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IDENTIFICATION OF *VIBRIO CHOLERAE, VIBRIO PARAHAEMOLYTICUS*, AND *VIBRIO VULNIFICUS* BACTERIA IN BATIK SHELLS (*PAPHIA UNDULATA*) CAUGHT AT PENGAMBENGAN BEACH, JEMBRANA REGENCY

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ABSTRACT

Indonesia is a maritime country with vast seas and beaches equipped with natural riches stored in them. Global warming causes several changes in the ocean, including the increase in bacteria commonly found in the ocean, such as *Vibrio sp*. bacteria which can infect various animals in the ocean. Several species of bacteria *Vibrio sp.* that can infect humans are *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* which can cause significant complaints in the human digestive tract, wound infections, and even sepsis. This research aims to determine the bacterial contamination of *Vibrio cholerae, Vibrio parahaemolyticus*, and *Vibrio vulnificus* in batik shell *(Paphia undulata)* caught at Pengambengan Beach, Jembrana Regency. This research is a quantitative observational study with a cross sectional research approach and uses convenience sampling as a sampling technique. Based on several research stages starting from cultivating bacteria in APW liquid media to conducting tests on TSIA media, the results showed that from 70 samples, 16 samples were found to be positive for Vibrio sp. consisting of 11 positive samples for Vibrio vulnificus and five positive samples for Vibrio parahaemolyticus. The results of this study showed that 16 positive samples of *Vibrio sp*. bacteria were found from batik shell (*Paphia undulata*) caught at Pengambengan Beach, Jembrana Regency.

Keywords: ***Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus***

INTRODUCTION

Indonesia has long been known as a maritime country because of its vast seas. Indonesia's ocean area is wider than Indonesia's land area. Several region in Indonesia are referred to as areas with a Minapolitan program, namely areas with a marine development concept with the principles of integration, quality, and high acceleration, such as Pengambengan Village which has the largest fishing port in Bali. The sea catch in Pengambengan Village at the end of 2015 was 17 thousand tons, with the catch in one sailing being 3-5 tons.[1]

One of the marine products that can be found easily on the surface of Pengambengan Beach is the batik shell (*Paphia undulata*). However, the catch from Pengambengan Beach cannot escape various pollution, including contamination by bacteria that live in the sea, such as *Vibrio sp.* bacteria, which are the bacteria most commonly found in the ocean.[2] *Vibrio sp.* bacteria found in marine animals and can infect humans include *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus*, which can cause complaints of gastroenteritis, especially diarrhea.[3] A person is said to have diarrhea if they experience defecation more than three times a day, and the stool consistency is runny or liquid.[4] The incidence of gastroenteritis in Indonesia is caused by consuming seafood, especially shellfish contaminated with *Vibrio sp.* found as much as 10-20%.[5] Climate change plays a role in the level of *Vibrio sp.* bacterial contamination in the ocean. As a result of global warming, the number of *Vibrio sp.* bacteria in the oceans has increased because *Vibrio sp.* bacteria are very sensitive to climate, *Vibrio sp.* bacteria are even said to be microbial barometers of climate change.[6]

Considering the severes health complaints that can be caused by *Vibrio sp.* bacteria and global warming which causes an increase in the number of *Vibrio sp*. bacteria contamination, and considering that Pengambengan Beach is a beach with a large fishing port, it is important to carry out research to identify *Vibrio sp.* bacteria in batik shells on Pengambengan Beach, Jembrana Regency. to prevent infections caused by *Vibrio sp.* bacteria.

