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Colony morphology and molecular identification of *Vibrio* spp. on green mussels (*Perna viridis*) in Yogyakarta, Indonesia tourism beach areas

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Abstract. *Hikmawati F, Susilowati A, Setyaningsih R. 2019. Colony morphology and molecular identification of* Vibrio *spp. on green mussels (*Perna viridis*) in Yogyakarta, Indonesia tourism beach areas. Biodiversitas 20*: *2891-2899.* Green mussels (*Perna viridis*) have filter feeder properties that allow pathogenic bacteria from the water environment to accumulate in relatively high levels. About 20% of foodborne diseases are caused by large quantities of seafood contaminated with bacteria. The purpose of this study is to determine the morphological characteristics, pathogenicity, identity, and the kinship of *Vibrio* species on green mussels in Yogyakarta coastal tourism areas. *Vibrio* spp. were grown on selective differential TCBS media. In this media, the suspected *Vibrio* spp. would produce yellow or green colonies. The ability of hemolysis of *Vibrio* was blood agar media, the species was molecularly identified using 16S rRNA gene sequence, and the phylogenetic relationship of the *Vibrio* spp., was analyzed using MEGA X Neighbor-Joining program. Based on morphological analysis, we obtained 23 bacterial isolates suspected to be *Vibrio* spp. Two Isolates (L_1K_2 6 and L_2K_2 13) were positive for α-hemolysis activity and 4 isolates (L_1K_1 3, L_2K_1 8, L_2K_2 16, and L_3K_2 22) were positive for β-hemolysis activity. The molecular analysis involved 18 *Vibrio* species, and 4 of them represented the *Vibrio* genus and 14 species represented 97-99% similarity species in accordance with the 16S rRNA sequence in database, namely: *Vibrio alginolyticus, Vibrio parahaemolyticus, Vibrio cholerae, Vibrio neocaledonicus, Vibrio mimicus, Vibrio azureus, Vibrio diabolicus, Vibrio tapetis, Vibrio natriegens,* and *Vibrio owensii.* The most dominant number of *Vibrio* isolates was *V. alginolyticus*, while the lowest was *V. owensii.* The highest number of *Vibrio* species in green mussels was found in Goa Cemara beach while the lowest was in Kwaru beach. *Vibrio* spp bacteria found in green clams in coastal tourism areas in Yogyakarta have close phylogenetic relationships with other *Vibrio* in seafood in Indonesian coastal waters.

Keywords: 16S rRNA gene sequence, filter feeders, foodborne diseases, Green mussels, TCBS media, *Vibrio*

INTRODUCTION

Yogyakarta Beach is the main culinary place that is visited by tourists. Green shellfish is a marine fishery product that is favored by tourists, tastes good and also has a high protein content. Green mussels (*Perma viridis*) are classified as soft-bodied animals (*Phylum Mollusca*). They are two-shelled animals and have a brownish-green color (Wati 2014). Green mussels have good nutritional values for consumption including 49.8% water, 15.5% fat, 18.5% carbohydrates, 4.3% ash, and 21.9% protein (Eshmat et al. 2014). Green mussels concentrate microorganisms from surrounding waters during the filter-feeding process, therefore microorganisms like pathogenic bacteria such as *Vibrio* spp. are easier to accumulate (Murdinah 2009).

Bacteria spread through seafood will lead to foodborne diseases. Foodborne diseases are diseases that occur after consuming seafood in very large quantities contaminated by pathogenic bacteria (BPOM 2008). The case of foodborne diseases can occur from a level that is not severe to the level of death. For example, foodborne diseases by *Salmonella* sp., *Vibrio cholerae*, and *Clostridium botulinum*. According to Davies et al. (2011), as many as 10-20% of cases of foodborne diseases transmitted through seafood are caused by the bacteria *Vibrio* spp. in large quantities *Vibrio* spp. is a type of bacteria that lives saprophytically in freshwater, seawater, and soil. These bacteria can also live in relatively high salinity of 20-40 ppt and will grow well in optimum pH conditions between 7.0 - 7.5 and optimum temperature growth of 37° C (Supardi and Sukamto 1999). *Vibrio* spp bacteria are gram-negative with a single cell form with a short stem that is bent (coma) or straight, the long size of the *Vibrio* spp bacteria. about 1,4-50nm and width 0,3-1,3, motile and have polar flagella (Felix et al. 2011).

Vibrio cholerae is often found in raw shrimp, raw fish, shellfish and fish. When *V. cholerae* enters the human body, they can cause foodborne disease. It is characterized by vomiting, diarrhea, dehydration (Kharirie 2013). *Vibrio alginolyticus* can be said to be a pathogenic bacteria that cause foodborne disease, it is proven that these bacteria result in gastroenteritis and peritonitis in humans, besides that they can also cause infection and lead to death (Campanelli 2008). *Vibrio parahaemolyticus* is flora in brackish water environment and one of the species *Vibrio* spp. which are pathogenic in commodity shrimp and in humans (De Paola et al. 2000). The presence of *V. parahaemolyticus* in fishery products causes foodborne diseases in humans through raw food consumption or imperfect processing. This can also be caused by cross-contamination between processed and raw foods or through washing with water containing *V. parahaemolyticus* (Daniels and Nicoll 2012).

