Prevalence of *bla*$_{PenA}$ and *bla*$_{OXA}$ in *Burkholderia pseudomallei* Isolated from Patients at Sappasitthiprasong Hospital and Their Susceptibility to Ceftazidime and Carbapenems

Marutpong Panya PhD*,
Suthinee Thirat MD*, Surasak Wanram PhD*,
Pawana Panomket PhD*, Jiraporn Nilsakul BSc**

** College of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani, Thailand*
*** Microbiology Laboratory, Sappasitthiprasong Hospital, Ubon Ratchathani, Thailand*

Background: *Burkholderia pseudomallei is a causative agent of melioidosis. Ceftazidime is the preferred drug of choice for treatment. However, the motility rate is high in endemic areas.*
Objective: *This study aimed to determine the susceptibility to four different antimicrobial agents and to detect the β-lactamase genes in B. pseudomallei isolates from patients admitted to Sappasitthiprasong Hospital.*
Material and Method: *85 B. pseudomallei isolates from patients admitted to Sappasitthiprasong Hospital between November 2010 and May 2011 were determined for antimicrobial susceptibility by standard disk diffusion and minimum inhibitory concentration (MIC). Real-time polymerase chain reaction (PCR) was used for the detection of bla*$_{PenA}$ *and bla*$_{OXA}$ *in β-lactamase genes.*
Results: *Almost all of the clinical isolates of B. pseudomallei were susceptible to ceftazidime and imipenem. Cefatzidime MIC was ≤1-16 μg/ml and imipenem MIC was ≤1-4 μg/ml. The real-time PCR revealed that more than 90% of B. pseudomallei isolates carried bla*$_{PenA}$ *and bla*$_{OXA}$*.*
Conclusion: *Although the clinical isolates of B. pseudomallei were susceptible to ceftazidime and imipenem, this study showed B. pseudomallei had a gene that produced beta-lactamase enzyme and may be poorly effective in the use of beta-lactam drugs.*

Keywords: *Burkholderia pseudomallei, Melioidosis, Ceftazidime, β-lactamase, bla*$_{PenA}$

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Burkholderia pseudomallei is a gram-negative bacillus that shows bipolar staining and is a causative agent of melioidosis. The disease can occur as an asymptomatic and/or acute infection and as severe sepsis, leading to death or chronic infection. Antibiotic treatment is limited because *B. pseudomallei* are able to resist aminoglycosides, first and second generation cephalosporins, and poor activity of fluoroquinolone. Ceftazidime is the preferred drug of treatment and reduces mortality by about 40%[1]. In vitro activity of ceftazidime antibiotic against *B. pseudomallei* was found to be 95 to 100% effective as determined by standard disk diffusion and minimal inhibitory concentration (MIC)[2]. However, the motility rate of melioidosis is high in endemic areas[2,3].

B. pseudomallei are resistant to first, second generation cephalosporins, and broad-expanded spectrum cephalosporins due to the production of beta (β)-lactamase. This enzyme can hydrolyse some cephalosporins, such as cephalothin, cefuroxime, and ceftotaxime. β-lactamase produced by gram-negative bacteria is variously secreted and active. There are four classes of β-lactamases based on a molecular study by the differentiation of nucleotide and amino acid sequences. β-lactamases are also classified into 4 functional groups based on their preferred substrates and inhibitors, cephalosporinase not inhibited by clavulanic acid, penicillinases and cephalosporinase inhibited by clavulanic acid, metallo-β-lactamase inhibited by EDTA, and penicillinase not inhibited

Correspondence to:
Panomket P, 85 Satonlamark Road, College of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani 34190, Thailand.
Phone: +66-81-2025723, Fax: +66-45-353928
E-mail: mdpawapa@ubu.ac.th, panomketp@yahoo.com

by clavulanic acid. Recently, the cloning of *B. pseudomallei* class A and D β-lactamase was reported[4-6], and high MIC of ceftazidime was found. However, the clinical isolates need to evaluate the genes related to β-lactamase production. It is anticipated that the results will be important in the understanding of the roles of antimicrobials in regard to *B. pseudomallei* and provide more information in the selection of appropriate drugs for patients with melioidosis.

