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Oanh, Dang Thihoang; Pedersen, Karl; Larsen, Jens Laurits

Published in:
Diseases in Asian Aquaculture IV

Publication date:
2002

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Oanh, D. T., Pedersen, K., & Larsen, J. L. (2002). Identification and characterization of Vibrio bacteria isolated from fish and shellfish in Vietnam. In *Diseases in Asian Aquaculture IV* (pp. 73-85). Asian Fisheries Society.

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Identification and characterization of *Vibrio* bacteria isolated from fish and shellfish in Vietnam

DANG THI HOANG OANH
Laboratory of Fish Pathology, Institute for Marine Aquaculture, College of Agriculture, Cantho University, 3-2 Street, Cantho City, Vietnam

KARL PEDERSEN and JENS LAURITS LARSEN
Laboratory of Fish Diseases, Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Bülowsvej 13, DK-1870 Frederiksberg C, Denmark

Abstract

A taxonomic relationship of thirty one Vietnamese bacterial isolates from different fish and shellfish together with twenty two reference strains was investigated by Euclidean distance with unweighted average linkage clustering. Comparison based on fourty seven phenotypic characters showed that these isolates mainly clustered in six groups of which four were equated with the well known *Vibrio* species, *V. alginolyticus*, *V. harveyi*, *V. cholerae* and *V. mimicus*. For the remaining two groups comprising twelve isolates, it was more difficult to establish any relationship with known species when compared with reference strains. Strains were also subjected to rRNA gene restriction pattern analysis (ribotyping), using *Mlu* I as restriction enzyme. Eleven ribotypes were detected among the Vietnamese isolates. Similarity of ribotyping patterns between strains supported the phenotypic identification. Twenty-eight strains were found to carry at least one plasmid and 12 different sized plasmids were detected ranging from less than 2 to 140 kb. The two *Vibrio* spp. groups performed high phenotypic and genotypic similarity within each group but were only remotely related with the reference strains. The evidence suggest that these strains may represent new *Vibrio* species.

Introduction

Vibriosis has been reported world wide and is a very important bacterial disease of marine fish and shellfish (Austin and Austin, 1993). *Vibrio anguillarum* and *V. ordalii* have been considered as the major causes of vibriosis, but other distinct species have also been isolated from disease outbreaks. *Vibrio vulnificus* was isolated from diseased eel (*Anguilla anguilla*) (Biosca *et al.*, 1991). *Vibrio alginolyticus* was reported as a pathogen of sea bream (*Sparus aurata*) and grouper (*Epinephelus malabaricus*) (Colorni *et al.*, 1981; Lee, 1995). Kiiyukia *et al.* (1992) isolated *V. cholerae* non-O1 from ayu

Oanh D.T.H., K. Pedersen and J.L. Larsen. 2002. Identification and characterization of *Vibrio* bacteria isolated from fish and shellfish in Vietnam. *In* Diseases in Asian Aquaculture IV. C.R. Lavilla-Pitogo & E.R. Cruz-Lacierda (eds.). Fish Health Section, Asian Fisheries Society, Manila.

(*Plecoglossus altivelis*). *Vibrio salmonicida* was involved in diseases of salmonid fish (Austin and Austin, 1993). *Vibrio harveyi* was reported as the causative bacterium of vibriosis in pearl oyster (*Pinctada maxima*), black tiger prawn (*Penaeus monodon*), kuruma prawn (*P. japonicus*) (Pass *et al.*, 1987; Lavilla-Pitogo *et al.*, 1990; Karunasagar *et al.*, 1994; Liu *et al.*, 1996; Leano *et al.*, 1998) while *V. damsela* (now *Photobacterium damselae*) was associated with diseased turbot (*Scophthalmus maximus*), rainbow trout (*Oncorhynchus mykiss*) and shark (*Orectolobus ornatus*) (Fouz *et al.*, 1992; Pedersen *et al.*, 1997).

