Asian J. Med. Biol. Res. 2016, 2 (2), 332-340; doi: 10.3329/ajmbr.v2i2.29078

Asian Journal of Medical and Biological Research
ISSN 2411-4472 (Print) 2412-5571 (Online)
www.ebupress.com/journal/ajmbr

Article

Isolation, molecular identification and antibiotic susceptibility profile of *Aeromonas hydrophila* from cultured indigenous Koi (*Anabus testudineus*) of Bangladesh

Shuvho Chakra Borty[1,3,4,5*], Fabaya Rahman[2], A.K.M. Ali Reza[3,4,5], Mst. Sharifa Khatun[3,4,5], Md. Luthful Kabir[1], Muhammed Hafizur Rahman[3,4,5] and Md. Shirajum Monir[6]

[1]Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh, Bangladesh
[2]Department of Pathology, Gonoshasthaya Samaj Vittik Medical College, Gono Bishwabidyalay, Savar, Dhaka, Bangladesh
[3]Department of Quality Assurance, Igloo Ice-Cream and Milk Unit, Kadamtali, Shampur, Dhaka-1204, Bangladesh
[4]Department of Quality Assurance, Frozen Food Unit-Igloo Foods Ltd., Kadamtali, Shampur, Dhaka-1204, Bangladesh
[5]Department of Quality Control, Igloo Dairy Ltd., Kadamtali, Shampur, Dhaka-1204, Bangladesh
[6]Freshwater Station, Bangladesh Fisheries Research Institute, Mymensingh-2201, Bangladesh

*Corresponding author: Shuvho Chakra Borty, Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh and Department of Quality Assurance, Frozen Food Unit-Igloo Foods Ltd., Kadamtali, Shampur, Dhaka-1204, Bangladesh. Mobile: +8801710500560; E-mail: shuvho.micro@gmail.com

Received: 07 June 2016/Accepted: 23 June 2016/ Published: 30 June 2016

Abstract: Fish play a crucial role in the Bangladeshi diet, providing more than 60% of animal source food, representing a crucial source of micro-nutrients and possessing an extremely strong cultural attachment. In this study isolation and identification of *Aeromionas hydrophila* was done by studying cultural properties, Gram's staining and biochemical properties of isolates of diseased indigenous Koi fish (*Anabas testudineus*) of different upazillas of Mymensingh district. Antibiogram profile of the isolated bacteria was studied by using wide range of commercially available antibiotics. Quantitative study of bacteria isolated from diseased indigenous Koi fish showed variation of number in different organ. Total bacterial load was found to be 1.90 × 105, 1.19 × 105, 3.21 × 105, 2.18 × 106 and 3.14 × 105 cfu/g in lesions; 2.52 × 107, 2.34 × 108, 5.41 × 108, 2.54 × 109 and 5.21 × 109 cfu/g in liver; 2.54 × 108, 2.41 × 108, 1.90 × 107, 3.65 × 107 and 3.45 × 108 cfu/g in spleen; 3.51 × 107, 5.28 × 107, 3.14 × 106, 1.85 × 107 and 4.52× 107 cfu/g in kidney in diseased Koi of Mymensingh sadar, Muktagacha, Tarakanda, Gouripur and Fulpur upazillas, respectively under Mymensingh districts. *Aeromonas hydrophila* was initially identified by their specific morphological, physiological and biochemical characteristics. Then molecular detection of *A. hydrophila* was done by PCR. PCR products of desired 760 bp were obtained for *A. hydrophila*. 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%).

Keywords: aquaculture; climbing perch; bacterial infection; Koi; bacterial load; *Aeromonas*

1. Introduction

The Climbing Perch fish *Anabas testudineus* (Bloch, 1792) is one of the important small indigenous species (SIS), fresh water fish of Bangladesh, which is locally known as Koi in different places of Bangladesh. SIS is generally considered to be those fishes which grow to a maximum length of about 25 cm (Felts *et al.,* 1996 and

