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# Antibacterial activities of bacteria asssociated with Marine sponges of *Axinella sp.* on Carbapenem-Resistant *Acinetobacter Baumannii* (CRAB)

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**Abstract:** *Carbapenem-resistant Acinetobacter baumannii* (CRAB) is a gram-negative bacilli that commonly causes nosocomial infection found in Indonesia. CRAB infection caused by *Acinetobacter baumannii* that is resistant to Carbapenem. Resistance occurred because bacteria that cause infections easily treated with antibiotics become difficult to treat due to the uncontrolled use of antibiotics. *A. baumannii* has been resistant against the carbapenem class of antibiotics; and therefore, antibiotics are required to be obtained from natural ingredients with optimal working power, such as from the marine sponges of *Axinella sp*. The purpose of this study was to determine the antibacterial activities of bacteria associated with marine sponges of *Axinella sp.* against CRAB. Methods: bacteria associated with *Axinella sp.* were isolated by differential dilution and cultures on Zobell 2216E media. Antibacterial activity test was performed using the overlay method. The antibacterial activity test was carried out to determine the presence of inhibition zones. The test results showed the bacteria associated with a marine sponge of *Axinella sp* strain 3 KD had antibacterial activity against CRAB growth with the formation of an inhibition zone of 16 mm. The results of the catalase test and oxidase test depicted that the isolates belong to the family of *Staphylococcaceae*. Conclusion: isolates bacteria associated with a marine sponge of *Axinella sp*. were potential antibacterial agents against CRAB growth.
**Keywords:** CRAB; Antibacterial activity; *Axinella sp*. isolate; overlay method; *Staphylococcaceae*

## INTRODUCTION

Nosocomial infections are defined as infections found in hospitals that attack patients and appear within 48-72 hours after treatments[1]. Microorganisms can live and reproduce in the hospital environment, on either the floor or medical and non-medical equipment[2]. In Indonesia, nosocomial infections caused by *A. Baumannii* reach 25.8%, one of the causes of nosocomial infection worldwide and the highest mortality rate worldwide can reach 52%[3].

Manifestations of nosocomial infections attributed to *A. baumannii* include pneumonia, secondary meningitis, bloodstream site infection (BSI), and urinary tract infection (UTI)[4]. Some strains of *A. baumannii* are highly resistant to antibiotics[5]. Inappropriate use of antibiotics can cause antibiotic resistance because infectious bacteria become difficult to treat[6]. Antibacterial therapy regimens currently considered to be potent in treating *A. baumannii*-related infection are carbapenem, sulbactam (with a beta-lactamase-inhibitor), and

colistin[7]. *A. baumannii* is resistant to carbapenemase antibiotics (Carbapenem-resistant *Acinetobacter baumannii* (CRAB)). Carbapenem is the main antibiotic used for treating patients with *A. baumannii*-related infection, causing resistance to many carbapenem antibiotics[8]. Antibacterial agents derived from biological sources include lactic acid bacteria[9], mushrooms[10,11], latex , fruits[12,13], and seeds[14]. Bioactive compounds from marine sources have been widely studied in previous studies. Currently, there are many studies examining bioactive compounds from marine resources.

Indonesia is one of the countries with a lot of potential marine resources; therefore, it is necessary to develop research on marine life to look for bioactive compounds with the ability as an effective antibacterial and few side effects[15]. Many researchers were interested in conducting studies on antibacterial from marine sponges[16].

Several studies have been reported on the antibacterial activity of microbes associated with marine sponge *Axinella* sp. Fungi associated with the marine sponge *Axinella* sp have potential as an antibacterial against *Staphylococcus aureus* and *Escherichia coli*[17]. Another study reported that bacteria associated with the fungus *Axinella* sp from the Mediterranean Sea had antibacterial activity against several non-resistant pathogenic bacteria[18]. There have been no reports of bacteria associated with sponge *Axinella* sp to CRAB. To minimize the research gap, the aim of this study was to evaluate the anti-CRAB potential of bacteria associated with *Axinella* sp.

