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ORIGINAL ARTICLE

Burkholderia pseudomallei in soil samples from an oceanarium in Hong Kong detected using a sensitive PCR assay

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Melioidosis, caused by *Burkholderia pseudomallei*, is an emerging infectious disease with an expanding geographical distribution. Although assessment of the environmental load of *B. pseudomallei* is important for risk assessment in humans or animals in endemic areas, traditional methods of bacterial culture for isolation have low sensitivities and are labor-intensive. Using a specific polymerase chain reaction (PCR) assay targeting a Tat domain protein in comparison with a bacterial culture method, we examined the prevalence of *B. pseudomallei* in soil samples from an oceanarium in Hong Kong where captive marine mammals and birds have contracted melioidosis. Among 1420 soil samples collected from various sites in the oceanarium over a 15-month period, *B. pseudomallei* was detected in nine (0.6%) soil samples using bacterial culture, whereas it was detected in 96 (6.8%) soil samples using the specific PCR assay confirmed by sequencing. The PCR-positive samples were detected during various months, with higher detection rates observed during summer months. Positive PCR detection was significantly correlated with ambient temperature ($P<0.0001$) and relative humidity ($P=0.011$) but not with daily rainfall ($P=0.241$) or a recent typhoon ($P=0.787$). PCR-positive samples were obtained from all sampling locations, with the highest detection rate in the valley. Our results suggest that *B. pseudomallei* is prevalent and endemic in the oceanarium. The present PCR assay is more sensitive than the bacterial culture method, and it may be used to help better assess the transmission of melioidosis and to design infection control measures for captive animals in this unique and understudied environment.

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INTRODUCTION

Burkholderia pseudomallei is an emerging, highly pathogenic, gram-negative beta-proteobacterium responsible for melioidosis, a potentially serious and fatal disease often manifesting as community-acquired pneumonia and sepsis. Although melioidosis is mainly endemic in Southeast Asia and northern Australia, the disease has been increasingly reported in countries outside the Asia-Pacific region, including India,[1,2] Mauritius,[3] South, Central and North America,[4–6] and West and East Africa,[7,8] suggesting an expanding geographical distribution and/or awareness. The illness can present as an acute, subacute or chronic process. Disease manifestations range from subclinical infection localized abscesses to severe pneumonia and fulminant sepsis, with case fatality rates of up to 19% in endemic areas.[9] The incubation period of melioidosis also varies widely from 2 days to 26 years.[10] Diagnosis of melioidosis can be difficult, as the bacterium may not be readily isolated from clinical specimens. Moreover, even with positive cultures, commercial bacterial identification kits often fail to distinguish between *B. pseudomallei* and closely related species such as *B. thailandensis* and *B. cepacia* complex.[11] Nevertheless, the advent of new molecular techniques has enabled the development of improved methods for more accurate species identification.[12–18] Treatment of melioidosis may be difficult, as *B. pseudomallei* is often resistant to multiple antibiotics, and a prolonged course of antibiotics is required to prevent disease relapse.[13,19] Unfortunately, in many of the endemic areas and countries, diagnostic and therapeutic resources are limited, hindering efforts to better assess the disease burden and improve treatment outcomes.

B. pseudomallei is a natural saprophyte that can be isolated from soil, groundwater, stagnant streams, rice paddies and ponds, which, together, are the major natural reservoirs of the bacteria.[20,21] Although its epidemiology and route of transmission are not fully understood, melioidosis is believed to be acquired through environmental contact with contaminated soil and contaminated water by percutaneous inoculation, inhalation of aerosols or ingestion.[22] Owing to its high mortality rates, antibiotic resistance and possible transmission by aerosols, *B. pseudomallei* is considered a potential agent of biological warfare and has been classified as a category B bioterrorism agent by the Center for Disease Control (Atlanta, GA, USA; http://www.bt.cdc.gov/agent/agentlist-category.asp). Human cases are often

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spatially and temporally clustered and may follow heavy rains and winds with exposure to soil and water.[23,24] *B. pseudomallei* also causes melioidosis in a wide range of animals in endemic areas.[25] In Hong Kong, melioidosis is an endemic disease not only in humans but also in captive marine mammals and birds, including bottlenose dolphins, California sea lions, pilot whales and zebra doves.[12] Strains of *B. pseudomallei* with closely related genotypes have been isolated from soil and water collected in the neighborhood of infected animals.[12] However, the environmental distribution of *B. pseudomallei* in Hong Kong is poorly understood.

