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Isolation of pathogens causing ulcer disease in farmed and wild fishes of Mymensingh

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Abstract

Studies were conducted to investigate bacterial and fungal pathogens causing ulcer diseases in farmed and wild water bodies in Mymensingh during October, 2000 to September, 2003. Fishes were found to be affected by ulcer diseases, especially in the months November to March usually peaked in January and February. Bacterial and fungal pathogens were isolated from the affected organs of the diseased fish and identified. The bacterial isolates were *Aeromonas hydrophila, A. veronii* biovar *sobria, A. veronii* biovar *veronii, Pseudomonas fluorescens* and *Edwardsiella tarda* and the fungal isolates were *Aphanomyces invadans*, *Saprolegnia* sp. and *Achlya* sp. In pathogenicity test, *A. hydrophila* was found to be more pathogenic than those of other isolates. In the case of fungi, *A. invadans* was detected as the most common fungal pathogen and was capable of producing characteristic lesion in the experimental fish. Experimental mixed infection with the bacterial pathogen, *A. veronii* biover *sobria* and fungal pathogen, *A. invadans* offered an interesting result producing similar lesions found in the naturally affected sampled fish. In histopathological observation, mycotic granulomas were a characteristic feature in the tissues of the EUS affected fishes. In contrast, no such feature was detected in the tissues of apparently healthy fish.

Keywords: Pathogen, Ulcer, Pathogenicity, Mixed infection

Introduction

Disease has become one of the alarming factors in reducing fish production in Bangladesh, It has been found in our previous research that the most common diseases in freshwater fishes are in the form of ulcers including epizootic ulcerative syndrome, EUS (Chowdhury,1998). EUS is a devastating disease affecting both farmed and wild fish in Asia and Australia. In the progress of the disease to Bangladesh where it was first reported in Chandpur district in February 1988 and then spread throughout the country (Hossain *et al.* 1992). It affected many fish species, with significant financial losses estimated at US$ 3.4 million in 1988 alone (Barua 1994). The disease created panic and most of the people did not even eat healthy fish due to fears of transmission of the disease to human and fish price dropped to 25-40% of their pre-disease level during the outbreaks in severely affected districts (Khan, 2001). Still now, the incidence of EUS has becoming a tremendous threat in reducing fish production in the country like other Asian countries. During the first EUS outbreaks in Bangladesh, Roberts *et al.* (1989) isolated two bacteria, *A. hydrophila* and *A. sobria* from affected fish. Chowdhury *et al.* (1994) isolated *Aeromonas, Vibrio, Pseudomonas, Micrococcus, Flavobacterium/ Cytophaga, Enterobacteria* and *Achromobacter* from EUS-affected fish. Iqbal *et al.* (1998) detected *A. hydrophila, A. veronii* biover *sobria* and *A. jandaei* as pathogenic bacteria in Japan, recovered from EUS affected mrigals collected from Bangladesh. A pathogenic *Aphanomyces* was isolated from mycotic granulomatosis affected fish in Japan by Hatai *et al.* (1977). But still it is not clear which is the first causative agent of EUS.

Information on causative agents of the ulcer diseases is very important for well management of fish ponds. In Bangladesh Fisheries, various ulcer diseases are often confused with the EUS, which are found to occur throughout the year in both farmed and wild fishes. But research on this aspect has not yet been done systematically in the country. Considering the importance, the present study was undertaken to isolate bacterial and fungal pathogens causing ulcer disease in farmed and wild fishes of Mymensingh.

Materials and Methods

Sampling

In each sampling 30 fishes of different species were caught by a cast net and grossly checked their disease condition with any abnormality or expression of lesions on which prevalence of disease outbreak was based. Among the diseased fishes, randomly selected fishes were sampled and immediately brought to the laboratory for isolation of suspective pathogenic bacteria and fungi.

Isolation of Bacterial Pathogens

a . Collection of bacterial swabs and their culture

Bacterial swabs were taken aseptically from the lesions and kidney of the disease affected sampled fish on the pre-prepared selective *Aeromonas* Agar Base and *Pseudomonas* Agar Base, SS Agar and then successive studies were performed in the laboratory. Pure cultures of *Aeromonas* spp., *Pseudomonas* spp. and *Edwardsiella* spp. were obtained from selective agar culture plate. The isolates were checked on Tryptone Soya Agar (TSA) and maintained in TSA slant at 4^0C for further studies.

b . Primary characterisation, identification and species determination

Various morphological characteristics of bacterial colonies such as shape, size and colour were recorded. The shape of individual bacterium was determined after Gram's staining taking fresh culture of 24 hrs. Gram's test was also done with 3% KOH. Motility test was performed by taking dilute suspension of fresh bacterial culture on clean glass slide with cover slip and observed under a microscope with closed circuit camera adjusted with a TV-monitor. Catalase test, Oxidase test, OF-test (Hugh and Leifson's test) was performed following the usual microbiological procedure. Suspective bacterial pathogens were identified up to genus level on the basis of the above characteristics.

