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EUS infected fishes and isolated bacteria from them in eastern Nepal

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Abstract
Present study deals with isolation, characterization and identification of bacteria from EUS infected fishes and pathogenicity of isolated bacteria from EUS affected fishes from eastern Nepal.
Out of six water bodies EUS outbreaks were recorded in three fish farms S_1, S_2 and S_3 from 2009-2015. Generally the outbreak of EUS takes place in the month of December and persists up to March. Altogether 444 naturally EUS affected fishes were collected and examined. The order of susceptibility was about 60% *Cirrhinus mrigala*, 30% *Labeo bata / L. rohita*, 7% *Catla catla*, 3% *Channa striatus, Puntius* sp., *Heteropneustes fossilis, Mystus tengara, Clarias batrachus* and *Lepidocephalichthys guntea* (rarely) among affected fishes.
Bacteria were isolated from the ulcers of naturally infected *Cirrhinus mrigala*, *Catla catla, Channa striatus, Puntius* sp., *Mystus tengara* and *Labeo bata.* Among 23 isolates, 2 belong to *Pseudomonas* sp. from Lb_1 and Cc_4, 2 belong to *Micrococcus* sp. from Cm_2 and P_4, 14 bacteria were of *Aeromonas hydrophila* isolated from Cm_1, Cm_3, Cs_1, P_1, P_3, Lb_2, Lb_3, Cc_1, Cc_2, Cc_3, Mt_1, Mt_2, Mt_3 and Mt_4. 1 belongs to *Moraxella* sp. from P_2, 1 belongs to *Aeromonas veronii biovar sobria* from Lb_4 and 3 belong to *Aeromonas caviae* from Cm_4, Cs_2 and Cs_3. All together 20 bacterial isolates were pathogenic and 3 were non- pathogenic. EUS (Epizootic Ulcerative Syndrome) is the major microbial fish disease in eastern Nepal.

Keywords: EUS, fish disease, bacteria, pathogenicity, baidya, babiya birta, tarahara, fish farm

1. Introduction
Various diseases of fish have been reported worldwide. Bacterial diseases such as furunculosis (Ghittino, 1972; Morrison and Plumb, 1994) [19,43] streptococciosis, bacterial gill disease (Lumsden *et al.*, 1994)[37], viral haemorrhagic septicemia (Meyers *et al.*, 1992)[40], fungal diseases (Noga *et al.*, 1991)[49], metazoan and protozoan diseases (Paperna, 1980) [53] are some of the fish diseases which have overwhelmed both wild and cultured waters throughout the world. The commonly occurring fish diseases in Nepal, India and Bangaladesh are dropsy, ulcerative disease, haemorrhagic septicemia, microsporidiasis, dactylogyrosis, ligulosis, argulosis and saprolegniosis (Kumar *et al.*, 1991; Das and Das, 1995)[29,14]. Fungal infection such as saprolegniasis, branchiomycosis (gill rot) and some fish pathogenic bacteria like *Streptococcus fecalis*, *Micrococcus* spp., *Pseudomonas* spp., *Aerococcus* spp. and *Flavibacterium* spp. were reported from Nepal (Rayamajhi and Bajracharya, 2005) [64].
Among fish diseases, epizootic ulcerative syndrome (EUS) caused a loss of about 15-20 % of total fish production during its initial outbreak in Eastern Terai of Nepal, in February 1989 and afterwards (Phillips, 1989) [54]. Lilley *et al.* (2002) [33] mentioned that among 37% of fish farms, 95% cases were of EUS affected and it was considered as the most destructive fish disease in Nepal (Phillips, 1989; ADB/NACA 1995) [2]. People are unwilling to start aquaculture activities due to the perceived high risk of diseases mainly EUS outbreaks and lack of knowledge of how to deal with fish disease (Callinan *et al.*, 1999) [6].
EUS is a disease affecting wild and farmed fish which was first appeared in summer months in farmed Ayu (*Plecoglossus altivelis*) in Japan in 1971 (Egusa and Masuda, 1971) [16]and named as mycotic granulomatosis (Miyazaki and Egusa, 1972) [41]. In 1972, a similar ulcerative disease in fish was reported from central Queensland, Australia with recurrence in subsequent years and the disease was known as red spot disease (RSD) (Rodgers and Burke, 1981) [65]. Then disease was reported in freshwater and estuaries fish in Asia-Pacific regions. It has spread across the entire south Asia extending from Papua New Guinea in the south east to Pakistan in the west and the disease is called epizootic ulcerative syndrome, EUS (FAO, 1986) [18]. Subsequently it spread to USA and Africa in 2006 and was confirmed as EUS in 2007 (Sosa *et al.*, 2007a; Mudenda, 2012) [69,44]. EUS is basically a disease of complex nature involving certain fungal and bacterial elements in its later stages, and probably one or more viruses (Chinabut, 1995) [10].
Although Mohan *et al.* (1999) [42] suggested that an invasive fungus *A. invadans* was the primary pathogen of EUS, several other bacterial pathogens also involved in EUS of fish. The disease had been named as red pest for *A. anguilla* (Schäperclaus, 1934) [68], red disease for *A. japonica* (Hoshina, 1962) [20] and *Cyprinus carpio* (Egusa, 1978) [15], red sore for *Micropterus salmonides* and Aeromonas disease in cyprinids (Takahashi, 1984b) [73]. Ulcer disease due to bacteria *Aeromonas hydrophila* has been reported in Indian major carps by Gopalakrishnan in 1963 and in *Catla catla* (Karunasagar *et al.*, 1986) [26].

