Molecular Characterization of Pathogenic Strains of Bacteria Causing Motile Aeromonas Septicemia in *Heteropneustes fossilis*: Implications for Potential Drug Development

Ph.D. Thesis

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Molecular Characterization of Pathogenic Strains of Bacteria Causing Motile Aeromonas Septicemia in *Heteropneustes fossilis*: Implications for Potential Drug Development

By

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This to certify that **Shirin Sultana** has conducted her Ph.D. thesis work entitled, "Molecular Characterization of Pathogenic Strains of Bacteria Causing Motile Aeromonas Septicemia in *Heteropneustes fossilis*: Implications for Potential Drug Development" under my supervision for the fulfillment of the degree of **"Doctor of Philosophy in Fisheries"** from the University of Dhaka. The work or any part of the thesis has not been submitted anywhere for any other degree.

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Dedications

To my dearest Parents

Md. Abdul Gafur

Ayesa Khatun

To my lovely Children

Nazifa Afrin Noma

Ahnaf Safin

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Abbreviation

°C=Degree Celsius

µl= Microliter

ACE = Abundance-based coverage estimator

AD = Average degree

ADME = Absorption, distribution, metabolism and excretion

AEBS = Benzenesulfonyl fluoride hydrochloride

aer = aerolysin

AHL = N-acyl homoserine lactones

Ala (A) = Alanine

ANOSIM = Analysis of similarity

ANOVA = Analysis of variance

APL = Average path length

APW = Alkaline peptone water

Arg (R)= Arginine

Asp (D) = Aspartic acid

BHI = Brain Heart Infusion

BioEdit = Biological Sequence Alignment Editor

BLAST= Basic Local Alignment Search Tool

Bp = Base Pair

CC = Clustering coefficient

CFU = Colony forming unit

Cys (C) = Cystatin

DFP = Diisopropyl fluorophosphate

EDTA = Ethylenediaminetetraacetic Acid

ESC = Enteric septicemia of catfish)

EUS = Epizootic Ulcerative Syndrome

FAO = Food and Agriculture Organization

FASTA = Fast Adaptive Shrinkage Threshold Algorithm

FISH = Fluorescence in situ hybridization

fla = polar flagella

Gln (Q) = Glutamine

Glu (E) = Glutamic acid

Gly (G)= Glycine

GRAVY = Grand average of hydropathicity

His (H) = Histidine

II = instability index

Ile (I)= Isoleucine

KEGG = Kyoto Encyclopedia of Genes and Genomes

LB = Luria-Bertani

LD 50 = Lethal dose 50

LDA = Linear discriminant analysis

Leu (L)= Leucine

Lip = lipase)

Lys (K) = Lysine

MAS = Motile Aeromonas septicemia

MD = Molecular dynamics

MEGA = Molecular Evolutionary Genetics Analysis

Met (M) = Methionine

Methyl red (MR)

mg= Milligram

MH = Mueller-Hinton

ML = Maximum Likelihood

NA = Nutrient agar

NCBI= National Center for Biotechnology Information

NGS = Next generation sequencing

NMDS = Non-metric multidimensional scaling

OUT = Operational taxonomic unit

PAINS = Pan-assay interference compounds

PBS = Phosphate buffered saline

PCoA = Principal coordinates analysis

PCR = Polymerase Chain Reaction

PDB = Protein data bank

Phe (F) = Phenylalanine

PICRUSt = Phylogenetic investigation of communities by reconstruction of unobserved states

Pro (P) = Proline

QIIME = Quantitative Insights Into Microbial Ecology

QS = Quorum sensing

RDP Ribosomal Database Project

RNA = Ribo-nucleic acid

RS = Rimler shotts media

SAR = Structure-Activity Relationship

SDF = Structure data file)

Ser (S) = Serine

Ser = serine protease

SMRT = Single molecule real-time sequencing

T/DGGE = Temperature/denaturing gradient gel electrophoresis

TAE = Tris-acetate-EDTA

Thr (T) = threonine

TPSA = Topological polar surface area

T-RFLP = Terminal restriction fragment length polymorphism

Trp (W)= Tryptophan

TSB = Tryptic soy broth

Tyr (Y) = tyrosine

UPGMA=Unweighted Pair Group Method with Arithmetic Mean

Val (V) = Valine

VP = Voges- Proskauer

Abstract

Background: Dysbiosis of harmonized microbial communities of fish are crucial to identify for better health management of aquaculture species. In addition to classical methods, high-throughput sequencing techniques are now frequently used to analyze the 16S rRNA gene amplicon to assess the shifting of microbial populations as biotic stressors for fish. This study elucidated the different microbiome community by both culture dependent and culture independent methods isolated from healthy and diseased stinging catfish (*Heteropneustes fossilis*) and associated environment. Moreover, the isolated pathogenic *Aeromonas hydrophila* strains from diseased fish skin were identified and confirmed by pathogenic gene amplification and experimental infection. In addition, an attempted was made to design potential inhibitor (drug like compound) against virulent serine protease.

Methods: Healthy and diseased stinging catfishes, soil and water samples were collected from fifteen locations of Bangladesh. Bacterial community from fish tissues and environmental samples were isolated and identified by conventional culture methods and then confirmed by 16S rRNA gene sequencing. Different microbiome community were identified and compared by 16S rRNA gene specific metagenomics approach. Pathogenic *Aeromonas hydrophila* was isolated from diseased fish skin lesions using selective Rimler Shotts medium with novobiocin supplement. Pathogenicity of isolated *A. hydrophila* was confirmed by PCR amplification of five pathogenic genes. After confirming virulence factors, two isolates (AGM2 and AYN7) were selected for experimental infection. The pathogens were later identified based on pathogenic gene specific markers and characterized by antibiogram profiling. One of the pathogenic genes coded for the serine protease was selected for identification of effective drug using *in silico* drug designing bioinformatics tools.

Results: Higher bacterial community was evident in healthy fish tissue than diseased fish; and found similarities with the communities of soil and water samples by culture dependent method. Significant difference of bacterial load was observed between healthy and diseased fish tissues. Similarly, the richer and diversified microbiome communities were found in healthy fish skin than diseased fish skin by culture independent 16S rRNA metagenomics. At the genus level, *Pseudomonas* had the highest richness in healthy groups and was almost completely absent in the diseased groups, but *Flavobacterium* was quite prevalent in diseased fishes. The diseased group harbored the *Aeromonas hydrophila* as minor dominant

which might reflect their opportunistic characteristics. Two isolated of *A. hydrophila* (AGM2 and AYN7) showed highest resistance in antibiogram test (9 and 6 antibiotics, respectively) against 11 antibiotics. In Mueller-Hinton (MH) agar media, the growth of these two isolates was inhibited by ceftriaxone, cefepime, gentamycin, azithromycin, and ciprofloxacin. Challenge test with AGM2 showed higher pathogenicity than AYN7 in post injection of healthy stinging catfish. Pathological signs and symptoms (lesions) and excess mucus (typical signs of MAS) were observed in injected fish with both isolates. The virulent gene *ahp*A encoded for serine protease was characterized and the homology modelling was performed to find potential inhibitor against the protein. An important parameter Ramachandran plot exhibited maximum amino acid residues (88.1%) in the favorable region which was also supported by the accepted G-factor value (overall score 0.38). The molecular docking of 3D structure showed the highest affinity (-7.6) with ceftriaxone from six selected compounds.

Conclusion: Fish health can be impacted by the highly varied microbial communities associated with skin across populations and species. Several factors can alter the community structure to make dominant as single or mixed pathogens. The findings of this study will improve the understanding of microbiota modulation and would act as a baseline information to develop possible drug (s) like compound that would be helpful to avoid indiscriminate use of antibiotic in aquaculture sector.

Chapter 1
Introduction

1.1 Background

Catfish production is recognized as one of the most significant achievements in aquaculture industry of Bangladesh. Catfish (stinging catfish, walking catfish, pabda, wallago catfish, shark catfish, striped catfish) species have a high nutritional value since they include a lot of protein, fats, ash, minerals, and vitamins. However, there are many challenges associated with the catfish aquaculture, for instance, lack of recycling of quality water, disease outbreak, poor quality seeds, poor quality feed, floods, natural disasters, water pollution by industrial effluents, excessive use of fertilizers, pesticides, disposal of excessive household wastes, invasion of alien species, pathogenic microorganisms and diseases. Pathogenic microorganisms are the major constraints of aquaculture as they cause disease outbreak in fish farms. Outbreaks of pathogenic diseases cause severe economic loss for single fish farmer, and sometimes make problems to the whole aquaculture production (Verschuere et al., 2000). Outbreak of Motile Aeromonas septicemia (MAS) and Epizootic ulcerative syndrome (EUS) disease was reported from catfish (shing, magur) farms in Bangladesh (Rashid et al., 2008; Sarkar and Rashid, 2012) but detailed microbiome structure was not explored. Therefore, this study focused on the microbiome community in stinging catfish and associated environment to identify pathogenic bacteria and to develop possible drug like compound against the virulent strains.

1.1.1 Present status of fish production in Bangladesh

Bangladesh is self-sufficient in fish production using fisheries and aquaculture resources and has gained global recognition as a leading producer of fish. Bangladesh is the 5th aquaculture producer in the world and is expected to continue the growing trend (FAO, 2020). Aquaculture has extended in many areas and 90% of the total pond areas are used for fish production in Bangladesh (Islam et al., 2016). Total fish production in Bangladesh was about 4.621 million metric tons in 2020-2021 fiscal year where aquaculture account for 57.10 percent of the total, making a significant contribution to the economy (DoF, 2022).

1.1.2 Contribution of fisheries in the socio-economic development of Bangladesh

Fish is the second most valuable crop in Bangladesh, and its production contributes to the livelihood and employment of millions of people. Fisheries and aquaculture directly or indirectly support more than 18 million people **(**Howell, 2020). Bangladesh has moved

from a low-income country category to a lower middle income country category, and aquaculture exports have played an important role in this transition. The country has shown continuous growth in aquaculture production which has become an important part of its macroeconomic framework. Bangladesh reduced its reliance on capture fisheries to meet domestic demand by aquaculture production.

1.1.3 Aquaculture species of Bangladesh

Aquaculture or fish farming has become very emerging sector in Bangladesh, which has a great potential due to its suitable climate and environment, diverse fish species, enormous water sources, diversified farming systems, government and non-government entrepreneurships and training facilities for fish farmers, and suitable fish markets. Freshwater aquaculture includes pond aquaculture of indigenous and exotic species under monoculture and polyculture systems. Native minor carps, Indian major carps, exotic carps, catfish, tilapia, snakeheads, java barb and other small indigenous species (SIS) are main contributors to the total fish production in Bangladesh (Islam et al., 2016). Catfish aquaculture is very important and lucrative in Bangladesh.

Stinging catfish (*Heteropneustes f*ossilis, Bloch, 1974) are distributed widely in Bangladesh, India, Myanmar, Laos, Nepal, Thailand and Sri Lanka (Talwar & Jhingran, 1991). In Bangladesh, it is known as shing or shinge inhabitants of ponds, swamps, ditches, and marshlands also found in muddy places (Froese and Pauly, 2015). This catfish is commercially important and has significant value as food fish. The species have high market value and is a target species for both large and small-scale fisheries because of its surviving ability to adverse environmental condition (Islam et al., 2021). The species is categorized as least concern species all over the world including Bangladesh (IUCN, 2017).

1.1.4 Importance of microorganisms in aquaculture

Microorganisms have great importance in fish farming where they get access naturally and artificially. Microorganisms are of three kinds: reviving, neutral and disintegrating (Zhou et al., 2009). Reviving microorganisms act as synergists enhance biological, physical and chemical properties of water and sediments. This type can change the neutral organisms to beneficial and they recycle the nutrient and degrade the organic compounds which are essential for cultured species and decrease the pollution. Beneficial microorganisms can synthesize acetic acid, lactic acid, and propionic acid those lowering

the pH in intestine facilitate the beneficial bacteria to become functionally active. Microorganisms produce different metabolic compounds that reduce NH_3, unionized ammonia and ammonium in the host intestine. Moreover, they secrete different digestive enzymes like lipase, amylase, proteinase and integrate Vitamin B type for aquatic animals. Beneficial microorganisms act as modulators of non-specific immune system that strengthen the macrophage activity and antibody level of aquaculture species. At the same time, they enhance resistance ability of aquatic species against diseases. But in some cases, beneficial microorganisms may turn into pathogenic forms through mutation leading to cause infection to the host. These disintegrating types microorganisms cause infection and diseases (Higa, 1999). Outbreak of these crumbling microorganisms have dire consequences in the economy. Because, the bacterial pathogens sometimes spread to entire sectors, and the sector goes to make enormous efforts to lower the quantity of pathogenic germs in its facilities (Bentzon-Tilia et al., 2016). Antimicrobial drugs are used to control this pathogenic outbreak in the farm with improper dose without any consideration resulting increased the resistant strain of *Aeromonas* in the environment as well as fish body (Guz et al., 2004). Indiscriminate use of antibiotics in aquaculture increases disease resistant bacteria, damages or changes usual microbiota of culture environment. This causes dysbiosis of microflora, as double contamination, make antibiotic residue that accumulates in the environment and aquatic products that are detrimental for human health.

1.2 Research gap

In sustainable and commercial aquaculture systems, it is essential to investigate pathogenic bacteria for specific identification, characterization and to take necessary measurements against any disease outbreak. Pathogenic community of aquaculture species and associated environment can be identified by both traditional and advanced methods most of which are culture independent methods for example, use of 16SrRNA, PCR and non-PCR based molecular methods, next generation sequencing (NGS) (Su et al., 2012). However, identified organism's pathogenicity or the ability to cause infection can be determined through challenge test. Experimental infection is the validation process of a pathogen's virulence factors and the degree of causing infection to the host. This method is very useful to develop effective vaccine, to find out immune activities, to determine an effective dose of antibiotic for controlling the infection resulting to reduce the antibiotic resistant bacteria of aquatic environment. Development of effective

inhibitors against virulence factors of an organism is another important therapy to control pathogenic infection. Because, pathogenic ability to cause infection of *Aeromonas* depend on the containing various virulence factors (hemolysin, aerolysin, serine protease etc.) produced by different secretion systems like type II, III, VI, biofilm formation (Rasmussen-Ivey et al., 2016). These inhibitory chemicals will target the multi-drug resistant sites and will inhibit the expression of virulence and pathogenesis related genes of bacteria (Sun et al., 2021). Different reported chemical compounds and natural plant extracts show inhibition activity against virulence factors and biofilm formation to control microbial infection (Sun et al., 2021). *In silico* analysis of identifying effective compounds as alternative therapeutics rather than antibiotics has been performed by many researchers in order to inhibit the virulence factors of *Aeromonas* (Wang et al., 2017; Aisiah et al., 2020). Although above-mentioned scientific advancements are available elsewhere in abroad for diagnosis and controlling disease outbreaks in fish, such kind of investigation and applications are yet to be practiced for aquaculture systems of stinging catfish in Bangladesh. Due to lack of proper identification and diagnosis of pathogenic bacteria of this fish species, both commercial and small-scale farmers have to count severe losses every year and fish health experts are unable to take specific preventive as well as curative measurements.

1.3 Aim and objectives of the study

Stinging catfish aquaculture is a major contributor of total production; therefore, a fundamental understanding of the diversity and dynamics of microbiome communities and their impact on fish health is emerging as a new need. The harmonized composition of the microbiome, including beneficial and opportunistic microbes from fish organs and the environment, must be studied to manipulate and operate beneficially in aquaculture. Comprehensive research on epidemiological identification of contemporary fish diseases and optimization of their solutions are essential. Clearly, more research is needed in future to improve and understanding of fish pathogen interactions and how they interact with fish host immune responses. This will deepen the understanding of disease processes and pathogenesis and be useful in disease management. Therefore, the aim of this PhD research was to study the detection of microbiome community especially pathogenic strain of *A. hydrophila* and its inhibitory drug like compound.

Considering the importance of microorganisms in catfish farming, the present study has been designed to achieve the following specific objectives:

1. To detect the bacterial diversity associated with *H. fossilis* and its environment
2. To analyze bacterial community in healthy & diseased *H. fossilis* by culture dependent and independent method
3. To isolate and characterize the causative agent of Motile Aeromonas Septicemia from diseased *H. fossilis* by challenge test and antibiogram
4. To design the possible drug like compound (inhibitor) against virulence factor (serine protease) causing MAS infection in *H. fossilis*

1.4 Study design

To attain the above-mentioned objectives, the present study was designed and conducted linking four experiments as chronologically and schematically depicted in Figure 1.1.

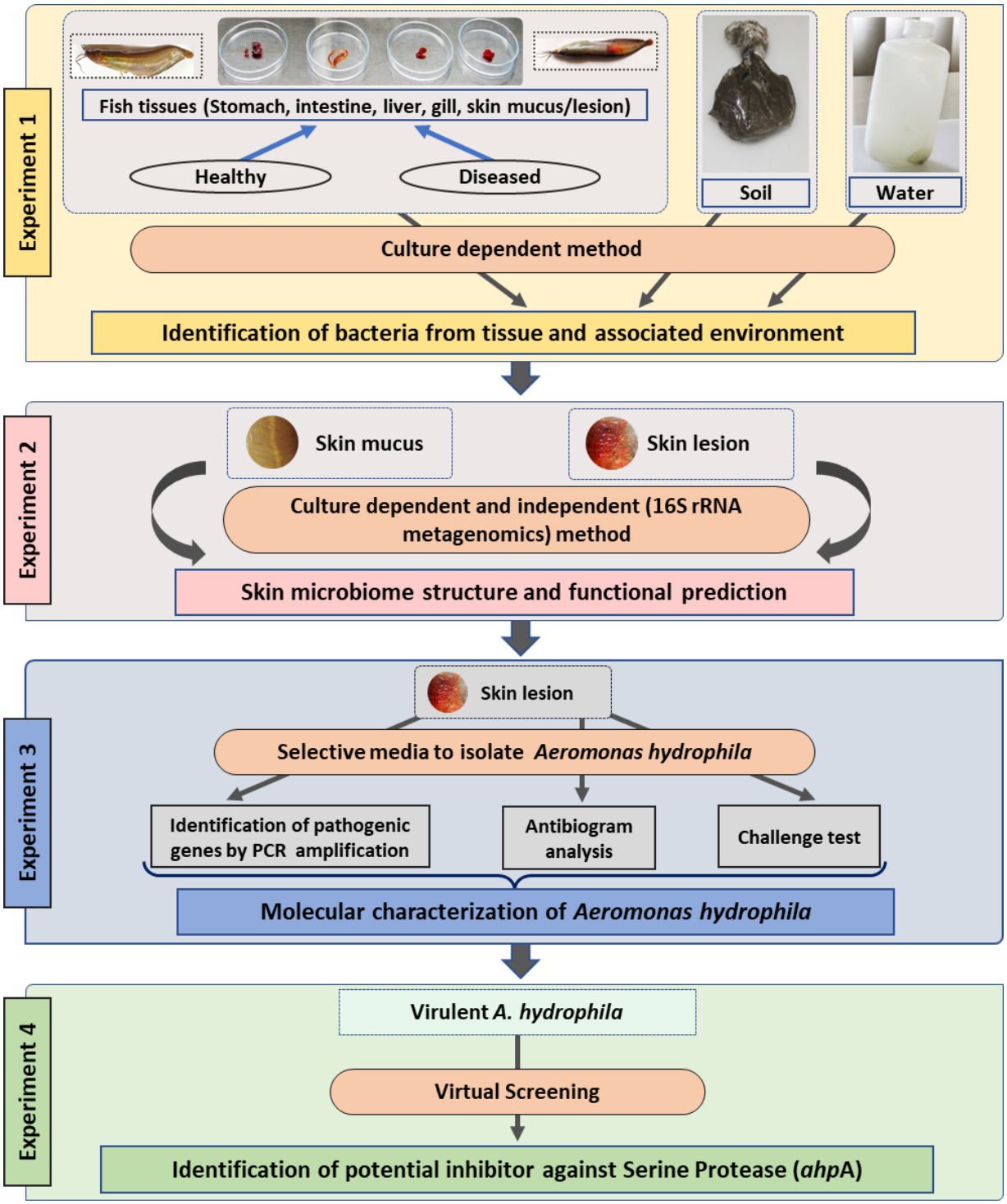

Figure 1.1: Layout of the overall study design

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Chapter 2
Review of Literature

2.1 Major diseases in fish and catfish farm

Disease is a major limiting factor for the cultivation of fish species, hindering economic and social development in many countries (Subasinghe et al., 2001). Various diseases have been described suffering from many aquaculture species (BFRI, 1999; Faruk et al., 2004; Karim and Stellwagen, 1998; Kawsar et al., 2022). Most farmers have no knowledge of the fish health issues and diseases in their systems. Significant impacts of fish disease have been observed in semi-intensive and traditional culture systems in Bangladesh (Aftabuddin et al., 2016). Different disease-causing organisms infected fishes such as bacteria, virus, fungi, protozoa, various parasites in aquaculture systems. Diseases such as epizootic ulcerative syndrome (Pal and Prodhan, 1990; Swann and White, 1991; Karunasagar et al., 1995; Sahoo et al., 1998; Chowdhury, 1998; DoF, 2002; Faruk et al., 2004; Rashid et al., 2008; Hasan et al., 2013), gill lesions, skin erosions, tail and fin rot are commonly observed in cultured fishes of Bangladesh (Faruk et al., 2004). Interaction between infectious agents and environment causes disease outbreak in fish farms (Noga, 2000). In catfish culture systems, high stocking densities, irregular feed supplies, unplanned feed and fertilizer utilization are the major source of stress for fish and increase infection for disease outbreaks. In catfish farm, bacterial diseases are the major threats, among them Motile Aeromonas Septicemia or hemorrhagic septicemia (Easa et al., 1983; Swann and White, 1991; Egusa, 1978; Cipriano et al., 1984; Chang et al., 2012; Hasan et al., 2013) enteric septicemia (Hawke et al., 1981; Hawke et al., 1998) and columnaries (Schachte, 1983; Decostere et al., 1998; Morley and Lewis, 2010) are the major diseases (Wagner et al., 2002; Plumb and Hanson, 2011).

2.2 Economic losses due to fish disease

The success of fish farming is subject to its good management, understanding the life cycle of fish and the environment. Fish diseases can be minimized through proper fish health management and/, or vaccination which will be helpful for preventing disease outbreak in aquaculture (Kawsar et al., 2022). In Bangladesh, annual average economic loss of BDT 20,615/ha/year (equal to US$ 344) in rural aquaculture due to fish disease which is equivalent to approximately 15% of the total production (Faruk et al., 2004). Pathogenic microorganisms mainly cause economic losses in aquaculture by infecting and killing fish (Abedin et al., 2020). Large-scale mortality in aquaculture occurred due to bacterial infestation (Bernoth, 1997; Hiney and Olivier, 1999; Sudheesh et al., 2012;

Hossain et al., 2014; Chandra et al., 2015; Zhou et al., 2018; Wise et al., 2021). Huge mortalities occurred in aquaculture farm due to MAS disease (Zhou et al., 2018).

Catfish production is pushed as one of the most successful developments in Bangladesh's aquaculture. Due to simple farming techniques, few high-value catfish such as stinging (*H. fossilis*), gulsha (*Mystus cavasius*), pabda (*Ompok pabda*) and magur (*Clarius batrachus*) are cultured nationwide. At the same time, few businesses and private organizations have made the technology commercially feasible by importing micronutrients and other essential technologies. As a result, production of these consumer-demanded fish increased by almost 75,000 metric tons in the last production year of 2019-2020 (Islam, 2021). These four species are especially nutritious because they are rich in protein, lipids, ash, minerals and vitamins. Unfortunately, some unidentified diseases in catfish farming ponds have caused a lot of serious mortalities and disrupted production. Each year, the production of thousands of tons of catfish is hampered by outbreaks of pathogens on farms. As a result, fish farmers are losing investment and interest in catfish farming, which is considered a major threat to the varied aquaculture practices in Bangladesh. Mortality rates in catfish ponds have grown out of control since 2015, and even fish farmers have taken some precautions. Farmers were unable to see the devastating mortality of fish in the ponds. Although they applied different antibiotics to control the mortality, the total mortality occurred within 7-10 days (Islam, 2021).

2.3 Concept of fish microbiome

Health status of fish and shellfish depend largely on the microbiome community (Li et al., 2018) exist in the different parts of body like skin, gill and intestinal tract (Bøgwald and Dalmo, 2014; Diwan et al., 2022). Two types of microbiomes based on existing period have been reported as permanent or temporary (Shade and Handelsman, 2012; Bhatt et al., 2018). The residential microbiota plays significant role for host with symbiotic relationship (Zhang et al., 2016), while temporary microbiome enters through feed into the gut and does not exist for long time (Diwan et al., 2022). For ecological adaptation, microbiome showed diversity depending on various biotic and abiotic stimuli which influences the composition and diversity (Elsheshtawy et al., 2021; Butt and Volkoff, 2019). Some commensal microbiotas are specific for some specific organs but credited to different functions (Pratte et al., 2018; Zhang et al., 2019; Wang et al., 2020).

Microorganisms interact with each other between species, genera, families (Röttjers and Faust, 2019) and interactions with host may be positive (commensalism, mutualism or synergism), negative (parasitism, predation or competition) or neutral (Ho et al., 2017).

2.4 Role of fish skin microbiome

Fish gut, skin and gill harbor high concentration of microbial communities contained high diversity and structure (Salinas et al., 2011; Xu et al., 2013) because these parts of fish body interact directly with environment. Direct contacted body parts like skin contains aerobic than anaerobic bacteria protect the host by defensing with invading pathogens (Zhang et al., 2018) and the microbial community is more diverse in skin than gut (Merrifield and Rodiles, 2015; Lowrey et al. 2015). Functional microbiome community in fish influenced by host species, host's health, genotypes, feed types, geographical locations and many factors (Webster et al., 2018; Stevens and Olson, 2015; Legrand et al., 2018; Boutin et al., 2014; Rasmussen & Sorensen 2001, Sylvain et al., 2020; Wang et al., 2010; Smith et al., 2007; Larsen et al., 2013, 2014; Horner-Devine et al., 2004; Sala et al., 2006, 2008).

Microorganisms maintain positive or negative relationship with the host and their genes functions are corelated with host metabolisms (Lynch and Hsiao, 2019; Loomis et al., 2021). Fish skin is the first barrier of protection against any environmental opportunistic invaders and diversified microorganisms exist on the fish skin (Zhang et al., 2018). Fish skin have no keratin and contain epithelial cells which secrets renewable continuous mucus that helps in constant contact with external environment (Schempp et al., 2009; Salinas et al., 2011), rendering its related community prone to physicochemical disturbances in comparison with the steady buffered intestine (Sylvain et al., 2016, 2017, 2019). These commensal organisms play vital role in protecting host by preventing infection (Balcázar et al., 2006), secreting compounds which have antagonistic effects (Boutin et al., 2012; Lowrey et al., 2015), competing for nutrients, space with pathogens (Chiarello et al., 2019) and by increasing immunity (Kelly and Salinas, 2017). Skin microbiome profiling might be useful in health management activities of fish and could be used as potential biomarker of fish health (Legrand et al., 2018; Wu et al., 2012).

Skin microbiome structure and their functions have been reported from many fish including catfish. Chiarello et al. (2019) showed the skin microbiome variation based on environmental condition; Wu et al. (2010) suggested bacterial community as an alternative indicator; Xu et al. (2013) studied teleost skin bacterial community that

produces similar gut immune response; Reid et al. (2017) reported that alpha virus infection causes dysbiosis of Atlantic salmon skin microbiome community; Sylvain et al. (2016) found the effect of pH drop on skin and gut microbiome in Amazonian fish tambaqui; Larsen et al. (2013) suggested skin microbiota for host species specificity; Larsen et al. (2015) found season driver for skin microbiome structure in gulf killifish; Llewellyn et al. (2017) reported parasites as disturber of mucosal community of Atlantic Salmon; Berggren et al. (2021) recommended fish skin microbiome varied among individual and population not within population; Rosado et al. (2019) characterized the skin and gill microbiome of farmed seabass; Tarnecki et al. (2019) studied the changed microbiome community of common snook during acclimatization to wild from hatchery; Doane et al. (2022) reported epidermal microbiome and their gene functions; Zhang et al. (2018) found relationship between skin microbiota and skin mucosal transcriptomic responses during parasitic infection; Legrand et al. (2018) recommended skin and gut microbiota as health indicator of fish; Xavier et al. (2020) studied the impacts of environment and ontogeny on skin microbiome of two Stegastes damselfishes; Caballero et al. (2020) described skin microbiome community of shark and stingray.

2.5 Motile Aeromonas Septicemia (MAS) disease in fish and catfish

Aeromonas hydrophila is a gram-negative, rod-shaped, mesophilic bacterium which causes Motile Aeromonas Septicemia (MAS), an important threatening agent for catfish aquaculture (Pridgeon and Klesius, 2011; Hossain et al., 2014; Pang et al., 2015). This bacterium infects a wide range of hosts including mammals, reptiles, amphibians and avians (Krieg and Holt, 1984) specifically fish (Ventura and Grizzle, 1987) including stinging catfish (Sarkar and Rashid, 2012; Ahammed et al., 2016; Rashid et al., 2008), Nile tilapia (*Oreochromis niloticus*) (Abd-El-Rhman, 2009), channel catfish (Zhang et al., 2016; Pridgeon and Klesius, 2011; Hemstreet, 2010), common carp (Alsaphar and Al-Faragi 2012; Harikrishnan et al., 2003; Robinson et al., 2012; Jeney et al., 2009). Several pathological signs and symptoms are considered for MAS infection as preliminary indicator of disease outbreak like ulcer, dropsy, lesions on skin, necrosis and hemorrhagic septicemia (Pridgeon and Klesius, 2011) observed on the lateral, ventral and dorsal parts of the body.

Virulent *A. hydrophila* was first reported in China in 1938 (Zhi-Hong et al., 1998); later, the aquaculture industry across the world experienced with rapid mortality by this

hypervirulent strain. This strain is known as primary pathogen in aquaculture and observed when the fish is in already stressed condition due to others environmental factors (Grizzle et al., 1993; Wise et al., 2021). *A. hydrophila* and other members of *Aeromonas* spp. also cause disease in catfish and other fish (Wise et al., 2021; Sudheesh et al., 2012). *Aeromonas* spp. are also regarded as serious emergent infectious pathogens for human beings (Figueras, 2005; Sudheesh et al., 2012).

2.6 Virulence factors of *Aeromonas* spp.

Some of the best studied regulatory functions are the link between quorum sensing (QS) and biofilm formation, suggesting that it may mediate not only the expression of virulence factors but also cell density (Swift et al., 1997; Lynch et al., 2002; Janda and Abbott, 2010). Quorum sensing is an intercellular signal communication system, which involve the production, secretion and subsequent recognition of signaling chemical substances. These signaling molecules are called autoinducers, pass through the communication system allows bacteria to regulate the expression of genes associate to virulence and pathogenesis (Sun et al., 2021). QS controls the pathogenic behavior of pathogenic bacteria broadly and participate in the formation of biofilm which is regarded as a key driver of resistant pattern against antibiotics (Sun et al., 2021). Thus, QS systems referred to as effective target point for antimicrobial therapy, because inhibitor can block QS system which in turn inhibit biosynthesis of N-acyl homoserine lactones (AHL) for gram-negative bacteria and oligopeptides for gram-positive bacteria and degrade the synthesized and/or inactive receptor protein (Belapurkar et al., 2014). Furthermore, polar flagella in *A. hydrophila* are expressed constitutively, there are also some well-regulated factors that can trigger the expression of lateral flagella, such as surface interaction and viscosity (Wilhelms et al., 2011, 2013). Another class of modulation includes the upregulation of pathogenic factors by lysogenic transformation. Many secretion systems like Type II Secretion System (Sandkvist, 2001; Peabody et al., 2003; Tseng et al., 2009; Pang et al., 2015), Type III Secretion System (Aguilera-Arreola et al., 2005; Pang et al., 2015), Type VI Secretion System (Bingle et al., 2008; Rasmussen-Ivey et al., 2016), biofilm formation (Costerton et al., 1995; Lynch et al., 2002), hemolysins (Wadstrom et al., 1976; Asao et al., 1984) performed *Aeromonas* spp. by which they secrete virulence factors (proteins, enzymes) for own its protections and to cause infection to the host.

2.7 Antibiotic resistance of *Aeromonas hydrophila*

Fish infection caused by *Aeromonas* strains is associated with various environmental factors as well as sudden changes of different parameters like temperature, dissolved oxygen level, carbon dioxide, mishandling, water and organic pollution, stress to fish, other pathogenic infection (Zdanowicz et al., 2020). These factors mainly decrease the immunity of fish and increase pathogenic colonization and infection (Cabello 2006; Naylor and Burke 2005). Growing concerns with bacterial disease outbreaks and widespread antibiotic use are caused by expanding intensive aquaculture systems (Daood, 2012). Uncontrolled antibiotic use for prevention and treatment of bacterial pathogens induces and increases resistant bacteria globally in fish farm (Mulyani et al., 2018; Patil et al., 2016). Resistant bacterial growth in the aquatic environment ultimately decreases the efficiency of many antibiotics for treating diseases and increases the likelihood of resistant bacterial growth in native fish species (Belém-Costa and Cyrino 2006). Furthermore, antibiotic resistance ability of bacteria will help to increase more virulent features with increased mortality outbreak in aquaculture farms (Zdanowicz et al., 2020).

Isolation and antibiotic susceptibility of *Aeromonas* spp. have been widely reported from fish, catfish, environmental sample like water, soil. Antibiotic resistant pattern reported in *A. hydrophila* isolated from *Oreochromis niloticus* fish (Belém-Costa and Cyrino, 2006); South African freshwater fish (Chenia, 2016); freshwater fish farm and farmed carp (Daood, 2012); freshwater animals (Deng et al., 2016); ornamental fish (Dias et al., 2012); farmed carp (Guz et al., 2004); farm raised fresh water fish (Hatha et al., 2005); diseased catfish (Kanchan et al., 2016; Laith and Najiah, 2014); retail fish Malaysia (Radu et al., 2003); marketed fish and prawn of South India (Vivekanandhan et al., 2002); African catfish (Wamala et al., 2018); tilapia and walking catfish (Ashiru et al., 2011); carp pond water (Zdanowicz et al., 2020).

2.8 Approaches and methods for identification of bacteria in aquaculture species

Ecological niche given by the fish for microbiome community affected diversity and structure and highly dependent on the environment (Sylvain et al., 2020). Proper profiling of microbiome community could be helpful in finding novel functions of microbes that assist the healthy status of fish. The fish microbiota has conventionally been studied by culture technique and subsequently identified based on the biochemical and morphological characteristics of the bacteria. The conventional or traditional usual

methods have been used since the beginning of microbiological analysis (Adzitey and Huda 2010; Adzitey and Nurul 2011). However, culture-dependent techniques are tedious, time consuming and have a limited understanding of the microbiome, as only a small fraction (1%) of fish bacteria can be cultured (Rimoldi et al., 2018). Effective investigation of pathogenic bacteria can be accomplished through a mixture of the traditional and some polymerase chain reactions (PCR)-based methods (Aurora et al., 2009; Loncarevic et al., 2008; Adzitey and Corry, 2011). The option for method utilization depends on the platform's technology available, the specific purpose to be identified, the complication of the samples to be tested, the required period and the bacterial load (Ferone et al., 2020). Most widely used culture independent methods for microbiome detection include Terminal restriction fragment length polymorphism (T-RFLP), temperature/denaturing gradient gel electrophoresis (T/DGGE), Fluorescence in situ hybridization (FISH) and high throughput next generation sequencing (NGS) of targeted samples. NGS technique offers rapid, cost-effective, in-depth and precise sequence of genomics, metagenomics, transcriptomics and other molecular biology research. Ion Torrent, pyrosequencing and latest Illumina are the update versions of NGS.

2.8.1 Culture dependent method

Traditional techniques to diagnose bacterial fish diseases have focused on attempting to culture pathogens from infected material and then using phenotypic data for identification and comparison with published diagnostic protocols, such as Bergey's Manual of Systematic Bacteriology. When infected tissue is exposed to the surrounding environment, such as skin ulcers, there is an innate risk of contamination and it is difficult to discern the relevance of bacterial growth resulting from the pathogen being a secondary invader. Culture methods include sample enrichment (selected organism enrichment and/or pre-enrichment) using different growth media viz, Tryptic soy agar, Nutrient agar, Luria-Broth (Kirk et al., 2004). Plating of the organism can be done by direct streaking of sample onto agar without enrichment and presumptive identification followed by biochemical test of the colonies (Corry et al., 2003; Adzitey et al., 2013). Conventional method has the advantage and still widely used because a specific or any viable bacteria can isolate, characterize and utilized for further study (Engberg et al., 2000). This method has preferences due to its sensitivity, efficiency, dependency but need to validate the phenotype with the genetic analysis (Ferone et al., 2020). Bacterial

communities have been detected by traditional viable cell count, enzyme activity test and genomic analysis (cloning and genomic DNA sequencing) (Bailón-Salas et al., 2017).

2.8.1.1 Biochemical tests

Biochemical tests are the traditional method but still used to date for the rapid identification of any specific bacteria. Microbial biochemistry tests speed up the process of identifying bacteria, save expenses, and guarantee or improve the precision of identifying an unidentified sample. Rapid commercial test kits for anaerobic bacteria have been available in recent years. The microbial biochemistry reaction plate comes with eight identification series, phosphate buffered saline (PBS), bacterial turbidity standard tube, and 30 biochemical matrices and their associated biochemical test markers (Peng et al., 2020). The readouts for the outcomes are diverse because different sorts of tests were conducted. The Minitek identification system using paper substrates, the API-20A system using dry powder substrates, the PIZYMAN-IDENT rapid enzyme activity assay system using primary materials, RaPID-ANA systems, and fully automated microbial identification systems are the most representative biochemical test kits (Peng et al., 2020).

2.8.2 Culture independent method

Beyond the culture dependent method, culture independent method regarded as rapid, more sensitive, less time consuming, less laborious and more effective (Magistrado et al., 2001; Keramas et al., 2004) specifically for fish disease diagnosis (Abu-Elala et al., 2015; Bartkova et al., 2017; Fernández-Álvarez et al., 2016; Keeling et al., 2013; Mooney et al., 1995; Yan et al., 2018). Høie et al., (1997) identified 10^3 and 10^4 colony forming unit of *A. salmonicida* with plasmid primers whereas Hiney et al., (1992) detected ~ 2 cells of *A. salmonicida* with 16S rRNA gene sequencing in kidney cell suspension based on polymerase chain reaction. Successive developments involved nested PCR (Taylor and Winton 2002), terminal-restriction fragment length polymorphism (RFLP) (Nilsson and Strom 2002), PCR–RFLP (Puah et al., 2018), multiplex PCR (Chapela et al., 2018), real-time PCR (Keeling et al., 2013), quantitative real-time-PCR (Du et al., 2017), real-time recombinase polymerase amplification (Pang et al., 2019) and reverse transcription-multiplex PCR (Rattanachaikunsopon and Phumkhachorn, 2012).

