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Seasonal Fluctuations of Motile Aeromonads and Pseudomonads in a Cultured Pond of Mrigal, *Cirrhinus mrigala*, in Bangladesh

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Abstract

Ecological studies on *Aeromonas* spp. and *Pseudomonas* spp. in mrigal, *Cirrhinus mrigala*, culture ponds were conducted in two fish farms in Bangladesh. The prevalence of *Aeromonas* spp. was high in pond water and in slime of the sampled fish in both fish farms during the winter season, when many diseases, including Epizootic Ulcerative Syndrome (EUS), occur. *Pseudomonas* spp. was also found in high percentages in the same sampling sites. High amounts of *Aeromonas* spp. and *Pseudomonas* spp. were also found in apparently healthy fish during winter. Frequent isolation of *Aeromonas* spp. from lesions of the sampled fish indicated a link between these bacteria and the outbreak of fish diseases in the country. The detected *Aeromonas* spp. was resistant to various antibacterial agents tested. A high percentage of *Aeromonas* isolates were found to be resistant to erythromycin and oxytetracycline. However, only a few were resistant to oxolinic acid and streptomycin.

Introduction

Fish plays an important role in the food and economic sectors of Bangladesh. However, in the last few years, diseases have threatened to reduce fish production in Bangladesh. Since 1988, Epizootic Ulcerative Syndrome (EUS) has broken out in several parts of the country, causing severe mortality

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in freshwater, including cultured and wild fishes every year particularly during the cold winter months.

A similar disease has been reported in Australia (1972), Papua New Guinea (1975), Indonesia (1980), Thailand (1980), Malaysia (1981), Kampuchea (1984), Laos (1984), Burma (1984), and Sri Lanka (1988).

A number of etiologies have been suspected for the outbreak of EUS. These include *Rhabdovirus* (Frerichs 1988), the fungi *Aphanomyces* (Willoughby *et al.* 1995), the parasite *Trichodina* (Subasinghe 1993), and bacteria such as *Aeromonas*, *Pseudomonas*, and *Micrococcus* (Lilley *et al.* 1991). Though the etiology of this disease is still uncertain, it is believed that *Aeromonas*, an opportunistic pathogen, significantly contributes to the pathogenesis of the lesions that are symptomatic of EUS. The observation that this bacterium is frequently isolated from EUS lesions (Anonymous 1986; Llobrera and Gacutan 1987), and the fact that it possesses the potential to produce a wide range of virulence factors, including cytotoxins, hemolysins, proteases and nucleases (Ljungh and Wadstrom 1986; Cahill 1990), support the above suggestion. As *Aeromonas* spp. and *Pseudomonas* spp. are constantly found in EUS -affected fish and have the potential to cause fish diseases, the present study was undertaken for the purpose of knowing the seasonal fluctuations of these bacteria and the action of some antibiotics on randomly selected *Aeromonas* spp. from *C. mrigala* at two fish farms in Bangladesh.

Materials and Methods

Sampling sites

Two fish farms, the government fish farm at Trishal and Jhalak fish farm at Gouripur, in the district of Mymensingh, Bangladesh, were selected as sampling sites. Mrigal, *Cirrhinus mrigala* and water samples were collected for a period of one year at monthly intervals from January to December 1994. Both ponds were about 2000 to 2500 m^2 in area and 1.5 to 2.0 m in depth. Stocking density was about 1 fish/m^2. The fish were given commercially available or prepared feed (wheat bran 40%, rice bran 15%, mastered oil cake 30%, fish meal 5%, flour 5%, molasses 4.8% and soybean oil 0.2%). The fish samples were transported to the laboratory in an oxygenated plastic bag while the water sample was transported using a sterile bottle in an icebox.

Bacterial isolation and identification

Consecutive decimal dilutions were made for the water samples with sterile physiological saline and were inoculated on Tryptic Soya Agar (TSA). Slime samples of the fish were collected by scraping with a sterile scalpel and serially diluting ten-fold dilutions with physiological saline. These were then inoculated on TSA plates. To collect kidney samples, the fish were soaked in 70% ethanol, their abdomens were opened aseptically and the kidneys taken out with sterilized forceps. The kidneys were then homogenized with sterile physiological saline diluted with the same solution and inoculated on TSA plates. When lesions were found on the body surface of the sampled fish, special attention was made to obtain a swab on TSA plates. All the plates were incubated at 25°C for 36-48 h. The total bacterial load of the pond water and respective fish organs were determined by standard plate count method. Representative numbers of the most numerous colony types were picked up, purified and maintained on TSA slants. The isolates were identified to generic level based on the scheme proposed by Cowan and Steel's Manual (Barrow and Fetham 1993) and were confirmed with help from Bergey's Manual of Systematic Bacteriology, Vol. 1 (Krieg and Holt 1984).

