

# **Chapter 1**

## **Introduction**



# **1. INTRODUCTION**

## **1.1. Bacterial success**

The single celled prokaryotes known as bacteria are found in huge numbers in all possible environmental conditions in air, water and soil, from freezing to quite high temperatures. The leading reason behind their evolutionary success is their capability to adapt to harsh environments. Human bacterial interactions occur throughout life. Many of them are beneficial to humans and are used in human welfare. While a small section of this may be pathogenic. This thesis is a study of a pathogenic mesophilic bacterium (*Aeromonas*) which is responsible for a variety of infectious diseases in humans, fishes and other animals. It is a leading cause of major economic losses in the pisciculture round the world every year. There is a rise in their population in freshwater bodies and drinking water supplies during the warmer months of the year (Khardori and Fainstein, 1988; Edberg *et al.*, 2007). The same seasonality is observed in the incidence of bacteraemia caused by *Aeromonas* with an elevation of gastrointestinal and extraintestinal infections in humans during the summer season (Kelly *et al.*, 1993; Llopis *et al.*, 2004; Tsai *et al.*, 2006). This can be attributed to the greater chance of exposure to the pathogen and subsequently the elevated threat of developing infections due to this bacterium with a seasonal rise in environmental temperature. *Aeromonas* can tolerate a high range of salinity, pH, temperature, conductivity and turbidity with the exception of extremely saline or polluted water and thermal springs which are not suitable for the growth of *Aeromonas*.

## **1.2. *Aeromonas* and Water**

The deep consortium between *Aeromonas* and aquatic environments has given many scientists the idea of using the genus *Aeromonas* almost synonymously with water (Janda and Abbott, 2010). The freshwater bodies are of prime importance along the banks of which our great civilizations have established. Most of the developmental activities such as agriculture, aquaculture, transportation, industrialization, etc are dependent on water. The natural water bodies contain two types of impurities, one being natural and the other being human induced. The impurities added to the water bodies due to human activities are more deteriorating for the environment than the natural impurities. These include dissolved gases, minerals,

pesticides, herbicides, microorganisms, antimicrobials, etc. Water bodies usually carry many microbes like protozoa, fungi, bacteria, etc. Among them bacteria utilize a major part of organic matter present in water. Contamination of the water bodies with the bacterial populations turn them into vectors for dissemination of water borne diseases raising the levels of mortality and morbidity caused due to these diseases. One of them is the enteric pathogen *Aeromonas* which is a major cause of diarrhea leading to considerable degrees of mortality and morbidity in human beings of different age groups (Black, 1993; Prado and O'ryan, 1994; Obi *et al.*, 1997). Good quality water is essential for all life processes on earth. Therefore, monitoring the microbial growth in the water bodies and specially, of the enteric pathogens is of utmost need.

### **1.3. Water Bodies of North Bengal**

The northern part of West Bengal is fortunate to have extensive natural freshwater bodies. The rivers of the Himalayan foothill region are at a risk of being polluted by surface run-offs from the agricultural fields, tea gardens, domestic and medical sewage surrounding the area. Thus, keeping a track of the water quality of the rivers of this region is of prime significance. The main rivers of this region are Teesta, Torsa, Mahananda and Balason while others being the tributaries and distributaries of these larger water basins. River Mechi lies in the Indo-Nepal border.

### **1.4. Effect of *Aeromonas* in Aquaculture**

During the past few decades, the aquaculture industry of the Asian subcontinent has evolved dramatically (Shariff, 1998). The aquaculture production has been significantly expanded by many countries. But this has also led to many environmental and socio-economic impacts, one of the leading being rise in fish health problems. India being situated in the monsoon belt is blessed with good rainfall. This has given us vast areas in the form of ponds, lakes and tanks with high aquaculture potential. Freshwater aquaculture of India accounts for almost 55% of total fish production of our country (Mishra *et al.*, 2017). Fish disease is a major limitation to the aquaculture industry and main constraint to the socio-economic growth of India and many other countries round the world (Bagum *et al.*, 2013; Sahoo *et al.*, 2013). Many of these diseases have greatly affected the livelihood of the fish

farmers. The sharp extension of aquaculture with divergent species and elevated stocking density has caused greater occurrence of viral, bacterial and parasitic pathogenesis in the fishes leading to higher mortality rates and declined production. Among these diseases bacterial infections are the most common and difficult to handle (Mishra *et al.*, 2017). Bacterial diseases may be fin and tail rot, ulcer, septicaemia, gill and systemic disease. *Aeromonas* is known to be associated with all the types of bacterial infections causing great economic loss to the fish culture industry round the world (Austin and Adams, 1996; Roberts, 1997; AlYahya *et al.*, 2018). It is considered as the major one among all the bacterial pathogens causing bacterial infections in fishes (Sahoo *et al.*, 2020). *A. hydrophila* is regarded as the dominant bacterial species for causing septicaemia in the freshwater cyprinids (Qian *et al.*, 1997) and it is considered as the most common pathogen among all the aeromonads responsible for disease outbreaks in aquaculture farms (Nielsen *et al.*, 2001; Sahoo *et al.*, 2020). *Aeromonas* always poses a threat to the freshwater fishes as it is widely distributed in freshwater environment and it also threatens public health specially, for those people who are exposed to diseased fish (AlYahya *et al.*, 2018).

