Journal of Agricultural Technology 2011 Vol. 7(3): 679-694
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Molecular characteristics of *Vibrio* sp causing Black Tiger Prawn (*Penaeus monodon*) disease in Sumatra and Java shrimp ponds by 16S rDNA sequencing

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Felix, F., Nugroho, T., Silalahi, S. and Octavia, Y.(2011). Molecular characteristics of *Vibrio* sp causing Black Tiger Prawn (*Penaeus monodon*) Disease in Sumatra and Java shrimp ponds by 16S rDNA Sequencing. Journal of Agricultural Technology 7(3):679-694.

Shrimp disease caused by *Vibrio* sp is one of the main limiting factors in the increasing production in shrimp farming. This disease may kill the shrimp and cause high loss in shrimp culture in South East and East Asia. Samples of ten individual of a ten months cultured giant tiger prawn as well as liter pond water and sea waters were collected from shrimp ponds in Bengkalis Island, Sumatra. Samples of shrimps were also collected from Jepara shrimp ponds in Central Java. They were selected by looking at their behaviour and unhealthy physical characteristics. Amplification, sequencing and bioinformatics analysis of 16S rDNA to identify *Vibrio* species were conducted in Biotech Center, BPPT Serpong Banten. The results of DNA sequencing of each bacteria isolate were compared to DNA sequences from GenBank, the international DNA bank database. Tracing was made by BLAST (Basic Local Alignment Search Tool) system accessed through the internet at http://www./ncbi.nlm.nih.gov/blast. The result found that of seven strains of V*ibrio* sp. Bacteria analyzed, five were 97% homologues to *V. alginolyticus, V. parahaemolyticus, V. harveyi, V. shilonii and V. vulnificus*. Meanwhile, another two strains isolated in this study were not found to be homologus to any of the Gen-Bank listed strain and therefore are considered as indigenous *Vibrio* sp bacteria from Indonesia.

Key words: Vibrio sp, bacteria, shrimp, aquaculture, fish disease.

Introduction

Shrimp farming contributes significantly to the production of Indonesian fisheries sector. Indonesia's exports of shrimp production once reached 50% of all fishery exports in 2002 and ranks fifth in the non-oil export commodities. In

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order to sustain the production of shrimp that has provided a major foreign exchange for the country, various factors that cause reduction of shrimp production must be addresed. Various failures that occur in Indonesian shrimp farming have become a phenomenon that is very detrimental. Such failures are usually caused by *vibrio sp* attack which results in the fast death of shrimp large numbers. *Vibrio sp* infected shrimp are generally characterized by clinical symptoms, in which the shrimp looks weak, dark red or pale, antennae and swimming legs are red. This is the type of pathogenic bacteria that infects and causes disease when shrimp conditions are weak and extreme environmental factors (Lopillo, 2000).

Due to the fact that Indonesia is one of the largest producers of shrimp in the world, it is very important to know what types of bacteria that causes death in shrimp. It is believed that there is a type of *Vibrio* sp originating from Indonesia that probably does not exist in other countries, because the diversity of *Vibrio* species in Indonesian waters is still very little studied and analyzed. Occurrence of shrimp mortality due to *Vibrio* sp attack makes the shrimp farmers suffered huge losses. The potential of such a large spread of *Vibrio* should be overcomed quickly by performing a variety of prevention efforts. In an effort to control the possibility of *Vibrio* sp attacks, the detection of *Vibrio* species needs to be done appropriately, because the waters in different locations can have a diversity of different *Vibrio* species as well. One of the best technologies that can identify *Vibrio* species is to analyse 16S rDNA sequences of isolated strains. In studying the shrimp disease-causing bacterium *Vibrio*, this technique is relatively a new technique that is often applied because it can be compared with the GenBank data base to determine the similarity of DNA homology with similar bacteria. Currently, many taxonomists accept that the study of molecular microbiology, particularly nucleic acid analysis, reliable method to indicate the species and determine phylogenetic relationships between different organisms. Analysis of DNA sequencing represents the last reference to identify subtypes within a species or microbial screening (Lusiano, 2007).

Data base for the bacteria of the genus *Vibrio* have been established and continued to grow with new species discoveries. This database can be accessed virtually in GenBank in the site http://www/ncbi.nlm.nih.gov/blast (Harth *et al.,* 2007). Each species of bacteria has molecular characteristics that can distinguish them from one species with other species in one genus (Andrito, 2007).

