sense of gratitude to him for his affectionate encouragement, multifarious help, untiring supervision, enthusiastic inspiration, constructive criticism, intimate association and valuable suggestions that have made this thesis work possible.

I would like to express my revering gratitude and proud gratefulness to the members of the Advisory Committee, Dr. G. Dash, Associate Professor and Head, Department of Aquatic Animal Health, Dr. T. K. Ghosh, Associate Professor and Head, Department of Aquaculture and Dr. T. S. Nagesh, Associate Professor, Department of Fishery Resources Management for their valuable suggestions, constructive ideas and constant encouragement.

I am grateful to the Dean, Faculty of Fishery Sciences, Kolkata for providing necessary help during the dissertation work.

I also like to express my gratitude and gratefulness to all the faculty members of FFSc., WBUAFS, Kolkata for their encouragement, immense inspiration and moral support rendered from time to time throughout the period of this study.

I would like to express my special thanks to the catfish farmers and traders of 24-Parganas (S), 24-Parganas (N), Burdwan and Nadia districts, West Bengal for their kind help and cooperation during the course of sample collection.

I am also grateful to ICAR-NAE programme for the laboratory and technological supports that played a major role in my research work.

I would like to express to my gratitude to Dr. Anjan, Dr. Agniswar, Dr. Sayani, Mr. Avijit, Mr. Haresh, Mr. Debajit, Mr. Debapriya for their valuable suggestions during the course of research work.

I extend my immense thanks to Mr. M. K. Das, Librarian, FFSc, WBUAFS and other members of the Library for their kind help during the needful time of dissertation work.

I wish to extend my indebtedness and heartiest thanks to my seniors Ms. Leesa, Mr. Biraj, Mr. Raghu, Mr. Rajesh and Ms. Farhana for their cooperation and constant support during entire survey period.

I would also like to extend my special thanks to my batchmates Mr. Tanmay, Tuhin, Sudipta, Partha, Suprabhat, Tushar, Tirtha, Aniruddha, Atanu, Prasanta, Abhishek Majhi, Anish, Bostanul, Abhishek Das, Saptarshi, Suman, Priyanka, Banasree, Sarita, Lopamudra, Kuheli, Debasmita, Sumana, Tara, Jayati and all other friends for their support and help.

Sincere thanks also extended to my juniors David, Vijay, Soham, Sudeshna, Tausif, Debadyuti, Julinta and Supradhnya for their kind support during the research work.

I would also like to extend my special thanks to Mrs. Tripti, Mr. Krishnendu, Mr. Biman, Ms. Sushama and Mr. Mrinal for their support and help.

Last, but not the least, I will fail from my duty, if I do not express my deep feelings, to my beloved parents and all the family members and relatives for their long patience, support, sacrifice, encouragement, guidance, constant inspiration and remarkable co-operation through all means.

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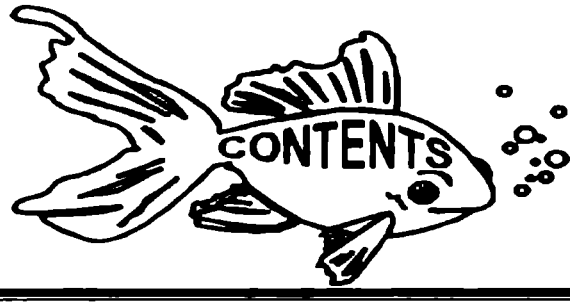
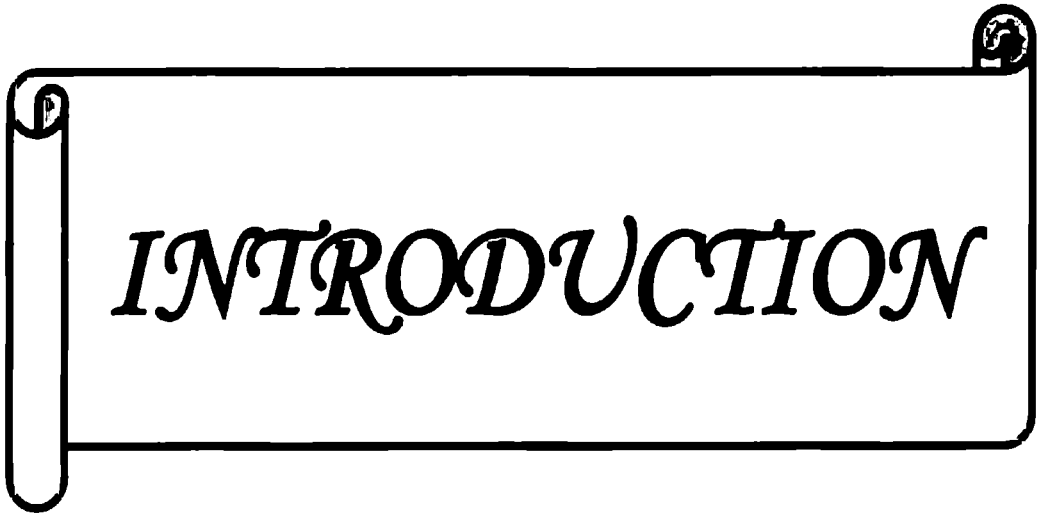


CHAPTER -1



1. INTRODUCTION

The fisheries sector, including aquaculture, is a fundamental source of food, livelihood, trade and recreation for people throughout the world. It plays a significant role in the economy by its contribution to income and wealth through the supply of nutritious food (Pillai and Kathia, 2004). Fish accounted for 16.7% of the world population's intake of animal protein and 6.5% of all protein consumed (FAO, 2014). World per capita food fish supply increased from an average of 9.9 kg in 1960 to 19.2 kg in 2012 (FAO, 2014). Fishery sector plays a major role in the socio-economic development of the country. It has been recognized as a powerful income and employment generator. It is a source of cheap and nutritious food and a foreign exchange earner. Development of fishery sector can ensure the food security as well as tackle the problem of unemployment. The FAO has defined "aquaculture" as the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Aquaculture continues to be the fastest growing animal food-producing sector and to outpace population growth. The per capita supply from aquaculture increased from 0.7 kg in 1970 to 9.4 kg in 2006, an average annual growth rate of 6.9% (FAO, 2006). Aquaculture contributed 48% of aquatic animal food for human consumption in 2007. According to FAO (2012), among total fish production of 154 million ton, aquaculture production comprise 41% (63.6 million ton) and capture fisheries production comprise 90.4 million ton. In 2010, the composition of world aquaculture production comprised of freshwater fish (56.4%, 33.7 million tonnes), molluscs (23.6%, 14.2 million tonnes), crustaceans (9.6%, 5.7 million tonnes), diadromous fish (6.0%, 3.6 million tonnes), marine fish (3.1%, 1.8 million tonnes) and other aquatic animals (1.4%, 814, 300 tonnes).

Production of freshwater fish has always been dominated by carps (71.9%, 24.2 million tonnes in 2010). Among carps, 27.7% are non-fed filter-feeders and the rest are fed with low-protein feeds. Production of tilapias has a wide distribution, and 72% are raised in Asia particularly in China and Southeast Asia, 19% in Africa, and 9% in America. Vietnam dominates production of omnivorous *Pangasius* catfish although there are other producers, such as Indonesia and Bangladesh. World production of *Pangasius* catfish may be understated because booming production in India has yet to be reflected in statistics. In 2010, Asia accounted for 73.7% of the production of other catfish species, America took its share to 13.5% with channel

catfish production, leaving 12.3% of production in Africa, dominated by North African catfish (FAO, 2012).

India produces a wide range of aquaculture product such as carp, tilapia, catfish, oysters, mussels, sea bass and shrimp. Fish culture is practiced in less than 30% of the total areas available. This sector has a potential to create huge market, provided fish cultivation is done on a scientific basis. India occupies the 2nd position in fish production next to China, which produced 90.62 lakh ton in 2012-2013. It also occupies the 2nd position in aquaculture production and contributes about 5.43% of global fish production. In India, fishery sector is the source of livelihood for 14.9 million people and contributed about more than Rs. 30,213.26 crores through export of marine products (MPEDA, 2014). Fishery sector share 0.78% of total GDP and 4.47% of total agricultural sector GDP (DAHDF, 2012).

As the demand of fish as food is increasing with time, aquaculture is needed to fulfil the demand. Along with carps, catfish farming system is popular throughout the world. The first efforts at raising catfish were made in the early 1900's at several federal and state fish hatcheries in USA. In the 1950's commercial catfish farming first started in Kansas and Arkansas in USA (http://aqua.ucdavis.edu/DatabaseRoot/pdf/1549MIS.PDF). Freshwater catfish are widely distributed throughout the world. They reach their greatest diversity in the continents spanning the equator, namely South America, Africa and Asia. The global production of catfish as food fish has increased from about 320, 000 tonnes in 1992 (Losordo *et al.,* 1998) to about 3.2 million tonnes (FAO, 2012). The USA is still the world's largest producer of catfish.

Catfish belong to the Kingdom: Animalia, Phylum: Chordata, Class: Actinopterygii, Order: Siluriformes. It is distributed in 32 extant families such as Akysidae, Amblycipitidae, Amphiliidae, Ariidae, Aspredinidae, Astroblepidae, Auchenipteridae, Bagridae, Callichthyidae, Cetopsidae, Chacidae, Clariidae, Claroteidae, Cranoglanididae,, Diplomystidae, Doradidae, Hypophthalmidae, Ictaluridae, Lacantuniidae, Loricariidae, Malapteruridae, Mochokidae, Nematogenyidae, Pangasiidae, Parakysidae, Pimelodidae, Plotosidae , Schilbeidae, Scoloplacidae, Siluridae, Sisoridae, Trichomycteridae and 1 extinct family,

Andinichthyidae. Nelson (1994) listed about 412 genera and 2,405 species in his book "Fishes of the World (Third edition)".

Catfish are generally characterized by (i) Elongated and compressed body, (ii) Often naked or covered with bony plates, (iii) Barbells present and (iv) Dorsal and pectoral fin often with spines, and pelvic fin without spines. They are generally hardy and air-breathing fishes. Catfish are efficient opportunists and survivors, equipped to exploit whatever resources are available. They have a wide tolerance to environmental extremes (Bruton, 1988). Catfish vary in size from tiny parasitic species with a total length of less than 5 mm to giant forms of 30 kg such as some of the *Pangasius* species and the African sharp tooth catfish (*Clarias gariepinus*). Catfish became an excellent aquaculture species, not only for their tolerance to environmental extremes, but also for their high annual production, high growth rate, high feed conversion rate (FCR). Feed conversion rates of up to 2.2-2.4 were found in experimental least cost diets containing 25% crude protein (Robinson *et al.*, 1998).

Catfish are currently produced worldwide using various production systems ranging from very low yielding extensive system to high yielding intensive systems. The choice of a system suitable for the species intended for production is probably the most important decision for any prospective aquaculture farmer, and may either result in the success or failure of any aquaculture business. Production systems can be categorized as stagnant pond, flow-through pond, recirculation pond production or raceway production. These production systems differ to a greater or smaller degree from each other in regard to the intensity of production, production costs and technical difficulty in operating and managing them. Catfishes are cultured only in some countries of the world for their high nutritive value which can be compared to some quality fishes, from both freshwater and marine. The most notable is the United States, where channel catfish *Ictalurus punctatus*, *I. cactus* and *I. furcatus*is found on a commercial scale. In Vietnam *Pangasius hypothalamus* and *P. bocourti* are cultured. In Africa, *Clarias gariepinus* and in the Philippines and Thailand *C. macrocephalus* and *C. batrachus* are cultured. In Bangladesh *Pangasius pangasius, C. gariepinus* and *C. batrachus* are the impotant culturable species.

Now a day's, catfish industry has been suffering from different constraints like lack of capital, high cost feed, scarcity of seed, environmental stressors and the most

effective one is diseases. Disease is one of the most important factors influencing the success of catfish culture. Disease in fish is the result of an interaction between at least three factors, viz., host susceptibility, pathogen virulence and suboptimal environmental conditions. Diseases of fish often occur as secondary infections following stress due to suboptimal environmental conditions such as poor water quality, nutritional deficiency and crowding. The main causes of disease can be summarized as: water quality deficiencies, bacterial and viral infections, protozoan parasites, fungal infections, monogenetic trematodes, digenetic trematodes, cestodes, nematodes and crustacean parasites. The pathogens associated with catfish seldom cause mortalities in the wild. However, in the "unnatural" environment of a high-density production system often associated with stressed fish, these pathogens are potentially deadly.

Disease outbreaks are constraint to catfish aquaculture production, thereby affecting both economic development of the country and socio-economic status of the fishers in many countries. For example,

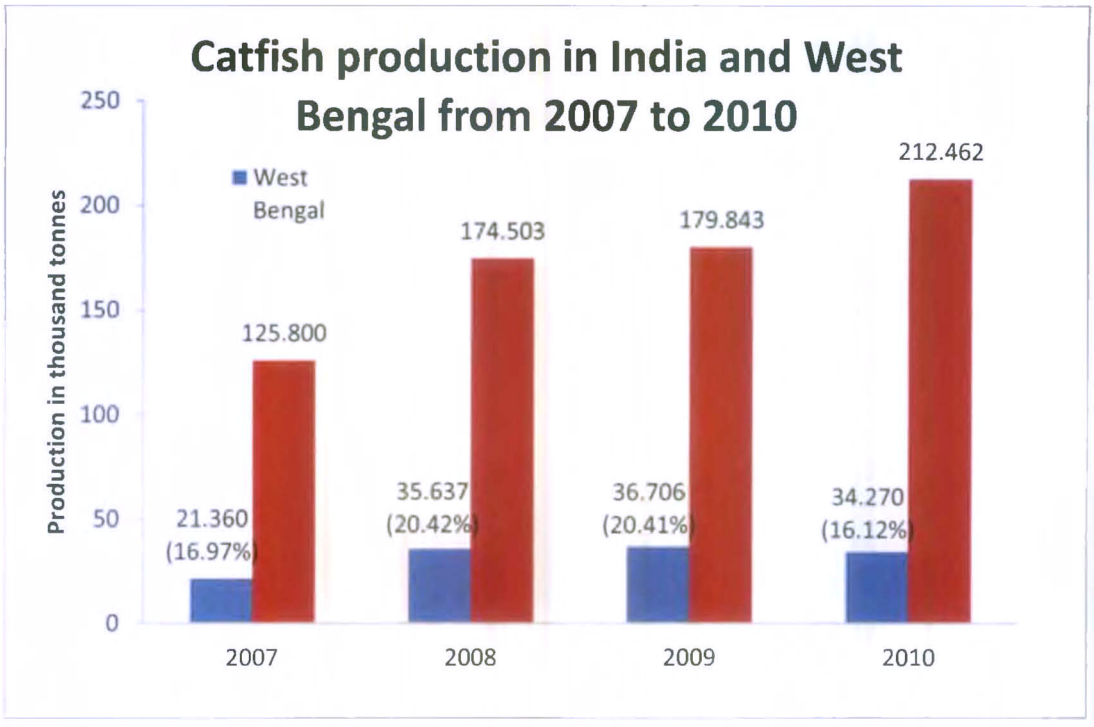
- The Catfish Journal (Anonymous, 1990) reported that 115 million catfish, valued US$ 8 million, were lost due to diseases during the first half of 1989.
- In Mississippi, USA, there was a loss of US$ 4-6 million due to enteric septicaemia of catfish (ESC) in the year 1990 (Fruend *et al.*, 1990).
- In Thailand, a loss of US$ 4.3-21.3 million due to jaundice in catfish in the year 1992 has been reported (Chinabut, 2002).
- In Mymensingh, Bangladesh, the reported loss due to *Aeromonas* infection in the year 2004-2005 was Tk 21,500/ha (Faruk, 2008).
- In Alabama, USA, the reported loss was US$ 40 - $60 million due to enteric septicaemia in *Ictalurus punctatus* (Channel catfish) in the year 2010 (Carrias, 2011).
- In U.S. catfish industry, the estimated annual loss was US$ 100 million due to diseases. (http://www.ars.usda.gov/research/projects/projects.htm?accn_no=418787)

Disease control in aquaculture has been achieved by following different methods using traditional ways, synthetic chemicals and antibiotics. However, the use of such expensive chemotherapeutants for controlling diseases has been widely

criticized for their negative impacts like accumulation of residues, development of drug resistance, immunosuppressant and reduced consumer preference for aqua-products treated with antibiotics. Many a time, the traditional methods are ineffective against controlling new diseases in large aquaculture systems. Therefore, to control fish diseases some prophylactic measures like vaccination, immunostimulant application and best management practices (BMP) are advocated. Application of herbal products also has a good efficacy in disease treatment.

The catfish farming sector in its present form is a relatively new development in the country. It became possible when the artificial propagation of the catfish species (Cacot, 1999; Cacot *et al.,* 2002) developed and were adopted in India. Among the different species of catfish, *C. batrachus, Heteropneustus fossilis, C. gariepinus, P. pangasius, P. sucthi, P. hypothalamus, Wallago attu* and *Ompok pabda* are mainly cultured in India. This development enabled the traditional small scale aquaculture practices that were dependent on wild caught seed stocks to shift to more intensified systems and dependent entirely on hatchery produced seed (Trong *et al.,* 2002). Also, over the last decade the farming of catfish took precedence, and pond farming became the dominant form because of its relatively faster growth rate, flesh quality and appearance, therefore marketability overseas (Phuong and Oanh, 2009).

In recent years, catfish farming is getting much importance in West Bengal. Catfish production of India and West Bengal has been on the rise due to high economic return with low input. The details of catfish production in India and West Bengal are depicted graphically below. The state West Bengal holds the 2nd position in catfish production and Andhra Pradesh holds the 1st position since 2008. The contribution of West Bengal's catfish production was in the range of 16 - 20% of the total catfish production of India since 2007. In 2010 catfish production in West Bengal has seen a slight reduction (DAHDF, 2012). Due to frequent occurrence of diseases, there has been considerable economic loss in Indian catfish aquaculture. Also, suitable remedial measures to combat the diseases have not yet been developed.



Therefore, the present study was undertaken to investigate the incidence of diseases in cultured catfish in West Bengal with the following objectives:

- To monitor the prevalence of infectious diseases in farmed catfish.
- To identify and characterize the etiological agents by conventional and molecular biological methods.
- To recognize pathomorphological and histopathological alterations in diseased catfish.
- To study the antibiotic sensitivity of bacterial flora of diseased catfish.

CHAPTER -2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Catfish culture systems

Ponds, raceways, cages and tanks are generally used for the commercial rearing of catfish especially channel catfish (*I. punctatus*). The type of system used is often determined by the location. Earthen ponds are by far most widely used culture system for catfish. Ponds are typically constructed by impounding natural basins in areas of uneven terrain. Commercial production ponds are 1-2 m deep and 2-8 ha or larger in surface area. An adequate water supply, catch basin and drain structure are essential. It is popular in USA, Bangladesh and India. There are two hydrobiological types of ponds in catfish farming. Watershed ponds are built in hilly areas by damming a small stream. In the long term, the major source of water is runoff from the drainage basin above the dam, although a source of pumped water is desirable to help off-set evaporation and seepage during droughts. Embankment ponds ponds are the most common types of ponds used in channel catfish farming. Embankment ponds are built on flat land by removing soil from the area that will be the pond bottom and using that soil to form levees or embankments around the pond perimeter. Ground water is generally lifted by using pumps. A well-managed pond which has been properly stocked with blue or channel catfish can produce from 400 to 1,000 pounds of catfish/surface acre/year (Lock and Steinbach, 2011).

Raceway culture system is familiar worldwide with its advantages of higher stocking densities, improved water quality, reduced manpower, easy feeding, grading, harvesting and precise disease treatments (Masser and Lazur, 1997). Raceways should be filled to the desired level and the desired water flow rate (eg., 100-400 gal/min depending on fish size or biomass) established before fish are stocked (Wyatt *et al.,* 2006). Fingerlings are stocked at approx. 6,000/ raceway of size 80 x 8 x 3.2 ft (Wyatt *et al.*, 2006). Circular tanks, constructed of plastic, concrete or steel, are widely used to culture aquatic species. Small 100 - 200L tanks are used to spawn fish, to maintain fry and fingerlings and to hold fish before sale. Circular tanks are used in flow through units and also in water reuse system. Cage culture is very common in USA, Japan and Taiwan. Usually the fingerlings of *I. punctatus* are stocked at densities of about 1,000 fish / cage of size 1.2 x 1.2 x 2.4 m (Eley *et al.*, 1972). Cage aquaculture allows an easy observation of rearing populations and better predation control. The production, expressed as the biomass gained per unit of time

and volume, was statistically greater. Cost of harvesting is comparatively less than any other culture system.

2.2 Catfish diseases

Disease is correlated with mainly six factors such as host that means species, age and strain, facilities or management practice of farm, environment of water body, nutritional status of fish, pathogen, its type and degree of pathogenicity, physiological status of host, its resistant power and immunological status (Plumb, 1999). A global estimate of disease losses to aquaculture by the World Bank in 1997 was USD 3 billion/annum (Alday, 2010). There are many diseases causing huge economic losses in catfish culture, most of which are caused by pathogens (virus, bacteria, fungi and parasites) and some due to nutritional deficiency and environmental factors. The lists of diseases are as follows:

2.2.1 Viral diseases

Very little is known about viral diseases in catfish. Two syndromes of unknown etiology have been recorded in African catfish. The first, which occurs mainly during the fingerling stage, concerns a rupture in the caudal part of the intestine. It is known as "Ruptured Intestine Syndrome". The second concerns the destruction of the arborescent organs (air-breathing organs), which leads to inflammation of the skull resulting in a lateral skull break. It is known as "Broken Head Disease". In Asia, a similar syndrome has been reported for *C. batrachus* and *C. macrocephalus* (http://www.oie.int/eng/normes/ fmanual/A_00023.html). In channel catfish, *I. punctatus* channel catfish viral disease is reported.

2.2.1.1 Channel catfish viral disease (CCVD)

Channel catfish virus (CCV) is host specific to the channel catfish, *Ictalurus punctatus*. The African catfish (C. *gariepinus*) and the Asian catfish (*C. batrachus*) have been tested for susceptibility and have been found to be resistant to CCV infection (Boon *et al.* 1988). Silverstein *et al.* (2008) tested the susceptibility of blue catfish (*Ictalurus furcatus*) and channel catfish (*I. punctatus*) against CCV and revealed that blue catfish was more resistant to it. Channel catfish viral disease is caused by a herpesvirus/*ictalurid herpesvirus* 1 (Plumb, 1986; Camus, 2004). Clinical signs of this disease are swollen abdomen, exophthalmus (pop eye), haemorrhaging of fins and ventral abdomen, haemorrhaging of the muscles, liver and kidney, dark and

enlarged spleen, fluid in the abdominal cavity, pale, enlarged kidney, which may be the only internal indication of disease in infected fish (Plumb, 1986; Camus, 2004; Silverstein *et al.*, 2008). The digestive tract contains no food, but may be filled with yellow fluid and mucus (Camus, 2004). Mostly outbreaks will occur in channel catfish juveniles during the first summer (June-September) when water temperatures are above 25°C (Plumb, 1986; Camus, 2004). Contradictory to the study Stingley *et al.* (2003) reported that adult catfish were more resistant to CCV than fry or fingerling. Vertical transmission of this disease is very common but horizontal transmission is also possible (DAFF, 2012).

A generalized viremia was established within 24 h after infection. The kidney, liver, spleen, and intestine became involved in virus replication 24-48 h after infection. Virus was isolated from brain tissue after 48 h. Virus titers were highest in the kidney and intestine 72 h after infection, and in the spleen, brain and liver after 96 h and comparatively low in the muscle (Plumb, 1986). Renal hematopoietic tissue was oedematous, and extensive areas of necrosis and cellular dissolution occur, coupled with an increase in macrophages. The liver developed regional oedema, necrosis and hemorrhages, and hepatic cells had eosinophilic intra-cytoplasmic inclusions. Pancreatic acinar cells were necrotic. The submucosa of the digestive tract was oedematous and had focal areas of macrophage concentration and haemorrhage. The spleen became congested with erythrocytes and lymphoid tissue became greatly reduced. Virus particles were seen in electron micrographs of the liver, kidney and spleen of infected fish (Plumb, 1986). According to Camus (2004) reducing environmental stress until the fish are 4 inches in length will minimize losses. CCVD developed into a hemorrhagic viremia after initially replicating in the kidney and then in the spleen. Thereafter, the virus was transported to the intestine, liver, heart and brain. Necrosis of the renal hematopoietic tissue and tubules; oedema, necrosis and congestion of the liver; intestinal oedema; congestion and haemorrhage in the spleen were characteristic histopathological findings. Skeletal muscle haemorrhage among experimentally infected fish was observed (Lio-Po *et al.*, 2001). It has the ability to establish latent infection in the host that will outbreak in ideal environment. Fish will get secondary invasion of external lesions *by Flavobacterium columnare*, *Aeromonas hydrophila* or aquatic phycomycetes may develop.

2.2.2 Bacterial diseases

2.2.2.1 Motile aeromonas septicaemia

Motile aeromonas septicaemia (MAS) also termed as red disease is caused by a group of motile aeromonads including *A. hydrophila, A. sobria, A. caviae* and other motile *Aeromonas* bacteria (Dung *et al.*, 2008; Kumar and Ramulu, 2013). Clinical signs of this disease are exophthalmia and distended abdomen (bloody ascites in the peritoneum, leading to swollen belly), deep dermal ulcers with haemorrhages and inflammation, haemorrhages on the head, mouth and base of fins, red, swollen vent, pink to yellow ascitic fluid, gas accumulation on gastro intestinal tract, haemorrhages on eye and after dissection internal organ necrosis can also be observed (Dung *et al.*, 2008; Huang *et al.*, 2010; Kumar and Ramulu, 2013). This infection often occurs at the change from dry to rainy season, especially during periods when fish are stressed during handling and transportation (Dung *et al.*, 2008). Infections can occur in any age fish, but losses are usually most severe in fry and small fingerlings. Environmental stress factors like high water temperatures, high ammonia and nitrite levels, pH, and low dissolved oxygen levels, overcrowding, high organic loads enhance the development of disease (Kumar and Ramulu, 2013). Severity of disease is influenced by a number of interrelated factors, including bacterial virulence, the kind and degree of stress exerted on a population of fish, the physiologic condition of the host, and the degree of genetic resistance inherent within specific populations of fish. Histopathology observations of internal organs revealed that kidney, liver and spleen cells were severely damaged. Degenerative changes consisting of focal necrosis and haemorrhage were detected in the spleen and kidney tubules. Cells were incoherent and bacterial cells were observed in the tubules. The tissues of the kidney and liver showed cellular degeneration. White nodules in the liver and kidney showed the presence of bacterial infection (Ly *et al.*, 2009).

2.2.2.2 Bacterial haemorrhagic septicemia

Bacterial haemorrhagic septicemia is caused by *A. hydrophila* (Jayavignesh *et al.*, 2011). Clinical signs of this disease are moribund condition, abnormal movement and loss of balance were observed, the posterior end of the body surface was found to develop greyish-white lesion that was extended up to caudal fin, anal region and the fin bases developed red colour, after dissection of the freshly dead fish, the liver was observed to be swollen, unsmooth, uneven and turned blackish in colour,

congestion/haemorrhage at vent (Sarkar and Rashid, 2012). After dissecting, haemorrhage/ congestion on all visceral organs including peritoneal lining and visceral fat, congested spleen, congested liver (reticular pattern), haemorrhage in muscle can be observed (Griffin, 2011). The organism was able to grow well at the optimum temperature of 37°C, but it also has the ability to grow at 4°C and it was identified that the growth at temperature range of 41°C was found to be less when compared to other ranges. The pathogen was able to grow well at both alkaline and acidic pH of 9.0 and 5.0, respectively (Jayavignesh *et al.*, 2011). Bacterial entry may be facilitated by injuries as catfish do not have protective scales and their sharp fins can easily tear the skin of another fish within the immediate vicinity (Alagappan, 2009). Mortality has also occurred due to pathological lesion such as necrosis and atrophy of hepatocytes, necrosis of sheathed arteries in the spleen and necrosis of renal tubules and glomeruli in the kidney (Alagappan *et al.*, 2009).

2.2.2.3 Infectious dropsy

Infectious dropsy is caused by *A. hydrophila* (Edun, 2007) and *Pseudomonas* sp. (AAHRI, 1995). Clinical signs of this disease are dropsy (clear or yellow liquid in the abdomen and also in many organs), swelling in the ventral region of the body, swelling at the pectoral fin base and yellowish fluid accumulation on the body cavity (AAHRI, 1995; Edun, 2007).

2.2.2.4 Bacillary necrosis of Pangasius

Bacillary necrosis of *Pangasius* is caused by *Edwardsiella ictaluri* (Fergusion *et al.*, 2001; Dung *et al.*, 2008). It is the most frequently occurring disease in *Pangasius* and have important factor in production systems of catfish of all ages, although especially fingerlings and juvenile fish seem to be affected (Dung *et al.*, 2008). Gross and clinical signs of this disease are fish swim slowly at the surface of the water, pale colour on skin and gills, petechial haemorrhages on eyes and fin bases, white spot in viscera, spleen, liver and kidney, necrosis of internal organs (Fergusion *et al.*, 2001; Dung *et al.*, 2008). Internally, white 1-3 mm diameter military lesions were observed under the capsular surface and throughout the parenchyma in the liver, kidney and spleen of diseased fish. On cut surface the muscle appeared yellow, often with petechial haemorrhages. The gills were pale and viscous because of the presence of increased mucus (Fergusion *et al.*, 2001). The disease occurs in all ages of fish,

although high mortalities of about 50-90% are reported in fingerlings and juvenile fish. The disease peaks during the rainy season, when temperatures drop below 28° C (Dung *et al.*, 2004). In general, crowding, mixing and adverse climate conditions are considered risk factors for the development and spread of BNP (Dung *et al.*, 2004). Careless treatment has unfortunately resulted in antimicrobial resistance in *E. ictaluri* treated with oxytetracycline or sulfonamides. In chronic infection fish will be affected by lesions in kidney, liver and spleen with a few multi-focal lesions in muscle. Renal tubules with multiple focally extensive areas of necrosis, progressing to pyogranulomatous lesions are reported (Fergusion *et al.*, 2001).

2.2.2.5 Enteric septicaemia of catfish

Enteric septicemia of catfish (ESC) has become one of the two most significant diseases of economic significance in the catfish industry. Enteric septicemia of catfish is considered the most important bacterial disease of cultured channel catfish. *Ictalurus punctatus* and is estimated to cost the industry US$40-60 million yearly in economic losses (Carrias, 2011). The causative agent of this disease is *E. ictaluri* (Hawke *et al.*, 1998; Chappell, 2008; Carrias, 2011). Catfish affected with ESC often are seen swimming in tight circles, chasing their tails. Whirling behaviour is due to the presence of the *E. ictaluri* in the brain (Hawke *et al.*, 1998). Affected fish will show the following characteristics, ulcers and petechial haemorrhage in body and head, ascetic fluid in intestine, exophthalmus, longitudinal, raised red pimples at the cranial foramen between the eyes, affected fish may display rigor or uncoordinated muscle twitching, resulting from the infection progressing to the brain (Hawke *et al.*, 1998; Chappell, 2008). Internal signs are clear, straw-colored or bloody fluid is often present in the fish body cavity. The liver typically has characteristic pale areas of tissue destruction (necrosis) or a general mottled red and white appearance. Petechial hemorrhages can be found in the muscles, intestine and fat of the fish. The intestine is also often filled with a bloody fluid (Hawke *et al.*, 1998). The highest losses occur in heavily stocked ponds that have experienced an environmental stressor within the 18–28°C temperature (Plumb and Shoemaker, 1995).

Edwardsiella ictaluri is one of the most important pathogens of channel catfish (*I. punctatus*) and other species of this family, like *I. furcatus, Ameiurus nebulosis, A. catus, Noturus gyrinus*. Disease is mainly transmitted through horizontal

transmission. Infection has been shown to occur by intestinal mucosa (oral uptake), and the olfactory mucosa (nasally). Recent evidence suggested that epidermal and branchial mucosa may be additionally important routes for transmission. In acute form kidney is the target organ and in chronic stage brain tissue are affected (http://www.baphiq.gov.tw/public/ Attachment/8123017304571pdf). *Edwardsiella ictaluri* was originally thought to be an obligate pathogen because it only survives for a short time in water; however, it was later demonstrated to survive for up to 95 days in sterile pond mud at 25°C (Hawake *et al.,* 1998). Histopathologic observation revealed necrotising myositis, locally extensive cellulitis in the head, involving connective tissue surrounding cranial bones and cartilage (DAFF, 2008).

Although the disease is primarily a disease of channel catfish, *I.* punctatus, but has been isolated from other species such as walking catfish *C. batrachus*, European catfish *Silurus glanis* and *P. pangasius* (Carrias, 2011). Pathogenesis studies have shown that *E. ictaluri* can enter through the gastrointestinal tract, the nares (nasal openings), and possibly through the gills (Hawke *et al.*, 1998). Two clinical forms of ESC occur in channel catfish: an acute form resulting in septicemia characterized by haemorrhage and necrosis of several organs, and a chronic form resulting in chronic encephalitis (Johnson, 1990). In latent state of the disease, infected fish become carriers of the pathogen. *Edwardsiella ictaluri* entering through the gut can cross the intestinal mucosa, enter the bloodstream, and migrate to the kidney within 15 min (Baldwin and Newton, 1993). Histological examination revealed a systemic infection of all organs and skeletal muscles, with the most severe changes being diffuse interstitial necrosis of the anterior and posterior kidney, and focal necrosis in the liver and spleen are also generally seen. In some cases gill inflammation, exophthalmia and anaemia may be observed (Blazer *et al.*, 1985; Carrias, 2011). In the chronic state, studies suggested that *E. ictaluri* enters the brain via the olfactory bulb, having colonized the olfactory sac, causing ulceration of the cranial vault (Miyazaki and Plumb, 1985; Morrison and Plumb, 1994; Carrias, 2011). *Edwardsiella ictaluri* is endemic, but it is considered an obligate pathogen as it requires a proper host for overall survival (Plumb and Quinlan, 1986). Under optimal conditions it can infect catfish of all ages (Francis-Floyd *et al.*, 1987). Horizontal transmission of ESC may occur through the shedding of bacteria into the aquatic medium by infected fish, and by cannibalism of infected fish where previously infected fish may serve as carriers of

disease and infect newly introduced fish (Hawke *et al.*, 1998; Tucker and Robinson, 1990; Wagner *et al.*, 2002).

