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Diversity of Colony Morphotypes, Biochemical Characteristics, and Drug Susceptibility Patterns of *Burkholderia pseudomallei* Isolated from Humans, Animals, and Environmental Sources in Thailand

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Abstract

Melioidosis is an infection that occurs in humans and a wide range of animals by *Burkholderia pseudomallei* as a causative pathogen, which inhabits environments of tropical and subtropical climate zones, particularly in Thailand. The standard laboratory diagnosis is by culture and biochemical characteristic differentiation. This study aimed to determine the diversity of colony morphology, biochemical characteristics, and drug susceptibility patterns of a *B. pseudomallei* collection isolated from humans, animals, soil, and water in Thailand. According to the previous characterization of colony morphotypes from Type I to VII, Type I is predominant with *B. pseudomallei* from humans and animals with a similar water source. Meanwhile, Type III is predominant from soil sources. The results confirmed the hypothesis that infection might be from water instead of soil exposure. The typical biochemical phenotypes were consistent among different isolate sources, including cytochrome oxidase (positive), triple iron sugar (K/N or A/N), sulfide (negative), indole (negative), non-motile, glucose oxidizing, and DNase (positive). The biochemical characteristic results supported the data for the selection of the prepopulated biochemical tests in clinical and research laboratories. The antimicrobial susceptibility patterns by standard disk diffusion showed mode values of inhibition zones for selected drugs that had lower trends in *B. pseudomallei* isolated from the soil than other sources. The results were consistent with MIC50 and MIC90 values of ceftazidime, which was highest in *B. pseudomallei* from the soil than other sources.

Keywords: Biochemical test, *Burkholderia pseudomallei*, Ceftazidime, Colony morphology, Drug susceptibility pattern, Melioidosis, Minimum inhibitory concentration

Introduction

Burkholderia pseudomallei is a Gram-negative bacterium found in tropical and subtropical climate areas of the entire world, especially Southeast Asia and northern Australia. It is a causative agent of melioidosis and classified as a Tier 1 select agent by the US Centers for Disease Control and Prevention (CDC) in regard to bioterrorism weapon usage. This bacterium is commonly found in environmental surroundings (soil and water) [1]. It resides underground; however, favorable topographies (e.g., rainfall) and climate and agricultural activities (e.g., farming) increase the risk of exposure to humans or animals through inhalation, ingestion, or skin abrasion leading to mild to severe symptoms [2]. Immune-compromised patients, such as those with diabetes mellitus or thalassemia, are specifically at risk [2]. Once the infection occurs, patients are usually admitted in sepsis with or without pneumonia or localized infection as significant symptoms depending on the route of infection. As *B. pseudomallei* is called mimicker, it can cause various manifestations due to various organ infections including the central nervous system, cardiovascular system, urinary tract system, head and neck, respiratory system, gastrointestinal system, skin and soft tissue, and musculoskeletal system. These symptoms can be mistaken for other diseases, such as leptospirosis or tuberculosis, causing trouble in diagnosis by medical staff [2]. Infection in domestic animals may be able to spread to humans by consumption or occupation. However, there are no actual reported

examples of zoonotic transmission. It supports the idea of the "One Health" concept that people, animals, and the environment are bound together in health through the ecosystem in which they coexist [3].

B. pseudomallei is a great patriarch of adaptation and survival. Even though the regulatory genes remain unclear, the bacterium is likely to change its molecular expression to tolerate hazardous environments [4]. Chantratita *et al*. showed that morphological changes were related to expression alteration including starvation stress, motility, biofilm formation, and cellular invasiveness. It has been characterized into seven morphotypes regarding surface texture at the center of the colony, outer edge, color, and colony diameter [5]. The Type I morphotype gives a rough center surface texture, irregular outer edge, and pale purple colony, which is the most common morphotype found in environmental and host specimens. It can switch to Type II and Type III during the starvation period and reverse its origin after stress removal [5]. The relationship between phenotypic variations seems to involve the antigenic variation and O-polysaccharide modification in the lipopolysaccharide, which is a potential target of further vaccine development [6].

B. pseudomallei infection can be acute, chronic, or latent, leading to recurrent infection after a period of time. Melioidosis can occur in any range of age [7]. It can hide inside macrophages and produce many kinds of virulence factors. Because of the bacterium's mechanism to evade an immune system, long-term treatment is needed to eliminate the pathogen. The drugs of choice for early intensive care include meropenem (MEM), imipenem (IMP), and especially ceftazidime (CAZ) that uses an intravenous route at least 10 - 14 days together with trimethoprim-sulfamethoxazole (SXT), co-amoxiclav (AMC), or doxycycline (DOX) orally for at least a further 3 months [8]. The antimicrobial susceptibility test for minimal inhibitory concentration is interpreted as belonging to the Clinical and Laboratory Standards Institute (CLSI) breakpoint including AMC, CAZ, IMP, DOX, tetracycline (TCN), and SXT [9]. In southeastern Thailand, the study of 1317 isolates collected from clinical isolates from 2015 to 2018 noted that there were 0.15 % CAZ resistance, 0.15 % low susceptibility to MEM, and 0.08 % AMC resistance in primary resistance. The relapse resistance indicated a lower susceptibility at 7.7 % resistance for CAZ and AMC and 15.4 % resistance for MEM in the range of low susceptibility [10]. The *B. pseudomallei* in animals from slaughterhouses in Nakhon Pathom and Ratchaburi provinces were collected from gross lesions from 2016 to 2017. Observation showed susceptibility for CAZ and IMP, but AMC and TCN were found to have 92.1 and 1.0 % susceptibility, respectively. Interestingly, SXT was found to have 34.8 % susceptibility [11]. For environmental specimens, there is no antimicrobial susceptibility test in Thailand; however, the study of antimicrobial susceptibility of Western hemisphere strains demonstrated one intermediate AMC resistance and intermediate resistance to TCN [9].