## MATERIAL AND METHOD

### Tools and Materials

The tools used in the research to test the antibacterial activity were autoclave (HMC Hirayama HICLAVE HVE-50), incubator (WTC Binder), laminar airflow cabinet (Labcono Purifier Class II Biosafety Cabinet), refrigerator (Sharp), petri dish, inoculation loop and needle, alcohol lamp, test tubes, and test tube racks.
The materials used in this study were Zobell 2216E media, MHB (Mueller Hinton Broth) antibacterial activity test media, MC media (Mac Conkey), BHI (Brain Heart Infusion), Standard Mc Farland, and physiological NaCl.

### Tested microorganisms

CRAB was isolated from wound sample collected from patients suffering from diabetes in RS. Dr. Kariadi Semarang, Central Java, Indonesia. Isolates were identified using MacConkey Agar (MCA) media, as well as biochemical tests with Vitek®MS and bacterial sensitivity tests using the Clinical Laboratory Standard Institute M100-S25 for minimum inhibitory concentration(MIC) interpretation (CLSI2019).

### Isolation of bacteria from *Axinella sp*.

*Axinella sp.* sponges were obtained from the waters of Tanjung Gelam Beach, Karimunjawa Islands, Jepara, Central Java, with a sampling technique at a depth of 0.5-1m. *Axinella* sp has been isolated from marine and fishery laboratory Universitas Diponegoro, Semarang. Then, isolation was performed by weighing ± 1 gram cleaned with sterile seawater, mashed, carried out a differential dilution of 10 to 10 4 then spread on Zobell 2216E media, incubated at a temperature of 35 ± 2 ° C for 48 hours, and then observed the incubation results. The Gram staining, catalase, and oxidase tests were used to know the bacterial family.

### Antibacterial Activity Test

The marine sponge-associated isolates were screened, using agar overlay assays[19] for antibacterial activity against CRAB. Pure isolates bacteria associated with sponge *Axinella* sp were grown on Zobell 2216E media by spotting them on the media and forming small spheres with a diameter of ± 2 mm. The isolates were incubated at 35 ± 2°C for 48 hours and results were observed. After that, a suspension of the test bacteria was made. 1 ml physiological NaCl was taken and put into soft agar media at the temperature of ± 40°C. It was homogenized using a

vortex and once homogeneous, it was poured into Zobell media containing pure isolates of bacteria from *Axinella sp* sponges in the form of spheres with a diameter of ± 2 mm. Then, it was incubated at 35 ± 2°C for 48 hours and the formation of an inhibition zone was observed.

## RESULTS AND DISCUSSION

### CRAB isolates from wound infections

CRAB was isolated from wound samples. The result of the sensitivity test of bacteria to antibiotics showed that *A. baumanii* from wound patients was a CRAB stain because it was resistant to Carbapenem (Meropenem) besides resistant to Ampicillin, Sulbactam, Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Amikacin Gentamicin, Ciprofloxacine, Tigecycline, Nitrofurantoin, Sulfamethoxazole showed that *A. baumanii* not only included in the CRAB strain but also in the Multidrug-Resistant Bacteria (MDR).

### Isolation of *Axinella sp.* Associated Bacteria

The isolation of bacteria from *Axinella sp* produced eight bacterial isolates, coded with 1 KA, 1 KB, 2 KA, 3 KA, 3 KB, 3 KC, 3 KD, and 4 KA.

