Development and validation of a triplex quantitative real-time PCR assay to detect efflux pump-mediated antibiotic resistance in *Burkholderia pseudomallei*

Jessica R Webb¹, Erin P Price^{1,2}, Nawarat Somprasong⁴, Herbert P Schweizer⁴, Robert W Baird³, Bart J Currie^{1,3} & Derek S Sarovich^{*1,2}

¹Global & Tropical Health Division, Menzies School of Health Research, Darwin, Northern Territory, Australia

²Faculty of Science, Health, Education & Engineering, University of the Sunshine Coast, Sippy Downs, Queensland, Australia

³Departments of Infectious Diseases & Pathology & Northern Territory Medical Program, Royal Darwin Hospital, Darwin, Northern Territory, Australia

⁴Department of Molecular Genetics & Microbiology, College of Medicine, Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA

*Author for correspondence: Tel.: +61 75 456 3748; Fax: +61 75 430 1111; dsarovich@usc.edu.au

Aim: To develop a probe-based triplex quantitative real-time PCR assay to simultaneously detect the up-regulation of the efflux pumps AmrAB–OprA, BpeAB–OprB and BpeEF–OprC in *Burkholderia pseudomallei* strains exhibiting increased minimum inhibitory concentrations toward meropenem, doxycycline or trimethoprim-sulfamethoxazole. **Methods:** The triplex assay was developed and subsequently tested on RNA isolated from eight clinical and eight laboratory-generated *B. pseudomallei* mutants harboring efflux pump regulator mutations. **Results:** The triplex assay accurately detected efflux pump upregulation in all clinical and laboratory mutants, which corresponded with decreased antibiotic susceptibility or antibiotic resistance. **Conclusion:** Rapid detection of antibiotic resistance provides clinicians with a tool to identify potential treatment failure in near real time, enabling informed alteration of treatment during an infection and improved patient outcomes.

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The development of antibiotic resistance in Gram-negative bacteria has become a global crisis as declared by the WHO [1] and CDC [2]. The Gram-negative bacterium *Burkholderia pseudomallei*, the causative agent of melioidosis, is one of the most intrinsically antibiotic-resistant bacteria [3–5], due to its diverse array of chromosomally encoded resistance mechanisms [6]. This inherent resistance limits melioidosis treatment options to a small number of antibiotics. Resistance emergence to any of these drugs during treatment is thus of great concern given the limited number of alternative treatments.

Melioidosis is arguably one of the most neglected tropical diseases of our time, with gross under reporting of cases, particularly in emerging endemic regions [7]. Recent modeling suggests that there are approximately 165,000 melioidosis cases worldwide annually, of which 89,000 are estimated to be fatal, rates that are similar to the much higher profile disease, measles [8]. There is currently no vaccine toward *B. pseudomallei*, with treatment fully reliant on antibiotic administration, which is biphasic and lengthy. Treatment typically involves a minimum of 10–14 days of intravenous ceftazidime (CAZ), with meropenem (MEM) used in severe cases or when resistance develops toward CAZ [9]. The subsequent eradication phase consists of at least 3 months of trimethoprim-sulfamethoxazole (SXT) or amoxicillin-clavulanate, with doxycycline (DOX) used in instances where patients develop impaired renal function, bone marrow suppression or skin reactions due to SXT intolerance [10]. The emergence of resistance during treatment is fortunately rare, but has now been documented for almost all antibiotics used to treat melioidosis [11–15], the exception being high-level (>12 µg/ml) MEM resistance. Alarming, cases of *B. pseudomallei* exhibiting

decreased susceptibility to MEM have recently been identified in Australian patients [16], and are associated with prolonged blood culture positivity and poorer patient outcomes [17].

B. pseudomallei encodes for three clinically relevant resistance-nodulation-cell division (RND) efflux pumps: AmrAB–OprA (*BPSL1802-BPSL1804*), BpeAB–OprB (*BPSL0814-BPSL0816*) and BpeEF–OprC (*BPSS0292-BPSS0294*), which give rise to resistance toward multiple antibiotic classes [18–20]. Each RND system consists of a membrane fusion protein (AmrA, BpeA and BpeE, respectively), an RND transporter (AmrB, BpeB and BpeF, respectively), an outer membrane protein (OprA, OprB and OprC, respectively), and one or more regulators (AmrR [BPSL1805], BpeR [BPSL0812], and BpeT [BPSS0290] and BpeS [BPSL0731], respectively) [21]. Over-expression of these efflux pumps can occur during the course of melioidosis treatment due to loss-of-function mutations in repressors or mutations leading to co-inducer independence of activators in their associated regulatory genes [17,22–24]. For instance, certain mutations affecting the TetR-type regulator gene, *amrR*, cause decreased MEM susceptibilities in melioidosis patients with prolonged infections [17], and in combination with mutations in the S-adenosyl-L-methionine-(SAM)-dependent methyltransferase gene *BPSL3085*, can lead to clinically significant DOX resistance [23]. The *bpeT* and *bpeS* genes encode two closely related LysR-type transcriptional regulators. Mutations that affect the carboxy-terminal co-inducer-binding domains of BpeT and BpeS, usually together with mutations in a tetrahydrofolate pathway-linked pterin reductase gene, *ptr1/folM*, have been associated with SXT resistance [22,24]. The *bpeR* regulator is less well characterized, although a mutation within this gene has been found in a clinical *B. pseudomallei* isolate that has intermediate resistance to multiple drugs, including MEM [17]. Importantly, all observed cases of MEM, DOX and SXT resistance have involved upregulation of one or more of these three RND efflux pumps [17,25].

