Genetic Diversity of Luminous *Vibrio* Isolated from Shrimp Larvae

*[1]Antonius Suwanto, [2]Munti Yuhana, [2]Emi Herawaty, [2]Sri Lestari Angka

[1]Department of Biology, Faculty of Science and Mathematics
[2]Department of Aquaculture, Faculty of Fisheries
Bogor Agricultural University, Bogor 16144, Indonesia.

ABSTRACT: A collection of 58 luminous vibrio isolated from shrimp farms in Java island, Indonesia were characterised and grouped by ribotyping and macrorestriction fragment-length polymorphisms (MFLP) or schizotyping employing pulsed field gel electrophoresis (PFGE). Restriction endonuclease *Not*I (5'-GCGGCCGC-3') digestion of luminous vibrio genomes yielded small numbers of DNA fragments with distributions that were readily interpreted for strain comparison. Several schizotypes (19) were distinguished following digestion of total genomic DNA with *Not*I and subsequent separation of DNA fragments using PFGE. The reproducibility of this method was 100%. Biochemical and physiological studies indicated that all of the isolates (except one which belonged to *Vibrio fischeri*) were identified as strains of *Vibrio harveyi*. Schizotype similarity was examined by cluster analysis, and four main groups with 19 different schizotypes were found. There was no correlation between schizotypes and geographical location of the shrimp farms or sample collection. Schizotyping was found to be a simple and reliable method for differentiating luminous vibrios. In addition, this method alone was able to reveal a high degree of genetic diversity within *V. harveyi* isolates from shrimp farms and larvae.

KEY WORDS: luminous, *Vibrio harveyi*, shrimp, ribotyping, schizotyping

INTRODUCTION

Tiger shrimp (*Penaeus monodon*) culture in Indonesia has become more intensive and extensive because of high demand and economic value of this export commodity. In fact, in the last five years, shrimp export has yielded the highest Indonesian income from the agricultural sector. Intensive aquaculture began to expand rapidly in the early 1980's with the acquisition of new technology. More than half of approximately 300,000 ha of shrimp ponds were located in Java island. Overall, Indonesia is currently the second largest producer of cultured shrimp, just behind Thailand (Winarno 1995). However, the exponential growth of shrimp culture is not supported by a sufficient supply of healthy fry, due to many complicated and inter-related problems in this area. Bacterial diseases have been implicated to be one of the most devastating diseases which can completely destroy hatchery productivity for extended periods (Lavilla-Pitogo et al. 1990).

Significant larval mortalities in Asian shrimp hatcheries, including Indonesia, are often associated with luminescent vibriosis which is caused by *Vibrio harveyi* or *V. splendidus* (Sunaryanto & Mariam 1986; Shariff & Subasinghe 1992). Midgut contents of broodstock shed into the water almost simultaneously with the eggs during spawning are suspected to be the main source of luminous vibrios (Shariff and Subasinghe 1992). In addition, the nearshore sea water may also be a major sources of infection (Lavilla-Pitogo et al. 1990).

Control of luminous vibrios by supplementation of antibiotics has become less effective due to the occurrence of bacterial resistance to a number of antibiotics. Tjahjadi et al. (1994) reported that most luminous vibrios isolated from shrimp hatcheries in Kalianget, East Java were resistant to a number of antibiotics tested except to rifampicin (50 mg/ml). Use of excessive antibiotics has also been implicated in shrimp growth retardation, abnormal morphogenesis and rejection of the exported shrimp due to the residuals.

Integrated control of luminous vibrios in tiger shrimp hatcheries requires more information on the their numbers, diversity, distribution, association with shrimp disease, population in nearshore sea water and larva-rearing water, and route of infection. The aims of this study were to investigate genetic diversity and relationships among luminous vibrios isolated from shrimp hatcheries and their nearby seawater environments in Java, Indonesia. DNA profiling employing rare-cutting restriction endonucleases and PFGE, designated as schizotyping (Suwanto and Kaplan 1992) as well as ribotyping was employed to reveal the genotypes of a number of luminous vibrios isolated.

MATERIALS AND METHODS

Bacterial isolates and growth media

A total of 55 luminous *Vibrio* strains were isolated from selected shrimp farms and sea water in coastal areas of

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*e-mail address:asuwanto@indo.net.id

Table 1. Isolates of luminous *Vibrio* used in this study.