HEADING

Vibrio cholerae

The morphology of *Vibrio sp.* bacteria is generally shaped like a slightly bent rod, is a gram-negative

bacterium, has a 2-4 μm length, and can grow well in waters with high temperatures.[6] The temperature these bacteria can accept ranges from 5-44°C, with the optimal temperature being 37°C, and the maximum temperature for bacteria being 50°C. *Vibrio sp*. bacteria can grow optimally at a pH between 7.0 to 7.5, and have facultative anaerobic properties namely when they are in areas where there is or is not oxygen, the bacteria are still able to live.[5,7]

When infecting individuals through the spread of toxins, *V. cholerae* releases cholerae toxin (CT) and coregulates philus toxin (TCP) which are produced by pili and outer membrane protein (OMP), and these toxins are encoded by the toxR gene**.**[8] Vibrio cholerae, by producing cholera toxin and colonizing the human small intestine, can cause cholera, namely severe secretory diarrhea accompanied by dehydration.[9] The spread of *V. cholerae* to humans and causing cholera can be through food or drink that has previously been contaminated by *V. cholerae*, or contact with cholera carriers, especially in densely populated areas with poor infrastructure**.**[10]

parahaemolyticus

Vibrio parahaemolyticus bacteria have two main hemolysin virulence factors, namely thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), where TDH and TRH have a role in pore formation which contributes to bacterial invasion in humans. When *V. parahaemolyticus* infects humans, it will cause gastroenteritis.[11] Gastroenteritis is a condition where inflammation occurs in the mucous membrane of the digestive tract with the main signs being diarrhea and vomiting. Gastroenteritis is related to the cleanliness of foods or drinks consumed by individuals, including food contaminated with *V. parahaemolyticus***.**[12] Apart from causing gastroenteritis, *V. parahaemolyticus* has also been reported to cause wound infections, ear infections, and even sepsis which can be life-threatening in patients with weakened immune systems.[11] Sepsis is a condition of organ failure that can be life-threatening due to dysregulation of the body's response to infection.[13]

vulnificus

The disease caused by *V. vulnificus* in humans is a food-borne disease, mainly through shellfish, where it can cause gastroenteritis and sepsis which can even be life-threatening in individuals with underlying predisposing conditions such as having previous comorbid diseases.[14] Apart from causing gastrointestinal complaints and sepsis, *V. vulnificus* can also cause wound infections which can potentially lead to death. Most infections in wounds are due to exposure to bacteria in pre-existing wounds, either through seawater or through shellfish.[15] Infections caused by *V. vulnificus* in wounds have an incubation period of 16 hours and require an incubation period of 26 hours to cause sepsis.[14]

Batik shell (*Paphia undulata*)

Batik clams are shellfish with shells that are elongated and elliptical in shape, have a greenish-brown color with a dark brown pattern and a zigzag shape on the shell, and have a thick shell type with a smooth and shiny surface, in contrast to the yellow inside of the shell. The body of the batik clam has a yellowish color and is only fused at the posterior part, has eulamellibranchia-type gills, extending from the posterior end of the body to the ventral umbo.[16] Batik shells are very easy to find on Pengambengan Beach, they can even be obtained without the help of any tools.

MATERIALS AND METHODS

This research is an observational study using a cross-sectional study research design. The samples used in this research were 70 batik shells that are elongated and elliptical, greenish-brown with a dark brown pattern and zig-zag pattern, and have a thick shell type with a smooth surface, taken from Pengambengan Beach, Regency. Jembrana uses a convenience sampling technique.

Samples have been selected according to the inclusion criteria: the catch of fresh batik shellfish found on Pengambengan Beach in less than 12 hours, characterized by the shell still being completely closed and not damaged or broken, and not having an overpowering fishy odor. The exclusion criteria in this study were catches of batik shellfish from Pengambengan Beach which were damaged during the journey to the Microbiology laboratory Faculty of Medicine, Udayana University as a place to observe bacteria, which are characterized by open or broken shells, and/or emitting a strong fishy odor. Samples were obtained from Pengambengan Beach fishermen who looked for batik shells at the Pengambengan Beach location at three different points, namely 23 samples at the eastern coast point as cluster A, 24 samples at the southern coast point as cluster B, and 23 samples at the southern coast point as cluster B. west as cluster C.

