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Identification of bacteria causing Vibriosis (*Vibrio sp*) on white snapper (*Lates calcarifer*) reared in the marine cultivation ponds

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Abstract

Vibrio is a group of bacteria that causes *Vibrio*sis in many aquatic biota cultivated in ponds. This research aims to determine the type of the *Vibrio sp*. which causes vibriosis disease in white snapper reared in the marine cultivation ponds at the Center for Marine Cultivation Fisheries (CMCF) Lampung, Indonesia. The research was conducted using investigative method by isolating *Vibrio* bacteria from the organ in white snapper, mud, and water. The bacterial isolates were indentified using biochemical test. The reseach parameters are TPC (*Total Plate Count*) *Vibrio sp*., water temperature, pH, salinity, DO, BOD and ammonia. The results showed there were three types of isolates that are suspected as the *Vibrio*sis bacteria in white snapper fish namely *Vibrio vulnificus*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. The three isolates obtained were known to be α-hemolysis. The enviromental quality parameters are as follows: water temperature of 29.8 °C, pH 7.8, salinity 33 psu, DO 4.4 mg / L, BOD 2.2 mg / L and ammonia 2.2 mg / L. The TPC-values of the bacteria *Vibrio sp*. isolated from the inlet, the main chambers, and the outlet of the pond are: <25 CFU/ ml, 7.6x104 CFU/ml, and 2.1x103 CFU/ml respectively. The levels of ammonia and calculation of TPC-values of *Vibrio* bacteria are exceeds the water quality standards for the fish farming.

Keywords: Vibriosis; Vibrio bacteria; White snapper; Lates calcarifer

1. Introduction

Microorganisms that cause disease in fish and shrimp are bacteria, fungi, and viruses. Pathogen bacteria often attack fish and it can cause mass deaths in fish and shrimp by *Vibrio sp*. [1, 2, 3]. The pathogen *Vibrio* bacteria can cause *Vibriosis* as a disease and it can result in deaths of more than 80% in fish in floating nets [4].

According to Taslihan et al. [5] *Vibrio alginolyticus* is the type of bacteria that it most often infects white snapper, causing mass death. These bacteria are very harmful both in seawater and brackish water fish farming. They can cause primary and secondary infections.

The environment is very important to support the success of fish farming. Unsuitable conditions can lead to disease infection in white snapper especially from *Vibrio* group bacteria. At present there is a mass death of white snapper in the pond of the Center for Marine Cultivation Fisheries (Balai Besar Perikanan Budidaya Laut, BBPBL) Lampung. Clinical symptoms of fish in the ponds indicate *Vibrio*sis infection in these fish such as anorexia, abnormal swimming behavior, loss of balance, the color of the body of the fish is red or black, and scales are peeling. Based on the description, it is necessary to research for the identification of *Vibrio sp*. causes of *Vibrio*sis in white snapper in ponds of Balai Besar Perikanan Budidaya Laut (BBPBL) Lampung. This study aims to determine the type of *Vibrio sp*. which causes disease in white snapper in pond of BBPBL- Lampung.

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2. Material and methods

2.1. Isolation and Characterization of the *Vibrio*

Vibrio sp. was isolated from internal organs (spleen, liver, kidney), fish wounds, mud and water of pond. Isolation was carried out from internal organs and wounds of fish by the streak method. Isolation from pond mud is done by pour plate method. Samples of 1 gram of sludge were put into 9 ml Alkaline Peptone Water (APW) and shaken until homogeneous. Then, the suspension is grown on TCBSA media with the spread method. Isolation from pond water is done by put in 1 ml sample to 9 ml APW media and shaken until homogeneous. After that, the suspension is grown on the Thiosulfate Citrate Bile Sucrose (TCBS) media (Yeast Extract, Bacto Peptone, Sodium Thiosulfate, Sodium Citrate, BTB, Agar, OX Bile, Sucrose) with the spread method. All samples were then incubated at 30-35^{o}C for 24 hours. After incubation for 24 hours, the bacteria were observed for colony characteristics, Gram staining, catalase testing, oxidase testing and motility testing in each isolate.

