Prevalence and Antibiotic susceptibility of *Aeromonas hydrophila* isolated from fresh water fishes

A dissertation submitted to the Department of Fisheries in partial fulfillment of the requirement for the degree of Master of Science (MS) in Fisheries

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DEDICATED

TO

MY BELOVED PARENTS

(Md. Harun Sarder and Rezia Begum)

Certificate

We certify that the research work embodied in this thesis entitled "**Prevalence and Antibiotic susceptibility of *Aeromonas hydrophila* isolated from fresh water fishes**" Submitted by **Halima Sarder**, Roll No.: Curzon-707, Session: 2014-15, Registration No.:2010-213-010/2010-11 has been carried out under our supervision.

This is further to certify that it is an original work and suitable for the partial fulfillment of the degree of Master of Science (MS) in Fisheries from the Department of Fisheries, University of Dhaka.

We wish every success in his life.

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Abstract

Aeromonas hydrophila is a microorganism that is a part of the normal bacterial flora of many animals. As an opportune organism, it is a secondary biological agent that contributes to the occurrence of a fish disease and its deterioration. Frequently, its presence is an indication of bad zoohygiene and zootechnical conditions in fish reservoir. *Aeromonas* is almost always present in clinical isolates and may be unjustly accused for bad health of fish. The research was undertaken to determine the prevalence of *Aeromonas hydrophila* from some selected freshwater fishes and to test antibiotic susceptibility.

In the present study, 5 different species of fishes were examined each with three replicates were collected from different fish market in Dhaka Metropolitan City. Samples were collected from three different organs such as muscle, gill and gut. Total Bacterial Count (TBC) and Total *Aeromonas* like colonies on NA and different Aeromonas Agar media were enumerated using serial dilution techniques. Bacterial isolates were characterized to confirm the presence of *Aeromonas hydrophila* using biochemical test and with comparison to reference strain (ATCC 7966). Further molecular method was used for the confirmation. The diffusion disk technique was used for testing antibiotic susceptibility.

Among organs, the highest incidence was found in the gill (54.5%) and lowest (8.8%) in the muscle. On the other hand, the lowest Aeromonas count was found $2.83\pm0.40x10^2$ in *Anabas testudineus* and the highest was $1.03\pm0.153x10^3$ in *Oreochromis mossambicus.* On market basis highest Aeromonas count was found in Ananda Bazar ($8.10\pm1.09x10^2$ cfu/g) and lowest in Hatirpool Bazar ($5.63\pm0.90x10^2$ cfu/g) with no significant difference. The isolates were tested for their susceptibility to 14 different antibiotics including Gentamycin, Amikacin & Chloramphenicol; and Maximum susceptibility to Tetracycline whereas all of the isolate showed resistant to a common used antibiotic Amoxyciline.

The obtained results point that antimicrobial susceptibility was more or less same regardless of the origin of the samples collected. All the selected fishes in this study contain *Aeromonas hydrophila* in their different organs.

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List of Symbols and Abbreviations

Symbols	Details
°C	Degree centigrade
AH	Aeromonas Hi-media Agar
AL	Aeromonas LAB Agar
AO	Aeromonas Oxoid Agar
APHA	American Public Health Association
BLAST	Basic Local Alignment Search Tool
cfu	Colony forming unit
cm	Centimeter
Co.	Company
ddH2O	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
Ed.	Edition
e.g.	Exempli grata (For exmple)
et al	Et alliori (And others)
etc.	Etcetra
Fe2+	Ferrous (Iron II) ion
FeCl3	Ferric (Iron III) chloride
Fig.	Figure
g	Gram
h	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide
HCl	Hydrochloric acid
i.e.	Id est (That is)
Inc.	Incorporation
is situ	In the original place
IUPAC	International Union of Pure and Applied Chemistry

K_2HPO4	Dipotassium hydrogen phosphate
kb	Kilo base
KCI	Potassium chloride
KOH	Potassium hydroxide
l	Liter
Ltd.	Limited
M	Molar
mA	Mili Ampear
mg	Milligram
$MgCl_2$	Magnesium Chloride
$MgSO_4.H_2O$	Aqueous Magnesium sulphate
min	Minute
ml	Millilitre
mM	Milimole
mm	Millimeter
µl	Microlitre
µM	Micromole
µm	Micrometer
M.R.	Methyl red
NA	Nutrient agar
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NH_3	Ammonia
$(NH4)_2$ HPO4	Diamonium hydrogen phosphate
nm	Nanometer
No.	Number
O_2	Oxygen
PS	Physiological Saline
PCR	Polymerase chain reaction
pH	Negative logarithm of hydrogen ion concentration
rDNA	Ribosomal deoxy-ribonucleic acid

rpm	Revolutions per minute
SAB	Society of American Bacteriology
SD	Standard Deviation
SEM	Standard Error of Mean
sp.	Species (Singular)
spp.	Species (Plural)
SPSS	Statistical Package for the Social Sciences
sq.	Square
U	Unit
USA	United States of America
UV	Ultra violate
v.	Version
viz.	Videly (Namely)
V.P.	Voges-Proskauer
W	West
w/v	Weight/volume
(-)ve	Negative
(+)ve	Positive
(-)ve	Negative
(+)ve	Positive

Chapter 1

Introduction

1.1 Background

Bangladesh is blessed with huge open water resources with a wide range of variations in nature. The total inland area at present is 47,03,658 hectare (DoF, 2012) that has a high potential for fisheries production.

Fishes are valuable sources of high quality protein and other organic products (McCance Rand Widdowson, 1960). Fishes occupy significant position in the socioeconomic fabric of the South Asian countries by providing the population not only nutrition but also income and employment opportunities (Rubbi et al., 2012).Basically fish takes a prominent place as a source of protein compared to other protein sources. Fish is very important food stuff in developing countries due to its high protein content and nutritional value. Fish provides more than 50% of the animal protein for the populations of 34 countries (Bhuiyan, 1987).

All populations of organisms, including, aquatic animals like fishes are limited partially or completely by diseases in their ecosystem (Real, 1996). Disease prevalence in the ecosystem is influenced by numerous environmental factors including infectious organisms and stressors (Nils kautsky et al., 2000).

Aeromonas species are facultative anaerobic Gram-negative bacteria that belong to the family Aeromonadaceae. These bacteria have a broad host spectrum, with both cold-and warm-blooded animals, including humans and are known as psychrophilic and mesophilic. Aeromonads are ubiquitous in fresh water, fish and shellfish, and also in meats and fresh vegetables (Boonyaratpalin, 1989).The epidemiological results so far are, however, very questionable. The organism is very frequently present in many food products, including raw vegetables, and very rarely has a case been reported. A sepsis caused by *Aeromonas* is indeed dangerous. The same *Aeromonas* species (primarily *A. hydrophila* HG1, *A. veronii* biovar sobria HG8/10, and *A. caviae* HG4) can cause self-limiting diarrhoea, particularly in children (Kirov et al., 2000). Up to 8.1% of cases of acute enteric diseases in 458 patients in Russia were caused by *Aeromonas* spp. (Demarta et al., 2000).

These bacteria have a broad host spectrum, with both cold-and warm-blooded animals, including humans and are known as psychrophilic and mesophilic. In fish, these bacteria cause hemorrhagic septicemia, fin rot, soft tissue rot and furunculosis. It was reported that epizootic ulcerative syndrome (EUS) caused by *Aeromonas sobria* resulted in great damage to fish farms in parts of southeast Asia such as in Bangladesh and India. *A. sobria* was also the virulence of *Aeromonas* spp. and was reported to causative agent of fish disease in a farm of perch, Perca. fluviatilis L, in Switzerland. In humans, *Aeromonas* causes diarrhea, gastroenteritis and extra enteritica reliable approach by which to identify potential conditions such as septicemia, wound infection, pathogenic Glycerophospholipid endocarditis, meningitis and pneumonia (Ali Aberoum and Hossein Jooyandeh, 2010).

Aeromonas hydrophila and other motile aeromonads are among the most common bacteria in freshwater habitats throughout the world, and these bacteria frequently cause disease among cultured and feral fishes (R.C. Cipriano, 2001). The bacterium is ubiquitous and occurs in most fresh water environments. It can be found both in the water column and in the top centimeter of sediment (Hazen, 1979). Motile aeromonads are adapted to environments that have a wide range of conductivity, turbidity, pH, salinity, and temperature (Hazen et al., 1978a).

Aeromonas hydrophila is species that secretes many extracellular proteins, including amylase, chitinase, elastase, aerolysin, nuclease, gelatinase, lecithinase, lipase and protease. These proteins are known as virulence factors that cause disease in fish and humans. Aerolysin is a representative virulence factor of *Aeromonas* and was reported to function as hemolysins and cytolytic enterotoxins (Ali Aberoum and Hossein Jooyandeh, 2010).

Aeromonas hydrophila is a microorganism widely distributed in nature: in water, soil and food. It is also part of the normal bacterial flora of many animals. While *Aeromonas* spp. is not considered fecal bacteria, they are present in the feces of healthy animals and humans, presumably as the result of ingestion of food and water containing these organisms (Holmes et al., 1996; Demarta et al., 2000). They are present in high numbers in sewage before and after treatment (Monfort and Baleux, 1991), thus they have been proposed as an indicator of sewage contaminated surface water. *Aeromonas* spp. may colonize drinking water distribution systems and produce biofilms that resist disinfection

(Holmes et al., 1996; Bomo et al, 2004). *Aeromonas sp* causes disease in both fish and in humans.

1.1.1 As a pathogen in human

Motile aeromonads can also cause disease in warm-blooded vertebrates. In immunocompromised human hosts, for example, *A. hydrophila* may cause septic arthritis, diarrhea, corneal ulcers, skin and wound infections, meningitis, and fulminating septi cemias (von Gravenitz and Mensch, 1968; Davis, 1978).

In humans *Aeromonas* causes different clinical symptoms i.e. septicemia, infection of wounds and of gastrointestinal tract. It is an opportune microorganism that may be primary or secondary infectious agent to create disease and may be a causative agent in terrestrial and aquatic animals (fishes) and also to humans. The knowledge on the mechanism of pathogenesis of this microbe is poor, but it is known that a number of different factors influence the development of the disease caused by it.

Human infections due to *Aeromonas* species occur predominantly during warm weather. The strains probably originate from water, soil, food or the human gastrointestinal tract. Four disease categories are known:

- Wound infection or cellulitis, related to exposure to water or soil
- A general infection in which the organisms spread throughout the body (septicaemia) in immunoc-ompromised individuals or individuals withvarious other significant illnesses
- Gastroenteritis (diarrhoeal disease)
- Extraintestinal infections such as meningitis, peritonitis, or otitis, or of sites such as the eye or urinary tract (Ali Aberoum and Hossein Jooyandeh, 2010).

The genus *Aeromonas* comprises important human pathogens causing primary and secondary septicemia in immunocompromised persons, serious wound infections in healthy individuals and in patients undergoing medicinal leech therapy, and a number of less well described illnesses such as peritonitis, meningitis, and infections of the eye, joints, and bones. Gastroenteritis, the most common clinical manifestation, remains controversial (Abbott et al,. 2003). Ascencio et al., (1998) have shown that *A. hydrophila, A. caviae*, and *A. sobria* can actually adhere to animal cell lines that have mucous receptors. Trust et al., (1980) also indicated that *A. hydrophila* had adhesive agglutination characteristics which facilitated attachment to eukaryotic cells.

1.1.2 As a pathogen in fish

Motile Aeromonas septicemia (MAS) caused by mesophilic *A. hydrophila* affects a wide variety of primarily freshwater fish species, including carp, tilapia, perch, catfish, and salmon. Epidemic disease outbreaks in fish caused by *A. hydrophila*, resulting in millions of dollars of lost revenue, have been reported worldwide.

Aeromonas infections are more common in warm water and temperate species than in coldwater fish. Infections can occur in any age fish, but losses are usually most severe in fry and small fingerlings.

Although motile aeromonads appropriately receive much notoriety as pathogens of fish, it is important to note that these bacteria also compose part of the normal intestinal microflora of healthy fish (Trust et al., 1974). Therefore, the presence of these bacteria, by itself, is not indicative of disease and, consequently, stress is often considered to be a contributing factor in outbreaks of disease caused by these bacteria. Such stressors are most commonly associated with environmental and physiological parameters that adversely fish under intensive culture.

Factors contributing to virulence include toxins, proteases, hemolysins, lipases, adhesins, agglutinins, and various hydrolytic enzymes (Janda et al., 1996). Virulence factors are present in two forms, cell-associated structures, and extracellular products. Among the cell-associated structures are pili, flagella, outer membrane proteins, lipo-polysaccharide, and capsules. The major extracellular products include cytotoxic, cytolytic, hemolytic, and entero toxic proteins. If cell-surface proteins are present i.e. adhezin or extracellular quorum sensing, protease, haemolysin and enterotoxins (acetilholinesterasisi) pathogenesis may develop. Burke et al., 1983, reported a 97% correlation between hemolysin production and enterotoxin production among *Aeromonas* species.

Motile aeromonads cause diverse pathologic conditions that include acute, chronic, and covert infections. Severity of disease is influenced by a number of interrelated factors, including bacterial virulence, the kind and degree of stress exerted on a population of fish, the physiologic condition of the host, and the degree of genetic resistance inherent within specific populations of fishes. Motile aeromonads differ interspecifically and intraspecifically in their relative pathogenicity or their ability to cause disease (R.C. Cipriano, 2001).

In the acute form of disease, a fatal septicemia may occur so rapidly that fish die before they have time to develop anything but a few gross signs of disease. When clinical signs of infection are present, affected fish may show exophthalmia, reddening of the skin, and an accumulation of fluid in the scale pockets (Faktorovich, 1969). There may also be a severe branchitis, as indicated by leukocytic infiltration and dilation of the central venous sinus Grizzle and Kiryu, 1993).

Intensification of fish farming in Bangladesh has increased the number of disease outbreaks in intensive production systems. Most etiological agents were not yet identified and their morbid processes still not studied. The *Aeromonas* complex are important fish pathogenic bacteria, causing septicemic infections and associated economic losses in fish culture worldwide (Holliman, 1993). Multiple antibiotic resistance (MAR) has been registered for *Aeromonas* hydrophila isolated from fresh water fish farms in association with a variety of drugs, commonly used as feed additives (Aoki et al., 1971; Pettibone et al., 1996; Son et al., 1997;Vivekanandhan et al., 2002). The main problem involving the use of antibiotics against *Aeromonas* infections is the development of resistance by these bacteria (Aoki and Egusa, 1971; Mitchell and Plumb, 1980), generally related to the presence of plasmids (Chang and Bolton, 1987; Ansary et al., 1992).

1.2 Rationale

The incidence of microbial pathogens, especially those of bacterial origin is one of the most significant factors affecting fish culture *(*Post, 1989*;* Zorrilla et al., 2003). Fishes are constantly exposed to bacteria and usually only succumb to an infection after being exposed to prolonged periods of stress. Environmental factors may act as stressors and can predispose a fish to bacterial diseases.

Most *Aeromonas* spp. isolates are psychrotrophic and can grow at refrigerator temperatures. This could increase the hazard of food contamination, particularly where there is a possibility of cross-contamination with ready-to-eat food products.

Olivier et al., 1981 indicated that both *A. hydrophila* and *A. sobria* produced enterotoxins, dermonecrotic factors, and hemolysins. Enterotoxins, haemolysins, proteases, haemagglutinins, and endotoxins produced by this complex of bacterial organisms have been the subject of much research (Cahill, 1990).

Some aeromonads are pathogenic for humans, and most human clinical isolates belong to HG-1, HG-4, HG-8, HG-9, HG-10, HG-12 or HG-14 (Janda and Abbott, 1998). HG-2, HG-3, HG-5, HG-6, HG-7, HG-11, HG-15, HG-16 and HG-17 are isolated from the environment or diseased animals, and they are not considered human pathogens (Janda et al., 1996). Pathogenic species causing human diseases are associated with a variety of infections such as septicemia, wound infections, meningitis, peritonitis, and hepatobilary infections. Capsule production has been reported for *A. salmonicida* and *A. hydrophila* serogroups (Martinez et al., 1995), but the function of capsule material is vague. It is presumed to resist complement activity and perhaps enhance adherence (Kirov et al., 2004). Some strains of Aeromonas produce enterotoxins responsible for causing gastroenteritis in humans; however, isolation of aeromonads from feces does not indicate pathogenicity, since these bacteria are widely distributed throughout the environment in water and foods, especially during summer months (Janda and Abbott, 1998).

Aeromonads (*A. hydrophila, A. sobria* and *A. salmonicida*) can be causative agents not only of human enteritis, but also of a fatal septicaemia as recorded in a 15-year old healthy girl; the causative agent was *A. sobria* (Demarta et al., 2000). Aeromonads septicemia most often caused by *A. hydrophila*, was described as a complication in 50 patients with liver cirrhosis and eye infection in immuno-compromised patients (Tamura and Hida, 2003). Fatal bacterial pneumonia in a 5-year old child was also caused by *A. hydrophila* (Demarta et al., 2000).

Aeromonas hydrophila and other aeromonads are among the most common bacteria in freshwater habitats throughout the world. Genus *Aeromonas* includes prominent micro-biota in freshwater reservoirs where they together with other microorganisms act as natural bio-filters and promote self purification of the water body. They are necessarily present in normal micro-flora and hydrobionts inhabiting fish reservoirs *(*Kompanets *et al.* 1992). However, they frequently cause problems in both feral and cultured fish (Cipriano, 2001).It is responsible for heavy economic losses caused by both high mortality and deterioration of product quality (Groff and Lapatra, 2000; Karunasagar et al., 2003).

1.3 Problem Statement

It has been known for decades that *Aeromonas* plays a causative agent role in fish diseases. The wide spread distribution in aquatic ecology systems indicates that interactions of *Aeromonas* species with fish are continuous and unavoidable, facilitating their opportunistic pathogenicity (Ottaviani et al., 2011; Hu et al., 2012).

Aeromonads have been reported as pathogens of fish, amphibians, and reptiles (Gosling, 1996b). Aeromonas spp. cause hemorrhagic disease, ulcerative disease, furunculosis, and septicemia in fish (Austin and Adams, 1996). Aeromonads cause pneumonia, peritonitis, abortion and other diseases in birds and domestic animals (Gray, 1984).

Aeromonas hydrophila has been recovered from a wide range of freshwater fish species worldwide (Austin and Adams, 1996).*A. hydrophila* was recorded widely infecting freshwater fish and marine fish species associated with skin lesions, tail and fin rot, haemorrhagic septicemia over the body and tissue destruction, epizootic ulceration and necrosis in the liver and kidney of fish (Austin and Adams, 1996; Doukas et al., 1998; Janda and Abbott, 2010). *A. hydrophila* has also been described as the dominant infectious agent of 'fish-bacterial-septicemia' in freshwater cultured cyprinid fishes.