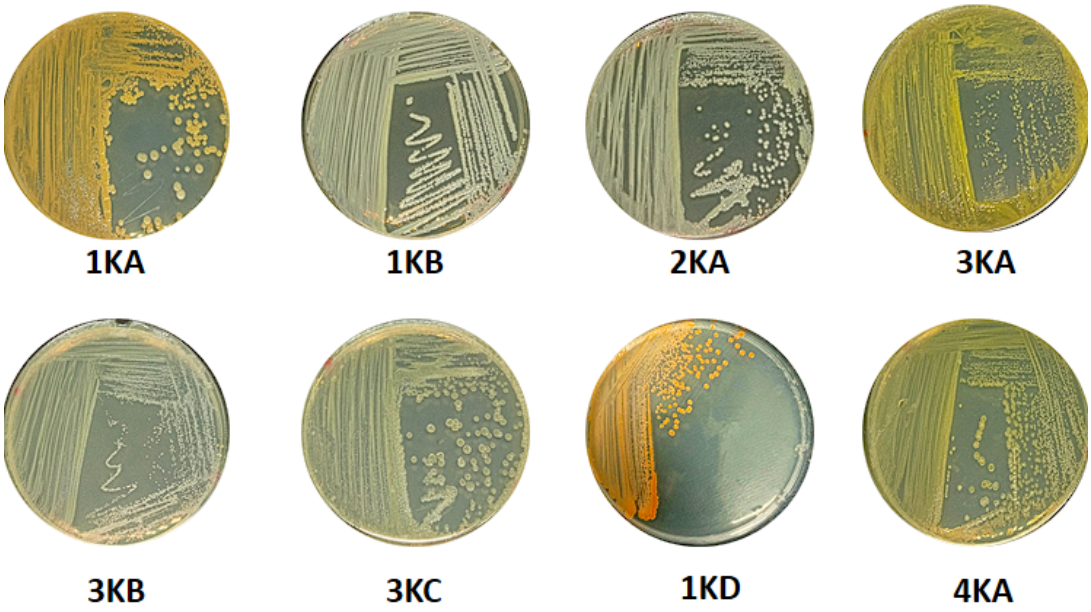


Figure 1. The results of pure bacterial isolation from *Axinella sp*

Table 1. Macroscopic Characteristics of Bacteria Isolates from *Axinella sp*.

| No. | Code of Bacterial Isolate | Characteristic of Colony | | | |
|---|---|---|---|---|---|
| | | Shape | Edge | Surface | Color |
| 1. | 1 KA | Round | Entire | Flat | Yellow |
| 2. | 1 KB | Round | Entire | Convex | White |
| 3. | 2 KA | Round | Entire | Pulvinate | White |
| 4. | 3 KA | Round | Entire | Convex | Yellow |
| 5. | 3 KB | Round | Entire | Raised | White |
| 6. | 3 KC | Round | Entire | Raised | White |
| 7. | 3 KD | Round | Entire | Convex | Orange |
| 8. | 4 KA | Round | Entire | Raised | Yellow |

The isolation of symbiont bacteria from *Axinella sp*. produced eight bacterial isolates with various characteristics. According to Dwijoseputro[20], macroscopic observations of colony morphology are: from above, the shape of the colony is dominantly round; from the side, the most dominant types of colony surface are flat, convex, pulvinate, and raised; from above, the colony edge is

entire; and the most dominant colors are yellow, white, and orange. Gram staining was performed on the pure isolates and microscopic observation was carried out to identify the cell shapes. Gram-positive bacteria were purple while gram-negative bacteria were pink.

Table 2. Macroscopic Characteristics of Bacteria Isolates from *Axinella sp*. with Gram Staining

| No. | Code of Bacterial Isolate | Gram | Cell Shape |
|---|---|---|---|
| 1. | 1 K A | - | Bacillus |
| 2. | 1 K B | - | Bacillus |
| 3. | 2 K A | + | Bacillus |
| 4. | 3 K A | - | Coccus |
| 5. | 3 K B | - | Bacillus |
| 6. | 3 K C | - | Coccus |
| 7. | 3 K D | + | Coccus |
| 8. | 4 K A | - | Bacillus |

Table 2 presents that the bacteria in the eight isolates were grouped into two, including two gram-positive bacterial isolates (2 K A) and six gram-negative bacterial isolates (1 K A, 1 K B, 3 K A, 3 K B, 3 K C and 4 K A). The microscopic observation identified coccus bacteria in two isolates and bacillus bacteria in six isolates.

**The Results of Inhibitory Test**

The inhibitory test showed the formation of an inhibition zone, producing a clear zone around the colony. The activities on the inhibition zone are presented in Table 3.

