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Antimicrobial Susceptibility of Western Hemisphere Isolates of *Burkholderia pseudomallei*: Phenotypic and Genomic Analyses

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Abstract

Current antimicrobial treatment recommendations for melioidosis, the disease caused by *Burkholderia pseudomallei*, are largely based on studies of strains isolated from the Eastern Hemisphere (EH), where most human cases are identified and reported. In this study, we evaluated the antimicrobial susceptibility of 26 strains in the CDC (Centers for Diseases Control and Prevention) collection from the Western Hemisphere (WH) isolated from 1960 to 2015. Minimal inhibitory concentration (MIC) values were measured by standard broth microdilution for 16 antimicrobials following Clinical and Laboratory Standards Institute (CLSI) guidelines. Twenty-four of the 26 WH strains were susceptible to the six antimicrobials with CLSI-defined MIC susceptibility interpretive criteria for *B. pseudomallei*: amoxicillin/clavulanate, ceftazidime, imipenem, doxycycline, tetracycline, and trimethoprim/sulfamethoxazole. One WH strain demonstrated intermediate amoxicillin/clavulanate resistance and another strain had intermediate resistance to tetracycline. For all antimicrobials tested, the susceptibility profiles of WH isolates were comparable with previously reported MIC results of EH strains. The overall similarities suggest that the same antimicrobials are useful for melioidosis treatment in both the WH and EH. Using *in silico* analyses of WH genomes, we identified a novel amino acid substitution P258S in the beta-lactamase PenA, which may contribute to decreased susceptibility to amoxicillin/clavulanate in *B. pseudomallei*.

Keywords

Burkholderia pseudomallei ; Western Hemisphere; antimicrobial susceptibility

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Disclosure Statement

No competing financial interests exist.

Introduction

Burkholderia pseudomallei is a Gram-negative motile bacterium that causes melioidosis in humans and animals. *B. pseudomallei* is found in soil and surface water in the environment but is intracellular upon host infection.[1] Approximately 165,000 human melioidosis cases are estimated to occur annually worldwide with an estimated fatality rate >50% in some countries, presumably owing to lack of proper therapy.[2]

The majority of melioidosis cases are reported in tropical areas of Australia and Asia (Eastern Hemisphere [EH]), where this bacterium is endemic.[1] Of interest, *B. pseudomallei* is being reported in the Americas (Western Hemisphere [WH]).[3] Major DNA sequence similarities are shared among the genomes of WH isolates analyzed to date and together these strains form a distinct phylogenetic clade compared with EH isolates.[4,5] The actual frequency of human melioidosis cases may be underrepresented in the WH as a result of misdiagnosis and/or reduced awareness by physicians.[6,7]

Several antimicrobials are used for melioidosis treatment, including beta-lactams such as cephalosporins (ceftazidime) and carbapenems (meropenem and imipenem), beta-lactam/beta-lactam inhibitor combinations (amoxicillin/clavulanate), folate pathway inhibitors (trimethoprim/sulfamethoxazole), tetracyclines (doxycycline), and sometimes phenicols (chloramphenicol).[1,8,9] Ceftazidime or meropenem are most often prescribed for initial intensive intravenous therapy, whereas trimethoprim/sulfamethoxazole, amoxicillin/clavulanate, or doxycycline are used for eradication oral therapy.[1]

Antimicrobial susceptibility patterns of regional strains are useful for laboratory isolation and subsequent identification of *B. pseudomallei*.[1] For example, intrinsic resistance of *B. pseudomallei* to gentamicin is used to isolate *B. pseudomallei* on Ashdown's agar, a selective culture medium that contains gentamicin.[10] Historically, the antimicrobials recommended for melioidosis treatment or for *B. pseudomallei* laboratory isolation and presumptive identification were selected based on the characteristics of EH strains. Reports of the antimicrobial susceptibility for *B. pseudomallei* strains from the WH are very limited,[11,12] and relevant differences with EH strains could alter human melioidosis treatment recommendations.

