ISOLATION, IDENTIFICATION AND ANTIBIOGRAM PROFILE OF BACTERIA ISOLATED FROM VIETNAMESE KOI (*Anabas testudineus)* OF SELECTED OUTBREAK AREAS IN MYMENSINGH DISTRICT

BY

MOST. SHAFI EARFAT JEBIN

EXAMINATION ROLL NO. 1605544

Session: 2016-2017

Semester: Jan- Jun, 2018

A Thesis

Submitted to the Department of Fisheries Technology

Hajee Mohammad Danesh Science and Technology University, Dinajpur

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE (MS)

IN

FISHERIES TECHNOLOGY

SEMESTER: JANUARY-JUNE, 2018

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Dedicated To
My
Beloved Parents

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ISOLATION, IDENTIFICATION AND ANTIBIOGRAM PROFILE OF BACTERIA ISOLATED FROM VIETNAMESE KOI (*Anabas testudineus)* FROM SELECTED OUTBREAK AREAS IN MYMENSINGH DISTRICT

ABSTRACT

Vietnamese Koi *(Anabas testudineus)* is considered as a high value fish species in Bangladesh. The fish contains high amount of iron and copper, which are nutritionally beneficial for hemoglobin synthesis in human. A total number of hundred seventy five of infected /dead V. Koi fishes were collected during outbreak period from seven different areas in Mymensingh district for conducting this study. The main purpose of this study was to isolate and identify the bacterial spp. responsible for mass mortality of V. Koi from selected outbreak areas in Mymensingh district and to know the pathogenicity of the isolated bacteria. The main clinical symptoms were hemorrhage in base of the fin, slight lesion on body, body and tail erosion. Post-mortem changes indicated congestion and enlargement with hemorrhage in liver, kidney and spleen .Isolation and identification of bacteria was conducted by studying cultural properties, gram staining and biochemical properties. Biochemical tests for bacteria which were performed by API kit indicated positive results by reacting with betagalactosidase, arginine dihydrolase, lysine decarboxylase, sodium citrate, tryptophan deaminase, sodium pyruvate, gelatinase, glucose, mannitol, sucrose, melibiose, amygdalin, and arabinose for all isolates. And showed negative result when tested for ornithine decarboxylase, H_2S production, inositol, sorbitol, and rhamnose. Among the various isolated bacteria, *Aeromonas hydrophila* and *A. caviae* were appeared as main pathogen in the infected fishes. Total bacterial load found at 1.13×10^6 to 3.21×10^8 cfu/g in lesions; 1.10×10^5 to 2.1×10^7 cfu/g in liver; 3.24×0^4 to 3.82×10^6 cfu/g in gill; 1.25×10^6 to 3.51×10^7 cfu/g in kidney and 3.74×10^4 to 2.43×10^6 in spleen of diseased Koi. Cumulative mortality rate was recorded the highest (89%) at the group 2 after 14 days of post inoculation because of the infection of *Aeromonas hydrophila.* Commercially available antibiotics were used for the determination of sensitivity test for the isolated bacteria. All the isolated bacteria were found sensitive to endrofloxacin, ciprofloxacine, levofloxacine, azithromycin and gentamicin. Ciprofloxacine and levofloxacine were highly effective against *A. hydrophila and A. caviae.* Ampicillin & penicillin did not show any activity against *Aeromonas* spp. Study indicated that motile *Aeromonas* sp. perhaps serve as the primary cause of hemorrhages, ulcerations and skin lesions as well as mortality of V. Koi. The result of this study will be useful for V. Koi aquaculture by applying right antibiotics against *Aeromonas* spp. in order to prevent mass mortality.

ABBREVIATIONS

ADH	Arginine dihydrolase
AIM	*Aeromonas* isolation media
AOAC	Association of official analytical chemist
API	Analytical profile index
BAU	Bangladesh Agricultural University
BBS	Bangladesh Bureau of Statistics
BFRI	Bangladesh Fisheries Research Institute
CCB	Calcium channel blocker
°C	Degree celsius
Cfu	Colony forming unit
CIT	Citrate
Clin	Clinical
CLSI	Clinical Laboratory Standard Institute
DO	Dissolved oxygen
DoF	Department of Fisheries
EDTA	Ethylene- diamine- tetra- acetic acid
Ed.	Edition
EUS	Epizootic ulcerative syndrome
FAO	Food and agriculture organization
FRSS	Fisheries resources survey system
G	Gram
GDP	Gross domestic product
H_2S	Hydrogen sulphide
H_2O_2	Hydrogen per oxide
IC	Inhibitory concentrations
IM	Intra muscular
IND	Indole
g/l	Gram per liter
IP	Intra peritoneal

L	Liter
Lab	Laboratory
LDC	Lysine decarboxylase
Log	Logarithm
Min	Minute
MAR	Multiple Antibiotic Resistance
Mg	Milligram
Ml	Milliliter
µg	Microgram
MOFA	Ministry of Food and Agriculture
MR	Methyl red
ND	Not detected
NaCl	Sodium chloride
OF	Oxidative fermentation
ppm	Parts per million
pAh	Polycyclic aeromatic hydrocarbon
PBS	Phosphate buffer saline
PS	Physiological saline
rpm	Rotation per minute
SEAFDEC	Southeast asian fisheries development centre
TDA	Tryptophan deaminase
TSA	Tryptone soya agar
TSI	Triple sugar iron
TVC	Total viable count
UN	United nations
IUCN	International union for conservation of nature
USA	United States of America
VP	Voges proskaur

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CHAPTER 1

INTRODUCTION

Fisheries sector is playing a key role among all the agricultural activities in Bangladesh. This sector is important to meet up the nutritional requirement of Bangladeshi people as well as to ensure food security, to create employment opportunity and thus generate income sources. Recently, it contributes 3.61% in national GDP, 24.41% to the agricultural GDP and 2.5% to the country's export earnings (Karugia *et al.,* 2014). Moreover, 16.5 million people are directly or indirectly dependent on fisheries related activities for their livelihood (DoF, 2017). Fish is contributing about 30% of annual protein supply for Bangladeshi people (Abisoye *et al.,* 2011).

Aquaculture is playing a leading role in the global fish production and contributing to meet up food demand for the growing population in the world (FAO, 2014). Bangladesh is surrounded with land of rivers as well as ponds (3, 72,397ha), lakes (5,488 ha) and other water bodies (DoF, 2016). Bangladesh secured 5th position in world aquaculture production (closed water body) which accounted more than half of the country's total fish production (55.15%) (DoF, 2016). Mainly ponds are acting as a key entity for aquaculture production (80%) which dominated by exotic fishes such as Vietnamese Koi, Pangas and Tilapia and indigenous carps such as Rui, katla, Mrigal.

Aquaculture in Bangladesh is very popular as it is profitable and good source of income. Nowadays fish growers tend to choose exotic and hybrid fishes for their rapid growth and easy maintenance. However, fishes like Koi **(***Anabas testudineus***)**, Shing and Puti are very popular for their delicate taste and flavor. These fishes are sometimes difficult to culture due their susceptibility to a wide variety of bacterial pathogens. These pathogens are capable of causing diseases (Lipp and Rose, 1997). The pathogens which are associated with fish can be classified mainly as indigenous and non-indigenous (Copp *et al.,* 2005). The non-indigenous pathogens contaminate the fish (FAO.1997). The indigenous bacterial pathogens are naturally abundant in fish habitat. On the other hand, nutritional deficiency, physical imbalance and other stressors such as poor water quality, overstocking may allow opportunistic bacterial pathogens to invade fish and cause infection (Mhango *et al*., 2010).

A wide range of pathogens (viruses, bacteria, parasites etc.), environmental factors (different water quality parameters such as pH, dissolved oxygen) can create fish diseases and thus cause fish mortality and hamper aquaculture production (Sarig 1971; Humphrey and Langdon 1985).

Among various freshwater fishes V. Koi (*Anabas testudineus*) is a famous exotic freshwater fish species in Bangladesh. V. Koi culture has become popular in Bangladesh since the expansion of induced breeding and mass seed production (Hossain *et al*., 2010). Compare to other aquaculture species in Bangladesh, V. Koi is relatively new. For the last several years, Koi fish, got high attention to fish farmers. Among the different Koi fishes Vietnamesee Koi that took the mantle of most exciting Koi breed, with the promise of the highest profits (Hossain *et al.,* 2010). However, the bottleneck is although the culture of this fish is profitable but not risk free from diseases that may destroy the whole investment. It was assumed that this disease occur due to bacterial infection. Most farmers (60%) produce V. Koi under polyculture while the leftovers (40%) practice monoculture (Charo-Karisa *et al.,* 2013). About 10% of farmers are involved in extensive/improved-extensive farming, while 60% and 30% practice in semi-extensive and intensive farming respectively. The total annual V. Koi production in Bangladesh was expected at 22,989 tons in 2010-11. The estimated total production of V. Koi revealed at around 37,400 tons in 2016-17 (DoF, 2016).

There are some special characteristics of V. Koi such as faster growth rate, shorter culture period and higher survival rate (Copp *et al*., 2005). It can endure adverse environmental conditions as for example, low oxygen due to presence of accessory air breathing organ, tolerance power of wide range of temperature and can even survive in poor water quality condition (Abisoye *et al.,* 2011). This particular fish (*Anabas testudineus*) is considered as an important food and suggested as a diet for the sick and convalescents as it contains high amount of iron, copper and easily digestible poly-unsaturated fats and many essential amino acids. However this fish is vulnerable to a wide variety of bacterial pathogens, which are competent of causing disease and are considered saprophytic in nature (Mhango *et al.*, 2010). The type of micro-organisms that are available in V. Koi depends on its habitat and classify as indigenous and non-indigenous bacterial pathogens.

Disease is considered as a primary constraint to the culture of many aquatic species, impeding both economic and social development in many countries (Hossain *et al.,* 2010). A number of diseases like epizootic ulcerative syndrome, skin erosion, gill damage, tail and fin rot are common in farmed Climbing Perch (*Anabus testudineus*) of Bangladesh. In pond aquaculture system, high stocking density and irregularly feed supply is very prone to disease outbreak. Many diseases of this hardy fish are secondary to environmental abuse and can be prevented through proper management by manipulating the ecosystem and the direction of selective antibiotics. However, there is lack of scientific information available for rural aqua-farmers. Thus, more study is required for getting good understanding of health and disease issues of V. Koi (Hasan *et al.,* 2013).

In order to produce disease free and healthy V. Koi, it is necessary to stop the outbreak of this fish due to infectious diseases. As pathogenic bacteria may cause the disease therefore isolation and identification is necessary to overcome the hindrance of V. Koi culture to prevent the disease (Copp *et al.,* 2005). This study was conducted to isolate and identify the potential pathogenic bacteria from the infected or dead fishes during outbreak time in selected areas of Mymensingh district. Later on study was conducted to characterize from the pathogenic bacteria as this will eventually help to reduce the fish diseases (Mhango *et al.,* 2010).

Therefore, the present study was conducted with the following objectives:

- To isolate and identify the bacterial spp. from V. Koi (*Anabas testudineus*) during mass mortality of selected outbreak areas in Mymensingh district.
- To study the distribution pattern of bacterial pathogens in the naturally infected/dead V. Koi fishes.
- To study the pathogenicity of the isolated bacteria by re-establishing experimental infection in the healthy V. Koi fish.
- To test the sensitivity of the isolated bacteria with different commercial antibiotics.

CHAPTER 2

REVIEW OF LITERATURE

2.1: Isolation and identification of bacteria

Borty *et al.*, (2016) reported about isolation and identification of *Aeromonas hydrophila* by studying cultural properties, Grams staining and biochemical properties of different strain of bacteria from diseased indigenous V. Koi (*Anabas testudineus*) collected from different upazilas of Mymensingh district.

Bhuvaneswari and Balasundaram (2009) conducted a study to evaluate the effect of compounds isolated from *Aeromonas calamus* in order to find out their inhibitory concentrations (IC) against *Aeromonas hydrophila.*

Crumlish *et al.*, (2002) studied about identification of *Edwardsiella ictaluri* from diseased freshwater fish, *Anabas testudineus*, cultured in the Mekong Delta, Vietnam. Bacterial strains were recovered from the liver of fish and grew only in tryptone soya agar medium.

Dananjaya *et al.*, (2015) studied on isolation and characterization of a virulent bacteriophage specific to *Aeromonas hydrophila.* The pAh-1 was isolated from stream water near Yuseong-gu, Daejeon, Korea after enrichment with *A. hydrophila* followed by soft agar overlay method. The isolated pAh-1 was morphologically classified as a member of Myoviridae family.

Zaman *et al.*, (2014) conducted a study for the isolation and identification of bacteria from V. Koi present in mud and water samples. Antibiotic sensitivity assay showed multi drug resistant profiles of *Pseudomonas* sp., *Aeromonas* sp., *Salmonella* sp. and *E. coli.* These bacteria are known to cause food borne illness in humans and spoilage of fish.