The main aims of this study were to determine the diversity of colony morphology, biochemical characteristics, and drug susceptibility patterns of a *B. pseudomallei* collection isolated from humans, animals, and soil and water in Thailand. The same predominate colony morphotypes from different sources supported the bacteria originating from the environment that was potent enough to infect humans and animals. The typical biochemical characters were used for the selection of the appropriate tests. Moreover, susceptibility patterns of different sourced isolates showed the distribution of drug-resistant *B. pseudomallei* not only in patients but that they may also be found in animals and the environment.

Materials and methods

Bacteria isolates

A total of 168 *Burkholderia pseudomallei* isolates were obtained from human clinical specimens (n = 90), environmental sources (n = 68), and animals (n = 10 from pig organ abscesses). All bacteria were stocked in Luria Bertani (LB) broth with 20 % glycerol in –70 °C at the Center of Excellence Research for Melioidosis (CERM), Walailak University, Nakhon Si Thammarat, Thailand. The bacteria from clinical specimens were collected in 2018 from four hospitals in Thailand including Maharaj Nakhon Si Thammarat Hospital (n = 18), Vachira Phuket Hospital (n = 44), Trang Hospital (n = 26), and Chaiyaphum Hospital (n = 2). Clinical specimens were composed of blood (n = 75), pus/wound (n = 7), sputum (n = 7), and catheterized urine (n = 1). Environmental isolates were collected in 2011 and 2018 from soil (n = 33) and underground water (n = 35) sources in Nakhon Si Thammarat, Thailand. Another 10 isolates were collected in 2018 from pig organs (liver abscess and lung abscess) of carrions, which were from slaughterhouses in Nakhon Si Thammarat, Thailand. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Human Research Ethics Committee Walailak University (Approval Number WUEC-20-140-01, date of approval 1 July 2020). However, permission from the IACUC was not considered applicable because the samples were from carrions from slaughterhouses, which generally should be discarded. All the experiments involving the living *B. pseudomallei* were performed in a BSL2-

enhanced level. The details of all *B. pseudomallei* isolates are in Supplementary Materials S1. A schematic representation of the study is shown in **Figure 1**.

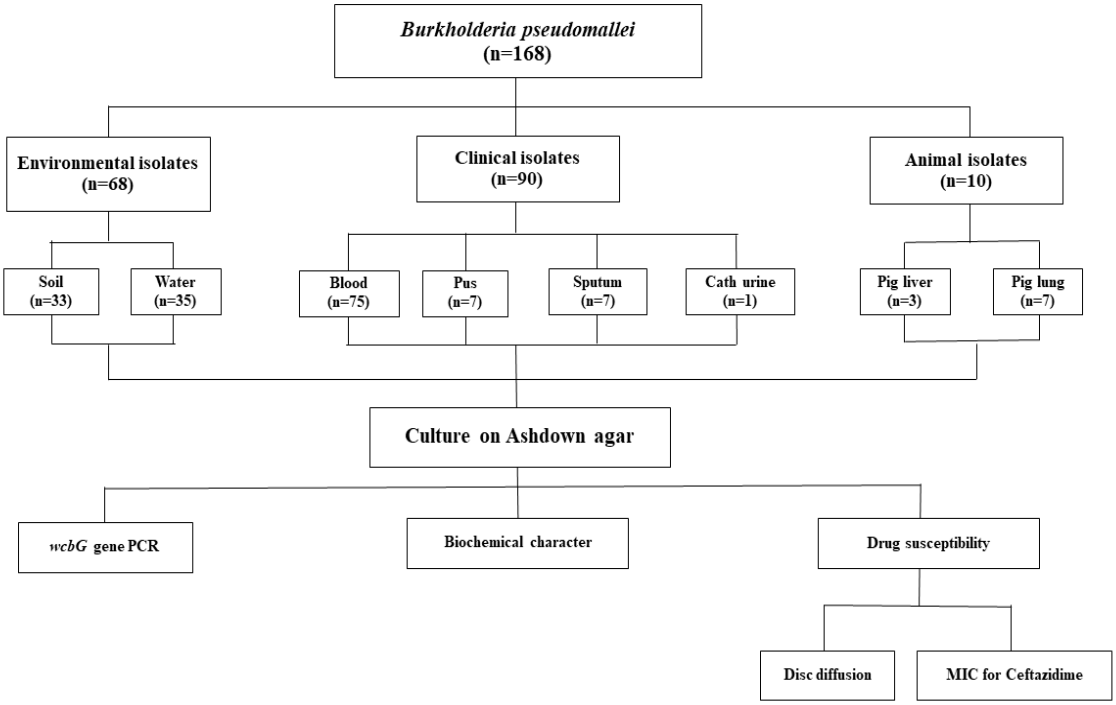


Figure 1 Schematic diagram of the study's design.

