Dynamics of initiation of disease in fishes through interaction of microbes and the environment

Fish is a precious source of protein as well as a means of livelihood, particularly for the people of developing countries. However, fishes are sometimes affected by different kinds of diseases which often become virulent, posing threat to their sustenance and conservation. EUS is one such disease which has been causing large-scale mortality among the freshwater fishes of India since 1988, after sweeping through Australia (1972), Papua New Guinea (1974), Indonesia (1980), Malaysia (1979, 1983), Thailand (1981, 1985), Kampuchea and Lao-PDR (1984), Myanmar (1984–85), Sri Lanka (1987) and Bangladesh (1988). Large hemorrhagic cutaneous ulcers, epidermal degeneration and necrosis, followed by sloughing of scales are the principal symptoms of EUS. Various microbes have been found to be associated with the pathogenesis of EUS[1–3]. Bacterial flora, notably *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Staphylococcus epidermitis*, and *Klebsiella* sp., have been isolated from EUS-affected fishes in specific culture media. All these bacteria have been found to be sensitive to commonly available antibiotics, notably Gentamycine, Chloramphenicol, Nalidaxic acid and Septran. However, culture of *A. hydrophila* in *Aeromonas* isolation medium with *Aeromonas* selective supplement revealed the presence of *aerolysin* gene, which has been confirmed through multiplex PCR (Table 1). Normal non-occurrence of EUS in fishes was revealed to be due to absence of *aerolysin* gene in *A. hydrophila* cultured from healthy fishes. The close association of these bacterial flora with fishes in water, vis-à-vis their isolation from diseased fishes prompted the present investigation in order to reveal the relationship, if any, between bacteria, fish and environment and any other pathogenic agent responsible for the initiation of the disease. Nevertheless, virological study from EUS-affected fishes revealed isolation of a virus in tissue culture which has been confirmed under electron microscope to be belonging to the Picobirna group (size 30–40 nm) which was further confirmed through techniques like immunofluoroscence assay and reverse transcription. The isolated psychrophilic acid-secreting bacterial flora under low temperature in the water bodies are known to multiply prolifically, thereby increasing acidity in water and lowering its alkalinity. The environment thus becomes congenial for viruses which are known to thrive under low alkaline condition[4]. Thus, in view of the above components, an experiment had been designed in order to reveal the initiation of disease in fishes (notably EUS) through interaction between environment (water temperature and pH), bacteria and suspected virus.

Bacterial flora was cultured from EUS-affected, air-breathing freshwater fishes (*Anabas testudineus*) in 10% Sheep Blood Agar (SBA). Further, the same isolates were cultured in specific *Aeromonas* isolation medium with *Aeromonas* selective supplement. The haemolytic transparent colonies seen on both types of media were then subjected to further biochemical analysis[5]. Confirmatory biochemical tests performed were triple sugar iron (TSI) utilization, citrate test, acid-gas production, DNAase test, MacConkey lactose agar (MLA), Vogues Proskaeur (VP) test, aesculine hydrolysis test, sugar breakdown test, oxidase test, etc. The haemolytic transparent colonies were able to break down the TSI for energy source and turned the slant yellow in colour. Bacterial isolates were found to be citrate and DNAase test-positive. The isolates were tested for acid-gas production by growing them into peptone water in a test tube with a Durham tube and incubated at 21°C overnight. The next morning gas bubbles as well as acid were observed in the test tube confirming them positive for acid-gas production. Colonies were also found to be positive for MLA and oxidase test. Phenol red plates spread with suspected plates were the experiment culture and then different sugar discs were placed on it. The bacterial colonies could break down the sugars and grow further. During aesculine hydrolysis test, plates with nutrient agar and aesculine were made and then the culture under study was spread on it. The cultures could break down the aesculine (sugar alcohol) and could react with ferric citrate added on it resulting in the formation of brown precipitate. The confirmed *A. hydrophila* were then cultured into nutrient agar tubes.

Table 1. Multiplex PCR data

Mix	Quantity (µl)
Master mix (2*x*)	12.5
Primers	
1F	0.5
1R	0.5
2F	0.375
2R	0.375
3F	0.375
3R	0.375
Double distilled water	7.51

Aliquot of 22.5 µl (Master mix + primers + double distilled water) in each tube and then 2.5 µl of template DNA was used.

