Research Article

Antimicrobial Susceptibility and Genetic Characterisation of Burkholderia pseudomallei Isolated from Malaysian Patients

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Burkholderia pseudomallei, the causative agent of melioidosis, is intrinsically resistant to many antibiotics. Ceftazidime (CAZ), the synthetic β-lactam, is normally used as the first-line antibiotic therapy for treatment of melioidosis. However, acquired CAZ resistance can develop in vivo during treatment with CAZ, leading to mortality if therapy is not switched to a different antibiotic(s) in a timely manner. In this study, susceptibilities of 81 B. pseudomallei isolates to nine different antimicrobial agents were determined using the disk diffusion method, broth microdilution test and Etest. Highest percentage of susceptibility was demonstrated to CAZ, amoxicillin/clavulanic acid, meropenem, imipenem, and trimethoprim/sulfamethoxazole. Although these drugs demonstrated the highest percentage of susceptibility in B. pseudomallei, the overall results underline the importance of the emergence of resistance in this organism. PCR results showed that, of the 81 B. pseudomallei, six multidrug resistant (MDR) isolates carried bpeB, amrB, and BPSS1119 and penA genes. Genotyping of the isolates using random amplified polymorphic DNA analysis showed six different PCR fingerprinting patterns generated from the six MDR isolates clusters (A) and eight PCR fingerprinting patterns generated for the remaining 75 non-MDR isolates clusters (B).

1. Introduction

Melioidosis, an infectious disease of major public health importance, is a multifaceted disease which is difficult to treat and results in high morbidity and mortality. It is mostly endemic in Southeast Asia, Northern Australia, the Indian subcontinent, and Central and South America [1, 2]. The causative agent of this fatal disease, Burkholderia pseudomallei, is an environmental saprophyte that can be isolated from soil and water and is known as a potential biological warfare agent [2]. Infection of B. pseudomallei is acquired mainly through wound inoculation, inhalation, or ingestion [2, 3]. Melioidosis may arise many years after exposure, commonly in association with compromised immunity. Overall, the mortality rate of melioidosis is about 50% in Northeast Thailand (35% in children) [3] and 19% in Australia [4]. However, in Malaysia, it was associated with 65% mortality especially in the septicaemic form in the 1980s and reduced to 19–37% in the past 20 years [1, 5–7]. Recurrence of infection is the most important complication in survivors despite prolonged antimicrobial treatment and this has been reported in 10% of Thai patients who survived the primary infection episode [4].

B. pseudomallei is intrinsically resistant to many antibiotics, including penicillin, first- and second-generation cephalosporins, macrolides, rifamycins, colistin, and aminoglycosides, but is usually susceptible to amoxicillin/clavulanic acid (AMC), chloramphenicol (CL), doxycycline (DOXY), trimethoprim/sulfamethoxazole (TS), ureidopenicillins, cefazidime (CAZ), and carbapenems [8]. CAZ, AMC, or the carbapenem antibiotics are used for the initial parenteral phase of the therapy, followed by a prolonged course of oral antimicrobial therapy with either TS with or without DOXY or AMC [9]. However, CAZ-resistant (CAZR) and/or AMC-resistant B. pseudomallei have emerged, ultimately leading to treatment failure [9]. The carbapenem antibiotics have been reported to have good bactericidal activities against B. pseudomallei and have been used effectively to treat patients with septicaemic
melioidosis [10, 11]. However, increased use of carbapenems may again give rise to resistance as observed with CAZ and AMC.

Owing to the difficulty in eradication of the organism following infection, prolonged antibiotic therapy is needed and a high rate of relapse was observed if the therapy is incomplete [12]. Furthermore, recurrence of infection is common despite adequate antimicrobial therapy [13]. It has been shown that B. pseudomallei isolated from relapse cases and persistent infections were resistant to antibiotics for treatment [3, 14].

