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Phenotypic Identification of Motile Aeromonads Isolated from Fishes with Epizootic Ulcerative Syndrome in Southeast Asian Countries

Mohammed Mahbub IQBAL, Kenichi TAJIMA and Yoshio EZURA

Abstract

A total of 44 strains of motile aeromonad isolated from fishes with epizootic ulcerative syndrome (EUS) in Malaysia, Thailand and Bangladesh including 14 reference strains of DNA hybridization groups further been investigated to identify them to the phenospecies level. Biochemical properties of the 44 isolates were compared with those of the 14 reference strains of *Aeromonas* species. Among the 44 strains reidentified phenotypically, 26 strains were placed into the phenon corresponding to *A. hydrophila* or related species, 12 strains were *A. veronii* biotype *sobria* and 5 strains were *A. jandaei*. The remaining 1 strain could not be able to place in any of the species of *Aeromonas* because of its too heterogeneity with the reference strains, which was designated *Aeromonas* sp. Properties of esculin hydrolysis, acetate utilization, Christensen's citrate, Jordan's tartrate and acid from sucrose and salicin partially differentiate the three identified species. Of 18 Malaysian strains identified phenotypically, 13 strains belong to *A. hydrophila*, 5 belong to *A. veronii* biotype *sobria*. Among the 15 strains from Thailand, 13 strains were identical to *A. hydrophila* phenospecies, one was *A. veronii* biotype *sobria*. Rest 1 strain was identified as *Aeromonas* sp. Of 11 Bangladeshi strains, 6 strains were identified as *A. veronii* biotype *sobria* and 5 were *A. jandaei*.

Key words: Phenotypic, Identification, Aeromonad, EUS, Southeast Asia

Introduction

Aeromonas spp. are recognized as an autochthonus inhabitants of aquatic environments (Hazen et al., 1978; Kaper et al., 1981; Larsen and Willeberg, 1984). Some of them are pathogenic for poikilothermal animals, such as frogs, snakes or fish and in homeothermal animals and humans (Altwegg and Geiss, 1989; Janda, 1991; Popoff, 1984). It has also been frequently isolated from the lesions of epizootic ulcerative syndrome (EUS) fishes (Anonymous, 1986; Llobrera and Gacutan, 1987; Torres et al., 1990; Subasinghe et al., 1990; Roberts et al., 1990). This disease is a serious threat to the freshwater fish production of Southeast Asian countries. It causes mass mortalities both cultured and wild fish species every year. Although EUS is now a major problem in Southeast Asian countries; but unfortunately the true etiology of the disease is still unclear. *Aphanomyces* fungus is believed to contribute the disease reported by Roberts et al. (1993). This fungus alone, however, can not initiate the disease because it is unable to breach the skin barrier (Willoughby et al., 1995). It has been also suggested that *Aeromonas* spp. contri-

Laboratory of Microbiology, Faculty of Fisheries, Hokkaido University
(北海道大学水産学部微生物学教室)

bute to the pathogenesis of the disease (Costa and Wejeyaratne, 1989). Certain phenons of *A. hydrophila* could induce EUS-like lesions reported by Lio-Po et al. (1990). Therefore, *Aeromonas* spp. in the fishes with EUS is of great importance as well as *Aphanomyces* fungus to know the true etiology of the disease. Recently a total of 44 aeromonads isolated from the fish with EUS have been identified to the species level based on a series of phenotypic traits and DNA-DNA hybridization (Iqbal et al., 1998). But their phenotypic traits were fastidious enough and did not correlate with the genetic identification in most cases. On the other hand, identification of bacteria in the microbiological laboratories mostly depends on their phenotypic properties. DNA-DNA hybridization or others molecular techniques to identify the bacterial flora are not still available in most laboratories. This study was therefore, undertaken to further identify the *Aeromonas* spp. isolated from EUS-affected fishes of Malaysia, Thailand and Bangladesh based on biochemical properties and to screen their phenotypic traits which might be helpful for identification of this species in the diagnostic laboratories.

