KORESPONDENSI PAPER

JUDUL : Application of Repetive Sequence-Based PCR on The Richness of Vibrio on The Tiger Shrimp (*Penaeus Monodon* Fab.) from Brackish Water Pond of Kendal, Central Java

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Prof. Ocky Karna Radjasa, Ph.D
Editor-in-Chief

APPLICATION OF REPETIVE SEQUENCE-BASED PCR ON THE RICHNESS OF VIBRIO ON THE TIGER SHRIMP (*Penaeus monodon* Fab.) IN BRACKISH WATER POND OF KENDAL

Sarjito[1] , Nestin E. W. Ningrum[1], Ocky Karna Radjasa[2] and S. Budi Prayitno[1]

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Abstract

A molecular-based study was conducted to estimate the richness of the causative agents associated with tiger shrimp (*Penaeus monodon* Fab.) from Mororejo extensive pond culture, Kendal, Central Java, Indonesia. Tiger shrimp were collected from the extensive pond culture and a total of 22 isolates were isolated from hepatopancreas and tail of tiger shrimp. Based on the repetitive sequence-based PCR (rep-PCR), three isolates were chosen for further sequencings. On the basis of sequence analysis, the data showed that the causative agents were closely related with *Vibrio* sp. Absa7 clone 423.1 and *Vibrio splendidus,* respectively. Present study highlights the effectiveness of rep-PCR in rapid grouping and estimating the richness of the causative agents of Vibriosis associated with the tiger shrimp.

Keywords: Rep-PCR, Vibriosis, Causative Agent, *Penaeus monodon* Fab

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, 1988). However, some more recently occurring disease syndromes of penaeid shrimp have been caused by *Vibrio* species which behave more like true pathogens than opportunistic invaders (Lightner *et al.,* 1992). In Thailand, vibriosis is the main cause of production loss due to bacterial disease in penaeid shrimp farms (Nash *et al.,* 1992). Vibriosis causes mortality in larvae, postlarvae, juveniles, subadults and also adults. At times, outbreaks cause mortality up to nearly 100% of affected populations (Lighner 1983). The gross signs of localized infection in the cuticle or subcuticle 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.

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 (Collins *et al.,* 1991). Most of the results indicate that phylogenetic relationships based on 16S rRNA sequences support the distinction of species among eubacteria, archaeobacteria, and eukaryotes. Because of this feature, the need for rapid diagnostic methods to identify aquatic environment and animal-borne pathogens makes the variable 16S rRNA regions attractive targets for synthetic oligonucleotide probes and PCR primers (Wayne *et al*., 1987). Thus, many laboratories have employed 16S rRNA-targeted hybridization and PCR amplification for the identification and detection of several marine bacteria (Heidelberg *et al.,* 1993). Moreover, because they are essential constituents of all living organisms, 16S rRNA genes are present in high copy numbers as essential and therefore, there is no problem with gene loss, which is a possibility for the cytotoxin–hemolysin gene (Brauns et al., 1991).

To best our knowledge, there has no report been documented so far on describing diversity of vibriosis, the causative agent of vibriosis associated with tiger shrimp from Indonesia culture using molecular tools. The research regarding the molecular diversity of vibriosis as the causative agent is important for creating health management of tiger shrimp culture. In this study, we reported the diversity of vibrioisis bacteria as a causative agent vibriosis isolated from tiger shrimp from Kendal traditional culture, Indonesia assessed by 16S rDNA approach.

MATERIALS AND METHODS

Sampling of Tiger Shrimp

The tiger shrimps were collected from traditional culture in Kendal District, Central Java, Indonesia and identify as tiger shrimp (*Penaeus monodon* Fab.). After collection, tiger shrimps were put into the cool containers and immediately brought to the Marine Laboratory of Fisheries and Marine Science Faculty, Diponegoro University in Semarang, Central Java for bacterial isolation.

Bacterial Isolation

Bacteria were isolated directly from hepatopancreas and telson of tiger shrimps by streak plate on TCBS agar (compotition (g L^{-1}); Peptone from casein 5.0; Peptone from meat 5.0; yeast extract 5.0; sodium citrate 10.0; sodium thiosulfate 10.0; ox bile 5.0; sodium cholate 3.0; sucrose 20.0; sodium chloride 10.0, iron (III) 1.0, thymol blue 0.04; bromothymol blue 0.004 and agar-agar 14.0).

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 plates (Madigan *et al,* 2000).

Repetitive – PCR

The procedure was carried out according a method previously described by Radjasa *et al.* (2007b). In the rep –PCR, BOX AIR (5'-CTACggCAAggCgACgCTgACg-3') (Versalovic *et al.,* 1994) was used. The REP 1R-I and REP 2-I primers contain the nuclutide inosine (I) at ambigious 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 95ºC for 5 minutes ; 30 cycled of denaturation (92°C for 1 minutes), annealing (50ºC for 1,5 minutes), extension (68ºC for 8 minutes) and final extension at 68ºC 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 (Muyzer *et al.,* 1995). Genomic DAN 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 e*t al.* (2007[b]). The determined DNA sequences of strains were then compared for homology to the BLAST database.

RESULTS

Characteristic of the Bacterial Isolates
The clinical features of tiger shrimps affected by vibriosis from Kendal Dictrict, Indonesia were white spot, pale abdoment, dark mouth and red color at telson.

Table 1. Characteristic of isolates associated with tiger shrimp form Kendal District, Indonesia.