The pathogenicity of *A. hydrophila* is usually considered to be multifactorial. Over the last 30 years, a number of virulence factors, including secretion systems, motility and adhesins, toxins, enzymes, quorum systems, iron acquisition and antibiotic resistance, have been identified. To date, most of the research on *A. hydrophila* has still focused on the so-called virulence factors. However, recently, increasing reports have proposed that animal environments that pathogens colonize have likely driven the evolution of new metabolic adaptations to maximize these new nutritional opportunities, and these adaptations may link with bacterial virulence. These suggest the known virulence factors may not be the only players in the bacterial infection process.

1.4 Research Needs

Conflicting views have been expressed concerning whether *A. hydrophila* is a primary pathogen of freshwater fish or a secondary opportunistic pathogen of compromised or stressed hosts. *A. hydrophila* has been associated with tail and fin rot, haemorrhagic

septicemia and epizootic ulcerative syndrome (EUS) (Austin and Adams, 1996; Roberts, 1997).

Poor personal hygiene and reuse of water have been shown to increase the risk of transmission. The risk of *Aeromonas* infections is significant for animals in aquaculture, where crowding promotes transmission (Austin and Adams, 1996).

Aeromonas hydrophila is ubiquitous in various aquatic environments, and has been considered as a pathogen of fish, amphibians, reptiles and mammals. Being an opportunistic pathogen, *A. hydrophila* could still cause the outbreaks of motile aeromonad septicemia (MAS) in fish. As has been reported, MAS has frequently caused huge economic losses in the cyprinid fish industry throughout China since 1989.The wide spread distribution in aquatic ecology systems indicates that interactions of *Aeromonas* species with fish are continuous and unavoidable, facilitating their opportunistic pathogenicity (Ottaviani et al., 2011; Hu et al., 2012).

Although motile aeromonads appropriately receive much notoriety as pathogens of fish, it is important to note that these bacteria also compose part of the normal intestinal micro-flora of healthy fish (Trust et al., 1974). Therefore, the presence of these bacteria, by itself, is not indicative of disease and consequently, stress is often considered to be a contributing factor in outbreaks of disease caused by these bacteria.

It was considered as a significant economic problem, particularly in China and India over the past decade (Citarasu et al., 2011). The existence and pathogenicity of *A. hydrophila* have been reported in a variety of freshwater species, comprising Salmo gairdneri (Peters et al., 1988), Clarias batrachus (Angka 1990), tilapia (Liu et al., 1999), Carassius auratus(Iqbal et al., 1999; Citarasu et al., 2011), Cyprinus carpio (Chirila et al., 2008; Citarasu et al., 2011), Oreochromis niloticus(Ibrahem et al., 2008), and Channa striata (Duc et al., 2013) in China. Nevertheless,to date the infection information of those fresh water fishes in Bangladesh is still limited. In present study, the efforts were conducted by a challenge test to confirm whether *A. hydrophila* is present into those selected fresh water fishes as haemorrhagic pathogen.

Wide use of antibiotics to treat bacterial infections and incorporation of sub-therapeutic dose of antibiotics into feeds for cultured organism resulted in a global increase in antibiotic resistance among pathogenic bacteria. The problem is more serious in

developing countries, where antibiotics are used widely. In India, antibiotics are extensively applied in animal husbandry and aquaculture. The use of antibiotics is the most important factor in amplifying the level of resistance in a given reservoir (Wegener and Frimodt-Moller 2000). Multiple antibiotic resistance (MAR) among *A. hydrophila* strains has been reported from many parts of the world (Pettibone et al., 1996; Son et al., 1997; Rajeswari Shome and Shome, 1999). Under these circumstances, it will be worthwhile to find out the prevalence of antibiotic resistance of the *Aeromonas* strains that may be considered as an emerging pathogen and to identify the high-risk source.

1.5 Objectives

Limited studies were carried out in Bangladesh regarding the prevalence of *Aeromonas hydrophila* infection in freshwater fishes. Therefore, this study was planned to fulfill the following objectives:

The overall objective was studying the prevalence of *Aeromonas hydrophila* in some freshwater fish species.

The specific objectives were

a) Identifying the isolated *A. hydrophila* through their biochemical activities
b) Characterization of pathogenic strain using PCR
c) Checking antibiotic susceptibility of the isolated strain

1.6 Scope and limitations

This study focused only on the prevalence of 5 freshwater fishes of Bangladesh. The main limitation was that fishes were collected from fish markets that were not representing the real environmental sources. Other factors included, if fishes play any role for the seasonality of *A. hydrophila* that was not covered in the present study. All activities were conducted in the Microbiology Laboratory and Aquatic Laboratory, Department of Botany and Department of Fisheries respectively, University of Dhaka, Dhaka. Molecular characterization was conducted into Life Science Division, Invent Technologies Ltd. Banani, Dhaka.

Chapter 2

Material and methods

2.1 Experimental fish

In the present study, 5 different fishes were examined each with three replicates (Table 2.1). The samples were collected from three markets (Ananda Bazar, Polashi Bazar and Hatirpool Bazar) of Dhaka Metropolitan City (Capital of Bangladesh, located on the banks of the Buriganga River.

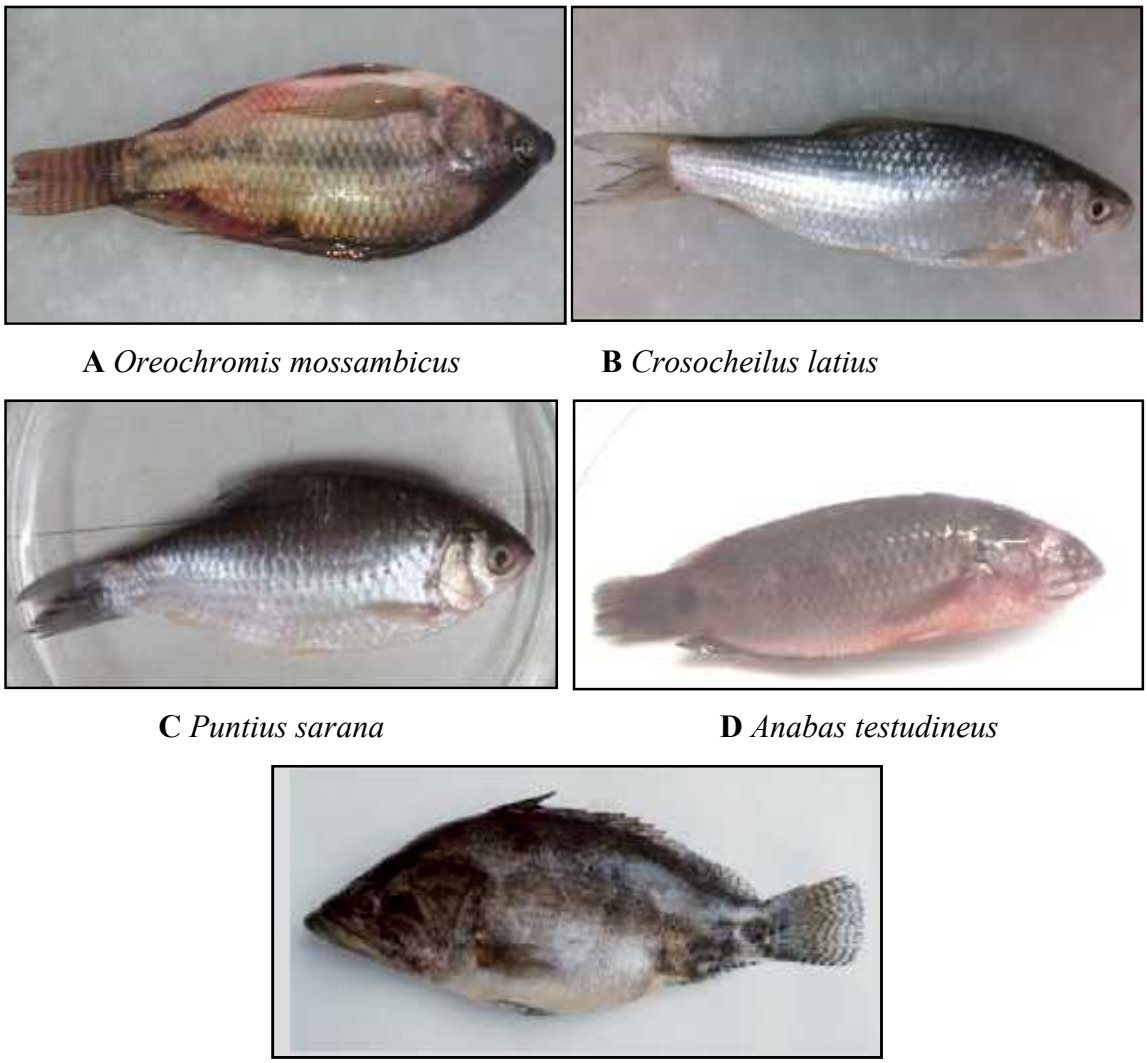
Samples from three different organs *viz*. muscle, gill, and gut were separately examined for each specimen.

Table 2.1 List of species of fishes used in this study

Scientific Name	English Name	Local Name	References
Puntius sarana	Olive barb	Sarpunti	Hmilton-Buchanon (1831)
Anabas testudineus	Climbing perch	Koi	Nargis & Hossain (1987)
Crosocheilus latius	Kala bata	Tatkini	Heckel (1838)
Oreochromis mossambicus	Tilapia	Tilapia	Shafi & Quddus (2001)
Nandus nandus	Mud Perch	Meni	Mustafa *et al*. (1980)

2.2 Sampling Procedures

Fish samples were collected in sterilized plastic bag aseptically following the methods of American Public Health Association (APHA) from different fish markets of Dhaka Metropolitan City early in the morning during the periods of June 2015 to November 2015. Samples were then transported in the laboratory within 30 minutes.



A *Oreochromis mossambicus* **B** *Crosocheilus latius*

C *Puntius sarana* **D** *Anabas testudineus*

E *Nandus nandus*

Plate 2.1: Photograph showing selected fishes for the isolation of *Aeromonas hydrophila*.

2.3 Laboratory of investigation

All the studies were carried out in the Microbiology Laboratory under Department of Botany and Aquatic Laboratory under Department of Fisheries, University of Dhaka. The molecular works were done on Invent Technologies Ltd., Life Science Division, Dhaka.

A. Muscle of Tilapia

B. Gill of Tilapia

C. Gut of Tilapia

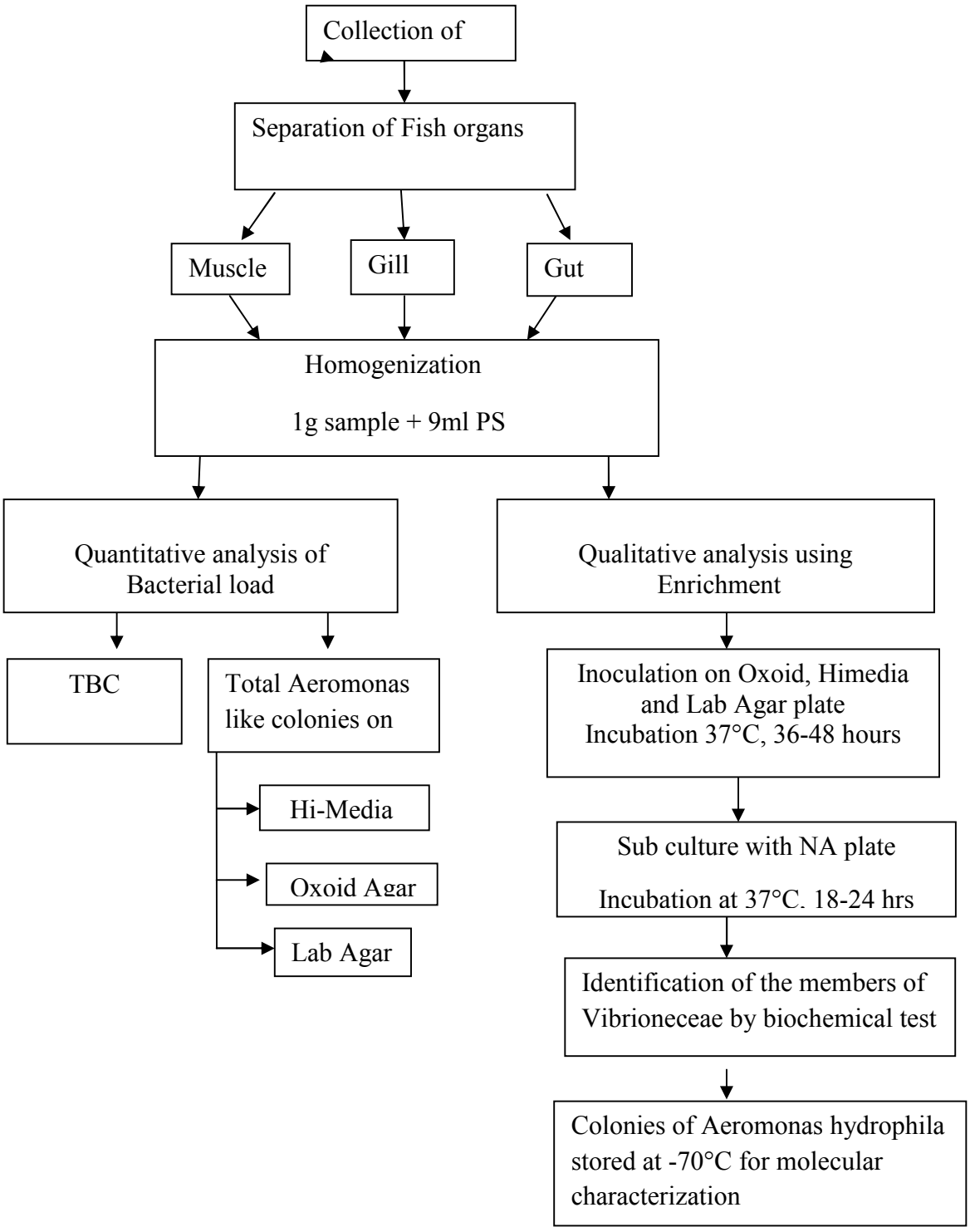
D. Organ solution of Tilapia

Plate 2.2: Photograph showing different organs of selected fish.

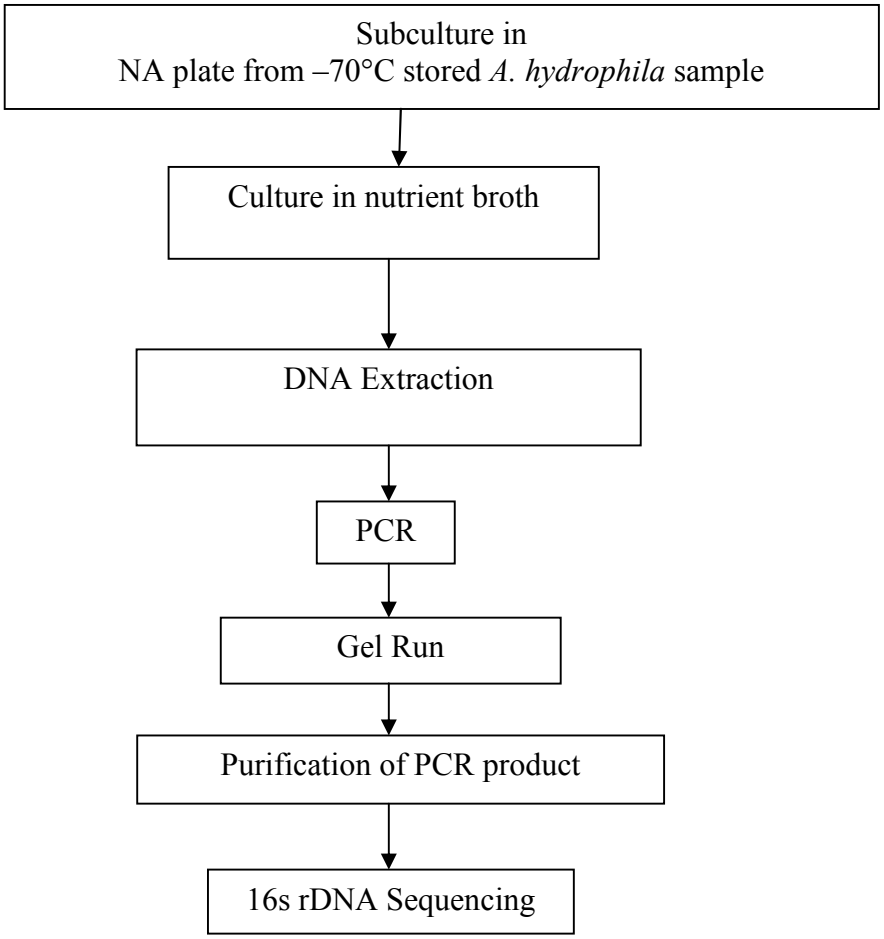
2.4 Processing of samples

The fish samples were processed within 2 h of collection following aseptic techniques. First, the samples were washed with sterile physiological saline (PS) to remove sand, detritus as well as microorganisms attached to the surface of fish. Then the muscle, gill and gut samples were collected aseptically following the method of APHA (1998). The collected samples were separately homogenized with PS solution using homogenizer and were then used for microbial load count and *Aeromonas* specific enrichment.

Flow chart 1 Processing of fish sample and identification of *Aeromonas hydrophila*



Flow chart 2 Molecular Characterization of *A. hydrophila*



2.5 Media and techniques for the enumeration and isolation of bacteria

2.5.1 Media used

Laboratory analyses were done within a week. Nutrient agar (NA) (Eklund and Lankford, 1967), Aeromonas Agar (Hi-media India), Aeromonas agar (Oxoid UK) and Aeromonas agar (LAB Switzerland) medium were used for the enumeration and isolation of total aerobic heterotrophic bacteria present in fish samples.

2.5.2 Techniques employed

Serial dilution technique (Greenberg et al., 1980) was used for the isolation of microorganisms. In fish sample, one gm was diluted with 9 ml sterile PS in a sterile test tube and shaken well. .This suspension was transferred to 9 ml of sterile PS for ten-fold

(1:10) dilution and further diluted up to 10^4 for the plating of NA medium and 10^3 dilutions for the plating of Aeromonas Agar (Hi-media, Oxoid and LAB) medium.

One ml of each of the diluted sample was taken in a sterilized Petri plate by sterilized pipette. Then molten agar medium poured and mixed thoroughly by rotating the Petri plate, first in one direction and then in the opposite direction. Plating in duplicated plates was made for each diluted sample. After setting the medium the plates were placed inversely and incubated at 37°C for 48 h in an incubator (Memmert GmbH + Co Kg 8540 Sehwabach).

2.6 Enumeration of bacteria

After 36-48 h of incubation the plates having well discrete colonies selected for counting from the respective culture plate. The selected plates placed on colony counter (Digital colony counter, DC-8 OSK 100086, Kayagaki, Japan) and the colonies were counted.

2.7 Isolation of bacteria

Based on their colonial morphology well discrete *Aeromonas* bacterial colonies were selected immediately after counting. The selected colonies were streaked on slant for further studies.