Material and Methods

Strains and culture conditions

A total of 44 *Aeromonas* isolates were studied. Among them, 18 strains from Malaysia, 15 strains from Thailand and 11 strains from Bangladesh (Table 1). Fourteen reference strains representing the different DNA hybridization groups (Janda, 1991) were also used in this study (Table 2). Isolates and reference strains were periodically subcultured on a nutrient agar (NA ; polypeptone 10 g, meat extract 5 g, NaCl 1.2 g, agar 15 g, distilled water 1,000 ml, pH 7.2) and stock cultures

Table 1. Origins of tested 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	*C. mrigala*	Trishal, Mymensingh	Lesion	1994

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

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

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

were maintained at −80°C in nutrient broth medium containing 20% glycerol (v/v).

Biochemical characterization

Urea hydrolysis, nitrate reduction, utilization of acetate and malonate, Jordan's tartrate, phenylalanine test, gas from glucose, methyl red, Voges-Proskauer, decarboxylase of lysine and ornithine properties were done according to "media for isolation-cultivation-identification-maintenance of medical bacteria, volume 1" (MacFaddin, 1985). Gelatin hydrolysis, esculin hydrolysis and lipase (corn oil) properties were done according to "Bacterial culture media, volume 1" (Sakazaki, 1978). Christensen's citrate test was done according to "DIFCO Manual, dehydrated culture media and reagents for microbiology, 10th edition (1984)". To know acid production from various carbohydrates, 0.5% of various carbohydrates were suspended into the dehydrated bacto OF basal midium (DIFCO) separately. All the experimental and the reference strains were investigated at 25°C and the results were read after 24 h unless otherwise indicated.

Determination of the GC contents of DNA

The cells were grown to mid log-phase at 25°C in 1 L nutrient broth in a shaker (Eyela, MMS-48GR). DNAs were then extracted from the bacterial cells and purified as described previously (Iqbal et al., 1998). The mole percent guanine-plus-cytosine (G+C) contents 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.

Results

All strains were Gram-negative, rod shaped motile bacteria which had positive reactions for oxidase and catalase, fermented glucose and were resistant to vibriostatic agent O/129, 2, 4-diamino-6, 7-diisopropylpteridine (Table 3). The DNA moles percent G+C contents of all the isolates ranged from 55 to 64 (Table 4). Variable biochemical properties of the 44 *Aeromonas* isolated strains (Table 4) were compared with those of the 14 reference strains (Table 5). Isolated strains, which differed by only 1 to 5 properties from reference strains, was placed into the same species. Among the 44 isolated strains reidentified phenotypically, 26 strains were placed into the phenon corresponding to *A. hydrophila* (HG1) or related HG2 group, 12 strains were *A. veronii* biotype *sobria* (HG8Y) and 5 strains were *A. jandaei* (HG9). The remaining 1 strain from Thailand (T8) was too heterogeneous with the reference strains (Table 5) and could not be able to place in any of the groups. But the strain expressed the basic phenotypic properties of the genus *Aeromonas*. Hence

Table 3. Phenotypic properties of the experimental and reference strains, which were invariably positive or negative or same results.

Traits	Results
Gram stain	−ve
Shape	Rod
Motility	Motile
Oxidase	+
Catalase	+
OF test	Fermentative
Acid from	
Glucose	+
Adonitol	−
D-Arabitol	−
Dulcitol	−
D-Galactose	+
Maltose	+
D-Sorbitol	−
Trehalose	+
Gelatin hydrolysis	+
Urea hydrolysis	−
Malonate utilization	−
Nitrate reduction	+
Growth in 0% NaCl	+
Vibriostatic agent (O/129)	Resistant

Table 4. Variable phenotypic characteristics of the experimental strains.

