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Bacteriological Studies on Fishes Affected with Epizootic Ulcerative Syndrome

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Abstract

Epizootic ulcerative syndrome is one of the most well known fish diseases that has caused disastrous effects throughout India after its first outbreak in 1988. Since then recurrences in varying intensities are reported every year. Bacteriological examination of the ulcerated area of affected *C. punctatus*, *Puntius sp.* and *Mystus sp.* revealed the presence of 16 strains of bacteria belonging to the genus *Pseudomonas, Aeromonas, Micrococcus, Bacillus, Vibrio* and *Moraxella* among which *Pseudomonas* and *Aeromonas* were the most common. Pathogenicity tests with all the isolates showed that only six strains belonging to the genus *Aeromonas* and *Pseudomonas* were pathogenic while the rest were nonpathogenic. The distinct inconsistency in the type of pathogenic bacteria isolated from the three fish species suggests that these are opportunistic pathogens that have invaded the fish secondarily.

Introduction

At present epizootic ulcerative syndrome (EUS) is one of the most common and most dreaded diseases of fresh and brackishwater fishes in India. Severe losses have been reported from all types of water bodies. Considerable amount of research activity is currently focused on the identification of major causative agents and their role in pathogenesis. Scientists working both in India and abroad claim that bacterial pathogens play a major role in the disease outbreak. Studies on affected fishes in different countries recorded a wide range of pathogenic bacteria, the most predominant being *aeromonads* and occassionally *pseudomonads* isolated from the ulcerated area and the internal organs such as kidney, liver, intestine and gills of affected fishes (Das 1997). Along with the bacteria, fungal species were also consistently isolated from the lesions of affected fishes (Reantaso 1992, Callinan et al. 1993, Roberts et al. 1993).

Soon after EUS struck India in 1988, the disease has resulted to insurmountable misery to the fishing community throughout the state of West Bengal (Das 1988, Jhingran 1990, Kumar et al. 1991, Pradhan 1992). Bacteria,

usually *aeromonads* and *pseudomonads*, were found to be associated with the skin lesions of the affected fishes (Pal and Pradhan 1990, Pradhan 1992).

EUS is endemic in many countries and is still extending its geographical range even into subtropical, subtemperate and temperate climates (Das 1997). At present, a declining trend has been observed mainly due to excessive application of various chemotherapeutic agents by cautious farmers. This helped check the severity of the disease outbreak although recurrences are still reported every year. Moderate to severe outbreaks are being reported from areas which used to be unaffected. Pal (1996, 1997) reported outbreaks of the disease in West Bengal every year since its first occurrence in 1988. This indicates that the primary source of the pathogen or the transmission factors are not yet controlled. Therefore, identification of the exact pathogen is of utmost importance to curb this dreaded disease. The main objective of this research was to study the bacterial species that may be present at the site of the ulcerative lesion of affected fishes and their pathological effects, if there are any, on healthy fishes.

Materials and Methods

Collection of diseased fishes

In November 1993, an outbreak of EUS was reported in some areas of the Midnapur District of West Bengal in *Channa punctatus*, *Puntius* sp. and *Mystus* sp. that were among the worst affected species. The severely affected fishes normally contained one deep ulcer and one or two moderate ulcers in the dorsal or tail region. Infected individuals of these three species with severe ulcerative lesions were collected from affected ponds and used for histopathological examination and bacterial isolation.

Histopathology

Tissue samples from the surface lesions were routinely processed for light microscopy and stained with haematoxylin and eosin (H-E) (Schäperclaus 1986).

