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Improved non-redundant species screening panels for benchmarking the performance of new investigational antibacterial candidates against Category A and B priority pathogens

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Background: NIAID has a programme for testing drug candidates against biodefense and emerging bacterial pathogens that uses defined strain panels consisting of standard laboratory reference strains and strains of clinical origin.

Objectives: The current studies were performed to assess the activity of standard-of-care drugs, determine benchmark criteria for new investigational antibacterial candidate prioritization and identify reduced non-redundant strain panels for candidate performance classification.

Methods: The susceptibilities of each strain in the screening panels to 40 standard-of-care drugs and clinical drug combinations were determined by percentage growth inhibition using multiple concentrations, a method commonly used in efficient high-throughput screening efforts. The drug susceptibility of each strain was categorized based on interpretive criteria to benchmark the activity of each standard-of-care drug and drug combination, followed by confirmation of select active drugs. Exact match and clustering analyses defined focused non-redundant species and pan-species screening panels.

Results: This process revealed a broad spectrum of susceptibilities among strains in each species, with important differences between the standard laboratory reference strains and strains of clinical origin. Exact match and clustering analyses identified subsets of non-redundant strains that can more efficiently classify drug activity resulting in individual species screening panels, a pan-species screening panel and a pan-species maximum resistance panel.

Conclusions: This study resulted in improved non-redundant species screening panels for benchmarking the performance of new investigational antibacterial candidates with the greatest potential for efficacy against clinically relevant Category A and B priority and emerging pathogens.

Introduction

Productive drug screening programmes are not necessarily intended to screen out drug candidates but, rather, efficiently categorize the activity of new investigational antibacterial candidates so they can be prioritized and further characterized through relevant secondary screens.[1] Often, this includes rapid activity assessment to inform and drive medicinal chemistry and lead optimization efforts. Drug screening begins with an evaluation of a drug candidate's potency, which is often performed against a single reference laboratory-adapted bacterial strain, a closely related model organism, or a panel of multiple reference strains from different species.[2–4] While laboratory or model strains provide a consistent reference for comparison of drug candidate performance; they do not adequately represent the spectrum of drug susceptibility often observed in clinical strains.[5] Further, for some bacterial species with unique or intrinsic resistance mechanisms, it is beneficial to include more than one reference strain to fully understand the potential of a drug candidate or pharmacophore series.[6–8] Therefore, drug susceptibility profiles against a spectrum of strains of clinical origin, in addition to reference laboratory strains, can provide superior information about a drug candidate's potential performance in a clinical setting and address the needs posed by drug-resistant strains.

Category A and B priority pathogens are included in the classification of emerging infectious diseases that have been defined

as diseases caused by organisms that are newly appearing or increasing in incidence.[9] To facilitate drug discovery, the National Institute of Allergy and Infectious Diseases (NIAID) has a drug candidate screening programme through their resources for researchers that provides evaluation against two screening panels of Category A and B priority and emerging bacterial strains.[10,11] The species in these panels are *Burkholderia pseudomallei*, *Burkholderia mallei*, *Francisella tularensis*, *Yersinia pestis* and *Bacillus anthracis*, represented by reference laboratory strains and strains of clinical origin.[10] Category A pathogens pose the highest risk to public health because they are difficult to treat and result in high mortality.[12] Category B pathogens pose less risk because of moderate morbidity rates and lower mortality.[12] Interest in these organisms is based on difficulty to treat, relapse rates and limited availability of clinically used standard-of-care (SoC) drugs and, in some cases, intrinsic resistance. Further, these Category A and B agents have been weaponized, presenting a concern for public health risk.[13] Therefore, improved throughput of new investigational antibacterial candidates is envisioned to expand the structural diversity and increase the number of compounds progressing through the drug discovery pipeline for difficult-to-treat medically important pathogens.