Experimental strains	DNA GC mole%	Esculin hydrolysis	Acetate utilization	Glucose (gas)	Voges-Proskauer	Methyl red	Phenylalanine	Christensen's citrate	Jordan's tartrate	Lipase (corn oil)	Lysine decarboxylase	Ornithine decarboxylase	Acid from Cellobiose	*myo*-inositol	Lactose	D-Manose	Raffinose	L-Rhamnose	Sucrose	Salicin	D-Xylose	Glycerol
A. hydrophila ATCC 7966^T (HG1)	62	+	−	+	−	+	+	+	−	+	−	−	−	−	+	+	−	−	+	+	−	+
Unnamed CDC 9533-76 (HG2)	61	+	−	+	+	+	+	−	−	+	−	−	−	−	−	+	−	−	+	+	−	+
M1	62	+	+	+	+	−	−	+	−	+	−	−	−	−	−	+	−	−	+	+	−	+
M24	63	+	+	+	+	+	+	+	−	+	−	−	−	−	+	+	−	−	+	+	−	+
M25	62	+	+	+	+	−	−	+	−	+	−	−	−	−	−	+	−	−	+	+	−	+
M26	59	+	−	+	+	+	+	+	−	+	−	−	+	−	−	+	+	+	+	+	−	+
M27	60	+	−	+	+	+	+	+	−	+	−	−	+	−	−	+	−	−	+	+	−	+
M29	61	+	+	+	+	−	+	+	−	+	−	−	−	−	+	+	−	−	+	+	−	+
M30	62	+	+	+	+	−	+	+	−	+	−	−	−	−	−	+	−	−	+	+	−	+
M31	57	+	+	+	+	−	+	+	−	+	−	−	−	−	+	+	−	−	+	+	−	+
M32	61	+	+	+	+	−	+	+	−	+	−	−	−	−	+	+	−	+	+	+	−	+
M33	62	+	+	+	+	−	+	+	−	+	−	−	−	−	+	+	−	+	+	+	−	+
M34	59	+	−	+	+	−	+	+	−	+	−	−	+	−	+	+	+	−	−	+	−	+
M71	58	+	−	+	+	−	+	+	−	+	−	−	+	−	+	+	+	−	−	+	−	+
M99	62	+	+	+	+	−	+	+	−	+	−	−	−	−	−	+	−	−	+	+	−	+
T2	55	+	+	+	+	−	+	+	−	+	−	−	−	−	+	+	−	−	+	+	+	+
T5	63	+	+	+	+	−	−	+	−	+	−	−	−	−	+	+	−	−	+	+	+	+
T7	62	+	+	+	+	+	+	+	−	+	−	−	−	−	+	+	−	+	+	+	+	+
T11	62	+	+	+	+	−	+	+	−	+	−	−	−	−	+	+	−	+	+	+	−	+
T15	64	+	+	+	+	−	−	+	−	+	−	−	−	−	−	+	−	−	+	+	−	+
T17	59	+	−	+	+	−	+	+	−	+	−	−	−	−	−	+	−	−	+	+	−	+
T18	62	+	+	+	+	−	+	+	−	+	−	−	−	−	−	+	−	−	+	+	−	+
T19	64	+	+	+	+	+	+	+	−	+	−	−	−	+	+	+	−	−	+	+	−	+
T20	61	+	+	+	+	+	+	+	−	+	−	−	−	+	+	+	−	−	+	+	−	+
T21	59	+	+	+	+	+	+	+	−	+	−	−	−	+	+	+	−	−	+	+	−	+
T25	61	+	+	+	+	−	−	+	−	+	−	−	−	−	−	+	−	−	+	+	−	+
T26	57	+	+	+	+	−	−	+	−	+	−	−	−	−	−	+	−	−	+	+	−	+
T28	62	+	+	+	+	−	+	+	−	+	+	−	−	−	−	+	−	−	+	+	−	+

Table 4. Contenued.

