CORRESPONDENCE PAPER

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

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Molecular Identification of Bacteria Associated with Shrimp's Vibriosis from Traditional Brackish Waterpond on the North Coastal of Central Java, Indonesia

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Molecular Identification of Bacteria Associated with Shrimp's Vibriosis from Traditional Brackish Waterpond on the North Coastal of Central Java, Indonesia

Abstract. Indonesian shrimp cultures are subject to threaten with vibriosis. Some traditional brackish water ponds along the north coast of Central Java is the only area remaining after the disease outbreaks destroyed the shrimp culture. The objective of this study was to discover the vibrio diversity associated with shrimp vibriosis in traditional brackish water ponds. Twenty-four shrimps, presumably infected vibriosis, were collected from 2 district regions on the north coast of Central Java in July-September 2018. Vibrios associated with the telson and inner part of the hepatopancreas of shrimp were isolated. Forty-one bacteria associated with vibriosis were obtained. Based on rep-PCR, representative five strains were selected for further study. The 16S rDNA sequence analysis showed that the JKP03, JKP05, JKP19, JKM01, and JKM06 isolates were closely related to *Vibrio rotiferianus*, *Vibrio diabolicus*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Shewanella algae*, respectively. Vibrio biodiversity in shrimp vibriosis was low. It confirmed that traditional shrimp farming was susceptible to vibriosis. Therefore, the findings of this study might be used as a basis for further disease prevention and control in traditional shrimp brackish water.

Keywords: *V. rotiferianus, V. diabolicus, V. parahaemolyticus, V. alginolyticus, S. algae*

INTRODUCTION

Shrimp is an important aquacultural species with high economic value and the most important export commodity from Central Java, Indonesia. Several brackish water ponds along the northern coast of Central Java are the only area remaining after the disease outbreaks destroyed the shrimp culture business in the 1990s (Hariati et al., 1995). In this area, the production of two species of shrimps, *Penaeus monodon* and *Litopenaeus vannamei* have still steadily increased. They are commonly cultured by using semi-intensive and traditional techniques. Most traditional shrimp farmers apply LEISA (low external input for sustainable aquaculture) by optimising local resources for instance using liquid compost from fermented organic waste from households for curing pond bottom and maintaining pond water quality (Ariyati et al., 2019). They also practice an integrated mangrove-shrimp aquaculture system, as Indonesian government's program, to rehabilitate and conserve mangrove forest. Although this system is more ecologically friendly compare to other types of aquaculture,

shrimp production is still low. Improper culture management, such as drainage of pond bottom and rely on a single sluice gate for water flow, causes viral and bacterial disease problems and result in mass mortality (Sarjito et al., 2012; Uddin et al., 2013; Liu et al., 2016). The emergence of antibiotic resistant bacteria due to excessive use of antibiotics that cause antibiotic residues in the environment. Bacterial disease outbreak, vibriosis, has still become a problem in shrimps culture in Indonesia (Isnansetyo et al., 2009; Istiqomah et al, 2020) and other countries (Abdelaziz et al., 2017; Mastan & Begum 2016).

Vibrio is the causative agent of vibriosis that common disease occurs in aquaculture worldwide (Akaylı & Timur, 2002). Crustaceans, molluscs, fish can be infected by these bacteria which results in mass mortality. Infection of vibrios in shrimps was characterized by pale hepatopancreas; reddish or pale on body carapace; reddish of uropod and telson; and red of antenna (Sarjito et al., 2018). Although *Vibrio* is a normal part of the bacterial flora in the estuarine and seawater environments, several vibrios are considered to be opportunistic pathogens in cultured shellfish, finfish, and shrimp (Austin & Austin 2007). Therefore, *Vibrio* may cause serious production loss in shrimp culture (Stalin & Srinivasan 2017). The vibriosis caused mortality in larvae, and adult stages by up to 50 % (Lightner 1996).

Most previous studies reported that vibriosis is related to vibrionacea. Most of them consist of genus Vibrio. However, two genera, namely *Shewanella algae* and *Listonella,* have been grouping on Vibrionaceae (MacDonell & Cowell 1985). Well known causative agents of vibriosis are *V. vulnificus, V. fluvialis, V. damsela, V .parahaemolyticus, V. fischeri, V.alginolyticus* (Chythanya et al., 2002; Kumar et al., 2014; Gopala et al., 2005). While Chandrakala and Priya (2017) have mentioned fourteen species Vibrio species known to be shrimp pathogens, namely *Vibrio harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbelli, V. fischeri, V. damsella, V. pelagicus, V. orientalis, V. ordalii, V. mediterrani, V. logei*.

There are many studies concerning genus vibrio in the aquaculture system (Liu et al., 2016; Sarjito et al., 2018; Gopala et al., 2005). However, most of the research was conducted on the mono species and in intensive cultured technology. The current study was commenced to elaborate on the shrimp medical symptoms which attacked by vibrios. A molecular approach was used to identify the causative agent of vibrios related to vibriosis. The study of vibrio diversity caused vibriosis in traditional brackish shrimps' pond is limited. The accuracy of the molecular method for identifying the genus *Vibrio* is very important for mitigating and designing disease prevention strategies for sustainable shrimp production. Considering vibriosis outbreaks has become an important limitation on shrimp production in Central Java.

In addition, these bacteria such as *Vibrio cholerae* can also cause human infections. Therefore, it is urgently needed to discover vibrio diversity associated with vibriosis in shrimps. The main objective of the current study was to develop the simple reliable molecular protocol to identify the genus Vibrio associated with vibriosis in shrimp of traditional brackish water pond on the north coast of Central Java.

MATERIALS AND METHODS

Shrimps sampling

Twenty four shrimps specimens with total lengths of 16,6 – 17,2 cm, presumably infected vibriosis, were sampled from Wonorejo dan Turunrejo subdistricts (Kendal district) and Margomulyo dan Puncel subdistricts (Pati district) in the north coast of Central Java (Figure 1). The samples were kept in an insulated container and brought to the laboratory for bacterial isolation. Eleven black tiger shrimps (*P. monodon*) and thirteen pacific white shrimps (*L. vannamei*) were used as material research.

Figure 1. The collection sites of the shrimps

Bacterial isolation

Bacterial isolation was done by scraping off the telson and inner part of hepatopancreas with a sterile scalpel. The tissues were serially diluted in 10^{-1}, 10^{-3} and10^{-5} CFU ml^{-1}, spread on the TCBS agar (Oxoid, England) and incubated at room temperature for 48 hours (Brock and Madigan 1991; Sarjito et al., 2018). Then, colonies with different morphological features were picked up and purified by serially streaking simple colony on agar plates.

Repetitive – Polymerase Chain Reaction (rep-PCR)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by 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 the *Vibrio* sp. Was adopted from previous methods (Brock & Madigan 1991; Sarjito et al, 2018). The rep–PCR oligonucleotide primers evaluated in this study were BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′), REP1R-I (5′-IIIICGICGICATCI 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 as described by the manufacturer. The reaction mixtures were denaturated at

95°C for 5 minutes followed by 30 cycles of amplification at 92°C for 60 seconds, annealing at 50°C for 90 seconds, followed by a final extension at 68°C for ten minutes. The 5 ul 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

Based on electrophoresis results on the 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

The GM3F and GM4R primers were used to amplify the 16S rRNA gene (Muyzer et al., 1995). Genomic DNA of vibrios strains was extracted from bacterial cells by freeze and thaw method. The 16S rRNA gene amplification, purification, and sequence were carried out by using a method that previously used by Radjasa et al. (2007) and Sarjito et al. (2018). PCR products were sequenced then compared by BLAST homology searching (Altschul et al., 1990; Widowati et al, 2020). 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 bacterial isolation The vibriosis signs were reddish and melanosis in telson (a), reddish in periopods and pleiopods (b), soft body (c) and reddish (Figure 2).

Figure 2. Shrimps with clinical signs of vibriosis

These clinical signs were similar to the results of vibriosis in the previous research (Raja et al., 2017; Sarjito et al., 2018). However, the clinical previously described by Mastan and Begum (2016) such as loss of appetite, red to brown gills, reduced feeding, empty gut, and general septicemia was not found in the present research.

The isolation succeed to isolate 41 pure bacterial strains based on the different morphology and type of growth on the TSBS-agar medium. Table 1 showed that bacterial strains were morphologically characterized by colony form (oval, circular, irregular) and color colonies (green, black, yellow, and white). Widanarni et al. (2003) reported the bacterial characteristics isolated from tiger shrimp larvae of Labuan, Pangandaran, and Lampung, Indonesia were gram-negative, short rod-shape, exhibited yellow colonies. Approximately 60% of bacteria associated with shrimp's vibriosis in Sri Lanka belonged to Vibrioceae (Raja et al., 2017). Moreover, the six species of Vibrio were collected from diseased shrimps in the culture ponds of Andhra Pradesh India (Jayasree et al., 2006). While *V. parahaemolyticus* was isolated from retail shrimp (Letchumanan et al., 2015).

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

Molecular identification and phylogenetic analysis

The rep-PCR analysis of 41 bacterial isolates showed that these isolates were classified into five 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 demonstrated that the *V. diabolicus* (16 isolates) and *S. algae* (10 isolates) were predominant species in shrimp cultured by traditional shrimp culture technology. Whereas, *V. alginolyticus* and *V. rotiferianus* were only represented with 7 isolates and 7 isolates, respectively. The lowest one was *V. parahaemolyticus* (1 strain). These results indicated that the vibrio diversity in the north coast of Central Java was higher than the diversity reported in white pacific shrimps, *L. vannamaei* Kendal (Sarjito et al., 2018), in *P. monodon* cultured in Sri Lanka (Heenatigala and Fernando, 2016) and South East Coast of India (Stalin & Srinivasan, 2017*).*The *V. rotiferianus, V. rotiferianus* and *Shewanella* sp. that 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).

Figure 3. Dendrogram of repetitive PCR of 41 isolated vibrios in shrimps

Table 2. 16S rDNA-based molecular identification of five Vibrio associated with Shrimp's vibriosis

The diversity of vibrios 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 *Vibrio rotiferianus* strain HDC47, *V. parahaemolyticus* strain SEM52, *V. alginolyticus* strain CX-71 and *Shewanella algae* strain SFH3, respectively (Figure 4). Some previous study reported that *V. rotiferianus* has been confirmed as a causative agent associated with shrimp *Fenneropenaeus chinensis* post larvae (Zhang et al., 2014). While *V. parahaemolyticus* has been found as a pathogenic bacterium in *P. monodon* (Alagappan et al., 2017), and *L. vannamei* (Kumar et al., 2015; Kongchum et al., 2017; 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), in the grow-out ponds of tiger shrimp (*P. monodon*) in India and Large Yellow Croaker (Liu et al., 2014; Santhyia et al., 2015; Selvin & Lipton 2004; Shanmugasundaram et al., 2015). Recently, *V. alginolyticus* bacterial species has been also isolated from corals, India (Deb et al., 2020), white shrimps in Central Java (Widowati et al., 2018), Bangladesh (Hannan et al., 2019), South India (Biju et al., 2016, Mastan & Begum 2016); Malaysia (Muthukrishanana et al., 2019) and it's related to the stress of white shrimps (Peng et al., 2019).

Figure 4. Phylogenetic-tree of the vibrios associated with vibriosis in shrimps from traditional brackishwater ponds of the northern coast of Central Java.

It was surprising that *V. diabolicus* was found in the present study. Limited reports are found regarding *V. diabolicus* associated with vibriosis in brackish water-cultured shrimp. This bacterium is commonly found as a bacterial pathogen in mussel *Bathymodiolus azoricus* (Barros et al., 2016), corals *Pacillopora verrucosa* (Deb et al., 2020), and green mussel (Susilowati et al., 2019). This bacterial species was firstly found from deep-sea hydrothermal vent polychaete (Deb et al., 2020) and in East Pacific Rise (Turner et al., 2018). The previous study 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* normally found in biofloc because it can be used to increase nutritional and disease resistance by application of probiotic (Far et al., 2013;

Goudenege et al., 2014; Interaminense et al., 2019). The present research also revealed that *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., 2010), *Cynoglossus seilaevis* (Han et al., 2017), *Haliotis diversicolor, Crassostrea angulate, Meretrix lusoria, Pena viridis, Geloina erosa* (Tseng et al., 2018) and freshwater-cultured whiteleg shrimp, *P. vannamei* (Cao et al., 2018). Since vibriosis disease still existed in farms and continues to grow, it is urgently needed to develop the control methods, such as the search for new vaccines, probiotics, and immunostimulant formulas for more potent efficacies.

CONCLUSION

Vibrio in traditional brackish water shrimp pond on the northern coast of Central Java, Indonesia is present in low diversity. However, this present study confirmed that shrimp cultured in traditional brackish water pond is susceptible to vibriosis. Therefore, the findings of this study might be used as basic information for further vibriosis prevention and control in shrimp cultured in the traditional brackish water pond.

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TABLES:

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

No.	Isolate code	Location	Source of organ	**Colony**		
				Colour	**Form**	**Characteristic**
1	JKP17	Pati	Hepatopancreas	Yellow	Oval	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	Hepatopankreas	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	Hepatopankreas	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	Hepatopankreas	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	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

Table 2. 16S rDNA-based molecular identification of five Vibrio associated with Shrimp's vibriosis

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

FIGURES:

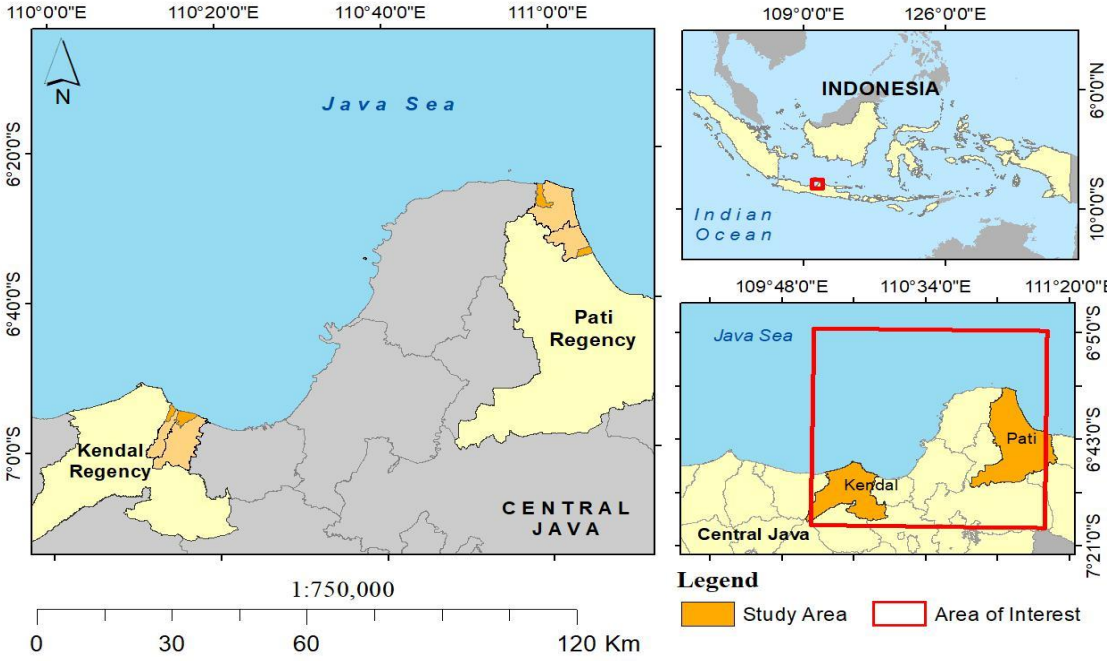


Figure 1. The collection sites of the shrimps

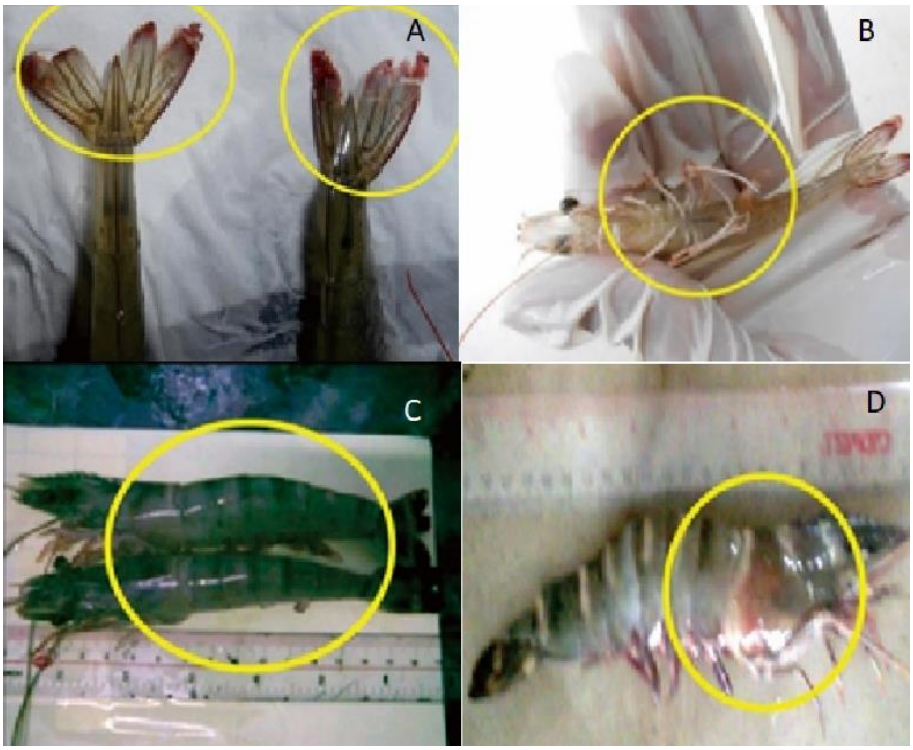


Figure 2. Shrimps with clinical signs of vibriosis (Note: A= reddish and melanosis in a telson, B= reddish in periopods and pleiopods, C= soft body, D= reddish carapace, periopods, pleopods, mouth, and telson carapace, periopods, pleopods, mouth, and telson (d).

