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Associated *Vibrio* Species in Shrimp Vibriosis from Traditional Brackish Water Pond in the North Coastal of Central Java, Indonesia

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Abstract

Indonesian shrimp cultures are threatened by vibriosis. Some traditional brackish water ponds remained along the north coast of Central Java after the disease outbreaks destroyed the shrimp culture. This study aimed to discover the *Vibrio* diversity associated with shrimp vibriosis in traditional brackish water ponds. An exploratory method with purposive sampling was used in this study. Twenty-four shrimps presumably infected with vibriosis were collected from two district regions on the north coast of Central Java in July–September 2018. The bacteria associated in shrimp vibriosis were isolated from the telson and inner part of the hepatopancreas with TCBS medium. Forty-one bacteria associated with shrimp vibriosis were obtained, and then repetitive-polymerase chain reaction (rep-PCR) was performed to obtain *Vibrio* strains. On the basis of rep-PCR results, five representative strains were selected for further study. The results of 16S rDNA sequence analysis showed that the JKP03, JKP05, JKP19, JKM01, and JKM06 isolates were closely related to *Vibrio rotiferianus*, *Vibrio diabolus*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Shewanella algae*, respectively. *Vibrio* biodiversity in shrimp vibriosis was high. These results confirmed that traditional shrimp farming was susceptible to vibriosis. Therefore, control methods such as vaccines, probiotics, and immunostimulant formulas must be developed to prevent and control the outbreak of shrimp vibriosis in traditional brackish water pond.

Introduction

Shrimp is an important aquacultural species with high economic value and the most important export commodity from Central Java, Indonesia. Some brackish water ponds remained along the northern coast of Central Java after the disease outbreaks destroyed the shrimp culture business in the 1990s (Hariati et al., 1995). The culture species in this area include *Peneaus monodon* and *Litopenaeus vannamei*, and their production has still steadily increased. They are commonly cultured using semi-intensive and traditional

techniques. Most traditional shrimp farmers apply low external input for sustainable aquaculture by optimizing local resources, such as using liquid compost from fermented organic waste from households for curing pond bottom and maintaining pond water quality (Ariyati et al., 2019). Farmers also practice an integrated mangrove-shrimp aquaculture system as Indonesian government's program to rehabilitate and conserve the mangrove forest. Although this system is more ecologically friendly than other types of aquaculture, shrimp production is still low. Improper culture management, such as drainage of pond bottom, and

relying on a single sluice gate for water flow, causes viral and bacterial disease problems and results in mass mortality (Sarjito et al., 2012; Uddin et al., 2013; Liu et al., 2016). Some farmers use chemicals, such as antibiotics, to combat vibriosis in shrimps. Antibiotic residues and antibiotic-resistant bacteria emerged in the brackish water pond environment because of excessive antibiotic dosages. Therefore, vibriosis outbreak has become a problem in shrimp culture in Indonesia (Isnansetyo et al., 2009; Istiqomah et al., 2020) and other countries (Abdelaziz et al., 2017; Mastan & Bism, 2016).

Vibrio is the causative agent of vibriosis, a common disease in aquaculture worldwide (Akaylı & Timur, 2002). It can infect crustaceans, mollusks, and fish, resulting in mass mortality. *Vibrio* infection in shrimps is characterized by pale hepatopancreas, reddish or pale body carapace, reddish uropod and telson, and red antenna (Sarjito et al., 2018). Although *Vibrio* is a normal part of the bacterial flora in the estuarine and seawater environments, several *Vibrios* are opportunistic pathogens in cultured shellfish, finfish, and shrimp (Austin & Austin, 2007). Therefore, *Vibrio* may cause serious production loss in shrimp culture (Stalin & Srinivasan, 2017). Vibriosis causes mortality in larvae and adult stages by up to 50% (Lightner, 1996).

