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Distribution of *Aeromonas* spp. Emphasizing on a Newly Identified Species *Aeromonas* sp. T8 Isolated from Fish and Aquatic Animals in Southeast Asia

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Abstract: Distribution pattern of a collection of 106 *Aeromonas* strains isolated from both healthy and epizootic ulcerative syndrome (EUS)-affected fish, septicaemic disease affected frog and turtle in Bangladesh, Japan, Malaysia, Philippines and Thailand was investigated. The study was conducted through the physio-biochemical characterization of the strains and subsequent confirmation by analysis of the 16S rDNA sequences of some randomly chosen representative strains from all identified phenotype. Special emphasis was given to confirm a group of strains, which belonged to a newly identified species *Aeromonas* sp. T8 by DNA-DNA hybridization method. The newly identified species *Aeromonas* sp. T8 group was particularly found in different species of EUS-affected fish in Philippines and Thailand. *A. hydrophila* subsp. *hydrophila* and *A. hydrophila* subsp. *ranae* was recovered from EUS of fish and septicaemic diseases of frog and turtle. *A. hydrophila* subsp. *hydrophila* was distributed in Bangladesh and Thailand while *A. hydrophila* subsp. *ranae* was found only in Thailand. *A. veronii* biotype *sobria* and *A. veronii* biotype *veronii* was found to be dispersed mostly in EUS-affected fish in different countries. *A. jandaei* was obtained from EUS-positive fish in Bangladesh and Malaysia but *A. media* from healthy fish in Bangladesh.

Key words: EUS, septicaemic disease, new species *Aeromonas* sp. T8, phenotypic identification, phylogenetic analysis

INTRODUCTION

Bacteria of the genus *Aeromonas* are wide spread in fresh, brackish, estuarine and marine water[1]. They are frequently isolated from both healthy and diseased fish as well as from other aquatic animals. They are also considered to be one of the most important bacteria among the etiological agents of fish diseases[2]. Particularly, motile aeromonads are associated with tail and fin rot, hemorrhagic septicaemia and epizootic ulcerative syndrome (EUS) in a variety of freshwater and marine fish of the world[3,4]. Moreover, they are repeatedly reported to cause diseases in amphibians and reptiles[5,6]. The outbreaks of motile *Aeromonas* associated diseases can reach epidemic proportions among the aquatic animals, leading to massive mortality rates[7]. Therefore, knowledge on the distribution of *Aeromonas* species in diseased fish and other aquatic animals is necessary for establishing the epidemiological pattern involved in the diseases of aquatic animals.

The taxonomy of the genus *Aeromonas* is changing continuously due to identification of newly described species. At present, the genus *Aeromonas* comprises 14 well-recognized genospecies[8]. Recently, Iqbal[9] identified an atypical motile *Aeromonas* strain (T8) isolated from EUS-affected fish of Thailand. It possessed distinct phenotypic characteristics and represented a cluster with *Aeromonas caviae* in the phylogenetic tree constructed on the basis of 16S rDNA sequences. However, it exhibited discrete DNA-DNA hybridization homologies with all recognized *Aeromonas* genospecies[9]. Thus, it was considered as a new species and designated as *Aeromonas* sp. T8. Isolation, identification and distribution of the bacteria in the Southeast Asian region is essentially needed.

The objective of this study was to determine the distribution pattern of a collection of 106 aeromonad strains, isolated from both healthy and EUS-affected fish, septicaemic disease affected frog and turtle in Southeast Asian countries by means of physio-biochemical and molecular identification methods.

MATERIALS AND METHODS

Bacterial strains: A total of 106 *Aeromonas* strains collected from fish, frog and turtle of Bangladesh, Japan,

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Malaysia, Philippines and Thailand were investigated in this study (Table 1). Most of them were collected from EUS-positive fish and only five (B33, J1, J2, J3 and J4) from healthy fish specimen. In addition, several strains were collected from frog (*Rana tigrina* and *Rana rugulosa*) and turtle (*Chrysemys scripta* and *Trionyx sinensis*) suffering from septicaemic disease.

In this study, the *Aeromonas* sp. strain T8 (IAM 14920, JCM 11177) and 14 reference strains representing different *Aeromonas* DNA hybridization groups[10] were also included. The reference strains were as follows: *A. hydrophila* ATCC7966^T, *A. bestiarum* CDC5933-76, *A. salmonicida* subsp. *salmonicida* ATCC14174, *A. salmonicida* subsp. *masoucida* ATCC27013^T, *A. caviae* ATCC15468^T, *A. media* JCM2385^T, *A. eucrenophila* NCMB74^T, *A. sobria* JCM2139^T, *A. veronii* biotype *sobria* ATCC9071^T, *A. jandaei* JCM8316^T, *A. veronii* biotype *veronii* JCM7375^T, DNA hybridization group 11 CDC1306-83, *A. schubertii* JCM7373^T and *A. torta* JCM8315^T.

Culture condition: All bacterial strains were periodically cultured in nutrient agar (NA; 1% Poly peptone, 0.5% Beef extract, 0.12% Sodium chloride and 1.5% Agar powder; pH 7.2). The stock cultures were maintained at –80°C in nutrient broth medium supplemented with 10% glycerol (V/V).

