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First Report of Two Bacterial Diseases from the Freshwater Fishes of the Andaman Islands, India

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ABSTRACT

Background: Studies on the bacterial diseases from the Andaman and Nicobar Islands are scanty and have only two reports recorded during 1996 and 1999. A case of emaciation and abdominal dropsy caused by different strains of *Aeromonas hydrophila* was recorded by earlier workers in the Andaman, Islands. In the present work, a similar disease syndrome was recorded in *Barbonymus gonionotus* and *Cyprinus carpio* var. *Koi.* The present study served to investigate the disease's cause and the pathogen associated with it.

Methods: This study details the isolation, biochemical and molecular characterization of three bacterial pathogens associated with emaciated, tail rot syndrome (ETS) and big belly syndrome (BBS) in *B. gonionotus* and *C. carpio* var. *Koi* respectively. Histology and re-infectivity study was also done for the infected host tissues and with *Catla catla* fingerlings.

Result: This study constitutes the first report of three bacterial pathogens from the Andaman Islands, India *viz*., *Aeromonas veronii*, *Aeromonas* sp. and *Pseudomonas alcaligenes* causing emaciation and abdominal dropsy, with biochemical, molecular, histopathological and experimental challenge study evidence.

Key words: 16S r-gene, *Aeromonas*, Dropsy, Pathogen, *Pseudomonas*.

INTRODUCTION

The Andaman and Nicobar Group of Islands (ANI) is a union territory of India, comprising 572 islands located in the Southeast of the Bay of Bengal, close to South East Asia. These Islands are known for their rich biodiversity containing unique, endemic flora and fauna. Biogeographically andaman and Nicobar Islands are very rich, harbouring unique endemic life forms. The islands are unique compared to mainland India in terms of the scope they offer for integrated development of all sectors of culture fisheries namely, freshwater, brackish water and mariculture (Saravanan *et al*., 2015). Freshwater aquaculture is one of the agro-allied activities in the Andaman group of Islands, which chiefly involves farming Indian major carp, Catfishes, Chinese carp and freshwater prawns (Kiruba Sankar *et al*., 2018).

There is an increasing demand for freshwater fish in the local market, due to which the food fish are also being imported from mainland India for consumption purposes.

At present, approximately 2500 minor irrigation ponds are available in the Andaman and Nicobar Islands which are used for irrigation purposes as well as for aquaculture. In recent times, ornamental fishes have also been imported into the Andaman Islands, especially in the capital city Port Blair due to the demand among aquarium hobbyists and for recreational purposes. Government and other agencies are planning to intensify aquaculture activity in these Islands; however, it is known that diseases are the major and primary constraints for any aquaculture activity (Assefa and Abunna, 2018). Worldwide *Aeromonas* sp. such as *A. hydrophila* and *A. veronii* are known to cause bacterial hemorrhagic septicemia, motile aeromonad septicemia and epizootic ulcerative syndrome (Özcan, 2023; Sharma *et al*., 2013). Similarly, *Pseudomonas* spp. has also been identified as the primary pathogen of several diseases causing septicaemia and haemorrhagic lesions on skin, fins and tail in farmed fish (Mishra *et al.,* 2017; Duman *et al.,* 2021). The very first fish disease reported from the freshwater fishes from Andaman Island is abdominal dropsy in Indian major carp (*Cirrhinus mrigala)* from South Andaman caused by a highly virulent strain of *Aeromonas hydrophila* (Shome *et al*., 1996); subsequently, Shome (1999) reported milder and chronic form of disease with thinning of musculature, fluid accumulation in the abdomen without hemorrhagic ulcers and erosion of the scales in *Catla catla* caused by *A. hydrophila*.

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Apart from these two earlier reports, there exists no report of bacterial diseases recorded in the freshwater fishes from the Andaman Islands. In this work, two bacterial diseases causing emaciation, scale loss and abdominal dropsy in *Barbonymus gonionotus* and *Cyprinus carpio* var. *Koi* is reported, constituting the first new report from these Islands.

MATERIALS AND METHODS

During May 2022, twenty-five specimens of *Barbonymus gonionotus* showing emaciation and tail rot syndrome (ETS) and three specimens of *Cyprinus carpio* var. *Koi* showing big belly syndrome (BBS) disease symptoms were collected from a fish pond and an aquarium outlet located in the South Andaman district, respectively. The diseased fishes were packed in a polythene bag filled with aerated water and transported to fisheries science division laboratory of ICAR-CIARI, Port Blair for investigation.