Isolation of bacteria

The severely ulcerated areas were dissected out aseptically from the fishes. Afterwards surface sterilization was done with 0.1% mercuric chloride (Pal et al. 1978). The dissected tissues were placed in 100 ml conical flasks containing 15 ml nutrient broth supplemented with glucose (Pradhan 1992). At least three individuals of each species were used for the isolation. The flasks were incubated at 30ºC for 72 hours. The tissues were then removed and the cultures were observed under the microscope. Different types of bacteria were found to be present in the cultures. Then, the cultures were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) and 1 ml of each dilution was

added to 20 ml nutrient agar in 90 mm diameter sterile petridishes. Single, separate colonies on the agar plates were selected at random according to standard methods and streaked on to nutrient agar slants and incubated for 24 h at 30ºC. Thus, isolated pure cultures were obtained. Code names were given to each of the isolates and stored at 4ºC for characterization and identification.

Morphological and biochemical tests for characterization of the isolated bacteria

For characterization of the bacteria that were isolated from the ulcers, a number of specific physiological and biochemical examinations were performed following standard methods (Cowan and Steel 1993).

Pathogenicity test

Experimental induction of skin ulcers by all bacteria that were isolated was attempted in *C. punctatus* (average weight 20 g) and *Heteropneustes fossilis* (average weight 15 g) according to the method of Pradhan and Pal (1990) with some modifications. The fishes, approximately one year old were collected from nearby fish farms of Sonapur and Gangarampur of the Darjeeling District of West Bengal that did not have any previous report of EUS outbreak. All fishes were maintained in glass aquaria measuring 90 x 35 x 35 cm. Water temperature was maintained at 28 to 30ºC. Intramuscular injection was given with 0.05 ml of cell suspension containing 1 x 10^7 cells ml^{-1} of each isolate in 0.85% NaCl. Each isolate was injected into 10 fishes of each species. The control set of fishes received 0.05 ml of sterile saline. The fishes were observed for 15 days and mortalities, if there were any, were recorded.

Results

Histopathological examination of the ulcer region of the affected fishes showed complete loss of the epidermis. Necrotic granulomatous response was noted in the dermal layer which confirmed that the disease was epizootic ulcerative syndrome (Fig. 1).

Five to six types of bacteria were isolated from the skin lesions of *C. punctatus* (Table 1), *Mystus* sp. (Table 2) and *Puntius* sp. (Table 3).

The *C. punctatus* isolates included four *Pseudomonas*, one *Bacillus* and one *Aeromonas*. The *Mystus* sp. isolates included three *Aeromonas*, one *Moraxella* and one *Pseudomonas*. The *Puntius* sp. isolates included one *Micrococcus* and one *Pseudomonas*. The rest were identified as motile *Aeromonas* spp., *Pseudomonas* and *Aeromonas* were among the most common bacteria present in the ulcers (Table 4).

Out of the 16 bacteria that were isolated only A01, A02, A03, A06, P02 and P06 were found to induce moderate ulcers in the healthy fishes of both

Table 1. Morphological and biochemical characteristics of bacteria isolated from ulcers of *Channa punctatus**.

	Bacterial isolates					
	A01	B01	P01	P02	P03	P04
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Occurrence	Mostly in singles	Singles, pairs, chains	Singles, pairs, chains	Singles, pairs, chains	Singles, pairs, chains	Singles, pairs, chains
Size	2.6-3.0 x 0.7-0.8 µm	2.7-3.5 x 0.75-0.8 µm	2.0-2.5 x 0.75-0.8 µm	2.0-2.5 x 0.75-0.8 µm	2.5-3.0 x 0.7-0.8 µm	2.2-0.3 x 0.7-0.8 µm
Spore	-	+	-	-	-	-
agar colonies	Circular, smooth, convex	Circular, smooth, convex	Circular, smooth, flat	Circular, smooth, flat	Circular, smooth, flat	Circular, smooth, flat
Culture in nutrient broth	Turbid	Turbid with pellicle and sediments	Turbid with pellicle	Turbid with pellicle	Turbid with pellicle	Turbid with pellicle
Gram reaction	-	+	-	-	-	-
Motility	+	+	+	+	+	+
Growth at :						
25ºC	m	m	m	m	m	m
30ºC	g	g	g	g	g	g
37ºC	g	g	g	g	g	g
42ºC	n	m	n	n	n	n
Indole production	+	-	-	-	-	-
M-R	+	-	-	-	-	-
V-P	+	-	-	-	-	-
Nitrate	+	+	+	+	-	-
Gas from glucose	+	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+
O-F Test	F	O	O	O	O	O
Acid from :						
Glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+
Sorbitol	-	-	+	-	+	+
Sucrose	+	+	+	+	+	+
m-inositol	-	-	+	-	-	+
Mannitol	+	+	+	+	+	+
Adonitol	-	-	-	-	-	-
Levan from sucrose	-	-	-	-	+	+
Arginine hydrolysis	+	-	+	+	+	+
H_2S from cystein	+	-	-	-	-	-
Citrate utilization	+	-	+	+	+	+
Pigment formation	-	-	+[a]	+[b]	+[b]	+[b]