Most previous studies reported that vibriosis is related to the Family Vibrionaceae, mostly of the Genus *Vibrio*. However, *Shewanella algae* and *Listonella* have been grouped in Vibrionaceae (MacDonell & Cowell, 1985). Well-known causative agents of vibriosis are *V. vulnificus*, *V. fluvialis*, *V. damsela*, *V. fischeri*, *V. parahaemolyticus*, and *V. alginolyticus* (Chythanya et al., 2002; Kumar et al., 2014; Gopala et al., 2005). Chandrakala and Priya (2017) have mentioned 18 *Vibrio* species acting as shrimp pathogens: *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*,

V. anguillarum, *V. vulnificus*, *V. campbelli*, *V. fischeri*, *V. damsela*, *V. pelagicus*, *V. orientalis*, *V. ordalii*, *V. mediterranei*, and *V. logei*.

Many studies focused on the Genus *Vibrio* in the aquaculture system (Liu et al., 2016; Sarjito et al., 2018; Gopala et al., 2005). However, most research involved mono species and intensive culture technologies. The current study aimed to elaborate on the medical symptoms of shrimp infected with *Vibrio*. A molecular approach was used to identify the causative agent of *Vibrio* related to vibriosis. Studies on the *Vibrio* diversity causing vibriosis in traditional brackish shrimp ponds are limited. The accuracy of the molecular method for identifying *Vibrio* is important to mitigate and design disease prevention strategies for sustainable shrimp production. Considering vibriosis outbreaks has become an important limitation on shrimp production in Central Java. In addition, bacteria such as *Vibrio cholerae* can also cause human infections. Therefore, to discover *Vibrio* diversity associated with vibriosis in shrimps is urgently needed. The current study aimed to develop a simple reliable molecular protocol to identify *Vibrio* associated with vibriosis in the shrimp of traditional brackish water pond on the north coast of Central Java.

Materials and Methods

Shrimps Sampling

Exploratory method with purposive sampling was used in this study. Shrimp specimens presumably infected with vibriosis were sampled from traditional brackish water of Wonorejo dan Turunrejo subdistricts (Kendal district) and Mulyo dan Puncel subdistricts (Pati district) in the north coast of Central Java (Figure 1). The samples were stored in an insulated container and brought to the laboratory for bacterial

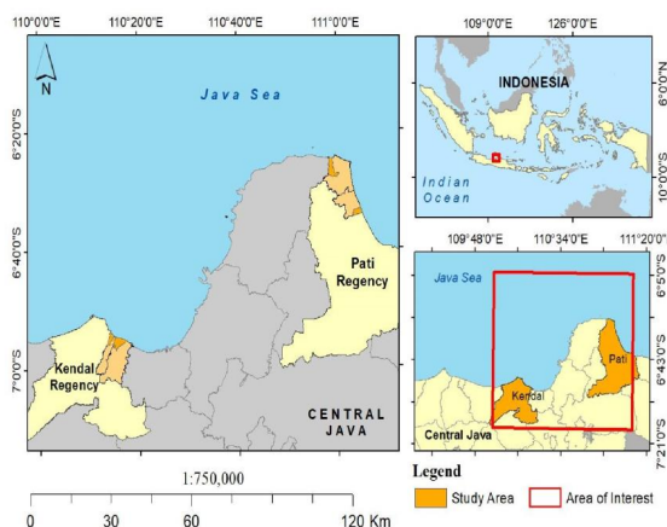


Figure 1. Collection sites of the shrimps

isolation. Shrimp samples were categorized ²² their length (16.9–17.2 cm). Then, three individuals of black tiger shrimps (*P. monodon*) and three individuals of pacific white shrimps (*L. vannamei*) from each location were sampled randomly. A total of 24 specimens (11 black tiger shrimps and 13 pacific white shrimps) were used as material research.

Bacterial Isolation

Bacterial isolation was performed by scrap ²³ off the telson and inner part of the hepatopancreas with a sterile scalpel. The tissues were serially diluted in 10^{-1} , 10^{-3} , and 10^{-5} of the resulting paste prepared in sterile water. Then, 50 μ L aliquots of each dilution were spread on TCBS agar (Oxoid, England) and incubated at room temperature for 48 h (Brock and Madigan, 1991; Sarjito ²⁴ al., 2018). Then, colonies with different morphological features were picked up and purified by serially streaking simple colonies on agar plates.