The presence of a large number of *V. parahaemolyticus*

is one of the causes of cases of septicemia and diarrhea in various regions of Southeast Asia (Merwad et al. 2011). Strains of *V. parahaemolyticus* that can cause disease are generally associated with the presence of virulence thermostable direct hemolysin (TDH) and thermostable direct hemolysin related hemolysin (TRH) factors. Thermostable Direct Hemolysin (TDH) is known as virulence factor because its β hemolysis activity which can lyse red blood cells is characterized by the presence of a clear zone in the blood agar media.

From the above explanation, research findings are expected to be used as information about the existence of *Vibrio* spp. on green mussels and as consideration for the safety of public consumption. The purpose of this study is to determine the morphological characteristics, pathogenicity, molecular identification, and kinship of *Vibrio* spp that can be expected to lead foodborne disease in green mussels found in the Yogyakarta coastal tourism area.

MATERIALS AND METHODS

Samples collection

The sampling was conducted on May 2018, taking place on three Yogyakarta, Indonesia tourist beach stations, namely, Depok Beach, Goa Cemara Beach, and Kwaru Beach with three conditions namely fresh, not fresh, and already through the cooking process. Nine samples of green mussels were taken at each beach site. The samples are then neatly stored into a sterile plastic clip containing the location and the condition of the shell after they were put into a Cooler Box containing Ice Gel. The samples were further tested for their pathogenicity, molecular identity and phylogenetic relationship at the Microbiology and Genetics Laboratory of Sebelas Maret University, Surakarta, Indonesia.

Isolation of *Vibrio*

Isolation is carried out in sterile rooms and conditions and uses personal protective equipment, so data is accurate. Samples of green mussels are removed from the shell carefully and remain sterile and then crushed by using sterile mortar until they are completely smooth. All parts of the green mussel organ are used because the size of the shells is less than 3 cm (Fitriatin and Manan 2015). The sample was diluted with three series of dilutions namely: 10^{-1}, 10^{-2}, and 10^{-3} use sterile aquadest, the sample is distributed by using a spread plate technique on sterile petri dishes using TCBS media namely selective media for *Vibrio* bacteria. The isolated samples were then wrapped in sterile paper and incubated at 37°C for 48 hours in the upside-down position. Furthermore, the colonies that grew on the media were taken by using sterile osseous needles and inoculated on TCBS media and then incubated again at 37ºC for 18 hours (Wayan 2015).

The morphological character of colonies of *Vibrio*

For detail morphological characterization, the colonies were taken using inoculation needles and transferred. with streak plate technique, into TCBS agar medium for further purification and then incubated at 37ºC. The pure colony was morphologically observed under a microscope. The characteristics that were observed including colony shape, color, size, edge, elevation (colony surface shape) and texture (Hidayat 2013).

Pathogenicity of *Vibrio*

Pathogenic bacteria suspected of being *Vibrio* bacterial isolates found in green mussels were carried out by using the hemolysis test in blood agar media (BAP). Media for blood or blood agar plate (BAP) is a growth medium for *Vibrio* bacteria that can distinguish pathogenic bacteria based on the effects of bacterial hemolytic exotoxins on red blood cells. *Vibrio* isolates were inoculated with streak plate technique on BAP media and then incubated in a place and sterile condition for 4 hours at 37^0C. Hemolysis activity is indicated by the zone of hemolysis in the BAP media which is seen when the light is on from behind. There are three types of hemolysis that could appear, namely β-hemolysis (when there is no zone of hemolysis around the growing colonies), α-hemolysis (some blood cells found in the zone of hemolysis or the presence of greenish discoloration around the colonies) and *ε-hemolysis* (no hemolysis) (Buxton 2003).

Genomic DNA extraction, DNA qualification, and quantification of *Vibrio*.

The DNA extraction was carried out using Presto™ Mini gDNA Bacteria Kit (Geneaid). The measurement of DNA quantity was carried out by using a bio-photometer at wavelengths of λ260 nm and λ280 nm. The purity of DNA can be measured by calculating the absorbance value on λ260 nm with λ280 nm (Ratio λ260: λ280). As much as 10 µL sterile aqua bidest was poured into cuvet in bio-photometer, then the blank button was pressed until number 0.0 appeared. Next, 1 µL aqua bidest was removed and replaced with 1 µL DNA. DNA was said to be pure and free of contamination if the ratio of absorbance ranges from 1.8 - 2.0 (Hasrida et al. 2016).

Amplification of 16S rRNA gene

Molecular identification was carried out by amplifying the 16S rRNA encoding gene using the PCR technique. The PCR reaction consisted of My Taq™ HS Red Mix PCR, 1 µL DNA template (100 ng), 2 µL primers with a concentration of 10 pmol (63 Forward and 1387 Reverse), and ddH_2O was added to a total volume of 25 µL. The used universal primer were pair of Forward 16S rRNA 63F (5'CAGGCCTAACACATGCAAGTC3') and reverse 1387R (5'GGGCGG WGTGTACAAGGC3') (Marchesi et al. 1998). The amplification was carried out in the following conditions: predenaturation at 94ºC for 5 min, 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 53ºC for 45 seconds, extension at 72ºC for 1,5 minutes, and a final extension at 72ºC for 5 minutes. PCR products were then electrophoresed.

DNA electrophoresis

As much as 2 µL loading dye as ballast and dye were mixed with 4 µL PCR products on parafilm. The solution was then inserted into a well made 1% agarose gel and

soaked in a buffer solution (50 ml TAE 10X solution + 950 ml sterile aqua dest) in the electrophoresis tank. The tank was then closed and connected to the power supply. The electrophoresis was run for 1.5 hours with Voltage 90 V. The agarose which has passed running process was soaked in ethidium bromide solution for 15 minutes and then soaked again in aqua dest for 10 minutes, afterward observation of DNA migration was carried out using UV transilluminator on Gel Doc (Fatchiyah et al. 2011).

