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Short Notes

Screening for Antibiotic-Producing Bacteria from Imbak Canyon Conservation Area (ICCA)

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Abstract

Antibiotic resistance is an escalating threat to public health. Therefore, there is an urgent need for new antibiotics. This study aims to screen for antibiotic-producing microorganisms from the forest soil of Batu Timbang. Soil samples were collected, diluted and spread plated onto 1/5 Nutrient Agar (NA) and Actinomycete Isolation Agar (AIA) for the isolation of antibiotic-producing microorganisms. A total of 180 bacterial isolates were screened for their antibiotic-producing ability, and ten were tested positive for inhibitory activity against one or more test pathogens via agar overlay assay (*Staphylococcus aureus* ATCC BAA-1717, *Enterococcus faecalis* ATCC 700802, and *Acinetobacter baumannii* ATCC BAA-1605). Ten bacterial isolates were subjected to 16S rRNA gene amplification and gene sequence analysis. The isolates were identified to be closely related to the genus *Variovorax*, *Streptomyces*, *Kitasatospora*, *Chromobacterium*, *Burkholderia*, *Pseudomonas* and *Massilia*. Three isolates (*Variovorax* sp. A5, *Variovorax* sp. A6 and *Kitasatospora* sp. H8) are potentially novel as these isolates form a different clade from their respective closely related species via phylogenetic tree analysis using reference sequences obtained from GenBank/EMBL/DDBJ databases. The antibiotics produced by the bacterial isolates might potentially be new, as novel species might possess unique biosynthetic gene clusters to produce new compounds. Nevertheless, further taxonomic identification and antibiotic isolation work is required. This study has revealed the potential of antibiotic discovery from Batu Timbang (Imbak Canyon Conservation Area) and its implications in tackling antibiotic resistance.

Keywords: Antibiotic, antibiotic-resistance, bio-prospecting, conservation, forest soil, Imbak Canyon Conservation Area

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Introduction

The extensive usage of antibiotics in the past few decades has caused an increase in the emergence and prevalence of antibiotic-resistant bacteria (ARB) (Holmes et al., 2016). The emergence of antibiotic-resistant bacteria is an inevitable event, as it is an evolutionary process during antibiotic therapy due to selection pressure. Some examples of ARB are methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, and *Acinetobacter baumannii* (Martens & Demain, 2017).

S. aureus is a major example of ARB that causes life-threatening infections. The first line therapy for *S. aureus* infection is beta-lactam antibiotics such as penicillin, cephalosporin and methicillin (Loubet et al., 2018). The emergence of methicillin-resistant *S. aureus* (MRSA) strains essentially indicates that these strains are resistant to all currently available beta-lactam antibiotics. Consequently, infections caused by MRSA must be treated with other non-beta lactam antibiotics such as vancomycin, linezolid and daptomycin (Kaur and Chate, 2015). However, studies have reported the emergence of *S. aureus* strains resistant to these non-beta lactam antibiotics, suggesting that *S. aureus* has gained resistance even to the last few options of antibiotic therapy (Silva et al., 2019; Yang et al., 2018).

Enterococci are another example of ARB which are known to be difficult to treat due to their intrinsic resistance and ability to acquire resistance determinants (Ong et al., 2017). Enterococci are currently ascendant nosocomial pathogens to cause bacteremia, nosocomial urinary tract and wound infections. Vancomycin has been consistently being relied on for the treatment of infections caused by multidrug-resistant enterococci (O'Driscoll and Crank, 2015). As vancomycin is used widely to treat MRSA and other gram-positive bacterial infections, certain enterococci species such as *Enterococcus faecalis* and *Enterococcus faecium* have gained resistance towards vancomycin, hence limiting the treatment options to other antibiotics, for instance linezolid, daptomycin, quinupristin/dalfopristin, and tigecycline (Ahmed and Baptiste, 2018).

A similar pattern of antibiotic resistant can be seen in gram negative bacteria such as *Acinetobacter baumannii*. This bacterium is resistant to most antibiotics that are currently being used to treat gram-negative bacterial infections globally (Asif et al., 2018). Despite this, the current progress on the development of new antibiotics is slow due to biological and pharmacological drawbacks (Brown &

Wright, 2016). Thus, there is a dire need to discover new antibiotics to treat ARB infections (Martens & Demain, 2017).