Thus, the aim of this study was to investigate the antimicrobial susceptibility profile of B. pseudomallei against a panel of medically relevant antibiotics including CAZ, AMC, DOXY, and TS. Evaluation using other antimicrobials such as CL, meropenem (MERO), imipenem (IMP), tigecycline (TGC), and clarithromycin (CLA) were also included. The antimicrobial susceptibility was performed using two determination methods, that is, the agar disc diffusion and Etest. Additionally, polymerase chain reaction (PCR) technique utilising specific primers was also used for detection of antibiotics resistant genes as this proves to be reliable for detection of β-lactam, aminoglycosides, and CAZR genes in multidrug resistant (MDR) isolates. Further investigation using random amplification of polymorphic DNA (RAPD) analysis was performed to identify the genetic relationships among the isolates and the mode of dissemination of the antibiotic resistance genes.

2. Materials and Methods

2.1. Ethics Statement. Ethics approval is not required in this study. No consent was required since no human participant was involved. All 70 clinical B. pseudomallei isolates used in this study were obtained from old archival bacterial collection and all the isolates used were anonymised. Since our institute is a teaching hospital, bacterial isolates obtained as a part of a diagnostic screening are archived and such cases are exempted from obtaining ethical clearances. The study however has an Institutional Biosafety Committee approval.

2.1.1. Bacterial Strains. A total of 81 B. pseudomallei isolates (70 clinical, 10 animal, and 1 soil) and one Burkholderia thailandensis (ATCC 700388) isolates were investigated in this study. Of the 70 clinical B. pseudomallei, 60 isolates were obtained from archival collections of strains isolated at the Medical Microbiology Diagnostic Laboratory, University of Malaya Medical Centre (UMMC, Kuala Lumpur), and 10 from Hospital Tengku Ampuan Afzan (HTAA, Kuantan, Pahang). The ten animal isolates were kindly provided by S Nathan (Universiti Kebangsaan Malaysia), Malaysia, and the soil isolate was obtained from the Bacteriology Unit, Institute of Medical Research (IMR), Malaysia. These isolates were confirmed using Gram-stain, morphological appearance on Ashdown agar, molecular identification using an in-house PCR method [18] and standard biochemical characterisation using API 20NE assay (Bio-Merieux, France). Escherichia coli (ATCC 25922) was used as control while B. pseudomallei K96243 was used as reference strain. The isolates used in this study were obtained from different sources, that is, soil, animal and human (blood, pus, sputum, urine, spleen, and lungs).

2.2. Antibiotic Susceptibility Testing

2.2.1. Disk Diffusion Test. In vitro antimicrobial susceptibility to nine antibiotics/antimicrobials, namely, CL, AMC, DOXY, MERO, IMP, CAZ, TGC, CLA, and TS, was determined using disc diffusion method according to the British Society of Antimicrobial Chemotherapy [19]. Nutrient agar plates were seeded with 100 μL of 10^6 colony forming unit per milliliter (CFU/mL) of the test isolates, which had been adjusted to 0.5 McFarland reading spectrophotometically at 600 nm. The agar plates were then incubated at 37°C for 24 hrs.

2.2.2. Minimum Inhibitory Concentrations (MICs). Antimicrobial MICs were determined using the broth dilution method and Etest. The broth dilution method was performed using Luria-Bertani (LB) broth (Difco, Lennox) for 24 hrs at 37°C with 10^5 CFU/mL of B. pseudomallei (previously washed using normal saline) as the inoculum. Interpretation of the broth dilution results was based on the NCCLS MIC breakpoints for non-Enterobacteriaceae and MIC for B. pseudomallei and B. thailandensis [19]. Inhibition of the bacterial growth was confirmed by spectrophotometric measurement at 600 nm and plating of the serial dilutions onto LB agar. Wells containing bacteria without antibiotics and fresh LB broth were included as positive and negative growth controls, respectively. E. coli ATCC 25922 was used as quality control organism in the antimicrobial MIC determinations. MIC determination using Etest strips (AB Biodisk, Sweden) was performed according to the manufacturer’s instruction.

