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Burkholderia pseudomallei Isolates from Sarawak, Malaysian Borneo, Are Predominantly Susceptible to Aminoglycosides and Macrolides

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Melioidosis is a potentially fatal disease caused by the saprophytic bacterium *Burkholderia pseudomallei*. Resistance to gentamicin is generally a hallmark of *B. pseudomallei*, and gentamicin is a selective agent in media used for diagnosis of melioidosis. In this study, we determined the prevalence and mechanism of gentamicin susceptibility found in *B. pseudomallei* isolates from Sarawak, Malaysian Borneo. We performed multilocus sequence typing and antibiotic susceptibility testing on 44 *B. pseudomallei* clinical isolates from melioidosis patients in Sarawak district hospitals. Whole-genome sequencing was used to identify the mechanism of gentamicin susceptibility. A novel allelic-specific PCR was designed to differentiate gentamicin-sensitive isolates from wild-type *B. pseudomallei*. A reversion assay was performed to confirm the involvement of this mechanism in gentamicin susceptibility. A substantial proportion (86%) of *B. pseudomallei* clinical isolates in Sarawak, Malaysian Borneo, were found to be susceptible to the aminoglycoside gentamicin, a rare occurrence in other regions where *B. pseudomallei* is endemic. Gentamicin sensitivity was restricted to genetically related strains belonging to sequence type 881 or its single-locus variant, sequence type 997. Whole-genome sequencing identified a novel nonsynonymous mutation within *amrB*, encoding an essential component of the AmrAB-OprA multidrug efflux pump. We confirmed the role of this mutation in conferring aminoglycoside and macrolide sensitivity by reversion of this mutation to the wild-type sequence. Our study demonstrates that alternative *B. pseudomallei* selective media without gentamicin are needed for accurate melioidosis laboratory diagnosis in Sarawak. This finding may also have implications for environmental sampling of other locations to test for *B. pseudomallei* endemicity.

Melioidosis is a potentially fatal disease endemic in Southeast Asia, northern Australia, and other tropical regions. Melioidosis is caused by the saprophytic bacterium *Burkholderia pseudomallei*, commonly found in the environment in regions of endemicity, with infection generally occurring from contact with contaminated water or soils (1). Clinical presentations of melioidosis are highly variable and can manifest as asymptomatic infections, localized skin abscess formation, acute or chronic pneumonia, genitourinary, bone, and joint infections, or severe systemic sepsis, with or without foci of multiple abscesses in internal organs, with a mortality of >90% in septic shock cases (2, 3). Due in part to the high virulence of this organism and increased concerns for transmission by aerosolization, *B. pseudomallei* was upgraded to a tier 1 select agent by the U.S. Centers for Disease Control and Prevention in 2012 (http://www.selectagents.gov/).

B. pseudomallei is intrinsically resistant to a wide range of antibiotics, including many β-lactams, aminoglycosides, and macrolides (4–6). This array of drug resistance is conferred through a variety of mechanisms, including inactivating enzymes, cell exclusion, and broad-range efflux pumps. Although almost all *B. pseudomallei* strains are resistant to the aforementioned antibiotics, there have been reports of rare (~0.1%) aminoglycoside and macrolide susceptibility in isolates from Thailand (7, 8) and in a chronic-carriage patient from Australia (9). Aminoglycoside and macrolide resistance in *B. pseudomallei* is thought to be conferred solely by the multidrug efflux pump AmrAB-OprA (10, 11).

In the present study, we identified aminoglycoside and macrolide sensitivity in *B. pseudomallei* isolates of clinical origin from a wide geographical region within Sarawak, Malaysian Borneo. Antibiotic susceptibility was confined to closely related isolates based on multilocus sequence typing (MLST). Using whole-genome sequencing (WGS), we identified a nonsynonymous mutation within the multidrug efflux pump, AmrAB-OprA. Reversion of this mutation restored aminoglycoside and macrolide resistance.

MATERIALS AND METHODS

Ethics. Bacterial strain collection and research were approved by the Medical Research Ethics Committee and registered with the National Medical Research Registrar and the Clinical Research Centre, Ministry of Health of Malaysia. All clinical isolates were from routine melioidosis laboratory diagnosis, and hence no written consent was obtained from patients.