No	Code	Source material	Location	Luminescence
1	Gc1B	Shrimp gut	Besuki, E Java	Luminous
2	Gc2B	Shrimp gut	Besuki, E Java	Luminous
3	GC3B	Shrimp gut	Besuki, E Java	Luminous
4	GC4B	Shrimp gut	Besuki, E Java	Luminous
5	GC5B	Shrimp gut	Besuki, E Java	Luminous
6	GC6B	Shrimp gut	Besuki, E Java	Luminous
7	U7.2B	Shrimp HP	Besuki, E Java	Luminous
8	P1.1B	PL1	Besuki, E Java	Luminous
9	P1.2B	PL1	Besuki, E Java	Luminous
10	P1.5B	PL1	Besuki, E Java	Luminous
11	P6.1B	PL6	Besuki, E Java	Luminous
12	P6.2B	PL6	Besuki, E Java	Luminous
13	P6.3B	PL6	Besuki, E Java	Luminous
14	P6.5B	PL6	Besuki, E Java	Luminous
15	W1.1B	Sea water	Besuki, E Java	Luminous
16	W1.3B	Sea water	Besuki, E Java	Luminous
17	W1.5B	Sea water	Besuki, E Java	Luminous
18	W1.6B	Sea water	Besuki, E Java	Luminous
19	W1.7B	Sea water	Besuki, E Java	Luminous
20	W1.8B	Sea water	Besuki, E Java	Luminous
21	W1.1P	Sea water	Puger, E Java	Luminous
22	S14.3B	Sediment	Besuki, E Java	Luminous
23	S16.8B	Sediment	Besuki, E Java	Luminous
24	S9.4P	Sediment	Puger, E Java	Luminous
25	W3.2B	Sea water	Besuki, E Java	Luminous
26	W3.4B	Sea water	Besuki, E Java	Luminous
27	W3.7B	Sea water	Besuki, E Java	Luminous
28	HHT	PL	Besuki, E Java	Luminous
29	Dan 10	PL	Besuki, E Java	Luminous
30	VTM1	PL	Besuki, E Java	Luminous
31	VTM2	PL	Besuki, E Java	Luminous
32	VTMR	PL	Besuki, E Java	Luminous
33	VhBk	PL	Balitkanwar, W Java	Luminous
34	B2H	Shrimp HP	Besuki, E Java	Luminous
35	AU2	Shrimp HP	Besuki, E Java	Luminous
36	BU2	Shrimp HP	Besuki, E Java	Luminous
37	BU3	Shrimp HP	Besuki, E Java	Luminous
38	P1.3L	PL1	Labuhan, W Java	Luminous
39	P2.1L	PL2	Labuhan, W Java	Luminous
40	P2.4L	PL2	Labuhan, W Java	Luminous
41	P5.3L	PL5	Labuhan, W Java	Luminous
42	M3.2L	Mysis	Labuhan, W Java	Luminous
43	M3.3L	Mysis	Labuhan, W Java	Luminous
44	M3.4L	Mysis	Labuhan, W Java	Luminous
45	Mys2	Mysis	Besuki, E Java	Luminous
46	Mys3	Mysis	Besuki, E Java	Luminous
47	Mys4	Mysis	Besuki, E Java	Luminous
48	Mys5	Mysis	Besuki, E Java	Luminous
49	Mys6	Mysis	Besuki, E Java	Luminous
50	Mys9	Mysis	Besuki, E Java	Luminous
51	Mys10	Mysis	Besuki, E Java	Luminous
52	HB3	Sea water	Pacitan, E Java	Luminous
53	TR1	Sea water	Pacitan, E Java	Luminous
54	PT3	Sea water	Pacitan, E Java	Luminous
55	AT2.3B	Shrimp HP	Besuki, E Java	Non luminous

Java island, Indonesia (Table 1). Several shrimp farms were sampled repeatedly at 2-4 month intervals, with samples comprising pond water, coastal water, sediment, shrimp, and shrimp larvae from nearby hatcheries. Luminous vibrios were isolated employing sea water complete (SWC) agar (750 ml sea water, 250 ml distilled water, 5 g bactopeptone, 1 g yeast extract, 3 ml glicerol, and 15 g agar).

Isolation of luminous vibrios from shrimp larvae was conducted by spreading suspensions of hepatopancreas or direct inoculation of shrimp larvae onto thiosulfate citrate bile-Salt sucrose agar (TCBS-agar, Oxoid). Characterization and identification of luminous vibrios were done as described by Baumann et al. (1984). This included *V. harveyi* B392 (ATCC 33843), *V. harveyi* B356, and *V. orientalis* ATCC 33934 for strain comparison. Isolated colonies were stored in 10% glycerol at -60°C.

Ribotyping

Total genomic DNA of vibrios was isolated as described for *E. coli* (Sambrook et al. 1989) with slight modification. DNA digestion with restriction enzymes and Southern blots were performed as described previously (Sambrook et al. 1989). For rDNA probes, a DNA fragment of approximately 7.5 kb containing the 16S ribosomal RNA gene of *E. coli* was isolated from pKK3535 (Brosius et al. 1981). DNA fragments were isolated from agarose gel using a Gene Clean kit (Bio 101, La Jolla, CA) and labelled with biotin using Bionick System (GIBCO-BRL). Labeled probes were purified employing column chromatogaphy (Sephadex G50). Detection of hybridized DNA was performed using the Photogene Detection System (GIBCO-BRL) as described by manufacturer.

Preparation of intact genomic DNA and restriction digestion

Purified isolates of luminous vibrios were grown in SWC agar for 24 hours at room temperature (28-31°C). Isolated colonies (3-4) were picked and suspended in sterile PIV solution (10 mM Tris-Cll pH 7.5, 1 M NaCl) such that the final cell concentration was approximately 2 x 10^9 cell/ml.

Intact genomic DNA isolation was performed by imbedding the bacterial cells in low melting point agarose blocks as described previously (Suwanto & Kaplan 1989). Digestion of intact genomic DNA using restriction endonucleases was performed as described by Suwanto and Kaplan (1989) as follows: restriction endonuclease digestions were performed in 150 ml of appropriate restriction buffer in a 1.5 ml micro tube for each piece of gel plug or gel insert. The gel plugs were equilibrated for 15 minutes on ice, the buffer was changed for new buffer and10 units of enzyme were added. The mixture was incubated on ice for 10-15 minutes to allow the enzymes to diffuse into the agarose plugs prior to digestion at 37°C. Digestions were performed in a shaking water bath for at least 4 hrs. After digestion, the buffer was aspirated and then 150 ml of ES solution was added. The mixture was incubated at 55°C for 10-15 minutes, and then the ES solution was removed by aspiration. The gel plug was dialyzed for at least 10 minutes by adding 1.5 ml 1x TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0) before placing the gel plug into the wells of the running gel.

