1 Taking the next-gen step: comprehensive antimicrobial resistance detection from *Burkholderia* 2 *pseudomallei* genomes

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28 Abstract

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

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

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

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

49 Introduction

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

56 Whole-genome sequencing (WGS) holds great potential for comprehensive AMR detection and
57 prediction from bacterial genomes by identifying all AMR determinants in a single genome or
58 metagenome [2], circumventing the need for multiple and often laborious diagnostic methods. Existing
59 bioinformatic tools such as ARG-ANNOT [3], Antibiotic Resistance Identification By Assembly (ARIBA)
60 [4], Comprehensive Antibiotic Resistance Database (CARD) [5], and MEGARes [2] can readily detect
61 AMR genes acquired from horizontal gene transfer events. Many bacterial pathogens also develop
62 AMR via chromosomal mutations, including missense single-nucleotide polymorphism (SNP)
63 mutations in β -lactamase-encoding genes, SNPs or nonsense insertion-deletions (indels) in efflux
64 pump regulators [6-8], gene amplification via copy-number variations (CNVs) [9], and functional gene
65 loss [6]. Recent improvements in AMR identification software mean that chromosomal mutations,
66 particularly SNPs, are now identifiable. For example, ARIBA can identify AMR-conferring SNPs and
67 indels in multiple species [4]. Nevertheless, other types of genetic variants –particularly gene loss or
68 truncation, inversions, and CNVs – remain poorly identified using existing tools, despite their crucial
69 role in conferring AMR [10].

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

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

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

97 **Methods**

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

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

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

115 **Minimum Inhibitory Concentrations (MICs).** MICs were determined using Etests (bioMérieux,
116 Murarrie, Australia). Sensitive, intermediate and resistant cut-offs were based on the Clinical and
117 Laboratory Standards Institute (CLSI) M100-S17 guidelines for *B. pseudomallei* (≤8/4, 16/8, and
118 ≥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,
119 ≥4/76 µg/mL for SXT). CLSI guidelines do not list MEM for *B. pseudomallei*; however, based on prior
120 work [15, 16], and recent proposed EUCAST breakpoints for *B. pseudomallei*, we categorised MEMr
121 as ≥3 µg/mL. Likewise, the CLSI guidelines do not list gentamicin (GEN) MIC values for *B.*
122 *pseudomallei* due to almost ubiquitous resistance (>16 µg/mL) towards this antibiotic; however, there
123 are notable exceptions [14, 17]. We chose a GEN-sensitive cut-off of ≤4 µg/mL, which also reflects
124 those strains unable to grow on Ashdown's agar, a selective medium for *B. pseudomallei* isolation that
125 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/rqi>; 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 (SXT_r) 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 (DOX_r) 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 [<50 bp], 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 (SXT_r; 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 SXT_r due to Ptr1_{W116R}, MetF_{Q28P}, and Dut_{V77A} variants, yet exhibited SXT sensitivity (Table 2). The cause of SXT_r 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 μ g/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 μ g/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_{ΔP81-H223} in MSHR1058; AmrR_{G149fs} in MSHR1174; AmrR_{ΔA128-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-R-A} and MetF_{Gln142*}) and 30% (AmrR_{ΔV62-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, respectively⁶ (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.

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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.

340 **Tables**

341

342 **Table 1. Antimicrobial resistance (AMR) determinants in 25 *Burkholderia pseudomallei* strains with verified AMR phenotypes, plus strains**