2.2.3. Polymerase Chain Reaction (PCR). The DNA of all the isolates tested was extracted using Qiagen Mini Amp Kit (Qiagen, USA) according to the manufacturer’s instruction. Primers (PenA-F and PenA-R) were used to identify the presence of penA gene from class A β-lactamase which causes the CAZR (Table I). Presence of the efflux pump, another main resistance mechanism, was also detected using the degenerate primers, MxBs, MxYs, and MxFs (Table I). PCR amplification was performed in 25 μL mixture containing 1X PCR buffer (MBI Fermentas, USA), 2 mM MgCl₂, 0.2 mM dNTP (MBI Fermentas, USA), 2.5 U Taq DNA polymerase (MBI Fermentas, USA), and 2 μL of DNA template. The PCR conditions used were as follows: initial denaturation at 95°C for 5 mins, 30 cycles of 95°C for 1 min, 55°C for 30 s and 72°C for 1 min, followed by a final extension for 10 mins at 72°C. The amplified PCR products were analysed using 1.5% agarose gel (Promega, USA) electrophoresis in 1X TBE buffer at 90 V for 1 hr and visualised using 3 μL SYBR safe (Life Technology, USA) staining under UV illumination. The limit of dilution was determined by subjecting the DNA of the targeted organisms to PCR after 10-fold serial dilution to produce DNA concentration ranging from 10 mg/mL to 10 fg/mL [17].
The highest reading for the Etest results (0.125–128 mg/L) was demonstrated for the MICs of CAZ, which also correlated with the most resistant isolate identified among the drug of choice for treatment of melioidosis. The MDR isolates had susceptibility towards MERO.

### 3. Results

#### 3.1. Antibiotic Sensitivity Testing

**3.1.1. Disk Diffusion.** The results indicated that, of the 81 *B. pseudomallei* isolates tested, six were found to be MDR (resistant to MERO, IMP, and CAZ). However, the remaining 75 isolates were resistant to at least one of the antimicrobial agents (non-MDR). The MDR isolates had susceptibility results that were distinct from other isolates tested (Table 2). The MERO- and IMP-resistant isolates had smaller inhibition zone (15.7 mm) compared to the sensitive isolates (20 to 25 mm). These isolates were also persistently mucoid and the inhibition zone diameter was not distinct. Among the 81 *B. pseudomallei* isolates, only 4.94% were CAZR and 2.47% showed intermediate susceptibility to CAZ, which is among the drug of choice for treatment of melioidosis. The CAZ isolates had no inhibition zone compared to the CAZ-sensitive isolates (24 to 29 mm).