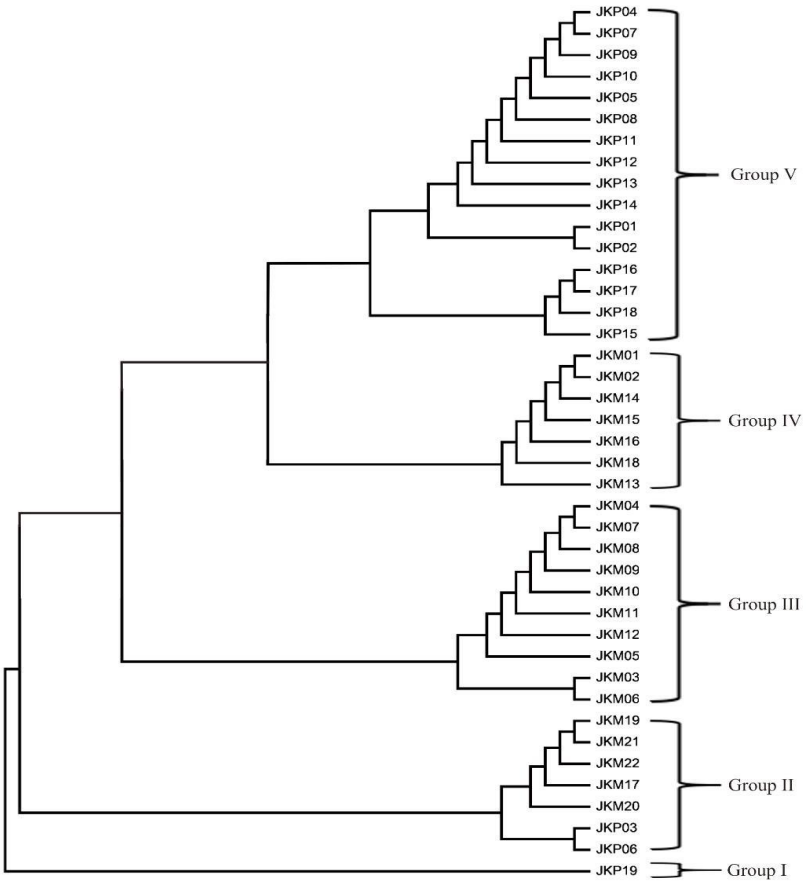
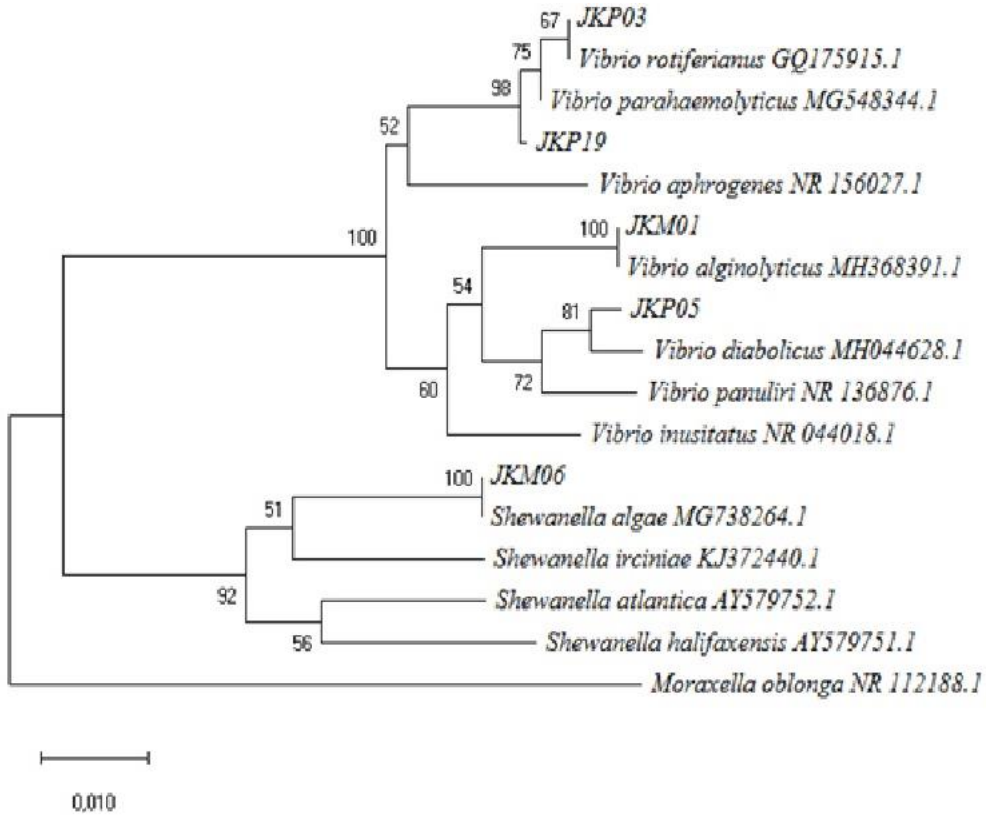


Figure 3. Dendrogram of repetitive PCR of 41 isolated vibrios in shrimps



554

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

559

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1. Reviewer Comments
Good effort

2. Reviewer Comments
Dear author,
Please explain why only 24 odd samples where selected for the study? How did you determine the CFU at the time of sampling (isolating tissue)?

Check the MS for grammar and language, add missing references and improve discussion.

All the best.

Reviewer 1

Date Invited:	Nov 18, 2020
Date Returned:	Nov 28, 2020
Check Revision:	No
Newness, currency and originality in the manuscript:	Good
Straightness and validity of material - method:	Good
Reliability, consistence of findings and power of discussion:	Good
Coordination of statement and wording and fluency of language:	Poor
Success in pursuing, selecting and presentation of references:	Middle

Manuscript category:	Research Paper
Note/Commend/Suggest About Abstract:	suggest change of title to ` Associated Vibrio Species in Shrimp Vibrosis in Traditional Brackish Water Pond in North Coast of Central Java, Indonesia` Abstract there is the need for some details and specificity in the method section of the abstract: 1 remove bacteria being general. use Vibrio instead (being specific). Only the Vibrio species was targeted using only the TCBS agar you cannot use bacteria in general term. 2. The 16S rDNA sequence analysis? you mean phylogenetic analysis of the 16S rDNA sequences? 3.Vibrio biodiversity in shrimp vibriosis was low? Specify what measure is low/ unit of lowness? 4.The conclusion here is not clear enough. Not entirely linked to results e.g.link disease prevention and control with specific statement in result. 5. Consider to change most of the keywords. They are mostly weak.
Note/Commend/Suggest About Introduction:	. There are challenges of wrong use of grammar, spelling and tenses. This occurred throughout the article. Kindly run grammar and spelling software for the entire manuscript. Sentence in line 38 is incomplete; line 51 add family to Vibrionaceae; delete one of the species in line 56.
Note/Commend/Suggest About Material and Methods:	line 77, use dot instead of comma to read 16.6-17.2 cm Justify choice of the location in this section; why were they studied? sampling procedure not detailed enough; you must provide information to justify choice of 11 and 13- how you arrived at these numbers, write out the reaction cocktail detail,
Note/Commend/Suggest About Results:	In line 135, provide discussion from past study why general septicemia may not be found and relate your result with authors In line 136, use either 41 Vibrio specimens /isolates. In line 137, use TCBS instead. Kindly rewrite the discussion to properly link the results in line 158 use use Vibrio species diversity line 161, V. volife.. was repeated Delete one Conclusions need to be more precise and based on th etarget of the key element discussed. Take note of figure and table caption to be self explanatory

Reviewer 2

Date Invited:	Nov 18, 2020
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Check Revision:	Yes
Newness, currency and originality in the manuscript:	Good
Straightness and validity of material - method:	Middle
Reliability, consistence of findings and power of discussion:	Middle
Coordination of statement and wording and fluency of language:	Poor
Success in pursuing, selecting and presentation of references:	Poor
Manuscript category:	Short Paper

Manuscript Information

Manuscript ID:	**GENAQUA-362**
Title:	Molecular Identification of Bacteria Associated with Shrimp's Vibriosis from Traditional Brackish Waterpond on the North Coastal of Central Java, Indonesia
Small Title:	Bacteria Associated with Shrimp's Vibriosis
Authors:	Sarjito Sarjito[1], Agus Sabdono[2]
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Keywords:	V. rotiferianus, V. diabolicus, V. parahaemolyticus, V. alginolyticus, S. algae
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Abstract

. Indonesian shrimp cultures are subject to threaten with vibriosis. Some traditional brackish water ponds along the north coast of Central Java is the only area remaining after the disease outbreaks destroyed the shrimp culture. The objective of this study was to discover the vibrio diversity associated with shrimp vibriosis in traditional brackish water ponds. Twenty-four shrimps, presumably infected vibriosis, were collected from 2 district regions on the north coast of Central Java in July-September 2018. Vibrios associated with the telson and inner part of the hepatopancreas of shrimp were isolated. Forty-one bacteria associated with vibriosis were obtained. Based on rep-PCR, representative five strains were selected for further study. The 16S rDNA sequence analysis showed that the JKP03, JKP05, JKP19, JKM01, and JKM06 isolates were closely related to Vibrio rotiferianus, Vibrio diabolicus, Vibrio parahaemolyticus, Vibrio alginolyticus, and Shewanella algae, respectively. Vibrio biodiversity in shrimp vibriosis was low. It confirmed that traditional shrimp farming was susceptible to vibriosis. Therefore, the findings of this study might be used as a basis for further disease prevention and control in traditional shrimp brackish water.

Manuscript Files

File Name	File Size	Date Created	Category	Description
GENAQUA-18186-9-title-page.pdf (../pdf-files/out/362-GENAQUA-18186-9-title-page.pdf)	20 KB	Nov 18, 2020	Title Page	Title page
GENAQUA-18186-1-table-1.pdf (../pdf-files/out/362-GENAQUA-18186-1-table-1.pdf)	45 KB	Nov 18, 2020	Table	Table 1
GENAQUA-18186-7-table-2.pdf (../pdf-files/out/362-GENAQUA-18186-7-table-2.pdf)	19 KB	Nov 18, 2020	Table	Table 2
GENAQUA-18186-6-figure-1.-sampling-site-locations.jpg (../pdf-files/in/362-GENAQUA-18186-6-figure-1.-sampling-site-locations.jpg)	334 KB	Nov 18, 2020	Figure	Figure 1
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GENAQUA-18186-4-main-document-manuscript-sarjito-ised.pdf (../pdf-files/out/362-GENAQUA-18186-4-main-document-manuscript-sarjito-ised.pdf)	1385 KB	Nov 18, 2020	Main Document	TRJS-18186-main document-sarjito-2020 revised

Second Reminder for Revision

From: GenAqua (info@genaqua.org)

To: sarjito_msdp@yahoo.com

Date: Tuesday, December 22, 2020, 10:42 PM GMT+7

ISSN: 2459-1831

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Dear Sarjito Sarjito,

We would like to remind you that the revised version of your manuscript entitled "Molecular Identification of Bacteria Associated with Shrimp's Vibriosis from Traditional Brackish Waterpond on the North Coastal of Central Java, Indonesia" has not been yet received although 23 days passed since the request has been sent to you. You can submit your revision through the online system. The site is located at http://www.genaqua.org/ Please upload the file containing your revised manuscript. The rebuttal letter should be placed in "cover letter" section. Please note that you should submit your revised letter by clicking on "revision requested" link, not as a new manuscript.

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Sincerely.

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From: Sarjito Sarjito (sarjito_msdp@yahoo.com)

To: info@genaqua.org

Date: Monday, December 28, 2020, 11:11 AM GMT+7

Subject: Submission of revised paper 362-GENAQUA-18186-4

Dear Editor,

Forgive us for being late resubmit our manuscript. We have difficulty in technical resubmit our revised manuscript using online system on deleting old files. Regarding to our manuscript anyway, we appreciate the careful review and constructive suggestions. We have carefully reviewed the comments and have revised the manuscript accordingly. Our responses are given in a point-by-point manner in the list (attached). Changes to the manuscript are shown in text highlight blue color (attached). It is our belief that the manuscript is substantially improved after making the suggested edits. We hope the revised version is now suitable for publication and look forward to hearing from you in due course.

Sincerely yours,

Sarjito


MAIN DOCUMENT.doc
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LIST OF COMMENTS AND MODIFICATIONS revised 1.docx
26.6kB


TITLE PAGE.docx
19.4kB

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

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LIST OF CORRECTIONS

REVIEWER-1

NO	COMMENTS	RESPONSE
1.	TITLE: suggest change of title to `Associated Vibrio Species in Shrimp Vibrosis in Traditional Brackish Water Pond in North Coast of Central Java, Indonesia`	TITLE: Title is changed as suggestion to be 'Associated Vibrio Species in Shrimp Vibrosis in Traditional Brackish Water Pond in North Coast of Central Java, Indonesia'
2.	ABSTRACT: There is the need for some details and specificity in the method section of the abstract:	ABSTRACT: We also added some sentences
	1. remove bacteria being general. use Vibrio instead (being specific). Only the Vibrio species was targeted using only the TCBS agar you cannot use bacteria in general term.	Let me think about it. Yes I agree that TCBS is a type of selective agar culture plate to isolate Vibrio species. However, some previous studies reported that there are any bacteria which can grow on TCBS apart from Vibrio spp. such as Shewanella sp. (Martin, 2015), Aeromonas (Hossain, 2018). Then, we use bacteria associated with vibriosis instead before 16S rDNA molecular identification.
	2. The 16S rDNA sequence analysis? you mean phylogenetic analysis of the 16S rDNA sequences?	Both homology BLAST and phylogenetic analysis were used to analyze the 16S rDNA sequences as presented in Table 2 and Figure 4.
	3.Vibrio biodiversity in shrimp vibriosis was low? Specify what measure is low/ unit of lowness?	Thanks for your question, we made mistake. We corrected to be: *Vibrio biodiversity in shrimp vibriosis was high*. After reanalysed using Shanon index, we found that the diversity value is 9.6 (not presented) $H' \leq 2$ = low; $2 < H' \leq 3$ = moderate; $H' > 3$ = high.
	4.The conclusion here is not clear enough. Not entirely linked to results e.g.link disease prevention and control with specific statement in result.	The conclusion changes as suggestion to be: Therefore, the findings of this study might require further development of the control methods such as vaccines, probiotics and immunostimulant formulas for shrimp's vibriosis outbreak prevention and control in traditional brackish water pond.