Table 3. The Results of Antibacterial Activity Test of Bacterial Isolates from *Axinella sp* on CRAB

| No. | Codes of Bacterial Isolates | Inhibition zone (mm) |
|---|---|---|
| 1. | 1 K A | 0 |
| 2. | 1 K B | 0 |
| 3. | 2 K A | 0 |
| 4. | 3 K A | 0 |
| 5. | 3 K B | 0 |
| 6. | 3 K C | 0 |
| 7. | 3 K D | 16 |
| 8. | 4 K A | 0 |

Table 3 details that the isolates of symbiont bacteria from *Axinella sp* coded 3 K D could form a clear zone around the colony with an average diameter of 16 mm.

Table 4. The Results of Antibiotic Control Test on CRAB

| Antibiotic Control | Inhibitor Zone Diameter (mm) |
|---|---|
| Gentamicin | 23 |
| Ampicillin | 25 |
| Meropenem | 0 |

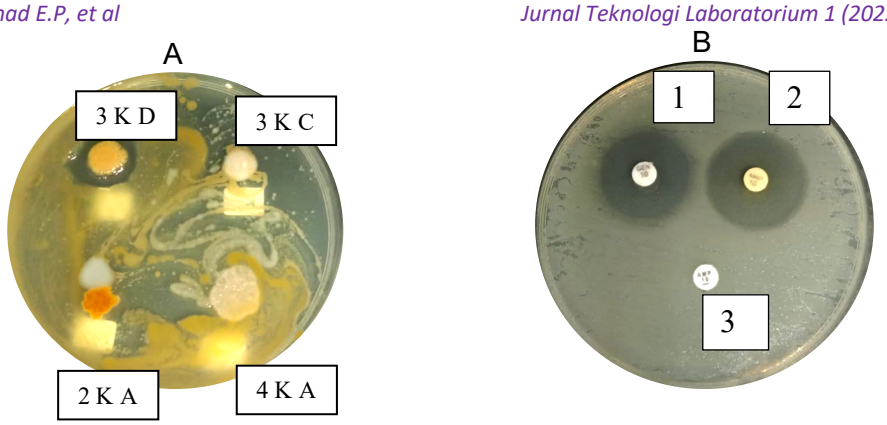


Figure 2. Inhibitory zone of (A) Isolates from *Axinella sp* Code P3-K9, (B) Antibiotics: 1. Gentamicin., 2. Ampicilin., 3. Meropenem

The results of the inhibitory test on isolates from *Axinella sp* (code: 3 K D) on CRAB, as presented in Table 4 and Figure 2, showed a clear zone around the colony with a diameter of 16 mm. The catalase test was carried out on the 3 K D isolate to determine its ability to produce catalase enzyme and its tolerance to oxygen. The catalase test was used to identify groups of catalase-producing bacteria. This test was performed to distinguish catalase-positive members of the *Micrococcaceae* or *Staphylococcaceae* from catalase-negative *Streptococccaceae*[21]. Meanwhile, the oxidase test was conducted to identify bacteria containing cytochrome c oxidase enzyme that could oxidize the reducing agents in the form of tetramethyl p-phenylenediamine (Kovac's reagent) or dimethyl p-phenylenediamine (Gordon and McLeod's reagent)[21]. The catalase test showed a positive result, indicated by the presence of foam or bubbles. Meanwhile, the oxidase test yielded a negative result with no blue-purple color change in the oxidase paper.

In this study, yellow marine sponges of *Axinella sp*. obtained from the waters of Tanjung Gelam Beach, Karimunjawa Islands, Jepara, Central Java. CRAB pathogenic bacteria causing nosocomial infections that were resistant to carbapenem antibiotics were also utilized. This study showed that one of eight isolates of *Axinella sp*. associated bacteria (code: 3 K D) have an antibacterial activity to inhibit CRAB bacteria by forming a clear zone around the colony. The previous studies explained that the *Axinella sp* contained bioactive compounds, including alkaloids and steroids[22]. Although identifications of compounds in *Axinella sp*. and the antibacterial activity of alkaloids and steroids were not performed in this study, another studies concluded that alkaloids could interfere with the peptidoglycan constituent components of bacterial cells so that the cell wall layer was not naturally formed, destroying the cytoplasmic membrane and bacterial cell wall[23]. The cell membrane turned into a fragile and lysed cell[24]. Bacterial cell walls were damaged, causing the disruption of bacterial cell metabolism and resulting in death[25]. Osmotic pressure happened and the cell walls became lysis, causing bacteria to die[26]. The results of the study showed that the *Axinella sp*. isolate (code: 3 K D) produced an inhibition zone of 16 mm.

