Isolation And Partial Characterization Of Bacteria Activity Associated With Gorgonian *Euplexaura* sp. Against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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Abstract. Methicillin-resistant *Staphylococcus aureus* (MRSA) infection has emerged in around the world and has been resistance to *ciprofloxacin*, *erythromycin*, *clindamycin*. The aims of this study were to isolate, to investigate and to characterize bacterial symbionts gorgonian having activity against MRSA. *Euplexaura* sp. was collected from Panjang Island, Jepara, Indonesia by snorkling 2-5 m in depth. Bacterias were isolated by using spesific media with dilution method. Bacterias were conducted by using the streak method. Antibacterial activity was investigated by overlay method. The potent bacteria was identified by using molecular identification (DNA extraction, electrophoresis, PCR and phylogenetic analysis using 16S rDNA genes with actinobacteria-spesific primers) and bio-chemical test (among 5 isolated bacteria from gorgonian showed activity against MRSA). The strain PG-344 was the best candidat that has an inhibition zone against MRSA. The result of sequencing bacteria is 100% closely related with *Virgibacillus salarius*. This becomes a potential new bioactive compounds to against MRSA that can be a new drug discovery.

Keywords: antibacterial, MRSA, *Euplexaura* sp., *Staphylococcus aureus*

1. Introduction

Antibiotic resistance is a problem for international hospitals when treating diseases caused by bacterial infection. Previous studies indicate that excessive use of antibiotic cause bacteria resistance [1][2]. *Staphylococcus aureus* has emerged recent decades and continues to be a threatening disease around the world. Multi-resistant cases have emerged in some parts of the world. The recent study showed that 50% of Methicillin-Resistant *Staphylococcus aureus (MRSA)* isolate have been resistant to *ciprofloxacin, gentamycin and clindamycin* [3]. MRSA medical treatments require more time and to conduct and are more expensive because of the irrational use of antibiotic.

Finding a new compounds structure to serve as an anti-MRSA agent is imperactive. Recent studies have shown that marine organisms such as sponges, bryozoans, tunicates and gorgonians produce bioactive compounds that have the potential to be used as a source of medicine. More than 1000 new bioactive compounds have been identified in the bacteria carried by these marine invertebrate. Bioactive compounds from marine invertebrate have biological properties which are essential in the development of new drugs. Bacteria associated with marine invertebrate indicated

having the same compound with its host, therefore it can be an alternative in the culture and synthetic drugs in the future [4].

Gorgonian is one species of marine invertebrates that contains various critical bioactive compounds, including them as cytotoxic, anti-bacterial, anti-fungal and immunostimulan compounds [5]. Secondary metabolites that are contained in gorgonian act as a defense when disturbed by environment. Secondary metabolites play an important role in maintaining habitats in the marine environment [6]. The previous study conducted by Tanaka [7] reveals that gorgonian *Isis hippuris* has potential compounds to produce a compound steroid polioxygen.

This study aims to isolate the bacteria associated with gorgonian *Euplexaura* sp. It is estimated to produce bioactive compounds similar to the host and to identify the antibacterial association against MRSA.

2. Material and Method

2.1 Euplexaura sp. Sampling

Gorgonian *Euplexaura* sp. samples were collected from Panjang Island, Jepara Indonesia by snorklers 2-5 m depth into the ocean. The samples were found around coral reefs and were collected into a sterilized bag to be carried out a surface immediately to a handling surface [8] . The collected samples were rinsed with sterile sea water and were stored in cool box. Gorgonian identification was done by identifying the sclerit.

2.2. Isolation and microbiological analysis

One gram sample was shattered delicately. The resulting homogenate was diluted with sterile seawater at four dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}). One mililiter of each dilution was inoculated into nutrient agar (pH 7,5) medium by spread plate method. The inoculated plates were incubated for 2 days at 29 ^{0}C. The association bacteria grew selectively within the medium. Purification bacteria was executed based on different color, texture and shape of colonies. The selective colonies transferred to nutrient agar by innoculum method.

2.3. Screening bacteria for antibacterial activity against MRSA

The secondary metabolites were screened for antibacterial activity by using overlay method [9]. The activity perfomed by innoculated bacteria on nutrient agar plate against Methicillin-Resistant *Staphylococcus aureus* (MRSA). The bacteria association were incubated overnight at 28^{0}C. *S. aureus* was innoculated on nutrient broth and was grown overnight at 27^{0}C according to the shaker condition. The concentration of *S.aureus* cultur was adjusted to 0,5 McFarland standards and were poured evenly on soft agar media. Antibacterial activity was initiated by pouring *S. aureus* on nutrient agar plate which contained grown bacteria. Antibacterial activity was indicated by the emergence the inhibiton zone surounding the bacterial association after 24 hours incubation at 28^{0}C. The active isolate created a stain that identified the biological morphology. It was observed at the magnification 100 X in microscope.