Sequencing

And sequenced. Next, the sequencing results were analyzed by comparing the sequence on the database in the NCBI (National Center for Biotechnology Information) with the Basic Local Alignment Search Tool (BLAST) (Ilmiah et al. 2012).

Phylogenetic analysis

The phylogenetic relationship was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) were shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the distances used to infer the phylogenetic tree. The distances were computed using the p-distance method and were in the units of the number of base differences per site. Phylogenetic relationship analyses were conducted in MEGA X software (Kumar et al. 2018).

RESULTS AND DISCUSSION

Morphological characteristics of suspected *Vibrio* spp colony

Based on visual observations of *Vibrio* spp. Which is found in shellfish in the coastal tourism area of Yogyakarta, *Vibrio* bacteria can be distinguished based on the color, shape, and size of colonies that grow on TCBS media. *Vibrio* spp. What was found was divided into two groups, namely the group that could ferment sucrose collected with yellow colonies and secondly, *Vibrio* spp. groups that cannot ferment sucrose are characterized by green colonies (Figure 1). Strengthened by the research of Mailoa and Sehtha (2011) which proved the color of green colonies in *Vibrio* is due to the nature of bacteria that cannot ferment sucrose, while the *Vibrio* yellow colony is able to ferment sucrose and can reduce pH on TCBS media.

Bacterial colonies in green mussel samples showed detection of *Vibrio* spp. in sufficient quantities. The highest number of bacteria is *Vibrio* spp. on Depok beach with non-fresh shellfish conditions with bacterial colonies of 0.686 x 10^5 CFU/g, while the lowest bacterial colonies with bacterial colonies of 0.002 x 10^5 CFU/g on Kwaru beach locations with boiled shells (Farida et al. 2019). All grown bacterial colony were grouped based on their morphological characteristics. In total, there are 23 bacterial isolates suspected as *Vibrio* colony were grouped based on their similarity of the macroscopic characteristics (Table 1). Morphological observations of bacterial colony show that all bacterial colony is circular, yellow or green color and the size of the diameter of bacterial colonies range from 2-5 mm. These characteristics are typical *Vibrio* colony. Some bacterial colonies found in the coastal tourism area of Yogyakarta have different morphologies as soon as they are green, but some are yellow in Depok beach, Goa Cemara, and Kwaru. While the conditions are not fresh, the morphology of the *Vibrio* bacteria found is yellow on the Depok coast and Goa Cemara beach, while the Kwaru beach is mostly green. Boiled green mussels besides a small amount for *Vibrio* are found to have green morphology on the beaches of Depok and Kwaru, and yellow policies on the Goa Cemara beach.

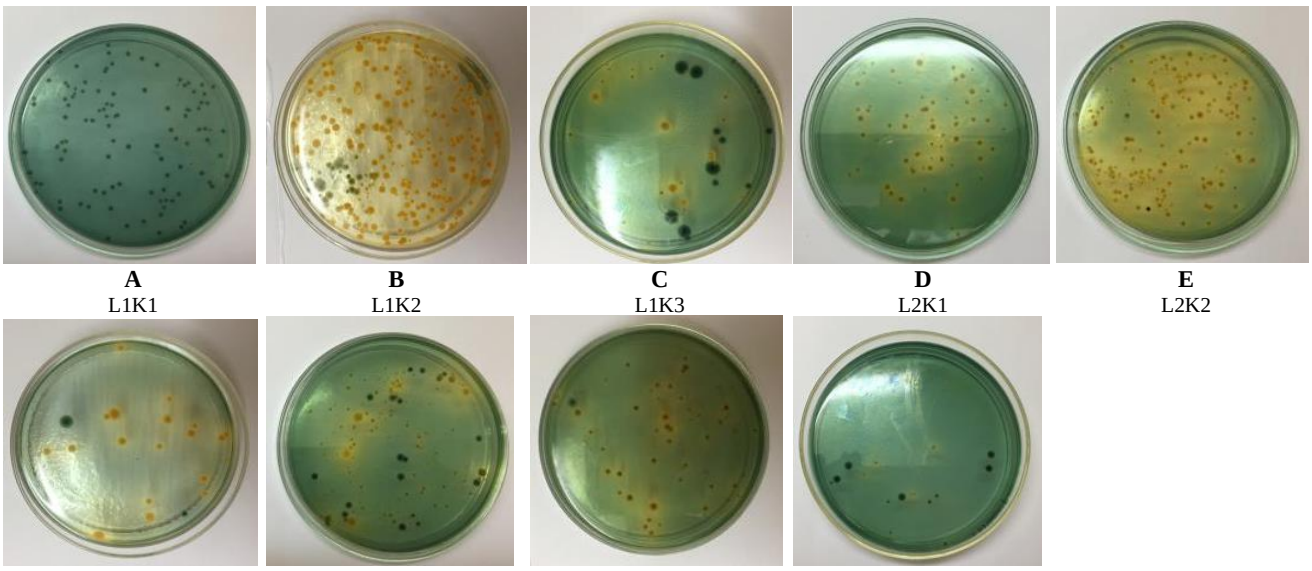
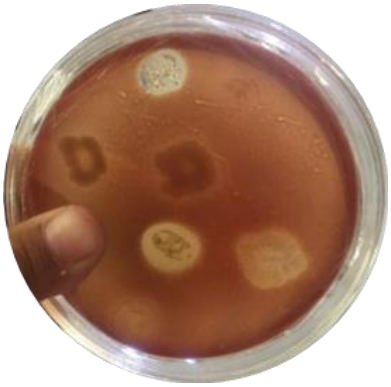