Antibiotic sensitivity

A total of 60 strains of motile aeromonads were randomly selected from water and fish organs to know their susceptibility to various antibacterial agents. A suspension of the individual isolate was prepared in sterile physiological saline and spread over the Iso-Sensi-Test Agar (Oxoid). In each inoculated plate, six antibiotic discs, viz. oxytetracycline (30 mg/disc), potentiated sulphonamides (25 mg), erythromycin (10 mg), chloramphenicol (30 mg), oxolinic acid (2 mg) and streptomycin (10 mg) were dispensed uniformly with the help of an Oxoid Unipath Disc Dispenser Mark-II and incubated at 25°C for 24 h. The results of the drug sensitivity tests were recorded with zone of growth inhibition measured in mm and expressed as resistant (R) when the growth was normal or when there was no inhibition of growth.

Results

The average total bacterial load of the three replicated samples of pond water, slime, and kidney of fish sampled from Trishal fish farm varied from $2.1x10^3$ to $2.6x10^5$ CFU/ml of water, $4.0x10^4$ to $4.7x10^7$ CFU/g of slime and 0 to $1.7x10^4$ CFU/g of kidney, respectively (Fig 1a). On the other hand, average total bacterial load of the samples from Jhalak fish farm varied from $3.2x10^3$ to $1.8x10^6$ CFU/ml of water, $4.8x10^3$ to $1.4x10^8$ CFU/g of slime and 0 to $3.0x10^4$ CFU/g of kidney (Fig 1b).

Patterns of monthly variation in prevalence of *Aeromonas* spp. and *Pseudomonas* spp. in pond water, slime and kidney are shown in Figs. 2, 3 and 4, respectively. Both bacteria were found either in pond waters or in the fish samples during the study period. Their percentages gradually increased after the summer months and remained high during the winter months.

The detected bacterial flora from the lesions of the sampled fish are summarized in Table 1. Many bacterial groups, including *Aeromonas, Pseudomonas, Moraxella, Staphylococcus, Corynebacterium* and *Bacillus,* were isolated from the surface lesions of fish sampled during the winter season. No fish with lesions were observed at the Trishal fish farm in April, May, June, July, September, and October; and at the Jhalak fish farm, in March, April, May, July, August, and September.

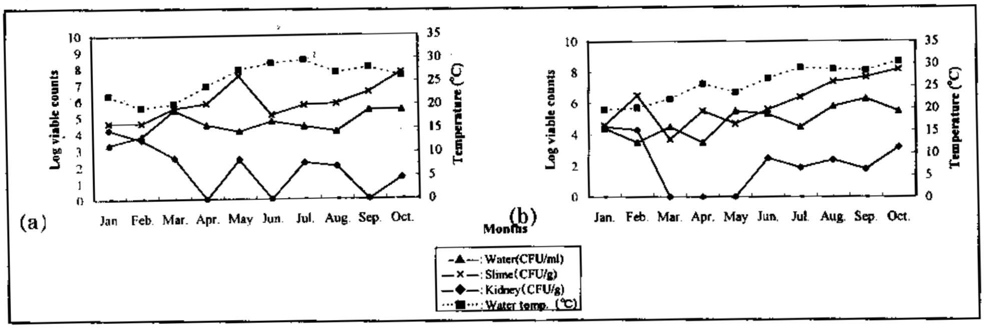

Fig. 1. Changes in bacterial load and pond water temperature at Trishal fish farm (a) and Jhalak fish farm (b).

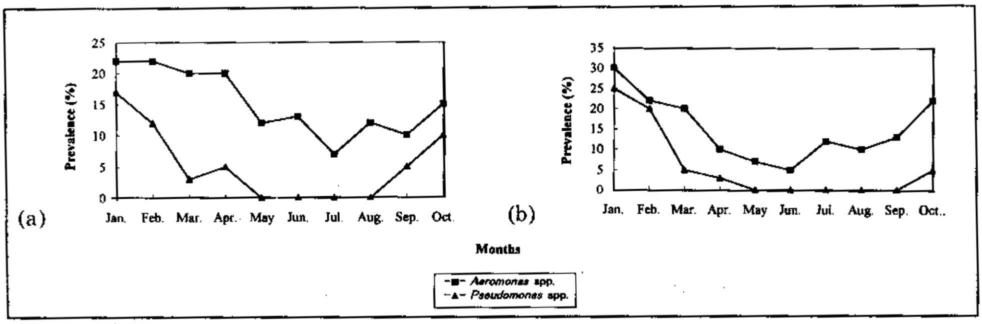

Fig. 2. Pattern of monthly variation in prevalence of *Aeromonas* spp. and *Pseudomonas* spp. isolated from the ponds at Trishal fish farm (a) and Jhalak fish farm (b).