**3.1.2. MIC Etest.** The highest reading for the Etest results (0.125–128 mg/L) was demonstrated for the MICs of CAZ, which also correlated with the most resistant isolate identified among the drug of choice for treatment of melioidosis. The MDR isolates had susceptibility towards MERO.
| Strain ID | Origin of isolation | Antimicrobial susceptibility testing | PCR detection |
|----------|--------------------|-------------------------------------|---------------|
|          |                    | CL  | CLA | DOXY | IMP | MERO | TGC | TS  | CAZ | AMC | PenA, bpeB, amrB & BPSS1119 | RAPD |
| Bp1      | Blood              | I   | S   | S    | S    | S    | R    | S   | S   | S   | S   | Negative | B   |
| Bp2      | Blood              | I   | I   | S    | S    | S    | R    | S   | S   | S   | S   | Negative | B   |
| Bp3      | Blood              | I   | I   | R    | R    | S    | R    | S   | S   | S   | S   | Negative | B   |
| Bp4      | Blood              | S   | I   | S    | S    | S    | R    | S   | S   | S   | R   | Negative | B   |
| Bp5      | Blood              | R   | R   | R    | R    | R    | R    | R   | R   | R   | R   | PenA, bpeB, amrB & BPSS1119 | A   |
| Bp6      | Blood              | S   | S   | S    | S    | S    | R    | S   | S   | S   | I   | Negative | B   |
| Bp7      | Blood              | I   | S   | S    | S    | S    | R    | S   | S   | R   | S   | Negative | B   |
| Bp8      | Blood              | S   | S   | S    | S    | S    | I    | S   | S   | S   | S   | Negative | B   |
| Bp9      | Blood              | R   | R   | S    | R    | S    | R    | S   | S   | R   | S   | Negative | B   |
| Bp10     | Blood              | S   | S   | S    | S    | S    | R    | S   | S   | S   | R   | Negative | B   |
| Bp11     | Urine              | S   | S   | S    | S    | S    | R    | S   | S   | R   | S   | Negative | B   |
| Bp12     | Blood              | I   | S   | S    | S    | S    | S    | S   | S   | S   | S   | Negative | B   |
| Bp13     | Blood              | S   | S   | S    | S    | S    | S    | S   | S   | S   | S   | Negative | B   |
| Bp14     | Blood              | S   | S   | S    | S    | S    | S    | S   | S   | S   | S   | Negative | B   |
| Bp15     | Blood              | S   | I   | S    | S    | S    | R    | S   | S   | S   | S   | Negative | B   |
| Bp16     | Sputum             | I   | I   | S    | S    | S    | R    | S   | S   | S   | R   | Negative | B   |
| Bp17     | Blood              | I   | S   | S    | S    | S    | S    | R   | S   | S   | I   | Negative | B   |
| Bp18     | Pus                | R   | I   | R    | R    | R    | R    | R   | R   | R   | R   | PenA, bpeB, amrB & BPSS1119 | A   |
| Bp19     | Blood              | I   | I   | S    | S    | S    | R    | S   | S   | S   | S   | Negative | B   |
| Bp20     | Blood              | S   | I   | S    | S    | S    | R    | S   | S   | S   | I   | Negative | B   |
| Bp21     | Blood              | I   | S   | S    | S    | R    | R    | S   | R   | S   | R   | Negative | B   |
| Bp22     | Blood              | S   | S   | S    | S    | S    | R    | S   | R   | S   | S   | Negative | B   |
| Bp23     | Blood              | I   | I   | S    | S    | S    | R    | I   | S   | S   | I   | Negative | B   |
| Bp24     | Lung               | S   | S   | S    | S    | S    | S    | R   | I   | S   | I   | Negative | B   |
| Bp25     | Blood              | I   | R   | R    | R    | R    | R    | R   | R   | I   | R   | PenA, bpeB, amrB & BPSS1119 | A   |
| Bp26     | Blood              | S   | S   | S    | S    | S    | S    | S   | S   | S   | I   | Negative | B   |
| Bp27     | Blood              | S   | S   | S    | S    | S    | S    | S   | R   | S   | I   | Negative | B   |
| Bp28     | Blood              | S   | I   | S    | S    | S    | S    | S   | I   | S   | I   | Negative | B   |
| Bp29     | Blood              | I   | S   | S    | S    | S    | S    | R   | S   | I   | S   | Negative | B   |
| Bp30     | Spleen             | I   | S   | S    | S    | S    | S    | S   | S   | S   | I   | Negative | B   |
| Bp31     | Blood              | S   | S   | S    | S    | S    | S    | R   | R   | S   | S   | Negative | B   |
| Bp32     | Blood              | I   | I   | S    | S    | S    | R    | I   | S   | R   | S   | Negative | B   |
| Bp33     | Blood              | I   | I   | S    | S    | S    | R    | S   | R   | S   | I   | Negative | B   |
| Bp34     | Blood              | I   | S   | S    | S    | S    | I   | S   | S   | S   | S   | Negative | B   |
| Bp35     | Blood              | R   | R   | R    | R    | R    | S    | R   | I   | S   | PenA, bpeB, amrB & BPSS1119 | A   |
| Bp36     | Blood              | S   | S   | S    | S    | S    | S    | S   | S   | S   | S   | Negative | B   |
| Bp37     | Blood              | R   | I   | R    | R    | R    | R    | R   | R   | R   | I   | PenA, bpeB, amrB & BPSS1119 | A   |
| Bp38     | Blood              | I   | I   | S    | S    | S    | S    | R   | I   | S   | S   | Negative | B   |
| Bp39     | Blood              | S   | S   | S    | S    | S    | S    | R   | S   | S   | S   | Negative | B   |
| Bp40     | Blood              | S   | S   | S    | S    | S    | R    | S   | S   | S   | S   | Negative | B   |
| Bp41     | Sputum             | I   | S   | S    | S    | S    | R    | S   | S   | S   | R   | Negative | B   |
| Bp42     | Blood              | I   | I   | S    | S    | S    | S    | I   | S   | S   | I   | Negative | B   |
| Bp43     | Blood              | I   | I   | S    | S    | S    | S    | S   | S   | S   | I   | Negative | B   |
| Bp44     | Blood              | I   | I   | S    | S    | S    | S    | S   | S   | S   | I   | Negative | B   |
| Bp45     | Blood              | I   | I   | S    | S    | S    | S    | S   | S   | S   | S   | Negative | B   |
| Bp46     | Blood              | I   | R    | S    | S    | S    | S    | S   | S   | S   | S   | Negative | B   |
| Bp47     | Blood              | S   | S    | S    | S    | S    | R    | I   | S   | R   | S   | Negative | B   |
| Bp48     | Blood              | I   | R    | S    | S    | S    | S    | S   | S   | S   | I   | Negative | B   |
| Bp49     | Blood              | S   | I   | S    | S    | S    | R    | S   | S   | R   | Negative | B   |
Table 2: Continued.

