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To cite this article: Rahmi *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **253** 012013

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## ***In Vitro* Pathogenicity of Bacterial Brown Band Disease on *Acropora* sp.**

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**Abstract.** This study aimed to determine which bacteria trigger brown band disease (BrB) through assessing the level of pathogenicity of bacteria present and microscopic histological analysis of coral tissue cells. The study was conducted from October 2014 to March 2015. The bacterial culture stage was conducted at the Faculty of Medicine Microbiology Laboratory, Universitas Hasanuddin, the histological analysis was conducted at the, Maros Veterinary Centre Pathology Laboratory, and pathogenicity tests of bacteria from brown band disease on healthy corals were conducted at the Marine Station Hatchery of the Faculty of Marine Science and Fisheries, Universitas Hasanuddin, on Barrang Lompo Island. Pathogenicity tests indicated that post-infection time before the appearance of BrB symptoms was different for each bacteria tested. The fastest-acting bacterial strain was *Acinetobacter* sp. RA3849 ORF, which caused disease symptoms in 5 hours, followed by *Streptococcus* sp. YM395 (9 hours), *E. faecalis* C56 (20 hours), and *Vibrio alginolyticus* H2X5 (22 hours). *Acinetobacter* sp. also produced the highest rate of infection spread in *Acropora* sp., reaching 5.05 cm/day. Histological observation showed tissue damage (necrosis) due to bacterial infection in the epidermal layer and hyperplasia of the gastrodermal layer, where there was an abnormal increase in abnormal tissue or organs due to an increase in the number of cells. The *in vitro* pathogenicity test showed that *Acinetobacter* sp. strain RA3849 reacts as a trigger of BBD disease.

### **1. Introduction**

Diseases that infect coral have been widely documented, including *Brown band disease* (BrB) [1]. Some of these coral reefs have been damaged by diseases and are predicted to increase every year. In recent years, reports of coral disease indicate increasing prevalence and severity [2,3,4,5,6].

Although the occurrence and symptoms of diseases that infect corals have been widely documented, e.g. black band disease [7], microbial infection [8], and brown band disease (BrB) [1], nevertheless, the aetiology of coral disease is not yet fully understood. Some of the diseases are caused by bacteria and fungi, others by biological agents which are as yet unknown, but which can affect coral health [9,10,11]. In some cases, the pathogens and/or the causal mechanism through which pathogenic organisms cause disease are not yet known for sure [6].

Macroscopic observation of coral colonies infected by brown band disease shows a dark brown band that occurs on both healthy tissue and white (bleached) tissue. This disease is active in warm temperatures,



especially during warm summers [12]. Despite the fact that this disease has been studied widely, it has been found difficult to identify the causative agent of this disease [13]. Observations made on Caribbean coral reefs have reported BBD infection advancing by as much as 6.2 mm/day [14]. while on Davies Reef the average tissue loss in *Acropora* coral colonies infected by BrB was 0.3 to 9 cm/day, in some cases even faster [15].

Despite the virulence of this disease, the mechanisms of bacterial pathogenicity underlying the emergence of brown band disease are poorly known. This study aimed to determine which bacteria trigger brown band disease (BrB), through assessing the level of pathogenicity of bacteria present and microscopic histological analysis of coral tissue cells.

## 2. Material and Methods

### 2.1. Time and Location

This study was conducted from October 2014 to March 2015. The bacterial culture stage was performed at the Faculty of Medicine Microbiology Laboratory at Hasanuddin University; histological analysis was carried out in the Pathology Laboratory of the Maros Veterinary Centre, and the brown band disease pathogenicity analysis (through challenging healthy corals) was undertaken at the Marine Station Hatchery on Barrang Lompo Island, belonging to the Faculty of Marine Science and Fisheries, Hasanuddin University.

### 2.2. Bacterial culture

Bacterial isolates obtained during stage 1 were cultured by growing a 1 oz. aliquot of each bacterial isolate on *sea water complete* (SWC) media, with the following composition: 5 g *bacto-peptone*, 5 g yeast extract, 3 mL glycerol, 250 ml distilled water, 750 mL sterile sea water, and 20 g *bactoagar*. The bacterial cultures were then incubated at 28 °C for 24 hours. Isolates cultured in solid media were grown on SWC liquid media, using a shaker incubator at 140 rpm and 28 °C, for 24 hours. The bacterial isolate concentrations were then calculated using the MacFarland method. The population of transmission bacteria should reach 10<sup>6</sup> CFU/mL [16].

### 2.3. Coral Acclimatization

Prior to the pathogenicity challenge test, the corals were acclimatized for 5 days following the protocol in [17]. The purpose of this acclimatization was to adapt the corals to new environmental conditions and ensure that there was no contamination with pathogenic microorganisms before the start of the experimental treatments. The test containers (aquaria) were equipped with aeration and water circulation. This test was carried out *in vitro* with aquaria 45 cm x 30 cm x 30 cm, and a sea water media volume of 80 litres.

