1. Introduction

1.1. Fish farming in India

Fisheries has become a major source of commercial export in India, as well as a viable means of livelihood. India is presently considered as a megadiversity rich country due to the tropical climate and wide-ranging geographical region (Gopi and Mishra, 2015). It is the second-largest producer of cultured fish in world after China which produces two thirds of globally cultivated fish (FAO, 2016).

According to the FAO's National Aquaculture Sector Overview for India, the first accounts of aquaculture in India originate from around 321 B.C. (FAO, 2005). Fish culture is discussed in both King Someswara's Manasottara (1127 A.D.) and Kautilya's Arthashastra (321–300 B.C.). In 350 B.C, temple tanks were stocked with fish and gained popularity over the years mainly in Bihar, West Bengal and Orrisa. In West Bengal, fish culture started in 1911 in backyard ponds and slowly spread to states like Tamil Nadu, Punjab, Uttar Pradesh (Rutaisire *et al.*, 2017).

In India, a total of 3231 fish species have been reported (Froese and Pauly, 2018) among which 788 species thrive in the fresh water reservoirs (Gopi and Mishra, 2015). The fisheries sector in India has witnessed an annual growth rate of 7% and freshwater aquaculture comprises of 95% of total annual production i.e., 5.77 million tonnes (Jayashankar, 2018).

Fish and fishery products are rich in proteins, vitamins and micronutrients like calcium, vitamin A, B12, and omega-3 fatty acids (Lauria *et al.*, 2018). These fatty acids have important role in metabolic functions, antinflammatory properties and reduces platelet aggregation. They form the structural composition of cell membranes, cardiovascular system, brain and nervous tissue. The presence of important minerals with health benefits like selenium, phosphorus and calcium has been found in fish (Khalili and Sampels, 2018). Hence it forms an important ingredient of the staple diet in few states of India (Marriott *et al.*, 2020). Fish is extensively bred in small tanks, ponds and streams using local and aboriginal techniques (Bhattacharya *et al.*, 2020). Fishes have an authentic taste and are in high demand on the global market (Paul, 2016). Moreover, there are vast quantities of diverse and plentiful resources, which have remained untapped due to a lack of infrastructure and technological capabilities (Das *et al.*, 2018). This industry has a tremendous potential that is yet to be realized. According to the FAO (2018a), aquaculture can contribute significantly to global food security and economic growth if developed and implemented in a sustainable manner.

1.2. Economy of aquaculture in sub-Himalayan West Bengal

West Bengal harbors around 251 species of freshwater fishes and fish farming forms an important source of livelihood due to its abundant natural resources (Patra *et al.*, 2017) and people's inherited fondness for fish (Paul, 2016). The Great Ganga divides West Bengal into North and South Bengal. The North Bengal further divides into two regions- 'Terai' and 'Dooars' region. The Terai comprises of marshy grasslands and forests whereas the Dooars forms the flood plains at the foothills of the eastern Himalayas. In North Bengal four districts -Jalpaiguri, Darjeeling, Alipurduar, and Cooch Behar lies on the foothills of the Himalayas (Barman and Das, 2014). Furthermore, it has been identified as a freshwater biodiversity hotspot based on the unique icthyodiversity and endemism of this region (Acharjee and Barat, 2012).

A total of 218 species have been enlisted from whole Himalayas (Menon, 1962) among which 65 species of fishes were reported from River Teesta and 125 species from Darjeeling Himalayan upland region (Acharjee and Barat, 2013). Apart from fish utilized for food, 176 indigenous ornamental fishes belonging to 98 genera were also reported. Agroforestry supported by various pond systems creates new alternative employment avenues for a large portion of rural population otherwise depended on seasonal agriculture farming (Das and Barat, 2014). Pala *et al.* (2019) reported that the fishes reared in homestead agroforestry fish ponds in gardens of the Cooch Behar district of sub-Himalayan West Bengal holds an economic benefit for the native people. Approximately, 150 homestead ponds out of which 44 were fish rearing ponds were reported in the same survey. Around 50.6% pond owners in Cooch Behar used pond reared fish for commercial purpose, whereas 37.3% fulfilled their nutritional requirement. Some tribal communities of Jalpaiguri district also rely on fish farming as a source of livelihood. The feeding material of the fish are also recycled agricultural waste like rice husk, mustard cake and cow dung and hence promotes nutrient recycling and concentration (Edwards *et al.*,1998). In order to meet the needs of supply chain management of commercial fish, rural infrastructures such as roads, markets, cold storage and electricity are developed (Paul, 2016). In farm ponds certain natural resources like nutrients and soil are conserved and it also acts as flood control systems (Wani *et al.*, 2003). Overall fish cultivation in ponds not only helps to fulfill the nutritional requirements of the rural population but also uplifts the economic condition of the farmers.