| StrainID | Origin of isolation | Antimicrobial susceptibility testing | PCR detection | RAPD |
|----------|---------------------|--------------------------------------|---------------|------|
| Bp50     | Blood               | I S S S S R I S R                   | Negative      | B    |
| Bp51     | Blood               | S S S S R I S R                     | Negative      | B    |
| Bp52     | Blood               | R R R S S S S S                    | Negative      | B    |
| Bp53     | Blood               | I R S S S S S                      | Negative      | B    |
| Bp54     | Blood               | R R S S S S S S                    | Negative      | B    |
| Bp55     | Blood               | R S S S S S S                      | Negative      | B    |
| Bp56     | Blood               | I S S S S S S                      | Negative      | B    |
| Bp57     | Blood               | I R S S S S S                      | Negative      | B    |
| Bp58     | Sputum              | S S S S S R R                     | Negative      | B    |
| Bp59     | Sputum              | S I S S S R I                      | Negative      | B    |
| Bp60     | Sputum              | S S S R R R R                     | Negative      | B    |
| Bp61     | Animal              | I S S S S I S                      | Negative      | B    |
| Bp62     | Animal              | I S S S S I S                      | Negative      | B    |
| Bp63     | Animal              | S S S S S S I                      | Negative      | B    |
| Bp64     | Animal              | S S S S S S S                      | Negative      | B    |
| Bp65     | Animal              | I S S S S S I                      | Negative      | B    |
| Bp66     | Animal              | I S S S S S I                      | Negative      | B    |
| Bp67     | Animal              | S S S S S S S                      | Negative      | B    |
| Bp68     | Animal              | I S S S S S S                      | Negative      | B    |
| Bp69     | Animal              | I S S S S S S                      | Negative      | B    |
| Bp70     | Animal              | I S S S S S S                      | Negative      | B    |
| Bt71     | Soil                | I I S S S S I                      | Negative      | B    |
| Bp72     | Blood               | I I S S S S R                      | Negative      | B    |
| Bp73     | Blood               | R I S S S S R                      | Negative      | B    |
| Bp74     | Blood               | I R S S S S R                      | Negative      | B    |
| Bp75     | Blood               | S S S S S S S                      | Negative      | B    |
| Bp76     | Blood               | R R S S S S R                      | Negative      | B    |
| Bp77     | Blood               | R I S S S S I                      | Negative      | B    |
| Bp78     | Blood               | R R I S S S R                      | Negative      | B    |
| Bp79     | Blood               | R R I S S S R                      | PenA, bpeB, amrB & BPSSII19 A |
| Bp80     | Blood               | R R I S S S R                      | Negative      | B    |
| Bp81     | Blood               | I S S S S S I                      | Negative      | B    |
| Bp82     | Blood               | I S S S S S S                      | Negative      | B    |

Foot note: R: resistant; I: intermediate; S: sensitive; CL: chloramphenicol; AMC: amoxicillin/clavulanic acid; DOXY: doxycycline; MERO: meropenem; IMP: imipenem; CAZ: ceftazidime; TGC: tigecycline; CLA: clarithromycin; TS: trimethoprim/sulfamethoxazole.
Intermediate is counted as resistant.

aBlood, pus, sputum, urine, spleen, and lungs are of human origin.

Bp: *B. pseudomallei*; Bt: *B. thailandensis*.

non-MDR isolates, only a single distinct cluster (B) was found (Table 2 and Figure 2).