*+ = positive; - = negative; g = good growth; m = moderate growth; n = no growth; O = oxidative; F = fermentative.

[a]reddish brown diffusible pigment produced in King's B medium.

[b]greenish yellow diffusible pigment produced in King's B medium.

Table 2. Morphological and biochemical characteristics of bacteria isolated from ulcers of *Puntius* sp*.

	Bacterial isolates				
	A02	A03	A04	C01	P05
Shape	Rod	Rod	Rod	Sphere	Rod
Occurrence	Mostly in singles	Mostly in singles	Mostly in singles	Singles, pairs, tetrads or in irregular clusters	Singles, pairs, chains
Size	2.5-3.2 x 0.7-0.8 µm	2.5-3.0 x 0.65-0.8 µm	2.8-3.2 x 0.75-0.8 µm	1.2-1.6 µm diameter	2.5-3.0 x 0.5-0.6 µm
Spore	-	-	-	-	-
agar colonies	Circular, smooth, convex	Circular, smooth, convex	Circular, smooth, convex	Circular, smooth, convex	Circular, smooth, slightly convex
Culture in nutrient broth	Turbid	Turbid	Turbid	Turbid with pellicle and sediments	Turbid with pellicle and sediments
Gram reaction	-	-	-	+	-
Motility	+	+	+	-	+
Growth at:					
25ºC	m	m	m	m	m
30ºC	g	g	g	g	g
37ºC	g	g	g	g	g
42ºC	n	n	n	n	m
Indole production	+	+	+	-	-
M-R	w	+	+	-	-
V-P	-	+	+	-	-
Nitrate	+	+	+	w	+
Gas from glucose	+	+	+	-	-
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+
O-F Test	F	F	F	O	O
Acid from:					
Glucose	+	+	+	+	+
Fructose	+	+	+	+	+
L-Arabinose	-	+	+	-	+
Sorbitol	-	-	-	+	-
Sucrose	+	+	+	+	-
m-inositol	-	-	-	+	-
Mannitol	+	+	+	+	+
Adonitol	-	-	-	-	-
Levan from sucrose	-	-	-	-	-
Arginine hydrolysis	-	+	+	-	+
H_2S from cystein	+	+	+	-	-
Citrate utilization	+	+	+	+	+
Pigment formation	-	-	-	Bright yellow colonies	+[a]

*+ = positive; - = negative; g = good growth; m = moderate growth; n = no growth; w = weak; O = oxidative; F = fermentative.

[a]greenish yellow diffusible pigment produced in King's B medium.

Table 3. Morphological and biochemical characteristics of bacteria isolated from ulcers of *Mystus* sp*.