Ashdown agar preparation

Ashdown agar is a selective medium for the isolation and characterization of *B. pseudomallei*. The preparation protocol followed that by Howard (2003). Ingredients were measured as follows: tryptone soya broth (10 g/L), agar (15 g/L), glycerol (40 mL/L), 0.1 % crystal violet (5 mL/L), and 1 % neutral red (5 mL/L). All components were mixed well with 1 liter of distilled water and autoclaved at 121 °C for 15 minutes. Finally, 4 mg of gentamicin was gently added while the mixture was chilled down to 50 °C before dispensing the agar into a sterile Petri dish [12].

Colony culture and observation of bacterial colony morphology

The bacteria from the glycerol stock were directly cultivated by scraping the top of the vial with a sterile loop and suspending them in sterile saline. The suspension was streaked on the Ashdown agar and incubated at 37 °C for 48 - 72 h. The colony morphotype identification chart by Chantratita *et al*. (2007) was used to classify the colony morphotype. Type I-VII colony morphotypes were categorized according to the surface texture at the center of the colony (i.e., smooth or irregular), outer edge (i.e., circumference) of the colony, presence of roughness in the outer half of the colony, colony color, and colony diameter [5].

Biochemical characteristics

The single colony from the Ashdown agar was tested for biochemical characteristics. The biochemical tests included the paper oxidase test, triple sugar iron agar (TSI), H_2S-indole-motile (SIM) semisolid tube, citrate utilization, urease, bile esculin hydrolysis, nitrate reduction test, DNase test agar, and sugar (glucose and maltose) oxidation-fermentation (OF) test. In addition, selected biochemical tests in this study were used to identify glucose non-fermentative Gram-negative bacilli in a clinical laboratory [13]. All biochemical tests were prepared, assessed for quality assurance by testing with recommended standard bacteria, performed, and interpreted according to recognized methods [13].

Latex agglutination test

Agglutination was performed on the colony from the Ashdown agar with a latex agglutination kit (Melioidosis Research Center, Khon Kaen University, Khon Kaen, Thailand) to detect *B. pseudomallei*. The principle of this latex agglutination kit involves the use of a monoclonal antibody against 200 kDa exopolysaccharide-coated latex beads [14].

Bacteria DNA extraction

Heat extraction was performed following the method of Dashti *et al.* (2009). First, two bacteria colonies on the Ashdown agar were put in a test tube containing 1 mL of sterile distilled water and boiled for 10 minutes in a water bath. Then, the tubes were centrifuged at 1000 rpm for 5 minutes and collected the supernatant. The DNA quality was determined by optical density at 260/280 nm, and 5 microliters of aliquot were used for PCR [15].

Polymerase chain reaction for *B. pseudomallei* confirmation

The *wcbG* gene (a putative capsular polysaccharide biosynthesis protein gene of *B. pseudomallei*) was amplified by standard PCR using a pair of primers: forward: 5'-AACGAGTCGGTCATTTCCCTGA-3' and reverse: 5'-CCGATATTGCCGACTTCCA CTGTGAT-3'. The total volume of PCR reaction was 50 µL, containing 5 µL of 10× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2.5 µL of dNTPs (1 mM each), 2.5 µL of each primer (10 µM each), 5 µL of DNA sample, 31.5 µL of dH_2O, and 1 µL (5 units) of *Taq* DNA polymerase. The amplification condition was initially set at 94 °C for 5 minutes, followed with 40 cycles at 94 °C for 30 seconds (denaturation), 60 °C for 30 seconds (annealing), and 72 °C for 45 seconds (extension) [16]. The 323 base-pair amplicon was generated in DNA gel electrophoresis, which was observed under UV light. Meanwhile, *B. pseudomallei* K96243 and *Escherichia coli* ATCC 25922 were used as the positive and negative controls, respectively.

Antibiotic drug susceptibility testing by the disc diffusion method

Susceptibility testing was performed using the Kirby-Bauer disc diffusion method [17]. The protocol, in brief, consisted of adjusting 0.5 McFarland suspension of each bacterial isolate with sterile, normal saline and measuring with a digital densitometer DEN-1 (Kisker Biotech, Steinfurt, Germany). Then, a lawn was made from the suspended bacteria using a sterile cotton swab on a 4 mm thick Mueller-Hinton agar (Oxoid Ltd., UK). The discs were applied on MHA and incubated at 37 °C for 20 - 24 h. The clear inhibition zone around the antibiotic disc was measured in millimeters. However, interpretative standards for zone sizes were not available for the *B. pseudomallei* in the Clinical and Laboratory Standards Institute [18]; thus, the threshold zone sizes for the *Enterobacteriaceae* family and *Pseudomonas aeruginosa* were recommended from a previous report [10]. The antibiotic discs were composed of ceftazidime (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), doxycycline (30 µg), ciprofloxacin (5 µg), meropenem (10 µg), and imipenem (10 µg) (Oxoid Ltd., UK). The interpretation of the inhibition zone sizes is shown in **Table 1**.

Table 1 Inhibition zone diameter interpretive standards for *B. pseudomallei*.