4. Discussion

In the current scenario, melioidosis is considered as a fatal disease with no effective vaccine available. Treatment also requires prolonged and high dosage antibiotic therapy to accomplish complete eradication. Nevertheless, prolonged therapy can lead to the development of resistance and to make matters worse, the causative agent, *B. pseudomallei*, is intrinsically resistant to a wide range of antibiotics. Thus, treatment options for melioidosis are limited to a small number of antimicrobial agents such as CAZ (primary treatment) and TS, DOXY, or AMC (secondary treatment) [21]. Despite many trials, CAZ remains as the drug of choice for treatment of severe melioidosis [22]. Carbapenems have also been shown to be highly active against *B. pseudomallei* [23]. As a result, resistance developed towards these antibiotics can pose a significant challenge in treatment of melioidosis.

In our study, comparison of Etest and broth microdilution against disk diffusion test for 81 *B. pseudomallei* strains and a *B. thailandensis* strain showed broad range of MICs and zone inhibition, respectively. Our findings may also be indicative of the increased antibiotic resistance in *B. pseudomallei* clinical strains. In short, it appears that either disc diffusion or Etest
### Table 3: Broth microdilution and Etest MICs of 81 *B. pseudomallei* isolates.

| Antimicrobial agent            | MIC (mg/L) | Broth dilution | Etest |  
|-------------------------------|------------|----------------|-------|
|                               |            | Range | 50% | 90% | Range | 50% | 90% |
| Chloramphenicol               |            | 4–16  | 8   | 16  | 0.25–24 | 3   | 8   |
| Amoxicillin/clavulanic acid   |            | 4–8   | 8   | 8   | 0.125–2 | 0.25 | 0.25 |
| Doxycycline                   |            | 0.5–1 | 0.5 | 1   | 0.19–16 | 0.19 | 16  |
| Meropenem                     |            | 3–4   | 2   | 4   | 1–4   | 1   | 1.5 |
| Imipenem                      |            | 0.5–1 | 0.5 | 1   | 0.094–8 | 0.094 | 0.125 |
| Ceftazidime                   |            | 2–64  | 2   | 4   | 0.125–128 | 0.250 | 0.5 |
| Tigecycline                   |            | 3–4   | 2   | 6   | 0.5–32  | 3   | 6   |
| Clarithromycin                |            | 4–16  | 4   | 16  | 1.5–48  | 1   | 32  |
| Trimethoprim/sulfamethoxazole |            | 4–64  | 16  | 64  | 0.003–0.25 | 0.032 | 0.125 |

Foot note: *multidrug resistant isolates.

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**Figure 2:** Random amplified polymorphic DNA (RAPD) polymorphisms of *B. pseudomallei* clinical isolates amplified by primer 272. The representative fingerprint patterns of two clusters RAPD were shown (i) RAPD cluster A (MDR) and (ii) RAPD cluster B (non-MDR).
could be applied for susceptibility testing in the case of CL, AMC, DOXY, MERO, IMP, CAZ, TGC, CLA, and TS in *B. pseudomallei* isolates (Table 2).

Additionally, in general, the Etest based antimicrobial susceptibility testing has the advantage of providing quantitative MICs results, which may be useful for clinicians to select appropriate treatments while the disc diffusion is a more reliable and cost effective technique for determining the prevalence of resistance among *B. pseudomallei* isolates in the more routine monitoring of melioidosis. A study by Piliouras et al., on the comparison of antibiotic susceptibility testing methods for TS with *B. pseudomallei*, also reported that the disc diffusion test was inappropriate for assessing the susceptibility of *B. pseudomallei* to TS. The study suggested that MIC based methods such as Etest may be a better choice for the determination of susceptibility for TS [24]. Difficulty in interpretation of disc diffusion results have also been implicated in previous studies which demonstrate varying susceptibility test results for TS with *B. pseudomallei* [25, 26]. This may be attributed to a few reasons including the imprecise endpoints for this combination of antimicrobial agent and organism as well as NCCLS guidelines being based on control standards of *Pseudomonas aeruginosa* and not *B. pseudomallei* [27, 28]. Thus, it is also evident that further creation of suitable internationally accepted MIC/zone inhibition breakpoints needs to be established through multilaboratory quality control. Several studies have been proposed for determination of the MICs, since these methods are cost effective and still able to produce reliable results [15, 29].