Since there are no comprehensive reports of drug activity against the strains in the NIAID screening panels, the current studies were performed to assess the activity of SoC drugs, determine benchmark criteria for drug candidate prioritization and identify non-redundant strain panels for candidate performance classification. Accordingly, the susceptibility of each strain to 40 SoC drugs and combinations were categorized based on benchmark criteria. Exact match and clustering analyses of the susceptibility data identified non-redundant strain panels for each species, a pan-species screening panel and a pan-species maximum resistance screening panel that improved drug candidate classification. The resulting susceptibility information and improved non-redundant strain panels can be used to streamline drug candidate throughput in novel drug discovery or repurposing efforts to rapidly prioritize and advance drug candidates with the greatest potential to treat infectious diseases caused by these pathogens.

Materials and methods

NIAID bacterial strains for drug candidate assessment

The strains used in this study are those specified in the defined panels of Category A and B pathogens used for drug candidate evaluation in the NIAID drug screening programme.[10] These panels include *B. anthracis* ($N=15$), *Y. pestis* ($N=5$), *F. tularensis* ($N=6$), *B. mallei* ($N=7$) and *B. pseudomallei* ($N=17$) (Table S1, available as Supplementary data at *JAC-AMR* Online). *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumannii* are also included in the panel as quality and testing control strains. CLSI interpretive criteria were applied to antimicrobial susceptibility testing results.[14]

Growth and maintenance conditions

B. anthracis

Tryptic soy broth (10 mL) (BD, Franklin Lakes, NJ, USA) was inoculated with 0.01 mL *B. anthracis* glycerol bacterial stock and incubated overnight at 37°C on an orbital shaker. Overnight cultures were diluted 1:100 into 10 mL tryptic soy broth and incubated for an additional 6 h. OD_{600} was taken, and cultures were diluted to a concentration of 1×10^6 cfu/mL in CAMHB (BD). Diluted cultures (0.05 mL) were used to inoculate 96-well plates.

Y. pestis

Brain heart infusion broth (50 mL) (BD) was inoculated with 0.05 mL *Y. pestis* glycerol bacterial stock and incubated for 48 h at 28°C. OD_{600} was taken, and cultures were diluted to a concentration of 1×10^6 cfu/mL in CAMHB (BD). Diluted cultures (0.05 mL) were used to inoculate 96-well plates.

F. tularensis

F. tularensis was streaked onto cystine heart agar supplemented with 2% haemoglobin (BD) and incubated at 37°C for 72 h. Bacterial suspensions were prepared in CAMHB supplemented with IsoVitalex (VWR, Radnor, PA, USA), 0.1% dextrose (Sigma–Aldrich, St Louis, MO, USA) and 0.025% ferric pyrophosphate (Sigma–Aldrich) (MMH) to an OD_{600} of ~0.5. Suspensions were diluted in MMH to a concentration of 1×10^6 cfu/mL, and 0.05 mL of diluted cultures were used to inoculate 96 well plates.

B. pseudomallei and B. mallei

LB broth (10 mL) was inoculated with 0.01 mL *B. pseudomallei* or *B. mallei* glycerol bacterial stock and incubated overnight at 37°C on an orbital shaker. Overnight cultures were diluted 1:100 into 10 mL LB and incubated for an additional 6 h. OD_{600} was taken, and cultures were diluted to a concentration of 1×10^6 cfu/mL in CAMHB (BD). Diluted cultures (0.05 mL) were used to inoculate 96-well plates.

SoC drug plate preparation

The compounds in the 40 clinical SoC drug panel represent the different drug families: aminoglycosides, macrolides, β-lactams, cephalosporins and quinolones. For benchmark evaluation, drug master plates were prepared in triplicate with 40 SoC drugs, separately or in combination, dissolved in appropriate solvents to concentrations of 0.8 mg/mL and 0.1 mg/mL, and stored at −20°C prior to testing. Master plates were used to prepare drug test plates at 64 mg/L and 8 mg/L in 0.05 mL CAMHB or MMH for *Francisella*. Once diluted 1:1 with inoculum, the final testing concentrations were 32 mg/L and 4 mg/L, respectively. Ten wells per 96-well plate contained CAMHB only as growth controls. For evaluation of MIC, drug plates were prepared with concentrations ranging from 64 mg/L to 0.031 mg/L in 0.05 mL CAMHB (MMH for *Francisella*). Once diluted 1:1 with inoculum, the final testing concentrations were 32 mg/L to 0.016 mg/L.