Identification using 16S rDNA techniques to get the bacteria on giant tiger prawns, pond water and sea water is dominated by the genus *Vibrio*. *Vibrio* is opportunistic pathogens, i.e. organisms that normally exist in environmental maintenance and then develop from the nature of the saprophyte into pathogenic because of environmental conditions make it possible.

The present study aims to determine the species of *Vibrio sp* that causes disease in Bengkalis (Sumatera island) and Jepara (java island) shrimp farming ponds at the site. Itispostulated that several Vibrio species is indigenous to Indonesia, and this study will enrich the Gene-Bank database.

Materials and methods

The research was conducted in May-July 2009. This included sampling of shrimp in shrimp aquaculture ponds Bengkalis of Sumatra island, and from August to December 2009 for shrimp and pond water from BBPBAP (Balai Besar Pangembangan Budidaya Air Payau / Central for Brackishwater Aquaculture Development) Jepara for sampling of pond water and sea water. Isolation of *Vibrio* was conducted at the Integrated Laboratory of Marine Science Department University of Riau. Furthermore, the amplification of PCR (Polymerase Chain Reaction) and DNA sequencing were performed at the Biotech Center, Research Institute for Development of Technology (BPPT) in Serpong, Banten Province.

The materials used are giant tiger prawns, sea water samples, pond water, TCBS gelatin Merck, the TSA gelatin Merck, TSI gelatin Merck, a solution of crystal violet, safranin, iodine, immersion oil, 3% hydrogen peroxide (H_2O_2), tetrametyl-p-phenylendiamein 1%, alcohol, aquabides, methylated, NaCl 0.9%, MR-VP broth, methyl red reagent, bacterial culture, fastPrep ® DNA Kit (USA), agarose, TAE buffer 1x, 6x loading dye, SYBR safe, DNA marker 1 kb DNA Ladder (Fermentas; # SM0311/2/3), Taq DNA polymerase, 10x PCR buffer, dNTPs mix, primers
9f (5'-GAGTTTGATCCTGGCTCAG-3 '),
765R (5'-CTGTTTGCTCCCCACGCTTTC-3'),
1114R, (5'-CCCGGAACCCAAAAACTTTG-3 '), 25 mM $MgCl_2$.

Tools that are used in this study include: Tomy MS-100R homogenizer, centrifuge machine Tomy/MX-301, PCR thermal Cycler (Takara Thermal Cycler Dice-TP model 600 v 2.00), UV Trans illuminator unit, BioRad electrophoresis and Gel documentation systems, and the AB 3130 Genetic Analyzer.

Samples of shrimp (*Panaeus monodon*) were taken in shrimp ponds in Bengkalis, Sumatra, Indonesia as many as 10 (ten) individuals about 2 months old. The similar type of shrimp samples were also taken from the Java Sea in Jepara Indonesia. Samples were taken by observing the behavior and physical characteristics of the shrimp. Characteristics of shrimp showed that they move slowly towards the given response, were weak, and had a paler color than the normally helthy shrimps. Shrimp samples were carried to thelaboratory in ice boxex. Samples of 1000mL sea water and ponds were collected from shrimp ponds and sea water on the beach on the island of Bengkalis. Shrimp samples were washed, dried, and weighed. The weight was 46.4 grams and was homogenated into 417.6 ml of sterile sea water (10^{-1}). This mixture was diluted upto 10^{-3} dilution. The samples were then plated by the serial dilution method to TCBS media on petri dishes. Colonies that grew were reinoculated in new media. Each different colony obtained were reinoculated and purified in three replications using TSA media.

The observation was directly conducted to identify morphologically among others: the observation on cell shape, colony color, colony size and type of colony. In addition, biochemical tests were also conducted to the bacteria test. Morphology Test and biochemical tests, namely: Gram stain, growth on TSI gelatin Medium, Catalase Test, oxidase test, Methyl Red test.

Polymerization Chain Reaction condition were : initial denaturation at 94^{o}C for 2 minutes, annealing at 50^{o}C for 40 second, polymerization at 72^{o}C for i minute, denaturation at 94^{o}C for 1 minute, 30 cycles. Sequencing of PCR samples were carried out by the ABI 3130 XL Genetic Analyzer Applied Biosystems. Mega BLAST analysis was carried out using the programs at www.ncbi.nlm.nih.gov, was done, The sequences were also analyzed and allign using the program package Clustal X, Genedoc, TreeView and Bioedit. The results were presented by descriptive analysis in the form of tables and figures. To obtain dendogram, NJ program was used in Clustal X with a level of 100 x bootstrap.