We also performed molecular level investigation to detect the presence of efflux pump and class A \( \beta \)-lactamase genes, which were found to be present in all the MDR *B. pseudomallei* isolates. Previous studies have associated the mutations in class A \( \beta \)-lactamase gene of *B. pseudomallei* to the resistance towards some cephalosporins and \( \beta \)-lactamase inhibitors [15]. It has been previously demonstrated that mutations in the *B. pseudomallei* class A \( \beta \)-lactamase coded by penA may confer resistance of the organism to CAZ. Both the low and high level of CAZR in the study of *B. pseudomallei* clinical isolates were confirmed by the detection of penA gene [15]. The presence of penA in *B. pseudomallei* used in our study matched the disc diffusion and MICs results.

Additionally, the necessity to characterise genes encoding for MDR efflux pump arises from the increased resistance of *B. pseudomallei* to a number of antibiotics. The presence of resistant nodulation division (RND) efflux pumps may contribute to the acquired resistance to fluoroquinolones and cross-resistant to unrelated antimicrobials [30]. This is not surprising as the efflux pumps can be associated with MDR isolates since they may be specific for one substrate or transport a wide range of antibiotics of multiple classes [31]. Primers MxBs, MxYs, and MxFs were used to amplify genes that encode proteins which are parts of the RND efflux pump [30]. The role of this efflux pump is associated with resistance towards a wide range of antibiotics including pefloxacin, ofloxacin, and CAZ in *B. pseudomallei* and accompanied by an increased resistance to aminoglycosides, \( \beta \)-lactams, macrolides, and CL [30, 31]. In this study, isolates that contained these genes (*bpeB*, *amrB*, and BPSSI119) were shown to be resistant to AMC, CLA, and CAZ as reported using the disc diffusion and MICs. The *bpeB*, *amrB*, and BPSSI119 genes used in our study focus on chromosomally encoded MDR efflux pumps, which have been the best developed and the most widely used for demonstrated efflux pump genes using primers [31].

In conclusion, our study demonstrated the antimicrobial susceptibility profiles of *B. pseudomallei* against antibiotics that are commonly used for treatment including CAZ, AMC, DOXY, and TS as well as other antimicrobials such as CL, MERO, IMP, TGC, and CLA. The antimicrobial susceptibility profiles did not show any significant correlation with the origin of the isolates as similarly reported in a previous study [32]. We also highlighted that the CAZR is due to the presence of *penA*, *bpeB*, *amrB*, and BPSSI119 in *B. pseudomallei*. However, except for the six blood isolates, none of the animal, soil, sputum, pus, lung, or spleen isolates were tested positive with the PCR detection assays for *penA*, *bpeB*, *amrB*, and BPSSI119. Thus, it may be possible that the resistant blood isolates were from relapse patients or patients who did not comply with antibiotic treatment. However, this remains to be elucidated. Horizontal dissemination of these genes may contribute to further emergence of CAZR in various other Gram-negative bacteria. Therefore, appropriate surveillance and control measures are essential to prevent further spread of the CAZR organisms. Further study using PCR-single-stranded conformational polymorphism (PCR-SSCP) to identify the point mutations which take place in the *B. pseudomallei* isolates will be used to compliment and facilitate better understanding of the impact of CAZR in clinical practice.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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**References**

[1] A. C. Cheng and B. J. Currie, “Melioidosis: epidemiology, pathophysiology, and management,” *Clinical Microbiology Reviews*, vol. 18, no. 2, pp. 383–416, 2005.

[2] N. J. White, “Melioidosis,” *The Lancet*, vol. 361, no. 9370, pp. 1715–1722, 2003.

[3] N. Moocherjee and R. E. W. Hancock, “Cationic host defence peptides: Innate immune regulatory peptides as a novel approach for treating infections,” *Cellular and Molecular Life Sciences*, vol. 64, no. 7–8, pp. 922–933, 2007.

