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Phenotypic and Genotypic Identification of Motile Aeromonads Isolated from Fish with Epizootic Ulcerative Syndrome in Southeast Asian Countries

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The phenotypic properties and genotypic characteristics of 44 aeromonads isolated from fish affected by epizootic ulcerative syndrome (EUS) in Southeast Asia were investigated. Among the 13 *A. hydrophila* phenospecies 9 were genotypically identical to *A. hydrophila* (HG1) and 4 were *A. veronii* biotype *sobria* (HG8Y) or *A. veronii* biotype *sobria*-related genospecies. All the 6 *A. veronii* biotype *sobria* phenospecies were placed in the same genospecies, *A. veronii* biotype *sobria*. Of the 12 *A. jandaei* phenospecies, 5 were *A. veronii* biotype *sobria* or *A. veronii* biotype *sobria*-related genospecies and 7 were *A. jandaei* genospecies. Of 13 *Aeromonas* isolates unspeciated by phenotyping 9 were genotypically identical to *A. hydrophila* or very similar to *A. hydrophila* and 2 isolates were *A. veronii* biotype *sobria* or very similar to *A. veronii* biotype *sobria*; 2 isolates could not be identified to genospecies level. These results strongly suggest that *Aeromonas* species from fish affected by EUS could not correctly be identified to the species level using various published biochemical schemes; it needs genetic identification like DNA-DNA hybridization.

Key words: *Aeromonas*, EUS, identification, phenotype, genotype

Epizootic ulcerative syndrome (EUS) of freshwater fishes is a serious threat to the fish production of Southeast Asian countries. It causes mass mortalities both cultured and wild fish species every year. Diseased fish develop one or more large dermal ulcers with varying degrees of destruction of underlying tissues in all parts of the body especially the head, abdomen and peduncle region. The syndrome appears to be seasonal (Tonguthai, 1985) and may be associated with environmental stress (Macintosh and Phillips, 1986) especially low water temperature during the winter season. The true etiology of the disease is yet unknown though some fish biologists suspect that an *Aphanomyces* fungus is associated with EUS (Roberts *et al.*, 1993). This fungus alone, however, can not initiate disease because it is unable to breach the skin barrier (Willoughby *et al.*, 1995). It has been also suggested that motile *Aeromonas* contribute to the pathogenesis of the disease (Costa and Wejeyaratne, 1989). Certain phenons of *A. hydrophila* induced EUS-like lesions when they were injected subdermally to snakehead and catfish (Lio-Po *et al.*, 1990). *Aeromonas* bacteria have frequently been isolated from EUS lesions (Anonymous, 1986; Llobrera and Gacutan, 1987; Torres *et al.*, 1990; Subasinghe *et al.*, 1990; Roberts *et al.*, 1990). This organism possesses the potential to produce a wide range of virulence factors including cytotoxins, haemolysins, proteases and nucleases (Ljungh and Wadstrom, 1986; Cahill, 1990). These evidences support that this bacterium may play an important role to cause EUS. However, till now, all the reports of *Aeromonas* associated with EUS were based on phenotypic identification which can not discriminate the species absolutely. According to Popoff *et al.* (1981) motile aeromonads consist of three species: *A. hydrophila*, *A. caviae* and *A. sobria* on the basis of biochemical traits. They also stated that each of these three species contains more than one-hybridization groups. In view of the consistent finding of *Aeromonas* in EUS and taxonomic complexity of the bacterium the present experiment was undertaken to identify the motile aeromonads isolated from EUS-affected fish of Malaysia, Thailand and Bangladesh.

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Materials and Methods

Strains and culture conditions

A total of 44 isolates of motile aeromonads were investigated in this study; 18 strains from Malaysia, 15 strains from Thailand and 11 strains from Bangladesh (Table 1). All isolates were Gram-negative, rod shaped motile bacteria which had positive reactions for oxidase and catalase, fermented glucose and were resistant to the vibriostatic agent O/129. Fourteen reference strains representing the different DNA hybridization groups were also used in this study (Table 2). Isolates and reference strains were periodically subcultured on nutrient agar slants and stock cultures were maintained at –80°C in 20% (v/v) glycerol/nutrient broth medium.

