นิพนธ์ต้นฉบับ . Original Article

การเปรียบเทียบวิธี Disk Diffusion และ Etest กับ วิธี Broth Microdilution สำหรับการทดสอบความไวของยาเซฟตาซิดิมและไตรเมโทพริม/ซัลฟาเมทอกซาโซลต่อเชื้อ *Burkholderia Pseudomallei* ที่แยกจากสิ่งส่งตรวจในภาคตะวันออกเฉียงเหนือของประเทศไทย

Sereywatana Chea[1], ณัฏฐญาณ์ ทองเหลา[2], ลำใย วงลคร[3], แววตา คู่วัจนกุล[3], สุทัศน์ สุทธิประภา[1], ศิรินทิพย์ บุญจรัสภิญโญ[4], อุมาพร ยอดประทุม[2], ภาวนา พนมเขต[5], โสรัจสิริ เจริญสุดใจ[2, 6*]

[1]สาขาวิชาเวชศาสตร์เขตร้อน คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จ.ขอนแก่น ประเทศไทย
[2]สาขาวิชาจุลชีววิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จ.ขอนแก่น ประเทศไทย
[3]หน่วยจุลชีววิทยาคลินิก งานห้องปฏิบัติการเวชศาสตร์ชันสูตร โรงพยาบาลศรีนครินทร์ คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จ.ขอนแก่น ประเทศไทย
[4]สาขาวิชาเวชศาสตร์ชุมชน เวชศาสตร์ครอบครัวและอาชีวเวชศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จ.ขอนแก่น ประเทศไทย
[5]วิทยาลัยแพทยศาสตร์และการสาธารณสุข มหาวิทยาลัยอุบลราชธานี จ.อุบลราชธานี ประเทศไทย
[6]ศูนย์วิจัยและบริการตรวจวินิจฉัยโรคติดเชื้อระบาดใหม่ มหาวิทยาลัยขอนแก่น จ.ขอนแก่น ประเทศไทย

A Comparison of Disk Diffusion and Etest with Broth Microdilution Methods for Susceptibility Testing of Ceftazidime and Trimethoprim/Sulfamethoxazole Against Clinical Isolates of *Burkholderia Pseudomallei* in Northeast Thailand

Sereywatana Chea[1], Nuttaya Thonglao[2], Lumyai Wonglakorn[3], Waewta Kuwatjanakul[3], Sutas Suttiprapa[1], Sirintip Boonjaraspinyo[4], Umaporn Yordpratum[2], Pawana Panomket[5], Sorujsiri Chareonsudjai[2, 6*]

[1]Department of Tropical Medicine, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[2]Department of Microbiology, Faculty of Medicine, Khon Kaen University, Thailand
[3]Microbiology Laboratory, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[4]Department of Community Medicine, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[5]College of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani, Thailand
[6]Research and Diagnostic Center for Emerging Infectious Diseases (RCEID), Khon Kaen, Thailand

Received: 11 October 2023 /Review: 18 October 2023 / Revised: 29 February 2024 / Accepted: 29 February 2024

*Corresponding author: Sorujsiri Chareonsudjai, E-mail: sorujsr@kku.ac.th

บทคัดย่อ

หลักการและวัตถุประสงค์: โรคเมลิออยด์ หรือโรคเมลิออยโดสีส เกิดจากแบคทีเรีย *Burkholderia pseudomallei* เป็นโรคติดเชื้อที่ถูกละเลยและมีอัตราการเสียชีวิตสูงในพื้นที่ที่มีการระบาด การทดสอบความไวต่อยาต้านจุลชีพมีความสำคัญสำหรับการเลือกใช้ยารักษาที่มีประสิทธิภาพเพื่อลดความรุนแรงของโรคและเพิ่มอัตราการรอดชีวิตของผู้ป่วย การศึกษานี้มีวัตถุประสงค์เพื่อเปรียบเทียบความคุ้มทุนและประสิทธิผลของการตรวจความไวต่อยาเซฟตาซิดิมและไตรเมโทพริม/ซัลฟาเมทอกซาโซลของ *B. pseudomallei* ด้วยวิธี disk diffusion และ Etest เทียบกับวิธี broth microdilution ด้วยเครื่องอัตโนมัติ

วิธีการศึกษา: นำ *B. pseudomallei* จำนวน 199 สายพันธุ์ที่แยกได้จากผู้ป่วยโรคเมลิออยด์ในภาคตะวันออกเฉียงเหนือที่รวบรวมจาก 3 แหล่ง มาทดสอบความไวต่อยาเซฟตาซิดิมและไตรเมโทพริม/ซัลฟาเมทอกซาโซล ด้วยวิธี disk diffusion และ Etest และวิธี broth microdilution ด้วยเครื่องอัตโนมัติและประเมินผลตามเกณฑ์ของสถาบันห้องปฏิบัติการทางวิทยาศาสตร์และการแพทย์ (Clinical and Laboratory Standards Institute)

