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Reduced susceptibility of *Burkholderia pseudomallei* following exposure to carbapenem

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Abstract. Reduced susceptibility in *Burkholderia pseudomallei* during carbapenem therapy may lead to treatment failure. We isolated a clinical strain that had developed reduced susceptibility to carbapenems while on treatment. After reviewing the patient's clinical notes, the initial isolate (BUPS01/14) was exposed to carbapenem *in vitro* to mimic the clinical scenario. The stability of susceptibility of the carbapenem-exposed strain (BUPS01/14R) was examined by serial subculture in antibiotic-free broth. Biochemical and morphological comparison was performed by the VITEK® system and electron microscopy. MICs increased 32-fold following carbapenem exposure and became stable in the antibiotic-free environment. On electron microscopic examination, the BUPS01/14R cells were smoother and less wrinkled compared to BUPS01/14 cells. This report highlights a potential anti-melioidosis treatment failure due to the emergence of resistance while on carbapenem monotherapy. Further study of this strain is necessary to understand the mechanism of resistance at a molecular level.

INTRODUCTION

Melioidosis, a life-threatening neglected tropical disease, is traditionally endemic in Southeast Asia and northern Australia (Besari *et al.*, 2012). However, *Burkholderia pseudomallei*, the causative pathogen of melioidosis is predicted ubiquitous throughout the global tropics which include Western sub-Saharan Africa and South America (Limmathurotsakul *et al.*, 2016; Birnie *et al.*, 2019). It is a natural saprophyte commonly isolated from soil, water and mud (Cheng & Currie, 2005), and can cause disease in human and animals (Kasantikul *et al.*, 2016). Transmission of the bacteria is usually through direct inoculation or inhalation of aerosolized particles and it often affects persons with underlying diabetes mellitus (Cheng & Currie, 2005). This organism exhibits intrinsic resistance to a wide range of antibiotics including early generation cephalosporins, penicillins, aminoglycosides, macrolides and polymyxin B (Lipsitz *et al.*, 2010), and increasing reports have been made on the development of resistance of this organism to important antimicrobial agents for treatment of melioidosis. The mortality rate of bacteremic melioidosis is ~40%, of which > 50% occur within 48 hours of emergency attendance (Yazid *et al.*, 2017).

In Malaysia, carbapenems (imipenem or meropenem) or ceftazidime, alone or in combination with trimethoprim/sulfamethoxazole, are the recommended antibiotics for intensive phase therapy of melioidosis (Malaysia Ministry of Health, 2014). While intravenous ceftazidime serves as the first-line treatment for

uncomplicated and mild acute infection, carbapenems are an alternative for more severe infections or persistent bacteremia during ceftazidime treatment (Lipsitz *et al.*, 2010). In addition, recent reports on *B. pseudomallei* isolates exhibiting resistance to imipenem (Bugrysheva *et al.*, 2015) and decreased susceptibility to meropenem (Price *et al.*, 2017) are of concern. In this study we report an isolate of *B. pseudomallei* with increased minimal inhibitory concentrations (MICs), equivalent to the non-susceptible range, following *in vivo* and *in vitro* exposure to carbapenem. This confirms the ability of this organism to develop reduced susceptibility following exposure to carbapenems which could potentially lead to treatment failure during the intensive phase of melioidosis therapy.

MATERIALS AND METHODS

Ethics approval

This study was approved by institution's Human Ethics Committee (Reference no. USM/JEPeM/16110488).

Minimal inhibitory concentrations (MIC)

E-test and broth microdilution methods were performed for MIC testing. E-test (bioMérieux, SA, France) was performed according to the manufacturer's instructions. In brief, inoculum concentration was adjusted to a turbidity equivalent to 0.5 McFarland standard using a densitometer (bioMérieux, SA, France). The suspension was inoculated onto Mueller-Hinton agar (Thermo Fisher Scientific, MA, USA) plates before the E-test strip was applied.

Broth microdilution assay was performed according to CLSI guidelines. The strain was tested against all recommended antibiotics for *B. pseudomallei*: imipenem/cilastatin (Merck Sharp & Dohme Corp., VA, USA), ceftazidime and amoxicillin/clavulanate (CCM Duopharma BioTech, Selangor, Malaysia), and doxycycline, trimethoprim and sulfamethoxazole (Sigma-Aldrich Co., Irvine, UK). Twelve tubes of 2-fold serial dilution of each antibiotic were prepared at a starting concentration of 512 µg/mL, before 100 µL of each solution was dispensed in a 96-well microplate.

