Taking the next-gen step: comprehensive antimicrobial resistance detection from *Burkholderia pseudomallei* genomes

Danielle E. Madden BSc (Hons)[1,2], Jessica R. Webb PhD[3], Eike J. Steinig BSc (Hons)[4], Bart J. Currie FRACP[3,5], Erin P. Price PhD[1,2,3^], and Derek S. Sarovich PhD[1,2,3^#]

[1]GeneCology Research Centre, University of the Sunshine Coast, Sippy Downs, Queensland, Australia

[2]Sunshine Coast Health Institute, Sunshine Coast University Hospital, Birtinya, Queensland, Australia

[3]Global and Tropical Health Division, Menzies School of Health Research, Charles Darwin University, Tiwi, Northern Territory, Australia

[4]Australian Institute of Tropical and Health Medicine, James Cook University, Townsville, Queensland, Australia

[5]Department of Infectious Diseases and Northern Territory Medical Program, Royal Darwin Hospital, Tiwi, Northern Territory, Australia

Keywords: ARDaP, antimicrobial resistance, comparative genomics, next-generation sequencing, bioinformatics

Running title: Antibiotic resistance detection from *B. pseudomallei* genomes

^Authors contributed equally

#Corresponding author

Derek S. Sarovich

dsarovich@usc.edu.au

+617 5456 3748

Locked Bag 4, Maroochydore DC, Queensland, 4558, Australia

Abstract

Background: Antimicrobial resistance (AMR) poses a major threat to human health. Whole-genome sequencing holds great potential for AMR identification; however, there remain major gaps in comprehensively detecting AMR across the spectrum of AMR-conferring determinants and pathogens.

Methods: Using 16 wild-type *Burkholderia pseudomallei* and 25 with acquired AMR, we first assessed the performance of existing AMR software (ARIBA and CARD) for detecting clinically relevant AMR in this pathogen. *B. pseudomallei* was chosen due to limited treatment options, high fatality rate, and AMR caused exclusively by chromosomal mutation (i.e. single-nucleotide polymorphisms [SNPs], insertions-deletions [indels], copy-number variations [CNVs], and functional gene loss). Due to poor performance with existing tools, we developed ARDaP (Antimicrobial Resistance Detection and Prediction) to identify the spectrum of AMR-conferring determinants in *B. pseudomallei*.

Results: CARD failed to identify any clinically-relevant AMR in *B. pseudomallei*, ARIBA cannot differentiate AMR determinants from natural genetic variation, and neither CARD or ARIBA can identify CNV or gene loss determinants. In contrast, ARDaP accurately detected all SNP, indel, CNV, and gene loss AMR determinants described in *B. pseudomallei* ($n \approx 50$). Additionally, ARDaP accurately predicted three previously undescribed determinants. In mixed strain data, ARDaP identified AMR to as low as ~5% allelic frequency.

Conclusions: We demonstrate that existing AMR software are inadequate for comprehensive AMR detection; ARDaP overcomes the shortcomings of existing tools. Further, ARDaP enables AMR prediction from mixed sequence data down to 5% allelic frequency. ARDaP databases can be constructed for any microbial species of interest for comprehensive AMR detection.

Introduction

Antimicrobial resistance (AMR) poses a major threat to human health worldwide and an increasing contributor to morbidity and mortality. Antibiotic use and misuse have resulted in an alarming increase in multidrug-resistant infections worldwide, resulting in an urgent need to improve global AMR detection and surveillance. Alongside pathogen identification, AMR detection is one of the primary goals of diagnostic microbiology, with far-reaching consequences for both infection control and effective treatment [1].

Whole-genome sequencing (WGS) holds great potential for comprehensive AMR detection and prediction from bacterial genomes by identifying all AMR determinants in a single genome or metagenome [2], circumventing the need for multiple and often laborious diagnostic methods. Existing bioinformatic tools such as ARG-ANNOT [3], Antibiotic Resistance Identification By Assembly (ARIBA) [4], Comprehensive Antibiotic Resistance Database (CARD) [5], and MEGARes [2] can readily detect AMR genes acquired from horizontal gene transfer events. Many bacterial pathogens also develop AMR via chromosomal mutations, including missense single-nucleotide polymorphism (SNP) mutations in β-lactamase-encoding genes, SNPs or nonsense insertion-deletions (indels) in efflux pump regulators [6-8], gene amplification via copy-number variations (CNVs) [9], and functional gene loss [6]. Recent improvements in AMR identification software mean that chromosomal mutations, particularly SNPs, are now identifiable. For example, ARIBA can identify AMR-conferring SNPs and indels in multiple species [4]. Nevertheless, other types of genetic variants –particularly gene loss or truncation, inversions, and CNVs – remain poorly identified using existing tools, despite their crucial role in conferring AMR [10].

The bacterium, *Burkholderia pseudomallei*, causes the often-fatal tropical disease melioidosis. Melioidosis severity ranges from mild, self-limiting skin abscesses to pneumonia, neurological disease, and septic shock. *B. pseudomallei* is naturally resistant to many antibiotics, including aminoglycosides, penicillins, macrolides, and polymyxins [11, 12]. Fortunately, human-to-human *B. pseudomallei* transmission is rare; almost all infections are acquired from the environment. As such, isolates collected prior to antibiotic treatment are almost universally susceptible to the following clinically-relevant antibiotics: ceftazidime (CAZ), amoxicillin-clavulanate (AMC), co-trimoxazole (SXT), doxycycline (DOX), meropenem (MEM) and imipenem (IPM) [13]. To prevent melioidosis relapse,

treatment involves prolonged (3-6 month) antibiotic therapy, which increases AMR risk and treatment failure [6]. AMR in *B. pseudomallei* has been reported for all clinically-relevant antibiotics [6], with novel AMR determinants towards these key antibiotics continuing to be uncovered.

Here, we tested 47 characterised *B. pseudomallei* genomes with known antibiotic phenotype profiles and associated AMR determinants, and three MEM-resistant (MEMr) strains with previously unidentified AMR determinants, against existing tools (ARIBA and CARD) to determine their AMR detection efficacy. Among the characterised strains, 25 were phenotypically-confirmed as resistant towards at least one clinically relevant antibiotic, 16 were sensitive, and the remainder encoded unusual sensitivity towards aminoglycosides and macrolides or stepwise AMR variants. Following testing against the current AMR tools, we developed a new tool, Antibiotic Resistance Detection and Prediction (ARDaP), to permit comprehensive AMR detection from microbial genomes. ARDaP was designed to meet four main aims: first, to accurately identify AMR determinants caused by a spectrum of mutational mechanisms (i.e. gene gain, SNPs, indels, CNVs, and functional gene loss); second, to predict enigmatic AMR determinants in isolates with phenotypically-confirmed AMR, third, to detect minor AMR allelic determinants in mixed (e.g. metagenomic) sequence data; and finally, to provide a user-friendly report that summarises the AMR determinants (if any) and associated AMR phenotypes, stepwise variants, unusual antimicrobial sensitivity determinants, and genetic variants associated with natural variation that do not confer AMR. Although we illustrate its utility in *B. pseudomallei*, ARDaP is amenable to AMR identification across all microbial species.

Methods

Ethics. Ethical approval was obtained as previously described [14].