Biochemical characterization has been the most commonly used method for identification of the isolates to species level (Bryant *et al.*, 1986 a, b; West *et al.*, 1986; Alsina *et al.*, 1994 a, b). Recently, genotyping methods have become widely used for bacterial typing. Ribotyping, which combines restriction endonuclease analysis with hybridisation using an rRNA probe, has been successfully applied for typing of bacteria and proved to be useful for epidemiological purposes and to support identification of isolates (Austin *et al.*, 1995, 1997; Dalsgaard *et al.*, 1995; Tiainen *et al.*, 1995). Plasmid analysis and restriction fragment length polymorphism of the plasmids have been used to characterize extra chromosomal DNA (Toranzo *et al.*, 1983; Olsen and Larsen, 1990; Pedersen and Larsen, 1995; Pedersen *et al.*, 1996; Austin *et al.*, 1997). However, these methods are limited to those strains carrying plasmid.

In Vietnam, serious disease outbreaks have recently occurred in many areas of intensive aquaculture causing a great loss of fish production (Dung *et al.*, 1997) and vibriosis was reported in various species of shrimp (Hao *et al.*, 1997). The basis of bacterial identification was mainly relied on conventional biochemical characters. Study on genetic characters of bacterial isolates from fish in Vietnam has not been reported yet. Thus, this study aimed to identify and characterise bacterial strains isolated from fish and shellfish in Vietnam to obtain information on fish vibriosis with regard to biochemical and physiological characteristics, ribotyping and plasmids.

Materials and Methods

Bacterial strains

Thirty one bacterial strains were isolated from disease outbreaks in 1996 and 1997 using Thiosulphate Citrate Bile-salt Sucrose (TCBS) agar. The strains were tested and presumptively equated with the genus *Vibrio*, and were studied together with twenty-two reference strains (Table 1). They were stored at - 80 °C in veal infusion broth (Difco) containing 15% glycerol and supplemented with 1% (w/v) sodium chloride.

Morphological and phenotypic characterization

Colony morphology and hemolysis were recorded after incubation for 2 days at 20°C on blood agar plates (Marine agar (Difco) with 5% calf blood). Cell morphology was studied by Gram-stained preparations from the same agar plates according to Hucker's modification method (Barrow and Feltham, 1993). Motility in broth (veal infusion broth

Table 1. Source, ribotyping pattern and plasmid of bacterial isolates (No. 1-31) and reference strains (No. 32-53) used in the study