Clinical history and strain selection

A *B. pseudomallei* strain with increased MIC to imipenem was isolated from the blood sample of a 60-year-old diabetic army camp worker who presented with fever and cough for four days. He was diagnosed with severe community-acquired pneumonia and treated with ceftazidime 1000 mg 8-hourly and azithromycin 500 mg daily upon admission. However, his condition deteriorated after Day-1 of admission and meropenem was initiated at a dose of 1 g 8-hourly. Multiple blood cultures resulted in the isolation of *B. pseudomallei*, which was confirmed by VITEK2 (bioMerieux, SA, France) (Table 1). Antimicrobial susceptibility testing was done at our clinical service laboratory which is ISO 15189 accredited. The result demonstrated a gradual increase in imipenem MIC between Day-1 and Day-10 of admission as shown in Table 1. Unfortunately, meropenem MICs were not monitored during patient admission due to limited resources and a lack of susceptibility interpretation in the Clinical and Laboratory Standard Institute (CLSI) guidelines. Eventually, the patient developed multi-organ failure on Day-7 and succumbed to melioidosis complicated with disseminated intravascular coagulation on Day-10 of admission. The bacterial isolates were kept at -80°C for further analysis.

Recovery of stored isolate

Following approval from the institutional review body and the identification of funding for further studies, the *B. pseudomallei* isolates were retrieved from -80°C after 11 months storage. Repeat MICs of retrieved isolates were performed in duplicate using broth microdilution assay method. Subsequently, *in vitro* exposure of the Day-1 isolate (labeled as strain BUPS01/14) in Mueller-Hinton broth containing increasing concentration of imipenem/cilastatin was undertaken to determine whether the development of reduced susceptibility of the strain to imipenem could be detected.

Table 1. Serial blood cultures from the patient and their MICs by E-test method

Date of Specimen	Day	Result	MIC (µg/mL)*				
			AMC	SXT	Doxy	CAZ	IPM
3rd March	1	*B. pseudomallei*	4	1.5	0.5	1.5	1.0
6th March	4	*B. pseudomallei*	4	0.25	0.38	1.5	6.0
8th March	6	*B. pseudomallei*	–	–	–	–	–
12th March	10	*B. pseudomallei*	4	1	0.75	1.5	16

*AMC, amoxicillin/clavulanate. SXT, sulfamethoxazole-trimethoprim. Doxy, doxycycline. CAZ, ceftazidime. IPM, imipenem.
Note: The MIC breakpoint based Clinical and Laboratory Standard Institute guideline for AMC susceptible ≤ 8; intermediate 16 and resistant ≥ 32; SXT susceptible ≤ 2 and resistant ≥ 4; Doxy susceptible ≤ 4, intermediate 8 and resistant ≥ 16; CAZ susceptible ≤ 8, intermediate 16 and resistant ≥ 32; IPM susceptible ≤ 4, intermediate 8 and resistant ≥ 16 µg/mL respectively.

In vitro exposure to carbapenem

BUPS01/14 was inoculated into Mueller-Hinton broth containing 0.25 µg/mL imipenem on the first day of exposure. After overnight incubation, the strain was resuspended in Mueller-Hinton broth containing 0.5 µg/mL imipenem. This procedure was repeated daily with increasing concentrations of imipenem, from 0.25 µg/mL to 8 µg/mL.

The isolate that was able to survive in 8 µg/mL was subcultured on blood agar and incubated for 16 hours at 35°C. Purity of the culture plate was checked before identification of the isolates by VITEK 2. The isolate were reconfirmed the identity as *B. pseudomallei* and labeled as BUPS01/14R strain. This strain was then passaged daily in antibiotic-free broth for 14 days, and subcultured daily onto both antibiotic-free and 4 µg/mL-imipenem-containing Mueller-Hinton agar to determine the stability of the imipenem MIC. In details, BUPS 01/14R colony was inoculated into 15 mL antibiotic-free Mueller-Hinton broth and incubated at 37°C in an orbital shaker run at 150 rpm overnight. On the next day, 0.1 mL of the overnight suspension was inoculated into another tube of 15 mL antibiotic-free Mueller-Hinton broth. Another 0.5 mL of the suspension was taken for counting by pipetting into 4.5 mL of normal saline solution for 1:10 dilution. Serial dilutions 1:10 was continued by adding 0.5 mL of each previous dilution to the next tube with 4.5 mL normal saline solution until $1:10^7$ dilution achieved. An amount of 100 µL from dilution tubes were pipetted and evenly lawned onto antibiotic-free and 4 µg/mL-imipenem-containing Mueller-Hinton agar. The Mueller-Hinton agar plates of serial dilutions were incubated overnight at 37°C. The number of colonies grew on agar at ranged of 20-200 colonies were counted and multiplied to the dilution factor for actual colony count. The daily passage were repeated for 14 days and similar dilution procedure were performed daily. The bacterial identity confirmation was done on every three to four days. The number of colonies (CFU/mL) of BUPS01/14R grown on 4 µg/mL-imipenem-containing Mueller-Hinton agar was plotted against the colonies on antibiotic-free agar. The antibiotic-containing Mueller-Hinton agar and broth were prepared daily prior to use.