Material and Method

Isolation and identification of Burkholderia pseudomallei

A total of 85 *B. pseudomallei* isolates from patients admitted to Sappasitthiprasong Hospital between November 2010 and May 2011 were included in this study. The isolates were grown on Ashdown's agar and incubated at 37°C for 48-72 hours. Identification of *B. pseudomallei* was performed by observation of colony appearance and cell morphology, phenotypic, biochemical, and immunological methods, sugar utilization by analytical profile index (API) (Oxoid, Basingstoke, Hants, UK), and latex agglutination described by Anuntagool N et al[7]. The single colonies were further sub-cultured on fresh Ashdown agar to confirm their purity. The purified colonies were stocked in skimmed milk containing 20% glycerol and were kept at -20°C until further use.

Antibiotic susceptibility test

Standard disk diffusion

The disk diffusion tests were performed on Mueller Hinton agar (Hardy Diagnostics, Santa Maria, USA) using the Kirby-Bauer method. *Pseudomonas aeruginosa* ATCC 27853 was used as a control with an expected inhibition zone of each antimicrobial. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI)[8]. Antimicrobial disks (Hardy Diagnostics, Santa Maria, USA) included ceftazidime (30 μg), ceftriaxone (30 μg), imipenem (10 μg), and ciprofloxacin (10 μg).

Minimal inhibitory concentration determination

MICs of ceftazidime, ceftriaxone, imipenem, and ciprofloxacin were tested by Sensititre gram negative plate (Trek Diagnostic Systems, Biosciences Inc., Magellan, USA) and performed according to the manufacturer's instructions. The concentrations of antimicrobials were 0.25-32 μg/mL. The MIC results of clinical isolates and controls were interpreted as recommended by CLSI[8].

Bacterial genomic DNA isolation

B. pseudomallei isolates were grown in Ashdown broth at 37°C for 24 hours. The bacterial cell pellets were harvested from the culture by centrifugation at 13,000 xg for 5 minutes. The bacterial cell pellets were washed three times with cooled sterile water and then subjected for genomic DNA isolation using Invitrogen™ kit (Lohne, Germany).

Primer design

Primers used in this study are listed in Table 1. The nucleotide sequences of *pen*A and *oxa* derived from *B. pseudomallei* were collected from the National Center for Biotechnology Information (NCBI) data base at http://www.ncbi.nlm.nih.gov, GenBanK accession No. CP000011.2 and ACOJ01000001.1 for bla_{PenA} and bla_{OXA}, respectively. The conserved regions on *penA* and *oxa* gene (OXA-57), which would not be present in other *Enterobacteriaciae* species, were compared with the corresponding genes by the multiple sequence alignment method through ClustalW program at http://www.ebi.ac.uk/Tools/msa/clustalw2. The conserved regions of either *penA* or *oxa* that were present in *B. pseudomallei* only were further used for primer design using the Primer 3 program at http://simgene.com/Primer3.

Detection of β-lactamase genes by real-time PCR

Real-time PCR was used to detect *penA* and *oxa*. The quantity of isolated genomic DNA of *B. pseudomallei* was verified by absorbance value at 260 nm and 280 nm ratio (OD_{260}/OD_{280}). The acceptable purity of DNA had OD_{260}/OD_{280} ratio values between 1.8 and 2.0 when tested by real-time PCR.

The total genomic DNA from each isolate of *B. pseudomallei* was diluted in DNase/RNase-free distilled water (Invitrogen™) and further used as DNA template in real-time PCR. The real-time PCR mixture of 25 μl was prepared using the SYBR® Green (LightCycler® 480 SYBER Green I Master, Roche, USA): 12.5 μl of SYBR Green PCR Master Mix, 3.82 μl PCR-grade water, 1.84 μl of each primer for *penA* and *oxa*, and 5 μl of template DNA. DNA amplification and detection were performed in LightCycler® 480 Real-Time PCR System (Roche, Germany). The thermal cycling protocol was as follows: initial denaturation for 10 minutes at 95°C followed by 40 cycles of 15 s at 95°C, and 10 s at 60°C. The fluorescent signal was measured at the end of each extension step at 60°C. After amplification, the threshold cycle number (C_T) of either *penA* or *oxa* was determined. The 16S rDNA was used

as a reference gene in this study. In addition, genomic DNAs isolated from *B. pseudomallei* K584 and K386 were utilized as positive controls and that from *B. pseudomallei* E908 was used as a negative control.

Results

Antimicrobial susceptibility tests

B. pseudomallei were susceptible to ceftazidime and imipenem when tested by standard disk diffusion test and broth dilution method for MIC determination. The highest susceptibilities were to ceftazidime and imipenem, as shown in Table 2. MIC range was ≤1-16, and ≤1-4 μg/ml for cefatzidime, and imipenem, respectively.

