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Article

Identification of pathogenic bacteria isolated from diseased stinging catfish, Shing (*Heteropneustes fossilis*) cultured in greater Mymensingh, Bangladesh

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Abstract: Stinging catfish, Shing (*Heteropneustes fossilis*) is been cultured in Bangladesh which is one of the most popular and highly valuable fish species. A total of 120 diseased *H. fossilis* were collected from twenty five fish farms in greater Mymensingh region, Bangladesh between November 2013 to February 2014. Remarkable clinical symptoms of the affected fishes were loss of equilibrium and, erosions at the bases of fins and tail, hemorrhages and skin lesions on body surface, profuse mucous secretion and congestion along with enlargement of most of the internal organs. Three different kinds of selective media were used for the isolation of total population of bacteria from the affected fishes. A number of biochemical tests were performed for the identification of the isolated bacteria. Commercially available panel of antibiotics were used for the determination of sensitivity of the isolated bacteria. A total of 85 bacterial strains were isolated and the isolated were *Aeromonas hydrophila, A. salmonicida, A. sobria, Pseudomonas angulliseptica, P. fluorescens* and *Vibrio anguillarum. Aeromonas* spp. appeared to be the main pathogen in the diseased fishes. Among the isolated spp of bacteria distribution of *Aeromonas* species was as follows: *A. hydrophila* 34 (40.00%), *A. salmonicida* 14 (16.47%), *A. sobria* 8 (9.41%) as well as 5 (5.88%) unidentified *Aeromons* strains. The other isolated of *Pseudomonas anguliseptica, P. fluorescens, Vibrio anguillarum* and *Vibrio* spp were distributed as 12 (14.11%), 4 (4.70%), 3 (3.52%) and 5 (5.88%), respectively in infected *H. fossilis*. However, results of antibiotic sensitivity test revealed that all the bacterial isolates of *H. fossilis* were found more or less resistant to all most all the antibiotics with the exception of ciprofloxacin and levofloxacinn which were found highly sensitivity against all the strains of *Aeromonas*s pp. and other species of bacteria.

Keywords: stinging catfish; skin lesion; pathogenic bacteria; antibiogram

1. Introduction

Among the different catfishes, Shing (*Heteropneustes fossilis*) is very popular and highly valuable fish species in Bangladesh. It is not only recognized for its delicious taste and market value but it is also highly regarded for medicinal aspects that contains high amount of iron (226 mg per 100 g) and fairly high content of calcium compared to many other catfishes (Saha and Guha, 1939). It is a very hardy fish that can survive for quite a few hours outside the water due to presence of accessory respiratory organs (Khan *et al.*, 2003; Monir and Rahman, 2015). This fish was abundantly available in open water of Bangladesh but presently, it is threatened due to over exploitation and various ecological changes in its natural habitat. Although, the appropriate breeding, nursing and rearing technology of fry and fingerlings of *H. fossilis* had been developed in few years ago but unknown

diseases of *H. fossilis* causes serious economic losses because of their high mortality. However, the production of *H. fossilis* is related to their aquaculture attributes which include ability to withstand handling stress, disease resistance, high growth rate, fecundity and palatability (Ikpi and Offem, 2011; Anyanwu *et al*., 2014).

