APPLICATION OF REPETITIVE SEQUENCE-BASED PCR ON THE RICHNESS OF VIBRIO ON THE TIGER SHRIMP (Penaeus monodon Fab.)

Sarjito¹, Nesti E. W. Ningrum², Ocky Karna Radjasa³ and Slamet Budi Prayitno¹

¹Aquaculture Study Program, Fisheries Department, Fisheries and Marine Science Faculty, Diponegoro University, Semarang 50275, Central Java, Indonesia
²Mandiri Bank, Jakarta Office Branch
³Marine Science Study Program, Fisheries Department, Fisheries and Marine Science Faculty, Diponegoro University, Semarang 50275, Central Java, Indonesia

Received: January, 5, 2012; Accepted: June, 5, 2012

ABSTRACT

A molecular-based study was conducted to estimate the richness of the Vibrio on tiger shrimp (Penaeus monodon Fab.) from brackish water pond of Mororejo, Kendal. Tiger shrimps were collected from the extensive brackish water pond and a total of 22 isolates were obtained from hepatopancreas and telson of tiger shrimp. Based on the repetitive sequence-based polymerase chain reaction (rep-PCR), it was found that two groups of Vibrio. To investigate the effectiveness of rep-PCR in estimating the richness of Vibrio on tiger shrimps, three isolates (JTW 01, JTW 03 and JTW 06) were chosen for further investigation. On the basis of sequence analysis, the result showed that the JTW 01, JTW 03 and JTW 06 were closely related to Vibrio sp. Absa7 clone 423.1, Vibrio splendidus and Vibrio splendidus, respectively. The result proved that two associated of Vibrio on tiger shrimp were Vibrio sp. Absa7 clone 423.1 and Vibrio splendidus. Therefore the present study highlights the effectiveness of rep-PCR in rapid grouping and estimating the richness of Vibrio on tiger shrimp.

Keywords: Rep-PCR; Vibriosis; Causative Agent; Penaeus monodon Fab

*Correspondence: Phone: +62-24-7474698; E-mail: sarjito_msdp@yahoo.com

INTRODUCTION

Tiger shrimp (Penaeus monodon Fab.) is a potential fishery commodity which has high economic value in domestic and international markets. While demanding of shrimp in the world is increasing, shrimp production is decreasing every year. Declining of shrimp production partly due to disease caused by bacterial disease like Vibriosis.

Vibriosis is a serious problem in the majority of penaeid shrimp culture operations. Vibrio species are a normal part of the bacterial flora in aquatic environments and formerly considered to be mostly opportunistic pathogens (Lightner, 1996; Myers, et al., 2006; Thompson, et al., 2003). However, some more occurring disease syndromes of penaeid shrimp have been caused by Vibrio species which behave more like true pathogens than opportunistic invaders (Gomez-Gil, et al., 2004; Kannaripan, et al., 2008). Vibriosis is the main cause of production loss due to bacterial disease in penaeid shrimp farms (Kannaripan et al., 2008). Vibriosis causes mortality in larvae, postlarvae, juveniles, sub-adults and also adults of shrimps. Outbreaks of the disease cause mortality up to nearly 100% of affected population (Sumaryanto and Mariyam, 1987). The gross signs of localized infection in the cuticle or sub-cuticle are called shell disease or black or brown spot disease and these superficial infections can develop into systemic infections under some circumstances. It is the systemic infections that cause mortality (Chen et al., 1992; Myers, et al., 2003; Suddess and Xu, 2001.)

Recently, many reports have also been published on the 16S rRNA gene sequences of
bacteria and the phylogenetic relationships deduced from analysis of these sequences (Radjasa, et al., 2001; Radjasa, et al., 2007a; Sabdono, 2001; Sarjito, et al., 2009). Most of the results indicate that phylogenetic relationships based on 16S rRNA sequences support the distinction of species among eubacteria, archaeobacteria, and eukaryotes (Radjasa, et al., 2001; Sabdono, 2001). Because of this feature, Cunningham (2002) suggested to use molecular methods for diagnostic of fish diseases. The molecular methods using 16S rDNA for detection of causative agent of Vibrios have been conducted in Turbot, Colistium nudipinnis, (Diggles, et al., 2000; Montes, et al., 2006), Brill, C. Guntheri, (Diggles, et al., 2000), Spotted Rose Snapper, Lutjanus guttatus, Steindachner, 1869 (Gomez-Gil, et al., 2004), groupers (Sarjito, et al., 2008; Sarjito, et al., 2009) and white shrimps, Litopenaeus vannamei, (Sarjito et al., 2011).