Bacterial strains. The *B. pseudomallei* strains used in this study are listed in Table 1. With the exception of MSHR7596, MSHR7597,

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TABLE 1 *B. pseudomallei* isolates used in this study

Strain	Source	Location or reference	ST[b]	GEN phenotype
MSHR5079	No clinical information	Kapit	50	GEN^r
MSHR5084	67-yr-old female with osteomyelitis; blood culture	Bintulu	658	GEN^r
MSHR5087[a]	3-yr-old female; abscess on scalp	Kapit	881	GEN^s
MSHR5089[a]	45-yr-old female	Kapit	881	GEN^s
MSHR5091	3-yr-old female; abscess	Kapit	881	GEN^s
MSHR5093	62-yr-old female with community-acquired pneumonia; blood culture	Kapit	881	GEN^s
MSHR5095	41-yr-old male with pneumonia; blood culture	Bintulu	881	GEN^s
MSHR5097	34-yr-old male; blood culture	Bintulu	881	GEN^s
MSHR5102[a]	17-yr-old male; neutropenic sepsis	Bintulu	658	GEN^r
MSHR5104[a]	53-yr-old female; blood culture	Bintulu	881	GEN^s
MSHR5105[a]	2-yr-old female; pus from left cervical lymphadenitis	Bintulu	881	GEN^s
MSHR5107[a]	18-yr-old male; pus from neck abscess	Kapit	881	GEN^s
MSHR6381	47-yr-old male; shoulder abscess	Kapit	881	GEN^s
MSHR6385	2-yr-old female with severe community-acquired pneumonia; blood culture	Kapit	881	GEN^s
MSHR6386	27-yr-old male with community-acquired pneumonia; blood culture	Kapit	881	GEN^s
MSHR6387	89-yr-old male with community-acquired pneumonia; blood culture	Kapit	881	GEN^s
MSHR6389	11-yr-old male; lower right eyelid and right cervical abscess, pus	Sibu	881	GEN^s
MSHR6390	30-yr-old male with community-acquired pneumonia	Sibu	881	GEN^s
MSHR6392	18-yr-old male; ankle abscess	Sibu	881	GEN^s
MSHR6401	61-yr-old male with community-acquired pneumonia; blood culture	Sibu	881	GEN^s
MSHR6404	12-yr-old female; pus from lower eyelid abscess	Sibu	881	GEN^s
MSHR6408	No clinical information	Sibu	881	GEN^s
MSHR6409	51-yr-old male with community-acquired pneumonia; blood culture	Sibu	881	GEN^s
MSHR6410	45-yr-old male with knee septic abscess; blood culture	Sibu	881	GEN^s
MSHR6411	27-yr-old male with community-acquired pneumonia; blood culture	Sibu	881	GEN^s
MSHR6414	41-yr-old male with ankle septic arthritis	Sibu	881	GEN^s
MSHR6415	54-yr-old female with sepsis secondary to right leg wound	Kapit	881	GEN^s
MSHR6418	15-mo-old female with occipital abscess; culture positive from abscess	Bintulu	881	GEN^s
MSHR6419	48-yr-old male with community-acquired pneumonia	Bintulu	50	GEN^r
MSHR6769	41-yr-old male with severe pneumonia with sepsis; blood culture	Bintulu	881	GEN^s
MSHR6774	14-yr-old male; pus from cervical abscess	Kapit	881	GEN^s
MSHR6778	11-yr-old female; neck abscess	Kapit	881	GEN^s
MSHR6780	31-yr-old male; pus	Kapit	881	GEN^s
MSHR6785	59-yr-old female; knee abscess	Kapit	881	GEN^s
MSHR6789	12-yr-old male; blood culture	Kapit	881	GEN^s
MSHR6793	37-yr-old female; blood culture	Kapit	997	GEN^s
MSHR6802	3-yr-old female; septic shock blood culture.	Sibu	881	GEN^s
MSHR6803	48-yr-old male	Sibu	881	GEN^s
MSHR6806	37-yr-old female; blood culture	Sibu	881	GEN^s
MSHR6816	34-yr-old male; blood culture	Sibu	46	GEN^r
MSHR6822	41-yr-old male with ankle septic arthritis	Sibu	881	GEN^s
MSHR6824	50-yr-old female with abscess and sepsis; blood culture	Miri	271	GEN^r
MSHR6828	Female; blood culture	Bintulu	881	GEN^s
MSHR6808	61-yr-old male with community-acquired pneumonia; blood culture	Sibu	881	GEN^s
MSHR7596	Spontaneous GEN^r mutant derived from MSHR6808	This study	ND	GEN^r
MSHR7597	Spontaneous GEN^r mutant derived from MSHR6808	This study	ND	GEN^r
MSHR7598	Spontaneous GEN^r mutant derived from MSHR6808	This study	ND	GEN^r
MSHR7599	Spontaneous GEN^r mutant derived from MSHR6808	This study	ND	GEN^r

[a] Whole-genome sequencing was performed on this isolate.
[b] Sequence type as determined by multilocus sequence typing. ND, not determined.