Repetitive-Polymerase Chain Reaction (rep-PCR)

⁴ The bacterial DNA was extracted from a 24 h-broth culture of isolate strains ⁴ using the chelex method with slight modification according to the procedure described by de Lamballerie (1992). The rep-PCR method used for typing strains belonging to *Vibrio* sp. was adopted from previous methods (Brock & Madigan, 1991; Sarjito et al., 2009; Sarjito et al., 2018). The rep-PCR oligonucleotide primers evaluated in this study were BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3'), REP1R-I (5'-IIICGICGICATCI GGC-3'), and REP2-I (5'-IIICGNCGNCATCNGGC-3') (Versalovic, 1994; Sarjito et al., 2012; Prayitno et al., 2015). The PCR product was purified by GoTaq[®]Green Master Mix (Promega) following the protocol described by the manufacturer. The reaction mixtures were denatured at 95°C for 5 min, followed by 30 cycles of amplification at 92°C for 60 s, annealing at 50°C for 90 s, followed by a final extension at 68°C for 10 min. The 5 μ L PCR amplicons were visualized and compared to molecular weight standards (100-basepair ladder) after electrophoresis on 1% agarose gels staining with ethidium bromide (Prayitno et al., 2015).

Isolate Grouping

On the basis of electrophoresis results on rep-PCR, the isolates were grouped by analyzing the band position on the gel with the Free Tree program (Hampl et al., 2001; Prayitno et al., 2015). Then, one isolate from each group in the dendrogram tree was chosen randomly for further identification.

Bacterial Identification

² The GM3F and GM4R primers were used to amplify the 16S rRNA gene (Muyzer et al., 1995). Genomic DNA

of *Vibrio* strains was extracted from bacterial cells by using the freeze-thaw method. The 16S rRNA gene amplification, purification, and sequence were carried out as previously described by Radjasa et al. (2007) and Sarjito et al. (2018). PCR products were sequenced and then compared by BLAST homology searching (Altschul et al., 1990; Widowati et al., 2018). The phylogenetic tree was constructed by using the Mega 7 software package (Sibero et al., 2017; Sibero et al., 2018).

Results and Discussion

Vibriosis Signs and Bacterial Isolation

Twenty-four shrimps with clinical signs of vibriosis were sampled for *Vibrio* isolation vibriosis signs were reddish and melanosis in the telson (a), reddish periopods and pleiopods (b), soft body (c), and reddish (Figure 2).

These clinical signs were similar to the results of vibriosis in previous research (Raja et al., 2017; Sarjito et al., 2018). However, the clinical signs previously described ¹³ Mastan and Begum (2016), such as loss of appetite, red to brown gills, reduced feeding, empty gut, and general septicemia, were not found in the present research. This result might be due to the difference ⁶ virulence degrees. In addition, the virulence degree of various *Vibrio* isolates depends on its ⁶ source and the pond environmental conditions. Even differences occur in the virulence degree of different *Vibrio* species and isolates. Jayasree et al. (2006) reported that *V. harveyi* isolated from LSS shrimp is the most virulent. Meanwhile, Soto-Rodriguez (2015) showed that *V. parahaemolyticus* is the most virulent. Furthermore, the virulence is generally dependent on the strain, density, infection route, exposure time, species ⁴³ derived, and age and general condition of the shrimp (Alday-Sanz et al., 2002; Saulnier et al., 2000).

Forty-one pure bacterial strains were isolated based on the different morphologies and types of growth on TCBS agar. Table 1 shows that bacterial strains were morphologically characterized by colony form (oval, circular, and irregular) and color (green, black, yellow, and white). Widana ¹⁹ et al. (2003) reported the bacterial characteristics isolated from tiger shrimp larvae ¹⁹ Labuan, Pangandaran, and Lampung, Indonesia are Gram-negative, short rod-shaped, yellow colonies. Approximately 60% of bacteria associated with shrimp vibriosis in Sri Lanka belong ¹⁵ to Vibrionaceae (Raja et al., 2017). Moreover, the six species of *Vibrio* were collected from diseased ⁴⁷ shrimps in the culture ponds of Andhra Pradesh India (Jayasree et al., 2006). Meanwhile, *V. parahaemolyticus* was isolated from retail shrimp (Letchumanan et al., 2015).