The clinical signs and symptoms were noted. The fishes were subjected to bacteriological investigation; organs like spleen, liver and kidney were used for bacterial isolation. TSA broth and agar (tryptic soy broth and agar) were employed for bacteria isolation for 24 h at 28°C and the dominant uniform bacterial colonies were purified by streaking onto the TSA agar plates twice. A single bacterial colony was selected and inoculated in TSA broth for 14 hr at 28°C, then preserved at -80°C in the TSA medium containing 20% (v/v) sterile glycerol.

Suspected bacterial colonies were identified and purified and the following biological and biochemical tests were carried out using a kit (Hiimedia): 1) Gram stain, shape, motility and colony pigmentation; 2) Growth in 0.00-3.00% NaCl; 3) Biochemical tests including catalase, oxidase, indole, H_2S, lysine decarboxylase, ornithine decarboxylase, methyl red, Voges-Proskauer, nitrate reduction, utilization of dextrose, maltose, sucrose, trehalose, mannitol, galactose, mannose. Bacterial DNA was extracted from the pure colonies using DNAZOL reagent (Thermo Fisher Scientific) following manufacturer protocol. The bacterial DNA was subjected to the PCR amplification of the 16S ribosomal RNA (16S rRNA) using the primers set (16S-F: AGAGTTTGATCCTGGCTCAG; 16S-R: TACGGCTACC TTGTTACGACTT) PCR cycling conditions of Weisburg *et al.* (1991). The amplified PCR products were sequenced using the same primers mentioned above using an ABI 3500DNA analyzer (Eurofins Pvt. Ltd., Banglore, India). The homology of the generated sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) program in the National Center for Biotechnology Information (Altschul *et al*., 1990) to find the closest sequences available in GenBank (www.ncbi.nlm.nih.gov). The generated sequences were submitted to GenBank under the accession numbers OK602698 (*Aeromonas veronii*), OK602699 (*Aeromonas* sp.), OP081425 (*Pseudomonas alcaligenes*). Additional sequences of the 16S rRNA gene sequences of bacteria were downloaded from GenBank to construct a phylogenetic tree using MEGA X (Kumar *et al*., 2018) (Appendix 1). The best-fit nucleotide substitution model was selected from 24 models based on the lowest Bayesian information criterion (BIC) score, which was considered to describe the best substitution pattern (Nei and Kumar, 2000). Model tests suggested the best-fit nucleotide substitution model be a general time reversible (GTR) model with maximum likelihood value (lnL) and gamma distribution (G) [BIC =4868.9, lnL = -2396.3, (+G) = 0.2]. The phylogenetic tree was constructed based on the maximum likelihood fits in MEGA X. Reliability of the phylogenetic tree was estimated using bootstrap values based on 1000 iterations.

The organs from infected fishes were preserved in neutral buffered formalin (NBF) for histopathological studies before being transferred into 70% ethanol. Embedding and sectioning of paraffin blocks were then carried out. The obtained slides were stained with hematoxylin and eosin (H and E). To fulfil Koch's postulate a re-infectivity study was also conducted in *Catla catla* fingerlings by intraperitoneal inoculation of live *A. veronii* and *P. alcaligenes* whole cell suspension (12 hr old) at a concentration of 1.5×10^9 CFU/0.2 ml/fish and PBS as a control. The bacteria was further isolated from dead and surviving fishes.

RESULTS AND DISCUSSION

The 25 specimens of *B. gonionotus* appeared emaciated with hazy eyes, ulcerated fins and loss of scales. The sick fishes isolated themselves from the crowd and also appeared pale. Dissection of the fish revealed a greenish fluid-filled abdomen, congested intestine, pale kidney and liver (Fig 1A). The fish underwent slow mortality over 8 days. The *C. carpio* var. *Koi* had scale loss and a bulged abdomen (Fig 1B). Dissection of the fish revealed a clear, fluid-filled abdominal cavity, pale kidney and liver. Two live specimens of BBS fish underwent mortality after 15 days. The tissue smears from both the host fishes resulted in three dominant colonies on BHI plates, *viz*., two colonies from ETS fishes and one colony from BBS fishes. The colony characteristics and biochemical results are provided in Table 1. Sequencing of the amplified PCR product revealed the two isolates from ETS fish to be *Aeromonas veronii* and an unknown *Aeromonas* sp. and one isolate from BBS fish to be *Pseudomonas alcaligenes* showing 100% BLAST similarity with the existing GenBank sequences. Phylogenetic analysis of the generated sequences further confirmed the species and genus identity of the isolated pathogen (Fig 2). The histology of ETS fish revealed hyperplastic and congested secondary gill lamellae with leuckocytic infiltration (Fig 3A); the liver tissue had hepatocyte vacuolar degeneration and granuloma (Fig 4A), the kidney tissue had dilated tubule and damage of basal lamina (Fig 5A); the heart tissue revealed vacuolization of cardiomyoctes (Fig 6A). The intestinal section revealed edema and hemorrhage of gastric submucosa with macrophage infiltration. The gill of BBS fish had edema of secondary gill lamellae (Fig 3B). The liver section revealed the disorganization of hepatic tissue architecture in perivascular region (Fig 4B). The kidney tissue had