Ringø and Gatesoupe (1998) conducted a study where they described about the isolation of seven viruses from various culture area during two years in laboratory. All isolates caused cytopathogenic effects in CCB and CaF-2 cell lines. Six isolates were sensitive to chloroform treatment, four isolates passed 100 nm filters and only one isolate were found to be sensitive against treatment with JUDR.

Rashid *et al.*, (2008) studied about isolation of *Aeromonas hydrophila* from EUS affected V. Koi (*Anabas testudineus)* of a fish farm in Mymensingh. *Aeromonas hydrophila* bacteria was isolated from the suspected EUS-affected Shing fish, *Heteropneustes fossilis* (Bloch). The disease investigations were primarily based on clinical signs and subsequently confirmed by the isolation of bacterial pathogen *Aeromonas hydrophila* from lesion of liver and kidney.

Sarkar *et al.*, (2012) studied about pathogenicity of the bacterial isolate *Aeromonas hydrophila* to catfishes, carps and perch. *Aeromonas hydrophila* recovered from naturally diseased shing fish was investigated against catfishes *(Heteropneustes fossilis* and *Clarias batrachus),* carps *(Labeo rohita, Catla catla* and *Cirrhinus cirrhosus)* and perch *(Anabas testudineus)* of average body. Injected *A. hydrophila* was re-isolated from liver, kidney and intestine of all the tested fishes.

Sabur (2006) studied about isolation of the pathogenic bacteria *Aeromonas hydrophila* in indigenous and exotic carps under their polyculture conditions. Amongst 50 bacterial isolates, the identified genera were *Aeromonas* sp., *Bacillus* sp., *Flavobacterium* sp., *Pseudomonas* sp., *Micrococcus* sp., *Streptococcus* sp., *Staphylococcus* sp., *Vibrio* sp. *and Salmonella* sp. being the most dominant genus.

Ahammed (2016) studied about isolation, identification and molecular detection of *Aeromonas hydrophila* from diseased stinging catfish V. Koi (*Anabas testudineus*). In this study isolation and identification of *Aeromonas hydrophila* was done by studying cultural properties, gram staining and biochemical properties of isolates of Shing fish (*Heteropneustes fossilis*) of different upazilas of Mymensingh district.

2.2: Histopathological findings and post-mortem changes

Saenphet *et al.*, (2009) studied on histopathological changes regarding the gills, liver and kidneys in *Anabas testudineus* (Bloch) fish living in unused lignite mine, li district, Lampung province, Thailand. Damage was observed in liver tissue with hemorrhages, blood congestion and necrotic cells with mononuclear cell infiltration.

Samanta *et al.,* (2016) investigated the histopathological changes in the stomach and intestine of Indian freshwater teleost, *Anabas testudineus* (Bloch, 1792). Pathological changes in the concerned fish organs namely stomach and intestine were assessed through light microscopy, scanning and transmission electron microscopy. Lesions observed under light microscopy also endorsed the findings of ultrastructural observations both in laboratory and field conditions.

2.3: Pathogenicity and experimental infection

Ahmed *et al.,* (2009) were investigated on the pathogenicity of *Anabas testudineus* was carried out on fresh water beel fisheries of Mymensingh, Bangladesh from April 2006 to March 2007.

Ahmed *et al.,* (2007) were investigated on health conditions of Thai V. Koi (*Anabas testudineus)* were carried out through clinical and histopathological observations from different farms of Mymensingh district for seven months during August 2006 to February 2007. Fish sampling and water quality parameters (temperature, dissolved oxygen and pH) were monitored on a monthly basis.

Dung *et al.,* (2008) isolated antimicrobial susceptibility pattern of *Edwardsiella ictaluri* from natural outbreaks of bacillary necrosis of V. Koi (*Anabas testudineus*) in Vietnam. The purpose of this study was to assess the in vitro susceptibility of 64 Vietnamesee isolates of *Edwardsiella ictaluri*, the causal agent of the infectious disease Bacillus Necrosis using the agar dilution technique. All isolates originated from different farms and were collected between 2002 and 2005.

Hasan *et al.,* (2013) conducted a study to know the culture strategies and fish health and disease problems in pond aquaculture in Mymensingh, Bogra and Pabna districts of Bangladesh. Questionnaire interview and participatory rural appraisal tools like focus group discussion (FGD) were conducted with selected fish farmers. The most prevalent diseases as reported by the farmers were pop eye (57.78%), ventral reddening (55.55%), tail and fin rot (48.89%), hemorrhagic lesion over the body surface (45.56%), dropsy (40%), gill rot (40%), white spot (40%) and epizootic ulcerative syndrome or EUS (33.33%).

Hossain *et al.*, (2013) were conducted a study to know the pathogenicity and LD50 of *Aeromonas hydrophila* isolated from diseased climbing perch *Anabas testudineus* against apparently healthy homologous fish and the distribution of the bacteria in the organs of the experimentally infected fish. In all the cases of intramuscular injection, external pathology was found. Reddish anal region and fin bases were observed.

Sarkar *et al.*, (2012) studied on pathogenicity of a bacterial isolate *Aeromonas hydrophila* recovered from naturally diseased Shing fish was investigated against catfishes (*Heteropneustes fossilis and Clarias batrachus*), carps (*Labeo rohita, Catla catla and Cirrhinus cirrhosus*) and perch (*Anabas testudineus*).

Velmurugan *et al.*, (2016) were conducted a study to evaluate the hematological effect of cypermethrin, a synthetic pyrethroid on *Anabas testudineus*. The results are statistically significant at $p<0.05$ level. These reports indicate that hematological parameters may be useful as a diagnostic test for cypermethrin exposure in *A. testudineus*.

Rahman *et al.*, (2010) studied on tail and fin rot disease occurred in Indian major carp, Catla (*Catla catla*) and climbing perch, V. Koi (*Anabas testudineus*) in fish farms located at two districts of Bangladesh. The affected fish showed lesion and erosion on the tail and fins. Approximately, 40% mortality was recorded in those farms.

Islam *et al.*, (2015) studied on serological Studies of *Aeromonas hydrophila* in Bangladesh. Total collected 36 *Aeromonas* isolates from various healthy fishes of different regions in Bangladesh were characterized for their species and serogroup designations. After different morphological and biochemical characterization, it was found that, 25 of them were *A. hydrophila.* Serological studies were done by performing slide agglutination tests followed by agglutination titration.

2.4: Antibiogram profile

Abraham *et al.*, (2004) conducted a study on antibiogram profile of 33 isolates comprising *Aeromonas* spp., *Pseudomonas* spp. and Gram-positive rods from diseased *Clarias auratus* and *Clarias* spp. which were screened against six broad-spectrum antibiotics viz. ciprofloxacin, chloramphenicol, co-trimoxazole, gentamycin, nitro-furantoin and oxytetracycline. Ciprofloxacin was the most effective in inhibiting bacteria at 0.05-0.10 µg/ml level.

Ahammed (2016) studied about antibiogram profile of the isolated bacteria by using wide range of commercially available antibiotics. The results of the antibiotic sensitivity test is exhibited that most of the bacterial samples were sensitive against ciprofloxacin (92%) and levofloxacin (84%), intermediate resistant against gentamicin (40%) and resistant against novobiocin (84%), ampicillin (100%) and penicillin (92%).

Lee *et al.*, (2010) performed an experiment on antibiogram profile of pathogenic bacteria isolated from moribund cage cultured V. Koi (*Anabas testudineus*) and red hybrid tilapia (*Tilapia* sp.) from Sungai Manir, Terengganu, Malaysia were studied and characterized. Sungai Manir was one of the famous rivers in Terengganu for its wide variety of cage cultured freshwater fish.

Wei *et al.*, (2011) was carried out a study to investigate the antibiogram profile and Multiple Antibiotic Resistance (MAR) index of *Edwardsiella tarda* isolated from freshwater-fish cultures. To date, the information on antibiogram of local *E. tarda* isolates is still lacking. Therefore, this study was conducted to reveal the most suitable choice of antibiotic for aquaculture use among six types of commonly-used antibiotics (ampicillin, kanamycin, tetracycline, nalidixic acid, furazolidone and sulphamethoxazole).

3.1: Brief description of the study outline

Seven Upazilas (Mymensingh sadar, Phulpur, Muktagacha, Trishal, Fulbaria, Bhaluka and Gaffargaon) of Mymensingh district were selected for this study. Five ponds were selected from each upazila for collecting samples. Sampling was started in the month of September 2017 and experiment was completed by April 2018. A total of hundred seventy five of infected /dead Koi fishes were collected from 35 ponds to complete this study.

Gross and post-mortem changes of different organs from dead fishes were recorded. Inoculation of infected tissue samples into nutrient broth medium was carried for the primary isolation of bacteria. Pure culture was conducted using selective media (*Aeromonas* isolation agar media, *Pseudomonas* isolation agar media). Primary identification of isolated bacteria were conducted by Gram staining method and motility test. Identification of bacterial strain was confirmed by studying their bio-chemical characterization (Oxidase, O-F, MR, VP, Catalase, Indole, H_2S, Nitrate reduction, Urease production, TSI, production of acid and gas in five basic sugars. Final confirmations of each isolate of bacteria were conducted by using API kit. Aquarium based experimental infection were conducted using dominant isolate of bacteria in healthy Koi fishes under six test groups including control. Each group consisting of five healthy fishes, which were inoculated with 0.2 ml (with the standard dose of toxin), bacteria (6.7×10^6 cfu/fish) and both (toxin+bacteria) through oral feeding and intramuscular routes of injection and control treated with 0.2 ml dose of PBS. Re-isolation and identification of pathogenic bacteria from the experimentally infected Koi fishes were completed using similar methods.

CHAPTER 3

MATERIALS AND METHODS

3.1: Outline of the study

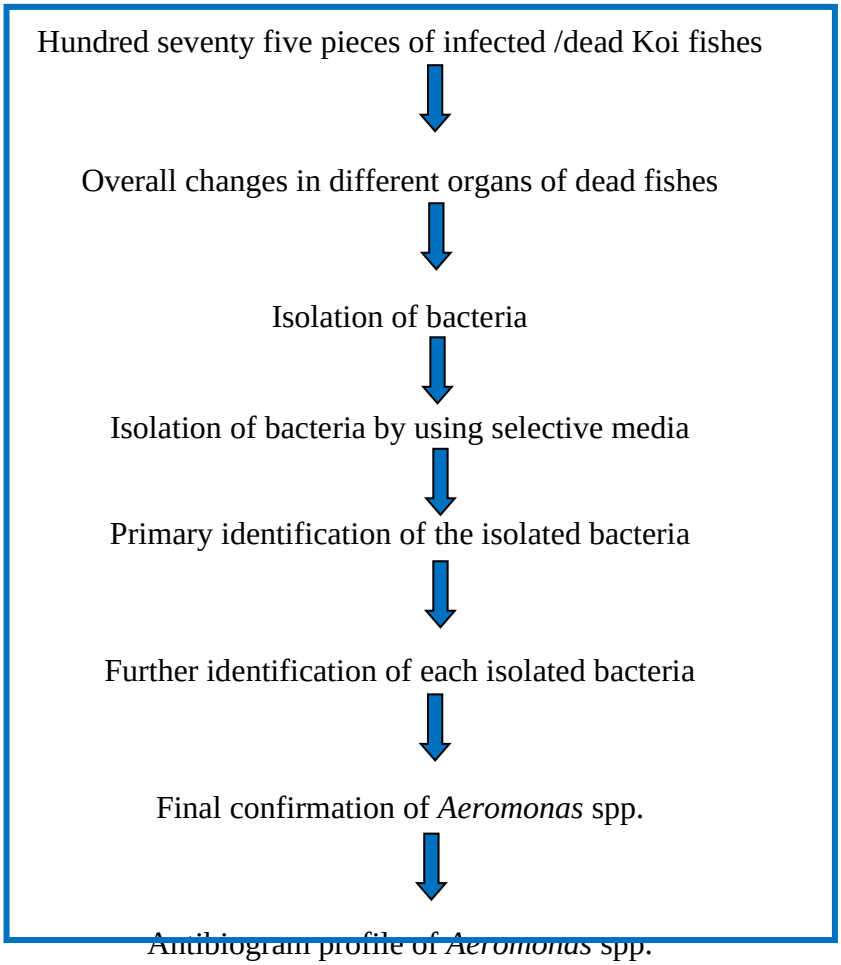

Figure 1: Flow chart showing the study outline

3.3: Study areas

Mass mortality of V. Koi fish were started in the month of August, 2017 in the cultured ponds of seven selected areas of Mymensingh districts namely Sadar, Phulpur, Muktagacha, Trishal, Fulbaria, Bhaluka and Gaffargaon.

Plate 1: Yellow stars indicating the sampling areas shown in the map during outbreak period

3.4: Species of fish samples

Rapid growing Vietnamese Koi fish (*Anabas testudineus*) is being cultured in different ponds of Mymensingh district since 2008.

Plate 2: Healthy Vietnamese Koi fish used in the study

3.5: Collection of dead fish samples

Diseased V. Koi fish were collected by using scoop net during sampling. The total number of collected fish samples was 175 fishes from 35 ponds. The collected fish samples were taken immediately with ice to the BFRI fish disease laboratory, Mymensingh.