Assessment of the environmental load of *B. pseudomallei* may help in estimating the disease risk and deciding possible preventive measures in endemic areas. Moreover, knowledge of its environmental distribution, in relation to specific habitats and factors such as climate change, is important for understanding the epidemiology of melioidosis. However, the gold standard for *B. pseudomallei* detection in environmental samples is culture, which lacks sensitivity and is time-consuming. Molecular methods based on detection of bacterial nucleic acids have the potential to overcome the problems of culture-based methods. Therefore, different polymerase chain reaction (PCR) assays have been reported to detect *B. pseudomallei*.[26–29] To detect *B. pseudomallei* DNA from environmental samples, a highly specific gene target is essential, as *B. pseudomallei* is phylogenetically closely related to *B. thailandensis* and other *Burkolderia* species that may be found in the same environment. Using a pan-genomic analysis approach in gene target selection, we previously developed a novel and specific PCR assay targeting a Tat domain protein for the identification and detection of *B. pseudomallei* from soil and simulated sputum samples.[30] In this study, we examined the prevalence of *B. pseudomallei* in soil samples from an oceanarium in Hong Kong where captive animals have been infected with melioidosis,[12] and we evaluated the sensitivity of the PCR assay compared to culture-based detection methods.

MATERIALS AND METHODS

Soil samples

Soil samples were prospectively collected each month from various sites in the oceanarium from June 2010 to August 2011, a period encompassing two wet seasons and one dry season. Briefly, a standard soil sampling technique was used,[31] with approximately 200 g of soil collected from a depth of 20–30 cm. Soil samples were sealed in plastic containers at the ambient temperature and immediately transported to the laboratory for enrichment and bacterial culture.

Culture, isolation and identification of *B. pseudomallei*

Bacterial culture and isolation of *B. pseudomallei* were performed according to previously published protocols with modifications.[32] Briefly, 100 g of each soil sample was mechanically homogenized with 100 mL of purified water. The mixture was left to settle at 25 ℃ overnight, and 1 mL of the resulting soil supernatant was collected for enrichment in 9 mL of modified Ashdown's broth containing 10 g/L tryptic soy broth (Oxoid, Basingstoke, Hampshire, UK), 40 mL/L glycerol (UltraPure, Waltham, Massachusetts, USA), 5 mg/L crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and 1 million international units (MIU)/L colomycin (Forest Laboratories UK Ltd., Dartford, Kent, UK) and in 9 mL Galimand's broth supplemented with 1 MIU/L colomycin (Forest Laboratories UK). The cultures were incubated aerobically at 42 ℃ for 10 days. Ten microliters of each enriched culture supernatant was plated on Ashdown's agar, containing 10 g/L trypticase soy broth (Oxoid), 40 mL/L glycerol (Ultrapure), 5 mg/L 0.1% crystal violet (Sigma-Aldrich), 50 mg/L neutral red, 5 mg/L gentamicin (Gibco, Waltham, Massachusetts, USA) and 15 g/L agar, and incubated aerobically at 42 ℃ for 48 h. The colonies gown on Ashdown's agar plates were screened for *B. pseudomallei* morphotypes. Suspected *B. pseudomallei* isolates were phenotypically identified by the API 20NE system (bioMérieux Vitek, Hazelwood, MO, USA) and Vitek 2 system (bioMérieux Vitek) supplemented by conventional biochemical methods.

