GENETIC DIVERSITY OF *Vibrio harveyi* ISOLATED FROM TIGER PRAWN *Penaeus monodon* HATCHERIES AND GROW OUT PONDS

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ABSTRACT

Vibrio harveyi is known as one among the most harmful bacteria infecting tiger prawn at every stage of its life's. The present research was aimed to reveal the genetic diversity of *Vibrio harveyi* isolated from tiger prawn (*Penaeus monodon*) culture. The samples of bacteria were collected from hatchery (brood-stock, larvae, natural feed, artemia, and larval rearing water) and grow-out (juveniles, water, shrimp, sediment, plankton, crab, mollusc, microalgae, and wild fish). The taxonomic identification of *Vibrio* spp. was performed based on the physiological and biochemical characteristic following the isolation by Thiosulphate Citrate Bile-salt Sucrose Agar (TCBSA) media. Amplified Ribosomoal DNA Restriction Analysis (ARDRA) for 16S-rRNA analysis and Macrorestriction Fragment Length Polymorphism (MLFP) analysis using Pulsed Field Gel Electrophoresis (PFGE) were applied to reveal the genetic diversity of *V. harveyi*. According to the taxonomic identification, of 361 isolates of *Vibrio* spp., 129 isolates (35.7%) were identified as *V. harveyi*. The result of ARDRA analysis showed that the 16S-rRNA gene of *V. harveyi* digested by *Rsa*I and *Hha*I enzyme, each generated three and four identical fragments respectively for the all samples. These meaned that ARDRA could not reveal any genetic variation on *V. harveyi*. The size range of all DNA fragment was less than 500 bp. This result indicated that the high genetic diversity of *V. harveyi* was revealed by MFLP-PFGE analysis. DNA fragment of *V. harveyi* was digested by *Not*I enzyme.

KEYWORDS: genetic, *Vibrio harveyi*, *Penaeus monodon*, ARDRA, PFGE

INTRODUCTION

Vibriosis is one of the major problem not only in tiger shrimp hatchery, but also in stunting pond and in grow-out pond culture. More than 25 species of vibrios have been isolated from tiger shrimp ponds in South Sulawesi (Nurhidayah, 2000). The most common *Vibrio* as causative agent on tiger shrimp hatchery, stunting pond, and grow-out pond culture is *V. harveyi* (Boer *et al.*, 1993; Karunasagar *et al.*; 1994) Tjahyadi *et al.*, 1994; Saulnier *et al.*, 2000). Another species of *Vibrio* infecting tiger shrimp in ponds were *V. parahaemolyticus*, *V. alginolyticus*, and *V. anguillarum* (Chanratchakool *et al.*, 1995).

V. harveyi, a gram-negative, luminescent, marine bacterium isolated both in a free-living state, and as a commensal organism in the enteric contents of marine animals, is known as one among the most harmful vibriosis infecting tiger prawn at every stage of its lines (Lavilla-Pittogo *et al.*, 1990). According to Yuhana (1999) luminescent *V. harveyi* infect tiger prawn larvae from adult (vertical infection) and larval rearing water and sea water (horizontal infection), because DNA profile of *Vibrio* isolated from sea water was identic by DNA profile of *Vibrio* isolated from tiger prawn larvae.

Genetic diversity analysis by PFGE-MFLP on genomic DNA can produce discret DNA fragment. The changing on any sequence in genom will make differentiation on DNA profile. According to Yuhana (1999), PFGE-MFLP analyisis is more discriminative to analysis genetic diversity of *Vibrio* spp. than that of PCR-RFLP analysis.

The research aims at identifying genetic diversity of *V. harveyi* isolated from tiger prawn (*Penaeus monodon*) culture using ARDRA and

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MFLP-PFGE analysis.

MATERIALS AND METHODS

Isolation of Vibrio *spp.*

Sample collection. *Vibrio* spp. were isolated from hatchery (brood-stock, larvae, food, artemia, larval rearing water, and juveniles) and grow-out (pond water, pond sediment, plankton, crab, mollusc, algae, and wild fish) of tiger shrimp in South Sulawesi. The samples ware collected using sterile bottles in fresh cool box for transporting to the laboratory of the Research Institute for Coastal Aquaculture (RICA).

