Molecular tracking investigation of melioidosis cases reveals regional endemicity in Hainan, China

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Abstract. Sporadic cases of melioidosis have been reported in Hainan, China for decades; however, to the best of our knowledge, there are no accurate source-identification investigations confirming that melioidosis is endemic. Four indigenous melioidosis cases were identified, which prompted the performance of contact microbiologic and molecular techniques to evaluate endemicity. Environmental samples were collected from various locations surrounding each patient's residence. The samples were screened for *Burkholderia pseudomallei* (*B. pseudomallei*) using Ashdown culture medium, and confirmed by polymerase chain reaction and 16S ribosomal DNA sequencing. Clinical and environmental isolates of *B. pseudomallei* were evaluated by multilocus sequence typing (MLST) and 4-locus multilocus variable number tandem repeat analysis (MLVA-4) for evidence of homology between them. Analysis by MLST indicated that one environmental sample and one clinical colony were sequence type-46, as well as type (8, 3, 11, 9) by MLVA-4. The evidence indicates a likely geographical and epidemiological association. Taken together, *B. pseudomallei* from the environmental samples in addition to the high molecular homology between the clinical and environmental isolates indicates, at least, regional endemicity of melioidosis in Hainan, China.

Introduction

Melioidosis is an often fatal infectious disease caused by *Burkholderia pseudomallei* (*B. pseudomallei*). It is known as the 'remarkable imitator' and the 'great mimicker' as the disease may mimic anything from a pyogenic bacterial infection to tuberculosis, with no one feature pathognomonic of melioidosis (1). The disease has an acute or subacute form and a chronic relapsing state that is associated with high mortality. Risk factors for infection include diabetes, alcoholism, renal insufficiency and chronic steroid use (2). The bacterium has a specific ecological niche, existing in soil and stagnant water in an area between the latitudes 20˚N and 20˚S of the equator; prominent endemic areas include Southeast Asia, northern Australia and areas of south America (3,4). The majority of patients are mainly affected by contact with contaminated soil or water, by either percutaneous inoculation or inhalation (5).

Hainan island (between the latitudes of 19˚ and 20˚N) is geographically close to Thailand, an endemic area, and has a population of ~8,600,000 individuals in an area of 35,400 km^2 with 1,823 km of coastline. This province is known as the Chinese vegetable basket and has >700,000 hectares of vegetable and paddy fields. More than one million individuals are at risk of contracting *B. pseudomallei*, which is a great public health concern and an important cause of community-acquired sepsis in Hainan. The first reported case of meliodosis in China was in 1990 in the south county of the Hainan province. Subsequently, a small number of additional cases were described in this region (6). However, the incidence may have previously been underestimated. In recent years, the number of sporadic cases has increased substantially (7). Although the results of these cases confirm that melioidosis is endemic in Hainan, no environmental *B. pseudomallei* isolates have been confirmed as the sources of infection for these melioidosis cases. Similarly, it is not clear whether there are certain natural reservoirs of *B. pseudomallei* in Hainan. Molecular typing methods for *B. pseudomallei* have been successful at assigning isolates into epidemiologically-associated groups. Multilocus sequence typing (MLST) and multilocus variable number tandem repeat analysis (MLVA) provide the ability to generate robust evolutionary hypotheses that enable tracking of *B. pseudomallei* in epidemiological outbreaks at fine phylogenetic scale (8,9). In the present study, a molecular investigation of four indigenous cases of melioidosis, which occurred in Hainan in 2015, is described.

Materials and methods

Samples. The four indigenous patients (who had never traveled outside the country) were admitted between June and August 2015

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Table I. Melioidosis cases identified in Hainan in 2015 (n=4).