### 2.4. Bacterial Pathogenicity Test

Once a bacterial concentration of 10<sup>6</sup> CFU/mL was reached for each type of bacteria isolated from corals infected with the BrB disease [16], healthy corals were exposed to (infected by) the bacteria. This challenge test was carried out on healthy corals, with 3-5 coral fragments per aquarium (n = 3). The bacterial pathogenicity test was performed on healthy corals using an immersion system. The bacterial concentration used was 10<sup>6</sup> CFU/mL, injected into the test maintenance medium (aquarium seawater). The test corals were observed for 144 hours to check for symptoms of disease transmission until infection was seen to have been successful. Water quality parameters (temperature, salinity, and dissolved oxygen) were maintained in the normal/ideal range (temperature 27-29 °C, salinity 30-32 ppt, and dissolved oxygen 4.8-

6.2 mg/L). The infected corals showed a colour change observed using Nikon Collfix P7100 underwater camera. The width of the discoloured area (in mm) was measured using callipers.

### 2.5. Preservation of samples and tissue

Diseased coral samples (1-2 cm) for histological analysis were first preserved through soaking for 12 hours in a solution prepared with 4% paraformaldehyde (wt/vol) and sterile PBS (Phosphate Buffered Saline) solution (pH 7.4) [18], then decalcified with BNF 10% (pH 8) [19] as a standard preparatory procedure for embedding in paraffin. The tissue was then processed through washing as follows: washing with 70% alcohol and 90% (once with each), followed by absolute ethanol for 1 hour (3 times) and then for 2 hours, washing with xylol for 1.5 hours, then washing with liquid paraffin (70 °C) three times for 1.5 hours, and finally stored in paraffin.

## 3. Result and Discussion

The results of the pathogenicity test showed that the lead time between the introduction of pathogenic bacteria and the appearance of BrB symptoms was different for each bacterial strain tested (Figure 1). The bacterial strain that caused symptoms of the disease to appear most rapidly post infection was *Acinetobacter* sp. RA3849 ORF (5 hours), followed by YM395 *Streptococcus* sp. (9 hours), *E. faecalis* C56 (20 hours), and finally *Vibrio alginolyticus* H2X5 (22 hours). *Acinetobacter* sp. strain RA3849 PHA is an opportunistic pathogenic microorganism [20] and some of the strains in this genus are obligate pathogens that are unable to live without a host [21].

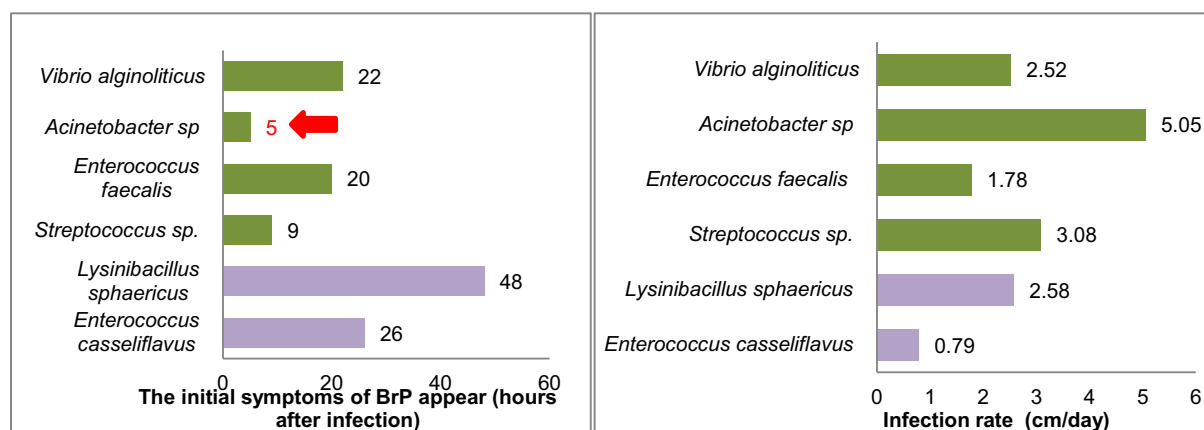


Figure 1. The time post-infection of initial appearance of BrB attacks on healthy *Acropora* sp. fragments challenged by bacterial isolates from the bands of BrB infected colonies. Bacteria isolated from healthy coral fragments (purple bars) and bacteria isolated from coral fragments on BrB bands (green bars) are shown. The earliest appearance of BrB symptoms is indicated by a red arrow.