PCR cycling	
Cycle 1	Step 1: 95°C for 5 min
Cycle 2 (*x* 35)	Step 1: 95°C for 0.5 min
	Step 2: 59°C for 0.5 min
	Step 3: 72°C for 0.5 min
Cycle 3	Step 1: 72°C for 10 min
Cycle 4	Step 1: 4°C for infinite

PCR ingredients are also shown. Total reaction volume is 25 µl.

Multiplex-PCR: Template DNA was prepared by suspending a loop full of biochemically confirmed *A. hydrophila* cultures in 100 µl of sterile double-distilled water. It was vortex-mixed and boiled for 10 min at 100°C in a boiling water bath. Then the suspension was centrifuged at 10,000 rpm for 10 min. The supernatant was used as template DNA. The primers used are given in Table 2.

A loop full of *A. hydrophila* was added to the water in an experimental unit (designated as 'exp') consisting of a close-walled sterile basket of size 42 × 26 cm containing sterile distilled water (1 l). A

Table 2. Primers used in multiplex PCR.

Primers used	Label	*n* mole	µl
AHH1F	(1F)	13.2	132 µl H_2O
AHH1R	(1R)	15.6	1556 µl H_2O
AH-aerAF	(2R)	12.8	128 µl H_2O
AH-aerAR	(2R)	15.9	159 µl H_2O
ASA1F	(3F)	12.7	127 µl H_2O
ASA1R	(3R)	15.4	154 µl H_2O

Table 3. Increase in bacterial colony count and decrease in pH during disease initiation in fish

Water temperature	Inoculation stage	Bacterial colony		pH		No. of fish added
		Control	Test	Control	Test	
28°	BI	0	0	6.7	6.7	2
	AI	0	0	6.7	6.67	
26°	BI	0	0	6.7	6.7	2
	AI	0	$8 \times 4 \times 10^{-3}$	6.7	6.61	
24°	BI	0	0	6.7	6.7	2
	AI	0	$12 \times 4 \times 10^{-3}$	6.7	6.57	
22°	BI	0	0	6.7	6.7	2
	AI	0	$17 \times 4 \times 10^{-3}$	6.7	6.53	
20°	BI	0	0	6.7	6.7	2
	AI	0	$34 \times 4 \times 10^{-3}$	6.7	6.51	
18°	BI	0	0	6.7	6.7	2
	AI	0	$63 \times 4 \times 10^{-3}$	6.7	6.49	
17°	BI	0	0	6.7	6.7	2
	AI	0	$75 \times 4 \times 10^{-3}$	6.7	6.47	
16°	BI	0	0	6.7	6.7	2
	AI	0	$152 \times 4 \times 10^{-3}$	6.7	6.43	
15°	BI	0	0	6.7	6.7	2
	AI	0	$212 \times 4 \times 10^{-3}$	6.7	6.4	
14°	BI	0	0	6.7	6.7	2
	AI	0	$260 \times 4 \times 10^{-3}$	6.7	5.38	
13°	BI	0	0	6.7	6.7	2
	AI	0	$294 \times 4 \times 10^{-3}$	6.7	5.1	
12°	BI	0	0	6.7	6.7	2
	AI	0	$412 \times 4 \times 10^{-3}$	6.7	5.04	
11°	BI	0	0	6.7	6.7	2
	AI	0	$503 \times 4 \times 10^{-3}$	6.7	4.9	Lesion developed
10°	BI	0	0	6.7	6.7	2
	AI	0	$480 \times 4 \times 10^{-3}$	6.7	4.98	

BI, Before inoculation; AI, After inoculation.

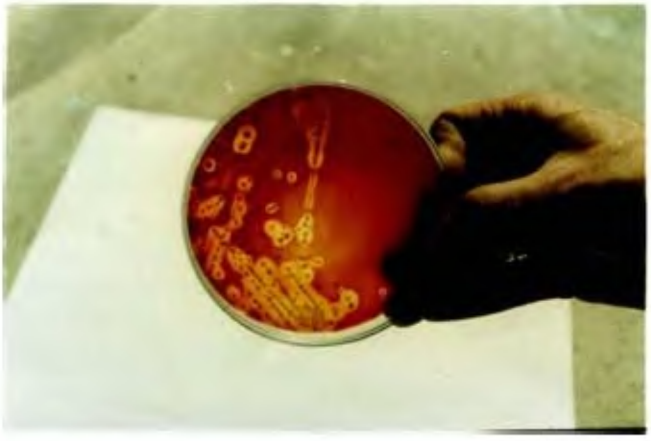

Figure 1. Haemolytic colonies grown on 10% SBA.

