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Surveillance of *Burkholderia* spp. in Bukit Merah Orang Utan Island (BMOUI), Perak, Malaysia

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Melioidosis is an infection caused by *Burkholderia pseudomallei* and it represents one of the most common infectious diseases in zoo and farm animals giving rise to high mortality, leading to biological diversity loss. We use the bacteriological and biochemical methods such as culture, biochemical test, Gram-staining and oxidase test to examine the presence of *B. pseudomallei* in 50 soils treated and non-treated with quicklime powder collected from Bukit Merah Orang Utan Island, (BMOUI), Perak, Malaysia. We observed mixed bacterial colony growth with various colony morphologies. Six of the isolates labelled as P2(25), P5(2), P5(3), P5(5), P6(1), and P6(6)2 showed pinkish purple colour with wrinkled characteristics similar to *B. pseudomallei* colony morphology after incubation for 48 h. The results showed that all putative soil-isolated organisms were Gram-negative bacteria, positive for oxidase test and negative for catalase test. In the VITEK® ID system and PCR sequencing analysis, no *B. pseudomallei* was identified. All six putative organisms were identified as in *B. cepacia* complex i.e.: *B. stagnalis* (four cases), *B. contaminans* (one) and *B. multivorans* (one). These preliminary findings demonstrate that a few species of *B. cepacia* group were successfully isolated from the soil of BMOUI using selective media for *B. pseudomallei*.

Keywords: *Burkholderia* sp., conservation, melioidosis, Orang Utan, soil

Orang Utan (*Pongo pygmaeus)* is a critically endangered species that needs serious attention to sustain its existence. Due to the massive activities such as logging, poaching and deforestation, Orang Utan has lost its natural habitat and being harmed by the activities. In order to overcome the declining number of Orang Utan, Bukit Merah Orang Utan Island (BMOUI) in Bukit Merah, Perak has been developed as an *ex situ* conservation centre and is currently housing 16 Orang Utan there.

The conservation of Orang Utan is a complex process since it involves both human and environmental factors (Hayashi *et al*., 2018). The health of Orang Utan must be well monitored to ensure the sustainability of the endangered species outside its natural habitat (Kasantikul *et al*., 2015). Disease attack has been documented as a major cause of extinction among many species either in the wild or in captive (Smith *et al*., 2006).

Generally, Orang Utan are primarily arboreal primates that build nests in trees, however they also spend more times on the ground, particularly after being introduced to the artificial environment (Kasantikul *et al*., 2015). Thus, Orang Utan are prone to infection caused by the soil bacteria as they have close intact with the soil. Soil is the reservoir for many opportunistic pathogens including *Burkholderia* spp. (Ginther *et al*., 2015). There are 19 species under the *Burkholderia* genus that play many essential roles in ecology and some of them may cause severe sepsis to the infected individual, such as *B. pseudomallei*, *B. mallei*, and *B. cepacia* (Inglis and Merritt, 2014; Hall *et al.*, 2015).

Melioidosis caused by *B. pseudomallei* is one of the deadly

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diseases threatening animals including Orang Utan which have been reported recently (Limmathurotsakul *et al.*, 2012; Kasantikul *et al*., 2015). In BMOUI, the frequency of melioidosis among Orang Utan is approximately 10% based on data from 2000 until 2018 as reported by Hayashi *et al*. (2018). Melioidosis is endemic in Southeast Asia and the reported seroprevalence of the disease among livestock animals in Malaysia is about 5.7% (Musa *et al*., 2012).

The acidic environment between pH 4.0 to 7.7 is optimum for the growth of *B. pseudomallei* in the soil. Meanwhile in the plantation industry, the quicklime or calcium oxide is widely used in managing the pathogenic disease due to its strong base property (Na-ngam *et al*., 2004). This study investigates the presence of *B. pseudomallei* in the soil of BMOUI and evaluates the effectiveness of quicklime used by BMOUI as preventive measure to control the risk of melioidosis infection among Orang Utan.