2.2. Hemolysis assay

Vibrio sp. was grown on the media of Blood Agar Plates (BAP) to determinate hemolytic or non-hemolytic bacteria. BAP media is made by adding 5% sheep blood to the total volume of media to be used (NA media). The bacteria were grown by the streak method, then it was incubated at a temperature of 30-35^{o}C for 24 hours. The hemolysis test results refer to Nelce and Setha [6] stating that there are 3 types of hemolysis: β-hemolysis (there is no blood around the colony), α-hemolysis (some blood cells found at the zone of hemolysis or some blood have a greenish color change around the colony) and γ-hemolysis (nonhemolysis).The γ-hemolysis shows that these microorganisms cannot have the potential as pathogenic bacteria [6].

2.3. Identification of *Vibrio sp*

Identification of *Vibrio sp*. performed on 27 isolates. Then, a series of biochemical tests conducted i.e. test of catalase, oxidase, motility, nitrate, lysine, ornithine, H_2S, glucose, Mannitol, Xylose, ONPG, indole, Urease, VP, citrate, TDA, Gelatin, Malonate, Inositol, Sorbitol, Rhamnose, Sukrose, Lactose, Arabinose, Adonitol, Raffinose, Salicin and Arginine.

2.4. Pond Water Quality assay

Water quality was observed by enumeration of pathogenic bacteria (*Vibrio sp*.) from pond environments. Physical parameters observed namely pond salinity and water temperature. Chemical parameters which observed namely pH, DO, BOD, and ammonia levels in pond water.

3. Results

3.1. Isolation and Characterization of Pathogenic Bacteria

Choosing fish samples is done by observing the clinical symptoms that appear in fish such as injury to the body, swimming abnormally, aloof, wrinkling on the fin decreases to stimulation, scales peeling, there are wounds on the body, exoptalmis (eyes protruding), tail fin wheeled backs, pale internal organs, excess slimy gills, liver experiencing multifocal necrosis, kidney swelling, swelling of the spleen and decreased appetite. Symptoms showed by fish infected with *Vibrio*sis (Figure 1).

Figure 1 Photograph of white snapper: (a) Clinical Symptoms of *Vibrio sp*. infection, (b) Healthy White Snapper Fish

Isolation and characterization of bacterial colonies were taken from white snapper, mud and pond water as many as 30 isolates. However, only four isolates with strong characteristics were suspected to be pathogenic and it cause disease in the white snapper (Table 1 and Table 2).

Table 1 The isolates of *Vibrio* bacteria

Source	**Characterization**			
	Quantity of Isolate	**Hemolysis Test**		**Biochemical Test (Identification)**
		Pathogen	**Non-Pathogen**	
Water	2	2	0	0
Kidney	6	5	1	1
Heart	9	7	2	1
Spleen	9	7	2	2
Wound	3	2	1	0
Mud	1	1	0	0
Total	30	24	6	4

Table 2 Characteristics of *Vibrio* colonies

Isolate	**Source**	**Characteristics of *Vibrio sp*. colonies on TCBSA medium**				
		Size	**Color**	**Morphology**	**Border**	**Elevasi**
14	Kidney	medium	Green	Irreguler	Undulate	Convex
16	Heart	medium	Green	Irreguler	Undulate	Convex
24	Spleen	medium	Yellow	Circular	Entire	Raised
25	Spleen	medium	Yellow	Irreguler	Undulate	Raised

The *Vibrio* isolate was grown on TCBSA media. The colonies are green and yellow (Figure 2).

