## ORIGINAL ARTICLE

# Screening of Pathogenic Bacteria From Sea Cucumber *Acaudina molpadioides* In Pulau Langkawi, Kedah

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**ABSTRACT**

**Introduction:** The outbreaks of foodborne diseases have been linked to the consumption of contaminated seafood. This research aims to screen the bacteria from the sea cucumbers *Acaudina molpadioides* collected from Pulau Langkawi. **Methods:** A total of 22 sea cucumber samples were collected randomly from Pulau Langkawi, Kedah, Malaysia. The samples were isolated and identified for the presence of bacteria using the conventional culture-based method. Presumptive bacteria colonies were subjected to various biochemical and antimicrobial susceptibility tests. **Results:** There were no bacterial growth in Hektoen Enteric (HE) agar and Thiosulphate-Citrate-Bile Salt (TCBS) agar. Positive samples were isolated from MacConkey (MAC) agar with 6 samples were Staphylococcus spp. (27.27%), 14 samples were Proteus spp. (63.63%) and 2 samples were Bacillus spp. (9.01%). Among these isolates, highest resistance was found against Ampicillin (45%) followed by Tetracycline (40%). **Conclusion:** The results indicate that the sea cucumbers *Acaudina molpadioides* were contaminated with potential bacteria. There is a need for adequate consumer protection measures.

**Keywords:** Sea cucumber, *Acaudina molpadioides*, Bacterial contamination

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## INTRODUCTION

Malaysia is one of the mega diverse countries that contain lots of marine taxa. There are more than 80 species of sea cucumber inhabit the marine and coastal waters of Malaysia (1). The sea cucumber is known as *timun laut, gamat, bat, balat*, and *brunok* for the locals (2).The body wall of the sea cucumber is the major edible part. It contains the numerous bioactive substances including acidic polysaccharides, collagen, triterpenoid saponins, cerberosides and gangliosides (3).

In Malaysia, particularly in Langkawi, the locals consume raw *Acaudina molpadioides* to make 'kerabu', a local delicacy famous at the state of Kedah (4). In Asia, sea cucumber is very popular seafood and its consumption is increased with recognition of its medicinal properties. However, the raw sea cucumber contains various microbes that may cause foodborne illnesses such as salmonellosis and gastroenteritis (5).

Food poisoning is a common illness which range from mild to serious and may cause life threatening conditions. Raw food has higher chances to become a ripe breeding ground for bacteria, which will multiply and release the toxins to cause harm to human body. In the past few years, food safety situation in Malaysia is still not considered as a vital issue although there were increased in the food poisoning cases (6). This study aims to determine the presence of bacteria from the body wall of the sea cucumber *Acaudina molpadioides*.

## MATERIALS AND METHODS

### Sample collection
A total of 22 sea cucumber samples were collected at the random sites in Pulau Langkawi, Malaysia on September 2018. Samples were identified by Prof. Dr. Ridzwan Hashim from International Islamic University Malaysia (IIUM) via the morphological features. The samples were placed in sterile sealed plastic bags before being transported to the laboratory in a cold box and analyzed immediately.

### Isolation and identification of samples
A ten-fold serial dilution was carried out after

homogenizing 5 g of *A. molpadioides* with 45 mL of alkaline peptone water (APW). Total of 100 µL aliquot of the APW-diluted samples (1 x $10^7$ cell/ml) homogenates were cultured onto TCBS, MAC, and HE agar. Incubation was then carried out at 37°C for 24 hours (7). Thereafter, the colonies that were presumed to be *Vibrio*, *Escherichia coli* (*E. coli*) and *Salmonella* were examined macroscopically on the basis of their color, size, and shape from TCBS, HE and MAC agar plates. Next, the colonies were examined microscopically with the aid of Gram staining and subjected to a series of biochemical tests such as catalase, oxidase, indole, methyl red, voges-proskauer and citrate tests. Then, the isolates were also subjected to antimicrobial susceptibility test.