Phenotypic identification

Biochemical tests were done according to "Media for isolation-cultivation-identification-maintenance of medical bacteria, volume 1" (MacFaddin, 1985) or "DIFCO Manual, dehydrated culture media and reagents for microbiology, 10th edition (1984)". All the experimental isolates and the reference strains were studied in the same conditions and the results were read after 24 h at 25°C unless otherwise indicated.

DNA extraction

Cultures were grown to mid log-phase at 25°C in 1 liter nutrient broth in a shaker (Eyela, MMS-48GR). DNAs were extracted from the bacterial cells and purified according to the procedure of Altwegg *et al.* (1990), with minor modifications. Briefly, cells were harvested, washed with 0.15 M saline containing 0.1 M EDTA and resuspended in 50 m*l* of lysing buffer [0.1 M Tris hydrochloride (pH 8.0), 0.1 M NaCl, 0.002 g proteinase K], sodium dodecyl sulfate was added to a final concentration of 1%. The cells were allowed to lyse at 50°C for 3 h. DNA was then extracted firstly with 20 m*l* saturated phenol, secondly with phenol-chloroform-isoamylalcohol (25:24:1) and then with chloroform-isoamylalcohol (24:1). The solvents were removed from the aqueous phase after each extraction by centrifugation at 8400 × *g* for 15 min at 4°C. The DNA was precipitated with two volumes of absolute ethanol. After spooling, DNA was washed with 70% ethanol and redissolved in 15 m*l* 10 mM Tris containing 1 mM EDTA (pH 8) buffer. DNA was then treated with 1.5 m*l* RNase (1 mg/m*l* in distilled water) and incubated at 37°C for 3 h. After the RNase treatment DNA was reextracted with phenol, phenol-chloroform-isoamylalcohol and chloroform-isoamylalcohol as described above. Then DNA was washed with 70% ethanol after precipitated with 16 ml of isopropanol and redissolved in 2 m*l* of 10 mM Tris

Table 1. *Aeromonas* isolates used in this study

Country	Code No.	Source	Geographic location	Organ	Year
Malaysia	M1, M4, M6, M88, M99	*Anabas testudineus*	Kangar	Lesion	1987
(n = 18)	M16	*Cyprinus* sp.	Kangar	Lesion	1987
	M56	*Clarias* sp.	Tanjung Karang	Spleen	1987
	M24, M25	*Puntius gonionotus*	Melaka	Spleen	1986
	M30	*Oreochromis* sp.	Sekichian	Spleen	1987
	M26, M27	*P. gonionotus*	Melaka	Liver	1986
	M32, M33	*Oreochromis* sp.	Salak South	Liver	1987
	M29, M31	*Oreochromis* sp.	Sekichian	Kidney	1987
	M34, M71	*Aristichthys nobilis*	Enggor	Kidney	1987
Thailand	T26	*Channa striatus*	Supanburi	Spleen	1994
(n = 15)	T19, T20, T21	*P. gonionotus*	Nakorn Sawan	Liver	1995
	T2, T8, T11	*Osphronemus groamy*	Uthaitiani	Kidney	1994
	T17, T18	*O. groamy*	Nakorn Sawan	Kidney	1995
	T5, T7, T15, T25, T28, T30	*Cl. macrocephalus*	Supanburi	Kidney	1994
Bangladesh	B1, B2, B3, B4, B5	*Cirrhinus mrigala*	Gouripur, Mymensingh	Lesion	1994
(n = 11)	B6, B7, B8, B9, B10, B11	*Cirrhinus mrigala*	Trishal, Mymensingh	Lesion	1994

Table 2. Reference strains of *Aeromonas* species used in this study

HG group	Phenospecies	Genospecies	Strain
HG1	*A. hydrophila*	*A. hydrophila*	ATCC7966
HG2		Unnamed	CDC9533-76
HG3		*A. salmonicida* subsp. *salmonicida*	ATCC14174
HG3'		*A. salmonicida* subsp. *masoucida*	ATCC27013
HG4	*A. caviae*	*A. caviae*	ATCC15468
HG5B		*A. media*	JCM2385
HG6		*A. eucrenophila*	NCMB74
HG7	*A. sobria*	*A. sobria*	JCM2139
HG8Y		*A. veronii* biotype *sobria*	ATCC9071
HG9		*A. jandaei*	JCM8316
HG10		*A. veronii* biotype *veronii*	JCM7375
HG11		Unnamed	CDC1306-83
HG12		*A. schubertii*	JCM7373
HG13		*A. trota*	JCM8315

ATCC: American Type Culture Collection, Rockville, MD, USA
NCMB: National Collection of Marine Bacteria, Aberdeen, Scotland
CDC: Centers for Disease Control, Atlanta, GA., USA
JCM: Japan Collection of Microorganisms, Saitama, Japan

containing 1 mM EDTA (pH 8) buffer.