	5. Consider to change most of the keywords. They are mostly weak	Keywords changed as suggestion. *Diversity, Vibrio, rep-PCR, Brackish water, North Coast of Central Java*
3.	INTRODUCTION	
	-There are challenges of wrong use of grammar, spelling and tenses. This occurred throughout the article. Kindly run grammar and spelling software for the entire manuscript.	The entire manuscript was already corrected by running grammar and spelling software (Grammarly)
	Sentence in line 38 is incomplete; The emergence of antibiotic-resistant bacteria due to excessive use of antibiotics cause antibiotic residues in the environment.	Corrected as In order to combat the vibriosis in shrimps, some farmer using of chemical substance, such as antibiotic. Due to the excessive antibiotics dosage applied that was resulted on antibiotic residues and emergence of antibiotic resistant bacteria in the brackish water pond environment. Therefore,..
	line 51 add family to Vibrionaceae; Most previous studies reported that vibriosis is related to vibrionacea.	Corrected as Most previous studies reported that vibriosis is related to family vibrionacea
	delete one of the species in line 56. While Chandrakala and Priya (2017) have mentioned fourteen species Vibrio species known to be shrimp pathogens, namely namely *Vibrio harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbelli, V. fischeri, V. damsella, V.* 58 *pelagicus, V. orientalis, V. ordalii, V. mediterrani, V. logei*.	Deleted to be …….*V. harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbelli, V. fischeri, V. damsella, V.* 58 *pelagicus, V. orientalis, V. ordalii, V. mediterrani, V. logei*
4.	MATERIALS AND METHODS	
	- line 77, use dot instead of comma to read 16,6-17,2 cm	Already corrected to be 16.6-17.2
	- Justify choice of the location in this section; why were they studied?	Kendal and Pati were choiced as studi sites because these locations are the only area remaining after the disease outbreaks destroyed the shrimp culture business in the 1990s
	sampling procedure not detailed enough; you must provide information to justify	Sampling procedure was corrected as suggestion to be:

	choice of 11 and 13- how you arrived at these numbers, write out the reaction cocktail detail,	Exploratory method with purposive sampling was used in this study. Shrimps specimens presumably infected vibriosis were sampled from traditional brackish water of Wonorejo dan Turunrejo subdistricts (Kendal district) and Margomulyo dan Puncel subdistricts (Pati district) in the north coast of Central Java (Figure 1). The samples were kept in an insulated container and brought to the laboratory for bacterial isolation. Shrimp samples were categories by their sizes of 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 locations were sampled randomly. Totally twenty four specimens (11 black tiger shrimps and 13 pacific white shrimps) were used as material research.
5.	RESULTS AND DISCUSSION	
	In line 135, provide discussion from past study why general septicemia may not be found and relate your result with authors In line 136, use either 41 Vibrio specimens /isolates.	Discussion was provided as suggested: It might be due to the different virulence degree. Besides, the degree of virulence of various Vibrio isolates depends on its source and the pond environmental conditions. Even differences occur in the degree of virulence of different species of *Vibrio* and also different isolates of the same species. Jayasree et al (2006) reported that *V. harveyi* isolated from LSS shrimp is the most virulent. While Soto-Rodriguez (2006) showed that *V. parahaemolyticus* is the most virulence. Further, the virulence is generally dependent on the strain, density, infection route, exposure time, species considered, and age and general condition of the shrimp (Alday-Sanz et al. 2002; Saulnier et al, 2000).
	In line 137, use TCBS instead. Kindly rewrite the discussion to properly link the results	Corrected as suggested to be TCBS

	in line 158 use use Vibrio species diversity line 161, V. volife.. was repeated Delete one The *V. rotiferianus, V. rotiferianus* and *Shewanella* sp. That.	.. results indicated that the vibrio species diversity on the north coast of Central Java The *V. rotiferianus,* and *Shewanella* sp. That
6	CONCLUSION	
	Conclusions need to be more precise and based on the target of the key element discussed. Take note of figure and table caption to be self explanatory	The conclusion is already corrected as suggested to be: The vibrios associated with vibriosis in shrimps from traditional brackishwater ponds of the northern coast of Central Java is present in high diversity. These bacterial associations are identified as *V. diabolicus , S. algae, V. alginolyticus and V. rotiferianus* and *V. parahaemolyticus*. Since vibriosis disease exists in farms and in high diversity, it is urgently needed to develop the control methods, such as the search for probiotics, new vaccines, and immunostimulant formulas for further vibriosis prevention and control of shrimp cultured in the traditional brackish water ponds.

REVIEWER-2

NO	COMMENTS	RESPONSES
1.	Please explain why only 24 odd samples where selected for the study?	Shrimps specimens presumably infected vibriosis were sampled from traditional brackish water of Wonorejo dan Turunrejo subdistricts (Kendal district) and Margomulyo dan Puncel subdistricts (Pati district) in the north coast of Central Java (Figure 1). The samples were kept in an insulated container and brought to the laboratory for bacterial isolation. Shrimp samples were categories by their sizes of 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 locations were sampled randomly. Totally twenty four specimens (11 black tiger shrimps and 13 pacific white shrimps) were used as material research. It was not appropriate time to get sample due to harvesting season. Too difficult to find shrimp's vibriosis on those size. For instance, we only found 2 shrimps of black tiger in one location.
	How did you determine the CFU at the time of sampling (isolating tissue)?	Mistake happen. We can't determine the Colony Forming Units (CFU) at the time of sampling. CFU omitted Correction: Bacterial isolation was done by scraping off the telson and inner part of the hepatopancreas with a sterile scalpel. The tissues were serially diluted in 10^{-1}, 10^{-3} and10^{-5} of the resulting paste that are prepared in sterile water. Fifty ul aliquots of each dilution were spread on the TCBS agar (Oxoid England) and incubated at room temperature for 48 hours (Brock and Madigan 1991; Sarjito et al., 2018). Then, colonies with different morphological features were picked up and purified by serially streaking simple colonies on agar plates.
2.	Check the MS for grammar and language, add missing references and improve discussion.	We have already checked the MS for grammar and language (GRAMMARLY software), checked the references and corrected as suggestion.

MANUSCRIPT
REVISED #1
28 DECEMBER 2020

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

Abstract. Indonesian shrimp cultures are subject to threaten with vibriosis. Some traditional brackish waters in Central Java is the only area remaining after the disease outbreaks. The research objective was to discover the vibrio diversity associated with shrimp vibriosis in traditional brackish waters. Exploratory method with purposive sampling was used in this study. Twenty-four infected vibriosis shrimps were collected from 2 district regions on the north coast of Central Java in July-September 2018. The bacteria associated in shrimp's vibriosis was isolated from the telson and inner part of the hepatopancreas with TCBS medium. Forty-one bacteria associated with shrimp vibriosis were obtained, then rep-PCR was done to get vibrios strains. Based on rep-PCR, representative five strains were selected for further study. The 16S rDNA sequence analysis showed that the JKP03, JKP05, JKP19, JKM01, and JKM06 isolates were closely related to *Vibrio rotiferianus*, *Vibrio diabolicus*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Shewanella algae*, respectively. Vibrio biodiversity in shrimp vibriosis was high. It confirmed that traditional shrimp farming was susceptible to vibriosis. Therefore, the findings of this study might require further development of the control methods such as vaccines, probiotics and immunostimulant formulas for shrimp's vibriosis outbreak prevention and control in traditional brackish water pond.

Keywords: Diversity, Vibrio, rep-PCR, Brackish water, North Coast of Central Java.

INTRODUCTION

Shrimp is an important aquacultural species with high economic value and the most important export commodity from Central Java, Indonesia. Several brackish water ponds along the northern coast of Central Java are the only area remaining after the disease outbreaks destroyed the shrimp culture business in the 1990s (Hariati et al., 1995). The culture species in this area mainly is *Peneaus monodon* and l*itopeneous vannamae*. The production of the two species of shrimps, *P. monodon* and *L. vannamei* have still steadily increased. They are commonly cultured by using semi-intensive and traditional techniques. Most traditional shrimp farmers apply LEISA (low external input for sustainable aquaculture) by optimising local resources for instance using liquid compost from fermented organic waste

from households for curing pond bottom and maintaining pond water quality (Ariyati et al., 2019). They also practice an integrated mangrove-shrimp aquaculture system, as Indonesian government's program, to rehabilitate and conserve mangrove forest. Although this system is more ecologically friendly compare to other types of aquaculture, shrimp production is still low. Improper culture management, such as drainage of pond bottom and rely on a single sluice gate for water flow, causes viral and bacterial disease problems and result in mass mortality (Sarjito et al., 2012; Uddin et al., 2013; Liu et al., 2016). In order to combat the vibriosis in shrimps, some farmer using of chemical substance, such as antibiotic. Due to the excessive antibiotics dosage applied that was resulted on antibiotic residues and emergence of antibiotic resistant bacteria in the brackish water pond environment. Therefore, bacterial disease outbreak, vibriosis, has still become a problem in shrimps culture in Indonesia (Isnansetyo et al., 2009; Istiqomah et al, 2020) and other countries (Abdelaziz et al., 2017; Mastan & Begum 2016).

Vibrio is the causative agent of vibriosis that common disease occurs in aquaculture worldwide (Akaylı & Timur, 2002). Crustaceans, molluscs, fish can be infected by these bacteria which results in mass mortality. Infection of vibrios in shrimps was characterized by pale hepatopancreas; reddish or pale on body carapace; reddish of uropod and telson; and red of antenna (Sarjito et al., 2018). Although *Vibrio* is a normal part of the bacterial flora in the estuarine and seawater environments, several vibrios are considered to be opportunistic pathogens in cultured shellfish, finfish, and shrimp (Austin & Austin 2007). Therefore, *Vibrio* may cause serious production loss in shrimp culture (Stalin & Srinivasan 2017). The vibriosis caused mortality in larvae, and adult stages by up to 50 % (Lightner 1996).

Most previous studies reported that vibriosis is related to family vibrionacea. Most of them consist of genus Vibrio. However, two genera, namely *Shewanella algae* and *Listonella*, have been grouping on Vibrionaceae (MacDonell & Cowell 1985). Well known causative agents of vibriosis are *V. vulnificus, V. fluvialis, V. damsela, V. parahaemolyticus, V. fischeri, V. alginolyticus* (Chythanya et al., 2002; Kumar et al., 2014; Gopala et al., 2005). While Chandrakala and Priya (2017) have mentioned fourteen vibrio species known to be shrimp pathogens, namely *V. harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbelli, V. fischeri, V. damsella, V. pelagicus, V. orientalis, V. ordalii, V. mediterrani, V. logei*.

There are many studies concerning genus vibrio in the aquaculture system (Liu et al., 2016; Sarjito et al., 2018; Gopala et al., 2005). However, most of the research was conducted on the mono species and in intensive cultured technology. The current study was commenced

to elaborate on the shrimp medical symptoms which attacked by vibrios. A molecular approach was used to identify the causative agent of vibrios related to vibriosis. The study of vibrio diversity caused vibriosis in traditional brackish shrimps' pond is limited. The accuracy of the molecular method for identifying the genus *Vibrio* is very important for mitigating and designing disease prevention strategies for sustainable shrimp production. Considering vibriosis outbreaks has become an important limitation on shrimp production in Central Java. In addition, these bacteria such as *Vibrio cholerae* can also cause human infections. Therefore, it is urgently needed to discover vibrio diversity associated with vibriosis in shrimps. The main objective of the current study was to develop the simple reliable molecular protocol to identify the genus Vibrio associated with vibriosis in 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. Shrimps specimens presumably infected vibriosis were sampled from traditional brackish water of Wonorejo dan Turunrejo subdistricts (Kendal district) and Margomulyo dan Puncel subdistricts (Pati district) in the north coast of Central Java (Figure 1). The samples were kept in an insulated container and brought to the laboratory for bacterial isolation. Shrimp samples were categories by their sizes of 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 locations were sampled randomly. Totally twenty four specimens (11 black tiger shrimps and 13 pacific white shrimps) were used as material research.

Figure 1. The collection sites of the shrimps

Bacterial isolation

Bacterial isolation was done by scraping off the telson and inner part of the hepatopancreas with a sterile scalpel. The tissues were serially diluted in 10^{-1}, 10^{-3} and10^{-5} of the resulting paste that are prepared in sterile water. Fifty ul aliquots of each dilution were spread on the TCBS agar (Oxoid, England) and incubated at room temperature for 48 hours (Brock and Madigan 1991; Sarjito et 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)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by 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 the *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′-IIIICGICGICATCI 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 as described by the manufacturer. The reaction mixtures were denaturated at 95°C for 5 minutes followed by 30 cycles of amplification at 92°C for 60 seconds, annealing at 50°C for 90 seconds, followed by a final extension at 68°C for ten minutes. The 5 ul 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

Based on electrophoresis results on the 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

The GM3F and GM4R primers were used to amplify the 16S rRNA gene (Muyzer et al., 1995). Genomic DNA of vibrios strains was extracted from bacterial cells by freeze and thaw method. The 16S rRNA gene amplification, purification, and sequence were carried out by using a method that previously used by Radjasa et al. (2007) and Sarjito et al. (2018). PCR products were sequenced 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 The vibriosis signs were reddish and melanosis in telson (a), reddish in periopods and pleiopods (b), soft body (c) and reddish (Figure 2).

Figure 2. Shrimps with clinical signs of vibriosis

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

The isolation succed to isolate 41 pure bacterial strains based on the different morphology and type of growth on the TCBS-agar medium. Table 1 showed that bacterial strains were morphologically characterized by colony form (oval, circular, irregular) and color colonies (green, black, yellow, and white). Widanarni et al. (2003) reported the bacterial characteristics isolated from tiger shrimp larvae of Labuan, Pangandaran, and Lampung, Indonesia were gram-negative, short rod-shape, exhibited yellow colonies. Approximately 60% of bacteria associated with shrimp's vibriosis in Sri Lanka belonged to Vibrioceae (Raja et al., 2017). Moreover, the six species of Vibrio were collected from diseased shrimps in the culture ponds of Andhra Pradesh India (Jayasree et al., 2006). While *V. parahaemolyticus* was isolated from retail shrimp (Letchumanan et al., 2015).

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

Molecular identification and phylogenetic analysis

The rep-PCR analysis of 41 bacterial isolates showed that these isolates were classified into five 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 demonstrated that the *V. diabolicus* (16 isolates) and *S. algae* (10 isolates) were predominant species in shrimp cultured by traditional shrimp culture technology. Whereas, *V. alginolyticus* and *V. rotiferianus* were only represented with 7 isolates and 7 isolates, respectively. The lowest one was *V. parahaemolyticus* (1 strain). These results indicated that the vibrio spesies diversity in the north coast of Central Java was higher than the diversity reported in white pacific shrimps, *L. vannamaei* Kendal (Sarjito et al., 2018), 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. that 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).

Figure 3. Dendrogram of repetitive PCR of 41 isolated vibrios in shrimps

Table 2. 16S rDNA-based molecular identification of five Vibrio associated with Shrimp's vibriosis

The diversity of vibrios 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 study reported that *V. rotiferianus* has been confirmed as a causative agent associated with shrimp *Fenneropenaeus chinensis* post larvae (Zhang et al., 2014). While *V. parahaemolyticus* has been found as 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), in the grow-out ponds of tiger shrimp (*P. monodon*) in India and Large Yellow Croaker (Liu et al., 2014; Santhyia et al., 2015; Selvin & Lipton 2003; Shanmugasundaram et al., 2015). Recently, *V. alginolyticus* bacterial species has been also isolated from corals, India (Deb et al., 2020), white shrimps in Central Java (Widowati et al., 2018), Bangladesh (Hannan et al.,

2019), South India (Biju et al., 2016, Mastan & Begum 2016); Malaysia (Muthukrishanana et al., 2019) and it's related to the stress of white shrimps (Peng et al., 2018).

Figure 4. Phylogenetic-tree of the vibrios associated with vibriosis in shrimps from traditional brackishwater ponds of the northern coast of Central Java.

It was surprising that *V. diabolicus* was found in the present study. Limited reports are found regarding *V. diabolicus* associated with vibriosis in brackish water-cultured shrimp. This bacterium is commonly found as a bacterial pathogen in mussel *Bathymodiolus azoricus* (Barros et al., 2016), corals *Pacillopora verrucosa* (Deb et al., 2020), and green mussel (Susilowati et al., 2019). This bacterial species was firstly found from deep-sea hydrothermal vent polychaete (Deb et al., 2020) and in East Pacific Rise (Turner et al., 2018). The previous study 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* normally found in biofloc because it can be used to increase nutritional and disease resistance by application of probiotic (Far et al., 2013; Goudenege et al., 2014; Interaminense et al., 2019). The present research also revealed that *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 seilaevis* (Han et al., 2017), *Haliotis diversicolor*, *Crassostrea angulate*, *Meretrix lusoria*, *Pena viridis*, *Geloina erosa* (Tseng et al., 2018) and freshwater-cultured whiteleg shrimp, *P. vannamei* (Cao et al., 2018). Since vibriosis disease still existed in farms and continues to grow, it is urgently needed to develop the control methods, such as the search for new vaccines, probiotics, and immunostimulant formulas for more potent efficacies.

CONCLUSION

The vibrios associated with vibriosis in shrimps from traditional brackishwater ponds of the northern coast of Central Java is present in high diversity. These bacterial associations are identified as *V. diabolicus*, *S. algae*, *V. alginolyticus*, *V. rotiferianus* and *V. parahaemolyticus*. Since vibriosis disease exists in farms and in high diversity, it is urgently

needed to develop the control methods, such as the search for probiotics, new vaccines, and immunostimulant formulas for further vibriosis prevention and control of shrimp cultured in the traditional brackish water ponds.