Isolates. Forty-seven *B. pseudomallei* strains were included in this study, including 25 with elevated MICs towards one or more clinically-relevant antibiotics (Table 1) and genotypically-confirmed AMR determinants. These isolates were selected as they represent the spectrum of known AMR determinants in *B. pseudomallei* (Table S1). Strains encoding unusual aminoglycoside- and macrolide-sensitivity, and stepwise mutations that lower the barrier to AMR development, were also examined (Table 1). A further 16 strains sensitive to all clinically-relevant antibiotics were included to test software efficacy (Table 2). Finally, three previously uncharacterised clinical strains exhibiting MEMr (MSHR1058 MIC=12 µg/mL; MSHR1174 MIC=6 µg/mL; MSHR8777 MIC=4 µg/mL; Table 3) were included to test the predictive capacity of ARDaP.

Culturing, WGS, and genome assembly. *B. pseudomallei* culture, DNA extraction, and WGS were performed as described elsewhere [9]. Genomic data for MSHR1058, MSHR1174, and MSHR8777 were uploaded to the NCBI Sequence Read Archive database (BioProject PRJNA641249). Accession numbers for all other genomic data are listed in Tables 1 and 2. For genomes lacking a publicly-available assembly, MGAP v1.1 (https://github.com/dsarov/MGAP---Microbial-Genome-Assembler-Pipeline) was used, with archetypal strain K96243 (RefSeq accessions NC_006350.1 and NC_006351.1) provided as the scaffolding reference.

Minimum Inhibitory Concentrations (MICs). MICs were determined using Etests (bioMérieux, Murarrie, Australia). Sensitive, intermediate and resistant cut-offs were based on the Clinical and Laboratory Standards Institute (CLSI) M100-S17 guidelines for *B. pseudomallei* (≤8/4, 16/8, and ≥32/16 µg/mL for AMC; ≤8, 16, ≥32 µg/mL for CAZ; ≤4, 8, ≥16 µg/mL for DOX and IPM; ≤2/38, nil, ≥4/76 µg/mL for SXT). CLSI guidelines do not list MEM for *B. pseudomallei*; however, based on prior work [15, 16], and recent proposed EUCAST breakpoints for *B. pseudomallei*, we categorised MEMr as ≥3 µg/mL. Likewise, the CLSI guidelines do not list gentamicin (GEN) MIC values for *B. pseudomallei* due to almost ubiquitous resistance (>16 µg/mL) towards this antibiotic; however, there are notable exceptions [14, 17]. We chose a GEN-sensitive cut-off of ≤4 µg/mL, which also reflects those strains unable to grow on Ashdown's agar, a selective medium for *B. pseudomallei* isolation that contains 4 µg/mL GEN.

AMR software parameters. The default RGI v5.1.0 database parameters of CARD v3.0.9 (https://card.mcmaster.ca/analyze/rgi; accessed 25Jun20), and ARIBA v2.14.5 (https://github.com/sanger-pathogens/ariba), were examined for performance across the *B. pseudomallei* genomes.

ARDaP AMR database construction. ARDaP is available at: https://github.com/dsarov/ARDaP. All reported *B. pseudomallei* AMR determinants, including stepwise AMR mutations and unusual antimicrobial susceptibility mutations (Table 1; Table S1), were annotated relative to K96243. The AMR determinants (as of version 1.7) are summarised in an SQLite database (Table S1; most up-to-date version available at: https://github.com/dsarov/ARDaP/tree/master/Databases/Burkholderia_pseudomallei_k96243). Briefly, CAZ resistance (CAZr) is caused by altered PenA β-lactamase substrate specificity [18-21], *penA* upregulation [7, 20, 22, 23] (including CNVs [9]), or loss of penicillin-binding protein 3 [24]; AMC resistance (AMCr) is caused by *penA* upregulation [14, 20]; MEMr is caused by AmrAB-OprA, BpeAB-OprB, or BpeEF-OprC resistance-nodulation-division (RND) multidrug efflux pump regulator loss-of-function [6]; SXT resistance (SXTr) is caused by cumulative mutations in core metabolism pathways coupled with AmrAB-OprA, BpeAB-OprB, or BpeEF-OprC RND efflux pump regulator loss-of-function [6, 9, 25]; and DOX resistance (DOXr) is caused by loss-of-function mutations within the SAM-dependent methyltransferase gene, *BPSL3085*, often in combination with AmrAB-OprA, BpeAB-OprB, or BpeEF-OprC regulator loss-of-function [26]. Our *B. pseudomallei* ARDaP database also includes AmrA and AmrB mutants that are associated with unusual aminoglycoside and macrolide susceptibility [14, 17]. To avoid poor-quality WGS data or incorrect species assignments, the database also includes two conserved genetic targets (Table S1) found only in this bacterium; strains lacking these loci are flagged for further user assessment.

ARDaP algorithm. To achieve high-quality variant calls, ARDaP incorporates several tools into its workflow (full list available at: https://github.com/dsarov/ARDaP). In addition to an organism-specific SQLite database (https://github.com/dsarov/ARDaP/tree/master/Databases), ARDaP requires WGS data, either genomes or metagenomes in paired-end Illumina v1.8+ FASTQ format, or assembled genomes in FASTA format, as input (Figure 1). For genomes in FASTA format, ARDaP first converts to synthetic Illumina v1.8+ reads. For genomes in FASTQ format, ARDaP performs quality filtering

followed by optional random down-sampling to a user-defined coverage (default=50x) to permit more rapid analysis. ARDaP then performs comparative genomic analysis to identify AMR determinants by mapping reads against an annotated reference, followed by alignment processing, SNP and indel identification, coverage assessment, and structural variant identification. High-quality genetic variants (SNPs, indels [<50bp], CNVs, gene gain, and gene loss or truncation) are then annotated. ARDaP next interrogates two databases: i) a customisable CARD [5] database is screened to identify horizontally-acquired AMR genes and to ignore conserved genes that do not confer AMR, and ii) a bespoke AMR determinant database (in this study, a *B. pseudomallei* database) containing species specific AMR determinants. ARDaP databases are created in SQLite and can be readily updated as additional AMR determinants are identified. This database also accommodates stepwise mutations and AMR conferred by ≥2 mutations. Finally, ARDaP can predict AMR by identifying novel high-consequence mutations (i.e. those resulting in a frameshift or nonsense mutations) in known AMR genes. These putative mutants can then be flagged for further investigation with phenotypic AMR testing. ARDaP outputs are presented in a comprehensive, human-readable report (Figure 3), which has been modelled from the report structure described by Crisan and colleagues .

Mixture detection. ARDaP incorporates a minor allelic variant analysis function to permit variant identification from mixed genomes/metagenomes, enabling the detection of emerging AMR determinants (down to 5% abundance). Minor-variant SNPs and indels are identified using the ploidy-aware HaplotypeCaller tool in GATK v4.1; deletions, CNVs, and structural mutations are identified with the ploidy-aware function of Pindel. *B. pseudomallei* strains with known AMR status were mixed at ratios of 5% increments ranging from 5:95 to 95:5, to 55-60x total depth. Two mixtures were created: MSHR0913 (sensitive to all clinically-relevant antibiotics) and MSHR0937 (MEMr; intermediate resistance to AMC and SXT), and MSHR0913 and MSHR8441 (SXTr; decreased susceptibility to CAZ, MEM, and DOX). MSHR0937 and MSHR8441 were chosen as they represent a wide spectrum of clinically-relevant AMR and mutation types (Table 4).