Table 1: Origin of *Aeromonas* spp. strains used in this study

Country	Code No.	Host species	Organ	Place
Bangladesh	B19, B24	*Anabas testudineus*	Lesion	Mymensingh
	B12, B13, B14, B17	”	Lesion	Mymensingh
	B31, B32	”	Kidney	Pabna
	B41, B42, B43, B44, B61	*Cirrhinus mrigala*	Lesion	Gazipur
	B25, B26, B45, B46, B47	*Clarias batrachus*	Lesion	Mymensingh
	B50, B51, B52, B53, B54	,,	Kidney	,,
	B33*	*Hypophthalmichthys molitrix*	Gill	Mymensingh
	B22, B27, B58, B60	*Labeo rohita*	Lesion	Mymensingh
	B18, B35	*Channa punctatus*	Lesion	Pabna
	B36, B37, B57,	,,	,,	Jessor
	B15, B20	*Channa striatus*	Lesion	Mymensingh
	B30, B38, B39, B40, B49	*Pangasius sutchi*	Lesion	Mymensingh
	B16, B21, B23	*Barbodes gonionotus*	Lesion	Sylhet
	B34, B55, B56, B59	,,	,,	Mymensingh
	B28, B29	*Nandus nandus*	Lesion	Mymensingh
Japan	J1*, J2*, J3*, J4*	*Oreochromis nilotica*	Intestine	-
Malaysia	M102, M106, M113	*Anabas testudineus*	Lesion	Kagar
	M107	*Aristichthys nobilis*	Kidney	Enggor
	M110, M115, M116	,,	Liver	,,
	M108,M111,M112, M118	*Clarias* sp.	Spleen	Tanjung
	M109, M117	,,	Kidney	,,
	M104	*Cyprinus* sp.	Lesion	Kagar
	M101, M103, M119,	*Channa striatus*	Lesion	Kagar
	M114	*Barbodes gonionotus*	Liver	Melaka
Philippines	P1	*Oreochromis* sp.	Spleen	Iloilo
	P2	*Clarias batrachus*	Spleen	Laguna de bay
Thailand	T9, T14, T27	*C. macrocephalus x C. gariepinus*	Kidney	-
	T4	*Osphronemus goramy*	Kidney	-
	T12, T13	,,	,,	-
	T31	*Pangasius sutchi*	Liver	-
	T32	*Datnioides microlepis*	Lesion	-
	T51, T54	*Astronotus ocellatus*	-	Bangkok
	T52, T53	*Rana tigrina*	-	Ayutaya
	T33, T34, T35, T36, T37,	*Rana rugulosa*	Kidney	-
	T38, T39, T40, T41, T42	,,	,,	-
	T43, T44, T45	,,	Liver	-
	T46, T47	,,	,,	Nakorn
	T49	,,	-	pathom
	T50	,,	-	Bangkok
	T55	,,	-	Chouburi
	T57	,,	-	Pigit
	T56	*Chrysemys scripta*	-	Bangkok
	T48	*Trionyx sinensis*	-	-

*: Isolated from healthy fish

-: Unknown

Phenotypic characterization: Each of the *Aeromonas* strains was tested for 44 physio-biochemical properties. Among these urea hydrolysis, nitrate reduction, gas production from glucose, utilization of acetate, malonate, Christensen's citrate, Jordan's tartrate and phenylalanine, decarboxylations of lysine and ornithine and arginine dihydrolase tests were conducted according to the "Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria"[11]. Gelatin hydrolysis, esculin hydrolysis, methyl red (MR), Voges-Proskauer (VP) tests were performed according to the "Cowan and Steel's Manual for the Identification of Medical Bacteria"[12]. Lipase (tributirin) activity was tested following the method described in the "Bacterial Culture Media"[13]. Acid production from various carbohydrates was examined as described by Iqbal[9]. All of the tests were performed at 25°C and the results were observed after 24 h unless otherwise indicated. Strains that differed by only 1 to 5 characteristics from the reference strains was placed into the same species according to the criteria described by Iqbal[9].

Sequence of 16S rDNA: All strains that possessed similar or very close phenotypic properties with the *Aeromonas* sp. strain T8 (IAM 14920; JCM 11177) and 1-2 randomly chosen bacterial strains representing each phenotye were further investigated for the 16S rDNA sequence similarity and subsequent phylogenetic analysis. Bacterial cells were grown in nutrient broth and genomic DNA was extracted by using Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instructions.

PCR for amplification of the targeted gene was performed with the universal primer set 24 F (forward primer) and 1540 R (reverse primer). Each PCR mixture contained 6 μl of 25 mM $MgCl_2$, 10 μl of 10×PCR buffer, 2.0 μl of each 10 mM deoxyribonucleotide tri-phosphate, 5.0 μl of 20 μM solution of each primers, 100-200 ng of DNA template, 0.5 μl of *Taq* DNA polymerase (Promega) and sterile double-distilled water in a total volume of 100 μl. The PCR amplification was performed with a Gene Amp 9700 PCR system (PE Applied Bio systems). The thermal profile for PCR amplification was followed as described by Iqbal[9]. The PCR amplicons were visualized on 1.5% agarose gel stained with ethidium bromide solution. The amplified PCR products were purified by using Wizard PCR Purification System (Promega, USA) following the manufacturer's instructions. The purified PCR products were then subjected to cycle sequencing. The primer set used for cycle sequence reaction was the same as mentioned by Iqbal[9]. The sequence reaction mixture contained 2 μl of Big Dye V 3.0 matrix standard (Applied Biosystems), 3 μl of 5 × sequencing buffer, 1 μl of primer (1.6 pmole), 100-200 ng of purified PCR product and sterile double-distilled water in a total volume of 20 μl. The cycle sequence was performed with a Gene Amp 9700 PCR system (PE Applied Biosystems) following the program described by Iqbal[9]. The extended products were precipitated and vacuum dried. The dried pellets were resuspended in 20 μl of template suppression reagent (Applied Biosystems), heated at 95°C for 2 min and immediately placed on ice. The solution was transferred in capillary tube and sequencing was done with an automated capillary type sequencer (Applied Biosystems).

16S rDNA sequence similarity: The 16S rDNA sequences of the representative strains were compared with the sequences of other experimental strains as well as with the sequences of reference strains found from the gene bank sequences of NCBI. The 16S rDNA sequence homology was determined following the method described by Iqbal[9].

Phylogenetic analysis: The 16S rDNA sequence data were aligned and phylogenetic analyses were performed by using neighbour-joining[14] method of CLUSTAL X 1.8 software program. The sequences determined in this study and the sequences obtained from NCBI database (Table 2) were used for the analysis. The phylogenetic tree was constructed by using the tree-view program for the windows.