Various molecular methods have been applied on Vibrio, such as: RAPD (Somarny et al., 2002) and rep-PCR (repetitive sequence based polychain reaction) (Sarjito, 2011). Rep-PCR was conducted in order to group a bacterial number isolates that produced complex fingerprint profiles from gram negative bacteria. Furthermore, rep–PCR has been applied on various diversity of associated sponge bacteria (Radjasa, et al., 2007a,b), psychrotrophic bacteria from Makasar straight (Radjasa, et al., 2007c) and causative agent of Vibrios (Sarjito, et al., 2009). However, to best our knowledge, there has limited report been documented so far on describing the application of rep-PCR on the richness of Vibrio on tiger shrimp from Indonesian extensive brackish water pond using molecular tools. The research regarding the diversity of Vibrio as the causative agent of vibriosis is important for creating health management of tiger shrimp culture. In this study, we reported the richness of Vibrio bacteria on tiger shrimp from extensive brackish water pond of Kendal, Central Java assessed by 16S rDNA approach.

**MATERIALS AND METHODS**

**Sampling of Tiger Shrimp**

The shrimps were collected from extensive culture in extensive brackish water pond of Mororejo village, Kendal Regency, Central Java, Indonesia and was identified as tiger shrimp (*Penaeus monodon* Fab.). After collection, tiger shrimps were put into the plastic containers and immediately brought to the integrated Marine Science Laboratory of Fisheries and Marine Science Faculty, Diponegoro University in Semarang, Central Java for bacterial isolation.

**Bacterial Isolation**

Bacteria Vibrio were isolated directly from hepatopancreas and telson of tiger shrimps by streak method on TCBS medium. Bacterial isolation was also conducted from the inner part of hepatopancreas and telson, which were scraped off with a sterile knife. The resultant tissues were serially diluted, spread on TCBS agar medium and were incubated at room temperature for 24-48 hours. On the morphological features, colonies were randomly picked and purified by making streak plate (Brock and Madigan, 1991).

**Repetitive – PCR**

The procedure was carried out according a method previously described by Radjasa et al. (2007b). In the rep–PCR, BOX AIR (5'-CTACgCgCgACgCTgACg-3') (Versalovic et al., 1994) was used. The REP 1R-I and REP 2-I primers contain the nuclutide inosine (I) at ambiguous potitions in the REP consensus. PCR reaction contained of 1 μL DNA template (diluted 100x), 1 μL primer, 7.5 μL Megamix Royal and sterile water up to total volume of 15 μL.

Amplifications were performed with a thermal cycler model Gene Amp PCR system 9700 with the following temperature conditions: initial denaturation at 95°C for 5 minutes ; 30 cycled of denaturation (92°C for 1 minutes), annealing (50°C for 1,5 minutes), extension (68°C for 8 minutes) and final extension at 68°C for 10 minutes. Five microliter aliquot PCR products were run using electrophoresis on 1 % ethibium bromade gel by using 1X TBE buffer.

**Grouping of Isolates**

Grouping was carried out according to a method of Radjasa, et al., (2007c) by making matrixes from the position of bands on the gel which were there analyzed by using Free Tree program by using UPGMA method for constructing the tree.
Resampling was performed by bootstrapping with 1000 replications.

**PCR Amplification and Sequencing of 16s rRNA Gene Fragments**

PCR amplification was carried out according to method of Radjasa et al., (2007a). Two primers, GM3F (5’AGAGTTTGATCMTGGC-3’) and GM4R (5’-TACCTTGTTACGACTT-3’) were used to amplify nearly complete 16S rRNA gene. Genomic DNA of causative agent of vibriosis strains for PCR analysis were obtained from cell materials taken from agar plate, suspended in steril water (Sigma, Germany) and subjected to five cycles of freeze (-80ºC) and thaw (95ºC). PCR amplification of partial 16S rRNA gene of bacteria, purification of PCR products and subsequent sequencing analysis were performed according to the method of Radjasa, et al. (2007b). The determined DNA sequences of strains were then compared for homology to the BLAST database (Atschul, et al., 1997).