Sample Preparation and culture media. The samples were washed by sterile sea water 2—3 times to prevent from out-side contamination before they were homogenized by vortex agitator. To obtain a single colony on Thiosulfate Citrate Bile Salt (TCBS) agar, appropriate dilutions (10^{-1}, 10^{-2}, 10^{-3}) ware performed by adding saline solution (0.85% (w/v) NaCl). A hundred microlitter of each dilution were spreaded on TCBS agar, and incubated at 25°C—28°C for 24 hours. The colonies on TCBS agar were morphologically identified according to the form, color, elevation, margin, and colony size (Austin, 1993; Austin & Austin, 1993; Hadioetomo, 1993; Atlas, 1997; Prescott *et al.*, 2002; Sung *et al.*, 2003), and than made a pure cultures from a completely separate colony by streak plates on Triptic Soy Agar (TSA) agar plate.

Physiological and Biochemical Test of Vibrio *spp.*

Physiological and biochemical test for identification of *Vibrio* spp. ware done through gram staining, oxidase, oxidatve-fermentative, motility, luminescence, swarming, voges-proskauer, arginin dehydrase, gas from glucose, growth at 40°C, lysine decarboxilase, pigmentation, amylase, sucorose, ornithine decarboxilase, putriscine, growth at ethanol 95%, heptanoate, xanthine, aminobutirate, arabinose, cellubiose, glucuronate, alfa-ketoglutarate, L-alanine, Leucine, and propionate (Austin, 1993; Austin & Austin, 1993; Hadioetomo, 1993; Alsina & Blanch, 1994; Muir, 1996). "Fortan Program" modified by Muir (1996) was used for grouping the *Vibrio* spp. according to the physiological and biochemical characterization.

16S-rRNA Gen Analysis of Vibrio harveyi *by Amplified Ribosomoal DNA Restriction Analysis (ARDRA)*

Genomic DNA Preparation. *V. harveyi* isolates were grown overnight in Sea Water Complte (sea water 750 mL, distilled water 250 mL, bacto pepton 5 g, yeast extract 1 g, and glicerol 3 mL) broth. Genomic DNA from each isolate was extrated by using the CTAB (cethylethylammonium bromide) protocol (Suwanto *et al.*, 2000).

Amplification of 16S-rRNA gen for ARDRA. Quantification of genomic DNA by using electrophoresis and gene quantification (Suwanto *et al.*, 2000) was done before the 16S-rRNA amplifield by PCR. Genomic DNA of *V. harveyi* was mixing in a microcentrifuge containing 18 mL ddH_2O; 0,05 mL dNTP 10mM; 1 mL Taq polymerase 5 U/mL; 1 mL buffer taq polymerase; 1 mL 63f primer (5'-CAGGCCTAACACATGCAAGTC) 5 pmol; dan 1 mL 1387r primer (GGGCGGWGTGTACAAGGC) 5 pmol (Marchesi *et al.*, 1998; Suwanto *et al.*, 2000). Amplification condition was programmed for 30 cycles, *pre start* 94°C 2 minutes, DNA denaturation 92°C 2 minutes, primer *annealing* 55°C for 30 seconds, extention 75°C for 1 minute, post PCR 72°C for 20 minutes, and hold in PCR at 4°C (Marchesi *et al.*, 1998; Suwanto *et al.*, 2000).

Restriction digest for 16S-rRNA gene. Restriction enzymes *Hhal* (5'-GCG'C), dan *Rsal* (5'-GT'AC) (New England, BioLabs^{O} Inc.) were used to generate 16S-rRNA gen from 30 isolates of *V. harveyi*. The raeaction condition for digesting was the mixture of 7 mL PCR product (gen 16S-rRNA), 2 mL of 10X buffer of restriction enzyme, 1 mL of restriction enzyme (2 units/mL), and 10 mL of ddH_2O. The reaction digest was placed into a floating ract and then incubated in a 37°C water bath for 24 hours. The reaction digest was then stopped by placing the samples in a 65°C water bath for 20 minutes. The samples were placed on ice and followed by heat inactivation before loaded into the gel electrophoresis for ARDRA prior to documentation purposes.

