Isolation and characterization of biofilm-mediated vibriosis-causing bacteria from *Macrobrachium rosenbergii*-aquaculture

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Abstract. Vibriosis is one of the major problems in prawn aquaculture leading to a significant loss in yield. The objective of the study was to isolate and identify the etiological agent of vibriosis from the giant freshwater prawn (*Macrobrachium rosenbergii*) aquaculture. The study was conducted by isolating the bacteria using selective media of thiosulfate-citrate-bile salts-sucrose (TCBS) agar followed by the morphological and biochemical characterizations, i.e., indole, methyl-red (MR), Voges-Proskauer (VP), citrate, urease, NaCl 0%, gelatinase and lysine decarboxylase tests. The isolated bacteria were further assayed for their standard growth-curve as well as the ability to form a biofilm on Congo-red agar (CRA). The results showed that the bacteria showed a yellow color in colony appearance and had negative Gram staining. On the biochemical assays, the isolates showed positive reactions on the indole, MR, VP, gelatine, and lysine decarboxylase; but had negative reactions on the citrate, urea, and NaCl 0% tests. The isolates were then identified as *Vibrio alginolyticus* and after further characterization, the isolates showed the ability to form a biofilm. This result signifies the urgency to overcome *V. alginolyticus* as the causing agent of vibriosis that was able to form a biofilm on the giant river prawn aquaculture.

1. Introduction

Giant river prawns (*M. rosenbergii*) are one of the Indonesian indigenous fishery commodities. The culture of giant river prawns is increasing due to its short period of maintenance which is 3-5 months as well as the low mortality rate which is less than 20% and the high production level reaching up to 230,000 t, with a value of US$1.1 billion in annual production globally [1]. However, the cultivated giant river prawns are more susceptible to disease, including vibriosis. Vibriosis infection can be manifested from larval stages until post larvae [2]. The presence of vibriosis infection in the adult prawns is one of the limiting factors in the giant prawn’s aquaculture because it provokes the decrease of the offspring. Besides the infection across individuals, vibriosis can also infect through the water from the maintenance tank by forming biofilm structure [3]. Therefore, it is necessary to conduct bacteriological examination on the water of the maintenance tank to determine the bacterial infection causing vibriosis in giant river prawn larvae.

This study aims to isolate and characterize morphologically and biochemically the bacteria that cause vibriosis in giant river prawn aquaculture. The study also aimed to qualitatively determine the ability of the isolate to form biofilm.

2. Methods

2.1. Isolation

The water sample used in this study was collected from the giant river prawn broodstock tank in the Freshwater Aquaculture Centre (BBAP) of Ujong Batee, Ministry of Marine and Fisheries, Indonesia. A total of 800 mL of water samples were collected and serially diluted (10⁻¹ and 10⁻²) using saline solution. A 0.1 mL of
the diluted samples were then transferred and inoculated into plates containing PCA and TCBS media before they were then subsequently incubated at 30 °C for 24 hours and 48 hours, respectively.

2.2. Identification and characterization
Identification and characterization of isolates were performed by the morphological and biochemical tests. The morphological identification was carried out by Gram staining, while biochemical identification and characterization were conducted using indole test, Methyl Red (MR) test, Voges Proskauer test (VP), citrate test, urease test, 0% NaCl test, gelatinase test, and lysine decarboxylase test.

2.3. Determination of growth rate
A loop of the isolate was inoculated into 50 ml of Tryptone Soya Broth (TSB) containing NaCl 2% and incubated in an orbital shaker at agitation speed at 120 rpm for 20 hours at room temperature. Serial dilution of 1 mL of bacterial inoculum was carried out up to 10⁻⁶ using 9 mL of trisalt solution (NaCl 0.184 g/L; KCl 0.75 g/L; MgSO4·7H2O 14.2 g/L) on each dilution. The tube was then homogenized followed by inoculation of 0.1 mL from each dilution on PCA 2% media and incubation for 20 hours at room temperature. The bacterial culture was then serially diluted into 1:16 and subsequently enumerated for their colonies and measured for its transmittance at a wavelength of 600 nm.

2.4. Qualitative assay of biofilm formation -
CRA medium was carried out by adding 0.048 g of Congo red dye and 2.16 g of sucrose and 0.702 g of NaCl into 60 mL of Trypticase Soy Agar (TSA). The biofilm formation test on CRA was then performed by inoculating the isolates from TSA medium to CRA medium and then incubated for 24 hours at 25 °C in aerobic conditions. Slime producing bacteria appeared as black colonies, whereas non-pigmented bacteria are not able to make biofilms [4].

3. Results and Discussion
Of the isolation, the colonies appeared in yellow-small-circular colonies with flat edges and the convex elevation. Moreover, on the Gram staining, the isolates were classified as Gram-negative comma-shaped (Vibrio) bacteria (Figure 1). Additionally, in the biochemical assays, the isolates showed positive reactions on the indole, MR, VP, gelatin, and lysine decarboxylase; but had negative reactions on the citrate, urea, and NaCl 0% tests (Table 1).