Results and Discussion

Performance of existing AMR tools in *B. pseudomallei.* The validated dataset of 47 *B. pseudomallei* isolates was used to assess the performance and capacity of existing AMR tools to identify AMR determinants in AMR but not antimicrobial-sensitive strains. According to CARD, all 47 genomes were found to harbour AMR determinants; however, all determinants corresponded with conserved genes in *B. pseudomallei* (Table S2). In addition, CARD failed to identify any clinically relevant AMR determinants in the 25 AMR strains. This finding is consistent with *B. pseudomallei* acquiring AMR through chromosomal mutation rather than gene gain, and an inability of current CARD software to identify AMR variants conferred by indels, CNVs, or gene loss/truncation. ARIBA outperformed CARD due to its ability to include missense (although not nonsense) SNPs in its database construction and to identify SNPs and indels in its report outputs. However, we encountered several shortcomings with ARIBA; notably, an inability to differentiate AMR determinants from natural variation, cumbersome and labour-intensive input file requirements, restrictions on database construction (e.g. reference genomes with indels and nonsense mutations cannot be included), and an inability to identify CNVs. In our hands, ARIBA provided comparable information to variant reporting outputs generated by comparative genomic pipelines. However, such efforts require extensive domain-specific knowledge to accurately uncover and interpret AMR determinants. In contrast, ARDaP uses a single and standardised SnpEff-based input, can differentiate natural gene variation from known and putative AMR determinants, can detect CNVs, and provides a user-friendly output that does not require domain-specific knowledge to interpret outputs.

ARDaP development and performance in *B. pseudomallei.* Due to shortcomings in existing AMR software, ARDaP was designed to both identify known AMR determinants and to ignore non-causal genetic variants (Table 2). ARDaP correctly identified all *B. pseudomallei* AMR determinants (Table 1); however, when tested against the 47 validated isolates, one false negative and four false positives were identified. The false negative result (SXT sensitivity rather than decreased SXT susceptibility) was due to an 800kb inversion in 354e that directly impacts *llpE*, a gene that resides between *bpeT* and *bpeEF-oprC* [7]. BpeT is a LysR-type transcriptional regulator that controls expression of BpeEF-OprC [27]. This inversion was not detected due to limitations in short-read Illumina data that led to this

structural variant not easily being identified by Pindel, coupled with the occurrence of this inversion outside of an AMR determinant.

The first of the false positives, chronic cystic fibrosis (CF) isolate MSHR5654, was predicted to be MEMr due to the presence of $BpeT_{Thr314fs}$. However, Etesting showed MEM sensitivity (2 µg/mL) in this strain, just below the MEMr threshold (≥3 µg/mL) [9, 23]. Although alterations in BpeT have been putatively linked with MEMr in MSHR1300 (4 µg/mL) [6] and 354e (6 µg/mL) [7], the role of BpeT mutations in conferring MEMr is contentious [25]. In support of this notion, MSHR1300 also encodes $AmrR_{K13fs}$, a TetR-family *cis*-acting repressor of the AmrAB-OprA RND efflux pump, which likely causes MEMr in its own right [6], and in 354e, the ~800kb inversion likely also affects other AMR-conferring genes besides *bpeT*. As such, the *B. pseudomallei* ARDaP database was updated to flag *bpeT* variants as stepwise mutations rather than solely conferring MEMr (Table 1; Table 2), thereby correcting the original false-positive call for MSHR5654. This issue highlights the complexity of unravelling AMR determinants, and in this case, the need for additional work to determine a role, if any, for *bpeT* mutations in conferring MEMr.

The remaining false-positive strains, MSHR5666, MSHR5669, and MSHR5670, were all obtained from a chronically-infected CF patient, CF9 [9]. All encode a SAM-dependent methyltransferase truncation ($BPSL3085_{A88fs}$) [23] and were predicted by ARDaP to be DOXr. We also observed $BPSL3085_{A88fs}$ in an unrelated DOXr chronic CF strain, Bp1651 [28] (Table 1). BPSL3085 mutations confer DOXr by altering ribosomal methylation patterns [9, 26]. However, all three strains remained DOX-sensitive (1.5 µg/mL) despite other CF9 strains encoding $BPSL3085_{A88fs}$ and being DOXr (MSHR5665: MIC=6 µg/mL; MSHR5667: MIC=48 µg/mL; Table 1) [9]. The higher DOX MIC in MSHR5667 is attributable to a second mutation ($AmrR_{L132P}$; Table 1). We postulate that MSHR5666, MSHR5669, and MSHR5670 encode an unidentified mutation that reverts them to a DOX-sensitive phenotype. Notably, all longitudinal CF9 isolates, including MSHR5666 and MSHR5669, encode *mutS* mutations, resulting in a hypermutator phenotype [9, 23]. Therefore, identifying the causal basis for this reversion is non-trivial due to the large number of mutations (range: 112-157) accrued by these hypermutator strains [9]. In addition, MSHR5670 was predicted to be SXTr due to $Ptr1_{W116R}$, $MetF_{Q28P}$, and Dut_{V77A} variants, yet exhibited SXT sensitivity (Table 2). The cause of SXTr reversion in this strain is also currently unknown.

Importance of including natural genetic variation in AMR databases. Accurate prediction of novel AMR determinants requires thorough cataloguing of both confirmed AMR-causing mutations and natural variation in AMR-encoding genes to avoid false positives. As an illustration, a PenA β-lactamase missense mutation (K96243 numbering: $PenA_{S78F}$; encoded by *BPSS0946*) has been linked to AMCr [18, 21, 28]. However, we found that $PenA_{S78F}$ alone is unlikely to cause AMCr due to its presence in genetically diverse AMC-sensitive strains (MIC=3-4 µg/mL in strains MSHR0291, MSHR0668, MSHR0848, MSHR0911, MSHR1711, MSHR2212, MSHR3902, MSHR4797, MSHR8392, and MSHR9887). Instead, AMCr is likely conferred by both $PenA_{S78F}$ and *penA* upregulation, the latter of which can be caused by mutations within the 5' untranslated region [20] or *penA* CNVs [9]. We therefore included $PenA_{S78F}$ as a putative stepwise mutation (Table 2), with an additional *penA* upregulation mutation required to confer the AMCr phenotype. In another example, we observed that both AMR and antimicrobial-sensitive strains can possess 3'-truncated *amrR* (Table 2). Multiple frameshift mutations and deletions in *amrR* are associated with MEMr [6] due to loss-of-repressor function (Figure 3). However, 3' region mutations (residues ~210-223) do not cause MEMr (Table 2). To accommodate this natural genetic variation, we coded ARDaP to ignore these non-causal 3' variants, thereby greatly reducing false-positive MEMr rates.

No evidence of IPM-resistant *B. pseudomallei*. In most melioidosis treatment guidelines, IPM has been replaced by MEM due to neurotoxicity concerns.[29] However, the recent discovery of MEMr *B. pseudomallei* has resurrected IPM as a treatment option due to a lack of cross-resistance between these carbapenems [6] and exceedingly low rates of reported IPM resistance (IPMr) [30]. The one study reporting an IPMr (MIC=8 µg/mL) *B. pseudomallei* strain, Bp1651, attributed this phenotype to a $PenA_{T147A}$ mutation (K96243 numbering: $PenA_{T153A}$) combined with upregulation due to a promoter mutation [28]. We subsequently refuted the role of the $PenA_{T147A}$ variant alone in conferring IPMr by identifying three genetically unrelated $PenA_{T153A}$-encoding strains that were IPM-sensitive [6]. Further, this variant is dominant (>50%) in publicly available *B. pseudomallei* genomes, most of which exhibit wild-type antimicrobial sensitivity. Given that $PenA_{T147A}$ occurs at a very high rate in the wild-type *B. pseudomallei* population, and none have been shown to exhibit IPMr, this mutant has not been included in our ARDaP database. However, this variant can readily be added as a stepwise AMR determinant should further evidence come to light about its role in conferring AMR.

