Antibiotic resistance profiles for the opportunistic pathogens *Burkholderia oklahomensis*, *Burkholderia ubonensis* and *Burkholderia vietnamensis*

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Abstract

Various bacteria belonging to the genus *Burkholderia* are recognized as emerging pathogens. Some of these have not yet been well studied. Here we determined the antibiotic susceptibility profiles of the three opportunistic pathogens *Burkholderia oklahomensis, B. ubonensis* and *B. vietnamensis*. All three bacterial species show resistance to carbenicillin, erythromycin and gentamicin and, with the exception of *B. ubonensis*, are most susceptible to tetracycline, trimethoprim and the carbapenems imipenem and meropenem. *B. ubonensis* was consistently the most resistant of the three bacteria and also exhibits increased resistance to tetracycline and carbapenems. Availability of antibiotic resistance profiles for these bacteria will facilitate future clinical, environmental and genetic studies with these opportunistic pathogens.

Introduction

There are over 40 different species of *Burkholderia* commonly found in surface soils and groundwater worldwide.[1] Although many of these species exhibit intrinsic antibiotic resistance, few have been studied for their antibiotic resistance profile. Understanding bacterial antibiotic resistance is a key factor in understanding the resistance mechanisms innate to bacteria. Developing antibiotic resistance profiles is also crucial for clinical, environmental and genetic studies.

Like many Gram-negative bacteria, mounting evidence indicates that multidrug efflux pumps of the resistance nodulation cell division (RND) superfamily play an important role in the multidrug resistance of *Burkholderia* species. *Burkholderia cenocepacia* expresses several RND pumps that contribute to drug resistance. [2, 3] Likewise, most *B. pseudomallei* strains are intrinsically antibiotic resistant due to AmrAB-OprA [4,5] and BpeAB-OprB [6, 7] efflux pump expression. *Burkholderia pseudomallei* is classified by the Centers for Disease Control and Prevention (CDC) as a bio-safety level (BSL) 3 organism and category B biothreat agent. *Burkholderia pseudomallei* is endemic to Southeast Asia, Northern Australia and other tropical and subtropical regions of the world.[8] In endemic regions it is of clinical importance as the etiologic agent of human melioidosis, a progressive disease with high mortality rates.[9,10]

Other *Burkholderia* species that have been studied include the BSL-2 *B. gladioli* and the *B. cepacia* complex (BCC). As opportunistic pathogens, these soil and water pathogens typically only affect immunocompromised or cystic fibrosis patients.[11, 12] The BCC contains at least ten closely related strains of *Burkholderia* species that are phylogenetically differentiable, but are phenotypically indistinguishable. Other species of *Burkholderia* are also intrinsically antibiotic resistant but there is little known or published for these organisms. The CDC lists the three *Burkholderia* species, *B. oklahomensis, B. ubonensis,* and *B. vietnamensis* as BSL-2 opportunistic pathogens. *B. oklahomensis* C6786 was isolated in 1973 from a wound infection after a farming accident in Oklahoma and initially named as the "Oklahoma" strain of *B. pseudomallei*. It was later determined, through gene sequencing, to be a novel species, *B. oklahomensis.*[13] Three more identical isolates were identified as sharing the same typical *Burkholderia* phenotypical features.[13] Four isolates have been obtained for an environmentally important species *B. ubonensis* that has been speculated to be the tenth genomovar of the BCC. Little is known about this bacterium other than it is found in surface soils and has not to date been associated with human infections.[14] The fifth genomovar of the BCC is known to be *B. vietnamensis*. *Burkholderia vietnamensis* is commonly isolated from surface soils and ground water and has been studied as a plant growth promoting bacterium and bioremediation agent for aromatic hydrocarbons.[15] It is a Gram-negative rod, motile and aerobic. *Burkholderia vietnamensis* is an opportunistic pathogen in humans often affecting cystic fibrosis patients.[16] As a prelude to future studies, we determined the antibiotic susceptibility profiles of *B. oklahomensis, B. ubonensis and B. vietnamensis*.

Materials and Methods

Bacterial strains and growth.