Materials and Methods

Study setting

Bukit Merah Orang Utan Island (BMOUI) at Peninsular Malaysia (5° 00’ 32.5” N, 100° 40’ 32.1” E) is formerly known as Pulau Panjang and is located in a 14-acre island covered with secondary tropical forest surrounded by 7000-acre of freshwater lake nearer to Bukit Merah Laketown Resort. It has climate and vegetation resemble that of Borneo and Sumatra nature, which is suitable for conservation of Orang Utan. BMOUI is established in February 2000 and officially opened for visitors. The visitors will walk through a secured tunnel while the Orang Utan are moving freely in open exhibit areas fenced with low electrical current. The overview of BMOUI is shown in Fig. 1.

Preliminary study on soil of BMOUI

A preliminary study was done to investigate the occurrence of melioidosis in BMOUI by referring to the medical history, clinical signs and post mortem reports of the Orang Utan. A set of questionnaires was also given to the veterinary officer of BMOUI to investigate the soil condition and soil management of the BMOUI conservation centre.

The consent approval was obtained from Bukit Merah Orang Utan Island Foundation (BMOUI), Bukit Merah Perak prior to sample collection. Beforehand, animal ethics approval was attained through Committee on Animal Research and Ethics Universiti Teknologi MARA (UiTM CARE: 289/2019) and Department of Wildlife and National Parks under research permit (JPHL&TN [IP]: 100-34/1.24 Jld 15[36]).

Fig. 1. The overview of Bukit Merah Orang Utan Island (BMOUI), Perak.

Soil sampling in BMOUI

Four outdoor exhibits where the Orang Utan were released during daytime and night holding enclosures were selected as sampling site. The sampling areas are shown in Fig. 2. The consensus guidelines for soil sampling as described by Limmathurotsakul *et al*. (2013) were followed with slight modification. The sampling area was divided into quicklime treatment area and non-quicklime treatment area as shown in Table 1. The soil of BMOUI was frequently treated with the quicklime powder by BMOUI rangers as a preventive measure. In every sampling area, the sampling site was divided into 5 ×

Fig. 2. The soil samples were collected from (A) deck-view exhibit, (B) lakeside, (C) near the holding enclosures and (D) outdoor exhibit area before the Orang Utan were released.

Table 1. The sampling sites treated with quicklimes and non-quicklimes areas as applied by BMOUI as preventive measure

Sampling site	Quicklime treatment area	Non-quicklime treatment area	
Lakeside	5	25	
Exhibition 1	5	-	
Exhibition 2	5	-	
Exhibition 3	5	-	
Cage area	-	5	
Total	20	30	50

5 grid squares which each length 5 m × 5 m apart. At the centre of each grid, approximately 40 g of soil was removed at a depth of 30 cm from the surface and was placed in the labelled sealable bag. The soil was protected from the sunlight and kept in ambient temperature. All soil samples were transported to Department of Medical Microbiology and Parasitology Laboratory, USM Hospital in Kelantan, Malaysia and were processed immediately for the identification of *B. pseudomallei*.

Isolation and screening for *B. pseudomallei*

Ten grams of soil was added into 10 ml of distilled water (DW) in the universal tube and the solution was allowed for sedimentation overnight. Ten microliters from the upper layer of the solution were collected and were plated onto Ashdown agar (Oxoid Ltd.). The agar was incubated at 37°C for seven days and was observed daily for presumptive *B. pseudomallei* colonies based on the characteristics as firstly, being flat and slightly dry pinkish purple in colour on the second day of incubation and secondly, being metallic sheen and wrinkled colonies after four days. Another millilitre from the upper layer of the solution was added into 9 ml of enrichment Ashdown broth containing 10 g of Tryptic soy broth, 40 ml glycerol, 5 ml of 0.1% crystal violet and 50 mg/L colistin. The broth was incubated for 48 h at 37°C. Then 10 µl from the solution was plated onto Ashdown agar (Oxoid Ltd.) and was observed as previously described (Limmathurotsakul *et al*., 2013). The presumptive *B. pseudomallei* isolates were tested with bacteriological tests such as Gram stain, catalase and oxidase test. Then, the positive colonies were screened with confirmatory test VITEK® automated instrument ID system (bioMerieux). The isolates were stored in BHI-glycerol broth at -20°C until further use.