DNA-DNA hybridization: Aeromonad strains that formed a cluster with *Aeromonas* sp. strain T8 were further investigated for DNA-DNA hybridization homology. Bacterial cultures were grown to mid log-phase at 28°C in 500 ml nutrient broth in a shaking incubator. DNAs were extracted according to the procedure of Altwegg *et al.*[15]. Purified DNA from *Aeromonas* sp. strain T8 was labelled with photobiotin (Vector Laboratories, USA) and DNA-DNA microplate hybridization was done at stringent condition following the method described by Iqbal[9].

RESULTS

Phenotypic identification: All bacterial strains were Gram-negative, rod shaped motile with polar flagella, positive for catalase, cytochrome oxidase, D-glucose fermentation and were resistant against vibriostatic agent 0/129 (Table 3). All of the strains were able to grow without the presence of NaCl and showed positive reaction for gelatin hydrolysis and nitrate reduction tests. On the other hand, all strains were negative for urea

Table 2: Gene Bank accession number of the 16S rDNA sequences of *Aeromonas* species used for the sequence similarity and phylogenetic analysis

Species name	Strain No.	NCBI accession No.
A. hydrophila subsp. *hydrophila*	ATCC 7966^T	X60404
A. bestiarum supsp. *ranae*	LMG 19707^T	AJ508766
A. bestiarum	CIP 7430^T	X60406
A. salmonicida supsp. *salmonicida*	NCIMB 1102	X60405
A. caviae	NCIMB 13016^T	X60408
A media	ATCC 33907^T	X60410
A. eucrenophila	NCIMB 74^T	X60411
A. sobria	NCIMB 12065^T	X60412
A. veronii	ATCC 35624	X60414
A. jandaei	ATCC 49568^T	X60413
Aeromonas sp. HG 11	ATCC 35941	X60417
A. schubertii	ATCC 43700	X60416
A. torta	ATCC 49657^T	X60415
Aeromonas sp. Group 501	CDC 2478-85	U88663
A. popoffii	LMG 17541^T	AJ224308
A. allosaccharophila	CECT 4199^T	S39232
A. encheleia	LMG 317541	AJ224309
A. enteropelogenes	DSMZ 6394^T	X 71121
A. culicicoli	NCINB 5147^T	AF 170914

ATCC: American Type Culture Collection, Rockville, MD, USA
CDC: Centers for Disease Control, Atlanta, GA, USA
CETC: Coleccion Espanola de Cultivos Tipo, Valencia, Spain
CIP: Collection bacterienne de l'Institut Pasteur, Paris France
DSMZ: Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH, Germany
LMG: Culture Collection of the Laboratorium voor Microbiologie, Ghent, Belgium
NCIMB: National Collection of Indusrtial and Marine Bacteria, Scotland
^{T}: Type strain

Table 3: Common phenotypic properties of *Aeromonas* strains

Traits	Results
Gram stain	-
Shape	Rod
Motility	Motile
Oxidase	+
Polar flagella	+
Catalase	+
O-F test	Fermentative
Growth in 0% NaCl	+
Vibriostatic agent (0/129)	Resistant
Gelatin hydrolysis	+
Urea hydrolysis	-
Malonate utilization	-
Nitrate reduction	+
Acid from	
Glucose	+
Adonitol	-
D-Arbitol	-
Dulcitol	-
D-Xylose	-
D-Galactose	+
Glycerol	+
Maltose	+
D-sorbitol	-
Threhalose	+

hydrolysis and malonate utilization tests. All strains produced acid from glucose, D-galactose, glycerol, maltose and threhalose but did not produce acid from adonitol, D-arbitol, dulcitol, D-sorbitol and D-xylose.

Differentiable phenotypic characteristics of the strains are shown in Table 4. These characteristics were compared with the characteristics of *Aeromonas* sp. strain

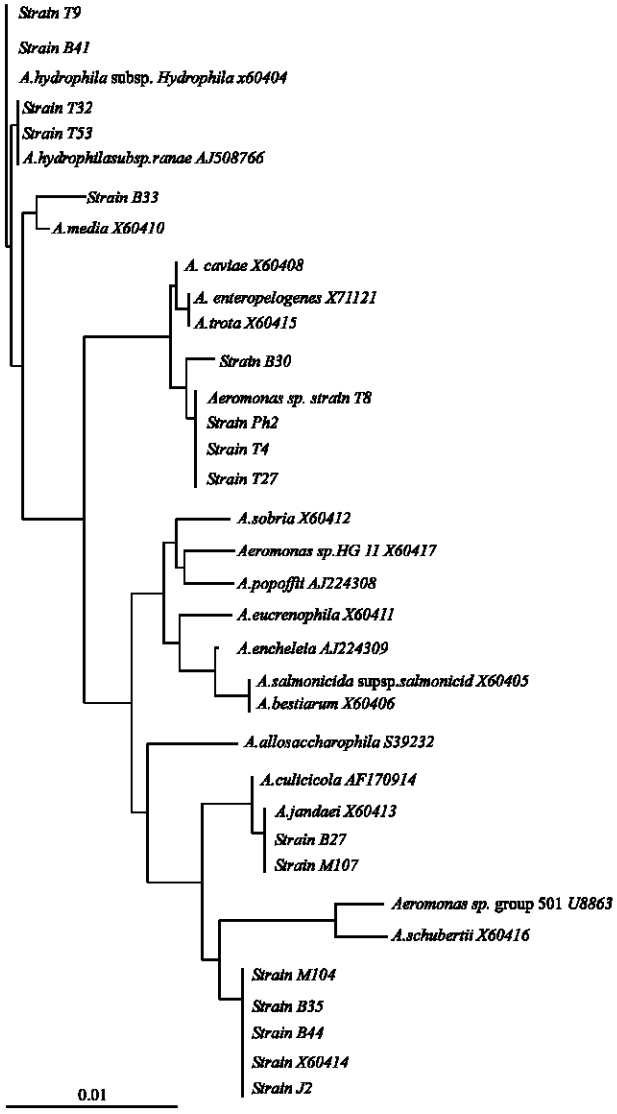

Fig. 1: 16S rDNA sequence-based phylogenetic relationship of Aeromonas strains. The unrooted phylogenetic tree was drawn using the neihbor-joining method in Clustal X. Scale bar 0.01 accumulated changes per uncleotide