V. harveyi *Genomic DNA analysis by Pulsed Field Gel Electrophoresis (PFGE)*

Preparation of bacterial suspention. *V. harveyi* isolates were grown overnight on Sea Water Complite. The separated colony was regrown on Luria Broth (10 g trypton, 5 g yeast

extract, 25 g NaCl and 1 L distilled water) at 28°C for 24 hours. Bacterial cell was picked up for several mL to make bacterial suspension with 10^9 cells/mL in concentration. One mL of the bacterial suspension was centrifuged at 5,000 rpm for 30 secods. The bacterial cell pellet was suspended in sterile PIV solution (10 mL Tris-HCl, pH 7,5; 1M NaCl) and sentrifuged at 5,000 rpm for 30 secods. The bacterial cell pellet was resuspended in 0.5 mL of PIV and prepared for intact geneomic DNA (Widanarni & Suwanto, 2000; Suwanto *at al.*, 2000).

Preparation for intact geneomic DNA. Preparation of intact genomic DNA was done by following procedure from Smith & Cantor (1987) and modified by Yuhana (1999), Suwanto *et al.*, (2000), Widanarni & Suwanto, (2000). Bacterial cell suspention (0.5 mL) in PIV solution were mixed up into 1% Low Melting point Agarose (LMA) in 1x TE solution (10 mL Tris-HCl and mM EDTA, pH 8.0) and embedding in plug agarose at 4°C for 10 minutes. The agarose plug was transfered into 25 mL erlenmenyer flask containing 5 mL EC solution (6 mM Tris-HCl, pH 7.5; 100mM EDTA; 1 M NaCl; 0.5% polyoxyethylene-2-ethileter; 0.2% natrium deoxycholate; 0.5% natrium lauril sarchosin added 1 mg/mL lysozime) and incubated in water shaker bath at 37°C, 80 rpm for over night. EC solution was aspirated and filled tube with 5 mL ESP solution (0,5% EDTA, pH 9,0°C—9,5°C; 1% natrium lauril sarcosin dan 200 mg/mL proteinase-K) and incubated in a shaker water bath at 55°C, 80 rpm for 48 hours. The ESP solution removed from tube and rinsed the plug 2 hourly with excess 1xTE buffer solution (10 mM Tris-Cl pH 8.0; 1 mM EDTA pH 8.0) 3 times and the plug ready for restriction endonuclease digestion.

Restriction endonuclease digesting for genomic DNA. The agar plug was cut into 2.5 x 10 x 1 mm size. The gell insert was equilibrated in 150 mL of 1x restriction buffer (1,335 mL of distilledwater; 150 mL of 10x restriction buffer; 15 mL 10 mg/mL of bovine serum albumin) at 4°C for 15 minutes. The restriction buffer was aspirated then filled with 150 mL of fresh restriction buffer and added 10—20 units of restriction enzyme and than incubated at 37°C for overnight. After digestion, the buffer aspirated and added 150 ES solution to inactivate restriction endonuclease and to clean DNA from DNA-binding proteins. The DNA was incubated at 55°C for 10 minutes (Suwanto & Kaplan, 1989; Suwanto *et al.*, 1998; Widanarni & Suwanto, 2000).

Separation of DNA Fragment using PFGE. Gel agarose for running gel PFGE were prepared by dissolving 1 g *high melting point agarose* in 100 mL 0.5 x TBE (50 mM buffer Tris Borat; 0.1 mM EDTA pH 8.0). After dissolved by heating on hot plate, the agarose solution was poured into agarose cast and let it until the gel sets at room temperature. The gel insert was placed into the well of agarose gel and covered with 1.5% LMA in 1 x TE buffer. The gel was put into PFGE chamber and run in 0.5 x TBE buffer, 5 volt, 14°C with ramping pulse time 10—80 seconds for 20 hours (Suwanto & Kaplan, 1989; Suwanto *et al.*, 1998; Widanarni & Suwanto, 2000).

DNA visualization. The gel was satained with ethidium bromide (EtBr) solution (2 mg/mL) for 10 minutes while shaked gently and washed water for 20—30 minute. The gel was visualized using trasillumiminator with UV leng of 280 nm. Photogaph was taken using gel documentation.

Data Analysis. The size of genomic DNA on gel electrophoresis was compared with standard DNA genomic (genomic DNA of *Rhodobacter spaeroides* digested with *ASe*I) (Sambrook *et al.*, 1989; Suwanto & Kaplan, 1989; Sambrook & Russel, 2001). The difference of bacterial strain was shown by number of band and position of migration.

