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Melioidosis in Timor-Leste: First Case Description and Phylogenetic Analysis

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Burkholderia pseudomallei, the causative agent of melioidosis, has not yet been reported in Timor-Leste, a sovereign state northwest of Australia. In the context of improved access to diagnostic resources and expanding clinical networks in the Australasian region, we report the first 3 cases of culture-confirmed melioidosis in Timor-Leste. These cases describe a broad range of typical presentations, including sepsis, pneumonia, multifocal abscesses, and cutaneous infection. Phylogenetic analysis revealed that the Timor-Leste isolates belong to the Australasian clade of *B. pseudomallei*, rather than the Asian clade, consistent with the phylogeographic separation across the Wallace Line. This study underscores an urgent need to increase awareness of this pathogen in Timor-Leste and establish diagnostic laboratories with improved culture capacity in regional hospitals. Clinical suspicion should prompt appropriate sampling and communication with laboratory staff to target diagnostic testing. Local antimicrobial guidelines have recently been revised to include recommendations for empiric treatment of severe sepsis.

Keywords. Timor-Leste; *Burkholderia pseudomallei*; melioidosis; phylogeny.

Melioidosis is caused by the gram-negative bacillus *Burkholderia pseudomallei*. Cases are classically characterized by pneumonia and multiple abscesses, although a wide range of presentations have been reported, making clinical diagnosis challenging. Reported case fatality rates have been 10%–40%, and as high as 90% in patients with severe sepsis [1, 2]. There is a strong association between melioidosis and poorer outcomes with risk factors, including diabetes mellitus, hazardous alcohol use, chronic lung disease, chronic renal disease, malignancy, immunosuppression, rheumatic heart disease or congestive cardiac failure, iron overload, and kava use [1, 3]. The current diagnostic standard is culture; however, *B. pseudomallei* can be misidentified as a culture contaminant or as another species (eg, *B. cepacia*, *Bacillus* spp., or *Pseudomonas* spp.), especially by laboratory staff unfamiliar with this organism [4, 5].

Melioidosis was first described in Burma (now Myanmar) as a newly recognized glanders-like disease in humans; reports from other Southeast Asian countries soon followed [6]. It is highly endemic in Northeastern Thailand and Northern Australia, where *B. pseudomallei* has been detected in soil and water. Melioidosis predominantly occurs in the tropics, at latitudes between 20° north and south of the equator [7]. Timor-Leste is a country located northwest of Australia, ~9° south of the equator. Timor-Leste is in the tropical wet and dry climate zone with a dry season from May to November and a wet season from December to April, very similar to Darwin in the Northern Territory of Australia [8]. Despite a climate anticipated to support the growth of *B. pseudomallei* and endemicity in surrounding countries, melioidosis has remained unrecognized in Timor-Leste [1, 9].

In 1999, a seroprevalence study reported that ~17% of East Timorese refugees had evidence of anti–*B. pseudomallei* antibodies [7]. As a developing sovereign state, the limited technical capacity of laboratories in Timor-Leste had, to date, prohibited the diagnosis of microbiologically proven disease. In 2020, the National Health Laboratory (NHL) of Timor-Leste was extended and re-equipped with support from a Fleming Fund Country Grant to improve antimicrobial use and encourage investment in surveillance of antimicrobial resistance [6, 10]. The contemporary laboratory was upgraded to PC2 standard and equipped with a BD Phoenix M50 (Becton Dickinson, Berkshire, UK) for automated antibiotic susceptibility testing and a matrix-assisted laser desorption/ionization-time of flight (MALDI-ToF) analyzer (Bruker Daltonic, Bremen, Germany) for bacterial identification, with participation in external quality assurance programs. The number of microbiology samples received has increased from ~24 specimens per month in 2017 to >400 specimens