Although *B. pseudomallei* is considered intrinsically resistant to multiple antimicrobials (including ampicillin, gentamicin, macrolides, and polymyxins), the majority of EH isolates are susceptible to the antimicrobials routinely prescribed for melioidosis treatment. Studies from Australia, Thailand, Bangladesh, Laos, and Cambodia showed that ≥96% of *B. pseudomallei* strains are susceptible to amoxicillin/clavulanate, ceftazidime, trimethoprim/sulfamethoxazole, carbapenems, tetracyclines, and chloramphenicol.[13–17]

However, susceptibility profiles may differ by geographical location. A recent study from China demonstrated that fewer *B. pseudomallei* strains (<93%) are susceptible to amoxicillin/clavulanate, ceftazidime, and trimethoprim/sulfamethoxazole.[18] Lower susceptibility rates to trimethoprim/sulfamethoxazole were also reported in India (94.1%)

and Vietnam (89.1%).[19,20] Misuse or overuse of antimicrobials for treatment may explain lower susceptibility rates in regions where increased resistance is reported.[18]

For trimethoprim/sulfamethoxazole, the differences in reported susceptibility may also be explained by challenges determining the minimal inhibitory concentration (MIC), which is read by subjectively identifying the broth microdilution (BMD) well with the lowest trimethoprim/sulfamethoxazole concentration in which there is ≥80% reduction in growth compared with the (growth) control, whereas the MIC for other antimicrobials is read as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism.[21]

Intrinsic and also acquired antimicrobial resistance, developed in response to treatment, have been described in *B. pseudomallei*.[22–26] Resistance to amoxicillin/clavulanate, ceftazidime, trimethoprim/sulfamethoxazole, chloramphenicol, tetracyclines, and carbapenems has been reported.[13,27–29] Some *B. pseudomallei* isolates exhibited significantly lower MICs to gentamicin to which the bacterium is intrinsically resistant. Isolates with low MICs to gentamicin were reported in East Malaysia and in cystic fibrosis patients.[28,30]

Point mutations or other genome rearrangements lead to antimicrobial resistance in *B. pseudomallei*.[23] Resistance to ceftazidime, amoxicillin/clavulanate, and carbapenems can result from mutations in the gene encoding the beta-lactamase PenA,[24,28,31–33] from the increased PenA expression owing to amplification of the gene itself[22,24] or a mutation inside its promoter region.[34] Deletion of penicillin-binding protein 3 (PBP3) also results in ceftazidime resistance along with significant growth deficiencies.[35] Resistance to trimethoprim/sulfamethoxazole is conferred by mutations in transcriptional regulators BpeT and BpeS leading to expression of the efflux pump BpeEF-OprC, and mutations in the dihydrofolate reductase (FolA) or in the pterin reductase (FolM) enzymes.[36]

Tetracycline resistance results from mutations in the S-adenosyl-L-methionine (SAM)-dependent methyltransferase, which is predicted to lead to altered ribosomal methylation at the binding site of tetracycline and from mutations in the repressor AmrR that leads to increased expression of the efflux pump AmrAB-OprA and possibly other efflux pumps.[37] The efflux pump AmrAB-OprA is expressed in most *B. pseudomallei* strains and is responsible for intrinsic gentamicin resistance.[38,39] Mutations leading to increased expression of the efflux pumps BpeEF-OprC[40] and, to a lesser degree of BpeAB-OprB,[41] increase chloramphenicol resistance in *B. pseudomallei*.

In this study, we report on antimicrobial susceptibility profiles for 26 WH *B. pseudomallei* strains from Centers for Diseases Control and Prevention (CDC) collection that were isolated from 1960 to 2015[4] and compare the data with published antimicrobial susceptibility results of EH isolates. We performed genome analyses of these WH strains to identify mutations that may contribute to resistance and correlate with the antimicrobial susceptibility profiles.

Materials and Methods

Bacterial strains, culture conditions, and biosafety

Twenty-six *B. pseudomallei* strains from the CDC collection were isolated in the WH from locations including North and South America and are previously described.[4] The WH isolates were from human infections and the environment. *B. pseudomallei* strains 1026b[42] and K96243,[43] included in this study as drug-susceptible "query" strains, were both originally isolated from the EH in Thailand. *B. pseudomallei* strains Bp1651[28] and MSHR1655,[44] resistant to multiple clinically relevant antimicrobials, were included as nonsusceptible "query" strains. These strains were isolated from Australia (EH) and associated with chronic infections. The *B. pseudomallei* strains used in the study are listed in Supplementary Tables S1 and S2.

Escherichia coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were included as quality control (QC) strains for antimicrobial susceptibility testing (AST). All strains were cultured on trypticase soy agar II with 5% sheep blood agar (SBA) plates (Becton, Dickinson and Company, Sparks, MD) at 35°C in ambient air.