Llobera and Gacutan (1987) [36] reported the association of *Aeromonas hydrophila* with necrotic ulcers and lesions in snakehead (*Ophiocephalus striatus*), Thai catfish (*Clarias batrachus*), crucian carp (*Carassius carassius*) and goby (*Glossogobius giuris*) in Laguna de Bay, Philippines from December, 1985 through February 1986. The bacteria were rarely isolated from the kidney and liver of carp and catfish. Boonyaratpalin (1989)[5] reported that the EUS involving both wild and cultured fish in Burma, Indonesia, Lao PDR, Malaysia, Singapore and Thailand was associated with bacterial pathogens, primarily *Aeromonas hydrophila* and occasionally *Pseudomonas* sp.. *A. hydrophila* was also reported to be associated with EUS affected fishes in Sri Lanka (Costa and Wijeyaratne, 1989; Subasinghe *et al.*, 1990) [11,72]. Jhingran and Das (1990) [21] induced the haemorrhagic ulcers inoculating pure bacteria isolated in healthy murrels. Kar *et al.* (1990) [24] also isolated *Pseudomonas aeruginosa* from the surface muscle lesions.

Four types of bacteria, two fluorescent Pseudomonads, one Aeromonad and one *Micrococcus* sp. were isolated from skin lesions of air breathing fishes by Pal and Pradhan (1990) [51] where Aeromonad showed strong resemblance with *Aeromonas caviae* (Pradhan, 1992) [58]. When a mixed culture of bacteria was inoculated in *Anabas testudineus* and *Channa punctatus*, severe ulcers were produced but pure cultures of the fluorescent Pseudomonads and Aeromonads induced only superficial ulcers while pure culture of *Micrococcus* sp. did not produce any ulcers (Pal and Pradhan, 1990; Pradhan and Pal, 1990) [51,57].

A. hydrophila was isolated from EUS affected fishes of more than 70 species by Chattopadhya *et al*. (1990) [9]. Several researchers reported associations of bacterial pathogens with EUS (Ali and Timuli, 1991; Mukherjee *et al*., 1991; Lio-Po *et al*., 1992) [3, 45, 34]. Torres *et al.* (1993) [74] performed virulence screening of 54 species of *Aeromonas* and found that *A. hydrophila* was the most pathogenic. Qureshi *et al*. (1995) [61] found *Aeromonad* and *Pseudomonad* were highly pathogenic while micrococcus and cytophagans were less pathogenic among eight bacterial isolates from EUS affected fishes. Lio-Po *et al.* (1998) [35] isolated four species of bacteria from EUS affected fishes from Philippines and Thailand and *A. hydrophila* was proved to be most pathogenic. Saha and Pal (2000) [66] isolated 16 strains of bacteria from the ulcers of infected fishes, *C. punctatus*, *Puntius* sp. and *Mystus* sp.. Only six strains of genus *Aeromonas* and *Pseudomonas* were pathogenic. Saha and Pal (2002) [67] tested the virulence of two florescent Pseudomonads and one Aeromonad isolated from the extraperitoneal lesions of diseased fishes injecting into the healthy *H. fossilis*. Das *et al*. (2007) [12] isolated eight pathogenic bacteria (seven *A. hydrophila* and one *A. caviae*) from the lesions of EUS affected fish *Cirrhinus mrigala* and 15 Aeromonas (Das *et al.*, 2009) [13] from Catla, Mrigal and Puntius of affected pond in Jalpaigudi district of West Bengal. More epidemiological studies are required to get an insight into the role of various environmental risk factors responsible for EUS (Pradhan *et al.*, 2014) [59].

So far no detailed works on the fish diseases have been carried out in Eastern Nepal. So it is considered worthwhile to study the fish diseases prevalent and to identify the bacterial fish diseases especially associated with EUS in the study areas.

Study Sites

Baidya fish farm (Site 1), Babiya Birta fish farm (Site 2) and Tarahara fish farm (Site 3) were selected in the disease prone areas of Eastern Nepal. Diseased fishes were collected from these sites during 2009-2015.

Site1 (S_1). Baidya Fish Farm, Tankisinwari, Morang (Fig.1).

It is located at latitude 26°31 '11.12" N and Longitude 87° 16' 25.64" E.

Site2 (S_2). Babiya Birta Fish Farm, Morang (Fig.1).

It is located at latitude 26°30' 23.85" N and Longitude 87° 26' 09.01" E.

Site3 (S_3). Tarahara Fish Farm, Sunsari (Fig. 1)

It is located at latitude 26°42' 05.77" N and Longitude 87° 16' 38.50" E.

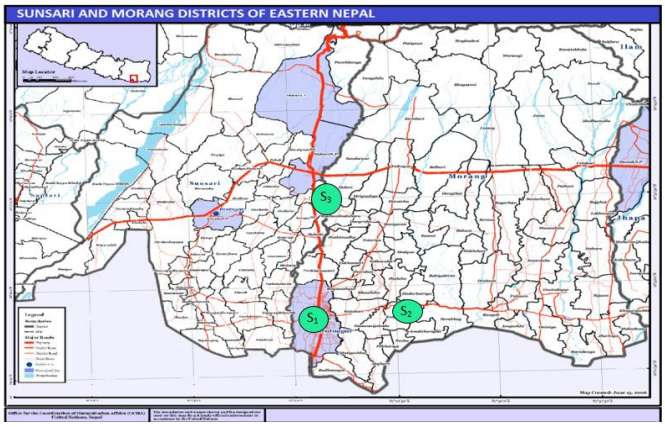


Fig 1: Map of Sunsari District showing sampling sites (1- 3) (Source: OCHA, UN, Nepal, modified)

2. Materials and Methods

2.1 Collection of diseased fish

Naturally infected fish *Catla catla*, *Cirrhinus mrigala*, *Channa striata* and *Puntius* sp., *Labeo rohita*, *Labeo bata*, *Clarias batrachus*, *Heteropneustes fossilis*, *Mystus tengara* and *Lepidocephalichthys guntea* showing ulcerative lesions were collected during winter months of the year 2009-2015 from different affected ponds in various locations of the Sunsari and Morang districts of eastern Nepal and were used for the isolation of bacteria. Infected fish were brought alive to the laboratory for further studies.