T8 as well as with other reference strains. Among the aeromonads, four strains possessed very close phenotypic characteristics with the *Aeromonas* sp. strain T8. The strains T4 and T27 revealed complete similarity with the phenotypic properties of strain T8. Whereas, strain P2 and B30 exhibited variable results with the strain T8 for 2 and 3 phenotypic characteristics, respectively. The rest of the aeromonad strains were identified as *A. hydrophila* subsp. *hydrophila* (37 strains), *A. hydrophila* subsp. *ranae* (22 strains), *A. media* (1 strain), *A. veronii* biotype *sobria* (24 strains), *A. veronii* biotype *veronii* (10 strains) and *A. jandaei* (8 strains).

Table 4: Differentiable phenotypic characteristics of Aeromonas strain

Experimental status	1	2	3	4	5	6	7	8	9	10	11	12	13	14	a	b	c	d	e	f	g	h	I
*A. hydrophila*subsp.*hydrophila* ATCC7966	+	-	+	+	-	+	+	+	-	+	+	-	-		-	-	+	+	-	-	+	+	+
B12	+	-	+	+	-	+	+	-	-	+	+	+	-		-	-	-	+	-	-	+	+	-
B13	+	+	+	+	+	-	-	+	-	+	+	+	-		-	-	-	+	-	-	+	+	-
B14	+	+	+	+	+	+	+	+	-	+	+	+	-		-	-	-	+	-	-	+	+	-
B15	+	+	+	+	+	-	-	+	-	+	+	+	-		-	-	-	+	-	-	+	+	-
B16	+	+	-	+	+	-	+	-	-	+	+	+	+		-	-	+	+	+	-	+	+	-
B17	+	+	-	+	+	-	+	+	-	+	+	+	-		-	+	-	+	-	-	+	+	+
B18	+	+	-	+	+	+	+	+	-	+	+	+	-		-	+	-	+	-	-	+	+	+
B19	+	+	-	+	+	-	+	+	-	+	+	+	+		-	-	-	+	-	-	-	+	+
B22	+	+	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	-	+	+	+
B23	+	-	+	+	+	-	+	-	-	+	+	+	-		+	-	-	+	-	-	+	+	+
B25	+	+	-	+	+	-	+	+	-	+	+	+	-		-	-	-	+	-	-	+	+	-
B26	+	+	-	+	+	-	+	+	-	+	+	+	+		-	-	-	+	-	+	+	+	+
B29	+	+	-	+	+	-	+	+	-	+	+	-	-		-	-	+	+	-	+	+	+	+
B31	+	-	+	+	+	-	+	+	-	+	+	+	-		-	-	-	+	-	-	+	+	+
B32	+	+	+	+	+	-	+	+	-	+	+	+	-		-	+	-	+	-	-	+	+	+
B37	+	+	+	+	+	-	+	+	-	+	+	+	-		-	-	-	+	-	-	+	+	-
B38	+	+	+	+	+	+	-	+	-	+	+	+	-		-	-	-	+	-	-	+	+	-
B41	+	+	+	+	+	-	-	+	-	+	+	-	-		-	-	-	+	-	-	+	+	+
B42	+	+	+	+	+	-	+	+	-	+	+	+	-		-	-	-	+	-	-	+	+	+
B43	+	+	+	+	+	-	+	+	-	+	+	+	-		-	-	-	+	-	-	+	+	+
B47	+	+	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	+	+	+	+
B48	+	-	+	+	+	-	-	-	-	+	+	+	-		-	-	-	+	-	-	+	+	+
B51	+	+	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	-	+	+	+
B52	+	+	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	-	+	+	+
B53	+	+	+	+	+	-	+	+	-	+	+	+	-		-	-	-	+	-	-	+	+	+
B55	+	+	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	+	+	+	+
B56	+	+	+	+	+	-	+	-	-	+	+	+	-		-	+	-	+	-	+	+	+	+
B59	+	+	+	+	+	+	+	+	-	+	+	+	-		-	-	-	+	-	-	+	+	-
B60	+	-	+	+	+	-	+	+	-	+	+	+	-		-	-	-	+	-	-	+	+	+
T9	+	+	+	+	+	+	-	+	-	+	+	+	-		-	-	-	+	-	-	+	+	+
T13	+	+	+	+	+	+	-	+	-	+	+	+	-		-	-	-	+	-	-	+	+	+
T14	+	+	+	+	+	+	-	+	-	+	+	+	-		-	-	-	+	-	-	+	+	-
T49	+	+	-	+	-	+	+	+	-	+	+	-	-		+	-	+	+	-	-	+	+	+
T51	+	+	-	+	-	+	+	-	-	+	+	-	-		+	-	-	+	-	-	+	+	+
T54	+	+	-	+	-	+	+	-	-	+	+	-	-		+	-	+	+	-	-	+	+	+
T55	+	+	-	+	+	-	+	-	-	+	+	+	+		-	-	-	+	-	-	+	+	-
T56	+	+	-	+	-	+	+	-	-	+	+	+	-		+	-	+	+	-	-	+	+	+
A. hydrophila subsp. *ranae* *	-	-	Nt	-	-	+	-	Nt	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T31	-	-	-	-	-	+	-	-	-	+	+	-	-		-	-	-	+	-	-	-	-	-
T32	-	-	-	-	-	+	-	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T33	-	-	-	-	-	-	+	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T34	-	-	-	-	-	+	+	-	-	+	-	+	-		-	-	-	+	-	-	-	-	-
T35	-	-	-	-	-	+	-	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T36	-	-	-	-	-	+	-	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T37	-	-	-	-	-	+	+	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T38	-	-	-	-	-	+	-	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T39	-	-	-	-	-	+	-	-	-	+	+	-	-		-	-	-	+	-	-	-	-	-
T40	-	-	-	-	-	+	+	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T41	-	-	-	-	-	+	-	-	-	+	+	-	-		-	-	-	+	-	-	-	-	-
T42	-	-	-	-	-	+	-	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T43	-	-	-	-	-	+	+	-	-	+	+	-	-		-	-	-	+	-	-	-	-	-
T44	-	-	-	-	-	+	+	-	-	+	+	-	-		-	-	-	+	-	-	-	-	-
T45	-	-	-	-	-	+	-	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T46	-	-	-	-	-	+	-	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T47	-	-	-	-	-	+	-	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T48	-	-	-	-	-	+	-	+	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T50	-	-	-	-	-	+	+	-	-	+	+	-	-		-	-	-	+	-	-	-	-	-
T52	-	-	-	-	-	+	-	+	-	+	+	+	-		-	-	-	+	-	-	-	-	-
T53	-	-	-	-	-	+	-	-	-	+	+	-	-		-	-	-	+	-	-	-	-	-
T57	-	-	-	-	-	+	+	-	-	+	+	-	-		-	-	-	+	-	-	-	-	-
A. media JCM 2385	+	-	-	-	-	+	-	-	-	+	-	-	-		+	-	+	+	-	-	-	+	+
B33	+	+	-	+	-	+	-	-	-	+	+	-	-		+	-	-	+	-	-	-	+	+
A. veronii biotype *sobria* ATCC 9071	-	-	+	+	+	+	+	-	-	+	+	+	-		-	-	+	+	-	-	+	-	+
B20	-	-	+	+	+	-	+	+	-	+	+	-	-		-	-	-	+	-	-	+	-	-