Reversions and unusual antimicrobial susceptibility. Aminoglycoside- and macrolide-class antibiotics are typically not included in melioidosis treatment regimens due to near-ubiquitous intrinsic resistance; indeed, GEN resistance is commonly used for *B. pseudomallei* selection [17]. However, rare cases of sensitivity have been documented, such as in ST-881 and ST-997 strains from Sarawak, Malaysian Borneo, which naturally encode $AmrB_{T368R}$, resulting in AmrAB-OprA loss-of-function and unusual aminoglycoside and macrolide susceptibility [17]. An AmrAB-OprA loss-of-function variant has also been described in Bp1651 ($AmrB_{A254fs}$) [28]. Although ARDaP detected *amrR* loss in MSHR1043, the co-presence of *amrA* loss ($AmrA_{L247fs}$) resulted in reversion of MEMr to a wild-type MIC (0.75 µg/mL). This reversion also causes unusual gentamicin (MIC=1 µg/mL) [14] and presumably kanamycin and azithromycin sensitivity. Given their confounding potential, we incorporated these reversions into ARDaP to more accurately reflect the true strain phenotype. In addition, strains encoding AmrAB-OprA loss-of-function variants are at far lower risk of developing MEMr than wild-type strains, supporting longer-term MEM use in such cases. These findings highlight the value of including sensitivity-conferring variants in AMR prediction databases by increasing the antibiotic arsenal in naturally multidrug-resistant pathogens where treatment options are limited.

AMR predictive capacity of ARDaP. We tested ARDaP's predictive capacity to identify the causative mutation/s in three clinical MEMr strains (MSHR1058, MSHR1174, and MSHR8777; MEM MIC range: 4-12 µg/mL; Table 3) with no previously reported AMR determinants. ARDaP identified novel *amrR* mutations in each strain, all of which resulted in AmrR loss-of-function ($AmrR_{\Delta P81\text{-}H223}$ in MSHR1058; $AmrR_{G149fs}$ in MSHR1174; $AmrR_{\Delta A128\text{-}H223}$ in MSHR8777; Figure 3). All patients received MEM treatment prior to isolate retrieval, consistent with selection pressure driving AMR. These findings provide further confirmation of the link between MEM treatment and potential treatment failure due to AmrR mutability [6], and demonstrate the value of ARDaP for predicting novel AMR determinants.

ARDaP performance on mixed sequence data. The ARDaP algorithm is mixture-aware, an important feature for detecting emerging AMR determinants in mixed strain data (e.g. non-purified colonies, culture sweeps, clinical specimens). To assess the performance of the mixture function in ARDaP, Illumina reads from antimicrobial-sensitive and AMR *B. pseudomallei* strains (Table 4) were mixed at 5% incremental ratios ranging from 5:95 to 95:5. ARDaP identified one AMR determinant down to the lowest tested ratio of 5% minor allele frequency: a *penA* 10x CNV from MSHR8441 (Table

4). The other determinants were identified by ARDaP when present at minor allele frequencies of 10% (BPSL3085$_{S130L}$), 20% (BpeR$_{Asp176Ala}$), 25% (Ptr1$_{A22_G23ins_R\text{-}R\text{-}A}$ and MetF$_{Gln142*}$) and 30% (AmrR$_{\Delta V62\text{-}H223}$) (Table 4). Although CNVs were readily identified at low allelic frequencies, and the detection of SNPs and indels ranged from 10 to 25%, gene truncations had the lowest sensitivity due to the challenge of discriminating gene loss from Illumina depth coverage variation, coupled with inherent limitations in short-read data mapping. Next, ARDaP was tested on a previously detected AmrR mixture from strain MSHR9021, which encodes AmrR$_{S166P}$ and AmrR$_{A145fs}$ variants at ~66% and ~33% allele frequencies, respectively [6] (Table 1). ARDaP detected AmrR$_{S166P}$ and AmrR$_{A145fs}$ at allele frequencies of 63% and 31%, respectively, thus closely reflecting their known proportions. Overall, ARDaP confidently identified all mixtures, albeit with varying sensitivities. Further validation on specific variant mixtures is recommended when new mixtures are identified to determine their sensitivity. Deeper sequencing (e.g. 100-500x) should enable more robust mixture detection at lower allele frequencies.

ARDaP reports. ARDaP generates an easy-to-interpret report that summarises the AMR determinants and associated antibiotic phenotype/s for each genome. This report summarises AMR findings for first-line, second-line, and tertiary antibiotics (Figure 2), along with instances of unusual antibiotic susceptibility, and has been designed to prioritise a clinical workflow. This report also lists stepwise AMR determinants, thereby informing early treatment shifts that mitigate the risk of AMR emergence and fixation. This easy-to-interpret report represents a major improvement over current software for AMR annotation such as ARIBA and CARD, both of which require an intimate understanding of AMR determinants to correctly interpret outputs and to ignore natural genetic variation. The AMR report produced by ARDaP represents an important step towards the incorporation of WGS as a routine tool for guiding best-practice AMR stewardship and personalised treatment regimens in the clinical diagnostic setting.

Funding

This work was supported by the National Health and Medical Research Council (1046812 and 1098337 to B.J.C., E.P.P., and D.S.S.; and 1131932 to B.J.C.), Advance Queensland (AQIRF0362018 to E.P.P, and AQRF13016-17RD2 to D.S.S.), the Australian Government (Research Training Scholarship to D.E.M.), and James Cook University (International Postgraduate Research Scholarship to E.S.). The funders had no role in study design; in the collection, analysis, or interpretation of data; in the writing of this report; or in the decision to submit for publication. The corresponding author had full access to all the data in the study and has final responsibility for the decision to submit for publication.

Acknowledgements

We thank Associate Professor Rob Baird and the microbiology staff at Royal Darwin Hospital for their support and expertise in identifying and characterising *B. pseudomallei* isolates, and Vanessa Rigas, Glenda Harrington, and Mark Mayo for isolate inventory support.

Author contributions

DSS conceived of the study; EPP and DSS designed the study; JRW, EPP, and DSS generated laboratory data and performed laboratory analyses; EJS and DSS wrote the software; DEM, EPP, and DSS performed data analysis, literature searches, figure generation, software testing, and feature development; BJC provided isolates; DEM, EPP, and DSS wrote the manuscript; and BJC, EPP, and DSS obtained funding for the study. All authors approved of the final manuscript.

Competing interests

The authors have no financial or non-financial competing interests.

Tables

Table 1. Antimicrobial resistance (AMR) determinants in 25 *Burkholderia pseudomallei* strains with verified AMR phenotypes, plus strains conferring unusual antimicrobial susceptibility and stepwise AMR variants[§].