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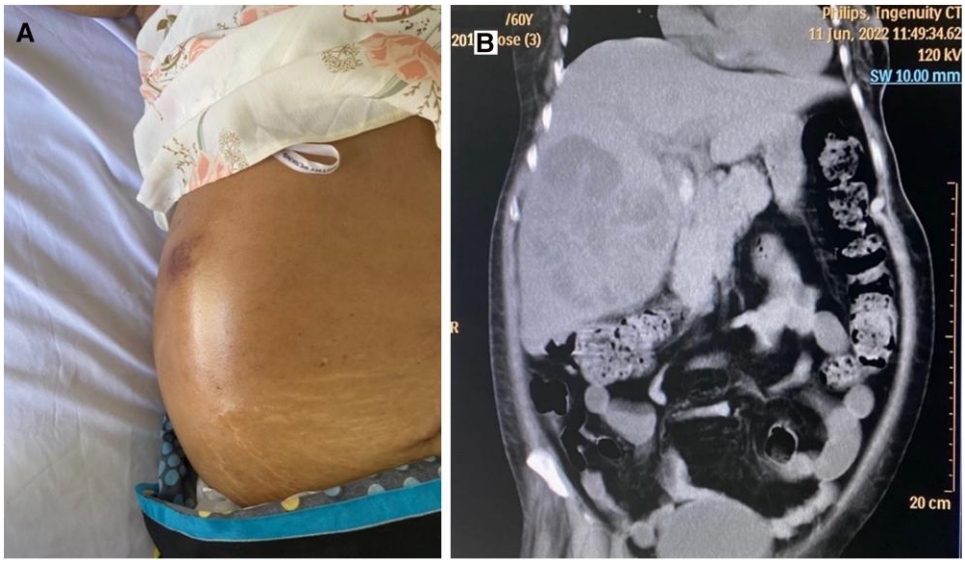

Figure 1. A (left), Clinically apparent abdominal distension. B (right), Computerized tomography demonstrated large, multiloculated hepatic abscess.

per month in 2022 [11]. In this context, we report the first 3 cases of culture-confirmed melioidosis in Timor-Leste.

PATIENT CONSENT

Cases 2 and 3 provided written consent for publication, and a waiver of consent was obtained from the ethics committee of Instituto Nacional de Saúde (National Health Institute) in order to describe Case 1, given the importance of raising awareness of melioidosis in Timor-Leste. The design of this work conforms to standards currently applied in Timor-Leste.

Case 1

In February 2022, a 38-year-old previously healthy male was admitted for 9 days with septic shock due to suspected acute gastroenteritis, requiring vasopressor support. Due to poor clinical response to empirical therapy with intravenous (IV) ceftriaxone 1 g 12-hourly, antibiotic therapy was broadened to meropenem 1 g IV 8-hourly, and blood cultures were collected. The patient rapidly improved, permitting discharge. Blood cultures subsequently demonstrated a lactose-fermenting gram-negative rod, which was identified by MALDI-ToF as *Burkholderia thailandensis*, with a likelihood score of 1.83, indicating "low confidence" in the identification. The isolate was retested with the BD Phoenix NID panel, identifying the organism as *Burkholderia cepacia* complex with 92% confidence. Finally, the API 20NE (bioMérieux SA, Marcy l'Etoile, France), version 8.0, identified *B. pseudomallei* with 99.8% confidence. As this was the first suspected *B. pseudomallei* isolate in Timor-Leste, and the patient had a clinically compatible illness, the sample was sent to the Royal Darwin Hospital (RDH) for *B. pseudomallei* Type III secretion system (TTS1) polymerase chain reaction (PCR) [12], which confirmed detection of *B. pseudomallei* DNA in April 2022. Following discharge, despite attempts to recall the patient, he was lost to clinical follow-up.

Case 2

In May 2022, a 61-year-old female with a history of type 2 diabetes mellitus and hypertension was admitted with fever, headache, and increasing right upper abdominal pain and distention (Figure 1A). Of epidemiological relevance, the patient reported that their home experienced flooding ~2 weeks prior. The patient was initially treated with IV ceftriaxone 1 g 12-hourly for suspected cholecystitis.

