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Bacteriological Studies on Fish Affected by Epizootic Ulcerative Syndrome (EUS) in Kerala, India

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Abstract

Bacteriological studies were conducted on fish affected by epizootic ulcerative syndrome (EUS) in Kerala, India, with emphasis on the hydrobiological features of the environment. Among the nine fish species analyzed, the most severe symptoms were observed in *Channa striatus*, *Wallago attu* and *Puntius*. The bacterial species isolated from the lesions and internal organs of these fishes showed a dominance of *Aeromonas hydrophila*. The frequency of isolation of *A. hydrophila* was low in fish showing mild symptoms and in apparently healthy fish collected from the affected areas. Repeated isolation of *A. hydrophila* from ulcerated parts of fish indicate that this organism could be responsible for the secondary infection. Water samples showed acidic pH, low salinity, and levels of mercury and cadmium within limits.

Introduction

Towards the end of August 1991, an outbreak of epizootic ulcerative syndrome (EUS) occurred in several parts of Kerala, in the southwestern part of India, causing largescale fish mortality. The disease was first observed in the freshwater lake of Kuttanad and rapidly spread to other freshwater lakes of Kerala. The disease was most severe in an area that had been receiving heavy rainfall between June and July, and was ravaged by floods four times in the season.

Fish varieties like *Etroplus suratensis*, *Puntius* sp., *Wallago attu* and *Channa striatus* were affected. Diseased fish developed large hemorrhagic lesions on the body surface, became sluggish and tended to keep to surface waters before dying.

A similar fish ailment had been reported from Australia (Burke and Rodgers 1981), Indonesia (Roberts et al. 1986), Sri Lanka (Frerichs 1988) and Bangladesh (Roberts et al. 1989). In India, reports of the occurrence of the disease came from Assam, the north eastern states of India (Das et al. 1990) and Uttar Pradesh (Chattopadhyay et al. 1990).

A number of aetiologies have been proposed for the outbreak of EUS in which the primary agent is thought to be virus, fungi, bacteria or parasites. Virus like *Rhabdovirus* (Frerichs 1988), fungi *Aphanomyces* (Callinan et al. 1990) and *Achlya* (Pichyangkura and Bodhalamik 1983), and chemoautotrophic

Nocardioform bacteria (Dastidar and Chakraborty 1992) have been frequently isolated from EUS-affected fish. Bacteria like *Aeromonas, Vibrio, Pseudomonas* and *Micrococcus* that are mostly opportunistic pathogens, have been associated with necrotic ulcers which are thought to be the secondary infections leading to death in severely ulcerated fish (Lilley et al. 1991).

The present paper is an account of the salient findings of the bacteriological and chemical studies conducted on diseased fish and the environment.

Materials and Methods

Fish samples were collected by cast nets from 11 locations where largescale fish mortality was reported. Both affected and apparently healthy fish were collected along with mud and water samples.

Altogether, 55 fish were obtained comprising nine species: *Puntius* sp., pearlspot (*Etroplus suratensis*), attuwalla (*Wallago attu*), oxeyed herring (*Megalops cyprinoides*), striped snakehead (*Channa striatus*), freshwater garfish (*Xenotodon cancila*), catfish (*Heteropneustes fossilis*), *Anabas* sp. and freshwater prawn (*Macrobrachium rosenbergii*). The fish were transported to the laboratory in sterile polyethylene bags under ice and examined immediately.

The specimens were examined for any gross external abnormalities before attempting bacteriological analysis. In the case of body lesions, Gram-stained smears were prepared for observation under microscope.

Bacteriological analysis of the fish followed the procedure outlined by Gillespie et al. (1974) and Frerichs and Hendrie (1985). The lesions material was directly inoculated into tryptic soy broth (Difco), and streaked on pre-set plates of tryptic soy agar (Difco), *Pseudomonas* isolation agar (Hi media), TCBS agar (Oxoid), Muellor Hinton agar and Rimler Shott agar (Hi media). The internal parts (muscle, liver and kidney) were dissected out and analyzed as in the case of lesions material.

The inoculated plates and tubes were incubated at 37°C for 18-20 h. Bacteria were recovered from the tubes by streaking loopfuls of suspension on plates of tryptic soy agar, *Pseudomonas* isolation agar, TCBS agar, Muellor Hinton agar and Rimler Shott agar. Representative numbers of the most numerous colony type were isolated, purified and maintained on tryptic soy agar at 29±1°C. The bacterial isolates were identified up to generic or species level based on the scheme proposed by Shotts and Bullock (1975). In addition to various confirmatory and supplementary tests mentioned in the scheme, the hemolytic and proteolytic ability of the isolates were also determined. The antibiotic spectrum of the isolates were determined by placing antibiotic discs (Pasteur Biological Laboratories [India], Gujarat) listed in Table 1 on a lawn of young culture, grown in nutrient agar incubating at 37°C and noting the diameter of the inhibitory zone around each disc.