PCR detection of *B. pseudomallei*

One milliliter of enriched soil culture supernatant from Ashdown's broth was harvested for bacterial DNA extraction using the QIAamp DNA mini kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions. A single-target PCR assay for *B. pseudomallei* was performed using *B. pseudomallei*-specific primers targeting a 189-bp fragment of a specific gene that encodes a Tat domain protein (locus BPSS0658 in the *B. pseudomallei* K96243 reference genome); the protocol was modified from our previously described multiplex PCR assay.[30] The PCR mixture (20 μL) contained purified DNA extract (1.0 μL) as template, 1.0 M betaine monohydrate (Fluka BioChemika, Steinheim, Germany), 0.5 μM primers (LPW13372: 5′-CAA GAA CGG TTT ATG CG-3′ and LPW13373: 5′-GAA GTG ATC CAT CAA ATG TC-3′), 2.0 μL 10× PCR buffer II, 2.5 mM $MgCl_2$, 200 μM of each dNTPs (GeneAmp, Applied Biosystems, Waltham, Massachusetts, USA) and 1.0 U *Taq* polymerase (Ampli*Taq* Gold; Applied Biosystems, Waltham, Massachusetts, USA). Thermal cycling was performed in an automated thermocycler (Veriti 96-well fast thermal cycler; Applied Biosystems, Waltham, Massachusetts, USA) with a hot-start at 95 ℃ for 10 min; 10 touch-down cycles of 95 ℃ for 30 s, annealing for 1.5 min at temperatures decreasing from 60 ℃ to 51 ℃ (with 1.0 ℃ decremental steps) and 72 ℃ for 1 min; 30 cycles of 95 ℃ for 30 s, 50 ℃ for 1.5 min and 72 ℃ for 1 min; and a final extension at 72 ℃ for 10 min. Five microliters of each amplified product was electrophoresed in 2.5% (w/v) agarose gel with a molecular size marker (GeneRuler 50 bp DNA Ladder; Fermentas, Pittsburgh, PA, USA) in parallel. Electrophoresis in Tris-borate-EDTA buffer was performed at 100 V for 45 min. The gel was stained with ethidium bromide (0.5 μg/mL) for 25 min, rinsed and photographed under ultraviolet light illumination. Standard precautions were taken to avoid PCR contamination, and no false-positive was observed in negative controls.

The PCR products were gel-purified using the QIAquick PCR gel extraction kit (QIAgen). Both strands of the PCR products were sequenced with an ABI 3130*xl* Genetic Analyzer according to the manufacturer's instructions (Applied Biosystems) using primers specific to each PCR product. The obtained DNA sequences were analyzed using a BLASTx search of the in-house *Burkholderia* pan-genome databases and by a BLASTn search against the NCBI online nucleotide collection (nr/nt) database to confirm their identities. The specificity of the PCR assay has been previously confirmed using pure isolates of closely related bacterial species, including *B. pseudomallei*, *B. thailandensis* and *B. cepacia*.[30]

Statistical analyses

Correlation of PCR detection with ambient temperature, relative humidity, rainfall and recent typhoon was performed using logistic regression. $P<0.05$ was regarded as statistically significant (IBM SPSS Statistics 19, Armonk, New York, USA).

RESULTS

Culture and isolation of *B. pseudomallei* from soil samples

A total of 1420 soil samples were collected from the oceanarium, comprising 90–120 samples per month during the 15-month study period. The samples were collected from various sites at three different

Table 1 Distribution of *B. pseudomallei* in different locations of the oceanarium

Area	Number of samples taken	Number of PCR-positive (%)
Lowland	750	35 (4.9%)
Headland	370	20 (5.4%)
Valley	300	41 (13.7%)

locations, including lowland, headland and valley areas situated at different altitudes (Table 1 and Supplementary Figure S1). Soil samples from the different locations all consisted of decomposed granite. Among the 1420 samples, nine (0.6%) samples were positive for *B. pseudomallei* by bacterial culture, ranging from 0% to 5.6% of samples taken in a given month (Figure 1). The positive isolates were detected in August 2010 (four isolates) in a valley area and in November 2010 (five isolates) in both lowland and headland areas facing the sea (Figure 1 and Table 1). No positive cultures could be recovered during the other months.

PCR for detection of *B. pseudomallei* from soil samples

Using a specific PCR assay targeting a *B. pseudomallei*-specific Tat domain protein-encoding gene, 96 (6.8%) of the 1420 samples showed positive bands of approximately 189 bp, corresponding to the expected PCR product size, by gel electrophoresis (Supplementary Figure S2). The positive detection rates ranged from 0% to 42.2% of samples taken in a given month (Figure 1). DNA sequencing of the positive PCR products confirmed that they originated from the target locus, with 100% nucleotide identities to the corresponding gene fragment of *B. pseudomallei* strain K96243 (GenBank accession NO BX571966). No positive reactions were found for any negative controls during the same PCR runs, thus excluding PCR contamination. The PCR-positive samples were detected during various months throughout the study period, with the exceptions of March, April and June 2011. Higher detection rates were observed during the summer months, when both ambient temperature and relative humidity were high (such as June–August 2010 and July–August 2011); the highest detection rate was recorded in June 2010 (42.2%) (Figure 1 and Table 2). By logistic regression, significant correlations were demonstrated between positive detection and ambient temperature ($P<0.0001$) or relative humidity ($P=0.011$) on the day of sampling, but not between positive detection and daily rainfall ($P=0.241$) or a typhoon within the 7 days prior to sampling ($P=0.787$). PCR-positive