Determination of the GC contents of DNA

The mole percent guanine plus cytosine (G + C) contents of DNA were determined by high-performance liquid chromatography (HPLC) of its nuclease P1 hydrolysate according to Kumagai *et al.* (1988). Standard mixtures of nucleotides (Yamasa, Choshi, Japan) were used as references for calibration of mole percent measurement.

DNA-DNA hybridization

The purified DNA from 3 phenotypic groups HG1, HG8Y, HG9 and 3 other aquatic motile aeromonad groups HG2, HG6 and HG7 (Janda and Kokka, 1991) were selected for photobiotinylation. Hybridization was performed in a microplate coated with DNAs extracted from either experimental isolates or reference strains according to the method of Ezaki *et al.* (1989), with minor modifications. Briefly, denatured DNA solutions at 40 μg/ml from reference strains or experimental isolates were immobilized on microplates. Then they were prehybridized with hybridization buffer containing carrier DNA (salmon sperm DNA) and then hybridized with hybridization mixture containing photobiotinylated DNA. DNA-DNA hybridization was carried out at T_m-25°C for 24 h and then streptavidin-conjugated beta-galactosidase was added to bind the biotinylated DNA. The enzyme was then reacted with 4-methylumbelliferyl-beta-D-galactopyranoside. After hybridization, the intensity of the resultant fluorescence was measured with a microplate photometer (Corona electric, MTP-22). The fluorescence intensity of the wells with salmon sperm DNA was calculated as 0%, and the intensity of the fluorescence for each biotinylated reference strain taken as 100%.

Results

Phenotypic identification

Phenotypic characteristics of the 44 *Aeromonas* isolates (Table 3) were compared with those of the 14 reference strains (Table 4). Isolates which differed by only 1 to 5 properties from a reference strain, was presumptively identified as the same phenospecies. Of the 53 phenotypic characteristics tested 31 were invariably positive or negative. Other 22 tests gave variable results as shown in Tables 3 and 4. Of the 44 isolates analyzed phenotypically, 13 were identified as *A. hydrophila* (HG1), 6 were *A. veronii* biotype *sobria* (HG8Y) and 12 were *A. jandaei* (HG9). The remaining 13 isolates could not be identified to species level and