The water samples were subjected to tests for pH, (Systronics, Bombay) dissolved oxygen and salinity (Stainton et al. 1974). For bacteriological analysis, appropriate dilutions of the water samples were spread plated on tryptic soy agar. After incubation at 37°C for 24 h, typical colonies were picked for identification (Gillespie et al. 1974). Mercury and cadmium in the water were also

estimated by wet digestion method, as these two elements are often suspected in this area (AOAC 1976). Mercury was analyzed using a mercury analyzer (ECIL model MA5800) by cold vapor absorption, and cadmium using GBC 902 Atomic Absorption Spectrophotometer.

Table 1. Sensitivity of the major bacterial isolates toward some common antibiotics.

	Antibiotic spectrum of the bacterial isolates				
Antibiotic	*A. hydrophila*	*A. calcoaciticus*	*E. coli*	*Pseudomonas*	*Arthrobacter* sp.
Amoxycillin	NA	NA	NA	NA	NA
Carbencillin	NA	NA	NA	NA	NA
Cephaloridine	NA	R	S	NA	NA
Cloxocillin	NA	NA	NA	NA	NA
Gentamycin	S	S	S	S	S
Kanamycin	M	S	M	NT	S
Neomycin	M	M	R	R	M
Nalidixic acid	S	S	S	R	S
Ampicillin	NA	S	S	NA	S
Bacitracin	S	NA	NA	NA	NA
Chloromycetin	S	M	S	R	M
Nitrofurantoin	S	R	S	NA	R
Sulphadiazene	R	M	NA	S	NA
Penicillin G	NA	NA	NA	NA	NA
Streptomycin	R	NA	NA	S	NA
Terramycin	S	S	S	NA	NA
No. of cultures tested	16	2	5	2	5

R - resistant, M - Intermediate, S - sensitive, NT - Not tested, NA - No action
Based on zone size interpretative chart, Span Diagnostics, Surat, India.

Results

The symptoms and the degree of infection of the fish collected from the affected areas differed considerably. Table 2 presents a description of their external appearance and major symptoms. Apparently healthy fish showing no external symptoms of the disease were considered as category A; fish with mild inflammation and degeneration of epidermis as category B. The most severe cases with massive inflammation or ulcerations were placed in category C and observed for *C. striatus, W. attu* and *Puntius* sp. (Fig. 1). All the fish in this group exhibited infection. In category B, all the fish were also infected, but the infection was milder and ulcerations were absent.

The internal organs of the clinical and subclinical cases failed to give evidence of a gross pathological condition except petechial hemorrhagic spots on the dorsal muscle in the case of *C. striatus*, and serum exudations in the visceral cavity of *E. suratensis*.

The bacterial species isolated from the fish collected from EUS-affected areas are summarized in Table 3. Of the nine fish species analyzed, six (66%) carried *A. hydrophila*. The lesion material consistently revealed the presence of *A. hydrophila*, but they were isolated from liver and muscle tissues. In category A fish, muscle, liver, blood and kidney were screened. But only the muscle portion yielded bacterial colonies.

Table 2. Description of the fish species collected from EUS-affected areas in Kerala, India, 1991.

Fish species	No. of fish examined*	Location	Category	Clinical symptoms
Puntius	2 (2)	Keettupadam	C	Hemorrhagic lesion on skin surface, putrid odor, degeneration of epidermal tissue, necrotic
Etroplus suratensis	2 (2)	Kollagiri, Kolathrapadam	B	No external lesions but petechial hemorrhagic spots on body, mouth and anal fin exophthalmia
Wallago attu	3 (3)	Kollagiri	C	No hemorrhagic lesions but massive degeneration of epidermis; highly putrid odor
Megalops cyprinoides	10 (1)	Kannadichal	A	No visible symptoms except hemorrhagic lesion on head of one fish
Channa striatus	2 (2)	Kannadichal	C	Large deep ulceration; highly necrotic with hemorrhagic spots; head eroded
Xenentodon cancila	8 (0)	Keettupadam	A	No visible symptoms
Heteropneustes fossilis	3 (3)	Keettupadam	B	Multiple inflammatory spots on head and fin; necrotic degeneration of epidermis
Anabas sp.	9 (0)	Keettupadam	A	Externally normal
Macrobrachium rosenbergii	16 (1)	Keettupadam	A	No visible symptoms except head erosion on one animal

* Values in parentheses show the number of infected fish.

