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The Diversity of Vibrios Associated with Vibriosis in Pacific White Shrimp (*Litopenaeus vannamei)* from Extensive Shrimp Pond in Kendal District, Indonesia

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Abstract. Vibriosis out breaks frequently occur in extensive shrimps farming. The study were commenced to find out the clinical signs of white shrimp that was infected by the Vibrio and to identify the bacterial associated with vibriosis in the pacific white shrimp, *Litopenaeus vannamei*. Bacterial isolates were gained from hepatopancreas and telson of moribund shrimps that were collected from extensive shrimp ponds of Kendal District, Indonesia and cultured on *Thiosulfate Citrate Bile Salts Sucrose Agar* (TCBSA). Isolates were clustered and identified using repetitive sequence-based polymerase chain reaction (rep-PCR). Three representative isolates (SJV 03, SJV 05 and SJV 19) were amplified with PCR using primers for 16S rRNA, and sequence for further identification. The clinical signs of shrimps affected by vibrio were pale hepatopancreas, weak of telson, dark and reddish coloration of smouth, patches of red colour in part of the body on the carapace, periopods, pleuopods, and telson. A total of 19 isolates were obtained and belong to three groups of genus *Vibrios*. Result of the 16S DNA sequence analysis, the vibrio found in this study related to vibriosis in white shrimps from extensive shrimp ponds of Kendal were closely related to *Vibrio harveyi* (SJV 03); *V. parahaemolyticus* (SJV 05) and *V. alginolyticus* (SJV 19).

Keywords: White Shrimp, Vibriosis, Rep PCR, 16S r RNA

10

1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is an important aquaculture species that has high economic value for export commodities from Kendal. This shrimp farms in this area usually use intensive and extensive technology to increase its production. However, inadequate intensive shrimp management or low quality management of extensively pond will cause disease problems, such as bacteria [1] and mass mortality [2]. Outbreaks of bacterial diseases, such as vibriosis, are still a problem in shrimp farming [3-4]. Vibrio infection in pacific white shrimp (*L. vannamei*) is

characterized by pale or reddish carapace, pale hepatopancreas, root and redish of telson; red and root antenna [1]. Although this is a normal part of the bacterial flora in the environment of estuary and marine waters [4-5], some species of vibrio are considered opportunistic pathogens against finfish, shellfish and shrimp [5]. Therefore, the genus Vibrio can cause production failure due to vibriosis in shrimp culture [3-4][6], resulting on death at the stage of zoe, larvae and adult [7].

Various studies have been conducted to identify vibrio associated with vibriosis in the aquaculture industry [2]. Some vibriosis agents in shrimp have been identified, such as: *V. harveyi* [8-9]; *V. alginolyticus* [2]; *V. parahaemolyticus* [2][7]; *V. fluvialis, V. damsela and V. vulnificus* [7]. Previous studies using biochemical methods to identify vibrio diversity in this area. Hence, the classification of bacteria with molecular approaches should be applied to develop early detection of disease caused by viruses, bacteria, including vibriosis in shrimp [9].

2. Materials and Methods

2.1. Sample of Shrimp

A total 31 moribound pacific white shrimp *(L. vannamei*), suspected having vibriosis infection, were collected from extensive brackish water pond in Kendal district located on Wakak River, Kendati, Indonesia. The identification of pacific white shrimp with vibriosis is based on the clinical symptoms of references suggested by Lighter [6]. This research applies explorato method with purposive random sampling. The isolation of bacteria in the sample was done at the Aquaculture Laboratory of Fisheries and Marine Science Faculty of Diponegoro University.

2.2. Isolation of Bacteria.

The vibrio bacteria were isolated fr n hepatopancreas and telson from pacific white shrimp (*L. vannamei*) with streak plate methods on Thiosulfate Citra Bile Salts Sucrose Agar (TCBSA) and it incubated at room temperature for 24-48 hours [10]. The colonies were randomly drawn and purified by making streak plates based on their morphological features [11].

2.3. Repetitive-PCR

Rep-PCR was done based on the method previously described by [10] which has bee modified by [13]. BOX AIR (5'-CTACggCAAggCgACgCTgACg-3') was applied in the rep–PCR. 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 a total volume of 05 µL.

Amplification was performed by thermal rotor model of Gene Amp PCR 9700 with the following temperature conditions as follows: initial denaturation at 95ºC for 5 minutes; 30 cycles of denaturation (92° C for 1 minute), 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 arried out using electrophoresis on 1% bromade gel ethers using 1X TBE buffer . The result was visualized in UV trans-illuminator and the image was captured by the gel documentation system (Biorad) [9].

