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Morphology, molecular identification, and pathogenicity of *Vibrio* spp. on blood clam (*Anadara granosa*) in Yogyakarta, Indonesia tourism beach areas

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Abstract. *Devi AR, Susilowati A, Setyaningsih R. 2019. Morphology, molecular identification, and pathogenicity of* Vibrio *spp. on blood clam (*Anadara granosa*) in Yogyakarta, Indonesia tourism beach areas. Biodiversitas 20: 2890-2896.* Seafood is very popular among Indonesian people, especially in coastal areas. In Bantul Yogyakarta, blood clams have become one of tourist's favorite, either cooked or raw. Blood clams are filter feeders that cause the clams to be vulnerable to contamination of pathogenic bacteria that can cause foodborne disease, including *Escherichia*, *Pseudomonas*, and *Vibrio*. The 10-20% cases of foodborne disease transmitted through seafood caused by *Vibrio* spp. Three species of *Vibrio* can cause foodborne disease in humans, i.e., *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. The purpose of this study was to determine the character of *Vibrio* using morphological and molecular identification and pathogenicity on blood clam (Anadara granosa). Blood clams samples were collected from Depok, Goa Cemara, and Kwaru beaches in Bantul, Yogyakarta, Indonesia. Isolation of *Vibrio* spp. from blood clams was done using selective differential Thiosulfate Citrate bile salts sucrose (TCBS) culture medium. The morphological characters of the isolate colonies were determined based on the color, shape, texture, and size of the colony. Hemolysis test was also performed to evaluate the pathogenicity by using blood agar media. Molecular identification of *Vibrio* species was made using 16S rRNA sequence. Phylogenetic analysis was performed using Neighbor-Joining method in Mega X software. Samples for the analysis came from DNA sequences of this study and those from the GenBank database. Of the total 15 isolates obtained, four isolates showed positive β-hemolysis, namely, isolate P_2S_2 -1bH, P_3S_1-1aH, P_2S_1-1aK and P_2S_2-1aK, and one isolate had positive α-hemolysis (P_3S_2-1aK). Seven species of *Vibrio* were identified *as V. algynolyticus*, *V. parahaemolyticus*, *V. diabolicus*, *V neocaledonicus*, *V. azureus*, *V. natrigens*, and *V. cholerae.*

Keywords: 16S rRNA, blood clams, molecular identification, morphology, molecular, *Vibrio*

INTRODUCTION

Seafood is very popular within the coastal, especially in seaside tourism areas. One of the famous tourism side in Indonesia that still shows massive improvement as a tourist destination is the beach area in Bantul, Yogyakarta. One of the most favorites is culinary. The shellfish is obtained directly from fishermen and breeder who live around the coastal area, and some were supplied from outside the city due to the lack of shellfish breeder in the area.

Fresh raw blood clams have nutritional values, including protein 19.48%, fat 2.50%, water 74.3%, and ash 2.24% (Nurjanah 2005). Although blood clams are rich in protein, if they are not adequately processed before consumption, they are contaminated by microorganisms that are dangerous for human health. Blood clams are filter feeders which filter to get food so that the clams are easy to get contaminated with microorganisms and can lead to the emergence of foodborne disease. Kanjanasopa et al. (2011) found that samples of marine products such as blood clams found in the south coast of Thailand are positively contaminan*ts* by *V. parahaemolyticus*, a bacteria that cause foodborne disease, and 30% of foodborne disease cases are from bacterial contamination. However, several studies show that the highest outbreaks and mortality in foodborne disease is caused by bacterial infections (Altekruse et al. 2008). Some of the bacteria that cause foodborne disease, including *Escherichia*, *Pseudomonas*, and *Vibrio*. *Vibrio* spp., makes up of 10-20% of foodborne diseases cases transmitted through seafood.