	Bacterial isolates				
	A05	A06	M01	P06	V01
Shape	Rod	Rod	Rod	Rod	Rod
Occurrence	Mostly in singles	Mostly in singles	Singles, pairs, chains	Singles, pairs, chains	Mostly in singles
Size	2.8-3.3 x 0.7-0.75 µm	2.5-3.0 x 0.7-0.8 µm	1.5-1.7 x 0.9-1.2 µm	2.7-3.5 x 0.75-0.8 µm	2.6-3.0 x 0.68-0.75 µm
Spore	-	-	-	-	-
agar colonies	Circular, smooth, convex	Circular, smooth, convex	Circular, smooth, convex	Circular, smooth, slightly convex	Circular, smooth, convex
Culture in nutrient broth	Turbid	Turbid	Turbid with sediments	Turbid with pellicle and sediments	Turbid with sediments
Gram reaction	-	-	-	-	-
Motility	+	+	+	+	+
Growth at:					
25ºC	m	m	m	m	m
30ºC	g	g	g	g	g
37ºC	g	g	g	g	g
42ºC	n	n	n	n	n
Indole production	+	+	-	-	-
M-R	+	+	-	-	-
V-P	+	+	-	-	-
Nitrate	+	-	-	+	+
Gas from glucose	+	+	-	-	-
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Gelatin hydrolysis	+	+	-	+	+
O-F Test	F	F	O	O	F
Acid from:					
Glucose	+	+	-	+	+
Fructose	+	+	-	+	+
L-Arabinose	+	-	-	+	+
Sorbitol	-	-	-	+	-
Sucrose	+	+	-	+	+
m-inositol	-	-	-	+	-
Mannitol	+	+	-	+	+
Adonitol	-	-	-	-	-
Levan from sucrose	-	-	-	-	-
Arginine hydrolysis	+	+	+	+	+
H_2S from cystein	+	-	-	-	-
Citrate utilization	+	+	-	+	+
Pigment formation	-	-	-	+[a]	-

*+ = positive; - = negative; g = good growth; m = moderate growth; n = no growth; O = oxidative; F = fermentative.

[a]greenish yellow diffusible pigment produced in King's B medium.

species and caused mortalities. The external clinical signs appeared in some fishes within 48 h of inoculation with the isolates. Initially, the area around the injection site turned reddish. It swelled gradually and around the small red spot (3 to 4 mm diameter) a zone of discoloration of the skin was noticed. The scales in *C. punctatus* were almost intact and only the mucous layer was affected. No notable change in the swimming behavior was observed at this stage. After 72 h, the red spots grew in size (15 to 20 mm in diameter), the skin and underlying muscle layer was eroded and it developed into a moderate ulcer, similar in appearance to that of the naturally affected fishes (Viswanath et al. 1997). The fishes became sluggish with irregular opercular movement. Hemorrhage at the injection site was observed in some fishes of both species injected with A06 bacteria. In the control set of fishes, no change was noticed within 15 days of observation. Healing was noted in all affected fishes after seven days of inoculation. Thus ulcers developed only up to moderate stage and did not proceed to the severe stage.

The percentage mortality in *C. punctatus* induced by isolates A01, A02, A03 and A06 were 10%, 5%, 10%, 15%, respectively while those induced by P02 and P06 were 10% and 0% respectively. (Fig 2). The percentage mortalities in *H. fossilis* induced by isolates A01, A02, A03 and A06 were 15%, 5%, 10%, 20%, respectively while those induced by P02 and P06 were 10%, and 5%, respectively. No mortality was recorded in fishes inoculated with the rest of the bacterial isolates in both species even after 15 days of observation.

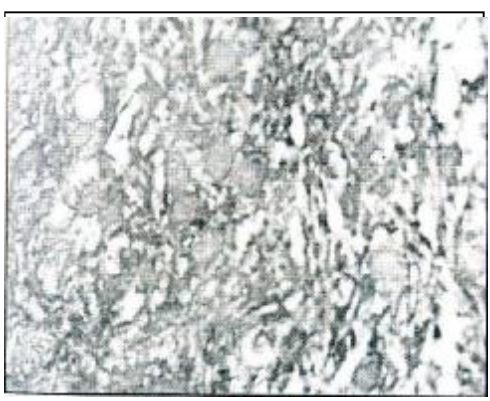

Fig. 1. A section of deep ulcer showing granulomatous and necrotic changes in the muscle of naturally infected *Channa punctatus*.

Discussion

The morphological features and biochemical profile of P01, P02, P03, P04, P05 and P06 reveal that these bacteria were gram negative motile

Table 4. Bacteria isolated from surface ulcers of naturally infected fishes.