2.4. Grouping of Isolates

The grouping of isolates was performed based on the method of making matrixes from the position of bands on the gel analyzed using the Free Tree program. This program uses UPGMA method for tree creation. Resampling was done by bootstrapping, 1000 replications [10]

2.5. PCR Amplification and Sequencing of 16 rRNA Gene Fragments

PCR amplification was initiated bas d on the method of Radjasa *et al.* [10][12] which has been modified by Sarjito *et al.* [13]. Two primers, GM3F (5'-AGAGTTTGATCMTGGC-3') and GM4R (5'-TACCTTGTTACGACTT-3') were used to amplify the early complete 16S rRNA gene [12]. The genomic DNA of the causative agent of strain vibriosis for PCR analysis was obtained from the cell material taken from the agar plate, which was suspended in sterile water (Sigma, Germany) and

subjected to five freezing cycles at -80ºC and melting 2 95ºC. PCR amplification of 16S partial bacterial rRNA gene [14], PCR product purification and subsequent sequencing analysis were performed in accordance with [10][12-13]. The sequence of DNA strains determined then compared to the homology to the BLAST database. To determine the relationship of the genus vibrios, the phylogenetic stage begins in accordance with the software Mega 1.

3. Results and Discussion

3.1. Result

3.1.1. Characteristic of the Bacterial Isolates

The clinical signs of shrimp affected by vibrios were pale hepatopancreas, weak of telson, dark and reddish colorozation of mouth, patches of red colour in part of the body, periopods, pleuopods and telson (Figure 1).

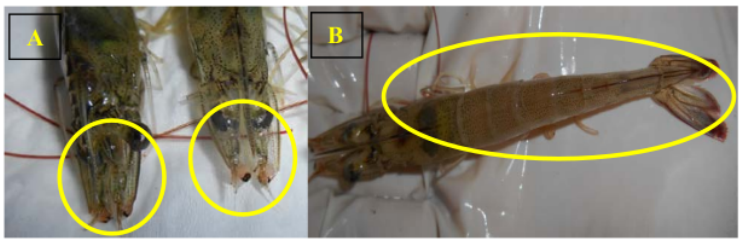

Figure 1. Clinical sign of pacific white shrimps, *L. vannamaei,* infected by vibriosis: (a.) Dark and reddish colorization of mouth; (b.) Reddish the carapace, periopods, pleuopods, mouth and telson.

Table 1. Characteristic of Isolates vibrios Associated with Vibriosis on Pacific White Shrimps, *L. vannaei,* from shrimp pond in Kendal District, Indonesia.

No.	Isolate code	Media	Source	Colony		
				Colour	Form	Characteristic
1	SJV01	TCBS	Hepatopancreas	Yellow	Rounded	Convex
2	SJV02	TCBS	Hepatopankreas	Yellow	Rounded	Convex
3	SJV03	TCBS	Hepatopankreas	Yellow	Rounded	Flat
4	SJV04	TCBS	Hepatopankreas	Yellow	Oval	Convex
5	SJV05	TCBS	Hepatopankreas	Yellow	Oval	Convex
6	SJV03	TCBS	Hepatopankreas	Yellow	Rounded	Convex
7	SJV03	TCBS	Hepatopankreas	Yellow	Rounded	Convex
8	SJV08	TCBS	Hepatopancreas	Yellow	Rounded	Convex
9	SJV09	TCBS	Hepatopancreas	Green	Rounded	Convex
10	SJV10	TCBS	Hepatopancreas	Yellow	Rounded	Convex
11	SJV11	TCBS	Hepatopancreas	Yellow	Rounded	Convex
12	SJV12	TCBS	Hepatopankreas	Yellow	Rounded	Convex
13	SJV14	TCBS	Hepatopankreas	White	Irregular	Convex
14	SJV05	TCBS	Hepatopancreas	White	Irregular	Rough
05	SJW17	TCBS	Hepatopankreas	Yellow	Rounded	Concave
16	SJV16	TCBS	Hepatopancreas	Yellow	Rounded	Rough
17	SJV17	TCBS	Hepatopancreas	Yellow	Oval	Convex
18	SJV18	TCBS	Hepatopancreas	Yellow	Rounded	Flat
19	SJV19	TCBS	Hepatopankreas	Yellow	Irregular	Rough

Bacterial isolation resulted in a total of 19 isolates were obtained from hepatopancreas, gill, haemolimph and carapaceous wounds from affected pacific white shrimp (*L. vannamei*) (Table 1)

3.1.2. Repetitive-PCR Analysis

Based on PCR-repetitive results and dendogram construction, there were three groups of Vibrios Associated with Vibriosis in pacific white shrimp, *L. vannamei*, from extensive shrimp pond in Kendal, Indonesia (Figure 2.)