Currently, identification of pathogenic *Vibrio* sp. has been made only by observing the clinical symptoms from the history of disease events, morphological characteristics, physiology, and biochemistry of the bacteria. One way to advance the identification method is by assessing the genetic properties using molecular. Some of the molecular identifications that currently being developed are through molecular markers of PCR-based techniques. The molecular identification of *Vibrio* taken from the blood clams proposed in this study is based on the 16S rRNA sequence. This research aimed to evaluate *Vibrio* content in blood clamps found in coastal areas in Bantul, Yogyakarta, Indonesia by performing pathogenicity test, morphological and molecular characterization.

MATERIALS AND METHODS

Sample collection

Blood clam samples were collected from three locations at Bantul beach area, Yogyakarta, Indonesia, namely Depok, Goa Cemara, and Kuaru Beaches. Nine individuals from each beach were collected and stored in a cooling box containing ice cubes until arriving in the laboratory (Widyowati 2008).

Isolation of *Vibrio*

The clam samples were crushed using a mortar, 5 gram of this was stored into a test tube and diluted using 9 mL of aquadest and diluted in three concentrations, 10^{-2} for the second and 10^{-3} for the third. Then 0.1 mL was taken from each dilution and spread using spread plate techniques on *Thiosulfate Citrate Bile Salt Sucrose Agar* (TCBS) that is commonly used in microbiology laboratories to isolates *Vibrio* species. Each dilution series was spread into three Petri dishes. All Petri dishes then were incubated at 37ºC for two days in an upside-down position. The growing colonies were then re-isolated into TCBS media and incubated at 37°C for colony purification. *Vibrio* single colony were re-isolated and grown on TCBS media with an incubation temperature of 37°C for 18 hours until the pure culture was obtained (Ilmiah et al. 2012).

Determination of morphology colony of *Vibrio* isolated from blood clams

The *Vibrio* colony that has already grown in TCBS media and incubated was then morphologically observed for its colony color, colony size, colony edge and elevation (the colony surface shape) (Hidayat 2013).

The pathogeny of *Vibrio* isolated from blood clams

The pathogenicity of *Vibrio* in blood clams was evaluated using the hemolysis test with the blood agar plate. The selected isolate was streak-scratched using inoculation needle on the media surface. It was then incubated for 24 hours at room temperature of 37°C. If the bacterial isolate induced hemolysis activity, a clear zone would form on the media. There are three types of hemolysis, i.e., β-hemolysis (there is no blood around the grown colonies), α-hemolysis (some blood cells found on the hemolysis zone or greenish discoloration around the colonies) and ε-hemolysis (no hemolysis) (Mailoa and Setha 2011)

Molecular characterization

The bacterial genomic DNA extraction was carried out using Presto™ Mini gDNA Bacteria kit (GeneAid). Sample of 2 µL of genomic DNA was added with aqua dest until the volume was 200 µL for 100 times dilution. Then the DNA was transferred into the bio-photometer cuvet. Stamps bio-photometer will read the absorbance of samples with a bio-photometer at λ260 nm and λ280 nm. DNA quality related to the purity of protein contaminants can be seen from the ratio of absorbance of DNA suspension at wavelength λ260 nm and λ280 nm. The OD260/280 ratio between 1.8-2.0 shows that DNA is relatively pure and free from contaminant (Ningsih et al. 2017). Predenaturation process was carried out at a temperature of 95º C for 1 minute. Denaturation at 95º C for 15 seconds. Annealing at 55ºC for 15 seconds. Extension or elongation was carried out at 72ºC for 10 seconds. Final extension at 72ºC for 5 minutes. The process of denaturation, annealing, and elongation each consist of 25 cycles. Then was stirred in the freezer box at -20º C and checked using electrophoresis (Marchesi et al. 1998; Stephanie and Waturangi 2011)

Sequencing and phylogenetic analysis

PCR product of 16S rRNA gene from colonies were sequenced in Genetika Sains (Jakarta) and result were compared with 16S rRNA sequences obtained from Genbank using BLAST. Phylogenetic tree was constructed using MEGA-X software based on neighbor-joining analysis with the Jukes-Cantor model and the bootstraps method.