**RESULTS AND DISCUSSION**

**Results**

**Characteristic of the Bacterial Isolates**

The clinical signs of tiger shrimps affected by Vibriosis from extensive brackish water pond of Kendal Regency, Indonesia were body melanosis, white spot, pale abdomen, dark mouth and red color at telson an tail.

Bacterial isolation resulted in total of 22 Vibrio isolates (JTW 1 – JTW 22) obtained from hepatopancreas of tiger shrimp (Table 1).

**Repetitive-PCR Analysis**

Based on the repetitive-PCR result and constructed dendogram of the Vibrio as causative agent of vibriosis on tiger shrimp, two groups were formed (Fig.1).

<table>
<thead>
<tr>
<th>No</th>
<th>Isolate Code</th>
<th>Isolate Source</th>
<th>Colony Color on TCBS</th>
<th>Colony Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>JTW 1</td>
<td>Hepatopankreas</td>
<td>Green</td>
<td>Round</td>
</tr>
<tr>
<td>2</td>
<td>JTW 2</td>
<td>Hepatopankreas</td>
<td>Yellow</td>
<td>Round</td>
</tr>
<tr>
<td>3</td>
<td>JTW 3</td>
<td>Hepatopankreas</td>
<td>Black</td>
<td>Round</td>
</tr>
<tr>
<td>4</td>
<td>JTW 4</td>
<td>Hepatopankreas</td>
<td>Yellow</td>
<td>Round</td>
</tr>
<tr>
<td>5</td>
<td>JTW 5</td>
<td>Hepatopankreas</td>
<td>Shiny yellow</td>
<td>Round</td>
</tr>
<tr>
<td>6</td>
<td>JTW 6</td>
<td>Hepatopankreas</td>
<td>Yellow</td>
<td>Round</td>
</tr>
<tr>
<td>7</td>
<td>JTW 7</td>
<td>Hepatopankreas</td>
<td>Dark yellow</td>
<td>Round</td>
</tr>
<tr>
<td>8</td>
<td>JTW 8</td>
<td>Hepatopankreas</td>
<td>Dark yellow</td>
<td>Round</td>
</tr>
<tr>
<td>9</td>
<td>JTW 9</td>
<td>Hepatopankreas</td>
<td>Dark green</td>
<td>Round</td>
</tr>
<tr>
<td>10</td>
<td>JTW10</td>
<td>Hepatopankreas</td>
<td>Black</td>
<td>Round</td>
</tr>
<tr>
<td>11</td>
<td>JTW11</td>
<td>Hepatopankreas</td>
<td>Yellow</td>
<td>Round</td>
</tr>
<tr>
<td>12</td>
<td>JTW12</td>
<td>Hepatopankreas</td>
<td>Shiny green</td>
<td>Round</td>
</tr>
<tr>
<td>13</td>
<td>JTW13</td>
<td>Hepatopankreas</td>
<td>Dark yellow</td>
<td>Round</td>
</tr>
<tr>
<td>14</td>
<td>JTW14</td>
<td>Hepatopankreas</td>
<td>Black</td>
<td>Round</td>
</tr>
<tr>
<td>15</td>
<td>JTW15</td>
<td>Hepatopankreas</td>
<td>Green</td>
<td>Round</td>
</tr>
<tr>
<td>16</td>
<td>JTW16</td>
<td>Hepatopankreas</td>
<td>Yellow</td>
<td>Round</td>
</tr>
<tr>
<td>17</td>
<td>JTW17</td>
<td>Hepatopankreas</td>
<td>Black</td>
<td>Round</td>
</tr>
<tr>
<td>18</td>
<td>JTW18</td>
<td>Hepatopankreas</td>
<td>Yellow</td>
<td>Round</td>
</tr>
<tr>
<td>19</td>
<td>JTW19</td>
<td>Hepatopankreas</td>
<td>Shiny yellow</td>
<td>Round</td>
</tr>
<tr>
<td>20</td>
<td>JTW20</td>
<td>Hepatopankreas</td>
<td>Yellow</td>
<td>Round</td>
</tr>
<tr>
<td>21</td>
<td>JTW21</td>
<td>Hepatopankreas</td>
<td>Black</td>
<td>Round</td>
</tr>
<tr>
<td>22</td>
<td>JTW22</td>
<td>Hepatopankreas</td>
<td>Shiny yellow</td>
<td>Round</td>
</tr>
</tbody>
</table>
**Repetitive-PCR Analysis**

Based on the repetitive-PCR result and constructed dendogram of the Vibrio as causatived agent of vibriosis on tiger shrimp, two groups were formed (Fig.1).