Molecular identification of *Burkholderia* spp.

The final confirmation of presumptive isolates was done using PCR amplification based on specific *B. pseudomallei* gene and 16S rRNA gene primers. Both primers were used to identify *B. pseudomallei* and other *Burkholderia* spp. isolated from the soil of BMOUI. Specific primers targeted type III secretion system gene cluster of *B. pseudomallei* BpTT4176 (forward, 5'-CGTCTCTATACTGTCGAGCAATCG-3') and BpTT4290 (reverse, 5'-CGTGCACACCGGTCAGTATC-3') as published by Novak *et al*. (2006). The DNA of presumptive isolates were extracted through boiling method. The PCR amplification was prepared in a total volume of 25 µl contained 5.5 µl of PCR water, 12.5 µl MyTaq Red Mix buffer, 20 µM of each primer and 5 µl DNA templates. A confirmed *B. pseudomallei* was isolated from the Department of Medical Microbiology and Parasitology Laboratory, USM Hospital in Kelantan, Malaysia and was used as positive control; while DW as negative control. The PCR amplification consisted of 30 cycles with the parameter set as follow: initial denaturation at 95°C for 3 min, followed by denaturation at 95°C for 30 sec, annealing time at 58°C for 30

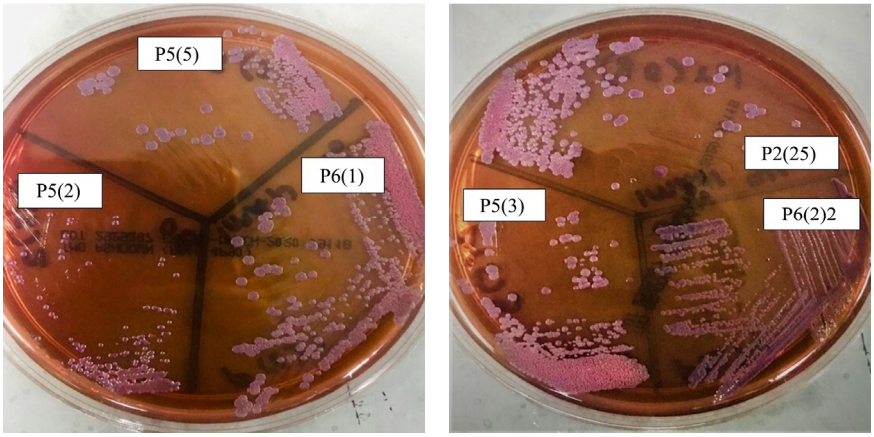

Fig. 3. The appearance of pinkish-purple colour with wrinkled characteristics of soil-isolates from BMOUI labelled as P2(25), P5(2), P5(3), P5(5), P6(1), and P6(6)2 on Ashdown agar after 48 h of incubation.

sec, elongation at 68°C for 1 min and a final extension at 72°C for 5 min. The amplicons were visualized by electrophoresis on a 1.5% agarose and the reference target size was 115 bp. For identification of isolates that showed a negative result for specific *B. pseudomallei* primer, the 16S rRNA gene was sequenced using universal 16S rRNA primers (Goldenberger *et al*., 1997) BakII-F (forward, 5' AGTTTGATCMTGGCTCAG 3') and BakII-R (reverse, 5' GGACTACHAGGGTATCTAAT 3'). The resulting amplicons were sent to 1st BASE Laboratories Sdn. Bhd. for sequencing. The sequence results were edited using Bioedit Sequence Alignment Editor version 7.2.5 and the species were identified using GenBank BLAST (https://blast.ncbi.nlm.nih.gov/).