Blood cultures grew *Burkholderia* species, identified by Biomerieux API 20NE as *B. pseudomallei*. The bacterial isolate was confirmed as *B. pseudomallei* at RDH by both lateral flow assay (InBios Active Melioidosis Detect; InBios, Seattle, WA, USA) for *B. pseudomallei* capsular polysaccharide antigen [13] and TTS1 PCR. Antibiotics were changed to meropenem, and the patient remained clinically stable. Computed tomography of the abdomen confirmed a large loculated liver abscess (Figure 1B). At this time, the patient opted not to receive further therapy and discharged herself despite medical advice. She required re-admission the following week due to increasing abdominal pain, and blood cultures were again positive for *B. pseudomallei*. In accordance with Australian treatment guidelines [13], meropenem 1 g IV 8-hourly and trimethoprim-sulfamethoxazole (TMP-SMX) 160/800 mg orally 12-hourly (adjusted for renal function) were commenced. TMP-SMX was withheld after 7 days due to worsening renal impairment. Percutaneous liver drainage was

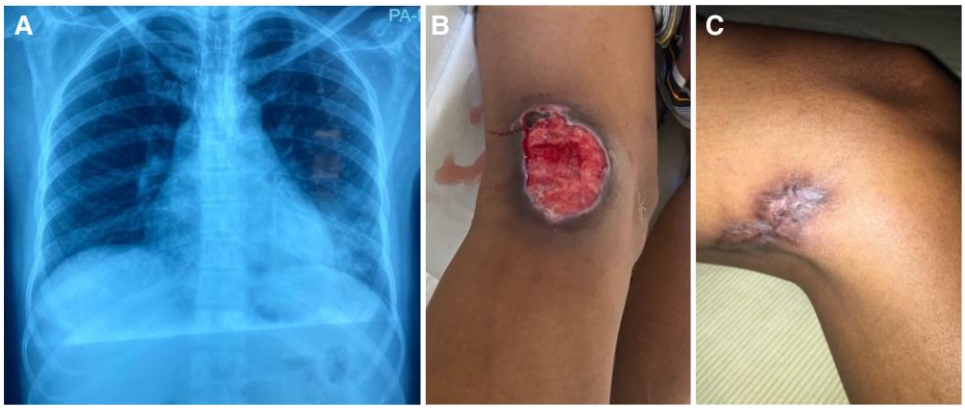

Figure 2. A (left), Chest x-ray demonstrating left lower lobar consolidation. B (middle), Skin and soft tissue infection over the left popliteal fossa. C (right), Healing and scar formation 3 months after commencement of therapy.

performed on day 7 of re-admission with aspirated pus culture positive for *B. pseudomallei*. A second drainage was required 4 weeks later, with pus again culture positive. TMP-SMX was re-started at the full dose, and the patient was prescribed a planned further duration of 4 weeks of IV meropenem 1 g 8-hourly as per guidelines [13], but again self-discharged. She was therefore prescribed oral TMP-SMX, but the patient unfortunately died 3 months later, after presenting to the emergency room with sepsis, which was presumed to be recrudescent melioidosis.

Case 3

In June 2022, a 39-year-old female homemaker without comorbidities was admitted with chest pain. Of epidemiological relevance, she reported regularly gardening but denied any recent traumatic injury. She reported the onset of fever, dyspnea, and dry cough 2 weeks before presentation, with progressive pleuritic chest pain. At admission, her temperature was 37.7°C, with normal blood pressure and oxygen saturation. The initial chest x-ray demonstrated left lower lobar consolidation (Figure 2A).

The patient also reported spontaneous abscess/ulcer formation in the popliteal fossa of the left knee ∼1 month before presentation (Figure 2B). The nonsuppurative lesion was painful and slowly progressive (7 cm in maximum diameter) with a clearly defined edge. Blood and wound cultures grew *B. pseudomallei,* identified by bioMérieux API 20NE. The bacterial isolate was again confirmed as *B. pseudomallei* at RDH by both InBios Active Melioidosis Detect lateral flow assay for *B. pseudomallei* capsular polysaccharide antigen [13] and TTS1 PCR. She was treated with IV meropenem 1 g 8-hourly for 3 weeks followed by TMP-SMX 12-hourly 240–1200 mg for a planned duration of 3 months. The popliteal abscess healed over the course of treatment (Figure 2C).