ผลการศึกษา: วิธี disk diffusion ของยาเซฟตาซิดิม (30 ไมโครกรัม) เมื่อเปรียบเทียบกับวิธี broth microdilution ให้ผลสอดคล้องหมวดหมู่ (categorical agreement) ร้อยละ 96 (191/199) มีผลผิดพลาดหลัก (major error) ร้อยละ 0.5 (1/199) และผลผิดพลาดเล็กน้อย (minor error) ร้อยละ 3.5 (7/199) วิธี Etest ของยาเซฟตาซิดิมเมื่อเทียบกับวิธี broth microdilution ให้ผลสอดคล้องหมวดหมู่ ร้อยละ 98.5 (196/199) ไม่มีผลผิดพลาดหลัก แต่มีผลผิดพลาดเล็กน้อย ร้อยละ 1.5 (3/199) อย่างไรก็ตาม วิธี disk diffusion ของยาไตรเมโทพริม/ซัลฟาเมทอกซาโซล (1.25/23.75 ไมโครกรัม) เมื่อเทียบกับวิธี broth microdilution แสดงผลสอดคล้องหมวดหมู่ ผลผิดพลาดหลัก ผลผิดพลาดเล็กน้อย เท่ากับร้อยละ 13.6 (27/199), 76.4 (152/199) และ 10.1 (20/199) ตามลำดับ ในขณะที่ Etest ของยาไตรเมโทพริม/ซัลฟาเมทอกซาโซล เมื่อเทียบกับวิธี broth microdilution ให้ผลสอดคล้องหมวดหมู่ ร้อยละ 46.2 (92/199) ผลผิดพลาดหลัก ร้อยละ 53.8 (107/199) และไม่มีผลผิดพลาดเล็กน้อย การเปรียบเทียบทั้งหมดไม่พบข้อผิดพลาดที่สำคัญมาก (very major error)

สรุป: วิธี disk diffusion และวิธี Etest ของยาเซฟตาซิดิม อาจเป็นประโยชน์ในพื้นที่ที่มีทรัพยากรจำกัดในการจัดการโรคเมลิออยด์ อย่างไรก็ตาม ในกรณีการทดสอบยาไตรเมโทพริม/ซัลฟาเมทอกซาโซล วิธี broth microdilution ควรใช้เพื่อประเมินประสิทธิผลของยาปฏิชีวนะเพื่อใช้เป็นแนวทางในการตัดสินใจรักษาโรคเมลิออยด์อย่างเหมาะสม

คำสำคัญ: เบอโคลเดอเรียสูโดมาลีอาย, การเปรียบเทียบวิธีทดสอบความไวของยาปฏิชีวนะ, เซฟตาซิดิม, ไตรเมโทพริม/ซัลฟาเมทอกซาโซล

Abstract

Background and objective: Melioidosis caused by *Burkholderia pseudomallei*, is a neglected infectious disease with a high mortality rate in endemic areas. Antimicrobial susceptibility testing (AST) offers crucial guidance for effective treatment to mitigate the severity of the disease and improve the survival rates. This study aimed to compare the cost-effectiveness and pragmatic viability of two ASTs, disk diffusion and Etest methods, to an automated broth microdilution method (BMD), against ceftazidime (CAZ) and trimethoprim/sulfamethoxazole (TMP-SMX).

Methods: A total of 199 clinical isolates of *B. pseudomallei* from melioidosis patients with culture-confirmed, were obtained from 3 collection sources in Northeast Thailand. These isolates were assessed using disk diffusion and Etest methods compared with an automated BMD based on the criteria set by the Clinical and Laboratory Standards Institute.

Results: The results revealed non-resistance isolates to CAZ and TMP-SMX by BMD. The disk diffusion for CAZ (30 µg) demonstrated a categorical agreement (CA) of 96% (191/199), with a major error (ME) of 0.5% (1/199) and minor error (MIE) of 3.5% (7/199) compared to the BMD. The Etest for CAZ demonstrated a CA of 98.5% (196/199), with no ME, and an MIE of 1.5% (3/199) compared to the BMD. However, the disk diffusion for TMP-SMX (1.25/23.75 µg) demonstrated the CA, ME, and MIE of 13.6% (27/199), 76.4% (152/199), and 10.1% (20/199) compared to the BMD, respectively. While the Etest for TMP-SMX demonstrated a CA of 46.2% (92/199), ME of 53.8% (107/199), and no MIE compared to the BMD. No very major error (VME) was detected in either test.

Conclusion: Disk diffusion and Etest methods for CAZ could be beneficial in areas with limited resources for the management of melioidosis. However, BMD is still recommended for TMP-SMX to assess the antibiotic's efficacy and guide appropriate treatment decisions for melioidosis.

Keywords: *Burkholderia pseudomallei,* broth microdilution, Etest, disk diffusion

Introduction

Melioidosis is an infectious disease caused by *Burkholderia pseudomallei* that is highly endemic in Southeast Asia and Northern Australia. The disease has been increasingly reported worldwide and was predicted to cause nearly 90,000 fatalities per year[1] but it is considered a neglected tropical disease due to the underestimated burden and mortality rate[2]. A high in-hospital mortality rate of melioidosis of 70% was reported in Cambodia[3]. While in developing countries such as Thailand, the mortality rate stands at 35%[4]. Whereas, developed countries such as Australia demonstrate a comparatively lower average of 14%[5]. This information reflects the limitations within healthcare systems and the economic constraints of numerous low-income and middle-income countries[2].