### **1.5. Discovery of *Aeromonas* and its evolution**

The first report of isolation of *Aeromonas* from experimental frogs dates back to 1891. It was named *Bacillus hydrophilus fuscus* (Sanarelli, 1891). The past of *Aeromonas* reflects the records of present-day microbiology, which ranges over a century from being perceived as a laboratory science in the beginning to the era of molecular biology today. The knowledge with regard to aeromonads among the researchers has also enhanced during this time period. Earlier, *Aeromonas* was thought to cause systemic illnesses in the cold-blooded animals. Now, aeromonads have been recognised not only as a virulent pathogen of poikilothermic/ectothermic animals, including fish, but also as the causative agent of a variety of infectious complications in both immunocompetent and immunocompromised humans. Aeromonads are also significant pathogens in humans after natural disasters. In a study 305 tsunami survivors were found to have been affected with skin and soft tissue infections. Twenty percent (20%) of the 641 bacterial isolates identified from these patients were found to be *Aeromonas* (Dixon, 2008).

## 1.6. Occurrence of *Aeromonas* in nature

Members of the genus *Aeromonas* are omnipresent and autochthonous, not only in aquatic habitats but are also found in soil, fish, domesticated pets and other vertebrate and invertebrates. They are found to colonize every possible environmental niche where bacterial ecosystem can exist. Aeromonads belong to genus *Aeromonas*, family *Aeromonadaceae* and share many characteristics with Enterobacteriaceae. The genus includes a number of genospecies. *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas sobria*, *Aeromonas veronii* and *A. schubertii* are the mesophilic aeromonads. *A. salmonicida* on the other hand are the psychrophilic ones. Most of the clinical manifestations in humans are caused by *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas veronii* by *sobria* (Janda and Abbott, 1998). The same trend is repeated in environmental samples with the inclusion of *A. salmonicida* as a dominant species in fish and aquatic habitats. Aeromonads show worldwide distribution and have been isolated from various sources like, freshwater fishes (Thune *et al.*, 1993; Austin and Adams, 1996), clinical samples (Kuhn *et al.*, 1997a), environmental samples (Nakano *et al.*, 1990; Huys *et al.*, 1996; Kuhn *et al.*, 1997a), drinking water supply (Burke *et al.*, 1984; Gadgil, 1998) and food, such as raw milk, vegetables, ice cream, meat and seafood (Abeyta *et al.*, 1986; Altwegg *et al.*, 1990a). *Aeromonas* have been isolated from the midgut of *Culex quinquefasciatus* and *Aedes aegyptii* mosquitoes, monkey faeces, and bivalve molluscs (Pidiyar *et al.*, 2002) and also from polluted waters. *Aeromonas* from invertebrates is identified by periodic surveys of their fecal content, retail foods such as meat, poultry and dairy products and scrutinization of episodes of *Aeromonas* mediated epizootic infections (Janda and Abbott, 2010).

## 1.7. Diseases caused by *Aeromonas*

Aeromonads have been implicated in septicaemia in variety of aquatic organisms and gastrointestinal and extra-intestinal diseases in humans (Janda and Duffey, 1988; Janda and Abbott, 1996). Mesophilic aeromonads have been associated with Epizootic Ulcerative Syndrome (EUS)-associated ulcerations in a wide variety of fishes (Vivas *et al.*, 2004). While the first *Aeromonas* strain was discovered as early as 1890, it took 60 more years to establish its human pathogenicity. *Aeromonas hydrophila* was first isolated from human faeces in 1937 (Miles and Halnan, 1937)

and over the past few decades, there has been a growing interest in the genus *Aeromonas* as an emergent human pathogen (Trust and Chipman, 1979; Altwegg and Geiss, 1989; Abbott *et al.*, 1998; Janda and Abbott, 1998; Joseph and Carnahan, 2000). Several aeromonads have been known to be involved in pathogenesis caused in humans, like surgical wound infections, cellulitis, nosocomial pneumonia, sepsis, hemolytic-uremic syndrome, peritonitis, urinary tract infections, meningitis and severe muscle degeneration. In all the cases, it appears that, pathogenic infections caused by *Aeromonas* occur both in immunosuppressed and immunocompetent patients (Wang *et al.*, 2003). In fishes also, it is observed that rate of infection in immunosuppressed fishes due to spawning or any environmental stress like high temperature or low water levels is higher. However, the elucidation of *Aeromonas*-mediated mechanism of pathogenesis in both aquatic organisms and in human subjects remains still elusive.