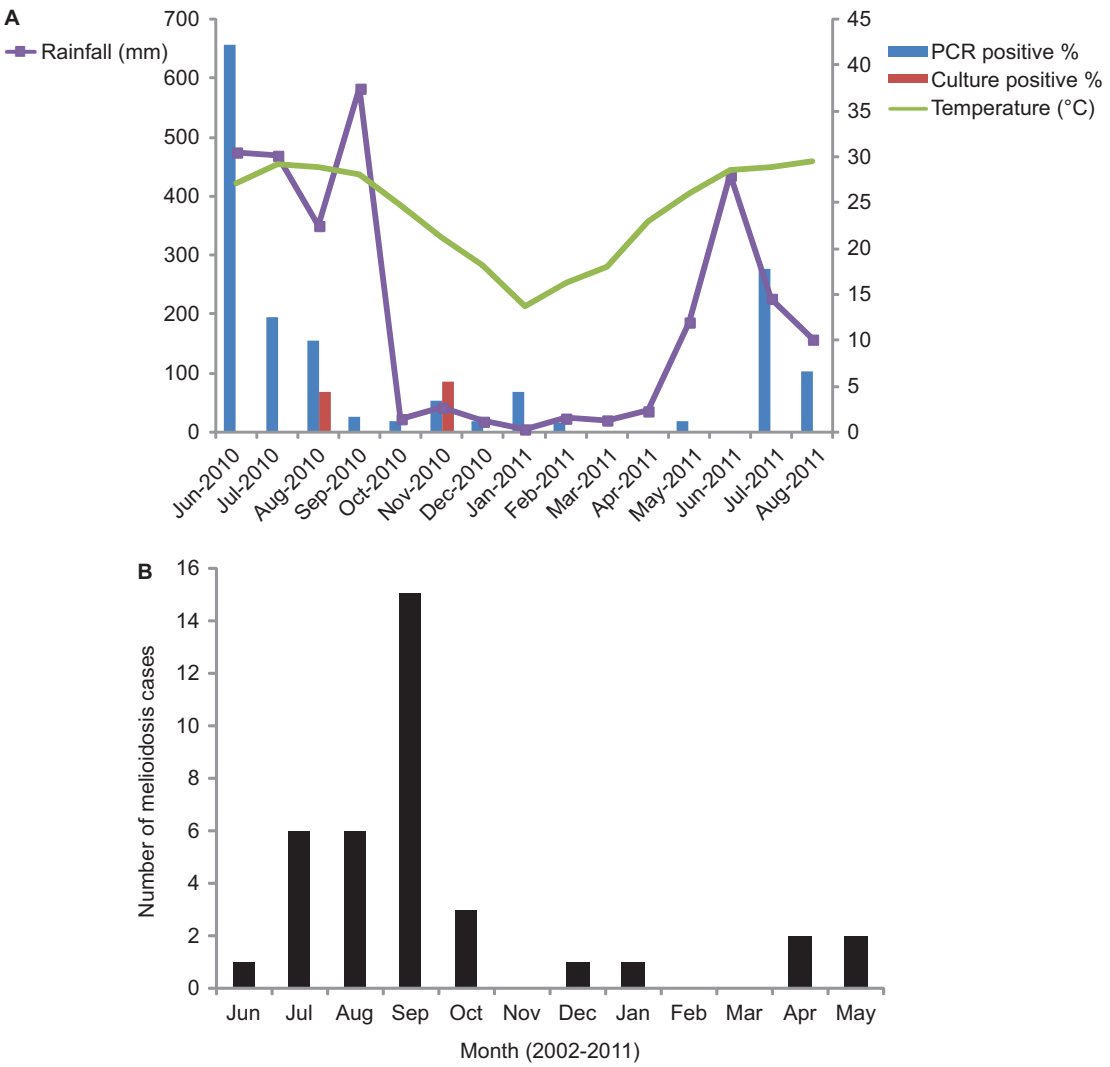

Figure 1 (**A**) Detection of *B. pseudomallei* from soil samples by PCR and culture during the study period and in relation to ambient temperature and rainfall. (**B**) Monthly number of melioidosis cases in captive animals in the oceanarium between 2002 and 2011. Data showed the cumulative cases in the respective months over a 10-year period (2002-2011).