2.8 Purification of the isolates

After initial selection on the basis of growth pattern, the selected isolates purified through repeated plating (by streaking plate methods). When a plate yielded only one type of colonies the organisms considered to be pure.

2.9 Maintenance and preservation of isolates

The purified isolates then transferred on Nutrient agar slant. The slants kept in polythene bags and preserved as stock culture in a refrigerator at 4°C for further study. Periodical transfers of isolates on agar slants were done for maintaining viability of the organisms.

2.10 Morphological observation of isolates

For the identification of selected isolates, following morphological characters were studied and recorded.

2.10.1 Colonial morphology

The bacterial colonies on plating medium were morphologically studied as their form, elevation, margin, surface, pigmentation, opacity, whether grown inside, at the bottom or on the surface of the medium and their rate of growth (Eklund and Lankford, 1967; Bryan, 1950).

2.10.2 Preparation for microscopic examination of isolated strains

Bacterial cells suspension made by using fresh culture with physiological saline. The prepared suspension was used to make smear. A good quality glass slide was used for this purpose. Thin smear was prepared on the clean and oil free slide. The smear allowed to dry in air and fixed by passing the slide over the flame of a spirit lamp. The following two different staining methods were employed to stain the fixed smear.

(i) Simple staining and
(ii) Differential staining method employed to stain the fixed smears.

2.10.2.1 Simple staining

Manual of Microbiological Methods (SAB 1957) was followed for simple staining. Basic dye safranin was used. The fixed smear flooded with a dye solution for one minute. The flooded smear washed off with water and dried in air.

2.10.2.2 Differential staining

Staining procedures that make visible differences between microbial cells or parts of cells termed as differential staining. In differential staining process, a combination of dyes was used that take advantage of chemical differences among cells (Claus, 1995). Among the differential stains most frequently used are the Gram stain, acid-fast stain and spore stain. For this purpose, fixed smear exposed to more than one dye solution to differentiate cell and or its parts. In this study, one differential technique (Gram Staining) was used.

Gram staining: This is one of the most important and widely used differential staining techniques which are considered as one of the important steps in identifying an unknown bacterium. For Gram staining, method described by (Claus, 1995) was followed.

Fixed smear treated with the following solutions and after application of each solution; the slide gently washed off with water.

- Crystal violet 60 sec.
- Lugol's iodine solution 60 sec.
- 95% Ethyl alcohol less than 30 sec.
- Mercurochrome solution 60 sec.

The slide was blotted dry and observed under microscope (Nikon Microphot, UFX-IIA, Japan).

The results were recorded as Gram positive (blue-violet) and Gram negative (light red).

2.11 Microscopic observation

The size and shape of vegetative cells of selected strains were observed. The arrangement of cells whether single or in chains or clusters were carefully recorded. Gram-reaction of the isolates were also studied and recorded. Photomicrographs of the observed cells as well as stage micrometer of the same magnification were taken using microscope (Nikon Microphot) with photographic attachment (Nikon, FX 35 WA, Japan).

2.12 Physiological and biochemical studies of the isolates

Following Bergey's Manual (Sneath et al., 1986), Manual of Microbiological Methods (SAB 1957), Microbiological Methods (Collins and Lyne, 1984) and Understanding Microbes (Claus, 1995) the following important physiological and biochemical tests of the isolated bacteria were carried out.

2.12.1 Oxidase test (Claus 1995)

The enzyme oxidase, present in certain bacteria catalyses the transport of electron from donor bacteria to the redox dye tetra-methyl-para-phenylene-diamine dihydrochloride.

The dye in the reduced state has a deep purple color. To perform this test filter papers soaked in 1% aqueous tetramethyl-phenylene-diamine dihydrochloride. Fresh young culture rubbed on the filter paper with a clean glass rod. Results were recorded within 10 seconds.

Blue color indicated a positive result.

2.12.2 Catalase test (Claus 1995)

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS).

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \uparrow$$

To demonstrate catalase activity, test organisms were taken by a sterilized loop on a glass slide and a drop of hydrogen peroxide was added to each of them.

The evolution of bubbles indicated the positive result *i.e.* the organism having the enzyme catalase.

2.12.3 Potassium hydroxide solubility test (Schaad 1988)

The test was done with a 3% potassium hydroxide (KOH) solution. One to two drops of 3% KOH placed on a clean and dried glass slide. A loop full of the bacterial cells from the edge of a 24 h old culture transferred and mixed thoroughly with the drops of KOH on the slide for 10 sec.

Viscous and slimy layer on bacterial strain indicated positive reaction.

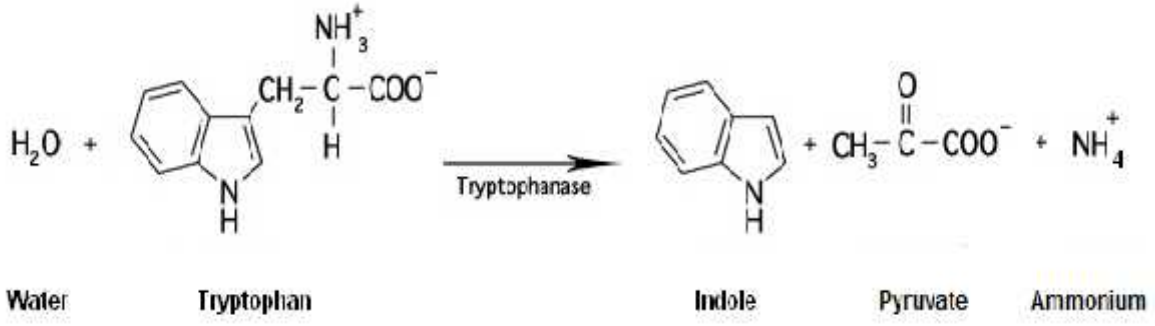
2.12.4 Methyl red test (Sneath *et al.* 1986)

Methyl red (M.R.) test is the test for mixed acid fermentation of glucose by microorganisms. Excreted acid contains large amount of formic, acetic, lactic and succinic acid and causes a major decrease in pH that can be detected by "Methyl Red" indicator. For this test V.P. broth was inoculated and incubated at 37°C for 5 days. After incubation 5 drops of methyl red indicator added to the culture broth.

Red color throughout the broth indicated positive reaction whereas yellow or any yellowish red indicated negative reaction.

2.12.5 Production of indole (Atlas 1997)

Indole is generated by reductive deamination from tryptophan via the intermediate molecule indole pyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine ($-NH_2$) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonia (NH_3) and energy.



For this test, Kovac's modification of Ehrlichs and Bohme method (1905) was followed. In this method 1% tryptone broth medium was used. The inoculated tubes incubated at 37° C for 3 days. After incubation 2 ml of the test reagent (Kovac's reagent) was added.

A rose pink color indicated formation of indole.

2.12.6 Voges-Proskauer (V. P.) Test (Sneath *et al.* 1986)

Voges-Proskauer (V.P.) test is a color reaction test for the production of a neutral product during glucose fermentation by microorganisms. Acetoin or acetyl-methyl carbinol oxidised to diacetyl, which reacts with creatine and forms a red complex.

For this test VP broth tubes inoculated and incubated for 5 days at 37°C. When sufficient growth observed, 3 ml of 5% alcoholic α-napthol solution added to each tube followed by 1 ml of 40% potassium hydroxide and 0.3% creatine solution. The tubes were then shaken vigorously and allowed to stand.

Development of crimson to ruby red color indicates a positive reaction that is the production of acetyl-methyl carbinol.

2.12.7 Brown water pigment solution (Sneath *et al.* 1986)

5ml of distilled water in test tube each was first autoclaved. Then 24 hour young growth of bacterial cell was taken using sterilized loop into the autoclaved water and bacterial cell suspension was made.

Brown color of bacterial cell into the water indicates positive result.

2.12.8 Growth response of isolates into nutrient broth at 37°C (Sneath *et al.* 1986)

To observe the growth response of the isolates, nutrient broth were made and into autoclave machine. Test tubes inoculated with 24 h bacterial culture and incubated at 37°C for 48 h.

Turbidity of inoculated broth indicated the growth of the isolates.

2.12.9 Hydrolysis of esculin (Collins and Lyne 1984)

To demonstrate esculin hydrolysis, esculin agar (0.1% esculin) slants were inoculated with fresh test cultures by streak method and incubated at 37°C for 24 hours.

Blackening of the medium indicated that the organisms were capable of hydrolyzing esculin. Uninoculated slant tubes were used as control.

2.12.10 Acid production from carbohydrate (Sneath *et al*. 1986)

Acid production of various carbohydrates is of great significance in differentiating species within a genus. For identification of the members of Aeromonadaceae, the following sugars and sugar-derivatives are used:

Monosaccharide

i) Pentose : Arabinose

ii) Hexose : Glucose

Disaccharide

i) Sucrose

Sugar-derivatives

i) Mannitol

ii) Inositol

iii) Xylose

The selected isolates were tested for their ability to produce acid from different carbon source. For this purpose inorganic nitrogen base agar medium was used. Bromocresol purple was added to this medium as an indicator and 1% carbon source was added to this medium. Above mentioned sugars were used as carbon sources. All those sugars were sterilized. The medium was poured into the sterilized petri-plates and allowed to solidify. Inoculation was done by point inoculation method and incubated at 37°C for 48 h.

Acid production from carbohydrate was determined by yellow color around the colony.

2.12.11 Gas production from carbohydrates (SAB 1957)

Gas production from carbohydrate or fermentation test is of considerable significance in the identification and classification of bacteria. In the study of fermentation, D-glucose (monosaccharide) was used.

Fermentation tubes with the above carbohydrate were made using bromothymol blue as an indicator. One Durham's tube was introduced in each of the test tubes.

Then the tubes were inoculated in duplicates with 24 hours old culture suspension with the help of sterilized pipette and incubated at 37°C for 48 h.

The change of color of the indicator from green to yellow indicated the production of acid. Presence of bubbles in the Durham's tube indicated the production of gas. No changes in color indicated negative reaction.

2.12.12 Kligler's Iron Agar test (KIA) (Atlas 1997)

Kligler's Iron Agar medium was used to differentiate gram negative enteric bacteria or their ability to ferment dextrose or lactose and their production of hydrogen sulfide.

Tubes of KIA media were inoculated by stabbing the butt and streaking the slant with inoculam of 24 h. The inoculated media then incubated at 37°C for 48 h.

Yellow color in the butt and slant indicated acid production while hydrogen sulfide production indicated by blackening of slant. Break in the medium indicated gas formation. Red color in the butt and slant indicated alkaline reaction.

2.12.13 Dihydrolysis of Arginine (Schaad 1988)

The test medium of Thornley was stab inoculated at the base of the medium and each tube instantly sealed with 3 ml of the molten (3%) agar and incubated at 37°C for 24 hrs.

A positive alkaline reaction was indicated by the development of deep red color.

2.12.14 Utilization of Citrate (Ronald M. Atlas 1997)

This test demonstrates the ability or inability of test organisms to use citrate as sole source of carbon for metabolism and growth. Tubes containing Simmon's citrate agar were inoculated and incubated at 37℃ for 7 days.

Utilization of citrate was established by changing the color from green to blue.

2.12.15 Motility test

Motility test was carried out by two ways as described below

i. Wet mount method
ii. Semi-solid medium method

Wet mount method (Sneath *et al.* 1986)

Bacterial suspensions were prepared using 24 h cultures with physiological saline (0.85%NaCl). On a clean and oil free slide a drop of bacterial suspension was taken and covered with a clean cover slip. The edge of the cover glass was sealed with petroleum jelly/nail polish and the bacterial cells examined with a phase contrast microscope. This were used for the study of vegetative cells, spores, sporangia and motility. Photomicrographs were taken with an advanced research microscope (Nikon Microphot, Japan) fitted with photo micrographic attachment (Nikon, FX 35 WA, Japan).

Movement of the bacterial cells revealed the motility of bacteria and the result was recorded.

Semi-solid medium method (Eklund and Lankford 1967)

In this method semi-solid agar medium were inoculated by stabbing the medium to a depth of 5mm with the help of a straight wire loop. The tubes were then incubated at 37C for 24 hrs.

Growth of the organisms throughout the medium indicated the motility of the organisms while non motile organisms were confined to the stab region.

2.13 Identification of the isolates

Following Bergey's Manual of Systematic Bacteriology Vol.1 (Kreig and Holt., 1984) Gram negative aerobic heterotrophic bacteria (*Aeromonas)* were identified.

2.14 Determination of resistance to antibiotics Kirby-Bauer disc diffusion susceptibility test (Hudzicki, 2012)

The Kirby-Bauer disc diffusion test is done to determine the sensitivity or resistance of pathogenic facultative anaerobic bacteria to various antimicrobial compounds. The pathogenic bacteria are grown on Muller Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit the organism.

- The isolates were grown in 5ml of nutrient broth.
- The Muller-Hinton agar plate was inoculated with the test organism by streaking the swab in a back and forth motion very close together as the plate was moved across down.
- The plate was rotated 60 and the action was repeated and the lid was slightly jarred allowing the plate to sit at room temperature for the surface of the agar plate to dry.
- Selected antibiotic discs were placed on the surface of the agar using forcep to dispense each disc at a time.
- The plates were placed at 37°C inversely for 24 h.

After 24 h the results were compared with the standard zones of inhibition for each antibiotic and the sensitivity, resistance or intermediary relationship of each of the selected bacteria was determined.

2.15 Molecular Techniques used for identification of *A. hydrophila*

2.15.1 The purity of DNA

Ultraviolet absorbance can be used to check the purity of a DNA preparation. With a pure sample of DNA the ratio of the absorbance at 260 nm and 280 nm (A 260/A 280) is

1.8. Ratios of less than 1.8 indicate that the preparation is contaminated, either with protein or with phenol.

2.15.2 Measurement of DNA concentration

DNA concentrations can be accurately measured by ultraviolet (UV) absorbance spectrophometry. The amount of UV radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. Usually absorbance is measured at 260nm, at which wavelength an absorbance (A 260) of 1.0 corresponds to 50 µg of double-stranded DNA per ml.

2.15.3 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is an in vitro method for synthesis of nucleic acid in which a particular segment of DNA can be specifically amplified. Primers hybridize with complementary strands of the target sequence and are oriented so that DNA synthesized by the polymerase proceeds across the region between the primers. Since the extension products themselves are also complementary and capable of binding primers, consecutive cycles of amplification essentially double the amount of the target DNA synthesized in the previous cycles. The result is an exponential accumulation of specific target DNA fragments approximately designated by 2^n where n is the number of cycles of amplification performed.

Requirements:

Following reagents are essential for performing PCR

DNA Isolation: Maxwell Cell Kit, Model: AS1030, Origin: Promega, USA.

PCR: Hot Stat Master Mix (dNTPs, Buffer, MgCl2, Taq Pol), Cat: M7432, Origin: Promega, USA.

Gel

1. Agarose, Cat: V3125, Origin: Promega, USA.
2. 1kb DNA Ladder, Cat: G5711, Origin: Promega, USA.
3. Ethidium Bromide Solution, Cat: H5041, Origin: Promega, USA.
4. TAE Buffer: Cat: V4251, Origin: Promega, USA.

In order to identify the isolates based on sequence comparison, partial amplification of 16S rDNA was necessary. For the partial amplification of 16S rDNA gene the following primer pairs were used:

Table 2.2 Primers investigated in this study

Primer	Sequences
27F	5'-AGAGTTTGATMTGGCTCAG
1492R	5'-GGTTACCTTGTTACGACTT 3'-AAGTCGTAACAAGGTAACC*

Preparation of Primer

Primers were dissolved in sterile miliQ water following the instruction product booklet to obtain 100 μM stock concentration of each primer. To make working concentration each primer was diluted 10× further.

Preparation of Template

All the bacterial isolates were cultured to grow single colony and one colony for each were resuspended in 50 μl sterile water and subject to heat lyses by heating in a boiling water bath for 5min. The lysed cell suspension were centrifuges for 1 min at 13,000 rpm in microcentrifuge and then the supernatants were used as the source of template DNA for PCR amplification of 16S rDNA gene.

The following components were used to prepare PCR cocktail. The total volume of PCR cocktail was 200 μl for 8 samples.

Table 2.3 Quantity of Primers with M7431 Master Mix (for 8 reactions)

Sl	**Items**	**Volume**	**Reaction Number**	**Total Volume**
1	Master Mix	12.5 ul	X 8	100 ul
2	T DNA (Concentration 25-65 ng/ul)	1 ul	X 8	8 ul
3	Primer F (Concentration 10-20 pMol)	1 ul	X 8	8 ul
4	Primer R (Concentration 10-20 pMol)	1 ul	X 8	8 ul
5	Water	9.5 ul	X 8	76 ul
	Total	25 ul	Total	200 ul

During the experiment, PCR buffer, dNTPs, Primers and DNA sample solution were thawed in ice from frozen stocks. PCR master mix was prepared for each primer to be tested by adding the components of PCR in the following order (mentioned above in

Table 2.3): ddH2O, Buffer, primer, dNTPS and Taq DNA polymerase mixed thoroughly and kept on ice. In the meantime the PCR tubes compatible with the thermal cycler were marked and aliquot the master mix to individual tube marked for each DNA samples. Template DNA (25 ng/µl) were pipetted (3.0 µl) into PCR tubes containing PCR mix. It was then mixed by tapering the tube following shortspin of the tubes. The total mixture was then recollected. The tubes were then sealed and placed in a thermal cycler and the cycling was started immediately. PCR amplification was done in an oil-free thermal cycler (Applied Biosystems® 2720 Thermal Cycler).

Equation for Annealing Temperature:

$$\text{Annealing Temp} = \frac{(Tm\,F + Tm\,R)}{2} - (1\ to\ 5)$$

PCR Product Size was around 200 base to 1000 base. And extension time was directly related with product sixe. Promega Taq Polymerase can amplify 1000 base per min. So that it was used 1min extension time for PCR Work.

Table 2.4 The optimum amplification cycle for PCR

Number of Cycle	Step Name	Temp	Time.
1	Pre Heat	95°C	3 min
32 to 35 Cycle	Denaturation	95°C	30 sec
	Annealing	As Listed as above Primer List Table	30 sec
	Extension	72°C	1 min
1	Final Extension	72°C	5 min
1	Hold	4°C	Over Night.

After completion of cycling program, the reactions were held at 4ºC.

2.15.4 Electrophoresis of the amplified products and documentation

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 0.4 g agarose powder containing ethidium bromide and 40 ml

1×TAE buffer. Agarose gel electrophoresis was conducted in 1×TAE buffer at 100 Volts and 300 mA for 50 mins. One molecular weight marker 1kb DNA ladder was electrophoresed alongside the amplified sample DNA.DNA bands were observed on UV-transilluminator and photographed by a Gel Documentation system.

2.16 Statistical Analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) v. 20.0 for windows (SPSS, SAS Institute Inc. Cary, USA). The data were analyzed to determine the descriptive statistics such as Standard Error of Mean (SEM), Standard Deviation (SD), Statistic Mean, Minimum and Maximum value and Ranges of variables. One way ANOVA was done to test the significance using 5% level of significance.