2.2 Collection and maintenance of healthy fish for experimental works

Healthy air breathing fish (*Heteropneustes fossilis*) collected from nearby fish farm with no history of EUS infections were used for experimental work. Fish were maintained in the laboratory in glass aquaria measuring 90 × 35 × 35 cm^3 in which the depth of the static water was 20 cm. Water temperature was maintained at 28 - 30°C. The fish were fed with chopped earthworms and acclimatized under laboratory conditions for at least 15 days before using them for experimental work.

2.3 Isolation of microorganisms from the ulcers of diseased fishes

2.3.1 Isolation of bacteria and culture

The ulcerated area of the diseased fish was dissected aseptically following Pal and Pradhan (1990) [51] and placed in a conical flask containing 15 mL of nutrient broth supplemented with glucose. The flask was incubated at 30°C for 72 hrs. Then 1 mL of each bacterial culture grown on nutrient broth was inoculated in a conical flask containing 20 mL of molten *Aeromonas* isolation medium supplemented with *Aeromonas* selective supplement and mixed thoroughly. The mixture was then poured on sterile petridish (90 mm diameter) and allowed to solidify for overnight at 30°C. Some colonies grown on the agar plates were selected and then streaked on to nutrient agar slants to incubate at 30°C for 24 hrs. Bacterial cultures grown on nutrient broth were also streaked on nutrient agar slasnts to incubate at 30°C for 24 hours. Each isolate was given a particular code name (Cm_1, Cm_2, Cm_3,C m_4,Cc_1, Cc_2, Cc_3, Cc_4, Cs_1, Cs_2, Cs_3, Mt_1,Mt_2,Mt_3 Mt_4, P_1, P_2 P_3, P_4, Lb_1, Lb_2,Lb_3 and Lb_4) and stored at 4°C. For routine experimental works, the isolates were sub cultured by growing in nutrient broth for 24hrs.at 30°C.Several media were used during the present study for isolation and maintenance of isolates and for biochemical tests.

2.4 Characterization of the isolated bacteria

To identify the bacteria, a number of physiological and biochemical tests (Barrow and Feltham, 1993) [4] were conducted following the identification scheme described by Popoff (1984) [55], Carnanhan *et al*. (1991) [7] and Abbott *et al.* (1992) [1].

2.5 Pathogenicity test of the isolated bacteria

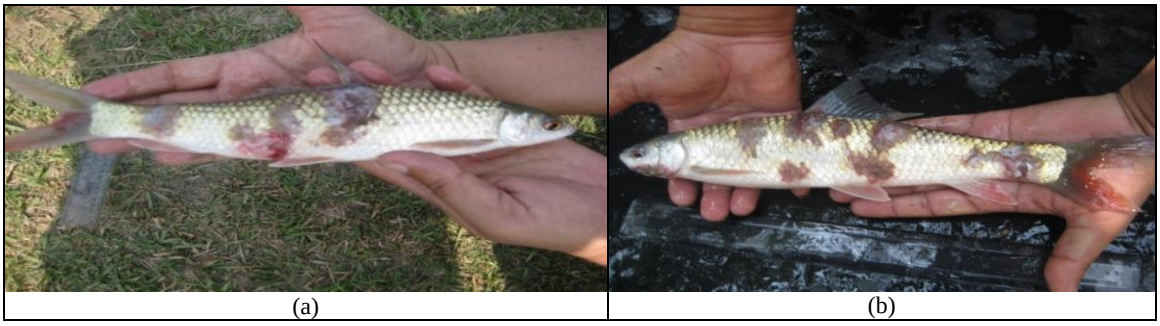
All the isolates were tested for their ability to induce ulcers in healthy *Heteropneustes fossilis* fish weighing of 50-60 g by intramuscular application of 0.5 mL of bacterial cell suspension (1×10^7 c.f.u. /mL) per 100 g of body weight in 0.85% NaCl. Each isolate was injected into a set of five fish. The control set of fish received 0.05 mL sterile saline. Fish were observed for changes in their behavioral patterns as well as development of hemorrhagic ulcers and tissue necrosis (Pradhan and Pal, 1990) [57]. Intramuscular injection was given at the trunk region on the right/left side of the fish from behind to the front at an angle of 20° to the body axis.

3. Results

3.1 Studies on the fish affected with epizootic ulcerative syndrome

A total 444 naturally infected fishes showing lesions on the body; 60% (262) *Cirrhinus mrigala*, 30% (130) *Labeo rohita* and *Labeo bata*, 8% (36) *Catla catla*, *Channa* spp., *Puntius* spp., *Clarias batrachus*, *Heteropneustes fossilis*, *Mystus tengara* and *Lepidocephalichthys guntea* (rarely) (Figs. 1a and b to 8a and b, 9 and 10) were collected during winter months of the year 2009-2015 from different affected ponds in various locations of the Sunsari and Morang districts of eastern Nepal and were used for the isolation of fungi. The infected fish were brought to the laboratory alive for further detailed observations.

In the early stage of lesion the fish showed single or multiple red spots on the body surface (Fig. 10). Some fishes showed moderate type of ulcer with erosion of the epidermis (Figs 3, 9). In the advanced stage ulcer became deep and necrotic with occasional haemorrhages (Figs. 1a and b, 5a and b).