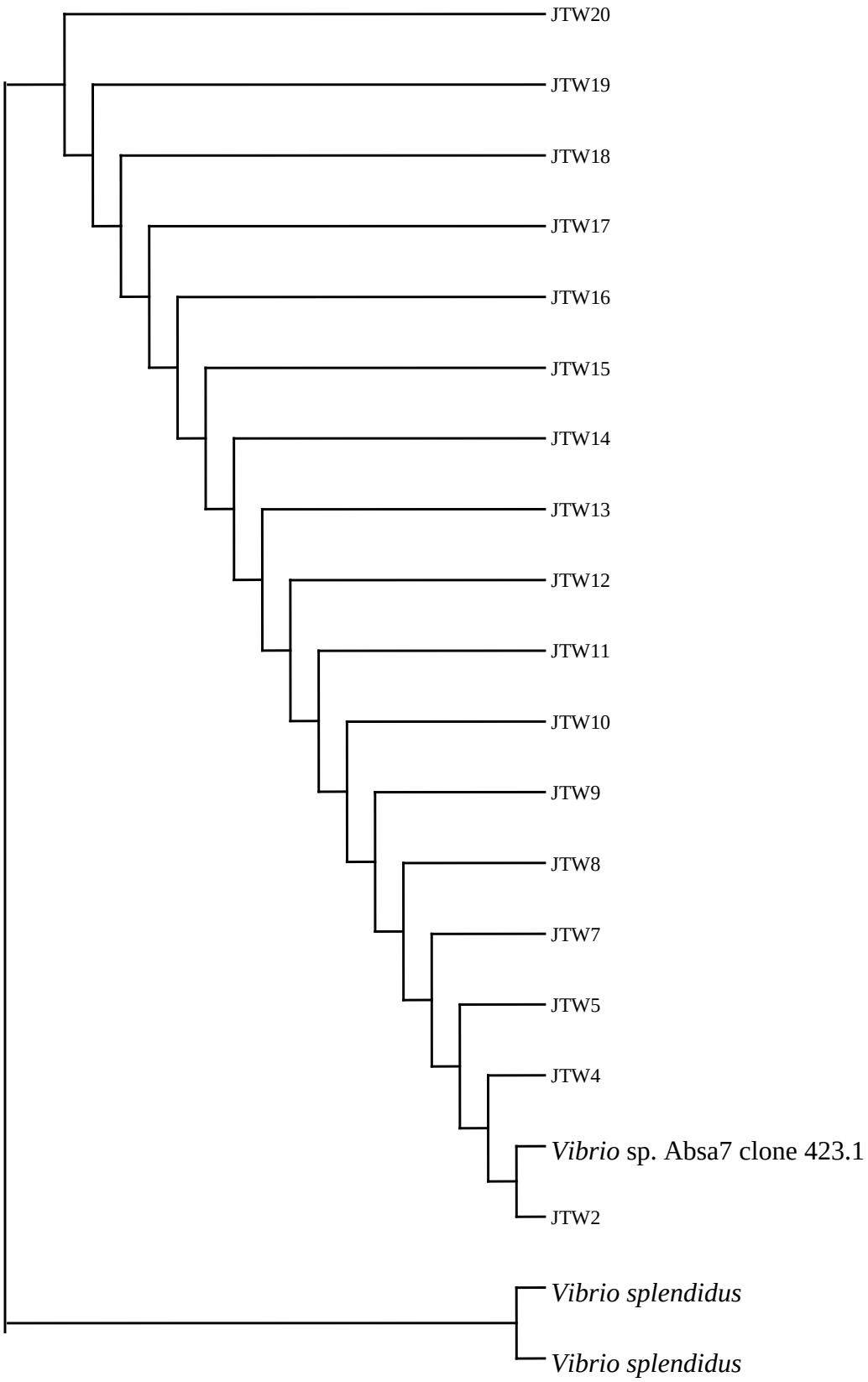
No	Groups	Colony Color on TCBS	Colony Form	Isolates Source	Isolates Code
1	*P. monodon*	Green	Round	Hepatopankreas	JTW1
2	*P. monodon*	Yellow	Round	Hepatopankreas	JTW2
3	*P. monodon*	Black	Round	Hepatopankreas	JTW3
4	*P. monodon*	Yellow	Round	Hepatopankreas	JTW4
5	*P. monodon*	Shinny yellow	Round	Hepatopankreas	JTW5

6	*P. monodon*	Yellow	Round	Hepatopankreas	JTW6
7	*P. monodon*	Dark yellow	Round	Hepatopankreas	JTW7
8	*P. monodon*	Dark yellow	Round	Hepatopankreas	JTW8
9	*P. monodon*	Dark green	Round	Hepatopankreas	JTW9
10	*P. monodon*	Black	Round	Hepatopankreas	JTW10
11	*P. monodon*	Yellow	Round	Hepatopankreas	JTW11
12	*P. monodon*	Shiny green	Round	Hepatopankreas	JTW12
13	*P. monodon*	Dark yellow	Round	Hepatopankreas	JTW13
14	*P. monodon*	Black	Round	Hepatopankreas	JTW14
15	*P. monodon*	Green	Round	Hepatopankreas	JTW15
16	*P. monodon*	Yellow	Round	Hepatopankreas	JTW16
17	*P. monodon*	Black	Round	Hepatopankreas	JTW17
18	*P. monodon*	Yellow	Round	Hepatopankreas	JTW18
19	*P. monodon*	Shiny yellow	Round	Hepatopankreas	JTW19
20	*P. monodon*	Yellow	Round	Hepatopankreas	JTW20
21	*P. monodon*	Black	Round	Hepatopankreas	JTW21
22	*P. monodon*	Shiny yellow	Round	Hepatopankreas	JTW22

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

Repetitive-PCR Analysis

Based on the repetitive-PCR result and constructed dendogram of the causative agent of the tiger shrimp, two groups were formed (Fig.1).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JTW5
JTW4
Vibrio sp. Absa7 clone 423.1
JTW2
Vibrio splendidus
Vibrio splendidus

Figure.1. Diagram of Group of Bacteria based on the Repetive Sequence-Based Polymerase Chain Reaction the 22 Isolates

Sequencing of Representative Causative Agents

Based on moleculer characterization it is showed that all isolates are the members of genus *Vibrio* as presented in Table 2.

Table 2. Molecular characterization of 3 representative of causative agents

No.	Isolates	Close Relative	Homology (%)	Acc. Number
1.	JTW 1	*Vibrio* sp. Absa7 clone 423.1	96	DQ357813.1
2.	JTW 3	*Vibrio splendidus*	100	GQ254509.1
3.	JTW 6	*Vibrio splendidus*	100	GQ254509.1

DISCUSSION

Vibrio species are natural habitants of seawater and brackish water widely distributed throughout the world (Sarjito *et al.*, 2009). However, some species have exhibited clinical significance for aquatic animal and are recognized as potential pathogens. The large number of vibrios fish 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 tradionally depend on their growth on Thio-SUlphate Citrate Bile Salt Sucrose selective medium and subsequent characterization by biochemical test (Diggles *et al.*, 2000).

The result of this study revealed that the application of Rep-PCR has been a reliable tool for strain rapid grouping and differentiation among the causative agents of vibriosis in tiger shrimp in Kendal District. This molecular approach may be used for the analysis of other vibrio species related to aquaculture disease.

Molecular identification of the causative agents of vibriosis shows that the identified strains were nicely in accordance with the dendogram constructed from the Rep-PCR analysis. The identify starins include of *Vibrio* sp. Absa7 clone 423.1. and *Vibrio splendidus.*

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

ACKNOWLEDMENTS

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review ms

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Date: Wednesday, March 7, 2012 at 09:13 AM GMT+7

To : Dr. Sarjito
Untuk diperbaiki manuskripnya. Mohon bisa diganti dengan referensi terbaru.
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Ocky

Ocky Karna Radjasa, Ph.D
Professor of Marine Microbiology
Department of Marine Science
Diponegoro University
Semarang 50275
Central Java
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MANUSCRIPT REVISION #1

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A molecular-based study was conducted to estimate the richness of the causative agents associated with tiger shrimp (*Penaeus monodon* Fab.) from Mororejo extensive pond culture, Kendal, Central Java, Indonesia. Tiger shrimp were collected from the extensive pond culture and a total of 22 isolates were isolated from hepatopancreas and tail of tiger shrimp. Based on the repetitive sequence-based PCR (rep-PCR), three isolates were chosen for further sequencings. On the basis of sequence analysis, the data showed that the causative agents were closely related with *Vibrio* sp. Absa7 clone 423.1 and *Vibrio splendidus*, respectively. Present study highlights the effectiveness of rep-PCR in rapid grouping and estimating the richness of the causative agents of Vibriosis associated with the tiger shrimp.

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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 95ºC for 5 minutes ; 30 cycled of denaturation (92°C for 1 minutes), annealing (50ºC for 1,5 minutes), extension (68ºC for 8 minutes) and final extension at 68ºC for 10 minutes. Five microliter aliquot PCR products were run using electrophoresis on 1 % ethibium bromade gel by using 1X TBE buffer.