Results and discussion

The sample of black tiger shrimp (*P. monodon*) were taken from ponds in Bengkalis, Riau, Sumatra and BPPT pond in Jepara Java Indonesia. Isolation of *Vibrio* from shrimp, pond water and sea water using TCBS resulted in 7 pure *Vibrio* isolates with respect to color, shape and size of the colony. To make it easier to identify during the study, those seven isolates were coded A, B, C, D, E, F, and G. In the next culturization, the media is gelatin TSA Merck. The seven isolates were studied; all colonies of bacterial isolates are comma-shaped (Table 1).

Table 1. Results of Staining, Morphological and biochemical observations

Isolate	Gram	Cell shape	Colony color	Motil	Oksi dase	Katal ase	Metil red	Glu kosa	Suk rosa	H2S	Kons. DNA (ng/µl)
A	-	comma	Yellow	+	+	+	+	+	-	+	108,43
B	-	comma	Blue Green	+	+	+	-	+	+	+	115,56
C	-	comma	Yellow	+	+	+	+	+	-	+	106,99
D	-	comma	Green	+	+	+	+	+	+	+	62,87
E	-	comma	Blue Green	+	+	+	-	+	-	-	62,87
F	-	Comma	Yellow	+	+	+	-	+	+	+	69,92
G	-	comma	Yellow	+	+	+	-	+	+	+	83,91

All Vibrio isolates showed oxidase and catalase positive. Tests using the medium for TSI to show the occurrence of glucose and sucrose fermentation and H2S gas by observing the color on for tilt and to erect. All isolates were positive, while for sucrose and glucose, A, C and E are negative and the rest were positive. Overall characteristics of each strain can be seen in Table 1.

Concentration of genomic DNA from each isolate that has been extracted can be known. This concentration compares the extracted DNA in units of micro-nano-grams per 1 liter of water, ranging between 62.87 ng / ug to 115.56 ng / ug, which represents the highest concentration and lowest concentration of D isolates are isolates H and I (Table 1).

Using DNA marker 1 Kb Ladder, all DNA fragments are visible on agarose gel size of 1500 bp. The size is consistent with the expected size of 16S rDNA genes of bacteria that is between 1500 to 1600 bp (Lusiano, 2007).

Electrophoresis results showed seven DNA fragment from Vibrio isolates that could continue through the process of sequencing, using primers 8F, 765R, and 1114R, performed in one direction as much as one-time use at every primary. The length of the DNA bases A obtained at 526 bp, 1015 bp D, E, 420 bp, 1020 bp H, and I 1020 bp.

The sequences obtained were submited to the GenBank in order to gain access number and get the code according to the desired strain by the researcher.

Table 2. BLAST and Submit to the GenBank

Isolate	Bacteria	Strain	Access Code	Homology (%)
A	*Vibrio alginolyticus*	FNS A08	FJ404761	98
B	*Vibrio parahaemolyticus*	FNS C08	FJ404763	99
C	*Vibrio harveyi*	FNS B08	FJ404762	98
D	*Vibrio shilonii*	FNS D08	FJ404764	98
E	*Vibrio vulnificus*	FNS E08	FJ404765	98
F	unculture bacterium 1	FNY F08	EU854942.1	93
G	Unculture bacterium 2	FNY G08	GQ075650.1	94

The result of BLAST (Basic Local Alignment Search Tool) via http / / www.ncbi.nlm.nih.gov/, showed that the five strains were *Vibrio alginolyticus, Vibrio parahaemolyticus, Vibrio harveyi, Vibrio shilonii,* and *Vibrio vulnificus*. It is believed that having sequence similarities exceeding 97% of the World Bank is in the genes (Table 2). Meanwhile, two other strains already believed to be the *Vibrio* sp, but the species is unknown. Strain the sixth has 93% similarity with the bacterium Vibrio 6G2. Meanwhile, the seven strains have percentage 94% homology with several candidates uncultured bacterium called bacteriu. Isolate the seven who had a top score is uncultured bacterium 16S ribosomal RNA clone nbw171g06c1 partial gene sequences. Based on information obtained by accessing the code indicates that the bacteria with access code is not the bacterium *Vibrio* sp but rather a species of bacteria obtained from sediment. But the morphology and biochemical test results showed that isolates the seventh is a bacterium of the genus *Vibrio* (Table 1), and supported also by this strain can body on TCBS medium, which is a specific media bacterium *Vibrio* sp.