Enterobacteriaceae constituted the second dominant member and included two species, *Escherichia coli* and *Enterobacter cloacae*, occurring in 33% each of the fish species. They were isolated mainly from the muscle enrichments. The *Arthrobacter* sp. were isolated from 44% of the fish species. The other bacterial species viz., *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa*, were present in 33% and 22% of the fish, respectively. *Micrococcus* sp. (22%) and *Bacillus* sp. (11%) were also isolated. Table 4 presents the rate of prevalence of various bacterial species in the different categories of fish. Out of 55 bacterial cultures isolated from the fish, *A. hydrophila* constituted the major component (40%) of the total isolates, followed in the order of prevalence by *Arthrobacter* sp., *Escherichia coli* and *Acinetobacter calcoaceticus*. The Gram positives formed only 25% of the total isolates.

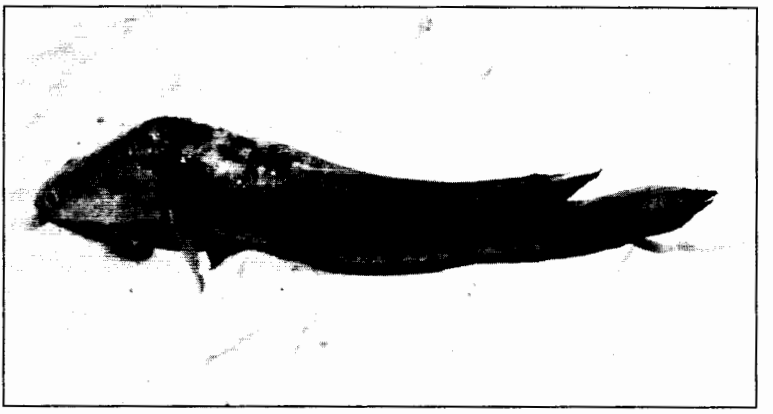

Fig. 1. Infected specimen of *Channa striatus* showing deep ulcerations in the head region.

Table 3. Occurrence of various bacterial species in fish collected from EUS-affected areas, Kerala, India.

Fish species	*A. hydrophila*	*E. coli*	*E. cloacae*	*Pseudomonas*
Puntius sp.	X			X
Etroplus suratensis	X	X	X	X
Wallago attu	X			
Megalops cyprinoides				
Channa striatus	X		X	
Xenentodon cancila	X	X		
Heteropneustes fossilis		X		
Anabas sp.				
Macrobrachium rosenbergii	X		X	

Fish species	*A. calcoaceticus*	*Arthrobacter* sp.	*Micrococcus* sp.	*Bacillus* sp.
Puntius sp.		X		
Etroplus suratensi	X	X		X
Wallago attu		X		
Megalops cyprinoides				
Channa striatus	X			
Xenentodon cancila		X	X	
Heteropneustes fossilis	X			
Anabas sp.				
Macrobrachium rosenbergii				

Table 4. Species-wise distribution of bacteria in the fish.

Bacterial types	No. of isolates	%
Aeromonas hydrophila	16	40
Escherichia coli	5	12.5
Enterobacter cloacae	2	5
Pseudomonas aeruginosa	2	5
Acinetobacter calcoaceticus	3	7.5
Arthrobacter sp.	7	17.5
Micrococcus sp.	2	5
Bacillus sp.	1	2.5
Others	2	5

The antibiogram of the major bacterial groups were determined (Table 1). Of the 16 antibiotics tested, chloromycetin was most effective to the *A. hydrophila* species. The other antibiotics, gentamycin, kanamycin, neomycin, bacitracin and nadidixic acid, were also effective.

Water collected from the locations showed the presence of *Arthrobacter, Micrococcus* and *Bacillus* species; and the mud carried *Pseudomonas, Arthrobacter, Bacillus* and *Aeromonas* sp. The values for pH were distinctly acidic, and salinity was very low. The levels of mercury and cadmium were also within limits (Table 5).

Table 5. Hydrographic conditions of the water from various EUS-affected areas.