B. pseudomallei, a causative agent of melioidosis is described as Gram-negative bipolar staining with 'safety pin' appearance, vacuolated and slender with rounded ends shape[6]. The organism demonstrates differing colonial morphology, with mostly smooth colonies initially and dry or wrinkled colonies on further incubations. The pathogen exhibits various antibiotic resistance mechanisms including efflux pump[7], gene duplication and amplification[8], gene mutation[9] and biofilm formation[10,11]. Therefore, effective management of melioidosis requires early diagnosis, appropriate administration of antibiotics, and access to critical care to mitigate the impact of the disease.

Melioidosis treatment follows established guidelines, typically involving the initial phase using intravenous administration of ceftazidime (CAZ) which is crucial to reducing the risk of mortality. Subsequently, a long-term oral therapy known as the eradication phase, where trimethoprim/sulfamethoxazole (TMP-SMX) is commonly used to prevent relapse[12]. Therefore, simple, and cost-effective antimicrobial susceptibility testing (AST) for *B. pseudomallei* may offer efficient methods for under-resourced laboratories and hospitals in endemic areas. However, previous studies have demonstrated the overestimation of *B. pseudomallei* resistance to TMP-SMX by disk diffusion including 58.7% in Australia[13], 46.5% in Khon Kaen, Thailand[14], 71% in Ubon Ratchathani, Thailand[15], and 53% in Malaysia[16].

The Etest method that provides a MIC value for TMP-SMX was also reported for erroneous results due to the misinterpretation of the 80% inhibition threshold[17]. One of the most significant observations suggested that true resistance in *B. pseudomallei* to TMP-SMX is very rare[13, 18]. In addition, the re-evaluation of *B. pseudomallei* to TMP-SMX by Etest in Thailand revealed the lower resistance observation due to the error in reading the 80% inhibition point in their previous study[17].

Comparisons between AST methods demonstrated poor agreement between disk diffusion and broth microdilution methods in determining the MIC susceptibility for TMP-SMX with *B. pseudomallei.* The agreement rates were of 53.5% and 84% in Thailand, respectively[14]. The observations in Australia revealed that disk diffusion showed 41.3% susceptibility to TMP-SMX while Etest was 97.5%[13]. Therefore, the disk diffusion method's tendency to overestimate resistance to TMP-SMX in *B. pseudomallei* raises interpretational concerns.

To date, only a limited number of comparisons between the disk diffusion, Etest, and broth microdilution for AST of *B. pseudomallei* have been identified. A strong correlation between Etest and BMD testing in evaluating *B. pseudomallei* susceptibility to CAZ has been well-documented. However, this correlation is not as pronounced in the case of TMP-SMX, thereby giving rise to certain concerns[19]. Moreover, a notable inconsistency was observed concerning the resistance to TMP-SMX when assessed through the disk diffusion method, in contrast to its sensitivity as determined by the MIC method[20]. Insufficient data of the directly compares TMP-SMX susceptibility results from disk diffusion and Etest methods with the standard broth microdilution method may limit treatment optimization and decision making. This study, therefore, aims to investigate the agreement among disk diffusion, Etest, and automated broth microdilution methods of CAZ and TMP-SMX against *B. pseudomallei*. The findings are expected to enhance decision-making processes offering valuable insights into the selection of appropriate testing techniques, particularly beneficial for low-income countries in endemic areas and may improve the management of melioidosis patients.

Methods

This study was approved by Khon Kaen University Ethics Committee for Human Research (Reference No. HE641251).

Burkholderia pseudomallei clinical isolates

A total of 199 *B. pseudomallei* clinical isolates from culture-confirmed cases, were selected from three different sources. These included collections from the Melioidosis Research Center, Khon Kaen University between 2006 and 2008, which reported TMP-SMX resistance by disk diffusion method (144 isolates: 90 from blood cultures, 30 from pus samples, 18 from sputum samples, and 6 from urine samples). Additionally, isolates were obtained from the Ubon Ratchathani University collections between 2013 and 2015 (15 isolates: 8 from blood cultures, 6 from pus samples and 1 from sputum sample). Furthermore, 40 isolates were obtained from routine diagnostics at Central Laboratory, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University in 2021: 17 from blood cultures, 5 from pus samples, 3 from urine samples, 2 each from sputum, ankle swabs and fluid samples, and one isolate each from the following sources: hip, eyes swab, knee swab, parotid swab, lymph node tissue, mediastinal mass, pericardium fluid, tracheal suction, and synovial fluid.

Escherichia coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls for CAZ and TMP-SMX.

Antimicrobial susceptibility testing

Disk diffusion, Etest, and broth microdilution were simultaneously conducted using the same bacterial suspension. All tests were performed according to the Clinical and Laboratory Standards Institute M45 guideline, 3rd edition (CLSI, 2016)[21]

All *B. pseudomallei* isolates were obtained from a -80 °C glycerol stock and streaked to Luria-Bertani agar (LB agar) plate and incubated at 37 °C for 48 hours. The bacteria were prepared by suspending colonies of *B. pseudomallei* into Mueller-Hinton Broth (Thermo Scientific, West Sussex, UK) and adjusted to an optical density using a densitometer (Densichek, BioMérieux, Durham, NC) of 0.5 McFarland in sterile 0.85% saline solution.