coagulative necrosis and haemorrhage (Fig 5B); the heart tissue had necrosis of cardiomyocytes and aggregation of inflammatory cells (Fig 6B). Brain section revealed degeneration of meninges and granular layer. Re-infectivity studies in *C. catla* fingerlings demonstrated the *A. veronii* and *P. alcaligenes* to cause 50% and 20% mortality after 5 to 6 days post-inoculation, respectively. The *C. catla* fingerlings injected with *A. veronii* demonstrated mild

Appendix I: List of GenBank sequences used for the phylogenetic analysis.

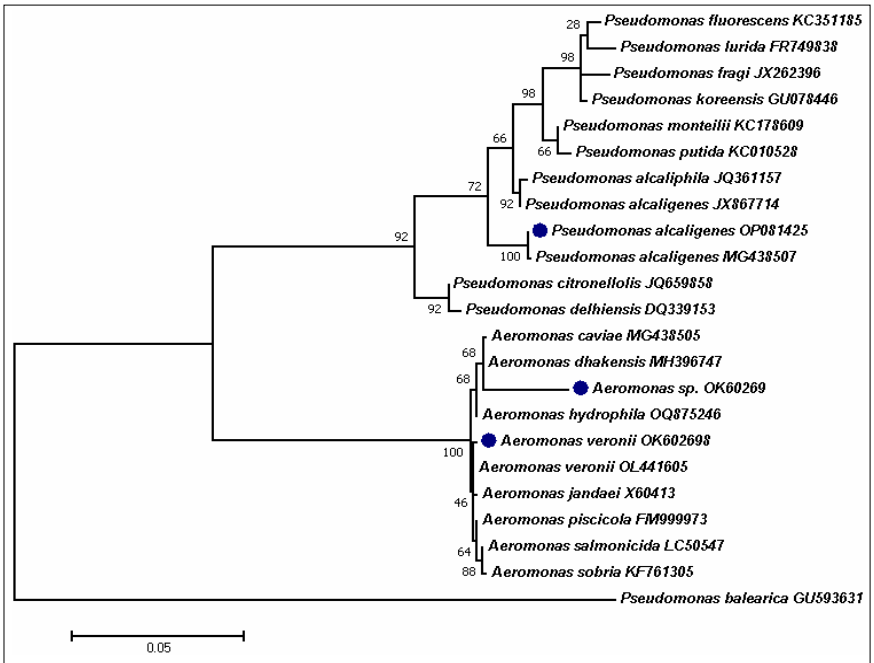
Species	Source	Country	GenBank accession number
Aeromonas veronii	*Barbonymus gonionotus*	Andaman Islands, India	OK602698
Aeromonas sp.	*Barbonymus gonionotus*	Andaman Islands, India	OK602699
Pseudomonas alcaligenes	*Cyprinus carpio* var. *Koi*	Andaman Islands, India	OP081425
Aeromonas caviae	*Betta splendens*	Thailand	MG438505
Pseudomonas alcaligenes	*Betta splendens*	Thailand	MG438507
Aeromonas dhakensis	pharmaceutical wastewater	Nigeria	MH396747
Aeromonas jandaei	-	-	X60413
Aeromonas salmonicida	-	-	LC50547
Aeromonas sobria	*Triplophysa siluorid*	china	KF761305
Aeromonas hydrophila	Grass carp	Pakistan	OQ875246
Aeromonas piscicola	*Oncorhynchus mykiss*	Spain	FM999973
Pseudomonas alcaligenes	*Betta splendens*	Thailand	MG438507
Pseudomonas fluorescens	Poplar rhizosphere	china	KC351185
Pseudomonas lurida	Nuclear fuel pool	Argentina	FR749838
Pseudomonas alcaliphila	Liquid	Poland	JQ361157
Pseudomonas monteilii	-	Czech	KC178609
Pseudomonas alcaligenes	River water	United Kingdom	JX867714
Pseudomonas citronellolis	Plant tissue	Singapore	JQ659858
Pseudomonas putida	Endospheric root	South Africa	KC010528
Pseudomonas balearica	Surface seawater	China	GU593631
Pseudomonas fragi	Copper mines	Poland	JX262396
Pseudomonas delhiensis	Fly ash dumping site of thermal power plant	India	DQ339153
Pseudomonas koreensis	Water column	Mexico	GU078446
Aeromonas veronii	*Micropterus salmoides*	China	OL441605