It is suspected that the two strains are *Vibrio* species native to Indonesia which is not in the genes World Bank. This is confirmed by Hagstrom *et. al.,* (2000) stated that isolates which have 16S rDNA sequence similarities greater than 97% may represent the same species. While the sequence similarities between 93% -97% can represent identity at the genus level but different at the species level.

Phylogenetic tree makes connecting the branching points (nodes), which is the taxonomic unit, such as species or genes, while the tree roots are the points that act as a (common ancestor) for all organisms under consideration (Pramana, 2007).

Allignment sample sequences with sequences from Gen Bank data base performed using the program Clustal X. To obtain a NJ phylogenetic tree used in the Clustal X program with a level of 100 x bootstrap, and then results in the form of phylogenetic trees can be seen on the program TreeView. The phylogenetic tree is useful to show the phylogenetic relationship of each species that visits based on molecular characteristics between species and among strains within the same species. Phylogenetic tree of several species of Vibrio bacteria can be seen in the picture below.

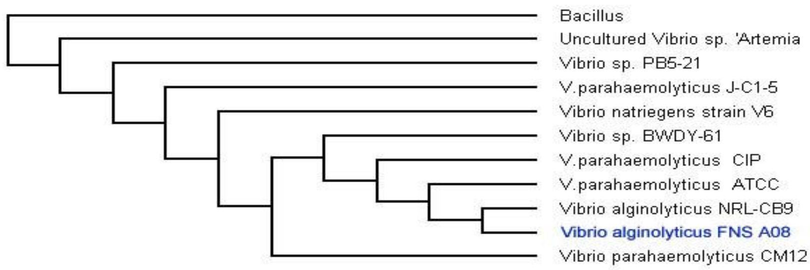


Fig 1. Phylogenetic tree Vibrio alginolyticus FNS A08

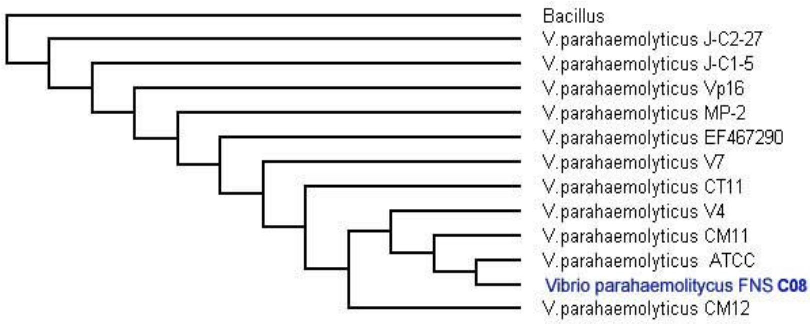


Fig 2. Phylogenetic tree Vibrio parahaemolyticus C08 FNS

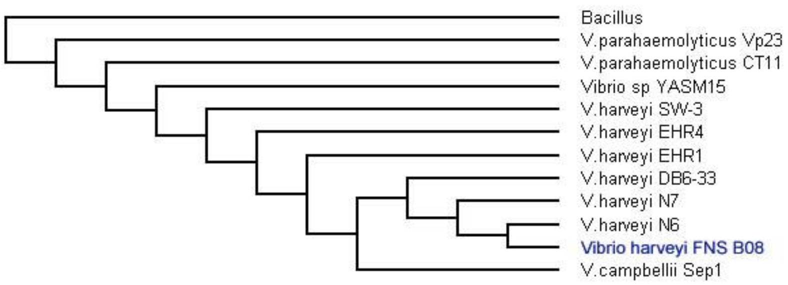


Fig 3. phylogenetic tree Vibrio harveyi FNS B08