Plate 3: Dead V. Koi fish sample

3.6: Duration of the study

The study was conducted for eight (8) months to complete the study which started since September 2017 to April 2018.

3.7: Preparation of culture media

Special culture media were used in the microbiological laboratory of BFRI to grow different kinds of bacteria. The media which were used to conduct this study are described below:

All the media was prepared as per the instruction of the manufacturer. However, brief description of all the medium are as follows according to CLSI (2007):

3.7.1: Tryptone soya agar media (TSA)

Fourty (40) grams of TSA was weighed and kept in a conical flask. Required amount of distilled water (DW) was measured by a measuring cylinder. One liter of DW was poured into the flask and shaked well to mix it up. The mouth of the flask was covered by an aluminum foil and the agar medium was autoclaved at 121^0C for 15 minutes under 15lb pressure per square inch for 15 minutes.

3.7.2: TSA slant

Required amount of TSA (40g/L) was weighed and one liter of distilled water was added as above to prepare agar mixture. The mixture was boiled for 5 min to melt the agar completely. Half of each sterile stock bottle was poured with sterile TSA medium and kept in a slant position till the medium became cool and solidified. The stock bottles were then kept at 4^0C in a refrigerator for future use.

3.7.3: Nutrient broth medium

Briefly, nutrient broth was prepared by dissolving 3gm of dehydrated nutrient broth into 100 ml of distilled water and was sterilized by autoclaving at 121^0C for 15 minutes under 15lb pressure per square inch for 15 minutes. Finally, the broth was dispensed into tubes (10 ml/tube and stored at 4^0C in the refrigerator until used).

3.7.4: Nutrient agar medium

Briefly, nutrient agar medium was prepared by dissolving 8 grams of agar in1000 ml of distilled water. Mixing was ensured by heating with frequent agitation. Boiling was done for one minute until complete dissolution. Dispensed into appropriate containers

and sterilize in autoclave at 121°C for 15 minutes. The prepared medium was stored at 2-8°C.

3.7.5: Peptone water suger medium

Briefly, peptone was used as a growth medium and as a base for carbohydrate fermentation media. 10.0 grams of peptone was suspended in 1000 ml distilled water. Heat applied if necessary to dissolve the medium completely. Dispensed in tubes and sterilize by autoclaving at 15 lbs. pressure (121°C) for 15 minutes.

3.7.6: Oxidative fermentative (OF) medium

Briefly, in 200 ml distilled water 3 gram of oxidative fermentation powder was used. Mixed well and dissolved by heating with frequent agitation. The solution was boiled for one minute until complete dissolution. Dispensed into appropriate containers and sterilized in autoclave at 121°C for 15 minutes. The prepared medium was stored at 2-8°C in a refrigerator.

3.7.7: Tryptone soya iodine agar (TSIA) medium

Briefly, at first 6.5 gram tryptone soya agar was taken in 100 ml distilled water. Then it was allowed for heating for one minute until complete dissolution. After cooling it was poured into 1 to 5 test tubes. It was sterilized by autoclaving at 15 lbs. pressure (121°C) for 15 minutes.

3.7.8: Triple sugar-iron agar medium

Briefly, 64.42 gram agar was suspended (the equivalent weight of dehydrated medium per liter) in 1000 ml purified/ distilled water. Heated for boiling to dissolve the medium completely. Mixed well and distributed into test tubes and Sterilized by maintaining at 10lbs pressure (115°C) for 30 minutes for as per validated cycle. The medium was allowed to set for further use.

3.7.9: Simmons citrate agar medium

Briefly, 3 gram oxidative fermentation powder was poured in 100 ml distilled water. Mixed well and dissolved by heating with frequent agitation. Boiled for one minute until complete dissolution. Dispensed into appropriate containers and sterilize in autoclave at 121°C for 15 minutes. The prepared medium was stored at 2-8°C.

3.7.10: Preparation of Physiological Saline

An amount of 0.87g NaCl was weighed and kept in a measuring flask. It was then filled distilled water to make the volume 100 ml. This was called physiological saline (PS=0.87% NaCl). Allocates of 0.9 ml and 5 ml were dispensed into test tubes and a stock of about 100 ml were kept in a flask. All the PS was autoclaved at 121°C for 15 and was kept at 4°C for future use.

3.8: Selective media

3.8.1: *Aeromonas* spp.

All the media were prepared as per the instruction of the manufacturer.

For this media preparation, at first 6 g *Aeromonas* isolation agar powder was taken into a conical flask. Medium was heated for boiling in order to dissolve completely. After heating 100 ml distilled water added into conical flask. Then the dissolved solution was poured into petridish and it was sterilized in autoclave at 121°C for 15 minutes.

3.8.2: *Pseudomonas* spp.

Briefly, at first 5 g of *Pseudomonas* isolation agar powder was taken into a conical flask. Then heated for dissolving the medium completely. After heating 100 ml distilled water added into conical flask. Then the dissolved solution was poured into petridish and it was sterilized in autoclave at 121°C for 15 minutes.

3.9: Morphological identification of bacteria

3.9.1: Gram's staining method

For gram staining method, at first a drop of distilled water was taken into sterilized glass slide with the help of syringe then a very small amount of cells were emulsified on slide and spread over large area. The smear was allowed to dry at room temperature and then heated to fix the cells passing through a flame. Then the smears were stained for 1 minute with aluminum oxalate crystal violate solution, then briefly rinsed with distilled water, immersed for 1 minute in iodine solution, washed lightly with distilled water and then dried. It was then counter stained for 30 second with

safranin solution. The slide was washed properly by running tap water. The slide image analyses were performed using a light compound microscope.

3.9.2: Motility test

For motility test, a single colony was mixed with 3 ml of PS. A drop of the suspension was taken on clean glass slide, covered with cover slip and placed under a luminous microscope. Bacterial motility was observed in a TV screen, adjusted with the microscope.

3.10: Identification of bacteria by various bio-chemical tests

3.10.1: Oxidative fermentative (O-F) test

Oxidative or fermentative glucose metabolism was examined using O-F basal medium containing bromothymol blue with 10% (w/v) dextrose, 10% (w/v) lactose and 10% (w/v) saccharose. Briefly, a single bacterial colony was stabbed into freshly prepared tubes of O-F medium in both anaerobic (with liquid paraffin) and aerobic (without paraffin) conditions. The color of the medium was examined after incubating the tubes at 24°C for 24 h.

3.10.2: 0/129 sensitivity test

The test was performed using commercially available diagnostic discs. Briefly, the suspended bacterial culture in sterile distilled water was carefully spread over the TSA plates (containing 1.5% NaCl) and let it dry for one minute. Then both 10g and the 150g discs of 0/129 was placed over the plate and pressed firmly downwards. The plate was inverted and incubated at 24°C for 24 h.

3.10.3: Oxidase test

A piece of filter paper was laid in a petridish. One or two drops of oxidase reagent were dropped on the center of the paper. A single colony was taken with a loop and smeared thoroughly on the reagent impregnated paper.

3.10.4: MR test

An isolate was inoculated into a tube with a sterile transfer loop. The tube was incubated at 35°C for 2–5 days. After incubation, 2.5 ml of the medium was transferred to another tube. Five drops of the pH indicator methyl red was added to this tube. The tube was gently rolled between the palms to disperse the methyl red. A negative reaction was indicated, the color of the medium was changed to yellow within a few minutes.

3.10.5: VP (Voges Proskaur) test

A tube of VP broth was inoculated with a pure culture of the test organism. Incubation was done for 24 hours at 35ºC. After incubation, 1 ml of broth was poured into clean test tube. Then 0.6ml of 5% alpha naphthol was added. The tube was gently shaken to expose the medium to atmospheric oxygen and to allow the tube to remain undisturbed for 10 to 15 minutes.

3.10.6: Catalase test

A small amount of bacterial colony was transferred into a clean, dry glass slide by using a sterilized loop. Then added a few drops of H_2O_2 onto the slide and mixed. A positive result was the rapid evolution of O_2 as evidenced by bubbling. A negative result was no bubbles or only a few scattered bubbles.

3.10.7: Indole test

To conduct this test, at first 2 ml peptone water was inoculated with 5 ml of bacteria culture and incubated for 48 hrs. 0.5 ml of kovac's reagent was added, dissolved through shaking and observed after 1 minute. Red colored in the reagent layer indicate positive indole test.

3.10.8: H_2S production

This medium was prepared with following reagents:

Ingredient	Amount
Beef extract	3g
Peptone	30g
Sodium thiosulfate	0.5g
Cysteine HCl	0.2g
Iron ammonium citrate	0.5g
Agar	5g
Distilled water	1000ml
pH	7.4

A 3 ml of the preparation was dispensed into each tube and autoclaved at 115°C for 15 min. Inoculation was done with a needle loop from a fresh culture and incubated at 25°C for 2 days. If the hydrogen sulfide was produced, the medium, especially upper layer, turned black.

3.10.9: Urease production

The broth medium was inoculated with a pure culture of the test organism; the surface of the agar slant was streaked with the test organism. Then the cap was left on loosely and the test tube was incubated at 35°C at ambient air for 18 to 24 hours; unless specified for longer incubation.

3.10.10: Nitrate reduction test

Nitrate broth was inoculated with a heavy growth of test organism using aseptic technique. Incubation was done at an appropriate temperature for 24 to 48 hours. Then one dropper of sulfanilic acid and one drop per full of α-naphthylamine were added to each broth.

- At this point, a color change to red indicates a positive nitrate reduction test.
- No color change indicates the absence of nitrite.

3.10.11: TSI (Triple sugar iron) test

The top of a well-isolated colony was touched with a sterilized inoculation needle. TSI Agar was inoculated by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant. Left the cap on loosely and incubated the tube at 35°C in ambient air for 18 to 24 hours.

3.10.12: Antibiotic sensitivity test

Eleven different discs of antibiotics namely ampicillin (10 µg), ciprofloxacin (5 µg), gentamicin (10 µg), oxytetracycline (10 µg), penicillin (10 µg), tetracycline (30 µg), levofloxacin (5 µg), azithromycin (10 µg), chlorotetracycline (25 µg), novobiocin (5 µg) and endrofloxacin (5 µg) were taken for this test. Briefly 200µl of suspended culture in distilled water was taken and spread over the surface of agar plate. After drying for little while in incubator, the antibiotic discs were placed over the plates and incubated at 24°C for 24 h. The degree of sensitivity was determined by measuring the zone of clearance/inhibition around the antibiotic discs.

3.11: Isolation of bacteria

Isolation of bacteria was carried out from the skin lesions and internal organs (liver, gill and kidney) of the diseased Koi fish. Smears from the skin lesions and internal organs were aseptically inoculated on nutrient broth as described by (Borty 2016). The overnight enriched broth was streaked onto various selective media such as: M-*Aeromonas* (MA) agar for *Aeromonas* spp. and incubated at 37°C for 24 hrs. Single colony was further sub-cultured until pure culture of bacteria was obtained.

3.12: Identification of bacterial pathogens

Bacterial colonies obtained from different culture plates were isolated and streaked on TSA slants and incubated overnight at 37^{o}C. Characterization of the pure isolates was performed by evaluating colonial characteristics, bacterial cell morphology by Gram's staining, motility test, biochemical tests such as catalase, glucose, sucrose and lactose utilization, indole, urease hydrogen sulfide production, TSI (gas production), methyl red (MR), voges praskaur (VP) Nitrogen reduction, oxidase, OF and antibiotic sensitivity tests.

3.13: API-20E microbiological identification

API-20E microbial identification kit (BioMérieux Marcyl'Étoile, France) was used to perform 20 biochemical tests at a time. The sample was prepared by suspending a single colony from a fresh culture into 5 ml of sterile saline solution. Then the suspension was carefully inoculated in all the tube sections according to the manufacturer instructions. The cupule sections of |CIT|, |VP| and |GEL| were completely filled up with the sample. For the ADH, LDC, ODC, H_2S and URE tests, the cupule sections were filled with sterile mineral oil to make an anaerobic condition. The prepared strip was covered with a lid and incubated for 2 days at 24°C. After incubation, all the reactions were recorded for identification of the isolated bacteria.

3.14: Total viable count (TVC)

Skin, gill and kidney of fish samples were minced and grinded individually in 1% peptone water. 0.5 ml of fish, mud and water samples were transferred into 4.5 ml of 1% peptone and to make a 10-fold serial dilutions up to 10^{-8}. 0.1ml of each of the 10^{-8} fold diluted sample was spread onto agar media and incubated at 37°C for 24-48 hrs. The mean number of colonies (30-300) of three plates in a particular dilution were multiplied by the dilution factor to determine TVC which was expressed as mean logarithm of the number of colony forming unit ($\log^{10}$ cfu/ml/fish).