![Diagram of Vibrio group based on the Repetitive Sequence-Based Polymerase Chain Reaction and 16S rDNA on the Tiger Shrimps.](image)

**Fig.1.** Diagram of Vibrio group based on the Repetitive Sequence-Based Polymerase Chain Reaction and 16S rDNA on the Tiger Shrimps.
**Sequencing of representative Vibrio as causative agent on tiger shrimps**

Based on molecular characterization it was showed that all isolates were the members of genus Vibrio as presented in Table 2.

<table>
<thead>
<tr>
<th>No.</th>
<th>Isolates</th>
<th>Close Relative</th>
<th>Homology (%)</th>
<th>Acc. Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>JTW 1</td>
<td>Vibrio sp. Absa7 clone 423.1</td>
<td>96</td>
<td>DQ357813.1</td>
</tr>
<tr>
<td>2</td>
<td>JTW 3</td>
<td>Vibrio splendidus</td>
<td>100</td>
<td>GQ254509.1</td>
</tr>
<tr>
<td>3</td>
<td>JTW 06</td>
<td>Vibrio splendidus</td>
<td>100</td>
<td>GQ254509.1</td>
</tr>
</tbody>
</table>

Based on the Fig. 2 and Table 2 showed that vibrio on tiger shrimp from extensive brackish water pond of Kendal regency was Vibrio sp. Absa7 clone 423.1 (JTW 01 - Groups I) and Vibrio splendidus (JTW 03; JTW 06 -groups II) with a homology of 96 % and 100 % respectively.

**Discussion**

It is widely known that the characterization and identification of bacterial isolates have traditionally been based on phenotypic traits, which are derived from biochemical test reactions. It is also noted that one disadvantage of these tests has been the requirement of strains to grow in order to produce a detectable reaction. The recent and rapid development of molecular biology techniques have been regarded as the solution to the problem of bacterial identification based on phenotypic approach.

In this work, a molecular biology approach based on 16S rDNA coupled with rapid grouping technique, rep-PCR was applied to estimate the richness of pathogens Vibrio associated with tiger shrimp from brackish waters of Kendal, Central Java.

Vibrio species are natural habitants of seawater and brackish water widely distributed throughout the world (Myers et al., 2003). However, some species have exhibited clinical significance for aquatic animal and are recognized as potential pathogens (Myers et al., 2006). The large number of Vibrio shrimp pathogens causing epizootic outbreaks in aquaculture has made it necessary to develop efficient, fast and sensitive methods for their detection. Both detection and identification of vibrios have been traditionally depend on their growth on Thio-Sulphate Citrate Bile Salt Sucrose (TCBSA) selective medium and subsequent characterization by biochemical test (Diggles et al., 2000).

Vibriosis in the tiger shrimps was characterized by melanosis of body, white spot, pale abdomen, dark mouth, red color at tail and lesion of the tail.

The result of this study revealed that the application of Rep-PCR has been a reliable tool for strain rapid grouping and differentiation / richness among the vibrio on the tiger shrimp in extensive brackish water pond of Kendal Regency. This molecular approach may be used for the analysis of other Vibrio species related to aquaculture disease. Molecular identification of Vibrio on shows that the identified strains were nicely in accordance with the dendogram constructed from the Rep-PCR analysis. The identify strains were Vibrio sp. Absa7 clone 423.1 (JTW 01) and Vibrio splendidus (JTW 03 and JTW 06). Vibrio sp. Absa 7 clone 42y 3.1. was identified by Schulze et al., (2006) on water of marine hatchery-abalone larvae from Vancouver Island waters, Canada. Whereas V. splendidus was reported by Panicker et al. (2004) on shell fish and water of Mexico Gulf.