Patient ID	Isolate	ST	Genome accession	Antibiotic MIC (µg/mL)[¥]	Stepwise variant[†]	AMR determinant	Reference/s
Thai patient	316c	17	SRR2975745	CAZr (64)	---	$PenA_{P173S}$	[20, 21]
Thai patient	354e	78	AHJD00000000.1	CAZi (6)	---	*penA* -78G>A	[7]
				SXTi (3)	BpeT structural variant[‡]	$Ptr1_{R21fs}$	
Australian patient	Bp1651	880	SRR2102060	CAZr (≥128)	*penA* -78G>A	$PenA_{D245G}$	[28]
				Unusual sensitivity to aminoglycosides (GEN, KAN) and macrolides (AZM)	---	$AmrB_{A254fs}$	
				DOXr (16)	---	$BPSL3085_{A88fs}$	
				AMCr (64/32)	*penA* -78G>A	$PenA_{S78F}$	
Pre-DPMS 89	MSHR0052	722	SRR5818275	MEMr (8)	---	$AmrR_{E190*}$	[6]
				DOXr (48)	$AmrR_{E190*}$	$BPSL3085_{V211M}$	
Australian patient	MSHR0292	236	SRR4254580	DOXr (16)	$AmrR_{S174P}$	$BPSL3085_{V40A}$	[26]
P215	MSHR0663	36	SRR2887062	SXTr (≥32)	$BpeT_{H278Y}$	$Ptr1_{R21fs}$	[6]; This study
	MSHR0937	36	SRR2886988	AMCi (12/6), MEMr (6)	---	$BpeR_{D176A}$	
				SXTi (3)	$BpeR_{D176A}$	$MetF_{Q142*}$	
P179	MSHR0678	114	SRR6075118	MEMr (3)	---	$AmrR_{E21D}$	[6]
	MSHR0800	114	SRR6075115	MEMr (6), DOXi (8)	---	$BpeR_{L85fs}$	
P337	MSHR1226	333	SRR9598635	CAZr (≥256)	---	$PenA_{C75Y}$	[20]
	MSHR1300	333	SRR6075114	CAZr (≥256)	*penA* -78G>A	$PenA_{C75Y}$	[20]
				MEMr (4)	---	$AmrR_{K13fs}$	[6]

				SXTi (3)	---	$AmrR_{K13fs}$	This study
P595	MSHR3683	144	SRR11678542	DOXi (12)	---	$BPSL3085_{A88fs}$	[9, 28]
P608	MSHR4083	36	SRR2887030	SXTr (24)	$AmrR_{\Delta A153\text{-}D156}$	Dut_{G91A}	[6]
				MEMr (6)	---	$AmrR_{\Delta A153\text{-}D156}$	
CF6	MSHR5654	1040	SRR3404570	CAZr (≥256)	PenA 30x CNV	$PenA_{C75Y}$	[9]
				CIPr (≥32)	---	$GyrA_{Y77S}$	
				SXTr (≥32)	$BpeT_{T314fs}$	$Ptr1_{R21fs}$	
CF9	MSHR5665	252	SRR3404582	SXTr (≥32)	$Ptr1_{W116R}$; $MetF_{Q28P}$	Dut_{V77A}	[9, 23]
				DOXi (6)	---	$BPSL3085_{A88fs}$	
	MSHR5666	252	SRR3404597	DOXs (1)[#]	---	$BPSL3085_{A88fs}$	
				SXTr (≥32)	$Ptr1_{W116R}$; $MetF_{Q28P}$	Dut_{V77A}	
	MSHR5667	252	SRR3404598	SXTr (≥32)	$MetF_{N162T}$; $AmrR_{L132P}$	Dut_{N99S}	
				MEMr (4)	---	$AmrR_{L132P}$	
				DOXr (48)	$AmrR_{L132P}$	$BPSL3085_{A88fs}$	
	MSHR5669	252	SRR3404599	DOXi (6)	---	$BPSL3085_{A88fs}$	
				SXTr (≥32)	$Ptr1_{W116R}$; $MetF_{Q28P}$	Dut_{V77A}	
P726	MSHR6755	975	SRR6075122	MEMr (3)	---	$AmrR_{\Delta V60\text{-}C63}$	[6]
P797	MSHR7587	437	SRR6075129	MEMr (4)	---	$AmrR_{G30D}$	[6]
	MSHR7929	437	SRR6075126	SXTr (4)	$AmrR_{G30D}$	$BPSL2263_{\Delta D110\text{-}G116}$	
				MEMr (4)	---	$AmrR_{G30D}$	
CF11	MSHR8441	46	SRR3382162	CAZi (12)	---	PenA 10x CNV	[6, 9]
				SXTr (≥32)	$AmrR_{\Delta V62\text{-}H223}$[c]	$Ptr1_{A22\text{-}G23ins_R\text{-}R\text{-}A}$	
				Decreased DOX susceptibility (4)	$AmrR_{\Delta V62\text{-}H223}$[c]	$BPSL3085_{S130L}$	
				Decreased MEM susceptibility (2)	---	$AmrR_{\Delta V62\text{-}H223}$[c]	
	MSHR8442	46	SRR3404603	SXTr (≥32)	$AmrR_{\Delta V62\text{-}H223}$[c]	$Ptr1_{A22\text{-}G23ins_R\text{-}R\text{-}A}$	
				DOXi (8)	$AmrR_{\Delta V62\text{-}H223}$[c]	$BPSL3085_{S130L}$	

				MEMr (3)	---	$AmrR_{\Delta V62\text{-}H223}$[c]	
Non-DPMS QP09	MSHR8481	1378	SRR6075123	MEMr (6)	---	$AmrR_{\Delta A70\text{-}H223}$[d]	[6]
P989	MSHR9021[a]	132	SRR6075127	MEMr (3)		$AmrR_{S166P}$; $AmrR_{A145fs}$[b]	[6]
Strains with unusual antibiotic sensitivity							
P314	MSHR1043[§]	131	SRR6380769	Unusual GEN sensitivity	---	$AmrA_{L247fs}$	[14]
Malaysian patient	MSHR5089[§]	881	SRR2975737	Unusual sensitivity to aminoglycosides (GEN, KAN) and macrolides (AZM)	---	$AmrB_{T368R}$	[17]
Strains with stepwise variants							
P179	MSHR0535	114	SRR6075120	DOXs (4)[§]	$BPSL3085_{R104fs}$	---	[6]
Thai patient	354e	78	AHJD00000000.1	AMCs (4/2)	*penA* -78G>A	---	[7]

Abbreviations: AZM, azithromycin; CAZ, ceftazidime; CIP, ciprofloxacin; CNV, copy-number variation; DOX, doxycycline; GEN, gentamicin; KAN, kanamycin; MEM, meropenem; MIC, minimum inhibitory concentration; SXT, co-trimoxazole

[¥]i, intermediate; r, resistant; s, sensitive

[†]Where applicable

[‡]Encodes an 800kb inversion that direct affects *llpE*, the intervening gene between the BpeT transcriptional regulator and its BpeEF-OprC efflux pump [7]

[#]False positive; isolate remained sensitive despite encoding a known AMR determinant. Cause of reversion unknown

[§]Strains MSHR1043 [14] and MSHR5089 [17] encode unusual antimicrobial susceptibility, and are susceptible to all five clinically-relevant antibiotics (i.e. AMC, CAZ, DOX, MEM, SXT), and strains MSHR0535 and 354e encode stepwise variants that elevate the DOX and AMC MICs, respectively, but do not exceed the established resistance cut-off for these antibiotics

[a]MSHR9021 was intentionally sequenced from a potentially mixed population to capture population diversity. Two AmrR mutants, $AmrR_{S166P}$ and $AmrR_{A145fs}$, were present at ratios of ~66% and 33%, respectively [6]. In the non-mixture mode, only the dominant variant, $AmrR_{S166P}$, is detected.

[b]Frameshift indel shortens protein length from 223 to 183 residues

[c]Frameshift indel increases protein length from 223 to 285 residues

[d]Frameshift indel shortens protein length from 223 to 117 residues

Table 2. *Burkholderia pseudomallei* strains phenotypically confirmed to be sensitive towards the five clinically-relevant antibiotics, and associated genome data.