The materials used in this research were batik shells, alkaline peptone water (APW) solution which was used as a bacterial fertilization medium, Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar as a selective medium for the growth of Vibrio colonies, Triple Sugar Iron Agar (TSIA) to carry out biochemical test, gram staining reagent used for the gram staining process and analyzing bacteria under a microscope at 1000x magnification. Meanwhile, the tools needed include a sterile pouch, cooler box, Bio Safety Cabinet type 2, incubator, micropipette, tweezers, scissors, mortar, petri dish, tube rack, test tube, bunsen, wire loop/loop, vortex, object glass, and microscope.

The batik shells that have been collected are put into a sterile pouch to minimize external contamination, then given identification such as sample code, place of collection, and time of collection, then put in a cooler box and given ice cubes so that the shells remain fresh during the trip to the laboratory of microbiology, Faculty of Medicine, Udayana University. The process continued by carrying out bacterial enrichment through of a 1 gram sample of batik clam meat which had been crushed first,

homogenized with 9 ml of APW media using a vortex, and then incubated for 24 hours at 37^0C. After the solution is incubated, the solution will turn cloudy, which indicates that bacteria can grow abundantly.[17] Next, using a hose, the resulting process of streaking the APW solution is carried out on TCBS agar medium, and re-incubating for 18-24 hours at a temperature of 37^0C.[18]

After finishing incubating the agar, yellow round bacterial colonies appear. The surface is slightly flat and smooth, appears opaque in the middle, and seems bright at the edges because it can ferment sucrose and lowers the pH of TCBS for V. cholerae bacteria, and round colonies with a soft green color for V. parahaemolyticus and V. vulnificus because they do not have the ability to ferment sucrose, with a colony size of 2-3 mm and a slightly sticky texture.[18,19,20] Next, the gram staining stage is carried out, and the results are observed under a microscope. When observed under a microscope, the morphological results for Vibrio bacteria are gram-negative bacteria with a bent rod shape.[6]

Besides being a selective medium for Vibrio bacteria, TCBS is also a differential medium for isolating others.[5] Differential media is a medium that can grow several types of bacteria and form a unique colony depending on the type of bacteria.[21] Bacteria that can grow on TCBS media and when observed with a microscope have the morphology of gram-negative rod bacteria such as *Vibrio sp.* bacteria including *Pseudoalteromonas, Pseudomonas, Aeromonas,* and *Shewanella*.[7,22] Because TCBS is also a differential medium, a TSIA biochemical test is needed to confirm the types of bacteria that colonize the TCBS medium.[5]

The TSIA biochemical test is carried out by taking a single colony of bacteria on TCBS agar then sticking it into the butt of the TSIA media, and also streaking it on the slope of the TSIA. After that, TSIA was incubated at 37^0C for 24 hours to observe the results whether there were changes in color on the slopes and bottom of the TSIA media, H_2S production which was indicated by the color changing to black, and gas in the TSIA media which was characterized by the separation of the slopes and the bottom of the TSIA. The bacteria *V. cholerae* and *V. parahaemolyticus* will produce a red color on the slopes and yellow at the base (K/A),[23] and *V. vulnificus* bacteria will produce a yellow color on the slopes and also the base (A/A), and all Vibrio do not produce gas and H2S.[24]

Primary data obtained from observations is then analyzed descriptively, namely through descriptions of research results, where the results will be displayed in descriptive statistical form through narratives and tables and using the Microsoft Excel application to help input data. This research has received ethical permission from the ethics commission of Udayana University with the number of ethics exemption letters: 2232/UN14.2.2.V.1/PT.01.01/2023

RESULT

The results of sample observations on APW media found that all 70 samples from three clusters could change APW to cloudy, indicating that the bacteria could grow well.