Location	Mercury (ppm)	Cadmium (ppm)	pH	Dissolved oxygen ($mg \cdot l^{-1}$)	Salinity (ppt)
Kollagiri	0.05	0.177	6	2.6	0.12
Kannakari	0.02	0.165	5.4	3.8	0.3
Kolathrapadam	0.05	0.038	6.5	3.0	0.17
Mankuzhi	0.09	0.139	4	2.9	0.26
Veliyam	ND	ND	5.2	3.4	0.3
Keettupadam	0.05	0.048	5.5	3.6	0.28
Kannadichal	0.10	0.170	4.8	2.8	0.16

ND - not determined

Discussion

The fish in Kerala exhibiting symptoms of EUS belonged to a wide variety and covered most of the fish species reported in India (Jhingran and Das 1990) and other Southeast Asian countries (Lilley et al. 1991). In the present study, *C. striatus* was found to carry the most severe symptoms.

The study revealed the dominance of *A. hydrophila* over other microbial species by the frequency of isolation from the fish samples and its abundance. This microorganism has been consistently isolated from lesion and muscle tissue of fish from India (Kumar et al. 1990) and countries like Thailand (Tonguthai 1985), Malaysia (Wong and Leong 1987), Indonesia (Dana 1987), Myanmar (Roberts et al. 1986) and Sri Lanka (Balasurya 1987). While *A. hydrophila* is a common inhabitant of healthy fish (Wong and Leong 1987) and the aquatic system (Kaper et al. 1981), it is also an established opportunistic pathogen infecting fish under physiological or environmental stress (Groberg et al. 1978; Lio-Po and Duremdez-Fernandez 1986).

Although *A. hydrophila* was isolated from lesions and internal organs of the fish, there were also instances where fish showing mild symptoms did not carry this bacterium. The authors failed to isolate this microorganism from category A, i.e., normal fish. This points to the possibility that the primary cause of the disease is agents other than *A. hydrophila*; but the ulcerative lesions may be due to *A. hydrophila*. We also observed that the *A. hydrophila* strains were highly proteolytic and β-hemolytic. Chattopadhyay et al. (1990) isolated (cytotoxic enterotoxigenic) strains of *A. hydrophila* from many ulcerated fish, but

none from normal fish. The pathogenic nature of *A. hydrophila* is further emphasized by the study of Lio-Po and Duremdez-Fernandez (1986) who showed that unscaled fish remained unaffected at a challenge dose of 10^5 cells·ml^{-1} of water medium, whereas descaled fish developed acute hemorrhage and septicemia at a lesser dose and in a shorter period. In the present study, the level of *A. hydrophila* in the water systems was low, being less than 1,000 cells·ml^{-1} (unpubl. data).

In addition to *A. hydrophila*, several other bacterial types were encountered in the present investigation. *P. aerugenosa, Acinetobacter calcoaceticus, Micrococcus,* etc., were present in the fish in Kerala. Austin and Austin (1985) in their review on bacterial pathogens, cited a variety of bacteria causing ulcerative diseases. *Pseudomonas* species have been frequently reported to cause ulcerative fish diseases (Bullock et al. 1965; Li and Flemming 1967; Wong and Leong 1987). In another study, *Micrococcus* sp., in association with *A. hydrophila, P. fluorescens* and *E. coli* was isolated, and the ulcerated lesions and hematopoetic tissues consistently showed the presence of *Micrococcus* sp. (Jhingran and Das 1990). The presence of *E. coli* and *E. cloacae* in the fish points to the possibility of sewage pollution. *Arthrobacter* and *Acinetobacter* strains are common inhabitants of soil and water and hence their presence in the fish is expectable. It has been estimated that 0.001% of the heterotrophic aerobic population of soil and water is composed of *Acinetobacters* (Baumann 1968). Most of the bacterial species reported in this study were isolated in previous studies from fish collected from Assam, Tripura, Meghalaya and West Bengal in India (Kumar et al. 1990). The sensitivity of the bacteria collected from these fish towards a large number of antibiotics indicates that the area is not frequently exposed to antibiotic residues.

The abiotic factors, such as temperature, eutrophication, sewage and pollution, that result in sublethal stress to fish are considered important in initiating the disease (Lilley et al. 1991). Acidic water may damage the gill structure and epidermis of fish and lower their resistance to infection. Low salinity, as observed in this study, makes fish less tolerant to environmental toxins.

Although the aetiology of EUS is still uncertain, it has been conclusively shown that the underlying cause is an infectious biological agent (Lilley et al. 1991). Repeated isolation of *A. hydrophila* from the lesions of infected fish in this study points to the possibility of a secondary infection due to *A. hydrophila* when the fish is under stress.

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