Patient ID	Isolate	ST	Genome accession	Antibiotic MIC (µg/mL)*					Reference/s
				AMC	CAZ	DOX	MEM	SXT	
Australian patient	MSHR0293	236	SRR4254579	2/1	1	1	0.5	0.4	[26]
P179	MSHR0492	114	SRR6075119	1.5/0.75	1.5	1	1.5	1	[6]
	MSHR0934	114	SRR6075116	2/1	2	1	0.75	1	
P337	MSHR1141	333	SRR2975732	1.5/0.75	1.5	1	0.75	0.75	[20]
P608	MSHR3763	36	SRR2887021	4/2	2	0.75	0.75	3	[6]
P726	MSHR5864	975	SRR6075121	3/1.5	1.5	1	0.75	1.5	[6]
P797	MSHR6522	437	SRR6075128	2/1	1.5	1	0.5	1.5	[6]
CF1	MSHR0913	279	SRR3404575	2/1	3	1	1	0.5	[9]
	MSHR1053	279	SRR3404578	1.5/0.75	2	1	0.5	1	
Malaysian patient	MSHR5093	881	SRR2975738	6/3	4	1.5	1	3	[17]
Malaysian patient	MSHR5104	881	SRR2975740	2/1	0.75	1	0.19	0.75	[17]
CF6	MSHR5651	1040	SRR3381886	1.5/0.75	1.5	0.38	0.5	0.75	[9, 23]
CF9	MSHR5662	252	SRR3381885	2	2	2	0.75	0.5	[9, 23]
	MSHR5670[#]	252	SRR3404600	1.5/0.75	2	2	0.75	0.5	
CF10	MSHR8438	442	SRR3382015	2/1	3	1	1	0.25	[9, 23]
	MSHR8440	442	SRR3404601	2/1	3	1	0.75	0.38	

Abbreviations: AMC, amoxicillin-clavulanate; CAZ, ceftazidime; DOX, doxycycline; MEM, meropenem; ST, multilocus sequence type; SXT, co-trimoxazole

*According to Etesting

[#]False positive; isolate remained sensitive despite encoding known AMR determinants towards DOX (BPSL3085$_{A88fs}$) and SXT (Ptr1$_{W116R}$; MetF$_{Q28P}$; Dut$_{V77A}$). Cause of reversion unknown

Table 3. ARDaP prediction in three meropenem-resistant *Burkholderia pseudomallei* isolates with previously unknown antimicrobial resistance (AMR) determinants.

Strain ID	ARDaP-predicted AMR determinant	MEM MIC (µg/mL)	Reference/s
MSHR1058	$AmrR_{\Delta P81\text{-}H223}$[a]	12 (MEM)	[6]; This study
MSHR1174	$AmrR_{G149fs}$[b]	6 (MEM)	This study
MSHR8777	$AmrR_{\Delta A128\text{-}H223}$[c]	4 (MEM)	This study

[a]Previously undescribed 3' *amrR* deletion shortens protein length from 223 to 130 residues.

[b]Previously undescribed 11bp insertion shortens protein length from 223 to 178 residues.

[c]Previously undescribed 3' *amrR* insertion increases protein length from 223 to 621 residues.

Table 4. ARDaP detection limits for AMR determinants in synthetic mixtures.

AMR determinant	K96243 gene ID	Mutation type	AMR minor allele detection (%)
Mixture #1: MSHR0913 (sensitive strain) and MSHR0937 (resistant to MEM; intermediate resistance to AMC and SXT)			
$BpeR_{Asp176Ala}$	*BPSL0812*	Missense SNP	20
$MetF_{Gln142*}$	*BPSL3288*	Nonsense SNP	25
Mixture #2: MSHR0913 (sensitive strain) and MSHR8441 (resistant to MEM and SXT; intermediate resistance to CAZ and DOX)			
$AmrR_{\Delta V62\text{-}H223}$	*BPSL1805*	Gene loss/truncation	30[†]
$BPSL3085_{S130L}$	*BPSL3085*	Missense SNP	10
$Ptr1_{A22\text{-}G23ins_R\text{-}R\text{-}A}$	*BPSS0039*	In-frame insertion	25
penA 10x	*BPSS0946*	Copy-number variation (10x duplication)	5

AMR, antimicrobial resistance; CAZ, ceftazidime; CIP, ciprofloxacin; DOX, doxycycline; MEM, meropenem; ND, not detected; SNP, single-nucleotide polymorphism; SXT, co-trimoxazole

[†]Represents the lowest allele frequency that was consistently detected in our mixed strain dataset.