2.2.2.6 Edwardsiella septicemia

The causative agent of this disease is *Edwardsiella tarda* (Bullock and Herman, 1985; Mohanty and Sahoo, 2007; Park *et al.*, 2012). The enormous loss caused by this pathogen is being felt in the USA, Japan, Europe and Asian countries; however, no exact figures are available to quantify (Mohanty and Sahoo, 2007). Clinical signs are spiral movement, dorsolateral petechial haemorrhage and cutaneous lesions, exophthalmus and ascites. Gross external lesions vary with species. Channel catfish often develop small, cutaneous ulcerations; in advanced cases, however, larger depigmented areas mark the sites of deep muscle abscesses (Meyer and Bullock, 1973; Ibrahem *et al.*, 2011).

Internally, the most common gross lesion consists of light-coloured nodules on the kidneys, spleen or liver. Large abscesses that develop in muscles of channel catfish and in internal organs emit a malodorous gas when punctured. Histopathologic observation showed lesions that are focal necrotic areas, often with abundant bacteria, both free and within macrophages. These lesions may be walled off by fibrocytes and epitheloid cells, or be invasive and spread into adjacent skeletal muscle (Bullock and Herman, 1985). *Clarias gariepinus* infected with *E. tarda* hepatocytes showed disorganization, disorientation and hydropic degeneration of hepatic plates, necrobiotic changes in renal tubules, depletion of lymphocytes and congestion of blood vessels and focal areas of necrosis in epidermal layer (Ibrahem *et al.*, 2010).

Because *E. tarda* is ubiquitous, many animals can serve as reservoir of infection (Bullock and Herman, 1985).*Edwardsiella* septicemia in fish is characterized by necrotic abscesses in the muscle that emit a putrid odour when incised. The skin that covers muscle abscesses can be pale or have petechia. Mortality of infected fish may be acute, but is often chronic depending upon the degree of stressful environmental conditions under which *E. tarda* infections occur. *Edwardsiella tarda* commonly affect the intestine of fish (http://www.cabi.org/isc/datasheet/84398). Histopathologic observation of affected Korean catfish, *Silurus asotus* showed in the liver, hepatocytes lost fat and underwent atrophy or necrosis. The spleen showed necrotized splenocytes and a haemorrhagic

pulp. In the kidney, glomerular destruction, degeneration of renal tubular epithelial cell and haemorrhage were observed (Yu *et al.*, 2009). This pathogen mainly transmitted through the water from an infected source (carrier animal faeces, water and mud). Carrion-eating birds may also be a source of infection (www.eaza.net/activities/tdfactsheets/080%20Edwardsiellosis.doc.pdf).

2.2.2.7 Enterobacter cloacae infection

Enterobacter cloacae have been associated with mortality of freshwater reared *Pangasianodon hypophthalmus*. The clinical signs were lesions on pectoral fin and the belly region. Gills were pale and viscous, ascetic fluid, internal organs were congested and pulpy. Incidences of fish mortalities of >50% were reported from two different culture ponds growing *P. hypophthalmus* in Bhimavaram region of Andhra Pradesh, India (Kumar *et al.*, 2013).

2.2.2.8 White patch disease/ Columnaris/ Saddle back disease

Columnaris, first described by Herbert Spencer Davis in 1922, is one of the oldest known diseases of warm water fish (Durborow *et al.*, 1998). It is one of the most common bacterial diseases in channel catfish, *I. punctatus* (Thomas-Jinu and Godwin, 2004, Beck *et al.*, 2012). The causative agents of this disease are *Flexibacter columnaris* (*Flavobacterium columnare*), *F. psychrophilia* and *Flavobacterium branchiophila* (Durbow *et al.*, 1998; Shoemaker *et al.*, 2008; Cai, 2013). A common clinical presentation of the disease is the pronounced erosion and necrosis of external tissues, with the gills typically being a major site of damage. Presumptive diagnosis of columnaris disease is based on the presence of the clinical signs and by the cell morphology (e.g., notably columnar aggregates referred to as "haystacks") of *F. columnare* in wet mount preparations of scrapings from infected tissues (Beck *et al.*, 2012; Arias *et al.*, 2012). The bacterium is capable of entering the blood stream and is routinely isolated from the internal organs. The disease commonly occurs in channel catfish when water temperatures are in the range of 25-32°C in the spring, summer and fall. Fish with columnaris usually have brown to yellowish-brown lesions (sores) on their gills, skin and/or fins (Durborow *et al.*, 1998; Darwish and Mitchell, 2009; Griffin, 2011). Columnaris signs include the development of pale or discoloured areas beneath the dorsal fin toward the pectoral fins and posteriad to the pelvic fin that evolved into typical saddleback lesions, skin became multifocal and diffuse (Grizzle and Rogers, 1976; Bullard *et al.*, 2011). In severe stage necrotizing dermatitis and

cutaneous sloughing exposed the underlining muscle and the gills had focal and multifocal necrotizing branchitis with yellowish pigment (Beck *et al.*, 2012).

The bacteria attach to the gill surface, grow in spreading patches, and eventually cover individual gill filaments and results in cell death. Skin lesions produced by columnaris initially are very shallow and may appear as an area that has lost its natural shiny appearance, a characteristic lesion produced by columnaris is a pale white band encircling the body, often referred to as saddleback condition. Stressful conditions favouring columnaris disease include low oxygen, high ammonia, high nitrite, high water temperatures, rough handling, mechanical injury and crowding.

Prevalence of *F. columnare* has been noted in the liver, gill and serum of infected fish (Welker *et al.*, 2005; Verma and Prasad, 2008) indicating its invasive nature. The infection will spread through the water column, and potentially can and will infect most fish, with which it comes into contact (http://www.fishvet.com/columnaris.htm). Cai (2013) reported the dynamics of biofilm formation by the fish pathogen *F. columnare* under static and flow conditions. Interactions between aquatic bacteria and surfaces occurred at the boundary layer and typically under flow conditions since few aquatic systems are static. As per the report of Beck *et al.* (2012) the ability columnaris bacteria to adhere to gill tissue or skin is thought to be a critical initial step in the pathogenesis of columnaris disease. Several *in vitro*, *ex vivo*, and *in vivo* studies have examined *F. columnare* adhesion and have collectively demonstrated that the virulence of different *F. columnare* isolates closely correlates with their respective ability to adhere and/or persist on host tissue. Potential mechanism of attachment may be through lectin mediated interactions. Lectins can be expressed by bacterial cells, including *F. columnare*, and by host epithelial cells which utilize lectins to bind and agglutinate bacteria by binding to surface glycol-conjugates (Beck *et al.*, 2012).

2.2.2.9 Red eye disease in *Pangasius*

Red eye disease in *Pangasius sutchi* was reported by Reddy and Mastan (2013). The causative agent of this disease was *Acinetobacter schindleri*. *Acinetobacter* members are found in water and act as common flora (Marian, 1990; Sousa and Silva-Souza, 2001). The fish show different symptoms like gill

impairment, erythrodermatitis, petechiae at lateral line, red mouth, redness at fin bases, swollen red colour anus, pop eye, red arched region around eye. This pathogen was highly virulent and capable of causing re-infection in *P. sutchi* and cause death (Reddy and Mastan, 2013).

2.2.2.10 Fulminant sepsis

Fulminant sepsis in *Pangasius sutchi* was reported by Reddy and Mastan (2013). The causative agent of this disease was *Wohlfahrtiimonas chitiniclastica*. *Wohlfahrtiimonas chitiniclastica* a new gamma-proteobacterium isolated from *Wohlfahrtia magnifica* (Toth *et al.*, 2008). The infected fish show different symptoms like erythrodermatitis, petechiae at lateral line, swollen red colour anus, exopthalmus, red arched region around eye, swollen enlarged liver in light yellow color, shrunken gastrointestinal tract and spleen, and hemorrhages on internal body cavity.

2.2.3 Fungal disease

2.2.3.1 Water mold /winter fungus disease

Fungi are a specific group that lack chlorophyll and are mainly secondary invaders of fish. Usually they can grow only on dead organic matter and often indicate there is something else wrong with the fish (Durbow, 2000). Many fungi cause diseases that can infect and kill channel catfish eggs, fry, fingerlings and adults (Durbow *et al.*, 2003). Causative agents are *Saprolegnia* sp., *Achyla* sp., *Branchiomyces* sp. (Durbow *et al.*, 2003). Most of the fungal infection occurs when there is rapid drop in water temperature (5°C in 24 h) and when the temperature is >15°C (Griffin, 2011). Clinical signs are brownish patches of cottony fungal growth, dry, depigmented skin, endophthalmia (sunken eyes), small, and focal infections spreading rapidly over body or gills (Durbow *et al.*, 2003; Griffin, 2011). Branchiomyces are producing hyphal stages in the gills (Eli *et al.*, 2011).This fungus is generally considered as a considered a secondary pathogen. *Saprolegnia* can also act as a primary pathogen (Neish, 1977; Willoughby and Pickering, 1977; Whisler, 1996). *Saprolegnia* causes tissue destruction and loss of epithelial integrity (Neish, 1991; Bruno and Poppe, 1996) due to cellular necrosis or dermal and epidermal damage (Pickering and Willoughby, 1982) including hyphae penetration of the basement membrane (Neish, 1991; Bruno and Wood, 1994). *Saprolegnia* fungi are necrotrophs when they grow on living sources and saprotrophs when they derive their

nutrition from non-living sources (Eli *et al.*, 2011). Saprolegnian fungi can act as primary invaders, in physiologically debilitated (example- decline in mucus production) and immunologically compromised fish (in "stress" situations) (Willoughby, 1978; Neish and Hughes, 1980). Branchiomyces infection in the blood vessels of the gill causes blockage, haemostasis and thromboses, which consequently cause extensive necrosis of the gill filaments. Areas of the gill filaments turn brown, due to haemorrhages and thromboses, and grey as a result of ischemia (Eli *et al.*, 2011).Abolude *et al.*, (2013) reported six genus of fungi commonly, *Penicillium* sp., *Acreomonium* sp., *Fusarium solani, Aspergillus* sp., *Mucor* sp., *Saprolegnia* sp. form broodstock of *C. gariepinus* as well as egg, where *Penicillium* sp. had 23% occurence, while *Saprolegnia* sp. had the least with 3% occurrence.

2.2.4 Parasitic diseases

2.2.4.1 Slimy disease

The causative agents are *Trichodina* sp., *Glossatella* sp., *Scyphidia* sp. and *Epistylis* sp. and among these, trichodinids are the most serious (AAHRI, 1995). *Trichodina* spp. has been isolated from skin, fins and gills of infested *C. gariepinus*. It has a large adhesive disc/saucer shaped body (Abo-Esa, 2008; Pádua *et al.*, 2012). It has been reported that its infestation is symmetrical with generous feeding rates (Durbow, 2003). *Epistylis* spp. often forms a branched colony on the skin and gills of infested catfish (Abo-Esa, 2008; Pádua *et al.*, 2012). *Trichodina* and *Epistylis* species, the most common parasites of *Pangasius* catfish, cause heavy infections leading to mortalities during the nursing period. Infections can be observed year round, but are heaviest when climate conditions are unstable, such as when downpours of rain are followed by periods of sunshine. *Glossatella* and *Scyphidia* are found more on the gill filaments than the skin and causes irritation, producing large amounts of mucus on the gills which in turn obstructs oxygen uptake, leaving the fish weak and vulnerable to bacterial infection (AAHRI, 1995). Clinical signs are anorexia, flashing, cloudy mucus secretion, whirling movement of fry, dark coloration with pale gills, petechial hemorrhages over the entire body surface and erosion of skin, fin and mouth (AAHRI, 1995; Durbow, 2003; Dung *et al.*, 2008). Infestation will be most in ponds with high stocking densities and poor water quality (Dung *et al.*, 2008).

2.2.4.2 Proliferative gill disease

Proliferative gill disease (PGD) is the 3rd most commonly diagnosed disease at the Aquatic Research and Diagnostic Laboratory (ARDL), Stoneville, MS (Griffin, 2011). The PGD was first reported in channel catfish *I. punctatus* at commercial farms in 1981 and the causative agents are *Aurantiactinomyxon* sp. and *Henneguya ictaluri* (Mitchell *et al.*, 1998; Griffin, 2011). *Henneguya exilis* was once believed to be the cause of PGD. Now, it is a problem in many cultured freshwater fish; channel catfish can be heavily infected. However, the evidence suggested that the interlamellar form of the parasite that evokes a serious inflammatory response is probably due to another Myxosporidean, *Aurantiactinomyxon* sp., or the extrasporogenic stage of the myxozoan *Sphaerospora ictaluri* (Moeller, 2014).

The complex life cycle of these parasites involves the oligochaete worm *Dero digitata*, where actinospores are developed and attack the fish. The actinospore stage of the parasite exposed to the catfish is responsible for significant gill damage, resulting in respiratory problems (Griffin, 2011). The PGD has become common in channel catfish farming because of its high mortality rate, which starts from a few dozen fish to 100% in less than 3 days (Mitchell *et al.*, 1998). This disease primarily occurs during spring season when the water temperature is between 15 and 25° C. Though the fingerlings are mostly affected, it also affects all other sizes too (Mitchell *et al.*, 1998; Griffin, 2011). It is also termed as Hamburger gill disease (Mitchell *et al.*, 1998) because this disease causes catfish to suffocate as a result of severe damage to the gills. Clinical signs are fish swim listlessly, catfish congregate all together, gills swell and become mottled red and white. Occasionally infection is found in internal organs. In advanced stages, the gill filaments do not lie flat and filaments on one gill arch are not distinct from filaments on other arches. The gills often look mashed and may bleed when touched or when the fish are simply lifted from the water (Mitchell *et al.*, 1998; Griffin, 2011). The number of trophozoite stages in the gills was shown to increase with the severity of the disease (Wise *et al.*, 2008).

2.2.4.3 White spot (ich) disease

The causative agent is *Ichthyophthirius multifiliis and m*ortalities are high in fry stage within five to seven days when water temperatures are 25°-28°C (Dung *et al.*, 2008; Padua *et al.*, 2012). The parasite is capable of killing large numbers of fish in a short period of time. *Ichthyophthirius multifiliis* has four stages namely tophont,

tomont, tomites and theorent in its life cycle (Matthews *et al.*, 1996). These theorants (also called swarmers) swim to a fish host and penetrate the fish epithelium using a penetrating gland and the strong swimming action of their cilia and developing trophont stage in the fish body, then at the stage of tomont they come out from the fish body (Durbow *et al.,* 1998). Clinical signs are flashing behaviour, pin point white spot in entire body surface and in later stages white patches on the skin and fin rot will be evident. White spots were also observed on the gill lamellae, gill slits and oral cavity (Durbow *et al.*, 1998; Padua *et al.*, 2012). The pathogenesis of infestation by *I. multifiliis* involves comprising of the tegument and gills, causing lymphocytic infiltration and focal necrosis, as well as different levels of epithelial proliferation. They diminish the respiratory capacity of the affected fish and depending on the intensity of the parasitism. They give rise to death in the affected fish due to asphyxia in association with opportunistic bacterial infections (Padua *et al.,* 2012).

2.2.4.4 Trematode infestation

2.2.4.4.1 Monogenetic trematodes

Thaparocleidus spp. is a monogenean parasite, and is identified in pangasid catfish from Malaysia. Clinical signs include sloughing of mucus, loss of appetite and haemorrhages (Whittington *et al.*, 2000).

2.2.4.4.2 Digenetic trematodes

The causative agent is cercariae and metacercariae stage of *Bolbophorus damnificus* and the optimum temperature for infestation is above 16°C (Griffin, 2011). It has complex life cycle involving American white pelicans, *Pelecanus erythrorhynchos* (final host) and rams horn snail, *Planorbis rubrum* (intermediate host); catfish (intermediate host). In channel catfish, *B. damnificus* cercariae penetrate the skin and form prodiplostomulum metacercariae in the superficial layers of the musculature (Doffitt, 2009). Haemorrhaging is often associated with cercarial penetration and metacercarial cyst development. In addition, kidney tubule necrosis and kidney inflammation may occur; however, the mechanism of this pathology is unknown (Overstreet *et al.,* 2002). *Bolbophorus damnificus* seemed especially pathogenic to the catfish compared with the other species. Infections of fingerlings could produce death within 5 min when infected by a few hundred individuals of the cercaria (Overstreet and Curran, 2004). Red and swollen gills and loss of appetite are the clinical signs.

2.2.4.5 Disease caused by cestode

A variety of tapeworms are parasitic in freshwater fish, but because their life cycles are complex and require one or two intermediate hosts, tapeworms are relatively uncommon in cultured fish. Adult and larval tapeworms can infect fish with adult infection always occurring in the intestine. Among cystode, some genus causes serious problem for catfish and they are as follows: *Polyonchobothrium clarias* (Barson and Avenant-oldewage, 2006), *Proteocephalus glanduliger, Ligula* and *Bothriocephalus* (AAHRI, 1995). *Lucknowia fossilis* and *Gangesia* sp. have been recorded from *C. macrocephalus* (FAO, 1981).

2.2.4.6 Diseases caused by nematodes (Round worms)

Nematode infections in catfish under natural conditions are very common. Fish are infected with these larvae by ingesting other infected fish. Ingested larvae subsequently migrate from the stomach to the viscera where their numbers can accumulate should the specific fish constantly feed on infected fish (FAO, 1981; AAHRI, 1995). Nematodes have been recorded from the intestine of *Camallanus macrocephalus* and *C. anabantus*. *Procallanus* sp. was isolated from the intestine of *C. batrachus* (FAO, 1981) and its infestation in *C. batrachus* showed histopathological alteration in the kidney on 15, 30, 45 and 60 days post-infection (Ruhela *et al.*, 2008). After 15 days the infected kidney showed variable sized glomeruli, cloudy swelling in tubules, vacuolar/atrophic degeneration, fibrosis, mild degenerative changes in distal convoluted tubules, enlarged Bowmen's capsule, necrotic changes as well as increased granulation and hyperplasia in proximal convoluted tubules. After 30 days of infection, the changes were rupture of Bowmen's capsule wall, degenerative changes, edema, necrosis, pyknosis, karyorrhexis and karyolysis in proximal and distal convoluted tubules, fibrosis, cloudy swelling and inflammatory lymphocytes, proliferation and shrinkage in glomeruli, and vacuolization in proximal convoluted tubules as well as cloudy swelling. After 45 days, the infected kidney showed cloudy swelling in glomeruli as well as variation in their size, infiltration of RBCs in intralobular vein and necrosis in proximal convoluted tubules, cloudy swelling in interstitium, vacuolization in the epithelial lining cells, necrosis in haemopoietic tissue and inflammatory edema. After 60 days post-infection, the changes were rupture of intralobular vein, cloudy swelling, necrosis in few proximal convoluted tubules, atrophy and shrinkage in glomeruli,

distinct inflammatory edema, pyknosis, karyorrhexis and karyolysis, aggregation of lymphocytes and dilation in blood vessels.

2.2.4.7 Myxosporean infestation

Myxobolus baskai and *Myxoblous pangasii* are myxosporeans that parasitise the gill capillaries and splenic serosa, respectively. *Hemigoides berlaudi*, *H. malayensis*, and *H. pangasii*are myxosporeans found on the gills and within the gill arteries and cartilage. *Henneguya shariffi* is also a myxosporean found on the gills. In channel catfish, *Henneguya* infections are broadly categorized with respect to the tissue parasitized and site of spore formation (Mccraren, 1975). Two types of branchial infection are known:

- Intralamellar: Cyst is developed within the lamellae.
- Interlamellar: Cyst is developed between the lamellae.

Myxospore also cause cutaneous infection in the form of,

i. Papillomatous form that causes large tumor-like lesions.
ii. Skin infection that causes formation of cysts on the external body surface.
iii. Rare projection form that localizes solely within the tissue of adipose fin.
iv. Development of cyst in the connective tissue surrounding the mandibular teeth.
v. Development of cyst in the gall bladder.

2.2.4.8 Diseases caused by acanthocephalans

Acanthocephalus sp. has been recorded from *C. macrocephalus*. Whilst in other species, this is generally considered of no significance. The penetration of the "thorny head" on attachment to the intestinal lining may well be a means of infection by enteric pathogens from the water (FAO, 1981).

2.2.4.9 Diseases caused by *Trypanosoma*

The flagellate blood parasites of the *Trypanosoma* species are frequently found in farm reared *P. hypophthalmus* (Dung *et al.*, 2008) and *C. gariepinus* (El-tantawy and El-sherbiny, 2010). *C. gariepinus* naturally infected with trypanosomiasis displayed paleness of the external body surface, emaciation and gasping with dullness, slack appearance and paleness of gills (Osman *et al.*, 2009). Internal examination showed splenomegaly and watery blood with paleness of the internal organs. Infection with *Trypanosoma* in *C. gariepinus* was reported in Turkey (Konas *et al.*, 2008). The parasitic infestation caused hematological changes by the reduction

of red as well as white blood cells. The reductions were highly significant as compared to the healthy sample ($p<0.05$) as noted by the red and white blood cell count, which dropped from $2.14\pm0.48x10^6$ to $1.62\pm0.27x10^6$ cells/ml and from $1.45\pm3.76x10^5$ to $2.42\pm0.78x10^4$ cells/ml blood in the infested samples, respectively (Supamattaya *et al.,* 2005). Similar trend was noted for hemoglobin and hematocrit which dropped significantly. Trypanosoma infection caused significant decrease in *C. gariepinus* blood serum total protein, albumin, globulin levels, albumin/globulin (A/G) ratio and cholesterol concentration. A significant increase in blood serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes activities have been noted (Osman *et al.,* 2009). Histopathological studies on liver, spleen, kidney as well as the gills from trypanosoma infected fish comparing with non infected fish revealed degenerative and infiltrative changes in hepatocytes and in renal tubular epithelium, while spleen showed vascular changes. Gills showed hypertrophy of epithelial cells in secondary lamella (Osman *et al.,* 2009).

2.2.4.10 Ciliate protozoa/ Protozoa infestation

Balantidium and *Ichthyonyctus* species have been found in the large and posterior intestines of juvenile and adult *Pangasius* catfish (Dung *et al.*, 2008). Pathogenesis of these organisms in *Pangasius* is unknown. El-tantawy and El-sherbiny (2010) identified different parasitic protozoa such as *Trypanosoma alhussaini*, *Amphileptus* sp., *Chilodonella hexasticha*, *Vorticella* sp. and *Tetrahymena* sp. from *C. gariepinus*.

2.2.5 Nutritional diseases

2.2.5.1 Jaundice disease

The disease, known as 'catfish jaundice', or 'yellow catfish disease,' was first recorded in central Thailand in early 1990. The severity of the disease outbreaks increased in the following years, with numerous farms reporting up to 100% mortalities in affected ponds (AAHRI, 1993). Fish were usually anorexic and moribund, yellow colouration to the skin and gill. The spleen, kidney and liver were enlarged and had pale-yellow coloration with yellow ascetic fluid accumulation. The gall bladder was also enlarged with yellowish bile. The cause of the disease was thought to be nutritional in origin, due to the feeding of rancid chicken viscera (Chinabut, 2002). The most obvious pathological changes were observed in the liver,

kidney and spleen. Examination of the liver revealed varying irregular vacuolation of the cytoplasm of the hepatocyte (Chinabut, 2002). Two types of pigment were found as deposits in the liver. One was amber in colour and scattered intercellularly throughout the liver parenchyma and also within the lumen of the bile duct and blood vessels. In addition, a lighter yellow-brown pigment, with a more granular texture, was observed accumulated in the macrophages, which were in clumps associated with the portal vessels. Pigment deposits were also widespread throughout the haemopoietic tissue of the kidney and spleen. Large accumulated deposits of yellow-brown pigment were observed within macrophages associated with the melanomacrophage centres of the spleen. Intracellular pigment was observed in individual cells scattered throughout the pulp. Degenerative changes in the cells associated with the melanomacrophage centres were observed in some sections. Kidney sections revealed the presence of amber pigment within the tubule lumen. Deposits of intracellular pigment of varying size were observed scattered throughout the haemopoietic tissue within individual cells and clumps of cells. Protein droplets had accumulated in the epithelial cells lining the renal tubules. Degeneration of the tubule walls was observed in some samples. These pigments were identified as ceroid and haemosiderin. Ceroid is a brown-yellow, acid-fast, lipid-positive pigment, resistant to organic solvents, which accumulates in the livers of fish during lipoid liver degeneration (LLD). Haemosiderin is a breakdown product of haemoglobin (AAHRI, 1993; Chinabut, 2002). Serum from jaundiced fish was bright yellow to orange in colour. Haematocrit levels from jaundiced fish were markedly reduced to 10-16% from normal level of 34-37% (AAHRI, 1993; Chinabut, 2002). Farmers were advised to stop feeding the fish at the first signs of disease and draining (20% of pond water) and replacing with freshwater. After the water change supplementary lime should be added to the pond at the rate of 120-300 kg/ha (AAHRI, 1993).

2.2.5.2 Fatty liver syndrome

Fatty liver syndrome is related to feeding high levels of rice and poor quality trash fish. The liver is unable to metabolize all of the fat taken in through the diet. The fat localizes in the liver, swelling and fatty infiltration of the liver cells, resulting in loss of liver function (FAO, 1981). The affected fish have large livers, often white or bronze in colour, pale gills due to concomitant anemia. Starvation followed by good

quality feeding will usually resolve the problem, but in the worst stages of the disease any handling leads to damage to the liver and possibly fatal haemorrhage.

2.2.5.3 Vitamin C deficiency syndrome

Clarias spp. in the wild generally obtain vitamin C from macrophytes and algae consumed with their prey (FAO, 1981). In the pond situation there is neither prey nor available plant vitamin C because the turbidity of the water prevents photosynthesis and thus any algal or macrophyte growth. Thus levels of vitamin C in the diet must be high to compensate. The three types of vitamin C deficiency are found:

i. Crack head

This is a condition of fast growing fish, where there is failure of ossification and under running of the osseous plates of the occipital bone, where it meets the body and, occasionally, the symphyses of the other skull bones, leaving a very ragged edge to the symphysis. The skin surface above this often becomes infected leading to serious under running necrosis of the skull. Affected fish appear greyish, and there is a dark roughened line where skin lesions occur. Knocking on the skull produces a very hollow sound, and when sections are cut off the skulls of affected fish, they appear less brittle than normal, and there is evidence of delayed calcification or rarefaction of the smaller skull bones, with necrotic inflammatory zones at the sites of secondary infection. The condition appears to be particularly associated with the feeding of boiled skinless rice as a high proportion of the diet and with poor water quality.

ii. Bone rarefaction spinal deformity

This condition results in severe spinal deformity with decalcification of the spinal vertebrate. The X-ray or microscopic examinations, often appear to be deformed or fractured and haemorrhaged. Affected fish grow very slowly and once deformed, even if the diet is improved, will remain thus. The condition has been associated with pelleted foods of known vitamin C deficiency and with poor trash fish, and can be a source of severe loss.

iii. Failure of fibrous wound healing

Vitamin C deficient catfish showed marked loss of efficiency at healing skin ulcers. This is due to failure of the scar tissue collagen to form. Also lead to severe secondary infection which can be fatal.

2.2.6 Environmental disease

2.2.6.1 Brown blood disease

Brown blood disease, also known as nitrite poisoning, is purely related with nitrite content of water (Durbow *et al.,* 1997; Durbow, 2000; Chappell, 2008). Nitrite concentration should be as low as 0.5 ppm to avoid problems (Durbow, 2000). The intensity of the condition can be evaluated by the color of the fish blood. Slightly affected fish have reddish-brown blood, whereas more acutely affected fish have chocolate brown coloured blood (Chappell, 2008). Nitrite, a product of the breakdown of ammonia by bacteria, is a compound that can enter the circulatory system of fish through the gills. Its presence at elevated levels can cause the fish blood to take on a dark brownish color, thus the name "brown blood." Chemically, nitrite can oxidize haemoglobin in the fish red blood cells and convert it into another compound called methaemoglobin. Methaemoglobin does not transport oxygen as is the function of normal haemoglobin and, as a result, affected fish show signs of low oxygen stress even in the presence of saturated levels of dissolved oxygen. The fish are actually suffocating because their blood cannot take up oxygen as is the case normally. Chlorides (Cl-) protect fish from nitrite toxicity. The ratio of chloride to nitrite needed to protect fish is about 10:1 (Durbow, 2000). So minimum of 60-100 ppm chlorides in water minimize the effect of ammonia. The chloride competes with nitrite at the fish gill surface for absorption (Chappell, 2008).

2.2.6.2 Non-infectious dropsy

The affected fish have swollen abdomen containing clear fluid, not yellow as in infectious dropsy. There is a change in the kidney tissues which indicates a malfunction of the fluid balance mechanism, as a result of which the fish are unable to release excess fluid from their bodies (AAHRI, 1995). The major predisposing factor to this disease is believed to be water pollution caused by high density of fish, overfeeding resulting in decaying food on the pond bottom and insufficient water changes. Under these conditions the amount of ammonia in the pond increases, affecting the gills and the filtration system of the kidney. Treatment of this disease is

difficult and can take from 7 to 10 days. Sick fish should be treated in 0.3 - 0.5% salt (3 - 5 kg of salt per 1000 l or 1 m^3 of water) for three days. The water should then be changed and the same amount of salt applied again. This procedure should be repeated until the fish are cured but it is only effective in the early stages of dropsy. As usual during treatment, the daily ration should be reduced and only live *Moina* sp. fed (AAHRI, 1995).

2.2.6.3 Gastro-intestinal blockage

Mainly fry stages of catfish are affected. Dead fish have swollen abdomen and normally more than half of the sick fish will die with a stomach full of food (AAHRI, 1995). Examination of fish with this disease reveals undigested planktonic diatoms in their stomach and intestines. The planktonic diatoms are associated with fry mortalities. Catfish fry affected by this disease have likely died due to diatoms blocking the digestive tract of fish. As yet there is no effective treatment for this disease. The best method is preventative, i.e., controlling the types of phytoplankton used for *Moina* culture.

2.2.6.4 Anorexic syndrome

Juveniles are mostly affected. Fish congregate at a place. The affected fish are darker than usual. Abdomen and body become thin. This disease often occurs at the beginning of the artificial breeding season for catfish hybrids, at this time some female brood stock are not yet mature, they are however injected with hormones to stimulate and increase the maturity of the eggs (AAHRI, 1995). Fry born from these immature female brood stocks are more likely to be weak, grow slowly and have dark, thin bodies. There is no means of curing these fry and most of them will die. Those that do survive will grow very slowly.

2.2.6.5 Visceral toxicosis of catfish

This syndrome is related to the toxin - botulium type E (Griffin, 2011). Mostly it is observed in early spring. Infected fish will show schooling on edge of bank. The signs include barbels quivering or muscle fasiculations, intestinal or gastric mucosa in oral cavity, chylous effusion, pale proximal intestinal tract with prominent blood vessels (congestion), intussusceptions, congested spleen and reticular pattern in liver.

2.3 Disease management

2.3.1. Antibiotics

Antibiotics are chemical substances produced by microorganisms that inhibit the growth of or kills other microorganisms. Durbow *et al.* (1998) reported that external and internal columnaris infections successfully treated with oxytetracyline HCl (Terramycin) medicated feed administered at 25 - 37.5 mg of active ingredient/pound of fish for 10 days. Antibiotics that have proven effective to control enteric septicaemia of catfish include oxytetracycline HCL (Terramycin, Pfizer) 16 mg active ingredient/kg of fish for 10 days, a combination of sulfadimethoxine and ormetoprim (Romet 30, Hofmann–LaRoche) at 50 mg/kg fish for 5 days and florfenicol (Aquaflor, Schering Plough) at 10 mg/kg fish for 10 days (Hawke *et al.*, 1998; Chappell, 2008). Outbreaks of *E. tarda* can be controlled by feeding terramycin at the rate of 2.5-3.0 g/100 lb of fish/day for 10 days (Bullock and Herman, 1985). Orbifloxacin, concentrations up to 50 mg/l in water, can control the *E. tarda* infection in *C. gariepinus* (Ibrahem *et al.*, 2010). *Aeromonas* infection of catfish had been treated with antibiotics (sulfamethoxazole + trimethoprim) or florphenicol or cefalexin (Ly *et al.*, 2009).

2.3.2. Herbal products

Antibiotics, vaccines and several other chemicals have been used in aquaculture operations as a remedy of various diseases. Even though they give positive effects, they have residual and other side effects. The alternative option is herbal bio-medicinal products in the aquacultural operations that have the characteristics of growth promoting ability and tonic to improve the immune system, act as appetite stimulators. In most cases, phenolics, polysaccharides, proteoglycans and flavonoids play a major role in preventing or controlling infectious microbes (Citarasu, 2010). Shangliang (1990) reported the antimicrobial activity of five Chinese herbal extracts, *Stellaria aquatica*, *Impatiens biflora*, *Oenothera biennis*, *Artemisia vulgaris* and *Lonicera japonica* against 13 bacterial fish pathogens where *A. salmonicida* and *E. ictaluri* were the most sensitive to these extracts. Ali *et al.*, 2014 reported that different herbs like *Tamarindus indica, Citrus aurantifolia, Terminalia bellirica, Terminalia chebula, Spondius pinnata* can resist the growth of *E. tarda.*

Ekanem and Obiekezie (2013) reported that extracts of *Carica papaya* and *Mucuna pruriens* were effective in the treatment of ichthyophthiriasis with high host

tolerance and ectoparasitic monogeneans were effectively dislodged from the gills and skin by the application of extracts of *Piper guineense* and *Artemesia annua* in *C. gariepinus, Phylanthus amarus*, *Allium sativum*, *A. annua*, Citrus lemon have the effective antibacterial activity against *Aeromonas* and *Pseudomonas* infection. Ginger, *Zingiber officiale* is effective to treat the ectoparasitic protozoa *Trichodina* and *Epistylis* spp. at dose 20 mg/l (Abo-Esa, 2008; Citarasu, 2010).

