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Exploration of bacteria associated with Nudibranchs to control *Vibrio* spp.

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Abstract. Shrimp culture process of *Litopenaeus vannamei* could not be separated from the disease threats. Vibriosis is the main cause of a decrease in aquaculture production. *Vibrio harveyi* is a pathogenic bacteria that can caused high mortality and *Vibrio parahaemolyticus* can also cause massive financial losses in shrimp culture. The use of antibiotics to control vibriosis, however, has a negative impact. For example, it resulted in the shrimp resistance to pathogenic bacteria. The potential of bacteria that are symbiotic with nudibranchs as anti *Vibrio* spp. has not been reported yet. The aim of this experiment was to examine the presence of bacterial symbiont in a nudibranch to inhibit or stop the growth of *V. harveyi*. Antibacterial activity test was done by the overlay method. The tested pathogenic bacteria were used *V. harveyi* and *V. parahaemolyticus*. Then the active bacteria were identified by morphological and molecular using 16S rRNA gene sequence. Isolation of bacterial symbiont in Nudibranch revealed 144 isolates. From those isolates only one isolate (SM-N-3(2)/7) was identified as having the ability against *V. harveyi* and *V. parahaemolyticus*. The isolate was gram negative rod bacteria. Based in molecular identification using 16S rRNA it was closely related to *Pseudoalteromonas piscicida* strain NBRC (99%).

1. Introduction

Vaname shrimp (*Litopenaeus vanamei*) is one of the brackish water fisheries commodities that have high economic value in the domestic and global markets, of which 65% are produced by Indonesia per year [1]. High profit from the vaname shrimp farming business has caused many farmers to try to cultivate this species [2]. *Vibrio* spp. can cause mortality greater than 50% [3]. The use of antibiotics to control vibriosis, however, has negative impacts on the environment and the shrimp. Therefore, it is important to explore alternative potential antibacterial products from natural sources such as nudibranch-associated bacteria. Oceans that are rich in biodiversity will continue to produce marine natural products, which may have antibacterial properties. These products can be used as an alternative in the health sector. As shown in the previous studies, marine microbes associated with macroorganisms can be the main source that produces bioactive compounds [4, 5, 6, 7, 8]. The



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bacteria-associated with these marine invertebrates can produce secondary metabolites that are active and also chemically diverse, such as anti-inflammatory, antibiotic, antitumor, anticancer, antibacterial and antifungal compounds. The nature of the secondary metabolites produced are very interesting to be a new drug discovery [9]. Many bacteria associated with nudibranch [10], and sponges [11] have been found to be anti *Vibrio* spp. in vaname shrimp [12]. Therefore research is needed to provide information about the nudibranch-associated bacteria that potential as anti *Vibrio* spp. to identify environmentally friendly alternatives to control *Vibrio* spp.

2. Methodology

2.1. Materials

This study was done from November 2018 until January 2019 that used culture collection of symbiotic bacteria from nudibranchs from Saparua, Manado. Vibriosis agents in this study were *V. harveyi* and *V. parahaemolyticus* from Center for Brackish Water Aquaculture (BBPBAP) Jepara, Central Java, Indonesia.

2.2. Methods

2.2.1. Nudibranch-associated bacteria isolation

Isolation of nudibranch-associated bacteria. Isolation of bacteria-associated nudibranch was done using a dilution methods [13]. Nudibranchs samples were cut into small pieces, and then crushed until smooth using mortar. After that, put the samples into sterile sea water. The dilution was done stratified, it began from 10^{-1} to 10^{-3} and 10^{-4} . Furthermore, inoculation of bacteria-associated with Nudibranch was done by doing the spread method [14]. The cultures from spread methods were incubated for 24 hours at 37°C [15]. Based on the morphological characteristics, separate colonies were purified into nutrient agar to obtain pure culture [16]. Last step was purified using streak methods in Agar media [17].