Isolate no.	Collection strain no.	Species identification	Source	Ribotyping patterns (a)	Plasmid content (kb)
1	98-05-59	*V. cholerae*	Larvae Hybrid catfish	6	30
2	98-05-70	*V. cholerae*	*Macrobrachium rosenbergii*/Larvae	7	109
3	98-05-72	*V. cholerae*	*M. rosenbergii*/Larvae	7	130, 16
4	98-05-74	*Vibrio sp.* (1)	*Osphronemus goramy*/Kidney	10	130
5	98-05-75	*V. harveyi*	*Pangasius bocourti*/Spleen	2	47
6	98-05-76	*V. alginolyticus*	*Penaeus monodon*/Postlarvae	3	76
7	98-05-77	*V. mimicus*	*P. bocourti*/Kidney	9	No plasmid
8	98-05-79	*Vibrio sp.* (1)	*M. rosenbergii*/Larvae	10	No plasmid
9	98-05-80	*V. harveyi*	*Penaeus monodon*/Postlarvae	1	47
10	98-05-81	*Vibrio sp.* (1)	*Penaeus monodon*/Postlarvae	11	98, 56
11	98-05-84	*V. harveyi*	*Penaeus monodon*/Postlarvae	2	47
12	98-05-87	*V. harveyi*	*Penaeus monodon*/Postlarvae	2	47
13	98-05-88	*V. harveyi*	*P. bocourti*/Kidney	2	47
14	98-05-89	*V. cholerae*	*M. rosenbergii*/Larvae	8	No plasmid
15	98-05-91	*V. harveyi*	*P. bocourti*/Spleen	2	47
16	98-05-96	*Vibrio sp.* (1)	*P. bocourti*/Liver	11	98, 70, 56
17	98-08-153	*Vibrio sp.* (2)	*Epinephelus spp.*/ Kidney	5	140
18	98-08-154	*Vibrio sp.* (2)	*Epinephelus spp.*/ Kidney	5	140, 17
19	98-08-156	*V. alginolyticus*	*Epinephelus spp.*/ Kidney	4	< 2
20	98-08-157	*V. alginolyticus*	*Epinephelus spp.*/ Kidney	4	< 2
21	98-08-158	*Vibrio sp.* (2)	*Epinephelus spp.*/ Kidney	5	140
22	98-08-159	*Vibrio sp.* (2)	*Epinephelus spp.*/ Kidney	5	140, 17
23	98-08-160	*Vibrio sp.* (2)	*Epinephelus spp.*/ Kidney	5	140, 17, < 6.9
24	98-08-161	*Vibrio sp.* (2)	*Epinephelus spp.*/ Kidney	5	140, < 6.9
25	98-08-162	*V. alginolyticus*	*Epinephelus spp.*/ Kidney	4	< 2
26	98-08-163	*V. alginolyticus*	*Epinephelus spp.*/ Kidney	4	< 2
27	98-08-164	*Vibrio sp.* (2)	*Epinephelus spp.*/ Kidney	5	140, 17
28	98-08-165	*V. alginolyticus*	*Epinephelus spp.*/ Kidney	4	< 2
29	98-08-166	*V. alginolyticus*	*Epinephelus spp.*/ Kidney	4	< 2
30	98-08-167	*Vibrio sp.* (2)	*Epinephelus spp.*/ Kidney	5	17
31	98-08-168	*V. alginolyticus*	*Epinephelus spp.*/ Kidney	4	< 2
32	*V. anguillarum* ATCC 19264^T	-	*Gadus morhua*	-	-
33	*V. fluvialis* NCIMB 2249^T	-	Human faeces	-	-
34	*V. furnissii* ATCC 35016^T	-	Human faeces	-	-
35	*V. natriegens* ATCC 33898^T	-	-	-	-
36	*V. pelagius* biovar I NCIMB 1900^T	-	Water	-	-
37	*V. pelagius* biovar II NCIMB 2253^T	-	-	-	-
38	*V. alginolyticus* ATCC 33838^T	-	-	-	-
39	*V. alginolyticus* AAHRI 94/93	-	*P. monodon*	-	-
40	*V. carchariae* ATCC 43516^T	-	-	-	-
41	*V. cholerae* 889, O1, Ogawa	-	-	-	-
42	*V. harveyi* ATCC 33866^T	-	-	-	-
43	*V. harveyi* AAHRI 94/A	-	*P. monodon*	-	-
44	*V. mimicus* ATCC 33653^T	-	Human ear	-	-
45	*V. tubiahii* NCIMB 1340^T	-	Larvae of hard clam	-	-
46	*V. splendidus* biovar I NCIMB 1^T	-	Fish	-	-
47	*V. splendidus* biovar II NCIMB 2251^T	-	Water	-	-
48	*V. ichthyoenteri* HWU P 8603	-	-	-	-
49	*V. hollisae* NCTC 11640^T	-	-	-	-
50	*V. penaeicida* KH-1^T	-	Shrimp	-	-
51	*V. ordalii* ATCC 33509^T	-	*Oncorhynchus kisutch*	-	-
52	*V. nigripulchritudo* LMG 3896^T	-	Water	-	-
53	*V. navarrensis* CIP 103193^T	-	-	-	-

ATCC: American Type Culture Collection; T: Type strain; NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, UK; AAHRI: Aquatic Animal Health Research Institute, Bangkok, Thailand; HWU: Heriot-Watt University, Edinburgh, UK; NTCC: National Type Culture Collection, Colindale, UK; LMG: Laboratorium voor Microbiologie, Rijksuniversiteit Gent, Belgium; CIP: Collection of the Pasteur Institute, Paris, France; (a) Ribotype patterns were given number from 1-11

(Difco) supplemented with 1% (w/v) NaCl) was studied using a drop of overnight culture on a slide and observed under light microscope.