Chapter 3

Results

In the present study, samples from the three fish market of Dhaka City were collected in three replicates for enumeration and isolation of bacteria having the similar characteristics with *Aeromonas hydrophila* bacteria the pathogenic, infectious that is the causative agent of Motile Aeromonads Septicemia belonging to those fresh water fishes available to the fish market and consumable to the people.

3.1 Length, weight and source of collected samples

The length, weight and color of the samples are shown in Table 3.1. Length of the collected samples ranged from10.0-21.2 cm. The weight of the samples ranged in between 9.20 and 207.19g. The maximum length (21.20 cm) was found in the fish of Hatirpul Bazar while the minimum (10.0 cm) was recorded in the fish of Ananda Bazar. Source is an important parameter for the variation of bacterial load in selected fish specimen.

Table 3.1 Length, weight and source of collected fishes

Name of fish	Replicate no	Date	Length(cm)	Weight(g)	Source
Sarpunti	1	29.06.2015	13.2	51.00	Ananda bazar
	2	02.11.2015	17.4	66.50	Polashi Bazar
	3	19.10.2015	17.8	81.32	Hatirpul Bazar
Tilapia	1	29.06.2015	18.1	113.42	Ananda Bazar
	2	03.08.2015	16.5	86.22	Polashi Bazar
	3	18.10.2015	21.2	207.19	Hatirpul Bazar
Tatkini	1	06.07.2015	18.2	64.25	Ananda Bazar
	2	03.08.2015	17.1	53.15	Polashi Bazar
	3	10.10.2015	14.3	34.20	Hatirpul Bazar
Koi	1	06.07.2015	10.0	20.60	Ananda Bazar
	2	03.11.2015	15.3	35.40	Polashi Bazar
	3	26.10.2015	14.7	49.77	Hatirpul Bazar
Meni	1	29.10.2015	11.4	9.20	Ananda Bazar
	2	01.11.2015	11.8	26.70	Polashi Bazar
	3	20.10.2015	12.4	38.90	Hatirpul Bazar

3.2 Bacterial load of the collected samples

The bacterial counts of the samples are shown in Table 3.2. A good number of bacteria were found to be associated with the samples collected from Hatirpul fish market. The two isolating media viz. nutrient agar (NA) and Aeromonas selective agar (i.e. Hi-media, Oxoid and LAB Agar media) were tested and both of the media were found to be suitable for enumeration and isolation. The total heterotrophic bacterial load ranged in between$1.60\pm0.252x10^5$ to$5.04\pm0.74x10^5$ respectively. The bacterial counts among the replicates were found to be varied. The maximum mean bacterial count was observed in *Nandus nandus* and the minimum was in *Oreochromis mossambicus.* On the other hand total Aeromonas count was found to be varied also. Maximum was $1.03\pm0.153x10^3$ *Oreochromis mossambicus* and minimum was $2.83\pm0.40x10^2$ in *Anabas testudineus.*

Table 3.2 Showing Bacterial density on NA and different Aeromonas agar of selected fish species. Means (± 1 SEM) within column and row for total for each species of bacteria without letters denote no significant differences (ANOVA, HSD, $p<0.05$).

Species	TBC	Total Aeromonas count
Puntius sarana	$3.34\pm0.63x10^{5ab}$	$9.72\pm1.21x10^{2bc}$
Oreochromis mossambicus	$1.60\pm0.252x10^{5b}$	$1.03\pm0.153x10^{3b}$
Crosocheilus latius	$2.28\pm0.46x10^{5b}$	$5.73\pm0.82x10^{2ab}$
Anabas testudineus	$2.69\pm0.42x10^{5b}$	$2.83\pm0.40x10^{2a}$
Nandus nandus	$5.04\pm0.74x10^{5a}$	$4.83\pm0.92x10^{2a}$

The table 3.2 describes the differences in mean according to row. The highest count of TBC was $5.04\pm0.74x10^5$ in *Nandus nandus* and lowest TBC count was $1.60\pm0.252x10^5$ in *Oreochromis mossambicuordings*. For the total Aeromonas count, it also shows differences in mean according to mean among the selected freshwater fishes. The highest Aeromonas count was $1.03\pm0.153x10^3$ in *Oreochromis mossambicus* and the lowest was $2.83\pm0.40x10^2$ in *Anabas testudineus.*

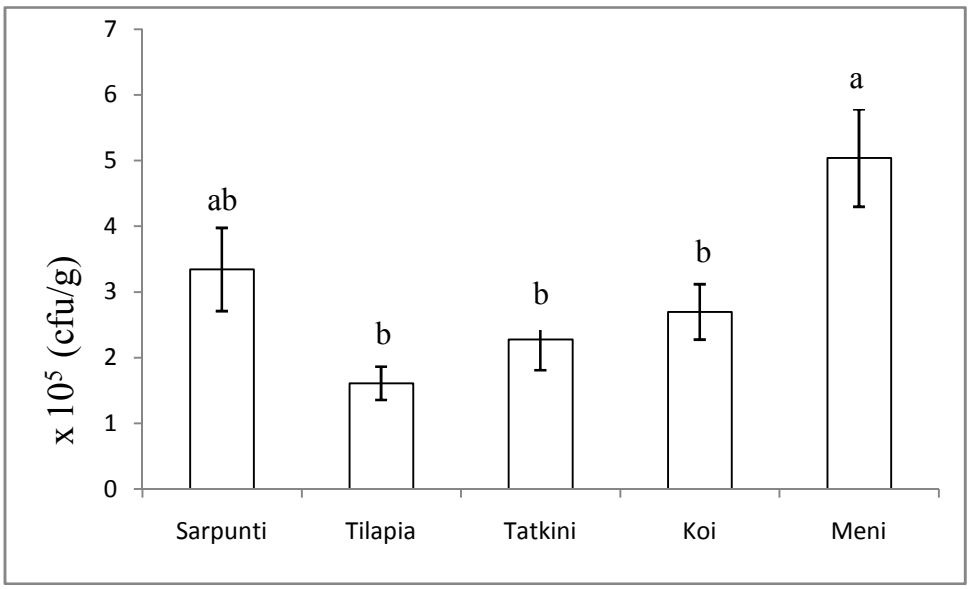


Figure 3.1 Graph showing variation in total bacterial count from selected freshwater fishes.

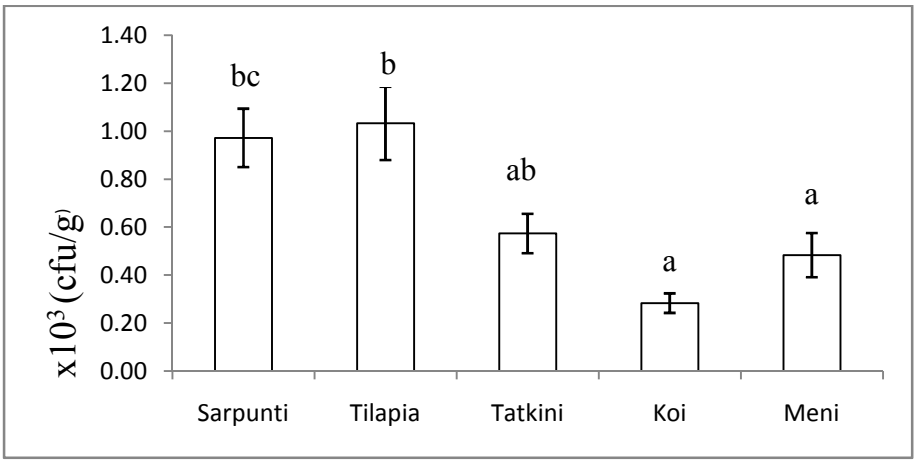


Figure 3.2 Graph showing variation (Mean±SEM) in total Aeromonas count from selected fresh water fishes.

Table 3.3 Showing bacterial density (cfu/g) on NA and Aeromonas agar (Hi-media) of selected organs of fish species. Means (± 1 SEM) within column and row for total for each species of bacteria without letters denote no significant differences (ANOVA, HSD, p<0.

Organs	TBC	Total Aeromonas Count
Muscle	$5.83\pm073x10^{4a}$	$1.76\pm0.24x10^{2c}$
Gill	$4.21\pm0.42x10^{5b}$	$1.09\pm0.103x10^{3a}$
Gut	$4.18\pm0.46x10^{5b}$	$7.38\pm0.66x10^{2b}$

The table 3.2 describes the differences in mean according to row. The highest count of TBC was $4.21\pm0.42x10^{5}$ in gill and lowest TBC count was $5.83\pm073x10^{4}$ in muscle. For the total Aeromonas count, it also shows differences in mean according to mean among the selected freshwater fish organ. The highest Aeromonas count was $1.09\pm0.103x10^{3}$ in gill and the lowest was $1.76\pm0.24x10^{2}$ in muscle.

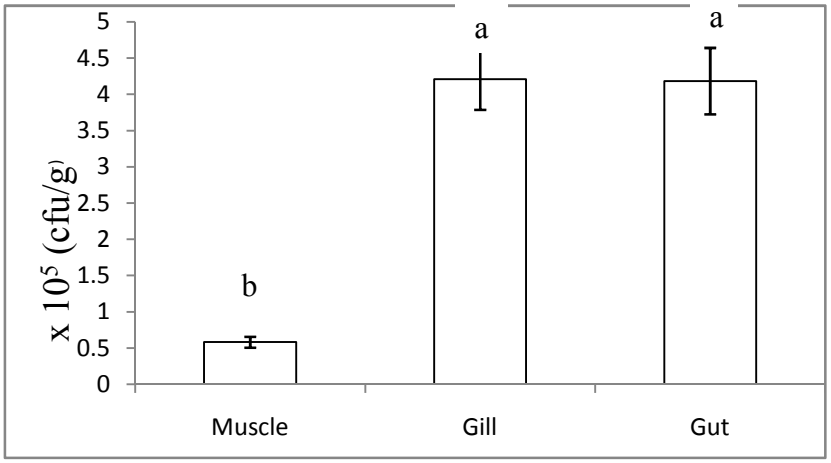


Figure 3.3 Graph showing variation in total bacterial count from selected fish organs.

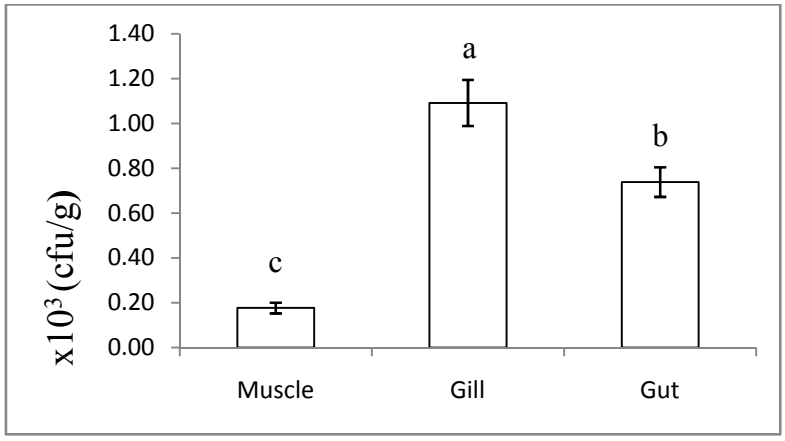


Figure 3.4 Graph showing variation (Mean±SEM) in total *Aeromonas* count from organs of selected fresh water fish.

Table 3.4 Showing bacterial count (cfu/g) on NA and Aeromonas Agar media of fish species collected from different fish market of Dhaka City. . Means (± 1 SEM) within column and row for total for each species of bacteria without letters denote no significant differences (ANOVA, HSD, $p<0.05$).

Market	TBC	*Aeromonas* Count
Ananda	$3.29\pm0.48x10^5$	$8.10\pm1.09x10^2$
Polashi	$3.33\pm0.47x10^5$	$6.33\pm0.67x10^2$
Hatirpool	$2.35\pm0.36x10^5$	$5.63\pm0.90x10^2$

The table 3.2 describes the differences in mean according to row. The highest count of TBC was $3.33\pm0.47x10^5$ in Polashi and lowest TBC count was $2.35\pm0.36x10^5$ in Hatirpool. For the total *Aeromonas* count, it also shows differences in mean according to mean among the selected freshwater fish organ. The highest *Aeromonas* count was $8.10\pm1.09x10^2$ and the lowest was$5.63\pm0.90x10^2$ in Hatirpool Bazar.

Figure 3.5 Graph showing variation in total bacterial count from selected freshwater fishes.

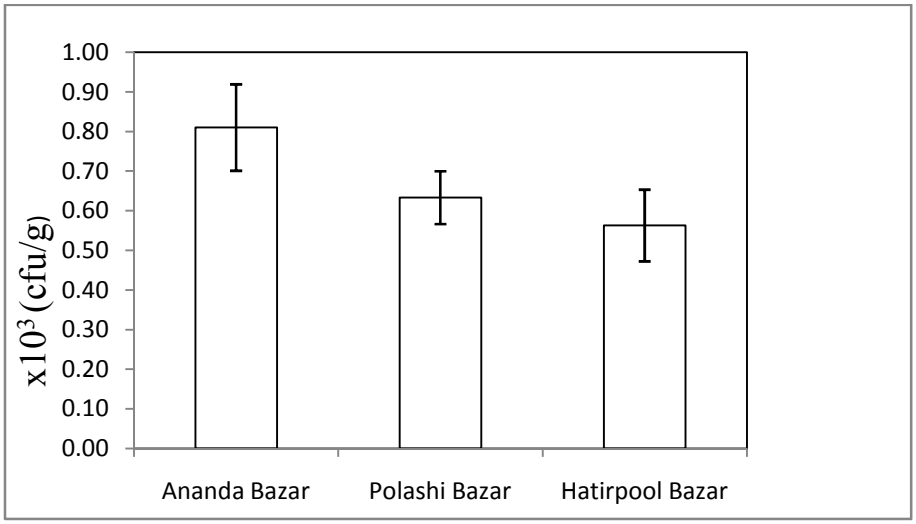


Figure 3.6 Graph showing variation (Mean±SEM) in total *Aeromonas* count from selected fresh water fishes collected from different fish market in Dhaka City.

Two other aeromonas agar (Oxoid and LAB agar media) was used for the identification and isolation of *Aeromonas* spp in some selected freshwater fishes collected from Polashi Bazar and Hatirpool Bazar.

Table 3.5 Showing bacterial count (cfu/g) on different Aeromonas Agar media of fish species collected from different fish market of Dhaka City. . Means (± 1 SEM) within column and row for total for each species of bacteria without letters denote no significant differences (ANOVA, HSD, p<0.05).

Species	AH	AO	AL
Puntius sarana	$1.04\pm0.16x10^{3a}$	$2.97\pm0.65\ x10^{3a}$	$3.59\pm1.07\ x10^{3a}$
Anabas testudineus	$2.68\pm0.49\ x10^{2b}$	$7.16\pm0.77\ x10^{2b}$	$2.35\pm0.24\ x10^{2a}$
Nandus nandus	$3.98\pm0.88\ x10^{2b}$	$6.13\pm1.06\ x10^{2b}$	$2.21\pm0.26\ x10^{2a}$

Table 3.5 showing variation of *Aeromonas* spp in different aeromonas agar media from some selected fish species.

Table 3.6 Showing bacterial count (cfu/g) on different Aeromonas Agar media of fish organs collected from different fish market of Dhaka City. . Means (± 1 SEM) within column and row for total for each species of bacteria without letters denote no significant differences (ANOVA, HSD, p<0.05).

Organs	AH	AO	AL
Muscle	$1.13\pm0.2 \times 10^{2b}$	$3.15\pm0.17 \times 10^{2b}$	$9.67\pm1.13 \times 10^{1b}$
Gill	$9.62\pm1.57 \times 10^{2a}$	$2.53\pm0.67 \times 10^{3a}$	$5.36\pm0.79 \times 10^{2a}$
Gut	$6.27\pm1.02 \times 10^{2a}$	$1.46\pm0.24 \times 10^{3ab}$	$1.82\pm0.25 \times 10^{2b}$

Table 3.6 showing variation of *Aeromonas* spp in different aeromonas agar media from some selected fish species.

Table 3.7 Showing bacterial count (cfu/g) on different Aeromonas Agar media of fish species collected from different fish market of Dhaka City. . Means (± 1 SEM) within column and row for total for each species of bacteria without letters denote no significant differences (ANOVA, HSD, p<0.05).

Market	AH	AO	AL
Polashi	$5.86\pm0.99 \times 10^{2}$	$1.58\pm0.48 \times 10^{3}$	$2.45\pm0.33 \times 10^{2}$
Hatirpool	$5.49\pm1.23 \times 10^{2}$	$1.28\pm0.23 \times 10^{3}$	$2.98\pm0.68 \times 10^{2}$

Table 3.7 Showing variation of *Aeromonas* spp in different aeromonas agar media from some selected fish species collected from two fish market in Dhaka Metropolitan City.

Table 3.8 A. Bacterial density (cfu/g) in Sarpunti *Puntius sarana,* Tatkini *Crosocheilus latius,* koi *Anabas testudineas*, tilapia *Oreochromis spp* and *Nandus nandus* measured from different organs of fishes. The fishes were sampled from different markets in Dhaka city with Means (±1 SD).

