Taxonomical and Pathological Studies on Motile *Aeromonas* Species Isolated from Fish with Epizootic Ulcerative Syndrome in Southeast Asian Countries*

By

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I. General introduction

Disease and aquaculture

Aquaculture is an important expanding enterprise in the world especially in Asian countries. Eighty four percent of the world's fish production through aquaculture comes from Asia according to FAO, 1995. Inspite of this, there is still a greater demand to increase the production of aquaculture products. Recently, the rapid growth of this enterprise was augmented by the development of intensification techniques in rearing aquatic animals. In practice, this means increasing the density of cultured fish populations to the tolerable maximum. This practice had resulted to increasing mass mortalities due to bacterial, viral and parasitic diseases in hatcheries and different culture systems on a scale never seen before.

The annual total losses through fish diseases are largely unrecorded but are very sustainable. In Asia, currently diseases have emerged as a major constraint to the sustainable growth of aquaculture in the region (FAO, 1995). It has been estimated that 15 Asian countries, having a total aquaculture output of over US$ 22.7 billion in 1990, lost US$ 1.36 billion the same year through disease (ADB/NACA, 1991).

History of Epizootic Ulcerative Syndrome (EUS)

EUS of freshwater fishes is a tremendous threat to the fish production of Southeast Asian countries. It causes mass mortalities both cultured and wild fish species every year in a cyclic manner. In Queensland, Australia, an epizootic of estuarine fish from the Burnett river characterized by shallow haemorrhagic ulcers occurred in 1972 with re-occurrence in subsequent years upto 1981 (Rodgers and Burke, 1981). The disease was named as red spot (RSD). Later, outbreaks of RSD have been reported in many species of estuarine and freshwater fish in New South Wales (Callinan et al., 1989), North Territory (Humphrey and Langdon, 1986; Pearce, 1990). Since 1972 the disease gradually spread to many countries in Asia-Pacific region. Papua New Guinea reported a similar type of disease characterized by dermal ulcer from the rivers of the south during 1975-76. The syndrome reached epizootic proportions to wild fish species such as snakehead, catfish, sand goby and kissing gouramy in natural water in Bogor, Indonesia in the early 1980's, which subsequently spread to West Central and Eastern Java (ODA, 1994). Malaysia reported the disease having red areas of ulceration all over the fish bodies during 1980-83. In early 1984, it was reported that an unidentified fish disease affected fish stock in all principal fishing areas of Kampuchea along with a significant decrease in the natural stock. In 1984, a similar disease was reported that southern and central parts of Laos. Myanmar also experienced the outbreak of the disease during 1984-85 affecting both wild and cultured fish stock. For Thailand, the disease was first reported in 1980 in the natural water. In 1982-83 a severe EUS outbreak in Thailand affecting snakehead *Channa striata* and many species of rice-field fish was reported (Tonguthai, 1985). Then the disease recurred every year upto 1992. In Sri Lanka, the disease was reported in 1988 in the river causing severe fish mortality. In the Philippines, the EUS was first observed in Laguna de Bay in late 1985 and spread to other parts quickly. After the severe flooding in 1988, the first EUS outbreak was reported in Bangladesh. Large scale

of fish mortality is still observed every year since its outbreak in Bangladesh. In the same year, EUS occurred in Northeast India and spread throughout the whole country (ODA, 1994). Nepal was also noticed by EUS in 1989 and 1990. By 1996 it was confirmed that EUS had spread to the upper part of Indus river in Pakistan (Kanchanakhan, 1996).

Fish species affected by EUS

Over 100 different species of both wild and reared fishes have been reported as susceptible to EUS. The main wild species are: *Channa striatus, C. punctatus, C. gachua, Clarias batrachus, Heteropneustes fossilis, Puntius sophore, P. ticto, Amblypharyngodon mola, Mystus vittatus, M. aor, Mastacembelus pancalus, M. armatus, Ambassis ranga, Nandus nundus, Callichorus pabda, Gadusia chapra* etc. The fishes affected in the culture systems are: *Cirrhinus mrigala, Cyprinus carpio, Catla catla, Labeo rohita, Puntius javanicus, Ctenopharyngodon idealla, Hypophthalmichthys molitrix* etc. Fish species most severely affected are the bottom dwelling genera like *Channa* spp., *Mastacembelus* spp., *Clarias* spp., *Heteropneustes* spp., *Cirrhinus* spp. and *Cyprinus* spp. etc.

Pathology of the disease

EUS outbreaks occur in a cyclical manner and appear to have some correlation with temperature, occurring normally during periods of lower water temperature (Roberts et al., 1990). Chinabut et al. (1995) stated that the disease occurred in average water temperature at 26°C in the tropical countries of south and Southeast Asia. The clinical pathology of the disease is similar in all species of fishes. Generally it appears more acute in smaller fishes. Snakehead fish in particular are severely affected but appear to be able to survive the disease process longer and so may show more chronic lesions. Although most snakeheads ultimately succumb, occasional fish with large, black, healed lesions are be found. However, the pathology described by Roberts et al. (1990) are given as follows: "Skin lesions vary from small areas of rosacea usually along the flank but occasionally on the side of the jaw or head to larger lesions, found anywhere of the body. These lesions are circumscribed ulcers with a bright haemorrhagic margin around a necrotic center, extending into the subjacent muscle, to large, grey/white, infarctive lesions often eroding deep into the cranial tissues or abdominal wall. Fish in the later stages of the disease are usually inactive or comatose, with severe hydration problems and often total erosion of the tail or part of the cranium. Internal organs are generally clinically normal except in the later stages when peritonitis, pale gills, discoloration of the liver and heart and enlargement and hyperaemia of the spleen are found". To avoid confusion between EUS and other ulcerative conditions of fishes, experts from various countries defined EUS as follows: "EUS is a seasonal epizootic condition of freshwater and estuarine warm water fish of complex etiology characterized by the presence of *Aphanomyces* infection and necrotizing ulcerative lesions typically leading to a granulomatous response".

Aetiology of EUS

Although EUS is now a major problem in Southeast Asian countries. But

unfortunately the true etiology of the disease is still unclear and perplexed. *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. contribute to the pathogenesis of the disease (Costa and Wejeyaratne, 1989). 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). Aeromonads produce a wide range of pathogenic factors such as pili, hemolysins, protease, invasin, serum resistance etc. 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. The genus *Aeromonas* was proposed by Kluyver and Niel (1936) to accommodate rod shaped bacteria possessing the general properties of the enteric group, but motile by means of polar flagella. The genus is now in the family of *Aeromonadaceae* (Colwell et al., 1986). The salient properties of the genus as described by by Popoff and Véron (1976) are however Gram-negative straight rods with rounded ends, generally motile by monotrichous polar flagellum or non-motile, facultative anaerobes, fermenting carbohydrates with formation of acid or acid and gas, reducing nitrates to nitrites, oxidase and catalase positive, resistant to vibriostatic agent 0/129 (2, 4-diamino-6, 7-diisopropyl-pteridine).

Identification of aeromonads from EUS

Identification of bacterial pathogen is the first step to understand its pathology. The general method of diagnosis of bacterial pathogens takes a few days and is not meaningful to the farmers who need quick results to save his crop. The conventional method sometimes however, can not identify the pathogens to the species level correctly (Abbott et al., 1992). Following determination of the structure of DNA by Watson and Crick (1953), studies in bacteriology have seen a major shift from functional to molecular techniques for identifying bacteria. Diagnosis by rapid and reliable detection methods like DNA-DNA hybridization, polymerase chain reaction (PCR) and DNA probes are now widely used in bacteriological laboratories. Ribosomal operons have acquired paramount relevance for the study of bacterial evolution and phylogeny. Small sub-unit of 16S rDNA gene are also used to know bacterial phylogeny and has been suggested to represent a potential target within the genome to find suitable sites for probes.

Unfortunately, the blessing of the recent molecular identification of bacterial species is however, absent in motile *Aeromonas* spp. of EUS affected fishes. All the literatures available to identify *Aeromonas* spp. associated with EUS are based on a set of biochemical property only. This identification can not discriminate the species at all which indicated in above.

Objectives of the present study

This study was therefore conducted based on the above premise and the following were its main objectives:

1. To characterize and identify motile *Aeromonas* spp. based on the

phenotypic properties isolated from fish with EUS in Southeast Asian countries;

2. To identify them based on DNA-DNA homology method;
3. To know the sequence of gene coding for 16S rRNA of identified *Aeromonas* species;
4. To know the phylogenetic position of motile *Aeromonas* spp. isolated from fish with EUS; and
5. To determine the virulence of the identified *Aeromonas* species.

II. Phenotypic identification of motile *Aeromonas* species

1. Abstract

A total of 44-strains of motile aeromonad isolated from fish with epizootic ulcerative syndrome (EUS) in Malaysia, Thailand and Bangladesh including 14-reference strains of DNA hybridization groups have investigated to identify them to the phenospecies level. Biochemical properties of the 44-isolates were compared with those of the 14-reference strains. Among the 44-strains identified 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*. The remaining one strain was identified as *Aeromonas* sp. Of 11 Bangladeshi strains, 6 strains were identified as *A. veronii* biotype *sobria* and 5 were *A. jandaei*.

2. 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. contribute 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. Phenotypic traits to identify *Aeromonas* species are fastidious enough and do not correlate with the genetic identification in most cases. Identification of bacteria in most microbiological laboratories depends on their phenotypic properties because of the unavailability of molecular techniques due to financial and technical reasons. For

Table 1. Diagnostic biochemical properties of motile *Aeromonas* species described by Popoff, 1984

Biochemical properties	*A. hydrophila*	*A. caviae*	*A. sobria*
Indole production	+	+	+
Sucrose fermentation	+	+	+
Mannitol fermentation	+	+	+
Salicin fermentation	+	+	−
Breakdown of inositol	−	−	−
Voges-Proskauer (Acetoin)	+	−	d
Gas from glucose	+	−	+
H_2S production	+	−	+
Esculin hydrolysis	+	+	−
Growth in KCN (Moller's)	+	+	−
Arginine utilization	+	+	−
Arabinose utilization	+	+	−

+ : Positive reaction; − : Negative reaction; d : Variable reaction

the simplified identification of the *Aeromonas* strains, Carnahan et al. (1991[c]) described an identification system, which was designated "Aerokey II". This system consisted seven tests to identify most frequently isolated human clinical species. In an evaluation using 60 well defined clinical and reference strains by Aerokey II system, Carnahan et al. (1991[c]) identified 97% strains to the species level. Later in another study Furuwatari et al. (1994) modified the Aerokey II and proposed "Aeroscheme" for the identification of clinical *Aeromonas* species. They identified a number of clinical and environmental strains based on their Aeroscheme correctly. The latest edition of Bergey's manual (Popoff, 1984) speciates the motile aeromonads into three entities by eight distinct biochemical characters (Table 1). Thus, on the basis of these properties, three motile aeromonads are recognized: *A. hydrophila*, *A. sobria* and *A. caviae*. This classification is, however, tentative pending further phenotypic and genotypic studies (Popoff, 1984). This taxonomic confusion further aggravates the role of motile aeromonads in EUS of fish. It is therefore imperative that the true identification of motile aeromonads must be clarified such that when EUS outbreaks occur, the causative agent may be dealt with promptly and efficiently.

This study was therefore, undertaken to 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.

3. Materials 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 2).

Table 2. Origins of tested isolates used in this study

Country	Code No.	Host species	Place	Organ	Year
Malaysia ($n=18$)	M1, M4, M6, M88, M99	*Anabas testudineus*	Kangar	Lesion	1987
	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 ($n=15$)	T26	*Channa striatus*	Supanburi	Spleen	1994
	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 ($n=11$)	B1, B2, B3, B4, B5	*Cirrhinus mrigala*	Gouripur, Mymensingh	Lesion	1994
	B6, B7, B8, B9, B10, B11	*Cirrhinus mrigala*	Trishal, Mymensingh	Lesion	1994

Table 3. 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
HG3′		*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

Fourteen reference strains representing the different DNA hybridization groups (Janda, 1991) were also used in this study (Table 3). 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 1000 ml, pH 7.2) and stock cultures 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

Table 4. 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 5. Variable phenotypic characteristics of the experimental strains