1.3. Problems limiting yield in aquaculture

In West Bengal, fish production has increased over the years; however, productivity of the fisheries has a slow growth rate due to infrastructure, quality of fish seeds and socioeconomic and environmental limitations (Roy, 2008). West Bengal has a higher demand of fish in comparison to its production. In order to meet the demands, West Bengal imports fishes from other states particularly Andhra Pradesh and Tamil Nadu (Ramsundar, 2011; Rahaman *et al.*, 2013).

On the other hand, fish farming without suitable scientific methodologies, results in large disease outbreaks that have a harmful impact on the quality of the fish produced. Fish diseases are considered to be a major impediment to the aquaculture industry of India and in many countries of the world (Mohan and Bhatta, 2002; Sahoo *et al.*, 2013; Bagum *et al.*, 2013).

Aquaculture practices vary from farm to farm, and this is thought to be one of the major contributors to changes in productivity. Various parameters including size of fish farms, fish seed source, stocking density, type of fish species, nursery management, pond preparation, harvesting frequency and awareness regarding adopted practices and fish disease management have a tremendous role in the fish yield (Abraham *et al.*, 2010). The present-day fish diseases are a result of intensive fish farming practices which leads to an imbalance in the host, pathogen an environment (Bondad-Reantaso *et al.*, 2005). The various types of infectious diseases in aquaculture includes parasitic infections, fungal infections, viral diseases and bacterial diseases. Protozoan fish parasites like *Ichthyophthirius* sp., *Trichodina* sp., monogenetic trematodes and *Argulus* sp. commonly leads to white spot or ich, gill flukes, skin flukes, disease which manifests by dermal ulcerations, osmotic imbalance, disrupts metabolism and results in high morbidity of fish. (Farhaduzzaman *et al.*, 2009; Sahoo *et al.*, 2013; Mishra *et al.*, 2015; Sandeep *et al.*, 2016). Fungal diseases are mainly caused by oomyctes fungi *Saprolegnia*, *Achlya* and *Aphanomyces* sp. Saprolgeniasis caused by *Saprolegnia* is manifested on surface of the skin which appear as white cotton wool like growths. Epizootic ulcerative syndrome (EUS) characterized as fish skin ulcers and haemmorhages is caused by *Aphanomyces* sp. Secondary infection is caused by other bacteria such as *Aeromonas* (Bagum *et al.*, 2013; Das and Mishra, 2014). Viral diseases such as Cyprinid HerpesVirus-2 (CyHv-2), Koi Rana Virus (KIRV), Megalocytivirus and Goldfish haemotopoetic virus necrosis herpes is prominent in ornamental fishes. However Indian aquaculture has not faced massive mortality in fishes due to viral outbreaks (Sahoo *et al.*, 2017)

Bacterial fish infections are quite prevalent and one of the most challenging health issues to overcome. Bacterial diseases including motile aeromonad septicemia (MAS) caused by *Aeromonas hydrophila*, edwardsiellosis caused by *Edwardsiella tarda*, *Pseudomonas* septicemia by *Pseudomonas putrefaciens*, flexibacteriosis by *Flexibacter columnar*, vibriosis by *Vibrio alginolyticus*, bacterial gill disease by *Vibrio parahaemolyticus* and enteric red mouth disease by *Yersinia puckeri* (Mishra *et al.*, 2017) are known to affect the fish culture. These bacteria are often saprophytic, becoming harmful only when fish are physiologically unbalanced, nutritionally inadequate, or when other stresses, such as poor water quality or overstocking, allow opportunistic bacterial infections to flourish. The changes in various physiological parameters such as temperature, salinity, pH, water conductivity and dissolved oxygen may trigger bacterial infections in cultured fishes in aquatic reservoirs (FAO, 2018b; Nadirah *et al.*, 2012). Motile aeromonad syndrome caused by *Aeromonas* (Anyanwu *et al.*, 2015) is one of the most common diseases severely affecting freshwater fish culture. Various members of this genus like *A.hydrophila*, *A. sobria*, *A. caviae*, *A. schuberti* and *A.veronii* have been identified in fish infections. The symptoms of the infection include reddish to grayish skin ulcers, swollen body, exophthalmia and tissue necrosis leading to mortality in fish.