2.4. PCR amplification and DNA sequencing

DNA extraction was obtained by diluting a sample with saponin, ddH_2O, chelex 20%. Supernatant which contains DNA used as a template for PCR analysis. The PCR mixture consisted of primers F (127) 1 µl, primers R (1492 R), DNA template 1 µl, PCR kit 12,5 µl, ddH_2O 9,5 µl. The total volume measured 25 µl. The PCR condition was denaturation at 45^{0}C for 1 minute, anneling at 53,9^{0}C for 1

minute, extention at 72^{0}C for 30 second and post cycling at 72^{0}C for 7 minute. All condition were repeated 30 times.

3. Result and discussion

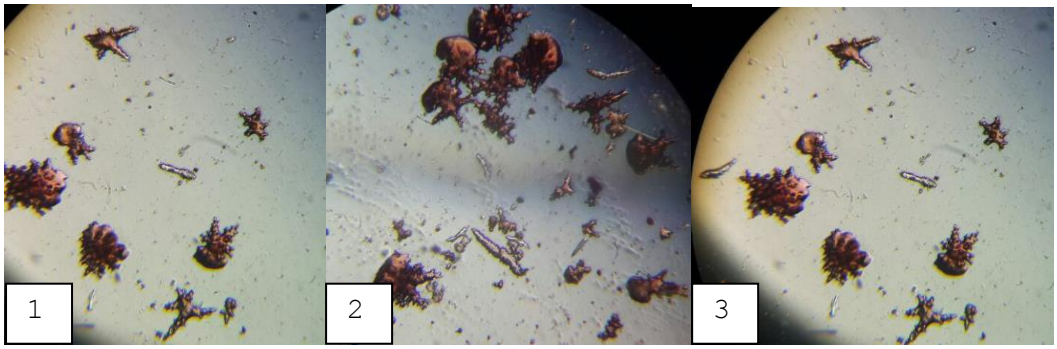

Figure 1. Sclerit of gorgonian *Euplexaura* sp. Note: 1=*strong club*; 2= *spindle*; 3= *rod*

Samples were identified by observation using a microscope at 10x magnification as presented in Figure 1. Forms sclerites observed and identified by [10]. *Euplexaura* sp. has 3 forms sclerites. They are *a strong club*, and *a spindle rod* and *a reddish color*. Each type has a gorgonian sclerites which is distinct. Basic forms sclerites include *double head*, *spindle*, *club*, *scaphoid* and *rod*. Sclerites form of a specific character possessed by each type of gorgonian can be used as a basic in determining taxonomic species. Therefore the name of the species can be recognized from the samples acquired.

Table 1. Zone of inhibition for antibacterial activity against MRSA

Bacteria	**Zone of Inhibition (diameter in mm)**										
	PG-341	PG-342	PG-343	PG-344	PG-345	PG-381	PG-382	PG-383	PG-384	PG-385	PG-386
MRSA Activiy	-	-	-	+	-	-	-	-	-	-	-

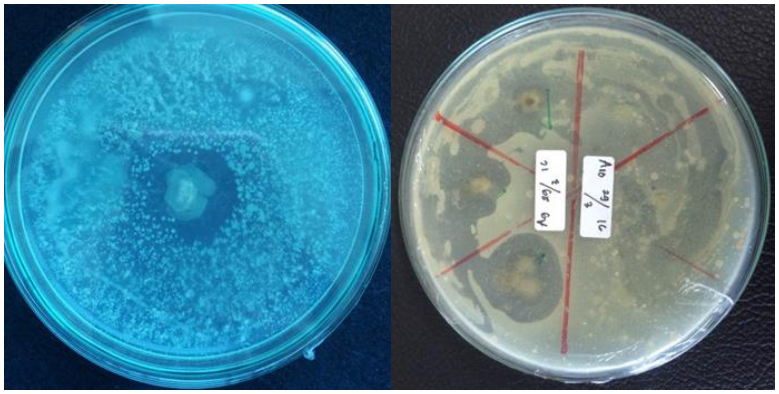

Figure 2. Antibacterial activity

The antibacterial activity of *S. aureus* was tested by overlay. The results in Table 1. showed, that one of the 11 *Euplexaura* sp. isolates possess antibacterial activity. Isolates PG-344 possess antibacterial activity as indicated by the inhibition zone around the isolates in Figure 2. Isolates PG-344 were able to inhibit the growth of *S. aureus* with an inhibition zone diameter of 21.86 mm. This is consistent with research done [11][12] that revealed that the gorgonian that contain the bioactive compound can function as an antimicrobial. Gorgonian produces a metabolism secondary to the environmental conditions in the crucial competitive interactions within a restricted environment [13].
Heindl [14] revealed that the bacteria that live in association with its host have the same activity of secondary metabolites. Isolates PG-344 have the ability to inhibit the growth of *S. aureus* activity caused by the presence of the active compounds. *Staphylococcus aureus* has experienced resistance to some antibiotics, such as *chlorampenicol, fosfomicin, tetraciclin* and *ciprofloxacine*. The clear zone that appears around the isolates indicates that there are bioactive compounds in isolates which are capable of inhibiting the growth of *S. aureus*.