Fishes	Organs		
	Muscle	**Gill**	**Gut**
Puntius sarana	$9.83\pm1.25\times10^4$	$10.2\pm1.99\times10^5$	$5.9\pm2.5\times10^5$
Oreochromis mossambicus	$2.67\pm1.53\times10^4$	$2.83\pm0.8\times10^5$	$14.7\pm4.42\times10^5$
Crosocheilus latius	$3.78\pm1.96\times10^4$	$3.3\pm2.6\times10^5$	$9.5\pm1.32\times10^4$
Anabas testudineus	$6.42\pm3.6\times10^4$	$1.68\pm0.5\times10^5$	$4.53\pm0.32\times10^5$
Nandus nandus	$6.67\pm1.71\times10^4$	$7.7\pm1.05\times10^5$	$9\pm0.79\times10^5$

The table A describes the differences in mean according to row and column. The highest count of TBC was $14.7\pm4.42\times10^5$ in gut of *Oreochromis mossambicus* and lowest TBC count was $2.67\pm1.53\times10^4$ in muscle of *Oreochromis mossambicus.*

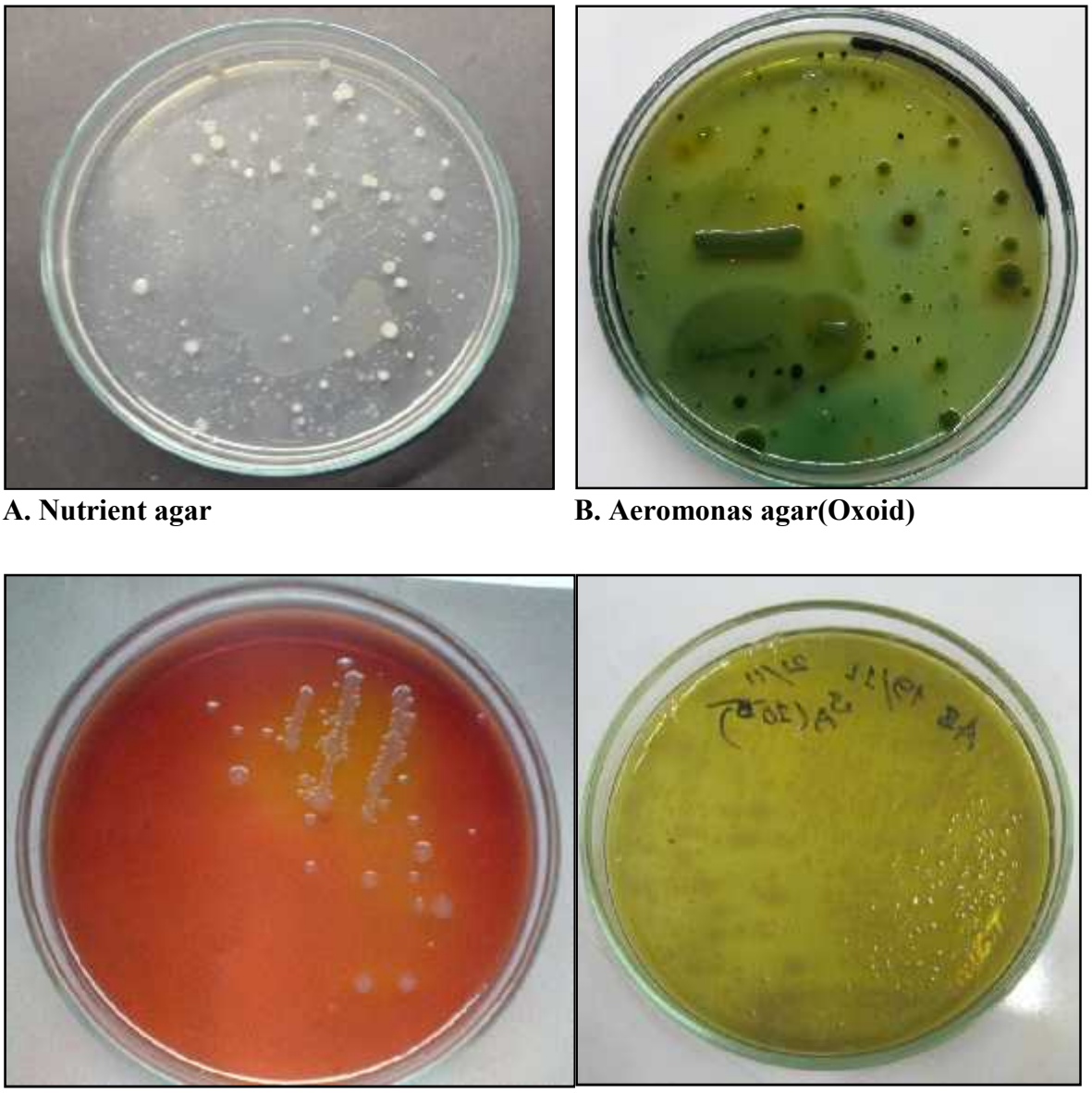
Table 3.8 B. Bacterial density (cfu/g) in Sarpunti *Puntius sarana,* Tatkini *Crosocheilus latius,* koi *Anabas testudineas*, tilapia *Oreochromis spp* and *Nandus nandus* measured from different organs of fishes. The fishes were sampled from different markets in Dhaka city with Means (±1 SD).

Fishes	Organs		
	Muscle	**Gill**	**Gut**
Puntius sarana	$1.96\pm0.41\times10^2$	$1.57\pm0.39\times10^3$	$1.10\pm0.17\times10^3$
O. mossambicus	$5.1\pm2.6\times10^2$	$2.08\pm0.99\times10^3$	$1.85\pm0.11\times10^3$
Crosocheilus latius	$1.76\pm1.67\times10^2$	$6.93\pm2.67\times10^3$	$7.73\pm0.66\times10^3$
Anabas testudineus	$3.33\pm5.77\times10^0$	$5.13\pm0.35\times10^2$	$3.66\pm0.55\times10^2$
Nandus nandus	$2.46\pm1.26\times10^2$	$1.4\pm0.11\times10^3$	$3.26\pm0.92\times10^2$

The table B describes the differences in mean according to row and column. The highest count of Total *Aeromonas* was $7.73\pm0.66\times10^3$ in gut of *Crosocheilus latius* and lowest TBC count was $3.33\pm5.77\times10^0$ in muscle of *Anabas testudineus.*

3.3 Isolation, selection and purification of the selected isolates

During this study a total of 45 colonies were primarily selected based on their colony morphology. These colonies comprised of all aerobic heterotrophic bacteria. Finally on the basis of their characters similar to *Aeromonas hydrophila* on the selected Aeromonas medium, 15 isolates were selected and purified for detail study towards identification and molecular characterization. Plate 3.1 and 3.2 show bacterial colonies developed in different steps of isolation and purification .On the other hand plate 3.3-3.6 shows comparison among strain of this study and reference strain (ATCC 7966).



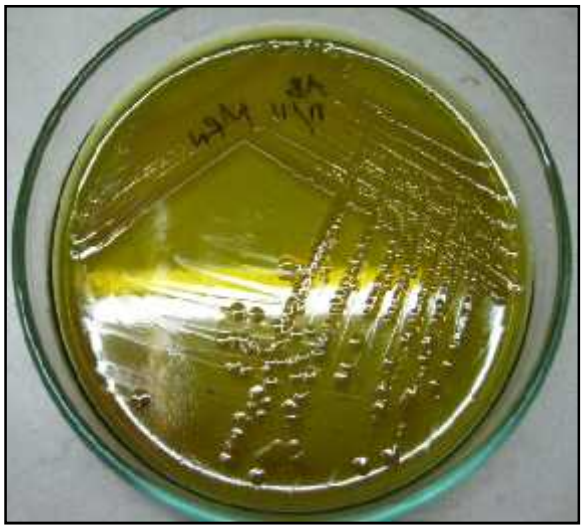
A. Nutrient agar **B. Aeromonas agar(Oxoid)**

C. Aeromonas agar(Hi-media) **D. Aeromonas agar(LAB)**

Plate 3.1 Photograph showing different bacterial colonies during enumeration and isolation of bacteria from *Puntius sarana* of Ananda bazar.

A. Sp 1 in aeromonas agar (Oxoid)

B. Tp 2 in aeromonas agar (Hi-media)



C. M 1 in aeromonas agar (LAB)

D. M 1 in nutrient agar

Plate 3.2 Photographs showing streak plate method for purification of the selected isolates.

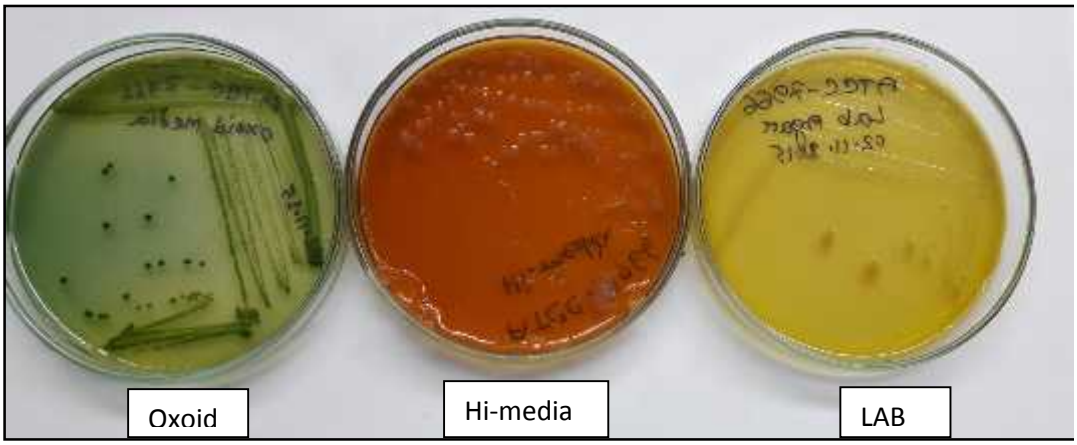


Plate 3.3 Photograph showing comparison among ref. strain (ATCC 7966) of different Aeromonas agar.

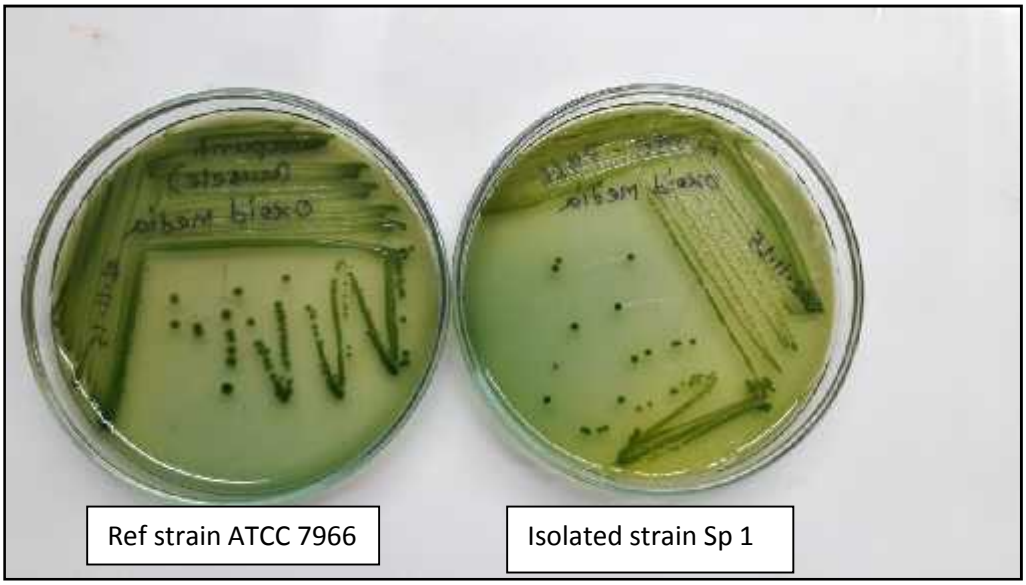


Plate 3.4 Photograph showing comparison between reference strain and Sp 1 strain in aeromonas agar (Oxoid Media).

Plate 3.5 Photograph showing comparison between reference strain and Sp 1 strain in aeromonas agar (LAB agar).

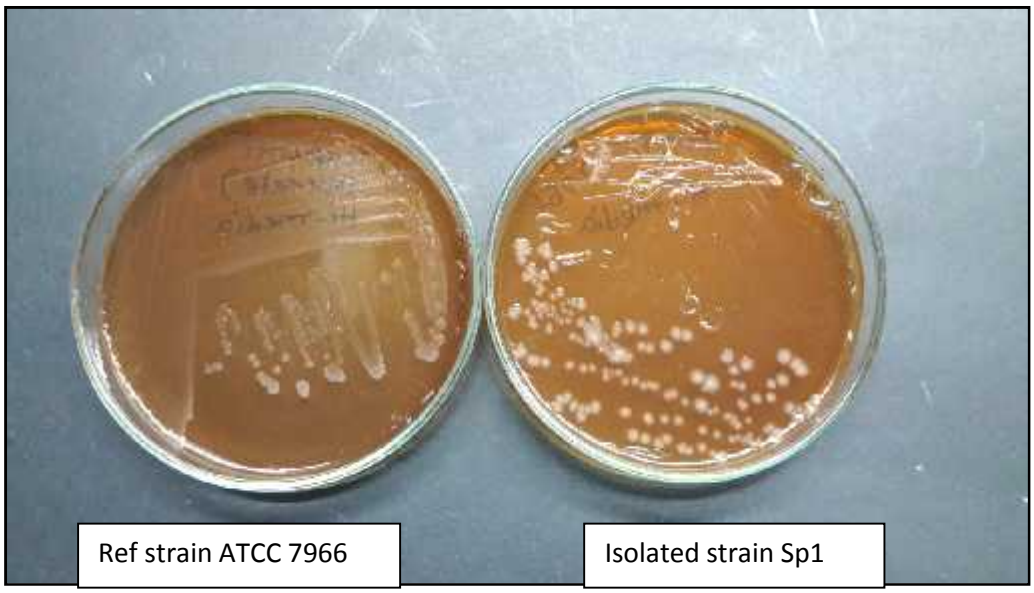


Plate 3.6 Photograph showing comparison between reference strain and Sp 1 strain in aeromonas agar (Hi- Media).

3.4 Colony morphology of the selected isolates

Colony morphology of the selected isolates was recorded in their form/shape, pigmentation, surface elevation, margin, surface and optical characteristics. The colonial morphologies of the selected isolates are presented in Table 3.9. All the colonies of the selected isolates were green in Oxoid agar media, pinkish in Hi-media, tan to buff color in Lab agar media and off white in NA, convex, smooth and opaque.

Table 3.9 Colony morphology of the selected isolates on NA

Isolate	Shape	Pigmentation	Elevation	Margin	Surface	Opacity
Sp 1	Circular	Off white	Convex	Entire	Smooth	Opaque
Sp 2	Circular	Off white	Convex	Entire	Smooth	Opaque
Sp 3	Circular	Off white	Convex	Entire	Smooth	Opaque
Tp 1	Circular	Off white	Convex	Entire	Smooth	Opaque
Tp 2	Circular	Off white	Convex	Entire	Smooth	Opaque
Tp 3	Circular	Off white	Convex	Entire	Smooth	Opaque
Tt 1	Circular	Off white	Convex	Entire	Smooth	Opaque
Tt 2	Circular	Off white	Convex	Entire	Smooth	Opaque
Tt 3	Circular	Off white	Convex	Entire	Smooth	Opaque
K 1	Circular	Off white	Convex	Entire	Smooth	Opaque
K 2	Circular	Off white	Convex	Entire	Smooth	Opaque
K 3	Circular	Off white	Convex	Entire	Smooth	Opaque
M 1	Circular	Off white	Convex	Entire	Smooth	Opaque
M 2	Circular	Off white	Convex	Entire	Smooth	Opaque
M 3	Circular	Off white	Convex	Entire	Smooth	Opaque

3.5 Liquid culture characteristics of the selected isolates in broth medium

The selected isolates were studied in nutrient broth medium. Growth responses with other characteristics are shown in Table 3.10.

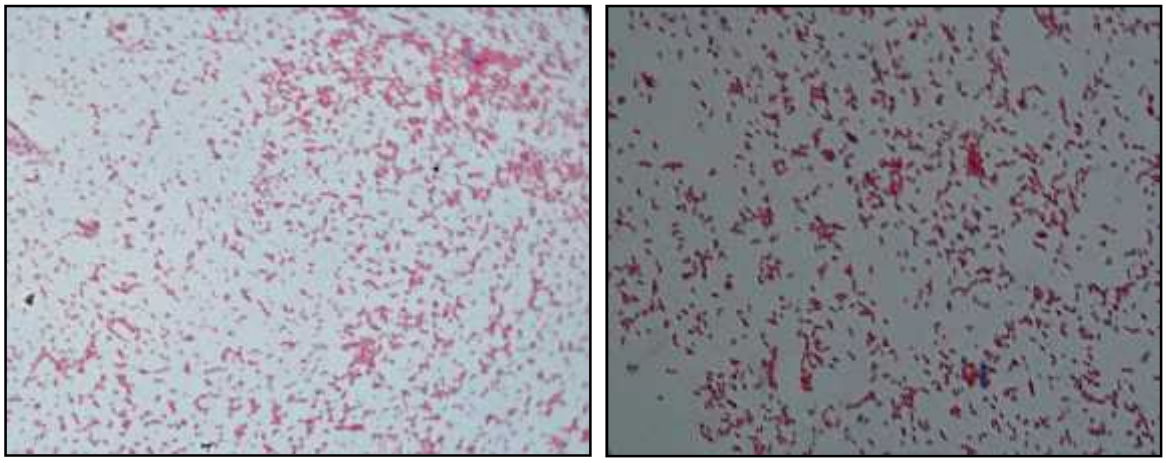
Table 3.10 Liquid culture characteristics of the selected isolates in nutrient broth

Isolate No	Growth amount	Surface growth	Sub-surface growth	Sediment
Sp 1	Abundant	No growth	Cloudy	Flaky
Sp 2	Abundant	No growth	Cloudy	Flaky
Sp 3	Abundant	No growth	Cloudy	Flaky
Tp 1	Scanty	No growth	Cloudy	Flaky
Tp 2	Abundant	No growth	Cloudy	Flaky
Tp 3	Scanty	No growth	Cloudy	Flaky
Tt 1	Abundant	No growth	Cloudy	Flaky
Tt 2	Abundant	No growth	Cloudy	Flaky

Tt 3	Abundant	No growth	Cloudy	Flaky
K 1	Abundant	No growth	Cloudy	Flaky
K 2	Abundant	No growth	Cloudy	Flaky
K 3	Abundant	No growth	Cloudy	Flaky
M 1	Scanty	No growth	Cloudy	Flaky
M 2	Abundant	No growth	Cloudy	Flaky
M 3	Abundant	No growth	Cloudy	Flaky

3.6 Microscopic observation of the selected isolates

All isolates were Gram negative. Photomicrographs of the selected isolates are shown in the plate 3.7.



(A) Simple Staining (B) Gram Staining

Plate 3.7 Photograph showing staining of isolated bacteria

3.7 Physiological and biochemical characteristics of the strains

Selected strains were studied for their biochemical characteristics. These features are essential for identification. The results were grouped into several tables for convenience of comparison. The results of the fermentation tests with the selected carbohydrates are shown in Table 3.11. Among the 15 isolates, 11 isolates could ferment all the tested carbohydrates and 4 couldn't whereas K 2 couldn't ferment Ababinose and Xylose, K 1 and M 2 couldn't ferment Sucrose and Xylose respectively, whereas all the isolates could ferment Glucose but 3 of the selected isolates *viz.* M 2, M 3 and Tp 3 couldn't produce gas during glucose fermentation (Plate 3.8).

For the inositol fermentation, 10 of the isolates couldn't ferment inositol but rest 5 isolates could whereas *Aeromonas hydrophila* (ATCC 7966) can't ferment inositol.

The results of the physiological and biochemical tests are given in Table 3.12. All the tested organisms were catalase negative but oxidase and KOH solubility positive. Among the isolates, 9 showed positive results for the V.P. test, 7 showed positive results for M.R. test and 10 showed positive result for indole production. The only 5 isolates to show negative results for the indole production were Tp 2, Tt 1, Tt 2, Tt 3 and K 1. Out of the 15 isolates only 3 isolates couldn't utilize citrate. 3 isolates viz. Sp 1, Tp 3 and M1 could not utilize citrate. Interestingly M 1 strain showed all result similar to reference strain but it showed different result for citrate production where it can't produce citrate and change the color from green to blue. For the esculin hydrolysis, only 2 strains (Tp 3 & Tt 1) couldn't hydrolyze while rest of the strain could hydrolyze esculin. All isolates showed motility in sloppy agar medium. Plate 3.9 shows different physiological and biochemical test.