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Field Code Changed

TABLES:

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

No.	Isolate code	Location	Source of organ	**Colony**		
				Colour	**Form**	**Characteristic**
1	JKP17	Pati	Hepatopancreas	Yellow	Oval	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	Hepatopankreas	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	Hepatopankreas	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	Hepatopankreas	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	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

Table 2. 16S rDNA-based molecular identification of five Vibrio associated with Shrimp's vibriosis

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

FIGURES:

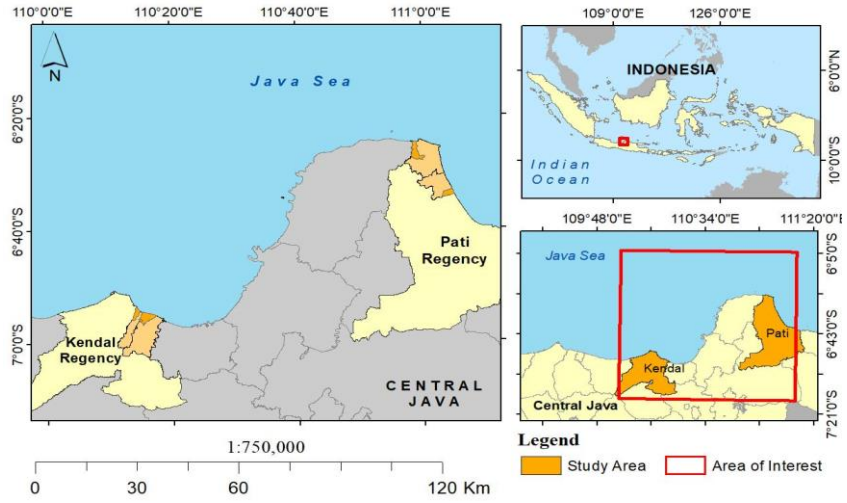


Figure 1. The collection sites of the shrimps

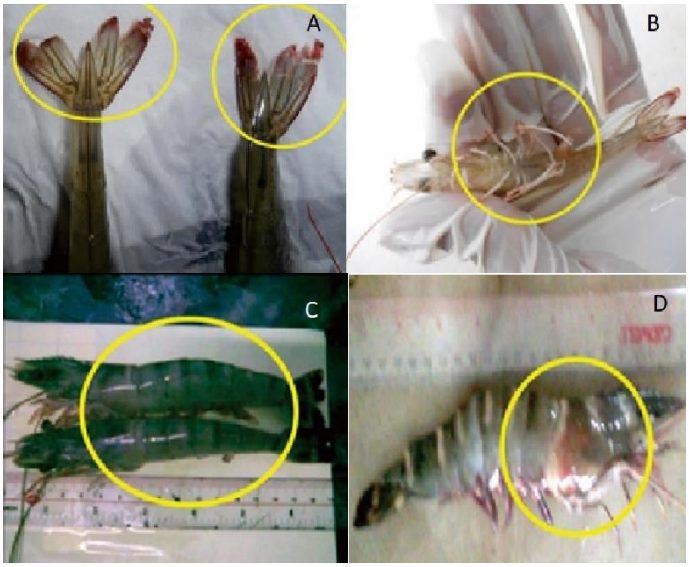
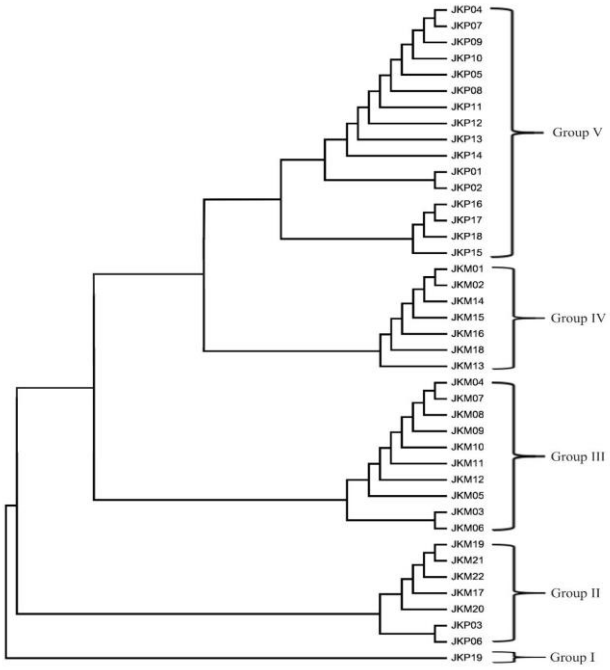


Figure 2. Shrimps with clinical signs of vibriosis (Note: A= reddish and melanosis in a telson, B= reddish in periopods and pleiopods, C= soft body, D= reddish carapace, periopods, pleopods, mouth, and telson carapace, periopods, pleopods, mouth, and telson (d).



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611 Figure 3. Dendrogram of repetitive PCR of 41 isolated vibrios in shrimps

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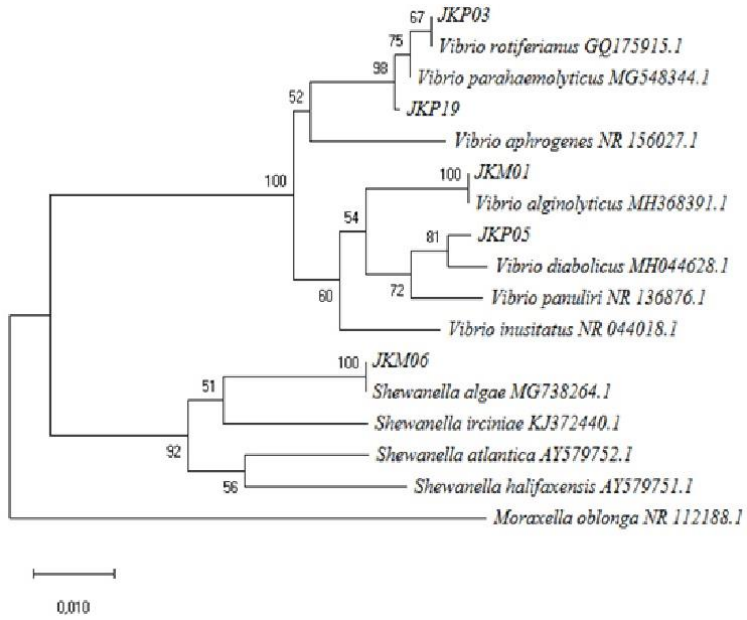

Figure 4. Phylogenetic-tree of the vibrios associated with vibriosis in shrimps from traditional brackishwater ponds of the northern coast of Central Java. The branching pattern was generated by the neighbor-joining method. Bootstrap values of 700 or more (from 1,000 replicates) are indicated at the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

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Manuscript Information

Manuscript ID: **GENAQUA-362.REV-1**

Title: Associated Vibrio Species in Shrimp Vibriosis from Traditional Brackish Waterpond in the North Coastal of Central Java, Indonesia

Small Title: Associated Vibrio Species in Shrimp Vibriosis

Authors: Sarjito Sarjito[1], Agus Sabdono[2]

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Keywords: VDiversity, Vibrio, rep-PCR, Brackish water, North Coast of Central Java

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Abstract

Indonesian shrimp cultures are subject to threaten with vibriosis. Some traditional brackish waters in Central Java is the only area remaining after the disease outbreaks. The research objective was to discover the vibrio diversity associated with shrimp vibriosis in traditional brackish waters. Exploratory method with purposive sampling was used in this study. Twenty-four infected vibriosis shrimps were collected from 2 district regions on the north coast of Central Java in July-September 2018. The bacteria associated in shrimp's vibriosis was isolated from the telson and inner part of the hepatopancreas with TCBS medium. Forty-one bacteria associated with shrimp vibriosis were obtained, then rep-PCR was done to get vibrios strains. Based on rep-PCR, representative five strains were selected for further study. The 16S rDNA sequence analysis showed that the JKP03, JKP05, JKP19, JKM01, and JKM06 isolates were closely related to Vibrio rotiferianus, Vibrio diabolicus, Vibrio parahaemolyticus, Vibrio alginolyticus, and Shewanella algae, respectively. Vibrio biodiversity in shrimp vibriosis was high. It confirmed that traditional shrimp farming was susceptible to vibriosis. Therefore, the findings of this study might require further development of the control methods such as vaccines, probiotics and immunostimulant formulas for shrimp's vibriosis outbreak prevention and control in traditional brackish water pond.

Manuscript Files

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GENAQUA-380-6-title-page.rev-1.pdf (../pdf-files/out/380-GENAQUA-380-6-title-page.rev-1.pdf)	19 KB	Dec 28, 2020	Title Page	Title page
GENAQUA-380-9-main-document.rev-1.pdf (../pdf-files/out/380-GENAQUA-380-9-main-document.rev-1.pdf)	1442 KB	Dec 28, 2020	Main Document	Main Document
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MANUSCRIPT
REVISED #2
15 FEBRUARY 2021

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

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 respresentative 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 diabolicus*, *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.

Keywords: Diversity, *Vibrio*, rep-PCR, Brackish water, North Coast of Central Java

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 *Litopeneous vannamae*, 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 & Begum, 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 Vibrionacea, 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. parahaemolyticus*, *V. fischeri*, and *V. alginolyticus* (Chythanya et al., 2002; Kumar et al., 2014; Gopala et al., 2005). Chandrakala and Priya (2017) have mentioned 14 *Vibrio* species acting as shrimp pathogens: *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. campbelli*, *V. fischeri*, *V. damsella*, *V. pelagicus*, *V. orientalis*, *V. ordalii*, *V. mediterrani*, 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 Margomulyo 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 isolation. Shrimp samples were categorized by 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.

Figure 1. Collection sites of the shrimps

Bacterial isolation

Bacterial isolation was performed by scraping 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 µL aliquots of each dilution were spread on TCBS agar (Oxoid, England) and incubated at room temperature for 48 h (Brock and Madigan, 1991; Sarjito et 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)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by 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′-IIIICGICGICATCI 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 denaturated at 95℃ for 5 min, followed by 30 cycles of amplification at 92℃ for 60 s, annealing at 50℃ for 90 s, followed by a final extension at 68℃ for 10 min. The 5 µL 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

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).

Figure 2. Shrimps with clinical signs of vibriosis

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 by 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 different virulence degrees. In addition, the virulence degree of various *Vibrio* isolates depends on its 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 considered, 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). Widanarni et al. (2003) reported the bacterial characteristics isolated from tiger shrimp larvae of Labuan, Pangandaran, and Lampung, Indonesia are Gram-negative, short rod-shaped, yellow colonies. Approximately 60% of bacteria associated with shrimp vibriosis in Sri Lanka belonged to Vibrioceae (Raja et al., 2017). Moreover, the six species of *Vibrio* were collected from diseased 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).

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

Molecular identification and phylogenetic analysis

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

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

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 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; Santhyia et al., 2015; Selvin & Lipton, 2003; Shanmugasundaram 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 (Muthukrishanana et al., 2019). In addition, they have been related to the stress of white shrimps (Peng et al., 2018).

Figure 4. Phylogenetic tree of *Vibrio* associated with vibriosis in shrimps from traditional brackish water ponds of the northern coast of Central Java.

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* has 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 seilaevis* (Han et al., 2017), *Haliotis diversicolor*, *Crassostrea angulate*, *Meretrix lusoria*, *Pena 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 and continues to grow, control methods such as new vaccines, probiotics, and immunostimulant formulas must be developed for more potent efficacies.

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.

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TABLES:

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	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	Hepatopankreas	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	Hepatopankreas	Yellow	Rounded	Convex
24	JKP10	Pati	Hepatopancreas	Yellow	Rounded	Convex
25	JKP19	Pati	Hepatopancreas	Yellow	Irregular	Rough
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27	JKM14	Kendal	Hepatopancreas	Black	Rounded	Convex
28	JKP08	Pati	Hepatopancreas	Yellow	Rounded	Convex

29	JKM13	Kendal	Hepatopancreas	Yellow	Rounded	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

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

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

FIGURES:

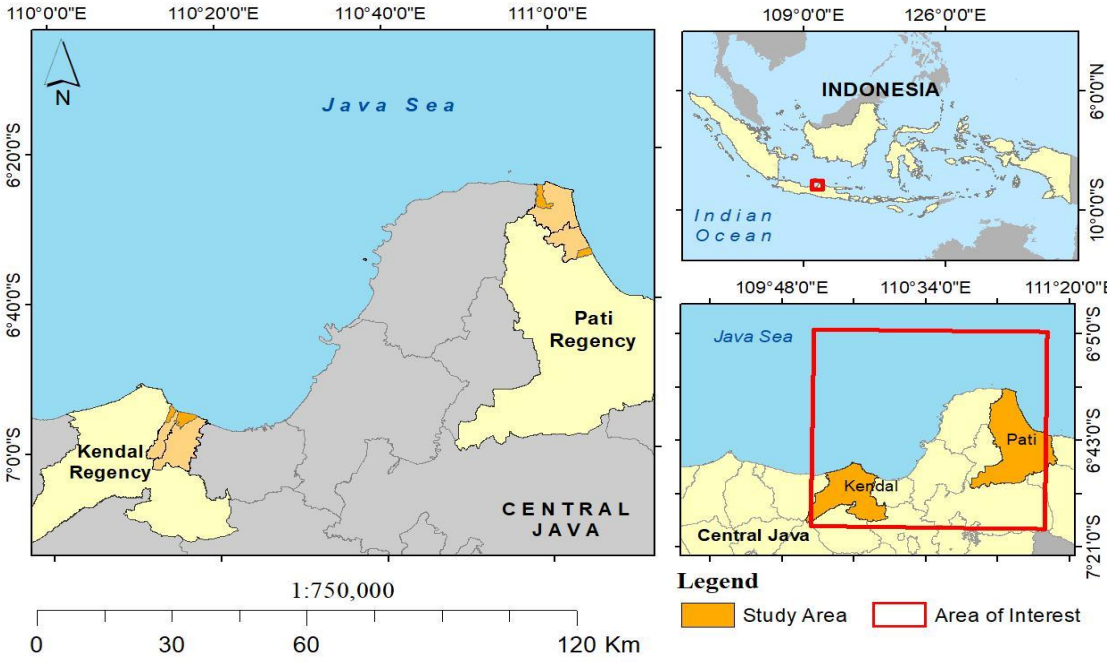


Figure 1. Collection sites of the shrimps

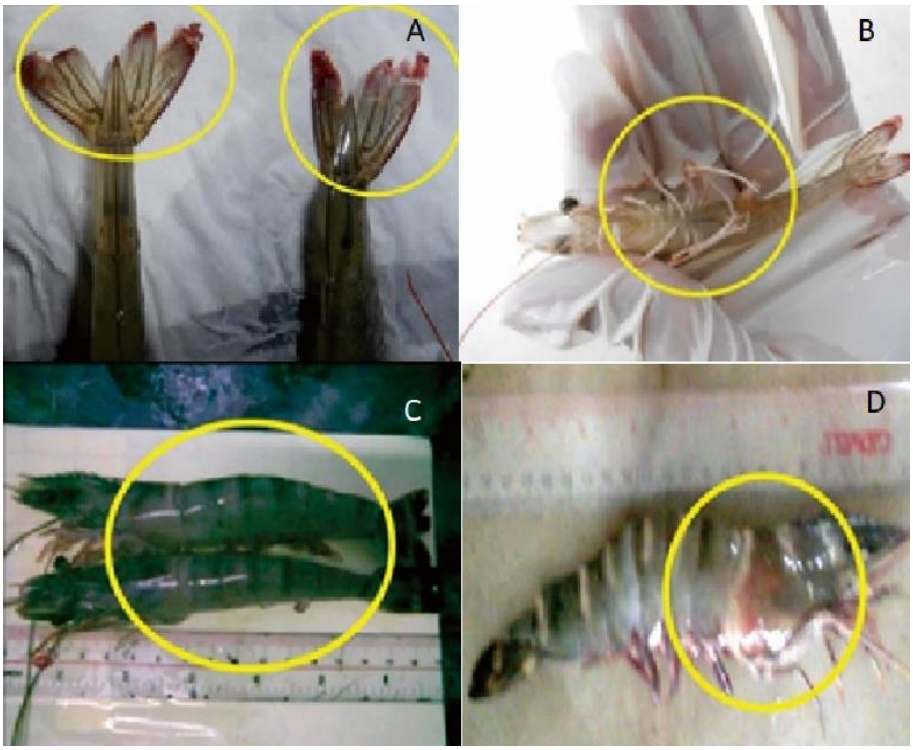


Figure 2. Shrimps with clinical signs of vibriosis (Note: A = reddish and melanosis in a telson, B = reddish in periopods and pleiopods, C = soft body, D = reddish carapace, periopods, pleopods, mouth, and telson carapace, periopods, pleopods, mouth, and telson (d).