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RESULTS

Characteristic of the Bacterial Isolates
The clinical features of tiger shrimps affected by vibriosis from Kendal Dictrict, Indonesia were white spot, pale abdoment, dark mouth and red color at telson.

Table 1. Characteristic of isolates associated with tiger shrimp form Kendal District, Indonesia.

No	Groups	Colony Color on TCBS	Colony Form	Isolates Source	Isolates Code
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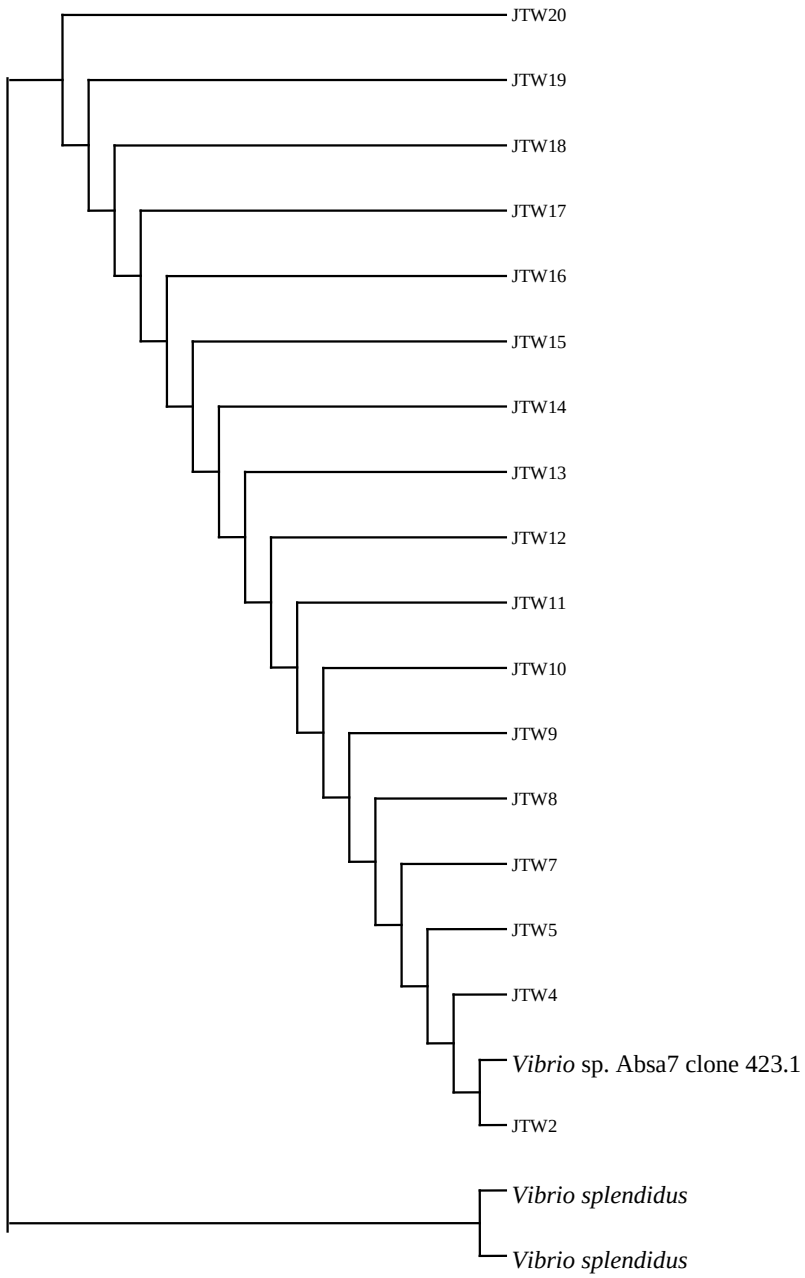
21	*P. monodon*	Black	Round	Hepatopankreas	JTW21
22	*P. monodon*	Shiny yellow	Round	Hepatopankreas	JTW22

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

Repetitive-PCR Analysis

Based on the repetitive-PCR result and constructed dendogram of the causative agent of the tiger shrimp, two groups were formed (Fig.1).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JTW14
JTW13
JTW12
JTW11
JTW10
JTW9
JTW8
JTW7
JTW5
JTW4
Vibrio sp. Absa7 clone 423.1
JTW2
Vibrio splendidus
Vibrio splendidus

Figure.1. Diagram of Group of Bacteria based on the Repetive Sequence-Based Polymerase Chain Reaction the 22 Isolates

Sequencing of Representative Causative Agents

Based on moleculer characterization it is showed that all isolates are the members of genus *Vibrio* as presented in Table 2.

Table 2. Molecular characterization of 3 representative of causative agents

No.	Isolates	Close Relative	Homology (%)	Acc. Number
1.	JTW 1	*Vibrio* sp. Absa7 clone 423.1	96	DQ357813.1
2.	JTW 3	*Vibrio splendidus*	100	GQ254509.1
3.	JTW 6	*Vibrio splendidus*	100	GQ254509.1

DISCUSSION

Vibrio species are natural habitants of seawater and brackish water widely distributed throughout the world (Sarjito *et al.*, 2009). However, some species have exhibited clinical significance for aquatic animal and are recognized as potential pathogens. The large number of vibrios fish 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 tradionally depend on their growth on Thio-SUlphate Citrate Bile Salt Sucrose selective medium and subsequent characterization by biochemical test (Diggles *et al.*, 2000).

The result of this study revealed that the application of Rep-PCR has been a reliable tool for strain rapid grouping and differentiation among the causative agents of vibriosis in tiger shrimp in Kendal District. This molecular approach may be used for the analysis of other vibrio species related to aquaculture disease.

Molecular identification of the causative agents of vibriosis shows that the identified strains were nicely in accordance with the dendogram constructed from the Rep-PCR analysis. The identify starins include of *Vibrio* sp. Absa7 clone 423.1. and *Vibrio splendidus.*

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

ACKNOWLEDMENTS

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MANUSCRIPT REVISED

APPLICATION OF REPETIVE SEQUENCE-BASED PCR ON THE RICHNESS OF VIBRIO ON THE TIGER SHRIMP (*Penaeus monodon* Fab.) FROM BRACKISH WATER POND OF KENDAL

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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 shrimp were collected from the extensive brackish water pond and a total of 22 isolates were isolated from hepatopancreas and telson of tiger shrimp. Based on the repetitive sequence-based polymerase chain reaction (rep-PCR) was found two groups of vibrio. To investige the efectiveness of rep-PCR to find out 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 was closely related with *Vibrio* sp. Absa7 clone 423.1, *Vibrio splendidus*, and *Vibrio splendidus* respectively. The result it was proved that two assosiated of vibrio on tiger shrimp was *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 the tiger shrimp.

Keywords: Rep-PCR, Vibriosis, Causative Agent, *Penaeus monodon* Fab

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, subadults and also adults of shrimps. Outbreaks of the disease cause mortality up to nearly 100% of affected population (Sunaryanto and Mariyam, 1987). The gross signs of localized infection in the cuticle or subcuticle 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.*, 1993; Myers *et al.*, 2003; Suddesh 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.*, 2007[a]; 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) sugested to use molecular methods for diagnostic of fish diseases. The moleculer methods using 16S rDNA for detection of causative agent of vibriosis have been conducted in Turbot, *Colistium nudipinnis,* (Diggles, *et al,* 2000; Montes *et al.*, 2006), Brill, *C. Guntheri,* (Diggles *et al,* 2000), Spotted Rose Snapperm, *Lutjanus guttatus*, Steindachner, 1869 (Gomez-Gil *et al.*, 2004), groupers (Sarjito *et a.l,* 2009) and white shrimps, *Litopeneus vannamei,* (Sarjito *et al.*, 2011).