Experimental strains	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 ATCC7966 (HG1)	+	−	+	−	+	+	+	−	+	−	−		−	−	+	+	−	−	+	+	−	+
Unnamed CDC9533-76 (HG2)	+	−	+	+	+	+	−	−	+	−	−		−	−	−	+	−	−	+	+	−	+
M1	+	+	+	+	−	−	+	−	+	−	−		−	−	−	+	−	−	+	+	−	+
M24	+	+	+	+	+	+	+	−	+	−	−		−	−	+	+	−	−	+	+	−	+
M25	+	+	+	+	−	−	+	−	+	−	−		−	−	−	+	−	−	+	+	−	+
M26	+	−	+	+	+	+	+	−	+	−	−		+	−	−	+	−	−	+	+	−	+
M27	+	−	+	+	+	+	+	−	+	−	−		+	−	−	+	−	−	+	+	−	+
M29	+	+	+	+	−	+	+	−	+	−	−		−	−	+	+	−	−	+	+	−	+
M30	+	+	+	+	−	+	+	−	+	−	−		−	−	−	+	−	−	+	+	−	+
M31	+	+	+	+	−	+	+	−	+	−	−		−	−	+	+	−	−	+	+	−	+
M32	+	+	+	+	−	+	+	−	+	−	−		−	−	+	+	−	+	+	+	−	+
M33	+	+	+	+	−	+	+	−	+	−	−		−	−	+	+	−	+	+	+	−	+
M34	+	−	+	+	−	+	+	−	+	−	−		+	−	+	+	+	−	−	+	−	+
M71	+	−	+	+	−	+	+	−	+	−	−		+	−	+	+	+	−	−	+	−	+
M99	+	+	+	+	−	+	+	−	+	−	−		−	−	−	+	−	−	+	+	−	+
T2	+	+	+	+	−	+	+	−	+	−	−		−	−	+	+	−	−	+	+	+	+
T5	+	+	+	+	−	−	+	−	+	−	−		−	−	+	+	−	−	+	+	+	+
T7	+	+	+	+	+	+	+	−	+	−	−		−	−	+	+	−	+	+	+	+	+
T11	+	+	+	+	−	+	+	−	+	−	−		−	−	+	+	−	+	+	+	−	+
T15	+	+	+	+	−	−	+	−	+	−	−		−	−	−	+	−	−	+	+	−	+
T17	+	−	+	+	−	+	+	−	+	−	−		−	−	−	+	−	−	+	+	−	+
T18	+	+	+	+	−	+	+	−	+	−	−		−	−	−	+	−	−	+	+	−	+
T19	+	+	+	+	+	+	+	−	+	−	−		−	+	+	+	−	−	+	+	−	+
T20	+	+	+	+	+	+	+	−	+	−	−		−	+	+	+	−	−	+	+	−	+
T21	+	+	+	+	+	+	+	−	+	−	−		−	+	+	+	−	−	+	+	−	+
T25	+	+	+	+	−	−	+	−	+	−	−		−	−	−	+	−	−	+	+	−	+
T26	+	+	+	+	−	−	+	−	+	−	−		−	−	−	+	−	−	+	+	−	+
T28	+	+	+	+	−	+	+	−	+	+	−		−	−	−	+	−	−	+	+	−	+

Table 5. Continued

Experimental strains	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* ATCC9071 (HG8Y)	−	−	+	+	+	+	−	−	+	−	−		−	−	+	+	−	−	+	−	−	+
M4	−	−	+	+	+	+	−	−	+	−	−		+	−	+	+	−	−	+	−	−	+
M6	−	−	+	+	+	+	−	−	+	−	−		−	−	+	+	−	−	+	−	−	+
M16	−	−	+	+	+	−	−	−	+	−	−		+	−	+	+	−	−	+	−	−	+
M56	−	+	+	+	+	−	−	−	+	−	−		−	−	−	+	−	−	+	−	−	+
M88	−	+	+	+	+	+	−	−	+	+	−		−	−	−	+	−	−	+	−	−	+
T30	−	+	+	+	−	+	−	−	+	−	−		−	−	−	+	−	−	+	−	−	+
B1	−	−	−	+	+	+	−	+	+	−	−		−	−	−	+	−	−	+	−	−	+
B5	−	−	−	+	+	−	−	+	+	−	−		−	−	−	+	−	−	+	−	−	+
B6	−	−	+	+	+	+	−	+	+	−	+		+	−	−	+	−	−	+	−	−	+
B7	−	−	−	+	+	−	−	+	+	−	−		−	−	−	+	−	−	+	−	−	+
B8	−	−	−	+	+	+	−	+	+	−	−		−	−	+	+	−	−	+	−	−	+
B11	−	−	+	+	+	+	−	+	+	−	−		−	−	−	+	−	−	+	−	−	+
A. jandaei JCM8316 (HG9)	−	−	+	+	+	+	+	−	+	−	−		−	−	+	+	−	−	−	−	−	+
B2	−	−	+	+	+	+	+	+	+	−	−		+	−	−	+	−	−	−	−	−	+
B3	−	−	+	+	+	+	+	+	+	−	−		+	−	−	+	−	−	−	−	−	+
B4	−	−	+	+	+	+	+	+	−	−	−		−	−	−	+	−	−	−	−	−	+
B9	−	−	+	+	+	+	+	+	+	−	−		+	−	−	+	−	−	−	−	−	+
B10	−	−	+	+	+	+	+	+	+	−	−		−	−	−	+	−	−	−	−	−	+
Aeromonas sp. T8	+	+	−	−	+	−	+	−	−	−	−		−	−	−	−	−	−	+	+	−	+

were read after 24 h unless otherwise indicated.

4. 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 4). Variable biochemical properties of the 44-*Aeromonas* isolated strains (Table 5) were compared with those of the 14-reference strains (Table 6). Isolated strains, which differed by only 1 to 5 properties from reference strains, was placed into the same species. Among the 44-isolated strains identified phenotypically, 26 strains were placed into the

Table 6. Variable phenotypic characteristics of the reference strains

Reference strains	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	+	−	+	−	+	+	+	−	+	−	−		−	−	+	+	−	−	+	+	−	+
HG2	+	−	+	+	+	+	−	−	+	−	−		−	−	−	+	−	−	+	+	−	+
HG3	+	−	+	−	+	+	−	−	−	+	−		−	−	−	+	−	−	−	+	−	+
HG3′	−	−	−	−	+	+	−	−	−	+	−		+	−	+	+	−	−	+	−	−	+
HG4	+	−	−	−	+	+	+	−	+	−	−		+	−	+	−	−	−	+	+	−	−
HG5B	+	−	−	−	+	−	−	−	−	−	−		+	−	+	+	−	−	+	+	−	+
HG6	−	−	+	−	+	+	+	−	−	−	−		+	−	+	+	−	−	−	−	−	+
HG7	−	−	+	+	+	+	−	−	+	−	−		+	−	+	+	−	−	−	−	−	+
HG8Y	−	−	+	+	+	+	−	−	+	−	−		−	−	+	+	−	−	+	−	−	+
HG9	−	−	+	+	+	+	+	−	+	−	−		−	−	+	+	−	−	−	−	−	+
HG10	+	−	−	+	+	+	+	−	+	−	−		+	−	+	+	−	−	+	+	−	+
HG11	+	−	−	−	+	+	−	−	+	−	−		−	−	+	+	−	−	+	+	−	+
HG12	−	−	+	+	+	+	+	−	−	−	−		−	−	−	+	−	−	+	−	+	+
HG13	−	−	+	−	+	+	+	−	−	−	−		+	−	−	+	−	−	−	−	−	+

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 one 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. It was designated *Aeromonas* sp.

Only a very few properties provided a satisfactory means to separate the three

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

Test	Percentage of isolates 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

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* 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.

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

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*. The remaining one 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*.

5. 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*.

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, 1991), (Table 3). 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., 1986[a,b]; Janda et al., 1984[a], 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 identified 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 one strain from Thailand (T8) was too heterogeneous with the reference strains and could not be able to place in any of the groups. The 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 with in 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 four more genospecies (Table 3). *A. hydrophila* was the dominating group followed by *A. jandaei* and *A. veronii* biotype *sobria* among the 44 isolates investigated in this study (Table 5). 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 three identified *Aeromonas* species with confidence. Only five phenotypic properties have been found to be able partially differentiate these three 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.

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 fish in the microbiological laboratories. 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.

III. Genotypic identification of motile *Aeromonas* species

1. Abstract

A total of 44 *Aeromonas* strains identified based on phenotypic traits in the preceding chapter were further identified by the DNA-DNA microplate hybridization method. Among the 26 *A. hydrophila* phenospecies 19 were genotypically identical to *A. hydrophila* (HG1), 5 were *A. veronii* biotype *sobria* (HG8Y) and 2 were *A. jandaei* (HG9) genospecies. All the *A. veronii* biotype *sobria* or *A. jandaei* phenospecies were placed in their own genospecies, *A. veronii* biotype *sobria* (HG8Y) or *A. jandaei* (HG9). The unspeciated one strain from Thai did not give any significant homology values with any of the reference strains used in this study. This strain was designated *Aeromonas* sp. 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.

2. Introduction

The identification of bacterial species in most bacteriological laboratories is based on phenotypic properties. Although this method has been quite successful, it has not been precise enough to distinguish closely related species. Bacterial genus, with wide phenotypic variations, the problem becomes aggravated resulting in confusion of the identification, which hampered its clinical significance as well as epidemiologic aspects. In view of these, there need new methods which give best reflection of the genetic make-up of organism that would be useful for identification.

Nucleic acid relatedness was applied to solve these problems. It was De Ley et al. (1970) who developed DNA hybridization technique, which provided the possibility of measuring the genetic relationship between bacterial strains. This method is now the official method to define a bacterial species (Joseph and Carnahan, 1994). Quantitative DNA-DNA hybridization between atypical strains and the type strains offer reliable data to determine whether the organism is identical or closely related to the type strain (Ezaki et al., 1988). According to this approach a genospecies is composed of strains whose DNAs are at least 70% relatedness at 60°C with a ΔT_m of 5°C or less (Wayne et al., 1987).

It has been pointed out in the preceding discussions that the taxonomy of motile aeromonads is in a state of confusion. It would seem that phenotypic identification based on routine procedures in most laboratories may not be sufficient to identify motile *Aeromonas* to the species level. Several authors stress the necessity of using molecular methods in addition to biochemical markers for the correct identification of strains with genomic species (Altwegg, 1993; Carnahan, 1993). The first application of this technique to the psychrophilic aeromonads by Popoff et al. (1981) demonstrated that the phenotypically circumscribed *Aeromonas* species, eg. *A. hydrophila*, comprised several genotypically different groups with genetic distances supporting species delineation. Since the study of Popoff et al. (1981) the following additional genospecies of *Aeromonas* so far have been described till now: *A. media*,

A. eucrenophila, *A. schubertii*, *A. veronii*, *A. jandaei*, *A. trota*, *A. enteropelogenes*, *A. ichthiosmia*, *A. allosaccharophila*, *A. encheleia*, *A. bestiarum* and *A. popoffii* (Allen et al., 1983; Carnahan et al., 1991[a,b], 1989; Hickman-Brenner et al., 1988, 1987; Schubert et al., 1990[a,b]; Martinez-Murcia et al., 1992[a]; Esteve et al., 1995; Ali et al., 1996; Huys et al., 1997).

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 taxonomic complexity of the bacterium the present experiment was undertaken to further identify the motile aeromonads isolated from EUS-affected fish of Malaysia, Thailand and Bangladesh based on DNA-DNA hybridization study and to know the relationship between phenotypic and genotypic identification.

3. Materials and methods

Strains and culture conditions

A total of 44-strains of motile aeromonads and 14-reference strains described previously were investigated in this study. The experimental strains and the 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 as described in previous chapter.

DNA extraction

Cultures were grown to mid log-phase at 20°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 ml of lysing buffer [0.1 M Tris-HCl (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 ml 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 8,400×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 ml 10 mM Tris containing 1 mM EDTA (pH 8) buffer. DNA was then treated with 1.5 ml RNase (1 mg/ml 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 ml of 10 mM Tris containing 1 mM EDTA (pH 8) buffer.

Determination of the GC contents of DNA

The mole percent guanine-plus-cytosine (G+C) contents were determined by high-performance liquid chromatography (HPLC) of its nuclease P_1 hydrolysate according to Kumagai et al. (1988). Briefly, DNA sample was dissolved in distilled water (EDTA free) at a concentration of 1 mg/ml. The DNA solution was denatured at 100°C for 5 min in a dry bath and then cooled rapidly in an ice bath for 30 min. A part of the denatured DNA solution was mixed with equal volume of nuclease P_1 (2 unites/ml of 40 mM sodium-acetate buffer containing 2×10^{-4} M $ZnCl_2$, pH 5.3), and incubated at 50°C for 1 h. Both the hydrolysate and the standard mixtures of nucleotides (Yamasa, Choshi, Japan) were applied to HPLC, separately. The G+C content (mol%) was calculated as follows:

$$GC(\%)=\{(Cx/Cs+Gx/Gs)/(Cx/Cs+Gx/Gs+Tx/Ts+Ax/As)\}\times100$$

Where, Cx, Gx, Tx and Ax are the peak areas of dCMP, dGMP, dTMP and dAMP in the nuclease P_1 digest of DNA, and Cs, Gs, Ts and As are the peak areas of dCMP, dGMP, dTMP and dAMP in the standard solution.

DNA-DNA hybridization

Purified DNAs from all phenotypic groups *A. hydrophila* ATCC7966 (HG1), Unnamed *Aeromonas* sp. CDC9533-76 (HG2), *A. veronii* biotype *sobria* ATCC9071 (HG8Y), and *A. jandaei* JCM8316 (HG9) were labelled with photobiotin (Vector Laboratories, USA). Hybridization was performed in a microplate immobilized with DNAs extracted from either experimental strains or reference strains according to the method of Ezaki et al. (1989), with minor modifications (Iqbal, 1999).

4. Results

GC mole percentage

The DNA moles percent G+C contents of all the 44 strains ranged from 55 to 64 (Table 9). The same values of all the reference strains of *Aeromonas* species varied from 57 to 62.