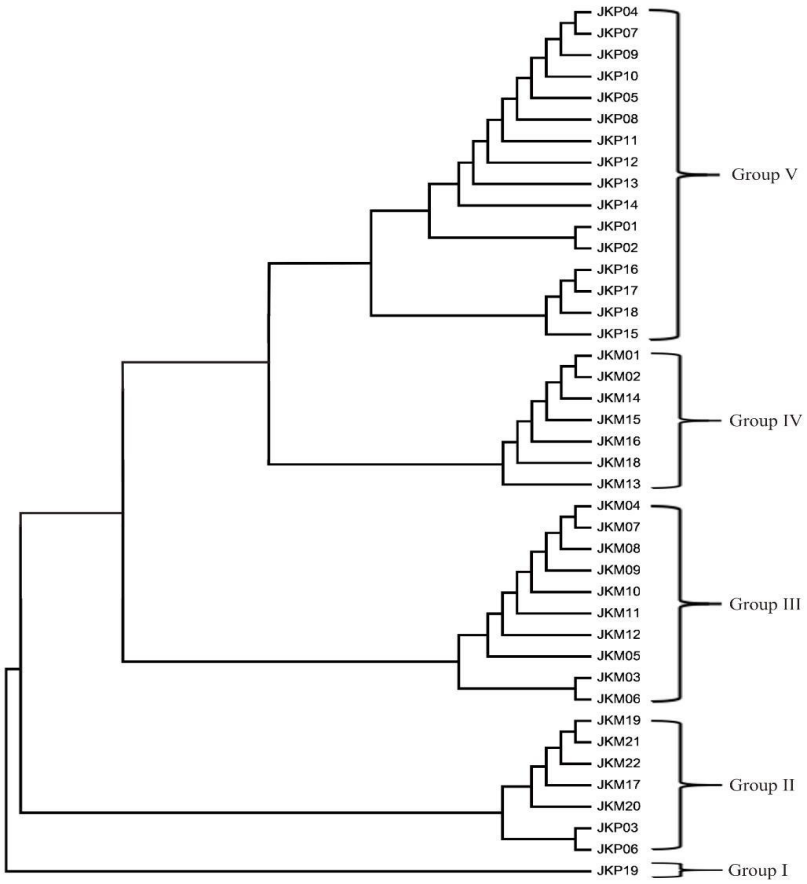
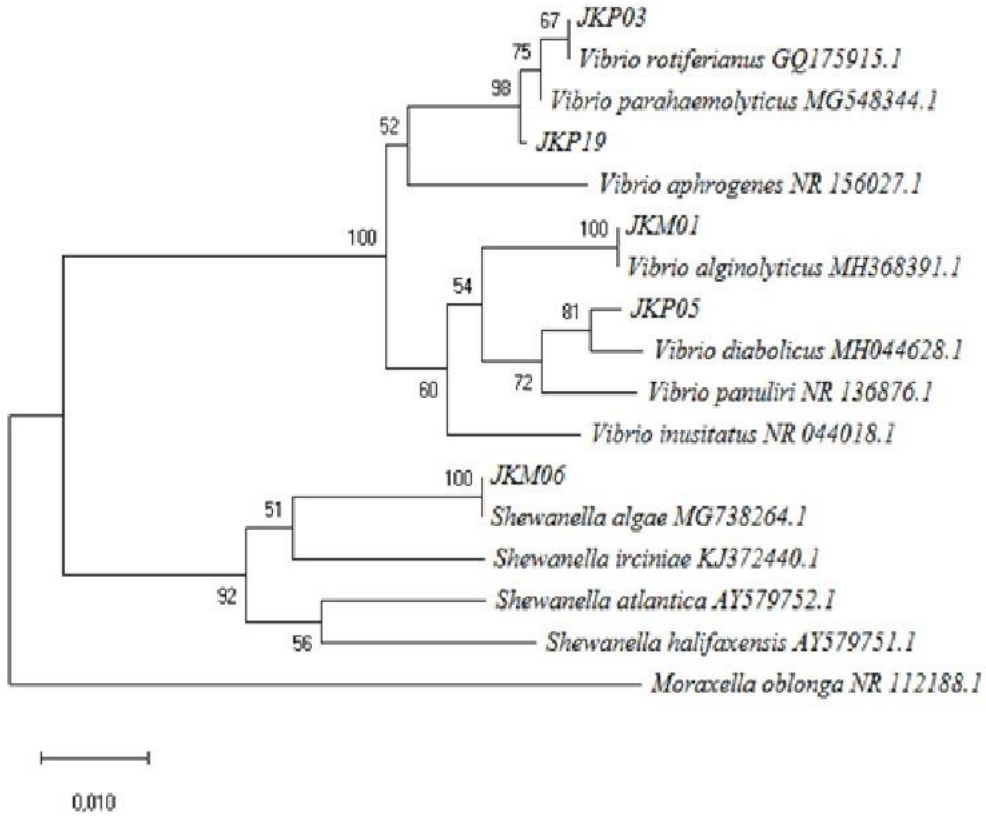


Figure 3. Dendrogram of repetitive PCR of 41 *Vibrio* isolates in shrimps



583

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

588

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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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North Coast of Central Java

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 respresentative 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 diabolicus*, *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 *Litopeneous vannamae*, 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 & Begum, 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 Vibrionacea, 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 14 *Vibrio* species acting as shrimp pathogens: *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. campbelli*, *V. fischeri*, *V. damsella*, *V. pelagicus*, *V. orientalis*, *V. ordalii*, *V. mediterrani*, 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 Margomulyo 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

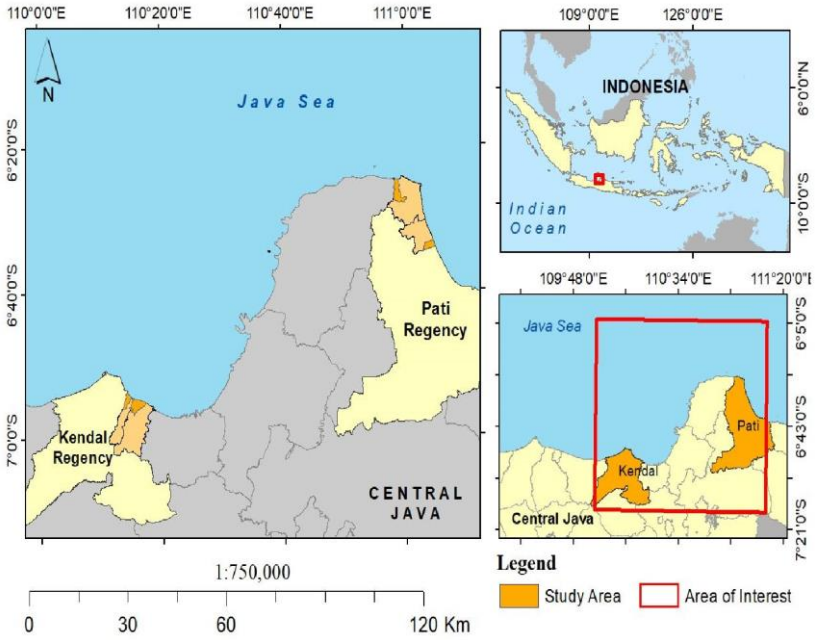

Figure 1. Collection sites of the shrimps

isolation. Shrimp samples were categorized by 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 scraping 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 µL aliquots of each dilution were spread on TCBS agar (Oxoid, England) and incubated at room temperature for 48 h (Brock and Madigan, 1991; Sarjito et 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)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by 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′-IIIICGICGICATCI 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 denaturated at 95℃ for 5 min, followed by 30 cycles of amplification at 92℃ for 60 s, annealing at 50℃ for 90 s, followed by a final extension at 68℃ for 10 min. The 5 µL 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

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 by 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 different virulence degrees. In addition, the virulence degree of various *Vibrio* isolates depends on its 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 considered, 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). Widanarni et al. (2003) reported the bacterial characteristics isolated from tiger shrimp larvae of Labuan, Pangandaran, and Lampung, Indonesia are Gram-negative, short rod-shaped, yellow colonies. Approximately 60% of bacteria associated with shrimp vibriosis in Sri Lanka belonged to Vibrioceae (Raja et al., 2017). Moreover, the six species of *Vibrio* were collected from diseased 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

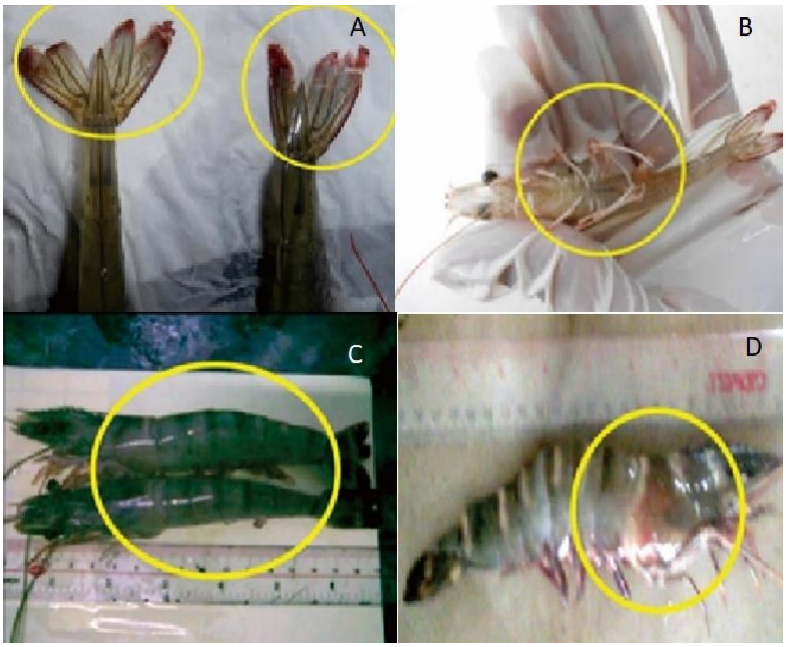

Figure 2. Shrimps with clinical signs of vibriosis (Note: A=reddish and melanosis in a telson, B=reddish in periopods and pleiopods, C=soft body, D=reddish carapace, periopods, pleopods, mouth, and telson carapace, periopods, pleopods, mouth, and telson (d).

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	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
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7	JKP03	Pati	Hepatopancreas	Yellow	Rounded	Flat
8	JKM11	Kendal	Hepatopancreas	Black	Rounded	Convex
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14	JKM01	Kendal	Hepatopancreas	Green	Rounded	Convex
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20	JKM06	Kendal	Hepatopancreas	Yellow	Rounded	Convex
21	JKM03	Kendal	Hepatopancreas	Black	Rounded	Convex
22	JKM19	Kendal	Hepatopancreas	Yellow	Rounded	Convex
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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	Convex
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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. vannamaei* 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

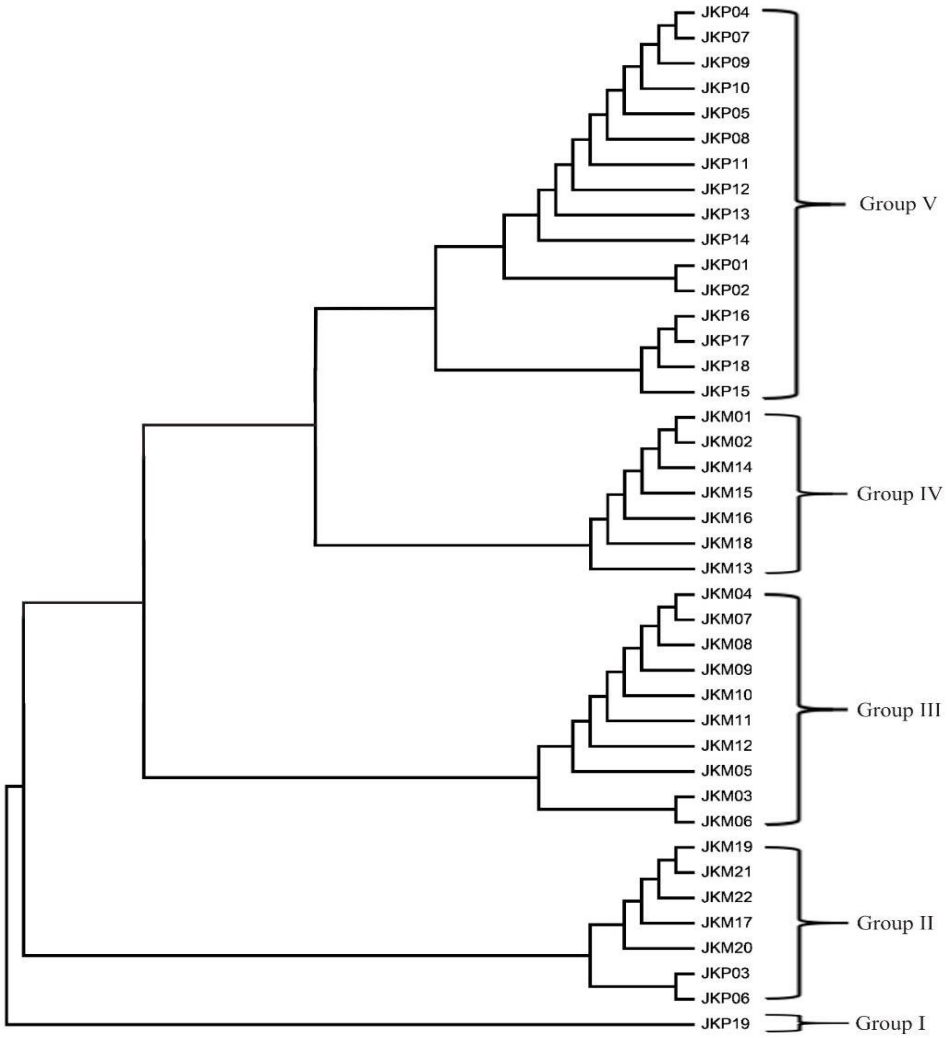

Figure 3. Dendrogram of repetitive PCR of 41 *Vibrio* isolates in shrimps

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

No.	Isolate	Closely Relative	Homology (%)	Acc. Number
1.	JKP03	*Vibrio rotiferianus*	100	GQ175915.1
2.	JKP05	*V. diabolicus*	99	MH044628.1
3.	JKP19	*V. parahaemolyticus*	94	MG548344.1
4.	JKM01	*V. alginolyticus*	97	MH368391.1
5.	JKM06	*Shewanella algae*	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; Santhyia et al., 2015; Selvin & Lipton, 2003; Shanmugasundaram 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 (Muthukrishanana 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* has 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 seilaevis* (Han et al., 2017), *Haliotis diversicolor, Crassostrea angulate, Meretrix lusoria*, *Pena 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 and continues to grow, control methods such as new vaccines, probiotics, and immunostimulant formulas must be developed for more potent efficacies.

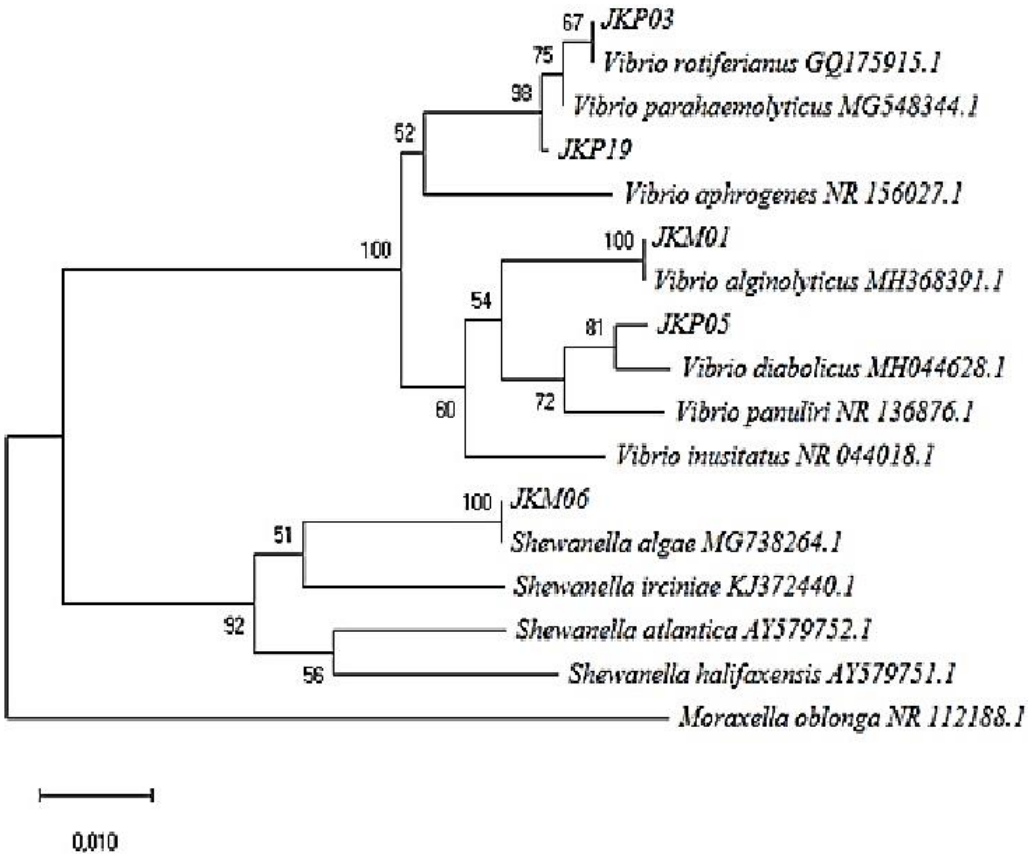

Figure 4. Phylogenetic tree of *Vibrio* associated with vibriosis in shrimps from traditional brackish water ponds of the northern coast of Central Java. The branching pattern was generated by the neighbor-joining method. Bootstrap values of 700 or more (from 1,000 replicates) are indicated at the nodes.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

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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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1.3. 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 Unites States cephalopods have been added 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. All surgical samples were performed under clove oil anesthesia and all efforts were made minimize suffering

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1.5. 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.*

Note : Sarjito (*SJT*) *; Agus Sabdono* (*AS*)

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

Abstract. Some traditional shrimp ponds remained along the north coast of Central Java, Indonesian 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. 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 respresentative 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*, *V. diabolicus*, *V. parahaemolyticus*, *V. 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.