Hossain *et al.,* 1991). This fish is native in Southeast Asian region, often found in fresh water sources of east India and south China (Chakraborty *et al.,* 2014). It is commonly found in open water (streams, lakes, floodplain and beels), paddy fields and swamps of Bangladesh and its preferred habitats are heavily-vegetated, stagnant waters. Total length is recorded 176 mm (Rahman *et al.,* 1989). The fish is very popular for its delicious taste and flavour. This species considered as a valuable item of diet for sick and convalescent (Kohonoor *et al.,* 2012). According to Saha *et al.,* 1971, the fish contain high values of physiologically available iron and copper essentially needed for hemoglobin synthesis. This fish was abundantly available in our open water system but due to over exploitation and various ecological changes in its natural habitat; this native species is declining. Indiscriminate destructive practices have caused havoc to aquatic biodiversity (Hussain *et al.,* 2001). International Union of Conservation of Nature (IUCN) enlisted A. testudineus as not threatened perch fish in Bangladesh. But due to rough and unplanned water management policy for irrigation, over exploitation, illegal practice of capture fisheries and various ecological changes in its natural habitat; this native species is threatened now (Chakraborty *et al.,* 2010). A decade ago, SIS was cultured in the pond as an additional crop while various large carp species were cultured as cash crop. Nevertheless, production systems are continuously changing (Rahman *et al.,* 2006). Nowadays, fish farmers culture SIS as a main cash crop (Jannat *et al.,* 2012)

In Bangladesh, a wide variety of SIS is available, among these Climbing Perch Koi *Anabas testudineus* (Bloch 1792), Taki *Channa punctata* (Bloch 1793), Veda *Nandus nandus* (Hamilton 1822), Pabda *Ompok pabda* (Hamilton 1822), Tengra *Mystus vittatus* (Bloch 1794), Mola *Amblypharyngodon mola* (Hamilton 1822), Puti *Puntius sophore*, Shing *Heteropneustes fossilis* (Bloch 1794), Magur *Clarias batrachus* (Linnaeus 1758), Chapila *Gudusia chapra* (Hamilton 1822), Chela *Salmophasia bacaila* (Hamilton 1822), Chanda *Chanda nama* (Hamilton 1822) are regarded as major SIS crop (Jannat *et al.,* 2012). Nowadays, among SIS climbing perch is the most popular aquaculture species and its aquaculture production is increasing very rapidly (Belton *et al*., 2011). Considering the importance of this species in nutritional, economical and biodiversity point of view, this species (*A. testudineus*) is being cultured in large scale across the country (Mondal *et al.,* 2010).

The current trend in aquaculture development is towards increased intensification and commercialization of aquatic production. Like other farming sectors, the likelihood of major disease problems increases as aquaculture activities intensify and expand (Hasan *et al.,* 2013). Disease is considered as a primary constraint to the culture of many aquatic species, impeding both economic and social development in many countries (Subasinghe *et al.,* 2001). A number of diseases like epizootic ulcerative syndrome, skin erosion, gill damage, tail and fin rot are common in farmed Climbing Perch of Bangladesh (Faruk *et al.,* 2004). In pond aquaculture system, high stocking density and irregularly feed supply is very prone to disease outbreak. Most pond fish farmers of Climbing Perch do not have a good understanding of health and disease issues in their system (Hasan *et al.,* 2013). Many diseases of this hardy fish are secondary to environmental insult, and can be prevented through proper management by manipulating the ecosystem and the administration of selective antibiotics. However, there is hardly such scientific information available from which rural pond aqua-farmers could be benefited.

The objectives of the present study were therefore isolation, identification and molecular detection of actual disease causing agent which is responsible for mass mortality of cultured indigenous Koi (*A. testudineus*) of Mymensingh district of Bangladesh.

2. Materials and Methods

2.1. Selection of fish farms and study area

Different Climbing Perch, Koi (*A. testudineus*) farms of Muktagacha, Tarakanda, Gouripur, Fulpur, Sadar upazillas under Mymensingh district located at 24°38′3″N 90°16′4″E of Bangladesh were selected to collect infected Koi fish samples for isolation, identification and molecular detection of actual pethogenic agent and evaluate their antimicrobial resistance patterns. The study was conducted from April, 2014 to October, 2015 to collect the samples in various seasones round the year.

2.2. Fish sample collection

Samples were collected from a total of 20 afflicted Koi farms depending on the availability of diseased fish from the study area in which Koi (*A. testudineus*) were suffering from tail and fin rot, reddish hemorrhagic external lesions and some asymptomatic causes. Moribund fishes were collected in clean sterile boxes containing ice packs and then transported to Fish Disease and Health Management Laboratory of Bangladesh Fisheries Research Institute, Mymensingh. The clinical signs and postmortem findings were recorded according to Rashid *et al.,* 2008 and Ahmed *et al*., 2009.