Whole-Genome Sequencing of *B. pseudomallei* Isolates

DNA extraction of the 3 *B. pseudomallei* isolates was as previously described [14], and the genomes were sequenced on an Illumina NovaSeq 6000 platform (SP Lane 300 cycles; Illumina, Inc., San Diego, CA, USA) at the Australian Genome Research Facility (AGRF).

Analysis of *B. pseudomallei* Whole-Genome Sequences

Variants based on 165 358 core genome single nucleotide polymorphisms (SNPs) were identified among 124 global *B. pseudomallei* genomes (Supplementary Table 1), and a core genome alignment was conducted using the default settings of Snippy, version 4.6.0 [15], with the closed *B. pseudomallei* genome K96243 as reference (N50 4 074 542 bp; 2 contigs; size 7 247 547 bp) [16]. A maximum likelihood phylogenetic tree was generated in IQ-TREE, version 2.2.0.3 [17], using the nucleotide substitution model TVM + F + I + I + R5 selected by the ModelFinder and lowest BIC score [18], and the final tree was rooted using MSHR0668, which was the most ancestral *B. pseudomallei* strain in a large phylogenetic study [19]. Bootstrapping was performed on 1000 replicates. The tree was visualized using Ggtree, version 3.6.1 [20], in R, version 4.1.2 (https://www.r-project.org/). Multilocus sequence types (MLSTs) were assigned in silico using the MLST assignment tool "mlst" (Seemann, T, https://github.com/tseemann/mlst) and the PubMLST website (https://pubmlst.org/) [21].

All 3 *B. pseudomallei* isolates were novel MLST sequence types, designated STs 2016, 2017, and 2021 (Table 1), with ST 2016 and 2017 being single-locus variants (6/7 shared identical alleles), with only the *narK* allele being different, and ST 2021 containing a novel *gmhD* allele and being quite distinct from STs 2016 and 2017. The phylogenetic tree showed that these *B. pseudomallei* isolates from Timor-Leste clearly reside within the Australian-PNG clade with a shared ancestry common to

Table 1. Description of Culture-Confirmed Melioidosis Cases, Including Risk Factors, Clinical Presentation, *B. pseudomallei* Multilocus Sequence Type, Treatment, and Outcome; All Isolates Were Susceptible in Vitro to Meropenem and to Trimethoprim-Sulfamethoxazole at the Doses Currently Recommended by Treatment Guidelines

	Case 1	Case 2	Case 3
Sex	Male	Female	Female
Age	38	61	39
Location	Dili	Dili	Dili
Presenting complaint	Fever and watery diarrhea	Fever and right upper abdominal pain	Fever, pleuritic chest pain, and right popliteal ulcer
Comorbidity	None	Type 2 diabetes mellitus, hypertension	None
Potential exposure events	Unknown	Flood	Gardening
Site of infection	Blood	Blood and hepatic abscess	Blood, lung, skin
Burkholderia pseudomallei MLST	ST 2021	ST 2017	ST 2016
Treatment	Limited duration of IV MEM, unable to commence eradication therapy	4 wk of IV MEM and abscess aspiration, then planned for 4 wk of IV MEM and TMP-SMX from last positive culture, followed by eradication: did not complete course	3 wk of IV MEM followed by eradication with TMP-SMX for 3 mo
Outcome	Lost to follow-up	Died	Full recovery

Abbreviations: MEM, meropenem; ST, sequence type, TMP-SMX, trimethoprim-sulfamethoxazole.