Keywords: Diversity, *Vibrio*, rep-PCR, Brackish water, North Coast of Central Java

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 *Litopeneous vannamae*, 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 & Begum, 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 Vibrionacea, 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. parahaemolyticus*, *V. fischeri*, and *V. alginolyticus* (Chythanya et al., 2002; Kumar et al., 2014; Gopala et al., 2005). Chandrakala and Priya (2017) have mentioned 14 *Vibrio* species acting as shrimp pathogens: *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. campbelli*, *V. fischeri*, *V. damsella*, *V. pelagicus*, *V. orientalis*, *V. ordalii*, *V. mediterrani*, 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 Margomulyo 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 isolation. Shrimp samples were categorized by 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.

Figure 1. Collection sites of the shrimps

Bacterial isolation

Bacterial isolation was performed by scraping 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 µL aliquots of each dilution were spread on TCBS agar (Oxoid, England) and incubated at room temperature for 48 h (Brock and Madigan, 1991; Sarjito et 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)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by 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'-IIIICGICGICATCI 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 denaturated at 95℃ for 5 min, followed by 30 cycles of amplification at 92℃ for 60 s, annealing at 50℃ for 90 s, followed by a final extension at 68℃ for 10 min. The 5 µL 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

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 pleopods (b), soft body (c), and reddish (Figure 2).

Figure 2. Shrimps with clinical signs of vibriosis

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 by 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 different virulence degrees. In addition, the virulence degree of various *Vibrio* isolates depends on its 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 considered, 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). Widanarni et al. (2003) reported the bacterial characteristics isolated from tiger shrimp larvae of Labuan, Pangandaran, and Lampung, Indonesia are Gram-negative, short rod-shaped, yellow colonies. Approximately 60% of bacteria associated with shrimp vibriosis in Sri Lanka belonged to Vibrionaceae (Raja et al., 2017). Moreover, the six species of *Vibrio* were collected from diseased 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).

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

Molecular identification and phylogenetic analysis

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

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

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 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; Santhyia et al., 2015; Selvin & Lipton, 2003; Shanmugasundaram 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 (Muthukrishanana et al., 2019). In addition, they have been related to the stress of white shrimps (Peng et al., 2018).

Figure 4. Phylogenetic tree of *Vibrio* associated with vibriosis in shrimps from traditional brackish water ponds of the northern coast of Central Java.

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* has 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 seilaevis* (Han et al., 2017), *Haliotis diversicolor, Crassostrea angulate, Meretrix lusoria*, *Pena 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 and continues to grow, control methods such as new vaccines, probiotics, and immunostimulant formulas must be developed for more potent efficacies.

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 Unites States cephalopods have been added 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. All surgical samples were performed under clove oil anesthesia and all efforts were made minimize suffering

FUNDING INFORMATION

Our Research Project was partially funded by Advance Research, Fisheries and Marine Science Faculty, Universitas Diponegoro of SUKPA (PNBP) 2018, No: 575–02/UN7.5.1/PG/2018 (30,000,000 IDR to SJT), "To investigate the Causative Agent of Diseases in Aquaculture Organism with Molecular Approach". So, the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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.*

Note : Sarjito (*SJT*) *; Agus Sabdono* (*AS*)

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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TABLES:

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	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	Hepatopankreas	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	Hepatopankreas	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	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

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

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

FIGURES:

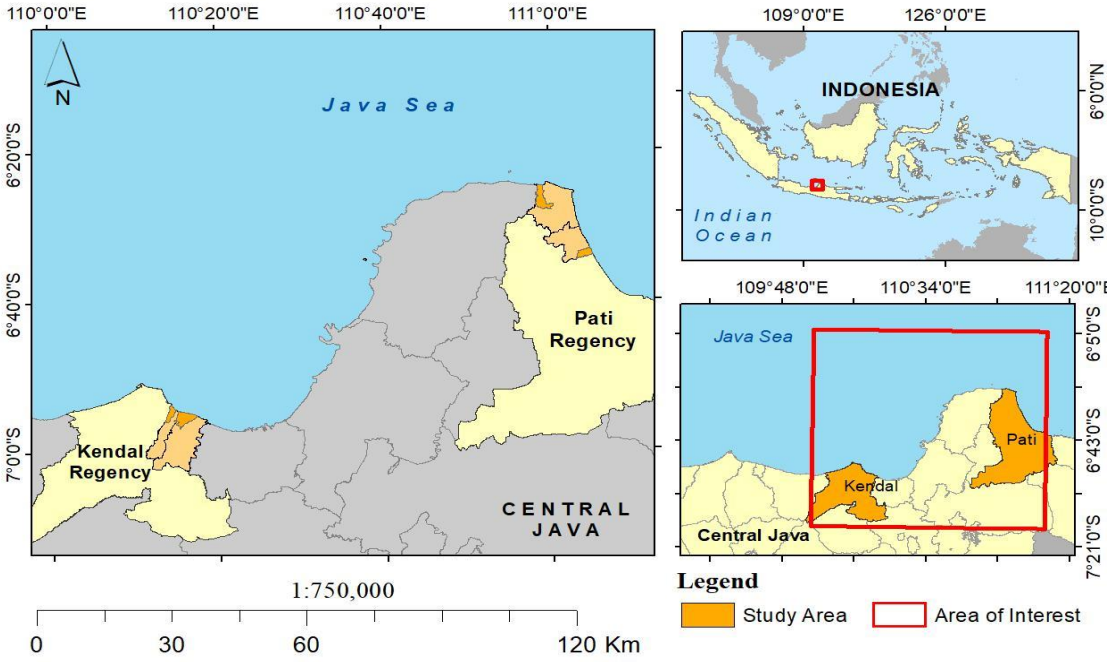

Figure 1. Collection sites of the shrimps

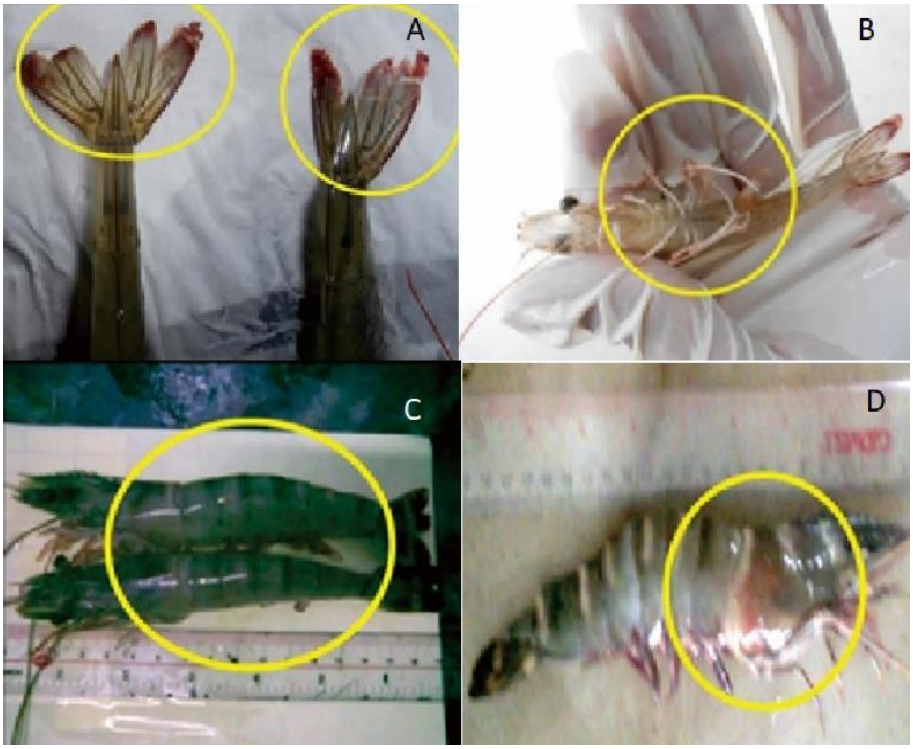

Figure 2. Shrimps with clinical signs of vibriosis (Note: A = reddish and melanosis in a telson, B = reddish in periopods and pleopods, C = soft body, D = reddish carapace, periopods, pleopods, mouth, and telson carapace, periopods, pleopods, mouth, and telson (d).

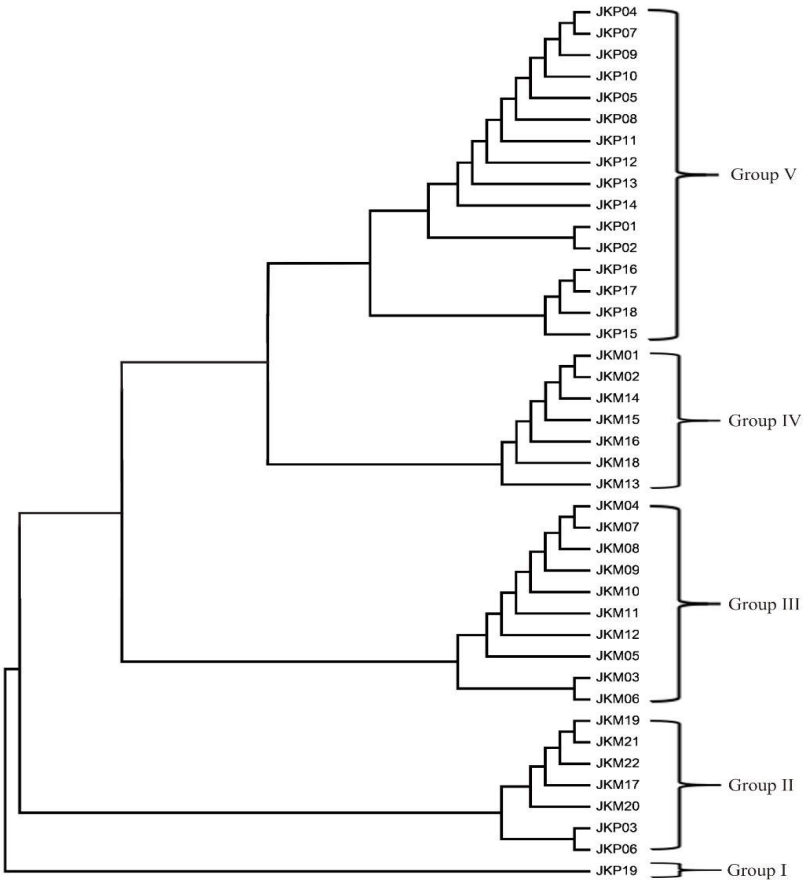

Figure 3. Dendrogram of repetitive PCR of 41 *Vibrio* isolates in shrimps

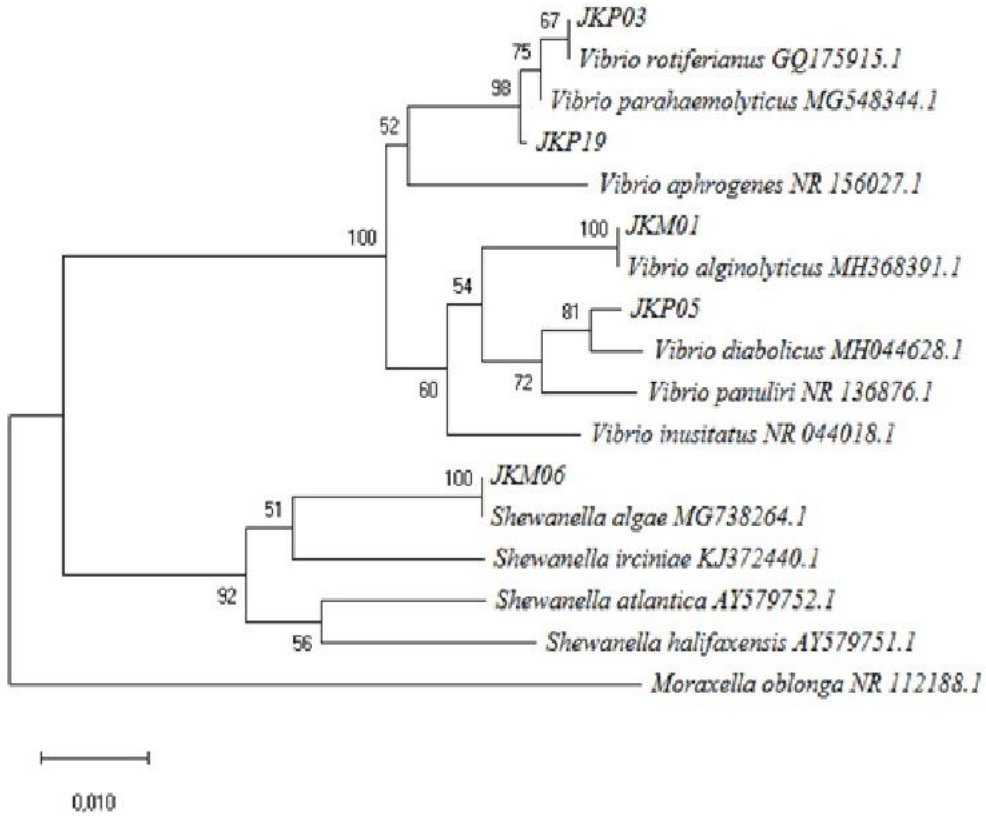

Figure 4. Phylogenetic tree of *Vibrio* associated with vibriosis in shrimps from traditional brackish water ponds of the northern coast of Central Java. The branching pattern was generated by the neighbor-joining method. Bootstrap values of 700 or more (from 1,000 replicates) are indicated at the nodes.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

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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 respresentative 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 diabolicus*, *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 *Litopeneous vannamae*, 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 & Begum, 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 Vibrionacea, 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 14 *Vibrio* species acting as shrimp pathogens: *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. campbelli*, *V. fischeri*, *V. damsella*, *V. pelagicus*, *V. orientalis*, *V. ordalii*, *V. mediterrani*, 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 Margomulyo 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

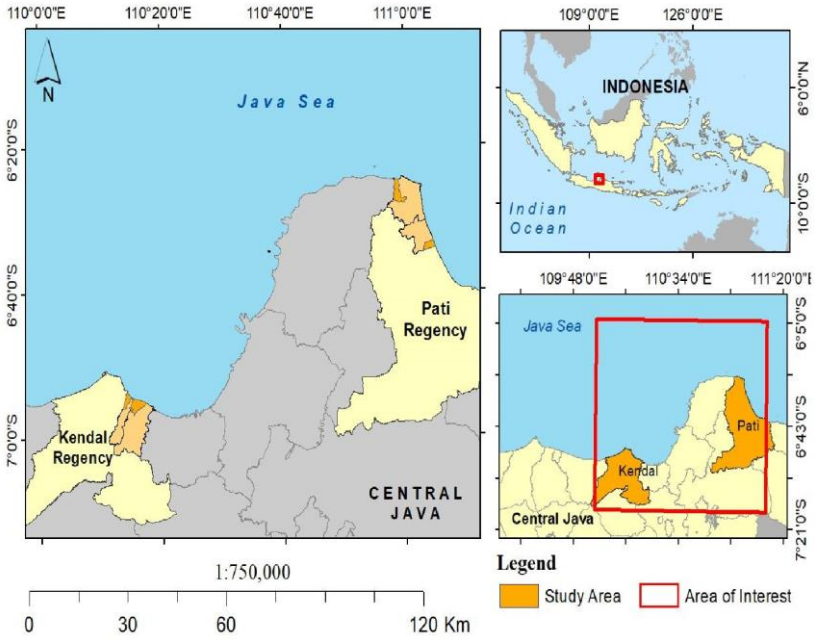

Figure 1. Collection sites of the shrimps

isolation. Shrimp samples were categorized by 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 scraping 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 µL aliquots of each dilution were spread on TCBS agar (Oxoid, England) and incubated at room temperature for 48 h (Brock and Madigan, 1991; Sarjito et 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)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by 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′-IIIICGICGICATCI 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 denaturated at 95℃ for 5 min, followed by 30 cycles of amplification at 92℃ for 60 s, annealing at 50℃ for 90 s, followed by a final extension at 68℃ for 10 min. The 5 µL 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

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 by 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 different virulence degrees. In addition, the virulence degree of various *Vibrio* isolates depends on its 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 considered, 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). Widanarni et al. (2003) reported the bacterial characteristics isolated from tiger shrimp larvae of Labuan, Pangandaran, and Lampung, Indonesia are Gram-negative, short rod-shaped, yellow colonies. Approximately 60% of bacteria associated with shrimp vibriosis in Sri Lanka belonged to Vibrioceae (Raja et al., 2017). Moreover, the six species of *Vibrio* were collected from diseased 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

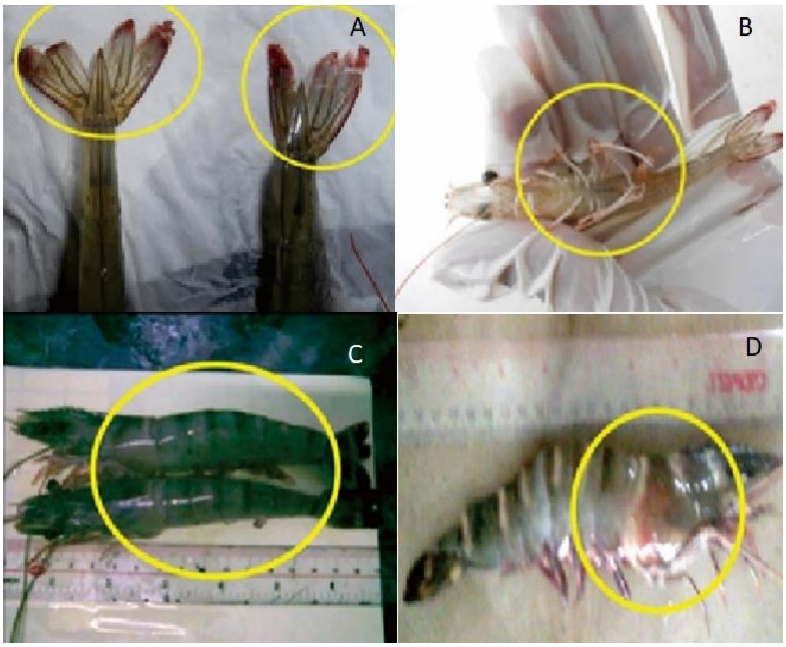

Figure 2. Shrimps with clinical signs of vibriosis (Note: A=reddish and melanosis in a telson, B=reddish in periopods and pleiopods, C=soft body, D=reddish carapace, periopods, pleopods, mouth, and telson carapace, periopods, pleopods, mouth, and telson (d).