1: Esculin hydrolysis, 2: Acetate utilization, 3: Gas from glucose, 4: Hydrogen sulphide, 5: VP, 6: MR, 7: Phenyl alanine, 8: Christensen's citrate, 9: Jordan's tartrate, 10: Lipase (tributyrin, 11: Arginine dihydrolase, 12: Lysine decarboxylase, 13: Ornithine decarboxylase, 14: Acid from: a Cellobiose, b: Myo-inositol, c: Lactose, d: D-Mannose, e: Raffinose, f: L-Rhamnose, g: Sucrose, h: Salicin, i: Arabinose

Table 4: Continue

Experimental status	1	2	3	4	5	6	7	8	9	10	11	12	13	14	a	b	c	d	e	f	g	h	i
B21	-	-	-	+	+	-	+	-	-	+	+	-	-		-	-	-	+	-	-	+	-	-
B24	-	-	-	+	+	+	+	-	-	+	+	+	-		-	-	-	+	-	-	+	-	-
B28	-	-	-	+	-	+	+	+	-	+	+	-	-		-	-	-	+	-	-	+	-	-
B34	-	-	-	+	+	+	+	-	-	+	+	+	-		-	-	-	+	-	-	+	-	-
B35	-	-	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	-	+	-	-
B39	-	-	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	-	+	-	-
B40	-	-	+	+	+	-	+	-	-	+	+	+	-		+	-	-	+	-	-	+	-	-
B44	-	-	+	+	+	-	+	+	-	+	+	+	-		-	-	-	+	-	-	+	-	-
B45	-	-	+	+	+	-	+	+	-	+	+	+	-		+	-	-	+	-	-	+	-	-
B46	-	-	+	+	+	-	+	-	-	+	+	+	-		+	-	-	+	-	-	+	-	-
B50	-	-	+	+	+	-	+	+	-	+	+	+	-		-	-	-	+	-	-	+	-	-
B61	-	-	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	-	+	-	-
M 102	-	-	+	+	+	+	+	+	-	+	+	-	-		+	-	-	+	-	-	+	-	-
M 103	-	-	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	-	+	-	-
M 106	-	-	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	-	+	-	-
M 108	-	-	+	+	+	+	+	+	-	+	+	+	-		-	-	-	+	-	-	+	-	-
M109	-	-	+	+	+	+	+	-	-	+	+	+	-		-	-	-	+	-	-	+	-	-
M 111	-	-	-	+	+	+	+	-	-	+	+	-	-		-	-	-	+	-	-	+	-	-
M 112	-	-	-	+	+	+	+	-	-	+	+	-	-		-	-	-	+	-	-	+	-	-
M117	-	-	-	+	-	+	+	-	-	+	-		-		-	-	-	+	-	-	+	-	-
M119	-	-	+	+	+	+	+	+	-	+	+	+	-		+	-	-	+	-	-	+	+	-
M120	-	-	+	+	+	+	+	-	-	+	-	+	-		-	-	-	+	-	-	+	-	-
P1	-	-	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	-	+	-	-
A. veronii biotype veronii JCM7375	+	-	-	+	+	+	+	+	-	+	+	+	+		+	-	-	+	-	-	+	-	-
B36	+	-	+	+	+	+	+	+	-	+	+	+	+		+	-	-	+	-	-	+	-	-
J1	+	-	+	-	-	+	-	+	-	-	-	-	+		+	-	-	+	-	+	+	-	+
J2	+	-	+	-	-	+	-	+	-	-	-	-	+		+	-	-	+	-	+	+	+	+
J3	+	-	+	+	-	+	-	-	-	-	-	-	+		+	-	-	+	-	+	+	-	+
J4	+	-	+	-	-	+	-	-	-	-	-	+	+		+	-	-	+	-	+	+	+	+
M 104	+	-	+	+	+	-	+	-	-	+	+	+	+		+	-	-	+	-	-	+	-	-
M110	+	-	+	+	+	-	+	+	-	+	+	+	+		+	-	-	+	+	-	-	+	-
M 113	+	-	+	+	+	+	+	-	-	+	+	-	+		+	-	-	+	-	-	+	+	-
M114	+	-	+	+	+	-	+	-	-	+	+	+	+		+	-	-	+	-	-	+	+	+
T12	+	-	+	+	+	+	+	+	-	+	+	+	+		+	-	-	+	+	-	+	+	-
A. jandaei JCM 8316	-	-	+	+	+	+	+	+	-	+	+	+	-		-	-	+	+	-	-	-	-	-
B27	-	-	+	+	+	-	+	+	+	+	+	-	-		-	-	-	+	-	-	-	-	-
B54	-	-	+	+	+	-	+	-	+	+	+	+	-		-	-	-	+	-	-	-	-	-
B57	-	-	+	+	+	+	+	-	+	+	+	+	-		-	-	-	+	-	-	-	-	-
B58	-	-	-	+	+	+	+	-	+	+	+	+	-		-	-	-	+	-	-	-	-	-
M 107	-	-	+	+	+	-	+	-	-	+	+	+	-		-	-	-	+	-	-	-	-	-
M 115	-	-	+	+	+	-	+	-	+	+	+	+	-		-	-	-	+	-	-	-	-	-
M 116	-	-	+	+	+	+	+	+	+	+	-	+	-		-	-	-	+	-	-	-	-	-
M 118	-	-	+	+	+	-	+	+	+	+	+	+	-		+	-	-	+	-	-	-	-	-
Aeromonas sp. T8	+	+	-	+	-	+	-	+	-	+	-	-	-		-	-	-	-	-	-	+	+	+
T4	+	+	-	+	-	+	-	+	-	+	-	-	-		-	-	-	-	-	-	+	+	+
T27	+	+	-	+	-	+	-	+	-	+	-	-	-		-	-	-	-	-	-	+	+	+
P2	+	+	-	+	-	+	-	-	-	+	-	-	-		+	-	-	-	-	-	+	+	+
B30	+	+	-	+	-	+	-	-	-	+	+	-	-		+	-	-	-	-	-	+	+	+