For disk diffusion and Etest, the bacterial suspension was evenly spread on the surface of a Muller-Hinton Agar (MHA) plate using a cotton swab and a plate rotator. After allowed to dry for 10 min, a CAZ disk of 30 µg (BD BBL Sensi-Disk, Becton, Dickinson and Company, MD, USA), and a TMP-SMX disk of 1.25/23.75 µg (BD BBL Sensi-Disk, Becton, Dickinson and Company, MD, USA) (one replicate) and E-test of CAZ (0.016-256 mg/L) (Liofilchem srl, Via Scozia, Italy) and TMP-SMX (0.002-32 mg/L) were applied to the plate (one replicate). The plates were incubated at 37°C for 24 h. The diameter of the inhibition zone was measured using a vernier caliper (Mitutoyo, Japan).

The disk zone growth of inhibition was measured according to the CLSI recommendation: CAZ (30 µg): S ≥ 18, I:15-17, R ≤14 and TMP-SMX: S ≥16, I:11-15, R ≤10. The MIC of Etest results were interpreted according to the criteria of the CLSI: CAZ: S ≤8 µg/mL, I:16 µg/mL, R ≥32 µg/mL and TMP-SMX: S ≤2/38 µg/mL, I: (-), R ≥4/76 µg/mL and the manufacturer's guideline (CAZ read the MIC at the point of complete inhibition of all growth and TMP-SMX read at 80% inhibition by the naked eyes). If Etest results were between twofold dilution, they were round to the next highest twofold dilution.

The broth microdilution (BMD) method was performed by diluting the bacteria in a Sensititre AIM™ Automated inoculation Delivery system (Thermo Scientific, West Sussex, UK) according to the manufacturer's procedure guideline before incubating with CAZ (0-32 µg/mL) and TMP-SMX (1/19-4/76 µg/mL) in the Sensititre ARIS® 2X (Thermo Scientific, West Sussex, UK) for 24 h at 37°C (one replicate). The results were recorded using the Sensititre ARIS 2X automated fluorometric plate reading system.

Data analysis

The regression analysis was applied to compare the results of the antimicrobial susceptibility. Categorical agreement (CA) was defined when the test result fell within the same susceptibility category as the reference method. Interpretative category errors were determined as follows: very major error (VME) denoted isolates that were resistant according to the reference method but deemed susceptibility by the tested method (false susceptibility); major error (ME)

referred to isolates classified as susceptible by the reference method but resistant by the tested method (false resistance); and minor error (MIE) indicated cases where the tested method yielded an intermediate result while the reference method classified the isolate as either or susceptible, or *vice-versa*.

Results

Comparison of disk diffusion to reference BMD for CAZ

Using the reference BMD method, the overall CA rate of the disk diffusion for CAZ was 96% (191/199) (Table 1), indicates that the disk diffusion method agreed with the BMD method in categorizing the majority of isolates as susceptible to CAZ. The ME rate was 0.5% (1/199), suggesting that the disk diffusion method rarely misclassified susceptible isolates as resistant. The result of no VME was found, indicating that the disk diffusion method did not misclassify any resistant isolates as susceptible. However, we observed 3.5% (7/199) of MIEs, indicating instances where the disk diffusion method classified isolates differently from the BMD method but within acceptable limits. Notably, none of the tested isolates demonstrated resistance to CAZ using BMD. The scattergram of 199 *B. pseudomallei* tested by disk diffusion and BMD for CAZ is shown in Figure 1.

Comparison of Etest to reference BMD for CAZ

The CA between Etest and the BMD reference method for CAZ was 98.5% (196/199). The high overall CA indicates that the majority of isolates were classified similarly by both the Etest and BMD methods regarding their susceptibility to CAZ. Neither VME nor ME was detected (Table 1), suggesting that the Etest method did not misclassify any resistant isolates as susceptible or vice versa. There was 1.5% (3/199) of ME was identified, indicating instances where the Etest method classified isolates differently from the BMD method, though still within acceptable limits. The scattergram comparing the Etest and BMD is shown in Figure 2.

Comparison of disk diffusion to reference BMD for TMP-SMX

The disk diffusion and BMD evaluation for TMP-SMX exhibited poor correlation due to the CA of 13.6% (27/199) (Table 1). This suggests that the disk diffusion method did not agree well with the BMD method in categorizing isolates as susceptible or resistant to TMP-SMX. However, the ME was high at 76.4% (152/199) suggesting a significant difference in the classification of isolates between the two methods. Additionally, the MIE was 10.1% (20/199) was observed, indicating instances where the disk diffusion method classified isolates differently from the BMD method, though still within acceptable limits but no VME was detected. The scattergram comparing disk diffusion and BMD is shown in Figure 3.

Comparison of Etest to reference BMD for TMP-SMX

The percentage of CA for Etest to BMD was 46.2% (92/199) (Table 1). This suggests that less than half of the isolates were classified similarly by both the Etest and BMD methods regarding their susceptibility to TMP-SMX. There was 53.8% (107/199) of ME, suggesting a significant discrepancy in the classification of isolates between the two methods. Notably, no VME and no MIE were detected, indicating that the Etest method did not misclassify any resistant isolates as susceptible or vice versa. The scattergram comparing Etest and BME is demonstrated in Figure 4.