Molecular Identification and Phylogenetic Analysis

The rep-PCR analysis of 41 bacterial isolates showed that these isolates were classified into five

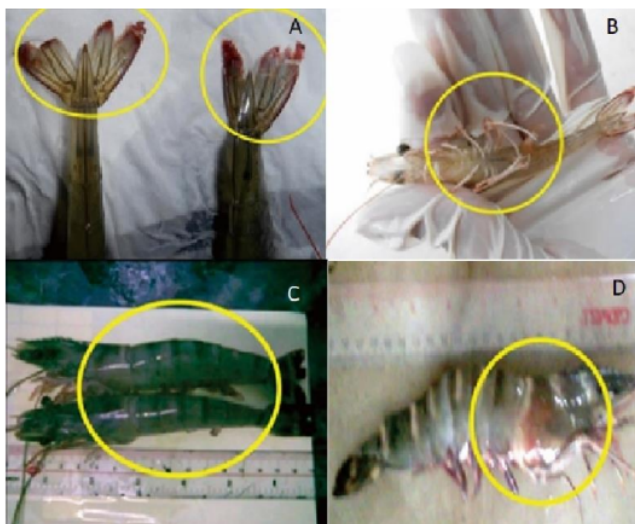


Figure 2. Shrimps with clinical signs of vibriosis (Note: A=reddish and melanosis in a telson, B=reddish in periopods and pleiopods, C=soft body, D=reddish carapace, periopods, pleiopods, mouth, and telson (d).

Table 1. Bacterial isolates obtained from the telson and inner hepatopancreas of shrimps with vibriosis clinical signs

No.	Isolate code	Location	Source of organ	Colony		
				Color	Form	Characteristic
1	JKP17	Pati	Hepatopancreas	Yellow	Oval	5 Convex
2	JKM05	Kendal	Hepatopancreas	Yellow	Rounded	Convex
3	JKM12	Kendal	Hepatopancreas	Green	Rounded	Convex
4	JKP11	Pati	Hepatopancreas	Yellow	Rounded	Convex
5	JKM18	Kendal	Hepatopancreas	Yellow	Rounded	Convex
6	JKP02	Pati	Hepatopancreas	Yellow	Rounded	Convex
7	JKP03	Pati	Hepatopancreas	Yellow	Rounded	Flat
8	JKM11	Kendal	Hepatopancreas	Black	Rounded	Convex
9	JKM17	Kendal	Hepatopancreas	Black	Rounded	Convex
10	JKP05	Pati	Hepatopancreas	Yellow	Oval	Convex
11	JKP12	Pati	Hepatopancreas	Yellow	Irregular	Flat
12	JKM07	Kendal	Hepatopancreas	Yellow	Rounded	Convex
13	JKM04	Kendal	Hepatopancreas	Yellow	Rounded	Convex
14	JKM01	Kendal	Hepatopancreas	Green	Rounded	Convex
15	JKP14	Pati	Hepatopancreas	White	Irregular	Convex
16	JKP15	Pati	Hepatopancreas	White	Irregular	Rough
17	JKM17	Kendal	Hepatopancreas	Yellow	Rounded	Concave
18	JKP18	Pati	Hepatopancreas	Yellow	Rounded	Flat
19	JKM15	Kendal	Hepatopancreas	Green	Rounded	Convex
20	JKM06	Kendal	Hepatopancreas	Yellow	Rounded	Convex
21	JKM03	Kendal	Hepatopancreas	Black	Rounded	Convex
22	JKM19	Kendal	Hepatopancreas	Yellow	Rounded	Convex
23	JKM20	Kendal	Hepatopancreas	Yellow	Rounded	Convex
24	JKP10	Pati	Hepatopancreas	Yellow	Rounded	Convex
25	JKP19	Pati	Hepatopancreas	Yellow	Irregular	Rough
26	JKP16	Pati	Hepatopancreas	Yellow	Rounded	Rough
27	JKM14	Kendal	Hepatopancreas	Black	Rounded	Convex
28	JKP08	Pati	Hepatopancreas	Yellow	Rounded	Convex
29	JKM13	Kendal	Hepatopancreas	Yellow	Rounded	5 Convex
30	JKP06	Pati	Hepatopancreas	Yellow	Rounded	Convex
31	JKM08	Kendal	Hepatopancreas	Yellow	Rounded	Convex
32	JKM09	Kendal	Hepatopancreas	Green	Rounded	Convex
33	JKP07	Pati	Hepatopancreas	Yellow	Rounded	Convex
34	JKP01	Pati	Hepatopancreas	Yellow	Rounded	Convex
35	JKM11	Kendal	Hepatopancreas	Yellow	Rounded	Convex
36	JKP09	Pati	Hepatopancreas	Green	Rounded	Convex
37	JKP04	Pati	Hepatopancreas	Yellow	Oval	Convex
38	JKM10	Kendal	Hepatopancreas	Black	Rounded	Convex
39	JKM02	Kendal	Hepatopancreas	Yellow	Rounded	Convex
40	JKM21	Kendal	Telson	Yellow	Rounded	Convex
41	JKM22	Kendal	Telson	Green	Rounded	Convex