343 **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}	
				CAZr (≥128)	penA -78G>A	PenA _{D245G}	
Australian patient	Bp1651	880	SRR2102060	Unusual sensitivity to aminoglycosides (GEN, KAN) and macrolides (AZM)	---	AmrB _{A254fs}	[28]
				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]
				CAZr (≥256)	penA -78G>A	PenA _{C75Y}	[20]
	MSHR1300	333	SRR6075114	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 _{ΔA153-D156}	Dut _{G91A}	[6]
				MEMr (6)	---	AmrR _{ΔA153-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 _{ΔV60-C63}	[6]
P797	MSHR7587	437	SRR6075129	MEMr (4)	---	AmrR _{G30D}	[6]
	MSHR7929	437	SRR6075126	SXTr (4)	AmrR _{G30D}	BPSL2263 _{ΔD110-G116}	
				MEMr (4)	---	AmrR _{G30D}	
CF11	MSHR8441	46	SRR3382162	CAZi (12)	---	PenA 10x CNV	[6, 9]
				SXTr (≥32)	AmrR _{ΔV62-H223} ^C	Ptr1 _{A22-G23ins_R-R-A}	
				Decreased DOX susceptibility (4)	AmrR _{ΔV62-H223} ^C	BPSL3085 _{S130L}	
				Decreased MEM susceptibility (2)	---	AmrR _{ΔV62-H223} ^C	
	MSHR8442	46	SRR3404603	SXTr (≥32)	AmrR _{ΔV62-H223} ^C	Ptr1 _{A22-G23ins_R-R-A}	
				DOXi (8)	AmrR _{ΔV62-H223} ^C	BPSL3085 _{S130L}	

				MEMr (3)	---	AmrR _{ΔV62-H223} ^c	
Non-DPMS QP09	MSHR8481	1378	SRR6075123	MEMr (6)	---	AmrR _{ΔA70-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

^aMSHR9021 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.

^bFrameshift indel shortens protein length from 223 to 183 residues

^cFrameshift indel increases protein length from 223 to 285 residues

^dFrameshift 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

362 *According to Etesting

363 #False positive; isolate remained sensitive despite encoding known AMR determinants towards DOX

364 (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 _{ΔP81-H223} ^a	12 (MEM)	[6]; This study
MSHR1174	AmrR _{G149fs} ^b	6 (MEM)	This study
MSHR8777	AmrR _{ΔA128-H223} ^c	4 (MEM)	This study

^aPreviously undescribed 3' *amrR* deletion shortens protein length from 223 to 130 residues.

^bPreviously undescribed 11bp insertion shortens protein length from 223 to 178 residues.

^cPreviously undescribed 3' *amrR* insertion increases protein length from 223 to 621 residues.

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

AMR determinant	K96243 gene ID	Mutation type	AMR minor allele detection (%)
<i>Mixture #1: MSHR0913 (sensitive strain) and MSHR0937 (resistant to MEM; intermediate resistance to AMC and SXT)</i>			
BpeR _{Asp176Ala}	BPSL0812	Missense SNP	20
MetF _{Gln142*}	BPSL3288	Nonsense SNP	25
<i>Mixture #2: MSHR0913 (sensitive strain) and MSHR8441 (resistant to MEM and SXT; intermediate resistance to CAZ and DOX)</i>			
AmrR _{ΔV62-H223}	BPSL1805	Gene loss/truncation	30 [†]
BPSL3085 _{S130L}	BPSL3085	Missense SNP	10
Ptr1 _{A22-G23ins_R-R-A}	BPSS0039	In-frame insertion	25
<i>penA</i> 10x	BPSS0946	Copy-number variation (10x duplication)	5

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

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

374

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 interrogated with the <i>Burkholderia pseudomallei</i> database
It is predicted to be resistant to Cefazidime, 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	Cefazidime	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 $\leq$ 32 μ g/mL			
penA/BPSS0946 Cys75Tyr CAZr Confers CAZ MIC $\geq$ 256 μ g/mL; also known as PenA Cys69Tyr			
BPSS0039 ptr1 Arg21fs SXT <PRECURSOR MUTATION>. Confers SXT MIC $\leq$ 32 μ g/mL in combination with BpeT Thr314fs, BpeT His274Tyr, or BpeT loss. Confers intermediate resistance by itself			
BPSS0290 bpeT Thr314fs SXT <PRECURSOR MUTATION>. Confers SXT MIC $\leq$ 32 μ g/mL in combination with Ptr1 Arg20fs			
penA/BPSS0946 upregulation CAZr 0 10x copy number confers MIC=12 μ g/mL; 2x copy number does not increase MIC (Viberg et al., 2017 MBio 8(2):00356-17)			
BPSS0039 ptr1 loss SXT 1 Confers SXT resistance in combination with efflux pump upregulation. Confers intermediate resistance by itself			
BPSS0290 bpeT loss SXT 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_{ΔP81-H223}, and AmrR_{ΔA128-H223}, all of which cause *amrR* loss-of-function, resulting in efflux pump overexpression and meropenem resistance.

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