2.2.2. Antibacterial tests

The purpose of screening antibacterial activity was to determine the antibacterial activity or biological potential of bacteria associated nudibranchs against pathogenic bacteria *Vibrio* spp. The method used in screening tests for this antibacterial activity was the overlay method [18]. The single colonies were inoculated on nutrient agar and were incubated for 24 hours. The bacteria pathogen density was standardized using a 0.5 McFarland. The plates were then overlaid with soft agar that has been mixed with 1% of the inoculated pathogens and incubated it for 24 hours at 37°C [19].

2.2.3. Identification of nudibranchs-associated bacteria

Identification of nudibranchs-associated bacteria that have the potential against *Vibrio* spp. was conducted using molecular identification, Polymerase Chain Reaction (PCR) and gram staining. PCR is a chain polymerase reaction, which is a reaction involving repeated polymerase enzymes. PCR is a simple test, which allows for amplification of specific DNA fragments from complex DNA collections [20]. The first step was DNA extraction using chelex 100 [21], and then all the ingredients together were mixed together. The PCR conditions used in this study were as follow, initial denaturation at 95°C for 3 minutes, 30 cycles of denaturation at 95°C for 1 minute, annealing at 53.9°C for 1 minute, and extension at 72°C for 90 seconds followed by final extantion at 72°C for 7 minutes. The next step was gel electrophoresis with a voltage of 100 volts for 30 minutes. Then the agarose gel was examined using the Gel doc to see the DNA tape from the PCR results. The next step was gel electrophoresis with a voltage of 100 volts for 30 minutes. Then the agarose gel was inserted into the Gel doc to visualize the DNA band from the PCR results. The PCR product was sent to PT. Genetic Science Indonesia Jakarta for sequencing analysis which was then followed by DNA chain reading using Mega X. The morphology under microscope was carried out by using Gram staining test and then using a light microscope with 1.0011 magnification. Gram staining is an important and a simple stage that can distinguish bacteria into Gram-positive and Gram-negative bacteria based on the reaction of the bacteria's cell wall to the reagent [22].

3. Results And Discussion

3.1. Nudibranch-associated bacteria isolation

Total 22 nudibranchs were collected from Saparua, Manado (Figure 1). The nudibranch-associated bacteria in this study were total 144 isolates bacteria.



Figure 1. Sample of Nudibranch

Morphology of 144 isolates bacteria associated nudibranch was showed in Table 1.

Table 1. Morphology of 144 isolates bacteria associated nudibranch

Shape	Colour	Diameter	Elevation	Colony edges
Circular (144)	Beige (143)	1 mm (89)	Convex (144)	Entire (135)
	Yellow (1)	2 mm (55)		Undulate (9)

The traditional methods that employ observation of either the morphology of single cells or colony characteristics remain reliable parameters for bacterial species identification [23]. Colony morphology is a characteristic of unique bacteria from each genus of bacteria to be identified [24]. Size (in millimeters), namely pinpoint (≤ 1 mm), small (2-3 mm), medium (4-5 mm), and large (> 5 mm), shape (circular, irregular, rhomboid, umbonate, filamentious or rhizoid), texture (opaque, transparent, opaque, translucent, metallic sheen, rough, wrinkled, mucoid), elevation (flat, convex, raised), margin (flat, filamentious, or ireegular), colour (yellow, golden yellow, white, green, bluish green or orange).

3.2. Antibacterial test

The result of antibacterial test or antagonist activity test, from 144 isolates nudibranch-associated bacteria, only one isolate (SM-N-3(2)/7) of them inhibited the growth of *V. harveyi* and *V. parahaemolyticus*. It can inhibit them for 7 days. The confirmation of bacteria associated with nudibranch activity against *Vibrio* spp. are performed in Figure 2.