RESULT AND DISCUSSION

Composition of Vibrio *spp.*

A total of 361 isolates of *Vibrio* spp. have been isolated from tiger prawn hatcheries and culture ponds in South Sulawesi, Indonesia. Physiological and chemical identification showed that a few of 15 species of *Vibrio* spp. have been isolated (Table 1.). Table 1 showed that *Vibrio* spp. was dominated by *V. harveyi* with 129 isolates (35.7%) of total isolates. Vandenberghe *et al.* (1999) reported that a total 351 of vibrios isolated from *Litopenaeus Vannamae* culture ponds, 57 isolates (16.24%) were identified as *V. harveyi.*

Another species of *Vibrio* isolated from hatchery and grow-out pond were *V. tubiashi* 41 isolates (11.4%), *V. alginolyticus* 39 isolates (10.8%), and *V. mimicus* 38 isolates (10.5%). Although *V. spilindidus, V. ordali, V. metzhinokovii, V. parahamolyticus, V.*

Table 1. Composition of *Vibrio* spp. isolated from hatchery and tiger prawn pond farming

Species	Number of isolate	Percentage (%)
V. harveyi	129	35.7
V. tubiashii	41	11.4
V. alginolyticus	39	10.8
V. mimicus	38	10.5
V. splendidus	29	8.0
V. ordalii	23	6.4
V. metschnikovii	18	5.0
V. parahaemolyticus	14	3.9
V. leiognathi	13	3.6
V. natriegens	6	1.7
V. cholerae	5	1.4
V. campbellii	3	0.8
V. anguillarum	1	0.3
V. fischeri	1	0.3
V. navariensis	1	0.3
Total	361	100

leognathi, V. natriegen, V. cholerae, V. fisheri, V. navarensis, and *V. anguillarum* were less than 10%. Hosseini *et al.* (2003) reported that from 770 pcs. of tiger shrimp was isolated several species of *Vibrio* spp i.e. *V. parahaemolyticus, V damsela, V. alginolyticus,* dan *V. fluvialis.*

Among 129 isolates of *V. harveyi,* 22 isolates (28.38%) were isolated from broodstock, 3 isolates (3.87%) from egg, 13 isolates (16.77%) from larvae, 6 isolates (7.74%) larval rearing water, 3 isolates (3.87%) from tiger shrimp post larvae, 2 isolates (2.58%) from larvae rearing water, 19 isolates (24.51%) from culture pond water, 12 isolates (15.48%) from culture pond sediment, 18 isolates (23.22%) from tiger shrimp culture, and 34 isolates (43.86%) from wild organisms cultured in ponds.

From 129 isolates of *V. harveyi,* 30 isolates were analyzed to 16S-rRNA gen using ARDRA and 100 isolates were analyzed by genomic DNA by Pulsed Field Gel Electrophoresis (PFGE).

ARDRA Analysis of V. harveyi *Isolates*

The result of ARDRA analysis showed that the 16S-rRNA gene of 30 isolates of *V. harveyi* digested by *Rsa*I and *Hha*I enzymes, each generated one identical fragment respectively for all samples. The 16Sr-RNA gen digested by *Rsa*I and generated three DNA fragments at the position size less than 500 bp (Figure 1). Furthermore, when the same isolates digested by *Hha*I, four DNA fragments were generated at the same size with the fragments generated by *Rsa*I (Figure 2). Different result has been reported by Yususf et *al.* (2002), where the analysis 16S-rRNA gene of 20 clone of isolates from soil by *RSa*I and *Hha*I generated 13 DNA fragments. This means that ARDRA could not detect any genetic variation on *V. harveyi* collected from different population. This might also indicate that the fragment visualized by *Hha*I and *RSa*I were species marker of *V. harveyi.*

Profile Genomic DNA of V. harveyi

PFGE results on 100 isolates of *V. harveyi* digested by *Not*I restriction enzyme produced 8—16 discret DNA fragments with 63—910 kb in size. Widanarni & Suwanto (2000) reported that PFGE electroforesis of 25 isolates of ampicilin-resistant *Vibrio* digested by *Not*I restriction enzyme produced 10—16 disccrete DNA fragments with sizes ranging from 31—910 kb. Representative discret DNA fragments from PFGE analysis for some samples were shown in Figure 3. The genomic DNA of *V. harveyi* digested by *Not*I restriction enzyme