One strategy to look for new antibiotics is by exploring untapped resources such as the deep-sea sediments and pristine forests (Wang et al., 2013; Ong et al., 2016). Microorganisms from these environments have always been the main source of new antibiotics, as they might have evolved to develop unique gene clusters for the biosynthesis of new compounds. When the competition is intense, microorganisms will tend to produce compounds such as antibiotics to secure their niches (Ong et al., 2016). As a result, the attempt to prospect for new antibiotics from unexplored environments remains a great research opportunity. A similar approach was adopted by Lo et al., (2000) and resulted in the positive isolation of actinomycetes from dipterocarp rainforest soils in Borneo. These actinomycetes possess various biological activities such as anti-cancer properties (Yip et al., 2010) and *Mycobacterium* isocitrate lyase inhibitor (Shin et al., 2009). Hence, by using the same technique, the objectives of this study were to isolate and to identify potential antibiotic-producing bacteria from the forest soil of Imbak Canyon Conservation Area (ICCA).

Methods and materials

Soil sampling

Soil samples were collected mainly in three localities: (1) the ICCA, as well as the area designated for the Batu Timbang scientific expedition which include the (2) Rafflesia track and (3) Lanap track. Each localities was sampled randomly six times with each sampling site being at least 100 m apart. The scientific expedition was carried out from 16 to 26 August 2017. The leave litters and top soils (~ 5 mm) were first removed, and approximately 50 g of soil samples were collected using sterile spatulas into sterile 50 mL Falcon tubes. The Falcon tubes with soil samples were then kept at room temperature in sealed polyethylene bags. All samples were transported to Monash University Malaysia for processing immediately after the expedition. Prior to processing, large roots and stones were first removed, followed by soil dilutions and cultivation experiments.

Isolation of bacteria and Actinomycetes

One gram of each soil sample was immersed in 0.85% (w/v) saline and serially diluted up to 10^{-8}. The diluents were then spread plated onto 1/5 NA (Merck, Germany) containing 100 µg/mL cycloheximide for the isolation of bacteria (Ong et al., 2015), and onto AIA (Becton Dickinson, UK) for the isolation of Actinomycete (Rashad et al., 2015). All media plates were incubated at 30°C

aerobically for 5 days. Ten representative colonies were randomly selected using the Harrison's disc method. These isolates were re-streaked onto their respective media and incubated at 30°C aerobically for 5 days to obtain pure cultures.

Isolation and screening for antibiotic-producing bacteria

The soil isolates were patched onto their respective plates by using sterile toothpicks and incubated at 30°C aerobically for 5 days. The inhibitory activity of the soil isolates against the test pathogen was assayed using agar overlay assay. The test pathogens that were used in this study include *Staphylococcus aureus* ATCC BAA-1717, *Enterococcus faecalis* ATCC 700802, and *Acinetobacter baumannii* ATCC BAA-1605. Briefly, 9 mL of 0.9% (w/v) tryptic soy agar (Merck, Germany) containing 100 µL of test pathogen strains (adjusted to 2.0 McFarland standard OD_{625} 0.32-0.40, approximately 6×10^8 CFU/mL) was overlaid on top of the patched plate and incubated at 37°C aerobically for 18-24 hours. The presence of a halo zone around the patched isolate indicates antibiotic activity against the test pathogen strains. The screening was carried out in triplicate and the annular radiuses of the inhibition zones were measured using a ruler.

DNA extraction, amplification and phylogenetic analysis

Microbial isolates with positive antibiotic activity were subjected to 16S rRNA gene sequence amplification and phylogenetic analysis. DNA extraction was performed by suspending bacterial colonies in 50 µL of sterile distilled water in PCR tubes and heated at 100°C for 3 min. The suspension was then centrifuged at 13000 × *g* for one min. The supernatant containing the bacterial DNA was transferred into a new PCR tube and used as DNA template for the following PCR reaction. The 16S rRNA gene sequences of the isolates were amplified using the universal primers 63F (5' – CAG GCC TAA CAC ATG CAA GTC – 3') and 1387R (5' – GGG CGG WGT GTA CAA GC – 3') (Marchesi et al., 1998). The PCR was set up as follow: 5 µL of DNA extract, 10 µL of 5 × MyTaq Red Reduction Buffer, 5 µM of forward primer, 5 µM of reverse primer and 1.25 U of MyTaq DNA polymerase. The reaction volume will be made up to 50 µL using sterile filtered milliQ water (Millipore, Germany). The PCR included an initial denaturation step at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 45 sec, and elongation at 72°C for 45 sec. PCR products were separated on a 1.5% (w/v) agarose gel in TAE buffer and the bands were visualized with 1 × GelRed. The 16S rRNA gene sequences of the isolates were aligned with sequences of closely related type strains retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL-X software (Thompson et al., 1997). The alignment was manually verified and adjusted prior to the