Molecular size markers

Rhodobacter sphaeroides 2.4.1 genomic DNA digested with *Ase*I (Suwanto & Kaplan 1989) was routinely used as a PFGE molecular size marker in this study. *1 Kb-DNA Ladder* (GIBCO-BRL) or a biotinylated Lambda DNA *Bst*EII digest (NEB, Beverly, MA) were used as DNA migration references in ribotyping studies.

DNA fragment separation by PFGE

CHEF-DR II (BioRad, Richmond, CA) was used to separate DNA fragments throughout these studies. We used a

1% (w/v) agarose gel (Pharmacia) or SeaKem GTG agarose (FMC Corp.) and various pulse times depending on the range of resolution needed. For *Not*I digestion, gels were typically run in 0.5x Tris-Borate-EDTA (TBE) buffer, 175 volt (0.06 - 0.09 mA), 14°C with a ramping pulse time from 10 - 80 seconds for 20 hrs.

Statistical Analysis

A matrix was constructed as the basis for determining the presence or absence of ribotyping or schizotyping bands at a given position over the size range from 1 to 20 kb for ribotyping and from 30 to 500 kb for schizotyping. A cluster analysis was carried out using the unweighted pair group method with arithmetic means (UPGMA clustering with simple matching coefficients) of similarity coefficients for all pairs of strains and a dendrogram was generated using a computer based taxonomy program (Numerical Taxonomy System, NTSYS-PC version 1.60 (Rohlf et al. 1990).

Materials

Bionick translation kits and the Photogene Detection System were purchased from GBCO-BRL. The Gene Clean kit for DNA purification was obtained from BIO 101 Inc. (La Jolla, CA), Proteinase K from Boehringer-Mannheim or SIGMA Chemicals, St. Louis, and X-ray film from Eastman Kodak Co., Rochester, N.Y. All chemicals were of reagent grade purity and were used without further purification.

RESULTS AND DISCUSSION

Physiological and biochemical characterisation of *Vibrio* isolates

A total of 55 isolates of luminous *Vibrio* were isolated from shrimp farms in Java, mainly from the Besuki area in East Java. There were 39 isolates from shrimp larvae with symptoms of luminous disease. Three isolates were from sea sand near shrimp farms, and 13 isolates from sea water in Besuki (East Java), Puger (East Java), Pacitan (East Java), and Labuhan(West Java). Designations for the bacterial isolates, source materials, and names of coastal sampling sites in Java are presented in Table 1.

With the exception of AT2.3B, that was isolated from a shrimp with symptom of tail erosion ("udang ekor gripis" in Indonesian), all isolates were luminous on either SWC or TCBS agar. All of the isolates, except *V. harveyi* B392 and B356, failed to ferment sucrose in the biochemical tests or were unable to produce acid from sucrose and so produced green colonies on TCBS agar. Therefore, in terms of sucrose fermentation, all of Java isolates have characteristics of *V. harveyi*. Moreover, all the luminous vibrios had similar morphological and physiological characteristics (i.e., Gram-negative, short rods, fermented glucose, oxidase and catalase positive, motile, produced H_2S and indole, did not grow in peptone at 4°C or 55°C, gave green colonies on TCBS at 28-37 °C, did not ferment lactose and sucrose, utilized L-serine and acetate as carbon sources, did not produce arginine dihydrolase, liquefied gelatin, produced amylase, and utilized L-tyrosine, glycine, and citrate as sole sources of carbon) (Table 2). According to these biochemical or physiological characteristics, all of the luminous *Vibrio* isolates, except W1.1P, were identified as *Vibrio harveyi* isolates. W1.1P was found to be an isolate of *Vibrio* (*Photobacterium*) *fischeri* (Table 2).

Schizotyping analysis

Restriction endonucleases *Apa*I (5'-GGGCCC-3'), *Eag*I (5'-CGGCCG-3'), *Not*I (5'-GCGGCCGC-3'), *Ase*I (5'-ATTAAT-3'), *Spe*I (5'-ACTAGT), and *Sma*I (5'-CCCGGG-3') were used in the preliminary experiment to screen for the most suitable restriction enzyme for PFGE analysis of genomic DNA. As shown in Fig. 1., *Not*I digested Gc1B genomic DNA into the fewest fragments ranging in size from approximately 33 kb to more than 410 kb.

Since *Vibrio* sp. genomic DNA contains low mol (G+C) (Logan 1994), restriction endonuclease *Ase*I which recognizes high A+T sequences might be expected to digest *Vibrio* genomes very frequently. Meanwhile, our studies indicated that *Sma*I and *Apa*I could be classified only as medium rare-cutter enzymes. Therefore *Not*I was chosen as the restriction endonuclease to be used in subsequent PFGE analysis of all *Vibrio* isolates (Fig. 1).

*Not*I macrorestriction fragment length polymorphisms (MFLP) or schizotyping (Suwanto and Kaplan 1992) of a number of isolates clearly demonstrated different genomic DNA profiles (Table 3), either in the number of DNA fragments generated or in the distribution of the fragments. *Not*I digested total genomic DNA into 6 - 19 resolvable fragments ranging in size from 18 - 1050 kilo base pairs (kb) (Fig. 2.). From analysis of a total 53 isolates, 19 different genomic DNA profiles were obtained by *Not*I schizotyping. These DNA profiles were unique and represented DNA fingerprints for a particular isolate or group of isolates (Fig. 3).

Table 3. Genomic DNA profiles of 58 *Vibrio* isolates by *Not*I schizotype.