The association process of marine sponges with their symbiotic bacteria began with the presence of bacteria in the waters of the surrounding environment[27]. The sponge will absorb specific bacteria through the sponge during filter-feeding and by vertical transmission of symbionts through the gametes of the sponge by the inclusion of the bacteria in the oocytes or larvae[28,29]. Sponges of the *Axinella* genus are known as producers of achemically diverse metabolites with excellent bioactive activity[30]. The metabolite compounds contained in the *Axinella sp*. sponge could kill or inhibit the growth of pathogenic bacteria[31]. According to Gunathilaka research, the bioactive compound from marine associated bacteria

has the potential to provide future drugs against sicknesses such as cancer, malaria, and inflammation[32]. Thus, bacteria with metabolites and bioactive compounds can perform as antibacterial agents.

The metabolites contained in the marine sponges of *Axinella sp*. are closely related to the metabolite compounds synthesized by the symbiotic microorganisms[33]. One of the isolates of the symbiotic bacteria of *Axinella sp*. is the potential to produce antibacterial compounds[34]. According to Konig research, several studies have reported that many bioactive compounds from marine biota are similar to the bioactive compounds from microorganism associated with these marine biotas[35]. They are true producers of bioactive compounds. Study by Gultom explained that isolates of bacteria associated with marine sponge *Axinella sp* contained alkaloid against *Staphylococcus aureus* and *Escherichia coli*[36]. According to Januar research that has found that the three compounds were hymeniadisine, 3-bromohymenialdiside, and dibromophakelin[37]. Zhang has been identified by detailed spectroscopic analysis reported 14-0-sulfate massadine has enhanced chemical stability that potential as antibacterial properties[38]. According to the research by Rastina, the ability of an antimicrobial to inhibit microbes depends on the concentration of antimicrobial material and the type of antimicrobial material produced[39]. The greater the concentration of an antimicrobial, the higher the concentration of bioactive substances, and thus, the inhibition will be higher, as indicated by an increase in the formed clear zone.

The antibacterial levels are divided into four categories: weak (<5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (>20 mm). The identification of marine microbial isolates associated with *Axinella sp*. included morphological observations, gram staining, catalase test, and oxidase test[40]. According to Henstsel, various types of microorganisms have been found in sponges, such as symbiotic bacteria, which are members of *Bacillus sp*., *Staphylococcus sp.,* and *Vibrio sp*[41]*.* The most common *Axinella sp*. symbiotic bacteria found were *Pseudomonas sp*., *Bacillus sp.*, *Vibrio sp*., and *Staphylococcus sp.* groups[33].

The antibacterial activity test confirmed the formation of a 16 mm inhibitory zone, signifying that symbiont bacterial isolates have a strong inhibitory power. Further catalase test to identify the isolates of *Axinella sp.* symbiotic bacteria showed a positive result, while oxidase test yielded a negative result, indicating that the *Axinella sp*. 3 K D symbiotic bacterial isolates belonged to the catalase-positive members of *Staphylococcaceae*.

## CONCLUSION

This research concluded that 3 K D bacterial isolates associated with *Axinella* sp. Are potential to be developed as antibacterial agents against the growth of CRAB, indicated by the formation of a clear inhibition zone around the colony with an average diameter of 16 mm. The bacteria are members of *Staphylococcaceae*. Further studies are recommended to be performed by incorporating different strains of pathogenic bacteria using *Axinella sp*. bacteria isolates and the overlay antibacterial test method. During the present research process, some obstacles occurred, such as uneven distribution of pathogenic test bacteria on the media. Therefore, proper homogenization with a vortex is required in further study. Moreover, media contamination also happened; and thus, it is recommended to use a biosafety cabinet to reduce the risk of contamination to obtain accurate and optimal results.

## AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

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## DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

## DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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