Figures

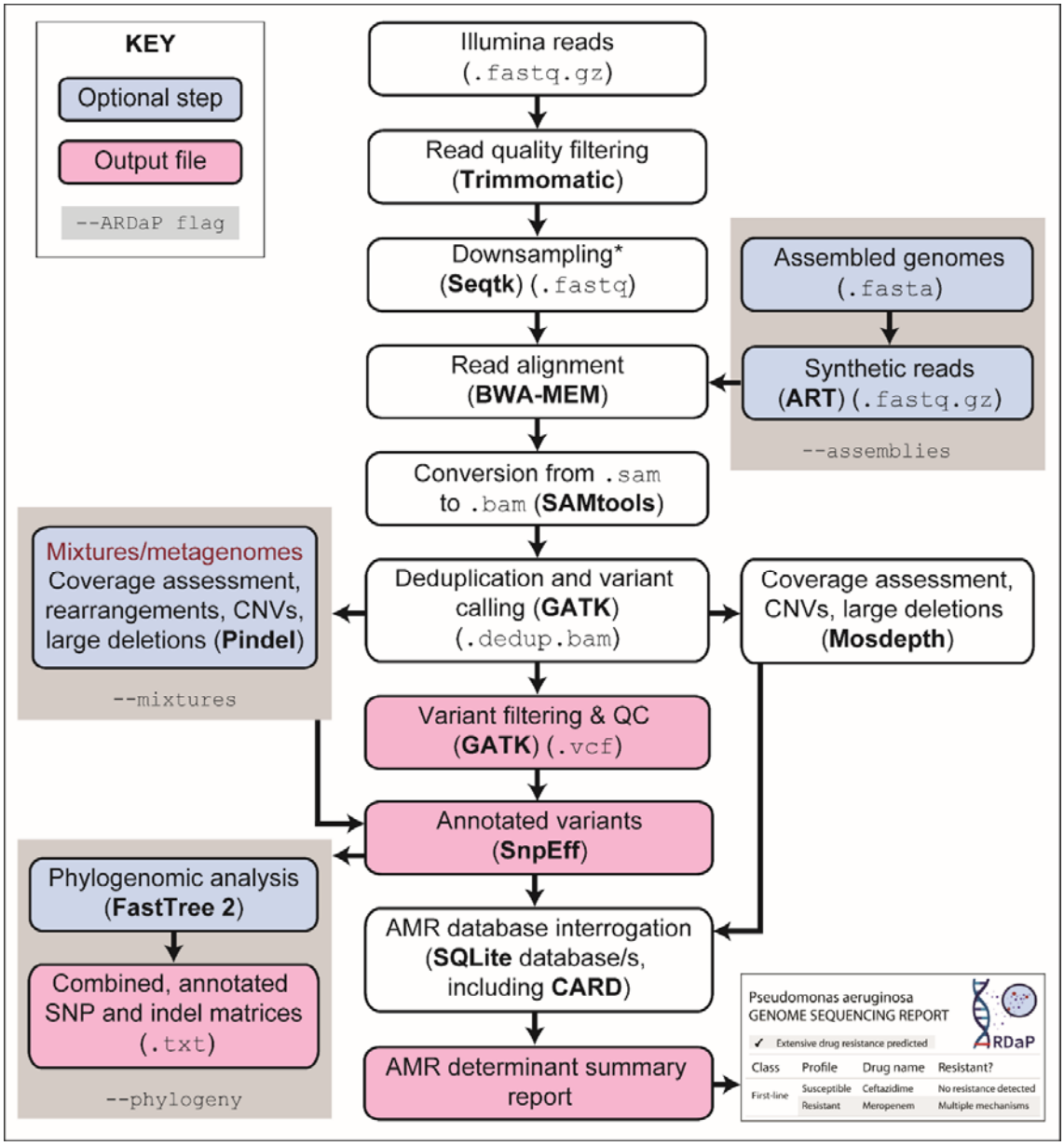

Figure 1. ARDaP pipeline. The user inputs assembled genome/s or raw sequencing reads, and a reference genome sequence. ARDaP then performs read alignment, read processing, deduplication, and variant identification. An optional phylogenetic analysis is also performed (if specified). Coverage assessment is undertaken on either single or mixed genomes (if specified); genetic variants are then annotated and antimicrobial resistance database/s interrogated. Finally, ARDaP produces a summary report of antimicrobial resistance determinants for each strain (Figure 2). *Downsampling is carried out by default but can be turned off using the `--size 0` flag in ARDaP.

BURKHOLDERIA PSEUDOMALLEI

GENOME SEQUENCING REPORT

NOT FOR DIAGNOSTIC USE

Patient Name	James Smith	Barcode	BARCODE
Birth Date	1/01/1990	Patient ID	MSHR5654
Location	Darwin	Sample Type	Blood
Sample Source	Blood	Sample Date	2020-07-30
Sample ID	MSHR5654	Sequenced From	Cultured isolate
Reporting Lab	RDH	Report Date/Time	2020-07-30
Requested By	Dr. Requestor Name	Requestor Contact	req_contact@genome.com

Summary

The specimen was interogated with the *Burkholderia pseudomallei* database

It is predicted to be resistant to Ceftazidime, Trimethoprim/sulfamethoxazole, Ciprofloxacin.

Drug Susceptibility

Resistance is reported when a high-confidence resistance conferring mutation is detected. "No mutation detected" does not exclude the possibility of resistance

- ☐ No drug resistance predicted
- ☐ Mono-resistance predicted
- ☐ Multi-drug resistance predicted
- ☑ Extensive drug resistance predicted

Drug class	Interpretation	Drug	Resistance determinant
First-line	Sensitive	Meropenem	No resistance detected
	Resistant	Ceftazidime	Multiple determinants
Second-line	Sensitive	Imipenem	No resistance detected
	Resistant	Trimethoprim/sulfamethoxazole	Multiple determinants
	Sensitive	Amoxicillin/Clavulanic acid	No resistance detected
	Sensitive	Doxycycline	No resistance detected

Extended/non-clinical drug susceptibility

Drug class	Interpretation	Drug	Resistance determinant
Tertiary	Resistant	Ciprofloxacin	gyrA BPSL2521 Tyr77Ser

Intrinsic drug resistance/unusual drug susceptibility

Drug class	Interpretation	Drug	Mechanism of sensitivity
Intrinsic	Resistant	Gentamicin	No sensitivity detected

Antimicrobial determinant details

gyrA|BPSL2521|Tyr77Ser|CIPr|Confers CIP MIC≥32 µg/mL

penA|BPSS0946|Cys75Tyr|CAZr|Confers CAZ MIC≥256 µg/mL; also known as PenA Cys69Tyr

BPSS0039|ptr1|Arg21fs|SXTr|<PRECURSOR MUTATION>. Confers SXT MIC≥32 µg/mL in combination with BpeT Thr314fs, BpeT His274Tyr, or BpeT loss. Confers intermediate resistance by itself

BPSS0290|bpeT|Thr314fs|SXTr|<PRECURSOR MUTATION>. Confers SXT MIC≥32 µg/mL in combination with Ptr1 Arg20fs

penA|BPSS0946|upregulation|CAZr|0|10x copy number confers MIC=12 ug/uL; 2x copy number does not increase MIC (Viberg et al., 2017 MBio 8(2):00356-17)

BPSS0039|ptr1|loss|SXTr|1|Confers SXT resistance in combination with efflux pump upregulation. Confers intermediate resistance by itself

BPSS0290|bpeT|loss|SXTr|0|<PRECURSOR MUTATION>. Isolates with this mutation are more likely to develop SXT resistance

Natural variation that does not confer antimicrobial resistance

amrR|BPSL1805|Val222fs|None|Known variation in amrR that does not cause antibiotic resistance

Figure 2. Example antimicrobial resistance (AMR) summary report produced by ARDaP. The final step in the ARDaP pipeline is the production of a user-friendly report that summarises patient and sample details, confirms that the given isolate is the expected species (in this case, *Burkholderia pseudomallei*), identifies any predicted AMR (including what mutation/s has/have been detected) and what antibiotic/s have been affected, identifies unusual antimicrobial sensitivity and natural variation that does not confer AMR, and identifies stepwise mutations that lower the barrier to AMR development.

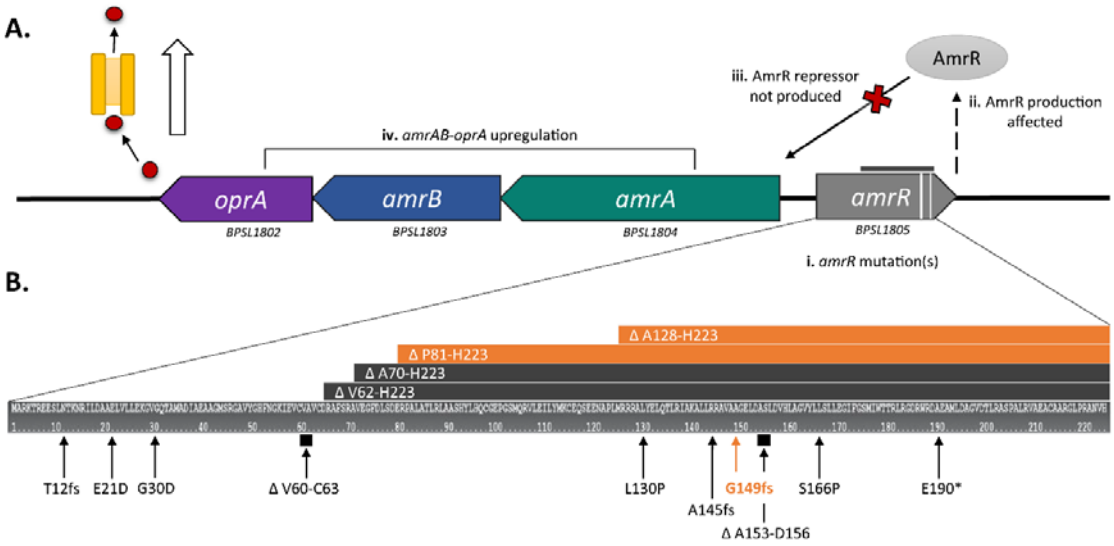

Figure 3. Operon organisation of the *Burkholderia pseudomallei* AmrAB-oprA resistance-nodulation-division efflux pump and loss-of-function mutations in its TetR-type regulator, AmrR. A. Transcriptional organisation of the *amrR* (*BPSL1805*), *amrA* (*BPSL1804*), *amrB* (*BPSL1803*) and *oprA* (*BPSL1802*) operon, and summary of how (i) *amrR* mutations cause (ii) loss-of-function of AmrR, which (iii) no longer represses expression of the resistance-nodulation-division AmrAB-OprA efflux pump, resulting in (iv) efflux pump over-expression and resistance to meropenem and aminoglycoside antibiotics. B. Distribution and annotation of *amrR* mutations. Eleven previously observed *amrR* mutations (in black) [6] have been augmented with three novel mutations identified in the current study (orange); $AmrR_{G149fs}$, $AmrR_{\Delta P81\text{-}H223}$, and $AmrR_{\Delta A128\text{-}H223}$, all of which cause *amrR* loss-of-function, resulting in efflux pump overexpression and meropenem resistance.