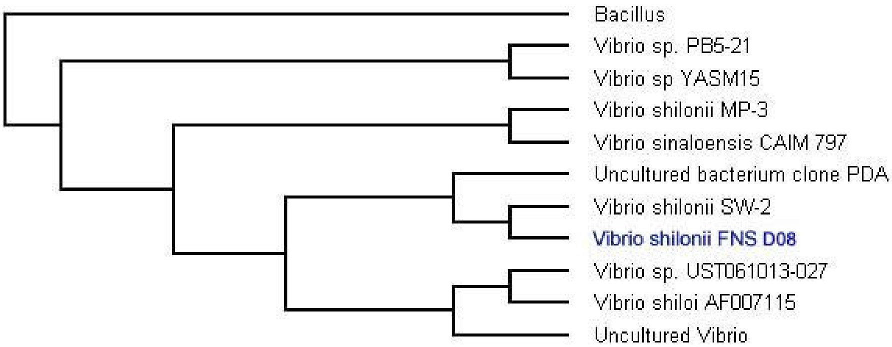


Fig 4. Phylogenetic tree *Vibrio shilonii* FNS D08

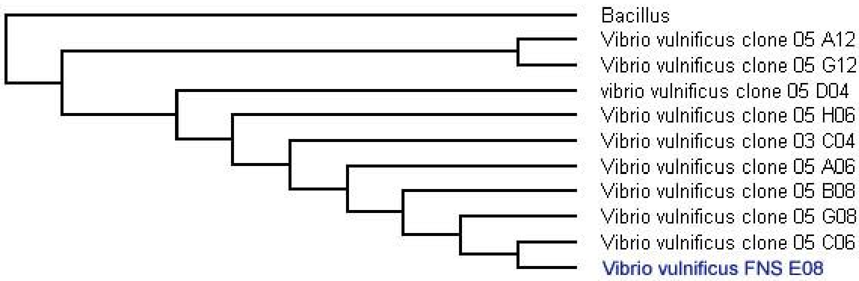
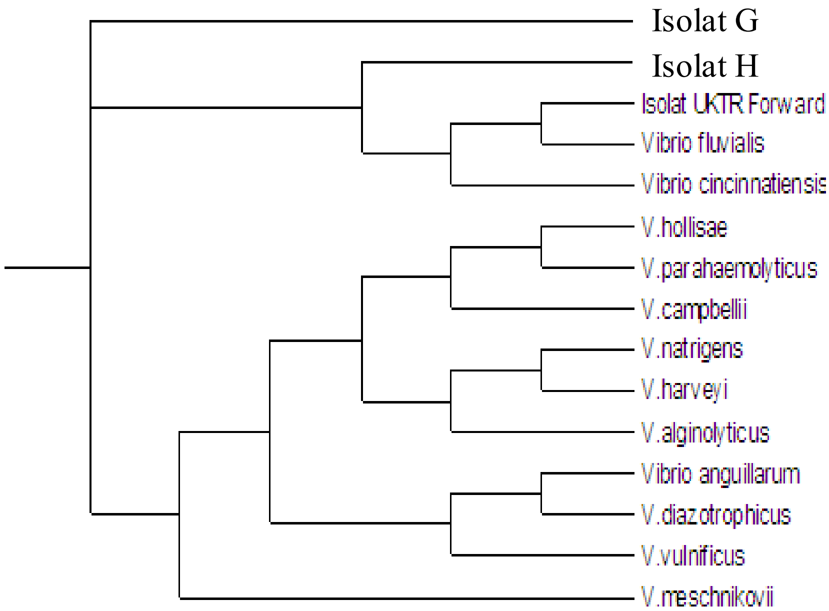


Fig 5. Phylogenetic tree *Vibrio vulnificus* FNS E08



Of the seven isolates, it could be seen from the phylogenetic relationship of the bacterium Vibrio sp, that five isolates was confirmed as existing isolate, but isolates G and H from the sequensing and philogenetic tree indicates tha they are not from reported bacteria Vibrio sp. Since results of morphology and biochemical test showed that the isolates G and H are bacteria of the genus Vibrio, it may therefore be provisionally concluded that isolates G and H aremost likely new species of *Vibrio*.

Based on the results of molecular identification, *V. alginolyticus* FNSA08 were obtained with 98% homology level with a base length of 526 bp and FNS strain A08. These bacteria were Gram negative, catalase positive, oxidase positive and included in motile bacteria.

V. alginolyticus growth is characterized by swarm on solid non-selective media. Another feature is, fermentation of glucose, lactose, sucrose, and maltose, forming a column measuring 0.8 to 1.2 cm in the yellow on TCBS media. Lethal toxin produced by *V. alginolyticus* strains isolated from sick kuruma shrimp (*Penaeus japonicus*) purified by Fast Protein Liquid Chromatography with hydrophobic interaction and gel filtration columns. The toxin is an alkaline serine proteases, showed maximum activity at pH 8 to 11 (Liu, and Lee, 1999).