Experimental strains	DNA GC mole%	Esculin hydrolysis	Acetate utilization	Glucose (gas)	Voges-Proskauer	Methyl red	Phenylalanine	Christensen's citrate	Jordan's tartrate	Lipase (corn oil)	Lysine decarboxylase	Ornithine decarboxylase	Acid from Cellobiose	*myo*-inositol	Lactose	D-Manose	Raffinose	L-Rhamnose	Sucrose	Salicin	D-Xylose	Glycerol
A. veronii biotype *sobria* ATCC 9071 (HG8Y)	59	−	−	+	+	+	−	−	+	−	−		−	−	+	+	−	−	+	−	−	+
M4	58	−	−	+	+	+	−	−	+	−	−		+	−	+	+	−	−	+	−	−	+
M6	58	−	−	+	+	+	−	−	+	−	−		−	−	+	+	−	−	+	−	−	+
M16	59	−	−	+	+	−	−	−	+	−	−		+	−	+	+	−	−	+	−	−	+
M56	58	−	+	+	+	−	−	−	+	−	−		−	−	−	+	−	−	+	−	−	+
M88	59	−	+	+	+	+	−	−	+	+	−		−	−	−	+	−	−	+	−	−	+
T30	61	−	+	+	+	−	+	−	−	+	−		−	−	−	+	−	−	+	−	−	+
B1	59	−	−	−	+	+	+	−	+	+	−		−	−	−	+	−	−	+	−	−	+
B5	58	−	−	−	+	+	−	−	+	+	−		−	−	−	+	−	−	+	−	−	+
B6	59	−	−	+	+	+	+	−	+	+	−	+	+	−	−	+	−	−	+	−	−	+
B7	59	−	−	−	+	+	−	−	+	+	−		−	−	−	+	−	−	+	−	−	+
B8	60	−	−	−	+	+	+	−	+	+	−		−	−	+	+	−	−	+	−	−	+
B11	59	−	−	+	+	+	+	−	+	+	−		−	−	−	+	−	−	+	−	−	+
A. Jandaei JCM 8316^T (HG9)	60	−	−	+	+	+	+	+	−	+	−		−	−	+	+	−	−	−	−	−	+
B2	60	−	−	+	+	+	+	+	+	+	−		+	−	−	+	−	−	−	−	−	+
B3	60	−	−	+	+	+	+	+	+	+	−		+	−	−	+	−	−	−	−	−	+
B4	60	−	−	+	+	+	+	+	+	−	−		−	−	−	+	−	−	−	−	−	+
B9	58	−	−	+	+	+	+	+	+	+	−		+	−	−	+	−	−	−	−	−	+
B10	60	−	−	+	+	+	+	+	+	+	−		−	−	−	+	−	−	−	−	−	+
Aeromonas sp.																						
T8	64	+	+	−	−	+	−	+	−	−	−		−	−	−	−	−	−	+	+	−	+

it belongs to the genus *Aeromonas* no doubt.

As the result of phenotypic identification, 13 unidentified *Aeromonas* strains in previous phenotyping (Iqbal et al., 1998), 11 strains were identified as *A. hydrophila* and 1 was *A. veronii* biotype *sobria* (Table 6). The remaining 1 strain yet to be identified to the species level of *Aeromonas*, which was designated *Aeromonas* sp. Among the 12 *A. jandaei* reidentified 5 of each were identical to *A. jandaei* and *A. veronii* biotype sobria and 2 were *A. hydrophila*. All the previously identified *A. hydrophila* and *A. veronii* biotype *sobria* strains were reidentified as the same species.

Only a very few properties provided a satisfactory means to separate the three groups *A. hydrophila* (HG1), *A. veronii* biotype *sobria* (HG8Y), and *A. jandaei* (HG9) (Table 7). These include hydrolysis of esculin, utilization of acetate and acid production from salicin, which separated *A. hydrophila* from *A. veronii* biotype *sobria* and *A. jandaei*. Christensen's citrate could be used to separate *A. veronii*

Table 5. Variable phenotypic characteristics of the reference strains.

Reference strains	DNA GC mole%	Esculin hydrolysis	Acetate utilization	Gas from glucose	Voges-Proskauer	Methyl red	Phenylalanine	Christensen's citrate	Jordan's tartrate	Lipase (corn oil)	Lysine decarboxylase	Ornithine decarboxylase	Acid from	Cellobiose	*myo*-inositol	Lactose	D-Manose	Raffinose	L-Rhamnose	Sucrose	Salicin	D-Xylose	Glycerol
HG1	62	+	−	+	−	+	+	+	−	+	−	−		−	−	+	+	−	−	+	+	−	+
HG2	61	+	−	+	+	+	+	−	−	+	−	−		−	−	−	+	−	−	+	+	−	+
HG3	59	+	−	+	−	+	+	−	−	−	+	−		−	−	−	+	−	−	−	+	−	+
HG3′	59	−	−	−	−	+	+	−	−	−	+	−		+	−	+	+	−	−	+	−	−	+
HG4	62	+	−	−	−	+	+	+	−	+	−	−		+	−	+	−	−	−	+	+	−	−
HG5B	61	+	−	−	−	+	−	−	−	−	−	−		+	−	+	+	−	−	+	+	−	+
HG6	60	−	−	+	−	+	+	+	−	−	−	−		+	−	+	+	−	−	−	−	−	+
HG7	57	−	−	+	+	+	+	−	−	+	−	−		+	−	+	+	−	−	−	−	−	+
HG8Y	59	−	−	+	+	+	+	−	−	+	−	−		−	−	+	+	−	−	+	−	−	+
HG9	60	−	−	+	+	+	+	+	−	+	−	−		−	−	+	+	−	−	−	−	−	+
HG10	59	+	−	−	+	+	+	+	−	+	−	−		+	−	+	+	−	−	+	+	−	+
HG11	62	+	−	−	−	+	+	−	−	+	−	−		−	−	+	+	−	−	+	+	−	+
HG12	62	−	−	+	+	+	+	+	−	−	−	−		−	−	−	+	−	−	+	−	+	+
HG13	62	−	−	+	−	+	+	+	−	−	−	−		+	−	−	+	−	−	−	−	−	+