2.8.3 Metagenomics

Due to the limitations of culture methods, the identification and measurement of microbes (including bacteria, viruses, fungi, and others) cannot be fully achieved. Microbial communities are important to the functioning of all ecosystems, but nonculturable microbes and their roles in natural ecosystems are not well understood. Metagenomics is based on genomic analysis directly from earth's diverse microbial samples also known as environmental genomics, community genomics and population genomics (Sabree et al., 2009). Existing reports have shown that there are only 0.001-0.1% of microorganisms in sea water, 0.25% in freshwater, 0.25% in sediments and soil contained only 0.3% which can be cultured (Ghazanfar and Azim, 2009). Metagenomic analysis include complex and larger genes or DNA segments to produce data from deep sea aquatic microbes, soil micro-flora and gut, skin, other environmental samples ecosystems of human and animals. Microbial research in aquaculture focuses on understanding the interrelationships between symbionts and antagonists with the aquatic organisms like fish, molluscs and crustaceans (Kaviarasu and Sudhan, 2016). This technology and bioinformatics analysis make possible to investigate and identify microbial diversity and richness, antibiotic resistant gene, pathogenic genes and their variable functions, probiotic bacteria and economically important novel gene (Kaviarasu and Sudhan, 2016). Target gene sequencing and metagenomic shotgun sequencing are two approaches of metagenomics for taxonomic diversity analysis.

Second-generation technology like Roche 454 or Ion Torrent efficiently generates longer read lengths (~700-1000 bp), but they are often not preferred due to the high cost of sequencing and homopolymer generation (Bharti and Grimm, 2021). Illumina platforms offer more cost-effective and higher accuracy; however, they only provide limited read lengths (~2 × 300 bp). Single molecule real-time sequencing (SMRT) platforms from Pacific Biosciences and Oxford Nanopore Technologies are currently preferred due to their longer read lengths of 15-100 and ~1000 kb, respectively (Bharti and Grimm, 2021). Third-generation technology like PacBio platform synthesize similar workflow of Illumina for sequencing which are fast, accurate, low cost and produce longer reads between 10 and 50 kbp with an average read accuracy of ~85% (Eid et al., 2009). The new PacBio Sequel System provides a significant longer read lengths (~0.5–10 Gbp) than PacBio RS II (Eid et al., 2009; Bharti and Grimm, 2021).

2.8.3.1 Target gene approach or 16S rRNA metagenomics

16S ribosomal RNA (rRNA) gene sequencing has been broadly used for microbiome species identification and taxonomical analysis (Petti et al., 2005; Schmalenberger et al., 2001). Bacterial 16S rRNA genes typically contain nine "hypervariable regions" that exhibit considerable sequence diversity between different bacterial species and can be used for species identification (Chakravorty et al., 2007). In most bacteria, hypervariable sections are flanked by conserved stretches that allow PCR amplification of target sequences using universal primers (Munson et al., 2004). Rapid methods to identify certain species-specific sequences within only one hypervariable region are also in common use (Varma-Basil et al., 2004). But, hypervariable regions of 16S rRNA gene show variable diversity of sequences, and no single region can be used for identification among all bacteria (Chakravorty et al., 2007). Small number (18,000–20,000) of raw reads generated from 16S rRNA gene sequencing referred to as Amplicon Sequence Variants (ASVs; DADA2 based) or Operational Taxonomic Units (OTUs;mothur based) (Durazzi et al., 2021). The OTU table generated after sequence processing and multiple pangenome alignments were done using customized databases such as SILVA, Greengenes and RDP of various genes families. Community richness, diversity and evenness can be analyzed through the set of sequence cluster analysis (Bharti and Grimm, 2021). Based on output from mothur or DADA2 functional predictions of microbiome communities can be predicted using Tax4Fun or PICRUSt tools (Durazzi et al., 2021). Both tools provide approximate functions of studied gene. Variations within and among samples can be determined from the OTUs dissimilarity indices analysis (Durazzi et al., 2021).

2.9 Tools of functional microbiome analysis

There are many available algorithm software packages to predict gene functions of microbiome communities. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) is a bioinformatics tool package that designed for functional prediction of metagenome from genetic marker (e.g., 16S rRNA) surveys and from full genomes. It is modified ancestral state reconstruction (ASR) method for functional prediction on profiling of microbiome communities by using 16S rRNA gene sequences (Langille et al., 2013). It predicts microbiome’s functional features from DNA sequences directly rather than detecting them. There is an interaction between

phylogenetic connection of organisms and associated functional genes of them (Segata and Huttenhower, 2011). PICRUSt is able to propose specific functions that may be the characteristics of an organism or of a particular habitat (Douglas et al., 2018). Moreover, it can identify same specific function from a distantly correlated organism. Hypothesis-generating PICRUSt tools has been proved its high accurateness (>90%) on paired 16S and metagenomic data analysis (Douglas et al., 2018). PICRUSt has been applied to wide range of samples collected from a varied range of habitats covering gastrointestinal tract of human, crops and environmental soils and aquatic habitat (Douglas et al., 2018). Based on the Operation Taxonomic Unit (OTU) tree in the Greengene database, bacterial functional profile of the common ancestor can construct a complete bacterial lineage (Yin and Wang, 2021). PICRUSt uses taxonomic information of 16S rRNA gene and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to predict functional characteristics of microbial communities (Koo et al., 2017).

Another important tool, Tax4Fun is an R package which linearly accumulates pre-computed functional profiling based on organism's taxonomic abundances using SILVA database as reference (Quast et al., 2012). It has more accuracy in profiling poor characterized phyla of large communities (Aßhauer et al., 2015). Besides, Piphillin is an analyzing tool that does not need phylogenetic tree or reference datasets of 16S sequences. It uses closest- neighbor algorithm for quick mapping of 16S to reference genomes (Douglas et al., 2018).

2.10 Experimental Infection

Challenge test studies are important to understand the rout, severity of infection and to know the pathophysiology of disease and new technology can be developed for treatment and management (Claudiano et al., 2020). This method will be efficient to decrease the damages and economical lose in the fish farm. Rashid et al., (2008) investigated different pathological changes in *H. fossilis* experimentally infected with *A. hydrophila* and found various clinical sign and symptoms. Sarker and Faruk (2016) found 100% mortality with intraperitoneal (IP) injection in juvenile pangasius (*Pangasianodon hypophthalmus*) after 15 days of challenge test. Many research have been conducted on the experimental infection with *Aeromonas* spp. to find effective routes, observe clinical signs, estimate mortality, observe histopathological changes in different organs with different doses and with various challenge routes in different catfish and other fishes for instance, *H. fossilis*

(Mostafa et al., 2008; Neowajh et al., 2017), yellow catfish (Zhai et al., 2021), channel catfish (Zhang et al., 2016), common carp (Alsaphar and Faragi 2012), blue tilapia (AlYahya et al., 2018).

Additionally, sometimes more than one pathogen can change the immune system of fish which is known as co-infection. Their interactions may be either antagonistic or synergistic. Co-infection have impacts on infection, disease severity, mortality and pathologies of host (Kotob et al., 2017). Synergistic interactions occurred when one pathogen starts immunosuppression of host and hampers the immune responses against following infections, increasing the severity of the disease and mortality (Bradley and Jackson, 2008). While in antagonistic relationship, both pathogens compete for nutrients and space and suppress the population of the pathogenic organism and in some cases, change the place of infection (Andrews et al., 1982). Sometimes first pathogen activates and modulates the immune system of host and hinders the other pathogen.

2.11 Structure and physico-chemical properties of serine protease

Bacteria synthesize a wide variety of endoproteases. Serine, aspartate, cysteine or metallo-proteases are the four classes of endopeptidases. Serine endo- and exo-peptidases occurred widely and have diverse function (Siezen and Leunissen, 1997). Serine protease has six groups of which chymotrypsin-like and subtilisin-like are the two largest groups (Siezen and Leunissen, 1997). More than 40 members of the subtilisin-like serine proteases, referred as “subtilases”, found in bacteria, Archaea, yeasts, fungi and in developed eukaryotes (Siezen et al., 1991). Temperature-labile serine protease showed highest activity at pH 7.5 and has a molecular weight and size of about 65000 and 30000, respectively (Yokoyama et al., 2002). The 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBS) and diisopropyl fluorophosphate (DFP) are two reported inhibitor of serine protease (Yokoyama et al., 2002). Serine protease produced by virulent *Aeromonas* spp. is the member of kexin subfamily, able to induce edema when the strain was injected subcutaneously (Takahashi et al., 2014). Serine protease is the most prevalent virulent gene having cytotoxic effects of *Aeromonas* strain (Skwor et al., 2013). The enzyme contained up to 1775 residues including N-terminal catalytic part having 268 to 511 amino acid residues and activation and/or signal-peptides having 27 to 280 amino acid residues (Siezen and Leunissen, 1997).

2.12 *In-silico* analysis of inhibitor against virulence factors

Over the last decade, new antibiotics development has declined abruptly, while drug-resistant pathogenic strains become harder (Gutiérrez-Barranquero et al., 2015). The detection of the bacterial quorum sensing (QS) system offers with promising approach to prevent and regulate microbial infections (Sun et al., 2021). In recent years, computer-aided drug design methods have been developed rapidly, and these device-based methods have attained high precision, allowing them to be regularly applied in the comprehensive interpretation of experiments and drug design activities (Śledź and Caflisch, 2018; Zhang et al., 2021).

Protein structure-based methods can be used to predict the binding mode of small molecules and their relative affinities. Prediction on binding affinity and mode of molecules can be done based on protein structure. High-throughput molecular docking based on implicit solvent force-field scoring of up to 106 small molecules can identify micro polar binding associates using firm protein targets very reliably (Zhang et al., 2021). Unambiguous solvent molecular dynamics (MD) simulation is a low-throughput methods used to describe non rigid binding sites and precisely evaluate binding paths, kinetics, thermodynamics. This technique can be applied to guide experiments and elucidate experimental phenomena (Zhang et al., 2021). MD simulations offer perceptions not only into crystal structures of protein dynamics, but also reveal new binding sites, covering the drug properties of targets molecules (Zhang et al., 2021).

One of the first methods complex relaxation schemes combines nanosecond-long molecules of all atoms simulations of target proteins to describe their conformational flexibility, while small molecules participate in fast docking of stored proteins in molecular snapshots dynamics (Amaro et al., 2008; Zhang et al., 2021). This technique has been effectively applied in cancer-related MDM2/MDMx-p53 (Barakat et al., 2010) interactions and HIV integrase (Schames et al., 2004). This *in-silico* analysis has been validated experimentally and approved to progress for development of enzyme inhibitor to work against infection caused by HIV (Hazuda et al., 2004). Yadav et al. (2021) suggested inhibitor against aerolysin virulence factor of *Aeromonas hydrophila* through molecular docking.

Quorum sensing inhibitors against *Pseudomonas aeruginosa* (Kamal et al., 2017), TMPRSS2 Inhibitors (Mahmudpour et al., 2021), inhibitor to block the peptidoglycan

glycosyltransferase of *A. hydrophila* (Aisiah et al., 2020), alanine racemase inhibitors of *A. hydrophila* (Wang et al., 2017) were recommended inhibitors identified with the help of various computer assisted drug design tools against different virulence factors in specific bacteria.

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Chapter 3
Comparative Analysis of Bacterial Communities in Cultured Stinging Catfish and Associated Environment

3.1 Abstract

Microorganisms are widely found in fish body and natural environment. In this study, the microorganism from different fish organs of healthy and diseased fish and their associated environments of fifteen locations in Bangladesh were identified. The bacterial load of different tissues such as skin, lesion, intestine, gill, liver were analyzed. The bacterial load in healthy fish tissues were found comparatively higher than diseased fish. The mean bacterial counts were $2.82\pm0.05\times10^8$, $3.23\pm0.04\times10^8$, $2.63\pm0.17\times10^8$ and $2.69\pm0.70\times10^8$ cfu/g in the skin, intestine, liver, and gills, respectively in healthy fish. In contrast, mean bacterial counts were $2.23\pm1.10\times10^8$, $2.96\pm0.12\times10^8$, $1.98\pm0.12\times10^8$, $1.80\pm0.27\times10^8$ cfu/g in the skin, intestine, liver, and gill of diseased fish, respectively. Maximum bacterial load ($3.85\pm0.26\times10^7$cfu) was found in Kurigram (Ulipur) pond soil samples and the highest load ($2.30\pm0.26\times10^7$cfu/ml) was found in Faridpur (Sadarpur) pond water sample. A total of 42 bacterial isolates were merged from 247 isolates and confirmed by biochemical and 16S rRNA gene sequencing. In healthy fish tissues, *Staphylococcus aureus*, *Pseudomonas aeruginosa* were the highest abundance and in diseased fish organs, *S. aureus*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter ludwigii*, *Aeromonas hydrophila* were the highest abundance. In pond soil sample, the dominant bacteria were *E. ludwigii* and *Bacillus amyloliquefaciens*. In water samples, the highest abundance of *S. aureus* was found. The highest diversity was observed in both healthy and diseased fish organs collected from Dinajpur (Jhanjira). In antibiotic sensitivity test, maximum bacteria showed sensitivity to Gentamycin (CN) (95.24%) and Chloramphenicol (C) (90.48%). The *Pseudomonas* sp. and *Enterobacter* sp. showed resistant traits to Amoxicillin, Ampicillin, Erythromycin, Tetracycline and Vancomycin. The study has implications for taking preventive measures against bacterial diseases and for providing baseline information regarding species specific antibiotic sensitivity and resistance which would be helpful for better health management in aquaculture systems.

3.2 Introduction

Aquaculture is a fast-growing food production sector in the world and Bangladesh is holding the third position among the top 10 fish producing countries in the world (FAO, 2022). Considering the recent culture potential of catfish, a sharp increasing trend has been observed on catfish production in Bangladesh. Among the catfish group, stinging catfish is popularly known as singhi, which is distributed in Bangladesh, India Laos, Myanmar, Nepal, Sri Lanka and Thailand (Talwar and Jhingran, 1991). It contains high amount of iron (2.2 mg/100 g of raw edible parts) and fairly high content of calcium compared to many other freshwater fishes (Bogard et al., 2015).

Significant losses in *H. fossilis* production occur due to various diseases caused by bacteria, virus, parasites and other abiotic stresses both in natural and culture ponds. Diseases in catfish farms occur mostly during the winter season (Begum et al 2013, Davis and Hayasaka, 1983). The common bacterial diseases in catfish are enteric septicemia of catfish (ESC), columnaris disease, and motile Aeromonas septicemia (MAS) (Wagner et al., 2002; Plumb and Hanson, 2011).

The different organs (gut, gill, skin, etc.) of fish body contain lysozyme and immunoglobulins which apparently act as defense mechanisms against bacteria. Bacterial community in the fish body (intestine, gill, skin, etc) and environment (water, soil) have significant influence on fish health and growth. Intestinal bacteria influence the development of pathogenic microorganisms (Huber et al., 2004). Different bacterial pathogens are the major cause of diseases and severe mortality occurred in aquaculture and in wild stocks (Sudheesh et al., 2012). The microbial community in the digestive tract of fish is quite dense and the abundance of microorganisms is higher in the surrounding water, indicating that the gastrointestinal tract provides favorable conditions for these organisms (Al-harbi and Uddin, 2005).

The bacteria including the genera *Pseudomonas, Staphylococcus, Flavobacterium, Vibrio, Micrococcus, Bacillus* and *Aeromonas* are the major isolates from catfish frequently found in pond water and large water bodies (Ogbukagu et al., 2021). Bacterial composition is strongly connected to the physico-chemical properties of the environments, which fluctuate in different geographical locations (Fierer and Jackson, 2006). When the environment changes, the bacteria adapt to the new ecosystem and there will be a chance to create new strains. The use of antibiotic compounds in fish feed and indiscriminate practice of

antibiotics in culture ponds increase the antibiotic resistant bacteria. Besides, different pathogenic multidrug resistant bacteria are common in apparently healthy and diseased fish because of industrial wastes and agricultural effluents are directly discharged into water bodies (Da Costa et al., 2013).

Phenotypic and biochemical tests were traditionally used for many years to identify bacteria. Biochemical method for identification of environmental bacteria may not be reliable because of inadequate information on environmental bacteria in computerized databases (Awong-Taylor et al., 2008). Additionally, uncultivable organisms, anaerobes and mycobacteria identification may not be possible with biochemical method (Woo et al., 2008). Molecular methods have been used for the routine identification of microbes, or for direct measures of abundance, diversity and phylogeny (Krieg 1994; Gevers and Coenye 2007; Liu and Stahl 2007).

In this study, prevalence of pathogenic bacteria was investigated, which are commonly present in healthy and diseased stinging catfish as well as associated soil and water samples. The bacterial isolates were screened based on both biochemical properties and molecular approaches (16S rRNA analysis). The objective of this study was to identify commonly present bacteria in healthy and diseased stinging catfish as well as associated soil and water samples. The result of this present study will be helpful for identification of the pathogenic strains in aquaculture species and ponds in the diagnosis and antibiotic sensitivity analysis.

3.3 Materials and methods

3.3.1 Study sites

The fish, sediment and water samples were collected from fifteen catfish farms of different locations in Bangladesh. Most of the farmers of the selected ponds practiced monoculture of shing and some farmers had mixed culture of shing and pabda fish. The major catfish culture area was in Mymensingh, Narsingdi and Jamalpur. The sampling locations and date of sample collections are presented in Figure 3.1 and details in Table 3.1. No aquatic vegetation was observed in the ponds, all ponds were well exposed to sunlight. Water source of the ponds was deep tube well. The sizes of the ponds ranged from 2-5 acres, and the depths of the ponds ranged from 4-7.5 feet. Farmers collected fry/fingerlings from the commercial hatcheries located in Mymensingh, Jashore, Bogura and some own-hatchery. The fry/fingerlings were stocked at 200-250/decimal and were cultured for 5- 6 months.

The farmers applied locally available readymade fish feed of different brands (CP, Narish, Ruposhi bangla, Mega, Amaan feed, catfish feed).

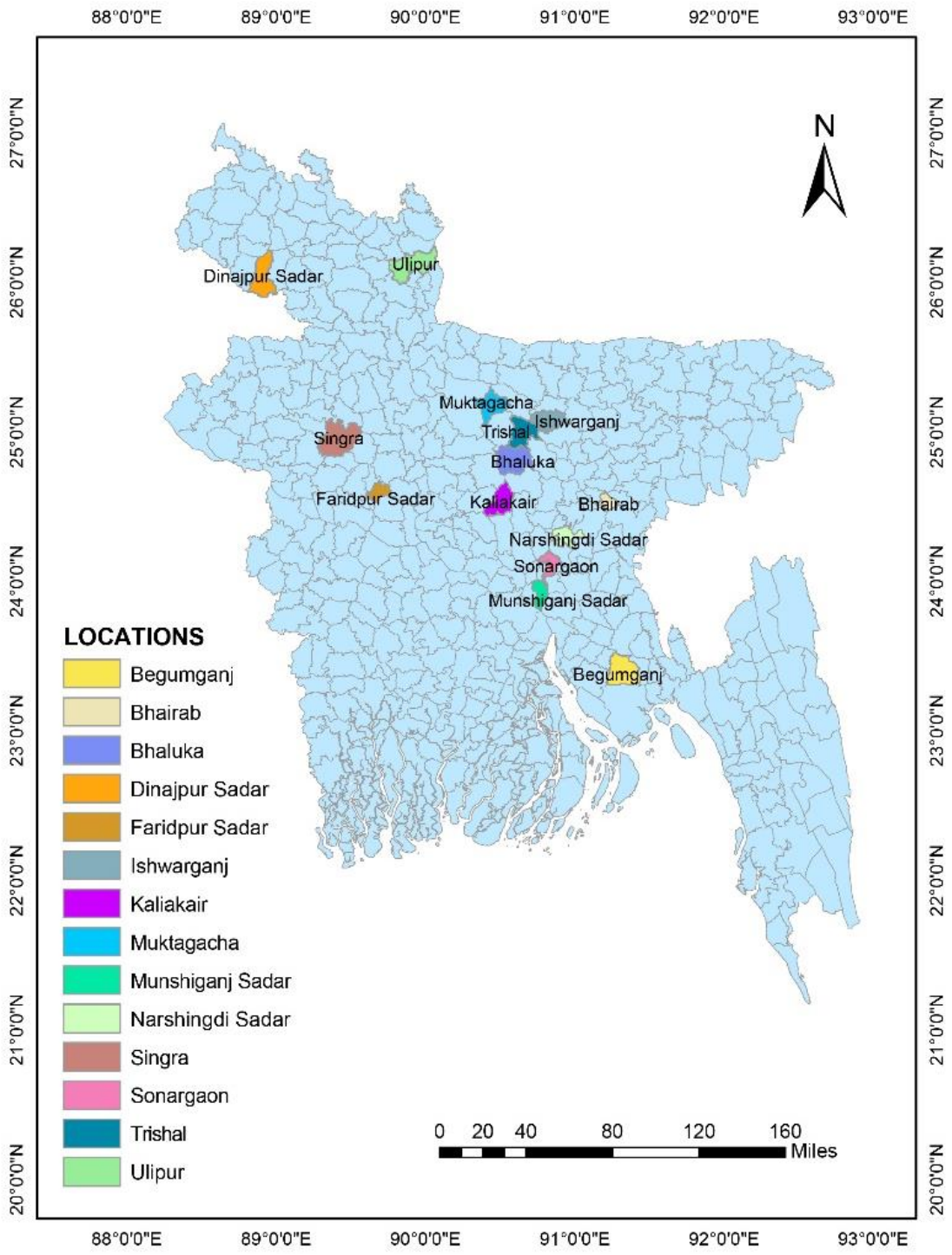

Figure 3.1: Fish, soil and water samples were collected from 15 locations in Bangladesh.

Table 3.1: Fifteen (15) locations of sample (fish, soil and water) collections and collection date

Sl. No.	Division	Farm Locations	Collection date	Coordinates
01	Chattogram	Noakhali (Begumganj)	2019 January	22°56'37.2"N, 91°06'56.7"E
02	Dhaka	Bhairab (Ghorakanda)	2018 October	24°02'45.6"N, 90°58'55.4"E
03	Dhaka	Sonargaon (Char-Kamaldi)	2018 December	23°43'17.7"N, 90°38'08.5"E
04	Dhaka	Munshiganj (Kanakshar)	2018 December	23°32'26.5"N, 90°31'48.4"E
05	Dhaka	Narshingdi (Bhelanagar)	2019 January	23°56'17.3"N, 90°42'32.4"E
06	Dhaka	Faridpur (Sadarpur)	2019 February	23°28'29.4"N, 90°01'36.4"E
07	Dhaka	Gazipur (Kaliakair)	2019 February	24°03'28.8"N, 90°11'55.1"E
08	Mymensingh	Muktagacha (Ghoga)	2018 October	24°02'45.6"N, 90°58'55.4"E
09	Mymensingh	Trishal (Bali Para)	2018 November	24°02'45.6"N, 90°58'55.4"E
10	Mymensingh	Bhaluka (Dhitpur)	2018 November	24°26'11.1"N, 90°27'11.7"E
11	Mymensingh	Ishwarganj (Madhupur)	2018 November	24°33'44.4"N, 90°33'35.9"E
12	Mymensingh	Ishwarganj (Barahit)	2018 November	24°38'34.3"N, 90°35'19.7"E
13	Rajshahi	Natore (Singra)	2019 February	24°30'07.4"N, 89°08'31.4"E
14	Rangpur	Dinajpur (Jhanjira)	2018 October	25°43'04.2"N, 88°43'47.9"E
15	Rangpur	Kurigram (Ulipur)	2019 January	25°39'34.2"N, 89°37'24.3"E

3.3.2 Collection of fish samples

A total of 45 apparently healthy fish (Figure 3.2A) and 45 diseased fish having pathological symptoms like lesion in different parts of the body (Figure 3.2B) were collected from fifteen sources. Average size of collected fish samples was 6-7 inches and average weight was 55-70 g. Healthy and diseased fish were collected from same pond three times by netting.

Figure 3.2: A-healthy fish; B-diseased fish showing lesion, black spot, tail and fin rot, eye protrusion symptoms observed in collected samples.

Each fish was kept in individual zipper bag with proper labelling. According to the standard microbiological practice, all samples were collected aseptically to avoid contamination. Fifteen soil and 15 water samples were also collected from those 15 locations. Different water quality parameters were checked and recorded for each pond. Fish and environmental samples of respective ponds were transported in an ice box to the laboratory and stored at -20°C temperature for further analyses.

3.3.3 Sample preparation

Tissue samples, soil and water were prepared and analyzed within 2-4 days of sample collections. From each fish selected organs such as the liver, intestine, gills, skin mucus and lesion were aseptically separated (Figure 3.3A) for bacteria isolation and further identification using biochemical and molecular analysis.

Dissected organs (liver, intestine, gills, skin mucus and lesion) were blended and mixed properly with 100 ml of alkaline peptone water (APW) in separate sterile conical flasks (Figure 3.3B). Separate surgical blades, scissors, forceps and dissection tray were used for each fish to avoid contamination. In case of environmental samples, 1 gm of soil and 10 ml of water were taken in separate conical flask after proper measuring and mixed with 100 ml and 90 ml alkaline peptone water respectively.

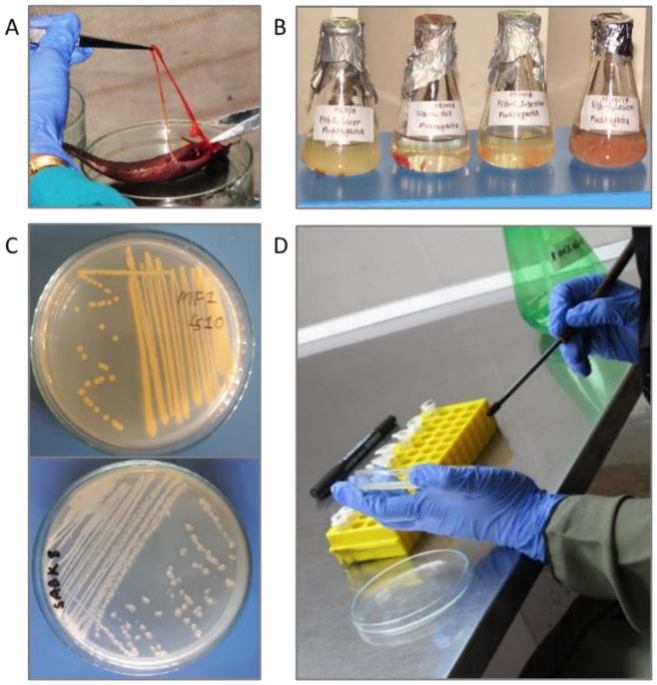

Figure 3.3: A: dissection of different organs; B: separated organs in alkaline peptone water for enrichment; C: Pure culture in LB agar; D: preservation of pure culture in 50% glycerol.

All samples were incubated at 37°C in a shaking incubator (Thermos Table IS-20R, Korea) for overnight at 120 rpm. After incubation, 1 ml of each enriched samples was transferred to 9 ml of physiological saline (0.9% NaCl) for 10-fold (1:10) dilution and further diluted up to 10^{-6}.

3.3.4 Measurement of water quality parameters

Water quality parameters including temperature, dissolved oxygen, pH, salinity were measured by EUTECH Instrument, PCD 650, Thermo fisher Scientific and ammonia was measured by ammonia kit (ammonia LR reagent, ion specific) of water samples collected from fifteen locations. All the values of the water quality parameters were compared with the standard values described by Wynne (2003); (Banerjea 1967), Swingle (1967) (Moyle 1949).

3.3.5 Counting of total viable bacteria

Diluted samples (100 µl) were inoculated on Tryptic soya agar and nutrient agar plate and incubated at 37°C for 24 hours for colony forming unit (CFU) counting and colony observation. Spreading of the sample in the plates were done with the help of sterilized spreader. Each colony was counted using digital colony counter (Labdex LX12CC) according to plate count method. Colony Forming Unit value was measured by the following formula-

$$\text{Viable bacterial count/ml} = \frac{\text{The total number of colonies} \times \text{Dilution factor}}{\text{The volume of the sample added to the agar plate}}$$

3.3.6 Isolation and preservation of bacteria

Each colony was carefully observed through eye observation, marked and numbered on the basis of pigment, shape, elevation, opacity and edges. After observation, each colony was sub-cultured in Luria-Bertani (LB) agar plates (Figure 3.3C) in aerobic condition at 37°C. Total culture on the plates was preserved in 50% glycerol in cryovials with proper labeling by using sterile loop. The cryovials were wrapped with parafilm and stored (Figure 3.3D) at -80°C (Utra-Low Temperature Freezer, Eppendorf, U725-86, Innova United Kingdom) for future study.

3.3.7 Morphological observation and grouping of bacteria

The isolates were morphologically observed and categorized into Gram-positive and Gram-negative through Gram's staining and string test of the colony on the basis of presence of

peptidoglycan and physical properties of the cell wall. This will reveal the bacteria in one of the two groups. Gram's stains are a widely popular and frequently painless method for detecting bacterial or fungal infections. In case of Gram's staining purple color formation indicated Gram-positive bacteria and pink/red color formation indicated Gram-negative bacteria. Further confirmation was done with positive string test result found for Gram-negative bacteria and negative string test result for Gram-positive bacteria.

3.3.8 Biochemical tests

Biochemical tests were performed for the identification of isolates based on their biochemical activities in response to various biochemical compounds. The identification was focused on the presence of essential nutrients secreted by the bacteria and the change of color of the medium, which ultimately changed the pH of the medium. The bacteria were tentatively identified following Bergey's Manual of Determinative Bacteriology (Bergey, 1994). Total six biochemical tests (oxidase, citrate utilization, methyl red (MR) test, Voges-Proskauer (VP) test, indole test and growth on MacConkey agar) were done for the presumptive identification of the bacteria.

3.3.9 Molecular identification

3.3.9.1 Genomic DNA extraction

For DNA extraction, single colony from LB agar media (Figure 3.4) was inoculated in 10 ml nutrient broth and incubated at 37˚C for overnight. From this culture, 1.5 ml content was centrifuged (KUBOTA 6200, 220V, Tokyo, Japan) in eppendorf tubes at 13000 rpm for 10 minutes. For disruption of cells, 350 µl lysis buffer and 20 µl of proteinase-K (20 mg/ml) was added and kept in water bath for 30 minutes at 65˚C. After that the tube was allowed to cool at room temperature and 3 µl of RNAse A solution was added and kept on heat block (Stuart, SBH130D, Bibby, UK) at 37˚C for 10 minutes. An equal volume (373 µl) of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the solution and mixed by vortex machine. After mixing, the suspension was centrifuged at 14000 rpm for 10 minutes. The aqueous layer (about 200 µl) from the top was transferred to a fresh microcentrifuge tube carefully to avoid any protein debris. Similarly, an equal volume of chloroform: isoamyl alcohol (24:1) was added to the tube and centrifuged at 14000 rpm for 10 minutes. The upper layer (150 µl) was taken to new eppendorf and 800 µl of ice cooled absolute ethanol was added to aqueous phase to precipitate the DNA (Figure 3.4) and inverted the tube by hand shaking and kept at -40°C for 1 hour. Then DNA was pelleted by

centrifugation at 13000 rpm for 10 minutes. The liquid was discarded and 1000 µl of chilled 70% ethanol with 20 µl of 7.5M ammonium acetate was added and kept at -20˚C for 30 minutes. Again, the content was centrifuged at 13000 rpm for 10 minutes and aqueous layer was eliminated and allowed to air dry. Finally, the 50 µl of TE buffer (pH 7.6) was added to dissolve the DNA by heating at 37°C for 10 minutes.

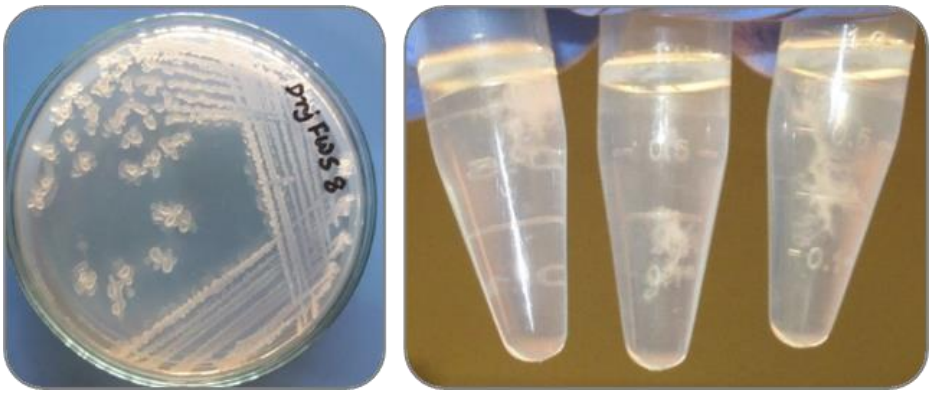

Figure 3.4: Pure culture in LB agar and extracted DNA from pure colony in 100% ethanol.

3.3.9.2 DNA quantification and quality confirmation of DNA

DNA concentration was checked by Nanodrop Spectrophotometer (ThermoScientific, USA) also checked the quality by running the DNA in 1.5% agarose gel. At first, 2 µl fresh TE buffer was placed on the lower pedestal of the machine and the lid was closed for blank measurement. After that pedestal was wiped properly and 2 µl of each DNA sample containing TE buffer was placed and measured the DNA concentration (ng/µl) and absorbance ratio. For 1.5% agarose gel preparation, 0.6 gm Ultrapure agarose (UA) (Invitrogen, life Technologies Inc., USA) was taken in 100 ml conical flask and 40 ml 1X Tris-acetate EDTA (TAE) buffer (pH 8.3) was added and dissolved by boiling in a microwave oven (SHARP, R-360H, Japan) for two minutes. Then it was allowed to cool to touch to bear hand then 3 µl SYBR Safe DNA gel stain (Invitrogen, USA) was added. After gentle mixing, the liquid was poured into the gel chamber after setting the comb. Then it was allowed to solidify for half an hour at room temperature. After removal of the comb, the gel was placed in the electrophoresis tank and 1X TAE-buffer was poured to cover the gel to a depth of about 1 mm. Then 3 µl DNA was mixed with 1 µl of 6X loading buffer and loaded in the well of gel. DNA marker (5 µl) (100 bp DNA ladder, Promega) was added to the specific well. Electrophoresis was carried out at 100 volts until the dye moved to the

half of the chamber. The gel was viewed and photograph was taken under a gel documentation system (ProteinSimple, FluorChem E System, USA).

3.3.9.3 PCR amplification with 16S rRNA and agarose gel electrophoresis

Polymerase Chain Reaction (PCR) was performed using 16S rRNA primer (27F: AGA GTT TGA TCC TGG CTC AG; 1492R: GGT TAC CTT GTT ACG ACT T) (Turner et al.1999; Lane, 1991).The reaction mixture for PCR, consisted of 12 µl of 2X premix (Maximo), 1 µl of forward primer, 1µl of reverse primer, 8.5 µl of nuclease free water and 2 µl of DNA template to a final volume 25 µl. PCR amplification was set as 1 cycle of initial denaturation at 95°C for 5 minutes which was followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 1 minute, extension at 72°C for 2 minutes and 1 cycle of final extension at 72°C for 10 minutes (Figure 3.5A). The PCR products were subjected to 1.5% agarose gel for electrophoresis (Figure 3.5B) and bands were observed in Gel doc system and compared with 100bp DNA ladder.

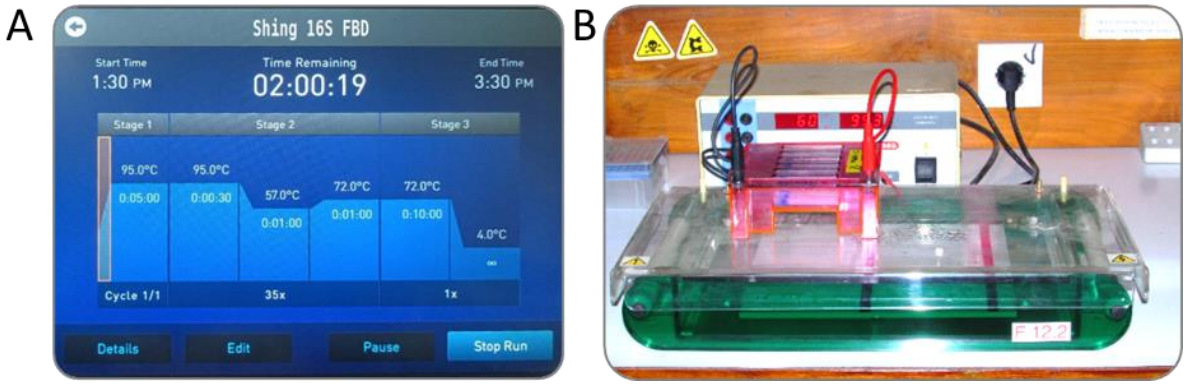

Figure 3.5: A-Polymerase chain reaction (PCR) condition for 16S rRNA gene amplification; B- Gel electrophoresis system for visualization amplified PCR products.

3.3.9.4 Purification of the PCR products

An amount of 15 µl PCR product was taken into purification column supplied by DNA purification kit (Thermo Scientific, USA). Binding buffer (60 µl) was added to the column and centrifuged at 10000 rpm for 2 minutes. After that, 600 µl of Wash buffer was added carefully and centrifuged again for 2 minutes at 10000 rpm. Then the lower part of the purification tube was removed and the upper part was transferred into a new tube and 50 µl of TE buffer was added to middle of the upper column and centrifuged at 10000 rpm for 10 minutes. Finally, the upper column was discarded and lower eppendorf contained the

purified DNA was stored at -20°C. The quality and purity of the purified DNA samples were measured using Nanodrop spectrophotometer.

3.3.9.5 PCR product sequencing and DNA sequences analysis

Purified PCR products were sequenced in Genetic Analyzer 3500 with 20 µl reaction volume containing approximately 10 ng of purified products as template, reaction premix (4.0 µl), BigDye terminator buffer (2.0 µl), forward and/or reverse primer(s) (0.32 µl) and remaining with ultrapure water up to make 20 µl final volume.