All laboratory work with *B. pseudomallei* were performed in a U.S. Federal Select Agent Program-registered biosafety level-3 laboratory inside a class II type A2 biological safety cabinet by trained personnel wearing personal protective equipment including a powered air-purifying respirator.[45]

Antimicrobial susceptibility testing

Standard BMD was performed following the Clinical and Laboratory Standards Institute (CLSI) guidelines.[46] In brief, bacteria were cultured overnight on SBA plates, each strain was used to prepare a cell suspension in sterile saline at a turbidity equivalent to a 0.5 McFarland standard, then diluted to achieve a final concentration of ~5×10^5 CFU/mL to inoculate 96-well BMD panels made in-house as detailed recently.[47] Wells of the BMD panels contained twofold serially increasing concentrations of an antimicrobial in 100 μL/well cation-adjusted Mueller Hinton broth (Becton, Dickinson and Company).

Antimicrobials were purchased from TOKU-E (Bellingham, WA): amoxicillin, aztreonam, clavulanate, imipenem, meropenem, sulfamethoxazole; from Sigma-Aldrich (St. Louis, MO): ceftazidime, chloramphenicol, doxycycline, gentamicin, tetracycline, trimethoprim; and from IHMA (Schaumburg, IL): avibactam. The concentration ranges for antimicrobials were as follows (in μg/mL): 0.06–128 for amoxicillin, aztreonam, ceftazidime, and gentamicin; 0.12–128 for tetracycline; 0.03–64 for clavulanate, imipenem, and meropenem; 0.06–64 for doxycycline; 0.12–64 for chloramphenicol; 0.5–1,024 for sulfamethoxazole when used alone or 0.3–608 for sulfamethoxazole when in combination with trimethoprim; and 0.15–32 for trimethoprim. The concentration of avibactam was 4 μg/mL. Amoxicillin/clavulanate was tested in 2:1 ratio and trimethoprim/sulfamethoxazole was tested in 1:19 ratio.

The inoculated BMD panels were incubated at 35°C in ambient air for 16 to 20 hours except for the strain MSHR1655, which was incubated for 43 hours. All MIC results were

read by assessing growth endpoints by eye. The MIC results were measured as the lowest concentration that inhibited ≥80% of bacterial growth for trimethoprim, sulfamethoxazole and trimethoprim/sulfamethoxazole, and at complete (100%) inhibition of growth for all other antibiotics.[21]

CLSI interpretive criteria, also known as breakpoints, were used, when available, to assess *B. pseudomallei* susceptibility results.[46,48] *B. pseudomallei* CLSI breakpoints were used for amoxicillin/clavulanate, ceftazidime, imipenem, doxycycline, tetracycline, and trimethoprim/sulfamethoxazole (Table 1). No CLSI MIC breakpoints are set for *B. pseudomallei* for other antimicrobials. European Committee on Antimicrobial Susceptibility Testing (EUCAST) *B. pseudomallei* breakpoints were used for interpretations of MIC results for chloramphenicol.[49] CLSI *B. pseudomallei* imipenem breakpoints were used for susceptibility interpretations of MIC results for meropenem. We used values for amoxicillin and clavulanate in *B. pseudomallei* amoxicillin/clavulanate CLSI breakpoints as individual amoxicillin and clavulanate breakpoints. We used CLSI breakpoints for Enterobacteriaceae for aztreonam, ceftazidime/avibactam, trimethoprim, sulfamethoxazole, and gentamicin. Enterobacteriaceae CLSI aztreonam breakpoints were used to assess aztreonam/avibactam susceptibility (Table 1).

Sequence analysis

Publicly available whole genome sequences (WGS) of the WH *B. pseudomallei* strains were used for analysis.[4] For each WH strain, the sequences of proteins (amino acid sequences were derived from DNA sequences) and DNA sequences of regulatory regions previously associated with *B. pseudomallei* antimicrobial resistance were compared with the sequences of susceptible “query” strains (1026b[41,42] and K96243[43]) and with nonsusceptible “query” strains (Bp1651[50] and MSHR1655[51]) using blastn, tblastn, and blastp at the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The *amrB* gene in WH strain FL2012 was sequenced by the Sanger method to demonstrate the absence of a point mutation found in WGS for this strain. In brief, bacterial lysates were prepared by treating cells with lysis buffer (0.05 M NaOH, 250 mM NaCl, 1 mM EDTA) prewarmed to 35°C followed by neutralization with 500 mM Tris-HCl pH 7.5, then subsequently filtered using a 0.1 μm polyvinylidene difluoride spin column filter (Millipore-Sigma, St. Louis, MO).[52] PCR was performed to amplify the DNA region using primers amrB_FL2002_F (CTC GCG CTG TCG CTC ACG CCG) and amrB_FL2002_R (GGC CCA TCG TCC AGT GCA GCG) and Q5 High Fidelity DNA polymerase with GC enhancer (New England Biolabs). The PCR product was visualized by 1.2% agarose E-gel electrophoresis (Invitrogen). Sanger sequencing reads were analyzed using the Applied Biosystems 3500XL Genetic Analyzer instrument as described previously[53] with primers amrB_FL2002_F and amrB_FL2002_R.