(a) (b)

Fig 1: a and b naturally EUS infected *Cirrhinus mrigala*

(a) (b)

Fig 2: a and b naturally EUS infected *Labeo rohita*

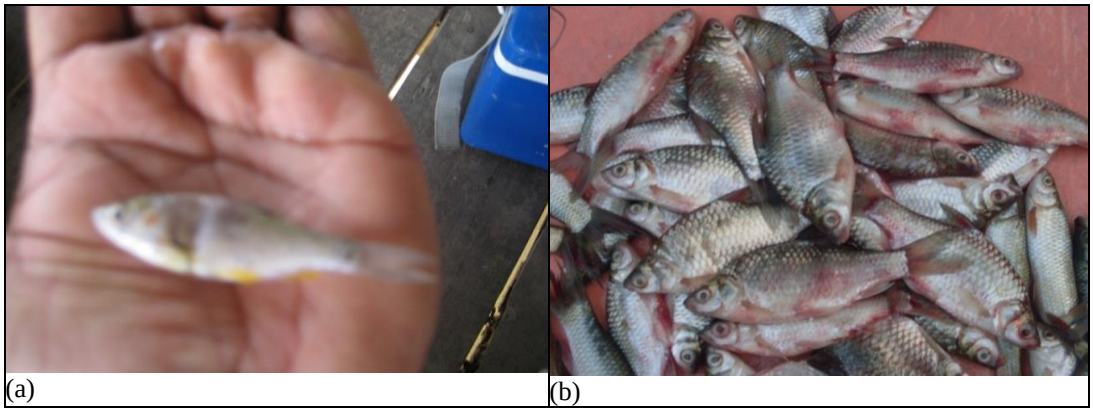
Fig 3: Naturally EUS infected *Catla catla*

(a) (b)

Fig 4: a and b naturally EUS infected *Labeo bata*

(a) (b)

Fig 5: a and b naturally EUS infected *Channa striata*



(a) (b)

Fig 6: a and b naturally EUS infected *Puntius* sp.

(a) (b)

Fig7: a and b naturally EUS infected *Mystus tengara*

(a) (b)

Fig 8: a and b naturally EUS infected *Clarias batrachus*

Fig 9: Naturally infected *H. fossilis*

Fig 10: Naturally infected *Lepidocephalichthys guntea*

3.2 Isolation of Bacteria and their characterization

Four types of bacteria were isolated from ulcers of *Cirrhinus mrigala* (Table 1). Four types of bacteria were isolated from ulcers of *Catla catla* (Table 2). Three types of bacteria were isolated from ulcers of *Channa striatus* (Table 3). Four types of bacteria were isolated from ulcers of *Puntius* sp. (Table 4). Four types of bacteria were isolated from ulcers of *Mystus tengara* (Table 5). Four types of bacteria were isolated from ulcers of *Labeo bata* (Table 6).

Results of the morphological observations (Figs. 11, 12, 13and 14) and biochemical test of the bacterial isolates from ulcers of different fishes are given in Tables 1- 6).

Altogether twenty three bacteria were isolated from the ulcers of six infected fishes, out of which fourteen were *Aeromonas hydrophila*, three were *A. caviae*, one was *A. veroni biovar sobria*, two were *Pseudomonas* sp., two were *Micrococcus* sp. and one was *Moraxella* sp.

Out of fourteen *A. hydrophila*, two $Cm_1$and Cm_3 from *Cirrhinus mrigala*, three (Cc_1, Cc_2 and Cc_3) from *Catla catla*, one Cs_1 from *Channa striata*, two (P_1 and P_3) from *Puntius* sp., four (Mt_1, Mt_2, Mt_3 and Mt_4) from *Mystus tengara* and two (Lb_2 and Lb_3) from *Labeo bata* were isolated. Out of three *Aeromonas caviae*, one (Cm_4) from *C. mrigala* and two (Cs_2 and Cs_3) from *C. striata* were isolated. *A. veroni biovar sobria*, was isolated only from *Labeo bata*. Two *Pseudomonas* sp. (Cc_4 and Lb_1) were isolated one each from *Catla catla* and *Labeo bata*. Two *Micrococcus* sp. were isolated one each from *Cirrhinus mrigala* (Cm_2) and *Puntius* sp. (P_4). One *Moraxella* sp. was isolated from *Puntius* sp. (P_2) (Table 7).

Table 1: Morphological and biochemical characteristics of bacteria isolated from the ulcers of *Cirrhinus mrigala.*

	Bacteria Isolates			
	Cm_1	**Cm_2**	**Cm_3**	**Cm_4**
Shape	rod	sphere	rod	rod
Occurance	single	single	single	single
	pairs	pairs		
		tetrads		
Size	2.8-3.2x0.75-0.8 µm	1.2-1.6µm diameter	2.8-3.2x0.75-0.8 µm	2.8-3.2x0.75-0.8µm
Spores	–	–	–	–
Agar Colonies	circular	circular	circular	circular
	smooth	smooth	smooth	smooth
	convex	convex	convex	convex
Gram reaction	–	+	-	-
Motility	+	-	+	+
Growth at:				
25°C	g	m	g	g
30	g	g	g	g
37	m	g	m	m
42	n	n	n	n
Growth at 6% NaCl	-	+	-	-
Indole Production	+	-	+	+
Resistance to Ch	-	-	-	+
VP	+	-	+	-
Nitrate	+	W	+	+
Gas from glucose	+	-	+	-
Oxidase	+	+	+	+
Catalase	+	+	+	+
O-F test	F	0	F	F
Acid from:				
Glucose	+	+	+	-
L-arabinose	+	-	+	+
Sucrose	+	+	+	+
Mannitol	+	+	+	+
Esculin hydrolysis	+	+	+	+
LDC	+	-	+	-
ODC	-	-	-	-
ADH	+	-	+	+
Pigment production	-	Bright yellow	-	-

+, positive; -, negative;0, neutral, g, good growth; m, moderate growth; n, no growth; Ch, cephalothin; VP, Voges-Proskauer reaction; O-F, Oxidation - Fermentation; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; ADH, arginine dihydrolase; w, weak.