Various molecular methods have been applied on vibrio, such as: RAPD (Sommary *et al*., 2003) 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. Futhermore, rep–PCR has been applied on various diversity of associated sponge bacteria (Radjasa *et al*., 2007[a.b]), psycrotrophic bacteria from Makasar straight (Radjasa *et al*., 2007[b]) and causative agent of vibriosis (Sarjito *et al.*, 2008; 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 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 identify 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, 2000).

Repetitive – PCR

The procedure was carried out according a method previously described by Radjasa *et al.* (2007[b]). In the rep–PCR, BOX AIR (5'-CTACggCAAggCgACgCTgACg-3') (Versalovic *et al.,* 1994) was used. The REP 1R-I and REP 2-I primers contain the nuclutide inosine (I) at ambigious 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 95ºC for 5 minutes ; 30 cycled of denaturation (92°C for 1 minutes), annealing (50ºC for 1,5

minutes), extension (68ºC for 8 minutes) and final extension at 68ºC 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.* (2007[c]) 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.*(2007[a]). 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 e*t al.* (2007[b]). The determined DNA sequences of strains were then compared for homology to the BLAST database (Atschul *et al.*, 1997).

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 abdoment, 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.).

Table 1. Characteristic of Isolates Associated with Tiger Shrimp (*Penaeus monodon*) form Extensive Brackish Water Ponds of Kendal Regency, Indonesia.

No	Isolates Code	Isolates Source	Colony Color on TCBS	Colony Form
1	JTW 1	Hepatopankreas	Green	Round
2	JTW 2	Hepatopankreas	Yellow	Round
3	JTW 3	Hepatopankreas	Black	Round
4	JTW 4	Hepatopankreas	Yellow	Round
5	JTW 5	Hepatopankreas	Shinny yellow	Round
6	JTW 6	Hepatopankreas	Yellow	Round
7	JTW 7	Hepatopankreas	Dark yellow	Round
8	JTW 8	Hepatopankreas	Dark yellow	Round
9	JTW 9	Hepatopankreas	Dark green	Round
10	JTW10	Hepatopankreas	Black	Round
11	JTW11	Hepatopankreas	Yellow	Round
12	JTW12	Hepatopankreas	Shiny green	Round
13	JTW13	Hepatopankreas	Dark yellow	Round
14	JTW14	Hepatopankreas	Black	Round
15	JTW15	Hepatopankreas	Green	Round
16	JTW16	Hepatopankreas	Yellow	Round
17	JTW17	Hepatopankreas	Black	Round
18	JTW18	Hepatopankreas	Yellow	Round
19	JTW19	Hepatopankreas	Shiny yellow	Round
20	JTW20	Hepatopankreas	Yellow	Round
21	JTW21	Hepatopankreas	Black	Round
22	JTW22	Hepatopankreas	Shiny yellow	Round

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).

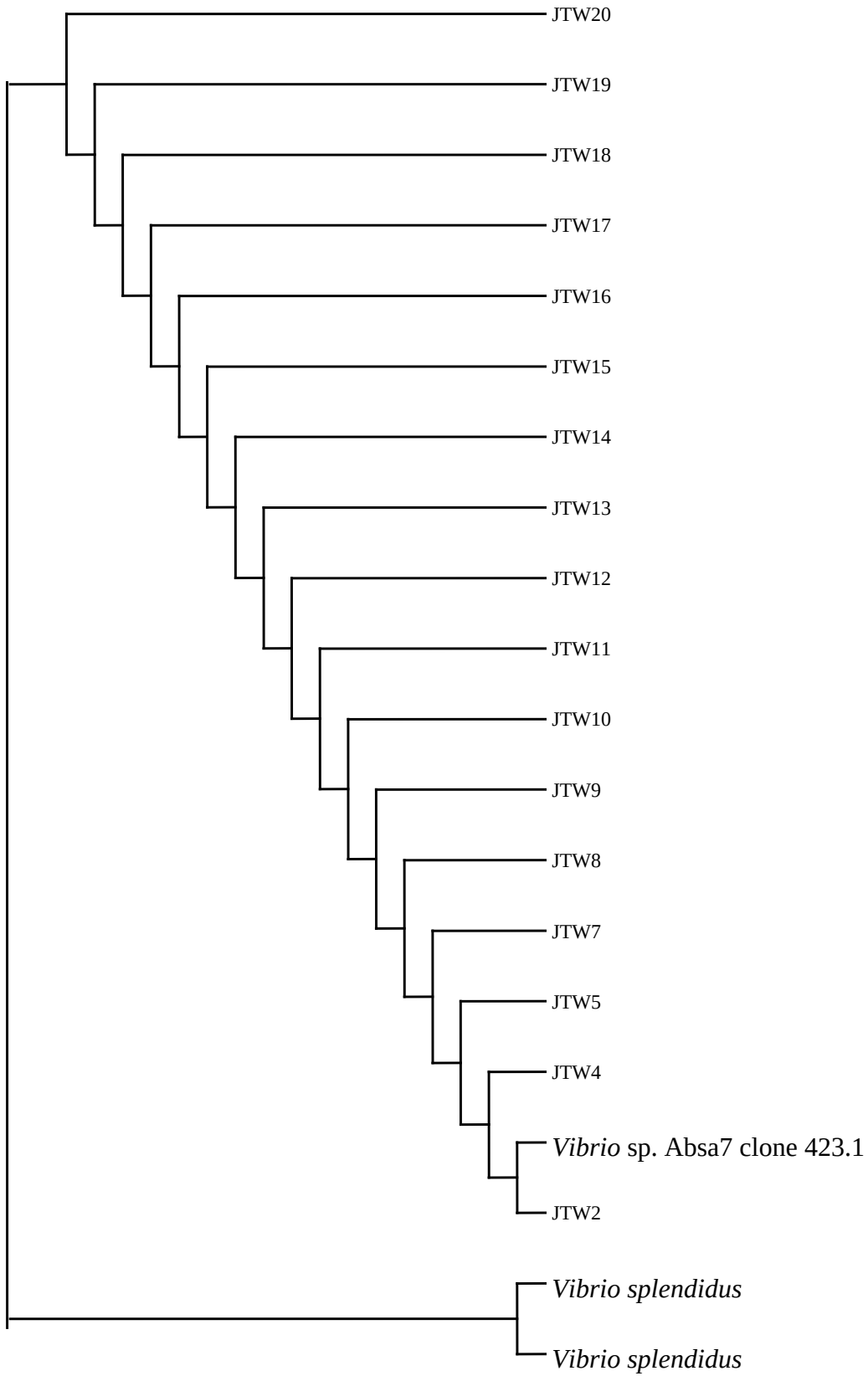
JTW20
JTW19
JTW18
JTW17
JTW16
JTW15
JTW14
JTW13
JTW12
JTW11
JTW10
JTW9
JTW8
JTW7
JTW5
JTW4
Vibrio sp. Absa7 clone 423.1
JTW2
Vibrio splendidus
Vibrio splendidus

Figure.1. Diagram of Vibrio group based on the Repetive Sequence-Based Polymerase Chain Reaction and 16S rDNA on the Tiger Shrimps.