Resazurin percentage growth reduction determination

Inoculated 96-well plates were incubated for 18 h at 37°C for *B. pseudomallei*, *B. mallei* and *F. tularensis* and 24 h at 37°C for *Y. pestis*. Resazurin sodium salt (Sigma–Aldrich) was dissolved in PBS (Sigma–Aldrich) at a concentration of 0.11 mg/mL and sterile filtered through a 0.2 μm filter. Ten microlitres was added to each well of the inoculated 96-well plate, and plates were incubated for an additional 4 h at 37°C. Metabolically active bacteria produce a colorimetric change by reducing resazurin to resorufin.[15] These colorimetric change is measured using extinction coefficients of resazurin to resorufin at 570 nm and 600 nm. Percentage growth inhibition of treated compared with untreated growth control is calculated using the following formula:

$$\lambda_1 = 570$$
$$\lambda_2 = 600$$
$$(\varepsilon_{ox})\lambda_2 = 117216$$

$$(\varepsilon_{ox})\lambda_1 = 80586$$

$$\left(\frac{[(\varepsilon_{ox})\lambda_2][A\lambda_1] - [(\varepsilon_{ox})\lambda_1][A\lambda_2] \textit{ of test agent dilution}}{[(\varepsilon_{ox})\lambda_2][A\lambda_1] - [(\varepsilon_{ox})\lambda_1][A\lambda_2] \textit{ of untreated positive growth control}}\right) \times 100 = \textit{percentage growth reduction}$$

Clustering and subgroup identification

Manhattan distance and the sum of absolute difference were used to assess differences in the susceptibility profiles between subtypes within the five organisms utilized and between all strains combined. Utilizing the calculated Manhattan distances and the unweighted pair group method with arithmetic mean (UPGMA) clustering method allowed the identification of similarities between the different subtypes per species and the combined subtypes of all species. Subtypes with a distance equal to zero, indicating an exact match of their susceptibility profiles, were identified and replaced with one representative subtype. To obtain the minimum number of subtypes that span all possible drug susceptibilities after removing exact matching susceptibility profiles, we first identified the subtype that included the maximum number of drug susceptibilities. We then added the subtype that would, along with the first subtype, provide the maximum number of drug susceptibilities, given that that newly added subtype is either disjoint or not completely overlapping with the first. We continued adding subtypes utilizing the conditions described until adding a new subtype resulted in no unique drug susceptibilities added to the spanning combined susceptibility profile.

Ethics statement

All studies performed at Colorado State University were conducted in a BSL3 facility dedicated to bacterial pathogen work under approvals and management of the Biosafety Official.