MSHR7598, and MSHR7599, which were derived from MSHR6808, all other strains were collected from melioidosis cases from four hospitals, located in Bintulu, Kapit, Miri, and Sibu, Malaysia. Strains were grown at 37°C for 24 h on tryptic soy agar (TSA) (Oxoid, Australia), regular Ashdown's agar (Oxoid), or Ashdown's agar without gentamicin (GEN) containing an additional 50 μg/ml colistin.

MLST. MLST was performed using previously published methods (12).

Antibiotic sensitivity testing. MICs were determined using Etests (bioMérieux, France) according to the manufacturer's instructions. *B. pseudomallei* strains were grown for 24 h before MIC determination. GEN MIC values for susceptible, intermediate, and resistant were defined as ≤4 μg/ml, 8 μg/ml, and ≥16 μg/ml, respectively (5, 6).

WGS and analysis. WGS was performed on six *B. pseudomallei* isolates (MSHR5087, MSHR5089, MSHR5102, MSHR5104, MSHR5105, and MSHR5107), using an Illumina GAIIx platform according to standard protocols. WGS data were aligned to the *B. pseudomallei* K96243 sequence (13) by using Burrows-Wheeler Aligner (14), with subsequent conversion to binary alignment map (bam) format by using SAMtools (15). Single nucleotide polymorphisms (SNPs) and indels were called by

using GATK (16) with previously published parameters (9). Mutations were assessed for change of function by using PROTEAN (17).

AS-PCR. A novel allele-specific PCR (AS-PCR) assay was designed to differentiate *amrB* mutants from wild-type *B. pseudomallei* isolates. Allele-specific PCR was performed as previously described (18), using one allele-specific forward primer (Invitrogen, Australia) (GmS_F_mut [5′-TCCGCGCGACGCTGATTCCGtG-3′] or GmS_F_wt [5′-TCCGCGCGACGCTGATTCCGtC-3′]) and a common reverse primer (GmS_R_com [5′-ACGTGAGCACGACGGTGATCC-3′]) in each reaction mixture. Underlined nucleotides represent SNP targets that match the wild-type or mutant allele, respectively. Lowercase nucleotides represent a deliberately incorporated penultimate mismatch to enhance allele specificity (19).

Restoration of aminoglycoside resistance. Aminoglycoside-sensitive *B. pseudomallei* isolates were grown in 50 ml tryptic soy broth (Oxoid) at 37°C with shaking at 180 rpm for 18 to 24 h. The bacterial culture was centrifuged at 4,000 rpm for 10 min to pellet cells. The majority of the supernatant was removed and the bacterial cells spread on TSA (Oxoid) containing 40 μg/ml gentamicin (Pfizer) and 30 μg/ml kanamycin (Sigma, Germany). Plates were incubated for 48 h at 37°C. Mutant colonies were purified and retained for further analysis.

Dideoxy sequencing. Dideoxy sequencing of *amrB* was performed by Macrogen (Seoul, South Korea). The following primers were designed and used for amplification and sequencing of this locus: AmrBseq_F (5′-GCCGGGCGTCAAGTACCAGATT-3′) and AmrBseq_R (5′-CTGATCTGCTTCATCGCCTTCAC-3′).

Primer design. All primers were designed using Primer3 software (20, 21).

Nucleotide sequence accession number. The nucleotide sequences for *amrB* have been deposited in the GenBank database under accession numbers KF548562 to KF548567.

RESULTS AND DISCUSSION

MLST of Sarawak isolates indicates a restricted geographical distribution. MLST was performed on all *B. pseudomallei* isolates collected in our study (Table 1), and sequence type (ST) profiles were submitted to the *B. pseudomallei* MLST database (http://bpseudomallei.mlst.net/). Thirty-seven (84%) isolates were ST881, with this ST forming the majority of samples from hospitals in the central region of Sarawak (i.e., Bintulu, Kapit, and Sibu). ST881 has been reported once previously, in a Chinese traveler returning from working in the Malaysian jungle (22). The sole isolate from the Miri hospital in northern Sarawak was ST271. The remaining six isolates belonged to ST46, ST50, ST658, and ST997 (Table 1). With the exception of ST997, which is a single-locus variant (SLV) of ST881, all other STs have been identified previously. ST658 has been found in Laos and Thailand. ST50 has been found in China and Thailand and is an SLV of ST46 and a double-locus variant of ST271. ST46 appears to be widespread and has been reported in historical samples collected from peninsular Malaysia in the 1960s (23) and from Bangladesh, Cambodia, India, Thailand, Vietnam, and the United States, most probably from a traveler to a region of endemicity. In contrast, ST271 appears rarely and, to date, has been found only in Malaysian Borneo.