groups (Figure 3). The five representative isolates, JKP03, JKP05, JKP19, JKM01, and JKM06, of each group were selected randomly for molecular identification (Table 2). Figure 3 and Table 2 demonstrate that *V. diabolicus* (16 isolates) and *S. algae* (10 isolates) were predominant species in shrimp cultured by traditional shrimp culture technology. *V. alginolyticus* and *V. rotiferianus* were only represented with seven isolates each. The lowest one was *V. parahaemolyticus* (1 strain). These results indicated that the *Vibrio* species diversity in the north coast of Central Java was higher than the diversity reported in the white pacific shrimp *L. vannamei* Kendal (Sarjito et al., 2018) and in *P. monodon* cultured in Sri Lanka (Heenatigala and Fernando, 2016) and South East Coast of India (Stalin &

Srinivasan, 2017). The *V. rotiferianus*, and *Shewanella* sp. found in the present study were also discovered in Sri Lanka, South East Coast of India, and Sical, Yucatan, Mexico (Heenatigala & Fernando, 2016; Stalin & Srinivasan, 2017; Rivera et al., 2019).

Table 2. 16S rDNA-based molecular identification of five *Vibrio* associated with shrimp vibriosis

The diversity of *Vibrio* related to vibriosis in shrimps cultured in traditional brackish water ponds in the north coast of Central Java showed that the JKP03, JKP05, JKP19, JKM01, and JKM06 isolates were closely related to *V. rotiferianus* strain HDC47, *V. parahaemolyticus* strain SEM52, *V. alginolyticus* strain CX-71, and *Shewanella algae* strain SFH3, respectively (Figure 4). Some previous studies confirmed