Table 1 Evaluation of categorial agreement between disk diffusion and Etest methods to BMD method for CAZ and TMP-SMX susceptibilities of *B. pseudomallei* clinical isolates

Sources	No. of isolates tested	No. of resistant isolates by BMD	Disk diffusion				Etest			
			No. (%) of CA	No. (%) of VME	No. (%) of ME	No. (%) of MIE	No. (%) of CA	No. (%) of VME	No. (%) of ME	No. (%) ofMIE
CAZ										
Melioidosis Research Center, KKU	144	0	143 (99.3)	0 (0)	1 (0.7)	0(0)	143 (99.3)	0 (0)	0 (0)	1 (0.7)
Central Laboratory, Srinagarind Hospital, KKU	40	0	37 (92.5)	0 (0)	0 (0)	3 (7.5)	39 (97.5)	0 (0)	0 (0)	1 (2.6)
Ubon Ratchathani University	15	0	11 (73.3)	0 (0)	0 (0)	4 (26.7)	14 (93.3)	0 (0)	0 (0)	1 (7.1)
Total	**199**	**0**	**191 (96.0)**	**0 (0)**	**1 (0.5)**	**7 (3.5)**	**196 (98.5)**	**0 (0)**	**0 (0)**	**3 (1.5)**
TMP-SMX										
Melioidosis Research Center, KKU	144	0	22 (15.3)	0 (0)	103 (71.5)	19 (13.2)	77 (53.5)	0 (0)	67 (46.5)	0 (0)
Central Laboratory, Srinagarind Hospital, KKU	40	0	1 (2.5)	0 (0)	39 (97.5)	0 (0)	9 (22.5)	0 (0)	31 (77.5)	0 (0)
Ubon Ratchathani University	15	0	4 (26.7)	0 (0)	10 (66.7)	1 (6.7)	6 (40.0)	0 (0)	9 (60.0)	0 (0)
Total	**199**	**0**	**27 (13.6)**	**0 (0)**	**152 (76.4)**	**20 (10.1)**	**92 (46.2)**	**0 (0)**	**107 (53.8)**	**0 (0)**

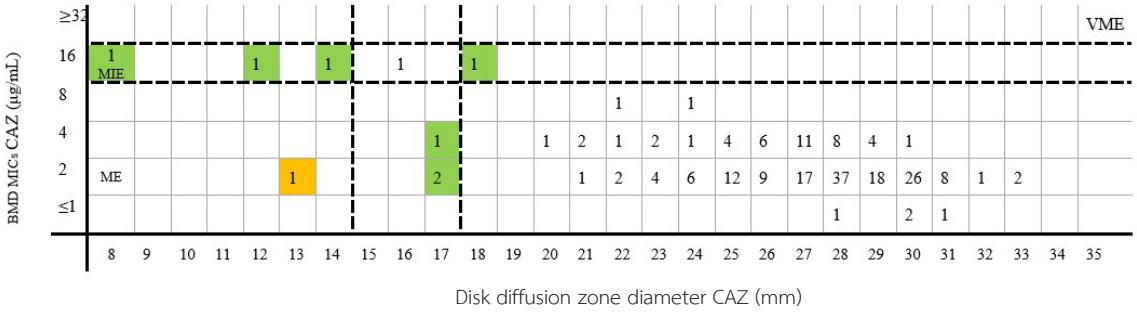

Figure 1 Scatter plot of CAZ zone diameter versus BMD MICs against 199 *B. pseudomallei* clinical isolates. The dotted lines represent the susceptibility breakpoint for CAZ. VME: very major error (false susceptible); ME: major error (false resistant); MIE: minor error (intermediate result by the tested method and resistant or susceptible by the reference method, or *vice-versa*). The yellow background indicates that one ME occurred when the disk diffusion was compared with the BMD. The green background shows that seven MIE occurred when the disk diffusion method was compared with the MIC of the BMD.

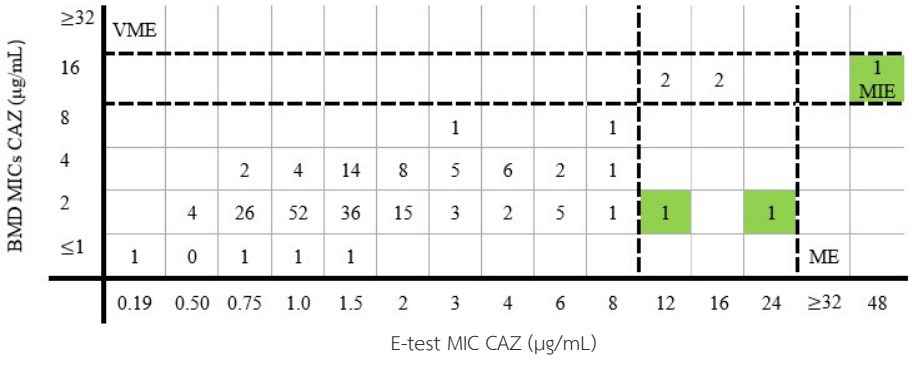

Figure 2 Scatter plot of CAZ Etest MICs versus BMD MICs against 199 *Burkholderia pseudomallei* clinical isolates. The dotted lines represent the susceptibility breakpoint for CAZ. VME: very major error (false susceptible); ME: major error (false resistant); MIE: minor error (intermediate result by the tested method and resistant or susceptible by the reference method, or *vice-versa*). The green background shows that three minor errors occurred in the MIC of the Etest compared with the MIC of the BMD.