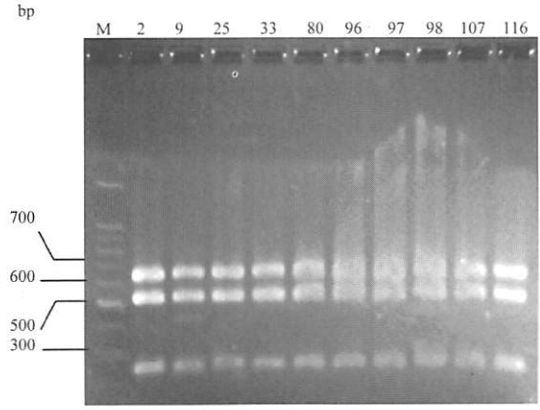

Figure 1. ARDRA-Rsal profile. M: Marker (100bp DNA ladder, Promega). 2: V. harveyi from post larvae of tiger shrimp. 9: V. harveyi from egg. 25: V. harveyi from brooders. 80: V. harveyi from wild crab. 96: V. harveyi from wild crab. 107: V. harveyi from brooders. 116: V . harveyi from wild fish 1

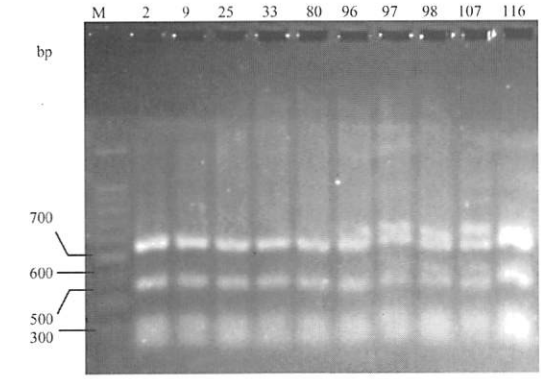

Figure 2. **ARDRA-HhaI** profile. M: Marker (100 bp DNA ladder, Promega). 2: *V. harveyi* from post larvae of tiger shrimp. 9: *V. harveyi* from egg. 25: *V. harveyi* from brooders. 80: *V. harveyi* from wild crab. 96: *V. harveyi* from mollusc. 97: *V.* brooders. 116: *V. harveyi* from wild fish

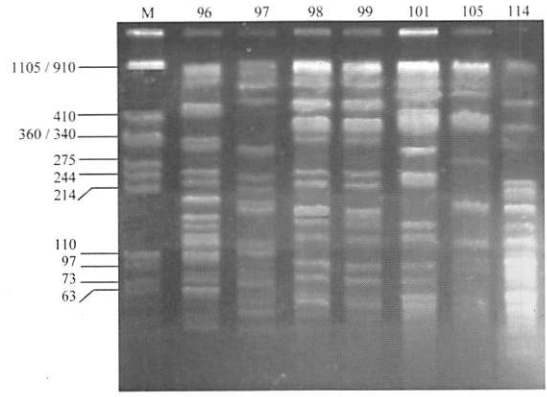

Figure 3. MFLP-PFGE analysis of *V. harveyi* genomic (M: marker R. sphaeroides 2.4.1 digest by AseI. Line 96-114: *V. harveyi* samples digest NotI). 96: *V. harveyi* from mollusc. 97: *V. harveyi* from mollusc. 98: *V. harveyi* from wild crab, 99: *V. harveyi* from wild fish. 101: *V. harveyi* from tiger shrimp, 105: *V. harveyi* from tiger, 114: *V. harveyi* from wild shrimp

showed difference profiles based upon the number of DNA fragment. This result indicated that strains of *V. harveyi* isolated from broodstock, egg, larvae, commercial size shrimp, and wild organism in shrimp pond culture were genetically heterogenous. It was proven that eventhough physiologically and biochemically similar, they genetically were different.

CONCLUSION

The result of genomic DNA of *V. harveyi* from PFGE analysis showed difference profiles based upon the number of DNA fragment. It conclude that strains of *V. harveyi* from different tiger prawn hatcheries and grow out ponds in South Sulawesi were genetically heterogeneous.

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