Results

Antimicrobial susceptibility

B. pseudomallei antimicrobial susceptibility results are given in Fig. 1 (MIC summary for all *B. pseudomallei* isolates), Table 1 (MIC_{50} and MIC_{90} for WH isolates), Supplementary Table S1 (individual MICs for WH isolates), and Supplementary Table S2 (individual MICs for susceptible and nonsusceptible query strains, EH isolates). Table 1 includes published CLSI and EUCAST breakpoints used for interpretation of the MIC values per antimicrobial.[46,48,49]

All 26 WH *B. pseudomallei* strains (100%) were susceptible to the 4 antimicrobials with CLSI *B. pseudomallei* susceptibility breakpoints (ceftazidime, imipenem, doxycycline, and trimethoprim/sulfamethoxazole) and also to meropenem. Twenty-five of the 26 WH strains (96%) were susceptible to amoxicillin/clavulanate, strain 724644 demonstrated intermediate resistance to amoxicillin/clavulanate (MIC = 16/8 μg/mL). Twenty-five of the 26 WH strains (96%) were susceptible to tetracycline, strain PB 1007001 demonstrated intermediate resistance to tetracycline (MIC = 8 μg/mL) but was susceptible to doxycycline (MIC = 2 μg/mL). All 26 WH isolates were resistant to chloramphenicol according to EUCAST criteria with MICs ≥16 μg/mL and had high MICs to gentamicin with (≥32 μg/mL).

Combination drug components amoxicillin and clavulanate, when tested separately, did not inhibit growth of the WH isolates at concentrations <32 μg/mL for amoxicillin and <16 μg/mL for clavulanate. In addition, aztreonam concentrations <32 μg/mL did not inhibit the WH strains. However, combining aztreonam and avibactam inhibited the growth: 25/26 WH strains had MICs in the susceptible range with only 1 WH strain (RI2013a) demonstrating intermediate resistance (MIC = 8/4 μg/mL). Clavulanate was the only beta-lactamase inhibitor tested without a beta-lactam antimicrobial because it has a known mechanism of resistance in *B. pseudomallei*. Trimethoprim and sulfamethoxazole separately inhibited growth of the WH isolates at concentrations as low as 2 μg/mL for trimethoprim and 16 μg/mL for sulfamethoxazole. Every WH strain (26/26) was sulfamethoxazole susceptible and 23 WH strains were susceptible to trimethoprim. Trimethoprim-resistant strains had MIC values equal to the breakpoint for resistance (Table 1 and Supplementary Table S1).

EH *B. pseudomallei* strain Bp1651 was resistant to all antimicrobial agents tested, except ceftazidime/avibactam and gentamicin (Supplementary Table S2). Ceftazidime/avibactam demonstrated potent activity (MIC = 4/4 μg/mL), whereas the ceftazidime MIC was >128 μg/mL. For Bp1651, aztreonam/avibactam MIC was high (64/4 μg/mL), demonstrating that avibactam did not protect aztreonam from degradation by beta-lactamase like it did for ceftazidime. Bp1651 was the only strain from this study with a low gentamicin MIC.

For strain MSHR1655, avibactam did protect aztreonam from beta-lactamase degradation. The aztreonam/avibactam MIC (4/4 μg/mL) was 32-fold lower than for aztreonam alone (MIC >128 μg/mL). MSHR1655 strain was ceftazidime susceptible (MIC = 4 μg/mL).

Genomic analysis of antimicrobial resistance markers

To better understand the phenotypic susceptibility results, we performed a comparative genomic analysis of *B. pseudomallei* study strains with unique susceptibility patterns. Genomic regions known to be responsible for antimicrobial resistance in *B. pseudomallei* were compared in the WH strains and the susceptible query strain 1026b. *B. pseudomallei* K96243 (susceptible strain), and nonsusceptible "query" strains Bp1651 and MSHR1655 were also included for this comparison.

The WH strain 724644 had intermediate resistance to amoxicillin/clavulanate. The MIC was ≥4 × higher than amoxicillin/clavulanate MICs of other WH strains. Blast analysis of the genomic region encoding beta-lactamase PenA, including 130-nucleotides upstream of *penA* gene translation initiation codon ATG known to affect susceptibility to beta-lactams, identified a novel and unique amino acid substitution (P258S, amino acids are numbered according to the Ambler scheme)[54] in strain 724644 compared with the susceptible query strain 1026b (Fig. 2 and Supplementary Table S3). The amino acid substitution P258S was also absent in PenA sequences in all *B. pseudomallei* strains listed in taxid 28450 when blastp analysis was performed.