By the increasing intensification of *H. fossilis* production and lack of health management measures have lead to many disease problems of bacterial, viral, fungal and parasitic origin. In most of the cases hemorrhages, septicemia, skin lesions are the common symptoms of the diseased fish. Skin of fish serves as organ of interaction with its environment and as the first site of attachment for different microorganisms (Noga, 2000; Anbuchezhian *et al.,* 2011). Skin lesions, often induced by attachment of microorganisms to fish skin, affect fish performance and productivity because of the crucial homeostatic functions such as osmoregulation, locomotion, respiration, thermoregulation, mechanical protective function and antimicrobial activities performed by the skin (Anyanwu *et al.,* 2014). Moreover, it serves as the first site of attachment for a plethora of microorganisms in the aquatic environment (Anbuchezhian *et al.*, 2011 and Austin 2011). Attachment of microorganisms to fish skin often induces skin lesions which, irrespective of the size, results in colonization by many opportunistic pathogens, life-threatening osmotic stress, increased energy costs from locomotion due to impairment of mucus production, swimming imbalance, increased predation due to colour change and deficiency in oxygen uptake (Mohanty and Sahoo, 2007; Declercq *et al.,* 2003; Toranzo *et al*., 2005; Ahamad *et al*., 2013). So, skin lesions adversely affect performance and productivity of the affected fish. As results, most of the fish farmers and different aquatic-drugs companies usually try to treat these infections using various antimicrobial chemicals as well as antibiotics but finally fail to stop mass mortalities and losses of *H. fossilis* due to development of resistance by the incriminated organisms. Bacteria associated with skin lesions and internal organs have been reported in fish reared in different region of Bangladesh and the reported bacteria which they were associated with include: *Edwardsiella tarda* in edwardsiellosis (Mohanty and Sahoo, 2007), *Flavobacterium columnare* in columnaris disease (Declercq *et al*., 2003), *Mycobacterium* species in mycobacteriosis (Toranzo *et al.,* 2005), *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* in motile *Aeromonas* septicaemia (Austin, 2011 and Ahamad *et al*., 2013), and *Aeromonas salmonicida* in typical furunculosis and *Psudomonas* spp. (Austin, 2011). However, there is little available literature about bacteria associated from skin lesion as well as the internal organs of diseased catfish and the antibiotic sensitivity profile of the bacterial isolates has not been reported from cultured fishes of Bangladesh yet. Therefore, the present study was undertaken to identify bacteria associated with skin lesions as well as internal organs of cultured *H. fossilis* and also their sensitivity against different antibiotics.

2. Materials and Methods

2.1. Sample collection

A total of 120 diseased *H. fossilis* were collected from twenty five fish farms in greater Mymensingh region, Bangladesh between November 2013 to February 2014. Minimum 10 diseased fish were collected by using scoop net from each affected fish farms. Most of the diseased fish was collected in alive condition and these fish were collected aseptically in sterile plastic box with ice. The fish samples were transported to the Fish Disease and Health Management Laboratory of Bangladesh Fisheries Research Institute, Mymensingh.

2.2. Isolation of bacteria

Isolation of bacteria was carried out from the skin lesions and internal organs of the diseased *H. fossilis*. Lesions of skin were disinfected with 70% ethanol for removing the surface contaminants. Smears from the skin lesions and internal organs were aseptically inoculated on nutrient broth as described by Ferdowsy *et al*., 2011; Monir *et al*., 2015. The overnight enriched broth was streaked onto various selective media such as: M-Aeromonas (MA) agar (for *Aeromonas* spp.), Acetamide agar (AC) agar (for *Pseudomonas* spp.), Thiosulfate citrate bile salt sucrose (TCBS) agar (for *Vibrio* spp.) and incubated at 37 °C for 24 hrs. Single colony was further sub-cultured until pure culture of bacteria was obtained.

2.3. 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 and involved colonial characteristics, bacterial cell morphology, motility test and biochemical tests of gram reaction, catalase test, glucose, sucrose and lactose utilization, citrate test, motility test, indole test, urease test, hydrogen sulfide production, TSI (gas production), methyl red (MR) test, voges praskaure (VP) test, coagulase test. The biochemical tests were carried out to identify the pathogens following Bergey's manual of Bacteriological classification (John *et al*., 1998).

2.4. Determination of antibiogram of bacterial isolates

Antibiotic susceptibility of the bacteria isolates was determined using the disc diffusion method as described by Finegold and Martin (1982). Stock cultures of the bacterial strains were grown in nutrient agar for 24 h at 37 ^{o}C. Then colonies of each of the isolate were adjusted to 0.5 McFarland's turbidity standard (equivalent to $1x10^{8}$ colony forming unit/ml) in sterile phosphate buffered saline (PBS) and the bacterial suspension was spread onto Mueller–Hinton agar (Oxoid). Antibiotic-impregnated discs were kept on the solid medium and the plates were incubated at 37 ^{o}C for 24 h. Zones of inhibition formed around the discs were measured and antibiotic sensitivity was assayed from the length of the diameter of the zones (in mm). The zone radius was actually scaled from the centre of the antibiotic disc to the end of the clear zone where bacteria could be seen growing. Zone diameters were interpreted as sensitive, intermediate and resistant according to the manufacturer's instructions.