Type of isolated bacteria	Source fish	No. of strains	Isolate numbers	Total no. of strains of each type of bacteria
Bacillus	*C. punctatus*	1	B01	1
Aeromonas	*C. punctatus*	1	A01	
	Puntius sp.	3	A02, A03, A04	6
	Mystus sp.	2	A05, A06	
Moraxella	*Mystus* sp.	1	M01	1
Pseudomonas	*C. punctatus*	4	P01,P02,P03,P04	
	Puntius sp.	1	P05	6
	Mystus sp.	1	P06	
Micrococcus	*Puntius* sp.	1	C01	1
Vibrio	*Mystus* sp.	1	V01	1

rods, catalase positive, oxidase positive, nonspore forming, utilized glucose oxidatively, and except for P01, all produced yellow-green pigment which diffused into the medium when grown in medium B of King. (Tables 1 to 3). Thus, they belonged to the genus *Pseudomonas* (Stanier et al. 1996, Palleroni 1984). P01 satisfied most of the characteristics of *Pseudomonas fluorescens* but it produced a reddish brown pigment instead of the usual yellow-green pigment and was capable of growing at 42ºC. So it was not regarded as *P. fluorescens*. Some strains of *P. aeruginosa* produce a dark red (Palleroni 1984) or brown (Cowan and Steel 1993) pigment and this bacteria is also capable of growing at 42ºC. However, P01 differed from *P. aeruginosa* in that it was capable of producing acid from sucrose, sorbitol and m-inositol. Ajellow and Hoadly (1976) have reported a fluorescent pseudomonad capable of growing at 41ºC but distinct from *P. aeruginosa*. Pal and Pal (1986) reported isolation of a fluorescent pseudomonad from the epithelial carcinoma of *Anabas testudineus*, which had similarities with *P. fluorescens* but was capable of growing at 42ºC. The biochemical tests that were done did not give any clue as to which species P01 may belong. Thus a further detailed study is awaited in order to decide its taxonomic status and nomenclature.

Pigment production, inability to grow at 41ºC and acid production from sucrose indicate that P02, P03, P04 and P06 resembled *P. fluorescens*. Denitrification ability, inability to produce levan from sucrose and acid from sorbitol and m-inositol suggested that P02 belonged to *biovar III*. However, this strain differed from *biovar III* in that it was able to produce acid from sucrose.