Results

Preliminary study on soil of BMOUI

Referring to the medical history of the Orang Utan living in BMOUI, there were three cases of melioidosis infection. Two of deceased Orang Utan named Pauline and Dinggo had been diagnosed with melioidosis based on post-mortem results from Veterinary Research Institute, Ipoh, Perak Malaysia and there is one of the Orang Utan named Jidin who survived from melioidosis after being treated with antibiotics. Meanwhile, the investigation of general information of soil management in BMOUI obtained from the questionnaires included the demography of the island, sources of food and drink, application of fertilizer, quicklime and pesticides and other soil-related activities in the island were helpful in understanding the preventive measures applied by BMOUI.

Morphological and biochemical characterization of bacterial isolates from soil in BMOUI

Out of 50 soil samples from BMOUI, 37 plates were successfully grown on Ashdown agar (Oxoid Ltd.) which is the selective medium to culture *B. pseudomallei*. All the 37 plates exhibited mixed growth of bacterial colonies with various morphologies observed. There were six of the isolates labelled as P2(25), P5(2), P5(3), P5(5), P6(1), and P6(6)2 that showed pinkish-purple colour with wrinkled characteristics after 48 h of incubation period as shown in Fig. 3. The isolates resembled *B. pseudomallei* based on the colony morphology observation and were selected as presumptive isolates. All six isolates were recovered from the soil untreated with quicklime powder in BMOUI based on soil management surveillance. The presumptive isolates were screened with Gram staining, biochemical test and VITEK test. The results of the tests are shown in Table 2. All isolates were Gram-negative and exhibited rod shape under the microscope observation. For biochemical tests, all isolates showed positive results when tested with oxidase test and negative results for catalase test. The isolates were further screened with VITEK® automated ID system confirmation test.

Burkholderia species identification

Based on identification via VITEK® automated ID system,

Table 2. The results for colony morphology observation, Gram staining, biochemical test, VITEK® automated ID system and 16S rRNA PCR sequencing

Isolates	GenBank accession number	Sampling area	Quicklime treatment	Colony morphology	Gram staining	Oxidase test	Catalase test	VITEK® automated ID system	16S rRNA PCR sequencing
P2(25)	ON714600	Lakeside	No	Wrinkled	Negative rod	Positive	Negative	*Burkholderia cepacia* group	*Burkholderia contaminans*
P5(2)	ON714602	Exhibit 3	No	Wrinkled	Negative rod	Positive	Negative	*Burkholderia cepacia* group	*Burkholderia stagnalis*
P5(3)	ON714608	Exhibit 3	No	Wrinkled	Negative rod	Positive	Negative	Unidentified	*Burkholderia stagnalis*
P5(5)	ON714634	Exhibit 3	No	Wrinkled	Negative rod	Positive	Negative	*Burkholderia cepacia* group	*Burkholderia stagnalis*
P6(1)	ON714640	Holding enclosure	No	Wrinkled	Negative rod	Positive	Negative	*Burkholderia cepacia* group	*Burkholderia stagnalis*
P6(2)2	ON714641	Holding enclosure	No	Wrinkled	Negative rod	Positive	Negative	Low discrimination	*Burkholderia multivoran*