RESULTS AND DISCUSSION

Morphology colony of the *Vibrio*

Fifteen bacterial isolates suspected to be *Vibrio* were obtained from blood clams tissue collected from the coastal area of Bantul (Table 1). Several bacterial isolates were unable to grow on TCBS media indicating that, as a selective medium, TCBS could inhibit a certain type of bacteria from growing. The identification of *Vibrio* isolates was carried out by colony morphological characterization or visual observation. *Vibrio* bacteria colonies that grow on TCBS media would vary in color, including green, yellow, transparent green, orange, and bluish green. According to Mailoa and Setha (2011), the green color of *Vibrio* colony is due to the nature of the *Vibrio* which is unable to ferment sucrose while the yellow colony is able to ferment sucrose and is able to reduce pH on TCBS media. The colony shape is circular, the colony edge is entire, and the colony elevation is various (law, convex, and effuse) (Ihsan and Retnaringrum 2017). TCBS plates were examined for the presence of either yellow color, rounded shape, 2-3 mm in diameter colonies (suspect: *V. cholera*, *V. fluvialis* or *V. alginolyticus*) or green in color, rounded shape and 2-3 mm in diameter (suspect: *V. parahaemolyiticus* or *V. vulnificus*).

The variation of the colony's color is likely due to several factors including abiotic factors (factors affecting *Vibrio* to maintain their survival) namely temperature, conductivity, acidity (pH), dissolved oxygen and total organic matter that was acting as host. In different condition/place, the *Vibrio*'s diversity will also be different according to the growing needs. Besides the abiotic factor, the biotic factors also affect the competition for food and interactions between *Vibrio* organisms or other species (Mudatsir 2007).

Pathogenicity of bacterial isolates

The hemolysis test of 15 isolates using blood agar showed that five isolates indicated positive hemolysis activity where four isolates indicated *β-hemolysis*, and one isolate indicated *α-hemolysis* (Table 2).

Table 1. Colony morphological characteristics of 15 bacterial isolates obtained from blood clams (*Anadara granosa*)

Location	Isolate code	Colony shape	Colony elevation	Colony size (mm)	Colony edge	Colony color
Depok beach	P_1S_1-1aK	*Circular*	*Convex*	4	*Entire*	Yellow
	P_1S_1-1aH	*Circular*	*Convex*	2	*Entire*	Transparent Green
	P_1S_2-1aK	*Circular*	*Flat*	2	*Entire*	Yellow
	P_1S_2-1aH	*Circular*	*Umbonate*	2	*Entire*	Green
Goa Cemara beach	P_2S_1-1aK	*Circular*	*Convex*	3	*Entire*	Yellow
	P_2S_1-1bK	*Circular*	*Flat*	4	*Entire*	Yellow
	P_2S_2-1aK	*Circular*	*Raised*	2	*Entire*	Shiny Yellow
	P_2S_2-1aH	*Circular*	*Raised*	2	*Entire*	Dark Green
	P_2S_2 -1bH	*Circular*	*Convex*	2	*Entire*	Dark Green
	P_2S_2-1aHB	*Circular*	*Convex*	2	*Entire*	Bluish Green
Kwaru beach	P_3S_1-1aH	*Circular*	*Convex*	2	*Entire*	Green
	P_3S_1-1aK	*Circular*	*Flat*	3	*Entire*	Dark Yellow
	P_3S_1-1bK	*Circular*	*Convex*	4	*Entire*	Yellow
	P_3S_2-1aK	*Circular*	*Raised*	3	*Entire*	Yellow
	P_3S_2-1aH	*Circular*	*Convex*	2	*Entire*	Green

Note: P=Location (1, Depok; 2 Goa Cemara beach; 3, Kwaru beach), S=Condition (1, Fresh; not Fresh)