Table 2 Seasonal distribution of *B. pseudomallei* in the oceanarium

	Mean air temperature (℃)	Total rainfall (mm)	PCR-positive (%)	Culture-positive (%)
Jun 2010–Aug 2010 (summer)	28.37	431.54	61 (20.3%)	4 (1.3%)
Sep 2010–Nov 2010 (autumn)	24.67	216.0	6 (2%)	5 (1.7%)
Dec 2010–Feb 2011 (winter)	16	15.84	6 (2.1%)	0 (0.0%
Mar 2011–May 2011 (spring)	22.3	81.07	1 (0.4%)	0 (0.0%)
Jun 2011–Aug 2011 (summer)	28.97	273.34	22 (8.1%)	0 (0.0%)

samples were derived from all the three sampling locations of the oceanarium, including the lowland, headland and valley areas, with the highest detection rate in the valley (Table 1 and Supplementary Figure S1).

DISCUSSION

The present study confirms that *B. pseudomallei* is endemic in the soil environment of the present oceanarium, where captive animals have been infected by the bacterium. Moreover, the present PCR assay is more sensitive than the culture method for detection of *B. pseudomallei* in soil samples. *B. pseudomallei* has been found in soil samples from endemic areas, including Thailand, southern China, Taiwan and northern Australia.[21,26,27,33,34] Although most previous studies have relied on the culture of viable bacteria from soil samples, there has been increasing interest in developing molecular detection methods. Although multiple PCR assays have been developed for such purposes, some assays still lack sensitivity or specificity.[28,29] Moreover, few studies have directly compared the sensitivities of PCR and culture methods. In one study, a quantitative PCR (qPCR) detection assay was developed and validated using 40 soil samples from northeast Thailand.[26] Among 26 of 40 soil samples that tested positive for *B. pseudomallei* by culture, all were also positive by qPCR.[26] Moreover, qPCR detected the bacterium in seven additional samples that were negative by culture. In another study from northern Australia, a real-time PCR assay was evaluated using enriched soil samples.[27] In addition to the 13 of 104 soil samples testing positive by both culture and qPCR, seven further samples were positive by qPCR but not by culture.[27] The present PCR assay also achieves higher sensitivity, with a detection rate >10-fold higher than culture methods for detection of *B. pseudomallei* from soil samples. However, it is difficult to compare the sensitivities of the different PCR assays used in different studies because different methods and gene targets were employed. Instead of the direct/enriched soil samples used in the two previous studies, the PCR assay in this study was performed using the enriched culture supernatant as the template to avoid the problem of PCR inhibitors that are often encountered in soils.[26,27] Similar methods using enriched culture supernatant for PCR have also been reported for groundwater samples.[21] This assay may offer a cheaper alternative to real-time PCR methods, which may not be available in some endemic areas or countries. The superiority of PCR-based assays over culture-based detection can be explained by the problems associated with the culture and isolation of *B. pseudomallei*. Cultivation depends on efficient selection of *B. pseudomallei* over other, often more rapidly growing environmental bacteria on the chosen selective media. Moreover, culture can only detect cultivable bacterial cells; it will not detect potentially viable but non-culturable cells, which may underestimate the *B. pseudomallei* bacterial load in environmental habitats. Although a positive PCR result does not imply the presence of viable bacteria, it represents a sensitive surrogate marker for the presence of *B. pseudomallei* in the environment. Nevertheless, as enriched culture supernatant was used in this study, a PCR-positive result in our soil samples may imply the presence of viable bacteria. Further studies on the application of the present and other molecular method-based assays are required to assess their usefulness for detecting *B. pseudomallei* in different environmental samples.