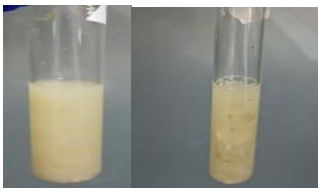

Figure 1. APW media after incubation

Then the results from the APW were continued with the bacterial culture process on TCBS media, and the following results were obtained:

Table 1. The results of colony identification on TCBS media

Name of cluster	Number of samples	The color of the bacterial colony results
Cluster A	23 samples	7 samples are yellow and green 10 samples are green 5 samples are yellow 1 samples is yellow & have H_2S
Cluster B	24 samples	5 samples are yellow and green 19 samples are yellow
Cluster C	23 samples	13 samples are green 2 samples are yellow 8 samples are green & have H_2S

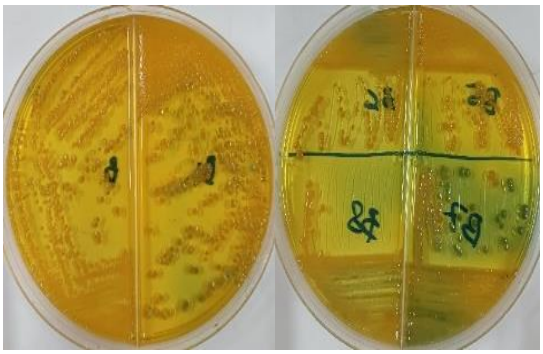

Figure 2. Growth of bacterial colonies on TCBS media

The morphology of the colonies on TCBS media was then observed under a microscope by previous gram staining and the following results were obtained:

Table 2. The results of morfology identification on gram staining number of samples

Name of cluster	Number of samples	Result
Cluster A	23 samples	13 Positive sample
Cluster B	24 samples	17 Positive sample
Cluster C	23 samples	6 Positive sample

Samples that matched the morphology of Vibrio bacteria were then continued with the TSIA biochemical test, and the following results were found:

Tabel 3. Results of bacterial incubation on TSIA media

Nama of cluster	Number of samples	Result
Cluster A	30 samples	16 samples A/A 2 samples K/A 9 samples A/A & H_2S 2 samples K/A & H_2S 1 samples A/A & H_2S & gas
Cluster B	29 samples	24 samples A/A 3 samples K/A 2 samples A/A & H_2S
Cluster C	23 samples	6 samples A/A 11 samples A/A & H_2S 2 samples A/A & H_2S & gas 3 samples K/A & H_2S & gas 1 samples H_2S

Figure 3. TSIA biochemical test results

Based on all stages of the research, a summary of the research results is obtained as follows:

Tabel 4. Results of identification of *vibrio* sp. bacteria in cluster A

Code of Samples	APW	TCBS	Gram-negative bacteria, twisted rod shape	TSIA	Result
B1	Cloudy	Yellow	-	A/A	-
B2	Cloudy	Yellow	√	A/A	-
B3	Cloudy	Yellow	-	A/A	-
		Green	√	K/A	*V. parahaemolyticus*
B4	Cloudy	Yellow	√	A/A	-
B5	Cloudy	Yellow	√	A/A	-
B6	Cloudy	Yellow	√	A/A	-
B7	Cloudy	Yellow	√	A/A	
		Green	√	K/A	*V. parahaemolyticus*
B8	Cloudy	Yellow	-	A/A	-
B9	Cloudy	Yellow	-	A/A	-
B10	Cloudy	Yellow	-	A/A	-
B11	Cloudy	Yellow	-	A/A	-
B12	Cloudy	Yellow	-	A/A	-
B13	Cloudy	Yellow	√	A/A	-
B14	Cloudy	Yellow	-	A/A	-
B15	Cloudy	Yellow	√	A/A	-
B16	Cloudy	Yellow	√	A/A	-
B17	Cloudy	Yellow	√	A/A	-
		Green	√	K/A	*V. parahaemolyticus*
B18	Cloudy	Yellow	-	A/A	-
B19	Cloudy	Yellow	-	A/A	-
		Green	√	A/A	*V. vulnificus*
B20	Cloudy	Yellow	-	A/A	-
		Green	√	A/A	*V. vulnificus*
B21	Cloudy	Yellow	-	A/A + H_2S	-
B22	Cloudy	Yellow	√	A/A	-
B23	Cloudy	Yellow	-	A/A	-
B24	Cloudy	Yellow	√	A/A	-
		Total positif :			**3 *V. parahaemolyticus*** **2 *V. vulnificus***