construction of phylogenetic tree using the neighbor-joining (Saitou & Nei, 1987) algorithm with the MEGA version 6.0 software (Tamura et al., 2011). The stability of the resultant tree topologies was evaluated by using the bootstrap resampling method (Felsenstein, 1985). The evolutionary distance for the neighbour-joining algorithm was computed using the Kimura's two-parameter model (Kimura, 1980).

Results and Discussion

A total of ten out of 180 isolates were tested positive for antibiotic activity against one or more test pathogens, as determined via agar overlay assay (Figure 1).

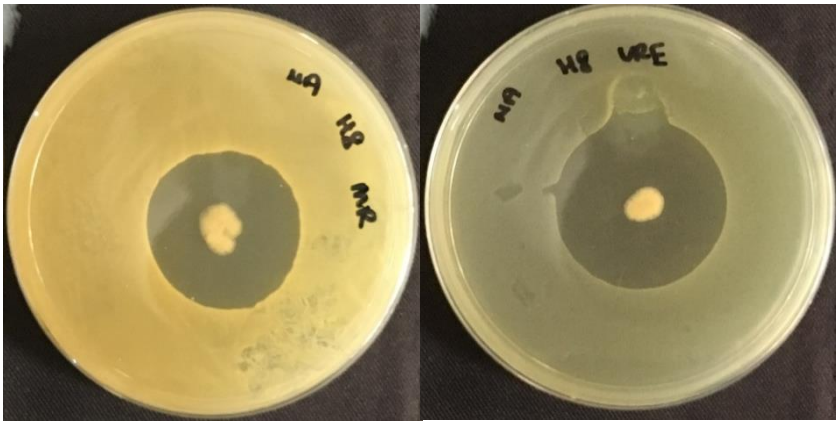

Figure 1. Antibiotic activity of isolate H8 against *S. aureus* ATCC BAA-1717 (left) and *E. faecalis* ATCC 700802 (right).

Staphylococcus aureus ATCC BAA-1717 is an opportunistic pathogen that is resistant to many conventional beta-lactam antibiotics, including methicillin, and has been reported to cause an epidemic in the USA (Tenover and Goering, 2009). Pathogens that are resistant to methicillin are resistant to most of the currently available beta-lactam antibiotics, hence limiting the treatment option to the last resort antibiotics such as vancomycin or daptomycin. Eight bacterial isolates (A5, A6, B8, D4, D7, E4, H2, and H8) were able to inhibit *S. aureus* ATCC BAA-1717 and could be potentially important species that can be further studied (Table 1).

Table 1. Annular radius of inhibition against three different test pathogens

Soil isolates	Annular radius of inhibition (mm)		
	S. aureus ATCC BAA-1717	*E. faecalis* ATCC 700802	*A. baumanii* ATCC BAA-1605
A5	2.7 ± 1.2	NA	NA
A6	2.7 ± 1.2	NA	NA
B8	6.0 ± 2.6	3.7 ± 1.5	NA
D4	2.3 ± 0.6	2.3 ± 0.6	NA
D7	3.3 ± 1.5	2.0 ± 1.0	NA
E4	1.7 ± 0.6	1.7 ± 0.6	NA
F1	NA	NA	7.0 ± 1.0
E9	NA	NA	4.7 ± 0.6
H2	14.3 ± 1.5	9.7 ± 1.2	NA
H8	14.0 ± 1.0	12.3 ± 2.1	NA

Values are reported as mean annular radius of inhibition zone ± SD in mm (n=3). NA = no activity.