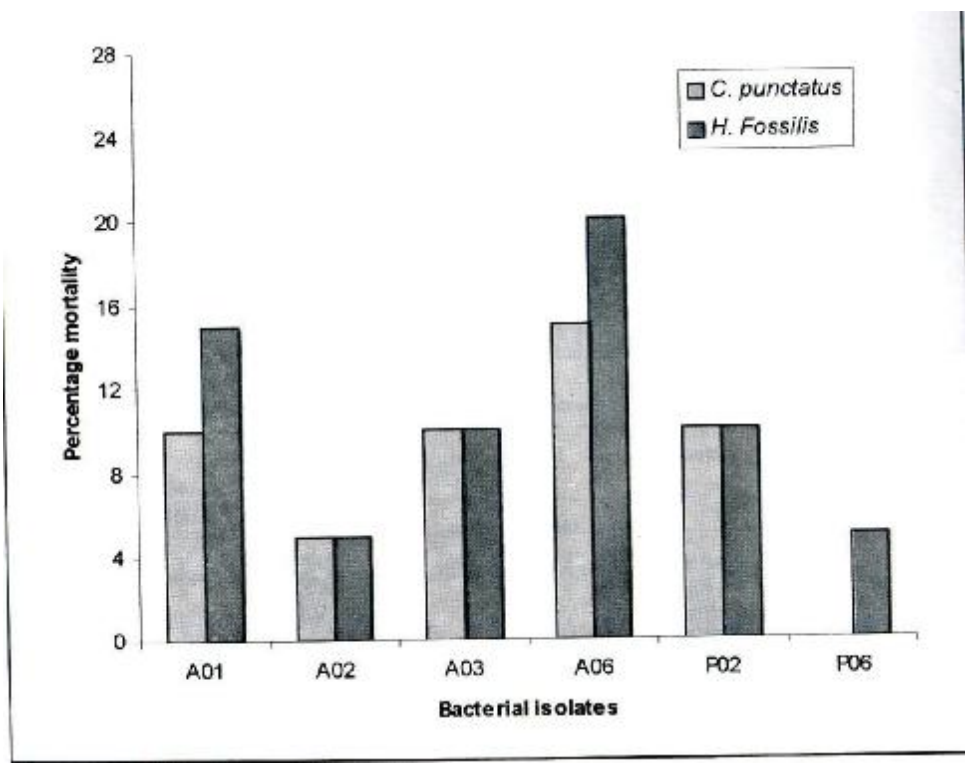

Fig. 2. Pathogenicity of the bacteria isolated from ulcers of fishes affected with EUS.

P03 and P04 could not reduce nitrate and was unable to produce acid from adonitol. The overall biochemical characters were similar to *P. fluorescens biovar V*. All reactions of P03 and P04 were similar except that P03 could not produce acid from m-inositol. P05 like P01 was capable of growing at 42ºC, but unlike P01, it was able to produce acid from sucrose, sorbitol, m-inositol and adonitol. Thus it resembled *P. aeruginosa* (Palleroni 1984) but it did not produce pyocyanin in King's A medium.

The biochemical profile of P06 indicated that it had similarities with *biovar II* and *IV* of *P. fluorescens* but based on the tests that were done, it was not clear to which of the two biovars it exactly belonged. Thus a more detailed characterization is required in order to specify its exact taxonomic status.

Isolates A01, A02, A03, A04, A05 and A06 were gram negative straight motile rods, catalase positive, oxidase positive, indole positive, reduced nitrate to nitrite and utilized glucose by fermentation. Thus they belonged to the genus *Aeromonas* (Popoff 1984). They were not considered to belong to the genus *Vibrio* because all the isolates produced gas from glucose. Species of *Vibrio* except *V. fluvialis* biotype II and *V. gazogenes* did not produce gas from glucose (Baumann and Schubert 1984). *V. fluvialis* biotype II was curved rod and gave a negative indole test, while *V. gazogenes* produced a red pigment and gave a negative oxidase test. Morphologically they were distinct from *Aeromonas salmonicida* in that all of them were motile straight rods mostly in singles, some in pairs and there were no chains or clumps of coccobacilli. Moreover, all the isolates were able to grow at 37ºC while none of the strains of *A. salmonicida* could grow at this temperature.

Among the *Aeromonas* strains isolated, A01, A03, A04 and A05 phenotypically resembled *A. hydrophila* while A02 resembled *A. sobria*. A06 was similar to A02 in all respects except that it showed a positive VP reaction.

The overall biochemical profile of V01, M01, B01 and C01 showed that they belonged to the genus *Vibrio, Moraxella, Bacillus* and *Micrococcus* respectively. V01 was not considered to belong to the motile *Aeromonas* group because it gave a negative indole test and it also did not produce arginine hydrolase in Thornley's medium (Baumann and Schubert 1984). The isolate M01 was both oxidase and catalase positive which distinguished it from *Acinetobacter* and *Kingella*, which are two other genera of the family *Neisseriaceae* (BØvre 1984). The genus *Neisseria* is also catalase and oxidase positive but morphologically they are coccoid (Vedros 1984). In isolate B01, the spore was in the central position of the bacterium and the sporangium was not swollen. It differed from other endospore forming bacteria belonging to other genera in that it was both catalse and oxidase positive (Claus and Berkeley 1986). Differentiation among species in the three genera, *Bacillus, Moraxella* and *Vibrio* was not attempted. Isolate C01 produced a yellow pigment and was able to grow on Simmon's citrate agar and thus resembled *Micrococcus varians* (Kocur 1986).