Antimicrobial Drug	Concentration (µg)	Inhibition Zone (mm)		
		R	I	S
Ceftazidime [a]	30	≤14	15-17	≥18
Trimethoprim/Sulfamethoxazole [b]	1.25/23.75	≤10	11-15	≥16
Doxycycline [b]	30	≤10	11-13	≥14
Ciprofloxacin [a]	5	≤15	16-20	≥21
Meropenem [a]	10	≤15	16-18	≥19
Imipenem [a]	10	≤15	16-18	≥19

mm: Millimeter, µg: microgram. [a]Inhibition zone interpretive standards of CLSI for *Pseudomonas aeruginosa* using the disc diffusion method. [b]Inhibition zone interpretive standards for *Enterobacteriaceae* using the disc diffusion method.

Determination of the minimum inhibitory concentration for ceftazidime

Susceptibility testing for ceftazidime was selected to determine the MIC by the E-test diffusion assay. The isolated colony in each sample was inoculated in cation-adjusted Mueller-Hinton Broth (CAMHB) (ThermoFisher Scientific, Massachusetts, United States) incubated overnight at 37 °C in aerobic condition and then adjusted to 0.5 McFarland by sterile 0.9 % NaCl with a digital densitometer DEN-1 (Kisker Biotech, Steinfurt, Germany). Then, a lawn was made from the suspended bacteria using a sterile cotton swab on a 4 mm thick Mueller-Hinton agar (Oxoid Ltd., UK). ETEST® CEFTAZIDIME 256 US (BioMérieux, Marcy-L'Etoile, France). Plates were incubated at ambient conditions at 37 °C for 18 - 20 hours. The MIC breakpoints for ceftazidime were interpreted as follows: susceptible ≤ 8 μg/mL, intermediate = 16 μg/mL, and resistant ≥ 32 μg/mL [19]. Quality control was performed using *Escherichia coli* ATCC 22952 and *Pseudomonas aeruginosa* ATCC 27853.

Statistical analysis

The morphotypes, biochemical tests, antibiotic susceptibility phenotypes were reported and calculated to percentages. Moreover, the diameter of inhibition zones was shown as mode values of respective distribution.

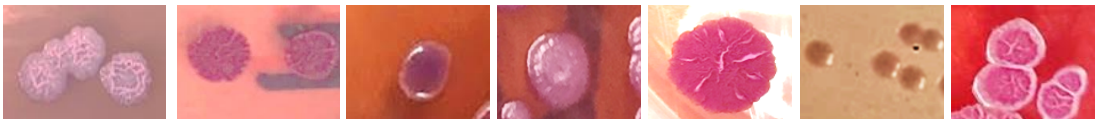
Results and discussion

In total, 168 *Burkholderia pseudomallei* isolates were collected from 90 human clinical sources, 10 animal organs, 33 soil sources, and 35 underground water sources; they were categorized into Type I-VII colony morphotypes according to the study by Chantratita *et al*. [5]. Two morphotypes were determined from four isolates from human clinical sources. All four isolates expressed the Type I morphotype and other morphotypes, including Type V for two isolates and Type II and III for each of the other two isolates. In addition, one isolate from animal organs was found with both Type I and VII morphotypes. The predominant colony morphology of *B. pseudomallei* in both the human clinical specimens (55.26 %) and animal organ abscesses (72.73 %) was Type I. Most of the human clinical sources were blood (83.3 %), followed by sputum (7.8 %), pus/wound (7.8 %), and catheterized urine (1.1 %). Surprisingly, Type I was also the major morphotype of *B. pseudomallei* from the underground water source (57.14 %). Meanwhile, Type III was the major morphotype of *B. pseudomallei* from the soil source (51.52 %). The summary data are shown in **Table 2**. The figure of colony morphology and characteristics for all isolates are in Supplementary Materials S2.

The *wcbG* PCR primers are specific to *B. pseudomallei* capsular gene. The expected PCR product was 323 base pairs. All isolates of tested collection from human, animal organs, and soil and water showed positive results. Negative DNA control *Escherichia coli* ATCC 25922 was not found in the *wcbG* specific band. Meanwhile *B. pseudomallei* K96243, which was a positive control, was also positive. Moreover, latex agglutination, which reacts specifically against exopolysaccharides of *B. pseudomallei*, reacted to positive agglutination to *B. pseudomallei*. All isolates were positive agglutination. The results indicated that all collected isolates in this study were *B. pseudomallei.*

The conventional tests for the determination of biochemical characteristics included 12 tests. The similarity to the typical characteristics of the *B. pseudomallei* collection from human isolates was 100 % for the oxidase, TSI (K/N or A/N), H_2S, indole, motility, esculin hydrolysis, nitrate reduction, and OF glucose tests. The nitrate reduction test of the soil isolates was not similar to that of the human isolates. The citrate and urease tests showed differences from the expected results in all isolate sources. The biochemical tests showed results consistent with the expected results in all *B. pseudomallei* for all sources for the oxidase, TSI, SIM, OF-glucose, and DNase tests. The data of biochemical test characteristics are shown in **Table 3**.

Table 2 Colony morphotypes *of Burkholderia pseudomallei* isolates from humans, animal organs, soil, and underground water grown on Ashdown agar.