Selection of biochemical and physiological characters followed the diagnostic scheme for *Vibrio* species associated with fish diseases described by Larsen and Pedersen (1999). Examinations of characters were performed according to the principles of Cowan and Steel's Manual (Barrow and Feltham, 1993) and methods of West and Colwell (1984). The phenotypic characters were coded in a binary format by scoring positive and negative character as 1 and 0 respectively. The data were examined using Euclidean distance with unweighted average linkage (UPGMA) clustering (Priest and Austin, 1993) using NCSS 97 computer program.

Ribotyping

Bacterial strains were propagated overnight in 10 ml of veal infusion broth containing 1% NaCl under gentle shaking. Total bacterial DNA was extracted from each isolate following the method described by Pedersen and Larsen (1993). DNA was digested by *Mlu* I restriction enzyme (Boehringer Mannheim). Restriction fragments were separated by electrophoresis in 0.8% agarose gel, using TAE buffer, pH 8.0 (40 mM Tris, 40 mM sodium acetate, 1 mM EDTA) for 18 hours at 25 volts. The DNA was vacuum-blotted and fixed (80°C for 30 minutes) onto a nylon membrane. Then hybridization with the digoxigenin-labelled probe complementary to 16S and 23S rRNA (Boehringer Mannheim) was performed overnight at 56°C. The membrane was then blocked and hybridised DNA fragments were labelled with alkaline phosphatase labelled anti-digoxigenin. Finally, the bands were detected by using colour substrate solution (90 μl NBT and 66 μl BCIP in 20 ml of staining buffer). Each isolate was visually coded for the presence (code 1) and absence (code 0) of each DNA fragment. The data were examined in the same way as the phenotypic data using Statistica version 5.0 program.

Plasmid profile

Propagation of bacterial strains was carried out following the same culture method as for bacterial DNA extraction. Plasmid DNA was extracted by the method of Kado and Liu (1981), separated by electrophoresis in 0.8% agarose gels in TAE buffer for 4 hours at 80 volts, stained with ethidium bromide and photographed under UV light. Plasmids from *Escherichia coli* 39R 861 and V 517 were used as size markers. Strains were tested 3 times. The size of plasmids from tested strains were determined as described by Tiainen *et al.* (1995).

Results

Phenotypic characteristics of Vietnamese isolates and reference strains are given in Table 2. A dendrogram showing phenotypic relationship between strains is illustrated in Figure 1. Euclidean distance was transformed into percentage of similarity. Thirty one Vietnamese isolates formed six groups, of which four groups were identified as *V.*

alginolyticus, *V. harveyi*, *V. cholerae* and *V. mimicus*, respectively. The other groups comprising twelve isolates could not be assigned to any species by comparing with the reference strains and were subsequently designated *Vibrio* spp. group 1 & 2.

Eleven ribotypes were detected within the Vietnamese isolates and were given numbers from 1 to11 (Table 1 and Fig. 2). Genetic relationship based on ribotyping data is presented in Figure 3 and described in further detail below. Twenty eight strains were found to carry at least one plasmid (Table 1) and 12 different sized plasmids were detected ranging from less than 2 to 140 kb.

On the basis of the combined phenotypic and genotypic data, the strains were assigned to species as indicated in Table 1.

Vibrio harveyi

Six Vietnamese isolates clustered together with the *V. harveyi* and *V. carchariae* type strains (Fig. 1). The strains did not develop swarming growth but they revealed hemolysis. They formed large sized (3-3.5 mm) colonies after two days incubation at 20 C on blood agar. The colonies were circular, shiny, smooth, adherent and greyish. Strains were positive in lysine and ornithine decarboxylase but negative for arginine dihydrolase. They produced indole but gave a negative VP reaction. They all produced acid from cellobiose, galactose, mannitol and trehalose but not from lactose or xylose.

Two ribotype patterns (ribotype 1 and 2) were found in this group and both clustered together with *V. carchariae* and *V. harveyi* type strains (Fig. 3). All strains carried a 47 kb plasmid.