Table 3. Some important phenotypic characteristics of the experimental isolates

Experimental isolates	Esculin hydrolysis	Acetate utilization	Glucose (gas)	Voges-Proskauer	Methyl red	Phenylalanine	Simmon's citrate	Jordan's tartrate	Lipase (corn oil)	Christensen's citrate	Lysine decarboxylase	Ornithine decarboxylase	Acid from L-arabinose	D-cellobiose	*myo*-inositol	lactose	D-mannose	raffinose	L-rhamnose	sucrose	salicin	D-xylose
A. hydrophila (HG1)																						
M24	+	+	+	+	+	+	+	−	+	+	−	−	+	−	−	+	+	−	−	+	+	−
M26	+	−	+	+	+	+	+	−	+	+	−	−	+	+	−	−	+	−	−	+	+	−
M27	+	−	+	+	+	+	+	−	+	+	−	−	+	+	−	−	+	−	−	+	+	−
M29	+	+	+	+	−	+	+	−	+	+	−	−	+	−	−	+	+	−	−	+	+	−
M30	+	+	+	+	−	+	+	−	+	+	−	−	+	−	−	−	+	−	−	+	+	−
M31	+	+	+	+	−	+	+	−	+	+	−	−	−	−	−	+	+	−	−	+	+	−
M32	+	+	+	+	−	+	+	−	+	+	−	−	+	−	−	+	+	−	+	+	+	−
T11	+	+	+	+	−	+	+	−	+	+	−	−	+	−	−	+	+	−	+	+	+	−
T17	+	−	+	+	−	+	+	−	+	+	−	−	−	−	−	−	+	−	−	+	+	−
T18	+	+	+	+	−	+	+	−	+	+	−	−	−	−	−	−	+	−	−	+	+	−
T19	+	+	+	+	+	+	+	−	+	+	−	−	+	−	+	+	+	−	−	+	+	−
T20	+	+	+	+	+	+	+	−	+	+	−	−	+	−	+	+	+	−	−	+	+	−
T21	+	+	+	+	+	+	+	−	+	+	−	−	+	−	+	+	+	−	−	+	+	−
A. veronii biotype *sobria* (HG8Y)																						
B1	−	−	−	+	+	+	−	+	+	−	−	−	−	−	−	−	+	−	−	+	−	−
B5	−	−	−	+	+	−	−	+	+	−	−	−	−	−	−	−	+	−	−	+	−	−
B6	−	−	+	+	+	+	−	+	+	−	−	+	−	+	−	−	+	−	−	+	−	−
B7	−	−	−	+	+	−	−	+	+	−	−	−	−	−	−	−	+	−	−	+	−	−
B8	−	−	−	+	+	+	−	+	+	−	−	−	−	+	−	+	+	−	−	+	−	−
B11	−	−	+	+	+	+	−	+	+	−	−	−	−	−	−	−	+	−	−	+	−	−
A. jandaei (HG9)																						
M4	−	−	+	+	+	+	+	−	+	+	−	−	−	+	−	+	+	−	−	+	−	−
M6	−	−	+	+	+	+	+	−	+	+	−	−	−	−	−	+	+	−	−	+	−	−
M16	−	−	+	+	+	−	+	−	+	+	−	−	−	+	−	+	+	−	−	+	−	−
M34	+	−	+	+	−	+	+	−	+	+	−	−	−	+	−	+	+	+	−	−	+	−
M56	−	+	+	+	+	−	−	−	+	+	−	−	−	−	−	−	+	−	−	+	−	−
M71	+	−	+	+	−	+	+	−	+	+	−	−	−	+	−	+	+	+	−	−	+	−
T30	−	+	+	+	−	+	+	−	+	+	−	−	−	−	−	−	+	−	−	+	−	−
B2	−	−	+	+	+	+	+	+	+	+	−	−	−	+	−	−	+	−	−	−	−	−
B3	−	−	+	+	+	+	+	+	+	+	−	−	−	+	−	−	+	−	−	−	−	−
B4	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	+	−	−
B9	−	−	+	+	+	+	−	+	+	−	−	−	−	+	−	−	+	−	−	−	−	−
B10	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	+	−	−
Aeromonas spp.																						
M1	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−	−	+	−	−	+	+	+
M25	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−	−	+	−	−	+	+	−
M33	+	+	+	+	−	+	+	−	+	+	−	−	+	−	−	+	+	−	+	+	+	−
M88	−	+	+	+	+	+	−	−	+	+	+	−	−	−	−	−	+	−	−	+	−	−
M99	+	+	+	+	−	+	+	−	+	+	+	−	−	−	−	−	+	−	−	+	+	−
T2	+	+	+	+	−	+	+	−	+	+	−	−	−	−	−	+	+	−	−	+	+	+
T5	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−	+	+	−	−	+	+	+
T7	+	+	+	+	+	+	+	−	+	+	−	−	+	−	−	+	+	−	+	+	+	+
T8	+	+	−	−	+	−	+	−	−	+	−	−	+	−	−	−	−	−	−	+	+	−
T15	+	+	+	+	−	−	+	−	+	+	+	−	−	−	−	−	+	−	−	+	+	−
T25	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−	−	+	−	−	+	+	−
T26	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−	−	+	−	−	+	+	−
T28	+	+	+	+	−	+	+	−	+	+	+	−	−	−	−	−	+	−	−	+	+	−