Table 2: Morphological and biochemical characteristics of bacteria isolated from the ulcers of *Catla catla.*

	Bacterial isolates			
	Cc_1	**Cc_2**	**Cc_3**	**Cc_4**
Shape	rod	rod	rod	Rod
Occurance	single	single	single	Single
				Pairs
	chains	chains	chains	or chains
Size	2.8-3.2x0.75-0.8 µm	2.8-3.2x0.75-0.8 µm	2.8-3.2x0.75-0.8 µm	2.2-0.3x0.7-0.8 µm
Spores	-	-	-	-
Agar Colonies	circular	circular	circular	Circular
	smooth	smooth	smooth	Smooth
	convex	convex	convex	slightly convex /flat
Gram reaction	-	-	-	-
Motility	+	+	+	+
Growth at:				
25°C	g	g	g	m
30°	g	g	g	g
37°	m	m	m	g
42°	n	n	n	n
Growth at 6% NaCl	-	-	-	-

Indole Production	+	+	+	-
Resistance to Ch	-	-	-	-
VP	+	+	+	-
Itrate	+	+	+	+
Gas from glucose	+	+	+	-
Oxidase	+	+	+	+
Catalase	+	+	+	+
O-F test	F	F	F	0
Acid from:				
Glucose	+	+	+	+
L-arabinose	+	+	+	+
Sucrose	+	+	+	+
Mannitol	+	+	+	+
Esculin hydrolysis	+	+	+	-
LDC	+	+	+	-
ODC	-	-	-	-
ADH	+	+	+	+
Pigment production	-	-	-	Yellowish green in King's B medium

Table 3: Morphological and biochemical characteristics of bacteria isolated from the ulcers of *Channa striata.*

	Bacterial isolates		
	Cs_1	**Cs_2**	**Cs_3**
Shape	rod	rod	rod
Occurance	single	single	single
Size	2.8-3.3x0.7-0.75µm	2.8-3.2x0.75-0.8µm	2.8-3.2x0.75-0.8µm
Spores	-	-	-
Agar Colonies	circular	circular	circular
	smooth	smooth	smooth
	convex	convex	convex
Gram reaction	-	-	-
Motility	+	+	+
growth at:			
25°C	g	g	g
30°	g	g	g
37°	m	m	m
42°	n	n	n
Growth at 6% NaCl	-	-	-
Indole Production	+	+	+
Resistance to Ch	-	+	+
VP	+	-	-
Nitrate	+	+	+
Gas from glucose	+	-	-
Oxidase	+	+	+
Catalase	+	+	+
O-F test	+	+	+
Acid from:			
Glucose	+	+	+
L-arabinose	+	+	+
Sucrose	+	+	+
Mannitol	+	+	+
Esculin hydrolysis	+	+	+
LDC	+	-	-
ODC	-	-	-
ADH	+	+	+
Pigment production	-	-	-

Table 4: Morphological and biochemical characteristics of bacteria isolated from the ulcers of *Puntius* sp.

	Bacterial isolates			
	P_1	**P_2**	**P_3**	**P_4**
Shape	rod	rod	rod	sphere
Occurance	single	single	single	single
	pairs		pairs	pairs

	chains		chains	tetrads or irregular clusters
Size	2.8-3.2x0.75-0.8µm	1.5-1.7x0.9-1.9 µm	2.8-3.2x0.75-0.8µm	1.2-1.6 µm diameter
Spores	-	-	-	-
Agar Colonies	circular	circular	circular	circular
	smooth	smooth	smooth	smooth
	convex	convex	convex	convex
Gram reaction	-	-	-	+
Motility	+	+	+	-
Growth at:				
25°C	g	m	g	m
30°	g	g	g	g
37°	m	g	m	g
42°	n	n	n	n
Growth at 6% NaCl	-	-	-	-
Indole Production	+	-	+	-
Resistance to Ch	-	-	-	-
VP	+	-	+	-
Nitrate	+	-	+	W
Gas from glucose	+	-	+	-
Oxidase	+	+	+	+
Catalase	+	+	+	+
O-F test	F	0	F	0
Acid from:				
Glucose	+	-	+	+
L-arabinose	+	-	+	-
Sucrose	+	-	+	+
Mannitol	+	-	+	+
Esculin hydrolysis	+	+	+	+
LDC	+	-	+	-
ODC	-	-	-	-
ADH	+	-	+	-
Pigment production	-	-	-	Bright yellow colonies

Table 5: Morphological and biochemical characteristics of bacteria isolated from the ulcers of *Mystus tengara.*

	Bacterial isolates			
	Mt_1	**Mt_2**	**Mt_3**	**Mt_4**
Shape	rod	rod	rod	rod
Occurance	single	single	single	single
	pairs	pairs	pairs	pairs
	chains		chains	chains
Size	2.8-3.2x0.75-0.8µm	2.8-3.2x0.75-0.8µm	2.8-3.2x0.75-0.8µm	2.8-3.2x0.75-0.8µm
Spores	-	-	-	-
Agar Colonies	circular	circular	circular	circular
	smooth	smooth	smooth	smooth
	convex	convex	convex	convex
Gram reaction	-	-	-	-
Motility	+	+	+	+
Growth at:				
25°C	g	g	g	g
30° C	g	g	g	g
37°C	m	m	m	m
42°C	n	n	n	n
Growth at 6% NaCl	-	-	-	-
Indole Production	+	+	+	+
Resistance to Ch	-	-	-	-
VP	+	+	+	+
Nitrate	+	+	+	+
Gas from glucose	+	+	+	+

Oxidase	+	+	+	+
Catalase	+	+	+	+
O-F test	F	F	F	F
Acid from:				
Glucose	+	+	+	+
L-arabinose	+	+	+	+
Sucrose	+	+	+	+
Mannitol	+	+	+	+
Esculin hydrolysis	+	+	+	+
LDC	+	+	+	+
ODC	-	-	-	-
ADH	+	+	+	+
Pigment production	-	-	-	-