3. Results

3.1. Clinical and post mortem findings

The clinical examination of diseased fish were loss of equilibrium and erosions at the bases of fins and tail, hemorrhages and skin lesions on body surface, profuse mucous secretion (Figures 1 and 2). Congestion and enlargement with hemorrhage was also observed in liver, kidney and spleen of the diseased *H. fossilis*.

3.2. Bacteria isolated from different organs of diseased *H. fossilis*

A total of 85 bacterial strains were isolated from 120 samples of diseased *H. fossilis*. The species composition and sources of these strains are presented in Table 1. The isolation frequencies of these 85 strains upon anatomical parts of infected *H. fossilis* were lesion (skin and fin) 30 (32.94%), gill 12 (14.11%), liver 10 (11.76%) and kidney 33 (38.82%). *Aeromonas* species were distributed as follows: *A. hydrophila* 34 (40.00%), *A. salmonicida* 14 (16.47%), *A. sobria* 8 (9.41%) as well as 5 (5.88%) unidentified *Aeromons* strains. On the other hand, only two species of *Pseudomonas* and one species of *Vibrio* were found from lesions and different organs of diseased *H. fossilis*. *Pseudomonas anguliseptica, P. fluorescens*, *Vibrio anguillarum* and *Vibrio* spp were distributed as 12 (14.11%), 4 (4.70%), 3 (3.52%) and 5(5.88%), respectively in infected *H. fossilis*. However, *Aeromonas hydrophila* appeared to be the main pathogen in the diseased *H. fossilis* rather than other bacterial species.

3.3. Morphological and biochemical tests for bacterial identification

Bacteria of the *Aeromonas hydrophila, A. salmonicida, A. sobria, Pseudomonas angulliseptica, P. fluorescens* and *Vibrio anguillarum* were identified from the lesion (skin and fin), gill, liver and kidney of the diseased *H. fossilis* (Table 2). The isolated *Aeromonas hydrophila* showed motile and oxidase, O-F, VP, catalase, indole, H_2S production, nitrate reduction test were positive whereas MR and urease production test were negative. *A. salmonicida* was non-motile and MR, indole, H_2S production, nitrate reduction test were negative. The strain of *A. sobria* showed motile and oxidative, O-F, VP, catalase, indole, H_2S production, nitrate reduction test were positive whereas MR and urease production test were negative. The isolated strain of *P. anguilliseptica* was oxidative, gram-negative rod, non–fluorescent, oxidative and catalase, indole, MR-VP postitive whereas H_2S production and nitrate reduction negative. The strain of *P. fluorescens* from infected *H. fossilis* was oxidative gram-negative rod, indole negative, H_2S negative, catalase positive, fluorescent positive, urease negative and MR-VP negative. While the strain of *Vibrio anguillarum* showed oxidative gram- negative rod and indole, H_2S production, nitrate reduction, urease production test negative but O/129 test was sensitive (Figures 3 and 4).

3.4. Susceptibility to antimicrobial agents in-vitro condition

In the study, all the isolated bacteria were sensitive to ciprofloxacine and levofloxacine, azithromycin and gentamicin. Ciprofloxacine and levofloxacine were highly effective against *A. hydrophila, A. almonicida, A. sobria, P. anguilliseptic* and *P. fluorescens* except *V. anguillarum* (Table 4). Novobiocine was highly effective against *V. anguillarum*, while azithromycin, gentamicin showed moderate effect against all the isolated bacteria. Ampicillin and penicillin did not show any effect against *Aeromonas* spp. and *Pseudomonas* spp. (Figure 5).

Table 1. Bacteria isolated from different organs of infected Shing (*H. fossilis*).