Table 3.11 Fermentation tests of the selected carbohydrates for identification of selected isolates comparison to Reference strain (ATCC 7966).

SL	Isolate	L-Arabinose	D-Manitol	D-Xylose	Inositol	Sucrose	Glucose	
							Acid	Gas
1	Sp 1	+	+	+	_	+	+	+
2	Sp 2	+	+	+	_	+	+	+
3	Sp 3	+	+	+	_	+	+	+
4	Tp 1	+	+	_	_	+	+	+
5	Tp 2	+	+	+	_	+	+	+
6	Tp 3	+	+	+	_	+	+	_
7	Tt 1	+	+	+	+	+	+	+
8	Tt 2	+	+	+	+	+	+	+
9	Tt 3	+	+	+	+	+	+	+
10	K 1	+	+	+	_	-	+	+
11	K 2	_	+	_	+	+	+	+
12	K 3	+	+	+	_	+	+	+
13	M 1	+	+	+	_	+	+	+
14	M 2	+	+	_	_	+	+	-
15	M 3	+	+	+	+	+	+	-
16	Ref	+	+	+	+	+	+	+

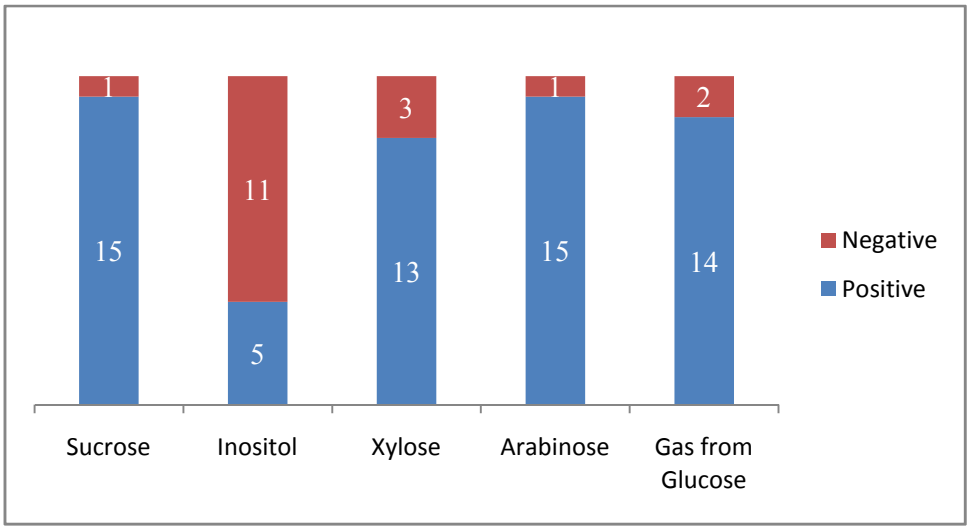


Figure 3.7 Comparison among different fermentation tests of the selected isolates.

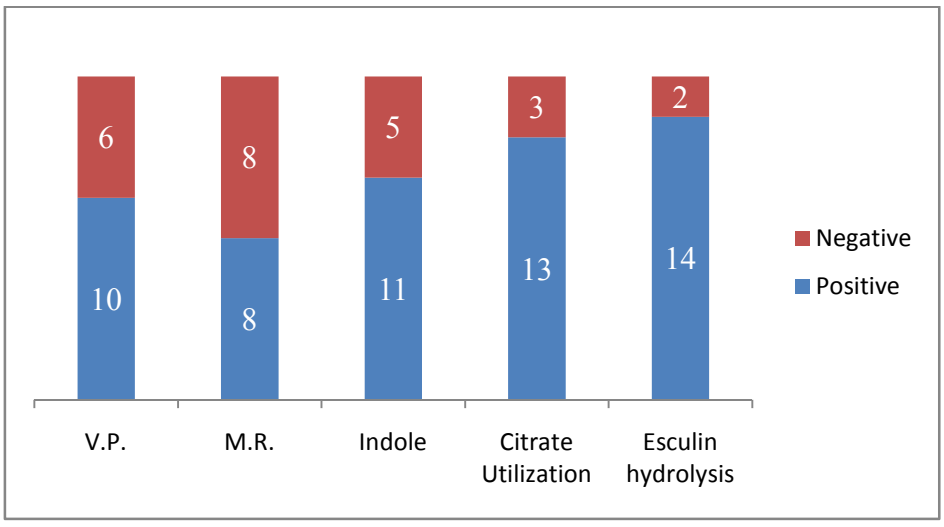
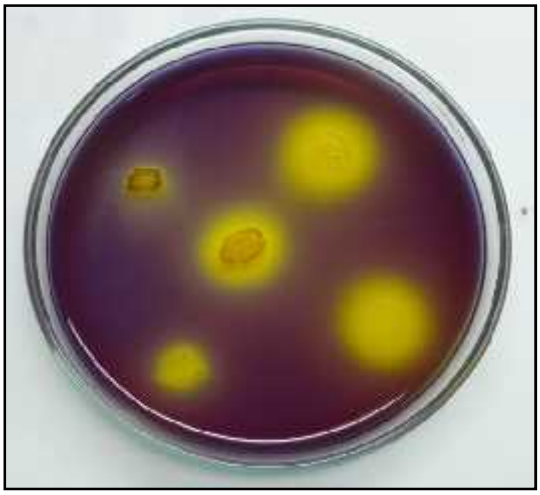


Figure 3.8 Comparison among different biochemical and physiological tests of the selected isolates.



(A) Mannitol

B) Inositol

C) Xylose

D) Sucrose

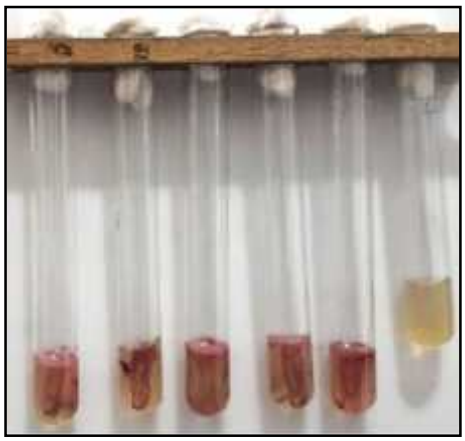
E) Arabinose

F) Glucose

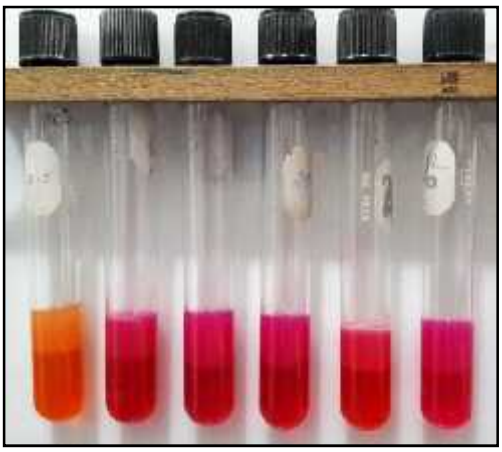
Plate 3.8 Photographs showing carbohydrate fermentation of the selected isolates.

Table 3.12 Biochemical test of selected isolates comparison to Reference strain.

SL No	Isolate No	Slant	Butt	H2S Production	Motility	Citrate	Esculin	Arginine	Indole	VP	Methyl Red
1	Sp 1	K	K	+	+	-	+	+	+	-	+
2	Sp 2	K	K	+	+	+	+	+	+	-	+
3	Sp 3	K	K	+	+	+	+	+	+	+	-
4	Tp 1	K	K	+	+	+	+	+	+	+	-
5	Tp 2	K	K	+	+	+	+	+	-	-	+
6	Tp 3	K	K	+	+	-	-	+	+	+	-
7	Tt 1	K	K	+	+	+	-	+	-	-	+
8	Tt 2	K	K	+	+	+	+	+	-	+	+
9	Tt 3	K	K	+	+	+	+	+	-	-	+
10	K 1	K	K	+	+	+	+	+	-	-	+
11	K 2	K	K	+	+	+	+	+	+	+	-
12	K 3	K	K	+	+	+	+	+	+	+	-
13	M 1	A	K	+	+	-	+	+	+	+	-
14	M 2	A	K	+	+	+	+	+	+	+	+
15	M 3	K	K	+	+	+	+	+	+	+	-
16	Ref	K	K	+	+	+	+	+	+	+	-



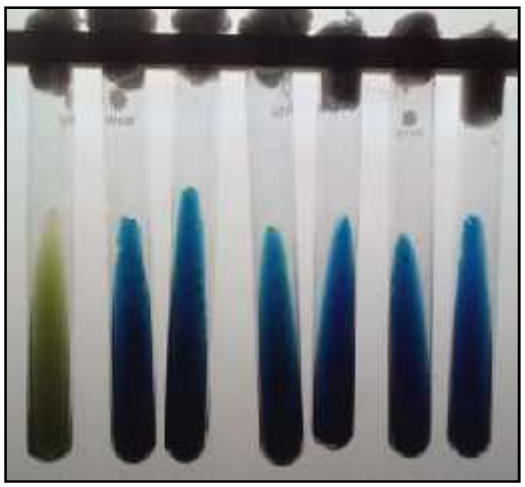
A. Motility test



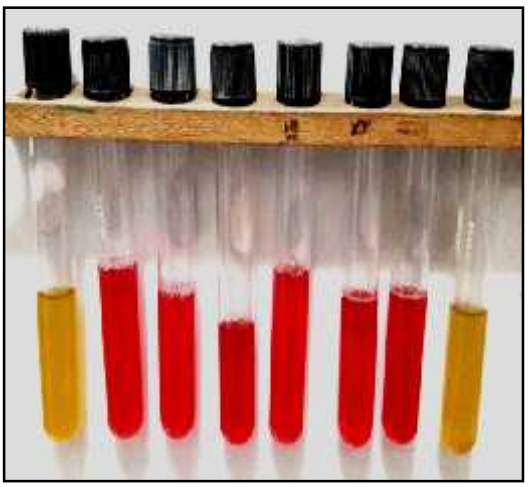
B. Arginine dihydrolysis

C. KIA test

D. Esculin hydrolysis



E. Citrate utilization test



F. Indole production

Plate 3.9 Photograph showing different biochemical test.

3.8 Culture and sensitivity test of the isolated bacteria

Results of culture of sensitivity test are shown in Table 3.13 and plate 3.10. During this study 16 bacteria including 1 reference strain (ATCC 7966) were tested against 14 common antibiotics viz. Amikacin, Amoxycilin, Ampicilin, Chloramphenicol, Ciprofloxacin, Erythromycin, Gentamycin, Kanamycin, Nalidixic Acid, Nitrofurantoin, Polymyxin B, Streptomycin, Sulphamethoxazole and Tetracycline.

In case of Ampicilin, only 3 (18.75%) strain showed sensitivity were Sp 1, Tp 3 and M 1. Rest of the strain including reference strain showed resistant.

All the strain showed resistant to Amoxycilin whereas all the strain showed sensitivity to Amikacin and Gentamycin.

In case of Chloramphenicol 2 (80%) strains (Tt 2, K 1) showed resistant and 1 (6.25%) (Sp 2) showed intermediate resistant result.

2 (80%) strain (Reference and Sp 3) showed intermediate and 2 (80%) strain (Sp 1 and Tp 3) showed sensitivity to Erythromycin. Rest of the strain showed resistance to this antibiotic.

Only Tt 2 (80%) strain showed resistance to Ciprofloxacin while rest of the strain showed sensitivity to this antibiotic. 4 (25%) strain showed sensitivity to Sulphamethoxazole were Sp 1, Sp 3, Tp 3 and M 1 while 1 strain Tp 2 showed intermediate resistant and rest showed resistant to this antibiotic.

Among 16 strain of Aeromonas including reference strain 7 (43.75%) strain showed sensitivity to Nalidixic Acid were Sp 1, Sp 3, Tp 2, Tp 3, K 3, M 1 and ATCC-7966 reference strain while rest strains showed resistant to Nalidixic Acid antibiotic.

In case of Tetracycline 6 (37.5%) strain *viz.* Sp 2, Tp 1, Tt 2, K 2, K 3 and M 2 showed whereas rest strain including K 1 (which showed similarity with reference strain ATCC 7966) showed sensitivity to this antibiotic.

Considering Chloramphenicol as standard Polymyxin B showed sensitivity only to Sp 3 strain and showed intermediate resistant to 6 strains including reference strain (ATCC 7966) while rest showed resistant to this Polymyxin B antibiotic.

All the strain except Tt 1 and Tt 3 showed sensitivity to Kanamycin while those two strain showed intermediate resistant to Kanamycin antibiotic.

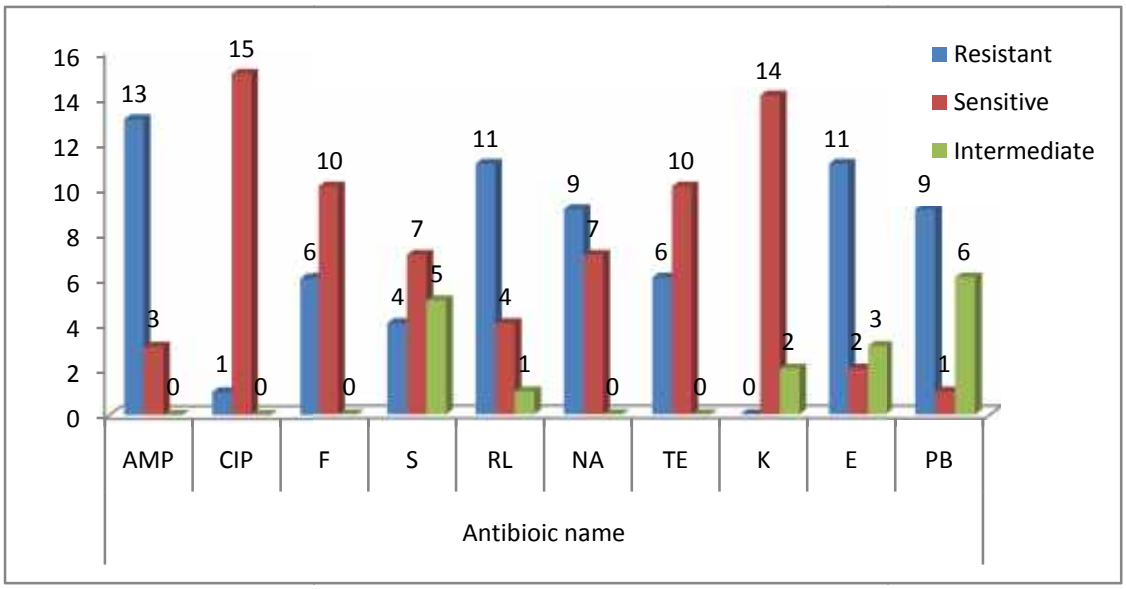


Figure 3.9 Graph showing variation in different antibiotic used.

Table 3.13 Results of culture and sensitivity test of the selected bacteria as performed by Kirby-Bauer disk diffusion susceptibility test (Hudzicki 2012)

Isolated strain	Antibiotic used in this experiment (µg)													
	AK 30	AML 10	AMP 10	C 30	CIP 5	E 15	GN 10	K 30	NA 30	S 10	RL 25	TE 30	F 300	PB 300units
Sp 1	S	S	S	S	S	S	S	S	S	R	I	S	S	R
Sp 2	S	R	R	S	S	R	S	S	R	I	R	R	S	I
Sp 3	S	R	R	S	S	I	S	S	S	S	S	S	R	S
Tp 1	S	R	R	S	S	R	S	S	R	R	R	R	S	R
Tp 2	S	R	R	S	S	R	S	S	S	I	S	S	R	R
Tp 3	S	R	S	S	S	S	S	S	S	S	S	S	S	I
Tt 1	S	R	R	S	S	R	S	I	R	S	R	S	R	R
Tt 2	S	R	R	R	R	R	S	S	R	S	R	R	R	R
Tt 3	S	R	R	I	S	R	S	I	R	R	R	S	S	I
K 1	S	R	R	R	S	R	S	S	R	R	R	S	R	I
K 2	S	R	R	S	S	R	S	S	R	S	S	R	S	R
K 3	S	R	R	S	S	R	S	S	S	S	R	R	S	R
M 1	S	R	S	S	S	S	S	S	S	I	R	S	S	R
M 2	S	R	R	S	S	R	S	S	R	S	R	R	S	R
M 3	S	R	R	S	S	I	S	S	R	S	R	S	R	I
Reference	S	R	R	S	S	I	S	S	S	S	R	S	S	I

A. Sp 3

B. Tp 1

C. M 1

D. Tt 2

Plate 3.10 Photograph showing the culture and sensitivity test of some selected isolates.

3.9 Provisional Identification

Consulting all morphological, biochemical and physiological characters of the isolated organisms, provisional identifications were done with the help of Bergey's manual of systematic bacteriology (Sneath et al. 1986). All the bacterial isolates belonged to a single genus *Aeromonas.*

The genus *Aeromonas* comprises many species and the selected isolates were provisionally identified on the basis of resemblance to that of standard strains. They belonged to Aeromonas complex.

Table 3.14 shows provisionally identified names of the bacterial isolates. *Aeromonas hydrophila* was found to be dominant among the isolates.

Table 3.14 Provisional identification of some selected isolates including Reference strain.

SL No.	Isolate No.	Provisionally Identified Bacteria
1	Sp 1	*Aeromonas hydrophila*
2	Sp 2	*Aeromonas hydrophila*
3	Sp 3	*Aeromonas hydrophila*
4	Tp 1	*Aeromonas hydrophila*
5	Tp 2	*Aeromonas hydrophila*
6	Tp 3	*Aeromonas hydrophila*
7	Tt 1	*Aeromonas hydrophila*
8	Tt 2	*Aeromonas hydrophila*
9	Tt 3	*Aeromonas hydrophila*
10	K 1	*Aeromonas hydrophila*
11	K 2	*Aeromonas hydrophila*
12	K 3	*Aeromonas hydrophila*
13	M 1	*Aeromonas hydrophila*
14	M 2	*Aeromonas hydrophila*
15	M 3	*Aeromonas hydrophila*
Referrence	ATCC- 7966	*Aeromonas hydrophila*

3.10 Molecular Characterization of the selected isolates

Using a pair of universal primer the 16S rDNA gene was amplified from eight unknown bacterial isolates. The PCR amplified DNA of the eight isolates (Sp 1, Sp 3, Tp 1, Tp 3, Tt 1, Tt 2, K 1 and M 1) were gel purified.