DNA-DNA hybridization

DNA-DNA homology results of the 44-*Aeromonas* strains and the reference strains are shown in Table 9. Of 44 motile *Aeromonas* strains 19 belonged to *A. hydrophila* genospecies (HG1), 17 belonged to *A. veronii* biotype *sobria* (HG8Y) and 7 belonged to *A. jandaei* genospecies (HG9). One strain designated *Aeromonas* sp. DNA relatedness of eight strains (M26, T7, T26, M4, M6, M56, M88 and B11) which were near to the borderline (62-69%) at T_m-25°C for species delineation exhibited significant homology values with the same species at T_m-15°C. T2 and T8 strains did not give any significant homology value with HG1, HG2, HG8Y or HG9. These two strains were then photobiotynalated, and hybridization was performed among DNAs of the two strains and all the reference strains at T_m-25°C. The strain T2 gave significant homology value with *A. hydrophila* ATCC7966^T (HG1) (Table 10). But the strain T8, which was too heterogeneous phenotypically, did not give significant homology value with any of the reference strains. The highest homology

Table 9. Levels of DNA relatedness of reference strains to the experimental isolates selected for the DNA-DNA hybridization at T_m-25°C

Unlabeled DNA	DNA GC mole %	Relatedness (%) to labeled DNA of			
		ATCC7966 (HG1)	CDC9533-76 (HG2)	ATCC9071 (HG8Y)	JCM8316 (HG9)
A. hydrophila ATCC7966^T	62	100	49	50	54
Unnamed CDC9533-76	61	59	100	35	51
A. salmonicida subsp. *salmonicida* ATCC14174	59	ND	ND	ND	ND
A. salmonicida subsp. *masoucida* ATCC27013^T	59	ND	ND	ND	ND
A caviae ATCC15468^T	62	51	25	27	35
A. media JCM2385^T	61	48	25	36	37
A. eucrenophila NCMB74^T	60	46	29	42	37
A. sobria JCM2139^T	57	50	27	35	43
A. veronii biotype *sobria* ATCC9071	59	53	28	100	64
A. jandaei JCM8316^T	60	54	32	54	100
A. veronii biotype *veronii* JCM7375^T	59	53	32	77	44
Unnamed CDC1306-83	62	51	38	36	42
A. schubertii JCM7373^T	62	6	13	21	8
A. trota JCM8315^T	62	54	34	49	28
A. hydrophila ATCC7966^T					
M29	61	94	37	37	47
M1	62	90	44	43	52
T28	62	89	33	35	41
M30	62	88	34	33	34
M25	62	86	39	36	45
T18	62	85	36	26	47
M99	62	82	32	40	44
T20	61	81	27	22	48
M33	62	79	ND	36	20
M32	61	75	26	28	40
T11	62	75	ND	35	21
T15	64	75	ND	41	28
T25	61	74	37	21	44
T19	64	73	ND	34	18
M24	63	71	ND	30	9
M31	57	70	ND	36	4
T7	62	69(73)	23	26	43
T26	57	69(73)	ND	23	18
T2	55	51(58)	26	21	34

Table 9. Continued

Unlabeled DNA	DNA GC mole %	Relatedness (%) to labeled DNA of			
		ATCC7966 (HG1)	CDC9533-76 (HG2)	ATCC9071 (HG8Y)	JCM8316 (HG9)
A. veronii biotype *sobria* ATCC9071					
T30	61	52	28	88	52
B1	59	66	32	88	62
B5	58	65	33	88	52
B8	60	61	30	83	56
B6	59	54	28	80	58
T17	59	54	27	76	52
B7	59	58	27	75	46
T5	63	30	ND	75	31
M27	60	40	ND	74	19
M16	59	46	26	73	57
T21	59	39	ND	71	21
M4	58	52	28	69(74)	57
M26	59	46	26	68(73)	51
B11	59	40	27	68(74)	45
M6	58	45	29	66(72)	57
M88	59	40	19	65(70)	47
M56	58	50	25	62(70)	49
A. jandaei JCM8316^T					
B2	60	56	28	59	93
B3	60	63	25	62	88
B4	60	60	29	57	87
B10	60	51	30	52	76
M34	59	45	20	39	76
M71	58	31	ND	51	73
B9	58	53	30	51	72
Aeromonas spp.					
T8	64	54	26	24	45

ND: Not detected; Number inside the bracket indicates DNA relatedness at T_m-15°C

value (59%) was found with *A. caviae* (HG4). Thus this strain recognized as a new species belongs to the genus *Aeromonas*. It was designated *Aeromonas* sp.

Relationship between phenotypic and genotypic identification

Correlation between phenotypic and genotypic identification is shown in Table 11. Of 26 phenotypic *A. hydrophila* 19 belonged to *A. hydrophila* genospecies (HG1) and five belonged to *A. veronii* biotype *sobria* (HG8Y), while 2 belonged to *A. jandaei* genospecies (HG9). All the phenotypic *A. veronii* biotype *sobria* and *A.*

Table 10. Levels of DNA relatedness of T2 and T8 isolates to the reference strains for the DNA-DNA hybridization at T_m-25°C and their DNA base composition

Unlabeled DNA	Relatedness (%) to labeled DNA of	
	Aeromonas sp. (T8)	*A. hydrophila* (T2)
A. hydrophila (T2)	29	100
Aeromonas sp. (T8)	100	58
A. hydrophila ATCC7966^T (HG1)	42	70
Unnamed CDC9533-76 (HG2)	39	60
A. salmonicida subsp. *salmonicida* ATCC14174 (HG3)	32	53
A. salmonicida subsp. *masoucida* ATCC27013^T (HG3′)	32	54
A. caviae ATCC15468^T (HG4)	59	52
A. media JCM2385^T (HG5B)	41	43
A. eucrenophila NCMB74^T (HG6)	28	45
A. sobria JCM2139^T (HG7)	37	51
A. veronii biotype *sobria* ATCC9071 (HG8Y)	32	46
A. jandaei JCM8316^T (HG9)	27	47
A. veronii biotype *veronii* JCM7375^T (HG10)	27	37
Unnamed CDC1306-83 (HG11)	34	43
A. schubertii JCM7373^T (HG12)	2	46
A. trota JCM8315^T (HG13)	32	44

Table 11. Correlation between phenotypic and genotypic identification

Phenotypic identification	Genotypic identification		
	A. hydrophila (HG1)	*A. veronii* biotype *sobria* (HG8Y)	*A. jandaei* (HG9)
A. hydrophila ($n=26$)	19	5	2
A. veronii biotype *sobria* ($n=12$)	0	12	0
A. jandaei ($n=5$)	0	0	5

jandaei belonged to their own genospecies, respectively.

Screening of biochemical properties

There was no single phenotypic test, which could be used to identify with confidence the three 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 12). *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.

Table 12. Selected biochemical properties based on the analysis of the phenotypic and genotypic identification

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

Table 13. Geographical distribution of the total identified *Aeromonas* genospecies

Country	*A. hydrophila*	*A. veronii* biotype *sobria*	*A. jandaei*	*Aeromonas* sp.
Malaysia ($n=18$)	9	7	2	0
Thailand ($n=15$)	10	4	0	1
Bangladesh ($n=11$)	0	6	5	0

Distribution of the identified Aeromonas species

All of the three genospecies were represented among the Malaysian strains dominantly *A. hydrophila* followed by *A. veronii* biotype *sobria* and *A. jandaei* (Table 13). Of the 15 strains of Thailand, 10 belonged to *A. hydrophila* and four strains belonged to *A. veronii* biotype *sobria*. The remaining one Thai strain was not identified to species level. Of 11 Bangladeshi strains, six were identified as *A. veronii* biotype *sobria* and five were *A. jandaei*.

5. Discussion

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 9). The DNA G+C mole percent of these 4 isolates were slightly out of the range. These variations may be due to the different methods to determine GC mole percentage in different laboratories. Since the GC mole percentage of the present experiment was detected using the most modern technique (High-Performance Liquid Chromatography, HPLC), so the reliability of the displayed GC contents for *Aeromonas* species are more reliable.

DNA-DNA hybridization has been used to identify bacterial genospecies as discussed previously. The technique wherein re-natured DNA is formed from separate single stranded samples is called DNA-DNA hybridization. This re-naturation technique can be done on either thin filters (membrane filters) or microdillution wells which bind single-stranded DNA very tightly. They provide an important technique for measuring hybridization. A sample of denatured DNA immobilized on either filter or microdillution well. The single strands bind tightly to the filter or well along the sugar-phosphate backbone, but the bases remain free.

The immobilized DNAs are then hybridized with labelled single stranded DNAs. The greater the degree of hybridization, the greater the degree of relatedness (Tortora et al., 1986).

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. According to the criteria, 19 strains were genotypically identical to *A. hydrophila*, 17 were *A. veronii* biotype *sobria* and 7 were *A. jandaei* (Table 9, 10). Two isolates from Thai (T2 and T8) did not give any significant homology value with HG1, HG2, HG8Y or HG9. These two strains were then photobiotynalated, and hybridization was performed among DNAs of the two strains and all the reference strains. The strain T2 gave 70% homology value with *A. hydrophila* ATCC7966^T (HG1) and thus it was identified as *A. hydrophila* genospecies (Table 10). But T8 strain did not give any significant homology value with any of the reference strain. Strain T8, is therefore, designated *Aeromonas* sp.

Kuijper et al. (1989) stated that phenotypic identification using the biochemical scheme of Popoff (1984) for *A. hydrophila*, *A. caviae* and *A. sobria* and published data on *A. veronii* and *A. schubertii* are often inadequate in that a strain biochemically identified as one species may belong to a different genospecies. In this study, strains were first identified comparing the biochemical properties with reference strains of *Aeromonas* genospecies rather than any published schemes. Then the phenotypic identification was confirmed by genotypic identification. Of 26 phenotypic *A. hydrophila* strains 19 belonged to *A. hydrophila* genospecies (HG1) and five belonged to *A. veronii* biotype *sobria* (HG8Y), while 2 belonged to *A. jandaei* genospecies (HG9) (Table 11). Seven strains from phenotypic *A. hydrophila*, were therefore, belonged to different genospecies. It appears from our study presented herein that there are not sufficient phenotypic properties, which can identify *A. hydrophila* with confidence. This finding is correlated with Kuijper et al. (1989). They conducted an experiment on clinical *Aeromonas* spp. and found variations in identification of *A. hydrophila* phenospecies and genospecies. Of their 26 *A. hydrophila* phenospecies only 15 were genotypically identical to *A. hydrophila* genospecies and rests were in different genospecis of *Aeromonas*.

A good correlation was found for phenotypically identified *A. veronii* biotype *sobria* and *A. jandaei*. All the phenotypic *A. veronii* biotype *sobria* and *A. jandaei* belonged to their own genospecies, respectively (Table 11).

T8 strain from Thailand did not give significant homology value with any of the reference strains used in this study. The highest homology value was found 59% with *A. caviae* (Table 10). The phenotypic properties of this strain were also too variable from any of the reference strains and thus it was designated *Aeromonas* sp. in the preceding chapter. This strain, is therefore, recognized a new species belongs to *Aeromonas* genus. Studies are necessary to find some other strains identical to T8 strain. Besides, 16S rRNA sequence study of it and other members of the genus will help to know taxonomic position of it as well as other three species of *Aeromonas* found in this experiment.

The phenotypic properties of the strains, 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 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 also found the differentiation between phenotypic and genotypic identification.

There was no single phenotypic test, which could identify the three identified *Aeromonas* species with confidence. Only four phenotypic properties were found to be able to partially differentiate these three species (Table 12). *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. These results correlate with the results of Abbott et al. (1992) and Altwegg et al. (1990).

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) as cited in the previous chapter. In fact, *A. sobria* phenospecies contains *A. veronii* biotype *sobria*, *A. jandaei* and four more genospecies. *A. hydrophila* was the dominating group followed by *A. jandaei* and *A. veronii* biotype *sobria* among the 44 isolates investigated in this study. Thus, the results of the present experiment are in agreement with the previous reports on *Aeromonas* species associated with EUS-affected fish.

As a result of genotypic identification, Malaysian strains represented all the three genospecies dominated by *A. hydrophila* and *A. veronii* biotype *sobria* (Table 13). Among the 15 Thai strains, 10 belonged to *A. hydrophila*, four belonged to *A. veronii* biotype *sobria* and one strain was designated *Aeromonas* sp. Six strains of Bangladesh were identified as *A. veronii* biotype *sobria* and five were *A. jandaei*. None of the strains from Thai were identified as *A. jandaei*. There was no *A. hydrophila* among the Bangladeshi strains. This may be because an insufficient number of strains were tested or absence of *A. jandaei* or *A. hydrophila* in the particular sampling environment or particular country. However, it needs further studies with sufficient number of strains from various localities.

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. A strain phenotypically identified as one species, but belonging to a different genospecies was found in the present study (Table 11). The present experiment has confirmed the necessity of genotypic identification like DNA-DNA hybridization of *Aeromonas* species.

IV. Phylogenetic study of experimental *Aeromonas* species

1. Abstract

The 16S rDNA gene sequences of two strains from each identified genospecies including T8 strain were determined. These sequences were then compared with all known aeromonads and some other out groups 16S rDNA sequences available from GenBank. A comparative sequence analysis showed that the three identified genospecies were almost identical exhibited 99.9% sequence similarity with only one base differences with the sequence of their own reference strain. Two strains in the same genospecies showed 100% sequence similarities with each other although they had different geographic origins. The results of 16S rDNA sequence studies were in agreement with those of DNA-DNA hybridization studies. The strain T8, which was too heterogeneous by phenotypic traits and did not give significant DNA relatedness with any of the reference strains of *Aeromonas* seemed to be relatively closer to *A cavaei*, *A. trota* or *A. enteropelogenes* from the dendrogram constructed using the 16S rDNA sequences. The data support a phylogenetic homogeneity of the three genospecies identified in the preceding chapter.