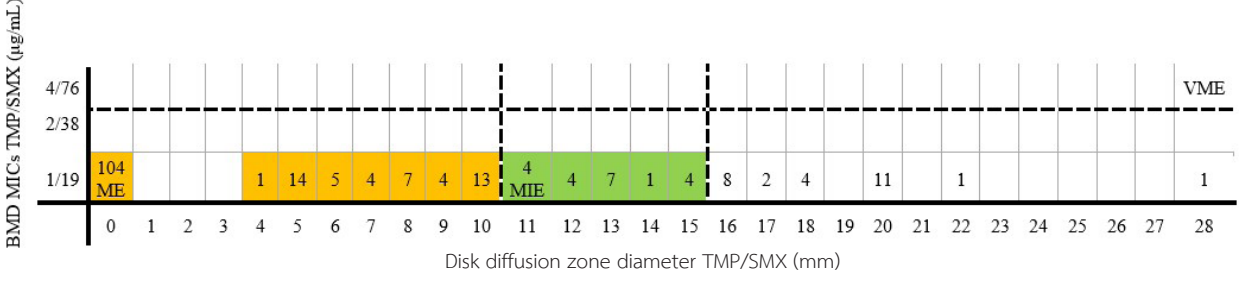

Figure 3 Scatter plot of TMP/SMX zone diameter versus BMD MICs against 199 *B. pseudomallei* clinical isolates. The dotted lines represent the susceptibility breakpoint for TMP/SMX. VME: very major error (false susceptible); ME: major error (false resistant); MIE: minor error (intermediate result by the tested method and resistant or susceptible by the reference method, or *vice-versa*). The yellow background indicates that one hundred and fifty-two ME occurred when the disk diffusion was compared with the BMD. The green background shows that twenty MIE occurred when the disk diffusion method was compared with the MIC of the BMD.

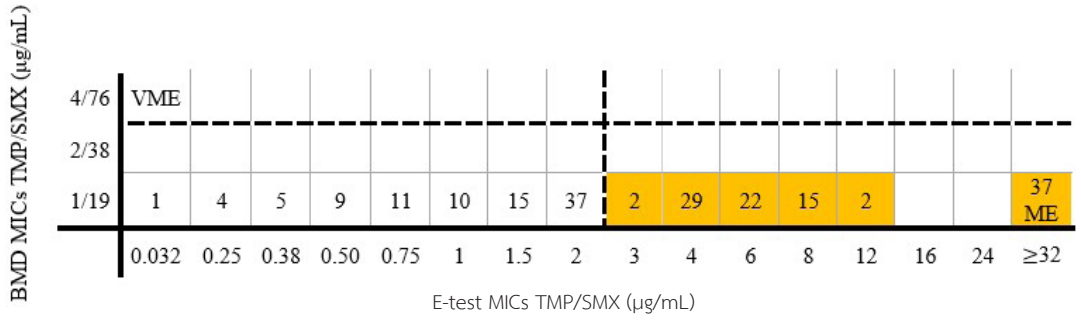

Figure 4 Scatter plot of TMP/SMX Etest MICs versus BMD MICs against 199 *Burkholderia pseudomallei* clinical isolates. The dotted lines represent the susceptibility breakpoint for TMP/SMX. VME: very major error (false susceptible); ME: major error (false resistant); MIE: minor error (intermediate result by the tested method and resistant or susceptible by the reference method, or *vice-versa*). The yellow background shows that one hundred and seven minor errors occurred in the MIC of the Etest compared with the MIC of the BMD.

Discussion

Prior studies have highlighted the importance of AST in the determination of appropriate antibiotic regimens for treating melioidosis. In alignment with this context, the present study compared two AST methods, disk diffusion, and Etest, to the reference method, an automated BMD, to assess the susceptibility of *B. pseudomallei* to CAZ and TMP-SMX for decision-making in resource-limited areas. This study presents a novel approach by comprehensively evaluating the performance of three commonly used methods for antimicrobial susceptibility testing: disk diffusion, Etest, and automated broth microdilution. The results revealed a strong correlation between disk diffusion and Etest methods for CAZ compared to the BMD. However, for TMP-SMX, both disk diffusion and Etest revealed not only the unsatisfactory category agreement but also high ME. Nevertheless, no VME for CAZ and TMP-SMX was observed. This comprehensive evaluation sheds light on the strengths and limitations of each method, offering valuable guidance for clinicians and researchers in selecting the most appropriate method for accurate, cost-effective and appropriate antimicrobial susceptibility testing.

These results corroborate the findings of a great deal of previous research on the comparison of AST for CAZ susceptibility testing of *B. pseudomallei.* This study demonstrates that the CA values of disk diffusion and Etest for CAZ exhibited excellent correlation, at 96% and 98.5%, respectively, when compared to the BMD method. This finding is consistent with that of Nhung et al., who demonstrated 100% susceptibility to CAZ using Etest for all *B. pseudomallei* isolates in Northern Vietnam[22]. Additionally, a comparison between the Etest and BMD of *B. pseudomallei* from 15 different countries revealed a strong correlation of Etest with the reference BMD for CAZ susceptibility[19].