No	Schizotype profile	Isolates with similar schizotype
1	*V. harveyi* B392	B392, B356
2	*V. orientalis*	*V. orientalis*, S14B, M3.2L, M3.3L, M3.4L, P1.3L, P2.1L, P2.4L, P5.3L
3	HHT	HHT
4	P6B / P1B	P1.1B, P1.2B, P1.5B, P1.6B, P6.1B, P6.2B, P6.3B, P6.5B
5	Mys	Mys2, Mys3, Mys4, Mys5, Mys6, Mys9, Mys10
6	VTM	VTM1, VTM2, VTMR
7	VhBk	VhBk
8	GcB	Gc1B, Gc2B, Gc3b, Gc4B, Gc5B, Gc7B, U7.2B
9	Dan10	Dan10
10	B2H	B2H, AU2, BU2, BU3
11	W1B	W1.1B, W1.2B, W1.5B, W1.6B, W1.7B, W1.8B
12	W3B	W3.2B, W3.4B, W3.7B
13	S16B	S16.8B
14	W1.1P	W1.1P
15	S9.4P	S9.4P
16	HB3	HB3
17	TR1	TR1
18	PT3	PT3
19	AT2.3B	AT2.3B

Table 2. Biochemical and physiological characteristics of the *Vibrio* isolates.

Characteristics	GcB	B2H	P1B	HHT	VTM	VhBk	MYS	AT23B	Dan10	W1B	W3B	S14B
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-
Glucose fermentation	F	F	F	F	F	F	F	F	F	F	F	F
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Indole formation	+	+	+	+	+	+	+	+	+	+	+	+
H2S formation	+	+	+	+	+	+	+	+	+	+	+	+
Luminescence	+	+	+	+	+	+	+	-	+	+	+	+
Gelatin liquifaction	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-	-
Amylase production	+	+	+	+	+	+	+	+	+	+	+	+
Colony color on TCBS	G	G	G	G	G	G	G	G	G	G	G	G
Fermentation to acid												
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-
Growth at												
4oC	-	-	-	-	-	-	-	-	-	-	-	-
28oC	+	+	+	+	+	+	+	+	+	+	+	+
37oC	+	+	+	+	+	+	+	+	+	+	+	+
55oC	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl at												
0%	-	-	-	-	-	-	-	-	-	-	-	-
0.50%	+	+	+	+	+	+	+	+	+	+	+	+
1%	+	+	+	+	+	+	+	+	+	+	+	+
3%	+	+	+	+	+	+	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+	+	+	+	+
7%	+	+	+	+	+	+	+	+	+	+	+	+
9%	+	+	+	+	+	+	+	+	+	+	+	+
10%	-	-	-	-	-	-	-	-	-	-	-	-
11%	-	-	-	-	-	-	-	-	-	-	-	-
Utilization of												
Citrate	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-
Glycine	+	+	+	+	+	+	+	+	+	+	+	+
L-Arginine	+	+	+	+	+	+	+	+	+	+	+	+
L-Tyrosine	+	+	+	+	+	+	+	+	+	+	+	+
L-Serine	+	+	+	+	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+	+	+	+	+
Identification	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh

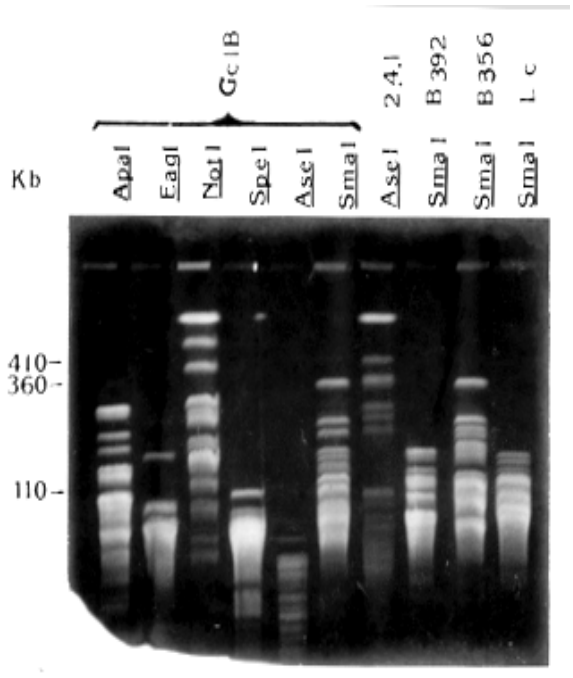

Figure 1. Pulsed-field gel electrophoresis of *Vibrio* isolates Gc1B, digested with *Apa*I, *Eag*I, *Not*I, *Spe*I and *Sma*I. 2.4.1 = R. sphaeroides 2.4.1 total genomic DNA digested with *Ase*I used as a molecular size marker. Running conditions: pulse time ramping 5-48 sec, running time 17 hr, 175 V (0.06-0.09 mA), running buffer temperature 14±1°C.

Table (cont'd). Biochemical and physiological characteristics of the *Vibrio* isolates.