2. Introduction

Developments in bacterial ecology and industrial biotechnology are severely hampered by the lack of reliable identification system (Bull et al., 1993, Goodfellow and O'Donnell, 1993). To obtain a phenotypic description requires long and fastidious work, which does not always warrant satisfactory identifications of bacterial species already described in previous chapter. Following determination of the structure of DNA by Watson and Crick (1953), studies in bacteriology have seen a major shift from functional to molecular techniques for identifying bacteria. Phylogenetic relationships among various organisms and their identification now can be derived from degree of DNA relatedness of their genomes : two closely related organisms share significant homologies, while distant organisms display low homologies which described in previous chapter. DNA-DNA relatedness is best suited for identification of closely related species or strains within a single species no doubt. Presently, a direct comparison of rRNA genes sequence is probably the most powerful tool for the identification of many bacteria (Stackebrandt and Goodfellow, 1991). These genes have acquired paramount relevance for the study of bacterial evolution and phylogeny. Indeed, rRNA genes (rDNA) are present and expressed in all bacterial species, are truly homologous in all organisms, are easily sequenced and now offer a large and ever increasing database of sequences and allow the identification of bacteria (Amann et al., 1994). The sequences of gene coding for 16S rDNA is also a powerful tool for deducing phylogenetic and evolutionary relationships among eubacteria, archaebacteria and eucaryotic organisms because of their high information content, conservative nature and universal distribution (Lane et al., 1985 ; Woese, 1987). The 16S rDNA sequence analysis is a standard method for the investigation of their phylogenetic relationships (Yamamoto and Harayama, 1995). Even though some closely related species may have only a few differences in

their 16S rDNA gene sequences, a phylogenetic tree can be established to give them an exact taxonomic position (Collins et al., 1991).

This study was therefore undertaken to sequenced 16S rDNA gene of some representative strains of *Aeromonas* identified in previous chapter in an attempt to i) clarify the intrageneric relationships of three identified aeromonad species and compare the sequence similarities with results of DNA-DNA hybridization; ii) to know the taxonomic position of the identified *Aeromonas* species especially strain T8 and iii) identify regions in 16S rDNA genes which will have a value to develop probe in future.

3. Materials and methods

Bacterial strains

Two strains from each genospecies including T8 strain listed in Table 14 were used in this study.

DNA extraction and purification

DNAs for PCR were prepared according to Enright et al. (1994). Briefly, bacteria were grown overnight in NA broth with shaking at 25°C and centrifuged at 14,000×g for 10 min at 10°C. The harvested cells were resuspended in 300 μl 10 mM Tris (pH 8.0) 2 mM EDTA to which 100 μl lysozyme (50 mg/ml), 10 μl RNase A (1 mg/ml), 10 μl proteinase K (5 mg/ml), 300 μl of a lysis solution containing 10 mM EDTA, 2% Triton X-100 and 50 mM Tris (pH 8.0) were added. After mixing, samples were incubated for 30 min at 37°C. Samples were extracted once with phenol and once with phenol-chloroform-isoamylalcohol (25 : 24 : 1, by vol.). The aqueous phase was extracted with an equal volume of chloroform-isoamylalcohol (24 : 1, by vol.) if necessary. DNA was then precipitated with ethanol at −20°C for 20 min in the presence of 5 M NaCl and collected by centrifugation at 14,000×g for 1 min at 4°C. The pellet was washed with 70% ethanol and resuspended in 10 mM Tris (pH 8.0) −1 M EDTA buffer.

Primers used in this study

Seven primers, complementary to the evolutionarily conserved portion of 16S rDNA, were used in the sequencing reactions (Table 15). All the oligonucleotide

Table 14. List of strains used in the 16S rDNA sequencing study

Code No.	Genospecies	Country
M29	*A. hydrophila*	Malaysia
T20	*A. hydrophila*	Thailand
M16	*A. veronii* biotype *sobria*	Malaysia
B1	*A. veronii* biotype *sobria*	Bangladesh
M34	*A. jandaei*	Malaysia
B10	*A. jandaei*	Bangladesh
T8	*Aeromonas* sp.	Thailand

Table 15. Sequencing primers used in the study

Code No.	Position*	Direction	Sequence (5′-3′)
24F	8-24	Sense	AGAGTTTGATCCTGGCT
800F	800-819	Sense	GTAGTCCACGCCGTAAACGA
1100F	1092-1112	Sense	AAGTCCCGCAACGAGCGCAAC
520R	528-509	Antisense	GCGGCTGCTGGCACGAAGTT
820R	821-802	Antisense	CATCGTTTACGGCGTGGACT
920R	928-909	Antisense	CCCCGTCAATTCCTTTGAGT
1190R	1176-1195	Antisense	GACGTCATCCCCACCTTCCT
1540R	1541-1522	Antisense	AAGGAGGTGATCCAGCCGCA

* Numbering was decided according to the 16S rDNA sequence from *Escherichia coli*

primers were purchased from Funakoshi co. Japan.

Amplification of 16S rDNA genes

Each PCR mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM each of four deoxynucleoside triphosphates (dNTPs), 0.2 μM each primer, 2.5 U of *Taq* DNA polymerase (Ampli*Taq* DNA polymerase; Perkin-Elmer, USA), and 100 ng of DNA template in a total volume of 100 μl. Control mixes

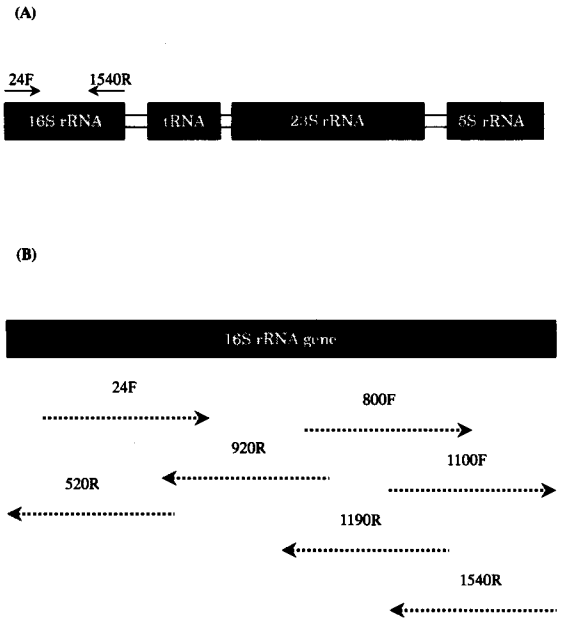


Fig. 1. rRNA operon indicating different genes and sequencing strategies.
A) amplification of 16S rDNA gene by 24F and 1540R primers; B) various segments of 16S rDNA gene elongated by various sense (F) and antisense (R) primers

containing no DNA were also used to know any contamination from reaction solutions or utensils. The amplification and sequencing primers were located at the highly conserved regions of the 16S rDNA gene (Fig 1). The 5′-and 3′-end amplification primers for 16S rDNA gene were 24F and 1540R, respectively (Table 15). The mixture was then layered with autoclaved mineral oil and subjected for amplification in a DNA thermal cycler (Perkin-Elmer 480, USA). The initial denaturation step consisted of heating the reaction mixture at 94°C for 4 min. The temperature was cycled to 94°C for 1 min for denaturation, then to 55°C for 1 min for annealing, 72°C for 1.5 min for extension for a total of 30 cycles. The PCR products were then visualized on 1.5% agarose gel stained with ethidium bromide with a molecular weight standard marker (pHY marker, TaKaRa, Kyoto, Japan). The amplification primers used in this study gave about 1.5 kb PCR product and corresponded to positions 25-1521 in the *E. coli* sequence.

Purification of PCR products and cycle sequencing

The PCR products, which produced a single band on agarose gels, were selected for sequencing and the mineral oil was removed on parafilm. Then they were purified by PEG (polyethylene glycol) 6000 precipitation according to the method Kusukawa et al. (1990). Briefly, 6 μl of 100 mM EDTA and 60 μl PEG/NaCl were mixed to 100 μl amplified products in an eppendrof tube and it was placed on ice bath for 15 min. The mixtures were centrifuged at 14,000×g for 10 min at 4°C and the supernatant was discarded carefully. The minute DNA pellet was rinsed with 500 μl of 75% ethanol, vacuum dried and resuspended in 10 mM Tris (pH 8.0) −1 mM EDTA buffer. Approximately 100 ng template was then directly sequenced by using a *Taq* FS Dye Terminator according to "ABI prism dye terminator cycle sequencing ready reaction kit" (The perkin-Elmer Corporation). Briefly, each sequencing mixture contained 8 μl Terminator ready reaction mixture, 3.2 pmole primer and 50-70 ng PCR products (Templete) in a total volume of 20 μl reaction mixture. The mixture was then overlaid with autoclaved mineral oil and subjected in a DNA thermal cycler (Perkin-Elmer 480, USA) for cycle sequencing. The initial denaturation step consisted of heating the reaction mixture at 96°C for 7 min. The temperature was cycled to 96°C for 30 sec for denaturation, then to 50°C for 15 sec for annealing, 60°C for 4 min for extension for a total of 25 cycles.

Purification of elongated products and electrophoresis

The mineral oil was removed by rolling the products on a parafilm and purified according to the protocol of "ABI prism dye terminator cycle sequencing ready reaction kit" (The perkin-Elmer Corporation). Briefly, 50 μl 95% ethanol and 2 μl 3 M sodium acetate (pH 5.2) was added to 20 μl crude extended product in a 1.5 ml microcentrifuge tube. The cocktail was then vortex and placed on ice for 10 min. The ethanol solution was aspirated with a micropipetter as completely as possible after centrifuges the cocktail at 14,000×g for 20 min. The minute pellet was rinsed with 250 μl 70% ethanol and centrifuge at 14,000×g for 2 min. The ethanol solution was carefully aspirated with a micropipetter as completely as possible and the pellet was vacuum dried and finally stored at −80°C under dark condition until applied to sequencer for electrophoresis.

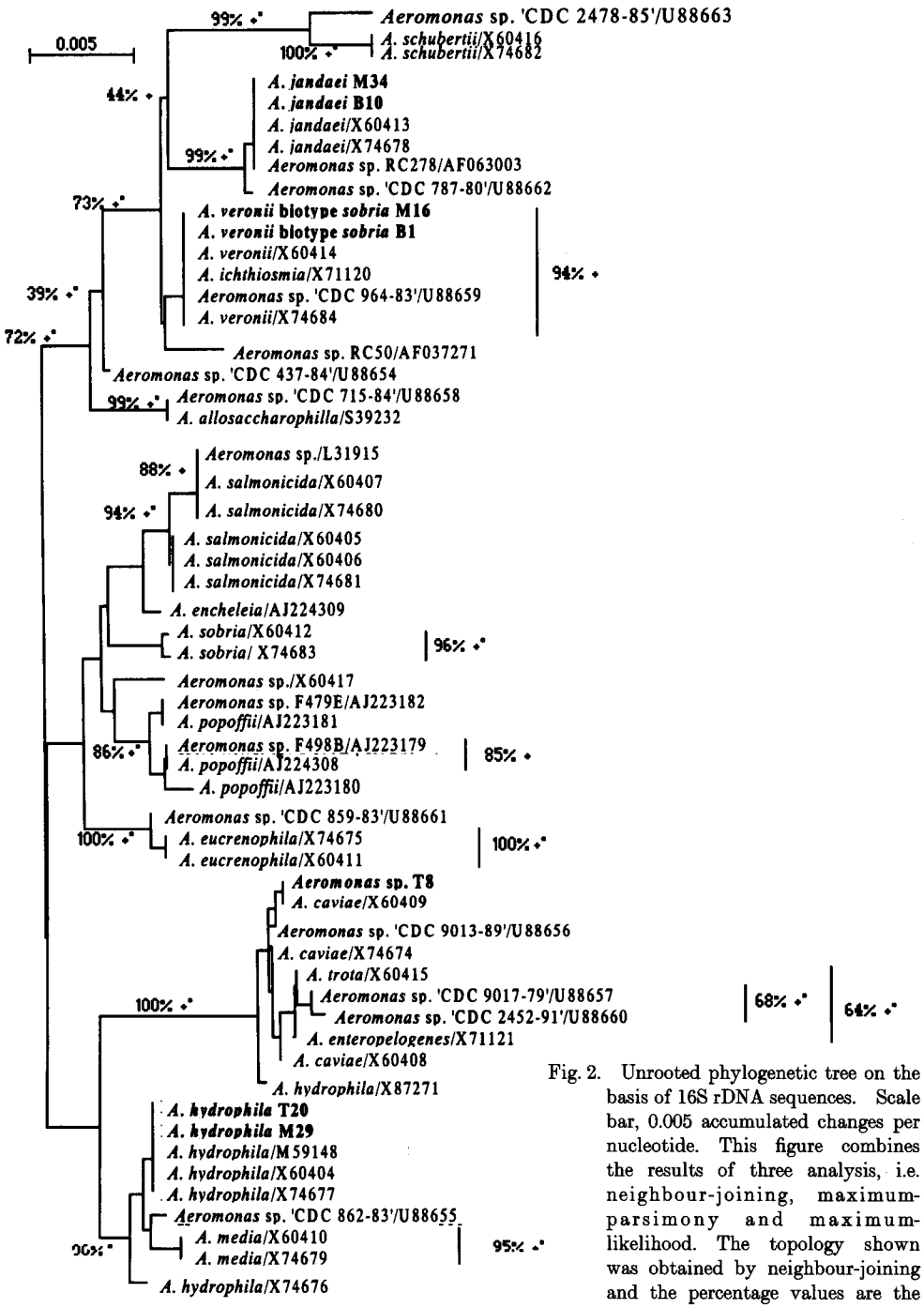


Fig. 2. Unrooted phylogenetic tree on the basis of 16S rDNA sequences. Scale bar, 0.005 accumulated changes per nucleotide. This figure combines the results of three analysis, i.e. neighbour-joining, maximum-parsimony and maximum-likelihood. The topology shown was obtained by neighbour-joining and the percentage values are the results of bootstrap analysis using 500 replications

The dried pellet was resuspended with 3 μl loading solution (5 : 1 deionized formamide : 50 mM EDTA containing 3% blue dextran), mixed well and centrifuge at 5,000 $\times$ g for 20 sec. The sample was heated at 90°C for two min and placed on ice until loaded to gel. The sequencing solutions were then loaded onto the wells of gel containing 4% acrylamide (acrylamide 3.8%, bis-acrylamide 0.2%) in 8.3 M urea and sequencing was done with an Applied Biosystems model 373A automated sequencer. 1 $\times$ TBE buffer was used as a running buffer.