Scanning electron microscopy (SEM)

Both BUPS01/14 and BUPS01/14R were prepared for observation under SEM, following the procedure provided by the Scanning Electron Microscope Laboratory, School of Health Sciences, Universiti Sains Malaysia. BUPS01/14R was prepared following incubation in 4 µg/mL-imipenem-containing media. Briefly, stationary-phase cells were fixed in McDowell-Trump fixative for at least 4 hours. Dehydration was performed using increasing concentrations of ethanol, before the cells were resuspended in hexamethyldisilazane (HMDS) and left to air-dry at room temperature before gold-coating.

Transmission electron microscopy (TEM)

Preparation of samples for TEM observation was done according to the procedure from the Electron Microscope Unit, Faculty of Medicine, University of Malaya. After

overnight incubation, BUPS01/14 and BUPS01/14R were fixed in 4% glutaraldehyde. Post-fixation was performed using 1% osmium tetroxide. The cells were then dehydrated in increasing concentrations of ethanol, followed by embedding in resin overnight.

RESULTS

MICs of stored isolate (BUPS01/14) and in-vitro carbapenem-exposed isolate (BUPS01/14R)

Repeat testing by E-test and broth microdilution methods revealed a reversion of the imipenem MICs to the susceptible range; i.e. 0.25 µg/mL for Day-1 isolate and 0.5 µg/mL for the isolates from Day-4 isolate and Day-10 isolate, respectively. The MICs of other antibiotics were similar to pre-storage result. The BUPS01/14R strain demonstrated imipenem MICs of 8 µg/mL and 6 µg/mL using broth microdilution and E-test methods, respectively. Increases in MICs up to 8-fold were also seen when the strain was tested against ceftazidime and amoxicillin/clavulanate. Table 2 shows the MICs of Day-1 strain (BUPS01/14) and *in vitro* imipenem-exposed strain (BUPS01/14R).

Stability of the carbapenem-exposed non-susceptible strain (BUPS01/14R)

The resistance acquired by BUPS01/14R were stable in antibiotic-free broth as shown by consistence proportion > 86% of viable bacterial count on 4 µg/mL-imipenem-containing Mueller-Hinton agar to antibiotic-free broth throughout the 14-day serial sub-culture (Figure 1).

Morphology comparison under electron microscopy

Scanning and transmission electron microscopic analyses demonstrated that the parent BUPS01/14 cells were ragged and roughly wrinkled, while the non-susceptible BUPS01/14R cells were smoother and less ragged (Figure 2).

DISCUSSION

Carbapenems (imipenem or meropenem) are recommended for intensive phase therapy of melioidosis, particularly in severe sepsis patients. However, resistance or reduced susceptibility of *B. pseudomallei* to carbapenems could affect treatment efficacy. Two recent independent reports of resistance of *B. pseudomallei* to carbapenems (Bugrysheva *et al.*, 2015; Price *et al.*, 2017), suggest that this can occur during treatment, with potentially serious clinical consequences. In agreement with these reports, our current studies demonstrated a further instance of the apparent development of reduced carbapenem susceptibility during melioidosis treatment, and an increasing MIC of *B. pseudomallei* to imipenem after *in vitro* exposure to carbapenem. Interestingly, the MIC of the original clinical isolates appears to have reverted to 0.25 µg/mL during -80°C storage. However, we were unable to identify the reasons underlying this phenomenon.