Isolated bacteria	Distribution (Number & %) of different bacterial strains (n=85) according to site of isolation				Total
	Anatomical parts of infected Shing				
	Infected area (skin & fin)	Gill	Liver	Kidney	
A. hydrophila	15 (17.64)	3 (3.52)	5 (5.88)	11 (12.94)	34 (40.00)
A. salmonicida	5(5.88)	2 (2.35)	2 (2.35)	5 (5.88)	14 (16.47)
A. sobria	3(3.52)	2 (2.35)	1 (1.17)	2 (2.35)	8 (9.41)
Unidentified *Aeromonas* spp	2(2.35)	0	1 (1.17)	2 (2.35)	5 (5.88)
P. anguliseptica	2(8)	3 (3.52)	1 (1.17)	6 (7.05)	12 (14.11)
P. fluorescens	1(1.17)	0	0	3 (3.52)	4 (4.70)
V. anguillarum	0	1(1.17)	0	2 (2.35)	3 (3.52)
Unidentified *Pseudomonas* spp.	2 (2.35)	1(1.17)	0	2 (2.35)	5 (5.88)
Total	30 (32.94)	12 (14.11)	10 (11.76)	33 (38.82)	85

Table 2. Morphological and biochemical characteristics of the isolated bacteria from infected Shing (*H. fossilis*).

Test name	*Aeromonas hydrophila*	*A. salmonicida*	*A. sobria*	*Psudomonas angulliseptica*	*P. fluorescens*	*Vibrio anguillarum*
Gram staining	-ve	-ve	-ve	-ve	-ve	-ve
Motility	+	-	+	+	+	+
Oxidase test	+	+	+	+	+	+
O-F test	+	+	+	O	+	+
MR test	-	-	-	d	-	d
VP test	+	-	d	+	-	+
Catalase test	+	+		+	+	d
Indole test	+	-	+	+	-	-
H_2S production	+	-	+	-	-	-
Nitrate reduction test	+	-	+	-	+	-
Urease production	-	-	-	+	+	-
TSI test	A/A	A/A	A/A	K/N	K/N	K/A
Production of acid from						
Glucose	+	+	+	+	+	+
Galactose	d	d		+	+	d
0/129 test (10 µg & 150 µg)	R	R	R	R	R	S

Note: d= variable reaction, O= oxidative, A= acid, K= alkaline, R= resistant, S= sensitive

Table 3. Antibiotics sensitivity test on isolated bacteria from infected Shing (*H. fossilis*).

Antibiotic (Cons/Disc)	*Aeromonas hydrophila*	*A. salmonicida*	*A. sobria*	*Psudomonas anguliseptica*	*V. anguillarum*
Ciprofloxacin (5µg)	+++	+++	+++	+++	++
Levofloxacin (5µg)	+++	+++	+++	+++	++
Gentamicin (10µg)	++	++	++	+++	++
Azithromycin (15µg)	++	++	++	++	+
Tetracycline (30µg)	+	+	+	+	+
Oxytetracycline (10µg)	-	+	+	+	+
Chlortetracycline (25µg)	-	-	+	+	+
Novobiocine (5µg)	-	-	-	-	+++
Ampiciline (10µg)	-	-	-	-	-
Penicillin (10µg)	-	-	-	-	-

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

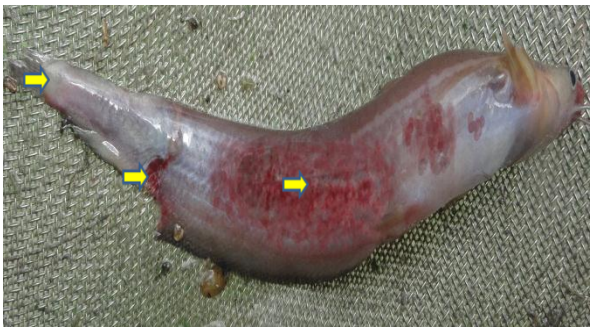

Figure 1. Hemorrhages on body surface and erosions at the bases of fins and tail in naturally infected *H. fossilis.*

Figure 2. Remarkable ulcerative skin lesion in naturally infected *H. fossilis.*

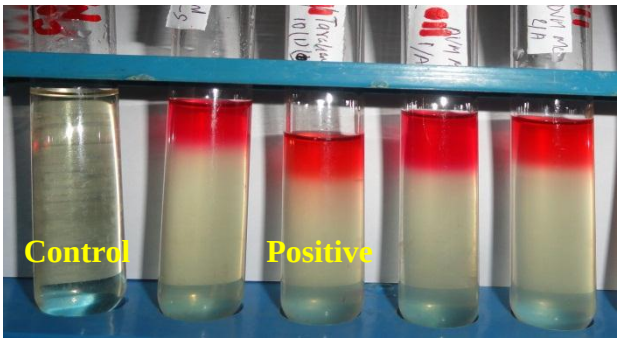

Figure 3. Methyl Red (MR) test showing positive result.