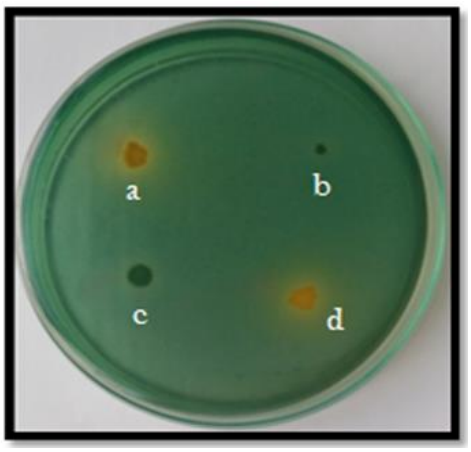
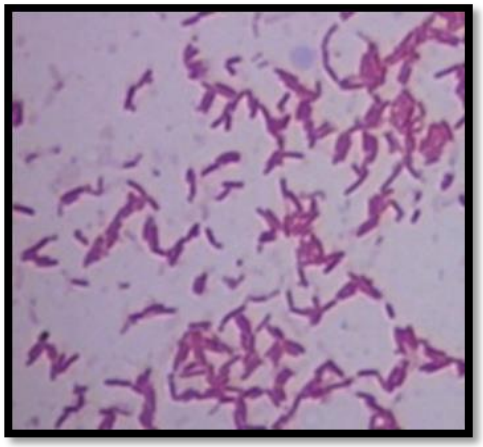


Figure 2 Isolate of *Vibrio* on TCBSA media; (a) Isolate-14; (b) Isolate-16; (c) Isolat-25; (d) Isolat-24

The results of Gram staining show that 30 isolates are Gram of negative and form of cocobasil or short stem. The motility test results in 30 motile isolates that showed an active movement (Figure 3)



Figur 3 Gram staining

Then, the observation results proved that 30 isolates produce enzyme of the oxidase and catalase. It can show that 30 isolates are group of *Vibrio sp*. (Figure 4)

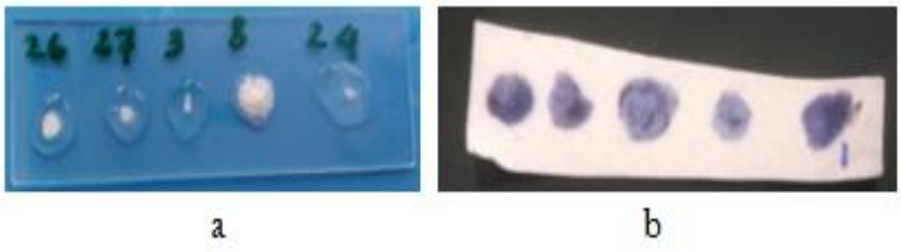


Figure 4 (a) Catalase activities; (b) Oxidase activities

Based on observations on the 30 isolates of bacteria, they were 24 isolates as α-hemolysis i.e: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22, 24, 25, 28, 29 and 30 isolates. Whereas the other 6 isolates were γ-hemolysis (nonhemolytic) i.e: 3, 20, 21, 23, 26 and 27 isolates (Figure 5).

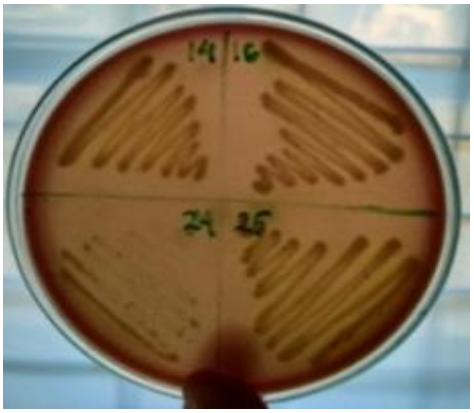


Figure 5 Hemolysis activities

3.2. Biochemical assays

It was found that isolates 14th from spleen organs identified as *Vibriovulnificus*. The bacteria were determinated by yellow colonies on TCBSA media because they were able to utilize sucrose compounds in the media. The bacteria created positively reaction to the indole, ONPG, glucose, and mannitol assay. However, the bacteria created reaction negatively to the hydrolysis of urea, lactose, arginine and ornitine assays (Table 3).