Figure 1. Morphology of 23 colonies of *Vibrio* isolates in green mussels (*Perna viridis*) found in the Yogyakarta, Indonesia coastal tourism. Note: L_1: Depok Beach, L_2: Goa Cemara Beach, L_3: Kwaru Beach and K_1: Fresh, K_2: Not Fresh, K_3: Boiled

Table 1. Morphological characteristics of the suspected *Vibrio* spp. Isolates in samples of green mussels (*Perna viridis*) found in the Yogyakarta coastal tourism

Location	Isolate code	Colony color	Colony form	Elevation of the colony	Size of the colony (mm)	Edge of the colony
Depok Beach	L_1K_1 1	Yellow	*Circular*	*Raised*	3	*Entire*
	L_1K_1 2	Green	*Circular*	*Raised*	3	*Calendar*
	L_1K_1 3	Bluish Green	*Circular*	*Umbonate*	2	*Entire*
	L_1K_2 4	Green Transparent	*Circular*	*Flat*	3	*Entire*
	L_1K_2 5	Bluish Green	*Circular*	*Umbonate*	5	*Entire*
	L_1K_2 6	Deep Yellow	*Circular*	*Convex*	5	*Entire*
	L_1K_2 7	Green	*Circular*	*Convex*	2	*Entire*
Goa Cemara Beach	L_2K_1 8	Yellow	*Circular*	*Raised*	2	*Entire*
	L_2K_1 9	Deep Yellow	*Circular*	*Convex*	4	*Calendar*
	L_2K_2 10	Deep Yellow	*Circular*	*Convex*	2	*Entire*
	L_2K_2 11	Green	*Circular*	*Convex*	3	*Entire*
	L_2K_2 12	Bluish Green	*Circular*	*Unbonate*	2	*Entire*
	L_2K_2 13	Yellow	*Circular*	*Raised*	2	*Entire*
	L_2K_2 14	Deep Yellow	*Circular*	*Convex*	3	*Entire*
	L_2K_2 15	Green	*Circular*	*Convex*	2	*Entire*
	L_2K_2 16	Bluish Green	*Circular*	*Umbonate*	2	*Entire*
Kwaru Beach	L_3K_1 17	Deep Yellow	*Circular*	*Convex*	5	*Entire*
	L_3K_1 18	Deep Yellow	*Circular*	*Flat*	3	*Calendar*
	L_3K_1 19	Yellow	*Circular*	*Flat*	2	*Calendar*
	L_3K_2 20	Yellow	*Circular*	*Flat*	2	*Entire*
	L_3K_2 21	Bluish Green	*Circular*	*Convex*	3	*Entire*
	L_3K_2 22	Deep Yellow	*Circular*	*Convex*	5	*Entire*
	L_3K_2 23	Yellow	*Circular*	*Convex*	2	*Calendar*

Note: (L_1: Depok Beach, L_2: Goa Cemara Beach, L_3: Kwaru Beach and K_1: Fresh, K_2: Not Fresh, K_3: Boiled)



L_1K_2 6

Figure 2. Hemolysis zone appears in the hemolysis test of *Vibrio* spp. L_1K_2

There are some types of *Vibrio* colonies in green mussels which are the result of various factors such as temperature, conductivity, acidity (pH), dissolved oxygen, and total available organic matter (Ilmiah et al. 2012). These factors affect the bacterial colony to maintain their survival. If green mussels are in different conditions or places, the types and numbers of *Vibrio* found in an area are likely to be different. Other factors that also influence the bacterial presence are competition for food in the region and interaction between *Vibrio* with other species there (Yital et al. 2007).

Pathogenicity of *Vibrio* spp.

Isolates with positive hemolytic activity showed the type of β-hemolysis and α-hemolysis can be seen in (Figure 2) which was marked by a clear zone in the surrounding colonies one example of Depok beach with a condition that is not fresh. The figure shows the formation of a lysis zone that clearly shows that isolates can lyse red blood cells. Really clear. The imperfect hemolysis process shows greenish-colored media, the imperfect or unreal lysis process causes no change in color in the media (Suryanto 2007).

Based on the results of pathogenicity test of *Vibrio* spp. in 23 isolates, 6 isolates showed a positive hemolysis activity, namely: isolates L_1K_1 3, L_1K_2 6, L_2K_1 8, L_2K_2 13, L_2K_2 16, L_3K_2 22 (Table 2). Bacterial colonies of L_1K_1 3 and L_2K_2 16 isolates have a bluish-green colony. While bacterial colonies L1K2 6, L_2K_1 8, L_2K_2 13, and L_3K_2 22 isolates have a yellow colony. The hemolysis test performed on blood agar media showed a different type of lysis. Qualitatively, the clear zone can be classified into three classes of lysis, and what was obtained in this research showed two classes of lysis. The first was α-hemolysis with medium lysis zone which had more different colors than other isolates. This including isolates with code L_1K_2 6 and L_2K_2 13. The second was β-hemolysis with a very powerful lysis zone where the isolate shows a very wide lysis zone indicated in isolates with code L_1K_1 3, L_2K_1 8, L_2K_2 16, and L_3K_2 22. Most of the bacteria that have the ability to hemolysis, namely *Vibrio* bacteria found in green mussels in a condition that is not

fresh and on the beach location of Goa Cemara. While in fresh conditions only a few of the total bacteria. Where both locations have bacteria with different hemolysis abilities.