2.3.3 Vaccines and immunostimulants

Vaccination

Vaccnination in aquaculture sector is very common, that reduce the improper use of antibiotic that produces resistant bacteria and also has residual effect on the product quality and human health (Dung, 2011). In the catfish industries of USA, vaccines are available for enteric septicaemia of catfish and columnaris disease. The vaccine AQUAVAC-ESC was used for the prevention of ESC disease caused by *E. ictaluri* (Shoemaker *et al.*, 2002). An attenuated mutant of *E. ictaluri* (RE-33) induced by passage in the presence of rifampicin has been licensed for use as a vaccine named Aquavac-ESC, Intervet and has been shown to provide protection when given at 7 days of age (http://www.baphiq.gov.tw/public/ Attachment/8123017304571pdf). The vaccine AQUAVAC-COL was used as an aid in the prevention of columnaris disease caused by *F. columnare* (Bebak and Wagner, 2012). In Vietnam, bacillary necrosis of pangasius was the most common disease caused by *E. ictaluri.* The vaccine ALPHAJECT$^{(R)}$ Panga 1 produced specific immune response against *E. ictaluri* (Dung, 2011).

Immunostimulants

Immunostimulant can activate the function of immune system even in immunosuppressive states caused by various toxins, adverse physic-chemical parameters, stressors or pathogens, then reverse the deleterious effect of stress (Anderson1992, Sakai 1999, Sahoo and Mukherjee, 2003). Common immunostimulants are lactoferrin, yeast β 1-3 glucan, levamisole hydrochloride and vitamin C (Kumari and Sahoo, 2006). Kumari and Sahoo (2006) used levamisole at 50 mg/kg feed for 10 days as an immunostimulant in Asian catfish (*C. batrachus*). Injection of β-1,3 glucan in channel catfish, *I. punctatus* greatly reduced the mortality from experimental infection with *E. ictaluri.* Anterior kidney of fish, receiving glucan had enhanced phagocytic and bactericidal ability (Chen and Ainsworth, 2006). Erazo-

pagador and Din (2001) reported that Ascorbic acid (vitamin C) @ 0.10-0.70 g/100 g feed promote specific growth rate, feed conversion ratio and enhance the wound healing mechanism. Because Ascorbic acid enhance the biosynthesis of collagen, that promote the wound healing mechanism (Ashley *et al.*, 1975). Kumer *et al.* (2014) reported that fish fed with *Ocimum tenuiflorum, Zingiber officinali* and *Allium sativum* showed resistance against *A. hydrophila.* Copper at a level of 0.01 mg/l protected fish from many different pathogens (http://chowan.ces.ncsu.edu/2009/08/copper-as-an-immunostimulant-for-fish-2/)

2.3.4 Best Management Practice (BMP) for grow-out

BMP's are defined as the management of activities to achieve an ongoing minimization of the activities' environmental harm through cost-effective and continually assessed measures. Best Management Practice (BMP) guideline should be used as the baseline to develop an environmentally sound and sustainable aquaculture sector (Hinrichsen, 2007). The BMPs relate to all aspects of fish farm design, operation, management, record keeping, environmental monitoring/ reporting and include rapid response plans for emergencies.

Clustering of farms as units in the management of common resources greatly enhances the results of application BMPs, leading to beneficial impacts on the individual farms, which could not have been achieved if, functioned individually. The second most important aspect of the application of BMPs is accurate record keeping on all aspects of the farming practices, including water quality criteria. Record keeping, though a cumbersome process and its use and application is not immediately evident, is the key to finding answers and solutions when calamities occur (Phoung *et al.*, 2011). Quality and quantity of available water are primary considerations for a production facility. A ground water source is preferred (Lewis and Shelton, 1994), as it reduces the risks of disease outbreak (Munro and Roberts, 1989). Using surface waters such as rivers and streams can introduce unwanted fish, parasites or diseases into production ponds (Kabata, 1985; Lewis and Shelton, 1994). Also, most surface waters vary seasonally in quality and quantity. Preferably, food fish production ponds should have a filling time of 10 days or less. Ponds used to grow stocker fish (fingerlings) should fill in five days or less (Durborow, 2000). Soils should be bored to check for quality (Masser *et al.*, 2005) and remove contaminated soil or incorporate

it into the outside of the levees so it will not pose a problem to catfish production (Masser *et al.*, 2005).

Pond preparation is essential to reduce risks of disease outbreaks, to obtain a healthy environment for growth of stock, and therefore attaining better overall productivity (De Silva and Dung, 2011). Ponds must be prepared for stocking by removing the existing fish populations because most pond fish, including sunfish and large shiners, compete with catfish for food and oxygen (Lock and Steinbach, 2011). The pond preparation duration should be sufficient enough to facilitate solar oxidation by allowing the pond bottom to dry and crack to a depth of 2-5 cm (Phoung *et al.*, 2011). Villalon (1991) and Kongkea, (1995) suggested a minimum period of 30 days for the pond preparation. Boyd and Tucker (1998) suggested pond preparation period of 15-45 days.

Monitor pond water quality and record keeping for water quality

Component	Recommended value or range
Dissolved oxygen	3 ppm or more in top 3 feet of water
Carbon dioxide	less than 20 ppm
pH	6 - 9.5
Total alkalinity	20 ppm or more
Total hardness	20 ppm or more
Un-ionized ammonia	less than 0.05 ppm
Nitrite	less than 0.5 ppm
Temperature change	less than 5 °F as rapid change (Efficient at 80-85°F)

Feeds containing 28-32% crude protein are adequate and the most economical (Masser *et al.*, 2005). The best times to feed catfish are between mid-morning and mid-afternoon (Phoung *et al.*, 2010). A maximum of 4% of body weight/day from the early stages to about 50-80 g/fish, and reduce the feeding rate as, to about 1-1.5% body weight/day (Phoung *et al.*, 2010). In general, twice a day feeding is necessary (Phoung *et al.*, 2010) and fingerlings should be fed 2 and 5 % of their body weight/day, and for broodfish, it is 1 to 2% of their weight (Chapman, 2009). During winter, feeding should be done at 1% body weight, when the temperature is at 12°C

and it should not be fed at temperatures of 10°C or less (Dorman and Torrans, 1987; Steeby and Brunson, 1997; Tucker, 1985).

In aquatic environment cure of disease outbreak is very difficult as pathogen spread rapidly through water, so if its entry can be prohibited then disease can be prevented. Management practice should be proper and BMP will be very effective in this aspect. BMPs will be most effective when adopted and operated through a cluster/ Associations system approach and it along with disease prevention promotes the growth and production of farming system.

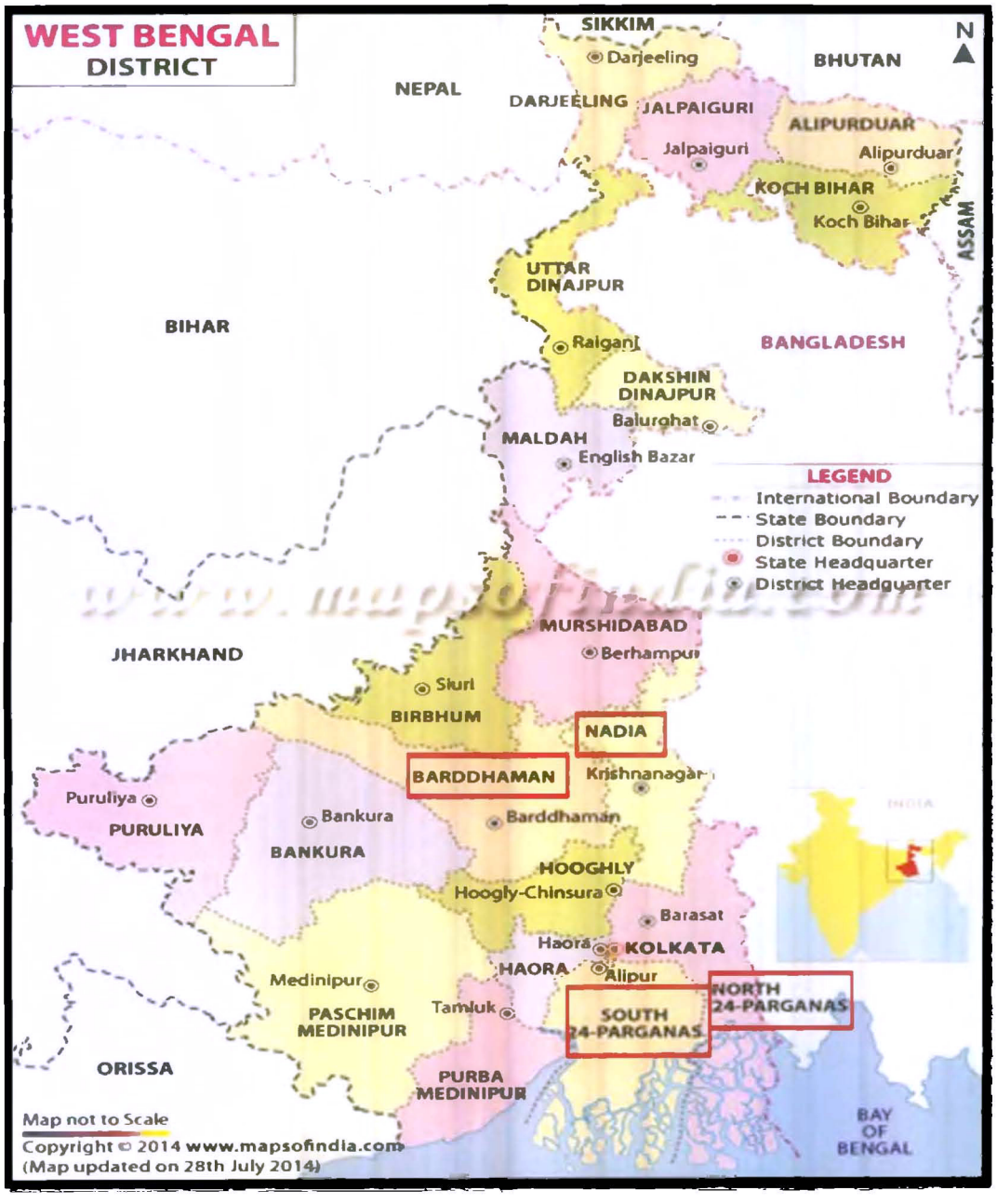
CHAPTER -3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Sampling area

Healthy and diseased catfish samples for this study were collected from different fish farms located in Garia and Canning of South 24 Parganas district, Deganga, Basirhat, Ramchandrapur and Naihati of North 24 Parganas district, Satyapole of Nadia district and Memari of Burdwan district, West Bengal, India. The locations of the sampling areas are presented in Table 1.



3.2 Materials

3.2.1 Sampling materials

Fish sampling sheets, leaflet of diseased fish and pathogens, vials, marker pen, dissection set, transport media, sterile swabs, slide, cover slip, alcohol, bouin's fixative, gloves, cotton, tissue paper, rubber band, cast net and camera for documentation were carried. Information on farming system, source of seeds, health status, use of organic or inorganic products, chemicals and drugs were gathered and properly documented in questionnaire.

3.2.2 Experimental fish

The experimental catfish of the present study are *Clarias batrachus, Clarias gariepinus* and *Pangasius pangasius,* cultured in semi-intensive farms of the above mentioned areas.

3.2.3 Microbiologial media

The bacteriological media used in this study include tryptone soya agar (TSA), tryptone soya broth (TSB), *Edwardsiella ictaluri* agar (EIA), *Edwardsiella ictaluri* broth (EIB), *Aeromonas* isolation agar (AIA), brain-heart infusion agar (BHIA), brain-heart infusion broth (BHIB), *Pseudomonas* isolation agar (PIA), Rimler-Shotts agar (RSA), glutamate starch phenol red agar base (GSPA), Mueller Hinton agar (MHA), transport media such as Hiculture™ transport swabs w/Amies medium w/charcoal and Hiculture™ transport swabs w/enteric pathogen transport medium. The above mentioned media except EIA and EIB were procured from Hi-Media, Mumbai (India). The *Edwardsiella ictaluri* medium, either as agar or broth, was prepared by mixing the individual components described in Shotts and Waltman II (1990). Colistin sulphate (Hi-Media, Mumbai) was added in to the sterile basal medium at a level of 10-µg/ml before pouring in to the sterile Petri plates. The media composition and antibiotic supplements used in culture media are presented in Annexure. Various other media for biochemical identification of bacteria were prepared with required ingredients as per the standard procedures (Collins *et al.,* 1989). The mycological media used in this study include corn meal agar (MEA) and rose bengal chloramphenicol agar - RBCA (Hi-Media, 2009). Physiological saline (0.85% (w/v) sodium chloride NaCl) solution was used as diluent for the bacterial enumeration and suspending bacterial cells. The pH of the media was adjusted to the

required level using 0.1 N sodium hydroxide (NaOH) solution or 0.1 N hydrochloric acid (HCl) solutions before sterilization.

3.2.4 Reagents and kits for electrophoresis, histopathology and molecular characterization

Reagents and chemicals used for electrophoresis and histopathology were procured from Hi-Media, Mumbai, India, Bioscience, Merck Millipore India Pvt Ltd, Mumbai, India and Stanbio Reagent Pvt. Ltd., Kolkata, India. Molecular grade chemicals, reagents and kits were procured from national and multinational companies such as Hi-Media, Mumbai, India, Bioscience, Merck Millipore India Pvt Ltd, Mumbai, India, Invitrogen Bioservices India Pvt. Ltd., Bangalore, India, Sigma Aldrich Chemical Co. Ltd., USA, Macherey-Nagel, Germany, Takara Biotech, Japan and Merck Biosciences, Germany.

3.2.5 Primers

Published universal primers were used for the amplification of 16S rDNA gene of bacterial isolates. The names of the primers, sequence and amplification size are given below:

Primers	Sequence (5'-3')	Amplification size	Reference
8F	AGAGTTTGATCCTGGCTCAG	1400 bp	Eden *et al.*, 1991
1492R	TACGGYTACCTTGTTACGACTT		

3.2.6 Antibiotic discs and sterile swabs

Antibiotic impregnated discs, viz., chloramphenicol (30 µg), ciprofloxacin (5mcg), clindamycin (2 mcg), gentamicin (10mcg), nitrofurantoin (300mcg), oxytetracycline (30 µg), co-trimoxazole (25mcg), vancomycin (30mcg), sulphafurazole (300mcg), gatifloxacin (5mcg), amoxyclav (30mcg) and erythromycin (15mcg), and sterile transport swab (Hiculture collecting device) on wooden stick (length 150 mm) were procured from HiMedia, Mumbai.

3.2.7 Minimal inhibitory concentration strip

HiComb MIC strips (HiMedia, Mumbai) were used to determine the minimal inhibitory concentration (MIC) of antibiotics such as chloramphenicol, ciprofloxacin,

gentamicin, nitrofurantoin, tetracycline and co-trimoxazole. The HiComb MIC strip was made with 8 extensions that carry the 4 mm discs of defined concentration of antibiotic, resembling the tooth of a comb. Each antibiotic consisted a continuous range of 16 two-fold dilutions on 2 different strips (Part A and B). Towards the stem of the strip MIC reading scale is given. This system provided a set of 16 different concentrations as follows: chloramphenicol (A: 240-0.01; B: 8-0.001), ciprofloxacin (A: 240-0.01; B: 2-0.001), gentamicin (A: 240-0.01; B: 5-0.001), nitrofurantoin (A: 240-0.01; B: 30-0.001), tetracycline (A: 240-0.01; B: 5-0.001) and co-trimoxazole (A: 240-0.01; B: 4-0.001).

3.2.8 Experimental fish for pathogenicity experiment

Pangasius pangasius of weight 57.33±6.11 g and length 20.05±1.49 cm and *Clarias gariepinus* of weight 22.69±2.49 g and length 13.92±0.82 cm were procured from grow-out farms in Garfa, South 24 Parganas district and Naihati, North 24 Parganas district, West Bengal, respectively.

3.3 Methods

3.3.1 Sterilization

Bacteriological media, biochemical test media, autoclavable labwares and glasswares were sterilized in an autoclave at 121°C for 15 min unless otherwise specified. Glass Petri plates, test tubes, pipettes, etc were sterilized in a hot air oven at 180°C for 2 h. Some specific media for bacteriological and biochemical tests were sterilized by steaming for an hour.

3.3.2 Sampling

On each sampling day, a minimum of 60 catfish were examined for diseases of irrespective species and size as per OIE guidelines (OIE, 2013). Informations like behavioural abnormalities, gross and clinical signs were recorded on the sampling sheet. Catfish with typical disease symptoms were sampled for bacteriology and histopathology. Clinical healthy catfish were also sampled for histology. Tissue samples of affected as well as healthy catfish were fixed in Bouin's fixative for histopathology. Tissues and/or inocula from affected external and internal parts of catfish were transferred in to the sterile transport media such as Hiculture™ transport swabs w/Amies medium w/charcoal and Hiculture™ transport swabs w/enteric pathogen transport medium (Hi-Media, Mumbai, India) and brought to the laboratory

in an insulated container. Morbid fish samples were also brought to the laboratory from nearby farms in oxygen filled polythene bags for analysis.

3.3.3 Bacteriological analyses

At the laboratory, inocula from each transport media or from the lesions or affected external and internal parts such as gills, intestine and kidney of morbid fish were streaked on to BHIA, PIA, EIA, GSPA, RSA and AIA plates, and incubated at 30±2^{0}C for 24-48 h. Inocula from each transport media or from morbid fish were also inoculated into the EIB and incubated at 30±2^{0}C for 12-16 h. Bacterial growth in the EIB makes the medium condensed and changes the colour from green to gray. After 12-16 h of incubation in EIB, loopful of inacula from each sample was streaked on to EIA plates and incubated at 30±2^{0}C for 24-48 h.

On EIA, both *Edwardsiella ictaluri* and *E. tarda* produce 0.5-1.0 mm green translucent colonies after 48 h. *Proteus* sp. produce 2-3 mm brownish green colonies that might swarm. *Serratia marcescens* produce 2-3 mm reddish colonies; *Aeromonas hydrophila* produce 2-5 mm yellowish-green opaque colonies; and *Yersinia ruckeri* produce 1-2 mm yellowish green colonies. Enterococci capable of growing on EIA produce tiny (approx. 0.5 mm) yellowish colonies (Shotts and Waltman II, 1990). Typical and distinct green translucent colonies of 0.5-1.0 mm size were picked randomly from EIA plates, purified by repeated streaking on TSA plates and maintained on TSA slants with proper markings.

On AIA, *Aeromonas* produce 0.5-3 mm dark green, opaque with dark centre, convex, glossy colonies after 18-24 h. *Pseudomonas* produce 0.5-1 mm blue/gray, translucent, convex, glossy colonies after 18-24 h. Typical and distinct dark green, opaque with dark centre colonies of 0.5-3.0 mm size were picked randomly from AIA plates, purified by repeated streaking on TSA plates and maintained on TSA slants with proper markings.

On RSA, *Aeromonas* produce 0.5-3 mm light yellow to light green, convex, glossy colonies after 18-24 h. *Citrobacter* produce 1-3 mm circular, convex, off white black centered colonies after 48 h incubation. *Proteus* produce 2-3 mm yellow colour, black centred, swarming colonies after 18-24 h. Typical and distinct light yellow to light green colonies of 0.5-3.0 mm size were picked randomly from RSA plates,

purified by repeated streaking on TSA plates and maintained on TSA slants with proper markings.

On GSPA *Aeromonas* produce 0.5-3 mm yellow, circular, convex, glossy colonies after 48-72 h. *Pseudomonas* produce 0.5-1 mm pink, convex, glossy colonies after 48-72 h. Typical and distinct yellow colonies of 0.5-3.0 mm size and pink colour colonies of 0.5-1mm were picked randomly from GSPA plates, purified by repeated streaking on TSA plates and maintained on TSA slants with proper markings.

On PIA, *Pseudomonas* produce 0.5-1 mm greenish yellow to blue-green, circular, convex, glossy colonies after 18-24 h.

3.3.4 Bacterial isolation and phenotypic characterization

Based on the dominance and definite colony morphology, representative colonies were picked from each plate and purified by repeated streaking on TSA and maintained on TSA slants. A series of biochemical reactions as described by Lechevallier *et al.* (1980) and Collins *et al.* (1989) were performed to identify bacteria up to genus level. Taxonomic keys proposed by Arcos *et al.* (1988) and the current literatures on *Aeromonas* spp. were followed for *Aeromonas* identification (Minana-Galbis *et al.*, 2009; Alperi *et al.*, 2010; Figueras *et al.*, 2011; Austin and Austin, 2012). Shewan *et al.* (1960), Bergey's manual (Holt *et al.*, 1994) and Collins *et al.* (1989) were consulted for *Pseudomonas* identification. Fisheries and Oceans Canada (2004) was consulted for *Edwardsiella* identification. Taxonomic keys proposed by University of Idaho, USA as per Bergey's Manual of Determinative Bacteriology (http://www.uiweb.uidaho.edu/microbiology/250/IDFlowcharts .pdf) were also consulted for the identification bacterial species. Identification of select bacterial isolates on the basis of biochemical characterization was done by an automated bacterial identification system (VITEK 2 - compact, BioMerieux, France).

3.3.5 Molecular characterization

3.3.5.1 Bacterial DNA extraction and PCR amplification of 16S rDNA gene

Genotypic characterization of select bacterial isolates was done by 16S rDNA sequencing. The genomic DNA of bacterial isolates was extracted by using Genomic DNA isolation kit (Macherey-Nagel, Germany) as per the manufacturer's protocol. The 16S rDNA gene was amplified through PCR reaction that was performed in a gradient thermal cycler (Eppendorf, Germany). The universal primers (forward primer

8F 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 1492R 5'-ACG GCT ACC TTG TTA CGA CTT-3') of amplification size 1400 bp were used. The reaction was carried out as below:

2X PCR TaqMixture (HiMedia, Mumbai)	12.5 µl
Forward primer 8F (10pMole/µl)	1.0 µl
Reverse primer 1492R (10pMole/µl)	1.0 µl
DNA template (50 ng)	1.0 µl
Molecular biology grade water	9.5 µl
Total reaction volume	25 µl

The PCR components were mixed and spinned shortly. The PCR reaction was set with the amplification condition as mentioned below. A total of 35 amplification cycles were performed.

Initial Denaturation		Denaturation		Annealing		Extension		Final extension	
95°C	2 min	94°C	45 sec	55°C	1 min	72°C	1 min	72°C	10 min
1 cycle				35 cycles				1 cycle	

3.3.5.2 Agarose gel electrophoresis

The PCR products were analysed on 1.2% agarose (Hi-Media, Mumbai) gels containing 0.5 µg/ml ethidium bromide in 1X Tris-acetate- EDTA (TAE) buffer.

3.3.5.3 DNA sequencing and analysis

The PCR amplified products were sequenced at the Genomics Division, Xcelris Labs Ltd, Ahmedabad, India. The consensus sequence was done with DNA Baser sequence assembly software version 4.20 (www.DnaBaser.com). Phylogenetic analysis was performed on a selection of 16S rRNA gene sequences that comprised the new sequence and closely related sequences determined by Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov). Data analysis and multiple alignments were performed by using ClustalX (MEGA5). Evolutionary trees for nucleotide sequences were drawn by using the neighbor-joining method with 1000 bootstrap replicates (Saitou and Nei, 1987).

3.3.6 Mycological analyses

At the laboratory, inocula from each transport media or from the lesions or affected external and internal parts such as gills, intestine and kidney of morbid fish were streaked on to CMA and RBCA plates, and incubated at 30±2^{0}C for upto 14 days.

3.3.7 Antibiogram

A total of 48 bacterial strains were screened for their sensitivity to 12 potential antibiotics by agar disc diffusion technique (Bauer *et al.*, 1966). Young cultures of bacteria (20 h old) from TSA slants were inoculated individually into TSB and incubated for 10-12 h at 30±2°C. Inocula from these 10-12 h grown cultures were taken separately using sterile cotton swabs and spread on to MHA plates. Antibiotic impregnated discs were placed aseptically on to the inoculated agar plates at least 15 mm away from the edge, at equal distance and sufficiently separated from each other to avoid overlapping of the zone of inhibition. The plates were then incubated for 24 h at 30±2°C and the diameter of zone of inhibition in mm was measured. Interpretation of sensitivity was based on the zone size interpretation chart provided by the manufacturer of the antibiotic impregnated discs.

3.3.7.1 Determination of resistance profile, multiple antibiotic resistance (MAR), MAR index and resistance pattern

The resistance profiles and resistance patterns for 12 potentially valuable antibiotics, viz., chloramphenicol, ciprofloxacin, clindamycin, gentamicin, nitrofurantoin, oxytetra-cycline, co-trimoxazole, vancomycin, sulphafurazole, gatifloxacin, amoxyclav and erythromycin were determined from antibiogram data. Multiple antibiotic resistance (MAR), i.e., (resistant to at least three antibiotic) and MAR index were calculated as per Orozova *et al.* (2010).

3.3.7.2 Determination of minimal inhibitory concentration (MIC)

The MICs of six antibiotics such as chloramphenicol, ciprofloxacin, gentamicin, nitrofurantoin, tetracycline and co-trimoxazole were determined against eight *Edwardsiella tarda* strains and one strain each of *Aeromonas sobria* and *Citrobacter ferundii* using HiComb MIC strips (Hi-Media, Mumbai) as per the manufacturer's instruction. The pure culture of bacteria were inoculated into TSB separately and incubated at 30±2°C for 2-8 h, until light to moderate turbidity

develop. Inocula taken through sterile cotton swabs were spread on to separate MHA plates. After 15 min of inoculation, the HiComb MIC strips were placed on the agar surface with the MIC scale facing upwards so that antibiotic discs should make a contact with the bacteria. The plates were incubated at 30±2°C for 18-24 h and observed for the zone of inhibition. The zone of inhibition will be in the form of an ellipse. The MIC value would be the value at which concentration the zone convenes the comb like projection of the strips. If there is no zone of inhibition observed, the MIC value is greater than the highest concentration on the strip. If the zone of inhibition is below the lowest concentration then MIC value is less than the lower concentration of the strip.

3.3.8 Pathogenicity of *Edwardsiella tarda* on *Pangasius pangasius* and *Clarias gariepinus* by intramuscular injection

Healthy catfish, as in section 3.2.8, were brought to the Faculty of Fishery Sciences, Chakgaria, disinfected with 5 ppm potassium permanganate for 15 min and stocked in 500 litre capacity fiberglass reinforced plastic (FRP) tanks containing 100 litre clean bore well water. The fish were acclimatized for about two weeks and during this period they were fed with commercial pellet feed (CP9931, CP Pvt. Ltd., Andhra Pradesh, India) at the rate of 2% body weight. The healthy fish were selected, released into the experimental tanks at the rate of 6 or 8 fish/tank and acclimatized for about 3 days.

3.3.8.1 Preparation of bacterial cell suspension

The *E. tarda* strain CGH9 maintained on TSA slant was streaked on to TSA plate and incubated at 30±2^{0}C for 24 h to get young culture. One young discrete colony was aseptically picked, transferred to 10 ml TSB and incubated at 30±2^{0}C for 24 h. Mass culture was done in 300 ml TSB at 30±2^{0}C for 24 h and centrifuged at 7500 rpm at 20^{0}C for 20 min. The cell pellet thus obtained was washed thrice with physiological saline and finally resuspended in 5 ml saline. The number of bacterial cells in suspension was determined by spread plating on TSA.

3.3.8.2 Experimental challenge test

For experimental challenge test with *P. pangasius* (weight 57.33±6.11 g and length 20.05±1.49 cm), ten numbers of glass aquaria of size (60L x 30H x 30W) cm were selected, disinfected, cleaned and dried for a week. All the tanks were filled with

clean bore-well water and labelled as per the dilution injected and control. The fish were introduced at rate of 6/tank containing 30 litre water. All the tanks were covered with nylon netting for adequate protection. Aliquots (0.1 ml) of *E. tarda* CGH9 cell suspensions from 10^{0} to 10^{-3} dilutions were injected intramuscularly beneath the dorsal fin region, in such a way so as to get 10^{9}-10^{6} cells/fish. Control fish received sterile saline. The challenged fish were maintained in their respective tanks for 22 days and fed daily with commercial pellet feed on demand. Observations on mortality, external signs of infections, cannibalism and behavioural changes were recorded daily.

For experimental challenge test with *C. gariepinus* (weight 22.69±2.49 g and length 13.92±0.82 cm), ten numbers of glass aquaria of size (60L x 30H x 30W) cm were selected, disinfected, cleaned and dried for a week. All the tanks were filled with clean bore well water and labeled as per the dilution injected and control. The fish were introduced at rate of 8/tank in 30 liter water. All the tanks were covered with nylon netting for adequate protection. Aliquots (0.1 ml) of *E. tarda* CGH9 cell suspensions from 10^{-1} to 10^{-4} dilution were injected intramuscularly beneath the dorsal fin region, in such a way so as to get 10^{9}-10^{6} cells/fish. Control fish received sterile saline. The challenged fish were maintained in their respective tanks for 22 days and fed daily with commercial pellet feed on demand. Observations on mortality, external signs of infections, cannibalism and behavioural changes were recorded daily.

3.3.9 Histopathology

The different organs of diseased and normal catfish were fixed in alcoholic bouin's fixative for 48-72 h. After fixation the tissues were transferred to 70% ethyl alcohol and kept overnight. Histopathological analysis was made as described by Roberts (2001) and the details of which are available in Annexure.

3.3.9.1 Tissue cutting and processing

Different tissues were taken out of the 70% alcohol and cut into small pieces. Then they were dehydrated by a series of ethyl alcohol with a gradually increasing concentration (85%, 90% and 100%). In each alcoholic concentration tissues were kept for 90 min with two changes. The tissues after dehydration were transferred to

xylene to make them transparent. Then the tissue was kept in liquid paraffin for at least 2 h 30 min. The paraffin was allowed to penetrate into the tissues.

3.3.9.2 Tissue embedding

Triple filtered matured paraffin (melting point 58-60°C) was used for this purpose. The organs were kept in melted paraffin contained in L-mould and the paraffin was allowed to solidify.

3.3.9.3 Preparation of blocks for sectioning

The solid paraffin blocks containing tissues, was trimmed into small square blocks and the tissues were exposed for proper sectioning.

3.3.9.4 Sectioning

The trimmed blocks were then fixed to the block holder of the microtome (Medimeas Model: MRM-RM 1191) and cut into sections or ribbons of 5-µm thickness. Good sections were carefully transferred to clean grease free glass slide. The grease free slides were layered with Mayer's albumin for better attachment of the tissues to the slides.

3.3.9.5 Stretching of tissues

The slides with ribbons of required lengths were transferred on hot plate containing warm water maintained at 50 - 55°C to render the wrinkled tissues stretched and flat.

3.3.9.6 Preparation of slides and staining

The dried slides were stained by haematoxyline and eosin staining (H&E) method described by Roberts (2001). Slides were permanently mounted using DPX (Dibutyl Phathalate Xylene) mountant.

3.3.9.7 Microscopy and photomicrography

The sections were screened with the help of monitor attached microscope. Colour microphotographs were taken from the selected slides at different magnification with advanced Trinocular Research Microscope (Olympus, Japan, Model: BX51).

3.3.10 Polypeptide profile of outer membrane protein of *Edwardsiella tarda*

3.3.10.1 Preparation of outer membrane protein (OMP)

The outer membrane protein antigen from *E. tarda* was prepared following the method of Chakraborty *et al.* (1982) with some modifications. The young culture of *E. tarda* was transferred to 300 ml TSB and incubated at 30±2^{0}C for 24 h. The cells were harvested by centrifugation at 7500 rpm for 20 min at 20°C. The cell pellet was washed and finally resuspended in 5 ml saline. This suspension was treated with 2% sodium dodecyl sulphate (SDS) and 2% mercaptoethanol for 20 min at 60°C for solubilisation. The extracts were then centrifuged at 7500 rpm for 20 min at 4°C and the supernatant was filtered, dialysed and concentrated. The filtrate obtained was stored at -20°C until further use. The protein concentration was estimated by the method of Lowry *et al.* (1951).

3.3.10.2 Polypeptide profile of OMP by SDS-PAGE

The OMP of *E. tarda* was analyzed by SDS-PAGE according to Laemmli (1970) using 12.5% polyacrylamide gel in a vertical mini slab gel electrophoretic apparatus. The samples were mixed with sample buffer in a proportion of 1:1 and subsequently the solution was heated at 100°C for 2 min. The amount of proteins applied was 50 µg/track.

CHAPTER -4

RESULTS

4. RESULTS

4.1 Details on the clinical signs on diseased catfish

Diseased catfish samples were collected from Garia and Canning of South 24 Parganas district, Deganga, Basirhat, Ramchandrapur and Naihati of North 24 Parganas district, Satyapole of Nadia district and Memari of Burdwan district, West Bengal, India. The details of location, species affected and their size and gross and clinical signs are tabulated in Table 1. The gross and clinical signs observed were sluggish behaviour, erratic movement, hanging, lethargy, anorectic, sliming, skin erosion, foul smell, pale gills, dull and red colouration of eyes, red lateral line, fin rot, tail rot, ulcer, dropsy, yellow fluid accumulation, abdominal haemorrhage, mouth and opercula haemorrhage, pectoral fin haemorrhage, discoloured and of liquefied internal organ. Haemorrhage and dropsy were the most common in all diseased catfish, but the severity of dropsy was more on *C. gariepinus*.