Table 2. Results of hemolysis test of *Vibrio* isolates

Isolate code	Hemolysis zone*	Hemolysis zone (mm)	Hemolysis type
P_1S_1-1aK	-	-	-
P_1S_1-1aH	-	-	-
P_1S_2-1aK	-	-	-
P_1S_2-1aH	-	-	-
P_2S_1-1aK	+++	1.87	*β-hemolysis*
P_2S_1-1bK	-	-	-
P_2S_2-1aK	+++	1.72	*β-hemolysis*
P_2S_2-1aH	-	-	-
P_2S_2 -1bH	+++	1.55	*β-hemolysis*
P_2S_2-1aHB	-	-	-
P_3S_1-1aH	+++	1.57	*β-hemolysis*
P_3S_1-1aK	-	-	-
P_3S_1-1bK	-	-	-
P_3S_2-1aK	++	1.98	*α-hemolysis*
P_3S_2-1aH	-	-	-

Note*: Hemolysis test was qualitatively done: +++: showing very strong lysed zone, + +: showing medium lysed zone, +: showing weak lysed zone

Hemolysin is a protein that can damage cell membranes and lyse red blood cells. Hemolysin spreads through blood circulation (Mangunwardoyo et al. 2009). The ability to produce extracellular toxins in the form of hemolysin is an indicator in determinating the virulence of *Vibrio*. The agar media with high salt concentrations made by Wagatzuma is used to detect *Vibrio* hemolytic activity (Figure 1).

The ability of β-hemolysis strains to multiply is faster in the gastrointestinal tract compared to α-hemolysis. This is an important factor in determining the virulence of these bacteria. While enterotoxin production by both β-hemolysis and α-hemolysis strain determines its pathogenic characteristic. B-hemolysis strain is more resistant than α-hemolysis. *Vibrio* that contains β-hemolysis are pathogenic to humans, but some bacteria are not invasive and do not enter the blood circulation and remain in the intestine. Bacteria containing β-hemolysis can produce enteroctine. Belkin and Collwell (2005) reported that the clear zone activity formed in the agar blood media shows the activity of β-hemolysin caused by thermostable direct hemolys*in (TDH)* gene. This gene determines virulence factors. *Vibrio* having TDH gene were mostly found in the aquatic environment. This is in accordance with the statement of Raghunath (2015), which shows that pathogenic *Vibrio* has low oxygen and high levels of organic components in the aquatic environment. This TDH gene and TRH gene (the thermostable direct hemolysin-related hemolysin) are active because there is a specific gene in *Vibrio*, that is the *toxR* gene. The work of the *toxR* gene is to activate other genes to produce toxins.

Identities of the *Vibrio* species based on the 16S rRNA encoding gene

Bacterial isolates are considered as different species if the similarities of rRNA sequence are lower than 97% (Stackebrandt and Goebel 1994). The taxonomy of modern bacteria is based on the 16S rRNA gene equation approach that 95% equation threshold for genus and 98,5% can be said as a species (Rossi-Tamisier et al. 2015).

Diform the database found in GenBank 10 out of 15 candidates (Table 3) were identified as *Vibrio*, then five other isolates were similar to bacteria from the genus *Rothia*, *Aeromonas*, and *Staphylococcus*. TCBS is highly selective media for *Vibrio* by providing a specific environment and source that only suitable for *Vibrio*. The presence of other species in this TCBS agar is most likely that they have a very similar environment requirement to grow as *Vibrio*. This is also the reason why further biochemical and molecular identification of grown isolates from TCBS media is needed. In this research, we used 16S rRNA encoding gene for isolates identification (Figure 2).