Apart from this, isolates B8, D4, D7, E4, H2, and H8 could inhibit the growth of *Enterococcus faecalis* ATCC 700802, which is a pathogen known for its ability to resist the action of vancomycin (Ahmed and Baptiste, 2018). This bacterium is difficult to treat as it is intrinsically resistant to many classes of antibiotic. Vancomycin-resistant enterococci account for one-third of the enterococcal healthcare-associated infections in the USA and for more than 20% of such infections in certain European countries (Balli et al., 2014).

Lastly, two isolates (F1 and E9) were able to inhibit the growth of *Acinetobacter baumannii* ATCC BAA-1605. *A. baumannii* can cause complications such as wound infections, urinary tract infections, and meningitis (Peleg et al., 2008). It is particularly pathogenic due to its intrinsic resistance mechanisms towards many antibiotics (Cerceo et al., 2016). Since the isolates obtained showed positive antibiotic activity against the three clinically-relevant pathogens, it was of our interest to carry out further work to determine their identities. This was achieved through 16S rRNA gene sequence amplification, followed by cross-referencing with the National Centre of Biotechnology Institute (NCBI) database. Seven out of the ten isolates had more than 99% gene sequence similarity to their corresponding top-hits, indicating that these isolates could belong to the same genus and species, but of a different strain. On the other hand, three soil isolates – A5, A6, and H8 showed gene sequence similarity with less than 99%, suggesting that these isolates could potentially be novel bacterial species (Table 2). Novel microorganisms thriving in the environment might develop unique biosynthesis gene clusters giving rise to potentially novel compounds (Imhoff et al., 2011). These compounds with antibiotic activity could be useful in the future to treat ARB infections. However, further work which include a complete

analysis on the phenotypic, genotypic, and chemotaxonomic of these isolates, as well as the isolation of the antibiotics are warranted.

Table 2. Location, top-hit, and percentage similarity of the 10 antibiotic-producing soil isolates

Isolates	Location (track)	Top-hit	Similarity (%)
A5	Rafflesia	*Variovorax guangxiensis* GXGD002	98.84
A6	Rafflesia	*Variovorax guangxiensis* GXGD002	98.76
B8	Lanap	*Streptomyces xanthocidicus* NBRC 13469	99.29
D4	IC centre	*Kitasatospora cystarginea* JCM 7356	99.84
D7	IC centre	*Kitasatospora cystarginea* JCM 7356	99.84
E4	IC centre	*Chromobacterium vaccinii* MWU205	99.60
F1	Rafflesia	*Burkholderia ubonensis* CIP 107078	99.60
E9	IC centre	*Pseudomonas nitritireducens* WZBFD3-gA2	99.76
H2	Rafflesia	*Massilia violacea* CAVIO	99.20
H8	Rafflesia	*Kitasatospora cheerisanensis* KCTC 2395	98.52

Since isolate A5, A6, and H8 showed percentage similarity lower than 99%, phylogenetic trees using neighbor joining based on 16S rRNA gene sequences were constructed to reveal the relationship of these isolates with published species of the known genus. The phylogenetic trees are shown in Figure 2 and Figure 3. Both isolate A5 and A6 were closely related to *Variovorax gossypii* JM310 and *Variovorax guangxiensis* GXGD002 (Figure 2).

Variovorax is a group of gram-negative bacteria that can tolerate high concentrations of toxic metals. Moreover, this group of bacteria are known to degrade organic pollutants, hence have been suggested for biotechnological use (Chen et al., 2013; Satola et al., 2012). However, the antibiotic-producing property of *Variovorax* species has not been described thus far. Isolate H8 was found to be closely related to *Kitasatospora cheerisanensis* KCTC395 (Figure 3). *Kitasatospora* are gram-positive bacteria, which belonged to the group of rare-actinomycetes. Members of *Kitasatospora* are known to produce antimicrobial compounds such as talosins (Yoon et al., 2006) and fuzanins (Aida et al., 2009). Nevertheless, isolate H8 could be producing a different type of compound with antibiotic activity. This is because it forms a distinct arm from its nearest relatives, hence signifying that it might be a novel species. This is an advantage because novel species tend to possess new gene clusters for the biosynthesis of new compounds. Nonetheless, further work on the species identification and isolation of the antibiotics are warranted.