Sarawak is located in Malaysian Borneo, with rugged topographies resulting in areas with limited access and the existence of subsistent indigenous communities. This ruggedness is especially true in the central region, where ST881 and ST997 were isolated. The apparent geographic restriction associated with limited sequence diversity of ST881 is similar to previous reports of *B. pseudomallei* isolates from Papua New Guinea, where low diversity of *B. pseudomallei* was observed in a geographically isolated region of Western Province (24, 25). The narrow genetic variability within the majority of the Sarawak isolates and those from Papua New Guinea could potentially reflect recent but independent introduction events in those two locations. More likely, however, the observed overlap of certain STs in Malaysian Borneo with other Asian regions supports the hypothesis of a more ancient dispersal of *B. pseudomallei* to Malaysian Borneo, with dispersal and diversity in Sarawak restricted by its remoteness, and potentially also by limited anthropogenic activities (25).

ST881 and ST997 isolates are susceptible to aminoglycosides and macrolides. All ST881 and ST997 isolates ($n = 38$) were shown to be susceptible to the aminoglycoside GEN, with MICs ranging from 0.5 to 1.5 μg/ml (see Table S1 in the supplemental material). In contrast, GEN MICs for wild-type *B. pseudomallei* are approximately 128 μg/ml (7). GEN is a selective component of Ashdown's agar media used for *B. pseudomallei* detection and isolation in regions of endemicity (26). In addition, the ST881 and ST997 isolates were sensitive to kanamycin (also an aminoglycoside) and azithromycin (a macrolide), with MICs of 1.0 and 2.0 μg/ml, respectively (see Table S1). All ST881 and ST997 isolates were susceptible to the antibiotics used in treating melioidosis, notably ceftazidime, meropenem, doxycycline, amoxicillin-clavulanic acid, and trimethoprim-sulfamethoxazole, demonstrating MICs similar to those for other wild-type *B. pseudomallei* strains. Non-ST881/ST997 isolates collected in this study had MIC values similar to those for other wild-type *B. pseudomallei* strains, with sensitivity to the clinically relevant antibiotics and resistance to GEN, kanamycin, and azithromycin (see Table S1).

WGS of four GEN-sensitive (GEN^{s}) isolates revealed a novel cytosine (C)-to-guanine (G) transition located within *amrB* (*amrB*-C1102G), which encodes the membrane-spanning component of the resistance-nodulation-division family (RND) efflux pump, AmrAB-OprA. This nonsynonymous SNP confers a threonine-to-arginine substitution (T367R) in a highly conserved region of the protein. This substitution was predicted to be deleterious by PROTEAN (17). This mutation has not previously been reported for any other sequenced *B. pseudomallei* isolate to date (NCBI database as of 15 May 2013) (see Fig. S1 in the supplemental material). To rapidly interrogate the *amrB*-C1102G SNP in the larger collection of isolates obtained in this study, we designed a novel allelic-specific PCR (AS-PCR) assay. AS-PCR was able to rapidly and accurately determine the SNP state at *amrB* position 1102 and confirmed that the mutation was restricted to *B. pseudomallei* isolates with the GEN^{s} phenotype. All strains in our study that were GEN^{r} possessed the wild-type nucleotide at this position.

Reversion of the C1102G mutation restores aminoglycoside and macrolide resistance. To verify the role of *amrB*-C1102G in aminoglycoside and macrolide sensitivity, we selected GEN^{r} mutant derivatives of a GEN^{s} strain (MSHR6808) and interrogated the *amrB*-C1102G SNP in the mutant strains. Of 95 GEN^{r} mutants, AS-PCR identified 4 that were potentially mutated at *amrB* position 1102. DNA sequence analysis confirmed that only MSHR7599 reverted to the wild-type sequence at *amrB* (*amrB*-G1102C) (R367T). Interestingly, MSHR7596, MSHR7597, and MSHR7598 all possessed a novel thymine transversion at this position (*amrB*-G1102T), which altered the codon to a third configuration, resulting in a methionine substitution (R367M). The GEN MICs for all four mutants were ≥256 μg/ml, whereas the original strain (MSHR6808) had an MIC of 0.75 μg/ml (see Table S1 in the supplemental material). Similarly, the MIC for kanamycin increased from 1.0 μg/ml to 128 μg/ml, and the MIC of azi-