**Antibiotics susceptibility testing**

The method of Kirby and Bauer disk diffusion was used in this study. The test was carried out on Mueller Hinton (MH) agar. Bacterial suspension was prepared by suspending colonies obtained from 24 h culture agar plate and adjusted to 0.5 McFarland standard. Each isolate was streaked onto the MH agar plate with the sterile swabs. The antibiotic disks were placed onto the surface of the agar. The plates were incubated at 37°C for 24 hours. The antibiotics used were streptomycin (10 µg), tetracycline (30 µg) and ampicillin (10 µg). The size of the zone of the inhibition were measured in millimeters (mm) after 24 hours. The results were interpreted as susceptible strain with clear zone more than 18 mm, intermediate resistance strain with clear zone 13 – 17 mm and resistance strain with clear zone less than13 mm (18). The negative control was distilled water. Inhibition zone was interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (8).

## RESULTS

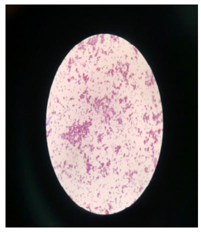
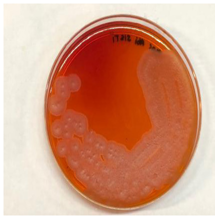
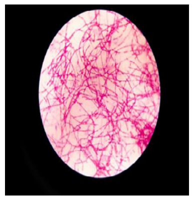
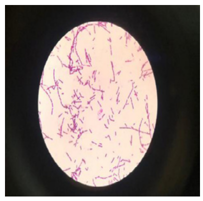
**Macroscopic and microscopic examinations**

In this study, 6 out of 22 of sea cucumber *A. molpadioides* isolates (27.27%) presented growth with the characteristics of small, wet and light pink colonies with lactose fermenter on MAC agar. These isolates displayed purple coloured cocci in cluster under microscopic examination with the aid of Gram staining, presuming as *Staphylococcus* species. There were 14 out of 22 isolates (63.63%) presented growth with characteristics of big, dry, non-lactose fermented with pale color colonies on MAC agar. They displayed pink colored and *Bacillus* in chain under microscopic examination, presuming as *Proteus* species. Dry, non-lactose fermenter with pale color colonies were observed on the MAC agar. There were 2 out of 22 isolates (9.1%), showing purple colored and *Bacillus* in chain under microscopic examination that were presumed as *Bacillus* species (Table I).

**Biochemical test**

There were 27.27% of the isolates presumed as *Staphylococcus* spp. appeared to be positive for catalase and methyl red tests, negative to indole, voges-proskauer and citrate tests. Next, 63.63% of the isolates presumed to be *Proteus* spp. appeared to give positive reaction to catalase, methyl red and voges-proskauer, while negative reaction was observed in indole and citrate tests. The remaining 9.1% of the presumed as *Bacillus* spp. presented positive reaction to catalase, methyl red and voges-proskauer, while negative to indole and citrate (Table II).

**Table I: The results of macroscopic and microscopic examination for *Staphylococcus* spp., *Proteus* spp. and *Bacillus* spp. isolates from *A. molpadioides***

| Bacteria Species | Macroscopic Examination (MacConkey agar) | Microscopic Examination (Gram Stain) |
|---|---|---|
| *Staphylococcus* spp. | <br>Small, wet and light pink colony, diameter 0.2 cm.<br>Non-lactose fermenter. | <br>Magnification: 100x<br>Gram: Positive<br>Color: Purple<br>Shape: Cocci in cluster |
| *Proteus* spp. | <br>Big, dry and pale colour colony, diameter 0.9 cm.<br>Non-lactose fermenter. | <br>Magnification: 100x<br>Gram: Negative<br>Color: Pink<br>Shape: Bacillus in chain |
| *Bacillus* spp. | <br>Dry and pale colour colony, diameter 0.6 cm.<br>Non-lactose fermenter. | <br>Magnification: 100x<br>Gram: Positive<br>Color: Purple<br>Shape: Bacillus in chain |

**Antibiotics susceptibility test**

Six samples presumed as *Staphylococcus* spp. were susceptible towards ampicillin and tetracycline. However, all of these isolates were intermediate resistance towards streptomycin. Comparatively, 14 samples presumed as *Proteus* spp. were susceptible

**Table II: The results of biochemical tests for *Staphylococcus* spp., *Proteus* spp., and *Bacillus* spp.**

| Bacteria Species | Biochemical Tests | | | | |
|---|---|---|---|---|---|
| | Catalase | Indole | Methyl Red | Voges-Proskauer | Citrate |
| *Staphylococcus* spp. | + | - | + | - | - |
| *Proteus* spp. | + | - | + | + | - |
| *Bacillus* spp. | + | - | + | + | - |

towards ampicillin while intermediate resistance towards streptomycin, and resistance towards tetracycline. Two samples presumed as *Bacillus* spp. were susceptible towards tetracycline, intermediate resistance towards ampicillin and resistance towards streptomycin. The results of antibiotics susceptibility tests for these isolates were shown in Table III.