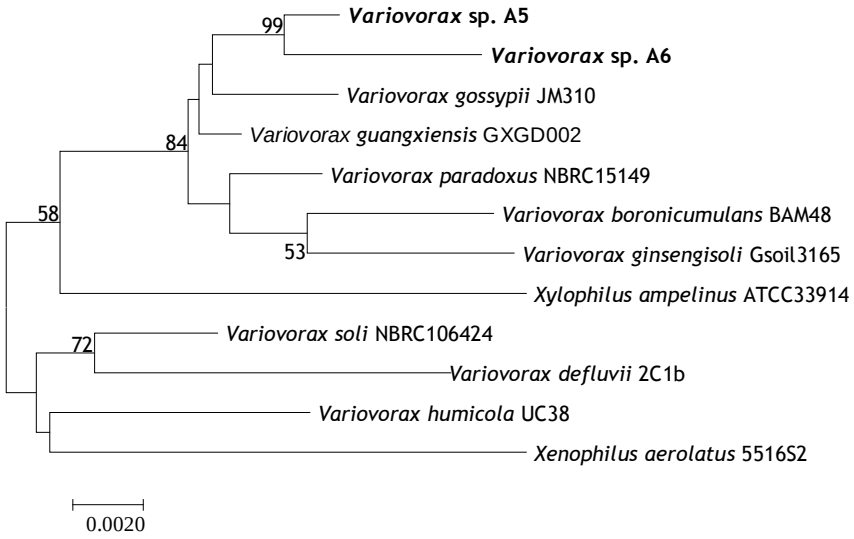

Figure 2. Neighbour-joining tree based on 16S rRNA sequences showing relationship between isolate A5 and A6 with their representatives related taxa. Bootstrap values (>50%) based on 1000 resampled datasets are shown at branch nodes. Bar, 2 substitutions per 1000 nucleotide positions.

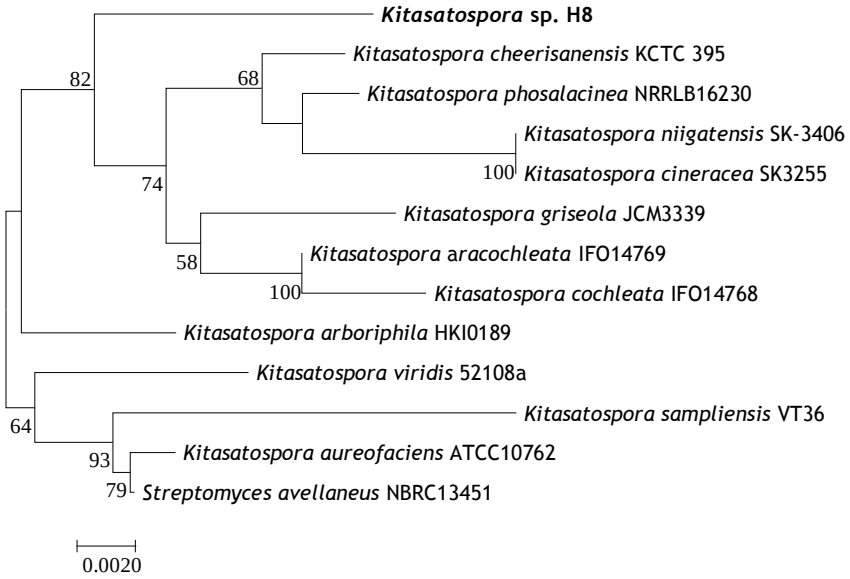

Figure 3. Neighbour-joining tree based on 16S rRNA sequences showing relationship between isolate H8 with their representatives related taxa. Bootstrap values (>50%) based on 1000 resampled datasets are shown at branch nodes. Bar, 2 substitutions per 1000 nucleotide positions.

Conclusions

The forest soils of Batu Timbang were found to be reservoirs for potentially novel bacteria with antibiotic producing ability. Three isolates – A5 and A6, as well as H8 were identified which belong to the genus *Variovorax* sp., and *Kitasatospora* sp., respectively. Preliminary work have shown that these isolates, along with their antibiotics could potentially be novel, hence, further work is required to fully determine their identities as well as to isolate the antibiotics. This study highlights the potential of antibiotic discovery from Imbak Canyon Conservation Area and its possible implications in addressing the issue of antibiotic resistance.

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