thromycin increased from 1.5 μg/ml to between 32 and 128 μg/ml (see Table S1). These MIC data indicate that methionine is an effective substitute for threonine at this position and demonstrate that *B. pseudomallei* has at least two different ways to develop GEN^r at *amrB* position 1102. Interestingly, 91 of the 95 mutants were unchanged at *amrB* position 1102; thus, multiple mechanisms exist for acquiring GEN^r in *B. pseudomallei*. The genetic bases for resistance in these mutants were not characterized in the current study. Importantly, the restoration of the *amrB*-1102C allele in one mutant, concomitant with acquisition of aminoglycoside and macrolide resistance, is evidence that the C→G substitution in ST881 and ST997 confers aminoglycoside and macrolide susceptibility.

Clinical and environmental significance of aminoglycoside- and macrolide-sensitive *B. pseudomallei* in Sarawak. The emergence and persistence of ST881 and ST997 in Sarawak are not fully understood. Our study has demonstrated that ST881 and ST997 can cause diseases in humans despite being susceptible to aminoglycosides and macrolides, suggesting that the loss of aminoglycoside and macrolide resistance has little consequence for virulence of these strains. However, it cannot be ruled out that this mutation enhances environmental survival of ST881 and ST997. Despite the aminoglycoside and macrolide sensitivity of the *B. pseudomallei* strains in our study, patients infected with these strains had similar clinical presentations and outcomes to those of patients infected with the GEN^r *B. pseudomallei* strains in this study (Table 1; see Table S1 in the supplemental material) and other studies (1, 27–29). This observation is consistent with the work of Trunck and coworkers, who reported unaltered virulence in rare GEN^s isolates from clinical cases (8).

Furthermore, we have shown that the distribution of GEN^s strains is not confined to a small geographic region; presumptive locations of *B. pseudomallei* acquisition for the patients infected with ST881 and ST997 strains span a large region within Malaysian Borneo, with an area of approximately 60,000 km^2 encompassing the Bintulu, Kapit, and Sibu regions (see Fig. S2 in the supplemental material). Although the GEN^s phenotype has previously been observed in *B. pseudomallei*, it has putatively been a result of niche adaptation and genome decay and is very rare (8, 9). To our knowledge, no other instances of clear phenotypic change have been associated with a specific *B. pseudomallei* lineage, possibly with the exception of *Burkholderia mallei*, the causative agent of glanders. *B. mallei* is a clone of *B. pseudomallei* which has undergone genome reduction and has become aminoglycoside sensitive (30, 31). Unlike *B. mallei*, which has lost the entire AmrAB-OprA efflux pump operon, our study identified a unique mechanism leading to aminoglycoside and macrolide susceptibility in ST881 and ST997. Further genomic analysis of these strains would be useful to determine whether they have undergone early stages of genome decay similar to that seen in isolates from chronic-carriage patients (9). In addition, the existence or persistence of these strains in the environment and whether susceptibility to aminoglycosides and macrolides affects their survival in the environment warrant further investigation.

Conclusion. In this study, we analyzed *B. pseudomallei* clinical isolates from Sarawak, Malaysian Borneo, to determine both the prevalence and molecular basis of GEN^s. The majority of these isolates were of a single ST and were sensitive to aminoglycosides and macrolides, a phenotype that is very rare in *B. pseudomallei* strains from other regions of endemicity. Aminoglycoside sensitivity was the result of a nonsynonymous SNP within *amrB*. Most selective media for *B. pseudomallei* growth contain GEN as a selective agent (26, 32, 33). Use of these media in Sarawak as a sole diagnostic tool for melioidosis is problematic because, based on our findings, approximately 86% of cases would not be diagnosed. Alternative selective media without GEN (for example, Ashdown's medium supplemented with colistin instead of GEN) may be a more appropriate option for laboratory diagnosis of suspected melioidosis cases in Sarawak. Laboratory scientists and clinicians in central Sarawak and surrounding regions should exercise caution in confirming *B. pseudomallei* infection by use of selective media containing GEN. Our findings have significant implications for laboratory diagnosis and environmental sampling of *B. pseudomallei*, particularly in Malaysian Borneo and other potential regions of endemicity where *B. pseudomallei* has yet to be uncovered.

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