Sources (Isolate /Morphotype)	Number of the Morphotype (%)						
	Type I	**Type II**	**Type III**	**Type IV**	**Type V**	**Type VI**	**Type VII**
Human (90/94)	51(54.26)	16(17.02)	7(7.45)	3(3.19)	11(11.70)	4(4.26)	3(3.19)
Animal (10/11)	8(72.73)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	3(27.27)
Soil (33/33)	1(3.03)	2 (6.06)	17(51.52)	0(0.00)	8(24.24)	3(9.09)	2(6.06)
Water (35/35)	20(57.14)	11(31.43)	0(0.00)	0(0.00)	4(11.43)	0(0.00)	0(0.00)
Figure and Features *							
	Rough center—irregular outer edge with pale purple color	Rough center—irregular outer edge with dark purple color and colony diameter < 5 mm	Smooth center—absent of surface roughness in outer half of colony and diameter ≥ 2 mm with variable color	Smooth center—present with surface roughness in outer half of colony	Rough center—irregular outer edge with dark purple color and colony diameter ≥ 5 mm	Smooth center—absent of surface roughness in outer half of colony and diameter < 2 mm with pale color	Rough center with smooth outer edge

*The features of colony morphology were recorded according to the colony's features including surface texture at the center of the colony (i.e., smooth or irregular), outer edge (i.e., circumference) of colony, presence of roughness in the outer half of the colony, colony color, and colony diameter following the study by Chantratita *et al.* [5].

Table 3 Biochemical characteristics of *Burkholderia pseudomallei* isolates.

Biochemical tests	Typical Characteristic	Number Similar to Expected Characteristic (%)			
		Humans (*n* = 94)	**Animals (*n* = 11)**	**Soil (*n* = 33)**	**Water (*n* = 35)**
Cytochrome oxidase	+	94(100.0)	11(100.0)	33(100.0)	35(100.0)
TSI	K/N	75(79.8)	8(72.7)	26(78.8)	25(71.4)
	A/N	19(20.2)	2(27.3)	7(21.2)	10(28.6)
H_2S	–	94(100.0)	11(100.0)	33(100.0)	35(100.0)
Indole	–	94(100.0)	11(100.0)	33(100.0)	35(100.0)
Motility	+	94(100.0)	11(100.0)	33(100.0)	35(100.0)
Citrate	+	47(50.0)	8(72.7)	30(90.1)	12(34.3)
Urease	–	41(43.6)	1(9.1))	16(45.7)	28(80)
Esculin hydrolysis	–	94(100.0)	8(72.7)	29(87.9)	33(94.3)
Nitrate reduction	+	94(100.0)	11(100.0)	28(84.8)	35(100.0)
OF glucose	Oxidizer	94(100.0)	11(100.0)	33(100.0)	35(100.0)
OF maltose	Oxidizer	71(75.5)	9(81.8)	26(78.8)	18(51.4)
DNase test	–	94(100.0)	11(100.0)	33(100.0)	35(100.0)

TSI, triple sugar iron agar; OF, oxidation-fermentation; +, positive; –, negative; K, alkaline; A, acid; N, neutral.

The patterns of antimicrobial drug resistance of *B. pseudomallei* from human sources consisted of nine patterns. Mono-resistance to trimethoprim/sulfamethoxazole was the highest in all sources, which ranged from highest to lowest as follows: 27.1 % from animals, 21.2 % from the soil, 13.8 % from humans, and 8.6 % from water. Interestingly, resistance to ceftazidime, which is one of the first drugs of choice for the initiation phase for melioidosis, was found in one isolate (3.0 %) of *B. pseudomallei* from soil sources, however, it was couple intermediate resistance with imipenem. Mono-resistance doxycycline was found only in one isolate (9.1 %) from animal sources. Meanwhile, intermediate resistance to ceftazidime was found in one isolate (1.1 %) from human sources. Double resistance to trimethoprim/sulfamethoxazole and ciprofloxacin was found in three sources, which ranged from highest to lowest as follows: 11.4 % from water, 3.0 % from the soil, and 1.1 % from humans. Double resistance to trimethoprim/sulfamethoxazole and doxycycline was found in two isolates from 1(3.0 %) soil and 1(1.1 %) human. Imipenem resistance was found in four isolates (12.12 %) from soil sources, which was coupled with resistance and intermediate resistance to trimethoprim/sulfamethoxazole and intermediate resistance to ciprofloxacin and meropenem. The data are shown in **Table 4**. Meropenem and imipenem showed 100 % susceptibility against *B. pseudomallei*, except for meropenem for human sources (98.9 %), meropenem in soil sources (97.0 %) and imipenem for soil sources (75.8 %). Trimethoprim/sulfamethoxazole had the lowest susceptibility percentage among the six antimicrobial drugs, which was 73.4, 45.5, 48.5, and 80.0 % for human, animal, soil, and water sources. The data are shown in **Figure 2**. The mode value of the antimicrobial drugs, including ceftazidime, trimethoprim/sulfamethoxazole, doxycycline, ciprofloxacin, meropenem, and imipenem, on *B. pseudomallei* isolated from humans had the highest trend among the sources of isolates, at 30, 6, 30, 22, 30, and 36 mm, respectively. In contrast, the mode value of the same antimicrobial agents showed the lowest trend in the soil isolates, at 26, 6, 26, 22, 26, and 20 mm, respectively. The data are shown in **Table 5**.