Analyses of 16S rRNA sequence data

The 16S rDNA sequences were aligned and studied using a set of programmes developed by R. Christen. In all phylogenetic analyses, we used the sequences determined in this study and small-subunit rDNA sequences obtained from the EMBL database. For Fig. 2, the following sequences were used: *Aeromonas* sp. CDC 2478-85 (U88663), *A. schubertii* DSM 4882^T (X60416), *A. schubertii* ATCC 43700^T (X74682), *A. jandaei* ATCC 49568^T (X60413), *A. jandaei* (X74678), *Aeromonas* sp. RC278 (AF063003), *Aeromonas* sp. CDC 787-80 (U88662), *A. veronii* NCIMB 13015^T (X60414), *A. ichthiosmia* (X71120), *Aeromonas* sp. CDC 964-83 (U88659), *A. veronii* ATCC 35624^T (X74684), *Aeromonas* sp. RC50 (AF037271), *Aeromonas* sp. CDC 437-84 (U88654), *Aeromonas* sp. CDC 715-84 (U88658), *A. allosaccharophilla* CECT 4199^T (S39232), *Aeromonas* sp. (L31915), *A. salmonicida* (X60407), *A. salmonicida* ATCC 27013^T (X74680), *A. salmonicida* (X60405), *A. salmonicida* (X60406), *A. salmonicida* ATCC 33658^T (X74681), *A. encheleia* (AJ224309), *A. sobria* NCIMB 12065^T (X60412), *A. sobria* ATCC 43979^T (X74683), *Aeromonas* sp. (X60417), *Aeromonas* sp. F479E (AJ223182), *A. popoffii* (AJ223181), *Aeromonas* sp. F498B (AJ223179), *A. popoffii* (AJ224308), *A. popoffii* (AJ223180), *Aeromonas* sp. CDC 859-83 (U88661), *A. eucrenophila* ATCC 23309^T (X74675), *A. eucrenophila* NCIMB 74^T (X60411), *A. caviae* (X60409), *Aeromonas* sp. CDC 9013-89 (U88656), *A. caviae* ATCC 15468^T (X74674), *A. trota* (X60415), *Aeromonas* sp. CDC 9017-79 (U88657), *Aeromonas* sp. CDC 2452-91 (U88660), *A. enteropelogenes* DSM 6394^T (X71121), *A. caviae* (X60408), *A. hydrophila* DSM 30187^T (X87271), *A. hydrophila* ATCC 7966^T (M59148), *A. hydrophila* NCIMB 9240^T (X60404), *A. hydrophila* ATCC 7966^T (X74677), *Aeromonas* sp. CDC 862-83 (U88655), *A. media* ATCC 33907^T (X60410), *A. media* ATCC 33907^T (X74679) and *A. hydrophila* ATCC 35654 (X74676).

Domains used to construct the dendogram shown in Fig. 2 were regions of the small-subunit rDNA sequences available for all sequences and excluding positions likely to show homoplasy: positions (45-1411, *E. coli* small-subunit rDNA sequence J01695 numbering). Phylogenetic analyses was performed by using three different methods, neighbour-joining (Saitou and Nei, 1987), maximum-likelihood (options QFYG, fast DNAml programme of Olsen, G.J., University of Illinosis, Urbana, USA) and maximum-parsimony (PAUP 3.0s for the Macintosh, heuristic search, Swofford, 1992). The robustness of each topology was checked by using the neighbour-joining method and 500 bootstrap replications with a distance correction according to the model Kimura two parameters (Felsenstein, 1985). Phylogenetic tree was drawn by using the njplot programme for the Macintosh (Gouy, M. CNRS URA 243, Universite Claude Bernard, Lyon, France).

4. Results

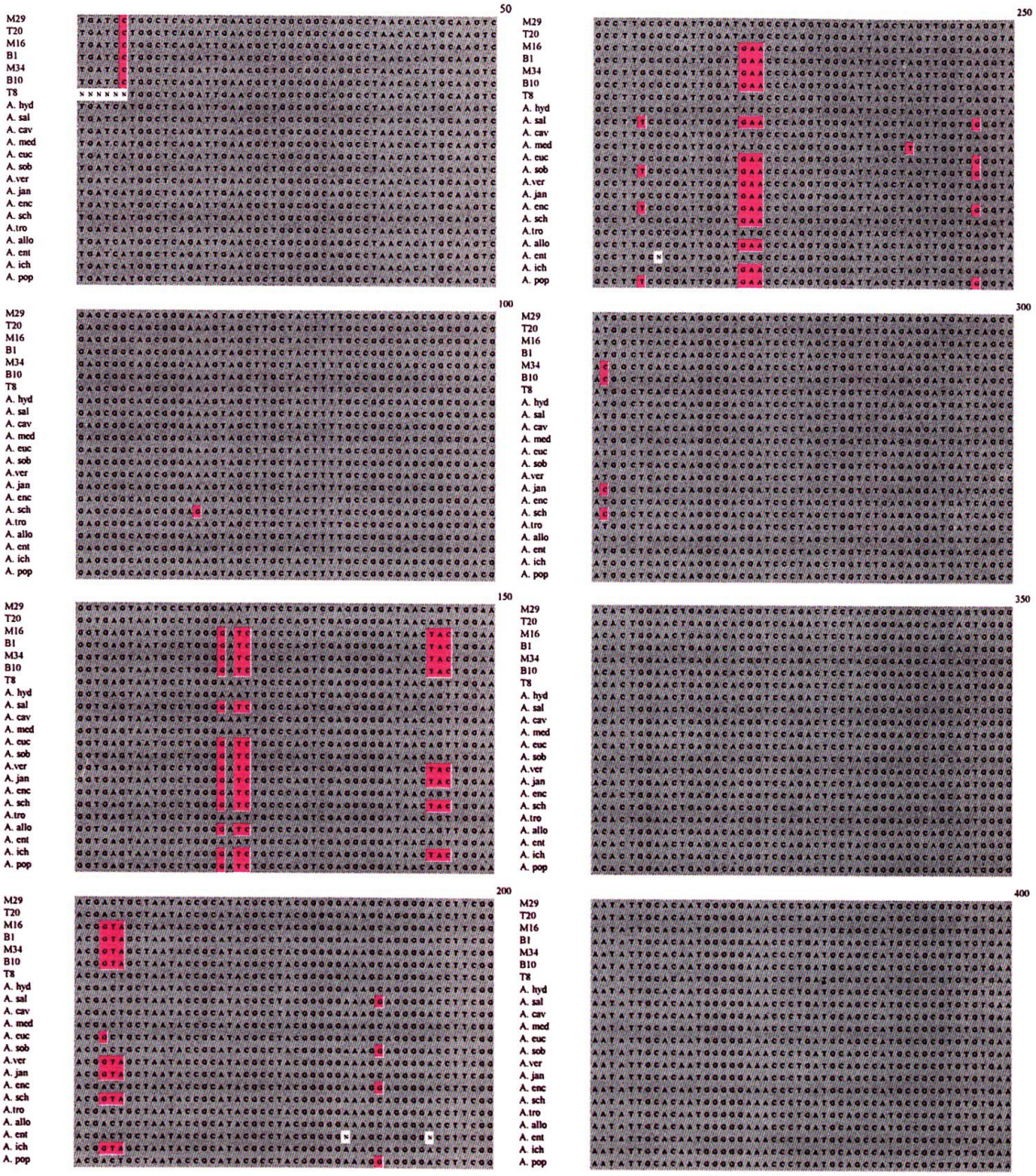
The 16S rDNA sequences of the selected strains from three identified genospecies including T8 were aligned and compared with those of all aeromonads found from the GeneBank (Fig. 3). The sequences of all two strains for a given genospecies were identical to each other. They showed only one nucleotide difference at positions six with their own reference strain found from GeneBank. It was found C nucleotide in that position for all three genospecies instead of A in the published data found from GeneBank (Fig. 3). It was not able to detect any nucleotide at the same position from T8 strain.

Nucleotide differences among *Aeromonas* species often occurred in the same regions of the molecule known as hypervariable regions. The major hypervariable regions were at positions 110 to 260, 435 to 470, 980 to 1010 and 1265 to 1335 (Fig. 3).

Table 16. Lavel of homology and numbers of nucleotide differences of 16S rDNA sequence from slected strains and all other *Aeromonas* species

Species	% of homology with/Number of nucleotide differences compared with						
	1	2	3	4	5	6	7
1. *A. hydrophila* (M29)	100.0/0						
2. *A. hydrophila* (T20)	100.0/0	100.0/0					
3. *A. veronii* biotype *sobria* (M16)	98.6/22	98.6/22	100.0/0				
4. *A. veronii* biotype *sobria* (B1)	98.6/22	98.6/22	100.0/0	100.0/0			
5. *A. jandaei* (M34)	98.4/25	98.4/25	99.4/9	99.4/9	100.0/0		
6. *A. jandaei* (B10)	98.4/25	98.4/25	99.4/9	99.4/9	100.0/0	100.0/0	
7. *Aeromonas* sp. (T8)	98.9/16	98.9/16	98.7/19	98.7/19	98.9/17	98.9/17	100.0/0
8. *A. hydrophila* (X60404)	99.9/1	99.9/1	98.5/23	98.5/23	98.3/26	98.3/26	98.9/16
9. *A. salmonicida* (X60405)	98.9/16	98.9/16	98.7/19	98.7/19	98.3/26	98.3/26	98.5/23
10. *A. caviae* (X60408)	99.0/15	99.0/15	98.5/22	98.5/22	98.9/16	98.9/16	99.9/2
11. *A. media* (X60410)	99.7/4	99.7/4	98.4/24	98.4/24	98.2/27	98.2/27	98.9/17
12. *A. eucrenophila* (X60411)	99.1/14	99.1/14	98.9/16	98.9/16	98.5/22	98.5/22	98.5/22
13. *A. sobria* (X60412)	98.7/20	98.7/20	99.0/15	99.0/15	98.8/18	98.8/18	98.7/19
14. *A. veronii* (X60414)	99.0/15	99.0/15	99.9/1	99.9/1	99.4/9	99.4/9	98.7/19
15. *A. jandaei* (X60413)	98.3/26	98.3/26	99.4/9	99.4/9	99.9/1	99.9/1	98.9/17
16. *A. schubertii* (60416)	97.7/34	97.7/34	98.7/20	98.7/20	98.6/20	98.6/20	98.0/30
17. *A trota* (X60415)	98.9/16	98.9/16	98.4/24	98.4/24	98.9/17	98.9/17	99.8/3
18. *A. encheleia* (AJ224309)	99.1/12	99.1/12	98.7/18	98.7/18	98.2/25	98.2/25	98.3/23
19. *A. popoffii* (AJ223180)	98.8/17	98.8/17	98.6/20	98.6/20	98.4/22	98.4/22	98.3/24
20. *A. ichthiosmia* (X71120)	97.5/38	97.5/38	99.5/8	99.5/8	98.9/16	98.9/16	98.7/19
21. *A. enteropelogenes* (X71121)	98.9/16	98.9/16	98.5/22	98.5/22	98.8/17	98.8/17	99.9/2
22. *A. allosaccharophila* (S39232)	98.6/21	98.6/21	99.1/13	99.1/13	98.7/19	98.7/19	98.8/18