Species determination of Aeromonads bacteria was performed following the Aerokey II system described by Carnahan *et. al.*(1991). All of the necessary characterizations were accomplished using API-20E system in addition to the conventional method described in Cowan and Steel's Mannual for the Identification of Medical Bacteria after Barrow and Feltham, 1993. The API-20E commercial kit is a standardised miniaturised version of conventional procedures for the identification of Gram-negative bacteria was used to confirm some of the characters. It is a microtube system enabling 20 standard biochemical tests to be carried out on a bacterial culture.

Isolation of Causative Fungi

a. Collection of fungal samples and culture

Fish with pale, raised lesions which was not completely ulcerated was considered for fungal isolation. Different types of affected fish (wild and farmed) were sampled form Mymensingh and immediately brought to the laboratory in the same water. Affected muscles were aseptically collected with the help of a sterile scalpel and immediately placed in petridish containing isolation medium, GP- PenOx broth. The fungus was transferred to fresh plates of GP-PenStrep agar until cultures were free of bacterial contamination. Then the fungus may be subcultured on GP-agar at intervals of no greater than 5 days.

b. Sporulation

In order to induce sporulation, an agar plug (3-4mm in diameter) was placed in a petridish containing GPY broth and incubated for four days at 22^0C. The nutrient agar out of the resulting mat was washed by sequential transfer through five petridishes containing autoclaved pond water (APW) and kept overnight at 22^0C in APW. After about 12 hrs. the formation of primary cysts and release of motile secondary zoospores were observed under microscope.

Pathogenicity of Bacterial and Fungal Pathogens

Detection of pathogenicity was performed selecting representative bacterial and fungal isolates. Healthy young *Barbodes gonionotus* of 20g to 30g in weight maintained in the aquarium were used as experimental fish. The fish were injected intramuscularly with 0.1ml of bacterial suspension of the pre-fixed dose of 2.5×10^6 CFU/ml at the base of dorsal fin of the experimental fish. In the case of fungal pathogens, fishes were exposed to 1×10^4 spores/ml of motile secondary zoospore suspension after a mild skin abrasion of the fish with a sterile scalpel. Control fish received only sterile physiological saline (o.85%NaCl). All the fish were maintained in aquarium at 22- 24^0C under aerated condition and in every 24h, 50% of water was exchanged with tap water. In the case of experimental mixed infection, the bacterial pathogen *A. veronii* biover *sobria* As-35 and fungal pathogen *Aphanomyces invadans* Ap-11 were used together. Appearance of lesions, moribund and death were considered as primary diagnosis of infection.

Histopathological Studies

a. Collection of tissue samples

The tissue samples of ulcer affected fish were collected carefully from the tissue surrounding the lesions and placed into 10% (neutral buffered) formalin in individual vial for fixation. The fixed tissues were step by step processed in the laboratory according to the standard histopathological protocol.

b. Tissue processing

The fixed and processed fish tissues were dehydrated and processed step by step in an Automatic Tissue Processor.

c. Sectioning and staining

Suitable sections (5µm) were obtained by cutting with microtome machine. The sections were then stained with haematoxylin and eosin (H&E) following the laboratory staining procedure described earlier (Thonguthai *et al.*, 1999).

d. Mounting and slide preparation

After staining, the sections were mounted with Canada balsam and covered by coverslips. Thus, the prepared permanent slides was kept in slide box and conveniently examined under a compound microscope.

Results and Discussion

In total, 28 bacterial isolates and 21 fungal isolates were recovered from several affected fish species collected from different diseased affected fish farm and wild water bodies in Mymensingh (Table 1). Among the investigated fishes, *Cirrhinus cirrhosus* and *Barbodes gonionotus* were found to be severely affected by ulcer disease. The isolated bacteria were categorised into 3 genera as *Aeromonas, Pseudomonas* and *Edwardsiella*. Among the total recovered bacterial isolates, number of *Aeromonas* were 16, *Pseudomonas* 07 and *Edwardsiella* 05. They were identified as *A. hydrophila, A. veronii* biovar *sobria, A. veronii* biovar *veronii, P. fluorescens* and *E. tarda*. The dominant bacterial species was *A. hydrophila*. The present finding supports the previous work done by Chowdhury, 1998 who reported the involvement of *A. hydrophila* in the ulcer diseases. Most of the isolates were recovered from EUS-affected fishes as coexisting bacteria and some were recovered frc n bacteria affected fishes. Bacteria including *Aeromonas* spp. are considered to be a secondary invader of the EUS disease (Supranee and Roberts, 1999). However, *Aeromonas* spp. is frequently isolated from EUS-affected fish (Subasinghe *et al.*, 1990 and Iqbal *et al.*, 1998). The recovered fungal isolates were primarily identified as *Aphanomyces*, *Saprolegnia* and *Achlya* (Table 2). Numbers of *Aphanomyces* were 11, *Saprolegnia* 06 and *Achlya* 04. Among these, only *Aphanomyces* could confirm up to species level and this was *Aphanomyces invadans* (Table 3).