Results and discussion

To identify a subset of non-redundant strains for each species that can be used to rapidly classify the activity of new investigational antibacterial candidates, each strain was tested against single SoC drugs ($n = 34$) and drug combinations ($n = 6$) and categorized as susceptible, intermediate or resistant at the clinically efficacious benchmark concentrations (Table S2). Our evaluation revealed considerable heterogeneity in drug susceptibility between clinical strains and reference strains with resistant or intermediate susceptibility to nearly 50% of the SoC treatments for the strains tested (Figure 1). It was found that the species tested were susceptible to doxycycline, tigecycline, tetracycline, chloramphenicol, piperacillin, imipenem, meropenem, rifampicin, norfloxacin, ciprofloxacin and levofloxacin, and piperacillin/tazobactam, imipenem/cilastatin, amoxicillin/clavulanic acid and ampicillin/sulbactam combinations. The majority of species were susceptible to streptomycin, gentamicin, kanamycin A, azithromycin, clarithromycin, solithromycin, erythromycin and ceftazidime. Compared with the other tested species, *B. anthracis* was uniquely susceptible to ampicillin, amoxicillin, carbenicillin and vancomycin. *F. tularensis*, *B. mallei* and *Y. pestis* species were susceptible to sulfadiazine, sulfamethoxazole, ceftazidime and trimethoprim. *B. pseudomallei* was found to be the most naturally resistant species being generally more resistant to chloramphenicol, clarithromycin, streptomycin azithromycin, erythromycin, ampicillin, amoxicillin, carbenicillin and vancomycin (Table S3). Even the combination of β-lactamase inhibitors, clavulanic acid and sulbactam, only slightly improved susceptibility of *B. pseudomallei*, further substantiating the role of drug efflux in the observed intrinsic drug resistance.[7,8] The majority of the strains in this study were susceptible to all the tetracycline and quinolone drug classes and resistant to the macrolide and penicillin classes. The penicillin drugs only showed activity when paired with a β-lactamase inhibitor.

Non-redundant species panels for drug screening

Screening efficiency can be much improved using a non-redundant screening panel for a species of interest or a non-redundant pan-species screening panel if evaluating broad-spectrum activity, particularly if a large number of new investigational antibacterial candidates are being assessed. Drug candidates that exceed screening criteria in this initial screen representing the full spectrum of drug susceptibilities can then be progressed to secondary screening panels or a pan-species max resistance screening panel. Exact match (Table S4) and hierarchical clustering analyses were performed using the susceptibility profiles, which provided clear demarcations for grouping strains by drug susceptibility and removal of strains that were an exact match based on drug susceptibility.

F. tularensis non-redundant screening panel

The non-redundant screening panel for *F. tularensis* clustered into two distinct drug susceptibility groups (DSGs) (Figure S1). DSG 1 contained strains WY96 and OR96, and DSG 2 contained strains SchuS4 and SchuS4 FSC237 in subgroup 2A and strains MA00 and KY99 in subgroup 2B. DSG 1 contained the most resistant strains distinguished by resistance to piperacillin, trimethoprim, cefpodoxime, imipenem, ceftazidime and meropenem. In general, *F. tularensis* strains were susceptible to quinolone antibiotics, tetracycline antibiotics, aminoglycoside antibiotics and rifampicin.

B. anthracis non-redundant screening panel

The non-redundant screening panel for *B. anthracis* organized into two DSGs and a singleton (Figure S2). *B. anthracis* DSG 1 consisted of strains A0318 and 46-PY-5, and DSG 2 consisted of strains WNA, CDC #3 (2010719149) and Vollum. *B. anthracis* strain CDC#1 organized as a singleton strain because it is resistant to the β-lactams amoxicillin, ampicillin, carbenicillin and piperacillin. *B. anthracis* strains showed general resistance to ceftazidime, colistin, bacitracin, cefpodoxime and trimethoprim, sulfadiazine, pyrimethamine, sulfamethoxazole combinations.

Y. pestis non-redundant screening panel

The *Y. pestis* non-redundant screening panel contained the fewest strains of the species tested and organized into a singleton and two DSGs with DSG 1 containing PEXU2 and PB6 and DSG 2 containing Nepal516 and ZE94-2122. *Y. pestis* CO92 organized as a singleton (Figure S3). *Y. pestis* CO92 was distinct due to reduced susceptibility to trimethoprim/sulfamethoxazole combination and resistance to ceftazidime, linezolid and cefpodoxime.