BioEdit Sequence Alignment Editor Software (version 7.0.9.0) was used to visualize the sequences. Sequence trimming was done to maintain high-quality fragments by removing some bases from initial and end portion. The forward and reverse sequences were used to make contig using DNA sequence assembler. The FASTA sequence of each contig were exported and compared with the GenBank database (www.ncbi.nlm.nih.gov/genbank) and was confirmed by performing BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) searching with percentage identity and query cover value comparison. A phylogenetic tree in Maximum Likelihood (ML) method was constructed using the nucleotide sequences and reference sequences from GenBank using MEGA software version X (Tamura et al. 2013). Thousand (1000) bootstraps were performed to assign confidence levels to the branch nodes of ML tree (Felsenstein, 1985). Nucleotide sequences were aligned using the program MEGA X.

3.3.10 Antibiotic sensitivity test

Antibiotic sensitivity test was used to determine which drug is more effective for treatment. Various antibiotic sensitivity tests were performed according to Kirby-Bauer disk diffusion method (Hudzicki, 2009) using Mueller-Hinton (MH) agar (Oxoid, UK). The tested antibiotics with their concentration per disk and standard zone diameter chart for sensitive and resistant pattern determination are shown in Table 3.2.

After 24 hours of inoculation, a pure culture from nutrient agar medium was picked with wire loop to 5 ml test tube containing Tryptic soy broth and incubated at 37˚C for overnight for optimum growth. Maximum growth was checked by observing the turbidity, which was compared with the control (contained only Tryptic soy broth without any bacterial colony). After that, a sterile cotton swab was dipped into the solution and streaked on Mueller-Hinton (MH) agar plate. Rubbing the agar plate with swab stick was repeated for two times for even distribution of the inoculums. Then, the agar plate was allowed to dry for 10

minutes. Then, antibiotic disks were placed in the plate surface (Figure 3.6) by using disk dispenser and incubated at 37˚C for 24 hours.

Table 3.2: Standard interpretive chart used for determination of the resistant and sensitive pattern by Kirby-Bauer disk diffusion method (Hudzicki, 2009)

Antibiotics name (dose)	Sensitive (S) mm	Moderately sensitive (MS) mm	Resistant (R) mm
Amoxicillin (30µg)	≥18 mm	14-17 mm	≤13 mm
Tetracycline (30µg)	≥15 mm	12-14 mm	≤11 mm
Erythromycin (15 µg)	≥23 mm	14-22 mm	≤13 mm
Vancomycine (15 µg)	≥12 mm	10-11 mm	≤9 mm
Azithromycine (15 µg)	≥18 mm	14-17 mm	≤13 mm
Ampicillin (2 µg)	≥17 mm	14-16 mm	≤13 mm
Chloramphenicol (30 µg)	≥18 mm	13-17 mm	≤12 mm
Cefixime (5 µg)	≥19 mm	16-18 mm	≤15 mm
Gentamycin (10 µg)	≥15 mm	13-14 mm	≤12 mm

Contact between the disk and agar surface was ensured gentle pressure so that the disk could not move once it was placed. Distance between the disks was maintained for 24 mm and 5 disks were placed in a 150 mm petri dish. Zone of inhibition was measured with the help of ruler which was graduated to 0.5 mm for determining the sensitivity, moderate sensitivity and resistance through unaided eye observation. Diameter of the zone of inhibition were recorded and compared with interpretative standard value for respective antibiotics.

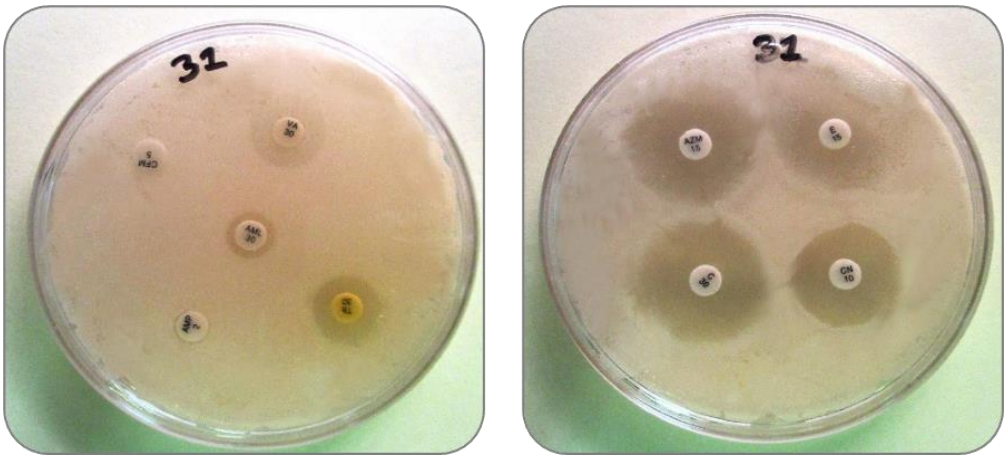

Figure 3.6: Inhibition zone produced by antibiotic disc in Mueller-Hinton (MH) agar plate.

3.3.11 Data analysis

The data were statistically analyzed using Statistical Package for the Social Sciences (SPSS) v. 22 (IBM). The mean and standard deviation were calculated to analyze significant relationship between bacterial genera of healthy and diseased fish, soil and water samples whether statistically significant or not. Analysis of variance (ANOVA) was carried out to determine significant differences of bacterial diversity between the locations.

3.4 Results

3.4.1 Water quality parameters

The mean values of temperature, pH, dissolved oxygen, ammonia and total dissolved solids were 20.67±3.54˚C, 7.18±0.38, 15.56±0.93 ppm, 1.73±0.37, and 320.33±41.09 ppm, respectively. The measurement values of the water samples were found within the range of standard values of water quality parameter for fish culture except with remarkable variation in ammonia (NH_3) content (**Appendix 5 Table A5.1**).

3.4.2 Bacterial load from fish tissue, soil and water samples

The variation of quantitative bacterial load from some organs of healthy and diseased stinging catfish, water and sediment samples of 15 locations were analyzed (**Appendix 6 Table A6.1)**.

The mean bacterial counts were found in healthy fish ranged from lowest $2.87\pm 0.04\times10^7$ to highest $3.23\pm 0.04\times10^8$cfu in Dinajpur (Jhanjira) and Bhaluka (Dhitpur), respectively (Figure 3.7). The highest mean bacterial load in skin, intestine, liver and gill in healthy fish were $2.82\pm 0.05\times10^8$, $3.23\pm 0.04\times10^8$, $2.63\pm 0.17\times10^8$ and $2.69\pm 0.70\times10^8$ cfu/g, respectively. The lowest mean bacterial load in skin, intestine, liver and gill in healthy fish were $2.87\pm 0.04\times10^7$, $8.63\pm 0.02\times10^7$, $4.40\pm 0.07\times10^7$ and $5.30\pm 0.90\times10^7$cfu/g, respectively.

Similarly in diseased fish, the mean bacterial counts were ranged from $8.17\pm 0.58\times10^4$ to $2.96\pm 0.12\times10^8$cfu in Dinajpur (Jhanjira) and Ishwarganj (Barahit), respectively (Figure 3.7). The highest mean bacterial load in skin, intestine, liver, gill in diseased fish were $2.23\pm 1.10\times10^8$, $2.96\pm 0.12\times10^8$, $1.98\pm 0.12\times10^8$, $1.80\pm 0.27\times10^8$cfu, respectively. The lowest mean bacterial load in skin, intestine, liver, gill in diseased fish were $1.06\pm 0.92\times10^6$, $1.15\pm 0.36\times10^5$, $8.17\pm 0.58\times10^4$, $1.81\pm 0.81\times10^5$, respectively.

The overall mean bacterial load on healthy fish were $15.6\pm8.70\times10^7$, $19.8\pm7.18\times10^7$, $14.2\pm6.90\times10^7$ and $15.2\pm6.66\times10^7$ cfu/g in skin, intestine, liver and gill, respectively. On

the other hand, in diseased fish, the mean bacterial loads were $4.94\pm7.47\times10^7$, $7.23\pm10.2\times10^7$, $5.07\pm7.10\times10^7$ and $4.87\pm5.28\times10^7$cfu/g in the skin, intestine, liver and gill samples, respectively. The bacterial load was highest in healthy fish organs because healthy fish harbors various types of microbiomes for the support of their different physiological functions (Ma et al., 2019).

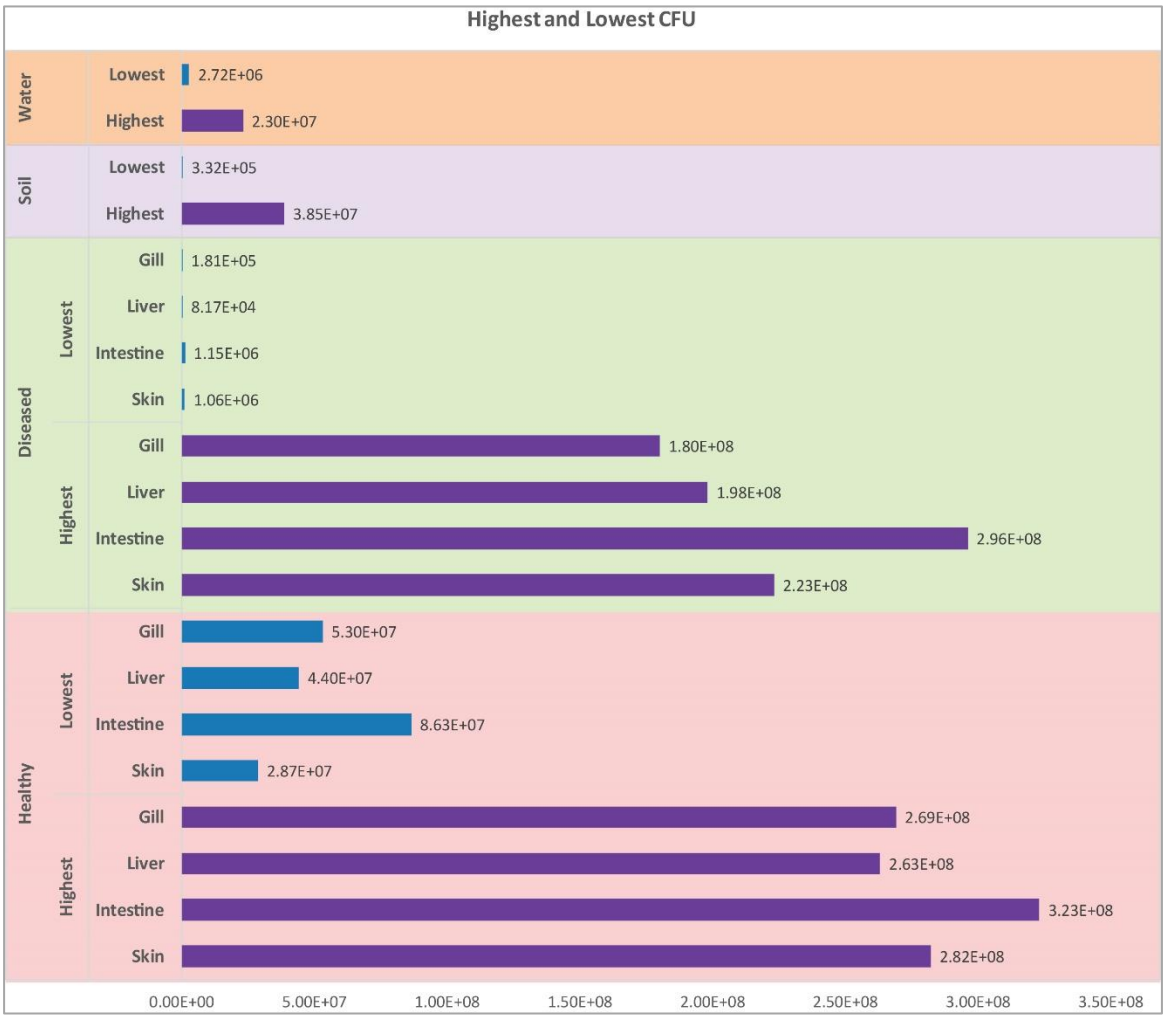

Figure 3.7: Highest and lowest mean cfu load observed in healthy and diseased fish tissue associated environmental sample.

The highest bacterial load ($3.85\pm0.26\times10^7$cfu/g) was found in Kurigram (Ulipur) from soil samples and in water sample, the highest load ($2.30\pm0.26\times10^7$cfu/ml) was found in Faridpur (Sadarpur). The mean load was $7.94 \pm2.76 \times10^6$ cfu/g in soil and the mean load was $1.25\pm0.24\times10^7$cfu/ml in water (Figure 3.7).

3.4.3 Morphological observation of isolates

A total of 247 isolates were isolated from different parts of fish body, water and soil samples. Out of 247 isolates, 42 different types were identified based on their morphology through eye estimation. The morphology and morphological characteristics of the 42 isolates are presented in Figure 3.8 (**Appendix 6 Table A6.2)**. Among 42 isolates, 28 isolates showed circular shapes phenotypic features, most are white in colored and opaque and few are translucent.

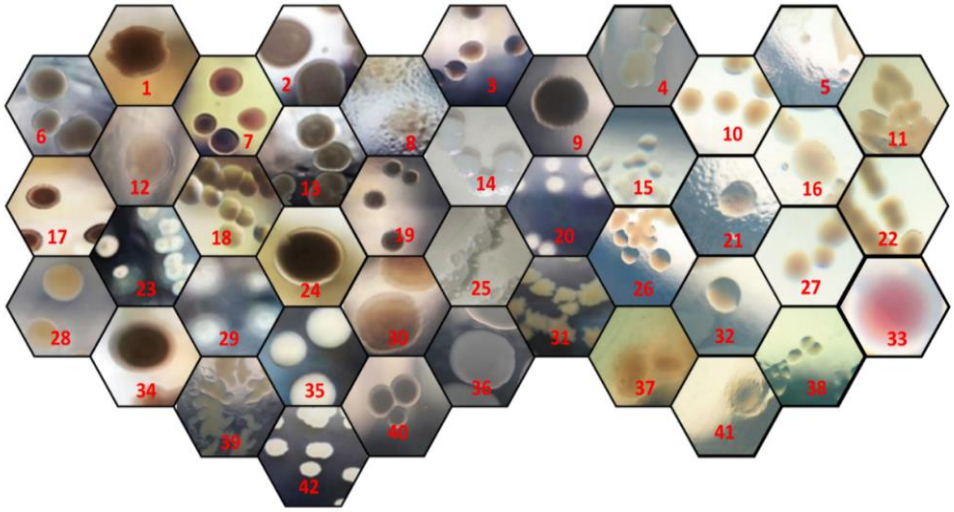

Figure 3.8: Morphology variation of 42 colony isolated from fish tissue, soil and water samples in nutrient agar media.

3.4.4 Results of biochemical tests

Biochemical tests were done for the presumptive identification of the 42 isolates **(Appendix 6 Table A6.3)**. From six biochemical test, 20 isolates were positive and 22 negatives for indole, 23 isolates were positive and 19 negatives for MR test, 14 isolates were positive and 28 negatives for VP, 19 isolates showed positive response for oxidase test while 23 negative, 25 isolates were capable to utilize citrate but 17 were unable, 20 isolates participated in lactose fermentation while 22 showed negative response in lactose fermentation process.

3.4.5 Molecular identification

Finally, the 42 isolates were confirmed by 16S rRNA gene sequencing. The list of 42 isolates confirmed by 16S rRNA gene sequencing are presented in phylogenic tree (Figure 3.9) **(Appendix 6 Table A6.4)**. Presumptive identification of 42 isolates by biochemical

methods were compared with molecular method and found 30.95% unmatched presumption with 16S rRNA gene sequencing confirmation. The 42 bacteria were found under the umbrella of three phyla; dominant Proteobacteria, Bacillota and Bacteroidota. The phylum Bacteroidota includes only one bacterium *Flavobacterium columnare*. Reported pathogenic bacteria like *Aeromonas hydrophila*, *Psudomonas* spp., *Enterobacter* spp., *Citrobacter freundii* were dominant under Proteobacteria phylum.

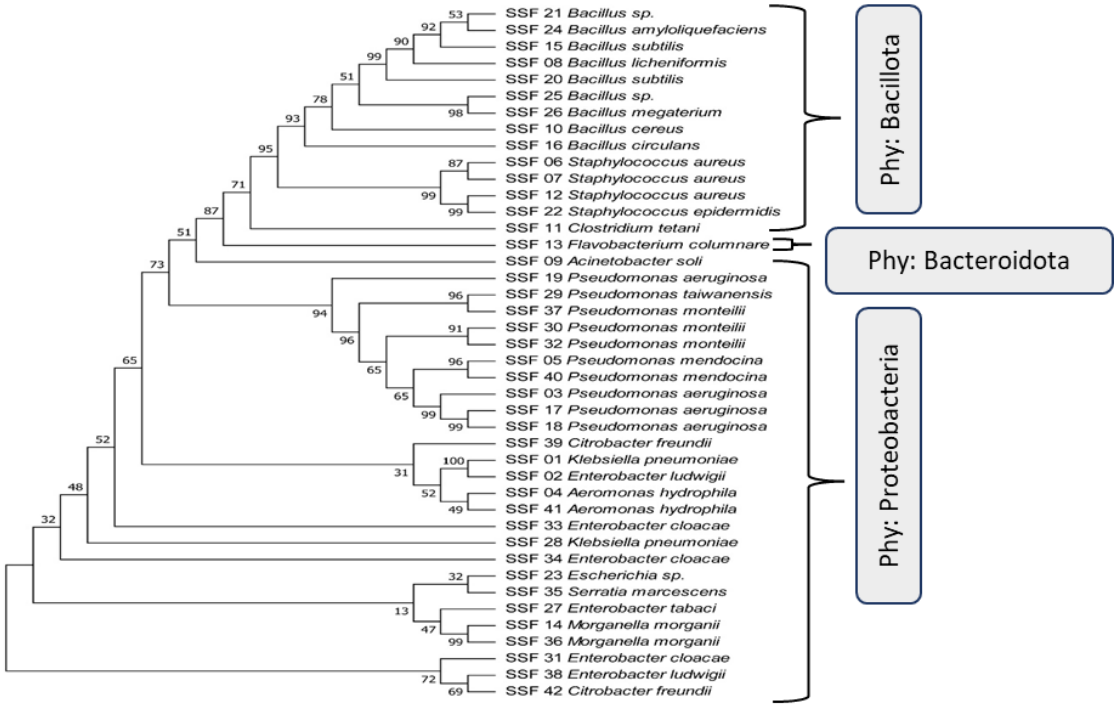

Figure 3.9: Phylogenetic tree of 42 bacteria obtained from healthy and diseased fish tissue, soil and water samples collected from 15 locations of Bangladesh.

3.4.6 Diversity of bacteria in fish tissue, soil and water

The gram (+) and gram (-), pathogenic and non-pathogenic bacteria were isolated from healthy and diseased fish organs. The prevalence and diversity in fifteen locations of isolated 42 bacteria from healthy and diseased fish organs and their diversity is presented in Figure 3.10.

The highest diversity was observed in Dinajpur (Jhanjira) and the lowest diversity was observed in Ishwarganj (Madhupur) in healthy fish organs. Whereas, the highest diversity was observed in Dinajpur (Jhanjira) and the lowest diversity was observed in Ishwarganj (Madhupur) in diseased fish. In the soil samples, the highest diversity was observed in Dinajpur (Jhanjira), Muktagacha (Ghoga), Bhaluka (Dhitpur), Sonargaon (Char-Kamaldi),

Munshiganj (Kanakshar) and the lowest diversity observed in Ishwarganj (Barahit), Noakhali (Begumganj). In water samples, the highest bacterial diversity was found in Dinajpur (Jhanjira) and the lowest diversity was found in Ishwarganj (Barahit).

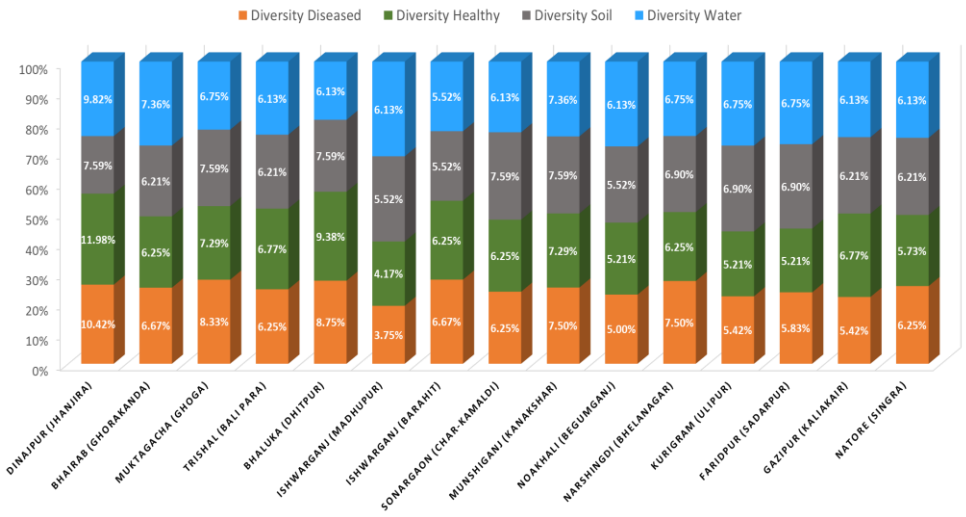

Figure 3.10: Diversity of bacteria isolated from healthy and diseased fish tissue, soil and water samples collected from 15 locations of Bangladesh.

The prevalence of 42 isolates in fish tissue (healthy and diseased), soil and water confirmed by 16S rRNA gene sequencing are presented in **Appendix 6 Table A6.5**. In the studied organs of healthy fish, *Staphylococcus aureus, Pseudomonas aeruginosa* have highest abundance and *Acinetobacter soli, Aeromonas hydrophila, Bacillus megaterium, Enterobacter tabaci, Flavobacterium columnare* showed lowest (Figure 3.11). In diseased fish organs, *S. aureus, P. aeruginosa, Klebsiella pneumoniae, E. ludwigii, A. hydrophila* were the highest in abundance and *B. licheniformis, B. megaterium* were the lowest in abundance (Figure 3.11).

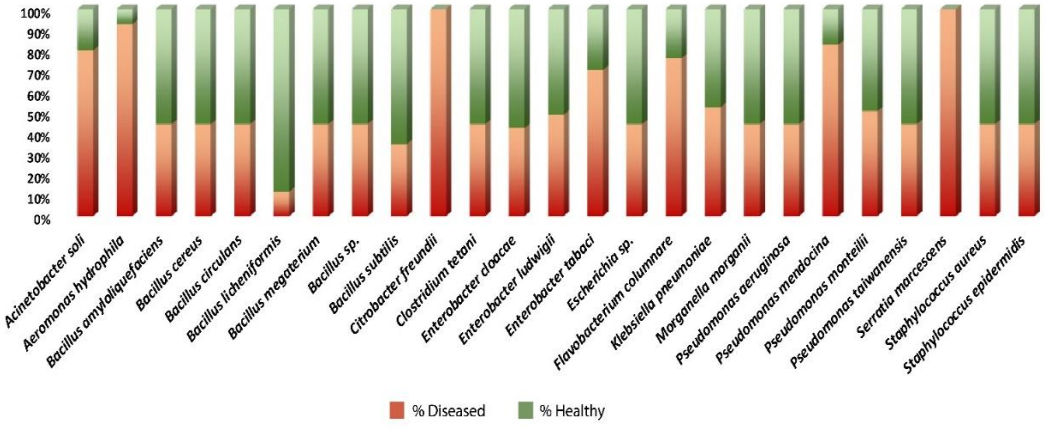

Figure 3.11: Prevalence of bacteria isolated from healthy and diseased fish tissue collected from 15 locations of Bangladesh.

In soil sample, the dominant bacteria were *E. ludwigii* and *B. amyloliquefaciens, Clostridium tetani, E. tabaci* had highest abundance (Figure 3.12). In water samples, the highest abundance of *S. aureus* was found whereas the lowest abundance showed by *E. tabaci* (Figure 3.12).

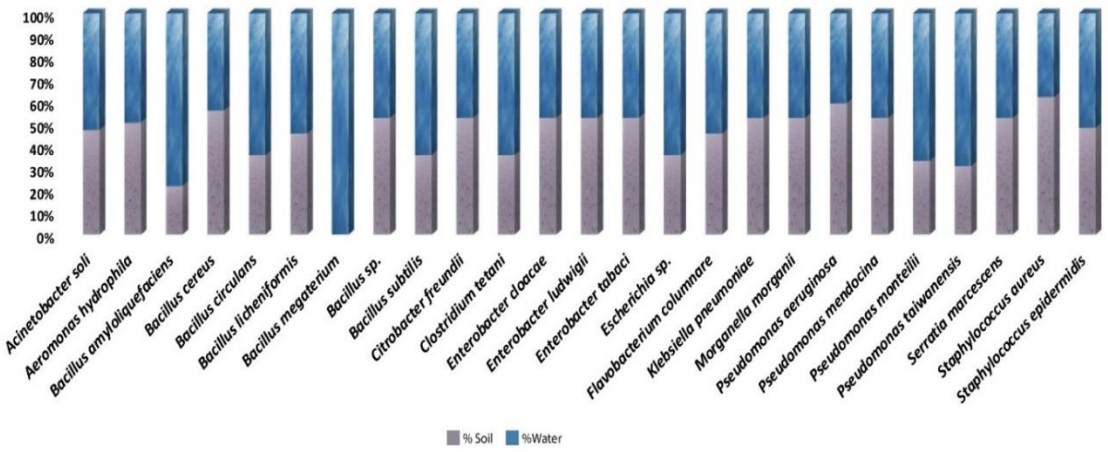

Figure 3.12: Prevalence of bacteria isolated from soil and water samples collected from 15 locations of Bangladesh.

3.4.7 Antibiogram profiling

Antibiotic sensitivity tests were done for the isolated 42 bacteria. The isolates showed different resistance patterns to various antibiotics. The results of antibiotic sensitivity of 42 isolates are presented in Table 3.3 **(Appendix 6 Table A6.6)**. All the isolates were susceptible to Azithromycin at 15 µg concentration except *B. licheniformis* (SSF_8). In case of Chloramphenicol, all the isolates except *P. taiwanensis* (SSF_29) were susceptible at 30 µg concentration. Similarly in case of Gentamycin all the isolates except *P. aeruginosa* (SSF_18) were susceptible to moderate susceptible at 10 µg concentration. Azithromycin, Chloramphenicol and Gentamycin exhibited more susceptible phenotypes than others.

Highest 95.24% isolates showed sensitivity to Gentamycin (CN) sensitivity to the bacteria followed by Chloramphenicol (C) (90.48%) (Figure 3.13). Bacterial isolates showed resistance phenotypes to the Ampicillin (AMP) (66.67%), Amoxicillin (AMP) (57.14%) and Cefixime (CFM) (57.14%). Bacteria from the genus *Pseudomonas* and *Enterobacter* showed resistant traits to Amoxicillin, Ampicillin, Erythromycin, Tetracycline and Vancomycin.

Table 3.3: Antibiotic sensitivity and resistant pattern of 42 bacteria. Zone of inhibition was measured in millimeter (mm)

Antibiotics	Isolates (N=42)		
	R (%)	MS (%)	S (%)
Amoxicillin (AML)	24 (57.1)	2 (4.8)	16 (38.1)
Ampicillin (AMP)	28 (66.7)	3 (7.1)	11 (26.2)
Azithromycin (AZM)	1 (2.4)	3 (7.1)	38 (90.5)
Chloramphenicol (C)	1 (2.4)	3 (7.1)	38 (90.5)
Cefixime (CFM)	24 (57.1)	2 (4.8)	16 (38.1)
Gentamycine (CN)	2 (4.8)	0 (0)	40 (95.2)
Erythromycine (E)	16 (38.1)	11 (26.2)	15 (35.7)
Tetracycline (TE)	12 (28.6)	8 (19.1)	22 (52.4)
Vancomycin (V)	21 (50)	0 (0)	21 (50)
Amoxicillin (AML)	24 (57.1)	2 (4.8)	16 (38.1)
Ampicillin (AMP)	28 (66.7)	3 (7.1)	11 (26.2)

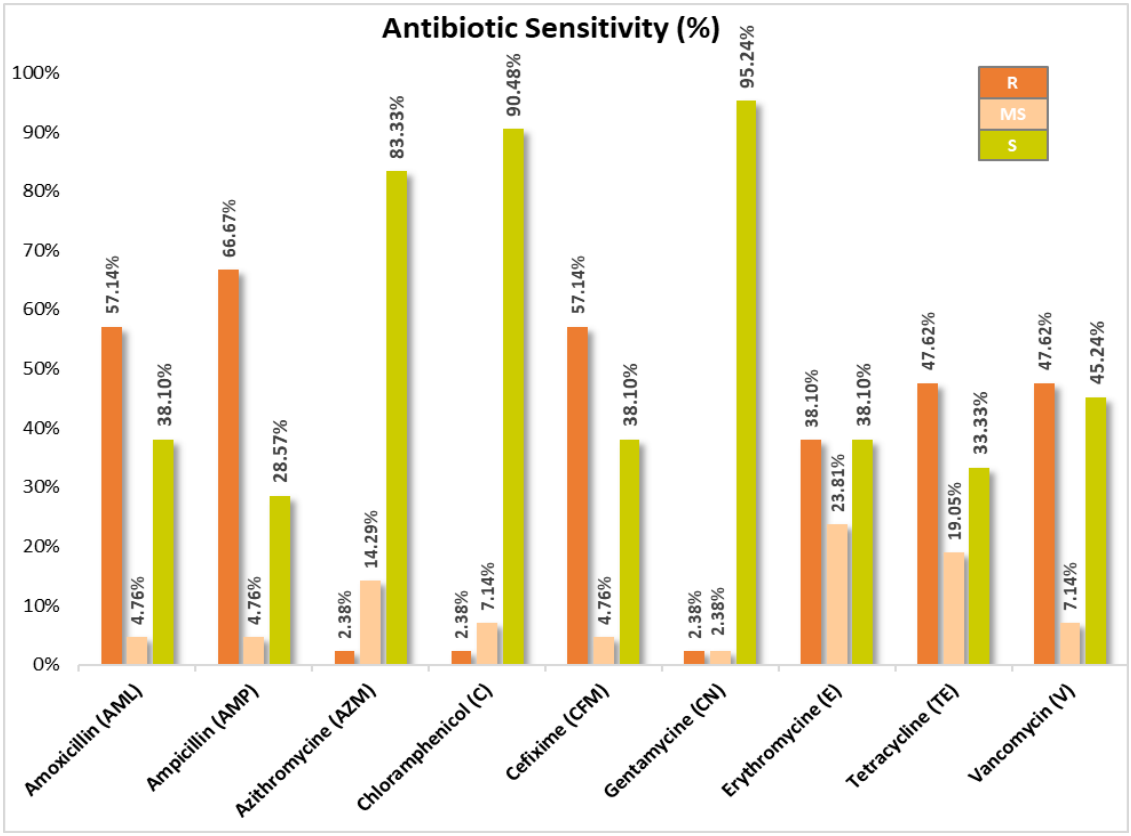

Figure 3.13: Antibiotic susceptibility results of 42 bacteria to 9 antibiotics isolated from fish tissue, soil and water sample in nutrient agar media.

3.5 Discussion

The aquaculture ponds have higher prevalence of microbial community compared to the wild or other systems (Wamala et al., 2018). Fish lives in aquatic environment and shelter different bacteria at different stages of their lifecycles. The high density of fish in aquaculture facilitates infection and transmission of bacteria (Penders and Stobberingh, 2008). Direct contamination of pond waters by bacteria from surrounding soils could also contribute to the observed high prevalence and diversity of bacteria in earthen ponds.

Microbial communities in fish organs are the reflection of its aquatic environment (Uchechukwu and Okoli, 2019; Hansen and Olafsen, 1999; Fernandez et al., 1996). The microbial communities play important role in different physiological functions like immunity development, digestion, disease resistance, etc (Wong and Rawls, 2012). Similarities of microbial communities were found between fish organs, sediments and culture water. Present study suggested that the fish obtained bacteria from the aquaculture systems is consistent with previous reports where they isolated bacteria from fish organs, soil and water (Ahmad et al, 2017; Austin, 2002; Fierer and Jackson, 2006). Isolates from water sample were high. In this study, 2.31×10^7 cfu/ml bacterial counts were found from the culture water whereas Chowdhury et al. (1994) observed a bacterial count of 1.3×10^4 to 5.6×10^5cfu/ml in *Clarius batrachus* (walking catfish) culturing pond water.

Bacterial load in healthy fish organs was higher than the diseased fish organs. Higher bacterial load was also reported from catfish organs by (Uddin and Harbi, 2012). Higher bacterial load in healthy fish play different physiological functions. Pathogenic bacteria in healthy fish exist as dormant condition when they got the favorable condition and infected fish and causes disease in fish. Highest bacterial load was observed in the intestine of healthy fish than diseased fish. Wu et al. (2010) found that the total viable counts were 3.4×10^8 cfu/g in the intestinal content and 2.1×10^7 cfu/g in the intestinal mucus of yellow catfish (*Pelteobagrus fulvidraco*). Hagi et al. (2004) observed bacterial loads of 1.9×10^9 cfu/g in the intestine of common carp. Higher microbial load was also observed in the slime of healthy fish than diseased fish. Chowdhury (1998) reported a bacterial load of up to 2.2×10^8 cfu/g in the slime of hybrid catfish (*C. batrachus* x *C. gariepinus*).

On the basis of morphological observation of the colonies, 247 isolates were merged into 42 unique isolates. Upon on molecular identification, *Staphylococcus aureus* and *P. aeruginosa* were found as predominant in both healthy and diseased fish. *Staphylococcus*

species are reported opportunistic potential pathogens widely present in the natural environment (Rong et al., 2017) and their dominant presence indicates the quality of fish (Ali, 2014). The dominant presence of *S. aureus* in the healthy fish tissues indicates their opportunistic pathogenic features which is consistent with the isolation of *S. aureus* from catfish *Silurus glanis* (Ali, 2014) and from aquatic products (Rong et al., 2017). Similarly, *P. aeruginosa* is reported pathogenic bacteria causing ulcer and hemorrhages and mortality in fish (Algammal et al., 2020). *S. aureus* and *P. aeruginosa* were found as dominant bacteria in healthy fish and water samples which may be of their opportunistic and dormant conditions.

C. freundii and *Serratia marcescens* were absent in healthy fishes but present in the diseased fish organs. In studied diseased fishes, the dominant bacteria were *K. pneumoniae, E. ludwigii, A. hydrophila.* In the soil samples, the dominant bacteria were *E. ludwigii.* In the water samples, the highest abundance of *S. aureus* was observed. The opportunistic pathogens commonly present in fish and in surrounding environment when fishes face stress condition then they infect fishes and cause diseases.

Different bacteria including *C. freundii, S. marcescens, K. pneumoniae, E. ludwigii, A. hydrophila* were well reported as pathogenic bacteria causing diseases with different signs and symptoms in fish (Junior et al., 2018; Dharmaratnam et al., 2017; Das et al., 2018; Preena et al., 2021; Mazumder et al., 2021). On the other hand, healthy fishes were free from any signs and symptoms but different opportunistic bacteria isolated from them indicated that the pathogens were ubiquitous in the aquatic environment depending on stress in fish to cause diseases (Wamala et al., 2018; Lio-Po and Lim, 2014). However, diseased fish and their tissue samples were used to isolate pathogenic bacteria in the present study.

Antibiotic susceptibility determination of important pathogenic bacteria is very essential for better health management of aquaculture species. In this study, maximum number of bacteria showed susceptible phenotypes to the nine antibiotics tests. However, the highest resistance was observed against Gentamycin (CN) and Chloramphenicol (C). High susceptibility was also reported against bacteria isolated from fish (González -Rey et al., 2004; Aravena-Román et al., 2012). Resistant patterns of the bacteria may be their intrinsic resistance and may be transfer to the progeny during cell division (Kümmerer, 2009). Amoxicillin, Ampicillin, Erythromycin, Tetracycline and Vancomycin were found

resistant to the bacteria under the genus *Pseudomonas* and *Enterobacter* which is the consistent of findings of antimicrobial resistant bacteria from farmed fish (Wamala et al., 2018; Newaj-Fyzul et al., 2008). Resistant bacteria can be naturally occurred in the aquatic environment (Cantas et al., 2013) and can transfer the antibiotic resistant genes to the fish bacteria. Resistant bacteria from humans and livestock could be transfer to fish bacteria (Wamala et al., 2018) as animal wastes are used to fertilize the ponds (Shah et al., 2012). Besides, unintentional use of antibiotics especially with feeds for fish growth enhancement or any other treatments might encourage the resistant bacteria in the ponds. In aquaculture, fishes are usually cultured at higher densities which increase the susceptibility to transfer resistant bacteria to fish bacteria.

3.6 Conclusion

Different bacteria were identified from healthy and diseased stinging catfish and associated pond soil and water from different locations of Bangladesh. Comparatively higher bacterial load was identified in healthy fish tissues than diseased fish. A total of 42 unique bacterial isolates were confirmed by biochemical and 16S rRNA gene sequencing. The *Staphylococcus aureus*, dominated both in the healthy and diseased fish organs and in associated water samples, whereas the *Enterobacter ludwigii* and *Bacillus amyloliquefaciens* dominated in the associated pond soil samples. Most of the bacteria showed sensitivity to Gentamycin (CN) and Chloramphenicol (C). On other hand, *Pseudomonas* sp. and *Enterobacter* sp. showed resistant traits to Amoxicillin, Ampicillin, Erythromycin, Tetracycline and Vancomycin. Information on the prevalence, antibiotic sensitivity and resistance patterns of bacteria in the studied aquaculture systems would be helpful for proper aquatic health management of important aquaculture species.

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Chapter 4
Skin Microbiome Structure and Functional Prediction in Healthy and Diseased *Heteropneustes fossilis*

(Based on this chapter an article entitled "Community structure and functional annotations of the skin microbiome in healthy and diseased catfish, *Heteropneustes fossilis*" has been published in the journal of "Frontiers in Microbiology" in 2022.)