Vibrio alginolyticus

Eight isolates clustered together with the *V. alginolyticus* type strain (Fig. 1). These isolates developed swarming growth on blood agar plate after 1 day of incubation at 37°C. The strains degraded blood but did not produce gas from glucose. They grew in 10% NaCl and at 37°C but not at 5°C. They produced acid from glucose, glycerol, mannitol, sucrose and trehalose but not from arabinose, lactose or xylose. All eight strains produced indole and were positive for lysine decarboxylase and VP, but tested negative for luminescence, urease, alginase and arginine dihydrolase.

Two ribotype patterns (ribotype 3 and 4) were detected within this group. All strains carried one plasmid. However, the 76 kb plasmid was detected only in one isolate (strain 98-05-76) and the rest carried a very small plasmid (smaller than 2 kb).

Vibrio cholerae

Four isolates clustered together with the *V. cholerae* reference strain (Fig. 1). These strains formed small sized hemolytic colonies (1-1.5 mm) after 2 days of incubation at 20°C on blood agar. The colonies were circular, entire, low convex and greyish. The surface of

Table 2. Phenotypic characters of studied strains and reference strains

	·*V. spp* 1 VN (n = 4)	*V. spp* 2 VN (n = 8)	*V. alg.* VN (n = 8)	*V. cho.* VN (n = 4)	*V. har.* VN (n = 6)	*V. mim.* VN 33838 (n = 1)	*V. alg.* ATCC 94/93	*V. alg.* AAHRI Ogawa	*V. cho.* 889, O1, 33866	*V. har.* ATCC 94/A	*V. har.* AAHRI 43516	*V. car.* ATCC 33653	*V. mim.* ATCC
Homolysis	+	+	+	+	+	+	+	+	+	+	+	+	+
Swarming	-	-	+	-	-	-	+	+	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Pellicle	-	-	-	-	-	-	-	-	-	-	-	-	-
Pigment	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine	-	+	-	-	-	-	-	-	-	-	-	-	-
Lysine-	-	+	+	+	+	+	+	+	-	+	+	+	
Ornithine	-	-	1	+	+	+	+	+	+	-	+	+	+
Growth in													
0% NaCl	-	-	-	+	1	+	-	-	+	-	-	-	+
3%	+	+	+	+	+	+	+	+	+	+	+	+	+
6%	+	+	+	+	+	+	+	+	+	+	+	+	+
7%	+	+	+	+	+	+	+	+	+	+	+	+	+
8%	+	-	+	-	4	-	+	+	-	+	-	+	-
10%	-	-	+	-	-	-	+	+	-	-	-	-	-
O test +	+	+	+	+	+	+	+	+	+	+	+	+	
F test +	+	+	+	+	+	+	+	+	+	+	+	+	
Urease	-	-	-	-	4	-	-	-	-	-	-	+	-
TCBS +	+	+	+	+	+	+	+	+	+	+	+	+	
0/129 150 µg	+	+	+	+	3	+	+	+	+	+	+	+	+
Luminescence	-	-	-	-	3	-	-	-	-	-	-	-	-
Citrate utilization	+	+	+	+	-	-	+	-	+	-	-	+	+
Aesculin	+	+	-	-	5	+	-	-	-	+	+	-	-
Indole production	,+	+	+	+	+	+	+	+	+	+	+	+	+
VP -	-	+	+	-	-	+	+	+	-	-	-	-	
Growth at 5 °C	-	-	-	-	-	-	-	-	-	-	-	-	-
20 °C +	+	+	+	+	+	+	+	+	+	+	+	+	
30 °C +	+	+	+	+	+	+	+	+	+	+	+	+	
37 °C +	+	+	+	4	+	+	+	+	-	-	-	+	
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+
Alginase	-	-	-	-	1	-	-	-	-	-	-	+	-
Amylase	+	+	3	+	2	-	+	-	+	-	-	+	-
Gelatinase	+	+	7	+	+	+	+	+	+	-	+	+	+
Tween 80	+	-	7	+	5	+	+	+	+	-	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas from glucose	+	-	-	-	4	-	-	-	-	-	+	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-	+	+	-
Cellobiose	+	+	1	1	+	-	-	-	-	+	+	+	+
Galactose	+	+	1	+	+	+	-	+	+	+	+	+	+
Glycerol	+	-	+	-	5	-	+	+	+	+	+	+	+
Lactose	-	-	-	1	-	-	-	-	+	-	-	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin +	+	3	-	+	-	-	+	-	-	+	+	-	
Sucrose	+	+	+	+	1	-	+	+	+	+	+	+	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	+	-	-	-	-	-	-	-	-	-	-	-	-