Characteristics	S16B	PL	W11P	S94P	HB3	PT3	B356	B392	Vo	Vs1	Vs11	Vp
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-
Glucose fermentation	F	F	F	F	F	F	F	F	F	F	F	F
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Indole formation	+	+	+	+	+	+	+	+	+	+	+	+
H2S formation	+	+	-	+	+	+	-	+	+	-	+	+
Luminescence	+	+	+	+	+	+	+	+	+	-	-	-
Gelatin liquifaction	+	+	-	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	+	-	-
Amylase production	+	+	-	+	+	+	+	+	+	+	+	+
Colony color on TCBS	G	G	G	G	G	G	Y	Y	G	Y	G	G
Fermentation to acid												
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-
Growth at												
4oC	-	-	-	-	-	-	-	-	-	-	-	-
28oC	+	+	+	+	+	+	+	+	+	+	+	+
37oC	+	+	+	+	+	+	+	+	+	+	-	-
55oC	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl at												
0%	-	-	-	-	-	-	-	-	-	-	-	-
0.50%	+	+	+	+	+	+	+	+	+	+	-	-
1%	+	+	+	+	+	+	+	+	+	+	-	+
3%	+	+	+	+	+	+	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+	+	+	+	+
7%	+	+	+	+	+	+	+	+	+	+	+	-
9%	+	+	+	+	+	+	+	+	+	+	+	-
10%	-	-	-	-	-	-	-	-	-	-	-	-
11%	-	-	-	-	-	-	-	-	-	-	-	-
Utilization of												
Citrate	+	+	-	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-
Glycine	+	+	-	+	+	+	+	+	+	+	+	+
L-Arginine	+	+	-	+	+	+	+	+	+	+	+	+
L-Tyrosine	+	+	-	+	+	+	+	+	+	+	+	+
L-Serine	+	+	+	+	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+	+	+	+	+
Identification	Vh	Vh	Vf	Vh	Vh	Vh	Vh	Vh	Vh	Vs1	Vs11	Vp

Remarks: + = positive; - = negative; G = green; Y = yellow; Vh = *V. harveyi*; Vf = *V. fischeri*; VsI = *V. splendidus* I; VsII = *V. splendidus* II; Vp = *V. penaecida*.

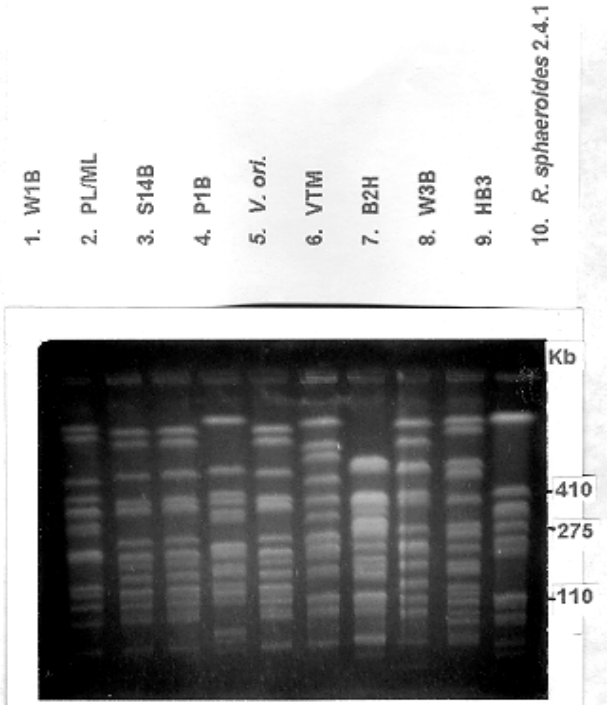

Figure 2. Pulsed-field gel electrophoresis of *Not*I digested genomic DNA from a number of *Vibrio* isolates. *Rhodobacter sphaeroides* 2.4.1 DNA digested with *Ase*I was used as the molecular size marker. Pulse time ramping 10-80 sec, running time 20 hr, 175 V (0.06-0.08 mA), running buffer temperature 14±1°C.