Sultana, S., Khan, M.N., Hossain, M.S., Dai, J., Rahman, M.S., and Salimullah, M. (2022) Community Structure and Functional Annotations of the Skin Microbiome in Healthy and Diseased Catfish, *Heteropneustes fossilis*. Frontiers in Microbiology. 13:856014. doi: 10.3389/fmicb.2022.856014

4.1 Abstract

The skin mucosa of fish is the main barrier against pathogenic organisms. Interactions between host and microbiome and its functional range have significant implications in aquaculture. This study exposed mucosal microbiome composition from healthy and diseased stinging catfish (*Heteropneustes fossilis*) and compared the inhabiting community clusters based on 16S rRNA metagenomics. Four major phyla Bacteroidota, Proteobacteria, Firmicutes and Actinobacteriota were found from operation taxonomic units (OTUs) annotation. The members from the predominant phyla Proteobacteria were abundant in healthy group but Firmicutes and Bacteroidota phyla significantly differentiated the two groups. The microbiome diversity and richness increased in skin of healthy fishes but did not differentiate significantly from the diseased groups. At genus level, *Pseudomonas* exhibited the highest richness in healthy groups and nearly absent in diseased groups, whereas the *Flavobacterium* was highly abundant in diseased fishes. Linear discriminant analysis (LDA) recognized two phyla (Firmicutes, Bacteroidota) and two genera (*Flavobacterium*, *Allorhizobium*) that were consistently labeled for the diseased fishes. The functional predictions by PICRUSt analysis defined the genes related to the activities of important physiological functions like metabolism, digestive and immune systems, environmental adaptations were expected to have higher expressions in diseased groups. Present study specified bacterial compositions, abundance and their genes functions could affect the health status of farmed stinging catfish. Aquaculture-originated pathogenic organism may be identified, and preventive measures can be taken for the surveillance of fish health

4.2 Introduction

Microbiome helps fish in growth, nutrient absorption, metabolism of dietary toxins, increase drought and thermal tolerance, disease resistance (Alberdi et al., 2016; Chevalier et al., 2015; Eddy and Jones, 2002; Wu et al., 2013). Diversified microbial communities are harbored by fish skin, gill and gut (Salinas et al., 2011; Xu et al., 2012) as these parts of fish body interact directly with environment. Among the directly exposed organs, microbial community is more diverse in fish skin compared to the gut. Skin is enriched with aerobic bacteria including Proteobacteria and Firmicutes than anaerobic community (Lowrey et al., 2015; Merrifield and Rodiles, 2015). This microbial community structure depends on a plethora of factors like host environment interactions, dietary compositions,

water chemistry as well as host genetics (Pérez et al., 2010; Ghanbari et al., 2015; Merrifield and Ringo, 2014). Also skin's microbiome diversity depends on fish species and in case of catfish, geographical locations was found to be the best factor for best microbiome structure (Chiarello et al., 2019).

In healthy fish, strong and balanced relations exist between the intestinal and mucosal bacterial community. The symbiotic bacteria in host skin helps in fighting against different pathogens to prevent infection (Lowrey et al., 2015). However, imbalance among the microbial communities causes immeasurable bacterial diseases (Ma et al., 2019). The genera, *Aeromonas*, *Pseudomonas*, *Flavobacterium* and *Francisella* from gram-negative bacterial community while the genera *Streptococcus* and *Lactococcus* from gram-positive species were reported responsible for high mortalities. Important pathogens responsible for breaking the harmony and causing high fish mortality within short time of infection includes both gram-negative and gram-positive bacteria (Sebastião et al., 2015). Mucosal immunity increased significantly after infection with opportunistic pathogens or parasitic infestation (Zhang et al., 2018).

The stinging catfish (*H. fossilis*) is highly economically important and popular aquaculture species that has been reported to be infected with different types of pathogens like bacteria, parasites, fungus (Sahoo et al., 1998; Sahoo and Mukherjee, 1997). Bacterial infection, particularly in stinging catfish caused heavy mortalities resulting severe economic loss.

Hitherto, pathogenicity studies based on traditional method characterized bacterial pathogens in catfishes such as in *H. fossilis* and *Clarias batrachus* (Ahammed et al., 2016; Sarkar and Rashid, 2012). In spite of the fact, traditional method could not reveal whole compositions; as a result, many unidentified causative agents for infection remained unknown. Only 1% bacterial community can be identified from culture-dependent method (Amann et al., 1995) while unculturable taxonomic bacterial groups can be efficiently revealed by next generation sequencing (NGS) (Ringø et al., 2016). The next-generation sequencing methods (metagenomics) allows the profiling of known and unknown microbiome communities in, on and around aquatic animals (Flegel, 2019). Nonetheless, the advantage of culture dependent method is that bacterial strains (whether pathogen or with probiotics potential) can be preserved for further experiments; on the other hand, the specific isolates may not be preserved from culture independent method.

Bacterial groups were identified from healthy and diseased *H. fossilis* based on culture-dependent and culture independent (16S rRNA metagenomes). Bacterial richness, diversity was investigated and compared between two groups. Since fish are associated with core microbiome communities, disease and important physiological functions related genes expression were also analyzed. This is the first report unfolding a comparison of mucosal microbiome communities in healthy and diseased stinging catfish.

4.3 Materials and methods

4.3.1 Sample collection and preparation

Fish samples (healthy and diseased) were collected from three districts of Bangladesh from October 2019 to January 2020. Before sample collection fish farms were selected when disease outbreak with skin lesion was observed on some fishes. Twenty-seven (27) healthy (Figure 4.1A) and 27 diseased (Figure 4.1B) fishes were collected from Mymensingh (M), Narsingdi (N) and Dinajpur (D) sources. Each fish was packed in sterilized zipper bag and labelled properly and brought into laboratory in an ice box. The details information of sample collection is presented in Table 4.1.

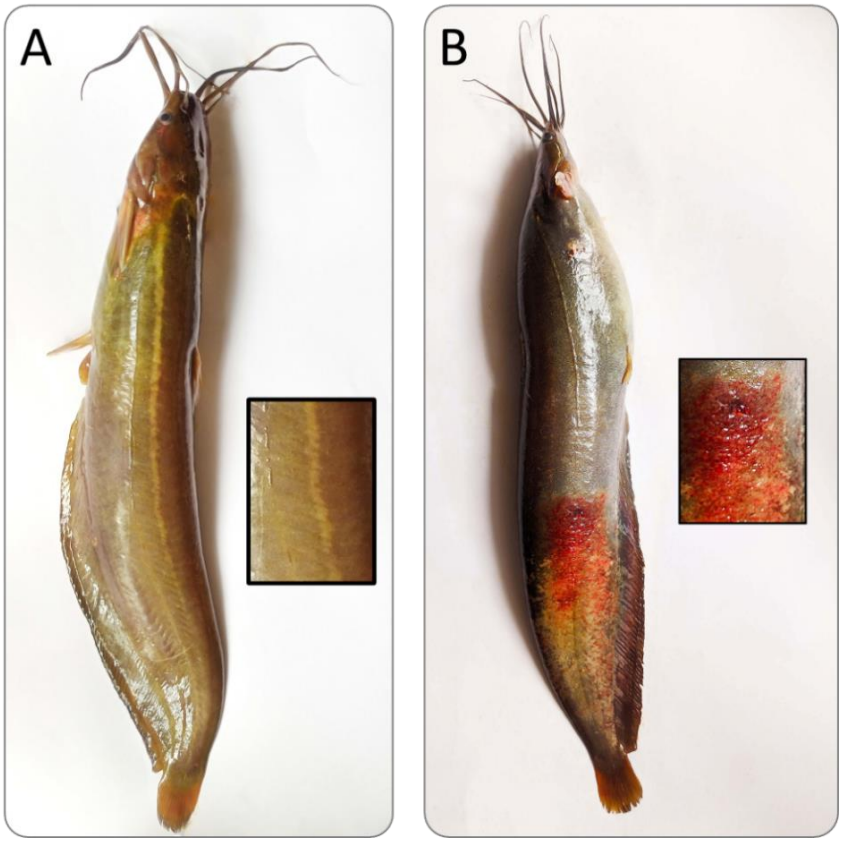

Figure 4.1: A-healthy fish and skin mucus; B-diseased fish and lesion in skin.

Table 4.1: Sample collection time and sources for metagenomic studies in *H. fossilis*

Sample collection date	Sources	Samples	Replications	Average length (cm)	Average weight (gm)
October, 2019 to January, 2020	Dinajpur (D)	Healthy	ND11, ND12, ND13, ND21, ND22, ND23, ND31, ND32, ND33	15.52	13.88
		Diseased	DD11, DD12, DD13, DD21, DD22, DD23, DD31, DD32, DD33	14.30	13.12
	Narshingdi (N)	Healthy	NN11, NN12, NN13, NN21, NN22, NN23, NN31, NN32, NN33	14.81	12.62
		Diseased	DN11, DN12, DN13, DN21, DN22, DN23, DN31, DN32, DN33	13.59	13.08
	Mymensingh (M)	Healthy	NM11, NM12, NM13, NM21, NM22, NM23, NM31, NM32, NM33	16.15	13.91
		Diseased	DM11, DM12, DM13, DM21, DM22, DM23, DM31, DM32, DM33	14.92	13.39

Different pathological signs and symptoms (lesions) were observed in different parts (tail, fin rot) of all diseased fishes collected from three locations. Similar clinical signs and symptoms were described in Indian catfish (Thomas et al., 2013), in stinging catfish (Rashid et al., 2008) and in Atlantic salmon (Karlsen, 2017); fishes without any clinical signs were defined as healthy fish.

4.3.2 Culture-dependent 16S rRNA gene sequencing

Skin mucus from healthy fish and lesions (wounds) from diseased fish were collected with the help of separate sterile scalpel in conical flask containing alkaline peptone water (APW). The sample then incubated in shaking incubator (ThermosTable IS-20R, Korea) at 37°C for enrichment (to increase a small number of desired organisms to detectable levels). After enrichment, samples were diluted with physiological saline (0.9% NaCl) serially.

Diluted samples were spreaded on nutrient agar (NA) (Oxoid, England). Each colony morphology (pigment, size, shape, elevation, opacity and edges) was observed carefully for merging and further sub-cultured in same media for glycerol stock and molecular identification.

For molecular identification, DNA was extracted from selected colony bacteria by the phenol chloroform method. Then the concentration and purity of isolated DNA were checked in Nanodrop spectrophotometer (Thermo Scientific, USA) at 260/280 absorption. For 16S rRNA gene amplification, universal primers (27F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R 5'-AAG GAG GTG ATC CAG CC-3') (Weisburg et al., 1991) were used. Total 25 µl reaction volume was used for thermal reaction consist of 12.5 µl of 2x premix (Maximo), 1 µl of forward primer/10 pmole, 1 µl of reverse primer/10 pmole, 8.5 µl of nuclease-free water and 2 µl (50 ng/µl) DNA. The Polymerase Chain Reaction (PCR) was initiated with initial denaturation of 1 cycle at 94 °C for 5 mins followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 1 min, extension at 72 °C for 2 mins and final extension at 72 °C for 10 mins. PCR products were confirmed in 1.5% agarose gel and photographed in gel documentation system. After confirming amplification all products were purified using purification kit (Thermo Scientific, USA). Purified PCR products were sequenced using a Genetic Analyser 3500. Sequences were visualized using BioEdit Software (version 7.0.9.0). Sequence comparison was performed in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank) with BLAST searching up to 97% similarity.

The confirmed sequences were submitted to NCBI GenBank under accession numbers MW857176-77,87,98, MW857245-59, 61-63, 71-73,77,79,81,89, 91, MW916538, and MW926915.

MEGA software version X (Kumar et al., 2018) was used for maximum likelihood (ML) phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000). The evolutionary history of the taxa was inferred using the neighbour-joining method (Saitou and Nei, 1987). The percentage of replicate trees was shown in which the associated taxa clustered with the sum of branch length (Felsenstein, 1985). The evenness of the tree was confirmed by bootstrapping (n =1000) with the MEGA program (Felsenstein, 1985).

4.3.3 Culture-independent 16S rRNA metagenomics

For the culture-independent method, DNA was extracted by following a previously described protocol (Han et al., 2018) with some modifications in cell disruption system. Around 30µl sample (skin mucus from healthy and lesion from diseased fish) was taken with the help of sterile swab stick (Figure 4.2A) in 2 ml Eppendorf tube containing 500 µl of distilled water.

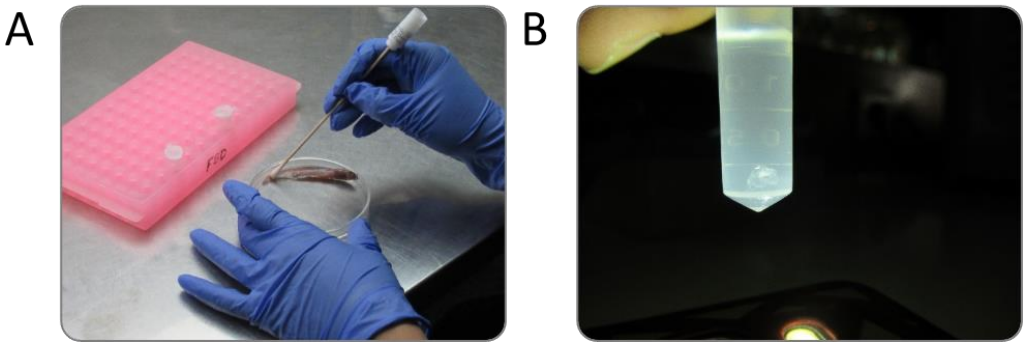

Figure 4.2: A-skin mucus collection from fish sample; B- cotton like DNA in 100% ethanol

The samples were then centrifuged at 2700×g for 5 minutes at 4°C and discard the liquid. Then resuspended in 1 ml of sterile phosphate buffered saline (PBS) vortexed for 2 minutes with 5 seconds of time interval. After that samples were centrifuged at 21500×g for 5 minutes at 4°C and eliminate the supernatant. Then 750µl of TE buffer and 50 µl of lysozyme were added and incubated at 60°C for 30 minutes. The samples then allowed to cool at room temperature and added 10 µl of RNAse-A and incubate at 30°C for 30 minutes. After this period, 30 µl of proteinase K was added and kept in water bath for 65°C for 60 minutes with invert shaking of the tube at 20 minutes of time interval for incubation. Then phenol chloroform method was used and DNA (Figure 4.2B) was suspended in 100 µl of TE buffer and stored at -20 °C after heating at 50 °C for 10 min. Three replications for each sample in the healthy groups (Narsingdi-NN, Mymensingh-NM, Dinajpur-ND) and the diseased groups (Narsingdi-DN, Mymensingh- DM, Dinajpur-DD) were used for the metagenomic analysis.

4.3.4 Metagenomics analysis

All DNA samples were sent for next-generation sequencing to Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) on an Illumina Nova sequencing platform. A specific primer with a barcode (515F-806R) was used to amplify the 16S rRNA gene (V4 region).

Fifty-four sequencing files were submitted to NCBI under accession numbers SRX10652409 to SRX10652462. The individual samples were assigned as paired-end sequencing based on unique barcodes and were truncated by cutting off the barcode and primer sequence. The obtained quality data were filtered according to Cutadapt (Martin, 2011). The UCHIME algorithm was applied to eliminate the chimera sequences (Edgar et al., 2011). Sequence analysis was performed using Uparse software, where ≥97% similarity was assigned to the same operational taxonomic unit (OTU) (Edgar, 2013). OTU-specific sequences were screened for further annotation by the Silva Database (Quast et al., 2012). The phylogenetic relationship of the dominant bacterial species in healthy and diseased fish groups was aligned using MUSCLE software (Edgar, 2004). The α and β diversity indices were estimated based on Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). Linear discriminant analysis (LDA) effect size (LEfSe) and biomarkers were identified to investigate different bacterial taxa to the genus level, as described by Segata et al. (2011). PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) software was used to align the sequences to infer the genes present in the samples and could be expressed (Langille et al., 2013). Mapping and functional annotations of the genes were performed at different KEGG levels (Kanehisa et al., 2016). Bacterial function differences between healthy and diseased fishes were calculated using Welch's t test.

4.4 Results

4.4.1 Metagenomics includes all culture-dependent bacterial isolates

By culture method total 143 isolates were isolated from healthy mucus and diseased lesion. Finally, all the isolates were combined into 32 isolates and confirmed at genus level by 16S rRNA sequencing and made a phylogenic tree (Figure 4.3A). Phyla Proteobacteria was exposed to 93.75% and 6.25% phyla was covered by Firmicutes. Total 26 and 14 OTUs were annotated by mucus and lesion up to genus level and both shared 8 OTUs (Figure 4.3B). Most common species were *Pseudomonas aeruginosa* and *Ralstonia pickettii.* A phylogenetic tree was constructed of the isolated 32 bacterial nucleotide sequences using the Neighbor-Joining method.

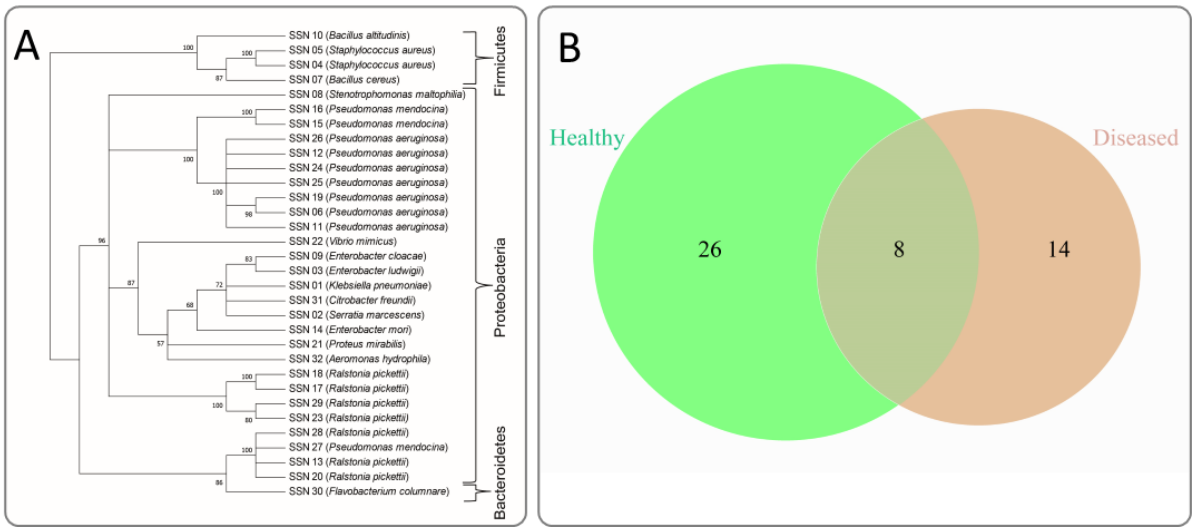

Figure 4.3: A-The Neighbor-Joining phylogenetic tree of the 32 bacterial isolates; B-number of bacterial genera identified by culture method from healthy and diseased *H. fossilis*.

Metagenomics revealed total microbiome and covered total phyla and genera from mucus and lesion. Microbiome composition, richness, top genera contributed mostly and predicted gene functions related to health indicator of fish were analyzed by metagenomic data.

4.4.2 Gene specific metagenomics exposes the richness of the skin microbiome

Illumina Nova sequencing platform was used for paired end sequencing where PCR free library was constructed. Conserved v3-v4 region of 16S rRNA gene generated 91,754 reads from 27 healthy and 27 diseased samples from three locations. After quality checking, the total sequence was grouped into 2,514 OTUs (Operating Taxonomic Units), which matched with the Silva132 database with 97% identity and 2,174 OTUs were annotated with a limit-level scoring percentage of 86.48 % by taxonomic analysis. A total of 1,066 (42.40%) OTUs scored at the genus level. The proportion at the level of phylum, class and order were 65.79%, 65.08%, 61.93%, respectively. The annotation ratio 54.85%, 42.40%, 12.05% was found for the family, genus and species level respectively (Table 4.2). The dominant genera were *Psychrobacter*, *Pseudomonas*, and *Flavobacterium* and *Psychrobacter*, *Pseudomonas* and *Flavobacterium.* The rarefaction curve reached more than 40,000 reads suggesting good sequence coverage (Figure 4.4A) while the species rank abundance curve (Figure 4.4B) also supported this good coverage by showing a higher relative abundance of the species.

Table 4.2: OTUs catalogue of 54 samples annotated to genus level for healthy and diseased *H. fossilis*

OTU catalogue	2,514
Annotated on database	2,174 (86.48%)
Annotated on Kingdom level	86.48%
Annotated on Phylum level	65.79%
Annotated on Class level	65.08%
Annotated on Order level	61.93%
Annotated on Family level	54.85%
Annotated on Genus level	42.40%
Annotated on Species level	12.05%

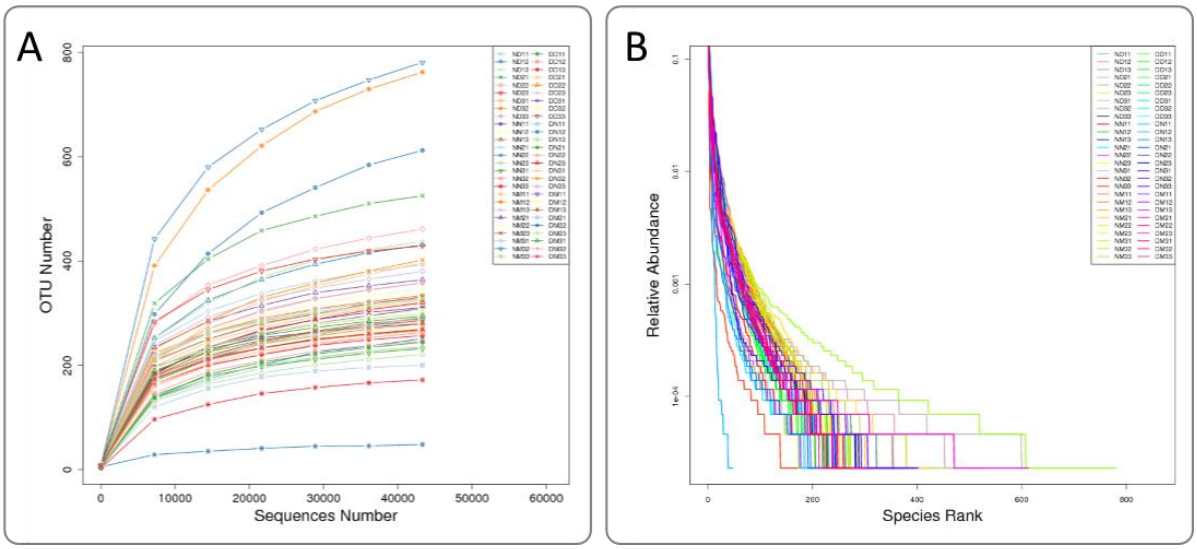

Figure 4.4: A- Rarefaction curve reached above 40000 reads (B) Species rank abundance curve showed the composition of species. The flatter the curve, the more evenness of species composition and the higher the curve, the richer species composition.

Total 1573 OTUs were generated from diseased fishes and 2108 OTUs were generated for healthy groups showed by Venn diagram (Figure 4.5A). Shared OTUs between healthy and diseased fishes were 1174; healthy group had more OTUs than infected groups. Higher number of exclusive OTUs were found in Dinajpur (ND) healthy fishes (Figure 4.5B).

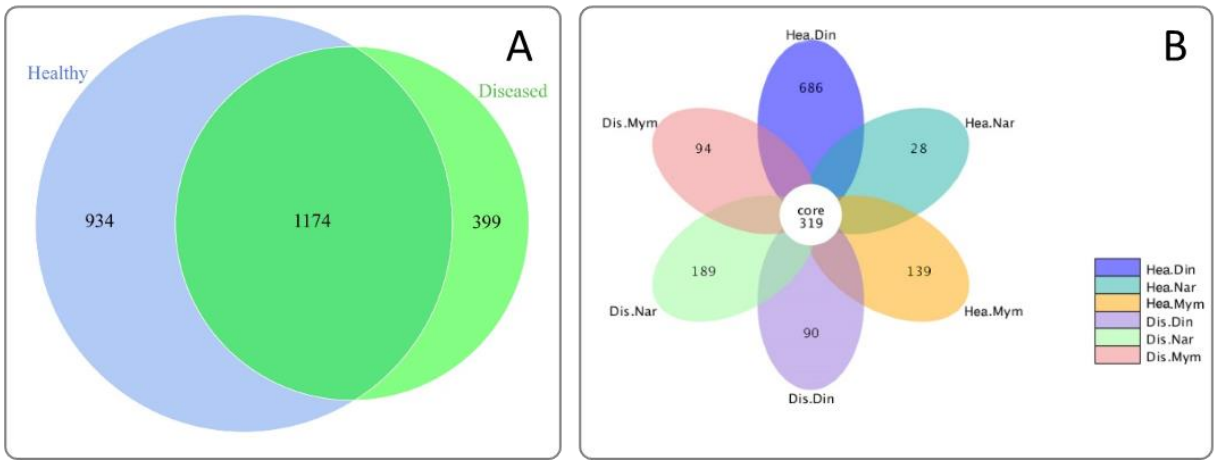

Figure 4.5: A-Healthy group formed 2108 OTUs OTUs and diseased group generated 1573 OTUs and both groups shared 1174 OTUs; B-In healthy group, Dinajpur sample had the highest OTUs (686); in diseased group, Narsingdi sample showed highest number of OTUs (189); (Dis: Disease; Hea: Healthy; Din: Dinajpur; Nar: Narsingdi; Mym: Mymensingh).

4.4.3 Healthy stinging catfish contains more diversified and richer microbiomes

Nine classifiable phyla were generated from all reads, viz., Proteobacteria, Firmicutes, Bacteroidota, Actinobacteriota, Cyanobacteria, Verrucomicrobiota, Planctomycetes, Fusobacteria, and Campilobacterota. Phyla found as dominant from healthy and diseased group were Proteobacteria (58.96% and 35.14%), Bacteroidota (29.89% and 17.82%), Firmicutes (4.32% and 2.57%), and Actinobacteria (6.83% and 4.07%). The relative abundance of the most dominant family was higher in healthy fishes (Figure 4.6A). Diverse bacterial classes were observed in Narshingdi (NN, DN) source than Mymensingh (NM, DM) and Dinajpur (ND, DD).

Additional analyses of alpha and beta diversity between healthy and infected groups were performed. The insignificant alpha diversity indices indicated that the healthy group had more diversity compared to the infected group. Species richness in healthy group was found higher through ACE (Figure 4.6B), Chao 1 (Figure 4.6C) analysis and non-significant differences were found in observed species. Shannon (Figure 4.6D) and Simpson's index (Figure 4.6E) estimators analysis also showed non-significant higher diversity in healthy group. A rarefaction curve based on OTUs was also constructed to estimate the microbiome diversity. The healthy groups were found to exhibit a higher level of microbiome richness than the infected groups.

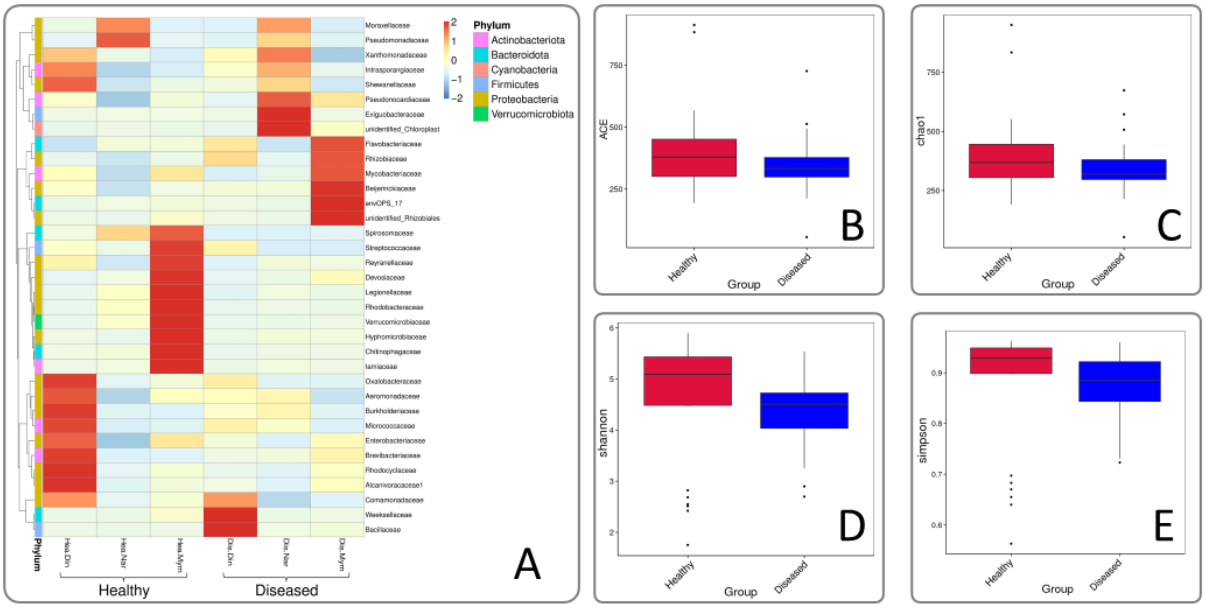

Figure 4.6: Relative abundance, richness and diversity of microbiome community in healthy and diseased *H. fossilis*. (A) Heatmap of OTU at family level with relative abundance. Columns represent groups of samples, rows indicate family OTUs. The color with key scale represents intensity of OTUs from lower (blue) to higher (red). Alpha diversity analysis parameters are shown by Boxplots (B-ACE, C-chao1, D- shannon, E-simpson). Non-significant higher microbial diversity was observed in healthy fishes.

The relative abundance of species diversity was also higher in the healthy group. In terms of location, the diversity and richness of healthy fish in Dinajpur (ND) was not significantly higher than that in Mymensingh (NM), and was significantly varied from Narsingdi (NN), while non-significant higher diversity and richness was found in diseased fish by alpha diversity parameters analysis. Significant differences were observed between healthy Narsingdi- disease Dinajpur (NN-DD), healthy Dinajpur- disease Dinajpur (ND-DD), and healthy Narsingdi- healthy Dinajpur (NN- ND) ($p<0.05$). In case of diseased samples, no significant differences were found ($p>0.05$).

Both weighted and unweighted UniFrac analysis were performed for beta diversity calculation. A three-dimensional scatter plot created using principal coordinates analysis (PCoA) showed the overlapping and dispersal of bacterial communities (Figure 4.7A), which was also consistent with the non-metric multidimensional scaling (NMDS) plot between healthy and infected groups. At the species level, PCoA presented that PC1 described 48.74% and PC2 explained 22.95% of the entire variance detected in the dataset.

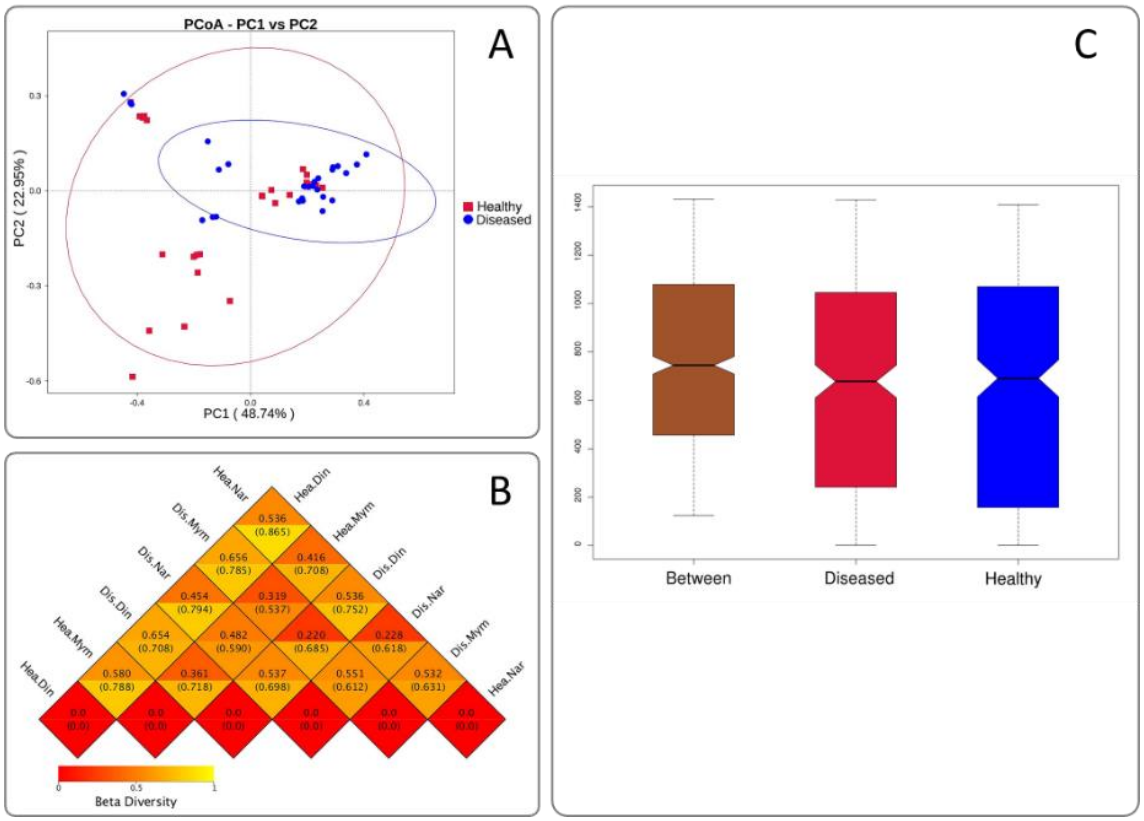

Figure 4.7: Beta diversity analysis of healthy and diseased group of *H. fossilis*. (A). Principal coordinates analysis (PCoA) of bacterial community based on Weighted UniFrac distance matrix. Each blue color dot represents infected fish and each red color dots indicate each healthy fish. (B). The heatmap of beta diversity index shows the distance values with lower distance (red) to higher (orange). The upper values represent the Weighted Unifrac distances, and the lower values represent the Unweighted Unifrac distances. (C). Intergroup and intragroup analysis of similarity (Anosim). R- value indicates dissimilarities between inter and intra groups and P value <0.003 shows that result was statistically significant.

Weighted Unifrac and Unweighted Unifrac distance Index values represent the coefficient of difference between healthy and diseased groups. In this study, the highest distance value (0.865) was observed in healthy fish of Narsingdi (NN) and Dinajpur (ND) samples (Figure 4.7B), followed by infected Narsingdi (DN) and healthy Dinajpur (ND) (0.794) and healthy Mymensingh and Dinajpur (NM and ND) (0.788).

One-way analysis of similarity (ANOSIM) between samples collected from different locations showed non-significant differences between healthy and diseased groups (R = 0.141, p = 0.003) (Figure 4.7C). R_{ANOSIM} values between 0.25 and 0.75 specify some degree of intersection, while values < 0.25 indicate that members of the two groups are nearly indistinguishable (Flynn et al., 2017). Pairwise differences within groups were found to be

significant except for healthy and diseased fish collected from Narsingdi (R = 0.1135, p = 0.109) (Table 4.3).

Table 4.3: Pairwise dissimilarities within the groups by one way analysis of similarities for 54 samples of *H. fossilis*

Group	R	P
Dis.Nar-Dis.Mym	0.7243	0.001
Hea.Din-Dis.Mym	0.9883	0.001
Hea.Din-Dis.Nar	0.608	0.002
Hea.Nar-Dis.Mym	0.7723	0.001
Hea.Nar-Dis.Nar	0.1135	0.109
Hea.Nar-Hea.Din	0.7809	0.001
Dis.Din-Dis.Mym	0.7889	0.001
Dis.Din-Dis.Nar	0.7171	0.001
Dis.Din-Hea.Din	0.9371	0.001
Dis.Din-Hea.Nar	0.7399	0.001
Hea.Nar-Dis.Mym	1.0	0.001
Hea.Mym-Dis.Nar	0.6663	0.001
Hea.Mym-Hea.Din	1.0	0.001
Hea.Mym-Hea.Nar	0.7414	0.001
Hea.Mym-Dis.Din	0.9705	0.001

4.4.4 Healthy and diseased stinging catfish maintain different microbiome profiles

The healthy groups generated more OTUs than the infected groups. At phylum-level analysis exhibited differences in abundance between two groups. Proteobacteria, Bacteroidetes, and Actinobacteria dominated the healthy group, while the diseased group was dominated by Proteobacteria, Bacteroidetes, and Firmicutes (Figure 4.8A).

Significant differences were observed in Bacteroidetes and Firmicutes between healthy and diseased groups. Both phyla were significantly enriched in both healthy and diseased groups ($p<0.005$). In the diseased fishes, the predominant Firmicutes contributed a significant microbiota in Narsingdi (DN) sources, which were also found in diseased fish in Dinajpur (DD) and a small number of microbiomes in Mymensingh (DM).

This phylum was rarely existed in healthy fish (NN, ND, NM). The healthy group showed significantly more abundance of Proteobacteria than the diseased group. This phylum was enriched in both healthy and diseased groups of Narsingdi (NN and DN) samples. Similarly, Actinobacteria predominated in healthy fish from Dinajpur (ND) and diseased fish from Narsingdi (DN). An unidentified phylum was rich in healthy fish from Mymensingh (NM), which was significantly varied from all other sources. Moraxellaceae

and Pseudomonaceae were highly abundant in the diseased fishes of Narsingdi (DN). The families Rhodobacteraceae, Reyranellaceae, and Spirosomaceae were highly abundant in healthy fishes.

This study suggested that common families and genera of bacteria are exist among fish samples in healthy and diseased fishes, but their abundances differed widely between two groups. This common phenomenon at the order and class level is also present in host species. Flavobacteriaceae, Moraxellaceae, Pseudomonaceae, Exiguobacteriaceae, and Weekellaceae families were abundantly observed in diseased fish from different sources. Flavobacteriaceae was slightly varied between diseased fishes of Dinajpur (DD) and Mymensingh (DM), but was hardly present in Narsingdi (DN). Moraxellaceae and Pseudomonaceae were highly riches in infected fishes of Narsingdi (DN). Rhodobacteraceae, Reyranellaceae, and Spirosomaceae families were highly abundant in the fishes of healthy group.

A network comparison and identification of putative genera were performed between the infected and healthy fishes at the genus level, showing that the healthy groups had a larger network diameter than the diseased groups (12 vs. 8) (Figure 4.8B and Figure 4.8C). The clustering coefficient (CC), average path length (APL), and average degree (AD) were higher in the healthy groups, indicating a more significant correlation between the genera of healthy groups. Members of the genera *Psychrobacter*, *Flavobacterium*, *Pseudomonas*, *Chryseobacterium*, *Exiguobacterium*, *Methylobacterium* and *Enterobacter* significantly contributed for diversity and distinguished healthy and diseased groups (Figure 4.8D). The genera *Flavobacterium*, *Chryseobacterium,* and *Methylobacterium* were significantly elevated in the diseased group ($p<0.005$) (Figure 4.8E).

In addition, different disease-causing genera such as *Citrobacter*, *Aeromonas*, *Ralstonia*, and *Rickettsia,* were exist in diseased fishes. In contrast, healthy fish from all three sources were enriched in communities of the genera *Psychrobacter*, *Enterobacter* and *Pseudomonas* (Figure 4.9A). *Psychrobacter* was highly abundant in Narsingdi (NN), absent in Dinajpur (ND) and rarely present in Mymensingh (NM). Similarly, *Pseudomonas* was enriched in Narsingdi (NN) and was rarely present in healthy fishes of Dinajpur (ND) and Mymensingh (NM). Table 4.2 shows the top 10 genera that mostly contributed to the healthy and diseased groups.