During the present study, *Pseudomonas* and *Aeromonas* were the predominant bacteria isolated from the diseased fishes. Other genera were also present but they seemed to be unrelated to the disease. Only strains of *Pseudomonas* and *Aeromonas* were pathogenic. Qureshi et al. (1995) also

found *Aeromonas* and *Pseudomonas* sp. to be pathogenic among the nine types of bacteria isolated from EUS positive fishes. In the present study, four of the six strains of *Aeromonas* isolates resembled *A. hydrophila* and two strains resembled *A. sobria*. Both strains of *A. sobria* and two strains of *A. hydrophila* were found to induce ulcers in healthy fishes. Among the pseudomonads, the two pathogenic strains resembled *P. fluorescens*.

There are some differences in the types of bacteria isolated from different fish species. While pseudomonads and aeromonads were consistently being isolated from all fishes, there were variations in other types of bacteria that were present at the ulcer site. Besides pseudomonads and aeromonads, *Bacillus* sp. and *Micrococcus* sp. were the only other bacteria isolated from *C. punctatus*, and *Puntius* sp. respectively. In the case of *Mystus* sp., two other bacteria, one *Moraxella* and one *Vibrio* were isolated. This wide difference may be explained by the fact that barring *Pseudomonas* and *Aeromonas*, the other bacteria reflect the microflora of the environment and bear no relationship with the disease. This explanation is supported by the nonpathogenecity of these bacteria. Other workers (Shewan 1961; Campbell and Buswell 1983) have also reported that fish microflora is affected by the environment.

One pathogenic pseudomonad and one pathogenic aeromonad each were isolated from *C. punctatus* and *Mystus* sp. These bacteria, however, differed phenotypically from each other. While A01, isolated from *C. punctatus* resembled *A. hydrophila*, A06 isolated from *Mystus* sp. resembled *A. sobria*. Karunasagar et al. (1995) isolated *A. sobria* and *A. hydrophila* from the internal organs and external lesions of EUS affected fishes. They also found these two bacteria in the internal organs during the asymptomatic stage of the disease. However, due to lack of homogeneity in the phenotypic characters and LD_{50} values of these isolates, they suggested that the role of *Aeromonas* sp. in the outbreak may be secondary in nature. However, Torres et al. (1993) found *A. hydrophila* to be the only pathogenic (both highly virulent and weakly virulent) bacteria. Subhasinghe et al. (1990) and Llobrera and Gacutan (1987) also found *A. hydrophila* to be consistently associated with the ulcers.

Among the pseudomonads, although both P02 and P06 isolated from *C. punctatus* and *Mystus* sp. resembled *P. fluorescens*, they differed in their biochemical characters. While P02 resembled *biovar III* of *P. fluorescens*, P06 was similar to *biovar II* and *IV*. On the other hand, no pathogenic pseudomonad was isolated from ulcers of *Puntius* sp. Both the pathogenic bacteria were aeromonads among which A02 resembled *A. sobria* and A03 resembled *A. hydrophila*. Thus, the inconsistency in the bacterial isolates from different fish species is distinct. This tends to suggest that these are opportunistic pathogens that have invaded the fish secondarily. Another notable feature is that none of the bacteria could individually induce severe ulcers. Several authors (Pradhan and Pal 1990; Okpokwasili and Okpokwasili 1994; Pal and Srivastava 1996) have reported that mixed cultures of two or more pathogenic strains were more virulent than individual bacteria. Since more than one pathogenic strain was isolated from each fish species, it is likely that by nature, these strains are acting *en masse* to produce deep severe ulcers.

Conclusion

Based on this study, it may be concluded that pathogenic strains of aeromonads and pseudomonads are associated with the ulcerative lesion of EUS affected fishes and reinfection studies show that they have a role in the formation of typical ulcerative lesion in fishes. However, the distinct inconsistency of the isolated strains in different fish species suggests that they are opportunistic pathogens and invade the fish secondarily.

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