The strains used in this study were the clinical *B. oklahomensis* isolate C6786 (laboratory stock number B94),[13] the environmental *B. ubonensis isolate* A1301 (laboratory stock number B180),[14] and *B. vietnamensis* H4102 (laboratory stock number B122)(obtained from Dr. Alex Hoffmaster, CDC Atlanta). All strains were grown at 37°C. Before use, these strains were struck for single-colonies on Lennox Luria-Bertani (LB) [17] agar (MO BIO Laboratories, Carlsbad, CA) plates. Single colony isolates were inoculated into Lennox LB broth in preparation for minimum inhibitory concentration (MIC) tests. For MIC tests, bacteria were then inoculated into 4 ml of Mueller-Hinton broth (MHB; Becton Dickinson, Sparks, MD) and grown overnight. The next day, the overnight culture was diluted into MHB and grown to log phase (A_{600nm} ~0.7). This culture was then diluted in sterile saline and adjusted to the density of a 0.5 McFarland equivalence turbidity standard (Remel, Lenexa, KS).

Antibiotics.

Table 1 lists the antibiotics used in the study by function, class and common name. Stock concentrations of antibiotics were made following standard protocol at concentrations of either 4,096 µg/ml or 32,768 µg/ml depending on the strain being tested and antibiotic. Antibiotics were either purchased as powders from Sigma, St. Louis, MO (carbenicillin, gentamicin, erythromycin, and tetracycline) or immobilized on Etest® strips from AB BIODISK, Solna, Sweden (trimethoprim, imipenem, meropenem).

MIC determinations.

A set of standard conditions set by the Clinical and Laboratory Standards Institute (CLSI) [18] must be followed when defining antibiotic resistance profiles. All procedures were performed in a biosafety cabinet (BSL-2+ conditions). The two methods used for MIC determinations were two-fold serial dilution in microtiter plates and Etest®.

The two-fold serial dilution method utilizes

Bactericidal			Bacteriostatic	
Class	**Representative tested**		**Class**	**Representative tested**
Aminoglycosides	Gentamicin		Tetracyclines	Tetracycline
Penicillins	Carbenicillin		Sulfonamides	Trimethoprim
Carbapenems	Meropenem Imipenem		Macrolides:	Erythromycin[1]

Table 1. Antibiotics tested in this study. Antibiotics are listed by function, class, and common name.
[1]Bacterial or bacteriostatic depending on concentration

96-well plates and two-fold serial dilutions of antibiotic concentrations. Each test was performed in triplicate with positive and negative controls. An antibiotic stock was made at twice the highest desired initial concentration of antibiotic to be tested in the dilutions. The antibiotic stock solution was distributed in 100 µl aliquots into the first column of the first four rows of a 96-well plate. Rows one through three constituted one triplicate experiment for one MIC test of a specific antibiotic. Rows four and five were controls to test the antibiotic stock and bacterial growth respectively to verify negative and positive growth controls. These controls allow visualization of any random growth that may occur in the wells and characteristics of the antibiotics (precipitate, color change, etc.).[18]
Mueller-Hinton broth was distributed (50 µl per well) to columns 2-12 of rows 1-5 and column 1 row 5. The antibiotic was then diluted two-fold throughout the plate. This was achieved by taking 50 µl from the first well in column 1, rows 1- 4, into the second column and mixing. This was then followed by taking 50 µl from this well into the next well, mixing and so on. Lastly, 50 µl aliquots were removed from the last wells in rows 1- 4. Next, prepared bacterial inoculant (50 µl; 0.5 McFarland turbidity standard) was added to each well in rows 1-3 and row 5. The plates were incubated for 24 h. 37°C and wells visually examined for growth. Growth in any well is considered a button of growth. The first concentration of antibiotic where no button of growth is visible is regarded the MIC in µg/ml.
Etest® strips are pre-loaded with a gradient of decreasing antibiotic concentrations. Each test was done in triplicate with three plates per test. Bacterial inoculant was prepared the same way as in the two-fold serial dilution method but once adjusted to a 0.5 McFarland standard, a sterile cotton swab was used to transfer the saline inoculant to MHA plates. The plates were struck for confluency (inoculant fully covers the plate), which produces a lawn of growth covering the agar. Etest® strips were placed carefully on the plate with sterile forceps avoiding bubbles and displacement. The plates were then incubated at 37°C for 24 hours. Etest® strips depict a different form of susceptibility showing an area of inhibition on a confluent lawn of growth around the strip labeled with antibiotic concentrations. Etest® MIC results are read by determining the end point of growth adjacent to the strip as seen by the naked eye for bactericidal antibiotics. Trimethoprim is bacteriostatic and thus Etest® protocols require that the results be read at 80% inhibition or the first point of significant inhibition as judged by the naked eye and not where the lawn is completely cleared.[18]