2.3. Bacteriological examination
Specimens from diseased Koi's skin, gill, liver and kidney were inoculated on Trypticase Soya Agar (TSA) plates and then incubated at 30°C for 24 hrs. The isolated bacteria were identified according to their biochemical characteristics (Sabur *et al.,* 2006, Narejo *et al.,* 2005).

2.4. Clinical observation
Collected fish were examined to observe their external lesion, injury or any other abnormalities and were recorded properly.

2.5.Total viable count of bacteria
At first each Koi fish was examined for its clinical sign of disease and disorders. A drop of blood was dissolved on TSA plate for colony counting. The fish was dissected immediately after clinical examination. The portions of skin, gill, kidney, liver and intestine were removed, weighed on an electric balance and kept in a sterilized pastel mortar for crushing. Each organ was crushed with physiological saline in the ratio of 0.1 g of organ: 0.9 ml of PBS to make stock solution. Eight decimal dilutions were prepared by transferring 0.1 ml from the earlier test tube to the next. These eight tubes were designated as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. Two samples of 0.1 ml from 10^{-2} and 10^{-3} in case of liver and kidney; 10^{-3} and 10^{-4} in case of intestine were transferred to Aeromonas isolation medium (AIM) to get only the colonies of *Aeromonas* spp.

Total bacterial load of each organ was calculated using the following formula used by Rashid *et al*. (2008).

$$\text{Total bacterial load} = \frac{\text{Avarage number of colonies on plates}}{\text{Dillution factor} \times \text{Volume plated}}$$

2.6. Identification of *Aeromonas* spp.
Identification of *Aeromonas* spp. was done based on detailed morphological, physiological and biochemical characterization of the isolates. At first, the bacteria were sub-cultured onto TSA plates to obtain fresh 24 hours culture. They were then streaked onto the selective Aeromonas isolation medium (AIM) for preliminary identification of the genus *Aeromonas* and discarding the others. Colonies grown on the selective medium were sub-cultured again onto TSA plates and subjected to biochemical tests using commercially available media after autoclaving at 121°C for 15 minutes.

2.7. Motility test
For motility test young and actively growing culture of the bacteria were collected from 24 h culture at 30°C. A single colony was mixed with 3 ml of PBS. 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 LCD monitor screan, adjusted with the microscope (OLYMPUS, Model CHS, Japan).

2.8. Physiological characterization
Physiological characters were studied by observing the growth of each isolate at temperature of 4°C, 37°C and 40°C. Growth of each isolate was observed in different concentrations of NaCl as 0%, 1%, 2%, 3%, 3.5% and 4%.

2.9. Biochemical characterization
Several biochemical tests were performed to evaluate the biochemical behavior of isolated bacteria. Biochemical tests are oxidase, catalase, oxidative-fermentative test, 0129 test, esculin hydrolysis test, acid and gas production from sugars: glucose, lactose, sucrose, manitol; methyl-red (MR) test, Voges-Proskauer (VP) test, indole and H2S production, decarboxylase test, citrate utilization test.

2.10. Molecular dectection of *Aeromonas hydrophila*
The genomic DNA was isolated as per the protocol described by Swaminathan *et al*. (2004). A single colony was inoculated in 10 ml of Nutrient broth (NB) and grown at 29°C overnight. Culture was centrifuged at 5000 rpm for 10 minutes. Four hundred microlitre of solution I (50mM Tris.HCl pH-8.0, 50mM EDTA pH-8.0, 25% sucrose, 1mg lysozyme), was added to the washed cell pellet and gently mixed and incubated at 37°C for 15 minutes. Thereafter 400ml of solution II (10mM Tris. HCl pH 8.0, 5mM EDTA pH-8.0, 1% SDS, 40µg Proteinase K) was added to the cells and incubated at 55 ^{o}C for three hours. The suspension was centrifuged at

6000 rpm for 10 minutes. The aqueous layer from the top was removed carefully to avoid any protein debris and transferred to a fresh microfuge tube. Double amount of chilled ethanol was added to aqueous phase so as to precipitate the DNA. The DNA was pellted by centrifugation at 12000 rpm for 10 minutes. The pellet, washed with 70% ethanol was dried and dissolved in 100 µl of TE buffer (pH 7.6). Primer used for the amplification of DNA as shown in Table 1. PCR was done as per the method described previously by Narjeo *et al.* (2005). Amplification was performed with a DNA thermal cycler (Mastercyclear, Eppendorf, Humburg, Germany) with some modifications as follows: The reaction mixture consisted of 1µl of Taq polymerase (1unit), 5 µl of 10X PCR amplification buffer (100 mM Tris-Hcl, 25 mM MgCl2, 500mM KCl, pH-8.3), 3 µl of deoxynucleoside triphosphate (100µM), 0.5 µl of each primer (100 pmoles) and double distilled water upto a final volume of 50 µl. A total of 40 PCR cycles were run under the following conditions: Initial denaturation at 94^0C for 4 minutes, denaturation at 94^0C for 1 minute, primer annealing at 65^0C for 1 minute, DNA extension at 72^0C for 1.5 minutes and final extension at 72^0C for 5 minutes.