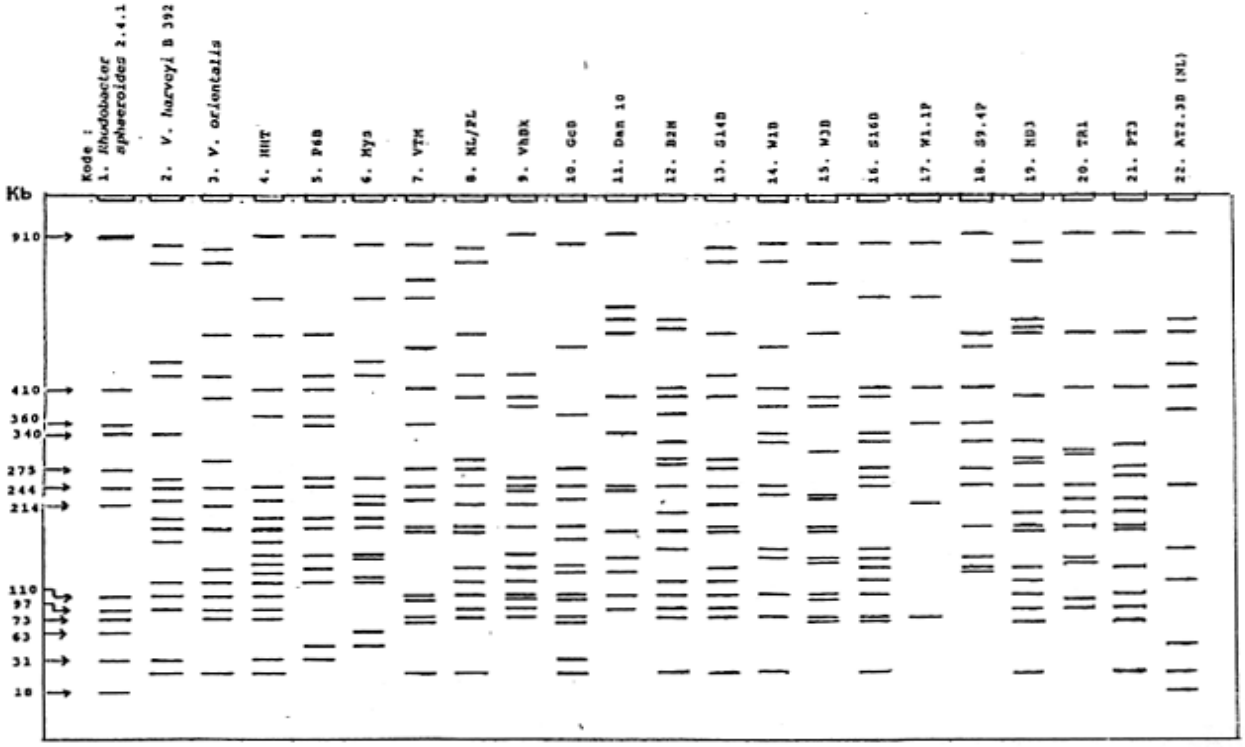

Figure 3. Idealized gel band plot of *Not*I digested genomic DNA of the *Vibrio* isolates together with estimated sizes.

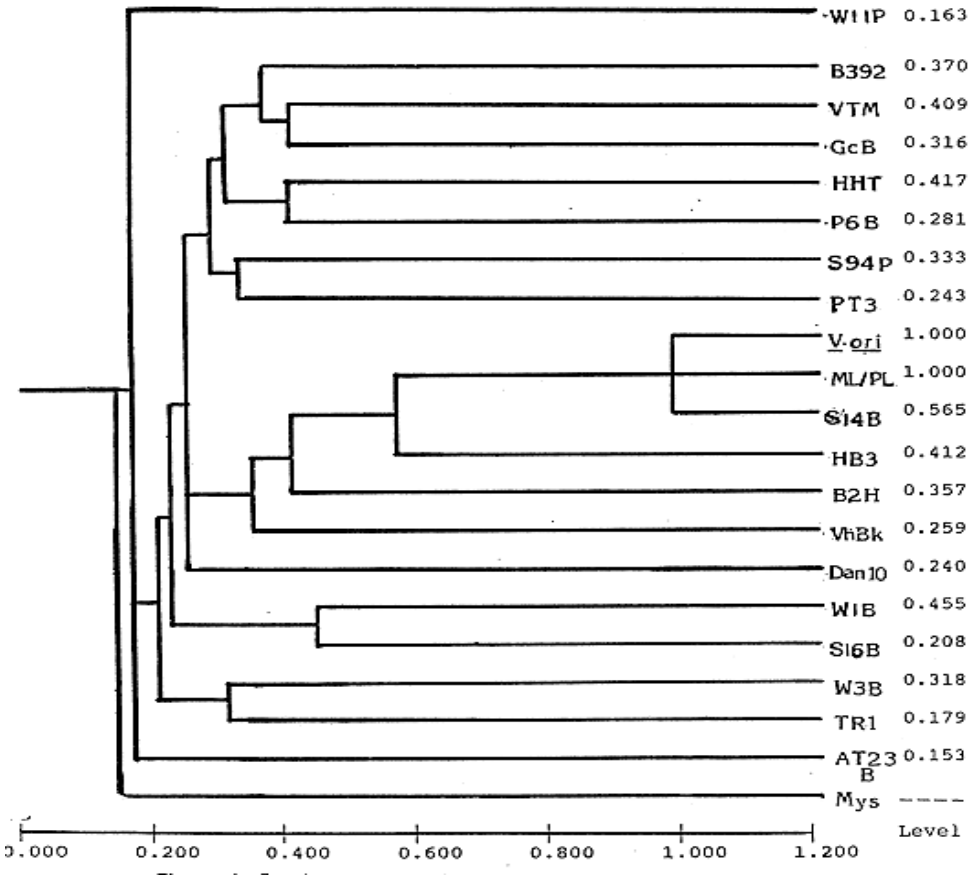

Figure 4. Dendrogram showing the relationships amongst *Vibrio* isolates based on PAGE analysis of genomic restriction fragments generated by *Not*I. similarities were calculated using Dice's coefficient, and clustering was achieved by UPGMA. A total of 55 isolates from shrimp farms and seashore areas on Java Island were studied together with 3 reference isolates from ATCC.

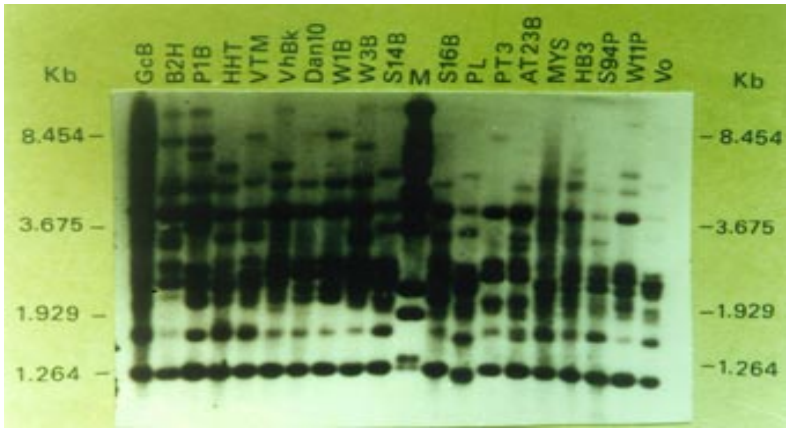

Figure 5. A representative gel of ribotypes for the *Vibrio* isolates.