Half the analyzed WH isolates, all susceptible to amoxicillin/clavulanate, carried the amino acid substitution T147A in PenA. This mutation was also present in the resistant EH query strain Bp1651, and was previously suspected to lead to decreased susceptibility to amoxicillin/clavulanate.[28] Based on these findings, the amino acid substitution T147A does not appear to affect amoxicillin/clavulanate susceptibility. For strain RI2013a, MIC for aztreonam/avibactam was ≥2 × higher than in other WH strains. The *penA* region in this strain did not have unique or previously reported mutations conferring resistance (Supplementary Table S3). The WH strains did not have additional *penA* mutations known to lead to *B. pseudomallei* resistance (Supplementary Table S3).

The *penA* gene sequence of strain CA2009 contains a stretch of six A nucleotides compared with five in strain Bp1651. The extra A is located 90 nucleotides from the beginning of the gene; and if true, this would result in a frameshift in PenA protein after first 30 amino acids. Strain CA2009 did not have modified susceptibility to beta-lactam antimicrobials, and the extra A may be a sequencing error. Genomic analyses revealed all isolates (26 WH and 4 query strains) contained the *pbp3* gene.

There were three WH strains (7894, Swiss2010, and TX2004) with trimethoprim MICs ≥2 × higher than those of other WH strains. The amino acid sequences of the dihydrofolate reductase FolA protein, a known target for trimethoprim, were identical in these three strains to that of the strain 1026b (Supplementary Table S4). FolA protein sequences were identical in all WH strains. Compared with 1026b, the amino acid sequence of FolM protein, pterin reductase, which may contribute to increased trimethoprim MICs in *B. pseudomallei*,[36] had a previously not reported amino acid substitution R53C in the three strains with elevated trimethoprim MICs but also in most of WH strains. This indicates that this mutation does not contribute to increased trimethoprim resistance. The three WH strains with elevated trimethoprim MICs did not have additional amino acid changes compared with strain 1026b in FolM (Supplementary Table S4).

The WH strain PB 1007001 had intermediate resistance to tetracycline, with an MIC ≥2 × higher than the MICs of the rest of WH strains (all tetracycline susceptible). Compared with strain 1026b, the strains PB 1007001 and all other WH strains analyzed here, carried a deletion of amino acids T278 and G279 in the SAM-dependent methyltransferase, the enzyme shown to contribute to resistance to tetracyclines in *B. pseudomallei*.[37] These two amino acids were also absent in both, the tetracycline-susceptible strain K96243 and the tetracycline-nonsusceptible strain MSHR1655. Because these deletions are present in both susceptible and nonsusceptible isolates, they alone cannot account for resistance to tetracyclines. Compared with strain 1026b, the resistant strain Bp1651 encoded a truncated version (coding for first 87 of 300 amino acids) of SAM-dependent methyltransferase protein (Supplementary Table S5), which may explain tetracycline and doxycycline resistance in Bp1651.

EH strain Bp1651 had low MIC to gentamicin (1 μg/mL), probably because of the truncation of transporter protein AmrB,[28] which is a part of the AmrAB-OprA efflux pump that confers resistance to aminoglycosides in *B. pseudomallei*.[38,55] All 26 WH strains and the 3 of 4 query EH strains (except Bp1651) had high MICs to gentamicin, ranging from 32 to >128 μg/mL (Supplementary Tables S1 and S2), indicating that AmrAB-OprA pump is expressed in these strains.

Unexpectedly, based on the WGS data, the *amrB* gene sequence of the WH strain FL2012 revealed a thymine insertion at position 1949 when aligned to the query strain 1026. This insertion would result in an AmrB protein frameshift after the amino acid F650 (of 1043 amino acids of full size AmrB protein), which could affect the function of the pump resulting in a gentamicin-susceptible phenotype. However, the WGS-predicted thymine insertion was in a homo-polymeric region of *amrB* gene and subsequent Sanger sequencing of this region did not confirm the insertion. The *amrB* gene sequence in FL2012 was identical to the 1026b susceptible strain by Sanger sequencing (Supplementary Table S6), which highlights the importance of confirming WGS data by a different sequencing method.

Discussion

The majority of WH *B. pseudomallei* isolates analyzed in this study were susceptible to antimicrobials commonly used for treatment of human melioidosis (amoxicillin/clavulanate, ceftazidime, meropenem, doxycycline, and trimethoprim/sulfamethoxazole). WH strains also demonstrated high MICs to gentamicin, which is used for *B. pseudomallei* identification. These included two WH strains, CA2010 and OH2013, which were isolated in the WH, but which genomes grouped with EH isolates.[4] The susceptibility results for WH strains are consistent with current recommendations for treatment and diagnosis of *B. pseudomallei* infections.