4.2 Bacteriology

4.2.1 Bacterial flora associated with diseased catfish

The bacterial flora associated with diseased catfish are presented in Table 1. *Edwardsiella tarda* and *Aeromonas* spp. were identified as the major associated flora of the diseases of cultured *C. gariepinus* in Deganga and Naihati, 24 Parganas North district. The diseased *C. gariepinus* of Basirhat was infected mainly with *Aeromonas* sp. Besides these, *Pseudomonas* sp., *Citrobacter freundii*, Entero-bacteriaceae and other bacterial flora like Gram positive rods and cocci were identified with the clinical signs of sluggish behaviour, vertical movement, anorectic, dropsy, yellow fluid accumulation, foul smell, focal cutaneous haemorrhage, pectoral fin haemorrhage, abdominal haemorrhage, ulcer, discolouration of internal organs, etc.

In *C. batrachus* of Canning, 24 Parganas South district and Memari, Burdwan district, motile *Aeromonas* spp., Enterobacteriaceae, *Pseudomonas* spp. and Gram positive rods and cocci were isolated where clinical signs like skin erosion, anorectic, eroded operculum, pale gills, vertical movement, reddening of mouth, haemorrhagic septicaemia, red lateral line and dropsy were observed.

From all the diseased *P. pangasius*, motile aeromonads such as *A. caviae, A. popoffii, A. hydrophila, A. sobria* and *A. schuberti* were isolated. *Edwardsiella tarda*

was isolated from the diseased pangas of Naihati. Along with motile aeromonads, Enterobacteriaceae, *Pseudomonas* spp., Gram positive rods and cocci were also isolated where clinical signs like erratic movement, sliming, dull and red eyes, haemorrhagic mouth and operculum, pale gills, fin rot, tail rot, abdominal haemorrhage, swelling and dropsy were observed.

The phenotypic characteristics of select bacterial strains as determined through an automated bacterial identification system (VITEK 2 compact, Biomerieux, France) are presented in Table 2. The selected bacterial strains were identified as *Edwardsiella tarda*, *Aeromonas sobria*, *Enterobacter cloacae*, *Serratia marcescens*, *Sphingomonas paucimobilis*, *Escherichia coli*, *Citrobacter freundii* and *Klebsiella pneumonia* on the basis of biochemical characterization.

4.3 Molecular characterization

4.3.1 16S rDNA analysis

Selected bacterial isolates were further identified and characterized through 16S rDNA analysis. This assay involved DNA isolation, amplification and sequencing of the gene coding for the 16S rDNA, i.e., the 1.5 kbp 16S rDNA from bacterium. The detailed information on the bacterial strains, host species, clinical signs, site of infection, GenBank accession numbers are presented in Table 3. The consensus sequence of amplified genes are presented in Table 4. In 1.2% agarose gel electrophoresis, approximately 1.5 kbp bands were obtained by PCR amplification (Fig. 1). Data analysis and multiple alignment of sequenced product were performed by using Clustal X (MEGA 5) and sequences were compared against the GenBank database of the National Institute of Biotechnology Information (NCBI) by using the BLAST (Basic Local Alignment Search Tool). The phylogenetic tree (Fig. 2) was constructed by using the Kimura-2 correction for evolutionary rate. All *Aeromonas* strains of diseased catfish were clustered together and separated from other groups. Likewise, all Enterobacteriaceae members were clustered together with three lineages. All *E. tarda* strains were clustered together within the Enterobacteriaceae group as a separate lineage.

4.4 Mycological analysis

None of the diseased catfish were found to have fungal infection, as there was no growth of fungus on CMA and RBCA during the incubation period of 14 days.

4.5 Antibiotic sensitivity testing by agar-disc diffusion assay

A total of 48 bacterial strains comprising *A. caviae, A. hydrophila, A. sobria, A. veronii, A. bestiarum, A. aquariorum, A. diversa, A. jandei, A. rivuli, A. schuberti, E. tarda, E. cloacae, M. morganii, K. pneumonia, S. marcescens* and *Bacillus* spp. from diseased catfish were subjected to antibiogram against twelve antibiotics, viz., amoxyclav (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), clinda mycin (2 µg), co-trimoxazole (25 µg), erythromycin (15 µg), gatifloxacin (5 µg), gentamicin (10 µg), nitrofurantoin (300 µg), oxytetracycline (30 µg), sulphafurazole (300 µg) and vancomycin (30 µg) and the results are depicted in Fig. 3 and Tables 5-7.

The bacterial strain of *P. pangasius* (Case 2) was sensitive to chloramphenicol, co-trimoxazole and sulphafurazole; while the strains of *P. pangasius* (Case 5) were only sensitive to chloramphenicol. The bacterial strains of *C. gariepinus* (Case 9) were sensitive to chloramphenicol, co-trimoxazole and gatifloxacin; while 60% bacterial strains of *P. pangasius* (Case 10) were sensitive to gatifloxacin. All the bacterial strains of *C. gariepinus* of Case 11 against chloramphenicol and *P. pangasius* of Case 12 against chloramphenicol, ciprofloxacin, gatifloxacin were sensitive. Majority (60%) of the bacterial strains from *C. batrachus* (Case 13) were sensitive to chloramphenicol, ciprofloxacin, co-trimoxazole, gatifloxacin and nitrofurantoin. All the bacterial strains from *C. batrachus* (Cases 14 and 15) were sensitive to chloramphenicol, ciprofloxacin, gentamicin, nitrofurantoin; while the strains of Case 16 were sensitive to nitrofurantoin (Table 5).

About 87% bacterial flora of diseased catfish (Fig. 3) were sensitive to chloram-phenicol followed by gatifloxacin (62%), oxytetracycline (53%), co-trimoxazole (47%), ciprofloxacin (40%), sulphafurazole (38%), nitrofurantoin (34%), gentamicin (32%), amoxyclav (13%), vancomycin (11%), erythromycin (4%) and clindamycin (2%). The bacterial flora of *P. pangasius* were sensitive to chloramphenicol (77.77%) and gatifloxacin (55.55%). The bacterial flora of *C. bnatrachus* was sensitive to chloramphenicol (71.42%) and nitrofurantoin (71.42%); while the bacterial flora of *C. gariepinus* were sensitive to oxytetracycline (93.33%) and chloramphenicol (86.66%).

All the *A. jandaei* and *A. veronii* strains were sensitive to chloramphenicol, co-trimoxazole, gatifloxacin, nitrofurantoin; while *A. diversa* were sensitive only to

gatifloxacin. The *A. popoffii* strain was sensitive to chloramphenicol, ciprofloxacin, gatifloxacin, gentamicin, nitrofurantoin; while all the strains of *A. rivuli* were also sensitive to the above said antibiotics except gentamicin (0%). All the *A. aquariorum* strains were sensitive to chloramphenicol, gatifloxacin, gentamicin; while the strains of *A. schuberti* were also sensitive to the above said antibiotics except gentamicin (50%). All strains of *A. sobria* were sensitive to co-trimoxazole, nitrofurantoin; while about 86% of *A. hydrophila* were sensitive to nitrofurantoin. About 80% of *A. caviae* were sensitive to chloramphenicol. *Aeromonas bestiarum* and *E. cloacae* were not sensitive to any of the tested antibiotics. All the *E. tarda* strains were sensitive to nitrofurantoin; while all other Enterobacteriaceae strains were sensitive to co-trimoxazole. *Bacillus* spp. were sensitive (83%) to chloramphenicol (Table 6). About 77% of the bacterial strains from *P. pangasius* were sensitive to chloramphenicol; while the 77% of the bacterial strains from *C. batrachus* were sensitive to chloramphenicol and nitrofurantoin. The bacterial strains of *C. gariepinus* were sensitive to oxytetracycline and chloramphenicol to the tune of 93.33% and 86.66%, respectively (Table-7).

4.5.1 Multiple antibiotic resistance index (MAR index) and multiple antibiotic resistance (MAR) profile of bacterial flora associated with diseased catfish

The details on MAR index and MAR profile are presented in Tables 8 and 9, respectively. The MAR index of bacterial strains was in the range of 0.416-0.916 for *Aeromonas* spp., 0.500-0.916 for *E. tarda,* 0.750-0.916 for other Enterobacteriaceae group and 0.083-0.833 for *Bacillus* spp (Table 8). All the strains of *Aeromonas* spp., *E. tarda* and other Enterobacteriaceae were of MAR group, i.e., were resistant to at least 3 antibiotics; while 83.33% of *Bacillus* spp. were of MAR group (Fig. 4).

4.5.2 Minimum inhibitory concentration (MIC) of antibiotics against *Edwardsiella tarda, Citrobacter freundii* and *Aeromonas sobria*

The results of MIC of antibiotics against *E. tarda, C. freundii* and *A. sobria* strains are shown in Table 10. The MICs of nitrofurantoin, co-trimoxazole, gentamicin, ciprofloxacin, chloramphenicol and tetracycline were observed to be in the range of 0.01 – 30 µg, 0.01 – >240 µg, 0.01 – 10 µg, 0.25 – >240 µg, 0.001 – 30 µg and 0.10 – 150 µg, respectively. The MIC values were high for the *E. tarda* strains followed by *A. sobria* strain from diseased catfish of West Bengal. The least values

were observed for *C. freundii* received from the Shrimp Disease Diagnostic Laboratory, Tamil Nadu Fisheries University, Chennai.

4.6 Pathogenicity of *Edwardsiella tarda* CGH9 on catfish *Pangasius pangasius* and *Clarias gariepinus* by intramuscular injection

Pathogenicity results of *E. tarda* CGH9 on catfish *P. pangasius* and *C. gariepinus* by intramuscular injection are presented in Tables 11 and 12. Cent percent mortality in *P. pangasius* was seen when injected with $6.00x10^{10}$and $6.00x10^{9}$ cells of *E. tarda* CGH9/fish. The LD_{50} value of *E. tarda* CGH9 on catfish *P. pangasius* was determined to be $1.77x10^{7}$ cfu/fish. Likewise, cent percent mortality in *C. gariepinus* was noted when injected with $3.07x10^{9}$cells of *E. tarda* CGH9/fish. The LD_{50} value of *E. tarda* CGH9 on catfish *C. gariepinus* was determined to be $5.75x10^{7}$ cfu/fish.

4.7 Histopathology

4.7.1 *Clarias gariepinus*

Histopathological observations on diseased *C. gariepinus* are depicted in Figs. 5 - 13. Histopathological alterations like inflammation of epidermal tissue and rough epidermal layer (Fig. 5), loosely packed red and white pulp and melanomacrophage aggregation on spleen (Figs. 6 and 7), degenerative changes of internal organs (Fig. 8), extensive degeneration, basophilic margination and disintegration of mucosal layer (Fig. 9), degenerative changes in cardiac glands (Fig. 10) were observed in *C. gariepinus* fries. In kidney, necrotised haematopoietic tissue, nephritic tubules with widened lumen and extensive necrosis (Fig. 11), karyolysis, cellular hypertrophy, pycnotic nuclei (Fig. 12), inflammation of epithelial tissue, vacuolization of tubular epithelium, hypoplastic haematopoietic tissue (Fig. 13) were observed.

4.7.2 *Clarias batrachus*

Histopathological observations on diseased *C. batrachus* fingerlings are depicted in Figs. 14 - 18. Histopathological alterations such as haemocyte infiltration and necrosis of muscle tissue (Fig. 14), inflammation of epidermal tissue, rough epidermal layer, extensive necrosis of muscle and fibrosis (Fig. 15), necrosis of kidney, cellular hypertrophy, nuclear hypertrophy, pycnotic nuclei and karyolysis (Fig. 16), loss of typical tubular epithelial lining (Fig. 17) and necrosis of tubular tissue, inflammation of kidney epithelial layer, hypoplastic haematopoietic tissue (Fig. 18) were noted.

4.8 Outer membrane protein (OMP)

The protein content of *E. tarda* OMP was estimated to be 3.846 mg/ml.

4.9 SDS-PAGE

The SDS-PAGE profile of *E. tarda* OMP antigen revealed a total of 18 bands comprising 13 major bands with molecular weight 83.6, 79.8, 75.2, 64, 49.7, 45.3, 42.3, 40, 36, 34.5, 31, 27.5 and 26.2 kDa and 5 minor bands with 96.7, 89, 84.8, 76.2 and 58 kDa (Fig. 19).

Table 1 – Details of gross and clinical signs, diagnosis and associated bacterial flora from diseased catfish

Case	Location	Species	Gross and clinical signs	Bacterial flora	Fungal infection	Diagnosis
1	Deganga, 24 Parganas North Latitude 22°41'01" N Longitude 88°39'10" E	*Clarias gariepinus* Weight: 15-20 g Length: 8-10 cm	Sluggish behaviour Anorectic Dropsy Yellow fluid accumulation Foul smell Abdominal haemorrhage Discolouration of internal organs	*Edwardsiella tarda* (1) *Providencia alcalifaciens* (1) *Corynebacterium* spp. (1) *Lactobacillus* sp. (1) Gram (−), stout rod (5)	-	Mixed bacterial infection
2	Garia, 24 Parganas South Latitude 22°27'55" N Longitude 88°24'24" E	*Pangasius pangasius* Weight:400-450 g Length: 35-40 cm	Haemorrhagic mouth Haemorrhagic abdomen Pale gills Dull eyes Fin rot Tail rot Sliming	*Morganella* spp. (2) *Escherichia coli* (1) Gram (+) short rod (3) Gram (+) cocci (2)	-	Mixed bacterial infection
3	Naihati, 24 Parganas North Latitude 22°88'81" N Longitude 88°45'23" E	*Pangasius pangasius* Weight:400-450 g Length: 35-40 cm	No symptoms externally	*Edwardsiella tarda* (1) *Aeromonas veronii* (1) *Pseudomonas* sp. (1)	-	Mixed bacterial infection

4	Ramchandrapur, Naihati, 24 Parganas North Latitude 22°54'01" N Longitude 88°24'48" E	*Clarias gariepinus* fry	Anorectic Muscle discolouration Focal cutaneous haemorrhage Gas accumulation Exophthalmia Distended abdomen- Kidney and visceral haemorrhage	*Aeromonas popoffii* (1) *Aromonas hydrophila* (1) *Edwardsiella tarda* (1) *Citrobacter freundii* (1) Enterobacteriaceae (2)	-	Mixed bacterial infection
5	Garia, 24 Parganas South Latitude 22°27'55" N Longitude 88°24'24" E	*Pangasius pangasius* Weight: 300-400 g Length: 30-35 cm	Haemorrhagic mouth Haemorrhagic operculum Red spot on eye Pale gills	*Aeromonas caviae* (2) *Pseudomonas* spp. (3)	-	Aeromoniasis and Pseudomoniasis
6	Satyapole, Nadia Latitude 22°15'03" N Longitude 88°15'44" E	*Pangasius pangasius* Weight: 10-20 g Length: 7-10 cm	Erratic movement Lethargy Haemorrhagic mouth Gill discolouration Haemorrhagic pectoral fin. Swollen abdomen	*Aeromonas popoffii* (8) Enterobacteriaceae (2) Gram (+) cocci (2)	-	Mixed bacterial infection
7	Ramchandrapur, Naihati, 24 Parganas North Latitude 22°54'01" N Longitude 88°24'48" E	*Clarias gariepinus* fry	Dark discoloration Fin/tail rot Whit spot on skin/gills Focal cutaneous haemorrahage Dropsy Abdominal ascites Gas accumulation Kidney an visceral haemorrhage	*Aeromonas veronii* (2) *Aeromonas sobria* (2) *Aeromonas trota* (1) *Pseudomonas alcalifaciens* (1) Enterobacteriaceae (2) *Sphingomonas paucimobilis*	-	Mixed bacterial infection

8	Naihati,24 Parganas North Latitude 22°88'81" N Longitude 88°45'23" E	*Clarias gariepinus* Weight: 15-20 g Length: 8-10 cm	Fin/tail rot Sloughing and protusion of scales Focal cutaneous lesions Pale skin and muscle	*Aeromonas sobria* (2) *Edwardsiella tarda* (1) Enterobacteriaceae (2)	-	Mixed bacterial infection
9	Topar char, Basirhat, 24 Parganas North Latitude 22°40'56" N Longitude 88°51'20" E	*Clarias gariepinus* Weight: 200-250 g Length: 25-30 cm	Lethargy Anorectic Foul smell Body haemorrhage Ulcer Dropsy with yellow fluid accumulation	*Aeromonas veronii* (4) *Aeromonas hydrophila* (2) *Aeromonas trota* (1) *Pseudomonas* spp. (3) Enterobacteriaceae (2) Gram (+) short rod (5) Gram (+) cocci (2)	-	Mixed bacterial infection
10	Topar char, Basirhat, 24 Parganas North Latitude 22°40'49" N Longitude 88°51'39" E	*Pangasius pangasius* Weight: 400-450 g Length: 35-40 cm	Haemorrhagic mouth Haemorrhagic operculum Fin rot Tail rot Pectoral fin haemorahage Pale gill Sliming	*Aeromonas schuberti* (2) *Aeromonas bestiarum (1)* *Klebsiella pneumonia* (1) *Enterobacter cloacae* (1) Enterobacteriaceae (5) *Pseudomonas* spp. (2) Gram (+) short rod (6)	-	Mixed bacterial infection
11	Deganga, 24 Parganas North Latitude 22°41'01" N Longitude 88°39'10" E	*Clarias gariepinus* Weight: 15-20 g Length: 8-10 cm	Vertical movement Focal cutaneous haemorrhage Ulcer Pectoral fin haemorrhage Dropsy with yellow fluid accumulation Gas accumulation Liquefied internal organ	*Edwardsiella tarda* (1) *Aeromonas schuberti* (1) *Aeromonas veronii* (1) *Aeromonas hydrophila* (1) Enterobacteriaceae (1) *Pseudomonas* spp. (3) Gram (+) short rod (1) Gram (+) cocci (6)	-	Mixed bacterial infection

12	Garia, 24 Parganas South Latitude 22°27'55" N Longitude 88°24'24" E	*Pangasius pangasius* Weight: 55-60 g Length: 15-20 cm	Sluggish behaviour Exophthalmia Pale gills Fin rot ,tail rot Abdominal haemorrhage Dropsy	*Aeromonas hydrophila* (2) *Aeromonas sobria* (1)	-	Aeromoniasis
13	Canning, 24 Parganas South Latitude 22°19'04" N Longitude 88°39'24" E	*Clarias batrachus* Weight: 10-20 g Length: 7-10 cm	Skin erosion Anorectic Eroded operculum Pale gills Vertical movement Reddening of mouth Haemorrhagic septicaemia	*Aeromonas caviae* (2) *Aeromonas rivuli* (1) *Aeromonas aquariorum* (2) *Aeromonas sobria* (1) *Aeromonas jandaei* (2) *Aeromonas fluvialis* (1) *Serratia marcescens* (1) *Pseudomonas* spp. (2) Gram (+) short rod (3) Gram (+) cocci (1)		Mixed bacterial infection
14	Memari, Burdwan Latitude 23°10'32" N Longitude 88°06'24" E	*Clarias batrachus* Weight: 15-20 g Length: 8-10 cm	Lethargic movement Exophthalmia Anotrectic Red colour lateral line Body haemorrhage Dropsy	*Aeromonas hydrophila* (1) *Aeromonas caviae* (1) Enterobacteriaceae (3) *Pseudomonas* spp. (1)		Mixed bacterial infection
15	Canning, 24 Parganas South Latitude 22°19'04" N Longitude 88°39'24" E	*Clarias batrachus* Weight: 10-20 g Length: 7-10 cm	Anorectic Erosion in operculum Pale gills Red colour lateral line Abdominal haemorrhage	*Aeromonas hydrophila* (2) Gram (+) short rod (2)	-	Mixed bacterial infection

16	Ramchandrapur, Naihati, 24 Parganas North Latitude 22°54'01" N Longitude 88°24'48" E	*Clarias gariepinus* fry	Lethargy and sluggish behaviour Erratic movement Hanging Anorectic Haemorrhage on body	*Aeromonas jandaei (2)* Gram (+) short rod (1)	-	Mixed bacterial infection
17	Deganga, 24 Parganas North Latitude 22°41'01" N Longitude 88°39'10" E	*Clarias gariepinus* Weight: 15-20 g Length: 8-10 cm	Vertical movement Focal cutaneous haemorrhage Ulcer Pectoral fin haemorrhage Dropsy with yellow fluid accumulation Gas accumulation Liquefied internal organs	*Edwardsiella tarda* (3) *Aeromonas sobria* (1)		Mixed bacterial infection

Densely stocked *Clarias gariepinus* fingerlings

Manuring of catfish pond

Bubble formation indicating poor pond water

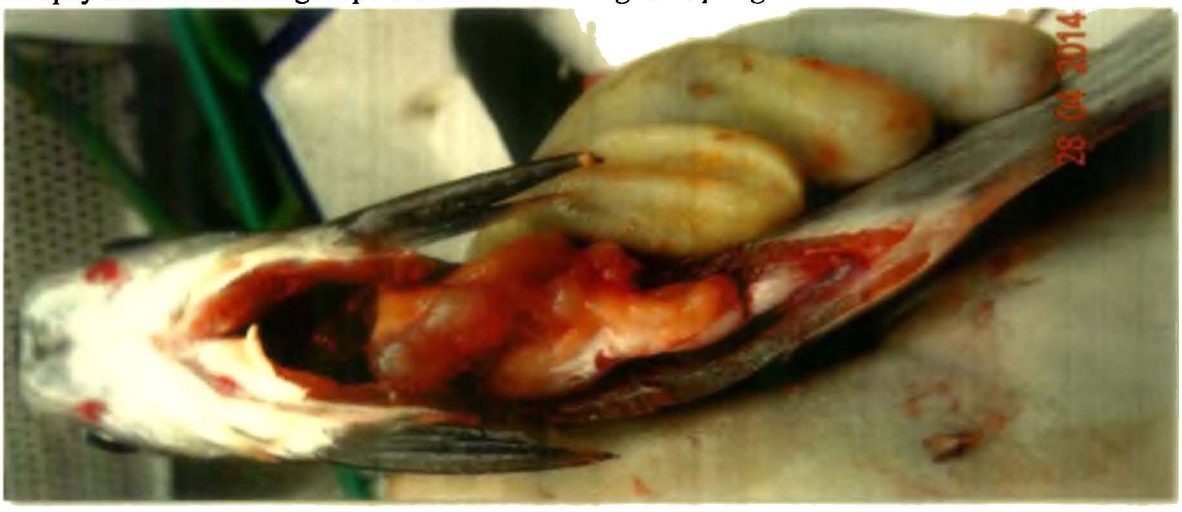
Harvesting of catfish

Reddish lateral line in diseased *Clarias batrachus*

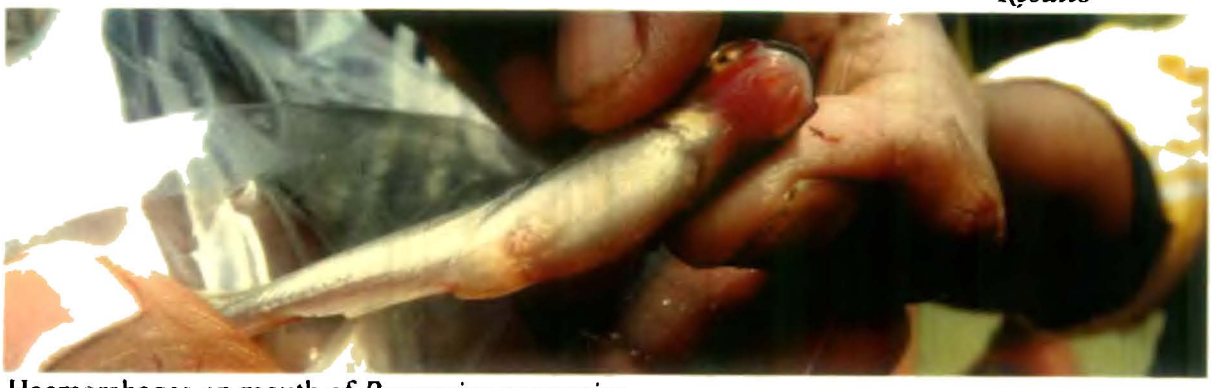
Ulcer in anorectic *Clarias gariepinus*

Haemorahages and yellow discolouration in diseased *Pangasius pangasius*

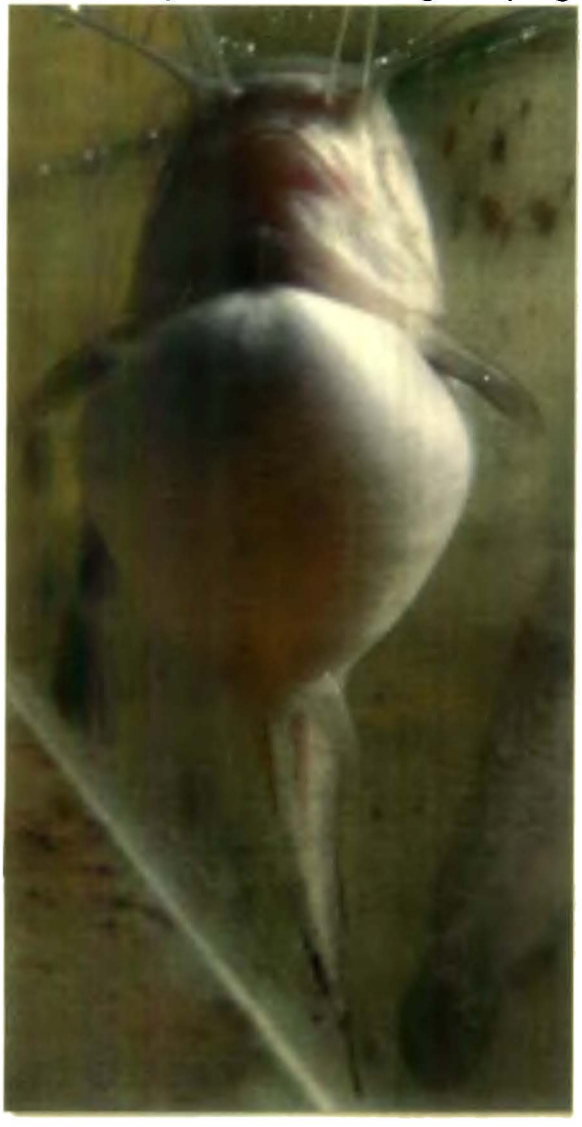
Dropsy and haemorahagic spots on diseased *Pangasius pangasius*



Fluid accumulation in intestine of diseased *Pangasius pangasius*



Haemorahages on mouth of *Pangasius pangasius*



Dropsy in diseased *Clarias gariepinus*

Abdominal fluid accumulation in diseased *Clarias gariepinus*

Table 2 – Biochemical characterization of bacterial isolates from diseased catfish through Biomerieux (Vitek 2-Compact)

Biochemical characteristics	Bacterial strains and Reactions														
	BR1	BR3	CGSB50	HMK1	PB46	CGH9	CGS215	CGB1	SWM1	SWI1	SWI2	SWI3	CGE4	CB2EB1	BG13
Adonitol (ADO)	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Alpha-glucosidase (AGLU)	-	(-)	-	-	-	-	-	-	-	-	-	-	-	-	-
Beta-glucoronidase (BGUR)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Beta-xylosidase (BXYL)	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Citrate (sodium) (CIT)	-	+	-	-	-	-	+	-	+	-	+	-	-	-	+
D-Cellobiose (dCEL)	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+
D-Glucose (dGLU)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Maltose (dMAL)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol (dMAN)	+	+	-	-	-	-	+	+	+	-	-	-	-	+	+
D-Mannose (dMNE)	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
D-Sorbitol (dSOR)	+	+	-	-	-	-	+	-	-	-	-	-	-	+	+
D-Tagatose (dTAG)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Trehalose (dTRE)	+	+	-	-	-	-	+	+	-	-	-	-	-	+	+
Fermentation/ glucose (OFF)	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
H_2S production (H_2S)	-	-	+	+	+	-	+	-	-	+	+	+	+	-	-
L-Arabitol (IARL)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lipase (LIP)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Malate assimilation (IMLTa)	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase (LDC)	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+
Orinithine decarboxylase (ODC)	-	+	+	+	+	+	-	-	-	+	+	+	+	+	-
Phosphatase (PHOS)	-	(-)	+	+	+	+	-	-	-	+	+	+	+	-	+
Saccharose/Sucrose (SAC)	-	+	-	-	-	-	+	+	+	-	-	-	-	+	+
Urease (URE)	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+

5-Keto D-gluconate (5KG)	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Ala-Phe-Pro-arylamidase (APPA)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alpha-galactosidase (AGAL)	+	+	-	-	-	-	+	-	-	-	-	-	-	-	+
Beta-alanine arylamidase pNA (BAlap)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Beta-galactosidase (BGAL)	+	+	-	-	-	-	+	-	+	-	-	-	-	-	+
Beta-glucosidase (BGLU)	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+
β-N-Acetyl-glucosaminidase (BNAG)	-	+	+	+	+	+	-	-	-	-	+	+	+	+	-
Coumarate (CMT)	+	-	+	+	+	+	-	-	+	+	+	+	+	+	-
Ellman (ELLM)	+	-	+	+	+	+	-	-	+	+	+	+	+	-	-
Gamma-glutamyl transferase (GGT)	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+
Glu-Gly-Arg-arylamidase (GGAA)	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Glutamyl arylamidase pNA (AGLTp)	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Glycine arylamidase (GlyA)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L Pyrrolydonyl-arylamidase (PyrA)	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+
L-Histidine assimilation (IHISa)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Lactate alkalinisation (ILATk)	+	+	-	+	-	+	+	+	-	-	-	-	-	+	+
L-Lactate assimilation (ILATa)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Proline arylamidase (ProA)	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-
Malonate (MNT)	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
O/129 Resistance (O129R)	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Palatinose (PLE)	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Succinate alkalinisation (SUCT)	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Tyrosine arylamidase (TyrA)	+	+	-	-	-	-	+	+	+	+	+	-	+	-	-
β-N-acetyl-galactosaminidase (NAGA)	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-

CGSB50, HMK1, PB46, CGH9, SWI1, SWI2, SWI3, and CGE4: *Edwardsiella tarda*; BG13: *Klebsiella pneumoniae*; SWM1: *Aeromonas sobria*; BR3: *Enterobacter cloacae*; CB2EB1: *Serratia marcescens*; CGB1: *Sphingomonas paucimobilis*; BR1: *Escherichia coli;* CGS215: *Citrobacter freundii*

Table 3 – Molecular characterization of bacterial strains isolated from diseased catfish.