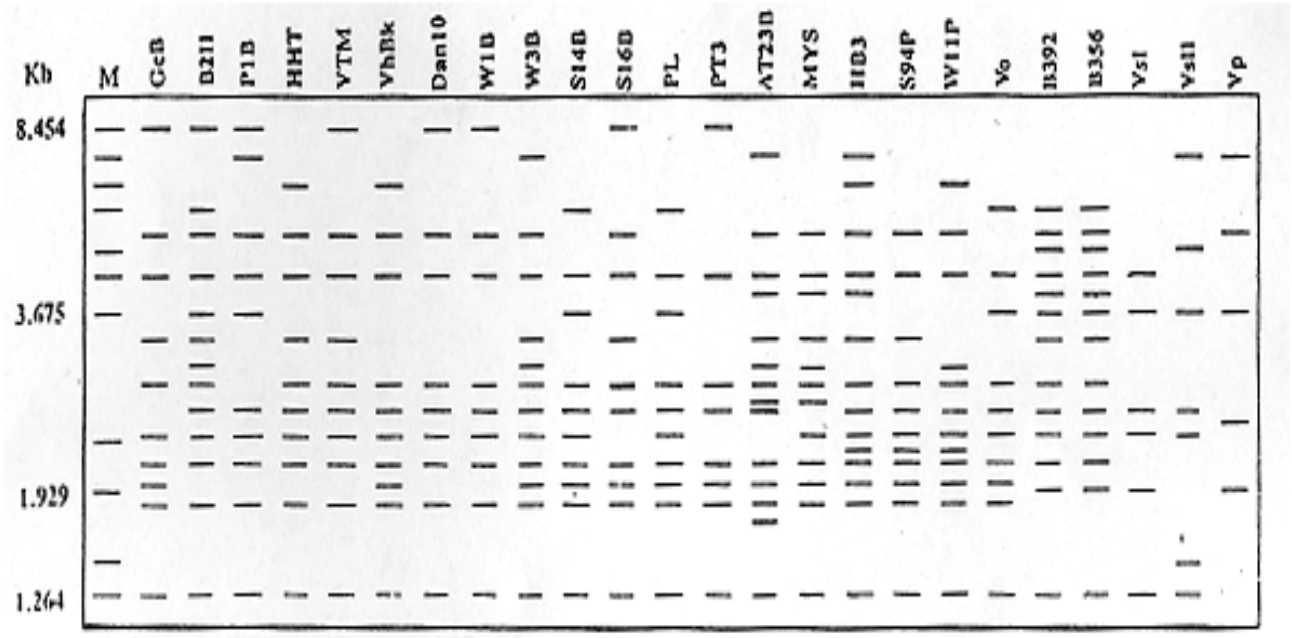

Figure 6. Interpretation diagram for all of the ribotypes of the *Vibrio* isolates studied.

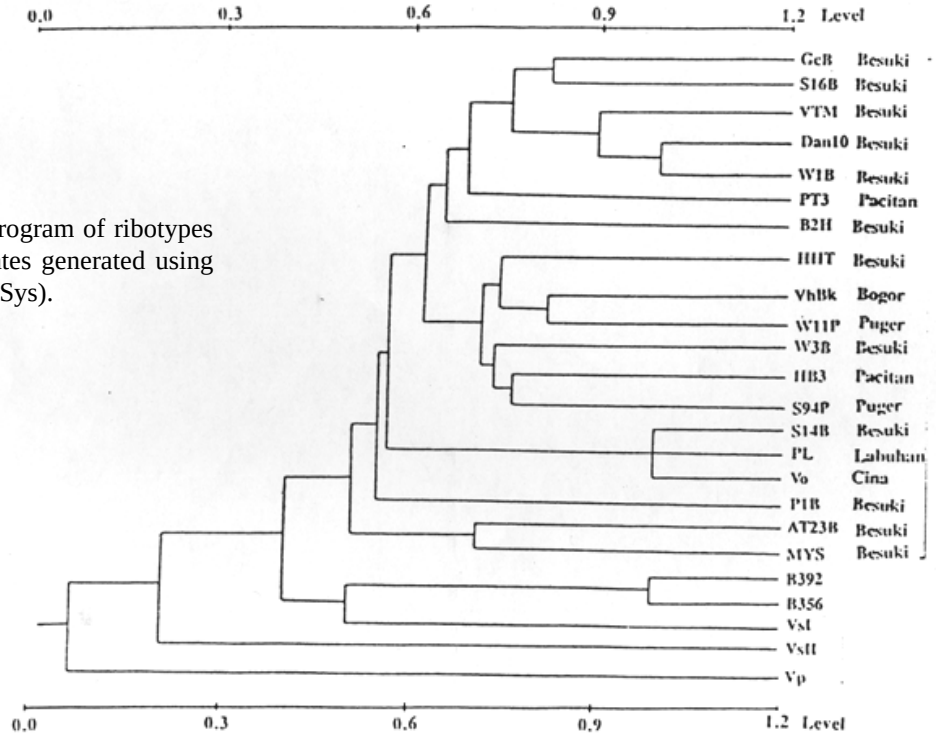

Figure 7. A dendrogram of ribotypes of *Vibrio* isolates generated using UPGMA (NT-Sys).