A. *Barbonymus gonionotus* showing pale eyes, emaciated body, tail rot, loss of scales and greenish fluid filled abdomen, B. *Cyprinus*

Fig 1: Symptoms of diseased fishes.

hemorrhagic septicaemia on the flanks and caudal peduncle, whereas in fishes injected with *P. Alcaligenes* mild dropsy was observed. The pathogens could also be re-isolated from the internal organs of both the dead and the surviving fishes.

Shome *et al.* (1996) recorded many sporadic cases of death in *C. mrigala* having symptoms of thinning of musculature giving a big head appearance, fluid accumulation in the abdomen without haemorrhagic ulcers



Blue dot indicate the GenBank number for the 16Sr DNA sequences generated in the present study.

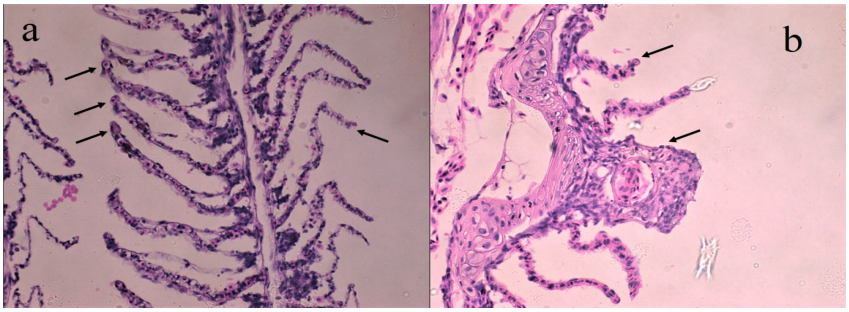
Fig 2: Phylogenetic analysis of *Aeromonas veronii*, *Aeromonas* sp. and *Pseudomonas alcaligenes* isolated from the diseased hosts.

Table 1: Biological and biochemical test performed for the three isolated pathogens.

Tests	*Aeromonas veronii*	*Aeromonas* sp.	*Pseudomonas alcaligenes*
Gram stain	-	-	-
Shape	Rod	Rod	Rod
Pigmentation	Cream coloured	Cream coloured	Translucent
Motility	+	+	+
Growth in 0.00-3.00% NaCl	+	+	+
Catalase	+	+	+
Oxidase	+	+	+
Indole	+	+	-
H_2S	+	+	-
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	-	-	-
Voges-Proskauer test	+	+	+
Methyl red test	-	-	-
Nitrate reduction	-	-	+
Dextrose	+	+	-
Maltose	+	+	-
Sucrose	-	-	-
Trehalose	+	+	-
Mannitol	+	+	-
Galactose	+	+	-
Mannose	+	+	-

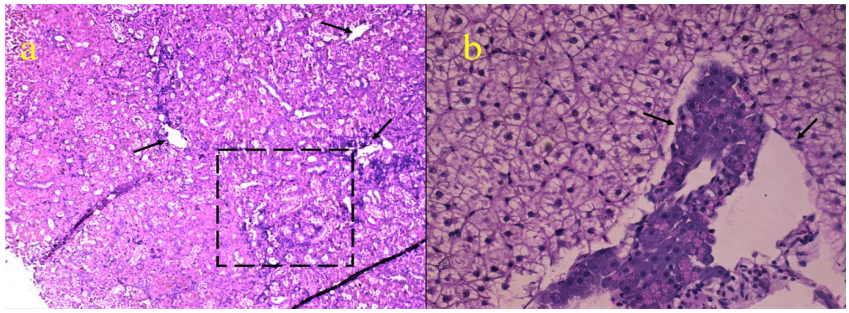
and erosion of the scales; subsequently, it was concluded that the causative agent of the disease to be a different strain of *A. hydrophila.*In the present work, probably the same disease case manifesting an emaciated body, thinning of the musculature, erosion of scales and fluid-filled abdomen is recorded in *B. gonionotus* with the main causative agent being *A. veronii* and an unknown *Aeromonas* sp. The pathogenic potential of these two isolates was further confirmed by the histopathological evidence on the host tissues.