In conclusion, the application of the Rep-PCR method is useful for rapid grouping and estimating of richness of vibrio as causative agents of vibriosis on tiger shrimp with high power and offers an alternative technique for grouping of numerous of marine bacterial isolates.

**ACKNOWLEDMENTS**

The authors thanks to Rector Diponegoro University for partial financial support under fundamental research (DIPA UNDIP No:
0363.0/023-04.2/XIII/2010 SK Rektor UNDIP
No : 134/SK/H/7/2010.

REFERENCES

Atschul, SF., T.L. Madden, A.A. Schaffer, J.
Zhang, Z. Zhang, W. Miller and D.J.
Lipman. 1997. Gapped BLAST and PSI-
BLAST: A New Generation of Protein
Database Search Programs. Nucleid
Acid Res. 25:3389-3402.

of Microorganisms. Prentice Hall,

Chen, S.C., S.L. Huang and G.H.
Kou. 1992. Studies on the Epizootiology and
Bacterial Infections in Cultured Giant
Tiger Prawns, Penaeus monodon
in Taiwan. In Disease of Cultured
Penaeid Shrimp in Asia and the United
States. pp.: 195 - 205.

Cunningham, C.O. 2002. Molecular Diagnosis
of Fish and Shellfish Diseases: Present
Status dan Potential Use in Diseases

Diggles, B.K, J. Carson, P.M. Hine, R.W.
Species Associated with Mortalities in
Hatchery-reared Turbot (Colistium
nudipinnis) and Brill (C. guntheri) in

Lightner, DV. 1996. A handbook of shrimp
pathology and diagnostic procedures for
disease of cultured penaeid shrimp.
World Aquaculture Society, Baton
Rouge, L.A. 35 P.

Gomez-Gil ,B., E.F. Avila, and F.G. Vargas.
2007. Vibrios of the Spotted Rose
Snapper Lutjanus guttatus Steindachner,
1869 from Northwestern Mexico. J.

Gomez-Gil, B., S. Soto-Rodriquez, A. Garcia-
Garca, R. Vazquez-Juarez, F.L.
Molecular Identification of Vibrio
harveyi Related Isolates Associated
with Diseases Aquatic Organisms
Microbiol., 150 : 1709-1777.

Kannaripan, E., Ravindran, J., R. Chandrasekar
and A. Kalaiarasi. 2008. Studies on
Luminous, Vibrio harveyi associated
with Shrimp culture system rearing
Penaeus monodon. Triveni Enterprise,
Lucknow, India.

Montes, M., R. Farto, J.M. Perez, S.P. Armada,
and T.P. Nieto. 2006. Genotype
Diversity of Vibrio Isolates Associated
with Turbot (Scopitalthmus maximus)

PCR detection of a newly emerged
pandemic Vibrio parahaemolyticus
O3:K6 pathogen in pure cultures and
seeded waters from the Gulf of Mexico
Appl Environ Microbiol., 69(4):2194-
200

Detection of pandemic Vibrio parahaemolyticus
O3:K6 serovar in Gulf of Mexico water and shellfish
using real-time PCR with Taqman
fluorescent probes, FEMS Microbiol

Rapid detection of Vibrio vulnificus
in shellfish and Gulf of Mexico water by
real-time PCR. Appl Environ Microbiol.,
70(1):498-507

Radjasa, O.K., H. Urakawa, K., Kita-
Characerization of psychotropic
bacteria in the surface and deep-sea
waters from northwestern Pasific
Ocean based on 16S ribosomal DNA

Radjasa, O.K., T. Martens, H.P. Grossart, T.
Brinkoff, A. Sabdono and M.Simon.
2007a. Antagonistic activity of a
marine bacterium Pseudoalteromonas
luteololacea TAB4.2. associated


Sabdono, A. 2001. Identifikasi dan Analisis Genetik Bakteri Karang Pendegradasi Senyawa Herbisida 2,4-Diklorofenoksi Asetat di Laut Jawa. Desertasi UGM. 162 hal. (In Indonesian)


Sarjito, 2011. Penggunaan Repetitive Sequence-Based Polychain Reaction (REP-PCR) untuk Pengelompokan Bakteri Vibrio yang Berasosiasi dengan Ikan Kerapu Sakit dari Perairan Karimunjawa. Ilmu Kelautan, 16(2) : 103 – 110. (In Indonesian)