Table 4. Some important phenotypic characteristics of the reference strains

Reference strains	Esculin hydrolysis	Acetate utilization	Gas from glucose	Voges-Proskauer	Methyl red	Phenylalanine	Simmon's citrate	Jordan's tartrate	Lipase (corn oil)	Christensen's citrate	Lysine decarboxylase	Ornithine decarboxylase	Acid from	L-arabinose	D-cellobiose	*myo*-inositol	lactose	D-mannose	raffinose	L-rhamnose	sucrose	D-salicin	D-xylose
HG1	+	−	+	−	+	+	+	−	+	+	−	−		+	−	−	+	+	−	−	+	+	−
HG2	+	−	+	+	+	+	+	−	+	−	−	−		+	−	−	−	+	−	−	+	+	−
HG3	+	−	+	−	+	+	−	−	−	−	+	−		+	−	−	−	+	−	−	−	+	−
HG3'	−	−	−	−	+	+	−	−	−	−	+	−		+	+	−	+	+	−	−	+	−	−
HG4	+	−	−	−	+	+	+	−	+	+	−	−		+	+	−	+	−	−	−	+	+	−
HG5B	+	−	−	−	+	−	+	−	−	−	−	−		+	+	−	+	+	−	−	+	+	−
HG6	−	−	+	−	+	+	+	−	−	+	−	−		−	+	−	+	+	−	−	−	−	−
HG7	−	−	+	+	+	+	−	−	+	−	−	−		−	+	−	+	+	−	−	−	−	−
HG8Y	−	−	+	+	+	+	−	−	+	−	−	−		+	−	−	+	+	−	−	+	−	−
HG9	−	−	+	+	+	+	+	−	+	+	−	−		−	−	−	+	+	−	−	−	−	−
HG10	+	−	−	+	+	+	−	−	+	+	−	−		−	+	−	+	+	−	−	+	+	−
HG11	+	−	−	−	+	+	−	−	+	−	−	−		−	−	−	+	+	−	−	+	+	−
HG12	−	−	+	+	+	+	−	−	−	+	−	−		−	−	−	−	+	−	−	+	−	+
HG13	−	−	+	−	+	+	+	−	−	+	−	−		−	+	−	−	+	−	−	−	−	−

were designated *Aeromonas* spp. (Table 3).

DNA base composition

DNA base composition of all the 44 isolates were almost agreed well with that set for the genus *Aeromonas* except T2, T8, T15 and T19 isolates (Table 5). Guanine plus cytosine values of these 4 strains were slightly out of the range.

DNA-DNA hybridization

DNA-DNA homology results of the 44 *Aeromonas* isolates and the reference strains are shown in Table 5. Of 13 phenotypic *A. hydrophila* isolates 9 belonged to HG1 and 4 belonged to HG8Y or HG8Y-related species. All the 6 phenotypic *A. veronii* biotype *sobria* belonged to HG8Y or HG8Y-related species. Among the 12 phenotypic *A. jandaei* isolates, 5 belonged to HG8Y or HG8Y-related and 7 isolates belonged to HG9. Among the 13 isolates of *Aeromonas* spp. group, which could not be identified based on the phenotypic characteristics, 9 were genotypically identical to HG1 or HG1-related species and 2 were HG8Y or HG8Y-related species. T2 and T8 isolates did not give any significant homology value with HG1, HG2, HG6, HG7, HG8Y or HG9.

Screening of biochemical properties

There was no single phenotypic test, which could be used to identify with confidence the 3 species, *A. hydrophila*, *A. veronii* biotype *sobria* and *A. jandaei* isolated from EUS-affected fish. Only four phenotypic properties were found which could partially differentiate these three species (Table 6). *A. hydrophila* could be separated from *A. veronii* biotype *sobria* and *A. jandaei* by their positive reactions in esculin hydrolysis, acetate utilization and acid production from salicin. On the other hand, *A. jandaei* could be differentiated from *A. veronii* biotype *sobria* by production of acid from sucrose.

Distribution of the identified Aeromonas *species*

All of the three genospecies were represented among the Malaysian isolates. (Table 7). Of the 15 isolates of Thailand, 9 belonged to *A. hydrophila* or *A. hydrophila*-related and 4 isolates belonged to *A. veronii* biotype *sobria* or *A. veronii* biotype *sobria*-related. The remaining 2 Thai isolates were not identified to species level. Of 11 Bangladeshi isolates, 6 were identified as *A. veronii* biotype *sobria* or *A. veronii* biotype *sobria*-related and 5 were *A. jandaei*.