From the MIC results shown in **Table 6**, all isolates of *B. pseudomallei* from humans, animals, soil, and water were 100 % susceptible to ceftazidime. The MIC50 for ceftazidime in the soil isolates was highest (2.0 µg/mL), followed by the human, water, and animal sources at 1.5, 1.5, and 1 µg/mL, respectively. The MIC90 for ceftazidime in the soil isolates was also highest (4.0 µg/mL), followed by the human, animal, and water sources at 2.0, 2.0, and 1.5 µg/mL, respectively. Both the MIC50 and MIC90 of all *B. pseudomallei* isolates were lower than the 8 µg/mL cut-off.

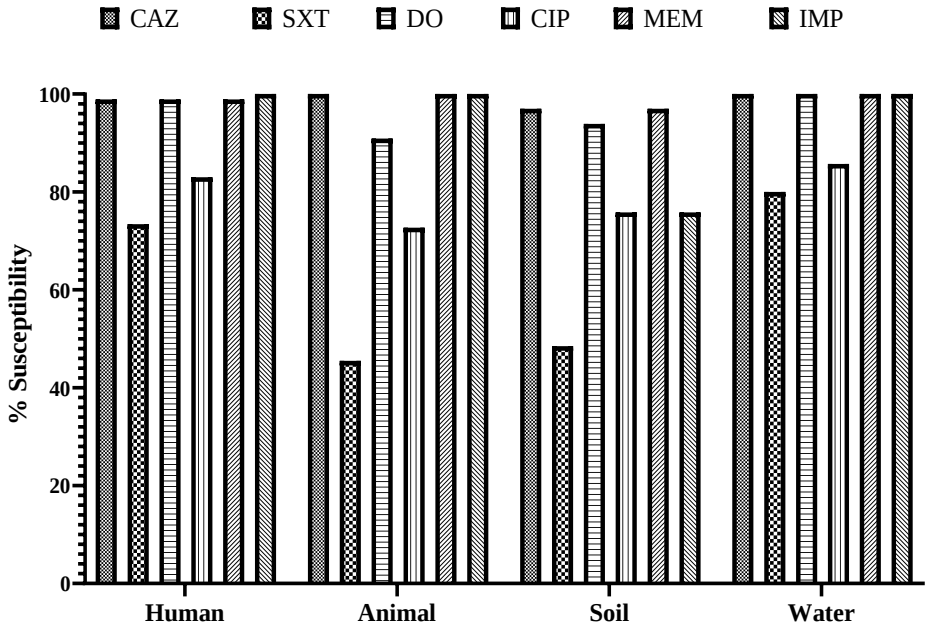


Figure 2 Percentage of susceptibility to antimicrobial drugs by *B. pseudomallei* isolates. CAZ ceftazidime (30 µg), SXT trimethoprim/sulfamethoxazole (1.25/23.75 µg), DO doxycycline (30 µg), CIP ciprofloxacin (5 µg), MEM meropenem (10 µg), and IMP imipenem (10 µg).

Table 4 Antimicrobial drug resistant patterns of *Burkholderia pseudomallei* isolates by the disk diffusion method.

Sources (n)	Number Resistant Isolate (%)	Number of Drug		List of drugs resistant/ intermediate (I)		
		R	I			
Human (94)	13(13.8)	1	0	SXT		
	3(3.2)	0	1	SXT (I)		
	8(8.5)	0	1	CIP (I)		
	1(1.1)	2	0	SXT	DO	
	1(1.1)	2	0	SXT	CIP	
	2(2.1)	1	1	SXT	CIP (I)	
	3(3.2)	0	2	SXT (I)	CIP (I)	
	1(1.1)	1	2	SXT	CAZ (I)	CIP (I)
	1(1.1)	1	2	SXT	CIP (I)	MEM (I)
Animal (11)	3(27.1)	1	0	SXT		
	1(9.1)	1	0	DO		
	3(27.1)	1	1	SXT	CIP (I)	
Soil (33)	7(21.2)	1	0	SXT		
	1(3.0)	0	1	CIP (I)		
	2(6.0)	0	1	IPM (I)		
	1(3.0)	2	0	SXT	DO	
	1(3.0)	2	0	SXT	CIP	
	1(3.0)	2	0	SXT	IPM	
	4(12.1)	1	1	SXT	CIP (I)	
	1(3.0)	1	1	CAZ	IPM (I)	
	1(3.0)	0	2	CIP (I)	IPM (I)	
	1(3.0)	1	1	CIP (I)	IPM	
	1(3.0)	2	1	SXT	CIP (I)	IPM
	1(3.0)	1	2	SXT	DO (I)	IPM (I)
	1(3.0)	1	2	SXT(I)	MEM (I)	IPM
Water (35)	3(8.6)	1	0	SXT		
	1(2.9)	0	1	CIP (I)		
	4(11.4)	1	1	SXT	CIP	

R, resistant; I, intermediate resistant; SXT, trimethoprim/sulfamethoxazole; CAZ, ceftazidime; DO, doxycycline; CIP, ciprofloxacin; MEM, meropenem; IMP, imipenem.