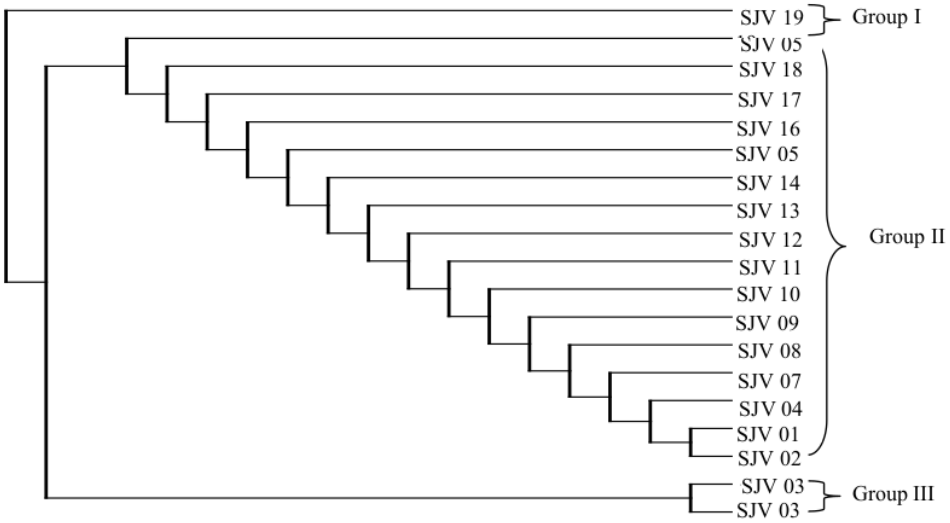

Figure 2. Dendogram of 19 Isolates of Vibrios associated in Pacific White Shrimps, *L. Vannamaei,* from Extensive brackish water Shrimp Ponds of Kendal, Indonesia

Three isolates (SJV 03, SJV 05 and SJV19) of 19 isolates (SJV01 - SJV 19) were selected for molecular identification. Based on molecular characterization, it was shown that all isolates belong to the Vibrio genus (Table 2)

Table 2. Molecular Characterization of Three Representative Of Genus Vibrio

No.	Isolates	Close Relative	Homology (%)	Acc. Number
1.	SJV 03	*Vibrio harveyi*	99	JF264473.1
2.	SJV 05	*Vibrio. parahaemolyticus*	98	HQ694830.1
3.	SJV 19	*V. alginolyticus*	99	EU055503.1

Vibrios associated with vibriosis in shrimp from extensive brackish water ponds in Kendal show a close relation with *Vibrio harveyi* (SJV 03); *V. parahaemolyticus* (SJV 05) and *V. alginolyticus* (SJV 19) with homology ranging from 98-99%. This was based on DNA sequence analysis of 16S. The complete phylogenetic of vibrio is shown in Figure. 3

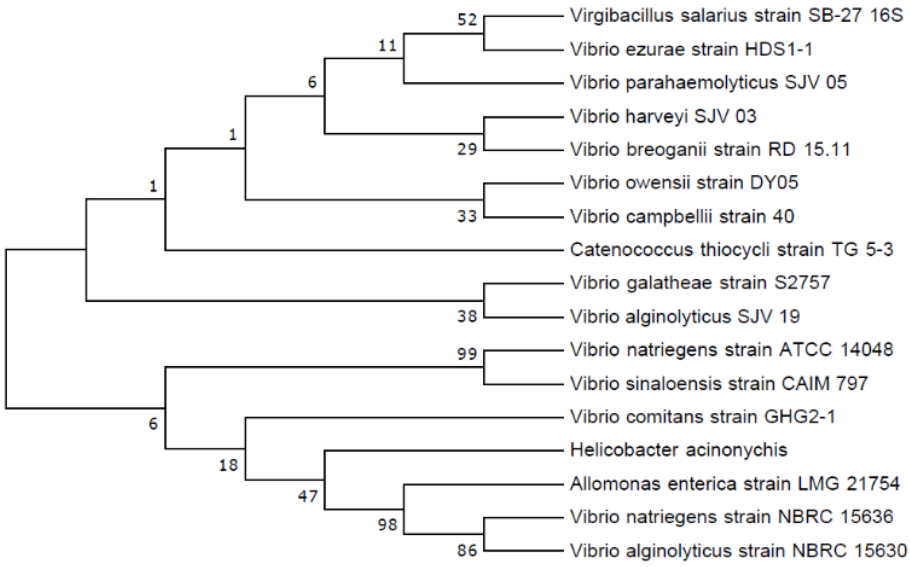

Figure 3. Phylogenetic of The Vibrios Related to Vibriosis in Pacific White Shrimps from Extensive Brackish Water Ponds of Kendal