Table 5. Levels of DNA relatedness of reference strains to the experimental isolates selected for the DNA-DNA hybridization at T_m-25°C and their DNA base composition

Unlabeled DNA	GC contents	Source of labeled DNA					
		HG1	HG2	HG6	HG7	HG8Y	HG9
HG1	62	100	49	36	43	50	54
HG2	61	59	100	25	44	35	51
HG3	59	ND	ND	ND	ND	ND	ND
HG3'	59	ND	ND	ND	ND	ND	ND
HG4	62	51	25	18	34	27	35
HG5B	61	48	25	21	29	36	37
HG6	60	46	29	100	53	42	37
HG7	57	50	27	57	100	35	43
HG8Y	59	53	28	24	30	100	64
HG9	60	54	32	21	39	54	100
HG10	59	53	32	23	34	77	44
HG11	62	51	38	17	24	36	42
HG12	62	6	13	17	14	21	8
HG13	62	54	34	58	52	49	28
A. hydrophila (HG1)							
M24	63	71	ND	ND	ND	30	9
M26	59	46	26	18	30	68	51
M27	60	40	ND	ND	ND	74	19
M29	61	94	37	26	35	37	47
M30	62	88	34	17	12	33	34
M31	57	70	ND	ND	ND	36	4
M32	61	75	26	19	23	28	40
T11	62	75	ND	ND	ND	35	21
T17	59	54	27	25	36	76	52
T18	62	85	36	22	23	26	47
T19	64	73	ND	ND	ND	34	18
T20	61	81	27	18	34	22	48
T21	59	39	ND	ND	ND	71	21
A. veronii biotype *sobria* (HG8Y)							
B1	59	66	32	27	27	88	62
B5	58	65	33	28	31	88	52
B6	59	54	28	26	23	80	58
B7	59	58	27	20	29	75	46
B8	60	61	30	19	36	83	56
B11	59	40	27	21	33	68	45
A. jandaei (HG9)							
M4	58	52	28	23	26	69	57
M6	58	45	29	18	34	66	57
M16	59	46	26	30	23	73	57
M34	59	45	20	14	26	39	76
M56	58	50	25	17	28	62	49
M71	58	31	ND	ND	ND	51	73
T30	61	52	28	19	28	88	52
B2	60	56	28	23	29	59	93
B3	60	63	25	21	35	62	88
B4	60	60	29	22	33	57	87
B9	58	53	30	17	31	51	72
B10	60	51	30	19	36	52	76
Aeromonas spp.							
M1	62	90	44	29	38	43	52
M25	62	86	39	21	24	36	45
M33	62	79	ND	ND	ND	36	20
M88	59	40	19	14	24	65	47
M99	62	82	32	28	39	40	44
T2	55	51	26	18	13	21	34
T5	63	30	ND	ND	ND	75	31
T7	62	69	23	17	27	26	43
T8	64	54	26	16	26	24	45
T15	64	75	ND	ND	ND	41	28
T25	61	74	37	19	37	21	44
T26	57	69	ND	ND	ND	23	18
T28	62	89	33	25	40	35	41

ND: Not detected

Table 6. Selected biochemical properties based on the analysis of the identified isolates studied in the experiment

Test	Percentage of isolates with positive result		
	A. hydrophila or *A. hydrophila* like	*A. veronii* biotype *sobria* or *A. veronii* biotype *sobria* like	*A. jandaei*
Esculin hydrolysis	100	29	29
Acetate utilization	100	29	0
Acid from sucrose	100	100	29
Acid from salicin	100	29	29

Table 7. Geographical distribution of the total identified *Aeromonas* species

Country	HG1	HG8Y	HG9
	A. hydrophila or *A. hydrophila* like	*A. veronii* biotype *sobria* or *A. veronii* biotype *sobria* like	A. *jandaei*
Malaysia (n = 18)	9	7	2
Thailand (n = 15)*	9	4	0
Bangladesh (n = 11)	0	6	5

* Two isolates from Thailand yet to be identified to the genospecies level correctly.

Discussion

According to the Bergey's manual of determinative bacteriology (Holt *et al.*, 1994), the basic morphological and biochemical properties of the genus *Aeromonas* are Gram negative rod shaped organisms with positive reactions for motility, oxidase, glucose fermentation and resistance to the vibriostatic compound (O/129). All of the reference strains and the experimental isolates were agreed with these descriptions of the genus *Aeromonas*.