V. parahaemolyticus FNS strain C08 has a characteristic blue to greenish-colored colonies, have the nature of Fermentative, glucose, lactose, sucrose and gas production is positive. Meanwhile, methyl red and H2S negative. Our isolated strain had 99% homology to the GenBank reported strain. *V. parahaemolitycus* has a diameter of 3-5 mm, greenish-blue colony color, dark green colony center, has many flagella (Richie, 2005). Bacteria V. parahaemolitycus is halophilic gram-negative bacteria are distributed in tropical coastal waters worldwide and causes gastroenteristis (De Paola *et al.,* 1998).

Thermostable direct haemolysin (TDH), is a major virulence factor of *V. parahaemolyticus*, was not toxic when heated at a temperature of 60-70 0C, but re-toxic when heated higher than 80 0C. Opposite phenomenon is known as the Arrhenius effect, been reminded of events that remain unexplained for 100 years. This suggests that this effect is associated with structural changes in proteins that produce fibrils (Fukui *et al.,* 2005).

V. harveyi B08 FNS has characteristic yellow colonies on TCBS media, have the nature of Fermentative, methyl red, glucose and sucrose positive. While lactose negative and H2S gas production. Based on the results of molecular identification, the bacteria isolated from our study had 98% homology levelto the GenBank strain with a base length of 420 bp.

Liu and Lee (1999) stated that the cysteine protease is a substance produced by pathogenic luminous bacteria *V. harveyii* 820 514, isolated from sick tiger shrimp (*Penaeus monodon*). Protease lethal to *P. monodon* with LD50 levels from 0.3 g protein pergram shrimp. Further stipulated that the cysteine protease is the main toxin produced by this bacterium. This protease is a cysteine protease first toxin found in Vibrio. In addition, *V harveyi* VIB 645, highly pathogenic to the species of salmon and extraseluler products with a high level of hemolytic activity towards fish erythrocytes, was found to contain two closely-related hemolysin genes (vhhA and vhhB). While the majority of the tested strains contained only a single hemolysin gene (Zhang *et. al.,* 2001).

Vibrio shilonii have the characteristic green colonies on TCBS media, have the nature of Fermentative, methyl red, glucose, lactose and sucrose positive. While the production of gas and H_2S negative. Based on the results of molecular identification, the bacteria, islate DO8 FNS had 98% homology level with the GenBank strain.

The model system of coral bleaching by bacteria has been studied extensively. Every summer, at least during the last 12 years, approximately 70% of reefs have shown the occurrence of bleaching. Organisms that cause coral bleaching is *V. shilonii*. These pathogens bind galactoside-containing receptor cells in the coral mucus, and then penetrate the rock layer, where bacteria grow, and reaching > 108 bakteri/cm3 network. *V. shilonii* produce a toxin (PYPVYAPPPVVP) that blocks intracellular zooxanthellae photosynthesis. In the winter, when seawater temperature drops below 20 ° C,

V. shilonii can not survive in the host coral and reefs to recover (Koren and Rosenberg, 2006).

Coral bleaching caused by infection of specific bacteria (as a refutation of the opinion of the influence of environmental stress) occurs when the zooxanthellae lost due to the influence of toxin produced by bacterial pathogens. Coral bleaching by bacteria occurs in the Mediterranean sea on scleractinian coral *Oculina patagonica* by *V. shilonii* (pathogens) and on Indian Ocean and the Red Sea on sea coral *Pocillopora damicornis* by the pathogen *Vibrio coralliilyticus* (Haim, 2002).

V. vulnificus has the characteristic blue to green colonies on TCBS media, have the nature of Fermentative and glucose positive. While methyl red, lactose, sucrose, production of gas and H_2S negative. From the molecular identification, the bacteria, we isolated (E08 FNS) had 98% homology level with the type strainreported in GenBank.

Bacteria *V. vulnificus* blue to green on TCBS, there are few who grow swarming (bacterial colonies grow over the entire surface of the flat (flat). Diameter of colonies reached 2-3 mm, grow well on TCBS medium at 37 0C (Prajitno, 1995). During infection, *V. vulnificus* reach the intestine and then attack the blood flow to penetrate the intestinal mucosal wall of the host resulting in septicemia. Lee *et. al* (2008). found that the toxin RtxA produced by *V. vulnificus* contributes to the cytotoxicity against intestinal epithelial cells.