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	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	Hepatopankreas	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	Hepatopankreas	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	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. vannamaei* 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

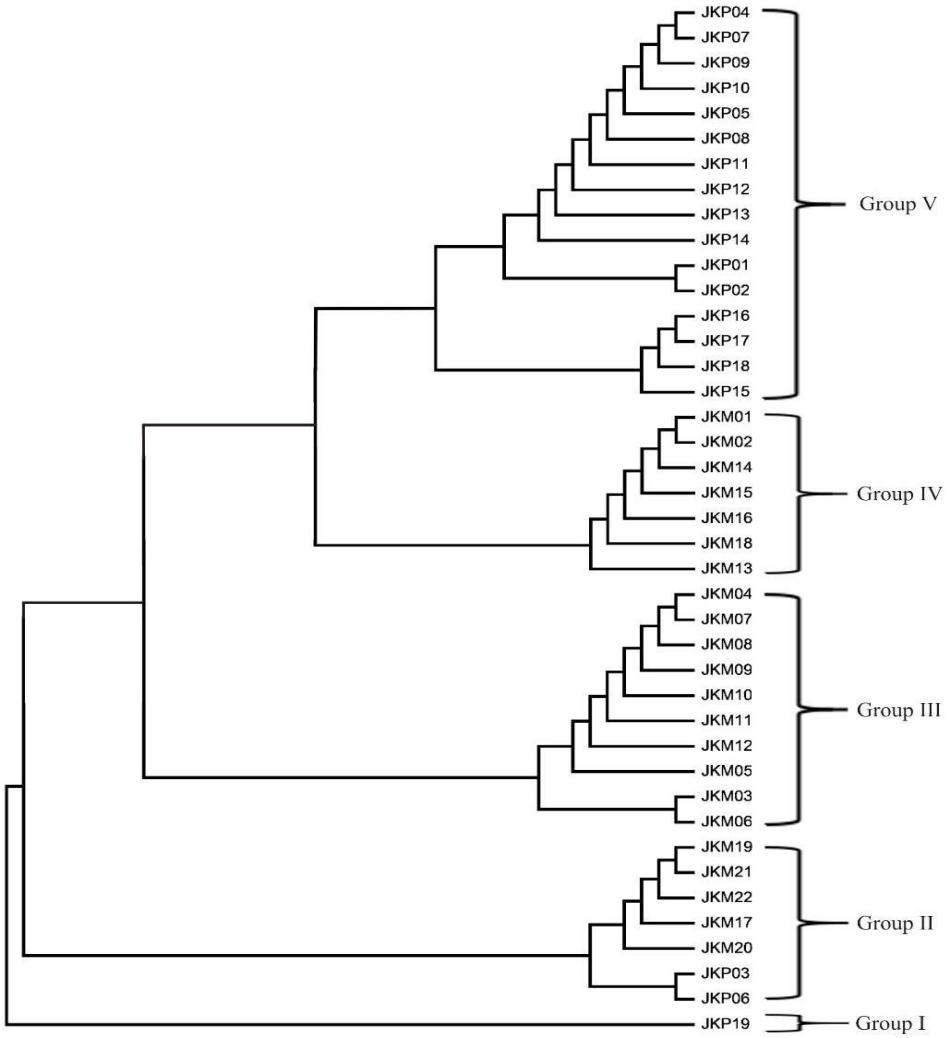

Figure 3. Dendrogram of repetitive PCR of 41 *Vibrio* isolates in shrimps

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No.	Isolate	Closely Relative	Homology (%)	Acc. Number
1.	JKP03	*Vibrio rotiferianus*	100	GQ175915.1
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3.	JKP19	*V. parahaemolyticus*	94	MG548344.1
4.	JKM01	*V. alginolyticus*	97	MH368391.1
5.	JKM06	*Shewanella algae*	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; Santhyia et al., 2015; Selvin & Lipton, 2003; Shanmugasundaram 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 (Muthukrishanana 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* has 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 seilaevis* (Han et al., 2017), *Haliotis diversicolor, Crassostrea angulate, Meretrix lusoria*, *Pena 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 and continues to grow, control methods such as new vaccines, probiotics, and immunostimulant formulas must be developed for more potent efficacies.

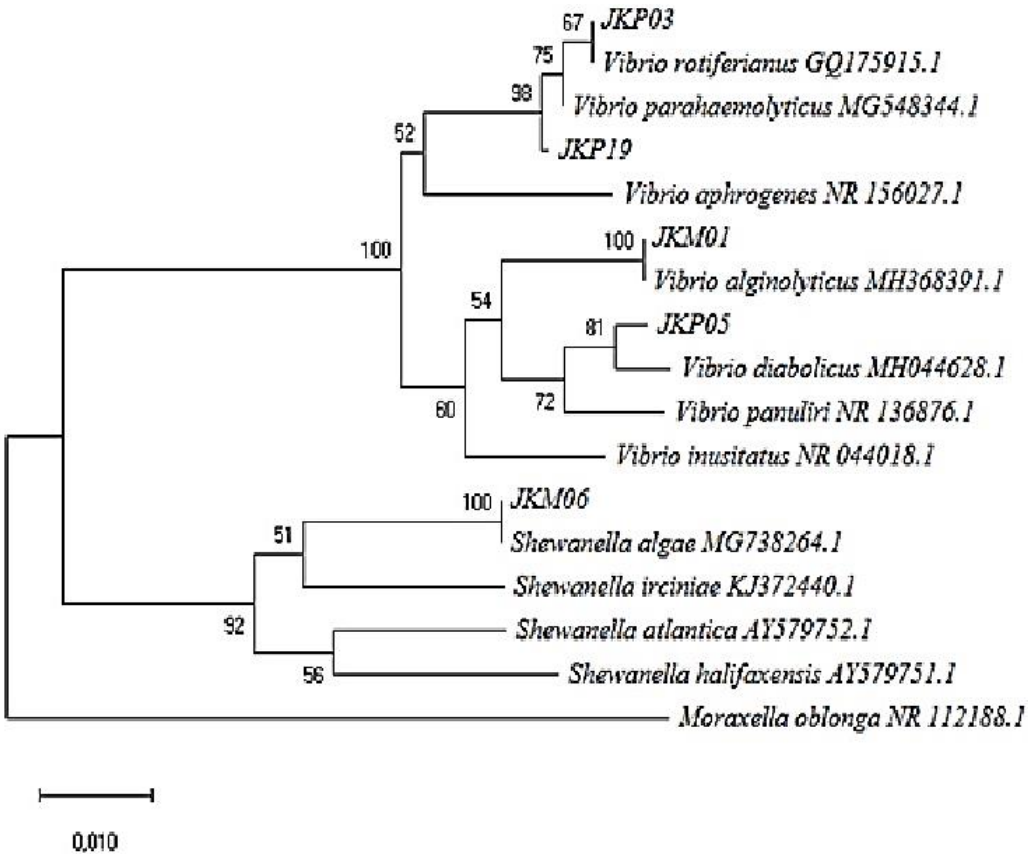

Figure 4. Phylogenetic tree of *Vibrio* associated with vibriosis in shrimps from traditional brackish water ponds of the northern coast of Central Java. The branching pattern was generated by the neighbor-joining method. Bootstrap values of 700 or more (from 1,000 replicates) are indicated at the nodes.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

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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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1.3. 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 Unites States cephalopods have been added 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. All surgical samples were performed under clove oil anesthesia and all efforts were made minimize suffering

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1.5. 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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1.6. 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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Associated *Vibrio* Species in Shrimp Vibriosis from Traditional Brackish Water Pond in the North Coastal of Central Java, Indonesia

Abstract. Some traditional shrimp ponds remained along the north coast of Central Java, Indonesian 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. 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 respresentative 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*, *V. diabolicus*, *V. parahaemolyticus*, *V. 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.

Keywords: Diversity, *Vibrio*, rep-PCR, Brackish water, North Coast of Central Java

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 *Litopeneous vannamae*, 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 & Begum, 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 Vibrionacea, 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. parahaemolyticus*, *V. fischeri*, and *V. alginolyticus* (Chythanya et al., 2002; Kumar et al., 2014; Gopala et al., 2005). Chandrakala and Priya (2017) have mentioned 14 *Vibrio* species acting as shrimp pathogens: *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. campbelli*, *V. fischeri*, *V. damsella*, *V. pelagicus*, *V. orientalis*, *V. ordalii*, *V. mediterrani*, 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 Margomulyo 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 isolation. Shrimp samples were categorized by 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.

Figure 1. Collection sites of the shrimps

Bacterial isolation

Bacterial isolation was performed by scraping 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 µL aliquots of each dilution were spread on TCBS agar (Oxoid, England) and incubated at room temperature for 48 h (Brock and Madigan, 1991; Sarjito et 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)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by 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'-IIIICGICGICATCI 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 denaturated at 95℃ for 5 min, followed by 30 cycles of amplification at 92℃ for 60 s, annealing at 50℃ for 90 s, followed by a final extension at 68℃ for 10 min. The 5 µL 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

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 pleopods (b), soft body (c), and reddish (Figure 2).

Figure 2. Shrimps with clinical signs of vibriosis

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 by 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 different virulence degrees. In addition, the virulence degree of various *Vibrio* isolates depends on its 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 considered, 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). Widanarni et al. (2003) reported the bacterial characteristics isolated from tiger shrimp larvae of Labuan, Pangandaran, and Lampung, Indonesia are Gram-negative, short rod-shaped, yellow colonies. Approximately 60% of bacteria associated with shrimp vibriosis in Sri Lanka belonged to Vibrionaceae (Raja et al., 2017). Moreover, the six species of *Vibrio* were collected from diseased 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).

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

Molecular identification and phylogenetic analysis

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

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

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 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; Santhyia et al., 2015; Selvin & Lipton, 2003; Shanmugasundaram 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 (Muthukrishanana et al., 2019). In addition, they have been related to the stress of white shrimps (Peng et al., 2018).

Figure 4. Phylogenetic tree of *Vibrio* associated with vibriosis in shrimps from traditional brackish water ponds of the northern coast of Central Java.

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* has 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 seilaevis* (Han et al., 2017), *Haliotis diversicolor, Crassostrea angulate, Meretrix lusoria*, *Pena 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 and continues to grow, control methods such as new vaccines, probiotics, and immunostimulant formulas must be developed for more potent efficacies.

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 Unites States cephalopods have been added 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. All surgical samples were performed under clove oil anesthesia and all efforts were made 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.*

Note : Sarjito (*SJT*) *; Agus Sabdono* (*AS*)

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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TABLES:

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	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	Hepatopankreas	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	Hepatopankreas	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	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

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

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

FIGURES:

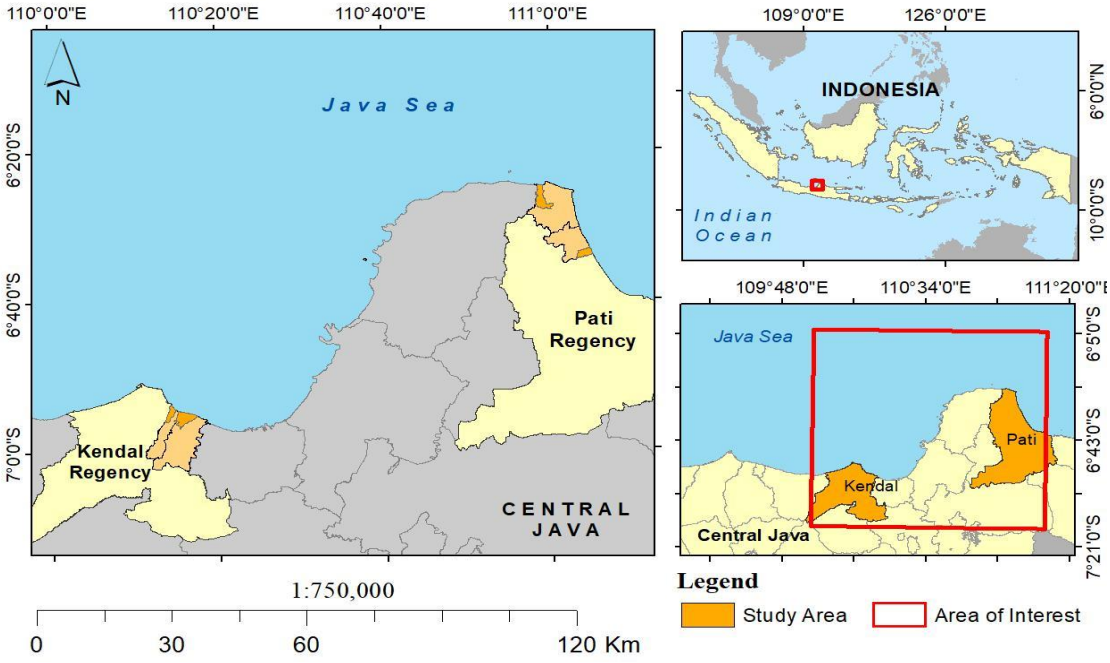

Figure 1. Collection sites of the shrimps

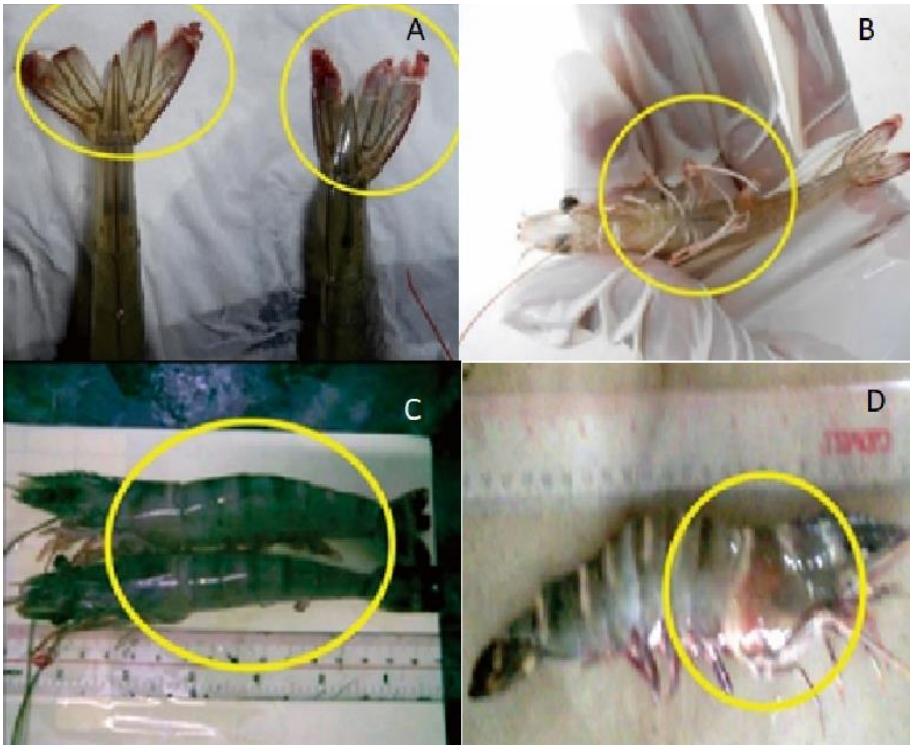

Figure 2. Shrimps with clinical signs of vibriosis (Note: A = reddish and melanosis in a telson, B = reddish in periopods and pleopods, C = soft body, D = reddish carapace, periopods, pleopods, mouth, and telson carapace, periopods, pleopods, mouth, and telson (d).