+', All strains positive; '-', all strains negative; numerical values indicate the number of positive strains. VN; Vietnamese strain

Fig. 1. Dendrogram based on the Euclidean distance of phenotypic data

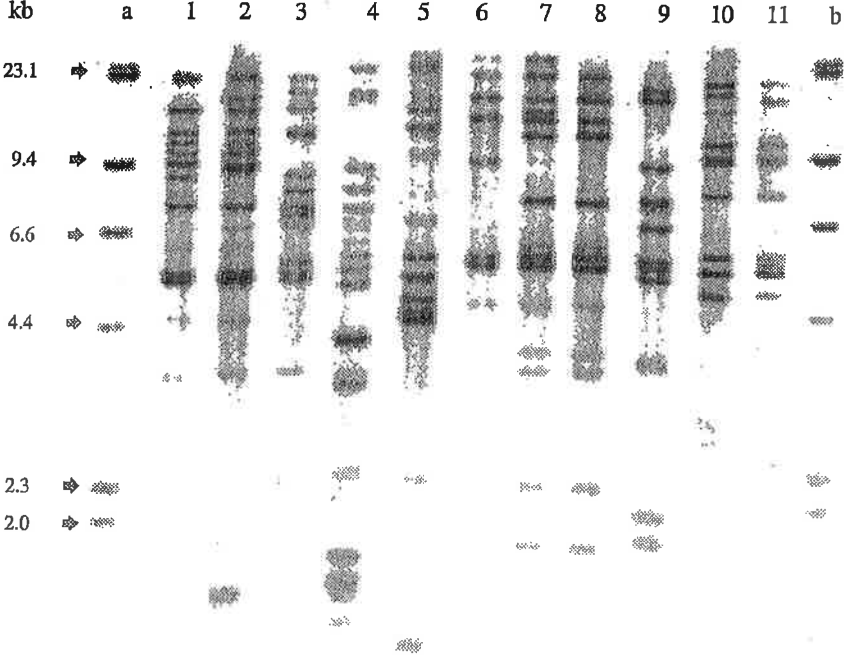

Fig. 2. Ribotyping patterns of studied isolates (lane 1-11: 98-05-80, 98-05-75, 98-05-76, 98-08-162, 98-08-153, 98-05-59, 98-05-70, 98-05-89, 98-05-77, 98-05-74 and 98-05-81). Lane a & b, *Hind* III-digested l DNA as molecular mass markers.

the colonies was smooth and shiny. All four isolates were positive in lysine and ornithine decarboxylase but negative in arginine dihydrolase. They grew in 1% peptone broth containing 0, 3, 6, and 7 % NaCl, but not 8 and 10%. They grew at 37°C but not at 5°C. All strains produced indole and acid from galactose, glucose, mannitol and trehalose but not from arabinose, salicin or xylose. Gas was not produced from glucose. They degraded gelatine and lipid but gave a negative result for alginase, luminescence, and urease. Variable reactions were observed in the fermentation of cellobiose, glycerol and lactose.

Three ribotype patterns (ribotype 6, 7 and 8) were detected within this group. They clustered together with the *V. cholerae* reference strain. Three strains carried at least one plasmid. The size of plasmids were 30 kb (strain 98-05-59); 109 kb (strain 98-05-70); 15 and 130 kb (strain 98-05-72).

Vibrio mimicus

There was only one Vietnamese isolate that clustered together with the *V. mimicus* type strain (Fig. 1). The size of the colonies was 3 mm after 2 days of incubation at 20°C and with hemolysis underneath the colony. The colonies were circular, entire, low convex, and whitish. The surface of the colonies was smooth and shiny. Biochemical and

Fig. 3. Dendrogram based on the Euclidean distance of ribotying data

physiological differences between type strain and tested strain were found in fermentation of esculin, cellobiose, glycerol and lactose. Ribotype 9 (strain 98-05-77) clustered with the *V. mimicus* type strain (Fig. 3). No plasmid was detected in this strain.