Table 1. List of primers used for *Aeromonas hydrophila* genome detection.

Primers	Sequences (5′ – 3′)	Amplicon size (bp)	Reference
Forward Primer	5′-AACCTGGTTCCGCTCAAGCCGTTG- 3′	760	**Nerjeo *et al.*, 2005**
Reverse Primer	5′-TTGCCTCGCCTCGGCCCAGCAGCT- 3′		

2.11. Antibiogram profile of *Aeromonas hydrophila*

All bacterial isolates were tested for their sensitivity to ten commercially available antibiotics by the disc diffusion method. 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), chlortetracycline (25 µg), novobiocin (5 µg). Tested bacterial strains were classified into three categories: sensitive, intermediate, and resistant and depending on the diameters of inhibition zones and standards supplied by Himedia Laboratories and comparing with other related references (Table 2). All tests were carried out in Fish Diseases and Health Management Laboratory of Bangladesh Fisheries Research Institute (BFRI), Mymensingh.

Table 2. Interpretation standards for disc diffusion susceptibility testing for *Aeromonas hydrophila* (CLSI 2012).

Sl. No.	Name of Antimicrobial agent	Disc concentration	Interpretation of results (zone in diameter in mm)		
			R	I	S
1	Ciprofloxacin	5 µg	≥ 16	12 – 15	≤ 17
2	Levofloxacin	5 µg	≥ 22	17 – 21	≤ 23
3	Gentamicin	10 µg	≥ 14	10 – 13	≤ 15
4	Azithromycin	15 µg	≥ 16	11 – 15	≤ 17
5	Tetracycline	30 µg	≥ 14	09 – 13	≤ 15
6	Oxytetracycline	10 µg	≥ 15	11 – 14	≤ 16
7	Chlortetracycline	25 µg	≥ 16	13 – 15	≤ 17
8	Novobiocin	5 µg	≥ 17	14 – 17	≤ 18
9	Ampicillin	10 µg	≥ 22	16 – 21	≤ 23
10	Penicillin	10 µg	≥ 14	10 – 13	≤ 15

Sl = Serial, No. = Number, µg = Microgram, mm = Millimeter, S = Susceptible, I = intermediately resistant, R = Resistant, ≥ = Greater than or equal to, ≤ = Less than or equal to.

3. Results

3.1. Clinical and post mortem findings

The clinical examination of diseased Koi (*A. testudineus*) exhibited: loss of equilibrium, slight lesion on body, body and tail erosion, hemorrhage in base of fin and edge of head, move with whirling and heavy mortalities of fish occur shortly after the advent of lesions. Congestion and enlargement in internal organs were appeared in postmortem examination.

3.2. Bacterial load in skin lesions, liver, spleen and kidney

Bacterial load in skin lesions, liver, spleen and kidney of infected Koi (*A. testudineus*) are calculated and then observed to have a variation. Total bacterial load was found to be 1.90×10^5, 1.19×10^5, 3.21×10^5, 2.18×10^6 and 3.14×10^5 cfu/g in lesions; 2.52×10^7, 2.34×10^8, 5.41×10^8, 2.54×10^9 and 5.21×10^9 cfu/g in liver; 2.54×10^8, 2.41×10^8, 1.90×10^7, 3.65×10^7 and 3.45×10^8 cfu/g in spleen; 3.51×10^7, 5.28×10^7, 3.14×10^6, 1.85×10^7 and 4.52×10^7 cfu/g in kidney in diseased Koi of Mymensingh sadar, Muktagacha, Tarakanda, Gouripur and Fulpur upazillas, respectively under Mymensingh district.

3.3. Morphological, physiological and biochemical test results

The isolated *Aeromonas hydrophila* from diseased Koi was finally identified by their specific morphological, physiological and biochemical characteristics. They were Gram negative, rod shaped, motile bacteria, positive for oxidase and catalase test. They fermented glucose and were resistant to vibriostatic agent 0129 test. The results of morphological, physiological and biochemical tests are presented in bellow Table 3,

Table 3. Results of biochemical characteristic of isolated bacteria.