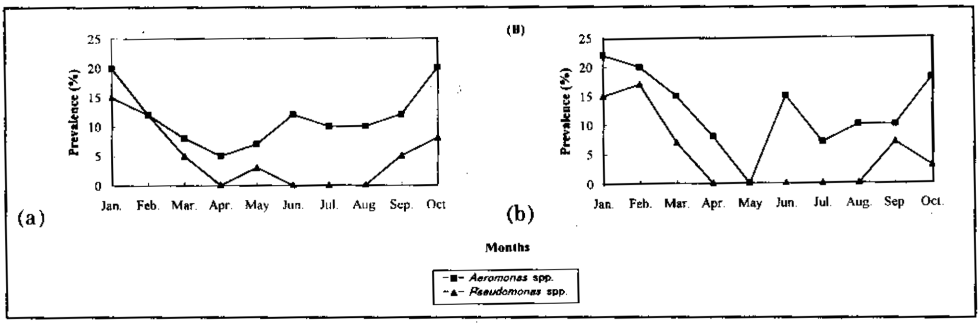

Fig. 3. Pattern of monthly variation in prevalence of *Aeromonas* spp. and *Pseudomonas* spp. isolated from the slime of sampled fish at Trishal fish farm (a) and Jhakal fish farm (b).

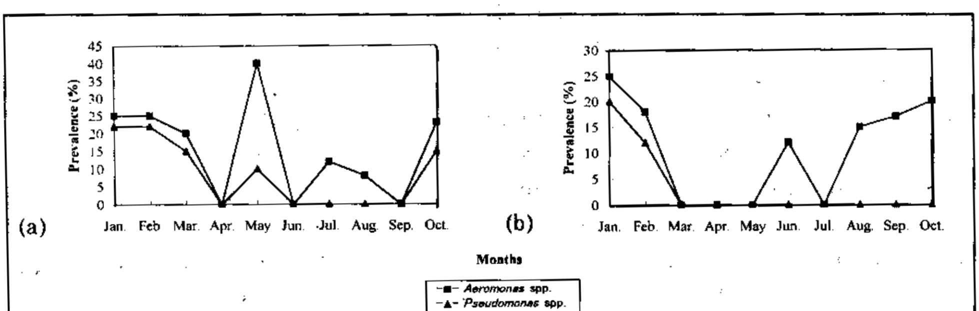

Fig. 4. Pattern of monthly variation in prevalence of *Aeromonas* spp. and *Pseudomonas* spp. isolated from the kidney of sampled fish at Trishal fish farm (a) and Jhalak fish farm (b).

Prevalence of resistant aeromonads to selected antibacterial agents is shown in Table 2. *Aeromonas* spp. showed about 80% resistance against erythromycin, 50% resistance against chloramphenicol and oxytetracylin, 30% resistance against sulphonamides and streptomycin and 15% resistance against oxolonic acid.

Discussion

Bacterial load in the pond water and on the body surface (slime) was heavy during the summer months. However, in the case of kidney, bacterial load was high during winter. In some cases, bacteria was not found from the kidney in high water temperatures (Fig. 1).

Dead organic matters in the water bodies increase in high water temperature. The higher the number of dead organic matters in the water environment, the greater the bacterial activities. The heavy bacterial load in the pond water may be due to the large amount of dead organic matters. Floods brought about by heavy rainfall during the summer months also may have allowed

Table 1. Bacterial genera/groups found in the lesions of fish samples from Trishal fish farm and Jhalak fish farm in January-December 1994.

Month	Trishal fish farm	Jhalak fish farm
January	*Aeromonas, Pseudomonas Moraxella*	*Aeromonas, Pseudomonas Micrococcus, Corynebacterium*
February	*Aeromonas, Pseudomonas, Moraxella, Staphylococcus*	*Aeromonas, Pseudomonas, Micrococcus*
March	*Micrococcus, Aeromonas, Bacillus Pseudomonas, Corynebacterium*	NL
April	NL	NL
May	NL	NL
June	NL	*Micrococcus, Aeromonas*
July	NL	NL
August	*Staphylococcus*	NL
September	NL	NL
October	NL	*Aeromonas*
November	*Aeromonas, Pseudomonas, Moraxella*	*Aeromonas, Pseudomonas*
December	*Aeromonas, Pseudomonas*	*Aeromonas, Pseudomonas, Micrococcus*

NL: No lesioned fish was found.

Table 2. Percentage of resistant *Aeromonas* spp. to selected antibiotics in Trishal fish farm and Jhalak fish farm found resistant to selected antibiotics.