The low CA values of 13.6% and 46.2% with high ME of 76.4% and 53.8% of TMP-SMX in disk diffusion and Etest methods, respectively, are consistent with data obtained in Wuthiekanun and colleagues' report, which demonstrated *B. pseudomallei* resistance to TMP-SMX at 71% by disk diffusion and 13% by Etest[23]. *B. pseudomallei* isolates in Northern Vietnam showed 10% resistance to TMP-SMX using Etest[22].

This finding contradicts previous studies that have suggested a very low primary resistance of *B. pseudomallei* to TMP-SMX when using the Etest method. Notably, these previous observations consistently indicated uncommon resistance of *B. pseudomallei* to TMP-SMX[17,18,24]. One possible explanation for this discrepancy could be the minor difference in reading at the 80% inhibition point of Etest, which often diffuses edges, leading to potential false interpretation as resistance[17, 19]. Additionally, the lack of standardized methodologies and interpretative criteria for disk diffusion and Etest in assessing TMP-SMX susceptibility with *B. pseudomallei* could lead to potentially misleading data, despite CLSI recommendations.

These results from this study underscore the importance of carefully selecting and validating AST methods, especially in regions with limited resources. The disk diffusion and Etest yielded a good CA with the automated BMD for CAZ susceptibility, suggesting that either of these methods can be employed in such settings with reasonable confidence. Nevertheless, caution is warranted when assessing TMP-SMX susceptibility, as both disk diffusion and Etest methods exhibited discrepancies when compared to the automated BMD, possibly indicating limitations in their accuracy for this specific antibiotic. However, most laboratories in melioidosis endemic regions in developing countries rely on disk diffusion while routine MIC is simply not practical or affordable. A multi-center study to establish epidemiological cut-off values for BMD and disk diffusion for eight clinically relevant antimicrobial agents against *B. pseudomallei* followed the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology suggested that CAZ remains the most appropriate first-line treatment during the initial parenteral phase even when it is reported as intermediate susceptible. Furthermore, TMP-SMX should also be prescribed for the eradication phase even though it is reported as intermediate susceptible that should be noted as "susceptible but requires high doses[25, 26]. Although true TMP-SMX resistance may be uncommon, implementing broth microdilution in resource-limited settings provides notable benefits in accuracy, reliability standardization that offers the accurate interpretation and guiding appropriate antibiotic therapy.

Conclusion

In conclusion, the disk diffusion and Etest methods showed acceptable performance as alternative methods to BMD for CAZ. While BMD to determine the MIC values is required for testing susceptibility to TMP-SMX.

Funding

Sereywatana Chea was supported by the Royal Scholarship under Her Royal Highness Princess Maha Chakri Sirindhorn Education Project to the Kingdom of Cambodia. This study was granted by the Faculty of Medicine, Khon Kaen University, Thailand (Grant Number IN64302).

References

1. Gassiep I, Armstrong M, Norton R. Human melioidosis. Clin Microbiol Rev 2020;33(2):e00006-19. doi:10.1128/CMR.00006-19
2. Savelkoel J, Dance DAB, Currie BJ, Limmathurotsakul D, Wiersinga WJ. A call to action: time to recognise melioidosis as a neglected tropical disease. Lancet Infect Dis 2022;22(6):e176-e82. doi:10.1016/S1473-3099(21)00394-7
3. Turner P, Kloprogge S, Miliya T, Soeng S, Tan P, Sar P, et al. A retrospective analysis of melioidosis in Cambodian children, 2009-2013. BMC Infect Dis 2016;16(1):688. doi:10.1186/s12879-016-2034-9
4. Hinjoy S, Hantrakun V, Kongyu S, Kaewrakmuk J, Wangrangsimakul T, Jitsuronk S, et al. Melioidosis in Thailand: present and future. Trop Med Infect Dis 2018;3(2):38. doi:10.3390/tropicalmed 3020038
5. Currie BJ, Ward L, Cheng AC. The epidemiology and clinical spectrum of melioidosis: 540 cases from the 20 year Darwin prospective study. PLoS Negl Trop Dis 2010;4(11):e900. doi:10.1371/journal.pntd.0000900
6. Cheng AC, Currie BJ. Melioidosis: epidemiology, pathophysiology, and management. Clin Microbiol Rev 2005;18(2):383-416.
7. Somprasong N, Yi J, Hall CM, Webb JR, Sahl JW, Wagner DM, et al. Conservation of resistance-nodulation-cell division efflux pump-mediated antibiotic resistance in *Burkholderia cepacia* complex and *Burkholderia pseudomallei* complex species. Antimicrob Agents Chemother 2021; 65(9):e0092021. doi:10.1128/AAC.00920-21
8. Chirakul S, Somprasong N, Norris MH, Wuthiekanun V, Chantratita N, Tuanyok A, et al. *Burkholderia pseudomallei* acquired ceftazidime resistance due to gene duplication and amplification. Int J Antimicrob Agents 2019;53(5):582-8. doi:10.1016/j.ijantimicag.2019.01.003
9. Sarovich DS, Price EP, Von Schulze AT, Cook JM, Mayo M, Watson LM, et al. Characterization of ceftazidime resistance mechanisms in clinical isolates of *Burkholderia pseudomallei* from Australia. PLoS One 2012;7(2):e30789. doi:10.1371/journal.pone.0030789