Chinabut *et al.* (1995) recovered saprophytic fungi consisting of *Saprolegnia*, *Achlya* and *Aphanomyces* spp. from the surface of lesion on fish including EUS lesion and stated that these may contribute to the diseases by acting as opportunistic parasites. Chowdhury and Muniruzzaman (2002) isolated *Aphanomyces* sp., *Saprolegnia* sp. and *Achlya* sp. from the fishes with ulcer type of diseases, which support the present study. List of identified bacterial and fungal isolates are shown in Table 4.

In the pathogenicity study, all of the *A. hydrophila* tested were found to be more pathogenic than *P. fluorescens* and *E. tarda* (Table 5). Among the three isolates of *A. veronii* biover *sobria*, two isolates As-35, As-45 were high pathogenic and As-52 was medium pathogenic. The two isolates of *P. fluorescens* Pf-9, Pf-I5 were medium pathogenic and Pf-10 was non-pathogenic whereas one isolate of *E. tarda* Et-16 was high pathogenic and two isolates Et-10, Et-11 were medium pathogenic. In the case of the fungal isolates, all of the *A. invadans* isolates (Ap-11, Ap-12, Ap-19) were found to be more pathogenic than that of *Saprolegnia* isolates (Sa-11, Sa-39). In this study, *A. invadans* was detected as a very common fungal pathogen, especially in the diseased fishes sampled in the month of January and February and produced characteristic lesion.

In the case of experimental mixed infection with the bacterial pathogen, *A. veronii* biover *sobria* As-35 and fungal pathogen, *A. invadans* Ap-11 offered an interesting result produci..g similar lesions found during sampling of naturally ulcer affected fish. It was evidence causing ulcer disease through mixed infection. The results supported the findings obtained by Lilley and Roberts (1997). The results correlate with the works done by Roberts (1994) and Chinabut *et al.* (1995) who reported that *A. invadans* failed to invade normal fish following the bath challenge. Chinabut *et al.* (1995) suggested that stress would be needed to make an invasion by the pathogen *A. invadans*. Balasuriya *et al.* (1990) and Cruz-Lacierda and Shariff (1995) reported that neither suspension of spores nor hyphae of *A. invadans* can produce typical EUS lesion unless the skin was abraded.

Table 1. List of bacterial and fungal isolates encountered in different diseased farmed and wild fish species

Fish species	Bacterial isolates			Fungal isolates		
	Aeromonas	*Pseudomonas*	*Edwardsiella*	*Aphanomyces*	*Saprolegnia*	*Achlya*
Farmed fishes						
Cirrhinus cirrhosus	A-28, A-29	P-9	-	Ap-11	Sa-10	Ac-5
Labeo rohita	A-31, A-32	P-10	-	Ap-12	Sa-11	-
Catla catla	A-33	-	-	Ap-13	-	-
Barbodes gonionotus	A-34, A-35	P-11	E-10	Ap-14, Ap-15	Sa-12	Ac-6
Pangasius hypophthalmus	A-37, A-38,	-	E-11	Ap-17	Sa-13	-
Wild fishes						
Anabas testudineus	A-39, A-40	P-13	E-14	Ap-18	Sa-14	Ac-7
Channa punctatus	A-42, A-43	P-14	E-15	Ap-19	Sa-15	Ac-8
Nandus nandus	A-44	P-15	-	A-20	-	-
Mastacembalus pancalus	A-45	P-16	-	Ap-21	-	-
Mystus vittatus	A-46	-	E-16	Ap-23	-	-

Table 2. Primary characterization of the recovered fungal isolates

Characters	Groups of fungal isolates		
	Type-A	Type-B	Type-C
Hyphae	Slender and much branched	Moderately stout and branched	Moderately stout and branched
Zoosporangia	Zoosporangia typically no wider than the hyphae	Zoosporangia short with terminal hyphal swelling	Zoosporangia short with terminal hyphal swelling
Zoosporangial renewal	Renewal by branching below empty sporangium	Renewal by internal proliferation	Renewal typically sympodial, branching from the hypha below the basal septum
Zoospores	The zoospore is not fully released from the sporangium and remain motile for a period longer than *Saprolegnia*.	Zoospores are fully released from the sporangium and remain motile for a short period.	Zoospores fully released from the sporangium and remain motile for a short period.