Burkholderia species non-redundant screening panels

The non-redundant screening panel for *B. pseudomallei* was the most diverse and consisted of nine strains organized into

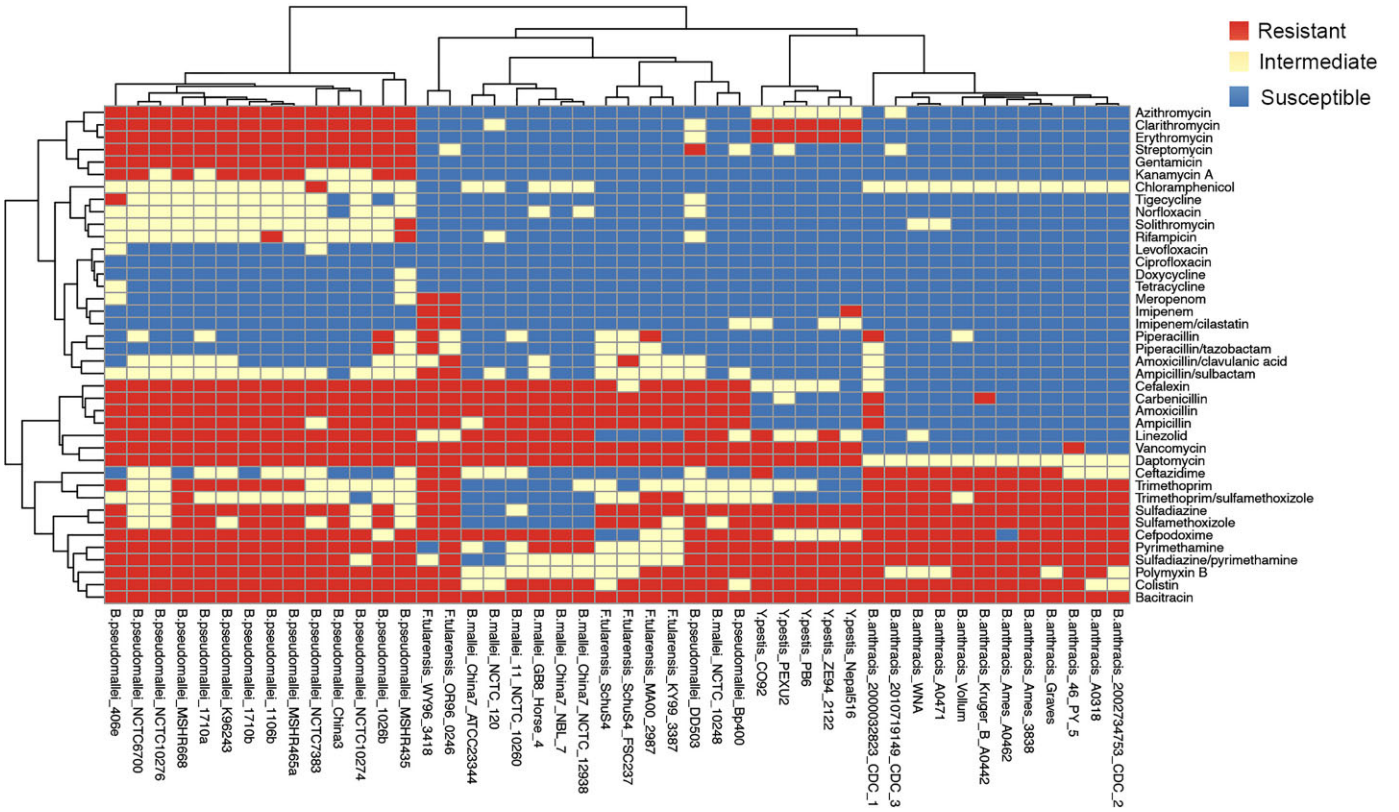

Figure 1. Pan-species susceptibility profile. Efficacious benchmark concentrations for each strain against single SoC drugs ($n = 34$) and drug combinations ($n = 6$), and categorization as susceptible (blue), intermediate (yellow) or resistant (red). Each bacterial strain was independently tested in replicates and grouped. Clustering lines are Manhattan distances and the UPGMA.