The susceptibility interpretation of WH strains for additional antimicrobials included in the study were overall similar to published EH strain results, with one exception: chloramphenicol. In the WH strains, MIC results for chloramphenicol (MIC_{50} = 16 μg/mL; Table 1) were 2 to 4 × higher (one or two dilution steps higher) than previously reported chloramphenicol MICs for EH strains (MIC_{50} = 4–8 μg/mL).[13,27,56]

In the absence of *B. pseudomallei* CLSI interpretative criteria, we used EUCAST chloramphenicol breakpoints.[49] Any *B. pseudomallei* strain with a chloramphenicol MIC >8 μg/mL is considered resistant (R) to this antimicrobial (Table 1). Every study strain tested here (26 WH and 4 EH) was chloramphenicol resistant according to the EUCAST criteria. On each day of testing, the QC strain *E. coli* ATCC 25922 performed as expected (MIC = 4 μg/mL chloramphenicol), indicating that the chloramphenicol used for testing was active and in correct concentration. Strain *P. aeruginosa* ATCC 27853 is not considered a useful QC strain for chloramphenicol.

Chloramphenicol MIC differences between our study and previously published works did not result from different AST methods used: while Dance *et al.*[27] and Jenney *et al.*[13] relied on agar dilution method to determine chloramphenicol MICs, Karatuna *et al.*[56] applied the same broth-based BMD used to determine MICs in our study. Any *B. pseudomallei* strain with a chloramphenicol MIC >0.001 μg/mL, but ≤8 μg/mL is considered "susceptible, increased exposure, I" by EUCAST.[49,56] This category is distinct from the EUCAST "susceptible, standard dosing regimen, S" category. Therefore, although most of the strains from previous works are not considered resistant to chloramphenicol, their MICs fall within the "increased exposure" and not the "standard dosing" susceptible category. Chloramphenicol is not currently included in the expert consensus recommendations for melioidosis treatment.[57]

The difference in testing methods may explain lower MIC values reported previously for strain Bp82 using agar-based E-test for trimethoprim/sulfamethoxazole, trimethoprim, and sulfamethoxazole (0.094, 0.75 and 4 μg/mL, respectively)[36] compared with MIC values for its parent strain 1026b determined by BMD in this study (0.5, 4, and 64 μg/mL, respectively). Susceptibility interpretations correlated poorly between agar-based E-test and broth-based BMD in *B. pseudomallei* using certain antimicrobials (tetracycline and trimethoprim/sulfamethoxazole) in a previous report, with E-test producing lower MIC values than BMD.[47] Difficulty to visually detect 80% growth inhibition can also contribute to different interpretation of MIC endpoints by different readers for these three antimicrobials.

Doxycycline inhibited growth of *B. pseudomallei* at lower concentrations than tetracycline with MIC_{50} by BMD of 1 and 4 μg/mL, respectively. A similar trend was observed previously in *B. pseudomallei* and other species.[27,47,56,58,59] In the EUCAST study, the epidemiological cutoff BMD MIC values for *B. pseudomallei* were two dilution steps lower for doxycycline (2 μg/mL) than for tetracycline (8 μg/mL).[56] The WH *B. pseudomallei* strain PB 1007001 (MIC = 8 μg/mL tetracycline and 2 μg/mL doxycycline) matches the susceptibility of majority wild-type strains for both antimicrobials according to the EUCAST study criteria. No mutations were identified in this strain compared with the strain 1026b to explain tetracycline MIC.

The CLSI MIC breakpoints for *B. pseudomallei* are currently identical for tetracycline and doxycycline (Table 1). EUCAST provides BMD breakpoints for doxycycline only[49] and only doxycycline but not tetracycline is recommended for eradication therapy for melioidosis.[1] However, doxycycline susceptibility may be inferred from tetracycline test

results.[56] This underlines the importance of susceptibility results for both antimicrobials. For these two antimicrobials, the difference in MICs for the same set of *B. pseudomallei* strains may warrant a revision of the CLSI interpretive criteria.