all isolates tested were negative for *B. pseudomallei.* However, four isolates labelled as P2(25), P5(2), P5(5), and P6(1) were identified as *B. cepacia* group, one isolate labelled as P5(3) was unable to be identified and one isolate labelled as P6(6)2 had low discrimination to determine the species. Using specific primers for *B. pseudomallei* targerted TTS1 genes, all the presumptive isolates were negative for *B. pseudomallei*, thus the identification of the isolates was performed using universal 16S rRNA primers. The 16S rRNA sequencing analysis revealed that unidentified organism P5(3) via VITEK system was *B. stagnalis* and P6(2)2 which was unsuccessfully identified via VITEK system due to the low discrimination factor was identified as *B. contaminans*. Meanwhile, the other three isolates P5(2), P5(5), and P6(1) identified as *B. cepacia* group via VITEK® automated ID system being identified as *B. stagnalis* via PCR analysis. 16S rRNA PCR analysis succeeded in recognizing the isolates at the species level. All these isolates are in *B. cepacia* complex group. The percentage of 16S rRNA gene sequence similarities for all isolates were 100% except for isolate P6(6)2 that showed 99.56% of gene sequence similarity.

Discussion

As the initial objective of this study is to investigate the presence of *B. pseudomallei* in the soil of BMOUI, thus the isolates were screened with culture method on selective Ashdown agar which is the gold standard method to isolate *B. pseudomallei* (Hemarajata *et al*., 2016). The isolates which exhibited colony morphology that resembled *B. pseudomallei* were selected as presumptive isolates. The 'wrinkled' characteristics are very similar to the appearance of *B. pseudomallei* colony that initially appears as smooth colony. It will develop dry and wrinkled-like corn-flower head in the agar after further incubation (Howard and Inglis, 2003). However, there was a research done by Chantratita *et al*. (2011) that reported the colony morphology of *B. pseudomallei* could be varied due to adaptation to the environmental stress, nutrient availability and temperature of incubation. During the screening process, some of the isolates that show less criteria of *B. pseudomallei* could be missed and being excluded. In addition, the misidentification of this bacteria also frequently occurred and reported particularly in the resource-limited setting laboratories and due to lack of the experiences by operating personnel (Brent *et al*., 2007).

In the confirmation by molecular PCR test, a specific *B. pseudomallei* TTS1 cluster gene and universal 16S rRNA gene were used to determine the bacterial species retrieved from the soil. No *B. pseudomallei* was identified in this study, however, other *Burkholderia* species were discovered namely *B. contaminans*, *B. multivorans*, and *B. stagnalis.* Nevertheless, Martina *et al*. (2018) stated that universal 16S rRNA gene which has been commonly used in bacterial identification somehow was less accurate to discriminate species level of *B. cepacia* group. Hence, Depoorter *et al*. (2020) suggested *recA* and *hisA* genes are more favourable to differentiate the species. Specific *B. pseudomallei* TTS1 cluster gene was used so as to fulfil the initial objective of this study to isolate *B. pseudomallei* from the soil samples of BMOUI. The BLASTN sequencing results

in this study showed the similarities of the 16S rRNA gene sequences percentages above the threshold value which were above 97% (Nguyen *et al*., 2016).

Beforehand, the presumptive isolates were run in the automated VITEK® instrument ID system and the results were also negative for *B. pseudomallei*. The species identification via VITEK® automated instrument ID system was unspecific and misidentification of *B. pseudomallei* to *B. cepacia* always occurred because of the biochemical profile in the VITEK® automated system database is not updated, yet *B. pseudomallei* was not in the list (Weissert *et al*., 2009). The accuracy of the VITEK® automated instrument ID system is in the range of 63% to 81% (Zhong *et al*., 2012). Currently, the *Burkholderia* genus consists of more than 80 species and divided into several group including *B. pseudomallei* group and *B. cepacia* group (Ginther *et al*., 2015; Depoorter *et al*., 2016). *Burkholderia pseudomallei* groups consists of *B. pseudomallei*, *B.mallei*, *B.oklahomensis*, and *B.thailandensis* while *B. cepacia* group consists of 23 species including *B. multivorans*, *B. contaminans*, and *B. stagnalis* (Inglis and Merritt, 2014; Furlan *et al*., 2019). Moreover, *B. cepacia* species in the group were described to have similar phenotypic with different genotypic characteristics, thus increasing the risk of misidentification of the species (Chung *et al*., 2003; Deepak *et al*., 2008).