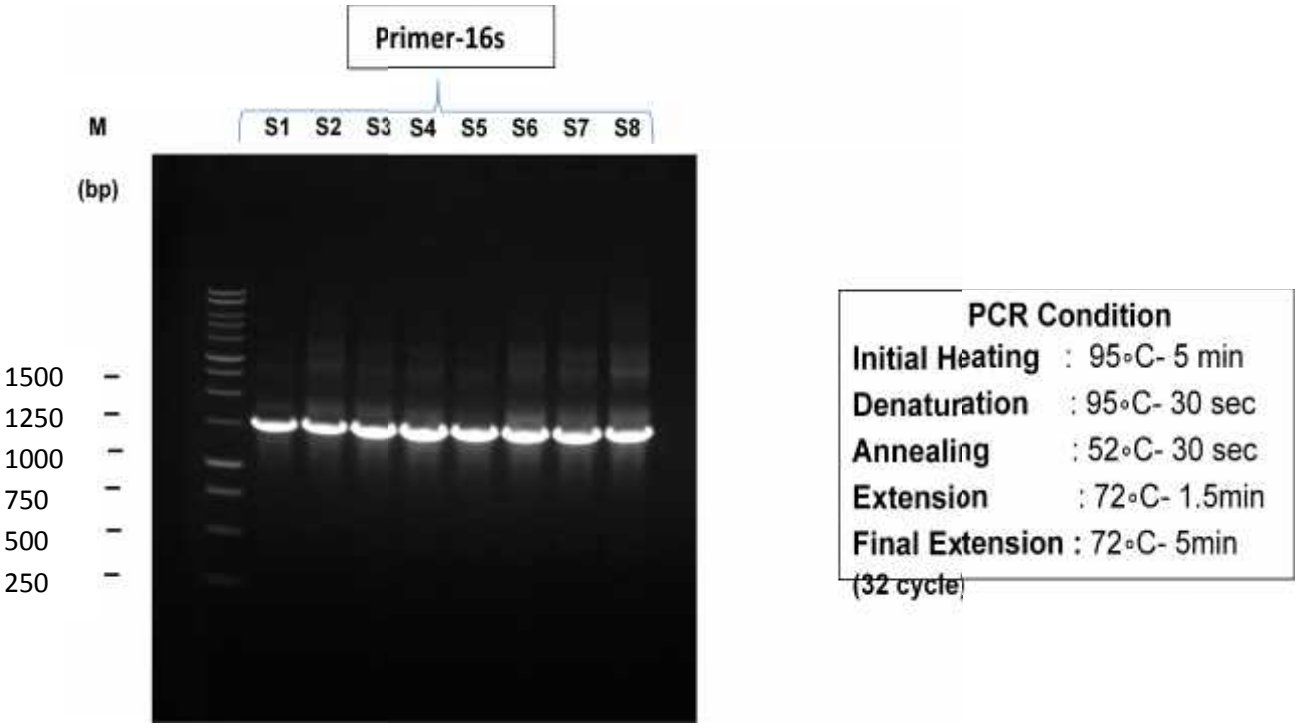


Figure 3.10 PCR Amplification of part of the 16S rDNA gene. Lane M=1.0kb ladder, lanes 1-4 are representing 4 different bacterial isolates viz. Sp 1, Sp 3, Tp 1, Tp 3, Tt 1, Tt 2, K 1 and M. In the gel approximate size of the amplified DNA band was 1250bp.

Chapter 4

Discussion

Aeromonas hydrophila was generally considered to be a secondary invader in red sore disease, in which the primary etiological agent was believed to be the protozoan ciliate *Epistylis* (Rogers, 1971). Motile aeromonads cause diseases wherever bait fishes or warm water or ornamental fishes are propagated. To a lesser extent, these bacteria also initiate disease in cold-water species. Although diseases associated with motile aeromonads are most severe among fish that are propagated under conditions of intensive culture, these bacteria may also affect feral fish and are common in the intestinal flora of apparently healthy fish (Trust et al., 1974). The bacterium is ubiquitous and occurs in most fresh water environments. It can be found both in the water column and in the top centimeter of sediment (Hazen, 1979). Certain algae (Kawakami and Hashimoto, 1978) and other protozoa (Chang and Huang, 1981) that are grazed upon by fish can harbor motile aeromonads. In the latter study, *Tetrahymena pyriformis* was experimentally shown to graze on populations of *Aeromonas hydrophila.* The bacterium, at concentrations of $1x10^6$ cells/mL co-existed with the protozoan.

Quality is the degree of excellence or grade of goodness. In simple terms, the quality of a food can be defined as those characteristics which make it acceptable to the consumers. For marketing of fish in fresh state, it is necessary to retain flavor, texture, odor and appearance. There are a number of parameters and standards (physical, chemical, microbial etc.) for the assessment of freshness quality of wet fresh fish. Bacteriological quality is of public health importance as it directly relates to spoilage of fish and becomes the cause of outbreak of food poisoning.

Raw fishes are highly perishable protein source that contain normal bacterial flora from their environments in addition to the contaminants occurred during harvesting and handling of the products. The living fishes carry populations of predominantly Gram negative psychrotrophic bacteria on their external skin, nearly 10^2-10^3 bacteria per gram (FAO, 1979).

The total bacterial load in fresh fish 11.8×10^7, 19.3×10^7 and 25×10^7cfu/g in muscle, gill and intestine respectively which is beyond the acceptable limit according to the ICMFS (ICMFS, 1998 and FDA, 2001). This might be due to contamination of source

water from where the fishes were caught or might be due to secondary contamination during the time of handling as well as storage of fishes in ice made from contaminated water. According to (Hatha et al., 2003) high microbial abundance might be due to contaminated source of water, poor hygiene and sanitation condition of processing.

Two types of bacteriological culture media *viz*. nutrient agar (a generalized complex medium) and aeromonas agar were used to assess the quantitative and qualitative study. The bacterial load of Sarpunti *Puntius sarana,* Tilapia *Oreochromis mossambicus*, Tatkini *Crosocheilus latius*, Koi *Anabas testudineus* and Meni *Nandus nandus* was found to be ranged between $9.83\pm1.25x10^4$ to $10.20\pm1.99x10^5$ cfu/g, $2.67\pm1.53x10^4$ to $14.7\pm4.42x10^5$ cfu/g, $3.78\pm1.96x10^4$ to$3.3\pm2.6x10^5$ cfu/g, $6.42\pm3.6x10^4$ to $4.53\pm0.32x10^5$ cfu/g and $6.67\pm1.71x10^4$ to$7.7\pm1.05x10^5$ cfu/g respectively on nutrient agar. On the other hand, the bacterial load of those fishes on aeromonas agar (Hi-media) ranged from$1.96\pm0.41x10^2$ to $1.57\pm0.39x10^3$ cfu/g, $5.1\pm2.6x10^2$ to $2.08\pm0.99x10^3$ cfu/g, $1.76\pm1.67x10^2$ to $7.73\pm0.66x10^3$ cfu/g, $3.33\pm5.77x10^0$ to $5.13\pm0.35x10^2$ cfu/g and $2.46\pm1.26x10^2$ to $1.4\pm0.11x10^3$ cfu/g respectively. The results clearly showed that Koi *Anabas testudineus* contains lowest number of *Aeromonas* (suspected) bacteria whereas Tatkini *Crosocheilus latius* contains the maximum number. The bacterial flora on newly caught fish depends on the environment rather than on the fish species (Shewan, 1961). Another source of contamination of harmful microorganism could be fishing vessel (Waheb et al., 2003). So this study proves the previous study (Shewan, 1961).

Fish taken from estuarine waters or rivers, ponds, lakes and canals may carry pathogenic bacteria such as *Salmonella* spp., *Shigella* spp., *Vibrio cholera, Aeromonas* spp. and other water borne pathogens. Fish of good quality should have counts of total bacteria of less than 10^5 per gram and faecal coliforms staphylococci should not exceed 10/gm and 100/gm respectively (FAO, 1979). This indicates human health risk due to consumption of fresh water fishes collected from pond, river, lake and canal etc. Therefore, precautions should be taken to prevent contamination during harvesting as well as post harvest handling of fishes. Depending on the habitat and other environmental factors, a wide range of variation in distribution of micro flora in fish has been reported (Shewan, 1976). The present study correlate with this finding and hence showed variation of bacterial count in different samples of fishes.

According to the International Commission on the Microbiological Specification of Foods (ICMSF, 1982) guideline, acceptable limit of total bacterial counts for giant prawns and

white fish are 10^6 and 5x 10^5 cfu per gram, respectively. In this study, total bacterial count was maximum 5.04±0.74x10^5 cfu per gram which shows acceptable limit. So this study clarified that the collected freshwater fishes from different fish market were consumable (ICMSF, 1982).

The lack of proper knowledge, facilities and carelessness of the fish retailers about microbiological quality of the freshwater fish is evident from the study. It was also observed that the fish species collected from local market were not preserved in ice. These were kept open in normal temperature. So, after a certain period of time or after a few hours the fishes comes to the market from the catch point, the microbiological condition of fish become lower and the quality deteriorate. The result also indicates that the hygienic condition and sanitation facilities are not good in the markets of Dhaka city.

The successful isolation and identification of *A. hydrophila* from extra-intestinal organs of naturally infected fish is in agreement with the results of Janda (1991) and Ali (1996) who reported that *A. hydrophila* isolates recovered from sterile extra-intestinal organs are considered to have originated from invasive disease and the acute MAS may result in localization of colonies identified as *A. hydrophila* within the hematopoietic tissue.

Bacteria associated with fish muscle and their great variation in the percentage has been reported by (Anwar et al., 1988). Depending on the habitat and other environment factors a wide range of variation in distribution of micro flora in fish has been reported (Lakshmy, 1999; Hess 1932 and Ito et al., 1993). It is important to note that when the total bacterial load reach 1.0x10^7 cfu/g or more in food and food products, these foods are considered as spoiled and (Taylor 1920).The total bacterial count was 5.83±073x10^4, 4.21±0.42x10^5 and 4.18±0.46x10^5 in muscle, gill and gut respectively and total *Aeromonas* count 1.76±0.24x10^2, 1.09±0.103x10^3 & 7.38±0.66x10^2 which means that the fishes collected from some markets was not harmful for consumption.

During this investigation a number of suspected *Aeromonas* were isolated and finally 15 were selected for detail study. After thorough characterization with the available facilities the organisms were compared with the standard description in the Bergey's Manual of Systematic Bacteriology volume I and II (Sneath et al., 1986 and Krieg and Holt 1984) and also with the reference strain (ATCC 7966). On the basis of similarity, isolates were provisionally identified. Gram negative (*Aeromonas hydrophila*) bacteria were found to be associated with the studied samples. According to (Huss et al., 1995),

Gram negative bacteria are more dominant bacteria in fish. However this depends on many factors such as fish species, location of fish body from where sample is taken, storage time after dying, habitat of fish etc.

The results of the biochemical characterization of the isolates were interpreted and found in agreement with those reported by Nieto et al., 1984 and Toranzo et al., 1986; in addition variable results were obtained in voges-proskauer reaction, citrate utilization, arabinose and amygadalin fermentation tests. It has been demonstrated that great variation in virulence exists within the motile *Aeromonas* species; few studies have been conducted to associate the biochemical characteristics of *A. hydrophila* species with virulence factors. Biochemical reactions such as voges-proskauer, arabinose and amygdalin fermentation and LDC test, have been correlated with virulence. Burke et al., 1982 and Santos et al., 1988 reported that a significant relationship was found between virulence of *A. hydrophila* for fish and production of acid from arabinose and sucrose and V.P. test, in addition to elastase and hemolytic activities. Virulent *A. hydrophila* can perform inositol and arabinose oxidation, and it is estimated to be 200 times more virulent to channel catfish than A. hydrophila is (Pridgeon and Klesius, 2011).In the present study 5 strain (Tt 1,Tt 2, Tt 3, K 2 and M 3) could perform inositol and all the strain except K2 perform arabinose oxidation which shows partial similarity with those previous works.

Glucose fermentation is a critical reaction that differentiates the motile aeromonads from species of *Pseudomonas* (Bullock, 1961).Although most strains of *A. hydrophila* produce gas during the fermentation of glucose, some motile aeromonads isolated from diseased fish are anaerogenic--that is, they do not generate gas (Ross, 1962). In the present study among 16 strains including reference strain (ATCC 7966), 3 of the strain can't produce gas which were isolated from fresh non-diseased fish.

Based on biochemical characteristics, it was determined that the strains of bacteria were homogenous, which is comparable to previous reports (Lee et al., 2000; Abbott et al., 2003). All strains grew on the previously described AH medium (Kaper et al., 1979), used for species identification, confirming that these bacterial strains are *A. hydrophila*.

Biochemically, *A. hydrophila* hydrolyzes esculin and ferments both salicin and arabinose, whereas *A. sobria* does not utilize these compounds (Lallier et al., 1981). In

our study all strain could ferment arabinose and except one strain all the strain could hydrolyze esculin. This study shows similarity with Lallier et al., 1981.

Motile aeromonads may be pleomorphic but generally produce circular, smooth, raised colonies on agar. Upon microscopic examination, the bacteria appear as short (0.5 X 1.0 ~m), gram-negative bacilli. Phenotypically, motile aeromonads are cytochrome oxidase positive, ferment glucose with or without the production of gas (R. C. Cipriano, 2001) which is comparable to this study.

Antimicrobial resistance of *Aeromonas* has been examined by many authors. Some of the authors point out that *Aeromonas hydrophila* isolated from water, food and clinical samples was not susceptible to many antimicrobial drugs. In previous studies, *A. hydrophila* was reported to be sensitive to chloramphenicol, erythromycin, kanamycin, neomycin (Boonyaratpalin 1989) and resistant to amoxicillin and clindamycin (Belem-Costa and Cyrino, 2006; Adanir and Turutoglu, 2007; Jayavignesh et al., 2011). The results from the present study were similar to these, but different from the results reported by Son et al., (1997, p. 480) and Vivekanandhan et al., (2002, p. 166), who found that *A. hydrophila* was resistant to chloramphenicol, erythromycin, kanamycin and tetracycline. In this study 10 strains including reference strain showed sensitivity to tetracycline and for the kanamycin no strain showed resistant but two strains (Tt 1 and Tt 3) showed intermediate resistant. Interestingly 1 strain (M 1) showed all similar result for the antibiotic sensitivity test with reference strain (ATCC 7966) but showed opposite result with sulphamethoxazole antibiotic.

More than 50% of the A. hydrophila strains was resistant to tetracycline and occurrence of tetracycline resistant strains of *A. hydrophila* from different sources was reported (Ansary et al., 1992; Ramteke et al., 1993; Pettibone et al., 1996; Son et al., 1997; Kampfer et al., 1999). In the present study 1 strain (M2) from Ananda Bazar was resistant to tetracycline whereas 2 strains (K 2, M 3) from Polashi Bazar and 3 strains (Tp 1, Tt 2 and K 3) from Hatirpool Bazar showed resistant to tetracycline.

Among the strains tested, most of the strains were resistant to erythromycin. This is partially supported with previous study (Ansary et al., 1992 and Son et al., 1997. However, Pettibone et al., 1996) have not reported any erythromycin resistant *A. hydrophila* strains. The variation in the drug resistance may well be related to the source

of the *A. hydrophila* isolates and the frequency and type of antimicrobial agents prescribed for treating *Aeromonas* infections, e.g. in cultured fish in different geographical areas (Son et al., 1997). The chloramphenicol resistant strains were few among *A. hydrophila* from fish. Except the strain isolated from Tatkini gill none of the strains isolated from selected fishes was chloramphenicol resistant. Similar findings have been recorded from Malaysian and American fish isolates (Ansary et al., 1992; Pettibone et al., 1996). Resistance towards chloramphenicol, erythormycin, kanamycin, nalidixic acid, streptomycin, sulphamethoxazole and tetracycline has been observed among *A. hydrophila* isolates from Tilapia mossambica (Son et al., 1997). In this study all the strain isolated from Tilapia showed sensitivity to Chloramphenicol and kanamycin whereas resistant to erythromycin, nalidixic acid, streptomycin, sulphamethoxazole and tetracycline. So it partially supports the study repoted by Son et al., 1997.

Moreover, it is known that the bacteria can receive and transmit antibiotic resistant genes from and to other gram-negative bacteria (Marchandin et al., 2003). Thus, aeromonad-infection in freshwater fishes may still be controlled by using correct drugs. Nevertheless, reduction of the use of chemicals in aquaculture is nowadays considered a good management practice. Certain types of chemicals, especially antibiotics, if used inappropriately, not only cause damages to animals and the environment, but also increase production costs and adverse consequences (Tonguthai, 2000). Furthermore, the observation of disease odd usually correlates with anorexia (Duc et al., 2013); hence the utilization of antibiotics at the disease outbreak time may not be effective. Also, the extensive use of antibiotic and other chemotherapeutics to prevent and treat fish diseases may cause drug-resistance phenomenon in cultured fish (Son et al., 1997). Vaccination treatment was recommended for use in disease prevention in cultured fish (Bakopoulos et al., 1995; Sommerset et al., 2005) and herbal concoction was used in the therapy of *A. hydrophila* infection in goldfish (Harikrishnan et al., 2009).

Molecular method for detecting of *A. hydrophila* was introduced and applied in numerous previous studies (Nielsen et al., 2001). Multiple studies have shown that, on a molecular level, VAH is similar to *A. hydrophila* with some variations.

Two universal primers, 27F and 1492R, were developed in amplification of genomic DNA genes and had allowed discriminating of identification up to the species level and typing of other bacteria (Jiang et al., 2006; Sarkar et al., 2012). It has been indicated

previously that levels of similarity between genomic DNA gene sequences higher than 97% suggest that the strains in question belong to the same species (Stackebrandt and Goebel, 1994)

In the present study characterization by biochemical methods were supported by the use of PCR. The assay was validated with motile, mesophilic *Aeromonas* sp. type strains. No amplification was seen in any *Aeromonas* strain other than *A. hydrophila* and no amplification products were obtained from negative controls. Thus, it ensured an improved identification of *A. hydrophila* isolated from freshwater fish in Bangladesh compared to identification based solely on phenotypic, biochemical testing which is comparable to previous study (Nielsen et al., 2001).

All isolated strains were identified as *Aeromonas hydrophila* in this study by biochemical and physiological test. Along with this all strains showed similarity in characterization with reference strain (ATCC 7966). Among those 15 strains, 8 strains were purified by gel.

Chapter 5
Conclusion and Recommendations

5.1 Conclusion

This research points out that *Aeromonas hydrophila* was present in all selected fish samples, regardless to their origin of collection.

These results show that the strains in all samples were exposed to antimicrobial drugs and developed resistance. This means that antimicrobial drugs are used inappropriately and a further development of the resistance may be expected, so the number of effective antimicrobial drugs is diminishing. Since this is a microorganism that may threaten human health, transmission of the reduced susceptibility may have negative consequences for humans.

This study demonstrated *A. hydrophila* as a potential pathogen, which can cause haemorrhagic septicaemia in fishes. In the antimicrobial susceptibility test, both tested bacterial strains showed resistant to most used drugs. The results revealed that the strains might have originated from high-risk source of contamination.