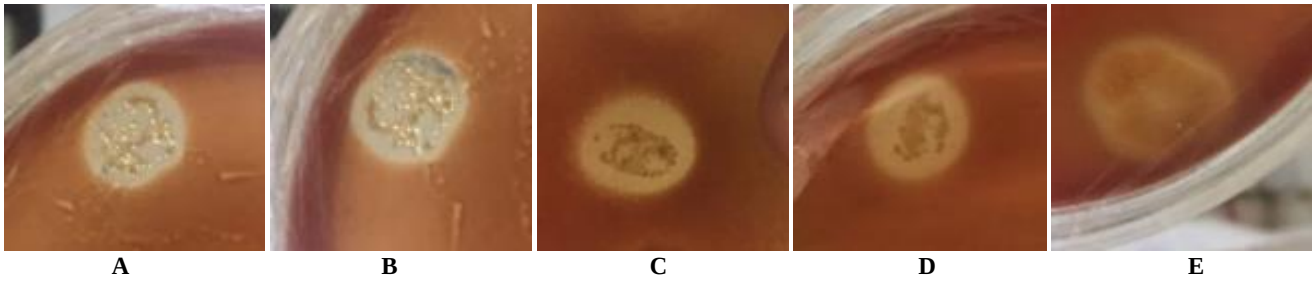

Figure 1. Morphological characters of the colony as resulted from the hemolysis test of *Vibrio* isolates. β-hemolysis: A. P_2S_2 -1bH, B. P_2S_2-1aK, C. P_3S_1-1aH, D. P_2S_1-1Ak; as well as α-hemolysis: E. P_3S_2-1aK

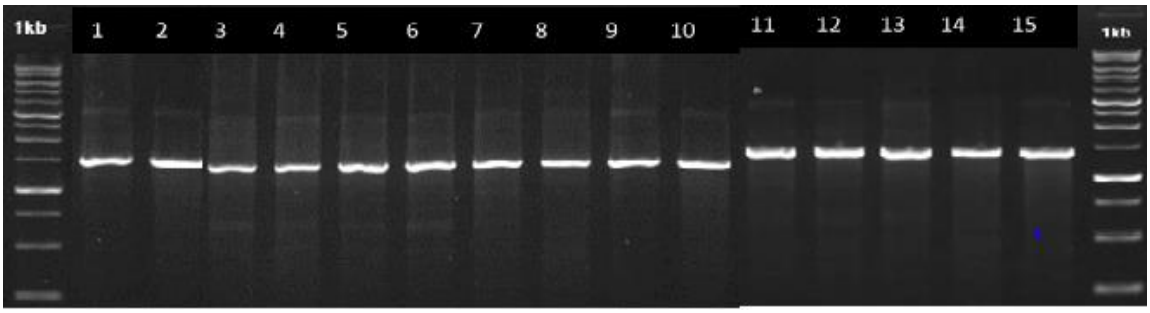

Figure 2. The electrophoresis gel of the 16S rRNA gene from the isolate amplified using primary 63F and 1387F from blood clams. Note: 1. P_1S_1-1aK, 2. P_1S_1-1aH, 3. P_1S_2-1aK, 4. P_1S_2-1aH, 5. P_2S_1-1aK, 6. P_2S_1-1bK, 7. P_2S_2-1aK, 8. P_2S_2-1aH, 9. P_2S_2 -1bH, 10. P_2S_2-1aHB, 11. P_3S_1-1aH, 12. P_3S_1-1aK, 13. P_3S_1-1bK, 14. P_3S_2-1aK, 15. P_3S_2-1aH

Table 3. The identity of *Vibrio* species based on the 16S rRNA sequence isolated from blood clams in Yogyakarta beaches area