biotype *sobria* from the other two groups. *A. jandaei* are somehow different from the other two species by Jordan's tartrate and acid from sucrose properties.

Of 18 Malaysian isolates, 13 strains belong to *A. hydrophila* and 5 belong to *A. veronii* biotype *sobria* (Table 8). Among the 15 isolates from Thailand, 13 strains were identical to *A. hydrophila* phenospecies, one was *A. veronii* biotype *sobria*. Rest 1 strain (T8) was identified as *Aeromonas* sp. Of 11 Bangladeshi isolates, 6 strains were identified as *A. veronii* biotype *sobria* and 5 were *A. jandaei*.

Discussion

The genus *Aeromonas* comprises a collection of oxidase and catalase-positive, glucose-fermenting, Gram-negative, rod-shaped, generally motile bacteria that are resistant to vibriostatic agent O/129 (Popoff 1984). In this study, all of the experimental isolates were agreed with these descriptions of the genus *Aeromonas*. DNA base composition of all the experimental isolates were almost agreed well with the range of 57–63 set for the genus *Aeromonas* except T2, T8, T15 and T19 isolates (Table 4). The DNA G+C mole percent of these 4 isolates were slightly out of the range.

According to the present taxonomy of motile aeromonads described by Popoff (1984) three phenospecies were recognized: *A. hydrophila*, *A. caviae* and *A. sobria*. Each of these species contains more than one DNA hybridization groups (Janda,

Table 6. Correlation between previous and current phenotypic identification of *Aeromonas* spp. isolated from EUS affected fishes.

Phenospecies	Strains identified in	
	Previous phenotyping	Current phenotyping
A. hydrophila	$n=13$ M24, M26, M27, M29 M30, M31, M32, T11, T17, T18, T19, T20, T21	$n=26$ M1, M24, M25, M26, M27, M29, M20, M31, M32, M33, M34, M71, M99, T2, T5, T7, T11, T15, T17, T18, T19, T20, T21, T25, T26, T28
A. veronii biotype *sobria*	$n=6$ B1, B5, B6, B7, B8, B11	$n=12$ M4, M6, M16, M56, M88, T30, B1, B5, B6, B7, B8, B11
A. jandaei	$n=12$ M4, M6, M16, M34, M56, M71, T30, B2, B3, B4, B9, B10	$n=5$ B2, B3, B4, B9, B10
Aeromonas spp.	$n=13$ M1, M25, M33, M88, M99, T2, T5, T7, T8, T15, T25, T26, T28	$n=1$ T8

Table 7. Selected biochemical properties based on the analysis of the identified strains studied in the experiment.

Test	Percentage of strains with positive result		
	A. hydrophila	*A. veronii* biotype *sobria*	*A. jandaei*
Esculin hydrolysis	100	0	0
Acetate utilization	81	25	0
Christensen's citrate	100	0	100
Jordan's tartrate	0	50	100
Acid from sucrose	92	100	0
Acid from salicin	100	0	0

Table 8. Geographical distribution of the total identified *Aeromonas* species.