Case	Fish species	Disease/Clinical sign	Site of infection	Strain code	Length (bp)	Gene Bank Accession number	Identification
Case 1	*Clarias gariepinus*	Abdominal haemorrhage	Abdomen	CGH9	1000	KC914626	*Edwardsiella tarda*
	Clarias gariepinus	Abdominal haemorrhage	Kidney	CGK3	1000	KC914627	*Providencia alcalifaciens*
Case 3	*Pangasius pangasius*	Cutaneous haemorrhage	Kidney	PB46	1057	KF853568	*Edwardsiella tarda*
Case 4	*Clarias gariepinus*	Cutaneous haemorrhage	Body	CGSB50	1416	KJ522790	*Edwardsiella tarda*
Case 5	*Pangasius pangasius*	Haemorrhagic mouth	Mouth	B	1055	KF481924	*Aeromonas caviae*
Case 6	*Pangasius pangasius*	Haemorrhagic septicaemia	Pectoral fin	PFC	1498	KF481927	*Aeromonas popoffii*
Case 8	*Clarias gariepinus*	Cutaneous haemorrhage	Kidney	HMK1	1463	KF853565	*Edwardsiella tarda*
Case 9	*Clarias gariepinus*	Cutaneous haemorrhage	Body surface	R1	1044	KF853562	*Aeromonas veronii*
	Clarias gariepinus	Cutaneous haemorrhage	Body surface	R9	1446	KF853563	*Aeromonas veronii*
	Clarias gariepinus	Cutaneous haemorrhage	Body surface	G18	1462	KF853564	*Aeromonas veronii*
	Clarias gariepinus	Cutaneous haemorrhage	Body surface	G17	1479	KF853566	*Aeromonas hydrophila*
	Clarias gariepinus	Cutaneous haemorrhage	Body surface	E20	1051	KF853567	*Aeromonas hydrophila*
Case 10	*Pangasius pangasius*	Cutaneous haemorrhage	Operculum	BG13	1412	KJ522785	*Klebsiella pneumoniae*
	Pangasius pangasius	Cutaneous haemorrhage	Operculum	BR3	1412	KJ522786	*Enterobacter cloacae*
Case 11	*Clarias gariepinus*	Dropsy	Abdomen	CGE4	1419	KJ522789	*Edwardsiella tarda*
Case 12	*Pangasius pangasius*	Abdominal haemorrhage	Abdomen	PFH1	1412	KJ522794	*Aeromonas hydrophila*
	Pangasius pangasius	Abdominal haemorrhage	Abdomen	PFH2	1423	KJ522795	*Aeromonas sobria*
Case 13	*Clarias batrachus*	Mouth haemorrhage	Mouth	CB2EB1	1420	KJ522787	*Serratia marcescens*

Table 4 - Consensus sequence of amplified 16S rDNA genes of bacterial strains of diseased catfish

Bacterial species and strain number	NCBI GenBank Accession number	Consensus sequence of 16S rDNA gene
Aeromonas caviae B	KF481924	AAAGGTCAGTGAGCTATATCTGCTGCTGTGACGATAGTCGCAAGAAGAAGCACTTGCTATTCGTGCCAGAAG CGCGTAATACGAGGGTGCAGCGTTATCGAATACTGGCCCAGTAAAGCGCACGCAGGCGGTGGATAAGATAGA TGTGATAGCCCACGCTGCACTGTGAATTGCATTTAAAACTCGGGTCCAGCGGTAGTCTCGTAGAGGGGGGTAG AATTAGCCAGGTGTAGCGGTGATAATGCGTAGAGATCTGGAGGAATAGCCGGTGTTGCGAAGGCGGCCCCCT CGACAAAGACTGACGCTCAGGTGCGAAAGCGTGCGGGAGCAAACACGATTAGATACTGCCCGGTAGTCCACG CCGTAATGCAACGATGTCGCATTTGGAGGCTGTGTCCTTGAGACGTGAAGCTTCCGGAGCTAACGCGTTAAAT CGACCGCCTGGAGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGCTGGGCCCGCACAAGCGGTGG AGCATGTGGTTTAATTCGATGCAAGCGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATCGGCTGTATGA GATACGGGAGTGCCTTCGGGAATCAGAACACAGGTGCTGCATTTAAGGCTGTCGTCAGCTCGTGTCGTGAGAT GTTGGGTTAAGTCCCAGGTGCAACGAGCGCAACCCCTGTCCTTTGTAAGAATGCCAGCACGTAATGGTGGGA ACTCAAGGGAGACTGCCGCTCAGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTA CGGCCAGGGCTACACACGTGCTAGCAATGGCGCGTACAGAGGGCTGCCCGAAGCTAGCGATAGTGAGCGAAT CCCAAAAAGCGCGGTCGTAGGTCCGGATTGGAGTCTGCTAACTCGACTCCATGAAGTCGGAATCGCTTGTAAT CGCAAATCAGAATGTTGCGGTGAATACGTTCCCGGGCCTGTACACACCGCCCGTCACACCATGGGAGTGGGTT GCACCAGAAGTAAAATAGCTT
Aeromonas popoffii PFC	KF481927	TCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGC TACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGATAACAGTTG GAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATG AACCCAGGTGGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGG

		ATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA
		TGGGGGAAACCCTGATGCAGCTATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAG
		GAGGAAAGGTTGTTGGCTAATATCCAGCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGC
		CAGCAGCCGCGGTAATACAGAGTGTGCCAGCGTTATGGAGATTTACTGGGTGATAAACGCGCACGCGTGCGG
		GTCGGATGTATTATGATGTGGAAGCCCCCGCTAGCTCCACCTGAGAATTGCATTTAAAAACTGTCCAAGCTAG
		AAGTCTCGTAGAGCGAGGTAGAATTCCCCAGGTGCAGCCGTGAAAGTGGAGTAGTAGATCATGGAGGGAATA
		CCCGGTGGCGATGGGCGGCCCCCCTGGACAGCAAGACTGACGCTCAGGTGCGAAACAGTGTGGGGAAGCAA
		ACAGCGATTAGATACCCTGGTAGTCCACGCGGTAAACGATGTCGATTTTGGAGGCTGTGTCGCTTGAGACGTG
		GCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGAGAGTACGGCCGCAAGGTTAAAACTCAAGCATGATTT
		GACGTGGCGCCCGCACACGCGGTGGAGCATGTGGTTTATTTCGATGCAACGCGAAGAACCTTACCTGGCCTTG
		ACATGTCTGGAATCCTGTAGAGATGCGGGAGTGCCTTCGGGAATCAGAACACAGGTGCTGCATGGCCGTTGTC
		AGCTCGTGTCGTGAGATGTTGGGTTAGAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAA
		TGCGTGGGAATCTCAAGGGAGACCGCCGGTGATAAAGCCCCCCGGAGGAAGGCTGGGGATGACGTGCAAGTC
		ATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGTCGGCTGCAAGCTAGCGAT
		AGTGAGACGAACTCCCAAAACTAGCGCGTCGTAGTCGACCGATCGGGGTCTGCAACTTGACTCCGTGAAGTT
		GATGAATCGCTGTAATCCGCAAATCATGGAATGTGCGGGAATGACGTTCCCGGGCCTCGTACACACCGCCCGT
		CACACCATGGGAGATCTGGGTTGCACCAACAAGTAGAATAGC
Aeromonas sobria PFH2	KJ522795	AGTCCGAGCGGCAGCGGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGG
		GGATCTGCCCAGTCGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATACGCCCTACGGGGGAAAGC
		AGGGGACCTTCGGGCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCAC
		CAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCT
		ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGA
		AGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGTAGCTAATAACTGCCAGCTGTGACGTT
		ACTCGCAGAAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCG

		GAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGA
		ATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA
		GAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGT
		GGGGGAGCAAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCT
		TGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAA
		ATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGGAAGAACCTTACC
		TGGCCTTGACATGTCTGGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATCAGAACACAGGTGCTGCATG
		GCTGTCGTCAGCTCGTGTCCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCC
		AGCACGTAATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGT
		CATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGGGCTGCAAGCTAGCGATA
		GTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCG
		CTAGTAATCGCAAATCAGAATGTTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGG
		GAGTGGGTTGCACCAGAAGTAGATAGCTTAACCTTCGGGAGGGCGTTACCCAACG
Aeromonas hydrophila PFH1	KJ522794	TGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCT
		GGGGATCTGCCCAGTCGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATACGCCCTACGGGGGAAA
		GCAGGGGACCTTCGGGCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTC
		ACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTC
		CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAG
		AAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGTAGCTAATAACTGCCAGCTGTGACGT
		TACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCG
		GAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGA
		ATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA
		GAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTG
		GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGA

		GACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG
		AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGC
		CTTGACATGTCTGGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATCAGAACACAGGTGCTGCATGGCTGT
		CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACG
		TAATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATG
		GCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCG
		AATCCCAAAAAGCGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAA
		TCGCAAATCAGAATGTTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGG
		TTGCACCAGAAGTAGATAGCTTAACCTTCGGGAGGGCGTTA
Aeromonas hydrophila E20	KF853567	TCTGTCTTACTCTCGTGCCAGCAAGCCGCTGGTAATACGGACGGGCTGCAGCGTTCATTGGGAATTACCGGGC
		GTAAGCGCACCGCGGGCGGTCTGATGAGTTAGATGTGAAAGCCCCCGCGCTCCACCTGGGAATTGCATTTAA
		AAACGACTGTCCATGCCAGATACGTCTTGTAGAGGGGGGCAGTAGAATTGCAGGTGTAGCGGTGAAGTGCGT
		AGAGATCTGGGGGAATACCTAGGTGGCGAAGTAGCGGCCCACCTGGACAAAGACTGACGCTCAGGTGCGAA
		AGTGTGGTGAGCAACACAGGATTAGATACCCTGGTAATCCACGCTCGTAAACGATGTCGATTTGGAGGCTGTG
		TGCTTGAGACGTGGCTTCTGGAGCTAACGCGGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGATTAAGA
		CTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTGCGATGCAACGCGAAGAACC
		TTACCTGGCCTATGACATGTCTGGAATCCTGTAGAGATGCGGAGAGTGCCTTCGGGAATCAGAACTACTAGGT
		GCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTGAAAGCCCTGGGTTAAGTCCCGCAACGAGCGCAACCC
		CTGTCCTTTGTTGCCAGCACGTAATGGTGGGTAGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGG
		TGGAGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTGACACACGTGCTACAATGGCGCGTACAGA
		GGGCTGCATTAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATTGGAGTCTTGCAACT
		CGACTCCATGAAGTCGGAATCGCTAGTCAATCGCAAATCAGAATGTTGCGGTGAATACGTTCCCGGGCCTTGT
		ACACACCGCCCATCACACCATGGGAGTGGGTTGCTACCAGAAGTAACGCGATAGCTTAACCTTCGGGAGGGC

		TTAGATCCATAGTTATCTATTCAAAAACAGGATAGACAC
Aeromonas hydrophila G17	KF853566	CGCCTGCAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATG CCTGGGAAATTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGG AAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATATGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGG CTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGA CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTG AAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTCAGTAGCTAATATCTGCTGACTGTGA CGTTACTCGCAGATAGTAGCACCGCTACTCCGTGCCAGCAGCGCAGTATACGAGGGTGCCAGCGTTATCGAAT TACTGGGCGTAAAGCGCACGCAGCGGTTGATAAGTTAGATGTGAACCAGCCCCGGGCTTCAACAGCTGAGAA CTGCATTTAAAACTGTCCACGCTAGAGTCTGTAGAGGGGGGTAGAATTCCAGGTGTAGCTGTGAAATGTGTAG AGATCTGGAGGAATACCTGGTTGGTGAAGGTGCGGCCCTCCTGGACAAAGACTGACGCTCAGGTGCGAAAGT GTGGGGAGCCACACAGGATTAGATACCCTGGTAATCCACGCTGCTAAACGATGTCGATTTGGAGGCTGTGTCC TTGAGACGTGGCTTCCGGAGCTAACGCGGTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTGCGATGCAACGCGAAGAACCTTAC CTGGCCTATGACATGTCTGGAATCCTGTAGAGATCGGAGAGTGCCTTCGGGAATCAGAACTACAGGTGGTGC ATGGCATGTCGTCAGCTCGTGTCGTGAGATGTGAAAGCCCTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGT CCTTTGTTGCCAGCACGTAATGGTGGGTAGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGA GGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTAACGCACACGTGCTACAACGTGGCGCGTACAGA GGGCTGCAAGCCCTAGCGATACACGCCGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATATGGAGTCT GCAACTCGACTCCATGAAGTCGGAATCGCCTAGTAATCGCAAATCAGAATGTTGCGGTTCGAATACGTTCACG GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGATAGCTTAACCTTCGGAG GGCATTAAACAAACAATATAAACCCAATCTCC

Aeromonas veronii G18	KF853564	ATACGATGAGGTCCACGGCTATAACATGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGC GAGCGGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGATAACTACTGGAAACGGTAGCTA ATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATGAACCCAGGTGGGA TTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACA CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCT GATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGG TAGCTAATAACTGCCGACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT AATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGAT GTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGA ATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGCGGCCCCCTGGACAAAG ACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT GTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTAC GGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAT GCAACGCGAAGAACCTTACCTGGCCCTTGACATGTCTGGAATCCTGTAGAGATACGGGAGTGCCTTCGGGAAT CAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA ACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAG GTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAG GGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGAC TCCGTGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGTTGCGGTGAATACGTTCCCGGGCCTTGTACACA CCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGATAGCTTAACCTTCGGGAGGGCGTTACCTACA GGAGGATTCCTGTCCGA
Aeromonas veronii R9	KF853563	CGCTGCGATCTTGCAGGGGCTTAGCTATGCAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCG AGCGGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGATAACTACTGGAAACGGTAGCTAA TACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATGAACCCAGGTGGGAT

		TAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACAC
		TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTG
		ATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGT
		AGCTAATAACTGCCAGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA
		ATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATG
		TGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAA
		TTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAG
		ACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
		GTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTA
		CGGCCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
		TGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATCCTGTAGAGATACGGGAGTGCCTTCGGGAAT
		CAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
		ACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAG
		GTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAG
		GGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGAC
		TCCGTGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGTTGCGGTGAATACGTTCCCGGGCCTTGTACACA
		CCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGATAGCTTAACCTTCGGAGGGCGTTCACACTATA
		TATCATTCTCTTAATTCA
Aeromonas veronii R1	KF853562	TAGCTTATGCTTGTGCCAGCAGCCCGTGGTAATACGTGAGGGTGCCAAGCGTTTATCGGAATTACTGGGCGTA
		AAGCGCACGCGGGCGGTCGGATGAGTTAGATGTGAAAGCCCCCGCGCTCAACCTGGGAAATTGCATTTAAAA
		GTGTCCAGTACCAGATACGTCTTGTAGAGGGGGGTAGAATTCCAGGTGCAGCTGTGAAGTGTGTAGAGATCG
		GGAGGAATTAACCGGTTGGTGAAGTAGCGGCGCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGG
		GAGCGAAACAGGGTTAGAGACCCTGGTAATCCACGCTCGTAAACGAGGTCGATTTGGAGGCTGTGTCCTTGA
		GACGTGGCTTCCGGAGCTAACGCGTGAAATCGACCGCCTGGGGAGTACGGCCGCAAGATTAAAACTCAAATG

		AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTGCGATGCAACGTGAAGAACCTTACCTGG
		CCTATGACATGTCTGGAATCCTGTAGAGATACGGAGAGTGCCTTCGGGAATCAGAACTACTAGGTGCTGCATG
		GCTGTCGTCAGCTCGTGTCGTGAGATGTGAAAGCCCTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTT
		TGTTGCCAGCACGTAATGGTGGGTAGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGAGGAT
		GACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTGACACACGTGCTACAATGGCGCGTACAGAGGGCTGCA
		AGCTAGCGATAGTGGTAGCGAATCCCAAAAAGCGCGTTGTAGTCCGGATCGGAGTCCTTGCAACTCGACTCC
		GTGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGTTGCGGTGAATACGTTAAACCCGGGCCTTTACACAC
		CGCCCATCACACCATGGGACATGTGGGTTGCACCAGAAGTAGATAGCTTAACCTTCGGAGGGCGTACCATAG
		TTTTATCTTTCCTAAAAAAGGAAAGCTCCGGA
Edwardsiella tarda HMK1	KF853565	ACCCTTGGGGGCAGGGCTATTACATGCAGTCGAGCGGTAGCAGGGAGAAAGCTTGCTTTCTCCGCTGACGAG
		CGGCGGACGGGTGAGTAATGTCTGGGGATCTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATA
		CCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGAACCCAGATGGGATTA
		GCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTG
		GAACTGAGACACGGTCCACACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGAT
		GCACCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTAGGGAGGAAGGTGTACGTG
		TTAATAGCACGTACAATTGACGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
		ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTTGGATGTG
		AAATCCCCGGGCTTAACCTGGGAACTGCATCCAAGACTGGCAAGCTAGAGTCTCGTAGAGGGAGGTAGAATT
		CCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCTCCTGGACGAAGAC
		TGACGCTCAGGTGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATG
		TCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGAAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACG
		GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATG
		CAACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAATCCTGTAGAGATACGGGAGTGCCTTCGGGAACG
		CTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAA

		CCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGT GGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAG AAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTC CATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACC GCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACTTTTTTT GGATTTTTAAGGTTGGGGG
Edwardsiella tarda PB46	KF853568	CACCTCGCTAACTCCGTGCCCAGCAGCCGCGTAATACGGAGGGTGCTAGCGTTAATGGGAATTAAGCTGGGC GTACACGCGCACGCTGGGCCGTCTGTTAAGTTGGATGTGAAGATCCCCCGGGCTTACCGGGATGAGAACTGC ATCCCAAGACTGGCAAGCTAGAGTCTCGTAGGACCAGGGAGGTAGAATTCCAGGTGTAGCGCCGTGAAATGC GTAGAGATCTGGAGGAATACCAGGTGGCGAAGGCGGCCTCCTGGACGAAGACTGACGCTCAGGTGCGAAAG CGTGGTGAGCAAAACAGGATTAGATACCCTGGTAGTCCACGCTGATAAACGATGTCGATTTGGAGGTTGTGCC CTTGAGGCGTGGCTTCCGAAGCTAACGCGTTAATATCGACCGCCTGGGGAGTACGGCCGCAAGATTAAATACT CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTACATTCGATGCAACGCGAAGAACCTT ACCTACTCTGTGACATCCAGCGCAATCCTGTAGAGATACGGGAGTGCCTATCGGGAACGCTGAGACTAAGGT GCATGCATGGCTGTCTGTCAGCTCGTGTTGTGAAATGTTCGGGTTAAGTCCCGCAACGAGCGCAACCCTTATC CTTTGTTGCCAGCGGTTCGTGCCGGGAACTCAAATTCGGAGACTGCCAGTGATAAACGTGGAGGAAGGTGGG GATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTGACACACGTGCTACAATGGCGTATACAAAGAGAA GCGAACTCGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCA TGAAGTCGGAATCGCTAGTAATGCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCG CCCGTCACACCATGGGAGTGGGTTCAAGCAAAAGAAGTAGGTAGCTTAACCTTCAAGCAGGGCGAGACCTTC ACCTACCATAGTGATCCAGCGACTCCCAAAAAAAAACAATGTGCCT

Edwardsiella tarda CGE4	KJ522789	ACCATGCAGTCGAGCGGTAGCAGGGAGAAAGCTTGCTTTCTCCGCTGACGAGCGGCGGACGGGTGAGTAATG TCTGGGGATCTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACC AAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGAACCCAGATGGGATTAGCTAGTAGGTGAGGTAATGG CTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGA CTTCCTACGGGAGGCAGCAGTGGGGAATATTGCACCAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTA TGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTAGGGAGGAAGGTGTGCGTGTTAATAGCACGTACAATT GACGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTT AATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTTAACCT GGGAACTGCATCCAAGACTGGCAAGCTAGAGTCTCGTAGAGGGAGGTAGAATTCCAGGTGTAGCGGTGAAAT GCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCTCCTGGACGAAGACTGACGCTCAGGTGCGAAA GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCC CTTGAGGCGTGGCTTCCGAAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTC AAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC CTACTCTTGACATCCAGCGAATCCTGTAGAGATACGGGAGTGCCTTCGGGAACGCTGAGACAGGTGCTGCATG GCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAG CGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATC ATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGACCTCGCGAGAGCAA GCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGT AATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTG GGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACC
Edwardsiella tarda CGSB50	KJ522790	ACCATGCAGTCGAGCGGTAGCAGGGAGAAAGCTTGCTTTCTCCGCTGACGAGCGGCGGACGGGTGAGTAATG TCTGGGGATCTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACC AAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGAACCCAGATGGGATTAGCTAGTAGGTGGGGTAATGG CTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGA

		CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATG
		AAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTAGGGAGGAAGGTGTGAACGTTAATAGCGCTCACAATTGA
		CGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAA
		TCGGAATTACTGGGCGTAAAGCGCACGCGGCGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTTAACCTGGG
		AACTGCATCCAAGACTGGCAAGCTAGAGTCTCGTAGAGGGAGGTAGAATTCCAGGTGTAGCGGTGAAATGCG
		TAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCTCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGT
		GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGA
		GGCGTGGCTTCCGAAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG
		AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTTCGATGCAACGCGAAGAACCTTACCTAC
		TCTTGACATCCAGCGAATCCTGTAGAGATACGGGAGTGCCTTCGGGAACGCTGAGACAGGTGCTGCATGGCT
		GTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCG
		GTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCAT
		GGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGAACTCGCGAGAGCAAGC
		GGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAA
		TCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGG
		TTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTACC
Edwardsiella tarda CGH9	KC914626	GGGCCTTGGGCGTCGGCCTTAACCATGCAAGTCGAGCGGTAGCAGGGAAGAAAGCTTGCTTTCTCCGCTGAC
		GAGCGGCGGAGGGGTGAGTAATGTCTGGGGATCTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTA
		ATACCGCATAACGTCGCAAGACCAATGTGGGGGACCTTCGGGCCTCATGCCATCAGATGACCCCAGATGGGA
		TTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACA
		CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAGTGGGCGCAAGCCT
		GATGCACCCATGCCGCGTGTATAAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTAGGGAGGAGGGTGTGA
		ACGTTAATAGCGTTCACATTTGACGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA
		ATACGGAGGGTGCAAGCTTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTTGGATG

		TGAAATCCCCGGGCTTAACCTGGGAACTGCATCCAAGACTGGCAAGCTAGAGTCTCGTAGAGGGAGGTAGAA
		TTCCAGGTGTAGCGGTAAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAGGGCGGCCTCCTGGACGAAG
		ACTGACGCTCATGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCGGGTAGTCCACGCTATACACTAT
		GTCTATGTGGAGGTTGTGCCCTTGAGGCGTGTCTCCCGACGCTAACGCGTTATATCCACCGCCTGGGAGAGTA
		CGGCCGCAGTGATAAAACTCAAATGATTGACGGGGGCCCGCACACGCGGTGGGAGCATGTGGTTTTAATTCT
		ATGCAACGCGAAGACCCTCACCTACTCTAGACCTCCAGCAAACTCCTGTAGAGATACGGGGA
Enterobacter cloacae BR3	KJ522786	AACCCATGCAGTCGTACGGTAGCACAGATAGCTTGCTCTCGGGTGACGAGTGGCGGACGGGTGAGTAATGTC
		TGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAA
		AGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCT
		CACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACT
		CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAA
		GAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATTGACG
		TTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATC
		GGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGG
		AACTGCATTCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG
		TAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGT
		GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTG
		AGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAT
		GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGG
		TCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTG
		TCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGT
		TCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG
		CCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGG
		ACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATC

		GTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTT
		GCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGC
Klebsiella pneumonia, BG13	KJ522785	TGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGA
		AACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGG
		GGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTA
		GGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG
		GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGC
		CTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTACCCG
		CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTA
		CTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCA
		TTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGAT
		CTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAG
		CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTG
		GCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGA
		CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGAC
		ATCCACAGAACTTTCAGAGATGGTTTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGC
		TCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCCG
		GGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTAC
		GACCAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCAT
		AAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATC
		AGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAA
		GAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACC

Providencia alcalifaciens CGK3	KC914627	GAAGGAAGAGGCACTGGTTTATTTCGTGCCAGGAGGCGGAGTAATAGGAGGGTGCTAGTGTTTATATGGAAT TACCGGGGGGCAACCCCCACGCGGGTGGTTGATTAATTAGATATGCAATGCCCCGGGCTCCACCTGGGAACT GCATCTAAGACTGTCAGGTTGAGTTTTGTAGAGGGGGGTAGAATTCCCGGAGGAGCGGAGAAATGAGTAGAG ATGAGGAGGAACACCGGTGGGGAAGGCCGCCCCCTGGACAAAGAATGACGCTCAGGTGAGAAAGTGTGGGG AACAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAAGATGTTGATTTGGAGGTTGCTTCCTTGAGGCG TGGCTTCCGGAGATAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATT GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTTGATGCAACGGGAAGAACCTTACCTAGCTTTG ACATCTCAGGAATCTTGCAGAGAAGCGTTAGTGCCTTTGGGAAATTGAAAACAGGTGGTGCATGGCTGTTGTC AGCTTGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAAT GGTGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCC CTTACGACCAGGGCTACACATGTGCTACAATGGCGCATACAAAGGGAAGCGACCTCGCGAGAGCAAGCGGAC TTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGT AAATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCTCCCGTCACACCATGGGAGTGGGTTGC ACCAGAAGTATATTAGTTTACTCTCTGGCTCAGATGAATCGCATAGTCTTGCCCCCGCCGT
Serratia marcescens CB2EB1	KJ522787	ACACATGCAGTCGAGCGGTAGCACAAGGGAGCTTGCTCCCTGGGTGACGAGCGGCGGACGGGTGAGTAATGT CTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCA AAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGC TCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGAC TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGA AGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAACTTAATACGTTCATCAATTGAC GTTACTCGCAGAAGAAGCACCGGCTAACTTCCGTGCCAGCAGCCGCGGTAATACGGGAGGGTGCAAGCGTTT AATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACC TGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAA TGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAA

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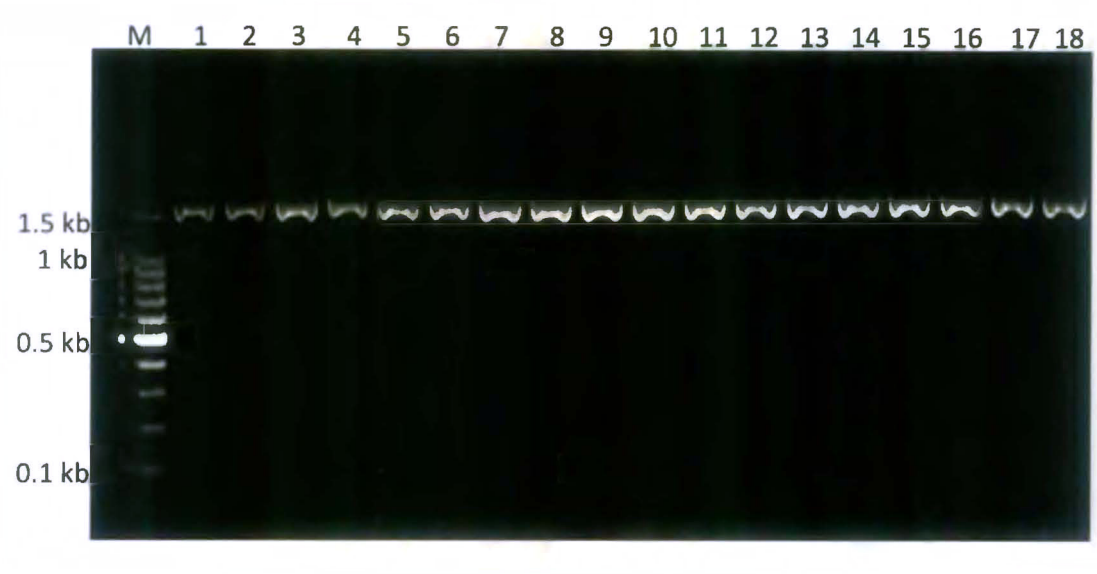
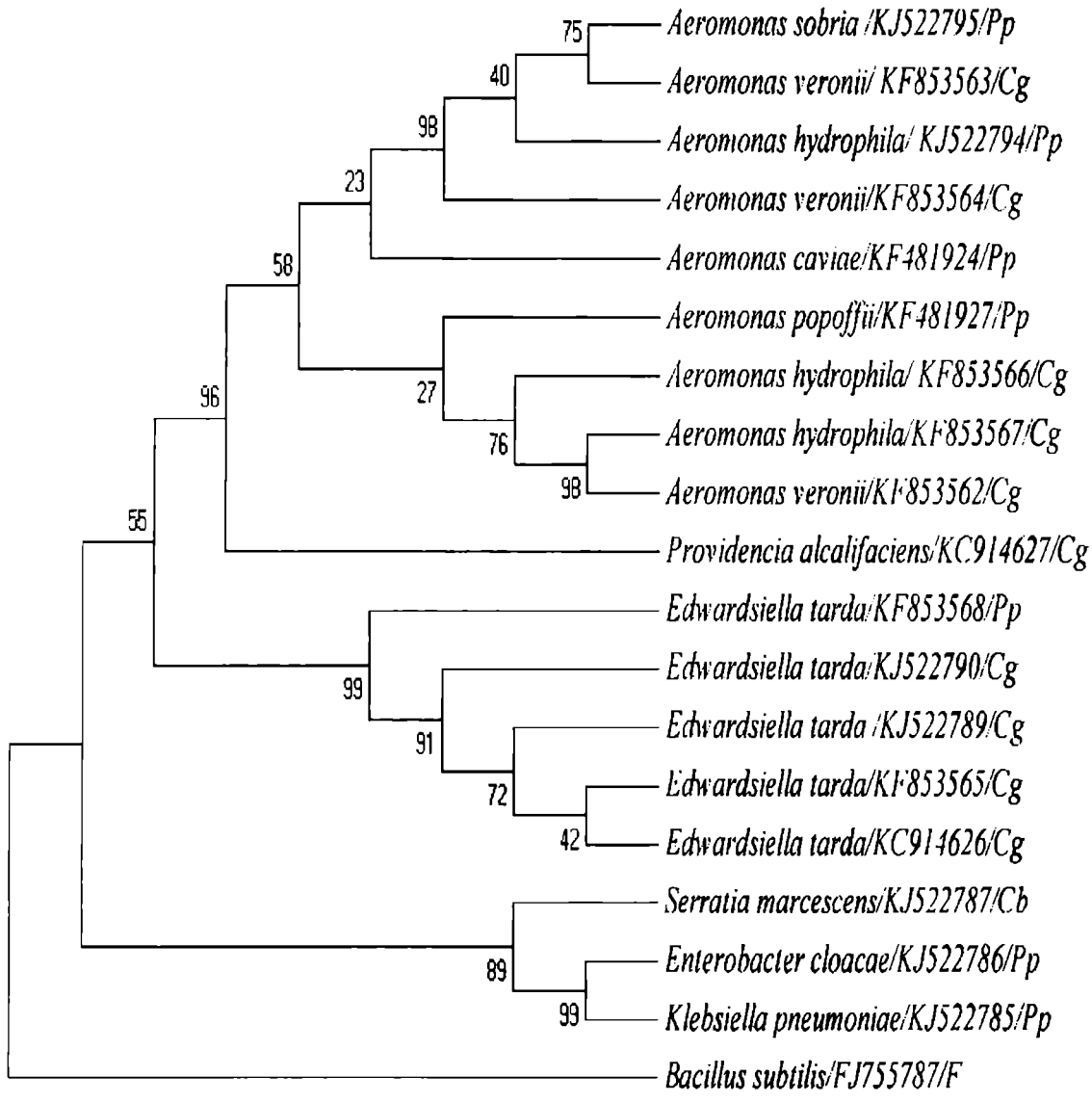


M: 100 bp molecular weight DNA marker

1	*Aeromonas caviae* B	2	*Aeromonas popoffii* PFC
3	*Aeromonas popoffii* PFC	4	*Aeromonas hydrophila* PFH1
5	*Aeromonas hydrophila* E20	6	*Aeromonas hydrophila* G17
7	*Aeromonas veronii* G18	8	*Aeromonas veronii* R9
9	*Aeromonas veronii* R1	10	*Edwardsiella tarda* HMK1
11	*Edwardsiella tarda* PB46	12	*Edwardsiella tarda* CGE4
13	*Edwardsiella tarda* CGSB50	14	*Edwardsiella tarda* CGH9
15	*Enterobacter cloacae* BR3	16	*Klebsiella pneumoniae* BG13
17	*Providencia alcalifaciens* CGK3	18	*Serratia marcescens* CB2EB1

Fig. 1 - Agarose gel (1.2%) showing 16S rDNA gene amplicons of bacterial strains of diseased catfish.



Pp: *Pangasius pangasius*; Cg: *Clarias gariepinus*; Cb: *Clarias batrachus*; F: Fish

Fig. 2 – Phylogenetic tree generated by neighbour-joining Kimura-2 parameter of the 16S rDNA sequence of bacterial strains isolated from diseased catfish.

Table 5 – Antibiotic sensitivity (%) of the bacterial flora associated with diseased catfish – Case wise

Antibiotics, concentration	Case study									
	Case 2	Case 5	Case 9	Case 10	Case 11	Case 12	Case 13	Case 14	Case 15	Case 16
Amoxyclav, 30 µg	0.00 (0)	0.00 (0)	33.33 (1)	0.00 (0)	0.00 (0)	33.33 (1)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
Chloramphenicol, 30 µg	100.00 (1)	100.00 (2)	100.00 (3)	40.00 (2)	100.00 (4)	100.00 (3)	60.00 (6)	100.00 (2)	100.00 (2)	0.00 (0)
Ciprofloxacin, 5 µg	0.00 (0)	0.00 (0)	33.33 (1)	20.00 (1)	25.00 (1)	100.00 (3)	60.00 (6)	100.00 (2)	100.00 (2)	0.00 (0)
Clindamycin, 2 µg	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
Co-trimoxazole, 25µg	100.00 (1)	0.00 (0)	100.00 (3)	40.00 (2)	25.00 (1	66.66 (2)	60.00 (6)	50.00 (1)	100.00 (2)	0.00 (0)
Erythromycin, 15 µg	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	33.33 (1)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
Gatifloxacin, 5 µg	0.00 (0)	50.00 (1)	100.00 (3)	60.00 (3)	75.00 (3)	100.00 (3)	60.00 (6)	50.00 (1)	100.00 (2)	0.00 (0)
Gentamicin, 10 µg	0.00 (0)	0.00 (0)	33.33 (1)	20.00 (1)	25.00 (1)	33.33 (1)	30.00 (3)	100.00 (2)	100.00 (2)	0.00 (0)
Nitrofurantoin, 300 µg	0.00 (0)	0.00 (0)	66.66 (2)	0.00 (0)	75.00 (3)	33.33 (1)	60.00 (6)	100.00 (2)	100.00 (2)	100.00 (2)
Oxytetracycline, 30 µg	0.00 (0)	0.00 (0)	33.33 (1)	20.00 (1)	0.00 (0)	33.33 (1)	50.00 (5)	100.00 (2)	50.00 (1)	0.00 (0)
Sulphafurazole, 300 µg	100.00 (1)	0.00 (0)	0.00 (0)	20.00 (1)	25.00 (1)	66.66 (2)	50.00 (5)	50.00 (1)	50.00 (1)	0.00 (0)
Vancomycin, 30 µg	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	10.00 (1)	50.00 (1)	0.00 (0)	0.00 (0)

Note: Values in parentheses are number of strains sensitive.