Location	Isolate code	Description	Max score	Total score	Quey cover	E value	Access No.	Ident
Depok Beach	P_1S_1-1aK	*Aeromonas enterpelogeneses*	1934	19262	90%	0.0	KX980472.1	97%
	P_1S_1-1aH	*Vibrio parahaemolitucus* strain HH101313	2156	2156	92%	0.0	MG386398.1	98%
	P_1S_2-1aK	*Vibrio alginolyticus* strain HH101307	2154	2154	92%	0.0	MG386392.1	98%
	P_1S_2-1aH	*Vibrio parahaemolyticus* strain SC2	1951	1951	88%	0.0	MK308579.1	98%
Goa Cemara Beach	P_2S_1-1aK	*Vibrio natrigens* strain Xmb012	1951	1951	88%	0.0	KT986142.1	98%
	P_2S_1-1bK	*Vibrio azureus* strain Xmb005	1951	1951	91%	0.0	KT986135.1	98%
	P_2S_2-1aK	*Vibrio alginolyticus* CZN-16S	1821	1821	98%	0.0	KR347254.1	98%
	P_2S_2-1aH	*Vibrio parahaemolyticus* strain VP-ABTNL	2159	2159	92%	0.0	MG589511.1	98%
	P_2S_2 -1bH	*Vibrio diabolicus* strain WAB2224	2154	2154	91%	0.0	MH169294.1	98%
	P_2S_2-1aHB	*Aeromonas dhakensis*	1934	1934	90%	0.0	MF953268.1	97%
Kwaru Beach	P_3S_1-1aH	*Rothia endhopytica* strain CF22	1951	1951	89%	0.0	KX062009.1	99%
	P_3S_1-1aK	*Vibrio cholerae* strain TP	2156	2156	92%	0.0	AY494843.1	98%
	P_3S_1-1bK	*Vibrio neocaledonicus* strain SCSIO 43731	1951	1951	88%	0.0	MH283815.1	98%
	P_3S_2-1aK	*Aeromonas caviae* strain 65H8M	1934	19013	90%	0.0	AP019195.1	97%
	P_3S_2-1aH	*Staphylococcus sciuri* strain SS3	2178	2178	91%	0.0	MG593973.1	98%

Note: P: Location of beaches and S: Condition blood clams (Fresh and not fresh)

P_2S_2-1aK and P_1S_2-1aK isolates were 98% similar to *Vibrio alginolitycus*; these bacteria are pathogenic and are halophilic bacteria. In TCBS media, these *Vibrio* bacteria form yellow colonies with 0.8-1.2 cm in diameter (Buwono 2004), and they can ferment glucose, lactose, sucrose, and maltose. *V. alginolyticus* is reported to be a pathogen but the infection and transmission causing foodborne disease is still being studied with allegations of transmission pathways from seawater that becoming parasites in marine species (seafood) or as opportunistic pathogens (Ben Kahla-Nakbi et al. 2007). *V. alginolyticus* has many virulence genes which are also found in other *Vibrio* species that live in seawater environments. Its contribution accelerates the development of wounds, intestinal diseases, and sepsis in humans (Ennaji and Cohen 2013). The deadly toxin produced by *V. alginolyticus* is *alkaline serine protease* (Felix et al. 2011). *V. algynolyticus* is proven to cause gastroenteritis and peritonitis in foodborne disease

patient. Besides, it also causes infection and death in immunocompromised patients (Campanelli et al. 2008). *Vibrio algynolyiticus* also infects in a short period of time.

P_2S_2-1aH and P_1S_2-1aH isolate has 98% similarities with *Vibrio parahaemolyiticus*. The colonies have a diameter of 3-5 mm with greenish-blue color with a dark green spot in the middle. The characteristic of *V. parahaemolyticus* (Richie 2005) has flagella, and it distributed on tropical beaches with fermentative biochemical of mining, lactose, sucrose, and positive gas production *V. parahaemolyticus* is a pathogenic bacteria that produce hemolysin called *Thermostable Direct Hemolysin* (TDH) and *Thermostable Direct Hemolysin-Related Hemolysin* (TRH). TDH is known as virulence factor because it has β hemolysis activity that leads to gastroenteritis in humans (Yennie et al. 2015). The level of virulence of *V. parahaemolyticus* does not depend on the amount of *V. parahaemolyticus*, instead very dependent on the toxin produced by the gene (Sujeewa et al. 2009). This TDH and TRH gene will act as toxic in the human digestive tracts. Thus, in certain numbers can cause dysentery (Anses 2012).