3.15: Calculation of total bacterial load

Total bacterial load of each organ was calculated by using the following formula:

$$\text{Total bacterial load} = \frac{\text{Average number of colonies on plates}}{\text{Dilution factor} \times \text{Volume of inoculum per plate}}$$

3.16: Propagation of bacteria and preparation of inoculum with bacteria and toxin

The collected stocks of *Aeromonas* spp. were grown on Tryptic Soya Agar (TSA) at 30°C for 24 hours and identification was confirmed using different biochemical characteristics prior to the experiment. The bacterial suspensions were prepared with 0.85% NaCl (physiological saline) that resulted in a concentration of 6.7×10^6 cfu/ml. The bacterial samples were centrifuged at 4500 rpm. After centrifuging the clear fluid of the upper layer of Eppendorf tube was collected and filtered by using Millipore filter (22µm) and finally taken as toxin of the bacterial sample. The remaining deposit was used as bacterial pallet for the intramuscular and oral injection.

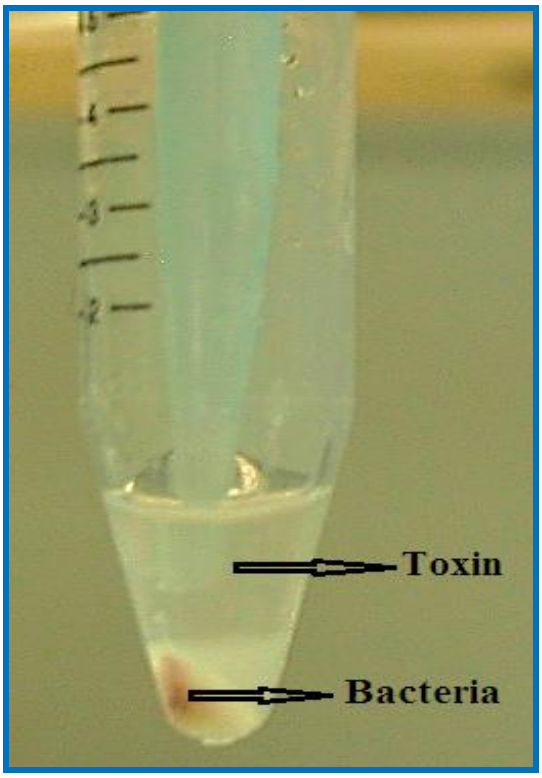

Figure 2: Eppendorf tube showing bacterial pallet and toxin

3.17: Set up of aquarium based experimental infection

A total number of 150 healthy V. Koi weighting 50-60 g were collected from disease free ponds of selected area. Prior to the artificial infection by selected motile *Aeromonas* spp., the collected fish were kept in aquaria for acclimatization at 28 to 30°C for 14 days by providing adequate feed and better aeration by recirculating water. The pathogenicity test was conducted at the Fish Disease and Health Management Laboratory of Bangladesh Fisheries Research Institute, Mymensingh. For challenge experiment, replicate groups of fish were placed in 14 rectangular 15-litre capacity well labeled glass aquariums. Each aquarium was aerated with aerator and one third of the water was replaced daily, dead fish were removed and debris was siphoned from the bottom of the aquarium.

3.18: Experimental infection

For the purpose of this study, intramuscular and oral injection was applied for generating the experimental infection in order to know the efficacy of the selected motile *Aeromonas* spp. to observe mortality of fishes. After acclimatization for 7 days, apparently healthy fishes were randomly assigned to 2 groups per bacterium for three different categories such as (bacteria, toxin and bacteria+toxin). Syringes (sterile and disposable) were used to inject intramuscularly and orally with 0.2 ml of pre-selected bacterial dose (6.7×10^6 cfu/fish). Water was exchanged three times in a week and residual feed was removed every two days by siphoning. The temperature, pH and dissolved oxygen, ammonia concentration of the water was kept at acceptable limit during the experiment. The injected fishes were then observed for clinical signs, symptoms and mortality daily up to 14 days.

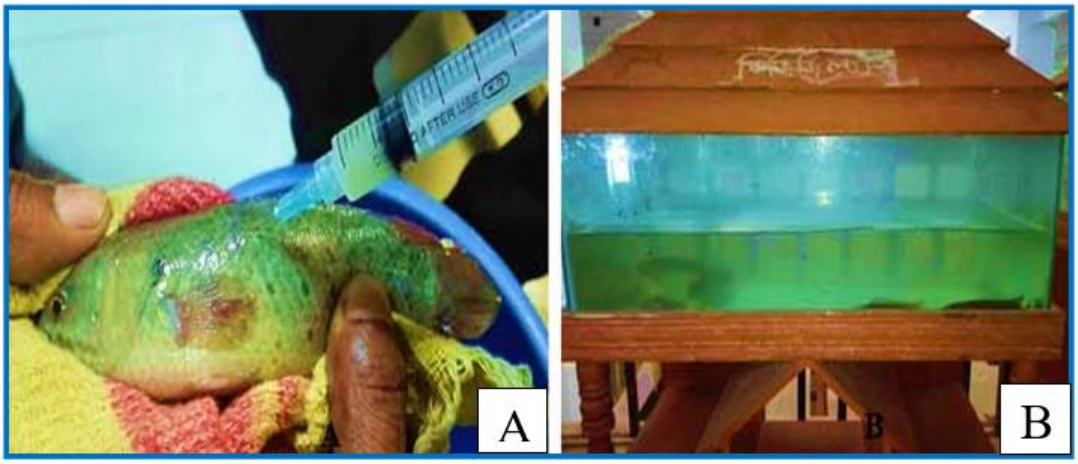

Figure 3: Experimental infection of V. Koi fish (A- Intramuscular injection of fresh Koi fish, B-Injected Koi fish kept in the aquarium)

3.19: Re-isolation of bacteria from experimentally infected V. Koi

Re-isolation and identification of pathogenic bacteria from the experimentally infected V. Koi fish was conducted by using similar method as followed for the field samples. A total of six test groups including control. Each group consist of 15 healthy V. Koi fishes. The fishes were inoculated with toxin (0.2 ml), bacteria (6.7×10^6 cfu/fish of inoculum) and both (toxin+bacteria) through oral and intramuscular routes of injection and the mock control group was inoculated with 0.2 ml of PBS.

3.20: Antibiogram profile of the *Aeromonas* spp.

The two bacterial isolates were tested for their sensitivity against eleven commercially available antibiotics by the disc diffusion method. Results of antibiotic sensitivity tests were recorded as sensitive, moderately sensitive and resistant depending on the diameters of inhibition zones and the guidelines of Clinical Laboratory Standard Institute (CLSI, 2007). The antibiotics, their codes and concentrations were as follows: ampicillin (10 µg), ciprofloxacin (5 µg), gentamicin (10 µg), oxytetracycline (10 µg), penicillin (10 µg), tetracycline (30 µg), levofloxacin (5 µg), azithromycin (10 µg), chlorotetracycline (25 µg), novobiocin (5 µg) and endrofloxacin (5 µg). All tests were carried out in Fish Diseases and Health Management Laboratory of Bangladesh Fisheries Research Institute (BFRI), Mymensingh.

3.21: Instrument and appliances

Instrument and appliances used in laboratory for this experiment was given below with there's figure:

Plate 4: A and B showing incubator and refrigerated centrifuge machine respectively

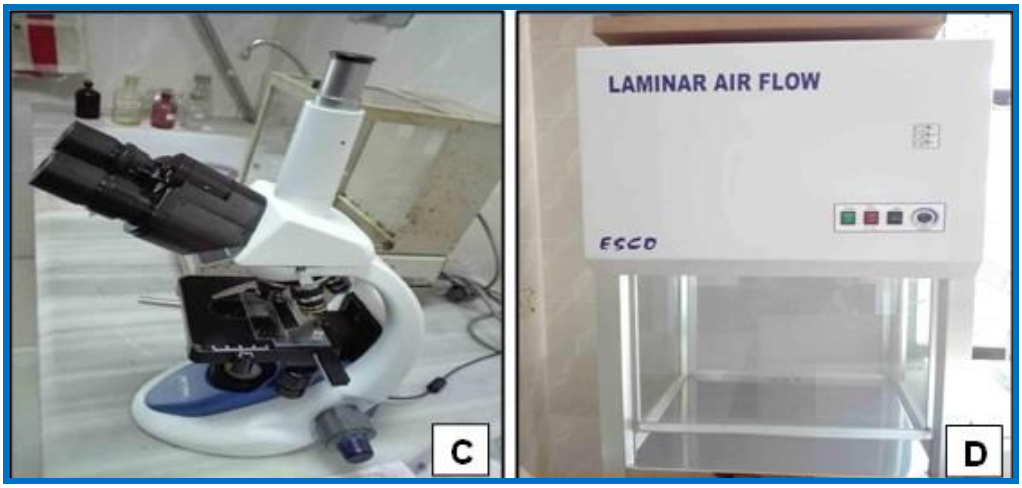

Plate 5: C and D showing compound microscope and laminar air flow

Plate 6: E and F showing autoclave and digital balance

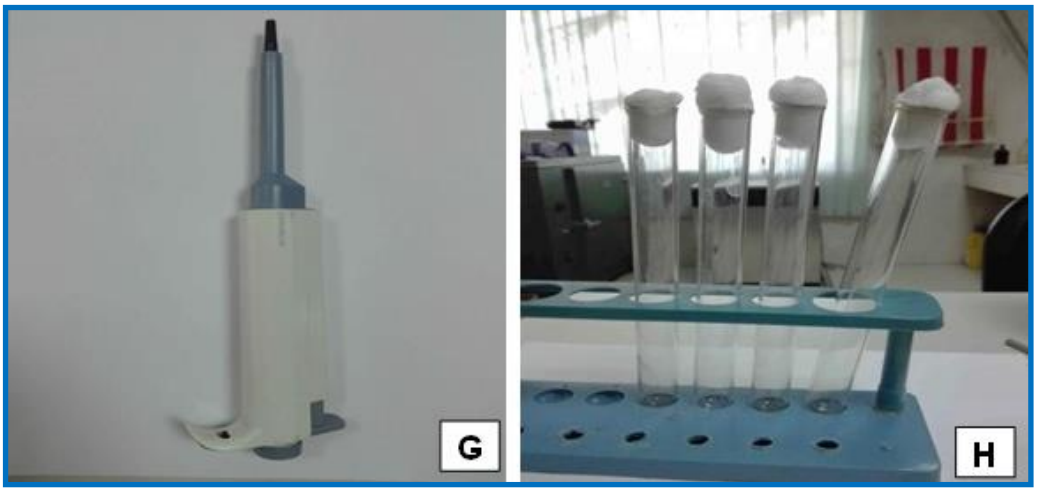

Plate 7: G and H showing micropipette and test tubes

Photos of Culture media

Culture media used to conduct this study are given below:

Plate 8: Different bacterial culture media used in the study

CHAPTER 4

RESULTS AND DISCUSSION

4.1: Gross changes of fish sample

Naturally infected V. Koi (*Anabas testudineus)* from seven sampling areas exhibited hemorrhage in base of fin, slight lesion observed in body and tail erosion were found. Heavy mortalities of fish were observed shortly after the advent of lesions at the time of clinical diagnosis. On the other hand normal fishes were found healthy, bright and good appearance in morphology.

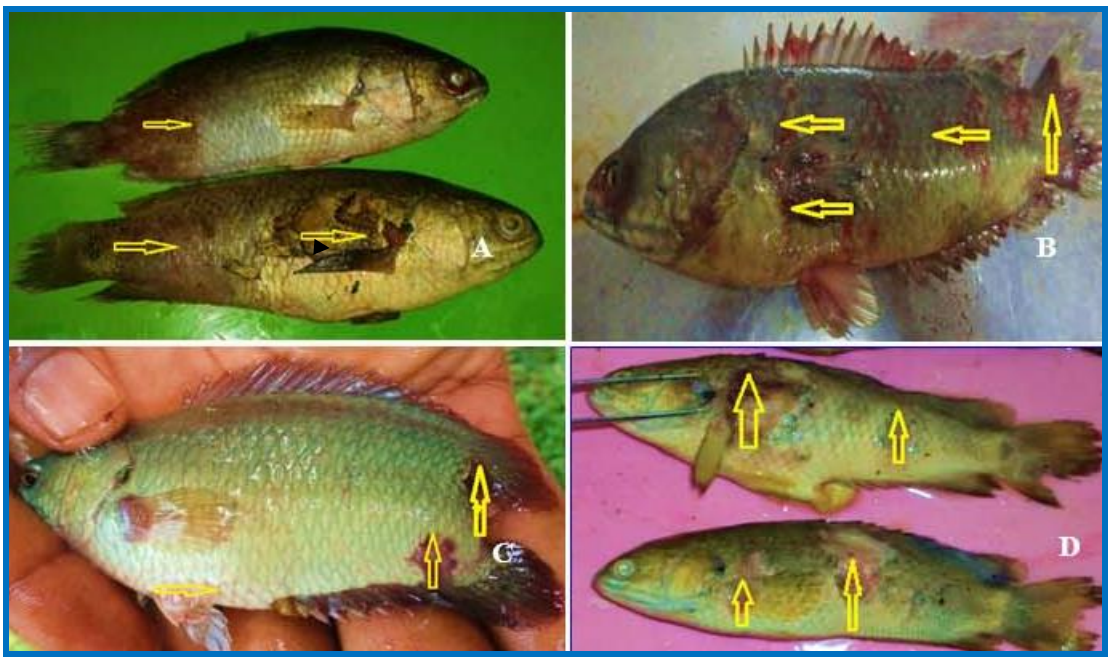

Plate 9: Diseased V. Koi fish sample (A- hemorrhage on the skin, B-hemorrhage on the fin base, C- erosion on tail, D- hemorrhage and lesion on the body)

Table 1: Gross changes of fish sample from seven Upazilas in Mymensingh district

Sampling areas (Upazilas)	Number of fish ponds per area	Number of fish sample per pond	Type of changes (%)		
			Hemorrhage	Ulceration	Erosion
Mymensingh Sadar	5	5	45	30	22
Muktagacha			43	25	28
Fulbaria			55	25	20
Bhaluka			35	30	25
Phulpur			45	21	18
Gafforgaon			43	28	20
Trishal			34	24	20
Total	35	175	-	-	-

4.2: Post-mortem changes of fish sample

Congestion and enlargement with hemorrhage were observed in liver, kidney and spleen of the diseased Koi after post-mortem examination. Among internal organs, liver was more affected than kidney in the present experiment.