Table 6 – Antibiotic sensitivity (%) of the bacterial flora associated with diseased catfish – Bacterial species-wise

Bacteria flora (n=48)	Antibiotics (concentration)											
	Amoxy clav (30µg)	Chloram phenicol (30µg)	Cipro floxacin (5µg)	Clinda mycin (2µg)	Co-tri-moxazole (25µg)	Erythrom ycin (15µg)	Gati floxacin (5µg)	Genta micin (10µg)	Nitro furantoin (300µg),	Oxytetra cycline (30µg)	Sulpha furazole (300µg)	Vanco mycin (30µg)
Aeromonas bestiarum (1)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aeromonas aquariorum (2)	0.00	100.00	50.00	0.00	50.00	0.00	100.00	100.00	0.00	0.00	0.00	0.00
Aeromonas caviae (5)	0.00	80.00	20.00	0.00	20.00	0.00	40.00	20.00	60.00	20.00	20.00	40.00
Aeromonas diversa (2)	0.00	50.00	50.00	0.00	50.00	50.00	100.00	50.00	0.00	0.00	0.00	0.00
Aeromonas hydrophila (7)	0.00	85.71	57.14	0.00	71.42	0.00	71.42	28.57	85.71	28.57	42.85	0.00
Aeromonas jandei (2)	0.00	100.00	50.00	0.00	100.00	0.00	100.00	0.00	100.00	50.00	50.00	0.00
Aeromonas rivuli (2)	0.00	100.00	100.00	0.00	0.00	0.00	100.00	0.00	100.00	0.00	0.00	0.00
Aeromonas schuberti (4)	25.00	100.00	50.00	0.00	25.00	0.00	100.00	50.00	0.00	50.00	25.00	0.00
Aeromonas sobria (2)	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	100.00	0.00	100.00	0.00
Aeromonas veronii (2)	50.00	100.00	50.00	0.00	100.00	0.00	100.00	50.00	100.00	50.00	0.00	0.00
Aeromonas popoffii (1)	0.00	100.00	100.00	0.00	0.00	0.00	100.00	100.00	100.00	0.00	0.00	0.00
Edwardsiella tarda (8)	22.22	88.88	11.11	0.00	22.22	0.00	44.44	22.22	100.00	0.00	22.22	11.11
Enterobacter cloacae (1)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Klebsiella pneumonia (1)	0.00	0.00	0.00	0.00	100.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00
Morganella morganii (1)	0.00	100.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00
Serratia marcescens (1)	0.00	0.00	100.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00
Bacillus spp. (6)	33.33	83.33	50.00	16.66	50.00	16.66	50.00	33.33	33.33	66.66	16.66	33.33

Note: Values in parentheses are number of strains tested.

Table 7 – Antibiotic sensitivity (%) of the bacterial flora associated with diseased catfish – Fish species-wise

Antibiotic, concentration	Fish species		
	Pangasius pangasius (n=18)	*Clarias batrachus* (n=14)	*Clarias gariepinus* (n=16)
Amoxyclav, 30 µg	22.22 (4)	0.00 (0)	13.33 (2)
Chloramphenicol, 30 µg	77.77 (14)	71.42 (10)	86.66 (13)
Ciprofloxacin, 5 µg	38.88 (7)	64.28 (9)	20.00 (3)
Clindamycin, 2 µg	5.55 (1)	0.00 (0)	0.00 (0)
Co-trimoxazole, 25µg	44.44 (8)	64.28 (9)	33.33(5)
Erythromycin, 15 µg	11.11 (2)	0.00 (0)	0.00 (0)
Gatifloxacin, 5 µg	55.55 (10	64.28 (9)	66.66 (10)
Gentamicin, 10 µg	22.22 (4)	50.00 (7)	26.66 (4)
Nitrofurantoin, 300 µg	22.22 (4)	71.42 (10)	13.33 (2)
Oxytetracycline, 30 µg	33.33 (6)	35.71 (5)	93.33 (14)
Sulphafurazole, 300 µg	50.00 (9)	50.00 (7)	13.33 (2)
Vancomycin, 30 µg	11.11(2)	14.28 (2)	6.66 (1)

Note: Values in parentheses are number of isolates sensitive.

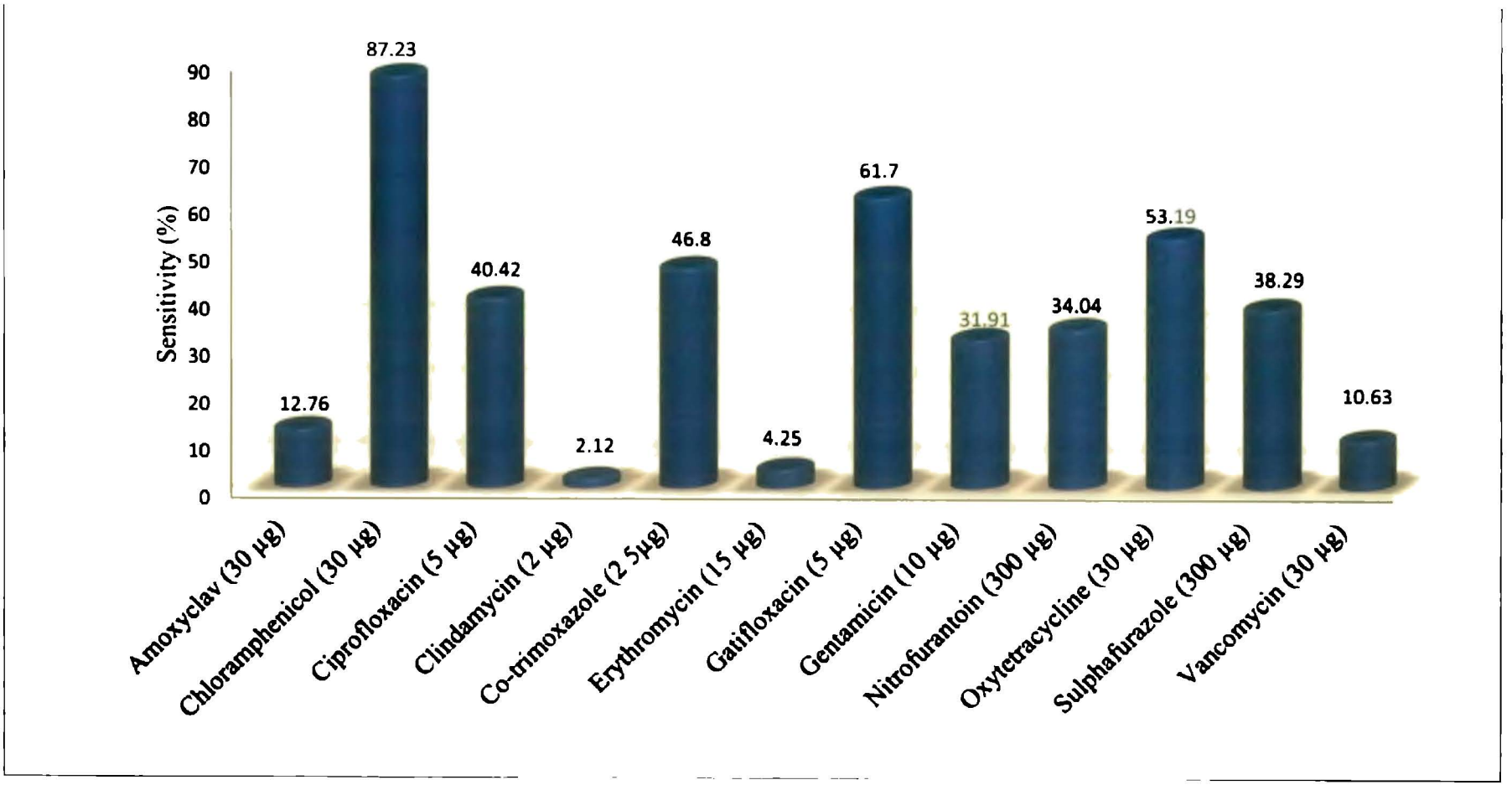


Fig. 3 – Antibiotic sensiivity of bacterial flora (n=48) associated with diseased catfish

Table 8 – Multiple antibiotic resistance (MAR) index of bacterial flora associated with diseased catfish

Bacterial species (n=48)	MAR Index
Aeromonas bestiarum (n=1)	1.00
Aeromonas aquariorum (n=2)	0.583
Aeromonas caviae (n=5)	0.416 - 0.833
Aeromonas diversa (n=2)	0.500 - 0.833
Aeromonas hydrophila (n=7)	0.416 - 0.916
Aeromonas jandei (n=2)	0.416 - 0.583
Aeromonas popoffii (n=1)	0.583
Aeromonas rivuli (n=2)	0.666
Aeromonas schubertii (n=4)	0.416 - 0.833
Aeromonas sobria (n=2)	0.750 - 0.916
Aeromonas veronii (n=2)	0.416 - 0.583
Edwardsiella tarda (n=8)	0.500 - 0.916
Enterobacter cloacae (n=1)	0.916
Klebsiella pneumoniae (n=1)	0.833
Morganella morganii (n=1)	0.750
Serratia marcescens (n=1)	0.750
Bacillus spp. (n=6)	0.083 - 0.833

MAR Index: Number of antibiotics to which the bacterium is resistant ÷ Total number of antibiotics tested

Table 9 – Multiple antibiotic resistance (MAR) profile of bacterial flora associated with diseased catfish

Bacterial species (n=48)	Number of strains	MAR*	Percentage
Aeromonas spp. (n=30)	30		100
Edwardsiella tarda (n=8)	8		100
Other Enterobacteriaceae (n=4)	4		100
Bacillus spp. (n=6)	5		83.33

MAR: Resistant to at least 3 antibiotics

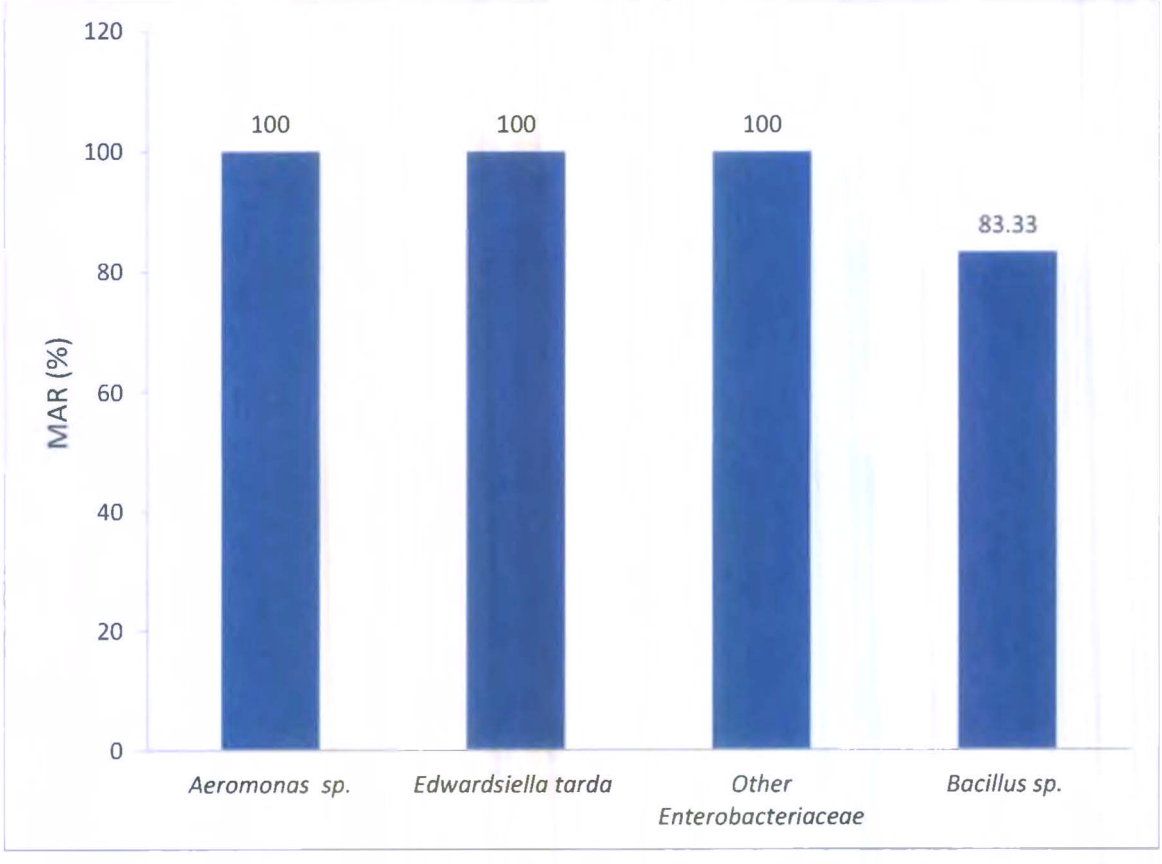


Fig. 4 - Multiple antibiotic resistance (MAR) in bacterial flora associated with diseased catfish.

Table 10 – Minimum inhibitory concentration (MIC) of antibiotics (in µg) against *Edwardsiella tarda* and other bacterial strains

Antibiotics	*Edwardsiella tarda* strains							*Citrobacter freundii*	*Aeromonas sobria*
	SWI3	SWI2	SWI1	HMK1	CGE4	CGSB50	CGH9	TNARC*	SWM1
Nitrofurantoin	0.01	3.00	1.00	30.00	30.00	5.00	1.00	3.00	0.10
Co-trimoxazole	>240.00	>240.00	>240.00	>240.00	0.01	>240.00	>240.00	0.01	30.00
Gentamicin	0.01	10.00	0.50	0.50	5.00	5.00	10.00	0.25	10.00
Ciprofloxacin	150.00	5.00	5.00	1.00	>240.00	2.00	1.00	0.25	>240.00
Chloramphenicol	0.001	0.001	0.001	8.00	30.00	0.10	0.001	0.001	30.00
Tetracycline	30.00	10.00	150.00	60.00	30.00	30.00	3.00	0.10	30.00

TNARC*: *Citrobacter freundii* from *Labro rohita*, received as gift from Shrimp disease diagnostic laboratory, Tamil Nadu Fisheries University, Chennai – 600061.

Table 11 – Pathogenicity of *Edwardsiella tarda* CGH9 on catfish *Pangasius pangasius* by intramuscular injection

Dilution	Number dead / Number injected		LD 50 value
	Trial 1	Trial 2	
10^{0}	6/6	6/6	$1.77x10^{7}$ cfu/fish
10^{-1}	6/6	6/6	
10^{-2}	2/6	0/6	
10^{-3}	0/6	0/6	
Control	0/6	0/6	

Note: The concentration of bacterial cells in suspension was $6.00x10^{11}$ cells/ml

Table 12 – Pathogenicity of *Edwardsiella tarda* (CGH9) on catfish *Clarias gariepinus* by intramuscular injection

Dilution	Number dead / Number injected		LD 50 value
	Trial 1	Trial 2	
10^{-1}	8/8	8/8	5.75×10^{7} cfu/fish
10^{-2}	3/8	2/8	
10^{-3}	0/8	0/8	
10^{-4}	0/8	0/8	
Control	0/8	0/8	

Note: The concentration of bacterial cells in suspension was $3.07x10^{11}$/ml

Fig. 5 - Light micrograph of diseased *Clarias gariepinus* skin showing inflammation of epidermal tissue (I) and rough epidermal layer (RE). X200 H&E staining

Fig. 6 - Light micrograph of diseased *Clarias gariepinus* spleen showing loosely packed red (←) and white pulp and dense melanomacrophage aggregates (MMA). X100 H&E staining

Fig. 7 - Light micrograph of diseased *Clarias gariepinus* spleen showing loosely packed red (←) and white pulp and melanomacrophage aggregates (MMA). X200 H&E staining

Fig. 8 - Light micrograph of stomach of diseased *Clarias gariepinus* showing degenerative changes marked by arrows. X40 H&E staining.

Fig. 9 - Light micrograph of stomach of diseased *Clarias gariepinus* showing extensive degeneration (ED), basophilic margination (BM) and disintegration of mucosal layer (DM). X100 H&E staining

Fig. 10 - Light micrograph of diseased *Clarias gariepinus* showing degenerative cardiac glands marked by arrows. X100 H&E staining

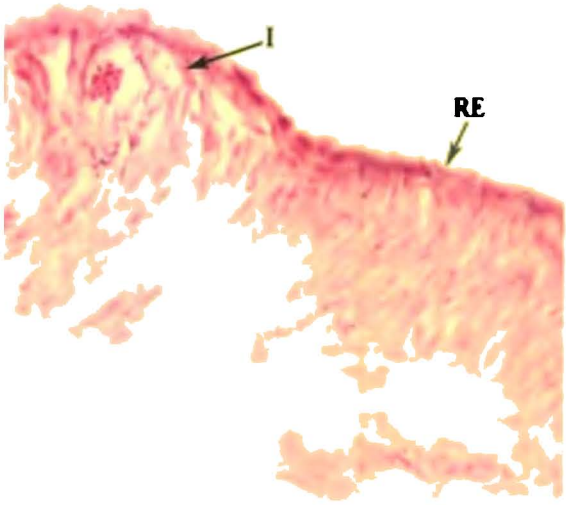


Fig. 5

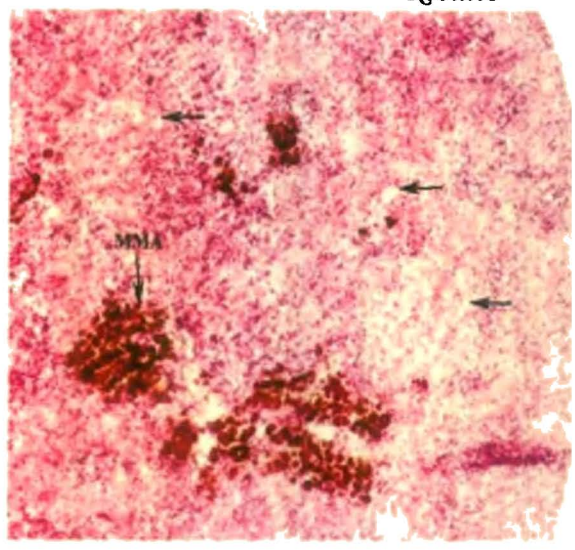


Fig. 6

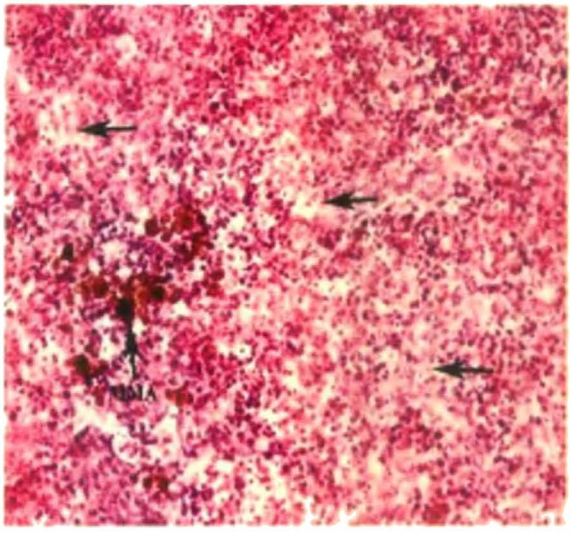


Fig. 7

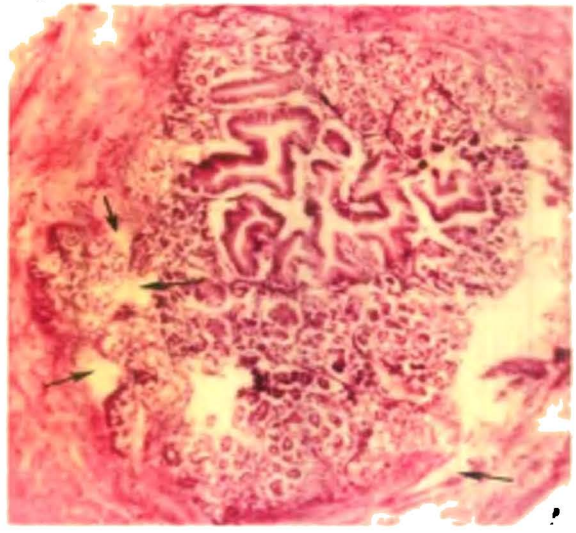


Fig. 8

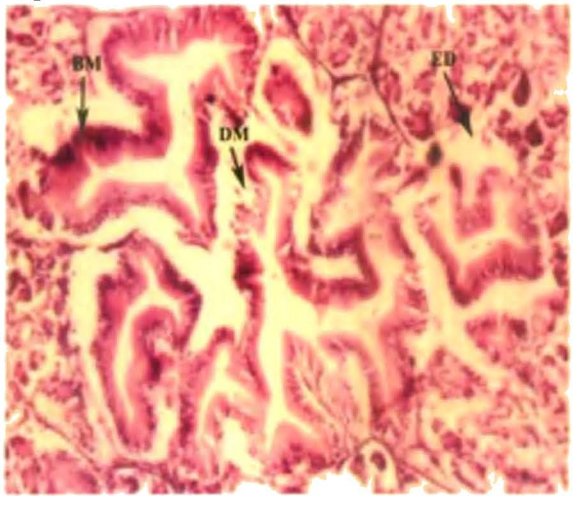


Fig. 9

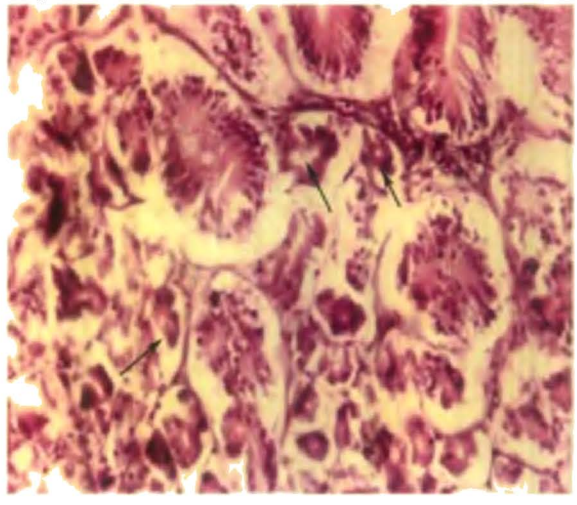


Fig. 10

Figs. 5 – 10: Histopathological alterations observed on diseased *Clarias gariepinus*

Fig. 11 - Light micrograph of diseased kidney of *Clarias gariepinus* showing necrosis (N), necrotised haematopoietic tissue (NHT) and nephritic tubules with widened lumen (WTL). X200 H&E staining

Fig. 12 - Light micrograph of diseased kidney of *Clarias gariepinus* showing necrosis (N), cellular hypertrophy (CH), pycnotic nuclei (P) and karyolysis (K). X400 H&E staining

Fig. 13 - Light micrograph of diseased kidney of *Clarias gariepinus* showing necrosis (N), inflammation of epithelial tissue (I), vacuolization of tubular epithelium (V) and hypoplastic haematopoietic tissue (HHT). X200 H&E staining

Fig. 14 - Light micrograph of diseased *Clarias batrachus* showing haemocyte infiltration (HI) and necrosis of muscle tissue (N). X200 H&E staining

Fig. 15 - Light micrograph of diseased *Clarias batrachus* showing inflammation of epidermal tissue (I) and rough epidermal layer (RE), extensive necrosis of muscle (EN) and fibrosis (F). X200 H&E staining.

Fig. 16 - Light micrograph of diseased *Clarias batrachus* kidney showing extensive necrosis (N), cellular hypertrophy (CH), nuclear hypertrophy (NH), pycnotic nuclei (P) and karyolysis (K). X400 H&E staining

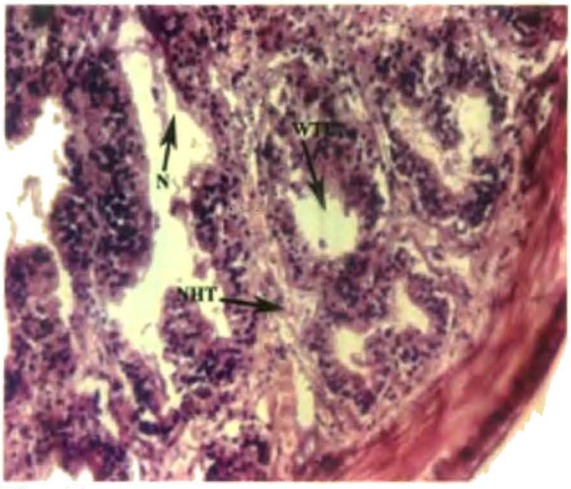


Fig. 11

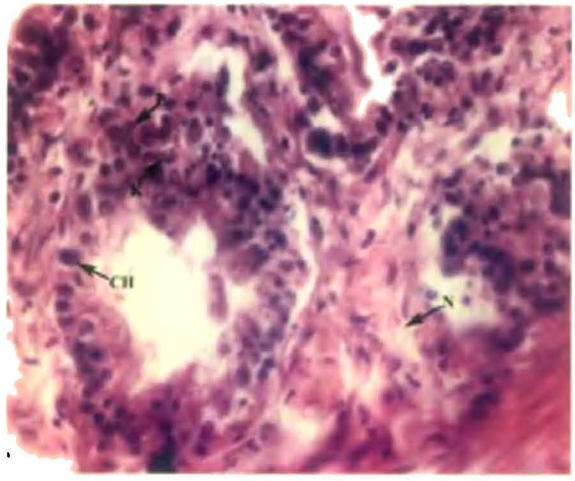


Fig. 12

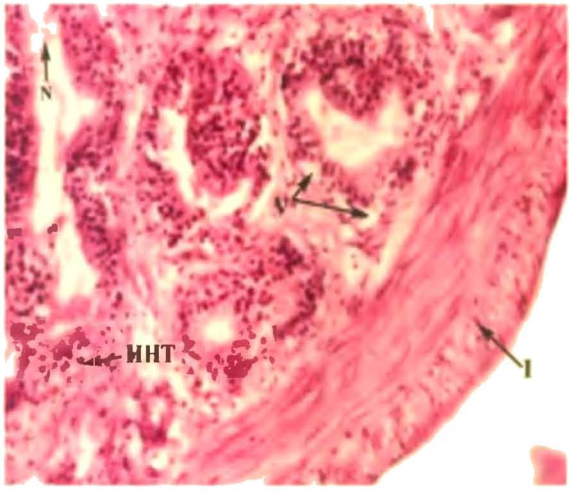


Fig.13

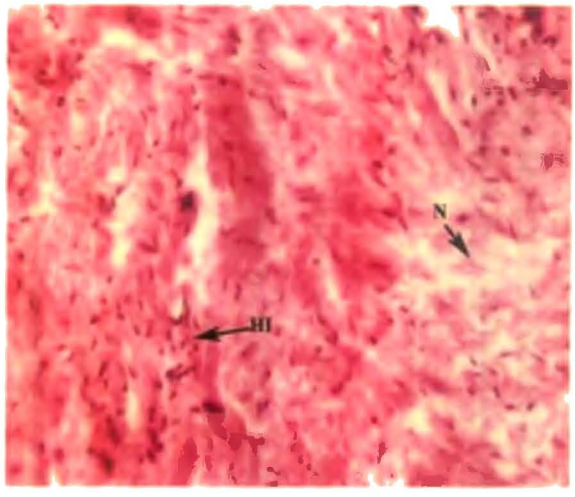


Fig. 14

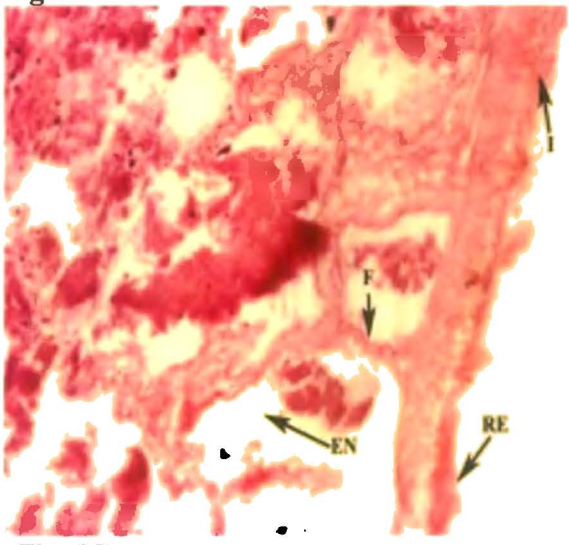


Fig. 15

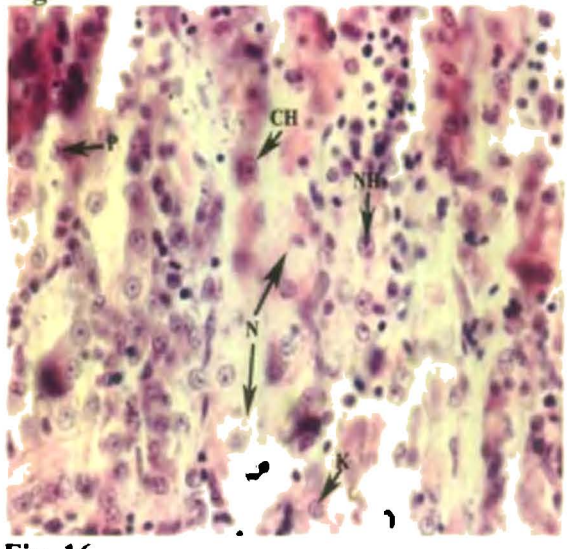


Fig. 16

Figs. 11– 13: Histopathological alterations observed on diseased *Clarias gariepinus*
Figs. 14 – 16: Histopathological alterations observed on diseased *Clarias batrachus*

Fig. 17 - Light micrograph of diseased *Clarias batrachus* kidney showing extensive necrosis (N) and loss of typical tubular epithelial lining (LTE). X400 H&E staining

Fig. 18 - Light micrograph of diseased *Clarias batrachus* kidney showing extensive necrosis (N), necrosis of tubular tissue (NT), inflammation of kidney epithelial layer (I), hypoplastic haematopoietic tissue (HHT). X400 H&E staining

Fig. 19 - Polypeptide profile of outer membrane protein of *Edwardsiella tarda*

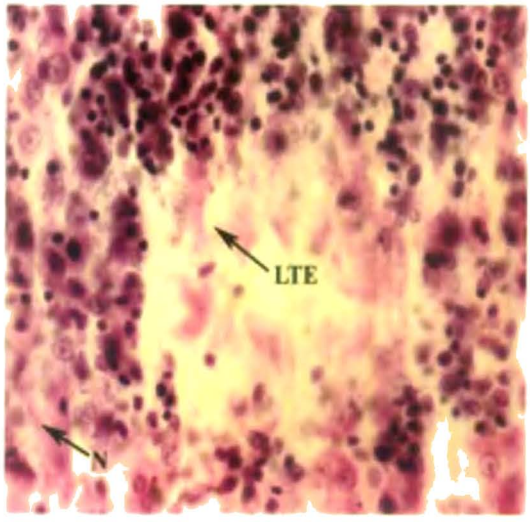


Fig. 17

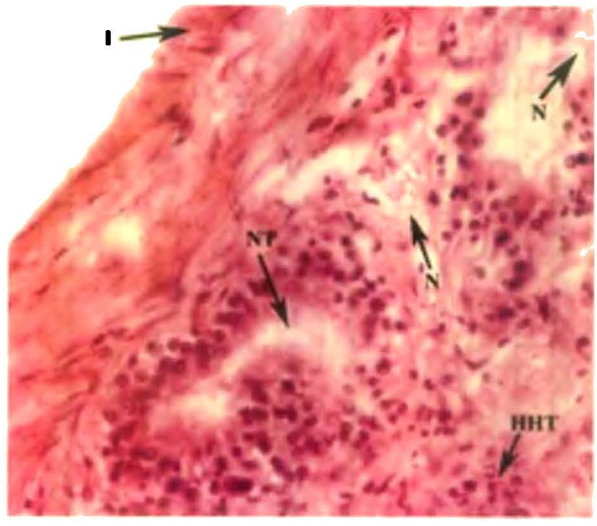
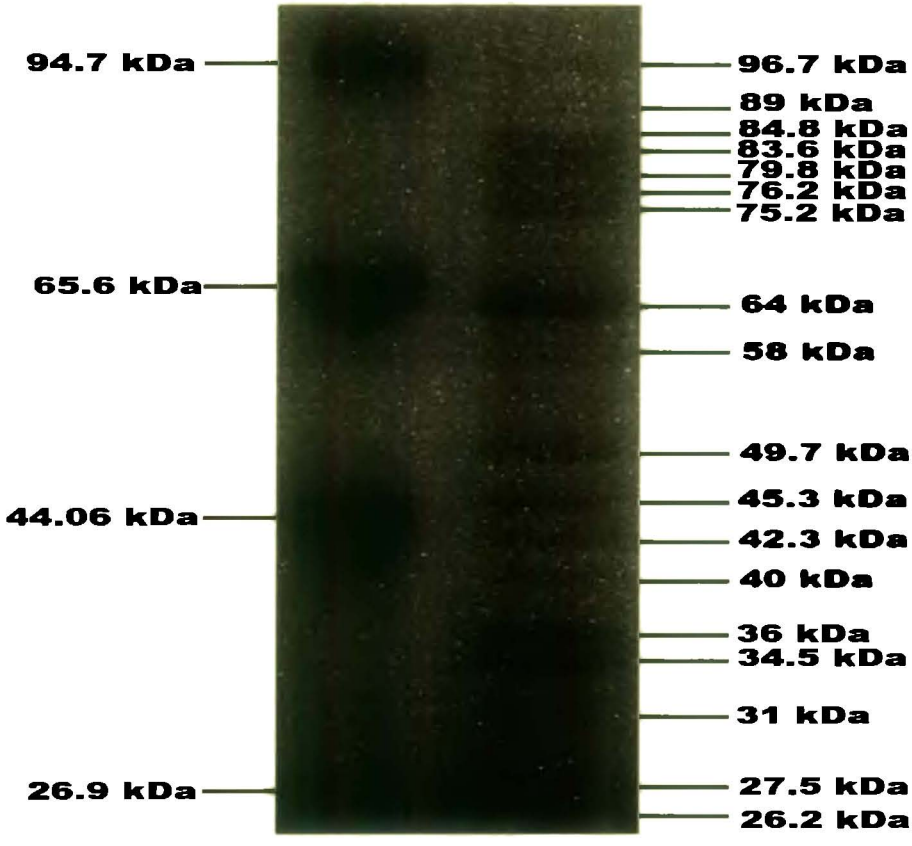


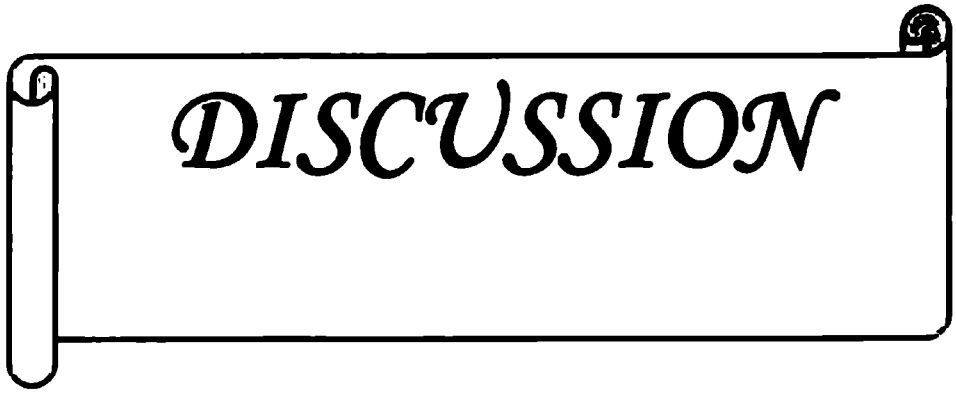
Fig. 18



Figs. 17– 18: Histopathological alterations observed on diseased *Clarias batrachus*

Fig. 19 - Polypeptide profile of outer membrane protein of *Edwardsiella tarda*

CHAPTER -5



5. DISCUSSION

In West Bengal different types of freshwater catfish are commercially cultured and these include *Clarias batrachus*, *C. gariepinus*, *C. macrocephalus*, *Heteropneustes fossilis*, *Pangasius sutchi*, *P. pangasius*, *Ompak pabda*, *Sperata gulio* and *S. tangra*. Among them, *C. batrachus* and *P. sutchi* are the most favourable from economical point of view and availability of seeds as induced breeding technique has been practiced for *C. batrachus* (Dhara and Saha, 2013) and *P. sutchi* (Chattopadhyay *et al.*, 2002). Development of grow-out technologies for *S. seenghala*, *S. aor*, *Wallago attu*, etc are also given greater importance in recent years in different parts of India due to their high consumer preferences and high nutritional value.