Figure 2. Bacterial growth and colony count.

pair of air-breathing fish (*Anabas testudineus*), weighing 20 g, were introduced into the basket. A control unit (designated as 'cont') was similarly set up. The experimental units were incubated at a series of temperatures: 28, 26, 24, 22, 20, 18, 17, 16, 15, 14, 13, 12, 11 and 10°C in a BOD incubator under aseptic condition. The results obtained are discussed below.

Bacterial flora obtained from EUS-affected fishes were grown on 10% SBA. Some of the colonies were found to be haemolytic (Figure 1). These grew as transparent colonies on specific *Aeromonas* isolation medium with *Aeromonas* selective supplement. The transparent colonies were found to be positive to certain confirmatory biochemical tests like TSI, oxidase, citrate, DNAase test, acid-gas production and sugar fermentation test. Conversely, the cultures were found

Figure 3. Initiation of disease lesion at 11°C.

to be negative to MLA test. Further, the cultures showed positive result to aesculine hydrolysis test as revealed from the production of brown colour[5]. The positive *A. hydrophila* cultures were then finally tested for slide agglutination with standard antiserum $AH_4(2)$ and found to be positive for the agglutination reaction.

The *A. hydrophila* cultures were then inoculated into closed-wall plastic baskets and incubated at different temperatures ranging from 28 to 10°C as indicated above for 48 h at each incubation temperature. After each incubation, the pH as well as colony counts (Table 3 and Figure 2) were checked; a decreasing trend in pH and an increasing trend in colony count were found. Surprisingly, at 11°C, the fish developed a reddish small circular lesion near the caudal fin (Table 3 and Figure 3).

The formation of a diseased lesion under experimental set-up seems to be due to the initiation of disease (notably EUS) in fishes through their interaction with bacteria, the environment and possibly viruses.

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Synedra ulna (Nitzsch) Ehrenberg: A new generic record in Schirmacher Oasis, Antarctica

The first systematic exploration of the Polar water of Antarctica was conducted by Englishman James Cook who circumnavigated Antarctica twice between 1772 and 1775. After that a number of expeditions were made to explore the diversity of marine coastal vertebrates and phytoplankton. Several workers studied distribution and taxonomical evaluation of algal flora of different places of Antarctic ice continent[1–7]. Since then about 700 algal taxa that have been reported from the Antarctic continent and off-shore islands became known to us from literature[8]. The first Indian expedition was launched to Antarctica in December 1981, with the participation of scientists from different organizations to study the algal flora of Schirmacher Oasis[9–12]. A total of 209 taxa of algae belonging to different classes have so far been reported from the Schirmacher Oasis and its surrounding area. Out of these, 10 genera belong to Bacillariophyceae[12].

Schirmacher Oasis (70°44′21″–70°46′04″S to 11°26′03″–11°49′54″E) is a group of low-lying hills, essentially a snow and ice-free high polar rock desert in the Eastern Dronning Maud Land, East Antarctica. It is 70 km away from Prince Astrid Coast. The Oasis oriented in east–west direction, lies between two types of large ice bodies, viz. glacier in the south and ice shelf at the north of the Oasis. It covers an area of about 34 km^2. The altitudes lie between the local zero level and 228 m with an average of 100 m. During the polar summer the ice melts and water flows in the form of streams often adding water to the lakes. The climate is relatively mild in the low altitude with air temperature over the glacier ice between –7.7 and +10.2°C during mid-summer (December 2003–January 2004) when melted water is abundant.

During the XXIII Indian Scientific Expedition to Antarctica in 2003–2004, the author collected 112 water samples from different lakes of Schirmacher Oasis, East Antarctica to study the diatom flora. Algae growths are abundant and readily visible on the surface of the rocks, boulders, weathered soils and