P_3S_1-1aK bacterial isolate has 98% similarities with *Vibrio cholerae*. *V. cholerae* is a bacterium that can grow in various media or selective media containing mineral salts and asparagine as a source of carbon and nitrogen. If it is grown in TCBS media, it will appear in the yellow colony. *V. cholerae* is a pathogenic bacterium and triggers foodborne disease in human through the digestive system (fecal-oral). *V. cholerae* increases the secretion of enterotoxins in the body and stimulates adenyl cyclase activity in intestinal cells. This results in the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) which causes electrolyte secretion into the intestinal cavity. It results in a large amount of fluid loss and electrolyte imbalance. This bacterial infection can result in gastroenteritis characterized by bloody bowel movements accompanied by vomiting blood. *V. cholerae* is not invasive and does not enter the bloodstream but persists in the intestinal tract. It enters the body with food and drink that positively contaminated by *V. cholerae*. After passing gastric acid, this bacterium will produce two virulence factors that cause a disease called cholera. The toxin is in the form of coregulated *pilus toxin* (TCP) and *cholerae toxin* (CT). Seafood is the most common source of *V. cholerae* contamination. The common preparation of seafood to prevent foodborne disease is heat cooking that kills all the bacteria (Paydar 2013).

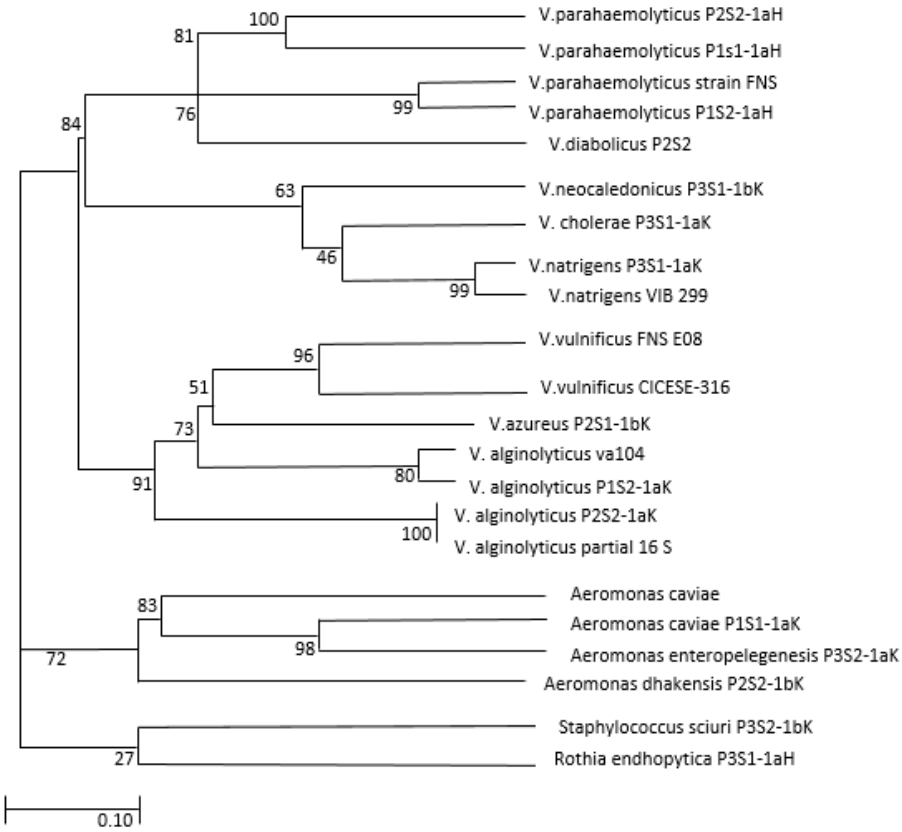

Figure 3. V*ibrio* spp. isolated from blood clams in Yogyakarta, Indonesia beaches area. Note**:** P_1S_1-1aK, P_1S_1-1aH, P_1S_2-1aK, P_1S_2-1aH, P_2S_1-1aK, P_2S_1-1bK, P_2S_2-1aK, P_2S_2-1aH, P_2S_2 -1bH, P_2S_2-1aHB, P_3S_1-1aH, P_3S_1-1aK, P_3S_1-1bK P_3S_2-1aK P_3S_2-1aH (*): *Vibrio* isolate from blood clams found in Yogyakarta, Indonesia. *V. parahaemolyticus* strain FNS C08, *V. natrigens* strain VIB 299, *V. vulnificus* strain FNS E08, *Aeromonas caviae, V. vulnificus* strain FNS E08, *V. vulnificus* strain CICESE-316, *V. vulnificus* strain FNS E08, *V. alginolyticus partial* 16S, *V. alginolyticus* strain va104: Isolate from finding previously in seafood are the cause of *foodborne disease*

P_3S_1-1bK isolate has a 98% similarity with *Vibrio neocaledonicus.* This bacterium is the latest strain identified in *Vibrio*. It is a gram-negative bacterium and is usually found in water with extreme conditions. In contrast to other *Vibrio*s, *Vibrio neocaldonicus* does not cause foodborne disease but known for its role in preventing metal degradation due to corrosion. It inhibits metal electrochemical corrosion by consuming oxygen through electron-transport proteins. This bacterium has been developed in China since 2014 because it is natural, non-toxic and effective in controlling the corrosion process and does not damage the marine ecosystem (Moradi et al. 2015)