10. Pibalpakdee P, Wongratanacheewin S, Taweechaisupapong S, Niumsup PR. Diffusion and activity of antibiotics against *Burkholderia pseudomallei* biofilms. Int J Antimicrob Agents 2012;39:356– 9. doi: 10.1016/j.ijantimicag.2011.12.010.
11. Sawasdidoln C, Taweechaisupapong S, Sermswan RW, Tattawasart U, Tungpradabkul S, Wongratanacheewin S. Growing *Burkholderia pseudomallei* in biofilm stimulating conditions significantly induces antimicrobial resistance. PLoS One 2010;5(2):e9196. doi:10.1371/journal.pone.0009196
12. Dance DA. Treatment and prophylaxis of melioidosis. Int J Antimicrob Agents 2014;43(4): 310–8. doi: 10.1016/j.ijantimicag.2014.01.005.
13. Piliouras P, Ulett GC, Ashhurst-Smith C, Hirst RG, Norton RE. A comparison of antibiotic susceptibility testing methods for cotrimoxazole with *Burkholderia pseudomallei*. Int J Antimicrob Agents 2002;19(5): 427-9. doi: 10.1016/s0924-8579(02) 00016-x
14. Lumbiganon P, Tattawasatra U, Chetchotisakd P, Wongratanacheewin S, Thinkhamrop B. Comparison between the antimicrobial susceptibility of *Burkholderia pseudomallei* to trimethoprim-sulfamethoxazole by standard disk diffusion method and by minimal inhibitory concentration determination. J Med Assoc Thai 2000;83(8):856-60.
15. Wuthiekanun V, Cheng AC, Chierakul W, Amornchai P, Limmathurotsakul D, Chaowagul W, et al. Trimethoprim/sulfamethoxazole resistance in clinical isolates of *Burkholderia pseudomallei*. J Antimicrob Chemother 2005;55(6):1029-31. doi:10.1093/jac/dki151
16. Hassan MR, Vijayalakshmi N, Pani SP, Peng NP, Mehenderkar R, Voralu K, et al. Antimicrobial susceptibility patterns of *Burkholderia pseudomallei* among melioidosis cases in Kedah, Malaysia. Southeast Asian J Trop Med Public Health 2014; 45(3):680-8.
17. Saiprom N, Amornchai P, Wuthiekanun V, Day NP, Limmathurotsakul D, Peacock SJ, et al. Trimethoprim/sulfamethoxazole resistance in clinical isolates of *Burkholderia pseudomallei* from Thailand. Int J Antimicrob Agents 2015;45(5): 557-9. doi:10.1016/j.ijantimicag.2015.01.006
18. Dance DA, Davong V, Soeng S, Phetsouvanh R, Newton PN, Turner P. Trimethoprim/sulfamethoxazole resistance in *Burkholderia pseudomallei*. Int J Antimicrob Agents 2014;44(4):368-9. doi:10.1016/j.ijantimicag.2014.06.003
19. Lonsway DR, Elrod MG, Kendrick N, Tiller R, Sullivan MM, Edwards JR, et al. Correlation between Etest and reference broth microdilution for antimicrobial susceptibility testing of *Burkholderia pseudomallei*. Microb Drug Resist 2020;26(4):311-8. doi:10.1089/mdr.2019.0260
20. Dutta S, Haq S, Hasan MR, Haq JA. Antimicrobial susceptibility pattern of clinical isolates of *Burkholderia pseudomallei* in Bangladesh. BMC Res Notes 2017;10(1):299. doi:10.1186/s13104-017-2626-5
21. Clinical and Laboratory Standards Institute. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2016.
22. Nhung PH, Van VH, Anh NQ, Phuong DM. Antimicrobial susceptibility of *Burkholderia pseudomallei* isolates in Northern Vietnam. J Glob Antimicrob Resist 2019;18:34-6. doi:10.1016/j.jgar.2019.01.024
23. Wuthiekanun V, Cheng AC, Chierakul W, Amornchai P, Limmathurotsakul D, Chaowagul W, et al. Trimethoprim/sulfamethoxazole resistance in clinical isolates of *Burkholderia pseudomallei*. J Antimicrob Chemother 2005;55(6):1029-31. doi: 10.1093/jac/dki151.
24. Crowe A, McMahon N, Currie BJ, Baird RW. Current antimicrobial susceptibility of first-episode melioidosis *Burkholderia pseudomallei* isolates from the Northern Territory, Australia. Int J Antimicrob Agents 2014;44(2):160-2. doi:10.1016/j.ijantimicag.2014.04.012
25. Dance DAB, Wuthiekanun V, Baird RW, Norton R, Limmathurotsakul D, Currie BJ. Interpreting *Burkholderia pseudomallei* disc diffusion susceptibility test results by the EUCAST method. Clin Microbiol Infect 2021;27(6):827-9. doi:10.1016/j.cmi.2021.02.017
26. Karatuna O, Dance DAB, Matuschek E, Ahman J, Turner P, Hopkins J, et al. *Burkholderia pseudomallei* multi-centre study to establish EUCAST MIC and zone diameter distributions and epidemiological cut-off values. Clin Microbiol Infect 2020;27(5): 736-41. doi:10.1016/j.cmi.2020.07.001