Vibrio spp. group 1

Four strains (98-05-74, 98-05-79, 98-05-81 and 98-05-96) were included in this group (Fig. 1). These strains gave negative results for amino acid degradation (arginine, lysine and ornithine). They did not develop swarming growth but showed hemolysis after 2 days of incubation at 20°C on blood agar. The colonies were large (3.5-4 mm), low convex, smooth, shiny, and whitish. All four isolates were positive in citrate utilisation, grew in 0, 3, 6, and 7 % NaCl, but not 8 or 10 %. They grew at 37°C but not at 5°C. All strains produced acid from cellobiose, galactose, glycerol, glucose, mannitol, salicin, sucrose, trehalose and xylose but not from arabinose or lactose. They produced gas from glucose, degraded starch, gelatine and lipid whereas tests were negative for alginase, luminescence and urease.

Two ribotypes (ribotype 10 and 11) were detected within this group which were not found to be similar with any reference strain. Three of four strains carried at least one plasmid. Plasmids of 98 and 56 kb were detected in two strains (98-05-81 and 98-05-96). Besides, strain 98-05-96 carried 70 kb plasmid. A 130 kb plasmid was found in strain 98-05-74 (Table 1).

Vibrio spp. group 2

This group included eight strains (98-08-153, 98-08-154, 98-08-158, 98-08-159, 98-08-160, 98-08-161, 98-08-164 and 98-08-167) (Fig. 1). The isolates showed identical phenotypic and genotypic characters. The colonies were entire, medium sized (2-2.5 mm) after two days of incubation at 20°C on blood agar, hemolytic and non-swarming. The surface of the colonies was smooth, shiny and whitish. They gave a positive result for arginine dihydrolase but negative for lysine and ornithine decarboxylase. They grew in 3, 6, and 7 % NaCl, but not 0 or 10%. They grew at 37°C but not at 5°C. All strains utilised citrate, degraded starch, gelatine and aesculin, produced acid from cellobiose, galactose, glucose, mannitol, salicin, sucrose and trehalose but not from arabinose, glycerol, lactose or xylose. They did not produce gas from glucose and gave negative response for luminescence, urease, lipase and alginase.

The eight strains had identical ribotype patterns (ribotype 5). They clustered distinct from any reference strain. One strain carried three plasmids, four strains carried two plasmids, and three strains carried one plasmid. Plasmids of 140, 17 and smaller than 6.9 kb were detected.

Discussion

All strains displayed the key phenotypical features of bacteria belonging to the genus *Vibrio*. Thus, all were motile, oxydase and catalase positive, Gram-negative rods which degraded D-glucose fermentatively, reduced nitrate to nitrite, grew on a *Vibrio* selective medium (TCBS) and were sensitive to the vibriostatic agent O/129 using 150 g discs

(West *et al.*, 1986). In addition, all strains grew abundantly at 20 and 30°C and also in 3 % NaCl. They all produced acid from mannitol and trehalose and gave positive result for indole production. The biochemical and physiological data were supported by ribotyping results. However, plasmid contents were heterogeneous even within each group.
Vibrio harveyi has been reported as an important disease agent in shrimp hatchery with high mortality (Leano *et al.*, 1998). This was also the case where most strains belonging to this group were isolated. Alsina and Blanch (1994) recorded that *V. harveyi* and *V. carchariae* were different in their utilization of arabinose as sole carbon source. All six strains within this group gave negative response for arabinose which was also the case for the *V. carchariae* type strain. Urease production has been suggested to be an important trait for virulence of *V. carchariae,* but also some *V. harveyi* isolates have been described as urease-positive (Bryant *et al.*, 1986; Pedersen *et al.,* 1998). This supports the identification of four urease-positive strain within this group. Furthermore, the ribotyping profile (ribotype 2) of these strains shared more bands with the *V. harveyi* type strain and they all carried a 47 kb plasmid. The detection of the plasmid is in accordance with the result of Pedersen *et al.* (1998) who found that *V. harveyi* was often carrying plasmids of approximately 40-50 and 70-80 kb in size. Moreover, Pedersen *et al.* (1998) provided taxonomic evidence that *V. carchariae* is the junior synonym of *V. harveyi*, which is also supported by the dendrogram (Fig. 1).