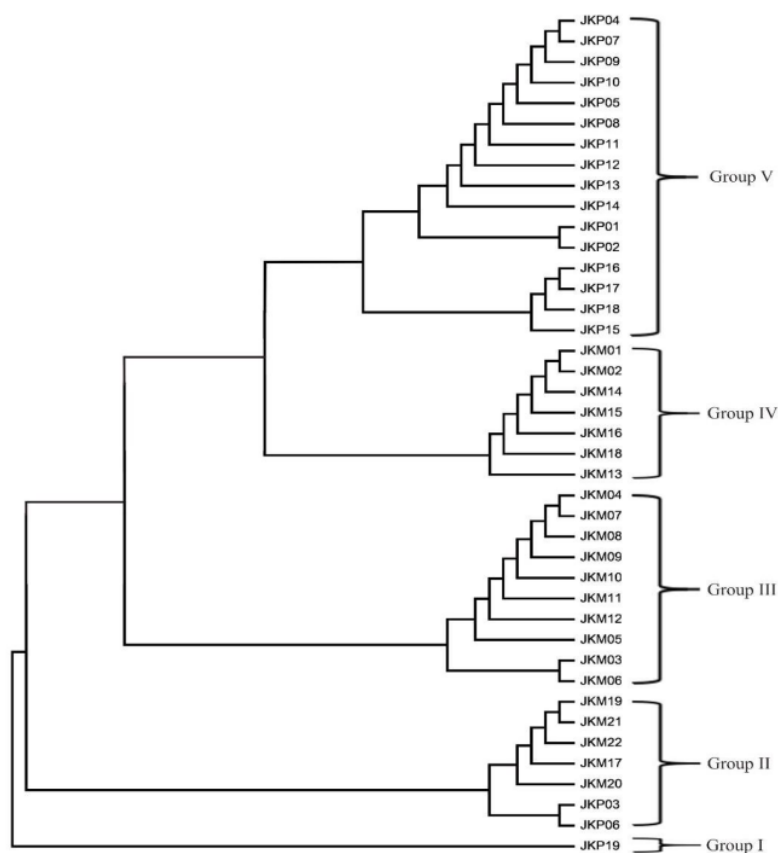


Figure 3. Dendrogram of repetitive PCR of 41 *Vibrio* isolates in shrimps

Table 2. 16S rDNA-based molecular identification of five *Vibrio* species associated with shrimp vibriosis

No.	Isolate	Closely Relative	Homology (%)	Acc. Number
1.	JKP03	<i>Vibrio rotiferianus</i>	100	GQ175915.1
2.	JKP05	<i>V. diabolicus</i>	99	MH044628.1
3.	JKP19	<i>V. parahaemolyticus</i>	94	MG548344.1
4.	JKM01	<i>V. alginolyticus</i>	97	MH368391.1
5.	JKM06	<i>Shewanella algae</i>	99	MG738264.1

that *V. rotiferianus* is a causative agent associated with shrimp *Fenneropenaeus chinensis* post larvae (Zhang et al., 2014). Meanwhile, *V. parahaemolyticus* is a pathogenic bacterium in *P. monodon* (Alagappan et al., 2017) and *L. vannamei* (Kumar et al., 2014; Kongchum et al., 2016; Raja et al., 2017). Further, *V. alginolyticus* has been found as a pathogenic bacterium in *L. vannamei* in Taiwan (Cheng et al., 2005; Chen et al., 2016; Liu et al., 2004; Wu et al., 2018) and in the grow-out ponds of tiger shrimp (*P. monodon*) in India and Large Yellow Croaker (Liu et al., 2014; Santhya et al., 2015; Selvin & Lipton, 2003; Shanmugasundara et al., 2015). Recently, *V. alginolyticus* bacterial species have been isolated from corals in India (Deb et al., 2020) and white shrimps in Central Java (Widowati et al., 2018), Bangladesh (Hannan et al., 2019), South India (Biju et al., 2016; Mastan & Begum 2016), and Malaysia (Muthukrishnan et al., 2019). In addition, they have been related to the stress of white shrimps (Peng et al., 2018).

Surprisingly, *V. diabolicus* was found in the present study. Limited studies reported about *V. diabolicus* associated with vibriosis in brackish water-cultured shrimp. This bacterium is commonly found as a bacterial pathogen in the mussel *Bathymodiolus azoricus* (Barros et al., 2016), coral *Pacillopora verrucosa* (Deb et al., 2020), and green mussel (Susilowati et al., 2019). This bacterial species was first found from deep-sea

hydrothermal vent polychaete (Deb et al., 2020) and in East Pacific Rise (Turner et al., 2018). Previous studies revealed that *V. diabolicus* might be isolated from polychaete (Raguenes et al., 1997; Rougeaux et al., 1999), horse mackerel, seawater sediment, dentex, oyster, and artemia collected from China, India, Greece, United States, East Pacific Rise, and Chile (Turner et al., 2018). According to the latest study by Susilowati et al. (2019), *V. diabolicus* is a known close genetic relationship with *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. fischeri*. *S. algae* is normally found in biofloc because it can be used to increase nutritional and disease resistance by application of probiotics (Far et al., 2013; Goudenege et al., 2014; Interaminense et al., 2019). In the present research, *S. algae* were recovered from shrimp vibriosis. Some previous studies also reported that this bacterium is a bacterial pathogen with massive mortality in *Carrasius auratus* (Altun et al., 2014), *Babylonia* spp. (Li et al., 2015), *Cynoglossus seilaensis* (Han et al., 2017), *Haliotis diversicolor*, *Crassostrea angulata*, *Meretrix lusoria*, *Perna viridis*, *Geloina erosa* (Tseng et al., 2018), and freshwater-cultured whiteleg shrimp *P. vannamei* (Cao et al., 2018). Given that vibriosis still exists in farms, continues to grow, control methods such as new vaccines, probiotics, and immunostimulant formulas must be developed for more potent efficacies.