Country	*A. hydrophila*	*A. veronii* biotype *sobria*	*A. jandaei*	*Aeromonas* sp.
Malaysia ($n=18$)	13	5	0	0
Thailand ($n=15$)	13	1	0	1
Bangladesh ($n=11$)	0	6	5	0

1991), (Table 2). These species can be identified by routine biochemical properties such as esculin hydrolysis, gas production from glucose, lysine decarboxylation, fermentation of arabinose and salicin etc. (Bryant et al., 1986a, 1986b ; Janda et al. ; 1984, Kuijper et al., 1989). In the present study 44 isolates from EUS affected fishes including 14 reference strains from all the hybridization groups were investigated to identify the *Aeromonas* species phenotypically. Among the 44 strains reidentified phenotypically, 26 strains were placed into the phenon corresponding to *A. hydrophila* (HG1) or related species, 12 were *A. veronii* biotype *sobria* (HG8Y) and 5 were *A. jandaei* (HG9). The remaining 1 strain from Thailand (T8) was too heterogeneous with the reference strains and could not be able to place in any of the groups. This strain was also unable to identify either any of the recognized *Aeromonas* species in previous experiment (Iqbal et al., 1998). Strain T8 was therefore designated *Aeromonas* sp. No isolated strain had been found which was 100% identical to any of the reference strains by phenotypic traits. Also, characteristics sometimes varied among the isolated strains in the same group. The isolated strains varied 1 to 5 biochemical traits from the reference strains within the same phenotypic group. These variations may be due to various sets of strains or isolates from different geographic areas used in this study or may be highly heterogeneity among the individuals. Besides, Plasmid DNA might be responsible for encoding some biochemical traits (Austin, 1988) ; as plasmid DNA may be lost in long time storage, resulting differences in characteristics may explain why isolates did not give 100% characteristics similarity within the same species.

A. hydrophila and *A. sobria* have frequently been isolated from the EUS-affected fish (Roberts et al., 1990 ; Lio-Po et al., 1990 ; Subasinghe et al., 1990 ; Llobrera and Gacutan 1987 and Tonguthai 1985). In fact, *A. sobria* phenospecies contains *A. veronii* biotype *sobria*, *A. jandaei* and 4 more genospecies (Table 2). *A. hydrophila* was the dominating group followed by *A. jandaei* and *A. veronii* biotype *sobria* among the 44 isolates investigated in this study (Table 4). Thus, the results of the present experiment are in agreement with the above mentioned reports on *Aeromonas* species associated with EUS-affected fish.

There was no single phenotypic test, which could identify the 3 identified *Aeromonas* species with confidence. Only 6 phenotypic properties have been found to be able partially differentiate these 3 species (Table 7). These results correlate with the results of Abbott et al. (1992) and Altwegg et al. (1990).

As a result of phenotypic identification, 13 Malaysian strains belong to *A. hydrophila* and 5 belong to *A. veronii* biotype *sobria* (Table 8). Among the 15 isolates from Thailand, 13 strains were identical to *A. hydrophila* phenospecies, one of each was *A. jandaei* and *Aeromonas* sp. Of 11 Bangladeshi isolates, 6 strains were identified as *A. veronii* biotype *sobria* and 5 were *A. jandaei*. There was no *A. jandaei* in the Malaysian and Thai isolates. Besides, none of the isolates from Bangladesh were identified as *A. hydrophila*. This may be because an insufficient number of isolates were tested or the particular species in the particular sampling environment was absent.

Of 13 unidentified *Aeromonas* strains in the previous phenotyping (Iqbal et al., 1998) 11 were identified as *A. hydrophila* or related species and 1 was *A. veronii* biotype *sobria* (Table 6). Among the 12 *A. jandaei* reidentified phenotypically 5 of each were identified as *A. jandaei* and *A. veronii* biotype *sobria* and 2 were *A.*

hydrophila. All the previously identified *A. hydrophila* and *A. veronii* biotype *sobria* strains were placed in the same species. Biochemical properties of the strains investigated in the present study were sometimes differed from that of the previous experiment (data are not shown). These variable or false properties were might be due to unintentional variations in pH of different media or length of incubation time. Besides, weak positive or negative reactions, which were difficult to determine visually, might be an another factor for these variations. However, it needs DNA-DNA hybridization or 16S rDNA sequencing experiment for further identification of the *Aeromonas* phenotypic species. These experiments will help to know the relationship between the phenotypic and genotypic identification as well as the phylogenetic position of the identified isolates.

In conclusion, *Aeromonas* species from different geographic origin do not give 100% characteristics similarity with those of reference strains. A false or weak positive and negative reaction, which often occurs in routine laboratories, leads a misidentification of *Aeromonas* species. The phenotypic properties presented herein, might be a useful phenotypic scheme to identify the three species of *Aeromonas* from EUS affected fishes in the microbiological laboratories.

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