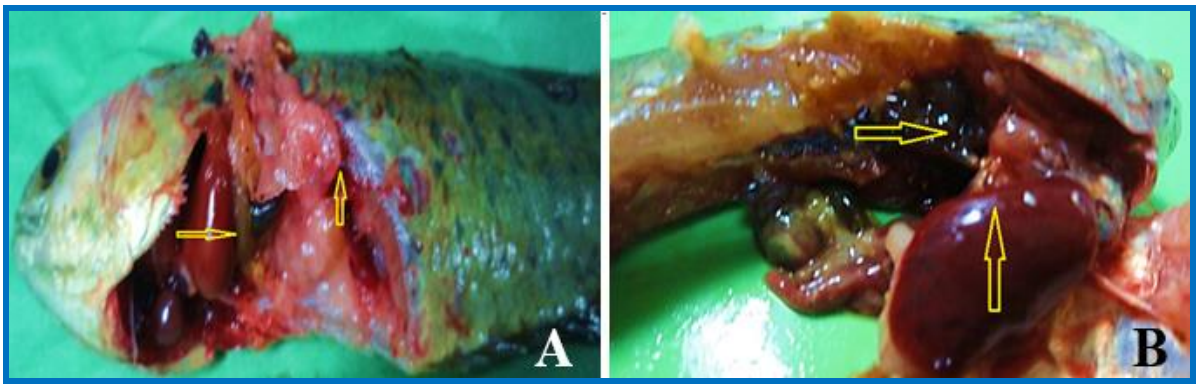

Plate 10: Changes of internal organs (A-Fresh liver and kidney, B-enlarge liver and kidney of V. Koi)

Table 2: Post-mortem changes of fish sample from seven Upazilas in Mymensingh district

Sampling areas (Upazilas)	Number of fish ponds per area	Number of fish sample per Upazila	Infected organs		
			Liver	Kidney	Spleen
Mymensingh Sadar	5	25	Swollen	Congestion	Splenomegaly
Muktagacha			Hepatomegaly	Congestion	Splenomegaly
Fulbaria			Swollen	Normal	Normal
Bhaluka			Normal	Congestion	Splenomegaly
Phulpur			Hepatomegaly	Normal	Splenomegaly
Gafforgaon			Normal	Congestion	Normal
Trishal			Swollen	Congestion	Splenomegaly
Total	35	175	-	-	-

4.3: Isolation of Bacteria

4.3.1: Bacteria (*Anabas testudineus)* isolated from different organs of infected V. Koi fish

A total of 5 bacterial strains were isolated from 175 samples of diseased Koi. Thisolation frequencies of these 5 strains in different parts of infected Koi described below:

Table 3: Mean distribution (%) of different bacterial strains in various organs of infected V. Koi (*Anabas testudineus)* fish from Mymensingh Sadar

Isolated bacteria	**Distribution of bacteria in different organs (%)**					**Total (%)**
	Skin	**Gill**	**Liver**	**Kidney**	**Spleen**	
Aeromonas hydrophila	20.17	17.69	20.23	12.76	12.12	81.86
Aeromonas caviae	26.99	11.59	11.08	13.07	17.83	80.58
Streptococcus sp.	22.24	11.05	11.05	9.53	10.59	64.48
Staphylococcus sp.	11.05	7.01	6.54	7.19	9.41	41.22
Escherichia coli	5.92	5.83	3.84	1.00	5.61	23.6

Table 4: Mean distribution (%) of different bacterial strains in different organs of infected V. Koi (*Anabas testudineus)* fish from Muktagacha

Isolated bacteria	**Distribution of bacteria in different organs (%)**					**Total (%)**
	Skin	**Gill**	**Liver**	**Kidney**	**Spleen**	
Aeromonas hydrophila	18.68	17.30	14.86	14.61	11.66	67.30
Aeromonas caviae	12.05	13.48	10.19	12.08	16.65	60.53
Streptococcus sp.	11.04	2.19	6.35	1.57	2.406	24.77
Staphylococcus sp.	9.77	7.46	0.00	12.05	0.00	25.69
Escherichia coli	7.46	0.00	11.04	0.00	7.46	22.67

Table 5: Mean distribution (%) of different bacterial strains in different organs of infected V. Koi (*Anabas testudineus)* fish from Fulbaria

Isolated bacteria	Distribution of bacteria in different organs (%)					Total (%)
	Skin	Gill	Liver	Kidney	Spleen	
Aeromonas hydrophila	20.17	17.83	22.21	15.18	13.48	86.29
Aeromonas caviae	32.09	11.04	12.05	11.43	17.72	84.21
Streptococcus sp.	20.17	10.66	11.48	10.22	11.08	61.68
Staphylococcus sp.	10.91	7.46	6.35	7.91	10.62	43.18
Escherichia coli	6.35	6.82	4.24	1.13	6.01	24.20

Table 6: Mean distribution (%) of different bacterial strains in different organs of infected V. Koi (*Anabas testudineus)* fish from Phulpur

Isolated bacteria	Distribution of bacteria in different organs (%)					Total (%)
	Skin	Gill	Liver	Kidney	Spleen	
Aeromonas hydrophila	21.69	18.00	15.37	16.45	11.08	82.60
Aeromonas caviae	16.32	15.62	10.48	12.05	18.38	71.86
Streptococcus sp.	11.66	2.06	6.54	1.90	2.32	24.50
Staphylococcus sp.	10.48	7.46	0.00	11.66	0.00	29.61
Escherichia coli	9.77	0.00	11.66	0.00	8.01	29.45

Table 7: Mean distribution (%) of different bacterial strains in different organs of infected V. Koi (*Anabas testudineus)* fish from Bhaluka

Isolated bacteria	Distribution of bacteria in different organs (%)					Total (%)
	Skin	Gill	Liver	Kidney	Spleen	
Aeromonas hydrophila	20.63	18.00	16.32	15.37	12.05	82.38
Aeromonas caviae	14.94	14.69	10.48	12.05	18.00	70.17
Streptococcus sp.	11.66	2.07	6.01	1.64	2.29	23.7
Staphylococcus sp.	10.48	8.01	0.00	11.66	0.00	30.16
Escherichia coli	10.04	0.00	11.66	0.00	8.01	29.72

Table 8: Mean distribution (%) of different bacterial strains in different organs of infected V. Koi (*Anabas testudineus)* fish from Gaffargaon

Isolated bacteria	Distribution of bacteria in different organs (%)					Total (%)
	Skin	Gill	Liver	Kidney	Spleen	
Aeromonas hydrophila	23.70	18.00	16.32	15.68	12.00	85.81
Aeromonas caviae	18.19	16.32	10.04	12.01	18.00	74.66
Streptococcus sp.	11.08	2.29	6.01	1.66	2.42	23.49
Staphylococcus sp.	10.48	8.01	0.00	11.66	0.00	30.16
Escherichia coli	9.77	0.00	11.46	0.00	8.01	29.25

Table 9: Mean distribution (%) of different bacterial strains in different organs of infected V. Koi (*Anabas testudineus)* fish from Trishal

Isolated bacteria	Distribution of bacteria in different organs (%)					Total (%)
	Skin	Gill	Liver	Kidney	Spleen	
Aeromonas hydrophila	18.19	20.63	15.15	12.05	12.01	78.13
Aeromonas caviae	20.17	15.37	10.04	12.27	18.50	76.36
Streptococcus sp.	11.46	2.42	6.01	1.68	2.29	35.51
Staphylococcus sp.	10.04	7.62	0.00	8.01	0.00	25.73
Escherichia coli	9.77	0.00	11.46	0.00	8.01	29.25

4.4: Morphological and bio-chemical tests of each bacterial isolate

The isolated *Aeromonas* spp. from diseased Koi was identified by their specific morphological, physiological and biochemical characteristics. The characteristics were found as Gram negative, rod shaped, motile and gave positive result for oxidase and catalase test. Moreover, they fermented glucose and were resistant to vibrio static agent 0/129 test. The results of morphological, physiological and biochemical tests are presented as follows:

Table 10. Biochemical characteristics of *Aeromonas* spp. determined by conventional methods

Test name	***Aeromonas hydrophila***	***Aeromonas caviae***
Gram staining	-ve	-ve
Motility	+	+
Oxidase test	+	+
O-F test	+	+
MR test	+	-
VP test	-	+
H_2S production	+	+
Nitrate reduction test	+	+
Urease production	+	-
TSI test	A/A	A/A
Production of acid from glucose	+	+
Galactose	D	+
0/129 test (10 µg & 150 µg)	R	R
Catalase test	+	D
Indole test	+	-

Note: F: Fermentative; (+): Positive response; (-): Negative response, D-Variable reaction, O-Oxidative, A-Acid, and R-Resistant

Table 11: Results of morphological and bio-chemical characteristics of isolated *Aeromonas* spp.

Characters	Characterization by Mostafa *et al.* (2008)	Characterization by Sabur (2006)	Present study result
Gram's stain	-	-	-
Shape	Rod	Rod	Rod
Motility	+	+	+
0/129	ND	ND	-
Oxidase	+	+	+
Catalase	+	+	-
OF test	F	F	F
Glucose	+	+	+
Lactose	+	+	+
Sucrose	+	+	+
Maltose	+	+	+
Manitol	+	+	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Esculin hydrolysis	+	+	+
Methyl-red test	-	-	-
Voges-Proskaur	+	+	+
Indole	+	+	+
H_2S production	+	+	-
Arginine decomposition	+	+	+
Lysine decarboxylation	-	-	-
Ornithine decarboxylation	-	-	-
Citrate utilization	+	+	+
TSI	ND	ND	'K' in slants but 'A' in butt
Growth at 4°C	-	-	-
5°C	+	+	+
37°C	+	+	+
40°C	-	-	-