Table 5 Zone diameter distribution of antimicrobial drugs to *Burkholderia pseudomallei* from humans, animals, and environmental sources.

Drugs (µg)	Sources (Number)	Number of Isolates in each Inhibition Zone Diameter																																		
		6	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
CAZ (30)	Human (94)									1					1	1		1		7	2	9	2	36		15	2	15		2							
	Animal (11)																1					3		5		2										
	Soil (33)						1					1			1	1	3	6	3	8		6		3												
	Water (35)																		1	9		4		16		3		2								
SXT (1.25/23.75)	Human (94)	19				1		4	1	4	2	2	2	9	1	12	5	8		13	2	5		3		1										
	Animal (11)	6								2								1								2										
	Soil (33)	13		3		1				1		1	1	1	1	2	1	3	3	1		1														
	Water (35)	7												3	1		2	2	1	3		7		2		7										
DO (30)	Human (94)					1								2		1	1	1	6	9		20	4	38	1	6		3		1						
	Animal (11)					1														1				7		2										
	Soil (33)		1						1	1				1		1	1	4	6	7		6	1	2		1										
	Water (35)																		1	10	5	11		7	1											
CIP (5)	Human (94)			1						4	2			15	3	28	8	17	5	8		1		1		1										
	Animal (11)													3		4		2		2																
	Soil (33)						1				2			6	2	11	4	1	3	2	1															
	Water (35)											1		3	1	5	5	5	6	4		5														
MEM (10)	Human (94)						1											3	2	16	8	15	3	37	1	4		3		1						
	Animal (11)																		2	3	4	2														
	Soil (33)										1		1	2		6		5	1	9	1	2		3		1		1								
	Water (35)																1	3		13		10	1	5		1		1								
IMP (10)	Human (94)																	1		3		2		3		6	1	17	3	33		12		12		1
	Animal (11)																		1									2		8						
	Soil (33)			1	1			1	1	2	2		1	6		2						2		1		3		4		5				1		
	Water (35)																1							2		4		6		15		3		3		1

Underlined values represent the mode of respective distribution. CAZ, ceftazidime; SXT, trimethoprim/sulfamethoxazole; DO, doxycycline; CIP, ciprofloxacin; MEM, meropenem; IMP, imipenem.

The recent results in our study might support the variation of morphotypes and biochemical characteristic phenotypes in environmental isolates, which indicate the variation of genotypes. Interestingly, *B. pseudomallei* isolates from water sources demonstrated the major morphotype with roughness at the colony center (I, II, and V) instead of smoothness (III, IV, and VI). Moreover, the highest proportion morphotype in water isolates was Type I (57.14 %), which was also the major morphotype found in human and animal isolates (54.26 and 72.73 %, respectively). Therefore, human and animal melioidosis infections might be exposed to the Type I morphotype due to the fact of exposure to contaminated water. Our findings are supported by a previous study by Kwanhian *et al.* (2020) that reported the matching of MLST genotypes isolated from visceral abscesses of pigs and an unchlorinated water supply at a pig farm in southern Thailand [3]. Another study that supports our findings is by Panomket *et al.* (2017), which reported mice infected with *B. pseudomallei* defective in biofilm production gave rise to less severe pathology [20]. In our study, the major morphotype of the soil isolates was a smooth Type III morphotype, followed by a smooth Type VI morphotype (9.09 %); however, the other rough morphotypes (Type I, II, VI, and VII) were sparse. Yet, there were four isolate samples in our collection that expressed two different morphotypes. Chantratita *et al.* (2007) reported that Type I has referential strains that could switch to other morphotypes [5]. Some conditions allow *B. pseudomallei* to change itself variedly as exemplified by long-term incubation (1 month in TSB), iron limitation, increased temperature, and sub-inhibitory concentrations of the antibiotic or human body as Type I morphotype is the preference. Clinical specimens from the same patient contained mixed morphology in one sample; moreover, some patients had different morphotypes of *B. pseudomallei* in specimens collected on the same day, which gave the idea of organism adaptation due to the fact of improper living conditions.

Conventional biochemical tests for *B. pseudomallei* identification are still used in routine laboratories from past to present, particularly in Thailand. Our collection of *B. pseudomallei* isolates was cultured on selective Ashdown agar supplemented with gentamicin, which can be resisted by *B. pseudomallei*. In addition, latex agglutination and *wcbG*-PCR were tested for confirmation. Therefore, all isolates, indeed, were identified as *B. pseudomallei*. However, the diversity of conventional biochemical characteristics was revealed. The results might support the selection of consistent biochemical tests to consider for use in diagnosis in clinical laboratories including the TSI, H_2S, indole, motility, OF-glucose, and DNase tests. On the other hand, some biochemical tests are not suggested for use in *B. pseudomallei* diagnosis. The results were not consistent in different sources and differed from the expected results such as the citrate, urease, esculin hydrolysis, and OF maltose tests. Nitrate reduction was different from the typical result in the soil isolates, as shown in **Table 3**.

