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Article

Isolation, identification and molecular detection of *Aeromonas hydrophila* from diseased stinging catfish shing (*Heteropneustes fossilis*)

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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 staining and biochemical properties of isolates of Shing fish (*Heteropneustes fossilis*) 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 Shing fish showed variation of number in different organ. 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 Shing 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; catfish; bacterial infection; bacterial load; *Aeromonas hydrophila*; PCR

1. Introduction

Aquaculture production in Bangladesh has got a considerable momentum in terms of production, nutritional supplement and livelihood improvement of a wide range of people. It has been progressively increased and its contribution to total fish production has gone up from 38.2% in 1998-99 to 52.9% in 2011-2012 (Hussain *et al.*, 2014). Currently, Bangladesh ranked 5th position in leading aquaculture producing countries in the world just after China, India, Vietnam and Indonesia (FAO, 2013). Sing (*Heteropneustes fossilis*) is considered as one of the high value species and can benefit fish farmers because of their high productivity and profitability. This fish is locally known as Shingi or shing. It is considered to be highly nourishing, palatable and tasty and well preferred because of its less spine, less fat and high digestibility in many parts of Indian subcontinent (Khan *et al.*, 2003). The species is very high content of iron (226 mg 100 g^{-1}) and fairly high content of calcium compared to many other freshwater fishes. Due to high nutritive value the fish is recommended in the diet of sick and convalescents. Being a lean fish it is very suitable for people for whom animal fats are undesirable (Rahman *et al.*, 1982). But now a day's indiscriminate destructive practices have caused havoc to aquatic

biodiversity in Bangladesh (Hussain and Mazid, 2001). Presently, *H. fossilis* is one of the threatened fish in Bangladesh (IUCN Bangladesh, 2000). Prevalence of disease is considered as one of the major reasons for poor fish production of the country. It is a great threat to achieve optimum production and become a limiting factor to economic success of aquaculture in Bangladesh. A total lack of mucus, edema, enlarged abdomen, growth of nodules on the body surface, presence of yellow, white or black spots on the skin, prolapsed anus and exophthalmia are all clinical signs of fish diseases (Plumb, 1994).

Only few studies have been conducted on the health and diseases of *H. fossilis* Bangladesh. Hasan (2007) reported that an established shing fish farm was found to be heavily affected by ulcer type disease in Mymensingh area and by investigation, *Aeromonas hydrophila* were isolated from the lesion. *H. fossilis* has been reported to be affected by some bacterial diseases (Sahoo and Mukherjee, 1997). Sahoo *et al*. (1998) found an outbreak of ulcer diseases in an organized farm having 10% of *H. fossilis* population with round ulcers on body surface. During the outbreaks of EUS in late 80s, *H. fossilis* was found to be one of the most severely affected species along with other air-breathing fishes (Barua *et al.,* 1994; Roberts *et al.,* 1994). It is still very vulnerable to EUS and other diseases. If this situation continues, these endemic fishes will be endangered in near future. There is also a sever lack of information regarding disease outbreak of *H. fossilis* under culture condition. Investigation on the diseases in *H. fossilis* is thus essential for developing its sustainable culture technology. With the countrywide rapid expansion of farming of *H. fossilis*, there has been reports of serious disease outbreaks and mortality of this fish under culture condition (Dehadrai *et al.,* 1998). As a result farmers are suffering from huge financial loss every year. They are getting frustrated and loosing in culturing in this fish. Farmers in Mymensingh area are in need for proper health management suggestion and appropriate treatment. Control of aquatic disease is complex and relies heavily on a combination of pathogen detection, disease diagnosis, treatment, prevention and general health management. Sustainable aquaculture production can only occur when fish are healthy and free from disease. Therefore, there is an urgent need to give immediate attention to investigate diseases of *H. fossilis* and develop appropriate health management strategies. The objectives of this study were to isolate and identify the causative agent of disease from Shing fish and evaluate their cultural sensitivity.

Therefore, the present study was designed with a view to achieve the following objectives:

a) Isolation and identification of *Aeromonas hydrophila* from diseased stinging catfish Shing *(H. fossilis)*
b) To determine the antimicrobial resistance patterns of the isolated bacteria.