Patient ID	County of residence	Possible exposures	Outcome	Clinical features
P1	Dongfang	Often barefoot on soil	Survived	Male student (age, 19 years) exhibited a left parotid abscess, which had been presentfor 10 days. Abscess pus was sampled on admission and was positive for *B. pseudomallei*
P2	Wenchang	Agricultural work	Succumbed	Diabetic male (age, 63 years) rice farmer exhibited chest pain, fever and weakness, which had been present for 7 days. Left lobar pneumonia developed on day 13 when a tracheal suction sample was identified to be positive for *B. pseudomallei*
P3	Qionghai	Agricultural work	Survived	Male (age, 37 years) rice farmer presented witha cough, neck pain and fever, which had commenced 2 days previously. Fulminant sepsis developed on day 3 when the blood sample was was positive for *B. pseudomallei*
P4	Haikou	Unknown	Succumbed	Diabetic housewife (age, 48 years) presented with left lower pleuritic-type and fever, chest pain which had commenced 1 day previously. Pneumonia developed on day 5 when a sputum sample was identified to be positive for *B. pseudomallei*

B. pseudomallei, *Burkholderia pseudomallei*.

Table II. Primers for polymerase chain reaction assays.

Assay	Primer	Forward	Reverse
TTS1	BpTT4176	CGTCTCTATACTGTCGAGCAATCG	CGTGCACACCGGTCAGTATC
YLF	YLF	CCGGGCCTTTCATGCTGTC	TGTTCGGTGATTTCGATTTGGA
BTFC	BTFC	CGAGCGCGTGAATCGAGTTG	CGACTGATCGCCAATTTCCA
cheB	cheB	ATCGGCCGGAGACGATTT	ACTACGCGAATCAATTCGTTTTC
wcbG	wcbG	ACACGCCCGCTGATTCCAA	GGTCCGGCATCGAGGATT
fhaB-1	fhaB-1	CGCCTTGGACGGTCACAT	TCGCTCAATACCGATGGGATG
$bimA_{Bm}$	bimA-Bm	AGCGCTTCGCGCATCTAC	CGCGTTAAACGCCGTACTTTC
$bimA_{BP}$	bimA-Bp	CTCGCTCGCCGGATCAAG	GCTTTGGCGTGCATATCGA
$BurkDiff_{BP}$	BD	CGAGCGCATCGTACTCGTA	CAAGTCGTGGATGCGCATTA

to Hainan People's Hospital (Haikou, China; Table I). The present study was approved by the Ethics Committee of Hainan Medical University (Haikou, China; CEEA2015-217) and all patients provided written informed consent. Samples of sputum, blood and pus were taken for culture, as appropriate. Colonies were identified by oxidative/fermentation glucose test as nonfermentative gram-negative Bacillus and were further identified using the VITEK-2 identification system (BioMérieux, Marcy-I'Etoile, France). The VITEK-2 suggested the isolate was *B. pseudomallei*, with an excellent level of identification (>99%), which was confirmed by 16S ribosomal DNA gene sequencing (Aiji, Ltd., Guangzhou, China). Each colony was stored at -20˚C and subcultured prior to DNA extraction for molecular analysis. Isolates were cultured on standard media at 37˚C overnight. Genomic DNA was extracted with the genomic DNA mini purification kit (Tiangen, Ltd., Beijing, China) according to the manufacturer's instructions for gram-negative bacteria.

Table III. Number of samples positive for *Burkholderia pseudomallei* from different sources.

	Location [positive samples/sample size (n)]			
Sample	P1	P2	P3	P4
Water	0/8	0/8	0/4	0/6
Soil	2/12	0/14	0/16	0/14

P, patient household site.

In August 2015, 20 soil and water samples were collected from various locations within a 300-m sampling radius of each patient's household. In total, 80 sampling sites were examined. Environmental samples were screened for the presence of

Table IV. Polymerase chain reaction assay panel and results used to profile the clinical and environmental isolates.

				Genomic target					
Strain	TTS1	YLF	BTFC	*cheB*	*wcbG*	*fhaB-1*	$bimA_{Bm}$	$bimA_{Bp}$	$BurkDiff_{Bp}$
P1	Pos.	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.	Pos.
P2	Pos.	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.	Pos.
P3	Pos.	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.
P4	Pos.	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.	Pos.
S1	Pos.	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.	Pos.
S2	Pos.	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.	Pos.