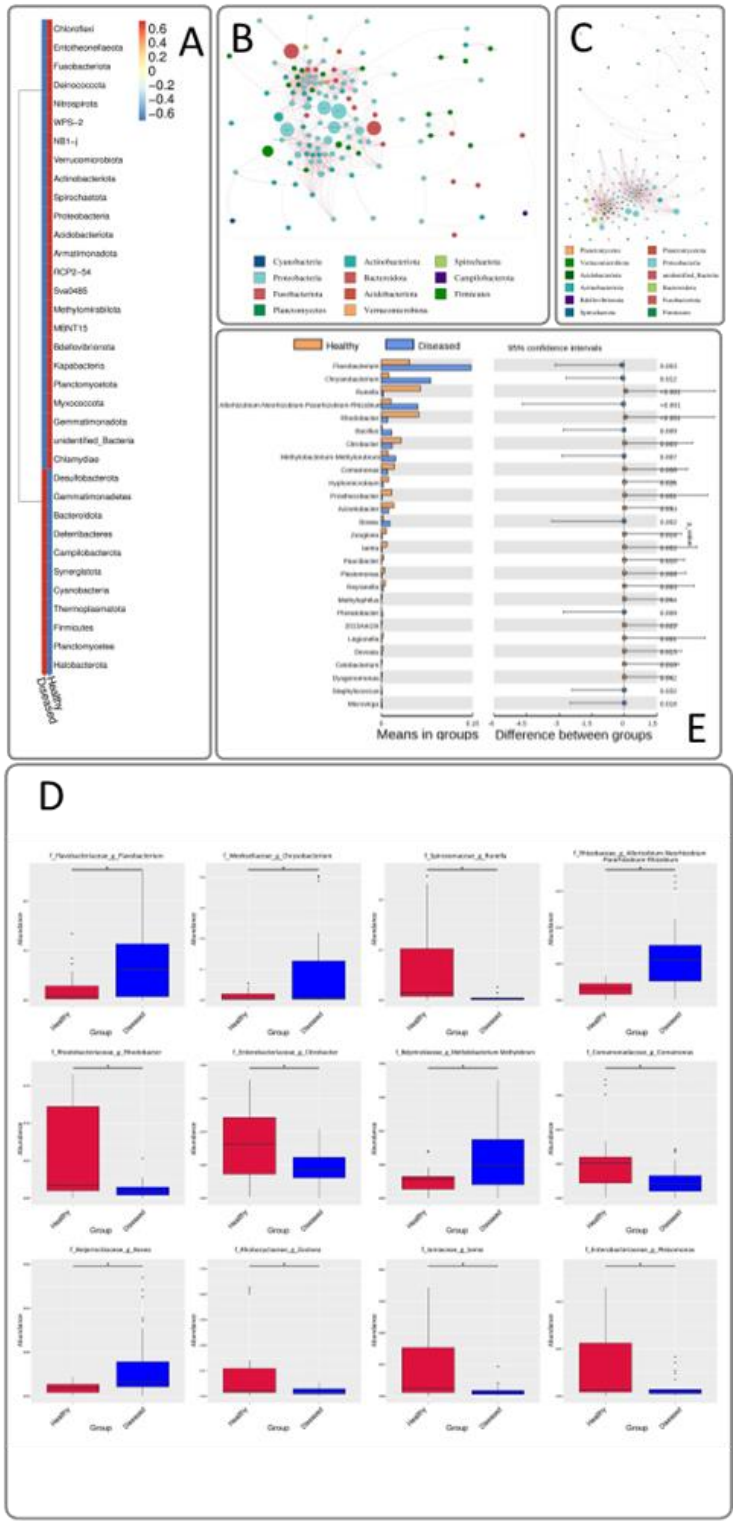

Figure 4.8: The difference between healthy and diseased of *H. fossilis* at phylum and genus level by network comparison and t-test. (A). The heatmap shows phyla abundance between groups. The key color scale showed the intensity range blue to red (lower to higher) of each phylum. Network comparison at genus level between diseased (B) and healthy (C). Each node represents each genus and node size indicates the abundance of the respective group. (D). The Boxplot showed the abundance and depletion of genera between groups. (E) The P values <0.05 define a statistically significant variation at phylum and genus level.

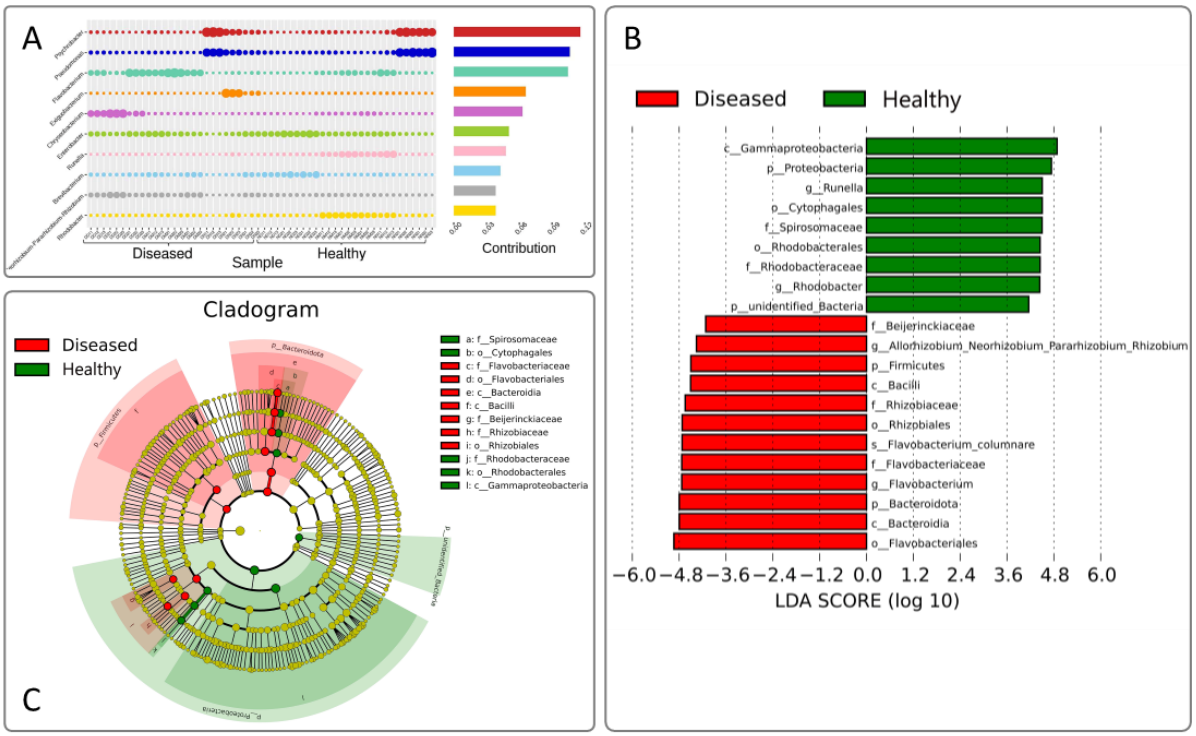

Figure 4.9: Most abundant bacterial communities that differentiate healthy and diseased *H. fossilis*. (A). The percentage similarity analysis (SIMPER) shows intensity of bacteria at the genus level. (B). Histogram based on Linear discriminant analysis (LDA) scores represents distinguishing bacterial communities. (C) Circular Cladogram showed taxonomic distribution of bacterial abundance between two groups.

Linear discriminant analysis (LDA) based on the LEfSe algorithm was calculated to classify the taxonomic variances between healthy and diseased fishes (Figure 4.9B). A logarithmic LDA (log 10) cutoff point of 4.8 indicated that healthy fish contained Proteobacteria, *Runella* and Rhodobacter, whereas higher abundances of Firmicutes, Flavobacterium and Flavobacterium *columnare* were found in the diseased groups (Figure 4.9C).

4.4.5 Pathogens from fish skin influence significant physiological functions

Functional variations were obvious due to variations in the microbiome composition between healthy and diseased fish. PICRUSt predicts the importance of these changes for disease causes and for potential benefit. KEGG Level 1 pathway showed that predicted functions associated to metabolic pathways and processing of environmental and genetic information did not differ significantly between groups (Figure 4.10A).

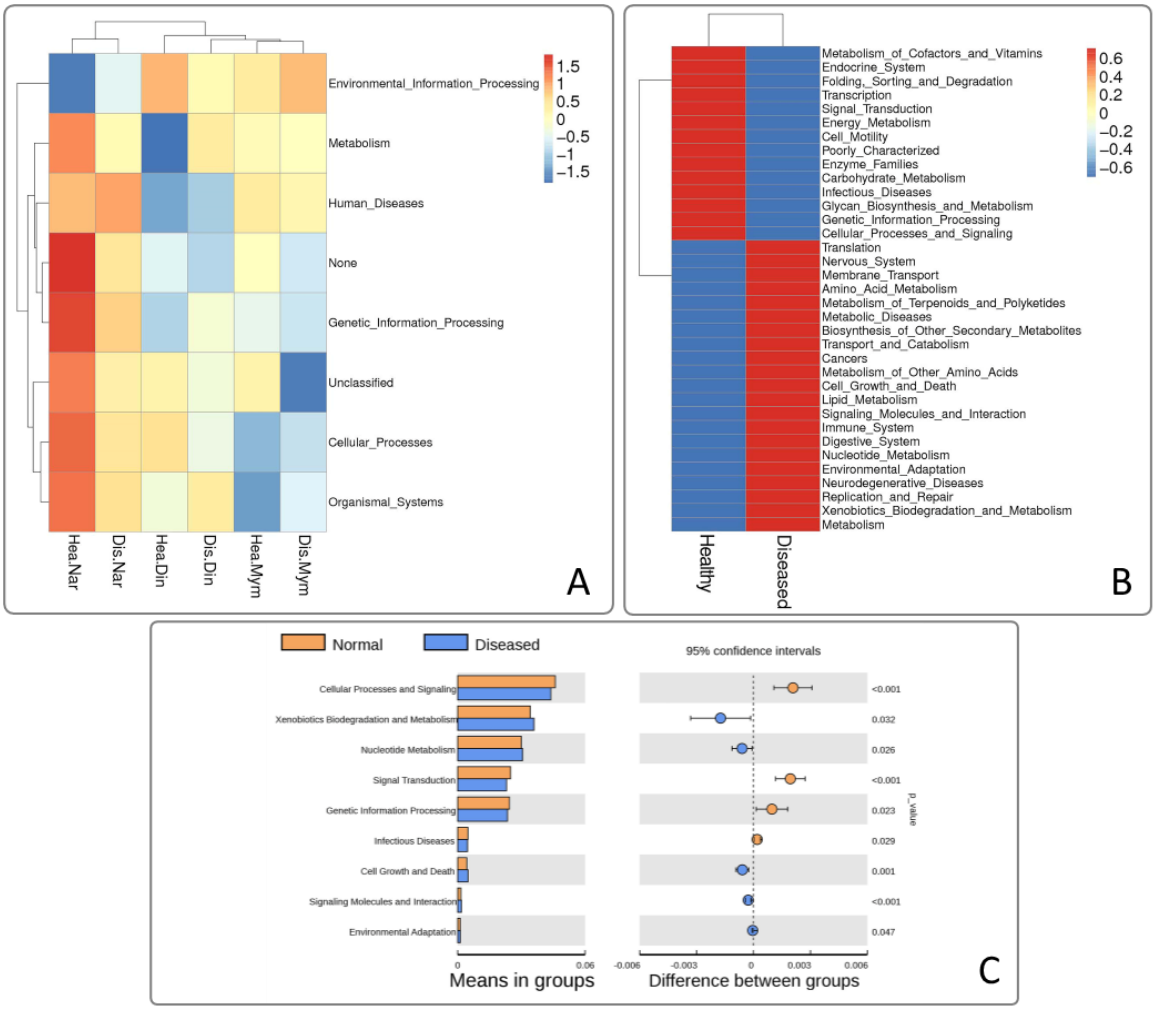

Figure 4.10: Functional annotations predicted by KEGG pathway analysis of the microbiomes in healthy and infected fishes. (A) Heatmap shows the changes in KEGG level 1 due to microbial functions. B) Comparisons of genes responsible for physiological functions between the groups analyzed by KEGG level 2. (C) The Welch's t-test of the functional predictions generated by the KEGG level 2. P values (<0.005) represents the statistically significant variation between the groups.

The relative richness of metabolic paths accounted for 47.88%, of which 23.90% of the genes represented the healthy metabolism of the fish, and 23.98% of the genes contributed to the metabolic activity of the diseased fish. The level 2 KEGG pathway showed that the predicted amino acid and carbohydrate membrane transport and metabolism genes were slightly higher in the diseased group, with an average of 13.96%, 10.23% and 9%.83%, respectively. Genes related to the digestive system, environmental adaptation, neurodegenerative diseases, and exogenous biodegradation expected to have more expression in diseased fish (Figure 4.10B). KEGG level 3 functional pathway analysis of genes predicted to be associated with different amino acids and enzymes could have higher expression in infected fish than in healthy fish. Pathways related to infectious diseases,

metabolic function, and genetic information processing were significantly higher in the healthy group ($p<0.05$) (Figure 4.10C).

4.5 Discussion

Any disease outbreak is closely related to the contact between fish pathogens and the environment (Noga, 2000). Single or mixed strains have been shown to be responsible for approximately 60% of transferrable diseases in fish (Tucker and Hargreaves, 2004; Mohammed and Peatman, 2018). In the preset study, a mixture of disease-causing bacteria was identified from lesions of diseased stinging catfish by a culture-dependent method, that was later confirmed by culture-independent 16S rRNA sequencing.

Catfish have high mortality rates due to bacterial diseases and they are prone to disease occurrences in winter due to suitable temperatures for disease outbreaks (Mohammed and Peatman, 2018). Total 2174 OTUs were annotated for genus from healthy and diseased fish of studied three sites. Healthy fish had more OTUs compared to disease among three locations. Generally, more diverse microbiome communities are found in healthy fish for different body functions, while diseased fish exhibited fewer and irregular physiological functions. No significant difference was reported in the number of OTUs between healthy and diseased Yunlong groupers (Ma et al., 2019).

The abundance of bacterial gammaproteobacteria was described from European catfish skin (Chiarello et al., 2019). Dominant four phyla Proteobacteria, Bacteroids, Actinobacteriota and Firmicutes were observed from mucus and lesion of healthy and diseased samples which is consistent with the reports from other fish and catfish skins (Wu et al., 2010; Lowery et al., 2015; Ma et al., 2019; Zhang et al., 2017). The major families of the dominant phyla, such as Enterobacteriaceae, Clostridiaceae and Bacillaceae were reported their pathogenesis and transmission through water to humans (Mukherjee et al., 2016). The observed phyla from studied healthy and diseased fish harbored both infectious and beneficial microbiome. The higher abundance of Firmicutes in diseased group suggested that bacteria of this phylum can cause lethal infections in *H. fossil* due to interactions between infective bacteria. The dominant phenomenon of phylum Firmicutes also reported by others in many fish species (Kim et al., 2017; Lowery et al., 2015).

Previous studies have reported harmonized microbiome communities in fish skin which covers opportunistic and helpful (probiotics) pathogens and their associated environmental

(Horsely, 1977; Georgala et al., 1958; Ahmed et al., 2018). Generally beneficial microbiome protests virulent pathogens prevalence by antimicrobial compounds secreted through a process antibiosis (Boutin et al., 2012; Lowrey et al., 2015; de Bruijn et al., 2018). Deviation from the balanced microbiome can be the causative agent of disease outbreak specially when fishes fight with different stressor under adverse condition (Sagvik et al., 2008; Turner et al., 2013; Stecher, Maier and Hardt 2013; Montalban-Arques et al., 2015; Romero, Ringø and Merrifield, 2014; Moya and Ferrer, 2016; de Bruijn et al., 2018).

Long-term exposure to stress can suppress the immune system and hasten potentially pathogenic microorganisms, thereby altering microbiome communities (Hu et al., 2021). In the current study, Flavobacterium, Exiguobacterium, Aureobacter, Rhizobium, Bacillus, and Rhodococcus genera exhibited dominant patterns in the diseased samples, while in the healthy group, *Runella*, *Rhodobacter*, *Pseudomonas*, *Citrobacter*, *Comamonas*, *Hyphomicrobium* and *Prosthecobacter* showed increased phenomena. Nonpathogenic Pseudomonas species secrets vitamin B12 (Fang et al., 2017), which helps to maintain growth and other physiological functions of host (Arai et al., 1972; Ma et al., 2019; Ikeda et al., 1988).

Increased mortality in diseased *H. fossilis* may be related to *Pseudomonas* genera depletion. Because *Pseudomonas* provides a natural environment free of toxic pollutants and produces compounds that are lethal to other species (Novik et al., 2015). Therefore, these useful bacteria identified from the mucus of healthy fish can be used as powerful probiotics. Beneficial microorganisms defend against pathogens by preventing the secretion of virulence factors or killing directly (van Hai and Fotedar, 2010; Kesarcodi-Watson et al., 2008) through suppression of pathogens quorum sensing (QS) capabilities (Flegel, 2019). The most dominant genera *Flavobacterium* in the infected group indicated that members of this genus may cause lesions in skin. This is not unexpected, as *Flavobacterium* has been reported to be a major fish pathogen (Chiarello et al., 2019), causing disease in wild and farmed fish and causing considerable losses (Wahli and Madsen et al., 2018).

Aeromonas, *Edwardsiella* and *Cylindromonas* are the main pathogens of catfish (Zhou et al., 2018). In our experiments, Exiguobacterium and *Chryseobacterium* detected in diseased fish, have also been reported as pathogens in fish, reptiles, birds, and mammals, including humans (Bernardet and Nakagawa., 2006; Loch and Faisal, 2015). Other reported pathogenic bacteria, such as *Citrobacter, Aeromonas*, *Morganella*, *Enterobacter*,

Rickettsia, *Roche*, *Pseudomonas*, and *Acinetobacter* were observed in healthy and diseased fish, suggested that they may be beneficial and opportunistic depending on physical and environmental factors. In diseased fish, bacteria can induce the proliferation of opportunistic bacteria and inhibit the growth of beneficial microbiota. To overwhelmed disease situations, probiotic bacteria can promote host immunity by secreting active antibodies and antibacterial agents from peptides derived from pathogenic microorganisms (Ángeles Esteban, 2012; Kelly and Salinas, 2017; Zhang et al., 2018).

Microbiome groups in fish are affected by multiple factors like geographical sites, seasons (Sullam et al., 2012; Zarkasi et al., 2014; Ye et al., 2014; Ray, 2016; Tarnecki et al., 2017). Non-significant differences observed in healthy and diseased groups by location, which is also common (Smith et al., 2015). The bacterial community members of Proteobacteria were uniform at all sites, but other dominant phyla, Actinobacteria, Bacteroidetes and Firmicutes showed different patterns of increases and decreases at different sites. However, at genus level different patterns were also identified in healthy and diseased fish samples. The genera *Pseudomonas, Chryseobacterium, Psychrobacter, Flavobacterium, Brevibacterium, Ralstonia* and *Enterobacter* were significantly varied in each site and in each part of the diseased and healthy fishes.

The LDA score based on LEfSe algorithm was analyzed to differentiate healthy and diseased groups and this bacterial community cluster analysis were also reported in healthy and diseased shrimp and Yunlong Grouper (Hossain et al., 2021; Ma et al., 2019). This analysis detected 1 phylum, 3 classes, 6 orders, 4 families and 4 genera; in diseased group, *Flavobacterium columnare* and Firmicutes showed the highest scores while in healthy fishes *Runella* and *Rhodobacter* had the highest LDA scores which is consistent with the findings of Zhang et al. (2018). In addition, principal coordinate analysis also non-significantly differentiated the microbial community from the diseased and healthy fishes.

Functional prediction through PICRUST analysis revealed many functional genes richness that are associated to metabolism, resistant systems, environmental adaptation, neurodegenerative diseases and environmental information processing. There are many reports on carbohydrate metabolism of the host contributed by gut microbiota in different species (Hooper et al., 2002; Turnbaugh et al., 2006; Brulc et al., 2009; Hess et al., 2011; Zhu et al., 2011). Consistent with other studies, genes associated with increased carbohydrate and amino acid metabolism in infected fish microbiota were found to be high

(Ni et al., 2014; Ma et al., 2019). The physiological procedure of carbohydrate absorption also increases with the onset of the disease. Differences were observed between healthy and diseased groups for carbohydrate metabolism in the present study but not significant as reported by Ma et al (2019) in intestinal microbiome functions in diseased and healthy Yunlong Grouper fishes. Functions of immune systems, environmental adaptations related genes were high in infected *H. fossilis* fishes indicating that the fishes need more energy to adapt with adverse environment (Zhang et al., 2018).

4.6 Conclusion

In the current study, the mucosal microbiome of healthy *H. fossilis* was more abundant and diverse than that of diseased *H. fossilis*. The useful microbiome protects host from infective microorganisms and plays vital role in different body functions. Under adverse environmental conditions, pathogenic microbes predominate on the skin and affect the whole microbiome structure of infected fish. These pathogens affect the function of genes related to fish metabolism, immune response and environmental adaptation, and inhibit fish growth and production. This phenomenon may be important for understanding the synergistic and antagonistic interactions between resident microorganisms and pathogens in aquaculture species. Overall, this study provides reference information for early detection of *H. fossilis* pathogens and facilitates preventive measures to prevent severe mortality in this species due to the presence of pathogenic microorganisms.

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Chapter 5
Isolation and Molecular Characterization of Pathogenic *Aeromonas hydrophila* from Diseased Stinging Catfish

5.1 Abstract

Gram-negative *Aeromonas hydrophila* is an important pathogen causing Motile Aeromonas Septicemia (MAS) in aquaculture species, especially in catfish. This major disease causes significant mortality within a very short time in catfish industry worldwide. This study was conducted to isolate and characterize *A. hydrophila* from lesion of diseased *Heteropneustes fossilis* using selective media. Three morphologically different isolates of *A. hydrophila* were identified and confirmed by 16S rRNA gene sequencing. One to five (1-5) pathogenic genes were identified from these isolates. Among them, two isolates AGM2 and AYN7 contained maximum five virulent genes. Then these two virulent isolates were used for antibiogram and experimental infection in healthy *H. fossilis*. Isolates AGM2 and AYN7 showed resistance to 9 and 6 antibiotics out of 11 antibiotics, respectively. Ceftriaxone, Cefepime, Gentamycin, Azithromycin and Ciprofloxacin inhibited the growth of these two isolates in Mueller-Hinton (MH) agar media. Challenge test with AGM2 showed highest pathogenicity at 4^{th} to 6^{th} day of post-injection in three phases. Stinging catfish was sensitive to AGM2 and 100% mortality was occurred within 10 days post injection. In case of AYN7, lethal dose (LD_{50}) was calculated as 6.77×10^6 cfu/ml by logit and probit analysis. Clinical signs and symptoms (lesions) and excess mucus (typical signs of MAS) were observed in fish used in the challenge test by both isolates. The findings of this experiment will help to identify rapidly proliferated pathogenic *A. hydrophila* and to take management measures for controlling in catfish farm.

5.2 Introduction

Disease outbreaks caused by bacteria, fungi, viruses, and parasites have become a greater hazard to catfish aquaculture in recent years due to environmental changes (Zhai et al., 2021). Among the bacterial diseases, Motile Aeromonas Septicemia (MAS), columnaris and enteric septicemia of catfish (ESC) are frequently occurred in catfish farms and their control measures mainly depends on the use of antibiotics (Zhai et al., 2021).

Aeromonas hydrophila is commonly considered as causative agent of hemorrhagic Motile Aeromonas Septicemia (MAS) for aquatic animals all over the world (Wang et al., 2017; Marinho-Neto et al., 2019). MAS disease comprises latent, chronic and acute forms (Korni et al., 2017), which depends on the pathogens, environmental factors and fish immunity. *Aeromonas* spp. are the rod shaped, gram-negative, motile, opportunistic pathogen cause infection when the host is under stress condition or injured. The motile aeromonads may

be transmitted by horizontal way and disrupt mucosal defense system not only in catfish but also in other fish species (Samayanpaulraj et al., 2020). This disease cause severe economic loss in fish farm. About 80% mortality may be occurred by this disease especially when the fish are under stressed condition (Riauwaty et al., 2020). The average loss of Tk. 20,615/ha/year (US$344) equivalent to 15% of total production due to fish diseases occurred in Bangladesh (Faruk et al., 2004). The common virulent *Aeromonas* spp. causes severe mortality in catfish farm every year in Bangladesh and worldwide with the influencing stressor (Abdelhamed et al., 2017). The pathogenicity depends on some extracellular proteins and several enterotoxins like aerolysins (aer), serine protease (ser), elastase (ahyB), DNases (exu), lipase (lip), polar flagella (fla) (Chen et al., 2019). After infection, fish showed many symptoms like skin lesion, tail and fin rots, pale gills, abnormal swimming etc. The internal organs kidney, spleen, pancreas, liver can be affected by this pathogen. The chronic signs of septicemia are ulceration, skin lesion and inflammation with crucial hemorrhages (Korni et al., 2017). Histopathological lesions include cell degradation, cell inflammation, necrosis due to this pathogenic infection. The presence of virulence factors must be identified in order to treat or manage the pathogenicity of the organism.

Disease control of MAS infection by using antibiotic is an alarming issue with increasing antibiotic resistant bacteria in the fish body and environment, which is ultimately becoming threat to human. The misuse of these antibiotics has resulted in long-lasting leftovers in aquatic habitats and soil sediments, this leads to antibiotic resistance. Multiple strains of *A. hydrophila* already reported as antibiotic resistant strains from many countries (Le et al., 2018). The capability of bacteria to withstand a variety of these antimicrobials enhances additional virulence factors, resulting in the appearance of multiple forms of antibiotic resistance (MAR) in *Aeromonas* spp., which may be transmitted to humans and animals through the consumption of contaminated fish products.

Bacterial co- infection of fish in nature is common, which occurred by different pathogens. Fish harbor diversified microorganisms including pathogenic and non-pathogenic and co-infection is frequently happened. During co-infection in nature, one pathogen may be increased and suppress other or vice versa. Alternatively, the load of both organisms may be increased during co-infection. But in co-infection, clinical signs and symptoms can not be identified to differentiate pathogen causing the infection (Wise et al., 2021). To identify a single causative agent responsible for disease outbreak by challenge test with a single

pathogen in laboratory is effective way to evaluate the pathogenicity of the field isolate (Zhang et al., 2016). Because sometimes one pathogen can alter the total immune system of fish moderated by another pathogenic organism. Therefore, the objective of this experiment was specific identification and characterization of the pathogenic strains of *A. hydrophila* by experimental infection and antibiotic sensitivity test.

5.3 Materials and Methods

5.3.1 Sample collection

Diseased fish with lesions (Figure 5.1) from the **experiment I (chapter 3)** were used in this study for the isolation of selective *Aeromonas* sp. bacteria. Diseased fish were collected from fifteen locations as described in the **chapter 3**. Fish samples were collected when the temperature started to fall in winter season. Different shing farms reported that the outbreak of disease mostly occurred during the winter season (Bagum et al., 2013).

Figure 5.1: Diseased fish with lesions in different parts of the body collected from different locations.

5.3.2 Isolation of selective bacteria

First, lesion part of diseased fish (Figure 5.2) was scrapped with sterile scalpel and enriched in alkaline peptone water at 37°C for overnight in the shaking incubator. After enrichment, the sample was diluted to tenfold (1:10) with physiological saline (0.9% NaCl) and serially diluted up to 10^{-6} times. Rimler shotts media (RS) (Himedia, India) was used to isolate the selective *Aeromonas* sp. RS medium is formulated for isolation and presumptive identification of selective *Aeromonas* sp. However, other gram-negative bacteria that may decarboxylate components like lysine and ornithine, ferment maltose, and create H_2S are able to grow in RS media (MacFaddin, 1985) like *Vibrio* sp. and *Citrobacter freundii*. Novobiocin supplement (antibiotic supplement) was used to suppress the growth of other

gram-negative bacteria. RS media with novobiocin supplement was used to isolate *Aeromonas* sp. for presumptive identification. One hundred µl of diluted sample was spreading on RS media and incubated for 24 hours at 37°C.

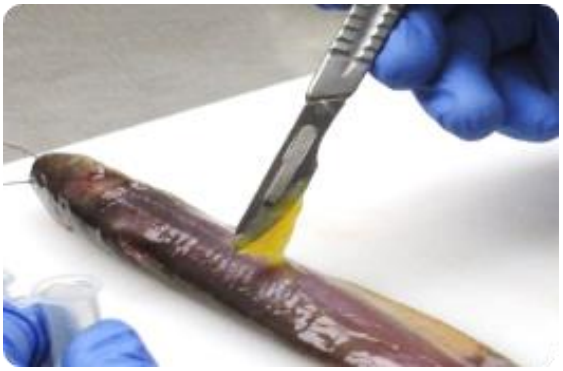

Figure 5.2: Mucus collection from lesion of diseased fish by sterilized scalpel.

5.3.3 Morphological characterization

The morphologies (color, shape, size, opacity, edge) of the colonies were observed in RS media under colony counter machine and recorded based on eye observation. Pure culture of each single colony was further sub-cultured on nutrient agar (NA) media for glycerol stock in cryovials. Two sets of each cryovials were carefully wrapped with parafilm and stored at -80°C (Utra-Low Temperature Freezer, Eppendorf, U725-86, Innova United Kingdom) and -40°C (Deep freezer, SANYO MDF-U5411, Japan) for further study.

5.3.4 Molecular confirmation of selected bacteria

Genomic DNA was extracted from each colony which showed similar morphology (color, shape) in RS media. Firstly, a single colony was cultured in Luria-Bertani agar media for pure colony culture. Then, a colony was picked with the help of inoculation loop in an eppendorf tube containing 450 µl lysis buffer, then cell disruption was done by heating 60 °C for 1 hour after adding 20 µl of proteinase-K (20 mg/ml) in water bath. During this time, hand shaking was done at 20 minutes interval. After that the sample was cooled at room temperature, and 5 µl RNAse A was added and kept at 37°C for 30 minutes. Then, an equal volume of (475 µl) phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed by hand shaking. The samples were then centrifuged at 10000 rpm for 15 minutes. About 300 µl of upper layer from the solution was transferred to new eppendorf tube with the same volume of chloroform: isoamyl alcohol (24:1) was added to the samples and centrifuged 10000 rpm for 10 minutes. Then, 150 µl upper layer was taken into a new eppendorf and 850 µl ice cooled ethanol was added to precipitate the DNA by hand shaking

and kept at -20°C for 40 minutes. The samples were centrifuged at 13000 rpm for 10 minutes to make a pellet of the DNA. The aqueous layer was discarded and 1000 µl of chilled 70% ethanol with 20 µl of 7.5M Ammonium acetate was added and centrifuged at 13000 rpm for 10 minutes. The liquid was eliminated and allowed to air dry. The DNA was dissolved with 50 µl of elution buffer (pH 7.6) by heating at 37 °C for 10 minutes.

DNA quality was checked by running in 1.5% agarose gel. Good quality DNA was amplified by 16S rRNA primer (27F: AGA GTT TGA TCC TGG CTC AG; 1492R: GGT TAC CTT GTT ACG ACT T) (Turner et al.1999; Lane, 1991). Total 25 µl reaction volume was amplified by 1 cycle of initial denaturation at 95°C for 5 minutes, which was followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 1 minute, extension at 72°C for 2 minutes and 1 cycle of final extension at 72°C for 10 minutes. The PCR products were visualized in 1.5% agarose gel by comparing with 100 bp DNA ladder.

After observing desired band pattern on agarose gel electrophoresis, the PCR products were purified with DNA purification kit (Thermo Scientific, USA). Then, the purified products were sequenced in Genetic Analyzer 3500 with forward and reverse primers. Then, the contig sequences were compared with BLAST searching in GenBank database (https://www.ncbi.nlm.nih.gov/genbank). Highest (99% to 100%) identity and query cover values were compared with the submitted sequence to confirm the isolates at genus level.

5.3.5 Amplification of virulent genes

Isolated DNA was amplified by different pathogenic genes of *A. hydrophila*. The primer list used for pathogenicity test is presented in Table 5.1.

Table 5.1: Selected pathogenic genes of *A. hydrophila* used to amplify studied isolates

Sl No	Gene	Size (bp)	Accession number
1	*ahp*A	911	KC812110.1
2	*ahp*B	224	AF193422.1
3	*lip*	383	AB237183.1
4	*fla*	593	AY424358.1
5	*aha*	1082	EF189590.1
6	*amp*	756	KP942407.1
7	*vas*H	509	DQ667172.1
8	*AHL*	483	AF485772.1

A total of 25 µl reaction mixture was prepared consisted of 12.5 µl master mix, 2 µl DNA (50 ng/µl), 1 µl of each forward and reverse primer, 8.5 µl nuclease free water. The reaction volume was amplified by gradient PCR with 1 cycle denaturation at 95°C for 5 minutes, initiation at 95°C for 30 seconds, annealing at 50°C to 60°C for 1 minute, elongation at 72°C for 2 minutes for 30 cycles and final elongation at 72°C for 10 minutes. The amplified products were checked on 1.5% agarose gel and confirmed the sizes by comparing with 50 bp (Biolabs Quickload and Promega).

5.3.6 Antibiotic sensitivity test of isolated *A. hydrophila*

Isolated *Aeromonas* sp. was used for sensitivity test with 11 available antibiotics following Kirby-Bauer disk diffusion method (Hudzicki, 2009) using Mueller-Hinton (MH) agar (Oxoid, UK). The antibiotics with their concentration per disk were Chloramphenicol (10 µg), Erythromycin (15µg), Gentamycin (10 µg), Cefipime (30 µg), Ampicillin (2 µg), Amoxicillin (30 µg), Cefixime (5 µg), Ceftriaxone (30 µg), Azithromycin (15 µg), Ciprofloxacin (5 µg), Tetracycline (30 µg). Pure culture of the *Aeromonas* sp. was inoculated in Tryptic soy broth (TSB) for overnight at 37˚C and checked turbidity for maximum growth. Sterile swab stick was used to streak the culture into Mueller-Hinton (MH) agar plate from broth culture. Each agar plate was kept at room temperature for 10 minutes to cool. Five (5) antibiotic disks were placed in each plate and kept at 37˚C for 24 hours for incubation. Inhibition zones were measured and differentiate into sensitive, moderate sensitive and resistant by comparing with standard value of respective antibiotics prescribed by Wikler (2006).

5.3.7 Experimental infection with *A. hydrophila*

One of the most useful techniques for evaluating an isolate's pathogenicity and aiding in the assessment of the economic damage to aquaculture is laboratory-based experimental infection. Effective prevention method can also be developed from challenge test. Induction of experimental infection was done by intraperitoneal injection for three phases to avoid error of experimental result.

5.3.8 Phase-I challenge test

The first phase was carried out in March, 2019 when the temperature was fluctuated around 21°C to 32°C. For first phase challenge test, fingerlings (Figure 5.3A) were collected from Catfish Agro Hatchery and Fisheries, Shambhuganj, Mymensingh. No pathological signs and symptoms were observed in the collected fish (Figure 5.3B).

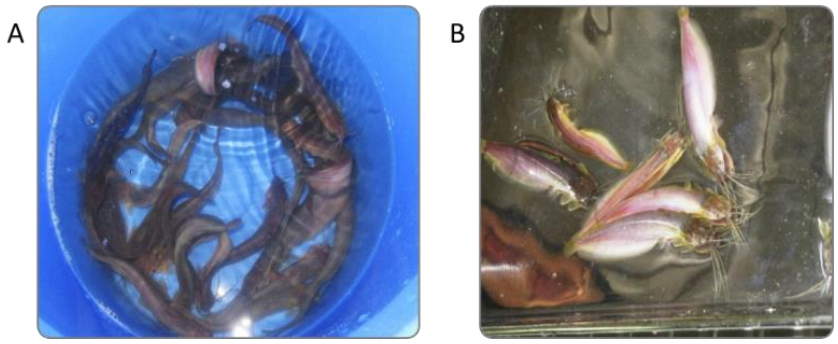

Figure 5.3: A- Collected *H. fossilis*; B- Morphological and clinical observations.

Fish weight and length were measured by weight machine (GIBBERTINI Italy, ser no. 153147). A blank small bowl was weighted and recorded the data. Then, the machine was adjusted to zero reading for previous bowl weight. After that, fish was kept on bowl and record the weight value and deducted the blank bowl weight to get fish weight. Fish length was measured by placing the fish in a laboratory tray, which was attached with a measuring scale.

Fish were divided into three treatments and one control (Figure 5.4A); 10 fish in each treatment and 10 fish were used as control. Twenty (20) liter water was placed in each tank from the overhead reservoir tank.

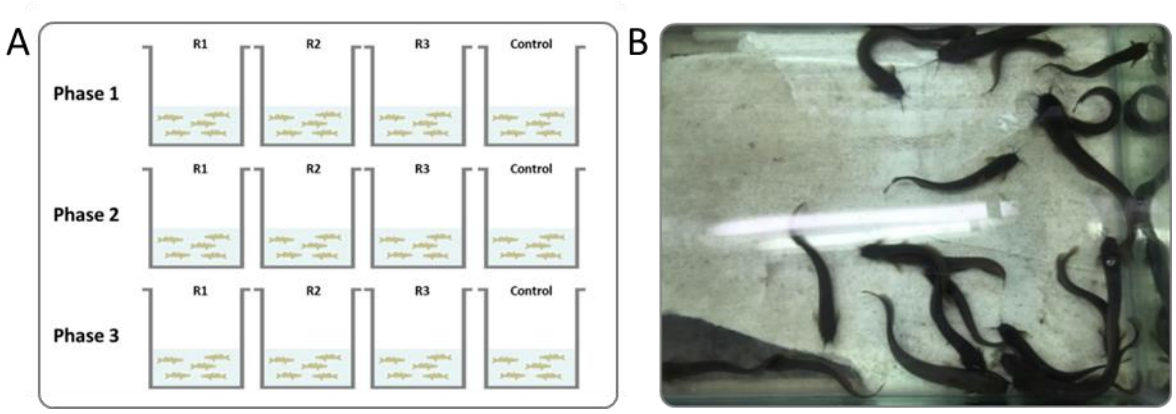

Figure 5.4: A-Schematic diagram on experimental infection of stinging catfish in three phases. B- Acclimatization of collected *H. fossilis* in aquarium for challenge test.