Table 6: Morphological and biochemical characteristics of bacteria isolated from the ulcers of *Labeo bata.*

	Bacterial isolates			
	Lb_1	**Lb_2**	**Lb_3**	**Lb_4**
Shape	rod	rod	rod	rod
Occurance	single	single	single	single
	pairs		pairs	pairs
	chains		chains	chains
Size	2.2-0.3x0.7-0.8 µm	2.8-3.2x0.75-0.8µm	2.8-3.2x0.75-0.8µm	2.5-3.0x0.7-0.8µm
Spores	-	-	-	-
Agar Colonies	circular	circular	circular	circular
	smooth	smooth	smooth	smooth
	convex	convex	convex	convex
Gram reaction	-	-	-	-
Motility	+	+	+	+
Growth at:				
25°c	m	g	g	g
30°	g	g	g	g
37°	g	m	m	m
42°	n	n	n	n
Growth at 6% NaCl	-	-	-	
Indole Production	-	+	+	+
Resistance to Ch	-	-	-	+
VP	-	+	+	+
Nitrate	+	+	+	+
Gas from glucose	-	+	+	+
Oxidase	+	+	+	+
Catalase	+	+	+	+
O-F test	0	F	F	F
Acid from:				
Glucose	+	+	+	+
L-arabinose	+	+	+	+
Sucrose	+	+	+	+
Mannitol	+	+	+	+
Esculin hydrolysis	+	+	+	-
LDC	-	+	+	+
ODC	-	-	-	-
ADH	+	+	+	+
Pigment production	Yellowish green in King's B medium	-	-	-

+, positive; -, negative;0, neutral, g, good growth; m, moderate growth; n, no growth; Ch, cephalothin; VP, Voges-Proskauer reaction; O-F, Oxidation - Fermentation; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; ADH, arginine dihydrolase; w, weak.

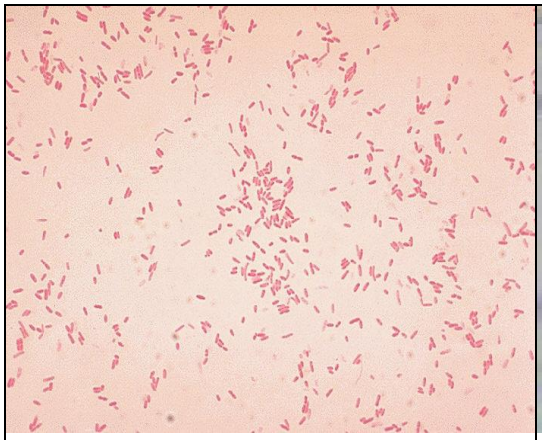


Fig 11: *Aeromonas caviae*, Cs_2(X400)

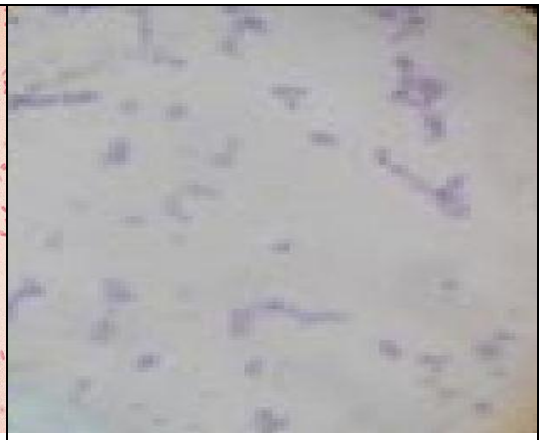


Fig 12: *Micrococcus* sp., P_4(x400)

Fig 13: *Pseudomonas* sp.,Cc_4 (X1000)

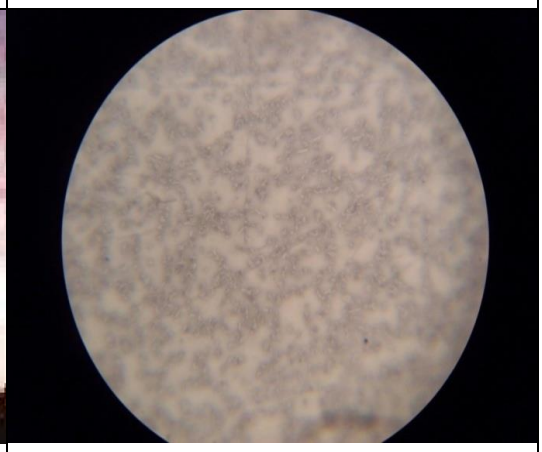


Fig.3.14. *Aeromonas hydrophila* Cm_1, (x400)

3.3 Pathogenicity test of the isolated bacteria

Among 23 bacterial isolates (Table 7), 20 were found to be pathogenic (86.95%) after intramuscular administration of these isolates to the healthy *Heteropneustes fossilis* fish. Two *Micrococcus* spp. (P_4 and Cm_2) and one *Moraxella* sp. could not induce any ulcer at the site of injection in healthy fish.

Moderate to severe ulcers were found at the injection site. Initially red patches appeared at the site of injection, it swelled gradually and after 72 hrs., the skin and underlying muscle layer eroded and it developed into ulcer. In control set, the fish received only saline suspension. No disease sign was noticed. All fish, in which ulcers developed, however did not die. The moderate ulcers were healed in some fish. No notable change of the swimming behabiour was also observed.

Table 7: Pathogenic and non-pathogenic bacteria isolated from EUS affected fish.