### **1.8. Virulence genes in *Aeromonas***

*Aeromonas* spp. has been regarded as "emerging pathogens" that possess multifactorial virulence genes and systems. To date, various virulence genes have been identified and described in this group of bacteria, namely, aerolysin, hemolysin, extracellular lipase, cytolytic enterotoxin, haemolytic toxin genes, acetylcholinesterase and proteases (Kingombe *et al.*, 1999 and references therein; Cascon *et al.*, 2000). Expression of these virulence factors permit the pathogenic aeromonads to attach, invade and destruct the host cells surpassing the host immune responses. Genome-level searches undertaken to identify virulence factors in aeromonads have indicated several open reading frames (ORFs), associated with only virulent aeromonads, and few putative genes like O-antigen and capsule, a Type III Secretion System (TTSS) gene cluster and phage-associated gene cluster. Several regions of the genome (called genomic islands or GIs) with unusual G-C content, have also been identified that carry mobility-associated genes (integrases or transposes) and other putative virulence genes (Yu *et al.*, 2005). Divergent multicellular behaviours exhibited by species of *Aeromonas* help them to grow and persist in varied natural and anthropogenic habitats. These adaptive strategies greatly enhance the virulence potential of *Aeromonas*. Ribonuclease R (Vac B) and quorum sensing (QS) gene homologs of *Aeromonas luxRI* have also been associated in

modulation and phenotypic expression of these virulence genes (Jangid *et al.*, 2007; Erova *et al.*, 2008). Quorum sensing molecules have been detected in some pathogenic species of *Aeromonas* like *A. Salmonicida* and *A. hydrophila* (Swift *et al.*, 1997). Challenging with quorum sensing inhibitors ensure higher survival rates in fishes, which further strengthen the evidence of quorum sensing molecules being involved in virulence potential of *A. hydrophila* and *A. salmonicida* (Natrah *et al.*, 2012). Aeromonads live in biofilms with an elevated cell density which enhances their ability to communicate with each other through QS systems (Talagrand-Reboul *et al.*, 2017). Quorum sensing make aeromonads capable of evading the host defence mechanisms which in turn helps them to establish the pathogenesis (Deep *et al.*, 2011).

## **1.9. Controversy**

The causative role of these virulence factors in developing diseases is controversial. Several species of *Aeromonas* have been isolated both from patients suffering from gastroenteritis and asymptomatic human subjects and the available evidences indicate that aeromonads may be a natural part of the intestinal bacterial population, either for a short term or through a prolonged period. A number of factors, including age, immune status of the subject and underlying illness, infection dose, and expression of sufficient virulence factors by the infecting organism, may be important in deciding the course of infection and the ability of *Aeromonas* spp. to cause disease.

## **1.10. Significance of the Study**

Species of *Aeromonas* have been discovered, isolated and studied from different origins like saline water, fresh water, deltas, oceans, chlorinated and unchlorinated drinking water supplies, highly polluted waters, poikilothermic and homeothermic animals worldwide and humans alike. Study on different antibiotic resistance and Minimum Inhibitory Concentration (MIC) of different *Aeromonas* sp. isolated from hospital effluents, natural water bodies, drinking water supply, etc has helped to provide us insights into the distribution of this bacteria group in this region. Therefore, the primary goal of the study was to isolate and characterize these pathologically-important bacterial strains in North Bengal region and also to investigate the presence of new specific species of this genus or species with novel

disease potential(s) in this part of the country. One hundred and twenty (120) water samples collected from 45 river sites, 6 hospitals sewage and 3 town drainage sites were screened for the presence of *Aeromonas*. Biochemical characterization as well as a PCR-based genotyping method (RFLP of 16S rDNA) was applied in order to identify endemic *Aeromonas* species from this region. The biochemical tests performed were catalase, indole, lipase, glucose fermentation, gelatinase, diastase, citrate utilization, methyl red, Voges Proskauer and oxidase tests. To further confirm the results of RFLP, sequencing of the 16S rDNA was done. This identified the *Aeromonas* isolates upto species level. Among all the twenty-one (21) *Aeromonas* isolates found in our study, four (4) *Aeromonas* species (*A. hydrophila*, *A. sobria*, *A. caviae* and *A. popoffii*) were identified; and to the best of our knowledge, *A. sobria* and *A. popoffii* have been reported for the first time from the aquatic bodies of this region.