Other than physiochemical property of the soil, proper and well management of the soil also influenced the presence of *B. pseudomallei* in the soil (Musa *et al*., 2016). Although no soil analysis was performed to the soil samples collected from BMOUI, we observed that the frequent quicklime application to the soil as a preventive measure successfully controlled the growth of *Burkholderia* spp. The quicklime powder was applied to the soil at BMOUI more frequently during rainy season. The presumptive of isolates in this study were recovered from the soil samples that were untreated with quicklime in the lakeside and holding enclosures areas. Based on the surveillance of the soil management in BMOUI, quicklime powder was applied frequently to the soil in three exhibit areas. These areas had the Orang Utan released from 9 am to 5 pm daily and afterwards the rangers will return them to the holding enclosures. In Thailand, quicklime was widely used in large amount to decontaminate the soil, particularly in farms and paddy fields. However, the post-effect of the quicklime application was still limited and needs to be explored (Na-ngam *et al*., 2004).

The presence of *B. cepacia* group members in the soil of BMOUI should not be taken lightly even though they are ubiquitous soil inhabitants. These *B. cepacia* group members have been reported to cause septicaemia known as “cepacia syndrome” mostly in cystic fibrosis patients (Martina *et al*., 2020). The infection caused by these *B. cepacia* group is very life-threatening and the mortality percentage is also high as reported by many hospitals. All the more serious, *B. cepacia group* infection is contagious and the infected individual must be isolated (Shommu *et al*., 2015). The treatment of this infection is difficult since the bacteria are resistant to many available antibiotics (LiPuma *et al*., 2002). Like *B. pseudomallei*, *B. cepacia* group members are opportunistic pathogens and able to survive in extreme environment for an extended period. Thus, Orang Utan with low immunity system has high risk to the infection. *Burkholderia cepacia* infections had been reported in animals as well and had raised awareness among veterinary centres (Berriatua *et al*., 2001).

It is compulsory to every veterinary and conversation centre to establish a standardized prevention protocol and soil management guidelines to ensure the welfare of the animals is maintained. The soil surveillance should be performed frequently including hygiene system, waste disposal, chlorination of the water supply and disinfectant of the soil surface (Kasantikul *et al*., 2015). In BMOUI, the health of every Orang Utan was monitored twice a year. A few feeding platforms were built as an effort to minimize the contact of Orang Utan with soil during feeding time. The water supply was routinely chlorinated and was boiled before given to the Orang Utan. Besides, the faeces of the Orang Utan were removed immediately in the morning from the holding enclosures area after they were released to the exhibit areas.

Although we were unable to isolate the *B. pseudomallei* which is the primary target organism in this study, a few species of *B. cepacia* group known as *B. multivorans*, *B. contaminans*, and *B. stagnalis* were successfully isolated from the soil of BMOUI. The presence of these opportunistic bacteria in the soil in BMOUI should be highlighted and concerned due to their ability to cause severe infection among animals. Based on the preliminary findings of the quicklime powder application in BMOUI, an extensively further study on the pre and post quicklime treatment

onto the soil should be emphasized in the future.

Nucleotide sequence accession number(s)

The 16S rRNA gene sequences of *B. cepacia* complex namely P2(25), P5(2), P5(3), P5(5), P6(1), and P6(2)2 isolated from BMOUI soil have been deposited in the GenBank database under accession numbers ON714600, ON714602, ON714608, ON714634, ON714640, and ON714641 respectively.

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Conflict of Interest

The authors declare that they have no competing interests.

Ethical Statement

The consent approval was obtained from Bukit Merah Orang Utan Island Foundation (BMOUI), Bukit Merah Perak prior to sample collection. Beforehand, animal ethics approval was attained through Committee on Animal Research and Ethics Universiti Teknologi MARA (UiTM CARE: 289/2019) and Department of Wildlife and National Parks under research permit (JPHL&TN [IP]: 100-34/1.24 Jld 15[36]).

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