The results showed that seven bacterial isolates from Bengkalis (sumatera Island) and Jepara (Java island) infected infected black tiger shrimp (*P. monodon* identified using analysis of 16S rDNA sequences, five strains were identified as *Vibrio alginolyticus, Vibrio parahaemolyticus, Vibrio harveyi, Vibrio shilonii,* and *Vibrio vulnificus,* with levels above 97% homology to reported GenBank sequences. The two other strains sequences are *Vibrio* strains sequences that have not reported in GenBank. It is believed that these two strains are Indonesian indigenous *Vibrio* species. More indepth study us needed to verify this.

Further studies are needed to determine the toxic levels and ability to harm caused by these two indigenous *Vibrio* species to the shrimps. Future studies are also required to understand how to prevent and understand the growth and infection of these bacteria to black tiger shrimp cultivation.

Acknowledgements

The autors thanks to general director higher education, director of reseach and community service Republic of Indonesia , for financially supporting this research.with contract :No142/SP2H/PP/DP2M/IV/2009 and No. 369/SP2H/PP/DP2M/IV/2010

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(Received 9 January 2011 ; accepted 1 April 2011)

Appendix. 16S rDNA gene sequences

Isolate	Sekuen Gen 16S rDNA
A	**5’** TGGAGAGTTTGATCCTGGCTCAGATTGAACGCT GGCGGCAGGCCTAACACATGCAAGTCGAGCGGAAACGAGTTATCTGAACCTTCGGGG AACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGAT GTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAG AGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGT GAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCAC ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA CAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGT AAAGCACTTTCAGTCGTGAGGAAGGTGTTAATAGCATTTGACGTTAGCGACAGAAGAA GCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA **3’**
B	**5’** AGAGTTTGATCCTGGCTCAGATTGAACGCTGGC GGCAGGCCTAAGACATGCAAGTCGAGCGGAAACGAGTTATCTTAACCTTCGGGGAACG ATAACGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGG GATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGA CCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAG GGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAC TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG CAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTT CAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATTTGACGTTAGCGACAGAAG AAGCACCGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATC GGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCG GGGCTCAACCTCGGAATAGCATTTGAAACCTGCAGACTAGAGTACTGTAGAGGGGGGT AGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAAT **3’**
C	**5’** TTTGGAGAGTTTGATCCTGGCTCAGATTGAAC GCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAAACGAGTTATCTGAACCTTCGG GGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGA TGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATGCCTACGGGCCAAAG AGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTG AGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACA CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACA ATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAA **3’**
D	**5’** AGAGTTTGATCCTGGCTCAGATTGAACGCTGG CGGCAGGCCTAACACATGCAAGTCGAGCGGAAACGAGTTAACTGACCCTTCGGGTGACGTT AACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCTGATGTGGGGGATA ACCATTGGAAACGATGGCTAATACCGCATAATGCCTTCGTGCCAAAGAGTGGGACCTTAGG GCCTCTCGCGTCAGGAGATGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAA GGCGACGATACCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAGTGAGACACGGTCC AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCC ATGCCGCGTGTGTGAAGATGGCCTTCGGGTTGTAAAGCACTTTCAGCAGTGAGGAAGGCGG GTACGTTAATAAGTGCTCGTTTGACGTTAGCTGCAGAAGAAGCACCGGCTAACTCCGTGCCA GCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATG CAGGTGGTTCGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACTGCATTTGAAAC TGGCGGACTAGCGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGCAATGCGTAGA GATCTGAAGGAATAC **3’**
E	**5’** CAGGCCTAACACATGTAAGTCGAGCGGCAGC ACAGAGAAACTTGTTTCTCGGGTGTCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTG CCCTGATGTGGGGGATCACCATTGGAAACGATGGCTAATACCGCATGATGCCCTTGATT ATAATGAACAGGAGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGAAATGCCCA GGCGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGT CTGAGAGGATGATCAGCCACACTGGAAGTGAGACACGGTCCAGACTCCTACGGGAGGC AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTG AAGAGGGCCTTCGGGTTGTAAAGCACTTTCAGTTGTGAGGAAGGGGGTGTTGTGAATA GCAGCATCATTTGACGTTAGCAACAGAAGAAGCACCGGCGAACTCCGTGCCAGCAGCC GCGGTAATACGGAGGGTGCGAACGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAG GTGGTTTGTTAAGTCACATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTGAGAC TGGCAAACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGTAATGCGT AGAGATCTGAAGGAATACCGGTGGCGAAGGCGTCCCCCTGGACAGATACTGACACTCA