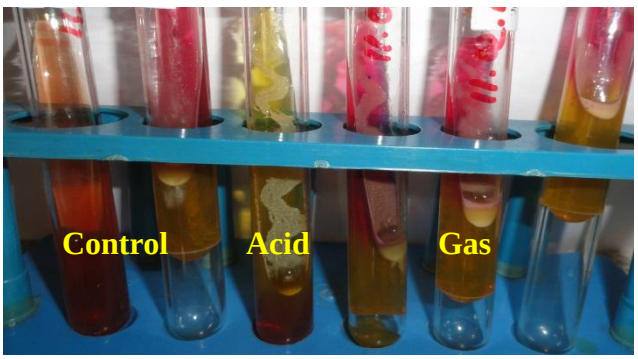

Figure 4. TSI test showing production of acid and gas.

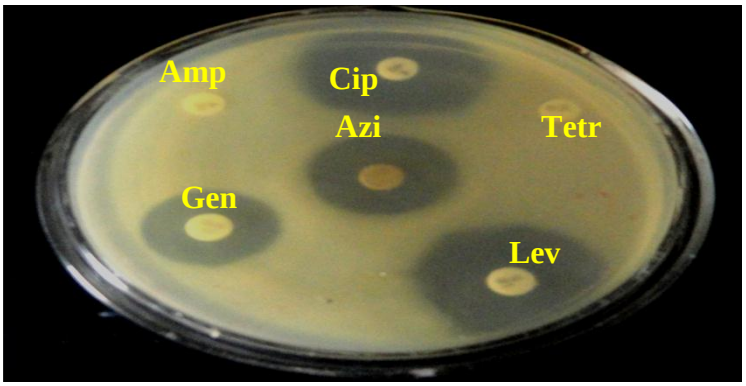

Figure 5. Antibiotic sensitivity and resistant pattern of bacteria isolated from infected *H. fossilis*.

4. Discussion

Shing (*H. fossilis*) is considered as a high valued species in Bangladesh. Although, *H. fossilis* is a hardy fish but production of it's in the cultured ponds has been affected by various factors including diseases caused by viral, bacterial and fungal pathogens. High mortality of young and adult *H. fossilis* was noticed in cultured ponds located in greater Mymensingh of Bangladesh. However, the clinical symptoms were loss of equilibrium, hemorrhages, skin lesions, body and tail erosion, mucous secretion, and congestion and enlargement with hemorrhage of the internal organs which were similar with the findings of Alicia *et al*. (2005); Khalil *et al*. (2010); Mastan (2013). Congested liver and internal organs were also observed in the diseased fishes by Ahmed and Shoreit (2001); Gamal *et al.* (2002).