Table 3 Data of biochemical assay

biochemical assay	**Isolate code**			
	14	**16**	**24**	**25**
Swarming	-	-	+	-
Growth at 37 ^{o}C	+	+	+	+
Growth with 0 % NaCl	-	-	-	-
Gram	-	-	-	-
Motility	+	+	+	+
Oksidase	+	+	+	+
Catalase	+	+	+	+
Moller's decarboxylases:				
Arginine	-	-	-	-
Lysine	+	+	+	+
Ornithine	-	-	-	-
Nitrate Reduction	+	+	+	+
Gas from glucose	-	-	-	-
Indole	+	+	+	+
ONPG	+	+	+	+
VogesProskauer	-	-	-	-
Acid from:				
Glucose	+	-	-	+
Sorbitol	+	-	-	+
Lactose	-	-	-	-
Raffinose	-	-	-	-
Mannitol	+	+	-	+
Rhamnose	-	-	-	-
Arabinose	-	-	-	-
Inositol	-	-	-	-
Sucrose	+	-	+	-
Adonitol	-	-	-	-
Xylose	-	-	-	-
Salicin	+	-	-	+
Gelatin Liquefaction	-	-	-	-
Citrate Utilization	+	+	+	+
Malonate Inhibition	-	-	-	-
H_2S Production	-	-	-	-
Urea Hydrolysis	-	+	+	+
Tryptophan Deaminase	+	+	+	+
Hemolysis	α	α	α	α
	84,00 %	95,34 %	87,33 %	97,15 %
Species	*V. vulnificus*	*V. parahaemolyticus*	*V. alginolyticus*	*V. vulnificus*

Isolate 16th origin from the liver was identified as *Vibrio parahaemolyticus* which is characterized i.e: its colonies are bluish green on TCBSA media. The bacteria can notutilize sucrose compounds on the media. The bacteria created positively reaction test of lysine, indole, ONPG, mannitol, and hydrolyze urea. The bacteria created negatively reaction test for arginine, ornitine, glucose, sorbitol and lactose (Table 3).

Isolate 24th origin from the kidney was identified as *Vibrio alginolyticus* which is characterized i.e: its colonies are yellow on TCBSA media. The bacteria can utilize sucrose compounds in the media, react positively to the test of lysine, indole, and ONPG. The bacteria created negatively reaction to the arginine test, ornithine, glucose, lactose and mannitol (Table 3).

Isolate 25thorigin from the kidney was identified as *Vibriovulnificus* which is characterized i.e: its colonies are bluish green on TCBSA media. The bacteria can notutilize the sucrose compounds present in the media, react positively to the test of lysine, indole, ONPG, glucose, sorbitol, and able to hydrolyze urea. The bacteria created negatively reaction to the test for arginine, ornithine, mannitol, and lactose (Table 3).

3.3. Pond Water Quality Measurement

Quantity of *Vibrio sp*. is obtained from the pond's abiotic environment. The other environmental data are pH, temperature, salinity, BOD, COD, and ammonia. The environments data are obtained from inlet water, pond water and outlet water (Table 4).

Table 4 Result of Pond Water Quality Measurement.

No	Parameter	Unit	Yield	Standart*
I	BIOLOGICAL			
	Pathogen bacteria (*Vibrio sp.*)			
	Water run in pond (*inlet*)	CFU/ml	<25	<25
	Water of pond	CFU/ml	$7.7x10^4$	$1x10^{2(A)}$
	Water run out pond (*outlet*)	CFU/ml	$2.2x10^3$	$1x10^{2(A)}$
II	PHYSICAL			
	Salinity	Psu	33	30-34
	Temperature	^oC	29.8	Normal
III	CHEMICAL			
	pH	-	7.89	7-8.5
	DO	mg/L	4.4	>4
	BOD	mg/L	2.26	<3
	Ammonia	mg/L	2.24	<0.4 (A)

*) Based on Sea Water Quality Standards for Marine Biota from Declaration of the Minister of Forestry and Environment No.51 in 2004 A: Abnormal

4. Discussion

Green colonies of bacteria were caused by the bacteria cannot ferment sucrose such as *Vibrio vulnificus* and *Vibrio parahaemolyticus*. The yellow colonies of bacteria were caused by the bacteria can ferment sucrose contained in TCBSA media.