The number inside the bracket indicates either strain number or GeneBank accession number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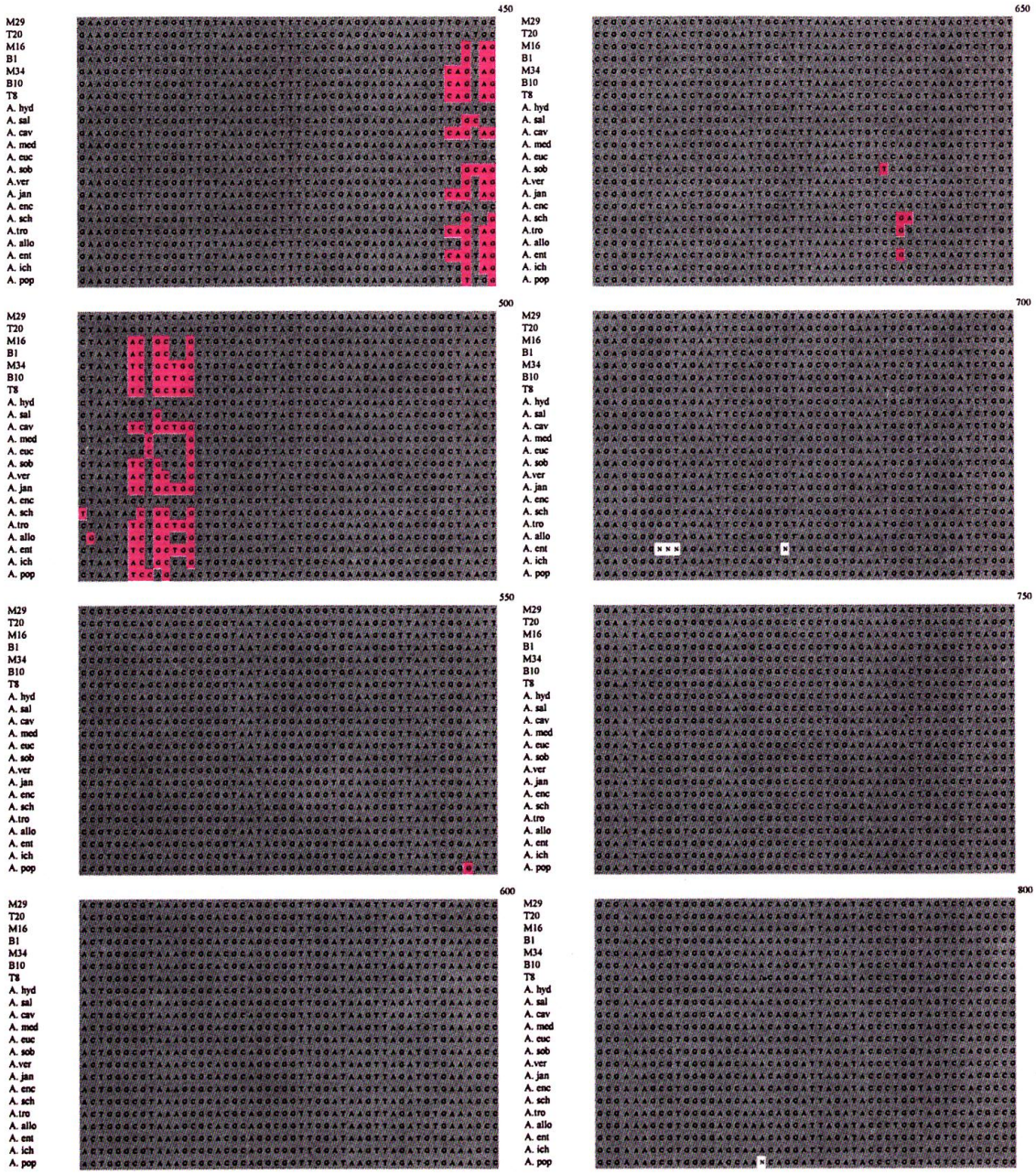
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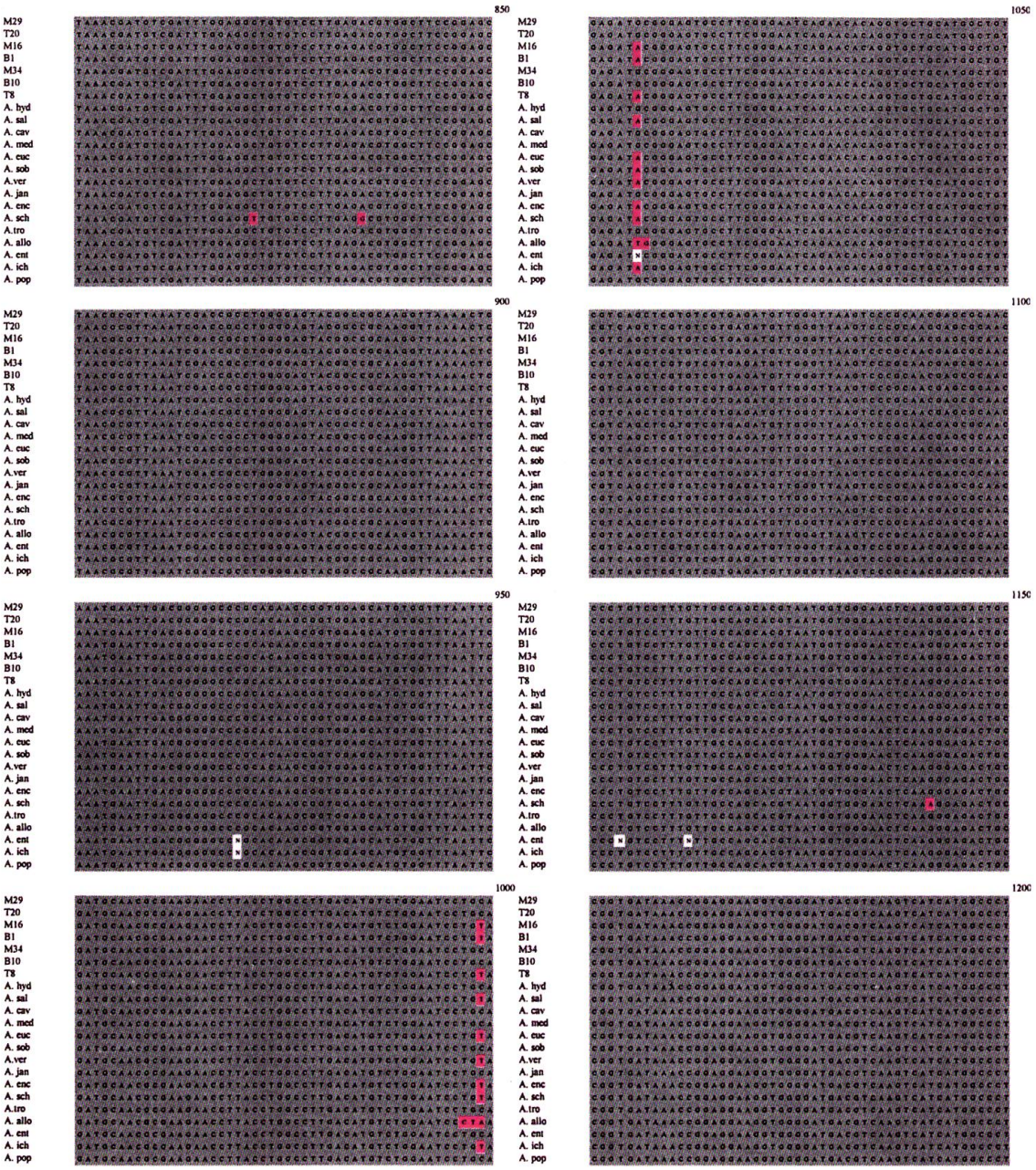
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A. pop

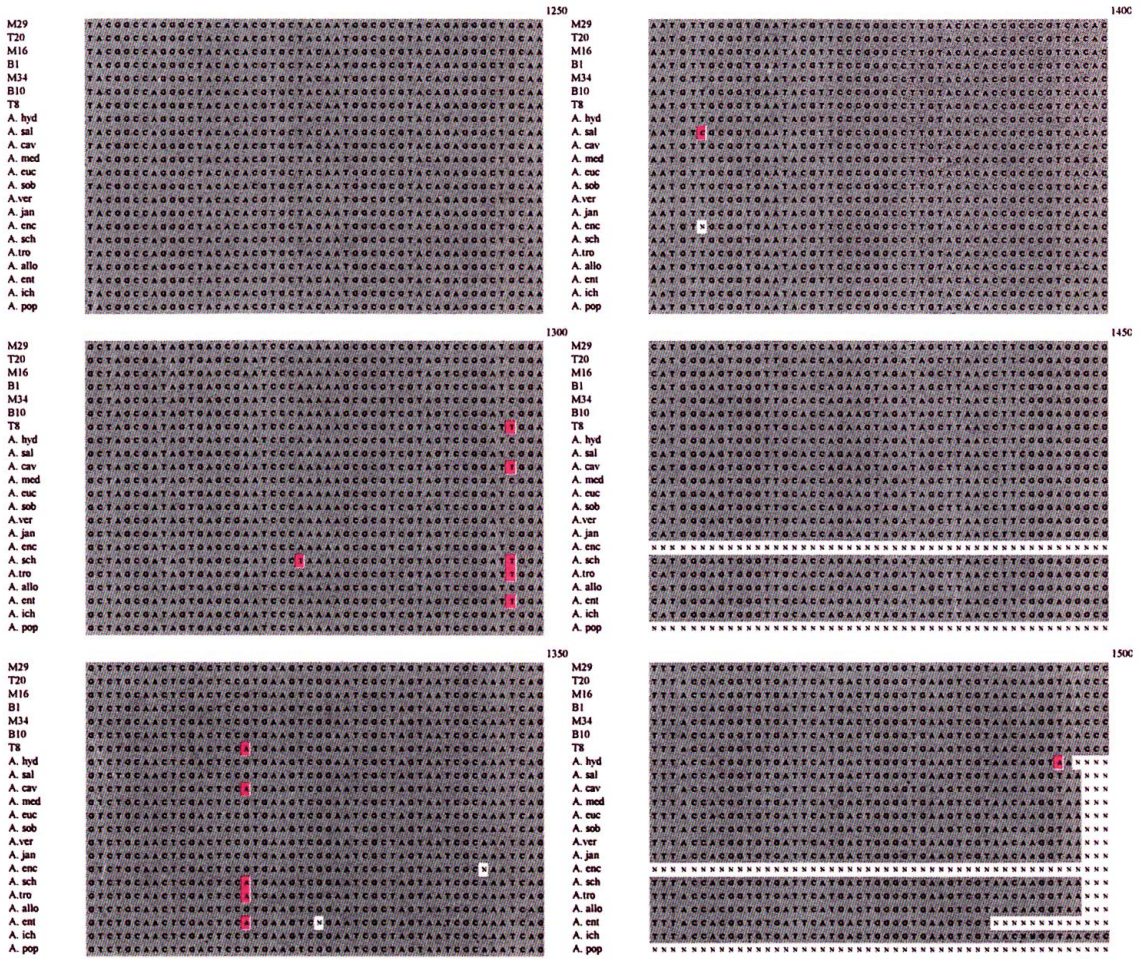

Fig. 3. 16S rRNA primary structures as determined by PCR DNA sequencing. A. hyd : *A. hydrophila* (X60404), M29 and T20 : *A. hydrophila*, A. sal : *A. salmonicida* (X60405), A. cav : *A. caviae* (X60408), T8 : *Aeromonas* sp., A. med : *A. media* (X60410), A. euc : *A. eucrenophila* (X60411), A. sob : *A. sobria* (X60412), A. ver : *A. veronii* (X60414), M16 and B1 : *A. veronii* biotype *sobria*, A. jan : *A. jandaei* (X60413), M34 and B10 : *A. jandaei*, A. enc : *A. encheleia* (AJ224309), A. sch : *A. schubertii* (X60416), A. tro : *A. trota* (X60415), A. allo : *A. allosaccharophilla* (S39232), A. ent : *A. enteropelogenes* (X71121), A. ich : *A. ichthiosmia* (X71120), A. pop : *A. popoffii* (AJ223180). The red pastes indicate positions where nucleotide differences are found in *Aeromonas* species

All the three identified genospecies were almost identical exhibited 99.9% sequence similarity with only one base difference with the sequence of each reference strain of their species (Table 16). T8 strain was closely related to *A. caviae*, *A. enteropelogenes* and *A. trota* exhibiting 99.8 to 99.9% sequence similarities with two to three bases difference (Table 16 and Fig. 3).

Phylogenetic analysis were performed by using three different methods, neighbour-joining (Saitou and Nei, 1987), maximum-likelihood (options QFYG, fast DNAml programme of Olsen, G.J., University of Illinosis, Urbana, USA) and maximum-parsimony (PAUP 3.0s for the Macintosh, heuristic search, Swofford, 1992). The tree was drawn by using the njplot programme for the Macintosh (Gouy, M. CNRS URA 243, Universite Claude Bernard, Lyon, France) is shown in Fig. 2. All the three genospecies of *Aeromonas* formed clusters with their own reference strains. Strain T8 clustered with *A. caviae* (Fig. 2).

5. Discussion

In the previous chapter it was described that the strains M29 and T20 were *A. hydrophila*, strains M16 and B1 were *A. veronii* biotype *sobria* while strains M34 and B10 gave significant DNA-DNA homology values with *A. jandaei*. In the present investigation it was confirmed that these strains were also phylogenetically clustered with their own reference strains (Fig. 2). Even though 16S rDNA sequence analysis of the present experiment were well agreed with that of DNA-DNA hybridization study, but it is very difficult to define species based on the similarities of sequences only. Because, *A. hydrophila* were 99.7% similar with only four bases difference with *A. media* as it was found in present investigation (Table 16). On the other hand, *A. veronii* biotype *sobria* were 99.4% similar sequence with nine bases difference with *A. jandaei* and vice versa (Table 16).

Nucleotide differences among the members of *Aeromonas* species occurred at positions 110 to 260, 435 to 470, 980 to 1010 and 1265 to 1335 known as hypervariable regions (Fig. 3). The remaining portions are almost identical to each other. These hypervariable regions were conserved for a given species, which might serve as targets to develop species-specific probes.

Strain T8 from Thailand is atypical in phenotypic properties described previously. It did not give significant DNA-DNA homology value with any of the reference strains of *Aeromonas*. But it is relatively related to *A. caviae*, *A. trota* and *A. enteropelogenes* as it was revealed from the analysis of 16S rDNA sequence (Table 16 and Fig. 2). This strain however gave the highest DNA relatedness (59%) to *A. caviae* and 32% to *A. trota* as described in the previous chapter. Bacterial genospecies, in fact, can be defined by DNA-DNA hybridization criteria not by 16S rDNA sequencing. DNA homology values of 70% at T_m-25°C with $\leq$5% divergence (Janda, 1991) are included in a given genospecies. According to the criteria T8 strain was different from either *A. caviae* or *A. trota* no doubt. On the other hand, phenotypic properties of strain T8 were so different from that of *A. enteropelogenes* (Table 17). Its phenotypic properties were also typical from any of the reference strains of *Aeromonas* species described previously. It also expressed the basic biochemical properties for the genus *Aeromonas*. For these instances, strain

Table 17. Phenotypic profiles of *A. enteropelogenes* and *Aeromonas* sp. (strain T8)

Phenotypic profiles	*A. enteropelogenes*[a]	*Aeromonas* sp. (T8)
Motility	+	+
Oxidase	+	+
Indole production	+	Nd
VP test	−	−
Esculin hydrolysis	−	+
Arginine dehydrogenase	+	Nd
Ornithine decarboxylate	−	−
Gelatin hydrolysis	+	+
Gas from D-Glucose	+	−
Acid from		
D-Glucose	+	+
L-Arabinose	−	Nd
Cellobiose	+	−
D-Sorbitol	−	−
Sucrose	−	+
Salicin	−	+
Oxidation of gluconate	−	Nd
Nitrate reduction	+	+
Hemolysis on sheep blood	+	Nd
O/129 resistance	+	+

[a] Schubert et al. (1990): Hyg. Med. 471-472
Nd: Not detected

T8 was designated a new species under the genus of *Aeromonas*.