5.1 Gross and clinical signs of diseased catfish

The diseased catfish, as presented in Table 1, exhibited sluggish behaviour, erratic movement, hanging, lethargy, anorectic, sliming, skin erosion, foul smell, pale gills, dull and red colouration of eyes, red lateral line, fin rot, tail rot, ulcer, dropsy, accumulation of yellow fluid, abdominal haemorrhage, mouth and opercula haemorrhage, pectoral fin haemorrhage, discoloured and liquefied internal organs. In diseased *C. gariepinus* gross and clinical signs such as sluggish behaviour, vertical movement, anorectic, dropsy, yellow fluid accumulation, foul smell, focal cutaneous haemorrhage, pectoral fin haemorrhage, abdominal haemorrhage, ulcer and discolouration of internal organs were observed. The diseased *C. batrachus* showed skin erosion, anorectic, eroded operculum, pale gills, vertical movement, reddening of mouth, haemorrhagic septicaemia, red lateral line and dropsy. In diseased *P. pangasius* gross and clinical signs like erratic movement, sliming, dull and red eyes, haemorrhagic mouth and operculum, pale gills, fin rot, tail rot, abdominal haemorrhage, swelling and dropsy were observed. Among all, haemorrhage was the most commonly observed clinical sign. Similar conditions were also noticed in motile *Aeromonas* septicaemic (MAS) catfish, *C. gariepinus* (Laith and Najiah, 2013), *C. batrachus* (Majumder *et al.*., 2007), *Pangasius* sp. (Dung *et al.*, 2008; Ly *et al.*, 2009; Kumar and Ramulu, 2013) and *Ictalurus lunetas* (Huang *et al.*, 2010).

Next to haemorrhage, dropsy was the most common sign. Along with dropsy other clinical signs like yellow fluid accumulation, discolouration and/or liquefaction of internal organs and gas accumulation were observed. Emanation of foul smell was

noticed in dissected fish. Similar clinical signs were reported for *Edwardsiella* septicemia caused by *E. tarda* (Bullock and Herman, 1985; Mohanty and Sahoo, 2007; Park *et al.*, 2012). Like *E. tarda, E. ictaluri* is also a common pathogen of catfish causing bacillary necrosis of *Pangasius* (Fergusion *et al.*, 2001; Dung *et al.*, 2008) and enteric septicaemia (Hawke *et al.*, 1998; Chappell, 2008; Carrias, 2011). The present study, however, did not find any association of *E. ictaluri* in diseased catfish.

5.2 Diagnosis of diseases and associated bacterial flora

Identification of bacteria is an important factor to diagnose the disease. In total, 17 disease cases of 3 different catfish species were investigated. Most of the diseased catfish were had mixed bacterial infection and bacterial species such as *Aeromonas caviae, A. hydrophila, A. sobria, A. veronii, A. bestiarum, A. aquariorum, A. diversa, A. jandaei, A. rivuli, A. schuberti, E. tarda, Enterobacter cloacae, Escherichia coli, Morganella morganii, Klebsiella pneumoniae, Serratia marcescens, Providencia alcalifaciens, Citrobacter freundii,* other members of Enterobacteriaceae, *Pseudomonas* spp. *Sphingomonas paucimobilis, Lactobacillus* sp., *Corynebacterium* sp., *Bacillus* spp., some unidentified Gram positive rods and cocci were isolated.

The phenotypic characteristics of select bacterial strains as determined through an automated bacterial identification system (VITEK 2 compact, Biomerieux, France) are presented in Table 2. The selected bacterial strains were identified as *Edwardsiella tarda, Aeromonas sobria, Enterobacter cloacae, Serratia marcescens, Sphingomonas paucimobilis, Escherichia coli, Citrobacter freundii* and *Klebsiella pneumoniae* on the basis of biochemical characterization. Nawaz *et al.* (2006) demonstrated that with the Vitek GNI system 81 strains isolated from the intestines of catfish (*I. punctatus*) collected from different geographical regions of United States were identified as *A. hydrophila* (n=23), *A. trota* (n=7), *A. veronii* (n=42), *A. caviae* (n=6) and *A. jandaei* (n=3). Several phenotypic approaches either conventional or commercial are still used but have limitations related to the influence of cultural conditions on morphological and biochemical characteristics and to the effect of environmental pressure on gene expression (Biovin-Jahns *et al.*, 1995; Maruyama *et al.*, 2000; Soutourina *et al.*, 2001; Figueras *et al.*, 2011). So along with phenotypic characterization, genotyping characterization should be followed to identify the taxonomic status of bacterial isolates (Alatossava and Alatossava, 2006; Nawaz et al.,

2006; Kozińska, 2007; Beaz-Hidalgo *et al.*, 2010). The 16S rDNA method is a popular technique used for genotypic characterization. This assay relies on the amplification of the gene coding for small -ribosomal RNA (16S rRNA), which is present in almost all bacteria and when combined with DNA sequencing the assay provides a definite identification of the infectious agents as the 16S rRNA contains conserved and highly divergent regions which serve for species specific identification (Martínez-Murcia *et al.*, 1992; Lee *et al.*, 2002; Vernon *et al.*, 2002; Alperi *et al.*, 2008). The 16S rDNA method is a scientific and objective method of identification of microorganisms (Tang *et al.*, 1998) and useful alternative when phenotypic characterization methods fail (Drancourt *et al.*, 2000). This method is widely used not only to define taxa, but also to identify organism present in clinical, veterinary and environmental samples (Biovin-Jahns *et al.*, 1995; Tang *et al.*, 1998; Drancourt *et al.*, 2000; Maruyama *et al.*, 2000).

In present study, selected bacterial isolates were further identified and characterized through 16S rDNA analysis. The detailed information on the bacterial strains, host species, clinical signs, site of infection, consensus sequence, GenBank accession number are presented in Table 3 and 4. In 1.2% agarose gel electrophoresis, approximately 1.5 kbp bands were obtained by PCR amplification (Fig. 1). Data analysis and multiple alignment of sequenced product were performed by using Clustal X (MEGA 5) and sequences were compared against the GenBank database of the National Institute of Biotechnology Information (NCBI) by using the BLAST (Basic Local Alignment Search Tool). The phylogenetic tree (Fig. 2) was constructed by using the Kimura-2 correction for evolutionary rate. All *Aeromonas* strains of diseased catfish were clustered together and separated from other groups. Likewise, all Enterobacteriaceae members were clustered together with three lineages. All *E. tarda* strains were clustered together within the Enterobacteriaceae group as a separate lineage.

The diseased *C. gariepinus* of Deganga (3 Cases) had mixed bacterial infection, where *E. tarda*, *Aeromonas* spp., *Pseudomonas* spp., Enterobacteriaceae, Gram positive rods and cocci were isolated. The diseased *C. gariepinus* of Basirhat, Ramchandrapur and Naihati also had mixed bacterial infection with the dominance of *Aeromonas* spp. Along with it, *E. tarda* was also found. Likewise, *E. tarda* was reported from diseased *C. gariepinus* (El-jakee *et al.*, 2008; Musa *et al.*, 20009;

Ibrahem *et al.*, 2010; Ali *et al.*, 2014) and *A. hydrophila* from diseased *C. gariepinus* (Laith and Najiah, 2013). Efuntoye *et al.* (2012) reported that majority of the bacteria associated with *C. gariepinus* belonged to the members of the family Enterobacteriaceae. Association of *Staphylococcus aureus* and *Staphylococcus* spp. (El-Sayyad *et al.*, 2010; Oladosu-Ajayi, 2011; Ajai, 2012; Udze, 2012; Efuntoye *et al.*, 2012), *Pseudomonas* spp. and *P. aeruginosa* were also reported in *C. gariepinus* (AAHRI, 1995). Association of *Pseudomonas* spp. and *A. hydrophila* was reported in diseased catfish with infectious dropsy (AAHRI, 1995; Edun, 2007). Different *Bacillus* spp. were also reported from *C. gariepinus* (Oladosu-Ajayi, 2011; Ajai, 2012; Udze, 2012), similar the observations of the present study.

The diseased *C. batrachus* from Canning, South 24 Parganas district (Case 13 and Case 15) and Memari, Burdwan district (Case 14), West Bengal showed multiple bacterial infection with the association of different bacterial species. Among them, motile *Aeromonas* spp. were the dominant. The results corroborate the observations of earlier studies (Majumder *et al.*, 2007; Sarkar and Rashid, 2012) where *A. hydrophila* and other motile *Aeromonas* spp. were reported as major pathogens of *C. batrachus.*

The diseased *P. pangasius* from Garia, South 24 Parganas district (Cases 2, 5 and 12), Basirhat (Case 10) and Naihati (Case 3), North 24 Parganas district and Satyapole, Nadia district (Case 6) exhibited three different kinds of bacterial infection. In Cases 2, 3, 6 and 10, infected fish had mixed bacterial infection; where Enterobacteriaceae, *Aeromonas* spp., Gram positive rods and cocci were detected. The Case 5 was diagnosed as Aeromoniasis and Pseudomoniasis, in which *A. caviae* and *Pseudomonas* spp. were detected. The Case 12 was diagnosed as Aeromoniasis, where *A. hydrophila* and *A. sobria* were identified. In an earlier study by Kumar and Ramulu (2013) association of three species of *Aeromonas,* viz., *A. hydrophila, A. caviae* and *A. sobria* with red disease in *P. hypophthalmus* was reported.

The present study recorded involvement of different species of motile *Aeromonas* in diseased catfish. *Aeromonas*, ubiquitous in all freshwater environments, probably cause the most common bacterial disease of freshwater fish (Noga, 2000), could be a potential candidate as indicator organism in order to monitor antimicrobial resistance in fish farming (Naviner *et al.*, 2006). Motile *Aeromonas* spp. were

reported as the causative agent of infectious disease in *H. fossilis* and *C. batrachus* (Sarkar and Rashid, 2012; Majumdar *et al.*., 2007), *I. punctatus* (Camus *et al.*, 1998; FAO, 2009), *I. lunetas* (Huang *et al.*, 2010), *P. hypophthalmus* (Dung *et al.*, 2008; Ly *et al.*, 2009; Kumar and Ramulu, 2013).

Pseudomonas spp. were reported to cause diseases in wide range of fish species including carps (Schaperclaus, 1959; Bauer *et al.*, 1973; Heuschmann-Brunner, 1978; Csaba *et al.*, 1981), tench (Ahne *et al.*, 1982) and trouts (Sakai *et al.*, 1989). *Pseudomonas* spp. was also reported in *C. gariepinus* (AAHRI, 1995) and *P. hypophthalmus* (Kumar and Ramulu, 2013). *Sphingomonas paucimobilis* was isolated from *C. gariepinus* (Case 7) in the present study. It is reported as a human pathogen causing septic arthritis (Rayan and Adley, 2010; Suoto *et al.*, 2012).

Among different pathogens, *E. tarda* infection was detected mostly in *C. gariepinus* in present study. The *E. tarda* infection in diseased *C. gariepinus* of the present study matched closely with the descriptions of *E. tarda* (Ewing *et al.*, 1965; Farmer *et al.*, 1984). Likewise, *E. tarda* infection in channel catfish, *I. punctatus* (Meyer and Bullock 1973; Wiedenmayer *et al.*, 2006), Korean catfish *Silurus asotus* (Yu *et al.*, 2009), *C. gariepinus* (Musa *et al.*, 2009; Ibrahem *et al.*, 2010), *P. pangasius* (Shetty *et al.*, 2014), *C. batrachus* (Sahoo *et al.*, 1998) was reported. Besides these, *E. tarda* infection in other fish like IMC (Das *et al.*, 2014), zebrafish (Pressley *et al.*, 2005), *Lates calcarifer* (Nadirah *et al.*, 2012) and mullet (Kusuda *et al.*, 1976) and salmon (Amandi *et al.*, 1982) was reported.

Among the Enterobacteriaceae group other than *E. tarda, E. cloacae, E. coli, M. morganii, K. pneumoniae, S. marcescens, P. alcalifaciens, C. freundii* and other members of Enterobacteriaceae were detected in diseased catfish of the present study. *Citrobacter* and *Proteus* were isolated from the intestines of freshwater fish and pond water (Niemi and Taipalinen, 1982; Apun *et al.*, 1999). Association of *Proteus* spp. was found in *I. punctatus* (Ramos and Lyon, 2000) and tilapia (Mhango *et al.*, 2010). Sharma *et al.* (2006) reported low prevalence of *Proteus mirabilis and P. vulgaris* in fish and so also in Okafor and Nzeako (1985) and Kumari *et al.*, (2001). Presence of these microbes has a fatal effect on human beings as different *Proteus* species were known to provoke diarrhoea and dysentery (Sharma *et al.*, 2006). *Citrobacter freundii* causes abnormal inflammatory changes in the intestine of trout and inflammatory and

necrotic changes in the internal organs of cyprinids (Drelichman and Band, 1985). Oladosu-Ajayi *et al.* (2011) reported association of enterobacteriaceae such as *C. freundii, P. mirabilis, E. cloacae, E. coli, K. Oxytoca* and *K. pneumoniae* from *C. gariepinus*. *Klebsiella pneumoniae* and *E. coli* were reported as the most predominant Enterobacteriaceae in *C. gariepinus* (Ajai, 2012; Udze, 2012). *Providencia stuartii* were reported as the aetiological agent of ulcerative disease of *L. rohita* (Ramkumar *et al.*, 2013). *Serratia marcescens* was reported as the causative agent of ampullary system infection and septicaemia in bonnet head shark, *Sphyrna tiburo* (Camus *et al.*, 2013). Species of *Serratia* genus exist in normal microbial flora of soil and water (Austin and Austin, 2012; Holt *et al.*, 1994), and the organs and intestines of fish (Markovic and Radojicic, 1996). *Serratia liquefaciens* is considered a pathogenic bacterium of fish, and it causes infection leading to heavy mortalities in Atlantic salmon populations (McIntosh and Austin, 1990; Austin and Austin, 2012). The strains of *Serratia fonticola* were isolated from freshwater and soil (Gavini *et al.*, 1979). Farmer *et al.* (1985) reported the isolation of *S. fonticola* as a contaminant in a wound and from the respiratory tract. Muller *et al.* (1986) suggested that one of the habitats of *S. fonticola* could be the digestive tracts of birds. Ritu Ranjan (2011) isolated *S. fonticola* in diseased *C. gariepinus* and *P. pangasius* in West Bengal where uncooked chicken wastes were used as feed. Efuntoye *et al.* (2012) reported that majority of the bacteria associated with *C. gariepinus* belonged to the members of the family Enterobacteriaceae, *S. aureus* and *P. aeruginosa*. *Enterobacter cloacae* was reported from *P. hypophthalmus* (Kumar *et al.*, 2013). *Klebsiella pneumoniae* present on skin and intestine may cause infection in catfish, *C. gariepinus* and can act as a vector of human pathogen (Udze *et al.*, 2012).

Bacillus spp. were also isolated in diseased catfish of the present study. *Bacillus* spp. are mostly found in the intestine of fish (Sugita *et al.*, 1998), usually non-pathogenic, which exhibit antimicrobial effect on bacterial pathogens (Kaynar and Beyatli, 2012; Natarajan and Rajikkannu, 2014). They also act as growth promoters for larval development (Arig *et al.*, 2013). They were also reported as fish pathogens. During 1989 to 1991, 10-15% mortalities were reported due to *Bacillus* infection in *C. carpis, C. gariepinus* and *C. nigrodigitatus* in Nigeria (Oladosu *et al.*, 1994). Similar to this study, association of different *Bacillus* species were reported earlier from *C. gariepinus* (Oladosu-Ajayi, 2011; Ajai, 2012; Udze, 2012).

Lactobacillus sp. was observed in the present study in diseased *C. gariepinus* from Deganga, North 24 Parganas District. It was reported earlier as the intestinal flora of African catfish *C. gariepinus* (Bucio *et al.*, 2006). Its antagonistic effect on fish pathogen like *Aeromonas* sp. and *Vibrio* sp. was also reported (Dhanasekaran *et al.*, 2010). Lactobacilli have also been reported as fish pathogens. Mitchel *et al.* (1986) reported *Lactobacillus piscicola* as an aetiological agent of 'pseudo-kidney disease' in yearling or older trout and salmon. Nzeh and Udze (2010) reported the incident of *L. delbruekii* infection on catfish *C. bidorsalis*.

Association of *Staphylococcus* spp. was found in silver carp (Shah and Tyagi, 1986), yellowtail and red sea bream (Kusuda and Sugiyama, 1981; Sugiyama and Kusuda, 1981). Along with other microbes, *Staphylococcus* spp. were reported from *C. gariepinus* as associated bacterial flora (El-Sayyad *et al.*, 2010; Oladosu-Ajayi, 2011; Ajai, 2012; Udze, 2012; Efuntoye *et al.*, 2012).

The present study addressed only the bacterial flora of disease catfish as no typical symptoms of fungal and viral diseases were observed during the survey period, Very little is known about the catfish virus. Diseases of unknown etiology have been reported in *C. gariepinus*. Nevertheless, the involvement of viruses in many of the disease Cases of the present study cannot be ruled out. This calls for screening of catfish for viruses and their diagnosis.

5.3 Antibiotic sensitivity of bacterial flora of diseased catfish

A total of 48 bacterial strains comprising *A. caviae, A. hydrophila, A. sobria, A. veronii, A. bestiarum, A. aquariorum, A. diversa, A. jandaei, A. rivuli, A. schuberti, E. tarda, E. cloacae, M. morganii, K. pneumonia, S. marcescens* and *Bacillus* spp. from diseased catfish were subjected to antibiogram against twelve antibiotics, viz., amoxyclav (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), co-trimoxazole (25 µg), erythromycin (15 µg), gatifloxacin (5 µg), gentamicin (10 µg), nitrofurantoin (300 µg), oxytetracycline (30 µg), sulphafurazole (300 µg) and vancomycin (30 µg). The bacterial flora exhibited varying degrees of sensitivity to different antibiotics (Fig. 3 and Tables 5-7). Among the bacterial pathogens tested, *A. bestiarum* isolated from *P. pangasius* was resistant to all the tested antibiotics. All the *A. jandaei* and *A. veronii* strains were sensitive to chloramphenicol, co-trimoxazole, gatifloxacin, nitrofurantoin; while *A. diversa* were sensitive only to gatifloxacin. The

A. popoffii strain was sensitive to chloramphenicol, ciprofloxacin, gatifloxacin, gentamicin, nitrofurantoin and all the strains of *A. rivuli* were also sensitive to the above said antibiotics except gentamicin (0%). All the *A. aquariorum* strains were sensitive to chloramphenicol, gatifloxacin, gentamicin and all the strains of *A. schuberti* were also sensitive to the above said antibiotics except gentamicin (50%). All strains of *A. sobria* were sensitive to co-trimoxazole, nitrofurantoin; while about 86% of the *A. hydrophila* were sensitive to nitrofurantoin. About 80% of *A. caviae* were sensitive to chloramphenicol. The results of this study are in agreement with Kaskhedikar and Chhabra (2010) and contradictory to the earlier observations (McPhearson *et al.*, 1991, Schmidt *et al.*, 2000, Bharathkumar, 2008). Most of the *Aeromonas* isolates were resistant to oxytetracycline. Rhodes *et al.* (2000) reported cent percent and Rahaman and Hossain (2010) reported 78.95% oxytetracycline-resistance among the mesophilic aeromonads from fish farm. The results of Ho *et al.* (2008) revealed that the isolates of *A. hydrophila* are sensitive to sulphamethoxazole, ciprofloxacin, oxytetracycline, enrofloxacin, erythromycin, sulpha-methoxazole/ trimethoprim, doxycycline and florfenicol and resistant to amoxycillin and ampicillin. According to Pendersab and Stobberingh (2008), the prevalence of resistance in *A. hydrophila* from catfish and eel farms in the southern part of the Netherlands was as follows: ampicillin and oxytetracycline 100%; sulfamethoxazole 24%; trimethoprim 3%; and ciprofloxacin and chloramphenicol 0%. The majority of samples showed a high degree of oxytetracycline resistance, implicating fish farms as a major reservoir of oxytetracycline resistance genes. The wide spread use of antimicrobial agents/therapeutants in aquaculture has been associated with increased antibiotic resistance in *A. hydrophila* (Aoki, 1992; De Paola *et al.*, 1995; Naviner *et al.*, 2006). Abraham *et al.* (2004) recorded both *Aeromonas* spp. and *Pseudomonas* spp. from freshwater fish culture systems of West Bengal as highly sensitive to broad-spectrum antibiotics. The differences in the frequency of resistance may be related to the source of the *Aeromonas* isolates and the frequency and type of antimicrobial agents used for treating diseases and for health management in that geographical area.

About 87% bacterial flora of diseased catfish (Fig. 3) were sensitive to chloram-phenicol followed by gatifloxacin (62%), oxytetracycline (53%), co-trimoxazole (47%), ciprofloxacin (40%), sulphafurazole (38%), nitrofurantoin (34%), gentamicin (32%), amoxyclav (13%), vancomycin (11%), erythromycin (4%) and

clindamycin (2%). The bacterial flora of *P. pangasius* was sensitive to chloramphenicol (77.77%) and gatifloxacin (55.55%). The bacterial flora of *C. bnatrachus* was sensitive to chloramphenicol (71.42%) and nitrofurantoin (71.42%), while the bacterial flora of *C. gariepinus* was sensitive to oxytetracycline (93.33%) and chloramphenicol (86.66%).

Ritu Ranjan (2011) also reported that about 88% of the bacterial strains isolated from *C. gariepinus* were sensitive to chloramphenicol. Tendencia *et al.* (2001) examined a total of 413 shrimp bacterial isolates for their resistance to four antibiotics and reported that maximum number of bacterial isolates were resistant to oxytetracycline. The study of Singh *et al.* (2009) revealed the presence of an important population of oxytetracycline-resistant bacteria in the microflora of Indian carp aquaculture farms. In a study by Sande and Madell (1985) all the isolates such as *Flavobacterium, Alcaligenes, Aeromonas, Pseudomonas, Escherichia, Serratia, Citrobacter, Enterobacter, Shigella* and *Proteus* were resistant to oxytetracycline, nitrofurazone and novobiocin, but sensitive to chloramphenicol, ciprofloxacin and gentamycin. Chloramphenicol has a wide spectrum of antimicrobial activity and it exerts marked therapeutic effects against sensitive organisms, although known to cause growth suppression. The observed differences in antibiotic resistance may be due to strain variation.

In the present study all the strains of *E. tarda* were sensitive to nitrofurantoin and 88.80% were sensitive to chloramphenicol. Ritu Ranjan (2011) reported that *E. tarda* strains were sensitive to gentamicin (100%) and chloramphenicol (85%). Contrary to the database of Stock and Wiedemann (2001), most of the *E. tarda* strains of the present study were resistant to oxytetracycline (89%) followed by co-trimoxazole (48%). Similar results were reported by Mallick (2008) and Pankajkumar (2009), who found all the 33 isolates of *E. tarda* were sensitive to ciprofloxacin. Ali *et al.* (2014) reported that *E. tarda* isolates were found to be sensitive against ciprofloxacin, streptomycin, chloramphenicol and gentamycin and were resistant to oxytetracycline, ampicillin and nalidixic acid. Pankajkumar (2009) recorded resistance to oxytetracycline to the tune of 69.70%, followed by nitrofurantoin (66.70%), gentamycin (54.50%), co-trimoxazole (18.20%) and chloramphenicol (6.10%) among *E. tarda* isolates in West Bengal. A high susceptibility of *E. tarda* to several antibiotics was documented in different studies (Muyembe *et al.*, 1973;

Reinhardt *et al.*, 1985; Bergan *et al.*, 1988; Vartian and Septimus, 1990; Clark *et al.*, 1991; Janda *et al.*, 1993; Reger *et al.*, 1993; Pankajkumar, 2009; Abraham, 2011).

Morganella morganii strain of present study from diseased *P. pangasius* was sensitive to chloramphenicol, co-trimoxazole and sulphafurazole. Ritu-Ranjan (2011) reported that *M. morganii* was sensitive to chloramphenicol and resistant to ciprofloxacin and oxytetracycline. Zalas-Wiecek (2011) also reported that most of the *M. morganii* strains were resistant to oxytetracycline. *Serratia marcescens* strains of present study from diseased *C. batrachus* were sensitive to ciprofloxacin, co-trimoxazole and sulphafurazole. But Ritu Ranjan (2011) observed in her studies that *S. fonticola* strains exhibited resistance to ciprofloxacin, co-trimoxazole and oxytetracycline to the tune of 40% and sensitive to nitrofurantoin (90%), chloramphenicol (90%) and gentamicin (80%). While *S. liquefaciens* were sensitive to oxytetracycline to the tune of 65%, chloramphenicol (80%) and gentamicin (70%) and showed maximum resistance to co-trimoxazole (40%) followed by nitrofurantoin (35%). According to the results of Aydin *et al.* (2001), *S. liquefaciens* were sensitive to quinolone group (norfloxacin, ofloxacin), gentamicin, netilmycine and potentiated sulphonamide. In the present study, *K. pneumoniae* was observed to be sensitive to co-trimoxazole and gatifloxacin. Amikacin (Sarathbabu *et al.*, 2012; Feglo, 2010) and imipenem (Ravichitra *et al.*, 2014) was reported as the most effective antibiotic against this pathogen.

In present study, *Bacillus* spp. were sensitive (83%) to chloramphenicol followed by oxytetracycline (66.66%), ciprofloxacin (50%). Earlier reports revealed that most effective antibiotics against *B. cereus* are gentamicin, ciprofloxacin, chloramphenicol followed by streptomycin, ofloxacin and nalidixic acid (Whong and Kwaga, 2007; Hafiz *et al.*, 2012).

5.3.1 Multiple antibiotic resistance index (MAR index) and multiple antibiotic resistance (MAR) profile of bacterial flora associated with diseased catfish

The details on MAR index and MAR profile are presented in Tables 8 and 9, respectively. The MAR index of bacterial strains was in the range of 0.416-1.000 for *Aeromonas* spp., 0.500-0.916 for *E. tarda,* 0.750-0.916 for other Enterobacteriaceae group and 0.083-0.833 for *Bacillus* spp. (Table 8). Wei *et al.* (2011) reported MAR index of *E. tarda* varied from 0.15-0.43 when tested against six antibiotics such as

ampicillin (10 µg), kanamycin (30 µg), tetracycline (30 µg), nalidixic acid (30 µg), furazolidone (15 µg) and sulphamethoxazole (25 µg). Orozova *et al.* (2010) studied the antibiotic sensitivity pattern of *Aeromonas* sp. and reported that MAR index varied from 0.26 to 0.53. In a study by Laith and Najiah (2013) the MAR index of *A. hydrophila* isolated from *C. gariepinus* varied from 0.1 to 0.5.

All the strains of *Aeromonas* spp., *E. tarda* and other Enterobacteriaceae were of MAR group, i.e., were resistant to at least 3 antibiotics; while 83.33% of *Bacillus* spp. were of MAR group (Fig. 4). The study by Abraham (2011) revealed comparatively high incidence of MAR in bacterial flora of catfish (76%) followed by miscellaneous fish (66%), sewage-fed farm grown carps (55%) and ornamental fish (48%) in West Bengal. Laith and Najiah (2013) reported the multiple antibiotic resistance (MAR) profile *A. hydrophila* isolated from *C. gariepinus.* According to them, all isolates showed resistance to at least one antibiotic. About 63% strains were resistant to at least 2 antibiotics; while 27.2% strains were resistant to at least 3 antibiotics. Contradictory with this report Mallick (2008) and Ritu Ranjan (2011) reported that about 68% and 69.50% bacterial isolates screened from diseased catfish were resistant to at least two antibiotics, respectively. Bharathkumar (2008) reported that about 86% of the MAR bacterial flora comprised of Enterobacteriaceae, *A. hydrophila* and *Pseudomonas* spp. from catfish hatcheries of West Bengal. Pankajkumar (2009) reported that 87% and 84% of the *E. tarda* strains from Bihar and West Bengal respectively were resistant to at least two antibiotics. Wei *et al.* (2011) observed that 61.11% of the *E. tarda* strains were resistant to at least 3 antibiotics. Probably, the high prevalence of MAR bacteria due to the abuse of antibiotics may be responsible for the repeated outbreak of diseases with high mortalities in certain catfish farms of the present study. The implications of this study are important for aquaculture industry and human health.

5.3.2 Minimum inhibitory concentration (MIC) of antibiotics against *Edwardsiella tarda* and other bacterial strains

The results of MICs of antibiotics against *E. tarda, C. freundii* and *A. sobria* strains are shown in Table 10. The results revealed wide variations in MIC values for the tested antibiotics, but always on the higher side. The MICs of nitrofurantoin, co-trimoxazole, gentamicin, ciprofloxacin, chloramphenicol and tetracycline were

observed to be in the range of 0.01 – 30 µg, 0.01 – >240 µg, 0.01 – 10 µg, 0.25 – >240 µg, 0.001 – 30 µg and 0.10 – 150 µg, respectively. Likewise, Pankajkumar (2011) observed high MIC values (≥200 µg/ml) to oxytetracycline and gentamicin, low or medium MICs to ciprofloxacin (0.20-0.39 µg/ml), chloramphenicol (3.13-25.00 µg/ml) and co-trimoxazole (3.13-50.00 µg/ml). The MIC values were high for the *E. tarda* strains followed by *A. sobria* strain from the diseased catfish of West Bengal. In an earlier study, Mallick (2008) reported MIC values above 200 µg/ml for oxytetracycline, gentamycin and trimethoprim against *E. tarda* strains from diseased catfish. Reinhardt e*t al.* (1985) reported MIC values (µg/ml) for 29 strains of *E. tarda* comprising the collections from Centers for Disease Control and ATCC in the range of ≤0.063 (all), 0.5 – 1.0 µg/ml and 6.25 - >200 µg/ml, respectively for oxytetra-cycline, gentamycin and trimethoprim. On the other hand, Stock and Wiedemann (2001) recorded low MIC for tetracycline (0.50-16.00 µg/ml and only one strain with >128 µg/ml), gentamycin (0.13-1.00 µg/ml), ciprofloxacin (≤0.03 µg/ml), co-trimoxazole (≤0.03-0.50 µg/ml), chloramphenicol (0.13-4.00 µg/ml) and nitrofurantoin (4.00-8.00 µg/ml) against 102 *E. tarda* strains from various sources including fish from different countries. Wei and Musa (2008) also recorded MIC values of tetracycline in the range of <0.02-0.39 µg/ml against 18 *E. tarda* isolates pathogenic to fish. Wei *et al.* (2011) reported that the MIC values of *E. tarda* isolated from *C. gariepinus* against six antibiotics varied and observed to be in the range of 1 - ≥128 mg/l. Prasad *et al.* (2013) observed the MIC value against *A. hydrophila, A. salminicida* and *E. tarda* noted as 50 µg/ml, 75 µg/ml, 100 µg/ml, respectively for oxytetracycline. Dung *et al.* (2008) reported *E. ictaluri* isolates were intrinsically resistant to the polypeptide antimicrobial agent colistin with MIC values equal to or >64 µg/ml. However, the least values ranging from 0.001 µg/ml for chloramphenicol to 3.0 µg/ml for nitrofurantoin were observed for *C. freundii* of Enterobacteriaceae group from Tamil Nadu. These results suggest that the bacterial flora associated with diseased catfish of West Bengal had developed resistance to potential antibiotics and is a cause for concern. Conceivably, the high MIC values for antibiotics may cause problems for chemotherapy in catfish aquaculture. The high MIC results indicated that pathogenic bacteria have developed resistance to antibiotics, which suggest that these antibiotics need to be applied in higher doses to reduce the bacterial infection. One of the important causes of this situation could probably due to the indiscriminate use of antibiotics irrespective of dose, mode and exposure. Therefore, to prevent these

pathogenic bacteria other options like use of herbal products (Shangliang, 1990; Citarasu, 2010), vaccines (Shoemaker *et al.*, 2011; Dung, 2011; Bebak and Wagner, 2012), immunostimulants (Anderson, 1992; Sakai, 1999; Sahoo and Mukherjee, 2003; Kumari and Sahoo, 2006; Chen and Ainsworth, 2006; Kumar *et al.*, 2014) and best management practices (Hinrichsen, 2007) are to be explored.