5.3.8.1 Acclimatization in laboratory aquarium

The fish were observed carefully to avoid any pathological sign and symptom (any lesion in body parts). Collected fish were optimized in laboratory aquarium (Figure 5.4B) for 7 days with measuring temperature, dissolved oxygen, pH of water daily. Catfish grower feed (Mega) was given twice in a day. Hundred percent (100%) water was changed every day.

5.3.8.2 Suspension preparation of *A. hydrophila*

The bacterial isolate (AGM2) isolated from a diseased fish (confirmed by 16S rRNA gene sequencing) having wounds in different parts of body was used for challenge test in three phases. Another strain (AYN7) isolated from diseased fish was also injected in the challenge test for one time and the lethal dose (LD_{50}) was determined. At first, pure culture of the selected strain was inoculated in Brain Heart Infusion (BHI) Broth (Biomark, India) and kept at 37˚C for 24 hours with continuous shaking at 120 rpm at shaking incubator. The optical density was measured at 600 nm (OD_{600}). The suspension was centrifuged at 14000 rpm for 5 minutes to make pellet. The pellet was resuspended with phosphate buffer saline (0.9% NaCl) for suspension (0.1 ml) preparation. The suspension was diluted for 10-fold (1:10) dilution and further diluted up to 10^{-6}. One hundred µl suspensions were streaked in nutrient agar plate to count the colony forming unit (cfu). Initially, suspension containing 2.08E+08 cfu was injected to the fish of three treatments and only PBS solution free of bacteria was injected to the fish of control group.

5.3.8.3 Selection of doses for injecting fish

Fish mortality significantly varied with cfu concentration and with the exposure time. Colony forming unit (cfu) for this study was selected based on previous literature. CFU was selected following the Zhang et al (2016) where they found lethal dose at 3.2×10^6 in channel catfish for *A. hydrophila*. For the present study, 2.08E+08, 3.28E+07, 4.06E+07 for phase I, phase II and phase III, respectively, was used for injecting shing fish intramuscularly.

5.3.8.4 Injection with *A. hydrophila* suspension

The fish were carefully handled with soft cotton to avoid any injury. Fish was placed in soft place and 0.1 ml suspension was injected (Figure 5.5) into muscle just behind the dorsal fin.

Figure 5.5: Handling and injecting *H. fossilis* with *A. hydrophila* suspension

5.3.8.5 Mortality observation

After injection, fish were observed carefully (Figure 5.6) and checked the mortality of each tank of every 6 hour for 10 days. At that time, no feed was given to the fish, water temperature, dissolved oxygen and pH were measured and 50% water was changed every day.

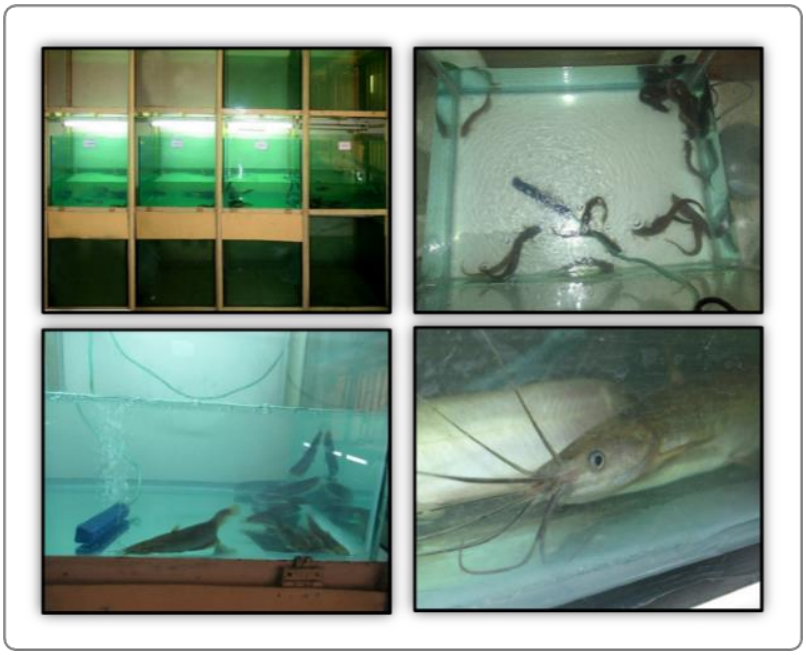

Figure 5.6: Injected *H. fossilis* with *A. hydrophila* suspension in aquarium.

Dead fish with signs and symptoms (Figure 5.7) were removed from the tank carefully and the data were recorded. Bacteria were re-isolated from the lesion of injected fish in RS media to confirm the species. The identification of the isolate was confirmed by 16S rRNA gene sequencing.

Figure 5.7: Clinical signs and excess mucus observation of infected *H. fossilis* with *A. hydrophila* in aquarium.

5.3.9 Phase-II and phase- III challenge test

Similarly, the challenge test with same strain and same procedure was done for second and third time. Fishes were collected from same source. Data regarding fish mortality from three phases was accumulated and calculated to make average.

5.3.10 Statistical analysis

Logit and probit were analyzed by the software R studio understanding the data, using descriptive data analysis through statistical packages, comparing the overall model fit, checking the model fit information, measuring the strength of association (calculating the pseudo-R-square) and parameter estimates.

5.4 Results

5.4.1 Isolation of *Aeromonas hydrophila*

Three different types of colony morphology were found in Rimler Shotts (RS) media with Novobiocin supplement. The morphology of these three colonies were dark green, yellow and black dot in center (Figure 5.8).

A total of 175 isolates of dark green, 120 isolates of yellow and 83 isolates of black dot in center was isolated and stocked in glycerol. DNA was extracted from each of the isolates. The isolates were detected to have pathogenic genes from *A. hydrophila* pathogen specific multiplex PCR and the highest pathogenic gene containing isolates were further confirmed as *A. hydrophila* through 16S rDNA gene sequencing.

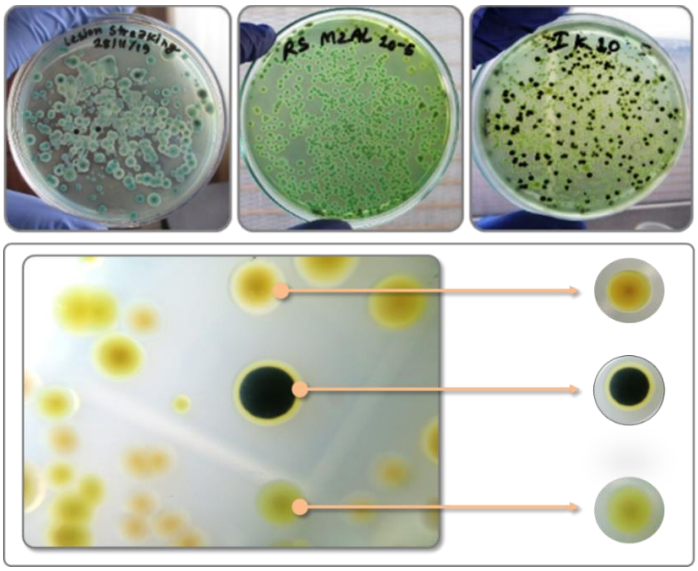

Figure 5.8: Green, yellow and black dot colony morphologies in RS culture media targeted to isolate *A. hydrophila*.

5.4.2 Pathogenicity of the *A. hydrophila*

A total eight genes were tested from which five pathogenic genes, *ahp*A (911bp), *lip* (383 bp), *fla* (593 bp), *vas*H (509 bp), and *AHL* (463 bp) were amplified by polymerase chain reaction. One green morphology *A. hydrophila* isolated of diseased fish from Mymensingh (AGM2) and one yellow color *A. hydrophila* isolated of diseased fish from Narsingdi (AYN7) responded to five pathogenic genes (Figure 5.9). Besides, black dot morphology (ABK9) isolates responded 1-3 virulent genes (Figure 5.9) in PCR amplification as a result, this isolate was not used for further antibiotic susceptibility test and challenge test.

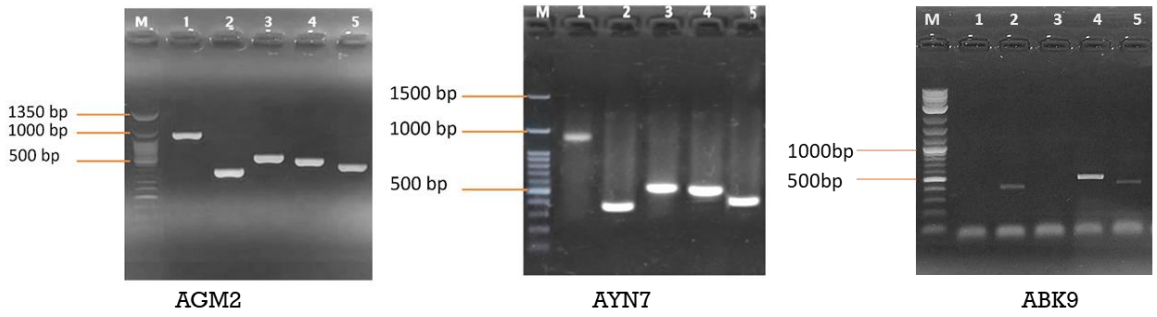

Figure 5.9: Amplification of five pathogenic genes by AGM2 and AYN7 and three pathogenic genes by ABK9 in Agarose gel, lane 1: *ahp*A (911bp), lane 2: *lip* (383 bp), lane 3: *fla* (593 bp), lane 4: *vas*H (509 bp), lane 5: *AHL* (463 bp M: marker 50bp).

5.4.3 Antibiotic sensitivity test

Two isolates (AGM2 and AYN7) confirmed for five pathogenic genes were used for antibiotic susceptibility test. A total of 11 antibiotics were selected from five generations for the antimicrobial spectrum. The green morphology AGM2 showed resistance to 9 antibiotics and sensitive to three antibiotics (Figure 5.10) according to the comparison of diameter of its respective inhibition zone with the standard value. Gentamycin, Cefepime and Ceftriaxone antibiotics inhibit the growth of AGM2. This isolate showed resistance to tested 1st to 2nd generation antibiotics and sensitive characteristics to 3rd and Next Generation Cephalosporins (Table 5.2).

On the other hand, the yellow isolate AYN7 showed a pattern of resistant to 6 antibiotics and sensitivity to 5 antibiotics. Gentamycin, Cefepime, Ceftriaxone, Azithromycin (covering 1st to 4th generation and next generation cephalosporins) (Table 5.2) showed sensitivity to AYN7 isolate.

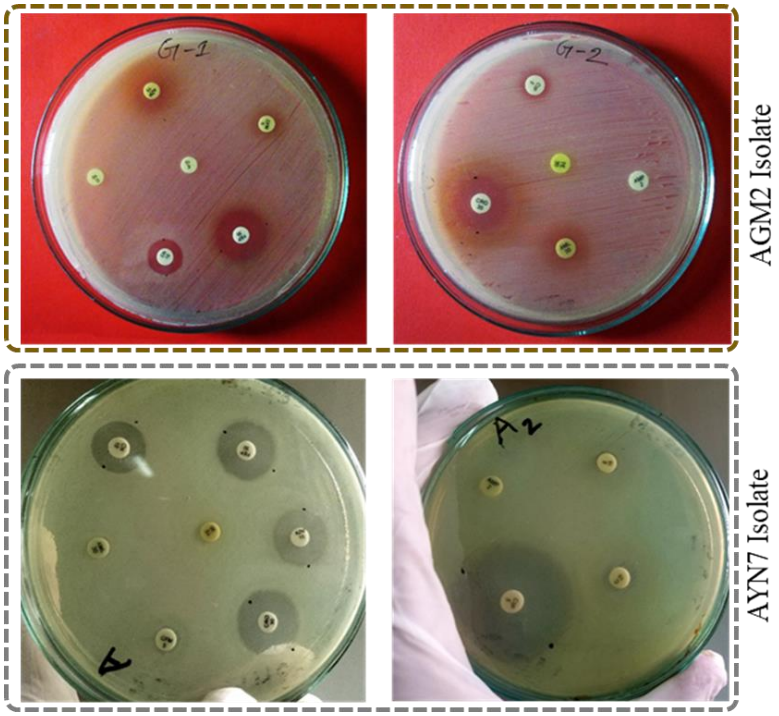

Figure 5.10: Antibiotic sensitivity test (zone of inhibition produced by AGM2 and AYN7 isolates) for *A. hydrophila* in Mueller-Hinton agar.

Table 5.2: Antibiotics list with group of generation used for sensitivity profiling of AGM2 and AYN7 isolates

Generation	Name of Antibiotics	*A. hydrophila* (AGM2-Green colony)		*A. hydrophila* (AYN7-Yellow colony)	
		Zone of diameter (mm)	Sensitivity	Zone of diameter (mm)	Sensitivity
NGC	Chloramphenicol (10 µg)	-	Resistant	-	Resistant
	Erythromycin (15 µg)	-	Resistant	-	Resistant
	Gentamycin (10 µg)	18	Sensitive	16.5	Sensitive
4th	Cefepime (30 µg)	20	Sensitive	19.5	Sensitive
3rd	Ampicillin (2 µg)	-	Resistant	-	Resistant
	Amoxicillin (30µg)	-	Resistant	-	Resistant
	Cefixime (5 µg)	-	Resistant	-	Resistant
	Ceftriaxone (30 µg	22	Sensitive	20	Moderately Sensitive
2nd	Azithromycin (15 µg)	-	Resistant	19	Sensitive
	Ciprofloxacin (5µg)	-	Resistant	30	Sensitive
1st	Tetracycline (30µg)	-	Resistant	-	Resistant

5.4.4 Challenge test with AGM2

The cfu of injecting suspension for fishes were 2.08E+08, 3.28E+07, 4.06E+07 for phase I, phase II and phase III, respectively.

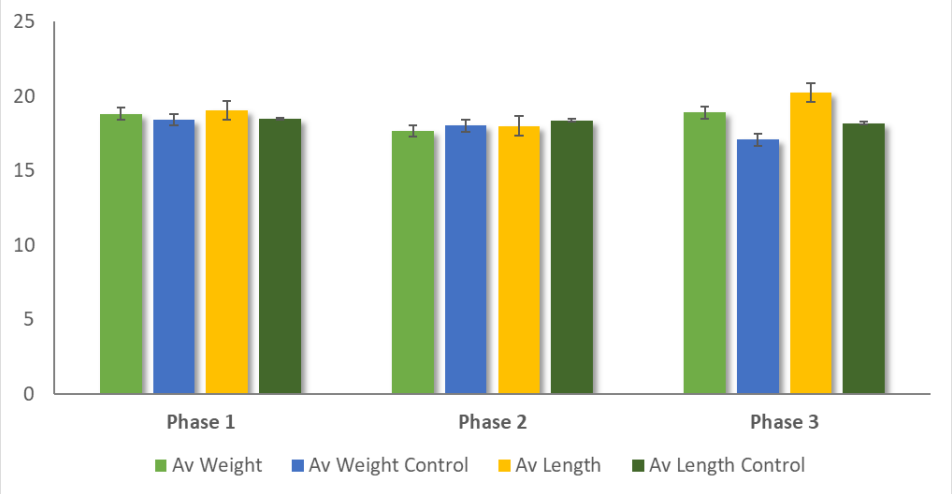

Figure 5.11: Average weight and length of fishes for challenge test in three different phases including controls.

Average weight and length of fishes for all phases is shown by Figure 5.11). There was no significant difference between the fish weight and length of three phases ($P > 0.005$).

5.4.5 Mortality of fish with AGM2 infection

Fish mortality was started after 16-17 hours of injection with lesions around injecting area, head and tail portions and excess mucus secretion. The survival and mortality of shing fish from three phase challenge test with AGM2 bacteria is presented in Table 5.3.

Table 5.3: Mortality of *H. fossilis* after challenge with AGM2 isolate in three phases for 10 days of post injection.

Strain	Differential concentration	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day	Total mortality	Mortality
AGM2	Phase I	1	2	6	7	6	2	4	1	0	0	29	1
	Phase II	0	1	4	12	10	3	0	0	0	0	30	0
	Phase III	1	5	6	7	6	3	2	0	0	0	30	0
	Control	0	0	0	0	0	0	0	0	0	0	0	30

In phase I and III, the highest mortality (23%) was observed in 4th day of post-injection. Maximum mortality (40%) was observed in 4th day in phase II. Severe mortality was observed in phase II and maximum mortality was occurred in 4th to 6th day for three phases. Total (100%) mortality was observed in phase II and III within 10th day of post injection Figure 5.12).

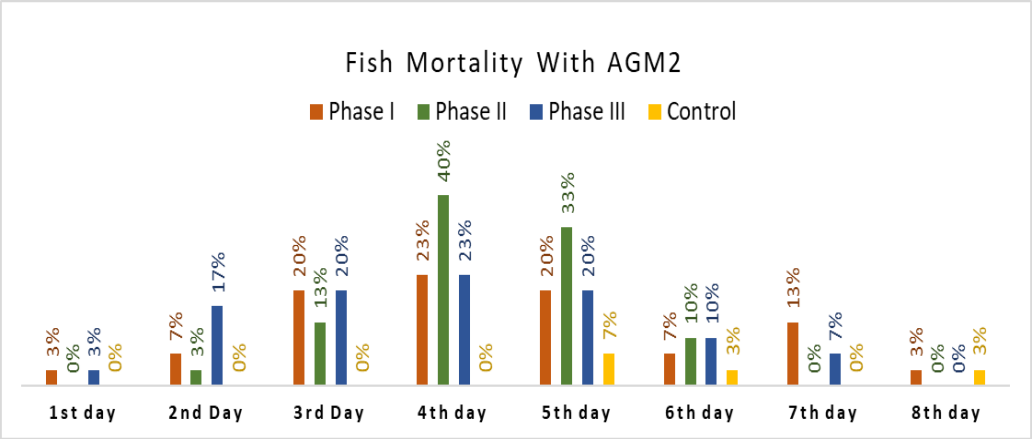

Figure 5.12: Fish mortality from challenge test with AGM2 in phase I, II and III.

5.4.6 Observation of signs and symptoms caused by AGM2

Dead fishes from challenge test showed signs and symptoms like lesions in different parts of body, tail and fin rot which have similarities with the diseased fishes collected from fish farm (Figure 5.13).

Figure 5.13: Signs and symptoms observed in dead fishes of three phases of challenge test with AGM2 isolate.

5.4.7 Re-isolation of AGM2 from infected fish

Bacteria was reisolated from the lesion (Figure 5.14A) of dead fish from challenge test with AGM2 by culture method and observed green colony in RS media (Figure 5.14B).

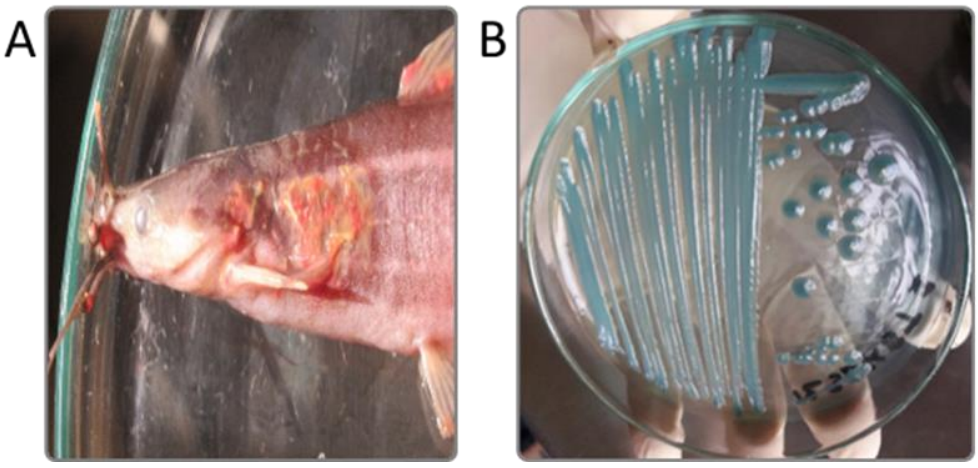

Figure 5.14: A-Lesion in experimentally infected fish; B- Reisolated bacteria from infected fish.

The isolate was again confirmed by 16S rRNA gene sequencing and found as *A. hydrophila* by BLAST searching in NCBI.

5.4.8 Challenge test with AYN7

Experimental infection with yellow color *A. hydrophila* (AYN7) was done for one time with three replications and controls (10 fishes in each replication and 10 fishes in each control). The injecting suspension contained 7.20E×10^6 cfu. Average weight and length of fishes is presented in Table 5.4.

Table 5.4: Average length and weight of *H. fossilis* fish used in challenge test with AYN7 isolate

Group	Average Weight (g)	Average Length (cm)
R1	12.18	14.26
R2	17.82	17.80
R3	19.82	18.94
Control	18.98	17.94

5.4.9 Calculation of LD_{50} for AYN7

Highest (90%) mortality was observed in replication 1 than replication 2 and 3. Lethal dose (LD_{50}) was calculated for this isolate and found as 6.77×10^{6} by logit and probit analysis (Figure 5.15A).

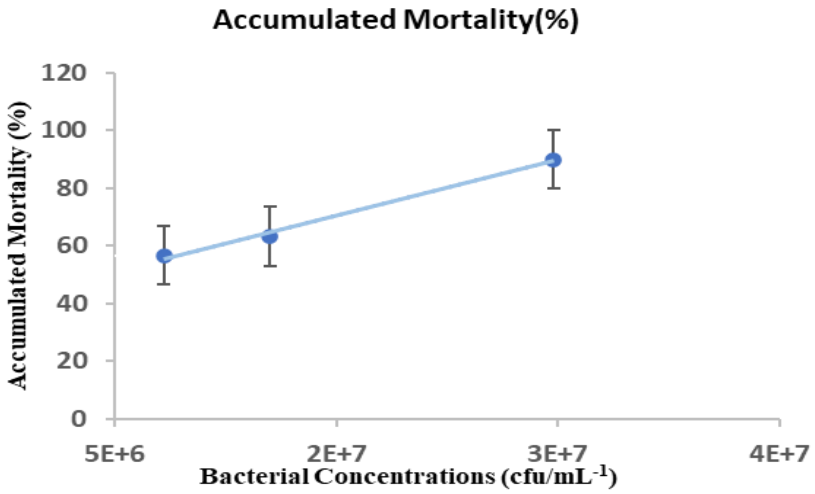

Figure 5.15: Accumulated mortality (%) in different bacterial concentrations.

5.4.10 Observation of signs and symptoms caused by AYN7

Lesions (Figure 5.16A) and excessive mucus were observed in dead fish body from experimental infection with AYN7.

5.4.11 Re-isolation of AYN7 from infected fish

Similarly, the isolate was reisolated (Figure 5.16B) and confirmed by observing yellow colony in RS media and 16S rRNA gene sequence analysis as *A. hydrophila*

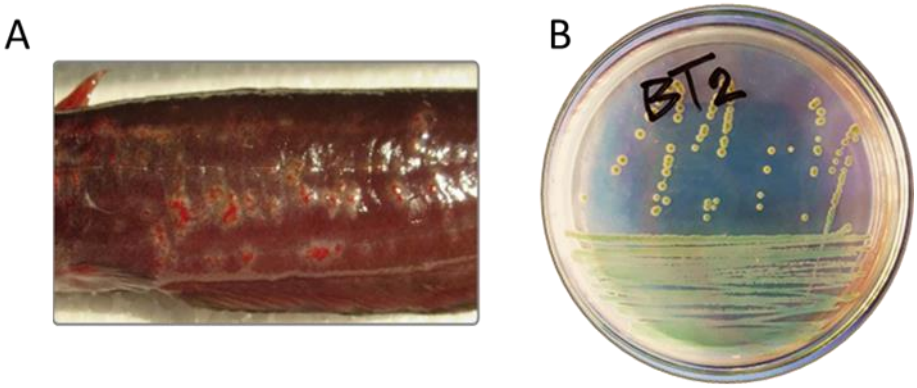

Figure 5.16: A-Lesions observed in artificially infected fishes, B-Re-isolation of yellow colony from infected fish.

5.5 Discussion

In this study, three isolates of *A. hydrophila* were isolated and confirmed by 16S rRNA gene sequencing. The green and yellow color *A. hydrophila* were found as virulent due to presence of pathogenic genes confirmed by PCR amplification. Virulent *Aeromonas* sp. were reported from freshwater fish and catfish by many researchers (Zhai et al., 2021, Zhang et al., 2016, Sarker and Faruk, 2016, Dong et al., 2017) though virulence factors of *Aeromonas* sp. varied among environmental and clinical strains (Zhang et al., 2014). Wound at the skin has been reported as primary sign and symptom caused by bacteria, virus and other damage (Gui et al., 2018). Wounded fish are more susceptible to virulent *Aeromonas* infection including clinical symptoms of MAS disease (Zhang et al., 2014).

A. hydrophila. showed resistant pattern to commonly used antibiotics. The AGM2 and AYN7 isolate exhibited resistance to Chloramphenicol, Erythromycin, Ampicillin, Amoxicillin, Cefixime, Azithromycin, Ciprofloxacin, Tetracycline which was also confirmed in carp (Guz and Kozinska, 2004; Harnisz and Tucholski, 2010), yellow catfish (Zhai et al., 2021). These common antibiotics are frequently used in shing aquaculture to control bacterial disease. Long time exposure of antibiotics in aquaculture will increase antibiotic resistant bacteria.

Challenge test with *A. hydrophila* was done to determine the pathogenicity of the isolate and to detect the single causative agent for MAS infection by injection method which was consistent with previous report (Alsaphar, 2012; Chen et al., 2019; Zhai et al., 2021). Intraperitoneal injection is effective and reproducible method for challenge test (Zhang et al., 2016) compared to immersion method though immersion method is more natural or closely related to natural infection. Moreover, the fish skin mucus protects host from pathogen which delay the infection during immersion (Le Guellec, Morvan-Dubois and Sire, 2004; Schadich and Cole, 2010). Medium virulent strain includes lethal dose value between 10^6 to 10^7 cfu/g body weight of fish according to previous report (Mittal et al., 1980). In this study, three different concentrations of AGM2 (2.08×10^8, 3.28×10^7, 4.06×10^7) were injected for three phases where the cfu concentration was very close to other studies with *Aeromonas* sp. in catfish and carp fish (Chen et al., 2010; Abdelhamed et al., 2017; Faruk et al., 2004). A lower dose (7.20×10^6 cfu) of AYN7 isolate was injected to the fish which is consisted with other reports (Zhang et al., 2014; Korni et al., 2017). No clinical signs were observed using 1.2×10^7 cfu in challenge test of *A. hydrophila* in

Piaractus mesopotamicus fish and 2.4×10^7 cfu/ml induced clinical signs and 1.5×10^8 cfu/ml caused the death of all fish within 24 h of inoculation (Carraschi et al., 2012). So, the dose which created pathological signs and symptoms is essential to determine because in this stage treatment can be started to establish protocol and to control.

Lesions and excess mucus secretion were occurred in the body surface of fish (around head, tail, fin) after infection caused by pathogenic AGM2 and AYN7 isolates. When infection occurs, other bacteria may enter in fish and worsen infection together with pathogenic bacteria although they are not pathogenic (Preena et al., 2019). Disease signs and symptoms including ulcer, tail and fin rot, eye protrusion, excess slime in the body were observed which showed the similarities with signs from experimental infection in catfish (Faruk et al., 2012; Sarker and Faruk, 2016; Nahar et al., 2016; Zhang et al., 2016; Abdelhamed et al., 2017) and other fish (Alsaphar, 2012; Khamees et al. 2013; Stratev et al., 2015; Chen et al., 2019, Kumar et al., 2016). Same *Aeromonas* strain can cause different symptoms and different strain can cause same clinical signs (Chen et al., 2019). Clinical signs and symptoms also varied on the fish immunity and the dose of bacterial concentration inoculated (Claudiano et al., 2020).

After infection, the fish showed signs of lesion in different parts of body. The bacterium was reisolated from the wounds of fish body by culture in Rimler shotts media. Color and other morphology were compared with the previous colony isolated from naturally infected fish. Similar morphology was found in reisolated colony from lesion of artificially infected fish. The bacterium was then sequenced of 16S rRNA gene and confirmed as *A. hydrophila*. Re-isolation of the *A. hydrophila* bacteria from artificially infected fish were also done in previous reports (Carraschi et al., 2012; Mazumder et al., 2021). Many reports proved that *A. hydrophila* cause infection with lesions in skin, inflammation (Alavinezhad et al., 2021). *Aeromonas* sp. are naturally occurring flora in water also in fish depend on certain conditions they become pathogenic for fish.

Clinical signs and symptoms were milder in collected diseased fish from farm than fishes infected with challenge test. Bacterial load during artificial infection was and confined area in aquarium may have effect on showing more pathological symptoms. Similar result was reported in *A. testudinius* fish by Mazumder et al., (2021). Increasing highly pathogenic *Aeromonas* strains in aquaculture farm become an alarming issue in many countries specifically for the spreading of virulent strain (Azzam-Sayuti et al., 2021). Mortality

ranged from 30-90% was recorded in African catfish challenged with *A. hydrophila* (Anyanwu et al., 2015). However, the identification and confirmation of pathogenic strains of *A. hydrophila* in *H. fossilis*, pathogenicity and sensitivity testing and challenge test against the pathogenic strains would be helpful to formulate any drugs for the effective health management and surveillance of fish diseases in the aquaculture systems.

Mortality of fish by challenge test also depended on the bacterial dose and host-pathogen interaction, the period fish exposed (Zhang et al., 2016). Mortality was maximum in AGM2 infection indicated its more pathogenicity and all fishes died within 10^{th} day of injection. Mortality started from 24 hours of post injection in case of both AGM2 and AYN7 strains. So cfu value and short incubation period indicated that both strains are moderately virulent for shing fishes. This result is consistent with the report of Chen et al., 2019 in carp fish. Lethal dose of 2.2×10^8 cfu/ml of *A. hydrophila* in *Pangasius bocourti* and *Ictalurus punctatus* was reported (Van Doan et al., 2013) which is higher than the dose used in this study. The LD_{50} of bacterial strain varied from 3.2×10^4 to $>10^8$ cfu depending on the strain. *A. hydrophila* dose 3×10^9 and 3.5×10^8 cfu/ml were injected intramuscularly and found the strain as lethal after 24 h injection (Boijink and Brandão 2001). Pathogenic *A. hydrophila* was reported in *Anabas testudinius* fish with 100% mortality by Hossain et al., 2011 at 9.2×10^7 cfu/fish. However, similar mortality was observed in *A. testudinius* with lower dose of 3.4×10^6 by Mazumder et al. (2021). Various factors such as fish species, fish size, age, and inherent genetical immunity, pathogenicity of bacterial strain, strain inoculation medium and challenge test model have effect on the lethal dose (Claudiano et al., 2020). *A. hydrophila* play role as both opportunistic (Vila et al., 2003) and primary infecting agent (da Silva et al. 2012; Pridgeon et al. 2013).

5.6 Conclusion

This experiment showed that healthy *H. fossilis* were infected heavily with the injected *A. hydrophila* in challenge test. This challenge test is effective to know the host-pathogen interactions. Appeared lesions in the body surface of fishes indicated the presence of virulence factors in the isolate. The present study suggested that stinging catfish is always under the risk of infection caused by pathogenic *A. hydrophila* strains since these species are frequently found in the aquatic environment.

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Chapter 6
Design of Potential Drug through Virtual Screening for Serine Protease (*ahpA*) against virulent *Aeromonas hydrophila*

6.1 Abstract

The *Aeromonas hydrophila* is an opportunistic pathogen causing severe mortality in fish within a week of infection. Expression of virulence factors by pathogenic *A. hydrophila* varied with environmental stress and immune system of host. Serine protease is one of the important virulence factor destruct host tissue and host defending proteins. Thus, the gene *ahp*A encoded for serine protease was characterized and homology modelling was done for finding potential inhibitor against the protein. The nucleotide sequences were translated for protein and found 160 amino acids sequences. Phylogenetic analysis showed maximum identity with serine protease of other *A. hydrophila* strains. The generated 3D structure showed acceptable results of verification parameters using protein structure verification server. Important parameter Ramachandran plot exhibited maximum amino acid residues (88.1%) in the favorable region which is also supported by the accepted G-factor value (overall score 0.38). The molecular docking of 3D structure showed highest affinity (-7.6) with ceftriaxone from six selected compounds. The top hit compound followed Lipinski's rule of five indicated the efficiency of physicochemical properties of compounds. Potential inhibitors (drug like compounds) against different virulence factors would be an efficient alternative way to control misuse of antibiotics in aquaculture industries.

6.2 Introduction

Bacterial diseases are plaguing rapidly growing aquaculture sector, causing economic losses estimated to be billions of dollars worldwide. *Aeromonas hydrophila* is known as the most important, common, and ubiquitous opportunistic pathogen causing disease in amphibians, fish, birds, reptiles, and mammals, the most common forms of which are gastroenteritis, septicemia, and necrosis fasciitis (Rasmussen-Ivey et al., 2016). Various factors affect virulence of *A. hydrophila* through the secretion and/or production of cytotoxins, adhesins, lipases, hemolysins and protease. Biofilm formation, utilization of definite metabolic pathways, expression of pathogenic factors by quorum sensing (Rasmussen-Ivey et al., 2016; Beaz-Hidalgo and Figueras, 2013) also have impacts on the disease-causing capacity of *A. hydrophila*. Environmental fluctuations like temperature, hypoxic conditions, level of other parameters beyond the standard are linked with the outbreak of *A. hydrophila* and the secretion of virulence factors (Swann and White, 1991; Mateos et al., 1993; Janda and Abbott, 2010). *A. hydrophila* isolated from the clinical sample can grow and survive greater than the strain's optimum growing temperature of

28◦C (Popoff and Veron, 1976); while environmental strains can adapt to low temperatures and can survive as low as 4◦C temperature, that inhibit the growth of medical isolates (Mateos et al., 1993).

The spectrum of virulence factors secreted by *A. hydrophila* comprises adhesion proteins, nucleases and toxins, catalysts, that can be expressed differently on different environmental conditions. *Aeromonas* spp. secrete a variety of degradative enzymes including collagenases, enolases, elastases, metalloproteases, lipases, and serine proteases that can lead to pathogenicity. Serine proteases (SPs) are a family of proteases that use single activated serine residues in the substrate-binding pocket to catalyze the hydrolysis of peptide bonds. The active site of all serine proteases contains three residues: serine, histidine, and aspartic acid. Serine protease secreted by *Aeromonas* spp. is a member of kexin subfamily and main extracellular proteases under serine proteases are able to induce edema (Takahashi et al., 2014). A novel thermostable serine protease was identified from *A. hydrophila* B32 that showed cytotoxic activity (Rodriguez et al., 1992). The gene *ahp*A of *A. hydrophila* which encode a 68-kDa temperature-labile serine protease have been reported to express efficiently (Esteve et al., 2004). Skwor et al. 2014 found most virulent serine protease gene among all other cytotoxic *Aeromonas* isolates which controlled by quorum sensing and Type II secretion systems. Serine protease (*ser*) associated with other virulent factors play vital role in *Aeromonas* pathogenesis. Hu et al., 2012 isolated *Aeromonas* strains from healthy fish, diseased fish, water samples and found diverse combinations of three or more virulence genes. Both serine protease and aerolysin showed cytotoxic effects and their presence is more frequent and common in diseased fish compared to healthy fish (Skwor et al 2014).

Characterization of *A. hydrophila* for assessing the pathogenicity through the usage of virulence factors as genetic indicators would improve the prevention strategies and control of this bacterial outbreaks (Li et al., 2011). Therefore, a promising method is essential to inhibit the growth of *A. hydrophila* which can disrupt the cell-to-cell communication (quorum sensing) and secretion of virulence factors, so that the pathogen can be eradicated from the host. Various chemicals, enzymes and natural products have been reported that interfere with quorum- sensing (QS) system. QS inhibition is an important possible way to control antibiotic resistant strains in aquaculture. Quorum sensing (QS) may be valuable and effective tool to control the synthesis and secretion of virulence factors and antibiotic resistant pathogen (Shaker et al., 2020). Structure-Activity Relationship (SAR) may be the

most significant perception to recognize the possible QS inhibitors (Shaker et al., 2020) for future drug development against serine protease (ser) of *A. hydrophila*. According to previous studies, compounds contained sulfur showed effective QS inhibitor and can eliminate the virulence bacteria (Shaker et al., 2020). This study focused on the identification of compounds that inhibit the secretion of serine protease and prediction on the compound's binding affinities through molecular docking based on *in silico* drug discovery technology.

6.3 Materials and methods

6.3.1 Amplification of *ahp*A gene

Aeromonas hydrophila strain (AGM2) was isolated from lesion of diseased fish collected from Mymensingh and confirmed by 16S rRNA gene sequencing in **Chapter 5**. The *ahp*A gene was amplified using the DNA isolated from *A. hydrophila* (AGM2) and used in molecular docking. Other four amplified virulent genes of AGM2 were not selected because of their small fragment size. The amplification condition was started with 1 cycle initial denaturation at 95°C for 5 minutes, initiation at 95°C for 30 seconds, annealing at 55°C for 1 minute, elongation at 72°C for 2 minutes for 30 cycles and final elongation at 72 °C for 10 minutes. The amplified product was run at 1.5% agarose gel for 911 bp size confirmation. Then the PCR product was purified by using the DNA purification kit (Invitrogen, USA). Purified product was subjected to sequence in Applied Biosystems 3130 Genetic Analyzer using 20 µl reaction volume containing approximately 10 ng purified products as template, ready reaction premix 4.0 µl, Big Dye terminator buffer 2.0 µl, primer 0.32 µl and ultrapure water to make 20 µl. Sequence of *ahp*A gene was aligned with 10 sequences of similar gene of *A. hydrophila* and other bacteria (retrieved from NCBI) and a phylogenetic tree was constructed for evolutionary analysis (Kumar et al., 2018; Saitou & Nei, 1987) using MEGA software version M11. The gene sequence was then submitted to GenBank (Submission ID-2613266). The amino acid sequence of serine protease (translated protein from *ahp*A gene) was inferred by using Expasy translate tool (http://www. expasy.org).

6.3.2 Physical and chemical properties analysis of serine protease

The encoded amino acid sequence of serine protease was checked for the physical chemical properties by using ProtParam (https://web.expasy.org/protparam/) and PredicProtein (https://predictprotein.org/) bioinformatics tools.

6.3.3 Sequence analysis of serine protease of *A. hydrophila* (AGM2)

Thirty amino acid sequences of serine protease of *Aeromonas* spp. were retrieved from NCBI for neighbor-joining tree construction. Then, retrieved and *A. hydrophila* (AGM2) amino acid sequences were subjected to multiple sequence alignment for constructing phylogenetic tree using MEGA software version M11.