Following carbapenem exposure, BUPS01/14R had stable MICs as demonstrated by the ability of the strain to persist on 4 µg/mL imipenem-containing agar after serial subculture in non-inducing condition. In addition, BUPS01/14R exhibited a degree

Table 2. The MICs of BUPS01/14 after -80°C storage and BUPS01/14R by broth microdilution method

Strain	MICs (µg/mL)*				
	AMC	SXT**	Doxy	CAZ	IPM
Day-1 parent strain (BUPS01/14)	4	4	1	2	0.25
Imipenem-exposed strain (BUPS01/14R)	32	4	1	16	8

* AMC, amoxicillin/clavulanate. SXT, sulfamethoxazole-trimethoprim. Doxy, doxycycline. CAZ, ceftazidime. IPM, imipenem.
** MICs by E-test method were 0.75 µg/mL for both BUPS01/14 and BUPS01/14R.

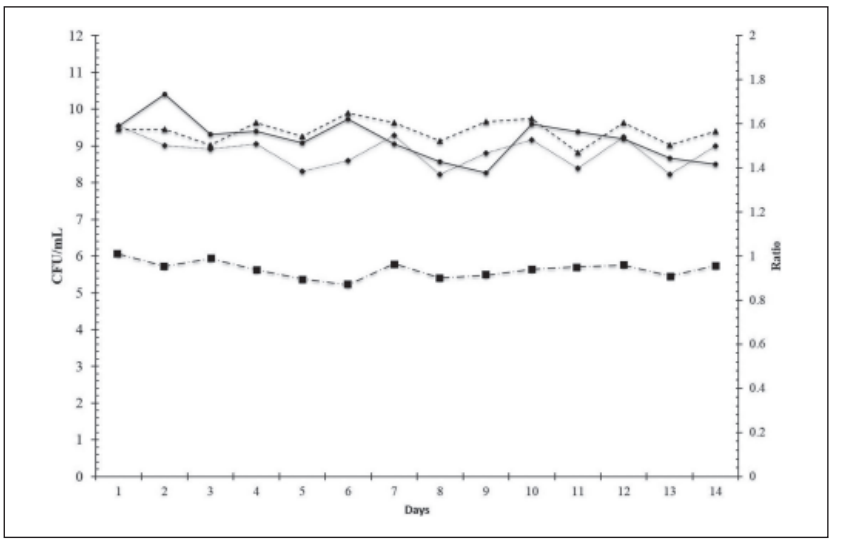

Figure 1. Graph of ratio of BUPS01/14R growth on antibiotic-free and 4 µg/mL-imipenem-containing MHA upon daily passages for 14 days. BUPS01/14 grown on antibiotic-free Mueller Hinton agar (MHA) (●); BUPS01/14R grew on antibiotic-free MHA (▲); BUPS01/14R grew on imipenem (4 µg/mL) containing MHA (♦); and the ratio between BUPS01/14R grown on antibiotic-free MHA to BUPS01/14R grown on imipenem (4 µg/mL)-containing MHA (■).

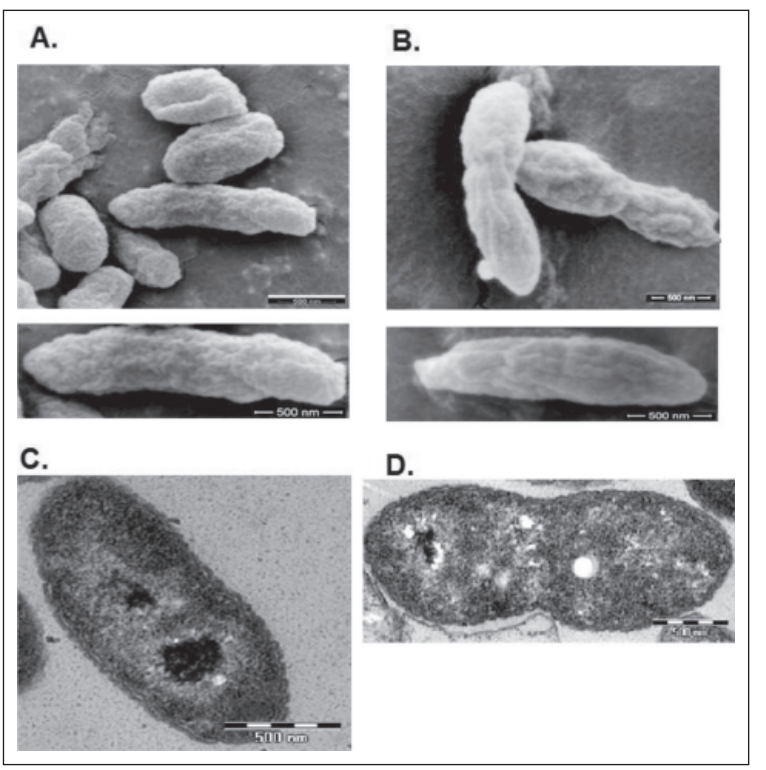

Figure 2. Scanning electron microscope view on (A) BUPS01/14 and (B) BUPS01/14R. (C) and (D) are transmission electron microscopy on the strains, respectively. BUPS01/14R had lost the rough and ragged contour of cell membrane.