**Table III: Antimicrobial susceptibility pattern of various bacteria isolated from the *Acaudina molpadioides***

| Bacteria Species | Ampicillin (10 μg) | Tetracycline (10 μg) | Streptomycin (10 μg) |
|---|---|---|---|
| *Staphylococcus* spp. | Susceptible | Susceptible | Intermediate |
| *Proteus* spp. | Susceptible | Resistance | Intermediate |
| *Bacillus* spp. | Resistance | Susceptible | Intermediate |

## DISCUSSION

### Macroscopic and microscopic examinations

In this study, there was no bacteria presence nor growth on the TCBS and HE agar. This could be due to the bacterial abundance, water temperature and marine environment (9). There were 6 out of 22 of the *A. molpadioides* isolates presented with growth of small, wet, light pink colonies with lactose fermenter on MAC agar. The microscopic examination results showed purple color and cocci in cluster bacteria. According to the Merck Millipore standard operation procedure (10), *Staphylococcus* spp. were appeared as small, opaque and isolated colonies. Palilu and Budiarso (2017) (11) stated that *Staphylococcus* can cause serious food poisoning which able to produce enterotoxin in food. In their study, Baird Parker Agar (BPA) was used to isolate the sample and BPA was a selective media to *Staphylococcus* spp., *Proteus* spp. and *E. coli.*

In this study, 14 out of 22 of the *A. molpadioides* isolates were presented with big, dry, non-lactose fermented and pale color colonies on MAC agar with pink colored *bacillus* in chain under microscopic examination. *Proteus* spp. is a non-lactose fermenter, appeared pale, convex, may swarm around the agar and have a strongly foul odor. They are Gram-negative and facultative anaerobic. In Palilu and Budiarso (2017) study, E. coli appeared as big brown black colored colony on the BPA, while *Proteus* spp. appears as brown-black colored colony on the BPA (11). *Proteus* spp. do not ferment the lactose and appear colorless colony on MAC. However, the *Proteus* colonies will appear a large, smooth and gray on blood agar. *Proteus* spp. often produce special characteristic, which will produce a foul smell and swarming on the agar plate (8).

Dry, non-lactose fermented and pale color colonies were observed on MAC agar. There were 2 out of 22 samples of *A. molpadioides* showed purple color *bacillus* in chain under microscopic examination. According to Lu et al., (2018), *Bacillus* spp. will show rough and colorless colonies (13). The study conducted by Tallent et al., (2012), *Bacillus cereus* on Mannitol egg yolk polymyxin (MYP) agar plates showed pink colonies while on the Bacara agar plates showed pink-orange colonies (14).

In this study we presumed that the isolated bacteria from the sea cucumber *A. molpadioides* were *Staphylococcus* spp., *Proteus* spp. and *Bacillus* spp. based on their morphological characteristics by referring to the Cowan et al., (2005) (15). To date, there was no study on screening of bacteria from *A. molpadioides* conducted before. This is the first study reporting on the isolation of these bacteria. The presence of Staphyloccus spp., *Proteus* spp. and *Bacillus* spp. in seafood may cause food poisoning and other infections in human. However, staphylococcal and other non-spore bacterial poisoning can be avoided by heat treatment of food. Ingestion of raw and undercooked sea cucumber is not advisable.

### Biochemical tests

In this study, 27.27% of the sea cucumber isolates appeared to be negative in indole, voges-proskauer and citrate tests while positive in methyl red and catalase tests. According to El-Hadedy and El-Nour (2012), *Staphylococcus* spp. were appeared to be positive in catalase, methyl red and voges-proskauer tests, while negative in indole test (16). From Chakraborty et al., (2011), all isolated *Staphylococcus* aureus showed positive results in urease test and 50% isolates showed positive in starch hydrolysis test (17). Total of 100% isolates ferment the lactose but not sucrose. Aryal et al., (2018) stated that *Staphylococcus* aureus was a non-capsulated bacteria and showed positive reaction in catalase test, citrate test, coagulase, methyl red test, voges-proskauer and urease test while negative in Indole test (18).