The presence of penA and oxa

All *B. pseudomallei* isolates carried bla_{OXA}, a class D beta-lactamase. The threshold cycle number (C_T) of bla_{OXA} ranged from 11.62 to 16.82, indicating a non-equivalent of gene copy number of bla_{OXA} in different strains. The same was found in regard to the *pen*A gene. Ninety-three percent (79 of 85) of the *B. pseudomallei* isolates showed the presence of the *pen*A gene. The threshold cycle number (C_T) *pen*A gene ranged from 15.26 to 24.93. In addition, the fluorescent signal could not be detected in one isolate, the negative strain of *pen*A and *oxa* gene, thus indicating the specificity of the oligonucleotide primers. The present study not only showed the prevalence of *pen*A and bla_{OXA} in *B. pseudomallei* but also revealed the ability to use real-time PCR as a rapid detection tool.

Discussion

Ceftazidime is the first drug of choice for the treatment of melioidosis but resistance to this agent has been observed in clinical and laboratory situations. In 2011, Panomket et al reported that *B. pseudomallei* were 100% susceptible to ceftazidime with MICs of 1-2 μg/ml[2]. Several laboratories generated a bacterial strain that contained mutations in either class A or class D β-lactamases[5,9,6]. The present study reported two β-lactamase producing encoding genes, *pen*A and bla_{OXA}, in clinical isolates of *B. pseudomallei*.

Bla_{PenA} is a chromosomal gene, which encodes a putative twin arginine translocase (TAT) and secretes a class A β-lactamase. Several reports described a role of this enzyme in acquired ceftazidime resistance in

Table 1. Primers utilized in this study

Olionucleotides	Sequence (5'-3')	Melting temperature (T_m) (°C)	Predictive product size (bp)
ESBL penA F	GTT CTG CAG CAC GTC CAA G	60.61	199
ESBL penA R	GGA GTT GTC GCT GTA CTG GAG	59.92	
ESBL Oxa F	TCG CGA TGT TCA AGA GTC AG	60.14	223
ESBL Oxa R	GCT CGA CGG ATA CGA TTT TG	60.61	
16s rRNA F	ATC TGA TCG GCC TCG ATG T	60.60	227
16s rRNA R	TCA GTA ATC GGC TTC CCA GT	59.69	

Table 2. Susceptibility tests to ceftazidime, ceftriaxone, imipenem, and ciprofloxacin by standard disk diffusion test and MIC

Antimicrobial agents	Ceftazidime	Ceftriaxone	Imipenem	Ciprofloxacin
Standard disk diffusion	Susceptible 100%	Susceptible 9%	Susceptible 99%	Susceptible 25%
MIC range (85 isolates)	≤1-8 μg/ml (84 isolates) 16 μg/ml (1 isolate)	≤8 μg/ml (78 isolates) 16-≥32 μg/ml (7 isolates)	≤1-1 μg/ml (82 isolates) 4 μg/ml (3 isolates)	≤0.25-0.5 μg/ml (46 isolates) 1-≥2 μg/ml (39 isolates)
	Susceptible 98.8% Intermediate 1.2%	Not have breakpoints for interpretive	Susceptible 100%	Not have breakpoints for interpretive

patients treated with ceftazidime[5,10,11]. This enzyme is susceptible to clavulanic acid inhibitor. *Pen*A confers resistance to numerous β-lactam antibiotics expressed in *Escherichia coli* and other gram-negative bacilli[4,5]. The nucleotide sequence of *B. pseudomallei pen*A has been reported[4]. In this study, *pen*A was found in more than 90% of *B. pseudomallei* clinical isolates from patients with melioidosis admitted to Sappasitthiprasong Hospital. These isolates were susceptible to ceftazidime and imipenem by standard disk diffusion and MIC. All patients were treated with ceftazidime and 30.3% were cured[2]. *Pen*A mutations were implicated in ceftazidime resistance in clinical isolates[12]. Strains with bla_{PenA} deletion become resistant to these antibiotics[12]. Moreover, *B. pseudomallei* were cultured in a low concentration of ceftazidime, and this condition activated the bacteria to be resistant to ceftazidime[5,6], and mutated some genes to be involved in the production of enzymes to destroy the antimicrobials. However, there is a need to investigate *pen*A mutations in the future.