1: Esculin hydrolysis, 2: Acetate utilization, 3: Gas from glucose, 4: Hydrogen sulphide, 5: VP, 6: MR, 7: Phenyl alanine, 8: Christensen's citrate, 9: Jordan's tartrate, 10: Lipase (tributyrin, 11: Arginine dihydrolase, 12: Lysine decarboxylase, 13: Ornithine decarboxylase, 14: Acid from: a: Cellobiose, b: Myo-inositol, c: Lactose, d: D-Mannose, e: Raffinose, f: L-Rhamnose, g: Sucrose, h: Salicin, i: Arabinose

* Results from Hyus *et al.*[38], Nt: Not tested

DNA sequence similarity: The 16S rDNA sequence similarity of the selected strains with other experimental and reference strains have been shown in Table 5. In the present study, the strains phenotypically belonged to any species showed highest sequence similarity with their respective type or reference strains. Among them, strains T4, T27 and P2 exhibited 100% sequence similarity while strain B30 exhibited 99.8% similarity with *Aeromonas* sp. strain T8 (Table 5).

Phylogenetic analysis: The aeromonad strains that were phenotypically identified as *Aeromonas* sp. T8 group, *A. hydrophila* subsp. *hydrophila*, *A. hydrophila* subsp. *ranae*, *A. media*, *A. veronii* biotype *sobria*, *A. veronii* biotype *veronii* and *A. jandaei* formed clusters with their respective type or reference strains in the phylogenetic tree (Fig. 1).

DNA-DNA hybridization: In the DNA-DNA hybridization experiment, the strains T4, T27 and P2 revealed 98, 111

Table 5: Level of homology of the 16S rDNA sequences of representative strains with other *Aeromonas* species

	% homology with						
Strain	1	2	3	4	5	6	7
Strain T4	100.00						
Strain B30	99.80	100.00					
Strain T9	99.10	99.30	100.00				
Strain T32	98.74	98.69	99.73	100.00			
Strain B33	98.67	98.72	99.53	99.40	100.00		
Strain B27	98.87	98.56	99.28	98.29	98.06	100.00	
Strain M104	98.74	98.43	98.54	98.49	98.26	99.40	100.00
Aeromonas sp. strain T8	100.00	99.80	99.10	98.74	98.67	98.87	98.74
A.hydrophila sub sp. *hydrophila*	98.73	98.67	99.93	99.73	99.53	98.27	98.47
A.hydrophila sub sp. *ranae*	99.93	98.55	99.66	99.87	99.25	98.13	98.35
A. bestiarum	98.30	98.50	98.86	98.67	98.73	98.27	98.73
A. salmonicida	98.50	98.50	98.86	98.67	98.73	98.27	98.73
A. caviae	99.90	99.50	98.93	98.93	98.86	98.93	98.54
A. media	98.90	99.50	99.73	99.53	99.60	98.20	98.43
A. eucrenophila	98.50	98.50	99.06	98.87	99.93	98.47	98.93
A. sobria	98.70	98.80	98.66	98.60	98.40	98.73	99.00
A. veronii	98.70	98.50	98.46	98.40	98.19	99.40	99.93
A. jandaei	98.90	98.80	98.20	98.20	97.99	99.93	99.46
DHG 11	98.54	98.47	98.60	98.54	98.32	98.60	99.13
A. schubertii	98.00	98.50	97.67	97.54	97.59	98.60	98.93
A. torta	99.80	98.40	98.89	98.87	98.66	98.87	98.47
A. popoffii	98.30	98.50	98.93	98.80	98.72	98.51	98.72
A. allosaccharophila	98.80	99.00	93.88	98.54	98.32	98.73	99.13
A. culiciocola	98.63	98.43	98.11	98.09	97.98	99.80	99.22
Group 501	98.80	98.60	97.75	97.68	97.60	98.84	99.00