Standard CLSI was used for antibiotic susceptibility in the disk diffusion method and MIC. Although the *B. pseudomallei* breakpoint was not established [19], the breakpoints for the *Enterobacteriaceae* and *Pseudomonas aeruginosa* were recommended from a previous report [10]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference was not applied in our study, although a breakpoint for *B. pseudomallei* by EUCAST v.11.0 was launched in 2021 [21,22]. However, ceftazidime and trimethoprim-sulfamethoxazole are not of susceptible standard (S) but only classified as I and R (susceptible, increased exposure and resistant, respectively). This stresses the need of using the highest suggested dose while treating melioidosis with these drugs [21,22]. Among mono-resistance to antimicrobial drugs, trimethoprim/sulfamethoxazole was found to have the highest rate in all *B. pseudomallei* from all sources, ranging from 11.4 - 27.1 %. The method that was used in our study was the disk diffusion assay. However, previous data have mentioned that the interpretation of the susceptibility test of *B. pseudomallei* to trimethoprim/sulfamethoxazole by disk diffusion was not observed, since bacteria might have a slight hazy growth within the zone of inhibition, which displays a blurring of the margin. Thus, a previous study suggested confirming trimethoprim/sulfamethoxazole susceptibility by MIC. The trend in MIC results usually has a higher rate than that of the disk diffusion method [23]. For ceftazidime, it is an antimicrobial drug for the parenteral route that treats melioidosis in the initiation phase. It was found that 3.0 % of the soil isolates were resistant by the disk diffusion method, although MIC results for ceftazidime showed 100 % susceptibility. The controversy of disk diffusion and MIC by ETEST® was indicated in present study. This may be from inaccuracies in the disk diffusion-based method that were reported in other susceptibility test in the other bacteria. Thus, the standard broth microdilution (BMD) method is still needed for reliable MIC results. However, the previous study indicated the high correlation (≥ 97 %) and essential agreement (≥ 93 %) MIC which determined by ETEST® and BMD of ceftazidime against *B. pseudomallei* [24]. Therefore, ceftazidime's susceptibility rate in the present study might be more reliable than the disk diffusion method. However, the low resistant rate to ceftazidime has been reported in many previous studies [23]. There were many patterns of antimicrobial drug resistance of *B. pseudomallei* in our study as shown in **Table 4**. Monitoring of antimicrobial resistance of *B. pseudomallei* isolates, not only in clinical specimens but also in animal and environmental isolates, needs to be conducted regularly to guide empiric therapy for human melioidosis, as it causes high mortality [25]. We found that mono-resistance to ceftazidime, the first drug of choice in the acute phase for melioidosis treatment, was 3.0 % of *B. pseudomallei* from soil sources. Double resistance to trimethoprim/sulfamethoxazole and ciprofloxacin ranged from highest to lowest in the water (11.4 %), soil (3.0 %), and human (1.1 %) isolates. Double resistance to trimethoprim/sulfamethoxazole and doxycycline was found from the soil (3.0 %) and human (1.1 %) isolates. Imipenem resistance was found in 6.0% of the soil sources, which was coupled with resistance and/or intermediate resistance to trimethoprim/sulfamethoxazole and intermediate resistance to ciprofloxacin. Although, meropenem and imipenem are used as an alternative to ceftazidime [26], meropenem and imipenem showed 100 % susceptibility, except for meropenem for human isolates (98.9 %) and imipenem for soil sources (90.9 %). The susceptibility rate for meropenem and imipenem in our study was higher than previous studies, which reported 100% susceptibility for imipenem [25]. Ciprofloxacin, a member of fluoroquinolones, was used individually or combined with doxycycline to treat acute melioidosis [27]. It can penetrate through phagocytic cells to kill intracellular bacteria [28]. However, recently, it was not recommended since individuals had a high relapse rate [27].

When the mode value of the inhibition zone was considered, the mode value of the antimicrobial drugs, including ceftazidime, trimethoprim/sulfamethoxazole, doxycycline, ciprofloxacin, meropenem, and imipenem, on *B. pseudomallei* isolated from humans had the highest trend among the sources of isolates, which were 30, 6, 30, 22, 30, and 36 mm, respectively. In contrast, the mode value of the same antimicrobial

agents showed the lowest trend in the soil isolates, which tended to be the same for the MIC50, MIC90, and MIC range for soil isolates with ceftazidime at the highest. Thus, the results indicated *B. pseudomallei* isolated from soil showed a trend in increasing ceftazidime resistance compared with other sources. Our results were consistent with Sadiq *et al*. study (2018) that reported *B. pseudomallei* ceftazidime resistance in soil isolates but not in animal and water isolates. They proposed that the different rates of antimicrobial drug resistance were associated with different genotypes of the isolates [25].

Conclusions

B. pseudomallei from humans, animals, and water isolates showed a predominant Type I colony morphotype. However, the Type III morphotype was predominant in soil sources. The biochemical characteristics of all isolates were consistent with the expected results including positive cytochrome oxidase, K/N or A/N triple iron sugar (TSI), non-H_2S, non-indole, motile, glucose oxidizer, and DNase production. In contrast, the citrate test result was not consistently positive in *B. pseudomallei* in all isolate sources. Antimicrobial drug susceptibility patterns by standard disk diffusion showed that the mode value of the inhibition zone of selected drugs had a lower trend in bacteria from soil than other sources. The results were consistent with the MIC50 and MIC90 values of ceftazidime, which was highest in *B. pseudomallei* from soil among the examined sources.

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