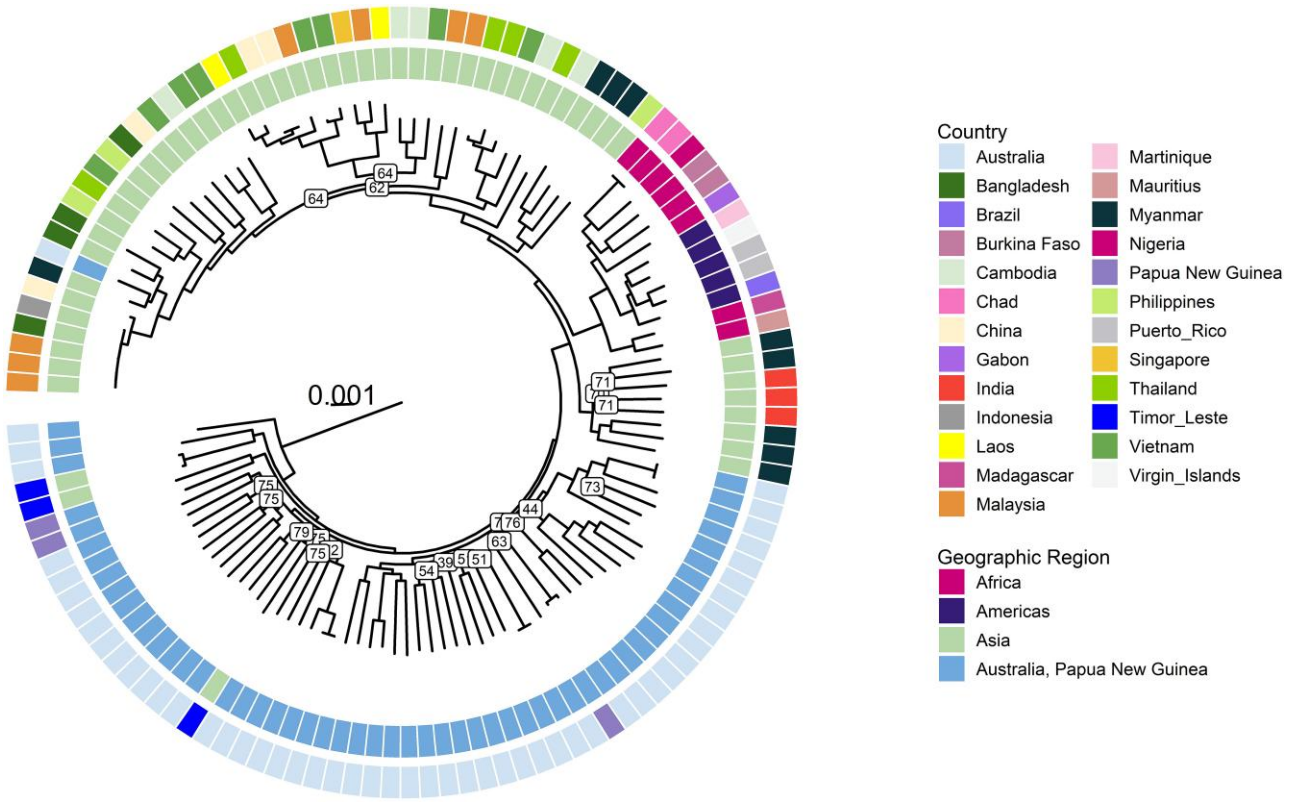

Figure 3. Phylogenetic tree of *B. pseudomallei* isolates. The scale bar indicates substitutions per site. Bootstrap support (based on 1000 replicates) for branches below 80% is shown.

the Australian isolates and are distinct from Asian/Southeast Asian *B. pseudomallei* (Figure 3). While bootstrapping revealed some deep nodes within the tree with lower support (<80%), which is common in *B. pseudomallei* global phylogenies, all main nodes associated with the Timor-Leste isolates were well supported (>90%). Isolates from cases 2 and 3 differed