Homology of one representative strain (except strain B30) from each species is presented in the Table. Homology of the representative strains belonged to a species was completely similar to each other

Table 6: Distribution pattern of *Aeromonas* species in Southeast Asian countries

		No. of isolates under different genospecies						
Country	Source	*Aero.* sp. T8	HG 1A	HG 1B	HG 5	HG 8	HG 9	HG 10
Bangladesh (n=49)	EUS positive fish	1*	29	-	-	13	4	1
	Healthy fish	-	-	-	1	-	-	-
Japan (n=4)	Healthy fish					-		4
Malaysia (n=14)	EUS positive fish	-	-	-	-	10	4	4
Philippines (n=2)	EUS positive fish	1	-	-	-	1	-	-
Thailand (n=33)	EUS positive fish	2	5	2	-	-	-	1
	Septicaemic frog	-	2	19	-	-	-	
	Turtle	-	1	1	-	-	-	
Total (n=106)		3 (+1*)	37	22	1	24	8	10

HG1A: *A. hydrophila* subsp. *hydrophila*, HG1B: *A. hydrophila* subsp. *ranae*, HG5: *A. media*, HG8: *A. veronii* biotype *sobria*, HG9: *A. jandaei*, HG10: *A. veronii* biotype *veronii*, *Aero.* sp. T8: *Aeromonas* sp. T8 group, * : *Aeromonas* sp. (close to *Aeromonas* sp. T8 group), n: Number of strains

and 70% DNA-DNA hybridization homology, respectively, whereas the strain B30 exhibited 36% hybridization homology with the *Aeromonas* sp. strain T8.

Distribution of *Aeromonas* spp.: A distribution pattern of the *Aeromonas* species identified in the present study has been summarized in Table 6. The newly identified species *Aeromonas* sp. T8 group was found to be distributed in Philippines and Thailand in EUS-affected fish. Among the other species, *A. veronii* biotype *sobria* and *A. veronii* biotype *veronii* were determined to be extensively distributed particularly in EUS-positive fish in Southeast Asia.

DISCUSSION

EUS is an extremely damaging fish disorder that has swept through the South and Southeast Asian region with varying intensity since 1980[16]. The aetiology of EUS is very complex and for a long time, the specific aetiological agent of the disease remained unclear. Recently, a fungus *Aphanomyces invadans* is widely considered as the primary causative agent of the disease[17]. However, the fungus alone cannot initiate the disease because it is unable to break the skin barrier of fish[18]. In addition, Lilley and Roberts[19] argued that the fungus could not be considered as the primary cause of

EUS unless the infective zoospore stage can be shown to breach the fish's skin unaided. Thus, a conclusive primary factor responsible for the outbreak of EUS is still unclear. On the other hand, *Aeromonas* spp. is frequently isolated from EUS-affected fish[20-23]. Some reports suggested that *Aeromonas* spp. might contribute to the pathogenesis of the disease[24]. Moreover, *Aeromonas* strains isolated from EUS-affected fish are often found virulent for fish upon artificial challenge experiments[9,25]. Although, the role of *Aeromonas* spp. in EUS-affected fish is still unclear, it's pathological importance in EUS should keep in concern.

Iqbal[9] identified an atypical *Aeromonas* strain, considered to be a new species *Aeromonas* sp. T8, which was isolated from a EUS-affected giant gourami (*Osphronemus goramy*) of Thailand. In a recent investigation, the strain was proved to be virulent for several tropical fish species; reproduced hemorrhagic and necrotic lesions and mortality in fish upon intramuscular challenge, but it was non-virulent for mammal (mouse) upon artificial challenge. Additionally, it exhibited higher hemolytic activity against fish blood cells compared to mammalian blood cells. Thus, whether the new species possess any significance in EUS and/or other *Aeromonas*-associated disease in aquatic animals is a new research concern. In the present study, the distribution pattern of a total of 106 *Aeromonas* spp. isolated mostly from different disease affected aquatic animals in Southeast Asian countries were determined emphasizing on the distribution of a new species *Aeromonas* sp. T8.

The experimental strains examined in this study, obviously fulfilled the basic morphological and biochemical properties of the genus *Aeromonas* as mentioned by Popoff[26]. Aeromonad strains can be classified up to species level by biochemical characteristics like esculin hydrolysis, gas production from glucose, lysine and ornithin decarboxylation, fermentation of arabinose, salicine and sucrose *etc.*[7,27,28]. In this study, the aeromonad strains isolated from healthy and EUS-affected fish, septicaemic disease affected frog and turtle were phenotypically identified as *Aeromonas* sp. T8 group, *A. hydrophila* subsp. *hydrophila*, *A. hydrophila* subsp. *ranae*, *A. media*, *A. veronii* biotype *sobria*, *A. veronii* biotype *veronii* and *A. jandaei*.

Identification of bacterial species in most ichthyo-pathological laboratories is still depended on the phenotypic identification method. This method is quite successful, but several researchers stressed on the necessity of using molecular methods, in addition to biochemical markers for more accurate identification[9,29,30]. Presently, a direct comparison of ribosomal RNA gene sequence is considered as a powerful tool for identification of many bacterial groups[31]. It is also a standard method for the investigation of phylogenetic relationship of bacteria[32]. Even though some closely related species might have only a few differences in their 16S rDNA sequences, a phylogenetic tree can be established to give them an exact taxonomic position[33]. Thus, we determined the 16S rDNA sequence similarity and subsequent phylogenetic analysis of all phenotypically identified *Aeromonas* sp. T8 like strains and representative strains from each phenotypes to confirm the accuracy of the phenotypic identification. During the investigation, it has been noticed that the strains phenotypically identified as *Aeromonas* sp. T8 formed a cluster with the *Aeromonas* sp. strain T8 in the phylogenetic tree constructed on the basis of 16S rDNA sequences. Among them, three strains (T4, T27 and P2) possessed 100% sequence similarity while, the strain B30 exhibited 99.8% similarity with the strain T8. The representative strains phenotypically belonged to *A. hydrophila* subsp. *hydrophila*, *A. hydrophila* subsp. *ranae*, *A. veronii* and *A. jandaei* revealed highest sequence similarity with only one nucleotide difference with their corresponding type or reference strains and also formed clusters with the respective type or reference strains in the phylogenetic tree. No nucleotide difference was observed among the strains phenotypically belonged to *A. veronii* biotype *sobria* and *A. veronii* biotype *veronii* and these strains took place in the cluster with the type strain of *A. veronii*. Martinez-Murcia *et al.*[34] also reported similar findings.