P, patient; S, soil; Bp, *Burkholderia pseudomallei*; Bm, *Burkholderia mallei*; Pos., Positive; Neg., Negative.

Table V. MLST and repeat copy number (MLVA-4 code) for six *Burkholderia pseudomallei* strains.

		VNTR loci (MLVA-4)			
Strain	MLST	RCN 2341	RCN 389	RCN 1788	RCN 933
P1	ST46	8	3	11	9
P2	ST30	3	4	8	2
P3	ST55	7	5	12	3
P4	ST1397	4	5	8	3
S1	ST46	8	3	11	9
S2	ST58	9	5	6	4

MLST, multilocus sequence typing; MLVA-4, 4-locus multilocus variable number tandem repeat analysis; VNTR, variable number tandem repeat; RCN, repeat copy number.

B. pseudomallei by culture using procedures described previously (10,11). The environmental isolates of *B. pseudomallei* were confirmed using the VITEK-2 identification system and 16S ribosomal DNA gene sequencing, as described above. Two *B. pseudomallei* isolates were identified during the course of the epidemiologic investigation of the 20 soil samples collected from around the home of one patient.

Polymerase chain reaction (PCR) profiling. Primers for the PCR assays are listed in Table II, as previously described (12). Briefly, PCR was conducted in a final reaction volume of 25 μl containing 1 μl template DNA, 250 nM of each primer, 12.5 μl Taq PCR master Mix (Tiangen, Ltd., Beijing, China), and adding distilled water to reach a final volume of 25 μl. Samples were separated by electrophoresis (100 V for 15 min), stained with ethidium bromide and visualized using the gel documentation system, Quantity One v4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

MLST and MLVA genotyping. Analysis by MLST was performed as previously described (13). Briefly, the alleles at each of the seven previously described loci (*ace*, *gltB*, *gmhD*, *lepA*, *lipA*, *nark* and *ndh*) were assigned for each isolate by comparing sequences with those on the *B. pseudomallei* MLST website (http://bpseudomallei.mlst.net). The allele numbers at each locus were used to define a sequence type (ST) based on the MLST database.

MLVA provided important insights into the genetic heterogeneity of individual strains of *B. pseudomallei* by offering fine-scale resolution, which is unattainable using MLST. Currie *et al* (14) described a simplified 4-locus multilocus variable number tandem repeat analysis (MLVA-4) for *B. pseudomallei* rapid typing (14). Briefly, four VNTR loci (2341, 389, 1788 and 933) were selected, the PCR products were subjected to capillary electrophoresis [20 V/cm (10 kV) for 20 min] using GeneScan 500 LIZ and DNA markers, and analyzed using the ABI software program, GeneMapper v4.1; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Results

Soil and water sampling. B. pseudomallei was isolated from one of the four sites, the results indicated that the geographical distribution of culturable *B. pseudomallei* was confined to a site, which was near the household of a patient with melioidosis (patient 1; P1), where two positive *B. pseudomallei* isolates were obtained from two soil samples. *B. pseudomallei* were not isolated from any of the 78 soil or water samples taken at the three sites where the other three patients lived (Table III).

PCR-profiling. As listed in Table IV, the detection assays (TTS1, YLF, $bimA_{Bp}$, *wcbG*, fhaB-1 and BurkDiff) were positive for all *B. pseudomallei* isolates except one isolate, which was negative for $bimA_{Bp}$ (P2), and the detection assays for Burkholderia mallei ($bimA_{Bm}$) and Burkholderia thailandensis (BTFC and *cheB*) were negative. The YLF-positive versus BTFC-negative results grouped the patient and soil isolates with Southeast Asian populations.