This condition makes decrease of the pH of the media which eventually results in a yellow bacterial colony such as *Vibrio alginolyticus* and *Vibrio fluvialis*. The *Vibrio* bacterial colonies that grow on TCBSA media are usually sticky like glue [6]. Another researcher, Felix et al. [1] states that the *Vibrio* group bacteria have gram negative properties, a short trunked cell shape with a size range of 2-3 µm. The bacteria of *Vibrio sp*. are motile and very active when in liquid culture. According to Bergey and Boone [7] *Vibrio sp*. has fermentative properties, produce oxidase and catalase enzymes, and

facultative of anaerobes. The results were Gram staining, motility test, oxidase test and catalase test showed that 30 of isolates as*Vibrio sp*. bacteria. Color changes became purple by the formation of cytochrome oxidase [8].

Vibrio bacteria are known to produce hemolysin enzymes. Haemolysis and leukosidine activities were caused by bacterial extracellular products (ECPs). ECPs as bacterial defense factors were used against host blood defenses because they can lyse blood cells. The bacteria that are able to survive will enter the bloodstream so that it spreads to all cells of the host's body as well as to the target organ [9].

Based on the ability to produce hemolysin, bacteria can be grouped into two positive Kanagawa Phenomena (KP) which will show β-hemolysis reactions, which the bacteria are characterized by colonies with clear areas around it, and negative Kanagawa Phenomena (KP) that the bacteria will show reaction-hemolysis shows by colonies which show signs of blanching. According to Nelce and Setha [6] the *Vibrio parahaemolyticus* bacteria were classified into positive kanagawa by showing clear areas around the colonies on agar media with high salinity to test *Vibrio* hemolytic activity. The bacteria *Vibrio parahaemolyticus* isolated from the feces of patients with gastroenteritis showed a positive Kanagawa Phenomenon (KP) reaction, while those isolated from fish, crabs, shrimp, shells, sand and mud on the edge of the rice were strongly suspected to be negative of Kanagawa Phenomenon (KP) reactions.

The density of *Vibrio* bacteria in ponds is very influential on fish farming. Total bacteria *Vibrio sp*. in pond water $7.65x10^4$ CFU / ml. This amount has exceeded the threshold, where the total *Vibrio* threshold recommended for the level of ponds / aquaculture ponds is $1x10^2$CFU / ml [10]. Total bacteria that exceeds the threshold will potentially increase infection. The emergence of disease infections, especially *Vibrio*sis, it is one of the problems in the effort of fish and shrimp cultivation because it can lead to death [11].

Vibrio bacteria are classified as opportunistic bacteria [12]. *Vibrio* bacteria can be pathogenic if the cells were the sufficient amount (quorum). Then, virulence factors expressed lead to death in fish. Quorum sensing is a mechanism in which bacteria coordinate the expression of certain genes in response to their population density by producing, releasing and detecting small signal molecules [13].

The pH value is 7.89, the salinity level shows a value of 33ppt, the water temperature shows a value of 29.8°C, the DO value shows a value of 4.4 mg / L, the BOD measurement shows the value of 2.26 mg / L indicates that it is still within the threshold suitable for conducting cultivation [14].

The results of ammonia measurements on white snapper ponds at BBPBL showed a value of 2.24 mg / L. According to the Declaration of the Minister of Forestry and Environment No.51 in 2004, ammonia levels for aquaculture activities must be <0.4 mg / L. That it shows that the ammonia value in the pond has exceeded the quality standard threshold because it shows a value of 2.24 mg / L. Ammonia levels that have overdone the threshold can cause pollution in these waters. The high amount of ammonia is assumed with the high percentage of feeding in the area of fish farming in the pond. The mode caused the waste remnants of metabolic products by fish, feces increase, thus affecting the high amount of ammonia in the pond.

The high ammonia levels also indicate indirectly that the levels of organic matter in these waters are high. The condition means that the higher the organic matter in a water, the higher the population of microorganisms in the waters. This situation will have an impact on the total *Vibrio sp*. in pond water which is 1.8×10^5 CFU / ml.

5. Conclusion

The research conducted at*Center for Marine Cultivation Fisheries* (CMCF) Lampung, Indonesia, found that there were three isolates of pathogenic bacteria indentified in fish cultivation ponds namely *Vibrio vulnificus, Vibrio alginolyticus* and *Vibrio parahaemolyticus*.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors declare no conflict of interest.

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