Twenty two variable phenotypic characteristics of the experimental isolates were compared with the characteristics of the reference strains. Among the 44 isolates 13 were presumptively identified as *A. hydrophila* (HG1), 6 were *A. veronii* biotype *sobria* (HG8Y) and 12 were *A. jandaei* (HG9). The remaining 13 isolates could not be identified to the species level and were designated *Aeromonas* spp. (Table 3). DNA-DNA hybridization was then performed to identify genotypically the three phenotypic and *Aeromonas* spp. groups. *Aeromonas* genospecies can be defined by DNA-DNA hybridization criteria. Homology values of 70% at T_m-25°C with ≦ 5% divergence (Janda, 1991) are included in a given genospecies. Isolates with homology values of ≧ 70% with any of the reference strains were considered the same species and 60–70% was designated respective species related in the present experiment. Of the 44 *Aeromonas* isolates tested in the present experiment, 18 were genotypically identical to *A. hydrophila* or *A. hydrophila*-related species, 17 were *A. veronii* biotype *sobria* or *A. veronii* biotype *sobria*-related species and 7 were *A. jandaei* (Table 5). Two isolates from Thai did not give any significant homology value with HG1, HG2, HG6, HG7, HG8Y or HG9. Studies are going on to perform DNA-DNA hybridization among these isolates and all the reference strains labeling T2 and T8 isolates separately. Besides, 16S rDNA sequence of these isolates and some other identified strains are also going on to know the phylogenetic relationship of these isolates with the identified strains.

The phenotypic properties of the isolates, which were identified to HG1, HG8Y and HG9 groups, did not coincide exactly with those of their corresponding reference strain. Also characteristics sometimes varied among the isolates in the same group. Identification of *Aeromonas* isolates to the species level based on the phenotypic properties is imprecise and strains that possess biochemical properties atypical for a given

Table 8. Correlation between phenotypic and genotypic identification

Phenospecies	Genospecies		
	HG1 *A. hydrophila*	HG8Y *A. veronii* biotype *sobria*	HG9 A. *jandaei*
HG1 (n = 13)	9	4	0
HG8Y (n = 6)	0	6	0
HG9 (n = 12)	0	5	7
Aeromonas spp. (n = 13)*	9	2	0

* Two isolates from Thailand yet to be identified to the genospecies level correctly.

genospecies are common. Such strains can not be identified accurately using a limited number of phenotypic properties. Kuijper *et al.* (1989), Hickman-Brenner *et al.* (1987) and Allen *et al.* (1983) reported that phenotypic identification of *Aeromonas* species using various published biochemical schemes or the diagnostic keys in Bergey's manual was substantially inadequate in terms of the genetic heterogenicity of most *Aeromonas* species. They found the differentiation between phenotypic and genetic identification.

There was no single phenotypic test which could identify the 3 identified *Aeromonas* species with confidence. Only 4 phenotypic properties were found to be able to partially differentiate these 3 species (Table 6). These results correlate with the results of Abbott *et al.* (1992) and Altwegg *et al.* (1990).

As a result of genotypic identification, Malaysian isolates represented all the 3 genospecies dominated by *A. hydrophila* and *A. veronii* biotype *sobria* (Table 7). Among the 15 Thai isolates, 9 belonged to *A. hydrophila* and 4 belonged to *A. veronii* biotype *sobria*. Six isolates of Bangladesh were identified as *A. veronii* biotype *sobria* and 5 were *A. jandaei*. None of the isolates from Thai were identified as *A. jandaei*. There was no *A. hydrophila* among the Bangladeshi isolates. This may be because an insufficient number of isolates were tested or absence of *A. hydrophila* in the sampling environment. However, it needs further studies.

From the data presented herein, it is apparent that correct identification of *Aeromonas* species isolated from the EUS of fish using a biochemical scheme is inadequate. An isolate phenotypically identified as one species, but belonging to a different genospecies was found in the present study (Table 8). The present experiment has confirmed the necessity of genetic identification like DNA-DNA hybridization of *Aeromonas* species and its value as a milestone to clarify the relationship between *Aeromonas* and EUS.

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