Determination of similarity coefficients and clustering by UPGMA of the 55 *Vibrio Not*I schizotypes and the reference strains suggested that there was no correlation between cluster membership and geographical distribution (Fig. 4.). For example, isolates S14B, ML/PL, and *V. orientalis* showed identical *Not*I schizotypes, although they were isolated from East Java (Besuki), West Java (Labuhan), and China (Yellow Sea), respectively. These results suggested that isolates of marine vibrios might have high mobility and be widely distributed as a consequence of their aquatic habitat or migration through the use of broodstock from other locations. This realization has consequences for shrimp farmers, because recognition of clonal lineages that conform to regional geographic boundaries is necessary for the formulation of disease prevention programs. Clearly, the fluctuation and widespread genetic diversity reported here may help explain the difficulty in obtaining broodstock resistant to luminous bacterial disease. In addition, the genetic diversity of luminous vibrios may cause difficulty in generating shrimp vaccines directed to a particular strain of luminous *Vibrio*. Therefore, the development of biocontrol or bioconditioners in shrimp farms should be an effective alternative approach to the prevention of disease, especially if it is combined with other practices such as low input sustainable agriculture.

Ribotyping

Fourteen isolates of *Vibrio*, including the non-luminous *V. penaeicida* (Ishimaru et al., 1995) were analysed using ribotyping. A representative result is depicted in Fig. 5., while an interpretation diagram from all of ribotypes is presented in Fig. 6. The dendrogram generated using UPGMA (NT-Sys) is shown in Fig. 7. The results indicated that strains of luminous vibrios (i.e., *Vibrio harveyi*) isolated from the sea and shrimp farms in Java were genetically heterogenous, as was demonstrated using *Not*I schizotyping. However, the degree of similarity in ribotyping analysis is relatively higher than the corresponding similarity using schizotyping. Interestingly, a number of isolates that were shown to be identical according to *Not*I schizotyping were also identical by ribotyping analysis (i.e., Dan10, W1B, S14B and PL/ML; *V. orientalis*, B392 and B356; VhBk and W1.1P). All the *V. harveyi* isolates showed distance relationship to *V. penaeicida*, a pathogen of Kuruma prawn isolated in Japan (Ishimaru et al., 1995).

In this study, we have been able to show that although ribotyping is not as highly discriminative as *Not*I schizotyping, it has sufficient discriminatory power to differentiate many genotypes of *V. harveyi* from Java island. This might be due to the fact that all of the members of the Enterobacteriaceae described to date carry seven rRNA operons. In contrast, bacteria such as *Xanthomonas campestris* pv. *glycines* with a similar chromosome size has only three rRNA operons. Strains of *X. campestris* pv. *glycines* could not be distinguished significantly using ribotyping. In other words, schizotyping is much more powerful than ribotyping in differentiating *X. campestris pv. glycines* isolates (Wahidin 1996, Rukayadi 1995).

The results of ribotyping analysis unambiguously demonstrated the enormous diversity of *Vibrio harveyi* isolates which might be important indicator for the occurance of shrimp and shrimp fry diseases in South East Asia. The ribotyping results support the conclusion obtained from *Not*I schizotyping, and altogether suggest that the development of microorganisms which can suppress the growth of *V. harveyi* might be a promising alternative for preventing luminous disease in shrimp

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Vibrionaceae Associated with the Larvae and Larval Rearing System of *Macrobrachium rosenbergii* : Systematics and Pathogenicity

Sarita G. Bhat, *I.S. Bright Singh

Environmental Microbiology Laboratory, School of Environmental Studies
Cochin University of Science and Technology, Fine Arts Avenue, Cochin 682 016, India.

ABSTRACT: Systematics of the family Vibrionaceae associated with the larvae of *Macrobrachium rosenbergii* in the hatchery has been worked out based on the principles of numerical taxonomy and the mole per cent G+C content of DNA. Altogether 313 isolates of Gram negative, motile/nonmotile, fermentative, Kovac's oxidase positive rods comprising 204 isolates from moribund larvae and 109 from apparently healthy larvae were subjected to the study along with 20 type strains. They were further divided as sensitive or resistant to 2,4 -diamino,6,7 di-isopropyl pteridine (0/129 compound) at 150 µg ml^{-1}. The operational taxonomic units were tested for 233 unit characters and the data thus generated were analysed using simple matching coefficient (Ssm) and unweighted average linkage by applying TAXAN. On completing the analysis, it was surprising to find that none of the type strains integrated with any of the isolated strains. Represntative strains from each phenon were segregated and the percentage G+C ratio was determined based on *Tm* values. Irrespective of the sensitivity of the phena to 0/129, the majority had G+C ratios falling within the range of those for *Vibrio* species while a few fell within the range of those for *Aeromonas* and *Photobacterium*. Suggested affiliations based on G+C ratios contrasted with gross phenotypic dissimilarities. Several strains had G+C ratios out of the range of Vibrionaceae and the newly constructed family Aeromonadaceae, even though they exhibited certain phenotypic traits of these families. It is postulated that the isolates studied here were either phenotypic variants or new species of the families mentioned above. This has to be further substantiated by DNA homology comparisons with existing type species. In any case, this study proved that sensitivity to 0/129 could not be used as a core character differentiating *Vibrio* and *Photobacterium* from *Aeromonas*. The study broadens the base of the familyVibrionaceae and Aeromonadaceae and opens up avenues for developing new taxonomic schemes for tropical isolates. Pathogenicity of 47 representative strains was determined based on the extent of larval mortality upon challenge with revitalized cultures as compared to mortality in positive and negative controls. With all the the test organisms, there was significantly higher larval mortality, indicating that the families Vibrionaceae and Aeromonadaceae as a whole are pathogenic to the larvae of *Macrobrachium rosenbergii.*

KEY WORDS: Vibrionaceae, Aeromonadaceae *M. rosenbergii*, Hatchery, Virulence, Taxan

Bhat SG, Singh ISB (1998) Vibrionaceae associated with the larvae and larval rearing system of *Macrobrachium rosenbergii* : systematics and pathogenicity. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.