N.B: -: Negative, +: Positive, F: Fermentative, ND: Not Defined

Morphological and biochemical tests for bacterial identification

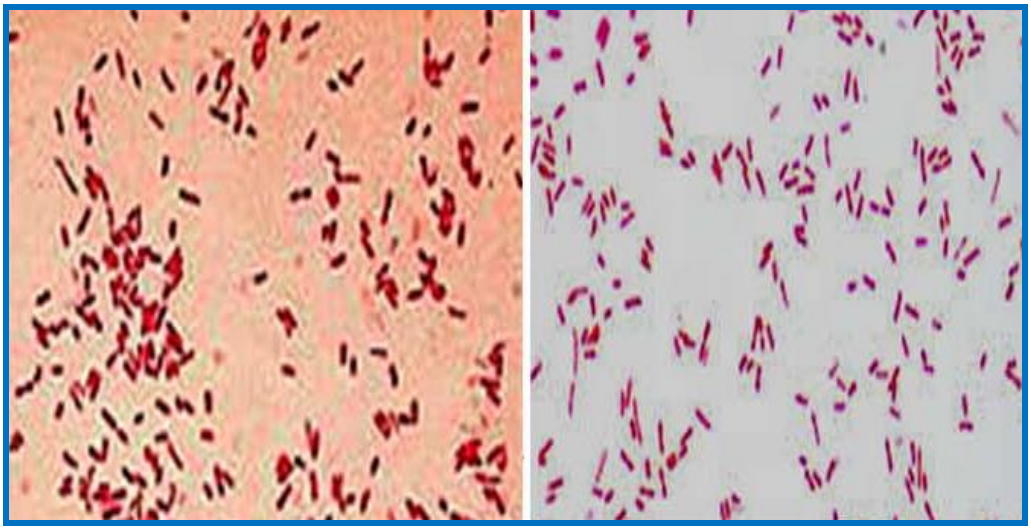

Figure 4: Gram negative rod shape bacteria

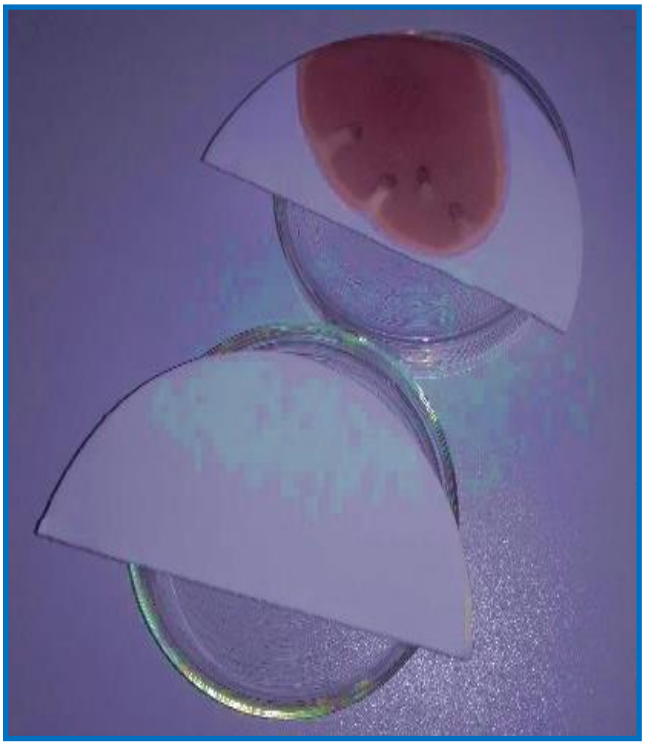

Figure 5: Oxidase test

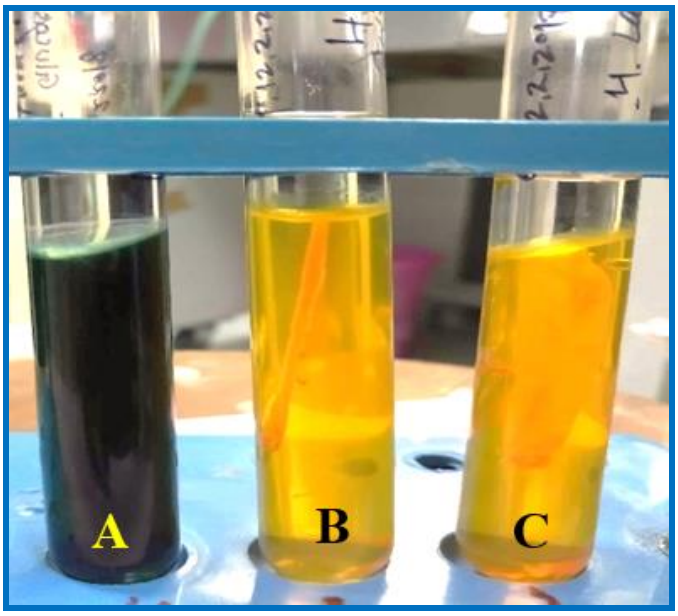

Figure 6: OF test (A-control, B-anaerobic condition, C-aerobic condition)

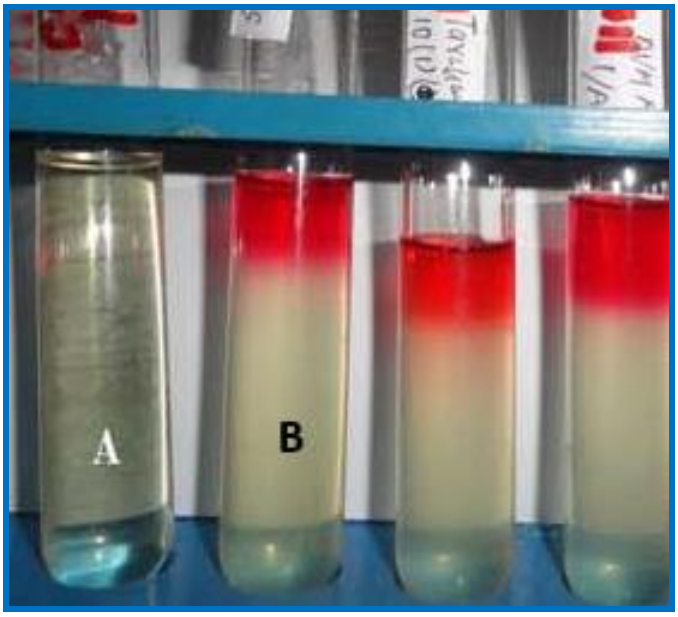

Figure 7: Methyl Red (MR) test showing positive result (A-control, B-positive)

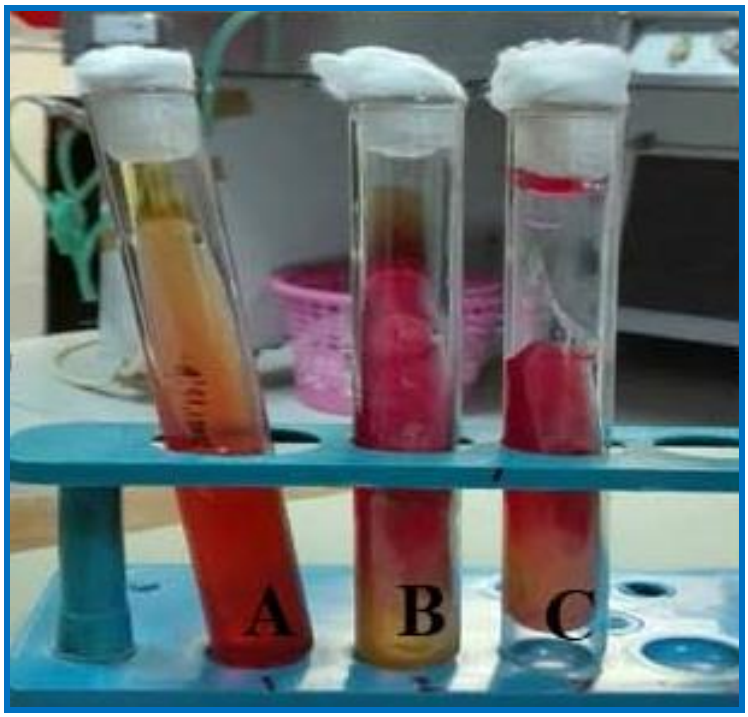

Figure 8: TSI test showing production of acid and gas (A-control, B-acid, and C-gas)

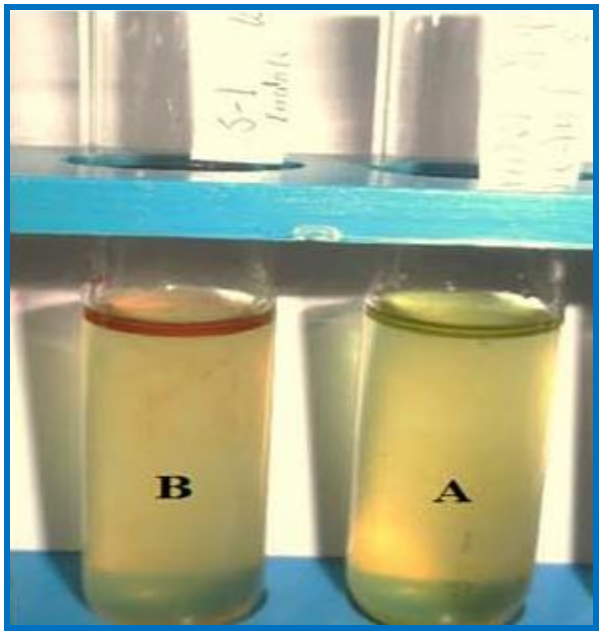

Figure 9: Indole test (A-control, B-positive)

Figure 10: Simmons citrate test (A-control, B-positive)

4.5: API-20E microbiological identification

Twenty biochemical tests were performed using the commercially available API-20E microbiological kit as described in methods. The positive or negative reactions of all isolates were recorded and summarized in the following table. All isolates gave positive results by reacting with betagalactosidase, arginine dihydrolase, lysine decarboxylase, sodium citrate, urease, tryptophan deaminase, tryptophane, sodium pyruvate, gelatinase, glucose, mannitol, sucrose, melibiose, amygdalin, and arabinose. However, negative reactions were shown for ornithine decarboxylase, H_2S production, inositol, sorbitol, and rhamnose.

Table 12: Biochemical characteristics of bacteria determined through the API 20E kit

Characteristics	**Response of bacterial isolates**	
	Aeromonas hydrophila	***Aeromonas caviae***
Beta-galactosidase	+	+
Arginine dihydrolase	+	+
Lysine Decarboxylase	+	+
Ornithine Decarboxylase	-	-
Citrate utilization	+	+
H_2S production	-	-
Urease production	-	-
Tryptophan deaminase	+	+
Indole production	+	+
Acetoin production	+	+
Gelatinase	+	+
Glucose	+	+
Mannitol	+	+
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Sucrose	+	+
Melibiose	+	+
Amygdalin	+	+
Arabinose	+	+

Note: (+): Positive response; (-): Negative response

Figure 11: A indication the picture of control and B indicating the picture of test API-20E kit which is showing the result of identification of bacteria

4.6: Total viable count (TVC) in the naturally infected V. Koi

Bacterial load was found from diseased Koi from seven Upazilas in Mymensingh districts. Commonly found bacteria were *Aeromonas hydrophila, Aeromonas caviae, Streptococcus* sp., *Staphylococcus* sp. and *Escherichia coli*. However, *A. hydrophila and A. caviae* were found mostly pathogenic, therefore, these two bacterial species were taken for further experiment. Total bacterial load was 1.13×10^6 to 3.21×10^8 cfu/g in lesions; 1.10×10^5 to 2.1×10^7 cfu/g in liver; 3.24×10^4 to 3.82×10^6 cfu/g in gill; 1.25×10^6 to 3.51×10^7 cfu/g in kidney and 3.74×10^4 to 2.43×10^6 in spleen of diseased Koi. *A. hydrophila* and *A. caviae* initially identified by their specific morphological, physiological and biochemical characteristics.

Table 13: Mean bacterial load in different organs from infected V. Koi

Name of the sampling areas (Upazilas)	**Total viable count in different organs**				
	Skin lesion cfu/g	**Liver cfu/g**	**Gill cfu/g**	**Kidney cfu/g**	**Spleen cfu/g**
Mymensingh Sadar	2.70×10^7	2.92×10^5	2.82×10^4	2.66×10^5	2.74×10^5
Muktagacha	2.54×10^8	2.88×10^7	2.37×10^5	2.43×10^6	3.24×10^6
Fulbaria	2.88×10^6	2.61×10^5	2.70×10^4	2.84×10^6	2.61×10^4
Bhaluka	2.63×10^7	2.70×10^5	2.61×10^6	2.70×10^7	2.82×10^5
Phulpur	2.61×10^6	2.37×10^6	3.10×10^5	2.57×10^7	2.46×10^6
Gaffargaon	2.89×10^7	3.88×10^5	2.63×10^4	3.18×10^6	2.98×10^5
Trishal	2.37×10^7	2.63×10^6	3.53×10^6	3.83×10^7	2.69×10^6

Table 14: Mean bacterial load in infected skin of V. Koi

Name of the sampling areas (Upazilas)	**Total viable count of isolated bacteria in skin of V. Koi**				
	Aeromonas hydrophila	*Aeromonas caviae*	*Streptococcus* sp.	*Staphylococcus* sp.	*Escherichia coli*
Mymensingh Sadar	3.21×10^8	2.10×10^7	5.43×10^5	1.25×10^6	1.54×10^6
Muktagacha	2.32×10^7	3.41×10^5	3.44×10^4	3.55×10^6	2.54×10^5
Fulbaria	2.00×10^7	3.41×10^5	3.40×10^4	3.15×10^6	2.44×10^5
Bhaluka	2.30×10^6	1.50×10^6	3.56×10^5	3.4×10^7	2.43×10^6
Phulpur	1.43×10^7	1.10×10^5	3.82×10^6	2.18×10^7	4.54×10^5
Gaffargaon	2.31×10^7	3.11×10^5	3.24×10^4	3.15×10^6	2.64×10^5
Trishal	1.13×10^6	2.76×10^5	3.24×10^4	2.31×10^6	2.44×10^4

Table 15: Mean bacterial load in infected liver of V. Koi

Name of the sampling areas (Upazilas)	**Total viable count of isolated bacteria in liver of V. Koi**				
	Aeromonas hydrophila	*Aeromonas caviae*	*Streptococcus* sp.	*Staphylococcus* sp.	*Escherichia coli*
Mymensingh Sadar	2.31×10^7	3.11×10^5	3.24×10^4	3.15×10^6	2.64×10^5
Muktagacha	2.00×10^7	3.41×10^5	3.40×10^4	3.15×10^6	2.44×10^5
Fulbaria	1.43×10^7	1.10×10^5	3.82×10^6	2.18×10^7	4.54×10^5
Bhaluka	3.21×10^8	2.10×10^7	5.43×10^5	1.25×10^6	1.54×10^6
Phulpur	1.13×10^6	2.76×10^5	3.24×10^4	2.31×10^6	2.44×10^4
Gaffargaon	2.00×10^7	3.41×10^5	3.40×10^4	3.15×10^6	2.44×10^5
Trishal	2.30×10^6	1.50×10^6	3.56×10^5	3.4×10^7	2.43×10^6