P_2S_1-1bK isolate has a 98% similarity with *Vibrio azureus* bacteria. This bacterium has similarity with *V. parahaemolyiticus*. It is luminous and translucent and has positive oxidation. In the sea, it is formed on sea agar with usually 5-7mm in size. *Vibrio azureus* is positive for indole glucose and amygdaline production and fermentation. Based on the 16S rRNA gene *V. azureus* strains forms a cluster on *Vibrio harveyi* (Yoshizawa et al. 2009).

P_2S_2-1bH isolate has 98% similarities with *Vibrio diabolicus*. This bacterium can cause pathogenic foodborne disease, and it lives in the deep sea. This species is very close to *Vibrio alginolyticus* and *V. parahaemolyticus* which are living in coastal areas. That *V. diabolicus* and *Vibrio antiquarius* are the same species. This species is a new member of *Vibrio. V. diabolicus* is confirmed to produces β-lactamase based on a cefinase test. It shows resistance to penicillin, ampicillin, and cephalothin and is susceptible to carbenicillin. This bacterium has a specific virulence gene called AcfA for colicin production. Colicin is a protein that stimulates the production of *Tox R, Tox S, Zonulaa occludens toxins (Zot)* and *Ace*. This gene makes a defense of toxic and metalloid and is resistant to antibiotics (Turner et al. 2018).

The limitation of the 16S rRNA equation used to identify species is only a big estimate. Several different species can have similarities up to 99% of the same sequence, as in *Bacillus globiporus* and *Bacillus psychrophilus* or with the same strain can have different copies of genes with a difference of 5% in some gene regions for example in *Escherichia coli* K12 (Nguyen et al. 2016).

The p of the *Vibrio* species found in this research and other *Vibrio* references was analyzed using Mega-X software. All species found in this research are closely related to other *Vibrio* species that have previously identified in Genbank (Figure 3).

Phylogenetic tree base on the contented gene sequences of the 16S rRNA (1500bp). This tree combines the result of both the neighbor-joining (NJ) method. Numbers at nodes denote the level of bootstrap support (%) based on 500 replicates. The root the *Vibrios* phylogenetic tree within the genus, we first reconstructed a phylogenetic tree from the closed genomes of 15 strains belonging to seven *Vibrio* species (*V. cholerae, V. alginolyticus, V. parahaemolyticus, V. diaolicus, V. neocalendonicus, V. natrigens,* and *V. azureus*).

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