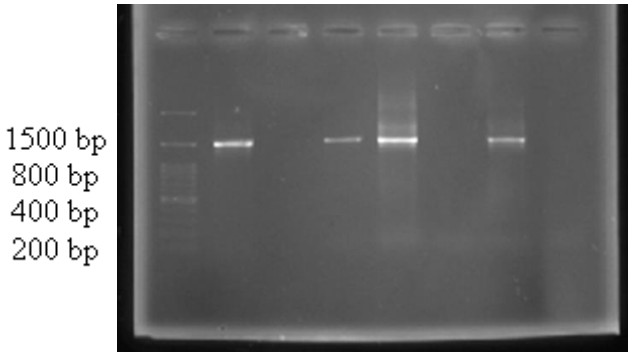

Figure 3. PCR Amplification 16S rDNA Isolate PG-344

Based on the electrophoresis results of PCR 16S rDNA, isolate PG-344 has a base length of 1500 bp. The results of DNA extraction have a band that is perfectly visible in Figure 3. Polymerase chain reaction (PCR) is a method that to detects microbial targets by amplifying the amount of target sequence inwith a relatively short time. PCR does not require a high number of DNA molecules to amplify target sequences. Only the target sequence needs to remain intact. Thus, PCR can amplify or partially damaged and denatured DNA [15].

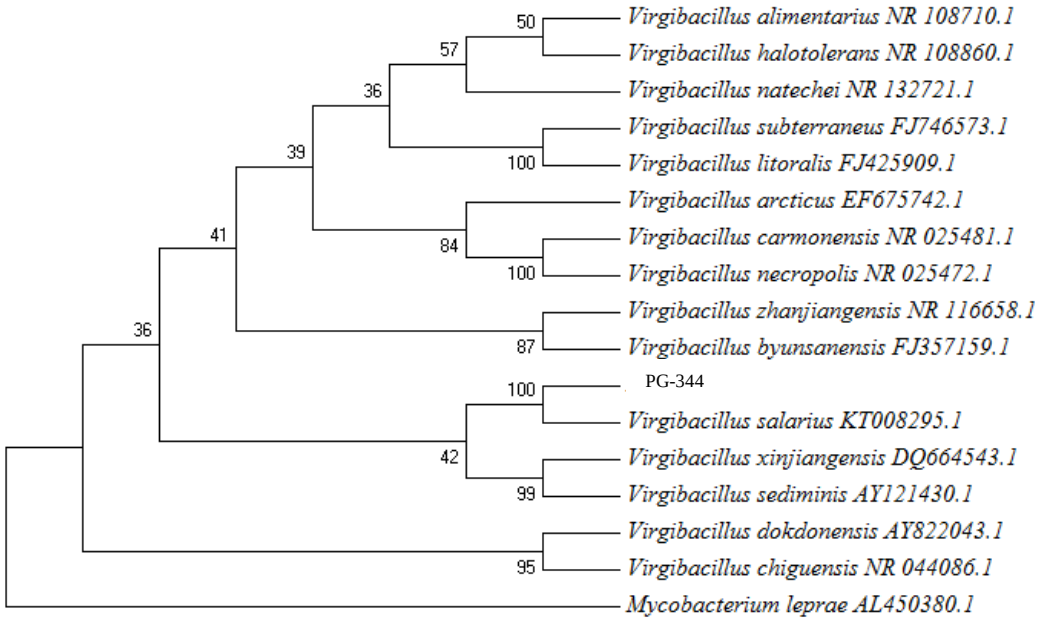

Figure 4. Filogenetic Tree

The bacteria comparator used in the phylogenetic tree are bacteria that have a closely related with the association *Euplexaura* sp. isolates. The Phylogenetic tree presented in Figure 4 was created by using the application MEGA 6.