	GATGCGAAAGCGTGGGGAGCAAACAGGTTTGCGAAGACGCAGGTGTGCCTTCGGGAGC TCTGAGACAG **3'**
F	5'TGCCATACGGNCCAAATGGGGGGACCTTCNGGCCTCTCCNCGTCAGGATATGCCTAAGTA GTGCGATTAGCTGAGTTGGNGAGGNAATGGCTCACCATATGGCACNATCCCTCAGCTGGTCT GACCAGGATGATGACCCCACNGGAACTGATACACGCTNCANACATCCTACGGAGAGGCCAC CTAGCGCGGGGAGATATTGCACAATGGGCACANCTGATGCTACCCTTGCCGCGTGTGATGAA TAAAGGCCTCTCGGGTTAGATAAAGCCACTTTCAATCTTGAGGCAAGGCTAGGCGTAGTTAA TACCTGCGTTACTTGACGTTCANCGANATAACAGGCACCGAGCCTAACATCCGTAGCCAGCA CCCTTCAGTAATACGGTAGAGTCCAGNGTTCATCNNAATTACTGGNCCTTAAAACNNATGCA TGTGNATTTGTTAAATACAATATGTGAAAANCCCGGGGTCTCAACCCTCGAAAATCACCTAT TTGA'3 3'AAATTTCCCGCGGGCATTCTGATCCAATTNTCCTAGCGAAGGAAAACTTCATGGAGTTTTN CCTGCAGACCTAAATACGGACTGGGAGCGCCTTTTTGGGGATTGGGTCACTATCTTTCCCTTG CTGCCTTCGGGTATGCGCCCTTGTAGCAGGGGGGTAGCCCTACTCGGAAGGGCCATGATGAC TTGACGTCCTCCCCACCTTCCTCCGGTTTATCACCGGGAGTCCTCCTGGAGTTCCCGACATTA ATCGCTGGCAAACAGGGATAAGGGTTGCGCTCGTTGCGGGAATTAACCCAAAATTTCGCAA CACGAGGTGACTACCTCCATGGGGCACCTGTTCTCAAAGTTCCCAAAGGCAAGAATCCATCT CCGGACTCCCCAGGCGGATCTAATTTAACGCGTTACTCCAGAAAGCCCCGGGCTCAAGGCCC CCAACCCTCCAAGATATACATGGTTTTAAGGGGTGGGACTACCCAGGGGTATCTAAACTCTG TTTTGCCCCCCAAAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAA ATTAAAACACATGCTTCCCCCGCTTTGTGCGGGCCCCCGTCAATTCATTTTGAGTTTTATACT TGCGACCGGTTAAGCTTTTCGGCATTCTGAAATGTTGAATATTCGGGGTCTAGGGGGGCCGT CCTTCCACCACGGGGGTAGTTCCTTTAAAATACCAAAAACCCATTTCCCCCCGCCTCCTCCCT GGAAATATTTGACCCCCCCTCCTGAGAGTGAATTCTAAAGGGTGCTGAGGTTTCAAAAGGGT TAAATCTCGCGAGGGGTGTAAGCCCCCGGCGGCTTATTTCCCACTCACGGAATTTAAAAAAA ACCCAACCTCGG[1]GGGGGGGGCCTTTTCAG'5
G	3'CGTTCCCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATATAGTCGAGTTGC AGACTACAATCCGAACTGAGAACAACTTTATGGGATTTGCTTGACCTCGCGGTTTCGCTGCC CTTTGTATTGTCCATTGTAGCACGTGTGTAGCCCAAATCATAAGAGGGCATGATGATTTGAC GTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCAACTTAGAGTGCCCAACTTAATGATG GCAACTAAGCTTAAGGGTTGCGCTCGTTGCGGGACTTAAACCAACATCTCACGACACGAGCT GACGACAACCATGCACCACCTGTCACTCTGTCCCCCGAAGGGGAAAACTCTATCTCTAGAGG GGTCAGAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTC CACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTACTCCCCAGGCGG AGTGCTTAATGCGTTAGCTGCAGCACT'5