Shome *et al.* (1999), reported *A. hydrophila* causing acute infectious abdominal dropsy and ulcerative diseases in Indian major carps from South and Middle Andaman Islands, however, in the present work, abdominal dropsy is reported in *C. carpio* var. *koi*, a cyprinid imported into Andaman for the aquarium trade and the pathogen isolated



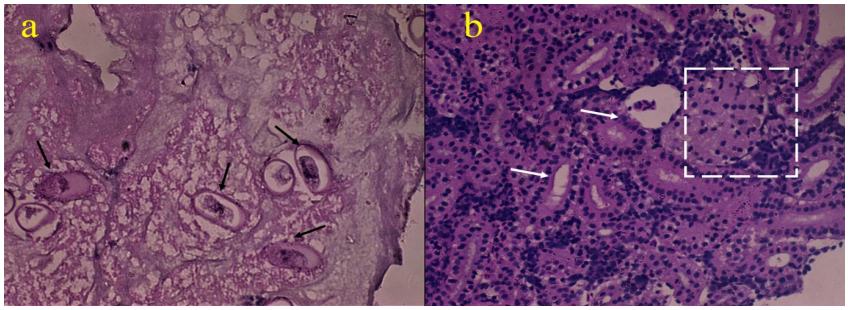
A. Telangiectasia condition of the primary gill lamella in *Barbonymus gonionotus* (arrows), B. hyperplastic and congested secondary gill lamellae with leuckocytic infiltration in *Cyprinus carpio* var. *Koi* (arrows).

Fig 3: Histology of gill sections.



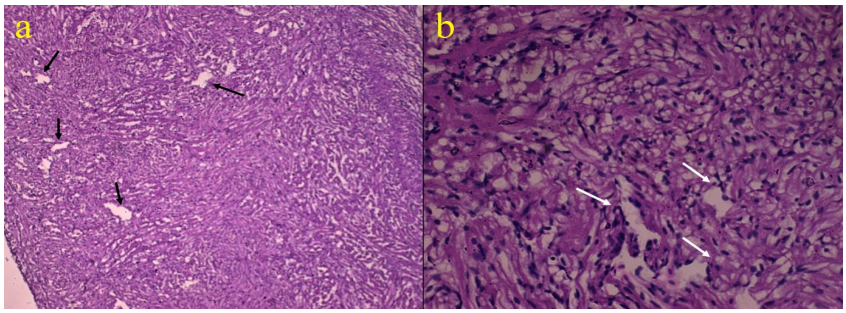
A. hepatocyte vacuolar degeneration (arrows) and granuloma (broken square) in *Barbonymus gonionotus*, B. Disorganization of hepatic tissue architecture in perivascular region (arrows) in *Cyprinus carpio* var. *Koi*.

Fig 4: Histology of liver sections.



A. Dilated tubule and damage of basal lamina (arrows) in *Barbonymus gonionotus*, B. Coagulative necrosis (broken square) and haemorrhage condition (arrows) in *Cyprinus carpio* var. *Koi.*

Fig 5: Histology of kidney sections.



A. Vacuolization of the cardiomyoctes (Black arrows) and aggregation of inflammatory cells (white arrows), A. *Barbonymus gonionotus and* B. *Cyprinus carpio* var. *Koi.*

Fig 6: Histology of heart sections.

being *P. alcaligenes*. *Pseudomonas alcaligenes* was also isolated from *Betta splendens* having big belly syndrome from Thailand (Dong *et al*., 2018). Worldwide, *Aeromonas* and *Pseudomonas* infections were known to be the causative agent for ulcerative syndrome, bacteria hemorrhagic septicemia, tail and fin rot; bacteria gill rot and dropsy (Snieszko and Bullock, 1968; Kazimierczak *et al*., 2019). Shome *et al.* (2005) also reported heavy dropsy outbreaks in the Indian major carp from Andaman, Meghalaya and Assam. The present record of *A. veronii*, *Aeromonas* sp. and *P. alcaligenes* in the two exotic cyprinids confirms that these pathogens are capable of causing infections in Indian major carps as evidenced in the re-infectivity studies in *C. catla* fingerlings. The present study also alerts fish farmers and aquarium hobbyists to take appropriate control and preventive measures for the management of ETS and BBS in fish. This study also offers scope for exploration of treatment protocols against these isolated pathogens.

CONCLUSION

The present work offers scope for the development of better management practices and treatment protocols for controlling ETS and BBS in freshwater fishes. The figures, test results and DNA sequences generated in the work will enable future researchers to work on the isolated pathogens.

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Conflict of interest: None.

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