The novel combination of beta-lactam antimicrobials with beta-lactam inhibitors (aztreonam/avibactam and ceftazidime/avibactam) had a good activity against WH *B. pseudomallei* strains (MIC_{90} = 4/4 and 1/4 μg/mL, respectively; Table 1). Avibactam restored the *in vitro* antimicrobial activity of aztreonam against EH *B. pseudomallei* strain MSHR1655 and activity of ceftazidime against EH *B. pseudomallei* strain Bp1651. Recent work showed that addition of avibactam reversed ceftazidime resistance in another EH strain of *B. pseudomallei*,[22] making ceftazidime/avibactam a potential option for treatment of ceftazidime-resistant *B. pseudomallei* strains.

Although EUCAST has published meropenem breakpoints for *B. pseudomallei*, for Fig. 1 we used CLSI imipenem breakpoints for interpretation of meropenem susceptibility. EUCAST breakpoints are equal for meropenem and imipenem ($S \leq 2$ μg/mL, $R > 2$ μg/mL)[49] but lower than CLSI imipenem breakpoints ($S \leq 4$ μg/mL, $I = 8$ μg/mL, $R \geq 16$ μg/mL, Table 1). CLSI breakpoints were applied to uniformly interpret susceptibility for these two carbapenems. Based on CLSI criteria, all WH *B. pseudomallei* strains are susceptible to imipenem and meropenem (Fig. 1 and Supplementary Table S1), but under EUCAST interpretation guidelines, one strain (724644) is susceptible to imipenem (MIC = 2 μg/mL) and is resistant to meropenem (MIC = 4 μg/mL). Future EUCAST and CLSI agreement on breakpoints could eliminate these inconsistencies. EUCAST *B. pseudomallei* clinical breakpoints were established using a multicenter study with 361 non-consecutive, non-duplicate *B. pseudomallei* isolates with and without suspected antimicrobial resistance.[56] The latest CLSI M45 document (2015) described the limitation that “very extensive microbiological, clinical, and pharmacodynamic databases normally used for setting breakpoints by CLSI do not exist” for *B. pseudomallei*.[46]

Very few mutations associated with antimicrobial resistance were detected in the WH study strains, and the antimicrobial susceptibility profiles of WH *B. pseudomallei* were typical. Although amino acid substitution P258S in PenA is unique to *B. pseudomallei* strain 724644, which has intermediate resistance to amoxicillin/clavulanate, this mutation is located outside the conserved regions important for enzymatic activity of PenA (Fig. 2). The functional contribution of the P258S amino acid substitution to intermediate amoxicillin/clavulanate resistance in *B. pseudomallei* requires further investigation.

In conclusion, confirming previous studies with larger panels of clinical EH isolates our results show that resistance to antimicrobials commonly used for treatment of *B. pseudomallei* infections is rare in WH isolates and treatments are expected to be similarly effective for EH and WH strains. As previously suggested, the antimicrobial combination of ceftazidime/avibactam may be a good option for treatment of ceftazidime-resistant *B. pseudomallei*. Multicenter evaluations of different antimicrobials are warranted, as well as the establishment of breakpoints for new drugs for melioidosis treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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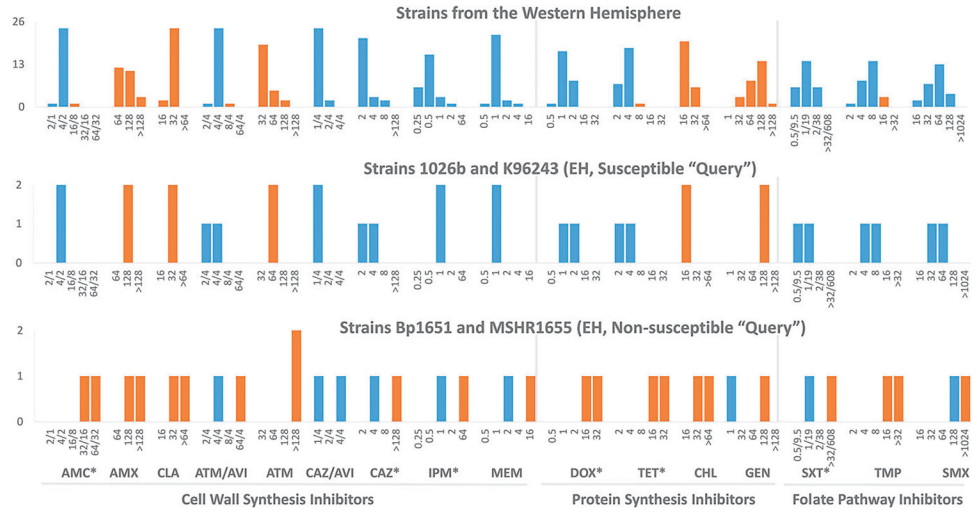

FIG. 1.