two DSGs (Figure S4). DSG 1 contained the most susceptible *B. pseudomallei* strains, which were characterized by the drug efflux incompetent laboratory strain Bp400 and strain DD503. *B. pseudomallei* DD503 is the most susceptible clinical strain with resistance limited to vancomycin, amoxicillin, streptomycin, macrolides and polypeptides. DSG 2 consisted of three subgroups. DSG 2 subgroup 2A included strains 406e, 1106b and MSHR668. DSG 2 subgroup 2B included strains NCTC7383 and NCTC10274. DSG 2 subgroup 2C contained strains 1026b and MSHR435. The *B. mallei* strain 10248 organized as a singleton, NCTC10260 and GB8 Hourse4 into DSG 1, and NCTC120 and China7 ATCC23344 organized into DSG 2 (Figure S5). This organization was driven by the resistance and intermediate susceptibilities to ceftazidime, chloramphenicol, sulfadiazine, ampicillin, colistin, polymyxin and pyrimethamine.

Identification of pan-species panels and a maximum resistance panel

Hierarchical clustering was performed on the entire strain susceptibility data set to identify a pan-species screening panel. The resulting pan-species screening panel consisted of 11 strains representing all the species evaluated. The value of the pan-species screening panel is that drug candidates can be screened against these 11 strains that are representative of the entire group of 50 strains across all five species (Figure 2). The resulting pan-species screening panel accurately classifies drug activity, thus affording a more efficient evaluation of a new investigational antibacterial candidate activity.

A pan-species maximum resistance screening panel was also identified based on the removal of strains that were identified as exact matches and from the greatest number of intermediate and resistant SoC treatments. The pan-species maximum resistance screening panel consists of *F. tularensis* OR96-0246, *B. anthracis* CDC#1, *Y. pestis* CO92, *B. pseudomallei* MSHR435 and *B. mallei* 10248, and is useful for evaluating the performance of a new investigational antibacterial candidate against the most resistant clinically derived strain in each species. However, the individual strains contained in the pan-species screening panels do not necessarily represent the entire susceptibility profile of the non-redundant species screening panels.

Classification of bacterial strains used in drug screening based on susceptibility to SoC drugs can guide drug screening efforts and provide information about the performance of drug candidates and benchmark their potential for clinical use, particularly for broad-spectrum candidates.[11] This study defined non-redundant species and pan-species drug screening panels to evaluate the performance of new investigational antibacterial candidates. Our group recently used these panels to screen two individual series of new investigational antibacterial candidates and successfully identified a novel rifampicin derivative with efficacy in the tularaemia murine infection model and a new drug