5.2 Recommendations

- Current study was conducted only in very short periods, so further studies are to be needed for better understanding of microorganisms associated with the fish samples.
- During the current study the prevalence of *A. hydrophila* in freshwater fishes in relation to sources were investigated irrespective of season. So, further study should be conducted on seasonal variation in the occurrences of these harmful bacteria.
- In the present study fishes were collected from fish market, therefore, more research are needed to compare *A. hydrophila* isolated from culture and wild freshwater fishes of Bangladesh.
- Because of shot time period in this study pathogenicity was not tested. So further studies are to be needed for testing pathogenicity by keeping fish in vitro condition.

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APPENDIX

Composition of the media and reagents used in this study are as follows.

1. Ammonium Crystal violet solution (SAB 1957)

Solution A

Crystal violet (85% dye content)	2.0 g
Ethyl alcohol (95%)	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80 ml

Solution A and B were mixed, Stored for 24 hrs before use.

2. Aeromonas Agar

Dehydrated form

3. Basal medium for fermentation (SAB 1957)

Beef extract	3.0 g
Amonium dihydrogen phosphate	1.0 g
Magnesium sulphate	0.2 g
Potassium chloride	0.2 g
Carbohydrate	10.0 g
Bromothymol blue	2.0 ml
Distilled water	1000 ml

4. Ferric chloride solution (Sneath *et al.* 1986)

$FeCl_3$	10 g
Distilled water	100 ml

5. Indole nitrate broth (Atlas 1997)

Tryptone	10.0 g
Distilled Water	1000ml
pH	7.2

6. Iodine solution (SAB 1957)

Iodine	0.33 g
Potassium Iodide	0.66 g

Distilled water	100 ml

7. Kligler's Iron Agar (KIA) Medium (Atlas 1997)

Peptone	20.0 g
Lactose	10.0 g
Glucose	1.0 g
NaCl	5.0 g
Ferric citrate	0.3 g
Beef extract	3.0 g
Yeast extract	3.0 g
$Na_2S_2O_3$	0.3 g
Agar	12.0 g
Phenol red	0.05 g
Distilled water	1000 ml
pH	7.4 ± 0.2 at 25°C

8. KOH-creatine solution (SAB 1957)

KOH	40.0 g
Creatinine	0.3 g
Distilled water	100 ml

9. Kovac's reagent (SAB 1957)

Para-dimethyl-amino-benzaldehyde	5.0 g
Butyl alcohol	75 ml
HCI (Conc.)	25 ml

10. Mercurochrome solution (SAB 1957)

Mercurochrome	0.5 gm
Distilled water	100 ml

11. Methyl Red/Voges-Proskauer broth medium (Sneath *et al.* 1986)

Protease peptone	7.0 gm
Glucose	5.0 gm
NaCl	5.0 gm
Distilled water	1000 ml

12. Methyl red solution (Bryan 1950)

Methyl red	0.1 gm
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

13. α-Napthol solution (Bryan 1950)

α –Napthol	15.0 g
Ethyl alcohol (95%)	100ml

14. Nutrient agar medium (Pelczar 1993)

Beef extract	3.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1000ml

15. Nutrient broth medium (Pelczar 1993)

Beef extract	3.0 g
Peptone	5.0 g
NaCl	5.0 g
Distilled water	1000 ml

16. Oxidase test reagent (Claus 1995)

Tetramethyl-p-phenylene-diamine dihydro-chloride	1.0 g
Distilled water	100 ml
Ethyl alcohol (95%)	100ml

17. Physiological saline

Sodium chloride	0.85 g
Distilled water	100 ml

18. Saffranin solution (SAB 1957)

Safranin	0.5 g
Distilled water	100 ml

19. Simmon's citrate agar (Atlas 1997)

$MgSO_4$. $7H_2O$	0.2 g
Mono-ammonium phosphate	1.0 g
Di-Potasssium phosphate	1.0 g
Na-citrate	2.0 g

NaCI	5.0 g
Bromo-thymol-blue	0.08 g
Agar	15.0 g
Distilled water	1000 ml.
pH	6.9 ± 0.2 at 25°C

SPSS output

Descriptives

TBC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Ananda Bazar	45	328777.78	319920.792	47690.976	232662.93	424892.62	25000	1000000
Palashi Bazar	45	333822.22	315593.329	47045.876	239007.49	428636.95	9000	930000
Hatirpool Bazar	45	235244.44	241853.276	36053.358	162583.68	307905.21	8000	800000
Total	135	299281.48	295952.766	25471.559	248903.18	349659.79	8000	1000000

Market wise

ANOVA

TBC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	277372637037.037	2	138686318518.519	1.598	.206
Within Groups	1145942466666 6.666	132	86813823232.323		
Total	1173679730370 3.703	134			

Descriptives

TBC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Puntius sarana	27	334222.22	329333.632	63380.287	203942.18	464502.27	10000	900000
Oreochromis mossambicus	27	160962.96	130988.103	25208.672	109145.80	212780.13	9000	440000
Crosocheilus latius	27	227666.67	241930.060	46559.462	131962.32	323371.01	20000	800000
Anabas testudineus	27	269666.67	218807.080	42109.442	183109.47	356223.86	8000	700000
Nandus nandus	27	503888.89	385413.198	74172.805	351424.51	656353.27	46000	1000000
Total	135	299281.48	295952.766	25471.559	248903.18	349659.79	8000	1000000

ANOVA

TBC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1842015007407.408	4	460503751851.852	6.050	.000
Within Groups	9894782296296.297	130	76113709971.510		
Total	1173679730370 3.705	134			

Multiple Comparisons

Dependent Variable: TBC
Tukey HSD

(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Oreochromis mossambicus	173259.259	75086.967	.149	-34464.24	380982.76
Crosocheilus latius	106555.556	75086.967	.617	-101167.94	314279.06
Anabas testudineus	64555.556	75086.967	.911	-143167.94	272279.06
Nandus nandus	-169666.667	75086.967	.165	-377390.17	38056.83
Puntius sarana	-173259.259	75086.967	.149	-380982.76	34464.24
Crosocheilus latius	-66703.704	75086.967	.901	-274427.20	141019.80
Anabas testudineus	-108703.704	75086.967	.598	-316427.20	99019.80
Nandus nandus	-342925.926*	75086.967	.000	-550649.43	-135202.43
Puntius sarana	-106555.556	75086.967	.617	-314279.06	101167.94
Oreochromis mossambicus	66703.704	75086.967	.901	-141019.80	274427.20
Anabas testudineus	-42000.000	75086.967	.981	-249723.50	165723.50

Nandus nandus	-276222.222*	75086.967	.003	-483945.72	-68498.72
Puntius sarana	-64555.556	75086.967	.911	-272279.06	143167.94
Oreochromis mossambicus	108703.704	75086.967	.598	-99019.80	316427.20
Crosocheilus latius	42000.000	75086.967	.981	-165723.50	249723.50
Nandus nandus	-234222.222*	75086.967	.019	-441945.72	-26498.72
Puntius sarana	169666.667	75086.967	.165	-38056.83	377390.17
Oreochromis mossambicus	342925.926*	75086.967	.000	135202.43	550649.43
Crosocheilus latius	276222.222*	75086.967	.003	68498.72	483945.72
Anabas testudineus	234222.222*	75086.967	.019	26498.72	441945.72

*. The mean difference is significant at the 0.05 level.

TBC

Tukey HSD

Species	N	Subset for alpha = 0.05	
		1	2
Oreochromis mossambicus	27	160962.96	
Crosocheilus latius	27	227666.67	
Anabas testudineus	27	269666.67	
Puntius sarana	27	334222.22	334222.22
Nandus nandus	27		503888.89
Sig.		.149	.165

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 27.000.

Descriptives

TBC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Muscle	45	58311.11	49540.077	7384.999	43427.62	73194.60	8000	220000
Gill	45	421111.11	284167.222	42361.148	335737.83	506484.40	100000	900000
Gut	45	418422.22	307338.316	45815.291	326087.57	510756.87	67000	1000000
Total	135	299281.48	295952.766	25471.559	248903.18	349659.79	8000	1000000

ANOVA

TBC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3919666237037.038	2	1959833118518.519	33.094	.000
Within Groups	7817131066666.666	132	59220689898.990		
Total	11736797303703.703	134			

Multiple Comparisons

Dependent Variable: TBC

Tukey HSD

(I) Organs	(J) Organs	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Muscle	Gill	-362800.000*	51303.320	.000	-484411.74	-241188.26
	Gut	-360111.111*	51303.320	.000	-481722.85	-238499.37
Gill	Muscle	362800.000*	51303.320	.000	241188.26	484411.74
	Gut	2688.889	51303.320	.998	-118922.85	124300.63
Gut	Muscle	360111.111*	51303.320	.000	238499.37	481722.85
	Gill	-2688.889	51303.320	.998	-124300.63	118922.85

*. The mean difference is significant at the 0.05 level.

TBC

Tukey HSD

Organs	N	Subset for alpha = 0.05	
		1	2
Muscle	45	58311.11	
Gut	45		418422.22
Gill	45		421111.11
Sig.		1.000	.998

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 45.000.

Descriptives

AH

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Ananda Bazar	45	810.44	731.241	109.007	590.76	1030.13	0	3000
Palashi Bazar	45	633.56	447.021	66.638	499.26	767.86	0	1700
Hatirpool Bazar	45	563.11	607.431	90.550	380.62	745.60	0	2100
Total	135	669.04	610.945	52.582	565.04	773.03	0	3000

ANOVA

AH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1461388.148	2	730694.074	1.986	.141
Within Groups	48554586.667	132	367837.778		
Total	50015974.815	134			

AH

Tukey HSD

Market	N	Subset for alpha = 0.05
		1
Hatirpool Bazar	45	563.11
Palashi Bazar	45	633.56
Ananda Bazar	45	810.44
Sig.		.133

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 45.000.

Descriptives

AH

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
1	45	176.67	161.330	24.050	128.20	225.14	0	600
2	45	1091.78	691.314	103.055	884.08	1299.47	150	3000
3	45	738.67	443.978	66.184	605.28	872.05	200	1800
Total	135	669.04	610.945	52.582	565.04	773.03	0	3000

ANOVA

AH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19169397.037	2	9584698.519	41.015	.000
Within Groups	30846577.778	132	233686.195		
Total	50015974.815	134			

Multiple Comparisons

Dependent Variable: AH

Tukey HSD

(I) Organs	(J) Organs	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-915.111*	101.912	.000	-1156.69	-673.53
	3	-562.000*	101.912	.000	-803.58	-320.42
2	1	915.111*	101.912	.000	673.53	1156.69
	3	353.111*	101.912	.002	111.53	594.69
3	1	562.000*	101.912	.000	320.42	803.58
	2	-353.111*	101.912	.002	-594.69	-111.53

*. The mean difference is significant at the 0.05 level.

AH

Tukey HSD

Organs	N	Subset for alpha = 0.05		
		1	2	3
1	45	176.67		
3	45		738.67	
2	45			1091.78
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 45.000.

Descriptives

AH

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
1	27	972.22	632.597	121.743	721.98	1222.47	110	2100
2	27	1032.59	797.195	153.420	717.23	1347.95	0	3000

3	27	573.70	427.409	82.255	404.63	742.78	0	1600
4	27	283.33	211.914	40.783	199.50	367.16	0	570
5	27	483.33	477.445	91.884	294.46	672.20	100	1700
Total	135	669.04	610.945	52.582	565.04	773.03	0	3000

ANOVA

AH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11243760.000	4	2810940.000	9.425	.000
Within Groups	38772214.815	130	298247.806		
Total	50015974.815	134			

Multiple Comparisons

Dependent Variable: AH

Tukey HSD

(I) Species	(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-60.370	148.635	.994	-471.56	350.82
	3	398.519	148.635	.062	-12.67	809.71
	4	688.889*	148.635	.000	277.70	1100.08
	5	488.889*	148.635	.011	77.70	900.08
2	1	60.370	148.635	.994	-350.82	471.56
	3	458.889*	148.635	.020	47.70	870.08
	4	749.259*	148.635	.000	338.07	1160.45
	5	549.259*	148.635	.003	138.07	960.45
3	1	-398.519	148.635	.062	-809.71	12.67
	2	-458.889*	148.635	.020	-870.08	-47.70
	4	290.370	148.635	.295	-120.82	701.56
	5	90.370	148.635	.974	-320.82	501.56
4	1	-688.889*	148.635	.000	-1100.08	-277.70
	2	-749.259*	148.635	.000	-1160.45	-338.07
	3	-290.370	148.635	.295	-701.56	120.82
	5	-200.000	148.635	.663	-611.19	211.19
5	1	-488.889*	148.635	.011	-900.08	-77.70
	2	-549.259*	148.635	.003	-960.45	-138.07
	3	-90.370	148.635	.974	-501.56	320.82
	4	200.000	148.635	.663	-211.19	611.19

*. The mean difference is significant at the 0.05 level.

Descriptives

AL

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Polashi Bazar	27	244.81	173.280	33.348	176.27	313.36	90	670
Hatirpool Bazar	27	298.52	355.438	68.404	157.91	439.13	0	1400
Total	54	271.67	278.281	37.869	195.71	347.62	0	1400

AH

Tukey HSD

Species	N	Subset for alpha = 0.05		
		1	2	3
4	27	283.33		
5	27	483.33		
3	27	573.70	573.70	
1	27		972.22	972.22
2	27			1032.59
Sig.		.295	.062	.994

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 27.000

ANOVA

AL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	38935.185	1	38935.185	.498	.484
Within Groups	4065414.815	52	78181.054		
Total	4104350.000	53			

Descriptives

AL

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Muscle	18	96.67	48.142	11.347	72.73	120.61	0	170
Gill	18	536.11	336.087	79.217	368.98	703.24	310	1400
Gurt	18	182.22	105.916	24.965	129.55	234.89	0	400
Total	54	271.67	278.281	37.869	195.71	347.62	0	1400

ANOVA

AL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1954011.111	2	977005.556	23.172	.000
Within Groups	2150338.889	51	42163.508		
Total	4104350.000	53			

AL

Tukey B

Organs	N	Subset for alpha = 0.05	
		1	2
Muscle	18	96.67	
Gurt	18	182.22	
Gill	18		536.11

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 18.000.

Descriptives

AL

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Sarpunti	18	359.44	453.995	107.008	133.68	585.21	0	1400
Koi	18	235.00	103.370	24.364	183.60	286.40	90	360
Meni	18	220.56	110.851	26.128	165.43	275.68	100	400
Total	54	271.67	278.281	37.869	195.71	347.62	0	1400

ANOVA

AL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	209911.111	2	104955.556	1.374	.262
Within Groups	3894438.889	51	76361.547		
Total	4104350.000	53			

AL

Tukey B

Species	N	Subset for alpha = 0.05
		1
Meni	18	220.56
Koi	18	235.00

Sarpunti	18	359.44

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 18.000.

Descriptives

AO

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Polashi Bazar	27	1583.33	2490.270	479.253	598.22	2568.45	150	8400
Hatirpool Bazar	27	1284.44	1199.200	230.786	810.06	1758.83	200	3900
Total	54	1433.89	1941.764	264.241	903.89	1963.89	150	8400

ANOVA

AO

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1206016.667	1	1206016.667	.316	.577
Within Groups	198627666.667	52	3819762.821		
Total	199833683.333	53			

Descriptives

AO

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Muscle	18	315.00	71.968	16.963	279.21	350.79	200	400
Gill	18	2526.11	2852.891	672.433	1107.40	3944.82	560	8400
Gurt	18	1460.56	1010.605	238.202	957.99	1963.12	150	3300
Total	54	1433.89	1941.764	264.241	903.89	1963.89	150	8400

ANOVA

AO

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	44020311.111	2	22010155.556	7.204	.002
Within Groups	155813372.222	51	3055164.161		

Total	199833683.333	53			

AO

Tukey B

Organs	N	Subset for alpha = 0.05	
		1	2
Muscle	18	315.00	
Gurt	18	1460.56	1460.56
Gill	18		2526.11

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 18.000.

Descriptives

AO

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Sarpunti	18	2971.67	2772.188	653.411	1593.09	4350.24	200	8400
Koi	18	716.67	325.847	76.803	554.63	878.71	200	1100
Meni	18	613.33	449.745	106.006	389.68	836.99	150	1700
Total	54	1433.89	1941.764	264.241	903.89	1963.89	150	8400

ANOVA

AO

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	63944633.333	2	31972316.667	11.999	.000
Within Groups	135889050.000	51	2664491.176		
Total	199833683.333	53			

AO

Tukey B

Species	N	Subset for alpha = 0.05	
		1	2
Meni	18	613.33	
Koi	18	716.67	
Sarpunti	18		2971.67

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 18.000.

Descriptives

AH

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Sarpunti	18	1036.67	699.235	164.811	688.95	1384.39	110	2100
Koi	18	268.33	210.915	49.713	163.45	373.22	0	530
Meni	18	398.33	375.488	88.503	211.61	585.06	130	1300
Total	54	567.78	575.492	78.315	410.70	724.86	0	2100

ANOVA

AH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6088233.333	2	3044116.667	13.541	.000
Within Groups	11464900.000	51	224801.961		
Total	17553133.333	53			

Multiple Comparisons

Dependent Variable: AH

Tukey HSD

(I) Species	(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Sarpunti	Koi	768.333*	158.044	.000	386.82	1149.85
	Meni	638.333*	158.044	.001	256.82	1019.85
Koi	Sarpunti	-768.333*	158.044	.000	-1149.85	-386.82
	Meni	-130.000	158.044	.691	-511.52	251.52
Meni	Sarpunti	-638.333*	158.044	.001	-1019.85	-256.82
	Koi	130.000	158.044	.691	-251.52	511.52

*. The mean difference is significant at the 0.05 level.

AH

Tukey HSD

Species	N	Subset for alpha = 0.05	
		1	2
Koi	18	268.33	
Meni	18	398.33	
Sarpunti	18		1036.67
Sig.		.691	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 18.000.

Descriptives

AH

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		

Muscle	18	113.33	85.406	20.130	**70.86**	**155.80**	0	220
Gill	18	962.22	670.194	157.966	628.94	1295.50	150	2100
Gurt	18	627.78	434.586	102.433	411.66	843.89	200	1600
Total	54	567.78	575.492	78.315	410.70	724.86	0	2100

ANOVA

AH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6582711.111	2	3291355.556	15.301	.000
Within Groups	10970422.222	51	215106.318		
Total	17553133.333	53			

Multiple Comparisons

Dependent Variable: AH

Tukey HSD

(I) Organs	(J) Organs	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Muscle	Gill	-848.889*	154.599	.000	-1222.09	-475.69
	Gurt	-514.444*	154.599	.005	-887.64	-141.25
Gill	Muscle	848.889*	154.599	.000	475.69	1222.09
	Gurt	334.444	154.599	.087	-38.75	707.64
Gurt	Muscle	514.444*	154.599	.005	141.25	887.64
	Gill	-334.444	154.599	.087	-707.64	38.75

*. The mean difference is significant at the 0.05 level.

AH

Tukey HSD

Organs	N	Subset for alpha = 0.05	
		1	2
Muscle	18	113.33	
Gurt	18		627.78
Gill	18		962.22
Sig.		1.000	.087

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 18.000.