Table 16: Mean bacterial load in infected gill of V. Koi

Name of the sampling areas (Upazilas)	Total viable count of isolated bacteria in gill of V. Koi				
	Aeromonas hydrophila	*Aeromonas. Caviae*	*Streptococcus* sp.	*Staphylococcus* sp.	*Escherichia coli*
Mymensingh Sadar	2.00×10^{7}	3.41×10^{5}	3.40×10^{4}	3.15×10^{6}	2.44×10^{5}
Muktagacha	1.13×10^{6}	2.76×10^{5}	3.24×10^{4}	2.31×10^{6}	2.44×10^{4}
Fulbaria	3.21×10^{8}	2.10×10^{7}	5.43×10^{5}	1.25×10^{6}	1.54×10^{6}
Bhaluka	1.43×10^{7}	1.10×10^{5}	3.82×10^{6}	2.18×10^{7}	4.54×10^{5}
Phulpur	3.00×10^{7}	3.41×10^{5}	3.40×10^{4}	3.15×10^{6}	2.54×10^{5}
Gaffargaon	2.30×10^{6}	1.50×10^{6}	3.56×10^{5}	3.4×10^{7}	2.43×10^{6}
Trishal	2.31×10^{7}	3.11×10^{5}	3.24×10^{4}	3.15×10^{6}	2.64×10^{5}

Table 17: Mean bacterial load in infected kidney of V. Koi

Name of the sampling areas (Upazilas)	Total viable count of isolated bacteria in kidney of V. Koi				
	Aeromonas hydrophila	*Aeromonas caviae*	*Streptococcus* sp.	*Staphylococcus* sp.	*Escherichia Coli*
Mymensingh Sadar	3.11×10^{8}	2.13×10^{7}	5.13×10^{5}	1.24×10^{6}	1.54×10^{6}
Muktagacha	2.62×10^{7}	3.31×10^{5}	3.64×10^{4}	3.56×10^{6}	2.58×10^{5}
Fulbaria	2.01×10^{7}	3.21×10^{5}	3.40×10^{4}	3.15×10^{6}	2.44×10^{5}
Bhaluka	2.38×10^{6}	1.70×10^{6}	3.26×10^{5}	3.45×10^{7}	2.73×10^{6}
Phulpur	1.13×10^{7}	1.30×10^{5}	3.22×10^{6}	2.38×10^{7}	4.84×10^{5}
Gaffargaon	2.39×10^{7}	3.81×10^{5}	3.94×10^{4}	3.15×10^{6}	2.64×10^{5}
Trishal	2.13×10^{6}	2.86×10^{5}	3.24×10^{4}	8.31×10^{6}	2.44×10^{4}

Table 18: Mean bacterial load in infected spleen of V. Koi

Name of the sampling areas (Upazilas)	**Total viable count of isolated bacteria in spleen of V. Koi**				
	Aeromonas hydrophila	*Aeromonas caviae*	*Streptococcus* sp.	*Staphylococcus* sp.	*Escherichia coli*
Mymensingh Sadar	3.31×10^{7}	3.71×10^{5}	3.24×10^{4}	3.45×10^{6}	2.54×10^{5}
Muktagacha	3.00×10^{7}	3.81×10^{5}	3.60×10^{4}	3.35×10^{6}	2.44×10^{5}
Fulbaria	1.43×10^{7}	1.60×10^{5}	3.42×10^{6}	2.28×10^{7}	4.34×10^{5}
Bhaluka	3.21×10^{8}	2.40×10^{7}	5.73×10^{5}	1.25×10^{6}	1.54×10^{6}
Phulpur	1.73×10^{6}	2.76×10^{5}	3.24×10^{4}	2.11×10^{6}	2.44×10^{4}
Gaffargaon	2.60×10^{7}	3.41×10^{5}	3.40×10^{4}	3.15×10^{6}	2.34×10^{5}
Trishal	2.60×10^{6}	1.50×10^{6}	3.56×10^{5}	3.4×10^{7}	2.43×10^{6}

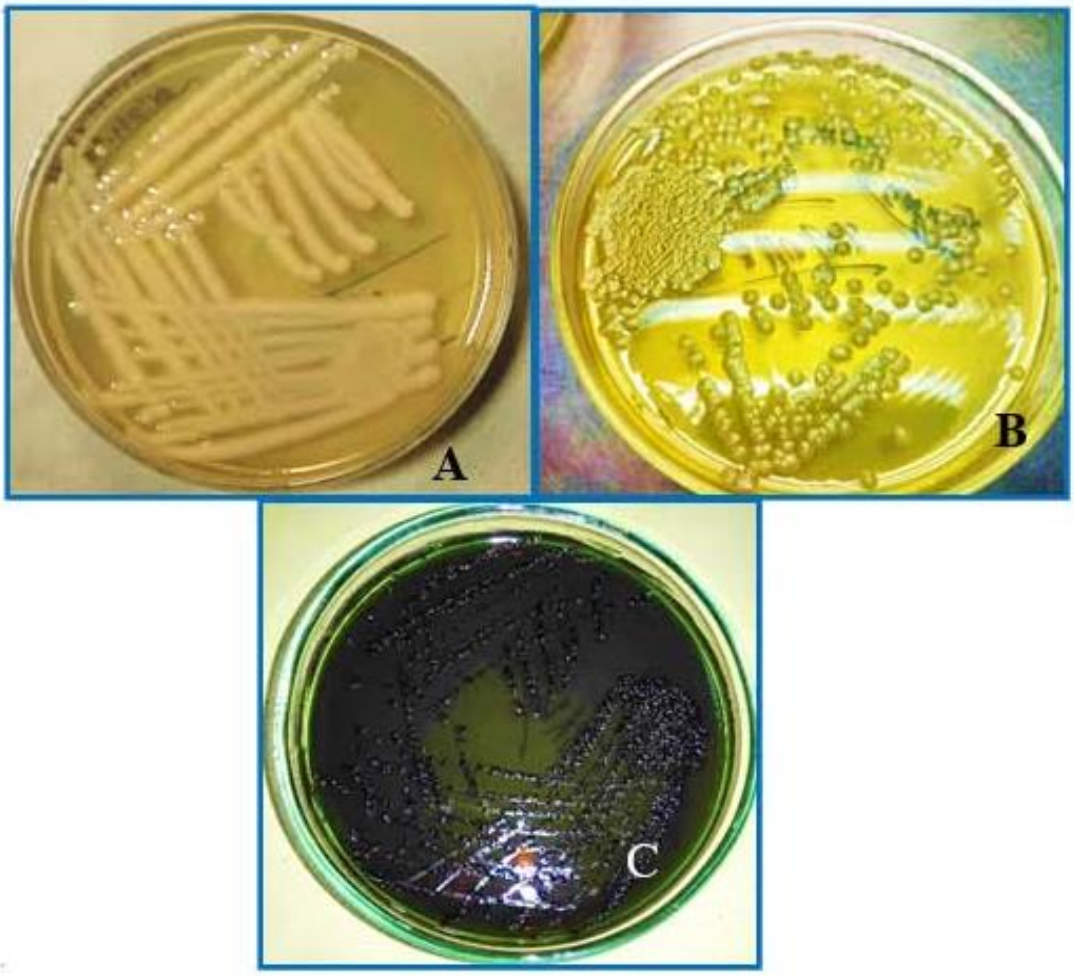

Plate 12: Media used for culture of bacteria (A- *Aeromonas hydrophila* on nutrient agar medium, B-Bacteria culture in TSA agar, C- *Aeromonas* selective media)

4.7: Effect of the field isolate of *Aeromonas* spp. on mortality of experimentally infected V. Koi

At the end of the experiment (after 14 days), the cumulative mortality rate was recorded highest (89%) in group 2 and lowest (73%) in group 1. However, the highest average of the occurrence of mortality was recorded about 89% in the groups 2.

Table 19. Cumulative progression of mortality rate in experimental V. Koi (*Anabas testudineus*) infected with *Aeromonas* spp.

Group	Infected bacteria	Number of healthy Koi used per group (Infection methods)	Mortality rate at days after oral and intramuscular routes of injection													Percentage (%)
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1	*Aeromonas hydrophila*	15 (Oral)	0	0	0	0	0	0	1	1	2	1	1	2	1	2	73
2		15 (Injection)	0	0	0	0	0	0	1	2	1	2	2	1	2	1	80
3	*Aeromonas caviae*	15 (Oral)	0	0	0	0	0	0	0	1	2	1	0	1	2	1	53
4		15 (Injection)	0	0	0	0	0	0	1	2	1	0	0	2	1	2	60
5	Control	15 (Oral)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6		15 (Injection)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

4.7: Antibiogram profile of isolated bacteria

The isolated *Aeromonas hydrophila* and *Aeromonas caviae* were tested against eleven commercially available antibiotics. The results were as follows:

Table 20: Antibiogram profile of isolated *A. hydrophila* and *A. caviae (*n=5) from infected V. Koi (%)

Antibiotic (Conc./Disc)	***Aeromonas hydrophila***	***Aeromonas caviae***
Endrofloxacin (5µg)	+++	+++
Ciprofloxacin (5µg)	+++	+++
Levofloxacin (5µg)	+++	+++
Gentamicin (10µg)	++	++
Azithromycin (15µg)	++	++
Tetracycline (30µg)	+	+
Oxytetracycline (10µg)	-	-
Chlortetracycline (25µg)	-	-
Novobiocine (5µg)	-	-
Ampiciline (10µg)	-	-
Penicillin (10µg)	-	-

Note: -: no inhibition, +: inhibitory zone between 5-12mm, ++: inhibitory zone between 13- 20 mm. +++: inhibitory zone between 21-30 mm

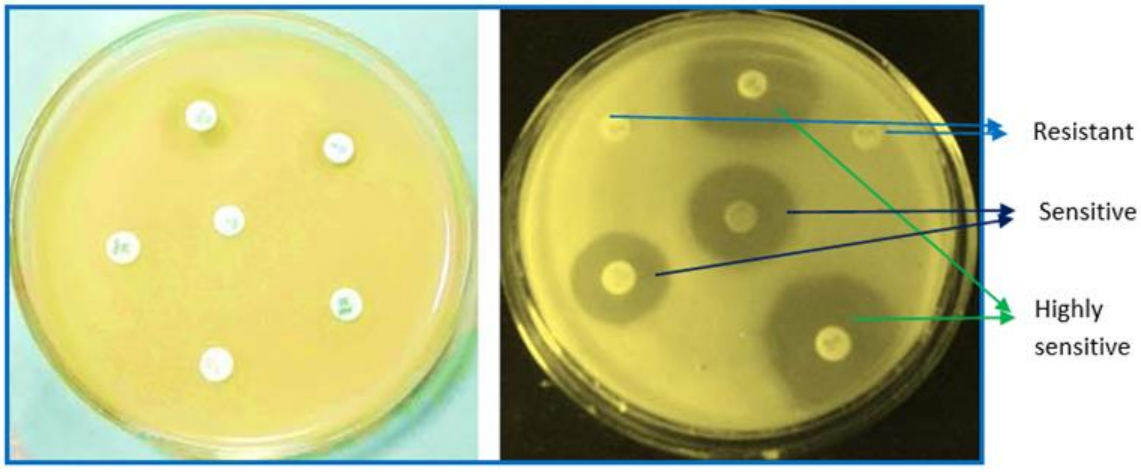

Figure 12: Antibiotic sensitivity test

Table 21: Month wise mortality rate (%) of V. Koi fish in the study areas during outbreak period

Sampling areas (Upazilas)	Mortality rate (%) of V. Koi during outbreak period					
	Sep	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**
Mymensingh Sadar	45	50	65	70	75	65
Muktagacha	40	46	55	72	66	69
Fulbaria	49	48	58	74	70	56
Bhaluka	38	52	60	62	64	63
Phulpur	42	50	62	68	53	54
Gaffargaon	34	48	66	70	82	62
Trishal	38	44	60	74	71	70

Table 22: Correlation between water quality parameters and mortality rate (%) of V. Koi at Mymensingh Sadar during outbreak period

Sampling period	Temperature (^{0}C)	pH	Dissolve Oxygen (ppm)	Mortality (%)
September	28	7.20	5.00	45
October	26	7.00	5.34	50
November	23	7.23	5.67	65
December	21	7.45	5.21	70
January	20	8.00	4.89	75
February	22	7.20	4.75	65

Table 23: Correlation between water quality parameters and mortality rate (%) of V. Koi at Muktagacha during outbreak period

Sampling period	Temperature (^{0}C)	pH	Dissolve Oxygen (ppm)	Mortality (%)
September	28	7.11	5.65	40
October	26	7.23	5.45	46
November	23	7.21	5.23	55
December	21	7.00	5.34	72
January	20	7.45	4.67	66
February	22	7.34	4.76	69

Table 24: Correlation between water quality parameters and mortality rate (%) of V. Koi at Fulbaria during outbreak period