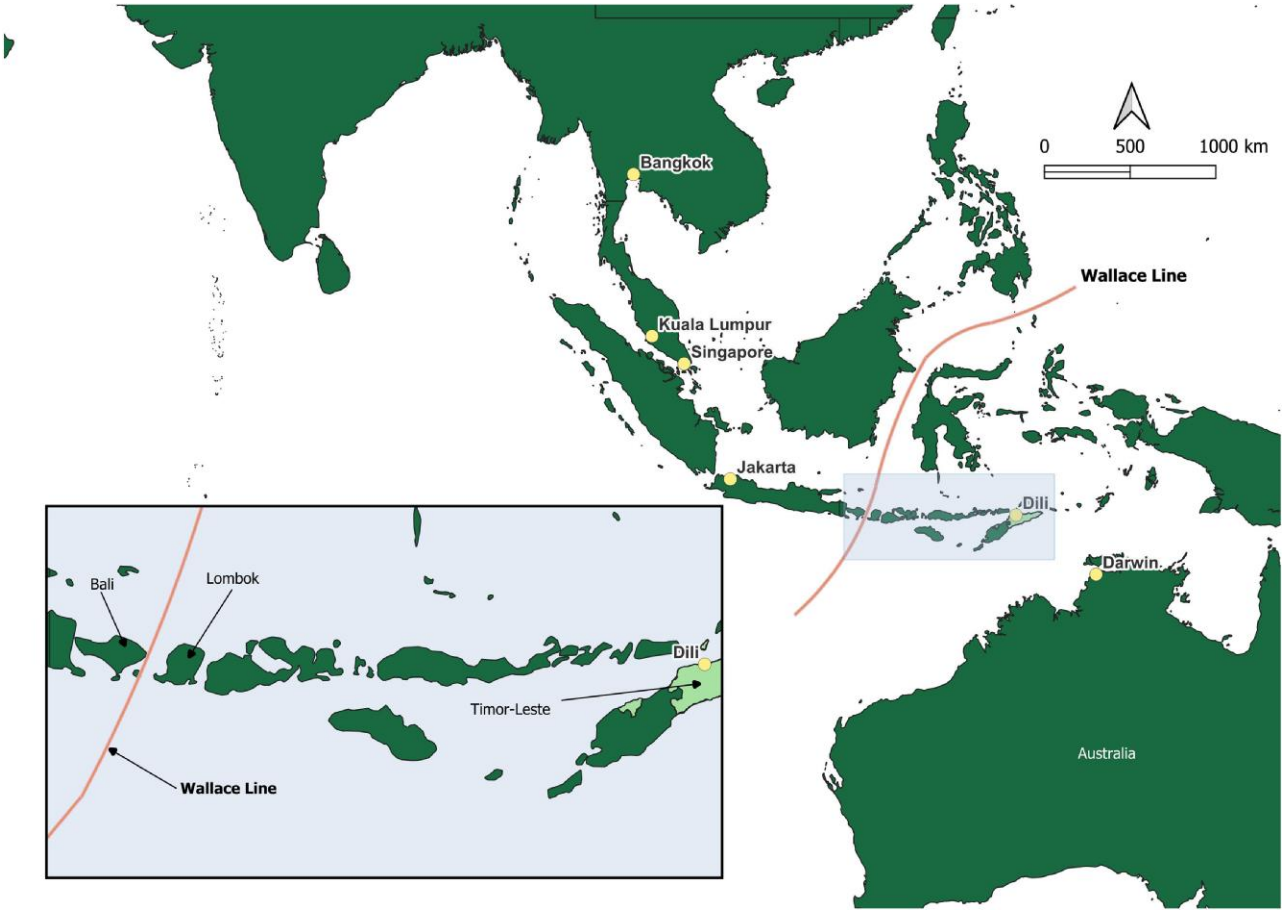

Figure 4. Timor-Leste in relation to the ecological boundary of Wallace's line.

by 1141 core genome SNPs, while there was a difference of >17 000 SNPs between these and the isolate from case 1.

DISCUSSION

This case series describes the first 3 patients in Timor-Leste with culture-confirmed melioidosis. This series highlights the broad clinical presentations of melioidosis, including septic shock, community-acquired pneumonia, deep-seated organ abscess, and cutaneous infection. Educational interventions are urgently needed to increase clinicians' awareness of this disease, promoting microbiological sampling in the appropriate clinical and epidemiological context and adapting antibiotic guidelines and local policy [1, 22]. These cases demonstrate the importance of ongoing investments in local diagnostic laboratory capacity in Timor-Leste. The National Health Laboratory has increased its capacity substantially in recent years; work is continuing to promote the use of diagnostic microbiological testing and to establish blood culture services in each of the municipality referral hospital laboratories in Timor-Leste.