6.3.4 Structural analysis of serine protease

To find out a suitable 3D structure of serine protease for homology matching, RaptorX (http://raptorx.uchicago.edu/ContactMap/) tool was used. This bioinformatics tool suggested five 3D structure for serine protease. The available reported 3D structure of serine protease was then searched in protein data bank (PDB) for the best alignment.

The generated 3D structure of serine protease was viewed using Discovery studio software and checked various parameters for protein structure validation using UCLA-DOE LAB—SAVES v6.0 server (https://saves.mbi.ucla.edu/) such as WHAT_CHECK (http://swift.cmbi.ru.nl/gv/whatcheck), Verify3D (Bowie et al., 1991, Lüthy et al., 1992), PROVE (Pontius, 1996). In the server systems, Ramachandran plot (Ramachandran & Sasisekharan, 1968) clarified various aspects of the model using PROCHECK (Laskowski et al., 1993). The energy of the generated model was calculated using PROSA software (Sippl, 1993).

6.3.5 Ligand screening and molecular docking for identification of suitable inhibitor

Based on the previous report, six antibiotics (Table 6.1) that are commonly used to control virulent *A. hydrophila* were selected to inhibit the serine protease activity and identify the important residues participated in the binding.

Table 6.1: Reported antibiotics used for antibiotic sensitivity test against *A. hydrophila*

Ligands	Molecular weight
Gentamycin	477.56
Azithromycin	748.98
Ciprofloxacin	331.34
Ceftriaxone	554.58
Cefepime	481.57
Capric acid	172.26

The 2D and 3D structure of these six molecules were retrieved from Pubchem (https://pubchem.ncbi.nlm.nih.gov/) and Drugbank (https://go.drugbank.com/). The online

SMILES Translator (https://www2.chemie.uni-erlangen.de/services/translate/) was used to convert the structure data file (SDF) to protein data bank (PDB) format.

Virtual screening of generated 3D structure of serine protease with six ligands was performed using AutoDock Vina compiled in PyRx (Dallakyan & Olson, 2015; Trott & Olson, 2010) bioinformatics tool. PyRx is an offline tool that allows virtual screening of several molecules for computational drug design or to check interactions between ligands and targets. The molecules were transferred into Open Babel within the PyRx and exposed to energy minimization. For visualization, Discovery Studio was used to view the interaction between generated molecule with target ligands.

6.3.6 ADME analysis of selected inhibitors

SwissADME (http://www.swissadme.ch/index.php) and PreADME online server (https://preadmet.qsarhub.com/druglikeness/) were used for important physicochemical properties (Absorption, Distribution, Metabolism and Excretion) analysis of the selected compounds. It is well known that 40% targeted drugs fail in the experimental trial due to poor physicochemical properties (Mahmudpour et al., 2021). ADME analysis based on 3D model to find or discard a potential drug in early stage helps in saving time and resources.

6.4 Results

6.4.1 Physico-chemical characteristics of *ahp*A serine protease through *in silico* analysis

The physicochemical properties analysis exhibited that the studied gene *ahp*A encoded a protein with 160 amino acid residues (Figure 6.1).

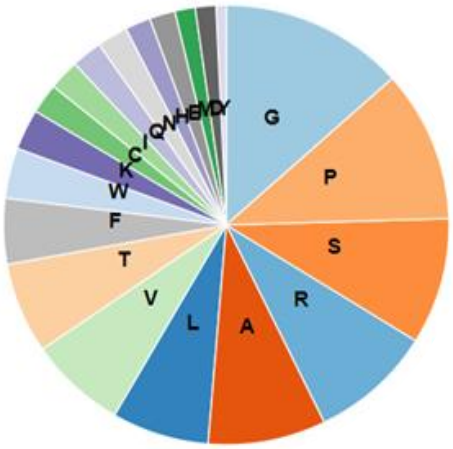

Figure 6.1: Amino acids residues in studied serine protease

The protein has molecular weight 16.85 KDa and theoretical pI 11.58, Alanine (11.9%) and Glycine (11.9%) content was highest followed by Serine (10.6%), Proline (10.0%) and Arginine (9.4%) (Table 6.2). Total number of negatively charged residues (Asp + Glu) was nine (3) and total number of positively charged residues (Arg + Lys) was 18. Atomic composition of the protein is presented in the Table 6.3.

Table 6.2: Amino acids composition in studied serine protease of *A. hydrophila*

Amino acids	Atom number	Content (%)	Amino acids	Atom number	Content (%)
Ala (A)	19	11.9%	His (H)	5	3.1%
Arg (R)	15	9.4%	Ile (I)	4	2.5%
Asp (D)	2	1.2%	Leu (L)	12	7.5%
Cys (C)	6	3.8%	Lys (K)	3	1.9%
Gln (Q)	6	3.8%	Met (M)	4	2.5%
Glu (E)	1	0.6%	Phe (F)	2	1.2%
Gly (G)	19	11.9%	Pro (P)	16	10.0%
Ser (S)	17	10.6%	Thr (T)	13	8.1%
Trp (W)	7	4.4%	Tyr (Y)	1	0.6%
Val (V)	8	5.0%			

Table 6.3: Atomic composition of the studied serine protease of *A. hydrophila*

Carbon	C	737
Hydrogen	H	1169
Nitrogen	N	231
Oxygen	O	204
Sulfur	S	10

A total of 4010 atoms was presented in the formula $C_{737}H_{1169}N_{231}O_{204}S_{10}$ of serine protease. The N-terminal of the sequence of the protein contained methionine (M) amino acid. The instability index (II) was 66.96 which indicated the protein was not stable. The protein showed Aliphatic index: 65.38, Grand average of hydropathicity (GRAVY): -0.191, Helix=10.4%, strand=23.4%, Loop=66.2%. The estimated half-life of the protein is 30 hours (mammalian reticulocytes, in vitro), >20 hours (yeast, in vivo), >10 hours (*Escherichia coli*, in vivo).

6.4.2 Phylogenetic tree analysis of serine protease

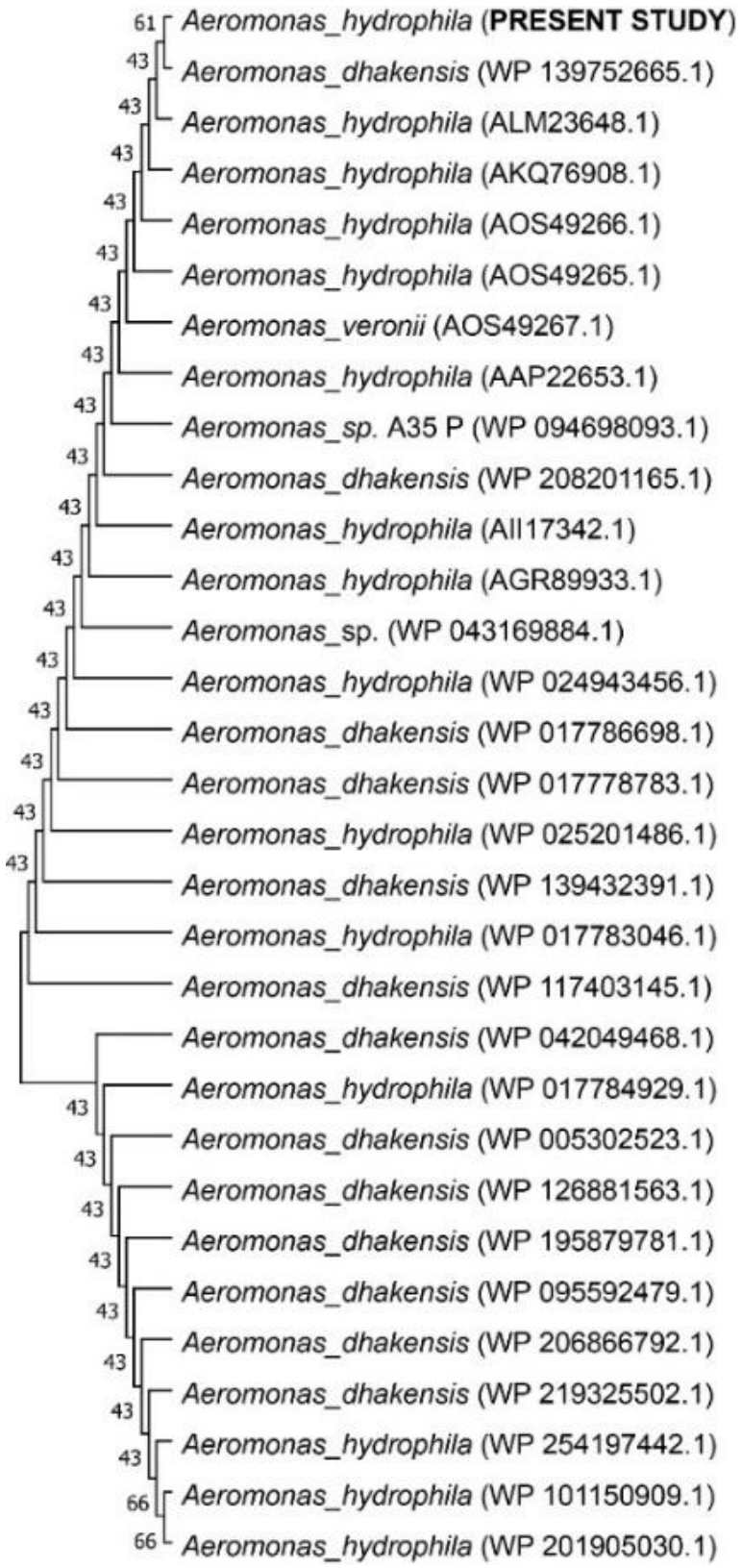

Figure 6.2: Neighbor joining tree of amino acid sequences of studied and 30 retrieved serine protease sequences of *A. hydrophila*.

The nucleotide sequence of *ahp*A from this study was aligned with other *ahp*A nucleotide sequences of *A. hydrophila* retrieved from NCBI. The studied nucleotide sequence showed 89-91% similarity with *ahp*A gene of other *Aeromonas* species. The studied serine protease gene was closest to the *A. dhakensis* (91% similarity) (Figure 6.2). The similarity of the nucleotide sequence of the strain (AGM2) with other *Aeromonas* species varied from 88-91% confirming that the strains of *Aeromonas* spp. were diverse. Different pathogenic strains of *Aeromonas* spp. contained the virulent gene with variable nucleotide sequences.

6.4.3 3D structure of serine protease

To build a suitable 3-D structure, the amino acids sequences of studied protein was submitted into the RaptorX (http://raptorx.uchicago.edu/). Five obtained suggestive 3-D model of the protein were presented (Figure 6.3).

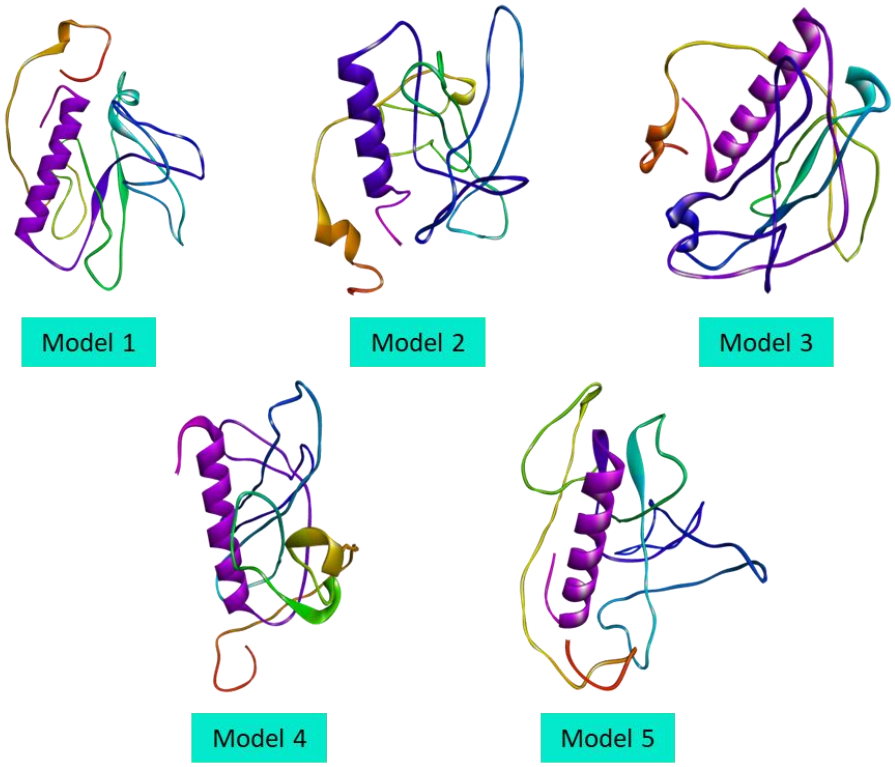

Figure 6.3: Suggestive five models obtained from RaptorX against serine protease of *A. hydrophila*.

The created 3D structure of the protein was visualized by BIOVIA Discovery Studio without any filtering and validate the structure features by using verification server (UCLA-DOE LAB — SAVES v6.0) like WHAT_CHECK, Verify 3D, PROCHECK and ERRATE.

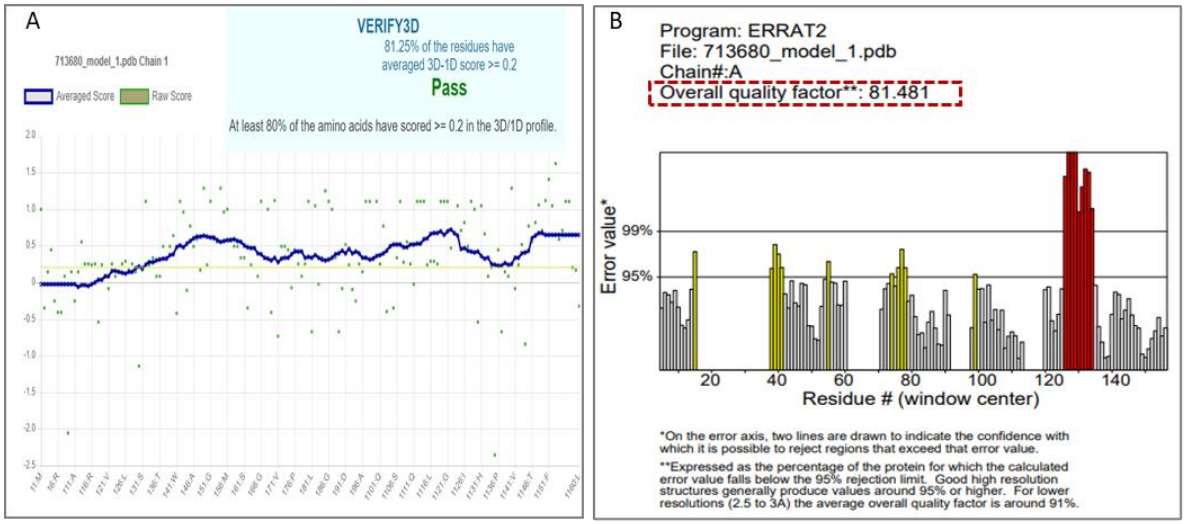

Figure 6.4: 3D structure validation serine protease of *A. hydrophila* by UCLA-DOE LAB — SAVES. A- Verify 3D; B- ERRATE2.

The selected 3D structure showed that 81.25% amino acid residues have an average 3 D-1D score > 0.2, which is approved and acceptable by this program (Figure 6.4A). The ERRATE2 expressed the percentage of the protein (81.48%) (Figure 6.4B), where high-resolution structures generally produced the values around 95% or higher.

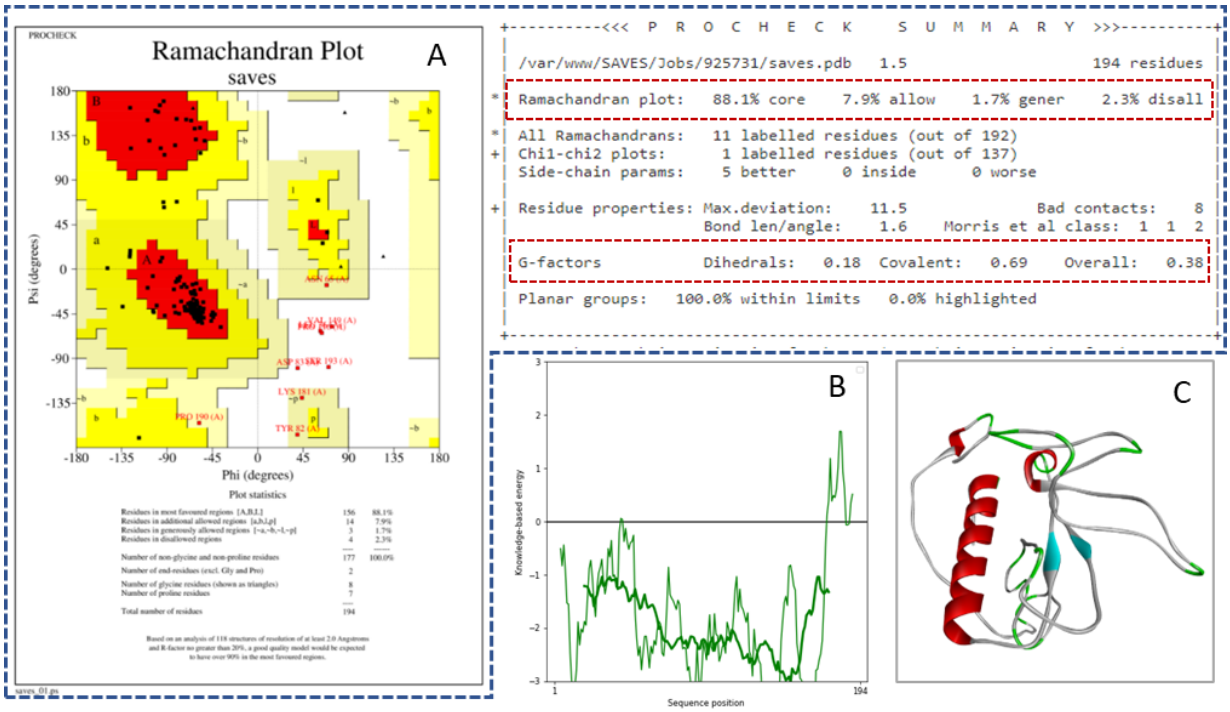

Figure 6.5: A-Ramachandran plot of amino acids of serine protease. Black dot indicates angles of distribution of amino acids in secondary structure region (red color). Yellow and light-yellow color indicate the additional allowed region and generously allowed region**;** B- Energy plot of selected model; C-Selected 3D model of serine protease.

But above 80% is the accepted for average overall quality factor. WHATCHECK showed that most of the amino acids were placed at the right positions and a few amino acids showed the displaced sites. Most important validating parameter is the Ramachandran plot showed that 88.1% amino acid residues were found in the most favorable region, 7.9% residues in additional allowed region, 1.7% residues in generously allowed region and 2.3% residues in disallowed region (Figure 6.5A). The score of the G-factor of the studied model was 0.18 for dihedral bonds, 0.69 for covalent bonds and overall score was 0.38 revealed by the Ramachandran plot (Figure 6.5A). The G score greater than -0.50 approve the model and within this limit of any model is acceptable and satisfactory. The energy of studied serine protease model (Z score) was found to be -6.51 determined by PROSA (Figure 6.5B). Based on the parameters analysis the selected 3D model of the serine protease is presented in Figure 6.5C.

6.4.4 Molecular docking analysis

Maximum binding affinities of the compounds were found from molecular docking and the best docked compound with serine protease was Ceftriaxone (-7.6 kcal/mol), Cefepime (-6.7 kcal/mol) and Ciprofloxacin (-6.7 kcal/mol) (Table 6.4). These compounds showed better affinity to bind with serine protease to inhibit its virulent activity.

Table 6.4: Results of molecular docking and effective inhibitors participate in the interaction against serine protease

Protein	**3P_*ahp*A: Serine Protease**					
Ligands	**Ceftriaxone**	**Cefepime**	**Ciprofloxacin**	**Azithromycin**	**Gentamycin**	**Capric Acid**
Formula	C18H18N8O7S3	C19H25N6O5S2	C17H18FN3O3	C38H72N2O12	C21H43N5O7	C10H20O2
Molecular Weight	554.58	481.57	331.34	748.98	477.56	172.26
No. of atoms	54	56	42	124	76	32
Binding affinity (kcal/mol)	-7.6	-6.7	-6.7	-6.1	-6.1	-5.1
Amino acid involved in interaction	Arg92(A), Gly121(A), Lys93(A), Leu102(A), Trp70(A), Gln101(A), Gly100(A), Arg69(A), Pro95(A)	Arg92(A), Gly121(A), Leu102(A), Gln101(A), Lys93(A), Trp70(A), Gly100(A)	Arg69(A), Lys93(A), Gln101(A), Pro95(A), Gly100(A)	Cys54(A)	Lys64(A), TRP70(A), Leu102(A)	Arg149(A), Ile18(A), Leu160(A), Phe152(A), Arg16(A), Val21(A)

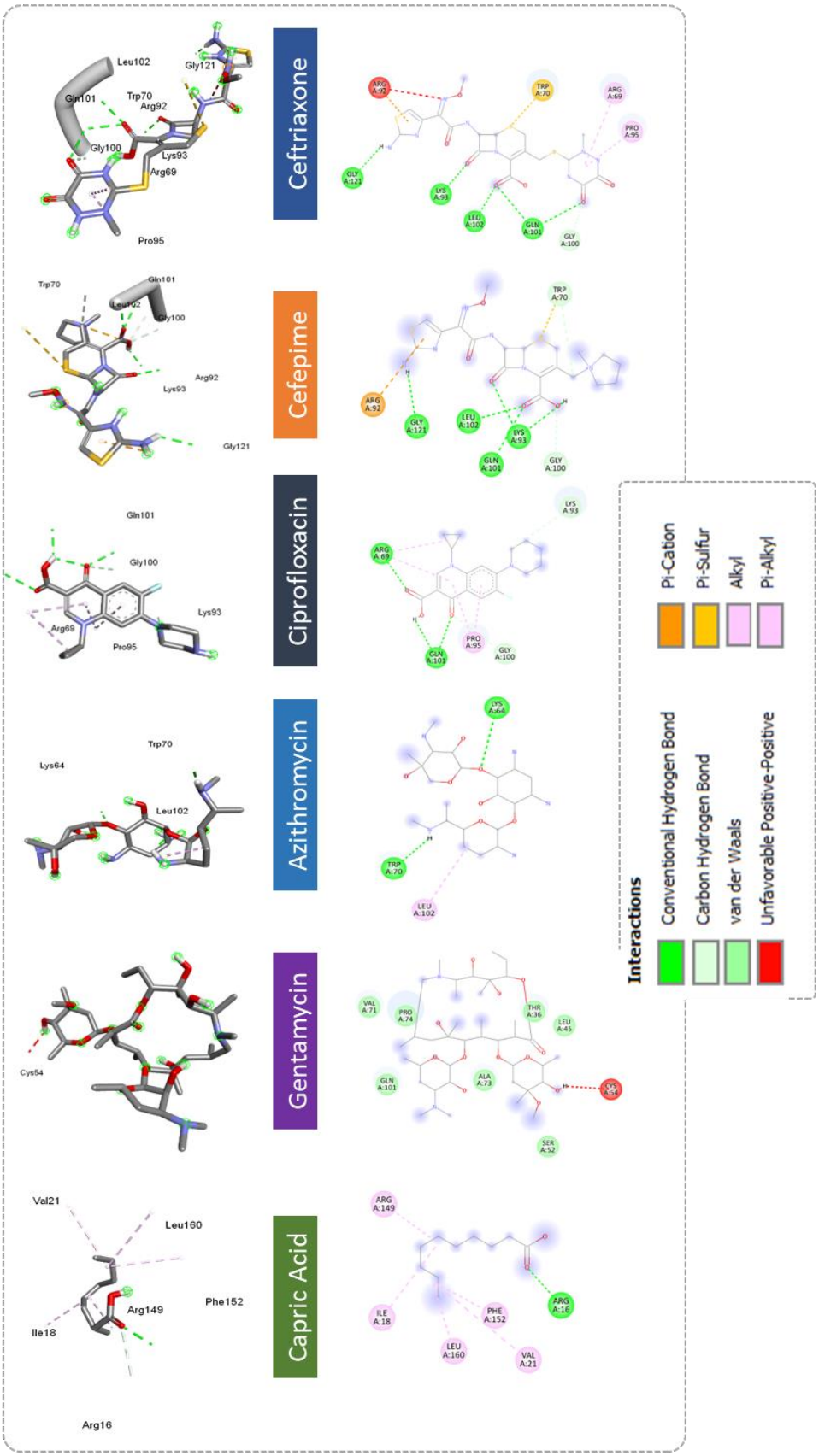

Figure 6.6: 3D and 2D visualization model of top five best docked complexes of serine protease of *A. hydrophila*.

The binding attitude of compounds with the protein model in 3D and 2D pose shown in Figure 6.6. Analysis exhibited that common residues were shared by the test compounds obtained from PyRx interaction. Amino acid residues Lys93(A), Arg92(A) and Gly121(A) were commonly present in serine protease and participated in interaction with the selected drugs.

The essential physicochemical properties of top hit compound were evaluated by using online bioinformatics server (SwissADME and PreADME). The Lipinski's rule of five, Lipophilicity, Pan-assay interference compounds (PAINS) evaluation, topological polar surface area (TPSA) and most important bioavailability properties (Table 6.5) assessed this compound as effective to proceed for experimental validation.

Table 6.5: Assessed physico-chemical properties of top hit compounds against serine protease of *A. hydrophila*

Compound	MW (g/mol)	Lipinsk' s rule of five	Lipophilicity	PAINS	TPSA (Å2)	Synthetic accessibility	Water solubility	Bioavailability score	Skin permeability (cm/s)	BBB Permeation
Ceftriaxone	554.58	0 violation	0.72	0 alert	293.80	5.06	Soluble	0.85	-10.58	No

6.5 Discussion

Proteases play a key role in various pathologies like pancreatitis, thrombosis and cancer; and low molecular compounds can act as inhibitor to fight against these disorders (enterotoxic activity) (Jedinák et al., 2006). Skwor et al. (2014) reported that the prevalence of aerolysin and serine protease virulent genes in *A. hydrophila* and *A. veronii* was comparatively high which was corresponded to pathological signs and symptoms. In the present study, the AGM2 isolate showed high pathogenicity by five pathogenic genes amplification and in experimental infection described in **Chapter 5**. Various reported antibiotics were used to control virulent *Aeromonas* spp. in aquaculture. Jedinák et al. (2006) suggested that the natural compounds of the flavonoid group showed high efficiency than other groups for protease inhibition. The development of drug design method using various bioinformatics tools increased rapidly for its high accurateness which allows to use

routinely in the interpretation of drug discovery and the experiments (Śledź and Caflisch 2018; Ge et al., 2017). Prediction methods on the binding modes and sites based on protein structure are helpful for discovery of small molecules and their binding affinities (Zhang et al., 2021). It has been stated that *in silico* drug discovery has been used to find drugs that inhibit a variety of enzymes, including alanine racemase and the AI-2 Biosynthesis of *A. hydrophila* (Ali et al., 2018, Wang et al., 2017).

Finding 3D structure of a target protein is the basic step in appropriate drug discovery. The 3D structures are used to know the structural facts and molecular functions and to determine effective inhibitors against target enzyme (Shaker et al., 2020). The 3D structure generally has N and C terminal domain and both domains are linked by a hinge (Gulick, 2009), which is consisted with the present study. Ramachandran plot analysis assessed the quality of the predicted model. In this study, most of the residues (88.1%) were grouped into the most favored region and only 2.3% residues were detected in outer region, which indicate the good quality of the predicted 3D structure. Shaker et al. (2020) found 77.5% residues in favored region of PqsA enzyme of *Pseudomonas aeruginosa* bacteria to block biofilm formation and Yadav et al. (2021) also reported 90.3% residues in the favored region of the studied aerolysin model of *A. hydrophila*. The secondary structure of the predicted model revealed helix=10.4%, strand=23.4%, loop=66.2% which is structurally more similar to the aerolysin model reported by Yadav et al., (2021).

In the present study, six reported compounds were used as reference inhibitor to find more potent compound. The sensitivity of these compounds except capric acid were tested against the studied *A. hydrophila* (AGM2) in antibiogram test that have been showed in the **previous chapter**. These compounds inhibited the growth of the studied *A. hydrophila*. The 3D model of these compounds was retrieved from Drug bank and Pubchem database. Docking between target protein and ligand plays an important role in accurate prediction and orientation of the ligands with the target proteins (Morris and Lim-Wilby, 2008). Two phase docking (docking with known inhibitors and docking with analog library) is more appropriate to obtain strong binding affinity and accurate binding poses (Shaker et al., 2020). Binding affinity includes internal energy, torsional energy and intermolecular force deducted from the boundless energy system (Shaker et al., 2020).

In this study, high binding affinity (-7.6) was found between the more potent ligand and target protein which indicate the stable bond required to inhibit protease enzyme causing

disorders. Three main chemical bonds: hydrophobic bonds, electrostatic bonds and covalent are exist between target protein (receptors) and compounds (ligands) interaction (Aisiah et al., 2020). Hydrophobic bonds are weak and important in interactions of receptors and compounds, which are soluble in fat including alkyl bonds. The covalent bonds are biologically irreversible and powerful bonds. The top hit compound Ceftriaxone showed five conventional hydrogen bonds with the significant residues, Gly121, Lus93, Leu102 and two bonds with Gln101; one carbon-hydrogen bond with Gly100; two pi-alkyl interactions with Arg69 and Pro95; one pi-sulfur bond with Trp70. In this study, the top hit ligand contained hydrophobic residue (leucine) on the surfaces which indicated the presence of antigenic determinants (Ali et al., 2018). Molecular docking results exhibited that interactive residues of six studied ligands exist in the pore of the target protein. Pore forming domain of protein helps in binding of the ligands (Yadav et al., 2021). The sharing structural motif of hit compounds participate in the interaction with the active conserved sites amino acid residues in similar docking mode (Shaker et al., 2020).

The physico-chemical properties analysis showed that the top hit compound was efficient enough to manage the infection caused by virulent bacteria. Though a drug should follow the Lipinski's rule of five (molecular weight to be <500 Da; to have less than ten H bonds for acceptor; less than five H bond for donor; high lipophilicity and molar refractivity to be between 40-130) (Lipinski, 2004). However, it is reported that an inhibitor can be efficiently orally utilized if it is not violated two or more predicting criteria of Lipinski's rule of five (Mahmudpour et al., 2021). Lipinski's rule of five is not fixed for the natural compounds (Lipinski, 2003) and they do not strictly follow the Lipinski's rule (Mahmudpour et al., 2021). The natural compounds try to keep low hydrophobicity along with their intermolecular H bonds during interactions (Ganesan, 2008).

6.6 Conclusion

The various parameters validated the compound Ceftriaxone as effective inhibitor against serine protease of *A. hydrophila* (AGM2) by different bioinformatics tools. Combination of both wet lab experiment and computer aided drug design method (screening of large-scale molecule) could be a useful, efficient way to find out potent inhibitor against protease and will be helpful for the development of novel antibiotics to control *A. hydrophila* in aquaculture systems.

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Chapter 7
Summary and Conclusions

7.1 Summary and Conclusion

Farmed stinging catfish are very much vulnerable to various microorganisms exist in their body and surrounding environment. Therefore, study of the microbiome in this aquaculture species is crucial for better health management. The current study started with the isolation of the bacterial community from healthy and diseased *H. fossilis* fish tissues and associated environment. The healthy fish tissues, particularly skin mucus showed comparatively higher bacterial load than that of diseased fish for performing various body functions. Both pathogenic and non-pathogenic bacteria were isolated which suggested that various environmental stress were actively involved in causing infection in fish body. Total 42 selected isolates were identified by classical and molecular methods. Most of them were sensitive to Gentamycin (CN) (95.24%) and Chloramphenicol (C) (90.48%). *Aeromonas* spp., *Klebsiella* spp., *Staphylococcus* spp., *Enterobacter* spp., *Bacillus* spp. were dominant in the fish tissue and in the environment.

The MAS infected fish exhibited red spot signs and necrotic tissues especially in the skin and fins, and had excess mucus in the whole body. Therefore, the study focused on the balanced and shifting microbiome community in the healthy and diseased stinging catfish skin that gives protection against pathogenic bacteria by culture dependent and culture independent metagenomics methods. Skin microbiome clustering for the healthy and diseased fish indicated that dominant beneficial bacterial community protected the healthy fish and abundant pathogenic community caused infection in diseased fish. The unbiased culture independent method was able to identify the whole bacterial community and their functions to maintain the health status of fish. The study showed the highly abundant presence of *Pseudomonas* sp. in the healthy skin though this organism plays both pathogenic and beneficial roles for fish. Pathogenic *Flavobacterium* sp., on the other hand was highly rich in diseased fish and was nearly absent in the healthy fish.

From the above experiments, the pathogenic bacteria *A. hydrophila* was reported in both fish tissue and environment. Molecular characterization of this specific pathogen demonstrated the pathogenicity (amplification of reported five pathogenic genes) and host-pathogen interactions (challenge test). The isolates showed high resistance pattern in antibiogram indicating an alarming threat for aquaculture. Two isolates (AGM2 and AYN7) of *A. hydrophila* caused infection in healthy stinging catfish in experimental infection. Clinical signs and symptoms (lesions, excess mucus) were observed in the post

injected fish. Re-isolation and confirmation of the two isolates were performed from experimentally infected fish by molecular method.

A potential inhibitor against the virulence factors of *A. hydrophila* could be very effective way to control as an alternative way of using uncontrolled antibiotics in catfish aquaculture. Besides, molecular docking was applied to find effective drug-like compounds. In docking, top hit Ceftriaxone exhibited very efficient compound (-7.6 binding affinity) to control the virulence factor (serine protease) of pathogenic *A. hydrophila*. The 3D structure of the predicted model was validated by several bioinformatics tools including Ramachandran plot analysis which is an effective assessment for selecting appropriate model. In addition, physicochemical properties of top hit compound defined the physical attributes properly and efficiently. The essential Lipinski's rule of five, Lipophilicity, PAINS evaluation, topological polar surface area (TPSA) and important bioavailability properties were also followed by the predicted model.

In conclusion, identification of pathogenic and beneficial bacteria from stinging catfish body and associated environment will be helpful in controlling pathogenic disease and for better fish health management. Metagenomics analysis based on 16S rRNA gene of healthy and diseased stinging catfish skin microbiome is reported as the first study in Bangladesh. The potential drug-like compounds identified from molecular docking and efficacy analysis could also be applicable as an effective alternative way of finding new drugs as well as to minimize the indiscriminate usage of antibiotic in aquaculture farms.

7.2 Limitations and further studies

This dissertation inspects on the beneficial and pathogenic microbiome community through culture dependent and independent methods and on the identification of possible inhibitors against virulence factor of pathogenic *A. hydrophila*. One major limitation of this study is the isolation and analysis of small number of bacteria from the studied samples. Although 16S rRNA metagenomics analysis of skin microbiome was performed through culture independent method but preservation of the identified bacteria is not possible for further analysis by culture process. Furthermore, dominant organisms in diseased fish lesion from culture independent method could not be verified by challenge test. So, a greater number of bacterial isolates should be analyzed from individual colony rather than merged colony, although, the process is expensive and time consuming. The pathogenic *A. hydrophila* was confirmed by 16S rRNA gene sequencing only, however, the virulent strains of this

pathogen need to decode by whole genome sequence for precise identification of pathogenic genes and their functional studies and outbreak patterns.

On the other hand, a limited number of inhibitory compounds was used for the *in-silico* drug design against serine protease domain of the virulent gene product. Bioactive compounds from herbal origin against various virulent factors of *A. hydrophila* should be identified via *in silico* analysis and validated through laboratory experiments. Histopathological study is essential to confirm the infection level in the body tissue of artificially infected and control fish used in the challenge test and it is also essential to confirm the specific antigen by immuno-histopathological analysis. It is planned to perform this kind of research experiments and in this regard, the infected and control tissue from the challenge test has been preserved in 10% formalin. Besides, experimental infection with other pathogenic bacteria is also essential to find out their pathogenicity. In addition, it is speculated that, imbalance between beneficial and pathogenic bacteria might cause various diseases; however, microbiome balance shifting from beneficial to pathogenic bacterial species should be experimented in a specific culture system of an aquaculture species.