Sequencing of Representative Vibrio as causative agent on tiger shrimps

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

Table 2. Molecular characterization of 3 representative of causative agents

No.	Isolates	Close Relative	Homology (%)	Acc. Number
1	JTW 1	*Vibrio* sp. Absa7 clone 423.1	96	DQ357813.1
2.	JTW 3	*Vibrio splendidus*	100	GQ254509.1
3.	JTW 06	*Vibrio splendidus*	100	GQ254509.1

Based on the Figure 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

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 vibrios 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 tradionally 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 srimps was characterized by melanosis of body, white spot, pale abdoment, dark mouth, red color at tail and lession 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 the vibrio of 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.

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Manuscript decision

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Date: Tuesday, June 5, 2012 at 01:42 PM GMT+7

Dear Dr Sarjito,

I am very pleased to inform you that your manuscript with a title of Application of Repetitive Sequence –Based PCR on the Richness of Vibrio on Tiger Shrimp (*Peneaus monodon* F) has been accepted for a publication in Journal of Coastal Development.

Our production staff will be in touch with you very soon.

Thank you for submitting you work to Jpurnal of Coastal Development.

Yours sincerely

Prof Ocky Karna Radjasa
Editor-in-Chief

Artikel yang mau diperbaiki

From: Ahmad Ronin (aron_nin@yahoo.com)

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Yth. Bpk Dr. Sarjito

Bersama ini saya kirimkan Artikel Bapak yg masih ada perbaikan.
Terima kasih.

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APPLICATION OF REPETIVE SEQUENCE-BASED PCR ON THE RICHNESS OF VIBRIO ON THE TIGER SHRIMP (*Penaeus monodon* Fab.) FROM BRACKISH WATER POND OF KENDAL

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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 shrimp were collected from the extensive brackish water pond and a total of 22 isolates were isolated from hepatopancreas and telson of tiger shrimp. Based on the repetitive sequence-based polymerase chain reaction (rep-PCR) was found two groups of vibrio. To investige the efectiveness of rep-PCR to find out 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 was closely related with *Vibrio* sp. Absa7 clone 423.1, *Vibrio splendidus*, and *Vibrio splendidus* respectively. The result it was proved that two assosiated of vibrio on tiger shrimp was *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 the tiger shrimp.

Keywords: Rep-PCR, Vibriosis, Causative Agent, *Penaeus monodon* Fab

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, subadults and also adults of shrimps. Outbreaks of the disease cause mortality up to nearly 100% of affected population (Sunaryanto and Mariyam, 1987). The gross signs of localized infection in the cuticle or subcuticle 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.*, 1993; Myers *et al.*, 2003; Suddesh 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.*, 2007[a]; 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) sugested to use molecular methods for diagnostic of fish diseases. The moleculer methods using 16S rDNA for detection of causative agent of vibriosis have been conducted in Turbot, *Colistium nudipinnis,* (Diggles, *et al,* 2000; Montes *et al.*, 2006), Brill, *C. Guntheri,* (Diggles *et al,* 2000), Spotted Rose Snapperm, *Lutjanus guttatus*, Steindachner, 1869 (Gomez-Gil *et al.*, 2004), groupers (Sarjito *et a.l,* 2009) and white shrimps, *Litopeneus vannamei,* (Sarjito *et al.*, 2011).

Various molecular methods have been applied on vibrio, such as: RAPD (Sommary *et al*., 2003) 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. Futhermore, rep–PCR has been applied on various diversity of associated sponge bacteria (Radjasa *et al*., 2007[a.b]), psycrotrophic bacteria from Makasar straight (Radjasa *et al*., 2007[b]) and causative agent of vibriosis (Sarjito *et al.*, 2008; 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 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 identify 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, 2000).

Repetitive – PCR

The procedure was carried out according a method previously described by Radjasa *et al.* (2007[b]). In the rep–PCR, BOX AIR (5'-CTACggCAAggCgACgCTgACg-3') (Versalovic *et al.,* 1994) was used. The REP 1R-I and REP 2-I primers contain the nuclutide inosine (I) at ambigious 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 95ºC for 5 minutes ; 30 cycled of denaturation (92°C for 1 minutes), annealing (50ºC for 1,5

minutes), extension (68ºC for 8 minutes) and final extension at 68ºC 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.* (2007[c]) 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.*(2007[a]). 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 e*t al.* (2007[b]). The determined DNA sequences of strains were then compared for homology to the BLAST database (Atschul *et al.*, 1997).

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 abdoment, 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.).

Table 1. Characteristic of Isolates Associated with Tiger Shrimp (*Penaeus monodon*) form Extensive Brackish Water Ponds of Kendal Regency, Indonesia.

No	Isolates Code	Isolates Source	Colony Color on TCBS	Colony Form
1	JTW 1	Hepatopankreas	Green	Round
2	JTW 2	Hepatopankreas	Yellow	Round
3	JTW 3	Hepatopankreas	Black	Round
4	JTW 4	Hepatopankreas	Yellow	Round
5	JTW 5	Hepatopankreas	Shinny yellow	Round
6	JTW 6	Hepatopankreas	Yellow	Round
7	JTW 7	Hepatopankreas	Dark yellow	Round
8	JTW 8	Hepatopankreas	Dark yellow	Round
9	JTW 9	Hepatopankreas	Dark green	Round
10	JTW10	Hepatopankreas	Black	Round
11	JTW11	Hepatopankreas	Yellow	Round
12	JTW12	Hepatopankreas	Shiny green	Round
13	JTW13	Hepatopankreas	Dark yellow	Round
14	JTW14	Hepatopankreas	Black	Round
15	JTW15	Hepatopankreas	Green	Round
16	JTW16	Hepatopankreas	Yellow	Round
17	JTW17	Hepatopankreas	Black	Round
18	JTW18	Hepatopankreas	Yellow	Round
19	JTW19	Hepatopankreas	Shiny yellow	Round
20	JTW20	Hepatopankreas	Yellow	Round
21	JTW21	Hepatopankreas	Black	Round
22	JTW22	Hepatopankreas	Shiny yellow	Round

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).