Considering these diagnostic challenges, cases have likely been unrecognized [22–25]. Smear-negative tuberculosis has a high attributable mortality in Timor-Leste, and we hypothesize that melioidosis could be one possible alternative etiology for chronic clinical presentations; with the ability to provide a more accurate microbiological diagnosis, the separation of tuberculosis and melioidosis is now possible [9, 22, 23, 25, 26]. It is anticipated that heightened awareness will result in increased requests for culture and susceptibility testing to confirm the clinical suspicion of melioidosis. Different methods were ultimately required to confirm these diagnoses, namely because the MALDI-ToF analyzer available at the time was unable to confidently speciate the organism. We have recently incorporated additional biochemical spectra to perform in-country confirmatory MALDI-ToF identity testing. Additionally, the recognition of these cases prompted the implementation of Ashdown's agar, a selective media that improves the detection of *B. pseudomallei* from nonsterile specimens, such as sputum [27].

The patients in this case series presented during, or soon after, the wet season in Timor-Leste. Although epidemiological

data are unavailable for the first case identified, two patients reported regular contact with soil and water. This corresponds with data from other endemic regions, where the incidence of melioidosis is higher in the wet season compared with the dry season [1, 22, 25]. One of these 3 patients had a history of diabetes and presented with a massive liver abscess, with a slow clinical response to meropenem, requiring serial percutaneous aspiration. This case is consistent with reports that diabetes mellitus is an important risk factor for poor outcomes in people with melioidosis [1, 22]. Unfortunately, in the context of a prolonged inpatient admission, the patient did not accept further treatment and died 3 months later due to sepsis, which is also the first reported death likely attributable to melioidosis in Timor-Leste.

To complement these clinical case descriptions, genomic sequencing of these isolates establishes the phylogenetic relationship of *B. pseudomallei* in Timor-Leste. In 2009, using bacterial whole-genome sequencing, Pearson et al. described an Australian origin for *B. pseudomallei*, postulating a single introduction event across the Wallace Line and into Southeast Asia [28]. Molecular clock estimates placed the timing of this "out of Australia" spread of *B. pseudomallei* to between 16 thousand years ago and 225 thousand years ago, during the last Ice Age when sea levels were much lower and land bridges joined many current islands to Australia and Papua New Guinea. Genomic sequencing of the Timor-Leste *B. pseudomallei* and subsequent phylogenetic analysis showed them to cluster with Australia and Papua New Guinea isolates, rather than isolates from Southeast Asia, which parallels the division of plant and animal populations by the ecological boundary of Wallace's line [29], which is west of Timor-Leste (Figure 4).

In a study of 276 globally distributed clinical and environmental isolates, Chewapreecha and colleagues used whole-genome sequencing to confirm the clear genetic distinction between isolates from Australasia and Asia and support the "out of Australia" hypothesis, as well as subsequent spread from Asia to Africa and then more recent spread from Africa to the Americas [30]. However, as with Timor-Leste, limited laboratory capacity in much of the tropical world makes for continuing uncertainty about the global footprint of *B. pseudomallei*. While the genetic diversity seen on whole-genome sequencing between ST 2021 and the other two *B. pseudomallei* isolates from Timor-Leste, and their deep branching, suggests unmasking of long-standing endemic presence of the bacterium in that country, the dynamic situation seen in the Americas suggests more recent expansion [31]. Understanding the role of anthropogenic and climate-related drivers in ongoing global dispersal of melioidosis requires improved global surveillance and collaboration [32].

In conclusion, awareness of the clinical spectrum of melioidosis is important and should prompt appropriate diagnostic microbiological testing and empirical treatment. Further research should be conducted to understand the epidemiology of melioidosis in Timor-Leste.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Potential conflicts of interest. All authors: no reported conflicts.

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