In conclusion, 16S rDNA sequence of *Aeromonas* species is an useful tool to know the phylogenetic relationships among the members of the genus. It may helps to identify the species with low confidence compared to that of DNA-DNA homology. In fact, it is not possible to identify *Aeromonas* species correctly to the genospecies level based on 16S rDNA sequence study only. It may be used as supplement not to alternate of DNA-DNA hybridization study. Developing primers from the data of 16S rDNA sequences presented herein will help to identify the three *Aeromonas* species associated with EUS of fish more easily and more quickly.

V. Pathogenesis of experimental motile aeromonads in goldfish

1. Abstract

The pathogenicity of *Aeromonas* species isolated from fishes with EUS was investigated at 20°C and 25°C by intramuscular injection and immersion method to goldfish. Median lethal doses (LD_{50}) were varied from $2\times10^{6.8}$ to 6×10^{8} CFU/fish at 20°C and $2.6\times10^{4.5}$ to 1.2×10^{8} CFU/fish at 25°C for intramuscular injection. Swelling at the site of injection were observed in almost all the experimental fish from the first day which gradually damaged and from where simultaneously scales protrusion appeared following dermal lesion from 2 to 3 days after injection. Internal haemorrhagic lesions on the abdominal cavity at the chronic stage were common instances. Infections were found significantly on the mechanically injured fish by bath challenge. Dermal lesions and haemorrhagic lesions on the abdominal cavity were also found at the chronic stages, which were almost identical to those of intramuscular injection. Infection rates at 25°C in most cases were higher than those at 20°C. Reisolates were significantly identified from kidney or lesions of dead, moribund, infected or sacrificed fish from both methods.

2. Introduction

Aeromonas spp. are recognized as pathogenic for poikilothermal animals, such as frogs, snakes or fish and in homeothermal animals and humans (Russell, 1898; Popoff, 1984; Altwegg and Geiss, 1989; Janda, 1991). These species, are however, considered to be opportunistic pathogens, which seemed a contradiction in terms. In the intensive or semi-intensive fish farming systems fish are easily stressed by mishandling, overcrowd, lack of nutrition or poor water quality. These conditions easily enhanced to induce disease on aquatic animals by *Aeromonas* species. Although the motile aeromonads occupy an important role as a cause of various diseases in fish, interest in aeromonads has been dominated by the non-motile *A. salmonicida*, generally regarded as its wide distribution to cause furunculosis and spticemia (McCarthy, 1977). There has been increasing evidence, however, of the importance of the motile aeromonads as causative agents of fish disease. Recently, *A. hydrophila* and *A. sobria* have frequently been isolated from EUS lesions (Anonymous, 1986; Llobrera and Gacutan, 1987; Lio-Po et al., 1990; Subasinghe et al., 1990; Torres et al., 1990) in Southeast Asian countries. This disease causes severe mortalities both cultured and wild fish species every year in a cyclic manner (Roberts et al., 1986, 1993). It has been suggested that motile *Aeromonas* contribute to the pathogenesis of the disease (Costa and Wejeyaratne, 1989). Three motile *Aeromonas* species have been identified by phenotypic and genotypic method from EUS affected fishes described in the previous chapters. The present study was therefore, undertaken to know the pathogenicity of the identified species to experimentally induced goldfish (*Carassius auratus*).

3. Materials and methods

Bacterial strains

A total of 44-strains of motile *Aeromonas* isolated from fishes with EUS have been identified to three genospecies : *A. hydrophila*, *A. veronii* biotype *sobria* and *A. jandaei* and one unidentified *Aeromonas* strain in the previous chapters. Two strains from each species including unidentified one (T8 strain), as listed in Table 18, were selected for this study.

Experimental fish

Goldfish (*C. auratus*) weighing 12–20 g, purchased from a local pet shop, were used in this study. Prior to the experiment, fish were acclimated to laboratory conditions at either 20°C or 25°C for at least two days.

Preparation of bacterial suspension

The strains were cultured on a nutrient agar (NA ; polypepton 10 g, meat extract 5 g, NaCl 1.2 g, agar 15 g, distilled water 1000 ml, pH 7.2) at 25°C for 24 h prior to the experiment. They were then suspended in sterile physiological saline (0.85% NaCl) and desired dilutions were prepared by ten-fold dilution method.

Pathogenicity test

The fish were lightly anaesthetized in MS222 (ethyl maminobenzoate methanesulfonate) prior to challenge. Then the fish were injected intramuscularly with 0.05 ml of three different concentrations of bacterial suspension in the flank, just below the anterior part of the dorsal fin. Negative control fish received 0.85% physiological saline instead of bacterial suspension. Each suspension of strain was injected to five fish. Each group was kept separately in a 3 l aquarium with aeration at 20°C and 25°C for 7 d and the water of each aquarium was changed at 24 h interval. No feed was allowed during the course of experiment. The mortality of fish were monitored daily. Finally the median lethal dose (LD_{50}) was calculated according to the method of Reed and Muench (1938).

One strain from each genospecies depends on relatively strong pathogenicity by intramuscular injection including the unidentified one were selected for the bath

Table 18. Details of EUS strains used in this study

Strain No.	Genospecies	Host fish species	Origin country	Organ	Year
M29	*A. hydrophila*	*Oreochromis* sp.	Malaysia	Kidney	1987
T20	*A. hydrophila*	*Puntius gonionotus*	Thailand	Liver	1995
B1	*A. veronii* biotype *sobria*	*Cirrhinus mrigala*	Bangladesh	Lesion	1994
M16	*A. veronii* biotype *sobria*	*Cyprinus* sp.	Malaysia	Lesion	1987
B2	*A. jandaei*	*Ci. Mrigala*	Bangladesh	Lesion	1994
M34	*A. jandaei*	*Aristichthys nobilis*	Malaysia	Kidney	1987
T8	*Aeromonas* sp.	*Osphronemus groamy*	Thailand	Kidney	1994

challenge. Five mechanically injured (some scales removed from the anterior part bellow the dorsal fin and one scratch with a sterile explorer) fish were immersed approximately in the concentration of median lethal dose of respected strain's suspension for 20 minutes. Then the challenged fish were washed with distilled water and maintained under the same condition as described above. Mortality and infection rate were monitored daily for two weeks. In the both challenges, the survived fishes were sacrificed 14 days post injection and the re-isolates were collected from the lesions or kidney by swabbing.

Re-isolation of challenged strains

The re-isolates from kidney and lesions of dead, moribund or sacrificed fish were purified on nutrient agar. Then colony hybridization was performed at T_m-25°C (optimal condition) and T_m-15°C (stringent condition) according to the method of Mass (1983) with minor modifications (Iqbal, 1999) to confirm the re-isolates. Hybridized DNAs were visualized by one step colorimetric detection method according to Keller and Manak (1989).

4. Results

Swelling at the site of injection were observed in almost all the experimental fish from the first day which gradually damaged and where simultaneously scales protrusion appeared following dermal lesion from 2 to 3 days after injection. Internal haemorrhagic lesions on the abdominal cavity at the chronic stage were common instances. The lesions and symptoms on the infected fish were almost similar to those of naturally occurred (Fig. 4). Median lethal doses (LD_{50}) were varied from $2\times10^{6.8}$ to 6×10^{8} CFU/fish at 20°C and $2.6\times10^{4.5}$ to 1.2×10^{8} CFU/fish at 25°C for intramuscular injection (Table 19). LD_{50} values of the tested fish kept at 25°C were approximately one order lower than those of fish were kept at 20°C. Respective re-isolates were identified from the kidney or lesions of dead, moribund or sacrificed fish. Neither any mortality nor any sign of infection were observed in the control groups.

Table 20 shows the results of the bath challenge of the respective strains. A

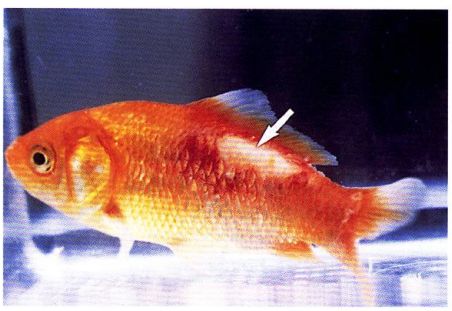

Fig. 4. External lesion (arrow) on the trunk region by intramuscular injection with strain M16 (*A. veronii* biotype *sobria*)

Table 19. Virulence of *Aeromonas* species after intramascular injection

Strain No.	Genospecies	Median lethal dose (CFU/ml) at	
		20°C	25°C
M29	*A. hydrophila*	$6\times10^{7.5}$	$9\times10^{6.3}$
T20	*A. hydrophila*	$6\times10^{7.3}$	$1.7\times10^{6.1}$
B1	*A. veronii* biotype *sobria*	$5\times10^{7.3}$	$1.8\times10^{6.4}$
M16	*A. veronii* biotype *sobria*	$2\times10^{6.8}$	$2.6\times10^{4.5}$
B2	*A. jandaei*	$2.3\times10^{7.5}$	$9\times10^{6.5}$
M34	*A. jandaei*	6×10^{8}	1.2×10^{8}
T8	*Aeromonas* sp.	$1.8\times10^{6.9}$	1.9×10^{6}
Control*			

* No mortality or any sign of infection were observed in the control group

Table 20. Infection rates, mortality and reisolation rates of goldfish challenged with *Aeromonas* species by immersion method

Strain No.	Genospecies	Infection rate (%) at		Mortality (%) at		Reisolation rate (%) at	
		20°C	25°C	20°C	25°C	20°C	25°C
T20	*A. hydrophila*	80	100	0	20	100	100
M16	*A. veronii* biotype *sobria*	100	100	0	40	100	100
B2	*A. jandaei*	80	100	0	20	80	100
T8	*Aeromonas* sp.	60	80	0	0	60	80
Control		0	0	0	0	0	0

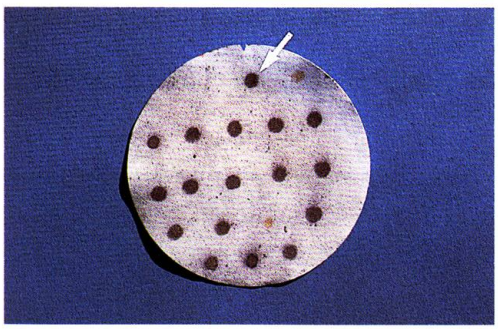

Fig. 5. A photograph of a Whatman 541 filter with colonies of re-isolates after colony hybridization with photobiotinylated M16 probe DNA. Arrow indicates the positive control; deep color (similar to positive control) indicates the positive results and fade color indicates negative results

significant number of fish were infected by the challenged strains. Dermal lesions and haemorrhagic lesions on the abdominal cavity were found at the chronic stages, which were almost identical to those of intramuscular injection. Infection rates at 25°C in most cases were higher than those at 20°C. Mortality did not occur at 20°C. On the other hand, only a few fish died at 25°C by either *A. hydrophila* or *A. veronii* biotype *sobria* or *A. jandaei*. Even though mortality was very low in immersion method but re-isolates were significantly identified from kidney or lesions of moribund or infected fish (Table 20, Fig. 5).

5. Discussion

It is evident from the results presented herein, that all the three genospecies of *Aeromonas* have the potentiality to cause EUS like lesions on the experimentally infected goldfish at 20 to 25°C (Table 19, 20). The median lethal doses varied depend on the strains used. The doses varied from $2\times10^{6.8}$ to 6×10^{8} CFU/fish at 20°C and $2.6\times10^{4.5}$ 1.2×10^{8} CFU/fish at 25°C. *A. veronii* biotype *sobria* was found relatively high pathogenic followed by unidentified *Aeromonas* strain (T8 strain), *A. hydrophila* and *A. jandaei*. Overall, the median lethal doses were about one log order lower in the fish kept at 25°C.

Even though there was no significant mortality by bath challenge but high infection rates both at 20°C and 25°C were recorded (Table 20). In almost all cases respective re-isolates were identified either from lesions or kidney of dead, moribund or sacrificed fish (Fig. 5). Existence of respective strains constantly in the lesions and kidney of dead, moribund or sacrificed fish indicated that they have the potentiality to cause EUS in goldfish. It is suspected that these bacteria may be penetrated into the kidney of tested fish through the artificial injury, which have the abilities to multiply and cause disease or mortality when the environmental conditions become worse and stressed.

The fish culture systems in Southeast Asian countries are now semi-intensive to intensive, where stocking densities are too high. It is very natural to damage the body barrier of the fish from their competition of feed, habitats or any other biological activities. Besides, in the open water system, water pollution from environment is very common in the Southeast Asian countries (Iqbal et al., 1996, 1999). The fish easily stressed when it is exposed to such environmental conditions and weakens the fish, which eventually succumbs to *Aeromonas*, the most common bacterial flora in the aquatic environments.

From the results of experimental infection at 20°C and 25°C, it was confirmed that the pathogenicity of the strains to goldfish was relatively strong at 25°C (Table 19, 20). According to Chinabut et al. (1995) an average water temperature of Southeast Asian countries is 26°C when the EUS outbreak occurs. The results of the present experiment, is therefore, corresponded to that of the temperature. This temperature may be enhanced the bacteria to increase themselves inside the hosts, influence to release various enzymes which in fact causes infection or death of the fish. So, the temperature may be one of the factors of high virulence of *Aeromonas* species. However, it needs further studies to clarify the temperature to produce different enzymes and virulent factors from the strains used in this study and their

roles to induce infections on fish.