Seven Vietnamese isolates clustered together with the *V. alginolyticus* type strain and performed typical phenotypic characters of *V. alginolyticus*, which are swarming growth on saline solid media, arginine dihydrolase negative, lysine decarboxylase positive, positive result for VP reaction, acid production from glycerol and sucrose, and growth in the presence of 8 and 10 % sodium chloride but not 0 % (Austin and Austin, 1993; Frerichs, 1993; Balebona *et al.*, 1998). However, strain 98-08-162 and 98-08-163 produced acid from salicin and the others gave negative result for degradation of starch which was different from the response of the type strain. Ribotyping patterns of these isolates had many bands in common with the type strain. All carried the same sized plasmid (smaller than 2 kb). This supported the hypothesis that they are *V. alginolyticus.*

Strain 98-05-76 also behaved like *V. alginolyticus,* but it did not utilize citrate and it produced acid from cellobiose, galactose and salicin. This may explain the fact that it clustered rather far distant from the reference strain (Fig. 1). Ribotype of this strain (ribotype 3) had only a few bands in common with *V. alginolyticus* type strain. However, it shared most bands with *V. alginolyticus* reference strain, AAHRI 94/93, which was also remotely related to the type strain. A 76 kb plasmid was only detected from strain 98-05-76. These evidence suggested to designate this strain *V. alginolyticus*-like.

Four Vietnamese isolates clustered together with the *V. cholerae* type strain. They showed slight differences in acid prodution from cellobiose, glycerol, and lactose but otherwise, their phenotypic traits were in agreement with the description of *V. cholerae* (Baumann *et al.*, 1984). They formed a heterogenous group with three ribotypes (ribotype 6, 7 and 8) and four plasmid profiles but their ribotyping patterns shared most bands with the *V. cholerae* type strain.

Strain 98-05-77 gave different responses in aesculin hydrolysis and acid production from cellobiose, glycerol and lactose compared to the *V. mimicus* type strain. However, its ribotype (ribotype 9) shared many bands with the *V. mimicus* type strain.
Vibrio spp. (group 1 & 2) performed idential phenotypic characters but these characters were a mixture between reference strains. The type strains used to compare these isolates were chosen according to their reactions in amino acid decarboxylases. *Vibrio* spp. group 1 clustered most closely to the *V. natriegens* reference strain but with low similarity (Fig. 1). The two groups were separated by their reaction in amino acid decarboxylases. The former gave negative result with arginine dihydrolase, lysine and ornithine decarboxylase while the latter gave positive result with arginine dihydrolase. Group 1 was gas producers and produced acid from xylose whereas these characters were not observed within any of the reference strains. Likewise, group 2 gave positive result for aesculin hydrolysis and produced acid from salicin but did not degrade Tween 80. Ribotyping profiles of each group were clearly distinct from any described ribotyping profiles of *Vibrio* reference strains. Plasmids were found heterogenerous especially within *Vibrio* spp. (group 1). Plasmids of 140 and 17 kb were detected in many strains within *Vibrio* spp. (group 2). Both phenotypes and genotypes of these two groups suggested that they may be new *Vibrio* species. To confirm this, further investigations regarding their phylogenetic relationship with recognised *Vibrio* species, such as rRNA gene sequences and DNA-DNA hybridization, should be carried out.

Acknowledgement

The authors are grateful to the Aquatic Animal Health Research Institute, Bangkok, Thailand, Research Institute for Aquaculture No. 1, Habac, Vietnam, and Laboratory of Fish Diseases, Institute for Mariculture, College of Agriculture, Cantho University, Vietnam, for the provision of strains. Technical assistance of Mrs. Maj-Britt Højgård, Laboratory of Fish Diseases, Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Denmark, is highly appreciated.

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