In the post-World War II period, rapid development and extensive use (or abuse) of a variety of antibiotics have given rise to resistant varieties of bacteria, thanks to the success and speed of bacterial adaptation. Antibiotic resistance was a major problem associated with the Gram-positive bacteria only around two decades back. However, in recent times, the Gram-negative bacterial population has become a reason of concern (Tan *et al.*, 2008; Cornaglia, 2009). Antibiotic resistance is posing a great challenge to the medical science in the treatment of infectious diseases raising the mortality rate to a great extent (Spellberg *et al.*, 2008). Bacteria apply various mechanisms of antibiotic resistance which include target substitution, target protection, antibiotic detoxification and blocking the intracellular invasion of antibiotics. A study (Walsh *et al.*, 2011) has reported the detection of much debated NDM-1 gene (*bla<sub>NDM-1</sub>*) in aeromonads from northern India (New Delhi). Although its emergence and distribution are controversial, the detection of NDM-1 gene in this clinically and agriculturally important bacteria group calls for a detailed surveillance of antibiotic resistance and also mode of transferability of NDM-1 gene in this bacteria group. Antimicrobial drug resistance was evaluated using twenty-nine (29) different antibiotics, in the *Aeromonas* isolates obtained during this study. The antibiotics chosen were the ones prescribed frequently by the doctors in the treatment of infectious diseases. All the twenty-one (21) *Aeromonas* isolates selected for further study were found to be resistant to ampicillin, amoxycylav and ampicillin/sulbactam, while variable degrees of resistance were observed for twenty (20) other antibiotics.

The data was also compared with previous reports of antibiotic resistance in this bacterial group of this region. While resistance to some antibiotics has reduced, resistance to some antibiotics has increased drastically over the years since their initial records.

Bacterial conjugative plasmids, transposable elements and integron systems are especially the panoply on which bacteria depend for their resistance to anti-bacterial compounds. Specially, plasmids serve as a platform for assemblage and dissemination of useful resistance genes (Dowson *et al.*, 1989; Bennett, 2008). Molecular characterization of *Aeromonas* plasmids was undertaken by several scientists with the view of addressing the problems of spread of genes conferring antibiotic resistance among the aeromonads (Toranzo *et al.*, 1983; Majumdar *et al.*, 2006; Rhodes *et al.*, 2000). Twenty-one (21) to sixty four (64) Kb plasmids have been reported earlier from diseased fishes of North Bengal region (Majumdar *et al.*, 2006; Das *et al.*, 2009). However, in the present study, plasmids ranging from 2 to 10 Kb in sizes have been detected/ isolated from the *Aeromonas* species obtained from the aquatic environment of this region. Plasmids less than 4 Kb have better transferability to other bacteria than the ones larger than 9 kb (Ohse *et al.*, 1995). Our study shows the presence of small sized plasmids in aeromonads which can impose a greater threat as they can easily spread among other pathogenic bacteria.

Presently, the development of resistance to multiple antibiotics in several bacterial species in our hospitals poses a great challenge to our medical science. Accordingly, understanding how antibiotic resistance develops and is spread by mobile genetic elements is a desirable pre-requisite for the design and development of prevention strategies intended to minimize the threat of bacterial infections. To get a better idea, the involvement of natural plasmids in anti-microbial drug resistance was evaluated in the aeromonads isolated in this region. Plasmids isolated from the *Aeromonas* species obtained during the study were used to transform *Escherichia coli* (JM109) and it was found that resistance to ampicillin, ampicillin/sulbactam, amoxycylav, cefuroxime, cephalothin, clindamycin, oxacilin, ticarcillin and vancomycin was transferred through plasmid from *Aeromonas* to *E. coli*.

It is difficult to combat infectious diseases caused by multidrug-resistant pathogenic bacteria and when these multidrug resistant genes spread to other bacteria it can impose greater difficulty to the human society. Therefore, we should resort to other means for fighting the infectious diseases. Application of predatory bacteria,

competitive exclusion of bacteria, bacteriocins and bacteriophage-therapy are some of the probable solutions to this problem in which active research is also being undertaken by several researchers.

## **1.11. Objectives**

1. Isolation, Biochemical and Molecular characterization of Aeromonads from varied sources like hospitals and domestic effluents, natural water bodies from selective sub-Himalayan regions of North Bengal.
2. Preparation of Antibigram of the bacterial isolates.
3. Isolation and Molecular Characterization of the Plasmid content and Plasmid-based resistance and/or virulence gene (s).
4. Investigation of Plasmid mediated transfer of Antibiotic Resistance and/or Virulence Gene(s).