1.4. *Aeromonas* as a pathogen

The genus *Aeromonas* is predominant in several natural resources especially in aquatic environments (Griffin *et al.*,1953; Latif-Eugenín *et al.*, 2016). Aeromonads are opportunistic pathogens and often detected as secondary pathogens in previously contracted infections (Martinez-Murcia *et al.*, 2008; Beaz-Hidalgo and Figueras, 2013). The expansion of world population increases the demand for food production and consequently natural livestocks are unable to meet the nutritional requirements. Therefore, intensive fish farming to increase the fish production is common, which often results in disease outbreaks due too poor culture techniques. Few species of the genus *Aeromonas* are reported to be isolated from the soil, meat and its derivatives, vegetables such as lettuce (Nishikawa and Kishi, 1988), milk and cheese etc. (Martins *et al.*, 2002; Palu *et al.*, 2006). *Aeromonas* causes various diseases in human mainly gastroenteritis and organ or tissue infection (Chen *et al.*, 2017b; Ku and Yu, 2017; Awan *et al.*, 2018a). Initially aeromonads were believed to act only as opportunistic pathogen. It was later proved to be directly involved in severe septicemia of immunocomprised individuals (Ku and Yu, 2017). It infects human via contaminated food, and water apart from direct contact with contaminated aquatic environments (Lai *et al.*, 2007; Bhowmick and Bhatacharjee, 2018). Due to secretion of variety of toxins and unique adaptability under unfavourable conditions, aeromonads can thrive in different habitats, and hence gain resistance to antibiotics (Rasmussen-Ivey *et al.*, 2016a; Awan *et al.*, 2018b; Ruppe *et al.*, 2018). Therefore, surveillance of the aeromonads in the environmental settings to monitor its disease-causing ability and increasing antibiotic resistance is necessary to address the public concern.

1.5. Antibiotic response in the aquatic environment

The emergence of fish diseases poses a threat to the aquaculture industry. In order to control the diseases, antimicrobial agents have been routinely used (Ibrahim *et al.*, 2020). All over the world, 67 antimicrobial compounds are used as prophylactics or therapeutics in aquaculture (Lulijwa *et al.*, 2020). Few classes of antimicrobial compounds such as tetracyclines, sulfonamides, quinolones, florfenicol and amoxicillin are frequently used to treat various infections in aquaculture. The administration of antimicrobials is usually done by techniques of feed medication, bioencapsulation, dipping baths and immersion baths (Smith *et al.*, 2008). Excessive use of the antibiotics causes these to be slowly released into the environment leading to development of resistance in resident microbes. These antibiotics are non-bio-degradable and persists in aquatic food chain. The consumption of fish and fish-derived products may lead to increase in antibiotic resistance among humans (Santos and Ramos, 2016). The antibiotic contamination in aquaculture, industrial waste, hospital waste and livestock waste cause accumulation of the anibiotics in sewage which is released into larger reservoirs (Kraemer *et al.*, 2019; Felis *et al.*, 2020). Being natural inhabitants of aquatic environments, aeromonads have acquired various mechanisms of antibiotic resistance including plasmids and antibiotic resistance genes (Hu *et al.*, 2019). In order to survive, the genus *Aeromonas* is also known to employ certain pathogenic mechanisms involving specific virulence factors. This metabolic diversity explains the ubiquitous nature of aeromonads (Mzula *et al.*, 2020).

1.6. Current scenario of *Aeromonas* sp. in sub-Himalayan West Bengal

In the year 1988, epizootic ulcerative syndrome (EUS) affected different fish species of North East India and North Bengal (Pal and Pradhan, 1990). Several researchers reported the presence of aeromonads in EUS affected fishes (Saha and Pal, 2000), in large aquatic reservoirs like rivers with sites contaminated by sewage and agricultural run offs (Roy and Barat, 2011), and in potable water, domestic and hospital effluents (Pal and Bhattacharjee,

2011) in North Bengal. In addition, some researchers found antibiotic resistance and its encoding genes (Mukherjee and Chakraborty, 2006; Bhowmick and Bhatacharjee, 2017) and plasmid bearing virulent types of pathogenic aeromonads from this sub-Himalayan region. (Majumdar *et al.*, 2006).

1.7. Objectives

With a lot of natural water resources, North Bengal has an immense potential for scaling up the fish production. Until now, very less knowledge exists regarding the characteristics of fish pathogenic aeromonads in this region. Also, investigations on the distribution of antibiotic resistance in this ubiquitous pathogen shall help to monitor level of antibiotic contamination in the environment. Therefore, it is important to understand the molecular mechanisms of its virulence and resistance. The zoonotic role of aeromonads also raises a concern regarding the public health.

Therefore, considering the above factors, the current study was undertaken with the following objectives:

1. Isolation of *Aeromonas* spp. from fish farming environments in sub-Himalayan West Bengal.
2. Biochemical characterization and phylogenetic analysis of bacterial isolates based on 16S rRNA gene sequence.
3. Study the virulence properties of the isolates of *Aeromonas* strains.
4. Antibiotic sensitivity profiling of the bacterial isolates.
5. Analyzing bacterial DNA for the presence of genetic determinants of resistance and virulence.
6. Study the mobility of antibiotic resistance coding genes by *in vitro* conjugation.