However, the strain B33, which phenotypically resembled to *A. media*, exhibited closest similarity with the sequence of type strain of *A. media* with 6 nucleotide differences. It formed a cluster with *A. media* but in a separate line in the phylogenetic tree. Most recently, Yanez *et al.*[35] reported a similar type of finding. Therefore, determination of the accurate taxonomic position of the mentioned strain will be worthwhile.

Although, 16S rDNA sequence analysis is an important tool for bacterial identification, DNA-DNA hybridization study is considered to be the best-suited method for identification of closely related species or strains of bacteria within a single species. Since, *Aeromonas* sp. T8 is a new species, special emphasis was given to confirm the *Aeromonas* sp. T8 related strains. On the basis of DNA-DNA hybridization study, strains T4, T27 and P2 were confirmed to be the member of the new species *Aeromonas* sp. T8. Whereas, the strain B30, which was phylogenetically near to *Aeromonas* sp. strain T8, did not show significant DNA-DNA hybridization homology with *Aeromonas* sp. strain T8. As a result, the

strain was assumed to be a different species that might be close to *Aeromonas* sp. T8. Thus, confirmation of the taxonomic status of this strain will be sensible.

During the investigation, the aeromonad strains isolated from EUS-positive fish were identified as *Aeromonas* sp. T8 group, *A. hydrophila* subsp. *hydrophila*, *A. hydrophila* subsp. *ranae*, *A. veronii* biotype *sobria*, *A. veronii* biotype *veronii* and *A. jandaei*. Iqbal[9] identified similar type of *Aeromonas* species, except *A. hydrophila* subsp. *ranae* from the EUS-affected fish. Several articles reported on frequent isolation of *A. hydrophila* and *A. sobria* from EUS-affected fish since the epizootic incidence of the disease[21,22,36]. At present, *A. sobria* phenospecies contains *A. veronii* biotype *sobria*, *A. veronii* biotype *veronii*, *A. jandaei* and four other genospecies. However, Iqbal[9] first reported on identification of the *Aeromonas* sp. strain T8 isolated from a EUS-affected fish of Thailand. The most important finding of this study is identification of more strains belonging to the new species from EUS-positive fish of Philippines and Thailand.

The septicaemic disease in frog, also occasionally known as 'red leg' disease, referring to hemorrhages in the leg muscles is reported to cause by motile *Aeromonas* species[5]. Recently, a new phenon *A. hydrophila* subsp. *ranae* has been reported as the causative agent of a septicaemic disease in frog of Thailand[37,38]. In the present investigation, the strains isolated from frog were identified as *A. hydrophila* subsp. *hydrophila* and *A. hydrophila* subsp. *ranae*. Until the present study, *A. hydrophila* subsp. *ranae* was reported to be isolated only from frogs of Thailand[37]. In this study, we also identified the bacteria from EUS-affected siamese tiger fish (*Datnioides microlepis*) and striped catfish (*Pangasius sutchi*) and from soft shell turtle (*Trionyx sinensis*). Since, *A. hydrophila* subsp. *ranae* is distributed in a wide range of hosts, determination of the pathological importance of the bacteria to fish and other animals will be of grate interest.

Motile *Aeromonas* spp. is also known to cause disease in turtle[5,6]. During this study, 2 aeromonad strains collected from red-eared turtle (*Chrysemys scripta*) and soft shell turtle (*Trionyx sinensis*) were identified as *A. hydrophila* subsp. *hydrophila* and *A. hydrophila* subsp. *ranae*, respectively.

Through the investigation, *A. hydrophila* subsp. *hydrophila* was detected as the dominant group followed by *A. hydrophila* subsp. *ranae* and *A. veronii* biotype *sobria*. However, *A. hydrophila* subsp. *hydrophila* and *A. hydrophila* subsp. *ranae* were found to be associated with different types of diseases like EUS in fish and septicaemic disease in frog and turtle. *A. hydrophila* subsp. *hydrophila* was distributed in Bangladesh and Thailand while *A. hydrophila* subsp. *ranae* was found only in Thailand. On the other hand, *A. veronii* biotype *sobria* and *A. veronii* biotype *veronii* were determined to be extensively distributed particularly in EUS-positive fish in Southeast Asia. *A. jandaei* was obtained from EUS-positive fish in Bangladesh and Malaysia but *A. media* from healthy fish in Bangladesh.

The newly identified species *Aeromonas* sp. T8 group was detected in EUS-affected fish collected from Philippines and Thailand. The strains of *Aeromonas* sp. T8 group were isolated from giant gourami (*Osphronemus goramy*), hybrid catfish (♂ *Clarias macrocephalus* × ♀ *Clarias gariepinus*) and walking catfish (*Clarias batrachus*). Giant gourami and walking catfish are commonly found in natural freshwater bodies in the tropical region especially in the Southeast Asian countries. All of these fish are also cultivated as popular commercial freshwater fish species in this area. Thus, we assume that the newly identified species *Aeromonas* sp. T8 might be widely distributed in Southeast Asia. To the best of our knowledge, this is the first report about the distribution of *Aeromonas hydrophila* subsp. *ranae* found in different diseased aquatic animals and also the distribution of a new species *Aeromonas* sp. T8 in EUS-affected fish in Southeast Asia.

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