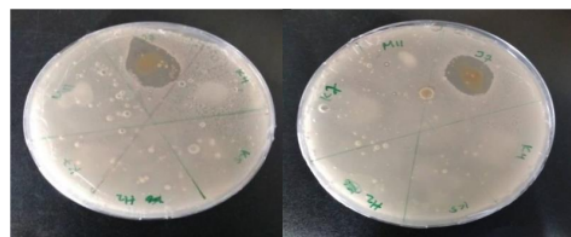


Figure 2. (a) Confirmation nudibranch-associated bacteria against *V. parahaemolyticus* (b) Confirmation nudibranch-associated bacteria against *V. harveyi*.

Table 2. Antibacterial activity of bacteria associated nudibranch against *Vibrio* spp. forming inhibition zone

No	Pathogenic Bacteria	Zone of Inhibition (mm)
1.	<i>V. harveyi</i>	19.4
2.	<i>V. parahaemolyticus</i>	10.63

The results of antagonistic activity screening have the average size of clear zone was 19.4 mm and 10.63 mm (Table 2). The response of the symbiont bacteria against *Vibrio* spp. was a strong. The literature stated that the classification of responses to bacterial growth barriers of 10-20 mm is strong [25]. The associate bacteria was also found or symbion with corals or sponges. It thought that produce antimicrobial compounds to protect the coral or sponge from pathogenic bacteria [26]. It usually providing carbon, nitrogen, biogeochemical cycles of sulfur and nutrients [27]. The secondary metabolites found in these marine animals, are formed due to the existence of competition between microbes present in the waters in the form of nutritional competition [28]. Inside of the marine organisms are microorganisms that will also synthesize secondary metabolites produced by their hosts or predators [29].

3.3. Potential nudibranch-associated bacteria identification

The isolate of 16S rRNA gene was analyzed by electrophoresis (Clever Scientific) of agarose gel 1% and used DNA Ladder to convinced whether 16S rRNA gene fragment size is ± 1500 bp (Figure 3.) The identification of potential bacteria based on 16S rRNA gene sequences has similarity 99% (Table 4) to *Pseudoalteromonas piscicida*.

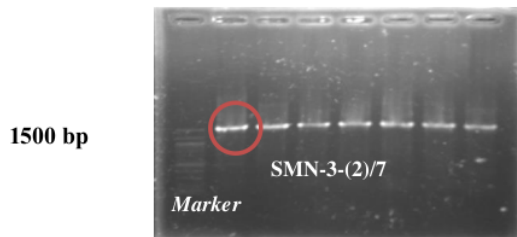


Figure 3. Amplification result using PCR 16S rRNA of Isolates SM-N-3(2)/7

Table 3. Homology of SM-N-3(2)/7 isolate using BLAST Systems

Isolate Code	Length of Nucleotides	Similarity to	Access Number (BLAST) NCBI	Homology
SM-N-3(2)/7	1458	<i>Pseudoalteromonas piscicida</i>	NR_114190.1	99%

The results of phylogenetic tree construction using the Mega X application are presented in Figure 4.

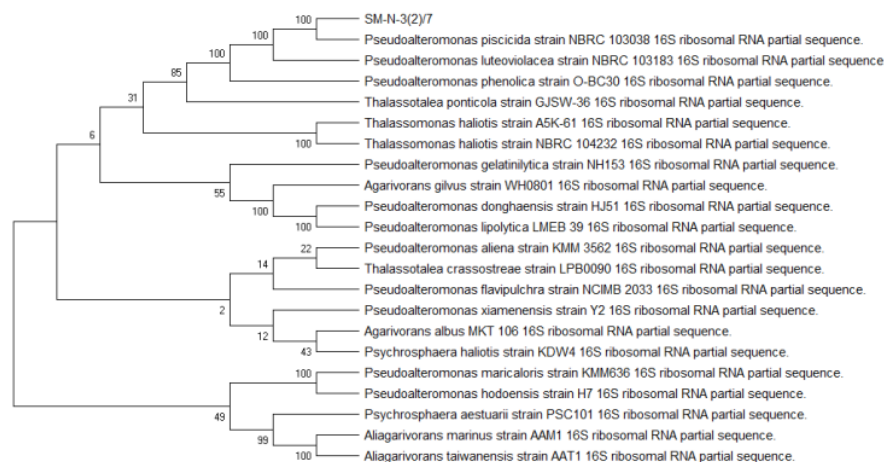


Figure 4. The phylogenetic of SM-N-3(2)/7 isolate based on 16S ribosomal RNA gene that was constructed using Neighbor-Joining analysis replicated in 1.000 bootstraps.