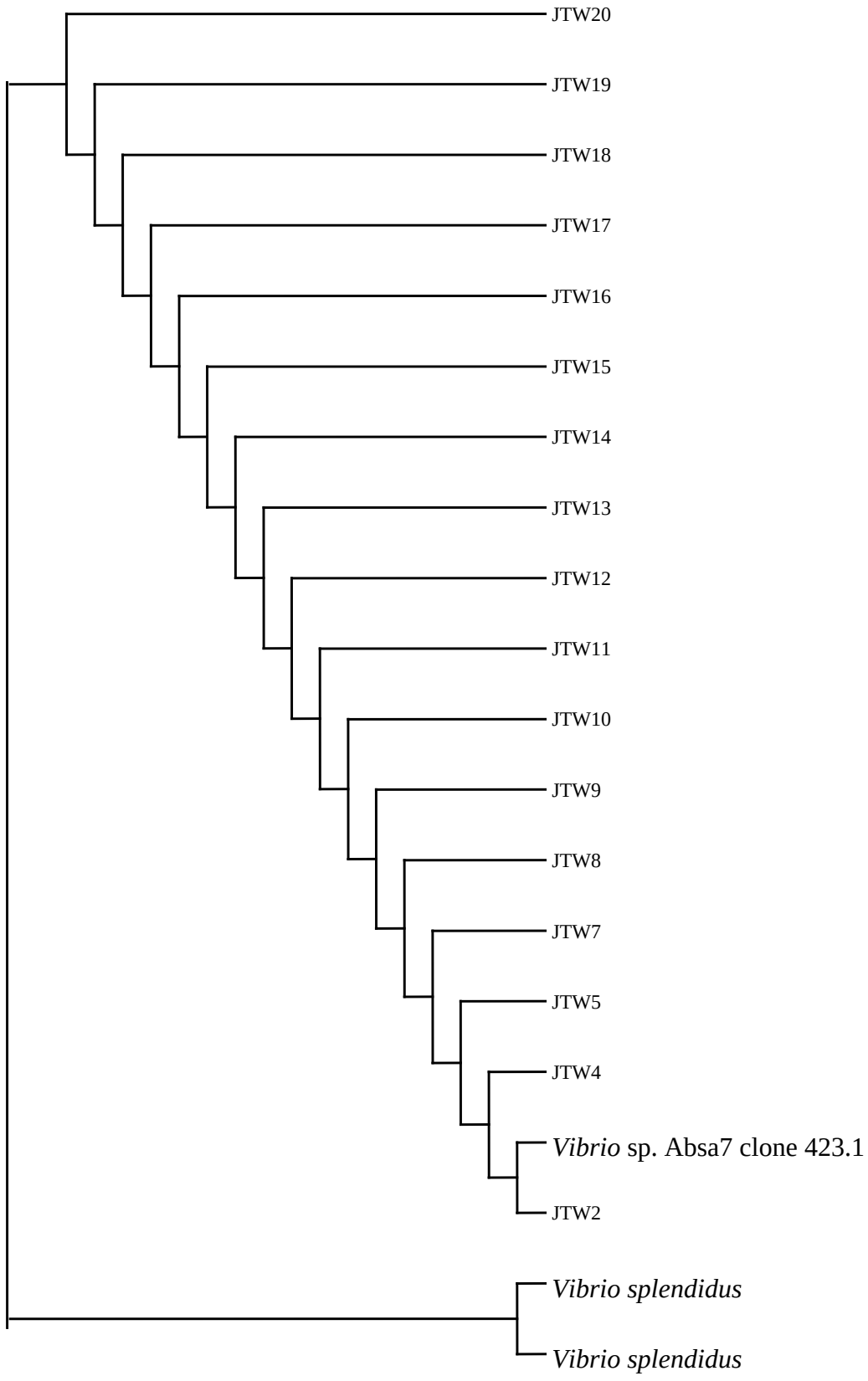
JTW20
JTW19
JTW18
JTW17
JTW16
JTW15
JTW14
JTW13
JTW12
JTW11
JTW10
JTW9
JTW8
JTW7
JTW5
JTW4
Vibrio sp. Absa7 clone 423.1
JTW2
Vibrio splendidus
Vibrio splendidus

Figure.1. Diagram of Vibrio group based on the Repetive Sequence-Based Polymerase Chain Reaction and 16S rDNA on the Tiger Shrimps.

Sequencing of Representative Vibrio as causative agent on tiger shrimps

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

Table 2. Molecular characterization of 3 representative of causative agents

No.	Isolates	Close Relative	Homology (%)	Acc. Number
1	JTW 1	*Vibrio* sp. Absa7 clone 423.1	96	DQ357813.1
2.	JTW 3	*Vibrio splendidus*	100	GQ254509.1
3.	JTW 06	*Vibrio splendidus*	100	GQ254509.1

Based on the Figure 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

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 vibrios 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 tradionally 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 srimps was characterized by melanosis of body, white spot, pale abdoment, dark mouth, red color at tail and lession 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 the vibrio of 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.

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Original Paper

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

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RESULTS AND DISCUSSION

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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 causatived agent of vibriosis on tiger shrimp, two groups were formed (**Fig.1**).

Table 1. Characteristic of isolates associated with tiger shrimp (*Penaeus monodon*) form extensive brackish water ponds of Kendal Regency, Indonesia.

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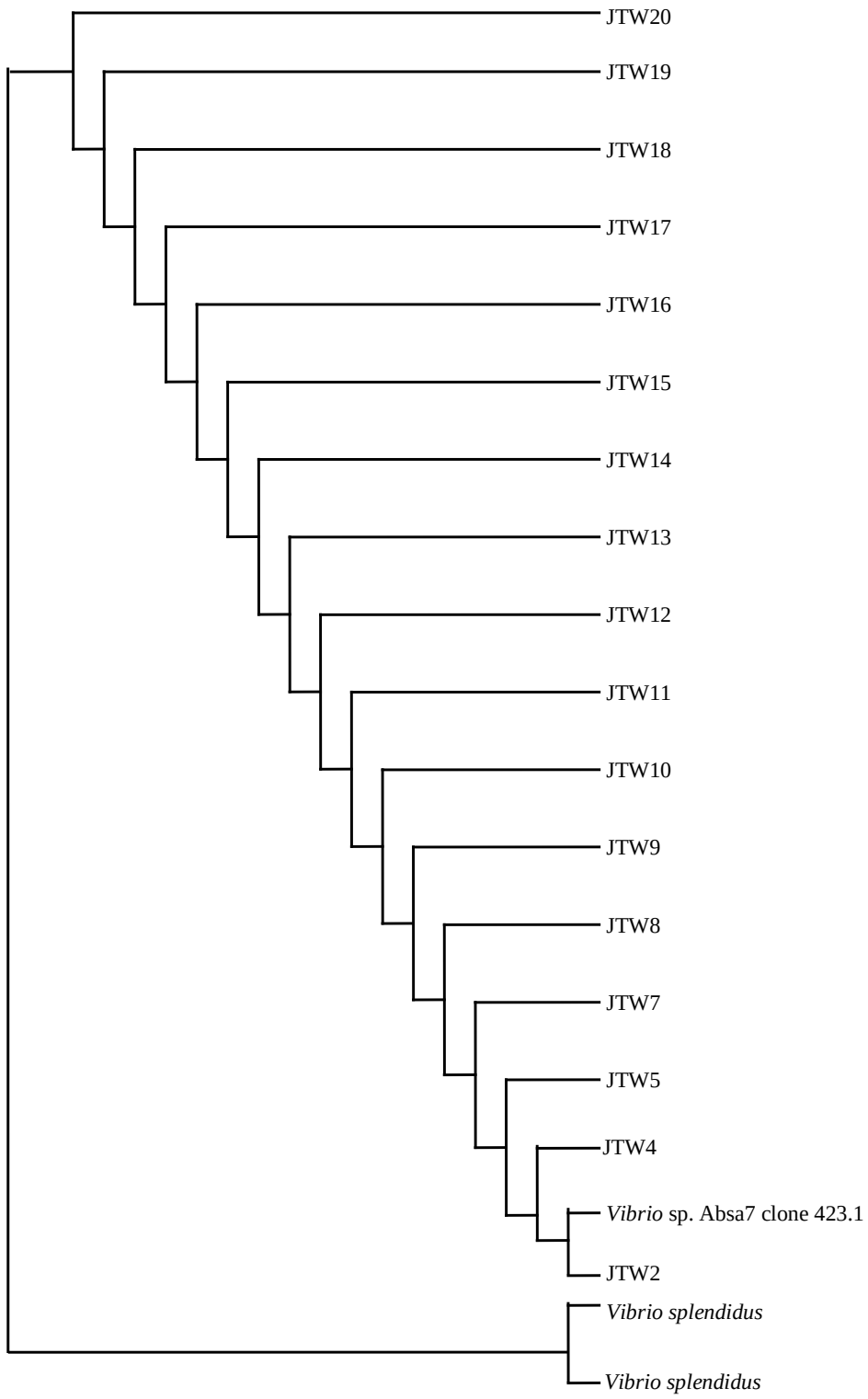

Fig.1. Diagram of Vibrio group based on the Repetive Sequence-Based Polymerase Chain Reaction and 16S rDNA on the Tiger Shrimps.