Appendices

Appendix 1. Media preparation for bacterial isolation

Table A1.1 Alkaline Peptone Water for 1 Liter (pH 8.6±0.2)

Name of the ingredients	Concentration
Peptone	10.0 gm
Sodium Chloride (NaCl)	20.0 gm
Distilled water	1000 ml

Table A1.2 Nutrient Agar for 1 Liter (pH 7.4±0.2)

Name of the ingredients	Concentration
Peptic digest of animal tissue	5.0 gm
Sodium Chloride (NaCl)	5.0 gm
Beef extract	1.50 gm
Yeast extract	1.50 gm
Agar	15.0gm
Distilled water	1000 ml

Table A1.3 Nutrient Broth for 1 Liter (pH 7.4±0.2)

Name of the ingredients	Concentration
Peptic digest of animal tissue	5.0 gm
Sodium Chloride (NaCl)	5.0 gm
Beef extract	1.50 gm
Yeast extract	1.50 gm
Distilled Water	1000 ml

Table A1.4 Tryptic Soy Agar for 1 Liter

Name of the ingredients	Concentration
Peptic digest of casein	15.00 gm
Enzymatic digest of soya bean	5.00 gm
Sodium Chloride	5.00 gm
Agar	15.00 gm

Table A1.5 Tryptic Soy Broth for 1 Liters

Name of the ingredients	Concentration
Peptic digest of casein	17.00 gm
Enzymatic digest of soya bean	3.00 gm
Sodium Chloride	5.00 gm
Di-potassium hydrogen phosphate	2.50 gm
Glucose	2.50 gm
Distilled water	1000 ml

Table A1.6 MR-VP Broth for 1 Liter (pH 7.0±0.2)

Name of the ingredients	Concentration
Peptone	10.0 gm
Sodium Chloride (NaCl)	20.0 gm
Distilled water	1000 ml
Name of the ingredients	Concentration
Peptone	10.0 gm
Sodium Chloride (NaCl)	20.0 gm
Distilled water	1000 ml

Table A1.7 Simmons Citrate Medium (pH 6.8±0.2)

Name of the ingredients	Concentration
Magnesium sulphate (heptahydrate)	0.20 gm
Ammonium dihydrogen phosphate	1.0 gm
Dipotassium phosphate	1.0 gm
Sodium citrate (dehydrate)	2.0 gm
Sodium Chloride	5.0 gm
Bromothymol blue	0.08 gm
Agar	15.0 gm
Distilled water	1000 ml

Table A1.8 Rimler-Shotts Medium Base (Final pH 7.0±0.2 @25°C)

Name of the ingredients	Concentration
Yeast Extract L-Lysine	3.00 gm
Maltose	3.50 gm
L-Cysteine hydrochlorie	0.30 gm
HydroChloride	5.00 gm
L-Omithine monohydroChloride	6.50 gm
Sodium thiosulphate	6.80 gm
Ferric ammonium citrate	0.80 gm
Sodium deoxycolate	1.00 gm
Sodium Chloride	5.00 gm
Bromothymol blue	0.03 gm
Agar	13.50 gm
Distilled water	990 ml

Table A1.9 MacConkey Agar (pH 7.1±0.2 @25°C)

Name of the ingredients	Concentration
Peptone	20.00 gm
Lactose	10.00 gm
Bile salts No.3	1.50 gm
Sodium Chloride	5.00 gm
Neutral red	0.03 gm
Crystal violet	0.001 gm
Agar	15.00 gm
Distilled Water	1000 ml

Appendix 2. Reagents and solutions for biochemical test

i. Solutions for Gram staining	
A. Crystal Violet Reagent (Primary stain) Solution I	
Name of the ingredients	**Concentration**
Crystal violet (certified 90% dye content)	2.0 gm
Ethanol, 95% (v/v)	20.0 ml
Crystal Violet Reagent (Primary stain) Solution II	
Ammonium oxalate (monohydrate)	0.80 gm
Distilled Water	80.0 ml
Solution I was mixed with solution II to obtain crystal violet staining reagent, stored for 24 hours and filtered through paper prior to use.	
B. Gram's Iodine (Mordant)	
Iodine	1.0 gm
Potassium iodide	2.0 gm
Distilled water	97 ml
This solution was stored in an amber bottle.	
C. Decolorizing Agent: Ethanol 95% (v/v)	
D. Safranin (Counterstain)	
• Stock Solution: 2.50 gm safranin dissolved in 100 ml ethanol (95%) (v/v) • Working Solution: 10 ml stock solution dissolved in 90 ml distilled water.	
ii. String Test Reagent (KOH 3%)	
KOH (1x)	3.00 g
Distilled Water	70.0ml
Catalase Test Reagent (3%H_2O_2)	
Methanol	9 ml
Hydrogen Peroxide $H_2O_2$30%(v/v)	1 ml
iii. Kovac's Reagent	
Amyl or isoamyl or butyl alcohol	150 ml

DMAB (para-dimethylaminobenzaldehyde)	10.0 gm
HCl (concentrated)	50 ml

iv. Methyl Red Solution

Methyl red	0.02 gm
Ethanol	95%(v/v) 60 ml

40 ml of deionized water was then added to make 100 ml of 0.05% (wt/vol) solution and stored at 4°c.

v. Voges-Proskauer Reagent

• Barrit's Reagent A: 5% (w/v) α-naphthol in absolute ethanol.
• Barrit's Reagent B: 40% (w/v) KOH in deionized water (this might be replaced by 40% (w/v) NaOH solution).
Above reagents must be prepared and used in fresh condition. They are also referred to as VP-1 and VP-2 or Vp-A and Vp-B

vi. Oxidase Test Reagent

Tetra--methyl-p-phenylenediaminedihydroChloride	1%
Distilled water	99 ml

Appendix 3. Reagents needed for DNA extraction

i) Lysis buffer

Required reagents
25mM TrisHCl, 100mM EDTA and 0.5% SDS

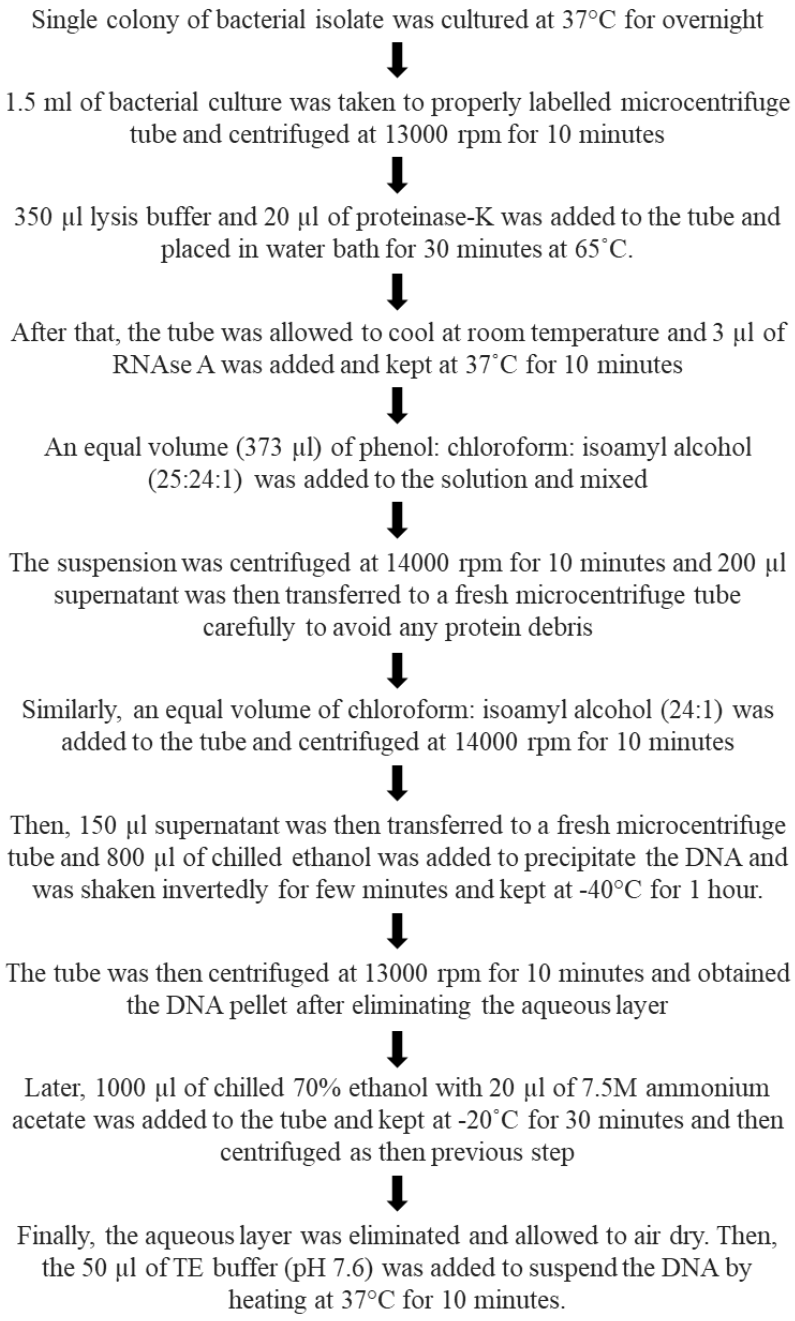
Stock solution (for 500ml)	
25mM TrisHCl	1.97 gram
EDTA	18.6 gram

Mixing was done with 400 ml distilled water properly than pH was adjusted to 8 with HCl if higher than 8 and NaOH if lower than 8. After that autoclaved the solution and added 2.5 gm SDS solution. Then distilled water was added to make 500 ml final volume

ii) Proteinase K-20mg powder was dissolved in 1 ml proteinase K buffer

iii) RNAase- Ready RNAase was used

iv) DNA extraction protocol



Appendix 4. Composition and Preparation of reagents for Agarose gel electrophoresis

i) **Electrophoresis buffer (50X TAE buffer)-** 1X TAE buffer was prepared from ready 50X TAE buffer

ii) Gel loading buffer

Bromophenol blue - 0.5%
Glycerol (mol. Grade)- 30%
Prepared in 1X TAE and stored at 4°C.

iii) Ethidium Bromide Solution

Ethidium bromide - 10 mg
Distilled water - 2 ml

iv) 1.5% agarose gel preparation

Agarose- 0.3 gm
TAE buffer (1X)- 20 ml

Appendix 5. Water quality parameters analysis

Table A5.1: Water quality parameters of water samples collected from 15 locations

Parameters / Locations	Temperature (°C)	Dissolved oxygen DO (ppm)	pH	Ammonia (NH_3)	Total dissolved solids (ppm)
Standard	28-30 (Wynn, 2003)	5-12 (Banerjea, 1967)	6.5-8.5 (Swingle, 1967)	0.05-0.15 (Moyle, 1949)	<400 (Wynn, 2003)
Dinajpur (Jhanjira)	25.93 ± 0.4	14.22 ± 0.4	6.57 ± 0.4	1 ± 0.5	381.83 ± 6.55
Bhairab (Ghorakanda)	25 ± 0.4	16 ± 0.4	7 ± 0.4	1 ± 0.5	376.6 ± 12.03
Muktagacha (Ghoga)	27.13 ± 0.42	15.17 ± 0.42	6.67 ± 0.42	1.5 ± 0.5	345.17 ± 13
Trishal (Bali Para)	22.07 ± 0.4	14.59 ± 0.4	7.13 ± 0.4	1.5 ± 0.5	314.55 ± 14.03
Bhaluka (Dhitpur)	23.07 ± 0.5	17.33 ± 0.5	7.23 ± 0.5	1.5 ± 0.5	307.37 ± 15
Ishwarganj (Madhupur)	20.93 ± 0.4	15.27 ± 0.4	8.07 ± 0.4	2 ± 0	311.85 ± 13.03
Ishwarganj (Barahit)	22.1 ± 0.36	15.2 ± 0.36	7.4 ± 0.36	2 ± 0	334.27 ± 12.51
Sonargaon (Char-Kamaldi)	20 ± 0.3	16 ± 0.3	7.3 ± 0.3	2 ± 0	313.83 ± 12.51
Munshiganj (Kanakshar)	17.9 ± 0.36	17.3 ± 0.36	7.7 ± 0.36	2 ± 0	357.4 ± 14.6
Noakhali (Begumganj)	16.07 ± 0.4	14.83 ± 0.4	6.83 ± 0.4	2 ± 0.5	309.63 ± 12.55
Narshingdi (Bhelanagar)	14.93 ± 0.5	15.32 ± 0.5	7.27 ± 0.5	2 ± 0	345.23 ± 11.56
Kurigram (Ulipur)	17.87 ± 0.51	16.13 ± 0.51	7.53 ± 0.51	2 ± 0.5	314.13 ± 12.51
Faridpur (Sadarpur)	17.9 ± 0.36	16.4 ± 0.36	6.9 ± 0.36	2 ± 0	282.27 ± 14.15
Gazipur (Kaliakair)	20 ± 0.4	14.5 ± 0.4	6.9 ± 0.4	2 ± 0	287.03 ± 12.09
Natore (Singra)	19.1 ± 0.36	15.2 ± 0.36	7.2 ± 0.36	1.5 ± 0.5	217.4 ± 13.5

Appendix 6. Bacterial load, morphology and identification

Table A6.1: Observed cfu of water, soil and fish (healthy and diseased) tissue (skin, liver, intestine, gill

Farm name	Soil (cfu/g)	Water (cfu/ml)	Fish organ (cfu/g)			
			Skin (H/D)	Intestine(H/D)	Liver (H/D)	Gill(H/D)
Dinajpur (Jhanjira)	$2.79 \pm 0.24\times10^{6}$	$3.24 \pm 0.24\times10^{6}$	$2.74 \pm 0.40\times10^{8}$/ $1.06 \pm 0.92\times10^{6}$	$2.69 \pm 0.80\times10^{8}$/ $1.15 \pm 0.36\times10^{5}$	$2.14 \pm 0.17\times10^{8}$/ $8.17 \pm 0.58\times10^{4}$	$2.69 \pm 0.70\times10^{8}$/ $9.60 \pm 0.95\times10^{5}$
Bhairab (Ghorakanda)	$5.14 \pm 0.24\times10^{6}$	$2.20 \pm 0.24\times10^{7}$	$2.39 \pm 0.39\times10^{8}$/ $1.05 \pm 1.02\times10^{8}$	$2.34 \pm 0.80\times10^{8}$/ $5.53 \pm 5.34\times10^{7}$	$1.78 \pm 0.17\times10^{8}$/ $4.88 \pm 0.84\times10^{7}$	$2.33 \pm 0.70\times10^{8}$/ $1.03 \pm 0.54\times10^{8}$
Muktagacha (Ghoga)	$2.27 \pm 0.24\times10^{6}$	$2.72 \pm 0.24\times10^{6}$	$2.82 \pm 0.05\times10^{8}$/ $1.43 \pm 1.07\times10^{7}$	$2.53 \pm 0.40\times10^{8}$/ $2.85 \pm 0.25\times10^{6}$	$8.16 \pm 0.34\times10^{7}$/ $1.45 \pm 0.33\times10^{6}$	$1.41 \pm 0.23\times10^{8}$/ $2.07 \pm 0.23\times10^{6}$
Trishal (Bali Para)	$4.62 \pm 0.24\times10^{6}$	$2.15 \pm 0.24\times10^{7}$	$2.71 \pm 0.41\times10^{8}$/ $1.19 \pm 1.03\times10^{7}$	$2.64 \pm 0.67\times10^{8}$/ $1.20 \pm 0.93\times10^{7}$	$1.81 \pm 0.24\times10^{8}$/ $2.48 \pm 0.17\times10^{6}$	$1.67 \pm 0.26\times10^{8}$/ $7.44 \pm 0.72\times10^{7}$
Bhaluka (Dhitpur)	$3.26 \pm 0.22\times10^{6}$	$3.71 \pm 0.22\times10^{6}$	$2.80 \pm 0.14\times10^{8}$/ $3.03 \pm 0.30\times10^{6}$	$3.23 \pm 0.04\times10^{8}$/ $2.63 \pm 0.67 \pm \times10^{6}$	$2.36 \pm 0.53\times10^{8}$/ $2.56 \pm 0.49\times10^{6}$	$2.61 \pm 0.22\times10^{8}$/ $9.31 \pm 0.79\times10^{6}$
Ishwarganj (Madhupur)	$5.57 \pm 0.19\times10^{6}$	$2.24 \pm 0.19\times10^{7}$	$1.01 \pm 0.13\times10^{8}$/ $1.31 \pm 0.57\times10^{6}$	$1.43 \pm 0.34\times10^{8}$/ $5.08 \pm 0.38\times10^{7}$	$9.00 \pm 0.06\times10^{7}$/ $1.11 \pm 0.51\times10^{6}$	$1.38 \pm 0.32\times10^{8}$/ $5.64 \pm 0.40\times10^{6}$
Ishwarganj (Barahit)	$2.70 \pm 0.19\times10^{6}$	$3.15 \pm 0.19\times10^{6}$	$1.23 \pm 0.15\times10^{8}$/ $1.96 \pm 0.96\times10^{8}$	$1.73 \pm 0.33\times10^{8}$/ $2.96 \pm 0.12\times10^{8}$	$1.21 \pm 0.04\times10^{8}$/ $1.98 \pm 0.12\times10^{8}$	$1.50 \pm 0.42\times10^{8}$/ $1.80 \pm 0.27\times10^{8}$
Sonargaon (Char-Kamaldi	$5.05 \pm 0.19\times10^{6}$	$2.19 \pm 0.19\times10^{7}$	$1.01 \pm 0.14\times10^{8}$/ $1.92 \pm 0.82\times10^{6}$	$1.49 \pm 0.08\times10^{8}$/ $7.45 \pm 4.99\times10^{5}$	$1.07 \pm 0.07\times10^{8}$/ $1.46 \pm 0.05\times10^{6}$	$1.13 \pm 0.14\times10^{8}$/ $8.43 \pm .63\times10^{5}$
Munshiganj (Kanakshar)	$3.32 \pm 0.19\times10^{5}$	$2.24 \pm 0.19\times10^{7}$	$7.46 \pm 0.14\times10^{7}$/ $2.46 \pm 0.57\times10^{6}$	$1.23 \pm 0.08\times10^{8}$/ $5.89 \pm 0.43\times10^{5}$	$8.09 \pm 0.08\times10^{7}$/ $3.50 \pm 3.04\times10^{7}$	$8.63 \pm 0.14\times10^{7}$/ $3.47 \pm 3.02\times10^{7}$
Noakhali (Begumganj)	$2.87 \pm 0.19\times10^{7}$	$3.21 \pm 0.19\times10^{6}$	$8.13 \pm 0.10\times10^{7}$/ $2.23 \pm 1.10\times10^{8}$	$8.63 \pm 0.02\times10^{7}$/ $1.73 \pm 0.86\times10^{8}$	$4.40 \pm 0.07\times10^{7}$/ $1.46 \pm 0.48\times10^{8}$	$5.30 \pm 0.90\times10^{7}$/ $6.51 \pm 0.63\times10^{7}$
Narshingdi (Bhelanagar)	$5.09 \pm 0.21\times10^{6}$	$2.19 \pm 0.21\times10^{7}$	$1.29 \pm 0.14\times10^{8}$/ $9.36 \pm .90\times10^{7}$	$1.77 \pm 0.08\times10^{8}$/ $4.90 \pm 0.45\times10^{7}$	$1.36 \pm 0.08\times10^{8}$/ $7.09 \pm 0.69\times10^{7}$	$1.41 \pm 0.14\times10^{8}$/ $5.93 \pm 0.58\times10^{7}$
Kurigram (Ulipur)	$3.85 \pm 0.26\times10^{7}$	$4.23 \pm 0.26\times10^{6}$	$1.36 \pm 0.11\times10^{8}$/ $1.14 \pm 1.01\times10^{6}$	$1.41 \pm 0.02\times10^{8}$/ $2.03 \pm 0.42\times10^{5}$	$9.89 \pm 0.07\times10^{7}$/ $5.47 \pm 2.76\times10^{7}$	$1.08 \pm 0.09\times10^{8}$/ $3.72 \pm 0.37\times10^{7}$
Faridpur (Sadarpur)	$6.13 \pm 0.26\times10^{6}$	$2.30 \pm 0.24\times10^{7}$	$1.11 \pm 0.11\times10^{8}$/ $3.01 \pm 0.23 \pm \times10^{6}$	$1.16 \pm 0.02\times10^{8}$/ $1.60 \pm 0.69\times10^{6}$	$7.34 \pm 0.07\times10^{7}$/ $7.00 \pm 0.53\times10^{5}$	$8.24 \pm 0.09\times10^{7}$/ $1.81 \pm 0.81\times10^{5}$
Gazipur (Kaliakair)	$3.26 \pm 0.26\times10^{6}$	$3.71 \pm 0.26\times10^{6}$	$1.11 \pm 0.86\times10^{8}$/ $8.02 \pm 0.78\times10^{7}$	$2.31 \pm 0.49\times10^{8}$/ $2.53 \pm 0.29\times10^{8}$	$2.63 \pm 0.17\times10^{8}$/ $1.93 \pm 0.13\times10^{8}$	$1.14 \pm 0.81\times10^{8}$/ $1.16 \pm 0.61\times10^{8}$
Natore (Singra)	$5.61 \pm 0.26\times10^{6}$	$8.68 \pm 0.26\times10^{6}$	$2.87 \pm 0.04\times10^{7}$/ $2.31 \pm 0.99\times10^{6}$	$2.82 \pm 0.81\times10^{8}$/ $1.86 \pm 0.94\times10^{8}$	$2.27 \pm 0.17\times10^{8}$/ $4.68 \pm 0.35\times10^{6}$	$2.26 \pm 1.16\times10^{8}$/ $4.65 \pm 0.45\times10^{7}$

Table A6.2: Characterization of 42 isolates based on their morphology obtained from healthy and diseased fish tissue, soil and water samples collected from 15 locations of Bangladesh

Sl. No.	Isolates ID	Size	Shape	Elevation	Pigment	Edges	Optical Property
1	SSF 1	Small	Circular	Convex	White	Entire	Opaque
2	SSF 2	Small	Circular	Convex	White	Entire	Translucent
3	SSF 3	Moderate	Circular	Raised	Tan	Entire	Opaque
4	SSF 4	Small	Circular	Convex	White	Entire	Opaque
5	SSF 5	Moderate	Irregular	Raised	Tan	Rhizoid	Translucent
6	SSF 6	Small	Circular	Raised	Yellow	Entire	Opaque
7	SSF 7	Punctiform	Circular	Raised	Yellow	Entire	Opaque
8	SSF 8	Large	Irregular	Pulvinate	White	Undulate	Opaque
9	SSF 9	Moderate	Irregular	Raised	Greenish	Filamentous	Translucent
10	SSF 10	Moderate	Irregular	Raised	White	Entire	Opaque
11	SSF 11	Moderate	Irregular	Raised	White	Lobed	Opaque
12	SSF 12	Small	Rhizoid	Raised	Yellow	Undulate	Opaque
13	SSF 13	Large	Irregular	Raised	Yellow	Entire	Opaque
14	SSF 14	Punctiform	Circular	Raised	White	Entire	Translucent
15	SSF 15	Large	Irregular	Convex	White	Entire	Opaque
16	SSF 16	Moderate	Irregular	Raised	Cream	Entire	Opaque
17	SSF 17	Large	Filamentous	Flat	Cream	Lobed	Translucent
18	SSF 18	Large	Circular	Raised	Greenish	Entire	Opaque
19	SSF 19	Moderate	Circular	Umbonate	Greenish	Entire	Opaque
20	SSF 20	Small	Irregular	Convex	White	Undulate	Opaque
21	SSF 21	Large	Irregular	Umbonate	White	Curled	Opaque
22	SSF 22	Large	Irregular	Raised	Yellow	Curled	Opaque
23	SSF 23	Small	Circular	Convex	White	Entire	Translucent
24	SSF 24	Moderate	Circular	Convex	Tan	Entire	Opaque
25	SSF 25	Moderate	Irregular	Raised	Yellow	Entire	Opaque
26	SSF 26	Small	Circular	Raised	White	Curled	Opaque

Sl. No.	Isolates ID	Size	Shape	Elevation	Pigment	Edges	Optical Property
27	SSF 27	Small	Circular	Raised	White	Entire	Translucent
28	SSF 28	Small	Circular	Convex	White	Entire	Opaque
29	SSF 29	Moderate	Circular	Umbonate	Greenish	Entire	Opaque
30	SSF 30	Moderate	Circular	Umbonate	Greenish	Entire	Opaque
31	SSF 31	Small	Circular	Convex	White	Entire	Translucent
32	SSF 32	Moderate	Circular	Umbonate	Greenish	Entire	Opaque
33	SSF 33	Small	Circular	Convex	White	Entire	Translucent
34	SSF 34	Small	Circular	Convex	White	Entire	Translucent
35	SSF 35	Small	Circular	Convex	Pink	Entire	Opaque
36	SSF 36	Punctiform	Circular	Raised	White	Entire	Translucent
37	SSF 37	Moderate	Circular	Umbonate	Greenish	Entire	Opaque
38	SSF 38	Small	Circular	Convex	White	Entire	Translucent
39	SSF 39	Moderate	Circular	Convex	White	Entire	Opaque
40	SSF 40	Moderate	Circular	Umbonate	Greenish	Entire	Opaque
41	SSF 41	Small	Circular	Convex	White	Entire	Opaque
42	SSF 42	Moderate	Circular	Convex	White	Entire	Opaque

Table A6.3: Results of biochemical tests of 42 isolates obtained from healthy and diseased fish tissue, soil and water samples collected from 15 locations of Bangladesh

Sl. No	Bacterial Types	Indole	MR	VP	Oxidase	Citrate	Lactose Fermentative
1	SSF 1	-	+	+	-	+	+
2	SSF 2	-	+	+	-	+	+
3	SSF 3	+	-	-	+	+	+
4	SSF 4	+	+	-	+	-	+
5	SSF 5	+	+	-	+	+	-
6	SSF 6	-	-	+	-	-	-
7	SSF 7	-	-	+	-	+	+
8	SSF 8	+	+	-	-	-	-
9	SSF 9	+	-	-	+	+	-

10	SSF 10	-	-	-	-	+	-
11	SSF 11	-	-	+	-	-	+
12	SSF 12	-	-	-	-	-	-
13	SSF 13	+	-	-	-	+	+
14	SSF 14	-	+	-	-	+	-
15	SSF 15	-	-	-	+	+	-
16	SSF 16	-	-	+	-	-	+
17	SSF 17	+	+	-	+	+	-
18	SSF 18	+	-	-	+	+	-
19	SSF 19	+	+	-	+	+	-
20	SSF 20	-	-	+	+	-	-
21	SSF 21	+	+	-	-	+	-
22	SSF 22	-	-	-	-	-	-
23	SSF 23	+	+	-	-	-	+
24	SSF 24	-	-	-	-	+	+
25	SSF 25	-	-	-	-	+	+
26	SSF 26	-	-	-	-	-	+
27	SSF 27	-	+	+	+	+	+
28	SSF 28	-	+	+	-	+	+
29	SSF 29	+	+	-	+	+	-
30	SSF 30	+	-	-	+	+	-
31	SSF 31	-	+	+	-	+	+
32	SSF 32	+	+	-	+	+	-
33	SSF 33	-	+	+	-	+	+
34	SSF 34	-	+	+	-	+	+
35	SSF 35	-	+	+	-	+	-
36	SSF 36	+	+	-	-	+	+
37	SSF 37	+	-	-	+	+	-
38	SSF 38	-	+	+	-	+	+
39	SSF 39	-	+	+	-	+	-
40	SSF 40	+	+	-	+	+	-
41	SSF 41	+	+	-	+	-	+
42	SSF 42	+	+	-	+	-	+

Table A6.4: Identification of 42 isolates by biochemical and molecular method obtained from healthy and diseased fish tissue, soil and water samples collected from 15 locations of Bangladesh

Isolate ID	Presumptive identification	Molecular identification	No. of base pairs used for molecular identification	Total score	Query cover (%)	E value	Identity matched (%)	Gen Bank Accession No. of corresponding sequence	GenBank Accession No. of isolates under present study
SSF 01	*Klebsiella sp.*	*Klebsiella pneumoniae*	994	1703	96%	0.0	98.75%	KR269806.1	OL336423
SSF 02	*Enterobacter spp.*	*Enterobacter ludwigii*	1011	1716	98%	0.0	97.99%	KF817747.1	OL336424
SSF 03	*Pseudomonas sp.*	*Pseudomonas aeruginosa*	1481	10545	100%	0.0	98.78%	CP093021.1	OL336425
SSF 04	*Enterobacter spp.*	*Aeromonas hydrophila*	971	1622	99%	0.0	97.20%	MF111291.1	OL336426
SSF 05	*Pseudomonas sp.*	*Pseudomonas mendocina*	1269	2318	99%	0.0	99.61%	MT094406.1	OL336427
SSF 06	*Staphylococcus sp.*	*Staphylococcus aureus*	1433	15862	100%	0.0	100.00%	CP095112.1	OL336428
SSF 07	*Staphylococcus spp.*	*Staphylococcus aureus*	1498	2663	100%	0.0	98.73%	OP364883.1	OL336429
SSF 08	*Bacillus sp.*	*Bacillus licheniformis*	1459	2595	99%	0.0	98.77%	AY842871.1	OL336430
SSF 09	*Flavobacterium columnare*	*Acinetobacter soli*	1023	10762	99%	0.0	91.38%	CP016896.1	OL336431
SSF 10	*Bacillus sp.*	*Bacillus cereus*	1539	42046	100%	0.0	99.68%	CP040334.1	OL336432

SSF 11	*Enterobacter spp.*	*Clostridium tetani*	1508	2785	100%	0.0	100.00%	NR_029260.1	OL336433
SSF 12	*Staphylococcus sp.*	*Staphylococcus aureus*	1455	2388	98%	0.0	96.44%	MK809243.1	OL336434
SSF 13	*Edwardsiella sp.*	*Flavobacterium columnare*	1472	2590	100%	0.0	98.38%	GU296112.1	OL336435
SSF 14	*Salmonella spp.*	*Morganella morganii*	1502	2712	99%	0.0	99.20%	KR094121.1	OL336436
SSF 15	*Bacillus spp.*	*Bacillus subtilis*	1498	2760	100%	0.0	99.87%	MT539995.1	OL336437
SSF 16	*Bacillus spp.*	*Bacillus circulans*	1517	2748	100%	0.0	99.34%	KC621293.1	OL336438
SSF 17	*Pseudomonas spp.*	*Pseudomonas aeruginosa*	1439	2553	100%	0.0	98.26%	KF769537.1	OL336439
SSF 18	*Pseudomonas sp.*	*Pseudomonas aeruginosa*	1474	2723	100%	0.0	100.00%	OP077207.1	OL336440
SSF 19	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	1411	2049	99%	0.0	90.25%	MF661883.1	OL336441
SSF 20	*Bacillus sp.*	*Bacillus subtilis*	1404	2588	100%	0.0	99.93%	MT043898.1	OL336442
SSF 21	*Bacillus sp.*	*Bacillus sp.*	937	1676	100%	0.0	98.83%	EU236745.1	OL336443
SSF 22	*Bacillus sp.*	*Staphylococcus sp.*	1455	2388	98%	0.0	96.44%	MK809243.1	OL336444
SSF 23	*Staphylococcus epidermidis*	*Escherichia sp.*	1489	2669	100%	0.0	98.72%	KR150992.1	OL336445
SSF 24	*Escherichia sp.*	*Bacillus sp.*	1475	2724	100%	0.0	100.00%	OL636030.1	OL336446
SSF 25	*Bacillus amyloliquefaciens*	*Bacillus sp.*	1480	2488	100%	0.0	96.86%	KP307834.1	OL336447

SSF 26	*Bacillus sp.*	*Bacillus megaterium*	1495	2758	100%	0.0	99.93%	KU605234.1	OL336448
SSF 27	*Bacillus megaterium*	*Enterobacter tabaci*	954	1718	100%	0.0	98.95%	MT613362.1	OL336449
SSF 28	*Enterobacter tabaci*	*Klebsiella pneumoniae*	1195	2165	100%	0.0	99.16%	MN166182.1	OL336450
SSF 29	*Klebsiella pneumoniae*	*Pseudomonas putida*	1007	1836	99%	0.0	99.60%	MZ203822.1	OL336451
SSF 30	*Pseudomonas taiwanensis*	*Pseudomonas monteilii*	1215	2237	100%	0.0	99.92%	MT605299.1	OL336452
SSF 31	*Pseudomonas monteilii*	*Enterobacter hormaechei*	1298	2329	99%	0.0	99.15%	MH029824.1	OL336453
SSF 32	*Enterobacter cloacae*	*Pseudomonas fluorescens*	1240	2278	100%	0.0	99.84%	MT605299.1	OL336454
SSF 33	*Pseudomonas monteilii*	*Enterobacter cloacae*	1085	1975	100%	0.0	99.35%	MT613377.1	OL336455
SSF 34	*Enterobacter cloacae*	*Enterobacter cloacae*	1261	2302	100%	0.0	99.52%	MT613371.1	OL336456
SSF 35	*Enterobacter cloacae*	*Serratia marcescens*	1271	2322	99%	0.0	99.76%	MN889394.1	OL336457
SSF 36	*Serratia marcescens*	*Morganella morganii*	1259	2302	100%	0.0	99.60%	MN744697.1	OL336458
SSF 37	*Morganella morganii*	*Pseudomonas monteilii*	1134	2002	95%	0.0	99.91%	MT605299.1	OL336459
SSF 38	*Pseudomonas monteilii*	*Enterbacter ludwigii*	1293	2338	100%	0.0	99.07%	JQ682629.1	OL336460
SSF 39	*Enterobacter ludwigii*	*Leclercia sp.*	980	1547	99%	0.0	93.20%	MZ707713.1	OL336461

SSF 40	*Citrobacter freundii*	*Pseudomonas mendocina*	1524	2752	100%	0.0	99.21%	MF321766.1	OL336462
SSF 41	*Pseudomonas mendocina*	*Aeromonas hydrophila*	1459	2656	100%	0.0	99.45%	MG428919.1	OL336463
SSF 42	*Aeromonas hydrophila*	*Citrobacter freundii*	1159	2135	100%	0.0	99.91%	MK909916.1	OL336247

Table A6.5: The prevalence of 42 isolates in fish tissue (healthy and diseased), soil and water confirmed by 16S rRNA gene sequencing

Isolate	Name	Healthy	Diseased	Soil	Water
SSF_01	*Klebsiella pneumoniae*	√	√	√	√
SSF_02	*Enterobacter ludwigii*	√	√	√	√
SSF_03	*Pseudomonas aeruginosa*	√	√	√	√
SSF_04	*Aeromonas hydrophila*	√	√	√	√
SSF_05	*Pseudomonas mendocina*	√	√	√	√
SSF_06	*Staphylococcus aureus*	√	√	√	√
SSF_07	*Staphylococcus aureus*	√	√	√	√
SSF_08	*Bacillus licheniformis*	√	√	√	√
SSF_09	*Acinetobacter soli*	√	√	√	√
SSF_10	*Bacillus cereus*	√	√	√	√
SSF_11	*Clostridium tetani*	√	√	√	√
SSF_12	*Staphylococcus aureus*	√	√	√	√
SSF_13	*Flavobacterium columnare*	√	√	√	√
SSF_14	*Morganella morganii*	√	√	√	√
SSF_15	*Bacillus subtilis*	√	√	√	√
SSF_16	*Bacillus circulans*	√	√	√	√
SSF_17	*Pseudomonas aeruginosa*	√	√	√	√
SSF_18	*Pseudomonas aeruginosa*	√	√	√	√
SSF_19	*Pseudomonas aeruginosa*	√	√	√	√
SSF_20	*Bacillus subtilis*	√	√	√	√
SSF_21	*Bacillus sp.*	√	√	√	√
SSF_22	*Staphylococcus epidermidis*	√	√	√	√
SSF_23	*Escherichia sp.*	√	√	√	√
SSF_24	*Bacillus amyloliquefaciens*	√	√	√	√
SSF_25	*Bacillus sp.*	√	√	√	√
SSF_26	*Bacillus megaterium*	√	√	×	√
SSF_27	*Enterobacter tabaci*	√	√	√	√
SSF_28	*Klebsiella pneumoniae*	√	√	√	√
SSF_29	*Pseudomonas taiwanensis*	√	√	√	√
SSF_30	*Pseudomonas monteilii*	√	√	√	√
SSF_31	*Enterobacter cloacae*	√	√	√	√
SSF_32	*Pseudomonas monteilii*	√	√	√	√
SSF_33	*Enterobacter cloacae*	√	√	√	√
SSF_34	*Enterobacter cloacae*	√	√	√	√
SSF_35	*Serratia marcescens*	×	√	√	√
SSF_36	*Morganella morganii*	√	√	√	√
SSF_37	*Pseudomonas monteilii*	√	√	√	√
SSF_38	*Enterobacter ludwigii*	√	√	√	√
SSF_39	*Citrobacter freundii*	×	√	√	√
SSF_40	*Pseudomonas mendocina*	√	√	√	√
SSF_41	*Aeromonas hydrophila*	√	√	√	√
SSF_42	*Citrobacter freundii*	√	√	√	√

Table A6.6: Antibiotic sensitivity and resistant pattern of 42 bacteria. Zone of inhibition was measured in millimeter (mm)

Sl. No	Species Name	Amoxicillin (AML)	Ampicillin (AMP)	Azithromycin (AZM)	Chloramphenicol (C)	Cefixime (CFM)	Gentamycine (CN)	Erythromycine (E)	Tetracycline (TE)	Vancomycin (V)
1	*Klebsiella pneumoniae*	R	R	S	S	S	S	R	MS	R
2	*Enterobacter ludwigii*	MS	R	S	S	R	S	MS	MS	R
3	*Pseudomonas aeruginosa*	R	R	S	MS	S	S	R	MS	R
4	*Aeromonas hydrophila*	R	R	S	S	R	S	S	S	S
5	*Pseudomonas mendocina*	R	R	S	S	R	S	S	S	S
6	*Staphylococcus aureus*	S	S	S	S	MS	S	S	S	S
7	*Staphylococcus aureus*	S	S	S	S	R	S	S	S	S
8	*Bacillus licheniformis*	S	MS	R	S	R	S	MS	S	S
9	*Acinetobacter soli*	S	S	S	S	R	S	MS	S	S
10	*Bacillus cereus*	R	R	S	S	R	S	S	S	S
11	*Clostridium tetani*	S	S	S	S	S	S	S	S	S
12	*Staphylococcus aureus*	S	S	S	S	R	S	S	S	S
13	*Flavobacterium columnare*	S	MS	S	S	S	S	R	S	R
14	*Morganella morganii*	S	S	S	S	R	S	MS	S	S
15	*Bacillus subtilis*	S	S	S	S	R	S	S	S	S
16	*Bacillus circulans*	S	MS	S	S	R	S	S	S	S
17	*Pseudomonas aeruginosa*	R	R	S	S	S	R	R	R	R
18	*Pseudomonas aeruginosa*	R	R	S	S	S	R	R	R	R
19	*Pseudomonas aeruginosa*	R	R	S	S	S	S	R	R	R
20	*Bacillus subtilis*	S	S	S	S	R	S	S	S	S

21	*Bacillus sp.*	S	R	S	S	S	S	S	S	S
22	*Staphylococcus epidermidis*	S	S	S	S	R	S	S	S	S
23	*Escherichia sp.*	MS	R	S	S	MS	S	MS	S	R
24	*Bacillus amyloliquefaciens*	S	R	S	S	R	S	MS	MS	R
25	*Bacillus sp.*	S	S	S	S	R	S	MS	MS	R
26	*Bacillus megaterium*	S	S	S	S	S	S	S	S	S
27	*Enterobacter tabaci*	R	R	S	S	S	S	R	S	R
28	*Klebsiella pneumoniae*	R	R	S	S	R	S	MS	R	S
29	*Pseudomonas taiwanensis*	R	R	S	R	S	S	R	R	R
30	*Pseudomonas monteilii*	R	R	MS	MS	R	S	R	R	R
31	*Enterobacter cloacae*	R	R	MS	S	R	S	R	R	R
32	*Pseudomonas monteilii*	R	R	S	S	R	S	MS	S	S
33	*Enterobacter cloacae*	R	R	S	S	S	S	R	R	R
34	*Enterobacter cloacae*	R	R	S	S	S	S	R	R	R
35	*Serratia marcescens*	R	R	S	S	S	S	R	R	R
36	*Morganella morganii*	R	R	MS	S	S	S	R	R	R
37	*Pseudomonas monteilii*	R	R	S	S	R	S	MS	MS	R
38	*Enterobacter ludwigii*	R	R	S	S	S	S	R	MS	R
39	*Citrobacter freundii*	R	R	S	S	R	S	MS	R	S
40	*Pseudomonas mendocina*	R	R	S	S	R	S	S	S	S
41	*Aeromonas hydrophila*	R	R	S	S	R	S	S	S	S
42	*Citrobacter freundii*	R	R	S	MS	R	S	R	MS	R

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