[4] S. Kanthawong, K. Nazmi, S. Wongratanacheewin, J. G. M. Bolscher, V. Wuthiekanun, and S. Taweechaisupapong, “In vitro susceptibility of *Burkholderia pseudomallei* to antimicrobial peptides,” *International Journal of Antimicrobial Agents*, vol. 34, no. 4, pp. 309–314, 2009.
[5] S. D. Puthucheary, N. Parasakthi, and M. K. Lee, "Septicaemic melioidosis: a review of 50 cases from Malaysia," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 86, no. 6, pp. 683–685, 1992.

[6] B. J. Currie, D. A. Fisher, D. M. Howard, et al., "Endemic melioidosis in northern Australian: a 10-year prospective study and review of the literature," *Clinical Infectious Diseases*, vol. 31, no. 4, pp. 981–986, 2000.

[7] S. H. How, K. H. Ng, A. R. Jamalludin, A. Shah, and Y. Rathor, "Melioidosis in Pahang, Malaysia," *Medical Journal of Malaysia*, vol. 60, no. 5, pp. 606–613, 2005.

[8] C. D. Ciornei, A. Egsten, and M. Bodilsson, "Effects of human cathelicidin antimicrobial peptide LL-37 on lipopolysaccharide-induced nitric oxide release from rat aorta in vitro," *Acta Anaesthesiologica Scandinavica*, vol. 47, no. 2, pp. 213–220, 2003.

[9] R. Perumal Samy, A. Pachiappan, P. Gopalakrishnakone et al., "In vitro antimicrobial activity of natural toxins and animal venoms tested against *Burkholderia pseudomallei*," *BMC Infectious Diseases*, vol. 6, article 100, 2006.

[10] F. M. Thibault, E. Hernandez, D. R. Vidal, M. Girardet, and J. D. Boyce, "The molecular and cellular basis of pathogenesis in melioidosis: how does *Burkholderia pseudomallei* cause disease?" *FEMS Microbiology Reviews*, vol. 33, no. 6, pp. 379–387, 2009.

[11] A. C. Cheng, D. A. Fisher, N. M. Anstey, D. P. Stephens, S. P. Jacups, and B. J. Currie, "Outcomes of patients with melioidosis treated with meropenem," *Antimicrobial Agents and Chemotherapy*, vol. 48, no. 5, pp. 1673–1675, 2004.

[12] A. J. H. Simpson, Y. Suputtamongkol, M. D. Smith et al., "Comparison of imipenem and ceftazidime as therapy for severe melioidosis," *Clinical Infectious Diseases*, vol. 29, no. 2, pp. 381–387, 1999.

[13] J. Suppiah, J. S. Thimm, S. H. Cheah, and J. Vadivelu, "Development and evaluation of polymerase chain reaction assay to detect *Burkholderia* genus and to differentiate the species in clinical specimens," *FEMS Microbiology Letters*, vol. 306, pp. 9–14, 2010.

[14] T. J. Inglis, F. Rodrigues, P. Rigby, R. Norton, and B. J. Currie, "Comparison of the susceptibilities of *Burkholderia pseudomallei* to meropenem and ceftazidime by conventional and intracellular methods," *Antimicrobial Agents and Chemotherapy*, vol. 48, no. 8, pp. 3099–3105, 2004.

[15] Y. Khosravi, S. T. Tay, and J. Vadivelu, "Analysis of integrons and associated gene cassettes of metallo-β-lactamase-positive *Pseudomonas aeruginosa* in Malaysia," *Journal of Medical Microbiology*, vol. 60, no. 7, pp. 988–994, 2011.

[16] F. V. Biot, E. Valade, E. Garnotel et al., "Involvement of the efflux pumps in chloramphenicol selected strains of *Burkholderia thailandensis* : proteomic and mechanistic evidence," *PLoS ONE*, vol. 6, no. 2, Article ID e16892, 2011.
[31] J. Turnidge and D. L. Paterson, “Setting and revising antibacterial susceptibility breakpoints,” *Clinical Microbiology Reviews*, vol. 20, no. 3, pp. 391–408, 2007.

[32] G. C. Ulett, B. J. Currie, T. W. Clair et al., “*Burkholderia pseudomallei* virulence: definition, stability and association with clonality,” *Microbes and Infection*, vol. 3, no. 8, pp. 621–631, 2001.