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Sequencing of representative Vibrio as causative agent on tiger shrimps

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

Table 2. Molecular characterization of 3 representative of causative agents

No.	Isolates	Close Relative	Homology (%)	Acc. Number
1	JTW 1	*Vibrio* sp. Absa7 clone 423.1	96	DQ357813.1
2.	JTW 3	*Vibrio splendidus*	100	GQ254509.1
3.	JTW 06	*Vibrio splendidus*	100	GQ254509.1

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 srimps was characterized by melanosis of body, white spot, pale abdoment, dark mouth, red color at tail and lession 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 of 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.

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Original Paper

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

Sarjito[1] , Nestin E. W. Ningrum[2], Ocky Karna Radjasa[3] and Slamet Budi Prayitno[1]

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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 investige the efectiveness 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 assosiated 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

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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 (Sunaryanto 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; Suddesh and Xu, 2001.).

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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 moleculer methods using 16S rDNA for detection of causative agent of Vibriosis have been conducted in Turbot, *Colistium nudipinnis,* (Diggles, *et al,* 2000; Montes, *et al.,* 2006), Brill, *C. Guntheri,* (Diggles, *et al.,* 2000), Spotted Rose Snapperm, *Lutjanus guttatus,* Steindachner, 1869 (Gomez-Gil, *et al.,* 2004), groupers (Sarjito, *et al.,*2009) and white shrimps, *Litopeneus vannamei,* (Sarjito, *et al.,* 2011).

Various molecular methods have been applied on Vibrio, such as: RAPD (Sommary *et al*., 2003) 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. Futhermore, rep–PCR has been applied on various diversity of associated sponge bacteria (Radjasa,*et al*., 2007a.b), psycrotrophic bacteria from Makasar straight (Radjasa, *et al*., 2007c) and causative agent of Vibriosis (Sarjito, *et al.*, 2008; 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, 2000).

Repetitive – PCR

The procedure was carried out according a method previously described by Radjasa *et al.* (2007b). In the rep–PCR, BOX AIR (5'-CTACggCAAggCgACgCTgACg-3') (Versalovic *et al.,* 1994) was used. The REP 1R-I and REP 2-I primers contain the nuclutide inosine (I) at ambigious 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.

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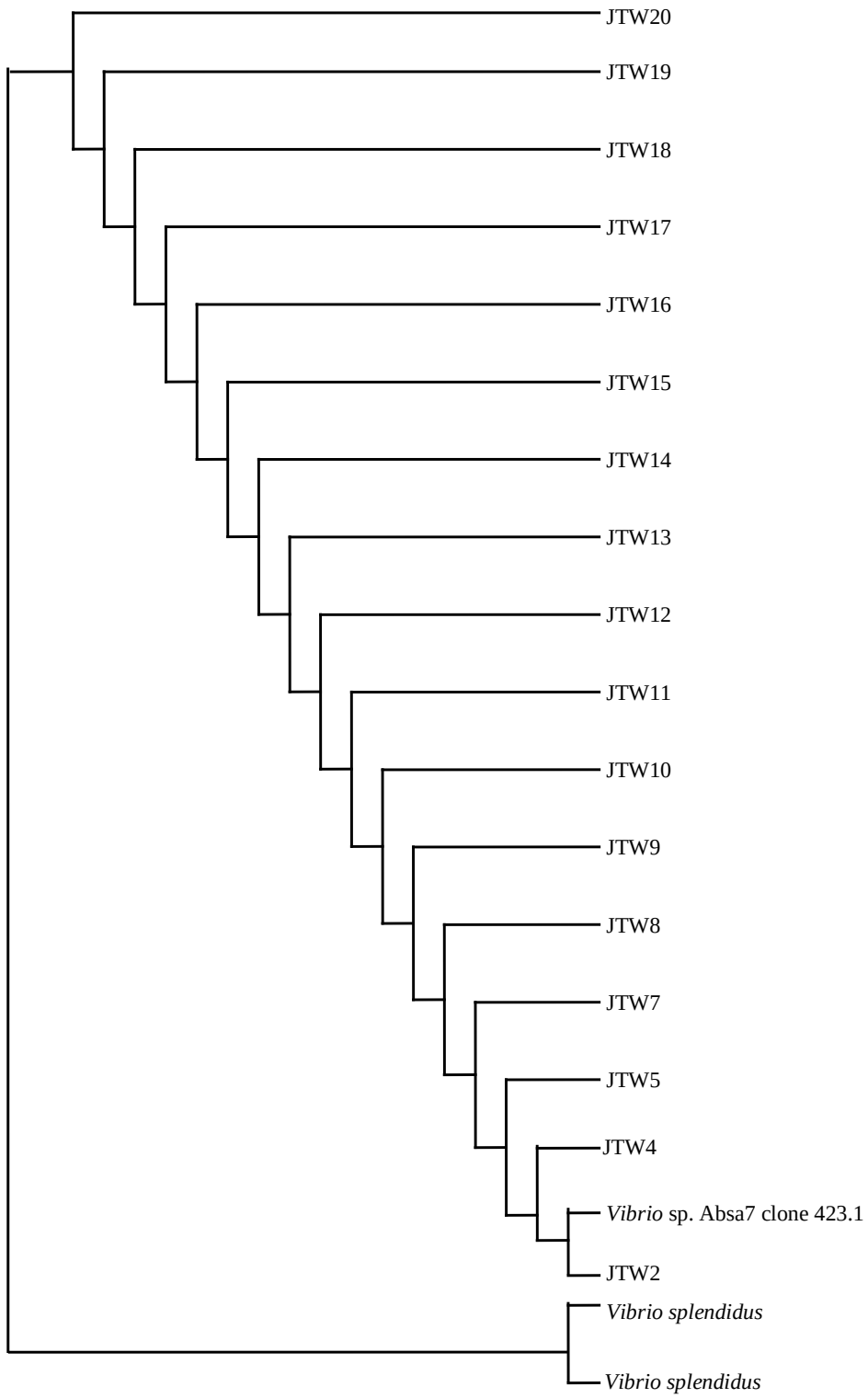

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Table 2. Molecular characterization of 3 representative of causative agents

No.	Isolates	Close Relative	Homology (%)	Acc. Number
1	JTW 1	*Vibrio* sp. Absa7 clone 423.1	96	DQ357813.1
2.	JTW 3	*Vibrio splendidus*	100	GQ254509.1
3.	JTW 06	*Vibrio splendidus*	100	GQ254509.1

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 srimps was characterized by melanosis of body, white spot, pale abdoment, dark mouth, red color at tail and lession 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 of 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.