3.2. Discussion

The genus vibrios are commonly found in marine and estuarine environments [4]. However, some species have demonstrated clinical significance for aquatic animals and are recognized as potential pathogens. Vibrio has been detected related to vibriosis in shrimp [9][15]. Therefore, the genus vibrio has the potential to cause vibriosis in shrimp [16]. These vibrio strains are found in aquatic ecosystems, such as water or sediment [17] or waters [18]. Most *Vibrio* spp. commonly reported pathogens for aquatic animals, but [2] reported that *Vibrio* sp. 33 is an avirulent strain.

The pacific white shrimp (*L. vannamei*) infected by the vibrio were identified by clinical signs as follows: pale hepatopancreas, weakened telson, darkness and redness colorization the mouth, and red spots on the carapace on the body, periopods, pleuopods, and telson. Similar clinical signs have been reported by Sarjito *et al.* [1].

The study also revealed that there were three Vibrio strains that infected pacific white shrimp in extensive brackish water pond, Kendal, i.e. *Vibrio harveyi* (SJV 03); *V. parahaemolyticus* (SJV 05) and *V. alginolyticus* (SJV 19). These results indicate that the vibrios diversity associated with vibriosis in pacific white shrimp, *L. vannamei*, was higher than the diversity reported on tiger shrimp, *Penaeus monodon,* [1]. However, this diversity was lower than the diversity found in grouper in Karimunjawa [13], pacific white shrimp, *L. vannamei*, in India [5].

V. harveyi is naturally found in marine and estuarine environments [16]. These bacteria are also known as pathogenic bacteria associated with black tiger shrimp, *P. monodon,* and the Pacific white shrimp *L. vannamei*, [20], Atlantic mediterranian invasive algae *Caulerpa cylindracea* [20], black tiger shrimp, *P. monodon,* from brackish water pond and coastal the region of India [16], marine invertebrate and vertebrate [3]. *V. harveyi* is also the main causative agent in luminous vibriosis [15] and vibriosis in shrimp ponds [22]. Furthermore, Thongkao [8] and Ramesh *et al.* [21] stated that *V. harveyi* is known as a bacterial pathogen in shrimp around the world that causes mortality up to 100% in the culture system.

V. parahaemolyticus has been reported as an important causative agent of disease in shrimps and resulted on mortality, and economical losses in this industry [22]. *V. parahaemolyticus* was also

detected in pacific white shrimp, *L. vannamei* [23]. According to Stalin and Srinivasan [4], *V. parahaemolyticus* is a pathogenic opportunistic causing symptomatic infection of shrimp. This can be attributed to outbreak diseas etween pacific white shrimp, *L. vannamei* cultures in India [16][23]. The vibrio was also known as the causative agent of vibriosis in pacific white shrimp *L. vannamei* [24]. The bacteria was also identified the dominant vibrio species associated with the mass mortality of juvenile Chinese shrimp, *Fenneropenaeus chinensis* [25].

Meanwhile, *V. alginolyticus* was reported as normal bacteria of estuarine and marine environment [27]. *V. alginolyticus* was reported as a pathogenic bacterium in a giant tiger shrimp growing pond, *P. monodon*, in India. Furthermore, Bunpa *et al.*[26] also stated that this bacterium was important bacterial and pathogenic bacteria associated with infections caused by seafood, fish diseases, shrimp diseases, sediment and seawater.

4. Conclusion

Clinical signs of shrimp infected by the genus vibrio associated with vibriosis from Kendal district, Indonesia were pale of hepatopacreas weaken of telson, dark and reddish colorization mouth, patches of red colour in part of the body on the carapace, periopods, pleuopods and telson. The study found that vibrios associated with vibriosis in shrimp from Kendal's extensive brackish water were closely to *Vibrio harveyi* (SJV 03); V. *parahaemolyticus* (SJV 05) and *V. alginolyticus* (SJV 19)

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