Bacteria with β-hemolysis have the ability to multiply faster in the digestive tract compared to α-hemolysis. This ability is an important factor in determining the virulence from *Vibrio* spp. The production of enterotoxin in both β-hemolysis and α-hemolysis can determine the pathogenicity. The strain with β-hemolysis can last longer than α-hemolysis.

The occurrence of foodborne disease is closely related to the pathogenicity factors of the bacteria, the ability to invade tissues, colonization, the speed of proliferation of pathogens, and host defenses system against pathogens (Tortora et al. 2001). The hemolytic activity makes the bacterial defense factors attack host defenses by lysing the host's blood cells. Bacteria that are able to survive will enter the bloodstream so that they spread to all cells of the host's body and the target organ (Fitriatin and Manan 2015).

PCR Amplification of 16S rRNA gene

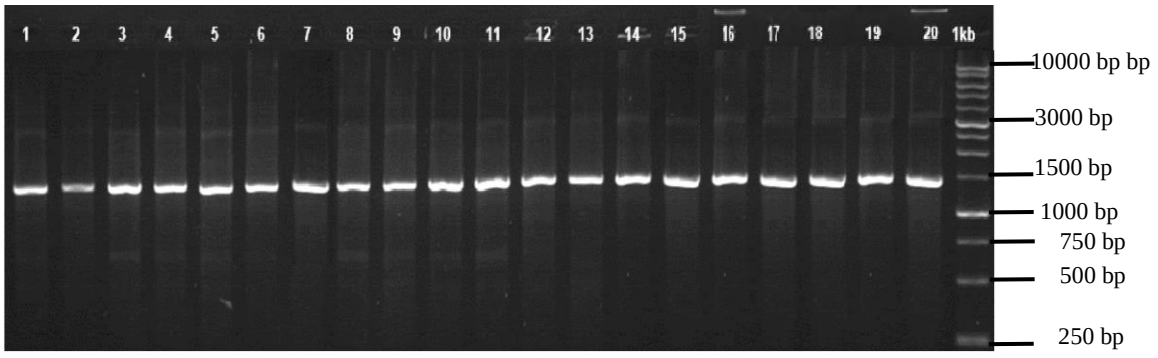
The gel electrophoresis shows that the 20 suspected *Vibrio* band's size is 1300 bp. Each amplified bacterial culture isolate was observed and documented in GelDoc. According to (Marchesi et al. 1998), if 16S rRNA gene of bacteria has been correctly amplified, then the length of the PCR product would be around ± 1300 bp (Figure 3).

The sequence analysis from the database in GenBank shows that 18 of the 20 isolates were identified to have similarities to the genus *Vibrio* and 2 isolates have similarities to the genus *Staphylococcus*. From 18 isolates, 10 different types of *Vibrio* species are found to have the 97-99%, similarity of 16S rRNA with *Vibrio* namely: *V. alginolyticus, V. parahaemolyticus, V. cholerae, V. neocaledonicus, V. mimicus, V. azureus, V. diabolicus, V. tapetis, V. natriegens,* and *V. owensii* (Table 3). Hagstrom et al. (2000) stated that isolates with a 16S rRNA sequence similarity greater than 97% could represent the same species.

Table 2. Hemolysis test results of *Vibrio* bacteria in green mussels found in the Yogyakarta coastal tourism area

Location	Isolate code	Hemolysis zone	Size of the hemolysis zone (mm)	Type of hemolysis
Depok	L_1K_1 1	-	-	-
Beach	L_1K_1 2	-	-	-
	L_1K_1 3	+++	1,85	β-hemolysis
	L_1K_2 4	-	-	-
	L_1K_2 5	-	-	-
	L_1K_2 6	++	1,57	α-hemolysis
	L_1K_2 7	-	-	-
Goa	L_2K_1 8	+++	1,96	β-hemolysis
Cemara	L_2K_1 9	-	-	-
Beach	L_2K_2 10	-	-	-
	L_2K_2 11	-	-	-
	L_2K_2 12	-	-	-
	L_2K_2 13	++	1,86	α-hemolysis
	L_2K_2 14	-	-	-
	L_2K_2 15	-	-	-
	L_2K_2 16	+++	2,11	β-hemolysis
Kwaru	L_3K_1 17	-	-	-
Beach	L_3K_1 18	-	-	-
	L_3K_1 19	-	-	-
	L_3K_2 20	-	-	-
	L_3K_2 21	-	-	-
	L_3K_2 22	+++	1,88	β-hemolysis
	L_3K_2 23	-	-	-

Note: "+++": very strong lysis zone, "++": Shows moderate lysis zone, "+": weak lysis zone, and "-": Shows negatife hemolysis.