of cross-resistance to ceftazidime and amoxicillin/clavulanate, which was not seen in the clinical isolates. Cross-resistance or secondary resistance to an antibiotic is not uncommon after *in vitro* exposure of an organism to an antibiotic with a similar mechanism of action (Niumsup & Wuthiekanun 2002; Viktorov *et al.*, 2008). Cross-resistance to the same class of antibiotics is most likely attributed to mutations that lead to resistance mechanisms that affect different members of the antibiotic class.

Mechanisms of *B. pseudomallei* resistance to ceftazidime have been described (Chantratita *et al.*, 2011; Sarovich *et al.*, 2012). This is particularly important considering the critical role of this antimicrobial in treatment of melioidosis. On the other hand, mechanisms of carbapenem resistance have only recently been in the spotlight as a result of reports on this phenomenon. This study was not designed to establish the mechanisms of resistance but rather to report on the phenotypic differences between BUPS01/14 and BUPS01/14R which may reflect the involvement of previously unknown mechanisms resulting in the reduction in susceptibility.

From the susceptibility profile it was noted that the decrease in susceptibility of BUPS01/14R was restricted to β-lactam antibiotics, ruling in the possible contribution of β-lactamase which was yet to be confirmed behind the incident. penA and Class D (Niumsup & Wuthiekanu, 2002) β-lactamases have been reported to contribute to *B. pseudomallei* antimicrobial resistance in many studies. Mutations of the *penA* gene have been correlated with resistance in *B. pseudomallei* to ceftazidime and recently it was reported that a mutation that caused amino acid substitution T147A of the gene led to non-susceptibility of the organism to imipenem (Bugrysheva *et al.*, 2017).

Examination by electron microscopy revealed changes in BUPS01/14R after exposure to imipenem. Under low resolution scanning electron microscopy, BUPS01/14R appeared mostly in clumps or aggregates, in contrast to the individually distributed cells of BUPS01/14. No formation of extracellular polymeric substance, as described by Chin *et al.* (2015), was seen in BUPS01/14R, which suggests that biofilm formation may not contribute to its decreased susceptibility.

In addition, single examination by electron microscopy of BUPS01/14 showed a ragged cell outline, but BUPS01/14R lost the ragged appearance, presenting a rough and more loosely wrinkled surface. Since β-lactam antibiotics act primarily by inhibition of cell wall formation (Kong *et al.*, 2010), these alterations may be due to adaptation and survival of cells in unfavorable conditions. However, this observation need to be repeated for confirmation of the changes. Other study has reported the phenotypic changes that correlated with resistance to ceftazidime in *B. pseudomallei*. The loss of function of the PBP-3 gene, leading to the development of elongated, unseptate and filamentous cells, has also been found to result in reduced susceptibility to ceftazidime (Chantratita *et al.*, 2011). We did not see a similar filamentation in BUPS01/14R, which is consistent with the fact that imipenem has low binding affinity to PBP-3 (Hashizume *et al.*, 1984). These differences in cell morphology may reflect other phenomena, which we are investigating further. For example, these might include changes in porins which reduce the penetration of the antibiotic through the bacterial cell membrane or efflux pumps which lead to antibiotic molecules being pumped out of the cells. Over-expression of efflux pumps has recently been described in *B. pseudomallei* exhibited decreased susceptibility to meropenem (Sarovich *et al.*, 2018).

In summary, reduced susceptibility to antibiotics during therapy is a known cause of treatment failure for many bacterial infections. This report emphasizes the need to be vigilant for the emergence of carbapenem resistant *B. pseudomallei* during treatment. In addition, this study has demonstrated that *B. pseudomallei* strains with reduced carbapenem susceptibility exhibited different phenotypic characteristics. Additional studies are

currently being carried out in this laboratory to elucidate the mechanisms underlying these differences.

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ETHICS APPROVAL
This study was approved by Universiti Sains Malaysia's Human Ethics Committee (Reference no. USM/JEPeM/16110488).

CONFLICTS OF INTEREST
The authors declare that they have no conflicts of interest.

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