Genotyping of clinical and environmental isolates. MLST and MLVA are commonly used to differentiate closely-associated bacterial strains within outbreaks (15). MLST and MLVA-4 were applied to all six isolates obtained from the four patients and two soil samples (S1 and S2) to determine genotypes. The results for the six isolate genotypes in the study are presented in Table V. MLST of one colony from

one soil sample supported the homogeneity of the genotype of P1 (ST-46). Unlike MLST, the higher-resolution MLVA technique discriminated between the within-patient isolates. Strains that appeared to be closely associated by MLST could, in certain cases, be seen to be linked using MVLA-4, which was able to discriminate between each ST. Between the four clinical isolates and two colonies that were obtained from the two soil samples, a total of four and two MLVA genotypes were identified in P1 to P4 and S1 to S2, respectively. Analysis by MLST indicated that one soil (S1) and clinical colony (P1) were ST-46, as well as type (8, 3, 11, 9) by MLVA-4, indicating that soil may have been the source of infection for this melioidosis case.

Discussion

Melioidosis is a tropical disease, which prevails in regions of Southeast Asia and northern Australia. However, in recent years, the known area of endemicity is expanding and extends to other locations around the globe, including regions of South America, certain countries in Africa, and various Pacific and Indian Ocean islands (4). *B. pseudomallei* is widespread in soil in the regions where it is endemic, such as Guangxi, China (16). Since 1990, cases of melioidosis have been sporadically reported from Hainan, China (17). Although the cases are common enough that Hainan is listed as one of the endemic areas for melioidosis, there is a paucity of information on the geographical distribution and the molecular investigations of *B. pseudomallei* in Hainan, China (18).

In the current study, the first molecular investigations into four cases of non-human-to-human locally acquired melioidosis infection in Hainan are described. There were only two *B. pseudomallei* strains isolated from soil samples obtained at the sites near to the household of one of the patients (P1). P1 was from the Dongfang district, which is situated in the tropical zone of China. This was a locally acquired case, as P1 had never travelled to any other country. The patient reported frequently working barefoot on soil prior to the onset of illness. In this case, with a possible mechanism of subcutaneous inoculation transmission, an in-depth molecular analysis was required to facilitate with understanding the possible nature of the exposure and the isolate. Molecular tracking data are useful for focusing the on-site epidemiological investigation towards possible sources of fomites associated with the exposure. Due to the limitations of MLST in determining the associations between STs, further refinement of the estimates of geographic origin could not be performed using this technique alone. MLVA is known to be highly accurate for defining isolate associations, and has previously been used to differentiate closely associated bacterial strains within outbreaks (19). Therefore, in the present study MLST and MLVA-4 were employed to achieve optimal molecular epidemiologic confidence for this unusual case of melioidosis. The on-site epidemiological investigations identified that isolates P1 and S1 shared the same MLVA-4 pattern and an identical ST. Furthermore, the molecular evidence indicates a likely geographical and epidemiological link. All six *B. pseudomallei* isolates contained the YLF marker, which is predominant in Southeast Asia (20) and one of the isolates amplified the *B. mallei* allele of $bimA_{Bm}$, with the other five having the *B. pseudomallei* allele.

In conclusion, to the best of our knowledge, this is the first molecular tracking investigation of melioidosis in China, and the first published isolation of *B. pseudomallei* from soil in Hainan. Due to the difficulty in identifying *B. pseudomallei*, it is likely that the reported melioidosis cases represent an underestimation of the true incidence of this infection, as there are few laboratories with experience in recognizing this organism. As a result, the actual incidence of melioidosis may be greater than is currently recognized. Whether this increase in the diagnosis of melioidosis in Hainan is due to the widespread existence of *B. pseudomallei* or a previous underdiagnosis of cases remains unclear. Thus, future epidemiological investigations to determine the geographical distribution and seroprevalence rate of *B. pseudomallei* in Hainan are required. Likewise, establishing epidemiological data with melioidosis at a national level using a appropriate surveillance system is considered to be important.

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