Results and Discussion

The results of multiple trials done in triplicate for accuracy have been condensed to arrive at the estimated minimum inhibitory concentration (MIC) values shown in Table 2. Carbenicillin was the only antibiotic for which MIC tests gave moderately varied results. However, the results were always greater or equal to the value listed in Table 2. For Etest® values that were between two markings on the strip, the upper value was used in accordance to the Etest® reading guide.
The CLSI determines breakpoints based on organism and antibiotic. Bacteria can be susceptible, intermediate, or resistant to antibiotics at different concentrations. After multiple trials (three to four) of each test performed in triplicate, we were able to confidently assign MIC values to each strain for all seven antibiotics used. Similar patterns of resistance and susceptibility can be seen between the three strains with respect to the different antibiotics tested. According to the CLSI breakpoint values for *Burkholderia* species, it can be concluded that *B. oklahomensis* exhibits resistance to carbenicillin, gentamicin and erythromycin, but susceptibility to tetracycline, trimethoprim,

Drug	MIC (µg/mL)[1]		
	B. oklahomensis C6786	*B. ubonensis* H4102	*B. vietnamensis* A1301
Carbenicillin	256	1,024	>512
Gentamicin	32	256	4
Erythromycin	128	64	32
Tetracycline	2	64	2
Trimethoprim	0.5	0.19	0.38
Imipenem	0.094	8	0.19
Meropenem	0.19	3	0.38

Table 2. Antibiotic Resistance Profiles for *B. oklahomensis*, *B. ubonensis* and *B.vietnamensis*. From the results of multiple trials, the following MIC values were determined. The protocols for MIC determination were performed as listed in the Materials and Methods section.
[1]MICs for trimethoprim, imipenem, meropenem and trimethoprim were determined using Etest®; all others established using the two-fold serial dilution method.

imipenem and meropenem. *Burkholderia ubonensis* exhibits resistance to carbenicillin, gentamicin, erythromycin, tetracycline, imipenem and meropenem, and is only susceptible to trimethoprim. *Burkholderia vietnamensis* exhibits resistance to carbenicillin and erythromycin, and is susceptible to tetracycline, trimethoprim, imipenem and meropenem, with possible resistance to gentamicin.

The values obtained were compared to previously determined values in our laboratory for *B. gladioli* pathovar *cocovenenans* and *B. pseudomallei*, where *B. gladioli* pathovar *cocovenenans* was found to be resistant to carbenicillin, erythromycin, tetracycline and imipenem and susceptible to gentamicin, trimethoprim and meropenem (unpublished observations). *Burkholderia pseudomallei* was determined to be resistant to carbenicillin, gentamicin and erythromycin and susceptible to tetracycline, trimethoprim, imipenem and meropenem.[7]

Generally, each species tested was resistant to older forms of penicillin drugs (carbenicillin) and appears to be susceptible to newer β-lactam antibiotics (imipenem and meropenem). However, *B. ubonensis* also shows resistance to imipenem and meropenem. In *B. pseudomallei*, resistance to older β-lactams is due to expression of the chromosomally encoded PenA β-lactamase, which shows little activity against imipenem and meropenem[19, 20] (D.A. Rholl and H.P. Schweizer, unpublished observations). *Burkholderia ubonensis* either encodes a similar enzyme with an extended substrate spectrum or the observed increased imipenem and meropenem resistance is due to another mechanism.

Of the three species examined in this study, *B. ubonensis* was consistently more resistant. All three bacterial species show resistance to carbenicillin, erythromycin and gentamicin and this resistance pattern is also observed with *B. pseudomallei* and *B. gladioli* pathovar *cocovenenans*. It has been well established that intrinsic aminoglycoside and macrolide resistance in *B. pseudomallei* is due to expression of the AmrAB-OprA efflux pump.[4, 5] While tempting to speculate that the same pump also operates in the *Burkholderia* species examined in this study, this remains to be experimentally confirmed.

Availability of the antibiotic resistance profiles determined in this study will facilitate future clinical, environmental and genetic studies with these opportunistic pathogens.

Acknowledgements

We acknowledge the technical advice of Nicole Podnecky and Dr. Takehiko Mima. We also thank Dr. Alex Hoffmaster from CDC Atlanta for providing strains. This work was supported by Colorado State University research funds provided to Dr. Herbert Schweizer.

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