Sampling period	Temperature (^{0}C)	pH	Dissolve Oxygen (ppm)	Mortality (%)
September	28	7.00	5.00	49
October	26	7.23	5.34	48
November	23	7.15	5.67	58
December	21	7.22	5.21	74
January	20	7.89	4.89	70
February	22	7.65	4.75	56

Table 25: Correlation between water quality parameters and mortality rate (%) of V. Koi at Bhaluka during outbreak period

Sampling period	Temperature (^{0}C)	pH	Dissolve Oxygen (ppm)	Mortality (%)
September	28	7.00	5.00	38
October	26	7.12	5.34	52
November	23	7.33	5.67	60
December	21	7.34	5.21	62
January	20	7.34	4.89	64
February	22	7.23	4.75	63

Table 26: Correlation between water quality parameters and mortality rate (%) of V. Koi at Phulpur during outbreak period

Sampling period	Temperature (^{0}C)	pH	Dissolve Oxygen (ppm)	Mortality (%)
September	28	7.67	5.65	42
October	26	7.87	5.45	50
November	23	7.34	5.23	62
December	21	7.12	5.34	68
January	20	7.65	4.67	53
February	22	7.34	4.76	54

Table 27: Correlation between water quality parameters and mortality rate (%) of V. Koi at Gaffargaon during outbreak period

Sampling period	Temperature (^{0}C)	pH	Dissolve Oxygen (ppm)	Mortality (%)
September	28	7.11	5.00	34
October	26	7.23	5.34	48
November	23	7.21	5.67	66
December	21	7.00	5.21	70
January	20	7.45	4.89	82
February	22	7.34	4.75	62

Table 28: Correlation between water quality parameters and mortality rate (%) of V. Koi at Trishal during outbreak period

Sampling period	Temperature (^{0}C)	pH	Dissolve Oxygen (ppm)	Mortality (%)
September	28	7.00	4.30	38
October	26	7.23	5.00	44
November	23	7.15	4.35	60
December	21	7.22	5.03	74
January	20	7.89	5.06	71
February	22	7.65	4.89	70

The climbing perch (*Anabas testudineus*), locally known as Koi in Bangladesh, is an important aquaculture fish item for its delicate taste and flavor. As this fish can withstand harsh environmental conditions such as low oxygen, wide range of temperature, therefore, important for aquaculture production (Kohinoor *et al*., 2009). This species is considered as a valuable item of food due to high amount of bio-nutritionally available iron and copper, which are essential component for hemoglobin synthesis in human body. In addition, the fish contains high amount of protein and easily digestible fat with many essential amino acids (Kohinoor *et al*., 2007).

Mymensingh district is famous for aquaculture production and many fish farms were developed during the last decades. However, mass mortality was observed recently due to outbreak of disease in many ponds which culture various fish species. Vietnamese Koi is one of the important culture species in Mymensingh district. Therefore it was necessary to isolate and identify the bacterial spp. during mass mortality of Vietnamese Koi (*Anabas testudineus*) of selected outbreak areas in Mymensingh district and to know the distribution pattern of bacterial pathogens in the naturally infected/dead V. Koi.

Mymensingh is a big district and recently declared as division. Therefore, in order to conduct this study seven areas in Mymensingh district namely- Mymensingh Sadar, Phulpur, Muktagacha, Trishal, Fulbaria, Bhaluka and Gaffargaon were selected. Vietnamese Koi culture was introduced in the selected areas long ago as this fish can easily adapt in the ponds with high yield (Sarker, 2011; DoFB, 2006). A number of fish hatcheries have been established in those areas to meet up the increasing demand of fish fry (Ahmed and Garnett, 2011).

Initially, clinical and gross changes of the collected fishes were examined. High hemorrhagic condition (45%) (Table 1) were observed particularly in Mymensingh sadar and Phulpur Upazila whereas lowest was found in Bhaluka (35%). In case of ulceration, Mymensingh sadar and Bhaluka (30%) were highly affected and minimum rate seen in Phulpur (21%). In contrast, erosion of the skin and other organs were found highest amount (28%) in Muktagacha. However, again the lowest (18%) was seen in Phulpur. Naturally infected V. Koi (*Anabas testudineus)* from seven sampling areas exhibited hemorrhage in base of fin, slight lesion on body, body and tail erosion, and edge of head and heavy mortalities of fish occur shortly after the advent of lesions at the time of clinical observation. On the other hand normal fishes were found healthy, bright and morphologically in good appearance.

Based on the clinical and gross changes it was confirmed that Mymensingh Sadar was the highly affected area at the time of the experiment. Previous study conducted by Ahmed and Shoreit (2001); Gamal *et a*l. (2002) found that liver and internal organs were affected in diseased fishes in this area. Moreover, the clinical and post mortem

findings of the diseased Koi fishes in the present study showed similar trend conducted in a previous study by Ahmed *et al*., (2009).

In the present study, congestion and enlargement with hemorrhage was observed in liver, kidney and spleen of the diseased Koi during post-mortem examination. Among different internal organs, liver was highly affected. In a previous study diseased fishes showed loss of equilibrium, hemorrhages, skin lesions, body and tail erosion, mucous secretion, and congestion (Khalil *et al.* 2010, Mastan 2013). All of the study showed that hemorrhages are the most common problem during the mass mortality of affected fishes.

The analytical profile index (API) is a classification of bacteria based on different tests which allow fast identification. Only known bacteria can be identified by this procedure. Twenty biochemical tests were performed using the commercially available API-20E microbiological kit as described the method section. The positive or negative reactions of all isolates were recorded and summarized (Table 12).

Bacterial load was found from diseased Koi in all areas (Table 13). The abundant bacteria were *A. hydrophila, A. caviae, Streptococcus* sp., *Staphylococcus* sp. and *E. coli.* Although five bacterial strains were isolated and identified as most common but *Aeromonas hydrophila* and *A. caviae* were emphasized for further study due to their high pathogenicity. The identification of bacteria was initially confirmed by their specific morphological, physiological and biochemical characteristics.

From the total viable count of isolated bacteria from infected V. Koi it was revealed that the highest number of the bacterial strains particularly *A. hydrophila* found in the kidney which was observed in Trishal upazila (3.83×10^7 cfu/g) and the lowest was observed in the gill at Mymensingh sadar (2.37×10^5cfu/g). Previous studies also observed similar phenomenon in various diseased fish species (Daskalov, 2006; Deng *et al*., 2009).

A. hydrophila was frequently observed diseased farmed and wild freshwater fishes in different locations in Bangladesh (Sarker *et al*., 2000). *Aeromonas* species have been reported to occur as commensals on fish skin where they cause opportunistic infections following immune-suppression. Anyanwu *et al.,* (2014) also reported that

the highest isolation (44%) of aerobic bacteria was obtained from fish skin lesion. This study also observed fish skin as an abundant source of *Aeromonas* sp.

After 14 days the cumulative mortality rate was recorded highest (80%) in group 2 and lowest (53%) in group 3 (Table 18).

The isolated *Aeromonas hydrophila* and *Aeromonas caviae* were tested against eleven commercially available antibiotics (Table 13). In this experiment all the isolated bacteria were sensitive to endrofloxacin, ciprofloxacine, levofloxacine, azithromycin and gentamicin. Ciprofloxacine and levofloxacine were highly effective against *A. hydrophila and A. caviae.* Ampicillin & penicillin did not show any effect against *Aeromonas* spp.

Pathogenicity test indicated that highest mortality rates (80%) were observed in Mymensingh sadar and the lowest mortality (53%) found in the fishes of Bhaluka caused by *Aeromonas hydrophila.*

In case of *Aeromonas caviae*, After 14 days of post inoculation (Table 21) of fish highest mortality rate were shown of the fish collected from Muktagacha (80%) and the lowest mortality (53%) found in the fishes of Mymensingh sadar, Fulbaria and Trishal.

To see the influence of water in disease formation thus influence on mortality, water quality parameters were monitored. Emphasis was given on temperature, dissolved oxygen, and pH as these parameters highly influenced to create fish diseases. Most of the parameters were found suitable for successful culture of Vietnamese Koi (Table 24). A study conducted by Muyen, Z. *et al.,* (2016) also confirmed that water quality parameters are not responsible for the mass mortality of the fishes of these areas.

Seasonal influence on mortality was also observed in the present study. And it was found that the highest mortality was observed in January whereas the lowest mortality was found in the September.

Infection caused by *Aeromonas* sp. is one of the most common bacterial diseases diagnosed in marine and cultured freshwater fishes (Vendrell *et al.,* 2006). *A. hydrophila* is found in diverse habitats, including soil, widely in fresh and salt water

also frequently found in chlorinated and non-chlorinated drinking water, and is pathogenic to warm and cold-blooded animals (Rahman *et al.,* 2005). The result of this study indicated that *A. hydrophila,* though opportunistic, was a severe pathogen for Koi. It can also be concluded from the result of this study that this pathogen easily invade to liver, kidney and intestine of Koi fish and caused infection.

The result of this study will be helpful for the fish farmers during the aquaculture of Koi for selecting effective antibiotics for disease prevention. Vaccination plays an important role in large-scale commercial fish farming. During the past 20 years fish vaccines have become an established, proven, and cost-effective method of controlling certain infectious diseases in aquaculture worldwide. Fish vaccination can be a good method to control and prevent fish diseases over antibiotic treatment. So, it is very important to develop vaccines against *Aeromonas* spp. since, bacteria is growing resistance against many commercial antibiotics.

CHAPTER 5

SUMMARY AND CONCLUSION

Vietnamesee Koi farming in Mymensingh district became very popular due to high profit. This Koi fish which scientific name is *Anabas testudineus* is one of the common exotic fish species in Bangladesh. This fish is nowadays considered to be the most economic and important fish species in Bangladesh because of its extreme market demand, having good nutritional value and luscious taste. Vietnamese Koi becomes very popular in Bangladesh due to the decline of native Koi. However, recently high mortality of Vietnamese Koi was reported due to pathogenic diseases. Generally, the presence of pathogenic bacteria in fish body indicates a poor quality of fish which is not easily acceptable by the consumers. Therefore, it was necessary to identify the pathogen which causing the disease. From this study, it was found that *Aeromonas hydrophila* and *Aeromonas caviae* were the two main bacterial strains which caused the pathogenicity in Climbing Perch, Koi (*A. testudineus*). In addition, bacterial sensitivity test indicated that antibiotics are capable to hinder this bacterial growth. Thus, Vietnamese Koi can still be a good candidate for aquaculture as this disease is preventable if proper antibiotics can be chosen. The culture of Vietnamese Koi individually or with other small indigenous species (SIS) can make a big role in poverty alleviation.

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APPENDICES

APENDIX-1

Composition of media:

1. *Aeromonas* isolation media:

Ingredients	**g/l**
Sodium thiosulphate	10.67
Sodium chloride	5.0
Ferric ammonium citrate	0.80
Bromothymol blue	0.04
Agar	12.5

2. Nutrient broth

Ingredients	**g/l**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25^0C)	7.4 ± 0.2

3. Nutrient agar

Ingredients	**g/l**
Beef extract	3.0
Peptone	5.0
Sodium chloride	5.0
Agar	20.0
Distilled water	1000 ml
Final pH	7.1±0.1

4. Simmons citrate

Ingredient	g/l
Magnesium sulphate	0.20
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromo thymol blue	0.08
Agar	15.0

5. *Pseudomonas* agar base

Ingredient	g/l
Pancreatic digest	16.0
Casein enzymic hydrolysate	10.0
Potassium sulphate	10.0
Magnesium chloride	1.40
Agar	11.0

6. OF basal medium

Ingredient	g/l
Casein enzymic hydrolysate	2.0
Sodium chloride	5.0
Dipotassium phosphate	0.30
Bromo thymol blue	0.08
Agar	2.0

7. TSI agar medium

Ingredients	g/l
Dextrose	1.0
Sodium chloride	5.0
Ferrous sulphate	0.20
Sodium thiosulphate	0.30
Phenol red	0.024
Agar	12

APENDIX-2

Preparation of reagents

1. Gram's stain solution

Ingredients	g/l
Stock crystal violet	10
Ethyl alcohol	1000
Ammonium oxalate	1
Distilled water	1000

2. Phosphate buffered saline solution

Ingredients	g/l
Sodium chloride	8.0
Disodium hydrogen phosphate	2.8
Potassium chloride	0.2
Potassium dihydrogen phosphate	0.2
Distilled water	1000

3. Peptone water

Ingredients	g/l
Peptone	1.0
Distilled water	1000

4. Kovac's reagent for indole preparation

Ingredients	g/l
p-dimethyl aminobenzaldehyde	5.0 g
Isoamyl alcohol	75 ml
Conc. HCl	25 ml

5. V-P reagent-1

5% alpha-naphthol in absolute ethyl alcohol.

V-P reagent-2

40% potassium hydroxide containing 0.3% creatine. The ingredient were dissolved by heating gently over a steam bath.

6. Methyl red solution

Ingredients	g/l
Methyl red	0.05
Ethanol (absolute)	28
Distilled water	22

7. Safranin solution

Ingredients	g/l
Safranin dye	2.5g
Ethyl alcohol (95%)	100ml
Distilled water	300ml