5.4 Pathogenicity of *Edwardsiella tarda* CGH9 on catfish *Pangasius pangasius* and *Clarias gariepinus*

Pathogenecity results of *E. tarda* CGH9 on catfish *P. pangasius* and *C. gariepinus* by intramuscular injection are presented in Tables 11 and 12. The LD_{50} values were determined to be $1.77x10^7$ cfu/fish and $5.75x10^7$ cfu/fish, respectively for *P. pangasius* and *C. gariepinus*. According to the degree of virulence described by Pu *et al.*, (2007) the virulent category include LD_{50} value of 10^2–10^5 cfu/fish and avirulent category include LD_{50} value of $>10^6$ cfu/fish. As per the above category and the results of the intramuscular injection trials, the present strain of *E. tarda* CGH9 was identified as avirulent.

Higher LD_{50} values have been reported for catfish in earlier studies. For example, Mallick (2008) reported LD_{50} value of two different *E. tarda* strains injected on *C. gariepinus* were $1.68x10^8$ cfu/fish and $8.52x10^7$ cfu/fish. Similarly, Sahoo *et al.* (2000) reported that the LD_{50} value for intraperitonially injected *Anabas testudineus* was $10^{7\,8}$ cells/ml. Virulent strains of *E. tarda* were also reported in many studies. Amandi *et al.* (1982) reported LD_{50} value for intramuscularly injected channel catfish to be only $4.0x10^5$ cells/fish. Mekuchi *et al.* (1995) reported that the LD_{50} value for intramuscularly injected Japanese flounder (*Paralichthys olivaceus*) was $7.1x10^1$ cells/fish. *Sparus macrocephalus* when injected with two virulent strains of *E. tarda* gave LD_{50} values in the range of $8.06x10^4$ - $8.31x10^4$ cells/fish (Wang *et al.*, 2012). Recently, Ali *et al.* (2014) reported wide variations in the LD_{50} values of *E. tarda* strains. In their study *C. mrigala* when injected with *E. tarda* gave LD_{50} values ranging from $1.3x10^3$ to $1.8x10^8$ cells/fish.

5.5 Histopathology

The histopathological observations on diseased *C. gariepinus* are depicted in Figs. 5 - 13. Histopathological alterations like inflammation of epidermal tissue and rough epidermal layer (Fig. 5), loosely packed red and white pulp and

melanomacrophage aggregation on spleen (Figs. 6 and 7), degenerative changes of internal organs (Fig. 8), extensive degeneration, basophilic margination and disintegration of mucosal layer (Fig. 9), degenerative changes in cardiac glands (Fig. 10) were observed in *C. gariepinus* fries. In kidney, necrotised haematopoietic tissue, nephritic tubules with widened lumen and extensive necrosis (Fig. 11), karyolysis, cellular hypertrophy, pycnotic nuclei (Fig. 12), inflammation of epithelial tissue, vacuolization of tubular epithelium, hypoplastic haematopoietic tissue (Fig. 13) were observed.

Infiltration of hemocytes and formation of gaps in between the muscle bundles due to loss of normal muscle striations in the muscle fibres of the ulcerated *C. gariepinus* were observed by Mallick (2008). *Clarias gariepinus* infected with bacterial disease revealed skin atrophy associated with disruption of mucous gland opening. Abnormal structure of both the epidermis and dermis led to impairment of its functional activity as well as partially decrease the respiration activity of the integument which is the main source of transported oxygen to internal organs (El-Sayyad *et al.,* 2010). According to Ibrahem *et al.* (2011) *E. tarda* infected *C. gariepinus* showed necrosis of epidermal layer with haemorrhage and congestion of dermal layer. The muscles showed Zenker's necrosis of muscle bundles in which the sarcoplasm stained deeply eosinophilic with losing of striation. Lalith and Najiah (2013) reported necrosis in the dermal layer and hypertrophy in *C. gariepinus* skin infected with *A. hydrophila*. The skin of *C. gariepinus* infected with *P. aeruginosa* showed vacuolar degeneration and necrosis in the epidermal cells with mononuclear inflammatory cells infiltration in between the epidermal cells (Hanna *et al.*, 2014). Pinghui (1974) recorded that the proteolytic enzymes produced by *P. aeruginosa* are responsible for the hemorrhagic and necrotic changes in the skin.

Histopathological alteration of spleen tissue in *C. gariepinus* infected by *E. tarda* showed marked haemorrhages associated with lymphocytic depletion in spleen tissue (Ibrahem *et al.*, 2010) and increase in numbers of melano-macrophages centers, congestion of blood vessels (Ibrahem *et al.*, 2011). Lalith and Najiah (2013) reported hyperplasia in the lymph follicles in spleen of *C. gariepinus* when infected with *A. hydrophila*. Ahmed and El-Refaey (2013) reported that spleen of bacteria infected *C. gariepinus* show area of depletion of haemobiotic element appears as honey comb. *Clarias gariepinus* infected by *P. aeruginosa* showed degeneration in spleen tissue as

clusters of melanomacrophage centers consisting of polyhedral large cells appeared brownish or dark brown in colour, increased both in number and extension in the splenic parenchyma, depletion of lymphocytic elements (Hanna *et al.*, 2014).

Intestine of bacterial infected *C. gariepinus* showed increase numbers of goblet cells with sever congestion of submucosal blood vessels and haemorrhagic with heavy leucocytic cells infiltrations (Ahmed and El-Refaey, 2013). Intestine of *C. gariepinus* infected by *P. aeruginosa* showed columnar epithelium of the intestinal villi and crypts undergo hypertrophy, where increased secretary activity with vacuolated clear cytoplasm and were infiltrated by inflammatory cells. A mass of necrotic debris, mucous, and desquamated epithelial cells were seen in the lumen (Hanna *et al.*, 2014). Islam *et al.* (2008) reported haemorrhages, atrophy, vacuolation and necrosis in the epithelial layer of intestine of moribund fish due to the abundance of *A. hydrophila* in *H. fossilis*.

The histopathological observations on diseased *C. batrachus* fingerlings are depicted in Figs. 14 - 18. Histopathological alterations such as haemocyte infiltration and necrosis of muscle tissue (Fig. 14), inflammation of epidermal tissue, rough epidermal layer, extensive necrosis of muscle and fibrosis (Fig. 15), necrosis of kidney, cellular hypertrophy, nuclear hypertrophy, pycnotic nuclei and karyolysis (Fig. 16), loss of typical tubular epithelial lining (Fig. 17) and necrosis of tubular tissue, inflammation of kidney epithelial layer, hypoplastic haematopoietic tissue (Fig. 18) were noted. In other fish too similar alterations have been reported due to *E. tarda* infection. Muscle tissue of zebra fish infected by *E. tarda* resulted in changes like sloughing of epithelial cells from the surface, thickening of epidermal layer, swelling and detachment of cells, muscle fibre detachment, pyknotic nuclei, necrotic or apoptotic cell death, club cells of the epidermis degeneration, extensive vacuolation around nuclei (Pressley *et al.*, 2005). Lymphocytic infiltration in the musculature of *O. niloticus* infected with edwardsiellosis (Nagla *et al.*, 2005) and liquefaction and gaseous necrosis in body musculature of *L. rohita* infected with *E. tarda* (Mohanty *et al.*, 2007) were also reported. The observations on loosely packed red and white pulp and collapsed melano-macrophage aggregation on spleen was similar to the bacterial infection caused by *A. hydrophila* in *Arius maculatus* (Alagappan *et al.*, 2009). Channel catfish (*I. punctatus*) infected with *Bacillus mycoides* revealed necrotic

muscle, scattered necrotic erythrocytes, and remnants of sarcolemma with chains remnants of sarcolemma (Goodwin *et al.*, 1994).

The *E. tarda* induced pathology in the kidney of *C. gariepinus* was similar to that of Mallick (2008) and Ibrahem *et al.* (2011). Mallick (2008) observed nephron with basophilic reaction and hypertrophoid nucleus, necrosis of nephric tubules, distinct melanomacrophage centre surrounded by infiltration of melanomacrophage cells. In some cases, kidney tubules were obliterated and disrupted with the formation of canals and heavy hemocytes response indicating the initiation of inflammatory response as first line of defence. Ibrahem *et al.* (2011) observed congestion of renal blood vessels with multi-focal areas of haemorrhages in kidneys of *E. tarda* infected *C. gariepinus*. The renal tubules showed different necrobiotic changes as cloudy swelling, hydropic degeneration and necrosis. *Aeromonas hydrophila* infected *C. gariepinus* exhibited degenerative changes in glomerular epithelium and inflammatory cells of kidney (Lalith and Najiah, 2013); while *A. hydrophila* infected *P. hypophthalmus* showed focal necrosis and cellular degeneration in kidney (Ly *et al.*, 2009). Kidney of *C. gariepinus* infected with *P. aeruginosa* revealed degenerative changes in the form of distinct vacuolization and necrotic changes in the tubular epithelium, hypertrophy of epithelial cells, disruption of hematopoietic tissues, widening of the blood vessels with severe hyperplasia, melanomacrophage cells aggregation (Hanna *et al.* 2014). Similar findings were detected by Amosu (2012) in *C. gariepinus* infected with *P. aeruginosa*. Diffuse necrosis along with massive atrophy hematopoietic tissue was observed in *A. hydrophila* infected *H. fossilis* (Islam *et al.*, 2008). Bacteria infected kidney of tilapia showing collapse of renal capsule and edema in bowman capsule, hyaline droplet degeneration (Ahmed and El-Refaey, 2013). Alagappan *et al.* (2009) reported histopathological alteration of *A. hydrophila* infected estuarine catfish, *A. maculatus* showing entirely destroyed tubules, lymphocyte proliferation and macrophage centre formation.

5.6 SDS-PAGE

The SDS-PAGE profile of *E. tarda* OMP antigen revealed a total of 18 bands comprising 13 major bands with molecular weight 83.6, 79.8,75.2, 64, 49.7, 45.3, 42.3, 40, 36, 34.5, 31, 27.5 and 26.2 kDa and 5 minor bands with 96.7, 89, 84.8, 76.2 and 58 kDa (Fig. 19) The result corroborates the observation of Adikesavalu *et al.* (2014). The OMP play a key role in protecting the bacteria from host immune

system. Being the outermost layer that comes first in contact with host, they also contribute in pathogenesis of bacteria by enabling host adhesion and invasion (Rao *et al.*, 2001; Maiti *et al.*, 2011). Kumar *et al.* (2007) examined the OMP of 10 *E. tarda* strains and reported one predominant protein band at 44 kDa. All the 10 strains had 9 other variable bands ranging from 20.3 to 38.8 kDa, similar to *E. tarda* ATCC 15947. In an another study on the analysis of protein profiles of 12 *E. tarda* strains by SDS-PAGE, Maiti *et al.* (2011) concluded that major bands were present between regions 36 and 44 kDa and a few low intensity bands like 15–30, 50, 70 and >100 kDa. The findings of the present study on *E. tarda* OMP antigen comprising a total of 18 bands with 5 minor and 13 major bands (Fig. 19) also revealed major 3 bands within the range of 36–44 kDa. The additional major bands were above 44 kDa (83.6, 79.8, 75.2, 64, 49.7, 45.3,), except 4 (34.5, 31, 27.5 and 26.2 kDa) bands. However, the polypeptide bands of 31, 58, 75.2 and 79.8 kDa may correspond to the low intensity bands reported by Maiti *et al.* (2011). The differences in OMP profiles among the *E. tarda* strains may be because of their heterogeneous nature. Darwish *et al.* (2001) and Panangala *et al.* (2006) have drawn a similar conclusion that *E. tarda* was relatively heterogeneous, after analyzing the OMP profiles of 10 and 18 *E. tarda* strains, respectively.

Outer membrane proteins (OMPs) play an important role in the interaction with hosts and in bacterial pathogenicity. Bacterial OMP play a significant role in virulence as they comprise the outermost surface in contact with host cells and immune defense factors (Buchanan and Pearce, 1979; Rao *et al.*, 2001; Neema *et al.*, 2011). The OMPs are used as an important component of serological diagnostic technique. To measure the specificity of antibody to the pathogenic bacteria, OMP can be used as antigen in the three- titers ELISA system. The OMPs are conserved regions and highly immunogenic and hence, researchers are interested in exploring the possibilities of OMP as a potential target for vaccine. A trial by El-Jakee *et al.* (2008) to create edwardsiellosis native vaccines revealed that *C. gariepinus* vaccinated through intraperitoneally injection with 0.1 ml/fish of the OMP of *E. tarda* containing 1.5 mg total protein/ml showing 100% survivability against *E. tarda* at the dose of 10^6 CFU/ml. The results of the present study would provide the basic information on the protein profile of *E. tarda* strain of catfish from Indian aquaculture system. Further research is, however, needed to identify the immunogenic protein

component of the OMPs and assess its potential in the development vaccine for edwardsiellosis or diagnostics for *E. tarda*.

In general, it was observed in many of the farms that the catfish were stocked at higher densities. The water quality, feed and health management measures were not addressed properly. Such poor management measures have led to stress and subsequently the infectious diseases and production loss. The catfish farmers can prevent or minimize economic losses due to infectious diseases by avoiding overstocking, ensuring good water quality, avoiding erratic/irregular feeding practices, feeding fish with balanced diet or diets supplemented with anti-stress agents such as probiotics, ascorbic acid, etc., preventing access of predators, avoiding over application of in-organic fertilizer or organic manure and aquadurgs, maintaining proper hygiene and sanitation, regularly monitoring catfish health adherence to better management practices to prevent the onset and spread of diseases.

CHAPTER -6

6. SUMMARY

The present study was carried out to investigate the diseases of catfish cultured in different districts of West Bengal, to isolate and identify the bacterial pathogens through phenotypic and genotypic methods and their antibiogram. Histopathological alterations of different tissues of diseased catfish were also observed.

The disease status of three catfish species such as *Pangasius pangasius*, *Clarias batrachus* and *Clarias gariepinus* were observed. The affected fish exhibited sluggish behaviour, erratic movement, hanging, lethargy, anorectic, sliming, skin erosion, foul smell, pale gills, dull and red colouration of eyes, red lateral line, fin rot, tail rot, ulcer, dropsy, yellow fluid accumulation, abdominal haemorrhage, mouth and opercula haemorrhage, pectoral fin haemorrhage, discoloured and of liquefied internal organs. Haemorrhage and dropsy were the most common in all diseased catfish, but the severity of dropsy was more on *C. gariepinus*.

The present study addressed only the bacterial flora of disease catfish as no typical symptoms of fungal and viral diseases were observed during the survey period.

Different types of bacterial species were isolated from diseased catfish and identified on the basis of biochemical characterization and also through an automated bacterial identification system (VITEK 2 compact, Biomerieux, France). These include *Aeromonas caviae, A. hydrophila, A. sobria, A. veronii, A. bestiarum, A. aquariorum, A. diversa, A. jandaei, A. rivuli, A. schuberti, Edwardsiella tarda, Enterobacter cloacae, Escherichia coli, Morganella morganii, Klebsiella pneumoniae, Serratia marcescens, Providencia alcalifaciens, Citrobacter freundii,* other members of the family Enterobacteriaceae, *Pseudomonas* spp. *Sphingomonas paucimobilis, Lactobacillus* sp., *Corynebacterium* sp., *Bacillus* spp., some unidentified Gram positive rods and cocci.

Selected bacterial strains were further characterized through 16S rDNA analysis. The PCR amplicons were sequenced and phylogenetic tree was constructed by using the Kimura-2 correction for evolutionary rate.

About 87% of the bacterial strains of diseased catfish were sensitive to chloramphenicol followed by gatifloxacin (62%), oxytetracycline (53%), co-

trimoxazole (47%), ciprofloxacin (40%), sulphafurazole (38%), nitrofurantoin (34%), gentamicin (32%), amoxyclav (13%), vancomycin (11%), erythromycin (4%) and clindamycin (2%).

The bacterial strains of *Pangasius pangasius* were sensitive to chloramphenicol (77.77%) and gatifloxacin (55.55%). The bacterial flora of *Clarias batrachus* were sensitive to chloramphenicol (71.42%) and nitrofurantoin (71.42%); while the bacterial flora of *Clarias gariepinus* were sensitive to oxytetracycline (93.33%) and chloramphenicol (86.66%).

The MAR index of bacterial strains was in the range of 0.416-0.916 for *Aeromonas* spp., 0.500-0.916 for *E. tarda,* 0.750-0.916 for other Enterobacteriaceae group and 0.083-0.833 for *Bacillus* spp. All the strains of *Aeromonas* spp., *E. tarda* and other Enterobacteriaceae were of MAR group, i.e., were resistant to at least 3 antibiotics; while 83.33% of *Bacillus* spp. were of MAR group.

The MICs of nitrofurantoin, co-trimoxazole, gentamicin, ciprofloxacin, chloram-phenicol and tetracycline were observed to be in the range of 0.01 – 30 µg, 0.01 – >240 µg, 0.01 – 10 µg, 0.25 – >240 µg, 0.001 – 30 µg and 0.10 – 150 µg, respectively against *E. tarda, C. freundii* and *A. sobria* strains. The MIC values were high for the *E. tarda* strains followed by *A. sobria* strain from diseased catfish.

The LD_{50} values of *E. tarda* CGH9 on catfish *P. pangasius* and *C. gariepinus* were determined as $1.77x10^7$ cfu/fish and $5.75x10^7$ cfu/fish, respectively.

Histopathological alterations on different tissues of diseased *C. gariepinus* were observed, which revealed inflammation of epidermal tissue and rough epidermal layer, loosely packed red and white pulp, melanomacrophage aggregation on spleen, degenerative changes of internal organs, extensive degeneration, basophilic margination and disintegration of mucosal layer, degenerative changes in cardiac glands, necrotised haematopoietic tissue, nephritic tubules with widened lumen and extensive necrosis, karyolysis, cellular hypertrophy, pycnotic nuclei, inflammation of epithelial tissue, vacuolization of tubular epithelium, hypoplastic haematopoietic tissue.

The diseased *C. batrachus* depicted histopathological alterations such as haemocyte infiltration, necrosis of muscle tissue, inflammation of epidermal tissue,

rough epidermal layer, extensive necrosis of muscle, fibrosis, necrosis of kidney, cellular hypertrophy, nuclear hypertrophy, pycnotic nuclei, karyolysis, loss of typical tubular epithelial lining, necrosis of tubular tissue, inflammation of kidney epithelial layer and hypoplastic haematopoietic tissue.

The SDS-PAGE profile of *E. tarda* strain OMP antigen revealed a total of 18 bands comprising 13 major bands with molecular weight 83.6, 79.8,75.2, 64, 49.7, 45.3, 42.3, 40, 36, 34.5, 31, 27.5 and 26.2 kDa and 5 minor bands with 96.7, 89, 84.8, 76.2 and 58 kDa.

CHAPTER -7

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7. REFERENCES

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CHAPTER -8

ANNEXURE

8. ANNEXURE

1. Composition of bacteriological media and reagent

Transport medium: Amies medium (HiMedia, 2009)

Ingredients	Grams/litre
Sodium chloride	3.00
Potassium chloride	0.20
Calcium chloride	0.10
Magnesium chloride	0.10
Monopotassium phosphate	0.20
Disodium phosphate	1.15
Sodium thioglycollate	1.00
Agar	4.00
Charcoal	10.00
Final pH (at 25^0C)	7.40 ± 0.20

Sterilized at 121^0 C (15 psi) for 15 min

Nutrient agar (HiMedia, 2009)

Ingredients	Grams/litre
Peptone digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Agar	15.00
Final pH (at 25^0C)	7.40 ± 0.20

Sterilized at 121^0 C (15 psi) for 15 min

Mueller Hinton agar (HiMedia, 2009)

Ingredients	Grams/litre
Beef, infusion from	300.00
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH (25^0C)	7.03 ± 0.20

Sterilized at 121^0C (15 psi) for 15 min

Tryptone broth (Collins *et al.*, 1989)

Ingredients	Grams/litre
Tryptone	10.00
Final pH (25^0C)	7.40 ± 0.20

Sterilized at121^0C for 15 min.

Brain heart infusion broth (HiMedia, 2009)

Ingredients	Grams/litre
Calf brain infusion solids	200.00
Beef heart, infusion solids	250.00
Proteose peptone	10.00
Sodium chloride	5.00
Disodium phosphate	2.50
Dextrose	2.00
Final pH (25^0C)	7.40 ± 0.20

Sterilized at 121^0C (15 psi) for 15 min

Brain heart infusion agar (HiMedia, 2009)

Ingredients	Grams/litre
Calf brain infusion from	200.00
Beef heart, infusion from	250.00
Proteose peptone	10.00
Sodium chloride	5.00
Disodium phosphate	2.50
Dextrose	2.00
Agar	15.00
Final pH (25^0C)	7.40 ± 0.20

Sterilized at 121^0C (15 psi) for 15 min

Tryptone soya broth (HiMedia, 2009)

Ingredients	Grams/litre
Casein enzymic hydrolysate	17.00
Papaic digest of soyabean meal	3.00
Sodium chloride	5.00
Dipotassium phosphate	2.50
Dextrose	2.50
Final pH (at 25^0C)	7.30 ± 0.20

Sterilized at 121^0C (15 psi) for 15 min

Tryptone soya agar (HiMedia, 2009)

Ingredients	Grams/litre
Casein enzymic hydrolysate	15.00
Papaic digest of soyabean meal	5.00
Sodium chloride	5.00
Agar	15.00
Final pH (at 25^0C)	7.30 ± 0.20

Sterilized at 121^0C (15 psi) for 15 min

***Edwardsiella ictaluri* agar (Shotts and Waltman II, 1990)**

Ingredients	Grams/litre
Bacto-tryptone	10.00
Yeast extract	10.00
Phenylalanine	1.25
Ferric ammonium citrate	1.20
Sodium chloride	5.00
Bromothymol blue	0.03
Bile salts	1.00
Distilled water, in ml	990.00
pH	7.00 ± 0.20
Agar	17.00
Mannitol*	3.50 g
Colistin*	10.00 mg

Sterilized at 121^{0}C (15 psi) for 15 min. *: A 10 ml solution containing 3.5 g mannitol and 10 mg colistin was filter sterilized and added to the medium prior to pouring in to the sterile Petri plates.

***Edwardsiella ictaluri* broth (Shotts and Waltman II, 1990)**

Ingredients	Grams/litre
Bacto-tryptone	10.00
Yeast extract	10.00
Phenylalanine	1.25
Ferric ammonium citrate	1.20
Sodium chloride	5.00
Bromothymol blue	0.03
Bile salts	1.00
Distilled water, in ml	990.00
pH	7.00 ± 0.20
Mannitol*	3.50 g
Colistin*	10 mg

Sterilized at 121^{0}C (15 psi) for 15 min. *: A 10 ml solution containing 3.5 g mannitol and 10 mg colistin was filter sterilized and added to the medium prior to pouring in to the sterile Petri plates.

Pseudomonas isolation agar base (HiMedia, 2009)

Ingredients	Grams/litre
Peptic digest of animal tissue	20.00
Magnesium chloride	1.40
Potassium sulphate	10.00
Triclosan (Irgasan)	0.025
Agar	13.60
Final pH (at 25^{0}C)	7.0 ± 0.2

Sterilized at 121^{0}C (15 psi) for 15 min

Phenol red agar base (HiMedia, 2009)

Ingredients	Grams/litre
Proteose peptone	10.00
Beef extract	1.00
Sodium chloride	5.00
Phenol red	0.025
Agar	15.00
Final pH (at 25^0C)	7.40 ± 0.20

Sterilized at 121^0C (15 psi) for 15 min.

Glutamate starch phenol red agar (HiMedia, 2009)

Ingredients	Grams/litre
L-Glutamate, sodium	10.00
Starch, soluble	20.00
Monopotassium phosphate	2.00
Magnesium sulphate	0.50
Phenol red	0.36
Agar	12.00
Final pH (at 25^0C)	7.00 ± 0.20
*Penicillin G@100IU/ml	

Sterilized at 121^0C (15 psi) for 15 min. * Normal sterile solution containing 100 IU/ml Penicillin G was added to the medium prior to pouring in to the sterile Petri plates.

Rimler-shotts (RS) medium (HiMedia, 2009)

Ingredients	Grams/litre
Yeast extract	3.00
Maltose	3.50
L-Cysteine hydrochloride	0.30
L-Lysine hydrochloride	5.00
L-Ornithine hydrochloride	6.50
Sodium thiosulphate	6.80
Ferric ammonium citrate	0.80
Sodium deoxycholate	1.00
Sodium chloride	5.00
Bromo thymol blue	0.03
Agar	13.50
Final pH (at 25^0C)	7.00 ± 0.20
*Novobiocin 1 vial	(5 mg/l)

Sterilized through steam sterilization. *1 vial of Novobiocin (5 mg) was added to the medium prior to pouring in to the sterile Petri plates.

Aeromonas isolation medium (HiMedia, 2009)

Ingredients	Grams/litre
Peptone, special	5.00
Yeast extract	3.00
L-Lysine hydrochloride	3.50
L-Arginine hydrochloride	2.00
Inositol	2.50
Lactose	1.50
Sorbose	3.00
Xylose	3.75
Bile salts	3.00
Sodium thiosulphate	10.67
Ferric ammonium citrate	0.80
Sodium chloride	5.00
Bromo thymol blue	0.04
Thymol blue	0.04
Agar	12.50
Final pH(at 25^0C)	7.00 ± 0.20

Sterilized by steam sterilization. *1 vial of Ampicillin (2.5 mg) was added to the medium prior to pouring in to the sterile Petri plates

Decarboxylase broth (HiMedia, 2009)

Ingredients	Grams/litre
Peptic digest of animal tissue	5.00
Beef extract	5.00
Dextrose	0.50
Pyridoxal	0.005
Bromo cresol purple	0.01
Cresol red	0.005
Pyridoxal	0.005
Final pH (at 25^0C)	6.00 ± 0.20

Sterilized at 121^0C (15 psi) for 10 min. L-Lysine, L-Arginine, L-Ornithine or other L-amino acids added at 1% concentration of total volume. After addition of L-Ornithine the pH was readjusted.

MR-VP medium (HiMedia, 2009)

Ingredients	Grams/ litre
Buffered peptone	7.00
Dextrose	5.00
Dipotassium phosphate	5.00
Final pH (at 25^0C)	6.90 ± 0.20

Sterilized at121^0C for 15 min

MOF medium (HiMedia, 2009)

Ingredients	Grams/litre
Casein enzymic hydrolysate	1.00
Yeast extract	0.10
Tris hydroxyl methyl aminomethane	0.50
Boric acid	0.001
Ammonium sulphate	0.50
Disodium phosphate	0.004
Ammonium nitrate	0.0008
Sodium chloride	9.70
Magnesium chloride	4.40
Sodium sulphate	1.60
Calcium chloride	0.90
Potassium chloride	0.275
Sodium bicarbonate	0.08
Potassium bromide	0.04
Strontium chloride	0.017
Sodium silicate	0.002
Sodium fluride	0.012
Phenol red	0.01
Glucose	10.00
Agar	3.00
Final pH (at 25^0C)	8.00± 0.20

Sterilized at 121^0C (15 psi) for 15 min.

Simmons citrate agar (HiMedia, 2009)

Ingredients	Grams/litre
Magnesium sulphate	0.20
Ammonium dihydrogen phosphate	1.00
Dipotassium phosphate	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Bromo thymol blue	0.08
Agar	15.00
Final pH (at 25^0C)	6.80± 0.20

Sterilized at 121^0C (15 psi) for 15 min.

Corn meal agar (HiMedia, 2009)

Ingredients	Grams/litre
Corn meal,infusion form	50.00
Agar	15.00
Final pH (at 25^0C)	6.0± 0.20
* Polysorbate 80 @ 1%	
Sterilized at 121^0C (15 psi) for 15 min	

Rose bengal chloramphenicol agar (HiMedia, 2009)

Ingredients	Grams/litre
Mycological peptone	5.00
Dextrose	10.00
Monopotassium phosphate	1.00
Magnessium sulphate heptahydrate	0.50
Rose bengal	0.05
Chloramphenicol	0.10
Agar	15.50
Final pH (at 25^0C)	7.2± 0.20
Sterilized at 121^0C (15 psi) for 15 min	

Gram staining reagents (Collins *et al.*, 1989)

I. A. Crystal violet	20.00 g
95% ethanol	20.00 ml
B. Ammonium oxalate	0.80 g
Distilled water	80.00 ml

Mix these two solutions (A and B), stand for 24 h and then filter.

II. Potassium iodide	2.00 g
Iodine	1.00 g
Distilled water	300.00 ml
III. Safranin	0.25 g
95 % ethanol	10.00 ml
Distilled water	90.00 ml

Grind safranin in a mortar with 10 ml of 95 % ethanol. Wash into flask and make up to 100 ml with distilled water.

Cytochrome oxidase reagent (Collins *et al.*, 1989)

Ingredients	
Tetramethyl-p-phenylene-diamine dihydrochloride (Oxalate)	1.00 g
Distilled water	100.00 ml

Whatman filter paper No.1 was cut into strips of 2.5 x 1.0 cm, dipped 1 % NNN'-tetramethyl-p-phenylenediamine dihydrochloride ($C_{10}H_{18}C_{12}N_2$) reagent, dried and stored in dark bottle at 4^0C.

Ehrlich reagent (Collins *et al.*, 1989)

Ingredients	
P-dimethyl amino benzaldehyde (DMAB)	1.00 g
Ethanol 95 %	95.00 ml
Hydrochloric acid, concentrated	20.00 ml

DMAB was dissolved in alcohol and then concentrated HCL was added slowly. The solution was stored in a refrigerator and used to perform the test whenever required.

2. Antibiogram: Zone size interpretative chart

Antibiotic	Disc content (µg/disc)	Diameter of zone of inhibition in mm		
		Resistant (≤)	Intermediate	Sensitive (≥)
Amoxyclav	30	13	14-17	18
Chloramphenicol	30	12	13-17	18
Ciprofloxacin	5	15	16-20	21
Clindamycin	2	14	15-20	21
Co-trimoxazole	25	10	11-15	16
Erythromycin	15	13	14-22	23
Gatifloxacin	5	14	15-17	18
Gentamicin	10	12	13-14	15
Nitrofurantoin	300	14	15-16	17
Oxytetracycline	30	11	12-14	15
Sulphafurazole	300	12	13-16	17
Vancomycin	30	14	15-16	17

3. Double staining (Haematoxylin and Eosine) composition

Haematoxylin	
Haematoxylin	1.00 g
Absolute alcohol	10.00 ml
Potassium alum	20.00 g
Distilled water	200.00 ml
Mercuric oxide	0.50 g
Glacial acetic acid	0.80 ml
Eosine	
Eosine-Y	1.00 g
90% alcohol	100.00 ml

4. Double staining (Haematoxylin and Eosine) schedule

Steps	Time duration
Xylene I	15 min
Xylene II	15 min
100% alcohol	5 min
90% alcohol	5 min
80% alcohol	5 min
70% alcohol	5 min
50% alcohol	10 min
30% alcohol	10 min
Distilled water	5 min
Haematoxylin	15 min
Distilled water	5 min
50% alcohol	1 min
70% alcohol	1 min
80% alcohol	1 min
90% alcohol	1 min
100% alcohol	1-3 min
Eosin	2-4 min
90% alcohol	1 min
100% alcohol (3 changes)	4-5 min
Xylene I	5 min
Xylene II	5 min

5. Reagent for protein estimation

Lowry's reagent

Reagent	Gram/100 ml
Solution A	
Sodium carbonate	2.8
Sodium hydroxide	0.56
Solution B	
Sodium potassium tartarate	2.85
Solution C	
Copper sulphate	1.42
Stock solution	
Solution A,B and C were mixed in the ratio of	100:1:1
Standard BSA	1.00 mg/ml
Folin reagent	
2 N Folin and Ciocalteu's reagent	5 ml
Distilled water	6 ml

SDS-PAGE

Reagents	
Solution A (30% acrylamide and 0.8% bis-acrylamide)	Gram/100 ml
Acrylamide	30.00 g
Bis-acrylamide	0.80 g
Distilled water	100.00 ml
Solutuion B	Gram/100 ml
Tris-HCl (pH 8.8)	18.17 g
SDS	0.40 g
Distilled water	100.00 ml
Solution C	Gram/100 ml
Tri-HCl (pH 6.8)	6.04 g
SDS	0.2 g
Distilled water	100.00 ml
Solution D (10% Ammonium persulphate)	Gram/ml
Ammonium persulphate	0.10 g
Distilled water	1.00 ml
10% SDS	Gram/10 ml
SDS	1.00 g
Distilled water	10.00 ml
Sample buffer	Gram/10 ml
Glycerol	5.00 ml
2-mercaptoethanol	0.25 ml
SDS	0.25 g

Solution C	2.5 ml
Bromophenol blue	0.01 g
Distilled water	2 ml
Electrode buffer	Gram/500 ml
Tris-base	1.5 g
Glycine	7.2 g
SDS	0.50 g
Distilled water	500.00 ml
Running gel (12.5%)	
Solution B	7.50 ml
Solution A	12.50 ml
Distilled water	6.60 ml
Glycerol	3.00 ml
APS	300.00 µl
TEMED	30.00 µl
Stacking gel (4 %)	
Solution C	2.50 ml
Solution A	1.30 ml
Distilled water	6.10 ml
10% SDS	0.10 ml
APS	100.00 µl
TEMED	10.00 µl
SDS-PAGE gel stain	Gram/200 ml
Coomassie brilliant blue (R250)	0.25 g
Methanol	100.00 ml
Acetic acid	20.00 ml
Distilled water	80.00 ml
SDS-PAGE destain	
Methanol	80.00 ml
Acetic acid	20.00 ml
Distilled water	100.00 ml