About 63.63% of the isolates in this study appeared to be negative in indole and citrate tests, positive to methyl red, voges-proskauer and catalase test. Based on Ahmed et al., (2006), *Proteus* spp. appeared to be positive for catalase and methyl red tests, negative in voges-proskauer, citrate and indole tests (19). According to Kishore (2016), *Proteus* penneri utilized citrate and produced hydrogen sulphide (20). Aryal et al., (2017) stated that *Proteus mirabilis* was non-capsuled, positive in catalase, hydrogen sulphide and citrate tests, have flagella and a Gram negative bacteria (21).

The remaining of 9.1% of isolates showed negative in indole and citrate tests, positive in methyl red, voges-proskauer and catalase tests. Al-Allaf et al., (2011) reported that *Bacillus* spp. appeared to be positive for catalase, voges-proskauer and citrate tests, while negative in methyl red and indole tests (22). Rajashekhar et al. (2017) reported that *Bacillus* spp. showed positive reaction to nitrate and catalase tests, it also can utilize the citrate as a carbon source (23). Aryal et al., (2017) also reported that *Bacillus subtilis* can ferment arabinose, positive in voges-proskauer, catalase and citrate tests, was flagellated, did not produce any gas and a Gram positive bacteria (24). However, *Bacillus sabtilis* was negative in indole and methyl red tests, was a motile organism that produced variable results in oxidase test. Biochemical tests of the bacteria isolated showed their unique characteristics for reactions on all the tests. However, in this study, all the bacteria can only be identified based on genus level and further tests using molecular methods should be conducted for species level identification.

**Antibiotics susceptibility test**

Previous study conducted by Chudobova et al., (2014) reported that *Staphylococcus* spp. were not resistant towards ampicillin, streptomycin, penicillin and tetracycline (25). In this study, all isolates presumed to be *Staphylococcus* spp. were susceptible towards ampicillin and tetracycline, intermediate susceptible towards streptomycin. The species isolated from this study and Chudobova et al., (2014) might be different although they have the same genus. Hence the difference in the antibiotics susceptibility tests result. Based on the study of Chakraborty et al., (2011), S. aureus were 20% resistant towards kanamycin and 46.6% resistant towards oxacillin (17).

Ahmed et al., (2006) found that *Proteus* spp. were resistant to ampicillin, streptomycin and tetracycline (19). On the other hand, this study discovered all 14 isolates presumed as *Proteus* spp. were susceptible to ampicillin, intermediate susceptible towards streptomycin but revealed resistant towards tetracycline. According to Kishore (2012), *Proteus penneri* has higher resistancy towards ampicillin, first and second generation of cephalosporins than *Proteus mirabilis* (20).

A study conducted by Agyare et al., (2018) reported that *Bacillus* spp. strains were susceptible to tetracycline (26). In this study, two isolates presumed as *Bacillus* spp. were susceptible to tetracycline, intermediate susceptible to ampicillin, however, resistant to streptomycin. According Rajashekhar et al., (2017) , *Bacillus* spp. isolated from insects were sensitive to streptomycin, tetracycline but resistant to ampicillin (23).

Antibiotic susceptibility and resistancy of these bacteria can represent as a preliminary findings as basis for bacterial infection treatment and in preventing the spread of antimicrobial resistance microorganism.

## CONCLUSION

In conclusion, the results indicate that sea cucumbers *A. molpadioides* were contaminated with potentially pathogenic bacteria such as *Staphylococcus*, *Proteus* and *Bacillus*. Thus, consumers are advised to avoid eating raw and undercooked sea cucumber *A. molpadioides*. The antibiotic resistant patterns also showed that there is a need to revise and reconsider the treatment for these bacterial infection. For future study, the sample size needs to be increased to investigate the prevalence of *Staphylococcus* spp., *Bacillus* spp., and *Proteus* spp. in *A. molpadioides* in Pulau Langkawi, Kedah, Malaysia. Rapid molecular techniques such as polymerase chain reaction (PCR) and serological methods should be conducted to confirm the identity of the bacteria species.

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