![Figure 1](image-url)

**Figure 1.** The observation of the Gram-negative comma-shaped (Vibrio) bacteria under the microscope with 1000x magnification. The bacteria are latter identified as *Vibrio alginolyticus*. The bar with the number underneath indicates the scale.
Table 1. The morphological and biochemical characteristics of the isolate of *Vibrio alginolyticus*

| Biochemical tests            | Result |
|-----------------------------|--------|
| Colony color                | Yellow |
| Gram                        | -      |
| Indole test                 | +      |
| MR test                     | +      |
| VP test                     | +      |
| Citrate test                | -      |
| Urease test                 | -      |
| Gelatinase test             | +      |
| Lysin decarboxylase test    | +      |
| NaCl 0% test                | -      |

Based on the results of morphological observation and biochemical tests, the isolate was confirmed as *V. alginolyticus*. The bacteria can also be distinguished from other Vibrios by isolating them in a Nutrient Agar (NA) medium so that their colonies will look swarming as these bacteria in the solid medium will synthesize many lateral flagella [5]. *V. alginolyticus* are halophilic Gram-negative bacteria that can use simple carbon source and energy without the requirement of vitamins or growth factors. Based on carbohydrate tests, *V. alginolyticus* were able to ferment some of the sugar media including glucose, sucrose, and mannitol. In this study, these bacteria also had positive results on MR tests and a negative result on VP test. Regarding habitat, *V. alginolyticus* live in a temperature range of 4–42 ºC and can stay for weeks in wet environments or lack nutrition condition. These characteristics explain why *Vibrio* is an effective opportunistic pathogen. *V. alginolyticus* infect fish and other organisms starting from the mucus of the body since it contains nutrients needed by these bacteria for their development [6]. Moreover, *V. alginolyticus* may excrete extracellular enzymes, such as gelatinase, lecithinase, and caseinase, [7] in addition to their virulence factors and antimicrobial resistance phenotypic features to assist their pathogenicity [8].

The standard curve of *V. alginolyticus* bacteria obtained from this study was \( y = 2 \times 10^9 x + 0.2975 \) with \( R^2 = 0.9127 \) (Figure 2). The standard curve in this study was used to estimate the number of cell/mL on biofilm formation. The estimated number of bacteria needed for the biofilm formation inhibition test is \( \sim 3 \times 10^5 \) CFU/mL [9]. The optical density value needed to obtain bacterial cell counts \((\sim 3 \times 10^5 \text{ CFU/mL})\) of *V. alginolyticus* is 0.2756 nm.

![Figure 2](image.png)

**Figure 2.** The standard curve of *V. alginolyticus*; the line represents the actual projection between the cell densities (CFU/mL) and optical densities; whereas the dotted line represents the linear regression of the standard curve.

The standard growth curve of bacteria is a standard curve which states the correlation between turbidity of bacteria culture and the number of organisms per mL of culture [10]. Based on these
estimates, the number of colonies that appear in the plate is an index for the number of organisms that can live and are contained in culture.

*V*. *alginolyticus* isolates in this study was also qualitatively evaluated for its ability to form biofilms. Qualitative assay of *V. alginolyticus* biofilm formation in Congo Red Agar (CRA) medium showed positive results. Positive results can be seen from the black color produced by the colony which shows that bacteria are able to form biofilms (Figure 3). For aquatic pathogenic bacteria, biofilm formation is one of the pathogenicity mechanisms to establish the infection within hosts [6].

**Figure 3.** The positive results of biofilm formation test on *V*. *alginolyticus* bacteria (black colony)

Biofilm formed in CRA media is induced by the addition of sucrose [11]. The sucrose has a role as a raw material for the formation of Extracellular Polymeric Substance (EPS). EPS contains exopolysaccharide which will react with Congo red dye so that the colonies appear in black. In addition to the sugar addition in CRA medium preparation, low-nutrients media also are effective in inducing the formation of biofilms. Some of *Vibrio* genera have the ability to duplicate faster on low-nutrient media because they have to compete with other competitors in absorbing nutrients.

4. Conclusion
The isolated bacteria from giant river prawns infected with vibriosis has been identified as *V. alginolyticus* which have a circular shape with a small size, appears in yellow, the edges are flat, convex and not transparent. The biochemical characteristics result of *V. alginolyticus* is positive on indole, MR, VP, gelatin, and lysine decarboxylase tests. However, the citrate, urea and 0% NaCl test result were negative. In addition, these bacteria can form biofilms that make these opportunistic pathogenic bacteria become a serious problem and require more attention in shrimp aquaculture.

Acknowledgement
This study was supported by the Institute of Research and Community Services (LPPM), Syiah Kuala University under the Assistant Professor Research Grant [No. 171/UN11.2/PP/PNBP/SP3/2018].

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