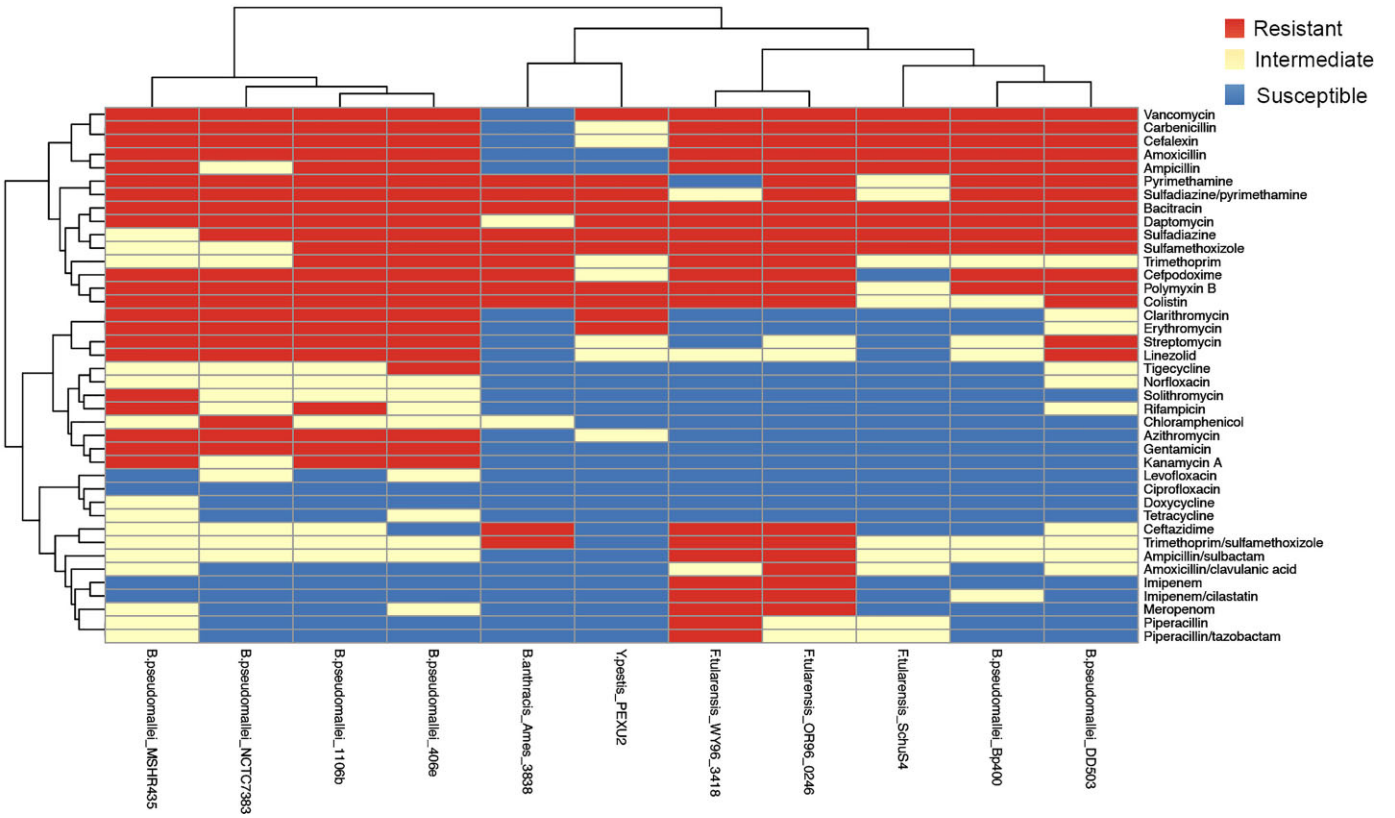

Figure 2. Pan-species non-redundant strain screening panel. Efficacious benchmark concentrations for each strain against single SoC drugs ($n = 34$) and drug combinations ($n = 6$), and categorization as susceptible (blue), intermediate (yellow) or resistant (red). Each bacterial strain was independently tested in replicates. Bacterial strains in each species with exact match susceptibility profiles were removed, and to obtain the minimum number of subtype strains that span all possible drug susceptibilities by the iterative contribution process we first identified the subtype strain with the maximum number of drug susceptibilities to serve as the foundation, and additional subtype strains were added if the newly added subtype was either disjoint or not completely overlapping with the first. This iterative process of adding subtype strains was continued until no new drug susceptibilities could be added to the spanning combined susceptibility profile for each species. Clustering lines are Manhattan distances and the UPGMA.

class diazabicyclooctane derivative with efficacy in the murine models of *Y. pestis* and *B. pseudomallei*.[16,17] The discovery of these broad-spectrum inhibitors unaffected by existing widespread drug resistance substantiates that these screening panels can accurately identify lead novel investigational antibacterial candidates with potential efficacy against clinically relevant NIAID Category A and B priority and emerging pathogens. Further, this information can also be used to guide treatment strategies for single and combination drug regimens that can be used to treat an infection when the agent is known or even before the specific pathogen is confirmed by laboratory testing.

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Transparency declarations

None to declare.

Author contributions

Overall study objectives were defined by R.A.S. The determination of MIC was performed by J.E.C., data structural analysis by Z.A. Study design and writing were performed by J.E.C., Z.A. and R.A.S.

Supplementary data

Tables S1 to S4 and Figures S1 to S5 are available as Supplementary data at *JAC-AMR* Online.

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