Based on Figure 1, one isolate (*Acinetobacter* sp. RA3849 ORF) was obtained which induced the symptoms of BrB very rapidly (5 h post-infection). Furthermore, the *Acinetobacter* sp. bacteria resulted in the highest infection spreading rate, 5.05 cm/day. This indicates that *Acinetobacter* sp. is a strong candidate as a pathogen causing or triggering BrB disease in *Acropora* sp. This group of bacteria has the ability to form colonies on the host, can successfully compete with other bacteria, is resistant to host defence mechanisms, and can produce pathogenic changes either directly or indirectly through stimulation of inflammation [22]. This type of bacteria has previously been reported as associated with BrB [22].

The highest infection spreading rate in *Acropora* sp. challenged by the bacterium *Acinetobacter* sp. was 5.05 cm/day, which is slower than the maximum average tissue loss rate in *Acropora* sp. infected with BrB reported from Davies Reef, which ranged from 0.3 to 9 cm/day, and in some cases tissue loss due to BrB infection can be even faster [15]. An example of the progress of the disease is shown in Figure 2.

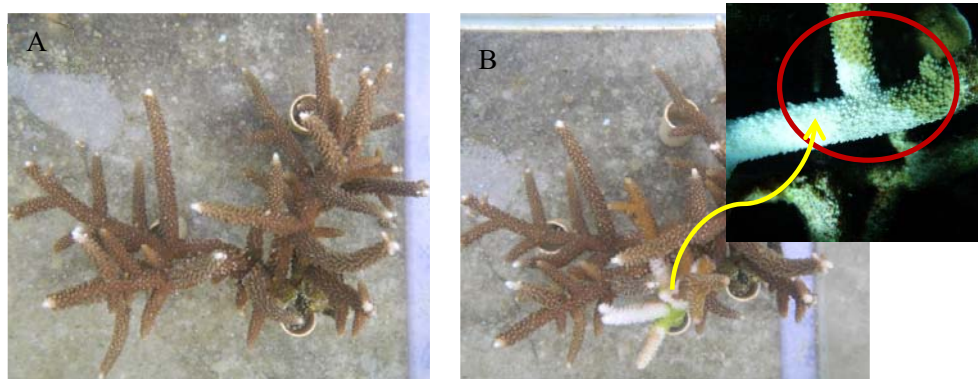


Figure 2. The initial appearance of BrB attacks on *Acropora* sp. fragments challenged with bacterial isolates of *Acinetobacter* sp. RA3849 ORF (A = Before the challenge test, B = after the challenge test).

The histological conditions of *Acropora* sp. tissue after challenge with BrB bacterial isolates (Figure 3) show the presence of bacterial aggregations forming an inclusion body in the mesoglea layer, as described by [1]. These aggregations of bacteria were found in the epidermal tissue of infected *Acropora* sp. specimens.

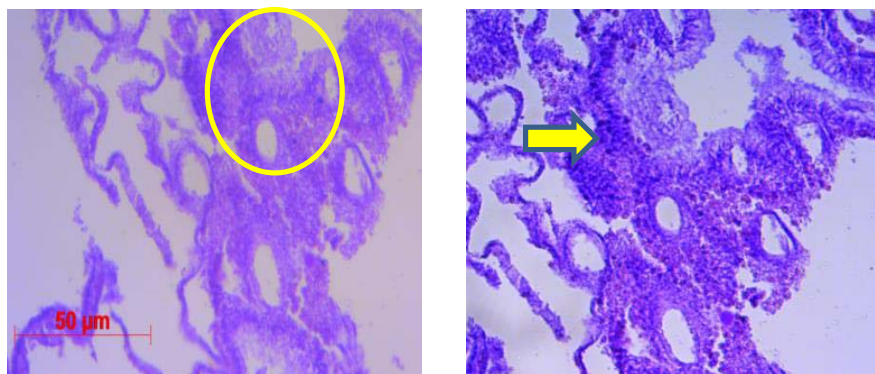


Figure 3. Aggregation of bacterial cells in *Acropora* sp. infected with BrB (400X magnification), yellow colour indicates bacterial aggregation.

These bacterial aggregations were not found in all of the samples analysed. This patchy presence is presumably due to the bacterial infection proceeding through several stages that can weaken *Acropora* sp. but are not identical between individual hosts. This is in line with reports that necrosis in *Porites* sp. only occurs in a proportion of samples [24]. Cell death in corals due to tissue loss has been widely reported [25,26,27], and is accepted as a common response to disease. Bacterial aggregations have also been found in *Acropora* spp. coral tissue infected with bacteria from other diseases [28], although bacterial aggregations are not always found to cause or be associated with injury or other diseases [18,29].

#### 4. Conclusion

The results of testing for pathogenicity *in vitro* showed that the bacterium *Acinetobacter* sp. strain RA3849 is a strong candidate as a trigger for BrB disease

#### Acknowledgements

This work was supported by a USAID, PEER Project grant. The authors also wish to thank ZMT Bremen Germany for the use of research facilities.

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