Bacteria	No. of isolates	Pathogenic	Non- Pathogenic
Aeromonas hydrophila (Cm_1,Cm_3,Cc_1,Cc_2,Cc_3,Cs_1,P_1,P_3,Mt_1,Mt_2,Mt_3, Mt_4, Lb_2 and Lb_3)	14	14	0
Aeromonas caviae (Cm_4, Cs_2, Cs_3)	3	3	0
A. veronii biovar sobria (Lb_4)	1	1	0
Pseudomonas sp. (Cc_4, Lb_1)	2	2	0
Micrococcus sp. (Cm_2, P_4)	2	0	2
Moraxella sp. (P_2)	1	0	1
Total	23	20	3

4. Discussion

In the present study, twenty three bacterial isolates were collected from the ulcers of six different EUS affected fish. The morphological features and biochemical profiles of eighteen isolates suggested that they were motile, non-spore forming, glucose fermenting, gram-negative bacilli. They were straight rods, grown in agar with 0% but not in 6% NaCl and reduced nitrate to nitrite (Tables 1, 2, 3, 4, 5 and 6). Thus, they all belonged to the genus *Aeromonas* (Popoff, 1984) [55]. Among the eighteen isolates, fourteen (Cm_1, Cm_3, Cs_1, P_1, P_3, Lb_2, Lb_3,Cc_1,Cc_2,Cc_3,Mt_1,Mt_2,Mt_3 and Mt_4) of them were positive to esculin hydrolysis test, sensitive to antibiotic cephalothin, produced gas from glucose and acid from arabinose (Carnanhan *et al*., 1991) [7]. These bacteria were

positive to lysine decarboxylase and arginine dehydrolase test but negative to ornithine decarboxylase test, produced acid from mannitol and sucrose and gave a positive to VP test (Abbott *et al*., 1992) [1]. Thus, these bacteria were regarded as *Aeromonas hydrophila*. The isolate Lb_4 was negative to esculin hydrolysis test, positive to indole test, produced acid from sucrose and gave a positive VP test (Carnanhan *et al*., 1991) [7]. It was tested positive to arginine dihydrolase, lysine decarboxylase and negative to ornithine decarboxylase, produced acid from arabinose and mannitol (Abbott *et al.*, 1992) [1]. Therefore, it belonged to *Aeromonas veronii biovar sobria.* The isolate Cm_4 and Cs_2 and Cs_3 (Tables 1, 3) were tested positive to esculin hydrolysis but could not produce gas from glucose (Carnanhan *et al*., 1991) [7] [7]. It was tested positive to arginine dihydrolase, negative to lysine decarboxylase and ornithine decarboxylase, gave a negative VP test and produced acid from arabinose, mannitol and sucrose (Abbott *et al.*, 1992) [1]. Thus these were identified as *Aeromonas caviae* (Fig. 11).

The morphological features and bio-chemical profiles of the isolated pathogenic bacteria, Cc_4 and Lb_1 (Tables 2, 6, 7; Fig. 13) revealed that these bacteria were gram negative, motile, catalase positive, utilized glucose oxidatively and produced yellowish green pigment in King's B medium. Thus they belonged to the genus Pseudomonas (Palleroni, 1984) [52].

The biochemical profile of Cm_2 and P_4 (Tables 1, 4, 7) showed that these bacterial isolates belonged to the genus Micrococcus (Fig. 12). These differed from the genus *Staphylococcus* and *Streptococcus* with respect to the breakdown of glucose. As these isolates utilized glucose oxidatively and produced a yellowish pigment, Cm_2 and P_4 resembled Micrococcus (Fig. 12). The sphere-shaped bacteria were gram positive, non-motile, non- spore forming, catalase positive, indole negative, oxidase negative and oxidative, occurring singly, in pairs, in tetrad, in short chain or in irregular cluster. Colonies were yellow and small, smooth, convex. It satisfied the characteristics of the species *Micrococcus varians* (Kocur, 1986) [28] e.g. oxidase negative, oxidative in metabolism, reduction of nitrate and nitrite, good growth between 25-37ºC and non-pathogenic. From the morphological and biochemical profile, P_2 isolate (Tables 5, 7) resembled Moraxella.

Fourteen *Aeromonas hydrophila* (Table 7, Fig. 14) were isolated from infected fishes e.g. *C. mrigala, C. catla, C. striata, Puntius* sp., *Mystus tengara* and *Labeo bata* and all are pathogenic. Three *Aeromonas caviae* were isolated from ulcers of *Cirrhinus mrigala* and *Channa striata* and one *A. veronii biovar sobria* was isolated from ulcer of *Labeo bata*. These are also pathogenic.

Two *Micrococcus* sp. were isolated from ulcer of *Cirrhinus mrigala* and *Puntius* sp., one *Moraxella* sp. was isolated from ulcer of *Puntius* sp. Two *Micrococus* sp. and one *Moraxella* sp. are non-pathogenic.

Globally, *Aeromonas* sp. is one of the most common bacteria associated with fish diseases. Although many strains are regarded as opportunistic pathogens, others are clearly primary pathogens in their own right (Trust, 1986) [75]. Fish diseases which involve *A. hydrophila* include red spot disease of European eel, *A. anguilla* (Schäperclaus, 1934) [68], red disease of Japanese eel, *A. japonica* and red disease of carp, *C. carpio* (Egusa, 1978) [15]. Jo and Onishi (1980) [22] isolated *A. hydrophila* from all diseased, cultured ayu, *Plecoglossus altivelis*. Rahim *et al.* (1985) [62] isolated *A. hydrophila* from the wounds of five species of fishes even before the outbreak of EUS in Bangladesh. Esteve *et al.* (1993) [17] isolated *A. hydrophila* and *A. jandaei* from diseased European eel from an eel farm in Spain. Two pathogenic *Pseudomonas* sp. were isolated from ulcer of *Catla catla* and *Labeo bata*. *P. anguilliseptica* was identified as the causative agent of red spot disease in Japan (Muroga *et al.*, 1973; Jo *et al*., 1975; Nakai *et al.*, 1985) [46, 23, 47], pond cultured eel, *A. japonica* in Taiwan (Kuo and Kou, 1978) [30], from *A. anguilla* in Scotland (Nakai and Muroga, 1982; Stewart *et al.*, 1983) [48, 71].