Type-A : *Aphanomyces* Type-B : *Saprolegnia* Type-C : *Achlya*

Table 3. Characterization of *Aphanomyces* isolates for identification

Characteristics	Description
Hyphal diameter	Rounded tips and branch almost at right angles to the main axis. 5-20 µm on GP agar.
Radial growth	Grows at temperature between 5-36°C, Salinity below 10 ppt NaCl. No growth on cornmeal agar, malt extract agar or Sabouraud dextrose agar. On GP agar (mm per 24h): 1.9 at 14°C 2.8 at 18°C 3.9 at 22°C 4.6 at 26°C 4.6 at 34°C No growth at 37°C
Zoosporangia	Equal diameter to mycelium (about 10 µm)
Zoosporangial renewal	Sympodial branching below empty sporangium.
Zoospore cyst	6-10 µm diameter, average 6.5 µm
Zoospore	Motile, subspherical and biflagellate. Released within 12hours of sporangial development at 22°C. No sporulation above 2 ppt NaCl

Species: *Aphanomyces invadans* - : No infection

Table 4. List of identified bacterial and fungal isolates

Isolates	Identified species
Bacteria	
Aeromonas sp. A-28, A-32, A-34, A-39, A-42, A-43, A-44, A-47, A-49, A-51	*Aeromonas hydrophila*
Aeromonas sp. A-29, A-35, A-45, A-52	*Aeromonas veronii* biover *sobria*
Aeromonas sp. A-31,A-40, A-46	*Aeromonas veronii* biover *veronii*
Pseudomonas sp. P-9, P-10, P-15, P-17	*Pseudomonas fluorescens*
Edwardsiella sp. E-10, E-11, E-16, E-30	*Edwardsiella tarda*
Fungus	
Aphanomyces sp. Ap-11, Ap-12, Ap-14, Ap-19, Ap-20	*Aphanomyces invadans*

Table 5. Detection of pathogenicity of bacterial and fungal isolates recovered from ulcer affected sampled fishes

Isolates	Response for infection
Bacteria	
Aeromonas hydrophila Ah-28	++
Aeromonas hydrophila Ah-32	++
Aeromonas hydrophila Ah-43	++
A. veronii biovar *sobria* As-35	++
A. veronii biovar *sobria* As-45	++
A. veronii biovar *sobria* As-52	+
Pseudomonas fluorescens Pf-9	+
Pseudomonas fluorescens Pf-10	-
Pseudomonas fluorescens Pf-15	+
Edwardsiella tarda Et-10	+
Edwardsiella tarda Et-11	+
Edwardsiella tarda Et-16	++
Fungus	
Aphanomyces invadans Ap-11	++
Aphanomyces invadans Ap-12	++
Aphanomyces invadans Ap-19	++
Saprolegnia sp. Sa-11	+
Saprolegnia sp. Sa-39	+
Achlya sp. Ac-50	+
Achlya sp. Ac-52	-

++ : High infection + : Medium infection -: Not detected

In histopathological observation, mycotic granulomas (Fig. 1) were found to be a characteristic feature of the ulcer affected fish tissues. Sometimes fungal hyphae were also observed in the sections of muscle tissues sampled from the affected fish, especially in the case of EUS affected fishes infected by *A. invadans*. In contrast, no such feature was detected in the tissues of apparently healthy fish. The results supported the findings obtained by Lilley and Roberts (1997). Ahmed and Haque (1999), Sarker (2000) and Khan (2001) obtained similar results.

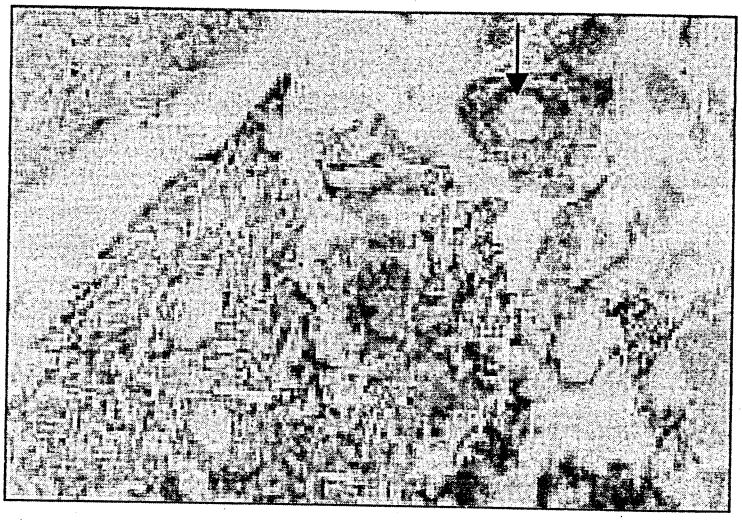

Fig. 1. Mycotic granuloma in the muscle tissue of EUS affected *Barbodes gonionotus*

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