Results of the Basic Local Alignment Search Tool (BLAST) showed that the bacterial isolates PG-34-04 have a 100% homology closely related to Virgibacillus salarius. Rosenberg [16], explains that the genus Virgibacillus is a gram-positive bacteria, with colonies that measure 0.5-5 mm. Cells occur independently, in pairs or as short chains. Endospores are round or elliptical and located in a position subterminal or terminal to sporangia. After 48 hours at 30-35 UC on a solid medium containing 10% NaCl, colonies formed that opaque and white. Additionally, these colonies were circular and covex with erose or margin that was slight stringy.

4. Conclusion

Test bacteria was selected by using a bioassay, which found one isolate, namely PG-344. PG-344 is caple of antibacterial activity that can protect against methicillin-resistant *Staphylococcus aureus* (MRSA). Selection of the test bacteria using a bioassay and gained 1 isolates, namely PG-344 which has antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Analysis of the isolate PG-344 showed that 93% of bacteria is closely related to *Virgibacillus salarius*.

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References

[1] Arana D M, Rubio M and Alós J I 2016 Evolution of antibiotic multiresistance in Escherichia coli and Klebsiella pneumoniae isolates from urinary tract infections: A 12-year analysis. *Enferm. Infecc. Microbiol. Clin.* no. xx

[2] de Matos P D M, de Oliveira T L R, Cavalcante F S, Ferreira D C, Iorio N L P, Pereira E M, Chamon R C and Dos Santos K R N 2016 Molecular Markers of Antimicrobial Resistance in Methicillin-Resistant Staphylococcus aureus SCCmec IV Presenting Different Genetic Backgrounds. *Microb. Drug Resist.* vol. 00. no. 00, pp. 1–7

[3] Lestari E S 2009 Antimicrobial Resistance in Indonesia Prevalence, determinants and genetic basis. ISBN 978-90-8559-606-6.

[4] Mayer A M S, Abimael D R, Roberto G S B, Nobuhiro F 2011 Marine pharmacology in 2007–8: Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous system, and other miscellaneous mechanisms of action. Comparative Biochemistry and Physiology, Part C 153. 191–222

[5] Manzo E, Ciavatta M L, Lopez Gresa M P, Gavagnin M, Villani G, Naik C G and Cimino G 2007 New bioactive hydrogenated linderazulene-derivatives from the gorgonian Echinogorgia complexa. *Tetrahedron Lett.* vol. 48, no. 14. pp. 2569–2571

[6] Melorose J, Perroy R, and Careas S 2015 No Title No Title," *Statew. Agric. L. Use Baseline*. vol. 1

[7] Tanaka J, Trianto A, Musman M, Issa H H, Ohtani I I, Ichiba T, Higa T, Yoshida W Y, and Scheuer P J 2002 New polyoxygenated steroids exhibiting reversal of multidrug resistance from the gorgonian *Isis hippurisTetrahedron*, vol. 58, no. 32, pp. 6259–6266

[8] Sabdono A, Radjasa O K 2008 Phylogenetic Diversity of Organophophorous Pesticide-Degrading Coral Bacteria from Mid-West Coast of Indonesia. Biotechnology 7 (4): 694-701. ISSN 1682-296X. Asian Network For Scientific Information

[9] Jenkins K, Jensen P R and Fenical W 1998 Bioassays with marine microorganisms. Methods in chemical ecology. Bioassay Methods, II, pp.1–38

[10] Janes M P 2008. Laboratory methods for the identification of soft corals (Octocorallia : Alcyonacea). Advances in Coral Husbandry in Public Aquariums. Public Aquarium Husbandry Series, vol.2., 2, pp.413–426

[11] Kim S K and Van Ta Q 2012 Bioactive Sterols from Marine Resources and Their Potential Benefits for Human Health 1st ed., Elsevier Inc. Available at: http://dx.doi.org/10.1016/B978-0-12-416003-3.00017-2

[12] Habbu1 P, Vijayanand W, Rajesh S, Smita M, Venkatrao H K 2016 Antimicrobial metabolites from marine microorganisms.*Chinese Journal of Natural Medicines*. 14(2): 0101.0116

[13] Zhang, G 2013 Cytotoxic 9,11-secosteroids from the South China Sea gorgonian *Subergorgia suberosa*. Steroids, 78(9), pp.845–850. 10.1016

[14] Heindl H 2010 Phylogenetic diversity and antimicrobial activities of bryozoan-associated bacteria isolated from Mediterranean and Baltic Sea habitats. Systematic and Applied Microbiology, 33(2), pp.94–104. Available at: http://dx.doi.org/10.1016/j.syapm

[15] Cavanaugh C M, Mckiness Z O P, Newton I L G, and Stewart F J 2006 *The Prokaryotes*. vol. 3, no. November 1902

[16] Rosenberg E 2014 Department of Molecular Microbiology and Biotechnology. Tel Aviv University. Tel Aviv, Israel