Tabel 5. Results of identification of *vibrio* sp. bacteria in cluster B

Code of Samples	APW	TCBS	Gram-negative bacteria, twisted rod shape	TSIA	Result
B1	Cloudy	Yellow	-	A/A	-
B2	Cloudy	Yellow	√	A/A	-
B3	Cloudy	Yellow	-	A/A	-
		Green	√	K/A	*V. parahaemolyticus*
B4	Cloudy	Yellow	√	A/A	-
B5	Cloudy	Yellow	√	A/A	-
B6	Cloudy	Yellow	√	A/A	-
B7	Cloudy	Yellow	√	A/A	
		Green	√	K/A	*V. parahaemolyticus*
B8	Cloudy	Yellow	-	A/A	-
B9	Cloudy	Yellow	-	A/A	-
B10	Cloudy	Yellow	-	A/A	-
B11	Cloudy	Yellow	-	A/A	-
B12	Cloudy	Yellow	-	A/A	-
B13	Cloudy	Yellow	√	A/A	-
B14	Cloudy	Yellow	-	A/A	-
B15	Cloudy	Yellow	√	A/A	-
B16	Cloudy	Yellow	√	A/A	-
B17	Cloudy	Yellow	√	A/A	-
		Green	√	K/A	*V. parahaemolyticus*
B18	Cloudy	Yellow	-	A/A	-
B19	Cloudy	Yellow	-	A/A	-
		Green	√	A/A	*V. vulnificus*
B20	Cloudy	Yellow	-	A/A	-
		Green	√	A/A	*V. vulnificus*
B21	Cloudy	Yellow	-	A/A + H_2S	-
B22	Cloudy	Yellow	√	A/A	-
B23	Cloudy	Yellow	-	A/A	-
B24	Cloudy	Yellow	√	A/A	-
		Total positif :			**3 *V. parahaemolyticus*** **2 *V. vulnificus***

Tabel. 6. Results of identification of *vibrio* sp. bacteria in cluster C

Code of samples	APW	TCBS	Gram-negative bacteria, twisted rod shape	TSIA	Result
C1	Cloudy	Green	√	A/A	*V. vulnificus*
C2	Cloudy	Green	-	A/A + H_2S	-
C3	Cloudy	Green	-	A/A + H_2S	-
C4	Cloudy	Green	-	A/A + H_2S	-
C5	Cloudy	Green	-	A/A + H_2S + gas	-
C6	Cloudy	Green	-	A/A + H_2S	-
C7	Cloudy	Green	√	A/A	*V. vulnificus*
C8	Cloudy	Green	-	K/A + H_2S	-
C9	Cloudy	Green	√	A/A	*V. vulnificus*
C10	Cloudy	Green	-	A/A + H_2S	-
C11	Cloudy	Green	-	A/A + H_2S + gas	-
C12	Cloudy	Green	-	A/A + H_2S	-
C13	Cloudy	Green	√	A/A	*V. vulnificus*
C14	Cloudy	Green + H_2S	-	A/A + H_2S	-
C15	Cloudy	Green + H_2S	-	K/A + H_2S	-
C16	Cloudy	Green + H_2S	-	A/A + H_2S	-
C17	Cloudy	Green + H_2S	-	A/A + H_2S	-
C18	Cloudy	Green + H_2S	-	K/A + H_2S	-
C19	Cloudy	Green + H_2S	-	A/A + H_2S	-
C20	Cloudy	Green + H_2S	-	A/A + H_2S	-
C21	Cloudy	Green + H_2S	-	H_2S	-
C22	Cloudy	Yellow	√	A/A	-
C23	Cloudy	Yellow	√	A/A	-
		Total positif :			**4 *V. vulnificus***