Condition:

Amount of DNA ladder loaded per lane	0.8 % Agarose gel
The volume of sample loaded per lane	0.1 µg Each
1Kb DNA ladder (bp)	1 µL Each
1Kb DNA ladder (ng/0,1 µg)	250 500 750 1000 1500 2000 2500 3000 4000 5000 6000 8000 9 6 4.6 18.4 4 6.8 6.8 18.4 3.6 5.6 5.6 5.6

Figure 3. 16S rRNA gene electrophoresis gel from isolates of suspected *Vibrio* spp. in samples of green mussels found in the Yogyakarta coastal tourism area (amplified using 63F and 1387R primers)

Table 3. Isolate of *Vibrio* spp. detected in the samples of green mussels found in the Yogyakarta coastal tourism area, identified using 16S rRNA gene sequence (around 1300 bp).

Location	Isolate code	Description	Max score	Total score	Query cover (%)	E-value	Indent (%)	Accession
Depok	L_1K_1 1	*Vibrio neocaledonicus* strain Xmb064	2028	2028	96	0.0	97	KT986172.1
Beach	L_1K_1 3	*Vibrio parahaemolyticus* strain AP407	1973	1973	88	0.0	99	MG575451.1
	L_1K_2 4	*Vibrio mimicus* strain AAMH02	2150	2150	92	0.0	98	KC549802.1
	L_3K_1 5	*Vibrio parahaemolyticus* strain SC2	1967	1967	88	0.0	98	MK308579.1
	L_1K_2 6	*Vibrio alginolyticus* strain *hq*-V141	1967	1967	88	0.0	98	MH553008.1
	L_1K_2 7	*Vibrio azureus* strain ECSMC16	1993	1993	95	0.0	97	KC210817.1
Goa	L_2K_1 8	*Vibrio cholerae* strain NIOT VC 06	2150	2150	92	0.0	98	MF692792.1
Cemara	L_2K_1 9	*Vibrio diabolicus* strain WAB2224	1967	1967	88	0.0	98	MH169294.1
Beach	L_2K_2 10	*Vibrio alginolyticus* strain WAB2135	1969	1969	88	0.0	98	MH169336.1
	L_2K_2 11	*Vibrio tapetis* strain P1	2148	2148	92	0.0	98	KU750805.1
	L_2K_2 13	*Vibrio cholerae* strain CECT 514	2145	2145	92	0.0	98	NR044853.1
	L_2K_2 14	*Vibrio neocaledonicus* strain CV (H) 31	2109	2109	95	0.0	98	MH643641.1
	L_2K_2 16	*Vibrio parahaemolyticus* strain Vp-x10	1967	1967	88	0.0	98	MH298548.1
Kwaru	L_3K_1 17	*Vibrio alginolyticus* strain hq-V173	1993	1993	95	0.0	97	MH553013.1
Beach	L_1K_2 18	*Vibrio cholerae* strain DL1	2150	2150	92	0.0	98	MG062857.1
	L_3K_1 19	*Staphylococcus saprophyticus* strain B9	2170	2170	92	0.0	99	MK073020.1
	L_3K_2 20	*Vibrio natriegens* strain BPRIST057	2028	2028	96	0.0	97	JF700507.1
	L_3K_2 21	*Vibrio owensii* strain F77007	2026	2026	96	0.0	97	HQ908739.1
	L_3K_2 22	*Vibrio alginolyticus* strain Va-x15	1973	1973	88	0.0	98	MH298577.1
	L_3K_2 23	*Staphylococcus xylosus* strain CMU-BE04	2170	2170	92	0.0	99	KX235339.1

Note: L_1: Depok Beach, L_2: Goa Cemara Beach, L_3: Kwaru Beach and K_1: Fresh, K_2: Not Fresh, K_3: Boiled

The isolates L1K2 6, L2K2 10, L3K117, L3K2 22, L2K1 8, L2K2 13, L1K2 18, L1K1 1, L2K2 14, L2K1 9, and L3K2 20 are yellow-colored colony which have 97-98% similarities with the 5 *Vibrio* species, namely: *V. alginolyticus*, *V. cholerae*, *V. neocaledonicus*, *V. diabolicus*, and *V. natriegens. V. alginolyticus* can be said to be a pathogenic bacterium that causes gastroenteritis and peritonitis in humans, besides that it can also cause an infection that leads to death in immuno-compromised patients (Campanelli 2008). When *V. cholerae* enters the human body, it could cause foodborne disease which is characterized by vomit, diarrhea, and dehydration (Kharirie 2013).

The green colonies are L1K1 3, L3K1 5, L2K2 16, L1K2 4, L1K2 7, L2K2 11, and L3K2 21 which have 97-99%. similarities with the also 5 *Vibrio* species that are *V. parahaemolyticus*, *V. mimicus*, *V. azureus*, *V. tapetis*, and *V. owensii*. This *Vibrio* belong to a pathogenic organism and can trigger foodborne disease because these bacteria can produce hemolysin. Seafood that contains *V. mimicus*, when is consumed directly in raw condition or cooked imperfectly, it can cause gastroenteritis and diarrhea (Spira et al. 1984). *V. parahaemolyticus* in fishery products causes foodborne diseases in humans through raw food consumption or imperfect processing (Lutz et al. 2013). This can also be caused by contamination of instant foods, raw foods, or by washing food with water contaminated with *V. parahaemolyticus* (Daniels and Nicoll 2012).

The highest type of *Vibrio* species in green mussels was found in Goa Cemara beach while the lowest was in Kwaru beach. From 10 types of *Vibrio* obtained in green clams, *V. alginolyticus* was found in all sampling locations in Yogyakarta coastal areas. The most dominant number of bacterial isolates is *V. alginolyticus*, and the second ones are *V. parahaemolyticus*, *V. cholerae*, *Vibrio neocaledonicus* and while the lowest is *Vibrio owensii*.