References

1. Didelot X, Bowden R, Wilson DJ, Peto TEA, Crook DW. Transforming clinical microbiology with bacterial genome sequencing. Nat Rev Genet **2012**; 13(9): 601-12.
2. Lakin SM, Dean C, Noyes NR, et al. MEGARes: an antimicrobial resistance database for high throughput sequencing. Nucleic acids research **2017**; 45(D1): D574-d80.
3. Gupta SK, Padmanabhan BR, Diene SM, et al. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrobial agents and chemotherapy **2014**; 58(1): 212-20.
4. Hunt M, Mather AE, Sanchez-Buso L, et al. ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. Microbial genomics **2017**; 3(10): e000131.
5. Jia B, Raphenya AR, Alcock B, et al. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic acids research **2017**; 45(D1): D566-d73.
6. Sarovich DS, Webb JR, Pitman MC, et al. Raising the stakes: Loss of efflux-pump regulation decreases meropenem susceptibility in *Burkholderia pseudomallei*. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **2018**.
7. Hayden HS, Lim R, Brittnacher MJ, et al. Evolution of *Burkholderia pseudomallei* in recurrent melioidosis. PloS one **2012**; 7(5): e36507.
8. Tristram S, Jacobs MR, Appelbaum PC. Antimicrobial resistance in *Haemophilus influenzae*. Clin Microbiol Rev **2007**; 20(2): 368-89.
9. Viberg LT, Sarovich DS, Kidd TJ, et al. Within-host evolution of *Burkholderia pseudomallei* during chronic infection of seven Australasian cystic fibrosis patients. mBio **2017**; 8(2).
10. Nicoloff H, Hjort K, Levin BR, Andersson DI. The high prevalence of antibiotic heteroresistance in pathogenic bacteria is mainly caused by gene amplification. Nat Microbiol **2019**; 4(3): 504-14.
11. Schweizer HP. Mechanisms of antibiotic resistance in *Burkholderia pseudomallei*: implications for treatment of melioidosis. Future Microbiol **2012**; 7(12): 1389-99.
12. Dance D. Treatment and prophylaxis of melioidosis. International journal of antimicrobial agents **2014**; 43(4): 310-8.
13. Limmathurotsakul D, Peacock SJ. Melioidosis: A clinical overview. Br Med Bull **2011**; 99(1): 125-39.
14. Price EP, Sarovich DS, Mayo M, et al. Within-host evolution of *Burkholderia pseudomallei* over a twelve-year chronic carriage infection. mBio **2013**; 4(4): e00388-13.
15. Crowe A, McMahon N, Currie BJ, Baird RW. Current antimicrobial susceptibility of first-episode melioidosis *Burkholderia pseudomallei* isolates from the Northern Territory, Australia. International journal of antimicrobial agents **2014**; 44(2): 160-2.
16. Maloney S, Engler C, Norton R. Epidemiological cut-off value of clinical isolates of *Burkholderia pseudomallei* from northern Queensland to meropenem, ceftazidime, trimethoprim/sulfamethoxazole and doxycycline by the microbroth dilution method. Journal of global antimicrobial resistance **2017**; 10: 291-4.
17. Podin Y, Sarovich DS, Price EP, et al. *Burkholderia pseudomallei* isolates from Sarawak, Malaysian Borneo, are predominantly susceptible to aminoglycosides and macrolides. Antimicrobial agents and chemotherapy **2014**; 58(1): 162-6.
18. Rholl DA, Papp-Wallace KM, Tomaras AP, Vasil ML, Bonomo RA, Schweizer HP. Molecular investigations of PenA-mediated β-lactam resistance in *Burkholderia pseudomallei*. Front Microbiol **2011**; 2: 139.
19. Sam IC, See KH, Puthucheary SD. Variations in ceftazidime and amoxicillin-clavulanate susceptibilities within a clonal infection of *Burkholderia pseudomallei*. Journal of clinical microbiology **2009**; 47(5): 1556-8.

20. Sarovich DS, Price EP, Limmathurotsakul D, et al. Development of ceftazidime resistance in an acute *Burkholderia pseudomallei* infection. Infect Drug Resist **2012**; 5: 129-32.
21. Tribuddharat C, Moore RA, Baker P, Woods DE. *Burkholderia pseudomallei* class A β-lactamase mutations that confer selective resistance against ceftazidime or clavulanic acid inhibition. Antimicrobial agents and chemotherapy **2003**; 47(7): 2082-7.
22. Chirakul S, Norris MH, Pagdepanichkit S, et al. Transcriptional and post-transcriptional regulation of PenA β-lactamase in acquired *Burkholderia pseudomallei* β-lactam resistance. Sci Rep **2018**; 8(1): 10652.
23. Price EP, Viberg LT, Kidd TJ, Bell SC, Currie BJ, Sarovich DS. Transcriptomic analysis of longitudinal *Burkholderia pseudomallei* infecting the cystic fibrosis lung. Microbial genomics **2018**; 4(8).
24. Chantratita N, Rholl DA, Sim B, et al. Antimicrobial resistance to ceftazidime involving loss of penicillin-binding protein 3 in *Burkholderia pseudomallei*. Proceedings of the National Academy of Sciences of the United States of America **2011**; 108(41): 17165-70.
25. Podnecky NL, Rhodes KA, Mima T, et al. Mechanisms of resistance to folate pathway inhibitors in *Burkholderia pseudomallei*: deviation from the norm. mBio **2017**; 8(5).
26. Webb JR, Price EP, Currie BJ, Sarovich DS. Loss of methyltransferase function and increased efflux activity leads to doxycycline resistance in *Burkholderia pseudomallei*. Antimicrobial agents and chemotherapy **2017**; 61(6).
27. Rhodes KA, Somprasong N, Podnecky NL, Mima T, Chirakul S, Schweizer HP. Molecular determinants of *Burkholderia pseudomallei* BpeEF-OprC efflux pump expression. Microbiology (Reading, England) **2018**; 164(9): 1156-67.
28. Bugrysheva JV, Sue D, Gee JE, et al. Antibiotic resistance markers in *Burkholderia pseudomallei* strain Bp1651 identified by genome sequence analysis. Antimicrobial agents and chemotherapy **2017**; 61(6).
29. Currie BJ. Melioidosis: evolving concepts in epidemiology, pathogenesis, and treatment. Seminars in respiratory and critical care medicine **2015**; 36(1): 111-25.
30. Wuthiekanun V, Amornchai P, Saiprom N, et al. Survey of antimicrobial resistance in clinical *Burkholderia pseudomallei* isolates over two decades in Northeast Thailand. Antimicrobial agents and chemotherapy **2011**; 55(11): 5388-91.