The Tat domain protein represents a sensitive and specific alternative target for PCR detection of *B. pseudomallei*. In previous studies using molecular detection of *B. pseudomallei* from environmental samples, the type III secretion system (TTSS) and, less commonly, the flagellin and BPSS1187 genes have been used as specific gene targets.[21,26,27,33,34] In studies from both Thailand and Australia, a 115-bp fragment of the single-copy TTSS1 gene was used as the gene target for amplification.[26,27] TTSS1 has been found to be ubiquitously present in *B. pseudomallei* but not in close relatives such as *B. thailandensis* or *B. mallei*.[35] In our previous study, different gene targets specific to *B. pseudomallei*, *B. thailandensis* and *B. cepacia* complex (the Tat-domain protein, a 70-kDa protein and a 12-kDa protein, respectively) were selected using a pan-genomic analysis approach.[30] Based on the three gene targets, a multiplex PCR assay was developed and found to be sensitive and specific for detection of the respective bacteria in simulated sputum samples.[30] A pilot study using 60 soil samples allowed the detection of *B. pseudomallei* in 19 (31.6%) samples and *B. cepacia* complex in 29 (48.3%) samples, with codetection of both bacteria in four (6.7%) samples. The apparently higher detection rate of *B. pseudomallei* in the pilot study than in the present study is likely due to the use of soil samples collected during the peak season, as opposed to the samples in the present study collected across different seasons. A single-target PCR assay based on the Tat-domain protein, found only in *B. pseudomallei* and not in *B. thailandensis* or *B. cepacia* complex, was chosen in place of the multiplex PCR assay for detection of *B. pseudomallei* in the present study. This strategy was designed to minimize the chance of false-negatives, which can occur in the multiplex assay as a result of interactions from the presence of *B. cepacia* DNA in the same soil sample. The results confirmed that the single PCR assay targeting the Tat-domain protein-encoding gene is specific for detecting *B. pseudomallei* and is more sensitive than culture methods.

Environmental detection of *B. pseudomallei* is important for disease anticipation and infection control measures to combat melioidosis in endemic areas, such as in the captive animals of the present oceanarium. The detection of *B. pseudomallei* in soil is believed to be related to the risk of developing melioidosis. For example, higher bacterial counts of *B. pseudomallei* from soil in the northeastern region of Thailand than in the central region may contribute to the higher incidence of reported melioidosis cases in the former region.[20] In a recent report from northern Australia, a *B. pseudomallei* strain cultured from an athlete with cutaneous melioidosis was identical by multilocus sequence typing and multilocus variable-number tandem repeat analysis to an isolate recovered from the soil at the location on the sports field where he was injured.[24] Such findings may alert clinicians to consider the possibility of melioidosis in persons from endemic areas with abrasion injuries that involve contact with soil.[24] However, as culture methods are more labor intensive and less sensitive, molecular detection should be the first-line method for environmental detection of *B. pseudomallei*; it can be supplemented by

culture-based methods for verification of positive results. Despite *B. pseudomallei* having been discovered nearly a century ago,[36] its geographical distribution and ecology in its natural habitat remains poorly understood. In China, a few reports have revealed the presence of *B. pseudomallei* in soil or water from southern coastal provinces, including Hainan, Guangdong and Guangxi.[34,37] The present assay serves as an alternative, sensitive molecular detection method to explore the soil distribution of *B. pseudomallei* in other potential endemic areas.

The higher PCR detection rate of *B. pseudomallei* in soil samples during summertime and the positive correlation of detection with ambient temperature and relative humidity may explain the seasonality of melioidosis in both humans and animals in Hong Kong, where sporadic cases or small outbreaks are mainly observed during summer. In our oceanarium, the seasonality of melioidosis cases among the captive animals from 2002 to 2011 also correlated with the monthly trend of PCR-positive soil samples in this study, with higher incidence during summer than winter months (Figure 1B). Although correlation with humidity has not been reported previously, studies have associated human melioidosis with rainfall, suggesting that the infection may result from acute exposure to the organism in the soil and water.[9,23,28,32,38,39] A case of fulminant infection was reported following exposure to stagnant floodwater in India.[1] Two fatal cases of human melioidosis have also been reported in Queensland, Australia, with disease onset preceded by unseasonal heavy rainfall.[39] A subsequent study in Queensland demonstrated that the timing and location of 47 melioidosis cases was generally correlated with rainfall across northern Australia, with a case cluster associated with post-cyclonic flooding.[40] In another study from northern Australia involving 318 cases, rainfall in the 14 days before hospital admission was found to be an independent risk factor for pneumonia, septic shock and death, suggesting that heavy monsoonal rains and winds may cause a shift toward inhalation of *B. pseudomallei*.[23] In northeast Thailand, the disease also showed a strong correlation with rainfall, and adults exposed to soil and water at work, such as rice farmers, had an increased risk of melioidosis.[38] Similar positive linear associations have also been demonstrated between monthly rainfall and melioidosis cases and/or deaths in Malaysia and India.[41,42] Although we did not find significant correlation between positive PCR detection and daily rainfall or recent typhoons, these factors may have caused delayed effects on bacterial load, or other factors may have had a greater impact on the ecology of *B. pseudomallei* in the unique environment of our oceanarium. Further studies are warranted to understand the role of climate changes, such as global warming, in the emergence of melioidosis in different endemic and non-endemic areas.

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