Phylogenetic analysis

Phylogenetic analysis of nucleotide sequences will usually be an important area in sequence analysis. Phylogenetic trees make branches that connect taxonomic unit points, such as species or genes, and the tree roots are points that act as ancestors for all organisms which are being analyzed (Felix et al. 2011). Phylogenetic relationship on those identified 18 *Vibrio* spp that are found in green mussels in the coastal tourism area of Yogyakarta is determined and shown in the phylogenic tree. Phylogenetic relationship was constructed by using the Neighbor-Joining method to correctly identify the tree in the neighboring position and also have branches as close as possible. The Vibrio species in this research were analyzed for their kinship relationship with other *Vibrio* species found in previous research.

In the phylogenetic tree *Vibrio* bacteria found in green mussels in the coastal tourism area of Yogyakarta can be seen trees showing two large groups (Figure 4), the first group was branched first, namely bacteria *V. alginolyticus* L_1K_2 6, *V. alginolyticus* L_3K_1 17, *V. alginolyticus* L_3K_2 22, dan *V. alginolyticus* L_2K_2 10 which was found in green mussels appeared to have a kinship relationship with the bacteria found in previous studies, namely strains *V. alginolyticus* strains of ATCC 17749 which cause foodborne diseases found in seafood. Based on the results of molecular identification, *V. alginolyticus* is obtained

with a homology level of 97-98% with a base length around 1300bp. the group found in this first branch has a similar morphology of yellow bacterial colonies. These bacteria are gram-negative, the catalase is positive, the oxidase is positive and belongs to the motile bacteria (Buwono 2004). Deadly toxins produced by *V. alginolyticus* strain Swy are originally isolated from diseased Curuma shrimp purified with Fast Protein Liquid Chromatography with the interaction of Hydrophobic (Phenyl Sepharose High Performance) chromatography and gel filtration columns. The toxin is an alkaline serine protease, showing maximum activity at pH 8-11 (Liu and Lee 1999).

Vibrio cholerae L_2K_2 13, *V. cholerae* L_2K_1 8, and *V. cholerae* L_1K_2 18 which was found in green mussels appeared to have a kinship relationship with the bacteria found in previous studies, namely strains *V. cholerae* strain V6. *V. cholerae* has yellow morphological features, including gram-negative bacteria, non-spores, facultative anaerobic life, and flagella. Clinical manifestations in the form of the disease will arise if a number of entering bacteria reach a certain amount. This amount is affected by the entry of bacteria into the digestive tract. *V. neocaledonicus* L_1K_1 1, *V. neocaledonicus* L_2K_2 14, and *V. tapetis* L_2K_2 11 which was found in green mussels appeared to have a kinship relationship with the bacteria found in previous studies, namely strains *V. Fluvialis* strain VL 5125 that cause foodborne disease found in seafood. *V. Tapetis* can cause vibriosis and foodborne disease in various marine organisms including mollusks (Pailard et al. 2004). *V. natriegens* L_3K_2 20, have a relationship with the bacteria found in previous studies, namely *V. shiloni* FNS D08 causes foodborne diseases found in seafood.