Characters	Characterization by Mostafa *et al.* (2008)	Characterization by Sabur (2006)	Present result
Gram's stain	-	-	-
Shape	Rod	Rod	Rod
Motility	+	+	+
0129	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	+	+	+
H2S production	+	+	-
Arginine decomposition	+	+	+
Lysine decarboxilation	-	-	-
Ornithine decarboxilation	-	-	-
Citrate utilization	+	+	+
TSI	ND	ND	'K' in slants but 'A' in butt
Growth at: 4°C	-	-	-
5°C	+	+	+
37°C	+	+	+
40°C	-	-	-

+: Negative; -: Positive; F: Fermentative; K: Alkaline; A: Acid, ND: Not done

3.4. Molecular detection of *Aeromonas hydrophila* by PCR

PCR products of desired size 760 bp were obtained in reaction mixture containing genomic DNA of the targeted organisms, *A. hydrophila* (Figure 1). No product was detected in control (Figure 1).

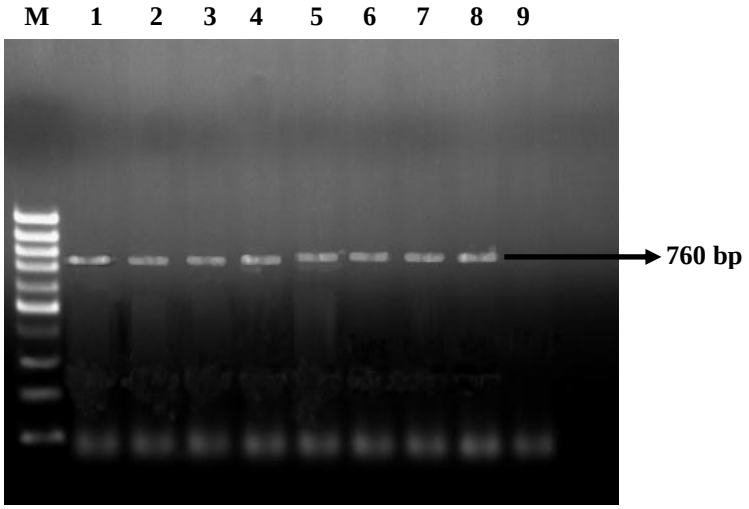

Figure 1. Agarose gel electrophoresis of PCR amplification generated by *Aeromonas hydrophila*. Lanes: (M) 100 bp DNA marker; (1-8) positive samples from field; (9) negative control.

3.5. Antibiotic sensitivity test

The isolated *Aeromonas hydrophila* were tested against ten commercially available antibiotics and the results of their sensitivity are presented in Table 4. Most of the bacterial samples were sensitive against ciprofloxacin (92%) and levofloxacin (84%), intermediate against gentamicin (40%) and resistant against novobiocin (84%), ampicillin (100%) and penicillin (92).

Table 4. Antibiogram profile of isolated *Aeromonas hydrophila* (n=25).

Antibiotics	No (%)		
	Sensitive	Intermediate	Resistant
Ciprofloxacin (5µg)	23 (92)	2 (8)	0 (0)
Levofloxacin (5µg)	21 (84)	4 (16)	0 (0)
Gentamicin (10µg)	15 (60)	10 (40)	0 (0)
Azithromycin (15µg)	12 (48)	8 (32)	3 (12)
Tetracycline (30µg)	4 (16)	12 (48)	9 (36)
Oxytetracycline (10µg)	1 (4)	10 (40)	14 (56)
Chlortetracycline (25µg)	3 (12)	13 (52)	9 (36)
Novobiocin (5µg)	0	4 (16)	21 (84)
Ampicillin (10µg)	0	0	25 (100)
Penicillin	0	2 (8)	23 (92)