So the results of bacterial contamination of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in batik shellfish caught at Pengambengan Beach, Jembrana Regency are as follows:

Tabel 7. The results of *Vibrio sp.* contamination on batik shells

Species	Amount	Percentage of contamination
V. cholerae	0	0%
V. parahaemolyticus	5	7,1%
V. vulnificus	11	15,7%
Positive Total:	**16**	**22,8%**

1. DISCUSSION

Global warming is havings impact on the oceans. The effects of global warming on the sea or coast include increasing acidity levels in the ocean, storms associated with the ocean, changes in coastal marine ecosystems, and increased contamination of *Vibrio sp.* bacteria in the sea, especially those that can infect humans such as *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*.[6,25]

The percentage of gastroenteritis cases that occur in Indonesia due to *Vibrio sp.* bacteria is 10-20%, and one of the marine products that is often the target of *Vibrio sp.* bacterial contamination is shellfish. Factors that can trigger gastroenteritis due to *Vibrio sp.* bacteria in shellfish are the selection of shellfish that are not fresh enough, and the process of ripening the shellfish not being carried out properly so that the bacteria can still survive. This factor arises because of the natural nature of *Vibrio sp.* bacteria which will grow well at high temperatures ranging from 5-44°C, with the optimal temperature being 37°C, however, these bacteria have a maximum temperature of 50°C so they will die at the proper ripening process.[5]

In previous research, the positive rate for *Vibrio sp.* in shellfish reached 24%.[26] Another study carried out in China's oceans in 2016 using various types of shellfish as samples, showed higher positive results for *V. parahaemolyticus* compared to *V. cholerae*, where the positive rate for *V. parahaemolyticus* was 27%, and 2% of samples were positive for *V. cholerae*.[2] The same results were also found in research conducted in 2018 located in the Yogyakarta Beach tourist area which used nine green mussels as samples, all samples were positive for *Vibrio sp.*[5]

The research carried out this time began with the process of fertilizing bacteria in APW liquid media. After incubation for 24 hours, the results showed that all the samples changed the APW's color to cloudy. The process

continued by streaking the resulting APW media on TCBS media and incubating again for 24 hours at 37°C.

The results of the culture on TCBS were then observed under a microscope to determine the morphology of the bacteria, and the final stage is a biochemical test on TSIA media. All the results from the series of tests that had been carried out were then sorted and five samples were positive for *Vibrio parahaemolyticus* bacterial contamination and 11 samples were positive for Vibrio vulnificus out of a total of 70 samples, and no positive numbers for Vibrio cholerae were found in the samples. The positive number for *Vibrio sp.* bacteria shows that it is necessary to provide education to the fishermen of Pengambengan Beach, Jembrana Regency regarding sanitation and good ripening processes when consuming batik shellfish or other seafood to help prevent gastroenteritis due to *Vibrio sp.* bacteria contamination.

2. CONCLUSIONS AND ADVICE

CONCLUSION

There was Vibrio bacterial contamination in batik shellfish (*Paphia undulata*) caught by fishermen at Pengambengan Beach, Jembrana Regency, 16 out of 70 samples or a percentage of 22.8%, with details of positive results for *V. parahaemolyticus* in five samples with a percentage of 7.1%, contamination *V. vulnificus* bacteria were 11 samples with a percentage of 15.7%, and no *V. cholerae* bacterial contamination was found.

ADVICE

Researchers recommend that further research be carried out regarding the identification of Vibrio bacteria in batik shellfish (*Paphia undulata*) on Pengambengan Beach, Jembrana Regency considering the quite high percentage of contamination obtained in this study, as well as considering the various limitations and weaknesses found in this study due to not using a test. Molecular microbiology and only identifies the presence or absence of bacteria without counting the number of colonies contained therein. With further evaluation and examination it will certainly be useful as a reference for subsequent research.

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