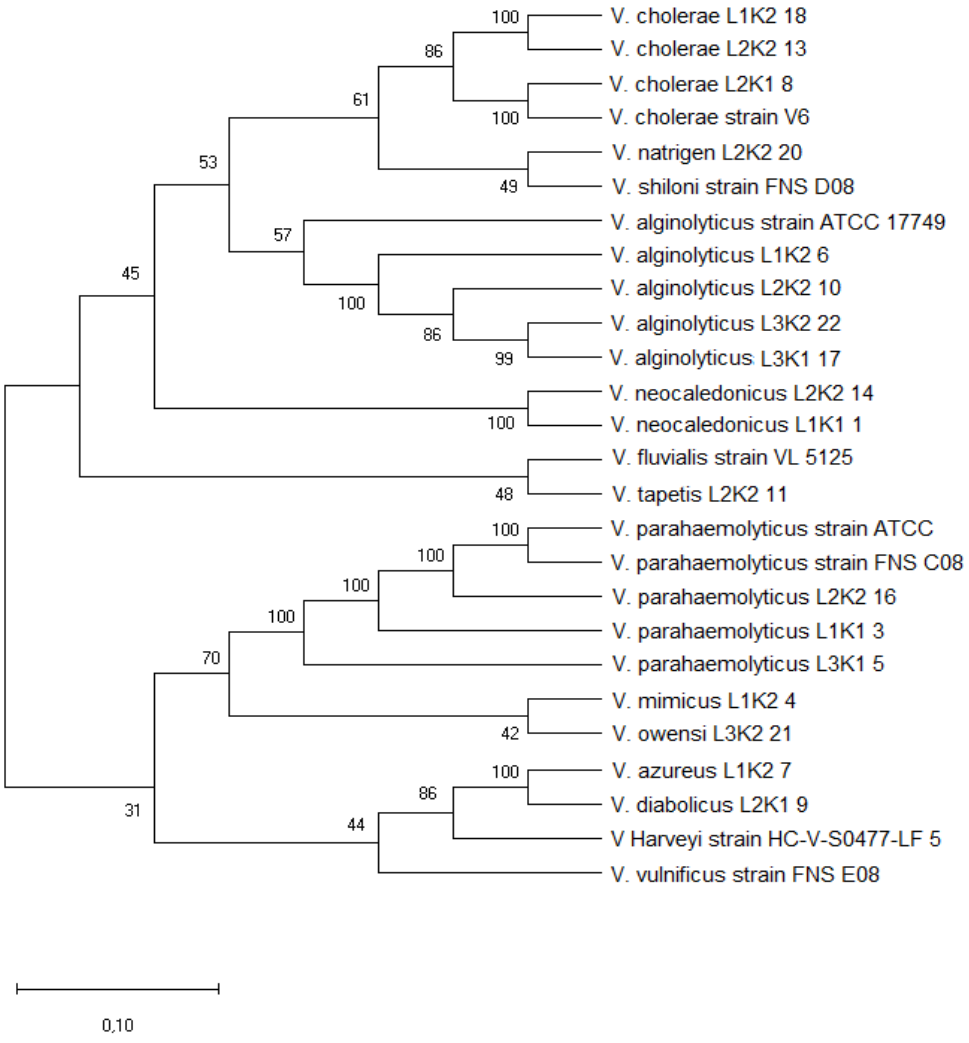


Figure 4. Phylogenic tree of *Vibrio* bacteria found in samples green mussels (*Perna viridis*)

In the phylogeny tree, the second group in the second branch is bacteria *V. parahaemolyticus* L_3K_1 5, *V. parahaemolyticus* L_2K_2 16, and *V. parahaemolyticus* L_1K_1 3 which was found in green mussels appeared to have a kinship relationship with the bacteria found in previous studies, namely strains *V. parahaemolyticus* strain FNS *C08*, *V. parahaemolyticus* strain ATCC 17802 that cause foodborne disease found on seafood. in the group, in this second branch, the morphology of the bacterial colonies is green in similarity. *V. parahaemolyticus* has the characteristics fermentative nature, glucose, lactose, sucrose, and with a positive gas production; whereas red methyl and H_2S are negative. The molecular identification of *V. parahaemolyticus* has 99% homology indicating that the pathogen has a base of about 1300bp. *V. parahaemolyticus* has a diameter of 3-5 mm, the center of the colonies is dark green, has a flagellum (Richie 2005). *V. parahaemolyticus* is a gram-negative halophilic bacteria that is distributed in tropical coastal waters throughout the world and causes gastroenteritis (De Paola et al. 2003).

Thermostable direct hemolysin (TDH) is the main virulence factor of *V. parahaemolyticus.* It is not toxic if heated at a temperature of 60^o-70^0C, but it will be toxic if heated higher than 80^0. This case is known as the Arrhenius effect, which explains that this effect is associated with structural changes in proteins that produce fibrils (Fukui et al. 2005). *V. diabolicus* L_2K_1 9 and *V. azureus* L1K2 7 which was found in green mussels appeared to have a kinship relationship with the bacteria found in previous studies, namely strains *V. harveyi* strain HC-V-S01-0477-LF 5 and *V. vulnificus* strain FNS E08. In term of virulence, these bacteria have a specific gene called AcfA for the production of colicin. Colicin is a protein that stimulates the production of ToxR, ToxS, Zonulaa occludens toxins (Zot) and Ace (Turner et al. 2018). *V. vulnificus* has a green colony color, fermentative properties, positive methyl red, glucose, lactose, and sucrose. During infection, *V. vulnificus* reaches the intestine and then attacks the bloodstream by penetrating the host intestinal mucosal wall which results in disease. Lee et al. (2008) found that RtxA *V. vulnificus* toxin released by RtxE transport plays a role in the cytotoxicity of *V. vulnificus* against intestinal epithelial cells.

In conclusion, twenty-three bacterial colonies, suspected as *Vibrio*, were isolated from TCBS media with characteristics including circular bacteria (irregular round), the diameter size of bacterial colonies ranges from 2-5 mm with yellow and green color. The hemolysis test performed on blood agar plate shows that the isolates of L_1K_2 6 dan L_2K_2 13 are α- hemolysis, while the isolates of L_1K_1 3, L_1K_1 3, L_2K_1 8, L_2K_2 16, and L_3K_2 22 are positive for β-hemolysis. The molecular analysis involved 18 *Vibrio* species, and 4 of them represented the *Vibrio* genus and 14 species represented 97-99% similarity species in accordance with the 16S rRNA sequence in database, namely: *V. alginolyticus*, *V. parahaemolyticus*, *V. cholerae*, *Vibrio neocaledonicus*, *Vibrio mimicus*, *Vibrio azureus*, *Vibrio diabolicus*, *Vibrio tapetis*, *Vibrio natriegens*, and *Vibrio owensii*. Most of *Vibrio* species in green mussels are obtained in Goa Cemara beach while the lowest is in Kwaru beach. From the 10 species of *Vibrio* obtained in green mussels, *V. alginolyticus* is spread in all locations of the Yogyakarta beach. *Vibrio* spp. bacteria found in green clams in coastal tourism areas in Yogyakarta have close phylogenetic relationships with other *Vibrio* in seafood in Indonesian coastal waters.

Further research is needed to determine the biochemical characteristics and toxic levels of each species of *Vibrio* spp. as the cause of foodborne disease. An effort to prevent the growth of *Vibrio* spp. in green mussels and fisheries, in general, is needed in order to reduce the risk of contamination. Then, it is necessary to do in-depth research to obtain *Vibrio* bacteria with strains which are assumed to be genuine Indonesian strains and it is expected to enrich the strain of *Vibrio* spp. in NCBI.

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