The distribution of MIC values measured for 26 *Burkholderia pseudomallei* strains from the WH, as well as for two susceptible and two nonsusceptible *B. pseudomallei* strains from the EH. EH strains were used as "query" strains for comparison of susceptibility and DNA sequences. Y axis depict number of strains, X axis show MIC values (in μg/mL). *Blue bars* indicate low-level MICs or MICs in the susceptible range, *orange bars* indicate high-level MICs or MICs in the nonsusceptible range. The *colors* were assigned based on MIC interpretive criteria described in CLSI M45,[46] M100,[48] and EUCAST[49] documents. *There are CLSI breakpoints for testing *B. pseudomallei* against AMC, CAZ, IPM, DOX, TET, and SXT. Because no CLSI breakpoints are currently defined for *B. pseudomallei* for other antibiotics tested, for interpretation of CHL results, *B. pseudomallei* breakpoints published by EUCAST were used; CLSI breakpoints for IPM were used for interpretations of MEM results; for interpretations of AMX and CLA alone, the individual components of the CLSI AMC drug combination breakpoints for *B. pseudomallei* were used; CLSI breakpoints for Enterobacteriaceae were used for ATM, CAZ/AVI, TMP, SMX, and GEN for interpretation of *B. pseudomallei* results; and CLSI Enterobacteriaceae ATM breakpoints were used for interpretation of ATM/AVI susceptibility, using the ATM value in ATM/AVI for this combination drug because there are no ATM/AVI breakpoints published by CLSI for any organism at this time. AMC, amoxicillin/clavulanate; AMX, amoxicillin; ATM, aztreonam; AVI, avibactam; CAZ, ceftazidime; CHL, chloramphenicol; CLA, clavulanate; CLSI, Clinical and Laboratory Standards Institute; DOX, doxycycline; EH, Eastern Hemisphere; EUCAST, European Committee on Antimicrobial Susceptibility Testing; GEN, gentamicin; IPM, imipenem; MEM, meropenem; MIC, minimal inhibitory concentration; SMX, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; WH, Western Hemisphere.

FIG. 2.

Diagram representing *Burkholderia pseudomallei* PenA protein. The positions of conserved regions are shown as *red bars* with labels on *top* of the diagram. The positions of known mutations for ceftazidime resistance—C69Y,[32,33] P167S[31,33] and D240G,[28] and for clavulanic acid resistance—S72F,[31,33] are shown with *red arrows* at the *bottom* of the diagram. The position of the newly identified substitution is shown with a *black arrow* at the *bottom* of the diagram. Amino acids are *numbered* according to the Ambler scheme.[54]

Table 1.

MIC_{50} and MIC_{90} Results for 26 Western Hemisphere *Burkholderia pseudomallei* Isolates and Minimal Inhibitory Concentration Interpretive Criteria

	***MIC_{50} and MIC_{90} for 26 WH* B. pseudomallei, μg/mL**		***CLSI MIC interpretive criteria, μg/mL***						***EUCAST MIC interpretive criteria for* B. pseudomallei, μg/mL[49]**	
			B. pseudomallei[46]			***Enterobacteriaceae* [48]**				
Antimicrobials	***MIC_{50}***	***MIC_{90}***	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**R**
Cell wall synthesis inhibitors										
AMC	4/2	4/2	≤8/4	16/8	≥32/16					
AMX	128	>128								
CLA	32	32								
ATM/AVI	4/4	4/4								
ATM	32	64				≤4	8	≥16		
CAZ/AVI	1/4	1/4				≤8/4	—	≥16/4		
CAZ	2	4	≤8	16	≥32					
IPM	0.5	1	≤4	8	≥16					
MEM	1	2								
Protein synthesis inhibitors										
DOX	1	2	≤4	8	≥16					
TET	4	4	≤4	8	≥16					
CHL	16	32							≤0.001	>8
GEN	128	128				≤4	8	≥16		
Folate pathway inhibitors										
SXT	1/19	2/38	≤2/38	—	≥4/76					
TMP	8	16				≤8	—	≥16		
SMX	64	128				≤256	—	≥512		

MICs were determined by broth microdilution.

AMC, amoxicillin/clavulanate; AMX, amoxicillin; ATM, aztreonam; AVI, avibactam; CAZ, ceftazidime; CHL, chloramphenicol; CLA, clavulanate; CLSI, Clinical and Laboratory Standards Institute; DOX, doxycycline; EUCAST, European Committee on Antimicrobial Susceptibility Testing; GEN, gentamicin; I, intermediate; IPM, imipenem; MEM, meropenem; MIC, minimal inhibitory concentration; R, resistant; S, susceptible; SMX, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; WH, Western Hemisphere.