The pathogenicities of the strains used in this study were not so strong in goldfish. But the respective re-isolates were consistently identified from either lesions or kidney of the tested fish (Fig. 5). Further studies are therefore, needed to know the pathogenicity and the pathogenic factors of the strains to EUS susceptible fish.

VI. General discussion and conclusion

"Epizootic Ulcerative Syndrome" is the name that was adopted by the Consultation of Experts on Ulcerative Fish Diseases (FAO, 1986) to describe an extremely damaging fish disorder that has swept through parts of Asia with varying intensity for over two decades. It is, as the name indicates, a condition characterized by large cutaneous ulcerative lesions that periodically results in the death of many species of wild and cultured freshwater fish. The occurrence of EUS in the region caused alarm among the fish farmers, consumers and the Governments. Most countries had never experienced a fish disease epizootic on such a large scale. Fish price tumbled and the consumers afraid to consume the affected fish owing to large and dirty looking ulceration in the body and head region. Losses due to mortalities and consumer rejection amounted to millions of US dollars (Tonguthai, 1985; Anonymous, 1986; Liobrera and Gacutan, 1987; Costa and Wijeyaratne, 1989).

Despite the inherent problems of investigating such a widespread and complex disease, advances are being made in ascertaining aetiological and epidemiological characteristics of EUS. Extensive studies are going on motile *Aeromonas* species and *Aphanomyces* fungus. *Aphanomyces invaderis* a new species of fungus has been identified by Willoughby et al. (1995) and 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). So, the question arises which factor initiate the disease in fact?

It has been also suggested that *Aeromonas* spp. contribute to the pathogenesis of the disease (Costa and Wejeyaratne, 1989). 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). Certain phenons of *A. hydrophila* could induce EUS-like lesions reported by Lio-Po et al. (1990). However, the precise role of motile *Aeromonas* species to cause EUS is still unknown as well as its taxonomy is ambiguous.

The taxonomy of motile *Aeromonas* species is confused with reports of phenotypic, antigenic and genotypic differences among the strains within a single species. Indications are such that there are several species those are genotypically separated but could not be phenotypically differentiated. Such indications can not reflect the true genospecies composing the genus *Aeromonas*. It prevented to understand the real role of certain species in EUS affected fish. A systematic investigation of the supposedly causative agent is, hence, imperative to adequately evaluate the role of motile *Aeromonas* species in EUS.

Using a set of phenotypic traits to differentiate 44-motile aeromonads from EUS affected fishes of Malaysia, Thailand and Bangladesh 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. It was designated *Aeromonas* sp. No isolated strain had been found which was 100% identical to any of the reference strains by phenotypic traits (Table 5, 6). 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 with in the same species. The emergence of *A. hydrophila* related species also reflects the phenotypic heterogeneity of the motile aeromonads.

The taxonomy of motile aeromonads has gone through many changes since the seventh edition of Bergey's Manual, which described in the review of literature section. Compared to the non-motile *A. salmonicida*, whose taxonomy has remained stable since its discovery, this is not true for the motile aeromonads in general. Because of this, the phenotypic properties of *A. hydrophila*, *A. veronii* biotype *sobria* and *A. jandaei* reported in the present experiment were not unique to their reference strain as well as among the individual strain in their own phenotypic groups.

In view of the above, it is essential to develop reliable techniques to identify motile aeromonads isolated from EUS affected fishes. The taxonomy of *Aeromonas* was markedly developed by the introduction of DNA-based techniques. DNA is the core of bacteria, which carries genetic information from one generation to the next. Genomic relatedness is therefore, tend to be reliable and stable, which may informs the ancestral relationships of the bacterial taxonomy. It was De Ley et al. (1970) who developed DNA hybridization technique, which provided the possibility of measuring the genetic relationship between bacterial strains. This technique is presently regarded as the official method to define a bacterial species (Joseph and Carnahan, 1994). According to this approach a genospecies is composed of strains whose DNAs are at least 70% relatedness at 60°C with a (ΔT_m of 5°C or less (Wayne et al. 1987).

To know the reliability of the phenotypic identification DNA-DNA hybridization was performed among the strains isolated from EUS affected fishes and all the recognized type strains of *Aeromonas* species. According to the criteria stated above, 19 strains were genotypically identical to *A. hydrophila*, 17 were *A. veronii* biotype *sobria* and 7 were *A. jandaei* (Table 9, 10). One strain from Thailand (T8) did not give any significant homology values with any of the reference strains of recognized *Aeromonas* species and was therefore identified it as a new species under the genus *Aeromonas*.

Of 26 phenotypic *A. hydrophila* strains 19 belonged to *A. hydrophila* genospecies (HG1) and five belonged to *A. veronii* biotype *sobria* (HG8Y), while 2 belonged to *A. jandaei* genospecies (HG9) (Table 11). Seven strains from phenotypic *A. hydrophila*, were therefore, belonged to different genospecies. It appears from our study presented herein that there are not sufficient phenotypic properties, which can identify *A. hydrophila* with confidence. This finding is correlated with Kuijper et al. (1989). They conducted an experiment on clinical *Aeromonas* spp. and found variations in identification of *A. hydrophila* phenospecies and genospecies. Of their 26 *A. hydrophila* phenospecies only 15 were genotypically identical to *A. hydrophila*

genospecies and the rests were in different genospecis of *Aeromonas*.

A good correlation was found for phenotypically identified *A. veronii* biotype *sobria* and *A. jandaei*. All the phenotypic *A. veronii* biotype *sobria* and *A. jandaei* belonged to their own genospecies, respectively (Table 11).

T8 strain from Thailand did not give significant homology value with any of the reference strains used in this study. The highest homology value was found 59% with *A. caviae* (Table 10). The phenotypic properties of this strain were also too variable from any of the reference strains and thus it was designated *Aeromonas* sp. This strain, is therefore, appeared to be a new species belongs to the genus *Aeromonas*.

There was no single phenotypic test, which could identify the three identified *Aeromonas* genospecies with confidence. Only four phenotypic properties were found to be able to partially differentiate these three species (Table 12). *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. These results correlate with the results of Abbott et al. (1992) and Altwegg et al. (1990).

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; Tonguthai, 1985). All of these studies were performed based on conventional phenotypic method, which can not identify *Aeromonas* species correctly as described previously. In fact, *A. sobria* phenospecies contains *A. veronii* biotype *sobria*, *A. jandaei* and four more genospecies. *A. hydrophila* have been found the dominating group followed by *A. jandaei* and *A. veronii* biotype *sobria* among the 44 strains investigated in this study. Thus, the results of the present experiment are in agreement with the above-mentioned reports on *Aeromonas* species associated with EUS-affected fish.

As a result of genotypic identification, Malaysian strains represented all the three genospecies dominated by *A. hydrophila* and *A. veronii* biotype *sobria* (Table 13). Among the 15 Thai strains, 10 belonged to *A. hydrophila*, four belonged to *A. veronii* biotype *sobria* and one strain was designated *Aeromonas* sp. Six strains of Bangladesh were identified as *A. veronii* biotype *sobria* and five were *A. jandaei*. None of the strains from Thai were identified as *A. jandaei*. There was no *A. hydrophila* among the Bangladeshi strains. This may be because an insufficient number of strains were tested or absence of *A. jandaei* or *A. hydrophila* in the particular sampling environment.

DNA-DNA relatedness is best suited for identification of closely related species or strains of bacteria within a single species no doubt. Presently, 16S rDNA gene sequencing is also a powerful tool for deducing phylogenetic and evolutionary relationships among eubacteria, archaebacteria and eucaryotic organisms because of their high information content, conservative nature and universal distribution (Lane et al. 1985; Woese, 1987). The 16S rDNA sequence analysis is a standard method for the investigation of their phylogenetic relationships (Yamamoto and Harayama, 1995). Even though some closely related species may have only a few differences in their 16S rDNA gene sequences, a phylogenetic tree can be established to give them

an exact taxonomic position (Collins et al., 1991).

Study was therefore undertaken to sequenced 16S rDNA gene of some representative strains of identified *Aeromonas* species to know the intrageneric relationships of three identified species, especially new identified *Aeromonas* species (T8 strain) and compare the sequence similarities with results of DNA-DNA hybridization. It may also helps to know the phylogenetic position of the strain T8, which seemed to be a new species of the genus *Aeromonas*.

All the three identified genospecies were almost identical exhibited 99.9% sequence similarity with only one base difference with the sequence of each reference strain of their species (Table 16). T8 strain was closely related to *A. caviae*, *A. enteropelogenes* and *A. trota* exhibiting 99.8 to 99.9% sequence similarities with two to three bases difference (Table 16 and Fig. 2). All the three genospecies of *Aeromonas* is therefore formed clusters with their own reference strains. Strain T8 clustered with *A. caviae*, *A. trota*, *A. enteropelogenes* and unknown *Aeromonas* species with relatively more closely related with *A. caviae* (Fig 2).Thus the results of 16S rDNA sequences completely correlated with the DNA-DNA relatedness study.

Nucleotide differences among *Aeromonas* species often occurred in the same regions of the molecule known as hypervariable regions. The major hypervariable regions were at positions 110 to 260, 435 to 470, 980 to 1010 and 1265 to 1335 (Fig. 3). These regions may help to develop oligonucleotide probes to identify three identified *Aeromonas* genospecies from EUS affected fishes.

Even though 16S rDNA sequence analysis of the present experiment were well agreed with that of DNA-DNA hybridization study, but it is very difficult to define species based on the similarities of sequences only. Because, *A. hydrophila* were 99.7% similar with only four bases difference with *A. media* as it was found in present investigation (Table 16). On the other hand, *A. veronii* biotype *sobria* were 99.4% similar sequence with nine bases difference with *A. jandaei* and vice versa (Table 16). Thus it may be used as supplement not to alternate of DNA-DNA hybridization study. Developing primers from the data of 16S rDNA sequences presented herein will help to identify the three *Aeromonas* species associated with EUS of fish more easily and more quickly in future.

Aeromonas spp. are recognized as pathogenic for poikilothermal animals, such as frogs, snakes or fish and in homeothermal animals and humans (Russell, 1898; Altwegg and Geiss, 1989; Janda, 1991; Popoff 1984). These species, are however, considered to be opportunistic pathogens, which seemed a contradiction in terms. In the intensive or semi-intensive fish farming systems fish are easily stressed by mishandling, overcrowd, lack of nutrition or poor water quality. These conditions easily enhanced to induce disease on aquatic animals by *Aeromonas* species. Recently, *A. hydrophila* and *A. sobria* have frequently been isolated from EUS lesions (Anonymous, 1986; Llobrera and Gacutan, 1987; Torres et al., 1990; Subasinghe et al., 1990; Lio-Po et al., 1990) in Southeast Asian countries. It has been suggested that motile *Aeromonas* contribute to the pathogenesis of the disease (Costa and Wejeyaratne, 1989). Three motile *Aeromonas* species have been identified by phenotypic and genotypic method from EUS affected fishes in the present experiment. So, the pathogenicity of the identified aeromonads has been attempted to experimentally induced goldfish (*C. auratus*).

It is evident from the results that all the three genospecies of *Aeromonas* have the potentiality to cause EUS like lesions on the experimentally infected goldfish at 20 to 25°C (Table 19, 20). The median lethal doses varied depend on the strains used. The doses varied from $2\times10^{6.8}$ to 6×10^{8} CFU/fish at 20°C and $2.6\times10^{4.5}$ 1.2×10^{8} CFU/fish at 25°C. *A. veronii* biotype *sobria* was found relatively high pathogenic followed by unidentified *Aeromonas* strain (T8 strain), *A. hydrophila* and *A. jandaei*.

The LD_{50} values of the strains were relatively high. The virulence of the strains possibly lost during primary culture or long storage in an artificial condition. Besides, naturally susceptible fishes were not available near to the research areas. Due to this reason, goldfish (*C. auratus*) has been selected as experimental induced fish, which is not naturally susceptible species in fact. So, this might be an another reason for relatively high LD_{50} values. Virulence study should be conducted to naturally susceptible fish species to know the role of identified aeromonads for inducing EUS lesions.

Even though there was no significant mortality by bath challenge but high infection rates both at 20°C and 25°C were recorded (Table 20). In almost all cases respective reisolates were identified either from lesions or kidney of dead, moribund or sacrificed fish. Existence of respective strains constantly in the lesions and kidney of dead, moribund or sacrificed fish indicated that they have the potentiality to cause EUS in goldfish. It is suspected that these bacteria may be penetrated into the kidney of tested fish through the artificial injury, which have the abilities to multiply and cause disease or mortality when the environmental conditions become worse and stressed.

The fish culture systems in Southeast Asian countries are now semi-intensive to intensive, where stocking densities are too high. It is very natural to damage the body barrier of the fish from their competition of feed, habitats or any other biological activities. Besides, in the open water system, water pollution from environment is very common in the Southeast Asian countries. The fish easily stressed when it is exposed to such environmental conditions which eventually succumbs to *Aeromonas*, the most common bacterial flora in the aquatic environments, which produce a wide range of virulence factors such as pili, hemolysins, protease, serum resistance and siderophore etc.

From the results of the present experiment, it is very evident that at least 4 motile *Aeromonas* genospecies, *A. hydrophila*, *A. veronii* biotype *sobria*, *A. jandaei* and *Aeromonas* sp. are associated with EUS. T8 strain from Thailand is recognized as a new species of *Aeromonas* genus. All the four identified *Aeromonas* genospecies have the potentiality to cause EUS lesions to experimentally induced goldfish (*C. auratus*). And it is not possible to identify *Aeromonas* species correctly to the genospecies level based on either phenotypic properties or 16 S rDNA sequence study only. They may be used as supplement not to alternate of DNA-DNA hybridization study.

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