Based on gram staining test, SM-N-3(2)/7 that has the potential to be anti *V. harveyi* and anti *V. parahaemolyticus* is gram negative bacterium due to the red appearance under the light microscope with magnification of 100x10. The shape of this bacterium is rod or bacill. The result of SM-N-3(2)/7 gram staining is presented in Figure 5.

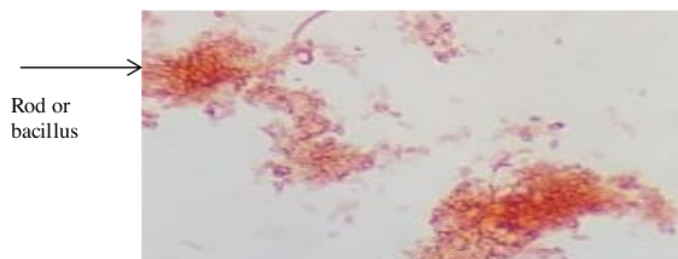


Figure 5. Gram Staining of SM-N-3(2)/7 isolate

Based on gram staining of SM-N-3 (2)/7 isolate was known that the form is short bacill or rod. The morphological characteristics of the SM-N-3 (2)/7 isolate is very similar to the characteristics possessed by bacteria of the genus *Pseudoalteromonas* [30]. Almost all marine bacteria are gram negative bacterial, and smaller than non-marine bacteria [31]. Prevalence of gram-negative bacteria in marine bacteria is greater than gram-positive bacteria [32]. Pathogens lysis process that occur because substances in *P. piscicida* not only inhibit the growth of these pathogens but also kill the pathogenic bacteria [33]. The substances contain alkaloids, polyketid [6], and also peptides [34]. *P. piscicida* produces antibacterials against *Aeromonas hydrophilla*, *Listonella anguillarum*, *Photobacterium damsela*, *Shewanella algae*, *Shigella sonnei*, *Staphylococcus aureus*, *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* [35]. Strain *Pseudoalteromonas* bacterial produces anti *V. parahaemolyticus* compounds during the exponential phase [33]. *P. piscicida* also show an antimicrobial spectrum and produces norharman (alkaloid a-carboline) with antibacterial activity against *Staphylococcus aureus* [34] [5]. These compounds are produced by microorganisms as well as their host [35]. The marine bacteria genus *Pseudoalteromonas* are recognized as the main producers of various biologically active meta [10] ites such as halogenated compounds, cyclic compounds, enzymes [36]. Therefore *P. piscicida* can inhibit the growth of *V. harveyi* and *V. parahaemolyticus*. The growth

the species will be disrupted because the other species produce compounds that can inhibit it [37]. Chemical interactions between different bacterial species will affect the production and secretion of antimicrobial secondary metabolites. *Pseudoalteromonas* species which is pigmented bacteria has been reported to having ability to secrete various extracellular compounds, including compounds with potential bioactivity against various pathogenic bacteria [38].

4. Conclusion

Isolation of associated bacteria in nudibranch revealed 144 isolates. From those isolates, only one isolate (SM-N-3(2)/7) was identified as active isolate against *V.harveyi* and *V. parahaemolyticus*. The rod-shaped gram negative bacteria was closely related to *Pseudoalteromonas piscicida* strain NBRC (99%) based on molecular screening using 16S rRNA gene.

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