4. Discussion

The clinical and post mortem findings of the diseased Koi fishes in this study is quite in consonance with those that reported by Ahmed *et al.*, 2009 and Chandra *et al.*, 1994. The *Aeromonas hydrophila* bacteria was isolated from diseased fish from different locations such as Mymensingh sadar, Muktagacha, Tarakanda, Gouripur and Fulpur upazillas. Total bacterial load was found to be 1.90×10^5, 1.19×10^5, 3.21×10^5, 2.18×10^6 and 3.14×10^5 cfu/g in lesions; 2.52×10^7, 2.34×10^8, 5.41×10^8, 2.54×10^9 and 5.21×10^9 cfu/g in liver; 2.54×10^8, 2.41×10^8, 1.90×10^7, 3.65×10^7 and 3.45×10^8 cfu/g in spleen; 3.51×10^7, 5.28×10^7, 3.14×10^6, 1.85×10^7 and 4.52×10^7 cfu/g in kidney of diseased shing fish of different upazillas of Mymensingh district consecutively. Rashid *et al.* 2008 and Hasan *et al.*, 2007 found 1.67×10^4 to 6.4×10^8 CFU/g, 1.71×10^3 to 1.18×10^9 CFU/g and 1.47×10^4 to 3.70×10^8 CFU/g of bacteria in liver, kidney and intestine of naturally infected Thai pangas respectively, those findings is partially similar with our study. Allison (2007) isolated *A. hydrophila* from Thai pangus, the bacterial load was found 2.6×10^6 to 3.6×10^7 CFU/g in liver, 4.8×10^6 to 7.2×10^7 CFU/g in intestine and 2.4×10^3 to 3.70×10^6 CFU/g in kidney, this is also in consonance with our study. Rahman and Chowdhury (1996) isolated *A. hydrophila* from kidney of carp fishes, total load of bacteria varied in the kidney of different sampled fishes were 2.6×10^5 to 1.7×10^6 CFU/g. Here the variations might be caused by different factors like temperature, p^H, chemical and gaseous composition etc. that influences the disease incidence. Ahmed (2009) was found total bacterial load to be 2.45×10^3 (koi) in blood and 8.70×10^6 (koi) CFU/g in

intestine these findings are almost similar to our study. The morphological and physiological characteristics of *A. hydrophilla* observed in this study was partially in consonance with those that found by Mostafa *et al.,* 2008 and Islam *et al.,* 2008. Hussain *et al.* 2014 also found focal necrosis haemorrrhages in the liver tissue, atrophy of the renal tubule in kidney and villi missing in intestine from the naturally infected shing fish by *Aeromonas hydrophila* in a mixed infection with the *Aphanomyces invadans* elicited EUS disease.

The biochemical characteristics of the isolated *A. hydrophilla* in this study are quite in consonance with those that reported by Mostafa *et al.,* 2008 and Sabur *et al.,* 2006. For the molecular detection of the bacterial causative agent of Koi fish diseases PCR was done by using gene specific primer according to Hasan *et al.,* 2007. The antibiogram profile of different antibiotics against *Aeromonas hydrophila* was found similar to those that previously reported by Hussain *et al.,* 2014, Sobur *et al.,* 2006, and Mostafa *et al.,* 2008. The result of this study will be beneficial for the fish farmers who are regularly culturing Climbing Perch, Koi for diagnosing and controlling diseases by the administration of specific antibiotics. Future research scopes are the pathogencity test of the bacteria for homologous susceptible fishes, identification of pathogenicity island in chromosome, production of antibiotics against *Aeromonas hydrophila*, serotyping of all *A. hydrophila* isolates.

5. Conclusions

The present study was conducted to identify the *Aeromonas hydrophila* from Climbing Perch, Koi (*A. testudineus*). In addition, clinical and bacteriological studies were carried out to examine disease status of cultured Shing fish of Mymensingh district. According to the farmer's opinion, the disease occurring seasons were early and late winter and frequency of disease occurrence was 1 to 2 times in a year. Koi was found to have high mortality rate. About 80-90% mortality occurs due to diseases. Massive pathological changes were found in different organs of diseased sample. Application of lime and salt in pond were the most common treatment followed by the use of antibiotics, potassium permanganate and copper sulphate. This study also identified fish health management problems which included poor understanding on fish disease and health management, lack of suitable therapeutics and their appropriate uses and lack of assistance regarding disease treatment. Therefore, more precautionary measures need to be taken at the onset of winter season to prevent diseases. So, attention should be drawn to maintain appropriate ambient for rearing Koi fish to avoid common diseases.

Acknowledgements

The authors are expressing cordial thanks and gratitude to all the stuffs of the Department of Microbiology and Hygiene of Bangladesh Agricultural University and Bangladesh Fisheries Research Institute, Mymensingh, Bangladesh to fulfill the experimental work successfully and also for providing continuous support and sincere cooperation.

Conflict of interest

None to declear.

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