Pal and Pal (1986) [50] reported induction of ulcer in *A. testudineus* by mixed culture of two bacteria, one fluorescent Pseudomonad and another coccus, *M. varians*.

P. anguilliseptica which caused hemorrhage in the mouth, opercula and ventral portion of the body of the fish was identified as the etiological agent of red spot disease in Japan (Wakabayashi and Egusa, 1972; Jo *et al.*, 1975 and Nakai *et al.*, 1985) [76, 23, 47]. Rahim *et al*. (1985) [62] observed that *A. hydrophila* was associated with the wounds of five species of fish in Bangladesh.

Llobera and Gacutan (1987) [36] reported the isolation of *A. hydrophila* from EUS affected fish. Boonyaratpalin (1989) [5] found primarily *A. hydrophila* and occasionally *Pseudomonas* sp. associated with the outbreak of EUS in Burma, Indonesia, Lao PDR, Malaysia, Singapore and Thailand. Association of *A. hydrophila* with EUS affected fish in Sri Lanka was also reported (Costa and Wijeyaratne, 1989; Subasinghe *et al.*, 1990) [11, 72]. Karunasagar *et al.* (1989) [26] recovered *A. hydrophila* and *A. sobria* more often.

Two virulent strains of *Pseudomonas* sp. and one virulent Aeromonad, *A. caviae* were isolated from ulcerative air breathing fish from North Bengal in 1988 and reported to be pathogenic to *A. testudineus* (Pal and Pradhan, 1990) [51] and *C. punctatus* (Pradhan and Pal, 1990) [57]. Likewise one aeromonad (*A. hydrophila*), two pseudomonads and one coccus (*Micrococcus varians*) were isolated from the ulcer tissues of *C. mrigala* (Pradhan *et al*., 1991) [56].

Besides *Aeromonas* sp. and *Pseudomonas* sp., some other types of bacteria were also found to be associated with EUS, *Micrococcus* sp. (Jhingran and Das 1990) [21], *E. coli* and *P. aerouginosa* (Kar *et al*., 1990) [24]. Chattopadhyay *et al.* (1990) [9]; Lio-Po *et al.* (1992, 1998) [34, 35]; Torres *et al.* (1993) [74] and Cartwright *et al.* (1994)[8] also reported the association of mainly *Aeromonas* sp. and *Pseudomonas* sp. with EUS. Ali and Tamuli (1991) [3] isolated three types of bacteria from ulcers from four species of affected fish and reinfection studies showed that *Aeromonas* sp. produced only mild infection. *Vibrio* sp. induced similar types of disease signs while *Micrococcus* sp. failed to induce any sign.

Mukherjee *et al.* (1991) [45] isolated five distinct strains of *A. hydrophila* from EUS affected fish. Torres *et al.* (1993) [74] isolated 54 strains of *Aeromonas* sp. from EUS affected fish. Karunasagar *et al.* (1995) [27] isolated *A. sobria* and *A. hydrophila* from EUS affected fish of Karnataka, India. Aeromonads and Pseudomonads isolated from EUS affected fish were found to induce EUS like lesion when injected intramuscularly to healthy snakehead (*O. striatus*) and

walking catfish (*C. batrachus*) (Lio-Po *et al.*, 1992; Leano *et al.*, 1995) [34,31]. Prasad *et al.* (1995) [60] observed that *C. mrigala* injected with virulent *A. hydrophila* strain isolated from EUS affected *M. armatus* was found to be highly pathogenic. Lio-Po *et al.*(1998) [35] isolated four types of bacteria associated with EUS, namely *A. hydrophila*, *Aquaspirillum* sp., *Pseudomonas* sp. and *Streptococcus* sp. Out of these bacteria, *A. hydrophila* was highly pathogenic. Saha and Pal (2000) [66]isolated 16 strains of bacteria from *C. punctatus, Puntius* sp. and *Mystus* sp. belonging to the genus *Pseudomonas, Aeromonas, Micrococcus, Bacillus, Vibrio* and *Moraxella*. Among these bacteria, only 6 strains of Aeromonads and Pseudomonads were pathogenic. Several workers have isolated different types of bacteria such as *Aeromonas* sp., *Micrococcus* sp., *Acinetobacter* sp. and *Streptococcus* sp. from the ulcer of affected fishes (Kar, 2000) [25].

Pal and Pradhan (1990) [51] isolated *A. caviae* isolated *A. hydrophila* and *A. sobria.* Karunasagar *et al.* (1995) isolated *A. sobria* and *A. hydrophila*, Lio-Po *et al.*(1992 ; 1998) [34,35] isolated *A. hydrophila* along with other bacteria. In the present studies *A. hydrophila, A. sobria* and *A. caviae* were isolated from different infected fish which produced ulcer when injected intramuscularly to healthy fish. EUS affected fishes often die because of bacterial septicemia caused by pathogenic aeromonads (Pal and Pradhan,1990; Rahman *et al.*, 2002) [51,63].

Mastan and Qureshi (2001) [38] reported that 17 species of common bacteria were isolated from the investigated water bodies and EUS affected fishes. Experimental infection trials suggested that *Aeromonas hydrophila* in association with *Pseudomonas fluorescens* may be playing the role of primary etiological agent in producing EUS in fishes. Das *et al*. (2009) [13] found that all the 15 strains *Aeromonas* isolated from the ulcers of EUS affected fishes *Catla catla*, *Cirrhinus mrigala* and *Puntius* sp.

From the experimental work, it was found that out of 23 bacterial strains isolated from infected fish fourteen *Aeromonas hydrophila,* three *Aeromonas caviae*, one *A. veronii biovar sobria* and two *Pseudomonas* sp. were pathogenic. Two *Micrococcus* sp. and one *Moraxella* sp. were non-pathogenic.

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