The OXA type β-lactamases refer to a class D β-lactamase. Most class D β-lactamases predominantly occur in *Pseudomonas aeruginosa*[13]. This type is produced by many gram-negative bacilli[14]. OXA β-lactamase hydrolyzes oxacillin, which is related to anti-staphylococcal penicillin. It is poorly inhibited by clavulanic acid[15]. Keith et al identified OXA-57 class D β-lactamase in clinical and environmental isolates of *B. pseudomallei* and *B. thailandensis*[16]. The mutation of OXA-57 implicated β-lactam resistance[16]. In this study all clinical isolates of *B. pseudomallei* harbored bla_{OXA}. However, further study of the mutation of bla_{OXA} is required. Mutation of bla_{OXA} can produce extended spectrum beta-lactamase (ESBL) enzyme. ESBL was reported to fail in β-lactam antibiotics used.

Conclusion

Eighty-five isolates of *B. pseudomallei* obtained from patients with melioidosis admitted to Sappasitthiprasong Hospital between November 2010 and May 2011, and were susceptible to ceftazidime and imipenem. Clinical isolates of *B. pseudomallei* carried bla_{OXA}, and Bla_{PenA} by PCR. The mutations of these genes need further investigation.

What is already known on this topic?

Clinical *B. pseudomallei* were isolated from patients admitted at Sappasitthiprasong Hospital between November 2010 and May 2011, susceptible to ceftazidime and imipenem and carried bla_{OXA}, and Bla_{PenA} by PCR.

What this study adds?

This study determined the susceptibility to four different antimicrobial agents including ceftazidime, ceftriaxone, imipenem, and ciprofloxacin and to detect the β-lactamase genes in *B. pseudomallei* isolates.

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Potential conflicts of interest

None.

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ความชุกของ bla_{PenA} และ bla_{OXA} ในเชื้อ *Burkholderia pseudomallei* ที่แยกได้จากผู้ป่วยในโรงพยาบาลสรรพสิทธิประสงค์ และความไวต่อยา ceftazidime และ ยา carbapenem

มารุตพงศ์ ปัญญา, ศุทธินี ธิราช, สุรศักดิ์ แว่นรัมย์, ภาวนา พนมเขต, จิราพร นิลสกุล

ภูมิหลัง: *Burkholderia pseudomallei เป็นสาเหตุของโรคเมลลิออยโดสีส ยา ceftazidime เป็นทางเลือกอันดับแรกในการรักษาแต่อย่างไรก็ตาม อัตราตายยังสูงในพื้นที่ระบาดของโรค*

วัตถุประสงค์: *ทดสอบความไวต่อยาต้านจุลชีพสี่ชนิดและตรวจหาจีนที่กำหนด เอนไซม์ beta-lactamase ในเชื้อ B. pseudomallei ที่แยกได้จากผู้ป่วยที่เข้ารับการรักษาพยาบาลในโรงพยาบาลสรรพสิทธิประสงค์*

วัสดุและวิธีการ: *เชื้อ B. pseudomallei ทั้งหมดจำนวน 85 สายพันธุ์ ที่แยกได้จากผู้ป่วยที่เข้ารับการรักษาที่โรงพยาบาลสรรพสิทธิประสงค์ตั้งแต่เดือนพฤศจิกายน พ.ศ. 2553 ถึงเดือนพฤษภาคม พ.ศ. 2554 ทำการทดสอบความไวต่อสารต้านจุลชีพด้วยวิธี standard disk diffusion และ minimum inhibitory concentration (MIC) และตรวจจีนที่กำหนดเอนไซม์ beta-lactamase ได้แก่ bla_{PenA} และ bla_{OXA} ด้วยวิธี real time PCR*

ผลการศึกษา: *ส่วนใหญ่เชื้อ B. pseudomallei ไวต่อ ceftazidime และ imipenem ค่า ceftazidime MIC ≤1-16 μg/ml และค่า imipenem MIC ≤1-4 μg/ml การทดสอบ real time PCR พบจีน bla_{PenA} และ bla_{OXA} ใน B. pseudomallei ที่แยกได้จากผู้ป่วยที่เข้ารับการรักษาที่โรงพยาบาลสรรพสิทธิประสงค์มากกว่าร้อยละ 90*

สรุป: *ถึงแม้ว่าเชื้อ B. pseudomallei ที่แยกได้จากผู้ป่วยจะไวต่อยา ceftazidime และ imipenem การศึกษานี้แสดงให้เห็นว่าเชื้อ B. pseudomallei มีจีนที่ผลิตเอนไซม์ beta-lactamase และอาจส่งผลต่อการใช้ยาในกลุ่ม beta-lactam ได้ไม่ดี*