2. Materials and Methods

2.1. Selection of fish farms and study area

Different Shing (*H. fossilis*) 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 Shing fish samples for isolation and identification of *Aeromonas hydrophila* to evaluate their antimicrobial resistance patterns. The study was conducted one year starting from July, 2013 to June, 2014.

2.2. Fish sample collection

Samples were collected from a total of 20 afflicted Shing farms depending on availability of diseased fish from the study area in which Shing (*H. fossilis*) were suffering from either suspected EUS lesion or suspected MAS disease symptoms exhibited by reddish hemorrhagic external lesions and were having mortality. 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 alterations were recorded according to Rashid (2008) and Ahmed *et al*., 2009.

2.3. Bacteriological examination

Specimens from diseased Shing'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

Two fish from each district were subjected to bacteriological investigation as follows. At first each 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 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 PS 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 AIM medium to get only the colonies of *Aeromonas.* Another two samples of same dilution were transferred to TSA for total bacterial colony counting.

2.6. Calculation of total bacterial load

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

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

2.7. 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 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.

2.8. Motility test

For motility test young and actively growing culture of the bacteria were collected from 24 h culture at 25°C. 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 screan, adjusted with the microscope (OLYMPUS, Model CHS, Japan).

2.9. 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%.

2.10. 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.

2.11. 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). Primers used for the amplification of DNA are 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 Reverse Primer	5′-AACCTGGTTCCGCTCAAGCCGTTG- 3′ 5′-TTGCCTCGCCTCGGCCCAGCAGCT- 3′	760	Nerjeo *et al.*, 2005

2.12. 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), chlorotetracycline (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	Chlorotetracycline	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 Shing (*H. fossilis*) 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 Shing (*H. fossilis*) 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 Shing of Mymensingh sadar, Muktagacha, Tarakanda, Gouripur and Fulpur upazillas, respectively under Mymensingh districts.

3.3. Morphological, physiological and biochemical test results

The isolated *Aeromonas hydrophila* from diseased Shing 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 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 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).

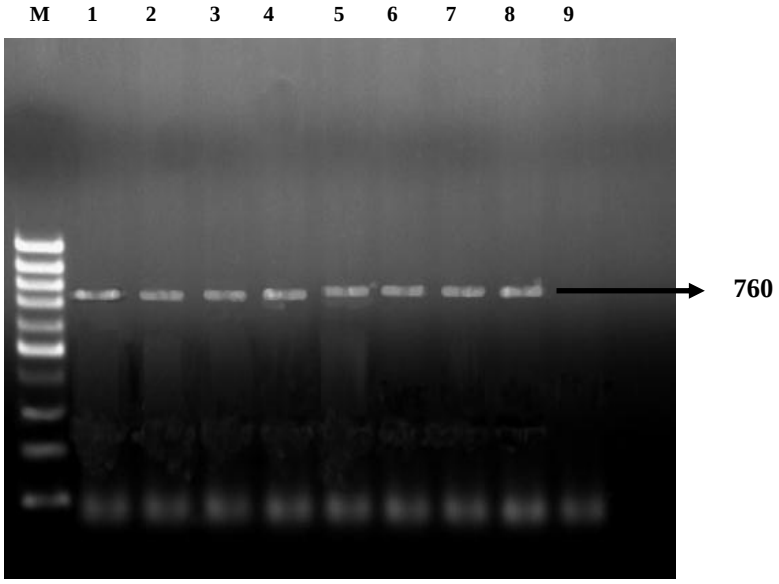

Figure 1. Agarose gel electrophoresis of PCR amplification generated by *Aeromonas hydrophila*. Lanes: (M) 100 bp DNA marker; (1-8) positive samples from field; (9) negative control.

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)

The isolated *Aeromonas hydrophila* were tested against ten commercially available antibiotics and the results of their sensitivity are presented in Figure 26 and Table 5. The results of the antibiotic sensitivity testing is exhibiting that most of the bacterial samples are sensitive against ciprofloxacin (92%) and levofloxacin (84%), intermediate against gentamicin (40%) and resistance against novobiocin (84%), ampicillin (100%) and penicillin.

4. Discussion

The clinical and post mortem findings of the diseased Shing 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

$\times 10^9$ 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 Shing 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 catfish 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 of catfish (*H. fossilis*). 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. Shing 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 Shing fish to avoid common diseases.

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Conflict of interest

None to declear.

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