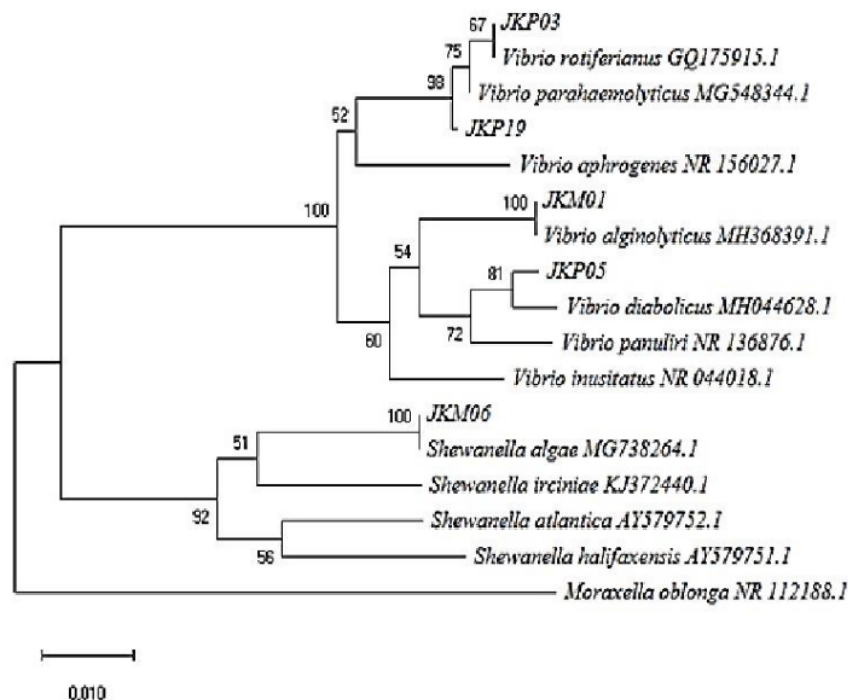


Figure 4. Phylogenetic tree of *Vibrio* associated with vibriosis in shrimps from traditional brackish water ponds of the northern coast of Central Java. The branching pattern was generated by the neighbor-joining method. Bootstrap values of 700 or more (from 1,000 replicates) are indicated at the nodes. *Moraxella oblonga* was used as an outgroup.

Conclusion

Vibrio associated with vibriosis in shrimps from traditional brackish water ponds of the northern coast of Central Java is present in high diversity. These bacterial associations were identified as *V. diabolicus*, *S. algae*, *V. alginolyticus*, *V. rotiferianus*, and *V. parahaemolyticus*. Given that vibriosis exists in farms and in high diversity, control methods such as probiotics, new vaccines, and immunostimulant formulas must be developed for further prevention and control of vibriosis in shrimp cultured in traditional brackish water ponds.

Ethical Statement

The research sample was moribund shrimps or shrimps with clinical symptoms of vibriosis. The shrimp was collected from the traditional brackish water ponds infected by vibriosis. In Indonesia, the ethical clearance commission has not regulated on invertebrates, but only vertebrate, while in the United States cephalopods have been addressed by the committee ethics. In accordance with the guidelines, EU directive 2010/63/EU for animal experiment and Indonesia regulation, so shrimp sampling, transportation and isolation bacteria from the samples were carried out by observing the principles of animal welfare. Surgical samples were performed under clove oil anesthesia and all efforts were made to minimize suffering.

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Author Contributions

Conceptualization: SJT, Data Curation: SJT and AS, Formal Analysis: AS, Funding Acquisition: AS, Investigation: SJT and AS, Methodology: SJT and AS, Project Administration: SJT, Resources: SJT, Software and Supervision: AS, visualization and writing original draft: SJT, Writing-review and editing: SJT and AS.

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Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

The authors have declared no conflict of interest.

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