Antibacterial agents	No. of isolates	Trishal Fish Farm	Jhalak Fish Farm
Chloramphenicol	60	40	50
Oxytetracycline	60	55	45
Potentiated sulphanomides	60	76	80
Erythromycin	60	76	80
Streptomycin	60	15	40
Oxolinic acid	60	5	30

various bacterial flora to enter the ponds since the embankments of these ponds were not so high. Since the body surface of fish is directly exposed to water, bacterial flora on the body surface (slime) was also relatively high during the summer months.

The amount of bacteria found in the kidney of sampled fish was high during the winter season, a seeming indication of an inverse relationship with pond water temperature. Generally, fish diseases, including EUS, occur during the winter months in Bangladesh. As there is no rainfall during the winter season, the water level of the pond decreases remarkably. So, fish are easily stressed by overcrowded or poor water quality during the winter season. These conditions expose aquatic animals to diseases caused by *Aeromonas*, *Pseudomonas* or any other bacteria. Large amounts of bacteria in the kidney reveal that the protective mechanisms of fish against bacteria may become weak during the winter season because of stress brought about by various adverse situations. In contrast, the bacterial load on the kidneys gradually declined or totally disappeared during summer, when the fish was apparently healthy.

The percentage of *Aeromonas* spp. and *Pseudomonas* spp. in pond waters and in fish organs (Figs. 2, 3 and 4) were found to be higher during winter. Usually, the kidney of healthy fish does not bear bacteria. But bacteria can be isolated from the fish when it is affected by disease. Large amounts of *Aeromonas* spp. and *Pseudomonas* spp. in the kidneys of sampled fish during the winter season reveal that the fish, even though apparently healthy, were really affected by disease. These *Aeromonas* spp. and *Pseudomonas* spp. may have contributed to the development of EUS or EUS-like lesions in the fish body. Jhingran and Das (1990) also isolated *A. hydrophila, P. fluorescens* and *E. coli* from the ulcerated lesions and hematopoetic tissues of EUS-affected fish, which correlates with the present study.

Though no lesioned fish was found in May, July, and October at Trishal fish farm, and in July, August, and September at Jhalak fish farm, the kidneys of the fish bore some bacteria, including *Aeromonas* spp. (Figs. 4 and 5), during these months. These results revealed that the bacteria might have invaded the host body at that time, though, from all outward appearances, the fish seemed to be healthy. The present study indicated the consistent association of *Aeromonas* spp. and *Pseudomonas* spp. with the lesions of disease affected fishes and suggest that these bacteria might have caused fish disease outbreak in Bangladesh. This is in agreement with other reports about EUS-affected fish. Lio-Po *et al.* (1990) investigated that certain phenons of *A. hydrophila* could induce EUS-like lesions in both snakehead and catfish in an experimental infection. Torres (1990) also reported that this bacterium is the most important pathogen in the EUS etiology. The fact that *A. hydrophila* adheres to fish cells and fish mucus and has the ability to invade epithelial cells (Krovacek *et al.* 1987) indicates the possibility of this bacterium causing disease in freshwater fishes.

The fishermen of Bangladesh have been extensively using various antibacterial agents in an attempt to control EUS since 1988, when the disease broke out in several parts of the country. But, in most cases, they do not follow the

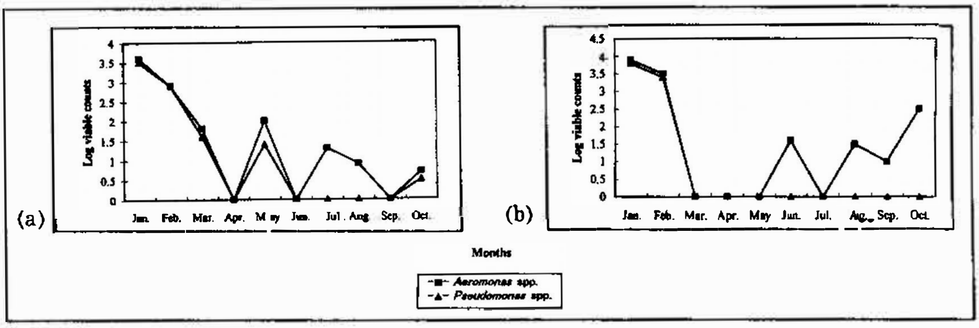

Fig. 5. Log number of viable counts of *Aeromonas* spp. and *Pseudomonas* spp. isolated from the kidney of sampled fish from Trishal fish farm (a) and Jhalak fish farm (b).

recommended dosages. This could be a cause for increased drug resistance to *Aeromonas* spp.

Fish biologists believe that *Aeromonas* spp. plays an important role in the development of EUS in fish. But, unfortunately, there are very few scientific studies on EUS and *Aeromonas* spp. The *Aeromonas* spp. that was studied in the present experiment has not yet been identified up to the species level. It needs further identification, including phenotypic and genotypic identification, to know the real species of *Aeromonas* associated with fish disease, including EUS, in Bangladesh.

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