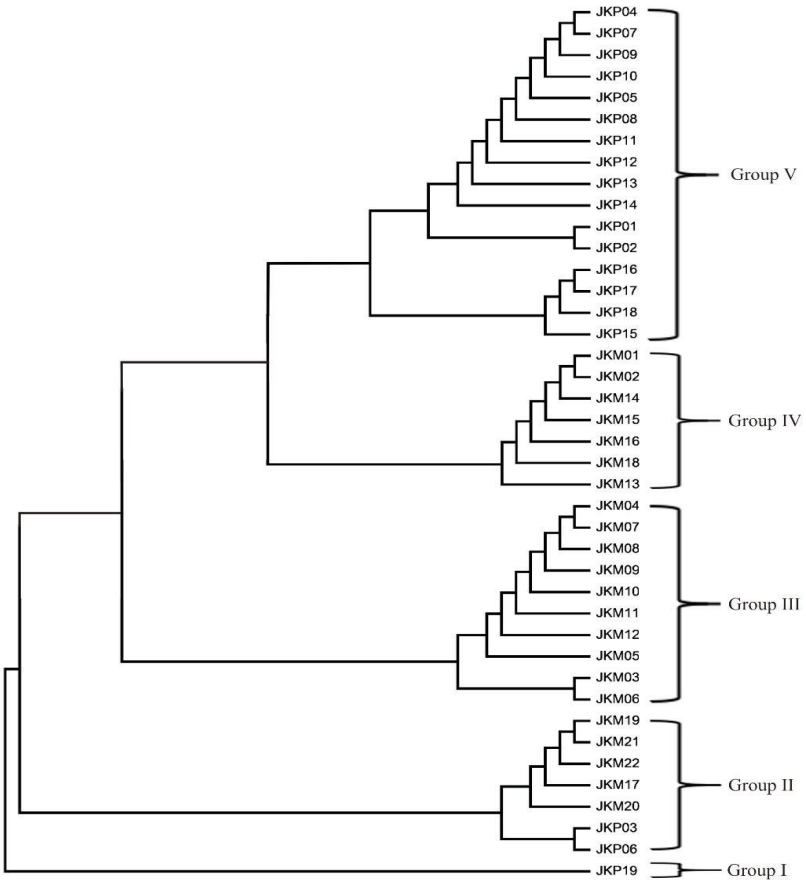

Figure 3. Dendrogram of repetitive PCR of 41 *Vibrio* isolates in shrimps

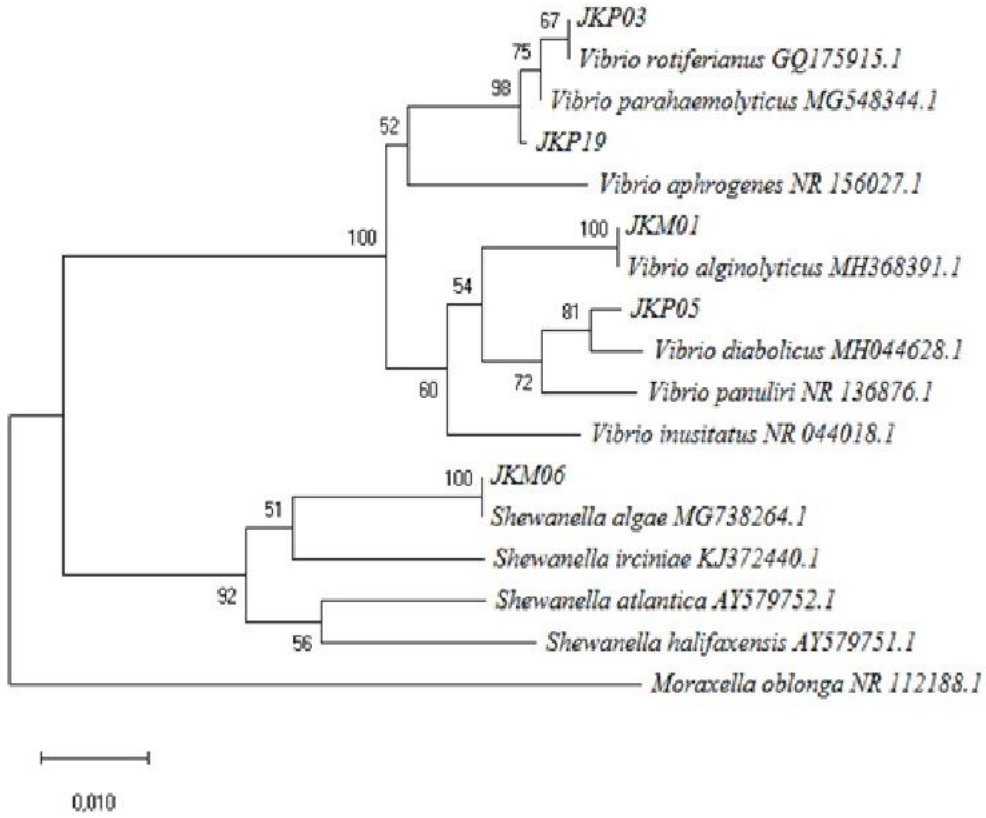

Figure 4. Phylogenetic tree of *Vibrio* associated with vibriosis in shrimps from traditional brackish water ponds of the northern coast of Central Java. The branching pattern was generated by the neighbor-joining method. Bootstrap values of 700 or more (from 1,000 replicates) are indicated at the nodes.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

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RESEARCH PAPER

GENAQUA
GENETICS OF AQUATIC ORGANISMS

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 respresentative 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 diabolicus*, *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 *Litopeneous vannamae*, 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 & Begum, 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 Vibrionacea, 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 14 *Vibrio* species acting as shrimp pathogens: *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. campbelli*, *V. fischeri*, *V. damsella*, *V. pelagicus*, *V. orientalis*, *V. ordalii*, *V. mediterrani*, 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 Margomulyo 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

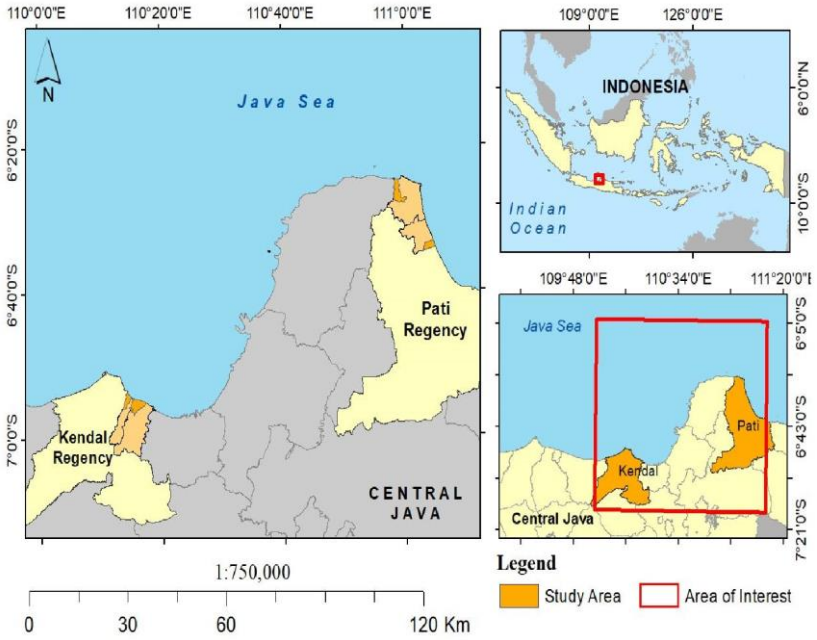

Figure 1. Collection sites of the shrimps

isolation. Shrimp samples were categorized by 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 scraping 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 µL aliquots of each dilution were spread on TCBS agar (Oxoid, England) and incubated at room temperature for 48 h (Brock and Madigan, 1991; Sarjito et 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)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by 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′-IIIICGICGICATCI 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 denaturated at 95℃ for 5 min, followed by 30 cycles of amplification at 92℃ for 60 s, annealing at 50℃ for 90 s, followed by a final extension at 68℃ for 10 min. The 5 µL 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

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 by 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 different virulence degrees. In addition, the virulence degree of various *Vibrio* isolates depends on its 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 considered, 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). Widanarni et al. (2003) reported the bacterial characteristics isolated from tiger shrimp larvae of Labuan, Pangandaran, and Lampung, Indonesia are Gram-negative, short rod-shaped, yellow colonies. Approximately 60% of bacteria associated with shrimp vibriosis in Sri Lanka belonged to Vibrioceae (Raja et al., 2017). Moreover, the six species of *Vibrio* were collected from diseased 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

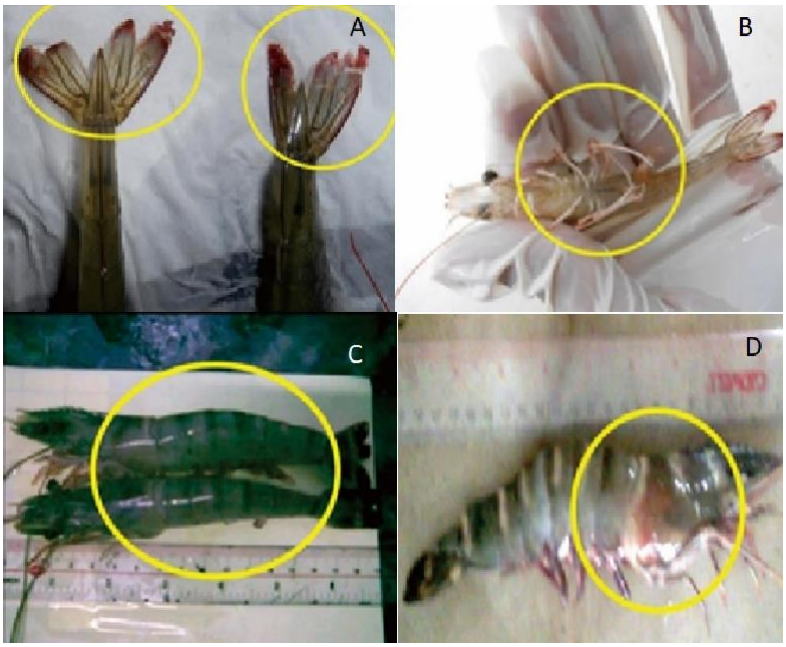

Figure 2. Shrimps with clinical signs of vibriosis (Note: A=reddish and melanosis in a telson, B=reddish in periopods and pleiopods, C=soft body, D=reddish carapace, periopods, pleopods, mouth, and telson carapace, periopods, pleopods, mouth, and telson (d).

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	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	Hepatopankreas	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	Hepatopankreas	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	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. vannamaei* 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

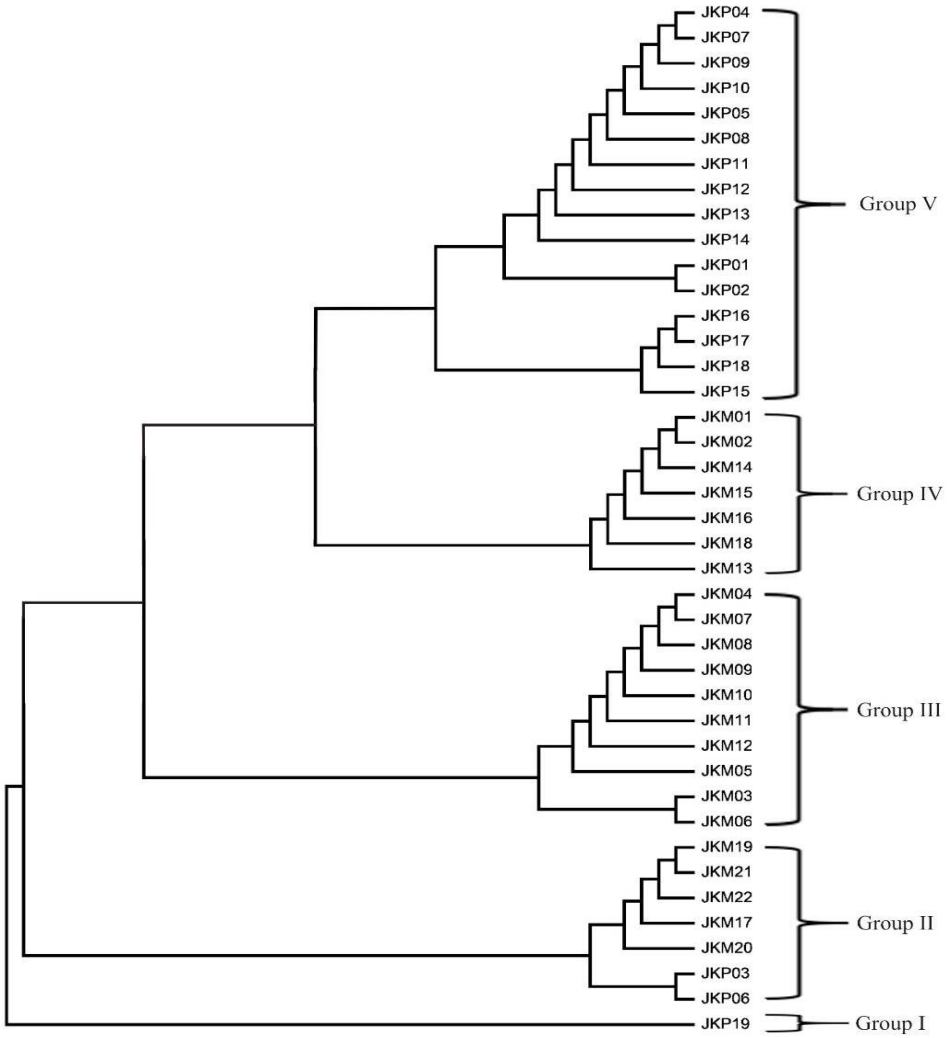

Figure 3. Dendrogram of repetitive PCR of 41 *Vibrio* isolates in shrimps

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

No.	Isolate	Closely Relative	Homology (%)	Acc. Number
1.	JKP03	*Vibrio rotiferianus*	100	GQ175915.1
2.	JKP05	*V. diabolicus*	99	MH044628.1
3.	JKP19	*V. parahaemolyticus*	94	MG548344.1
4.	JKM01	*V. alginolyticus*	97	MH368391.1
5.	JKM06	*Shewanella algae*	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; Santhyia et al., 2015; Selvin & Lipton, 2003; Shanmugasundaram 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 (Muthukrishanana 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* has 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 seilaevis* (Han et al., 2017), *Haliotis diversicolor, Crassostrea angulate, Meretrix lusoria*, *Pena 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 and continues to grow, control methods such as new vaccines, probiotics, and immunostimulant formulas must be developed for more potent efficacies.

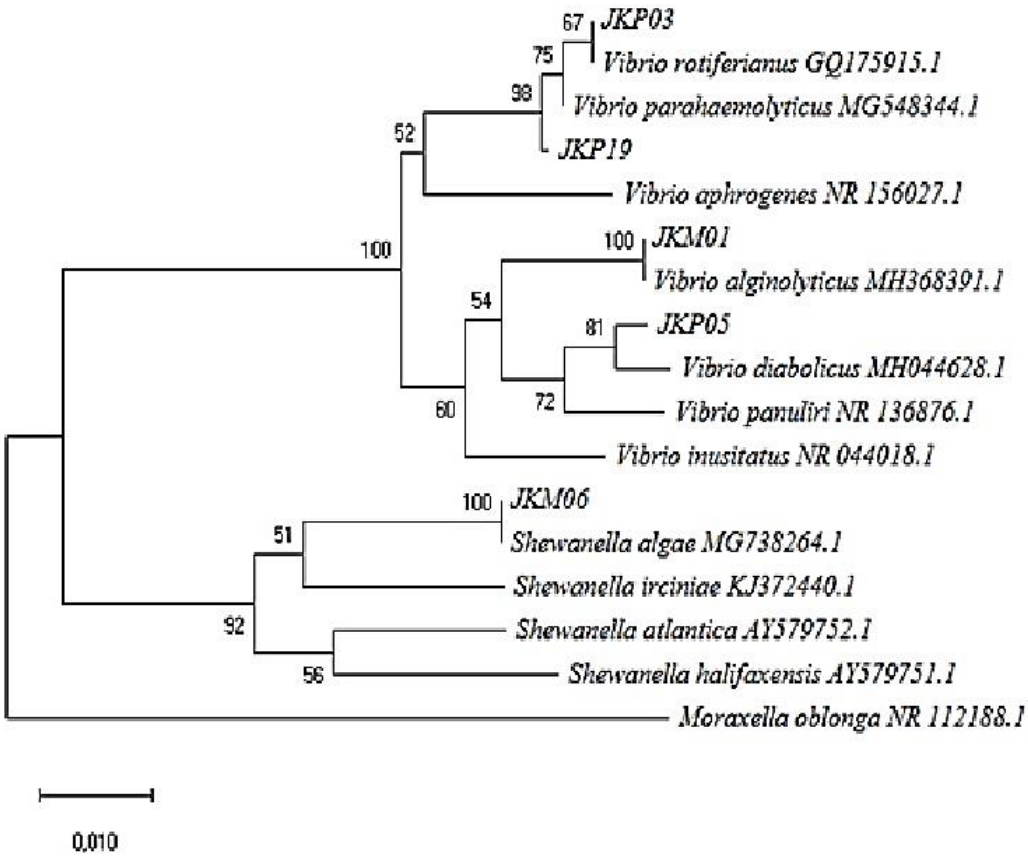

Figure 4. Phylogenetic tree of *Vibrio* associated with vibriosis in shrimps from traditional brackish water ponds of the northern coast of Central Java. The branching pattern was generated by the neighbor-joining method. Bootstrap values of 700 or more (from 1,000 replicates) are indicated at the nodes. *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 Unites States cephalopods have been added 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. All surgical samples were performed under clove oil anesthesia and all efforts were made 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.*

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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