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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 moleculer methods using 16S rDNA for detection of causative agent of Vibriosis have been conducted in Turbot, *Colistium nudipinnis*, (Diggles, *et al.,* 2000; Montes, *et al.*, 2006), Brill, *C. Guntheri*, (Diggles, *et al*., 2000), Spotted Rose Snapperm, *Lutjanus guttatus*, Steindachner, 1869 (Gomez-Gil, *et al.,* 2004), groupers (Sarjito, *et al.*, 2008; Sarjito, *et al.*, 2009) and white shrimps, *Litopeneus 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. Futhermore, rep–PCR has been applied on various diversity of associated sponge bacteria (Radjasa,*et al*., 2007a.b), psycrotrophic bacteria from Makasar straight (Radjasa, *et al*., 2007c) and causative agent of Vibriosis (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'-CTACggCAAggCgACgCTgACg-3')
(Versalovic *et al.,* 1994) was used. The REP 1R-I and REP 2-I primers contain the nuclutide inosine (I) at ambigious 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 95ºC for 5 minutes ; 30 cycled of denaturation (92°C for 1 minutes), annealing (50ºC for 1,5 minutes), extension (68ºC for 8 minutes) and final extension at 68ºC 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.

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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, e*t 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 abdoment, 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 causatived agent of vibriosis on tiger shrimp, two groups were formed (**Fig.1**).

Table 1. Characteristic of isolates associated with tiger shrimp (*Penaeus monodon*) form extensive brackish water ponds of Kendal Regency, Indonesia.

No	Isolate Code	Isolate Source	Colony Color on TCBS	Colony Form
1	JTW 1	Hepatopankreas	Green	Round
2	JTW 2	Hepatopankreas	Yellow	Round
3	JTW 3	Hepatopankreas	Black	Round
4	JTW 4	Hepatopankreas	Yellow	Round
5	JTW 5	Hepatopankreas	Shinny yellow	Round
6	JTW 6	Hepatopankreas	Yellow	Round
7	JTW 7	Hepatopankreas	Dark yellow	Round
8	JTW 8	Hepatopankreas	Dark yellow	Round
9	JTW 9	Hepatopankreas	Dark green	Round
10	JTW10	Hepatopankreas	Black	Round
11	JTW11	Hepatopankreas	Yellow	Round
12	JTW12	Hepatopankreas	Shiny green	Round
13	JTW13	Hepatopankreas	Dark yellow	Round
14	JTW14	Hepatopankreas	Black	Round
15	JTW15	Hepatopankreas	Green	Round
16	JTW16	Hepatopankreas	Yellow	Round
17	JTW17	Hepatopankreas	Black	Round
18	JTW18	Hepatopankreas	Yellow	Round
19	JTW19	Hepatopankreas	Shiny yellow	Round
20	JTW20	Hepatopankreas	Yellow	Round
21	JTW21	Hepatopankreas	Black	Round
22	JTW22	Hepatopankreas	Shiny yellow	Round

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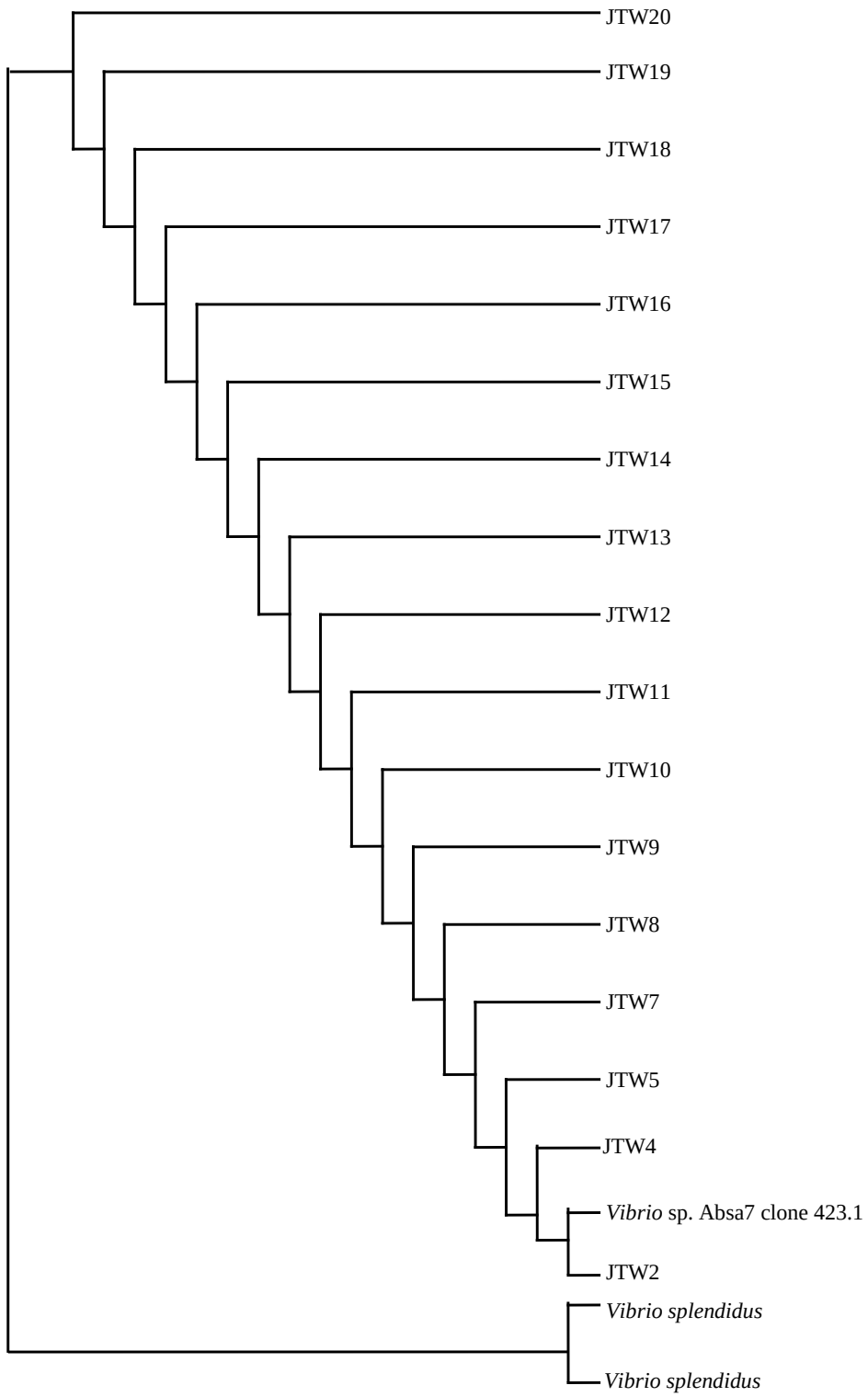

Fig.1. Diagram of Vibrio group based on the Repetive Sequence-Based Polymerase Chain Reaction and 16S rDNA on the Tiger Shrimps.

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Sequencing of representative Vibrio as causative agent on tiger shrimps

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

Table 2. Molecular characterization of 3 representative of causative agents

No.	Isolates	Close Relative	Homology (%)	Acc. Number
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