The isolated bacteria were *Aeromonas hydrophila, A. salmonicida, A. sobria, Pseudomonas angulliseptica, P. fluorescens* and *Vibrio anguillarum.*. Similar results and isolates were recorded by Ahmed and Shoreit (2001); Laila El-Seedy *et al*. (2004). Lewis and Plumb (1979) and Monir *et al*. (2015) observed that among the bacterial diseases, the motile *Aeromonas* caused mass mortality in catfish and induced serious epidemics of ulcerative disease in fish in Southeast Asia and other regions of the world (Angka *et al*., 1988; Areerat 1987; Roberts *et al*., 1992; Anyanwu *et al*., 2014). Furthermore, Yesmin *et al.* (2004) and Chowdhury and Baqui (1997) noticed that *Aeromonas* is a very common pathogen in carps and other freshwater fishes. Hossian and Chowdhury (2009) also reported that *Aeromonas* and *Pseudomonas* are the important bacterial pathogens frequently isolated from the diseased fishes throughout the world as well as in Bangladesh. *A. hydrophila* was frequently observed in various species of diseased farmed and wild freshwater fishes in different locations of Bangladesh (Sarker *et al.*, 2000). It was recognized as a causative agent of ulcer type disease occurred in farmed fishes (Chowdhury, 1998). Moreover, Sabur (2006) isolated and identified five species of *Aeromonas* spp. from the diseased fishes of *Labeo rohita, Cyprinus carpio, Cirrhinus cirrhosus, Catla catla* and *Hypophthalmichthys molitrix. A. hydrophila* were frequently isolated from various lesions of epizootic ulcerative syndrome (EUS) of different fishes (Roberts *et al.*, 1990; Sarkar and Rashid 2012). Chowdhury (1998) isolated and identified some *Pseudomonas* strains from diseased farmed fish of Bangladesh. Paul *et al*. (1998) reported isolation of Pseudomonads from diseased Rajpunti (*Barbodes gonionotus*). Hossain *et al*. (2011) also identified several Pseudomonad isolates recovered from diseased fish from different types of water bodies. *P. anguilliseptica* is a causative agent of red spot disease, causes severe mortalities in pond cultured fishes in Japan (Zhang *et al*. 2009). *P. anguilliseptica* was also isolated from *O. niloticus* fishes affected with Pseudomonas Septicemia in Bangladesh (Hossain *et al.*, 2009). Fayed (1997) and Swain (2007) also isolated *P. fluorescens* from various diseased fishes. However, the highest isolation frequencies of the bacterial strains were 30 (32.94%) from lesion of skin and fin. Anyanwu *et al.*, (2014) also reported that the highest isolation (44%) of aerobic bacteria was obtained from fish skin lesion. Yanong (2011) found that most skin lesion-causing organisms in freshwater fish are gram-negative bacteria. Many researchers (Sugita *et al*., 1997; Shewan and Hobbs, 1990; Shewan, 2000; Okaeme, 2006) have isolated different species of bacteria from the skin of the fresh water fish (catfish) including *Bacillus* species from the skin of warm water fish. The isolated aerobic bacteria could have caused the skin lesions following immune-suppression of the fish or they could also have contaminated wounds following mechanical injury. *Aeromonas* species have been reported to occur as commensals on fish skin where they cause opportunistic infections following immune-suppression (Janda, 2010). All bacterial infections were found as mixed infections. Mixed bacterial infections with *Aeromonas* spp. & *Pseudomonas* spp. was also reported by Ahmed and Shoreit (2001).

In this study, *Aeromonas* sp. and *Pseudomonas* sp. isolates were conducted by disk diffusion method against ten antibiotics where, all of the isolates were found to be sensitive to ciprofloxacine and levofloxacine, azithromycin and gentamicin but, most of the isolates were found resistant or less sensitive to tetracycline, chlorotertacycline and oxyteracycline. Ciprofloxacine and levofloxacine were highly effective against *Aeromonas* sp. and *Pseudomonas* sp. where, ampicillin and penicillin were fully resistant. Since tetracycline, chlorotertacycline and oxyteracycline are indiscriminately used in fish culture ponds as well as farmers don't maintain recommend applying dose and resulting in transfer of resistance genes to the isolated bacterial strains. Furthermore, Sahoo and Mukherjee (1997) noticed that tetracycline group has been reported to enhance the production of plasmid-mediated resistance in aquatic bacteria resulting in increased frequency of new tetracycline resistant isolates. However, Truong *et al.* (2008) found two isolates of *Aeromonas hydrophila* were sensitive to sulphamethoxazole and ciprofloxacin. As regards to *V. anguillarum*, this was sensitive to novobiocine while azithromycin, gentamicin showed moderate effect against all the isolated bacteria. These findings were almost similar to the findings of other researchers (Hossain, 2006; Rahman *et al*., 2008).

5. Conclusions

This study has shown that bacterial diseases could be a major cause of considerable economic loss to Shing (*H. fossilis*) farmers in greater Mymensingh, Bangladesh. *Aeromonas hydrophila, A. salmonicida, A. sobria, Pseudomonas angulliseptica, P. fluorescens* and *Vibrio anguillarum* was the major cause of bacterial disease of this species. *Aeromonas* spp. and other isolates from the affected *H. fossilis* have developed multidrug resistance to antibiotics probably due to indiscriminate use of these antibiotics in fish cultured ponds. Isolation and identification of causative agent and determination of the antimicrobial profile of bacterial agents associated with skin lesions and internal organs is necessary for effective antimicrobial treatment. However, disease prevention of Shing (*H. fossilis*) should be carried out by means of the better culture practices and health management to ensure the optimum yields and the best quality of the products.

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Conflict of interest

None to declare.

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