Despite the critical role that these RND efflux systems play in conferring acquired antibiotic resistance in *B. pseudomallei*, no methods are currently available to quickly and simultaneously detect their altered expression. A number of singleplex PCR assays have been published [24,26–28]; however, these assays are SYBR Green-based and thus cannot be multiplexed, making them more time consuming and costly to perform. Additionally, SYBR Green-based assays are typically less robust and less specific than probe-based PCR assays due to the nonspecific nature of the fluorogenic SYBR Green dye, which detects all double-stranded DNA molecules. Therefore, the aim of this study was to develop a robust and highly specific fluorogenic probe-based triplex quantitative real-time PCR (qPCR) assay to simultaneously detect the upregulation of the RND efflux pumps AmrAB–OprA, BpeAB–OprB and BpeEF–OprC in strains exhibiting increased minimum inhibitory concentrations (MICs) toward DOX, MEM and SXT. The triplex assay was first validated to determine the limits of quantitation (LoQ), detection (LoD) and linearity, and then tested against a panel of genetically characterized, antibiotic-resistant *B. pseudomallei* isolates.

Methods

Ethics statement

This study was approved by the Human Research Ethics Committee of the Northern Territory Department of Health and the Menzies School of Health Research (HREC 02/38).

Melioidosis patients & corresponding *B. pseudomallei* clinical isolates

Eight Australian melioidosis cases were examined in this study. Seven cases have previously been described, including one (Pre-DPMS 89) that lacks the initial isolate (wild-type [WT]) of the isogenic pair. Prior comparative genomic analysis and functional characterization showed that all non-WT strains from these patients encode RND efflux pump regulatory mutations that cause increased MICs (either resistance or decreased susceptibility) toward SXT, DOX or MEM [16,17,22,23].

During this study, an adult patient, P1048, presented with acute pneumonia to Royal Darwin Hospital, Northern Territory, Australia. Initial treatment involved MEM for 4 weeks, followed by 6 weeks of CAZ and then a 3-month eradication course of DOX due to SXT intolerance (neutropenia). MSHR9766 was cultured from sputum upon initial admission, and MSHR9872 was cultured from sputum during the final week of MEM treatment. P1048 reverted to being culture negative after 2 months of treatment and has since recovered from their infection. MIC testing showed that MSHR9766 was sensitive to MEM (0.5 µg/ml), whereas MSHR9872 had decreased susceptibility toward MEM (6 µg/ml), although both isolates were sensitive toward the other clinically relevant antibiotics (DOX, SXT and CAZ). Due to the development of an elevated MEM MIC in a latter isolate from this patient, we included this patient in our study.

The strain details for these eight cases, including regulatory mutations and MIC data, are shown in Table 1.

Table 1. *Burkholderia pseudomallei* clinical isolates and laboratory-generated mutants used in this study.

Patient	Strain	Regulatory mutation	DOX	TMP	SMX	SXT	CAZ	MEM [‡]	Ref.
Clinical strains									
Pre-DPMS 89	MSHR0052	AmrR _{E190} [†]	48 (r)	ND	ND	ND	ND	8 (i)	[17]
Non-DPMS 00	MSHR0293	WT	1	ND	ND	0.38	1	0.5	[23]
	MSHR0292	AmrR _{S174P}	16 (r)	ND	ND	0.50	1	2	
CF6	MSHR5651	WT	0.38	ND	ND	0.75	1.5	0.5	[22]
	MSHR5654	BpeT _{T314fs}	3	ND	ND	≥32 (r)	≥256 (r)	2	
P215	MSHR0664	WT	ND	ND	ND	2	0.75	1.5	[17]
	MSHR0937	BpeR _{N176A}	4	ND	ND	2	8 (i)	6 (i)	
P1048	MSHR9766	WT	ND	ND	ND	ND	ND	0.5	This study
	MSHR9872	AmrR _{G50E}	ND	ND	ND	ND	ND	6 (i)	
P797	MSHR6522	WT	1	ND	ND	1.5	1.5	0.5	[17]
	MSHR7929	AmrR _{E30D}	2	ND	ND	4 (r)	1.5	4	
P608	MSHR3763	WT	0.75	ND	ND	3 (i)	2	0.75	[17]
	MSHR4083	AmrR _{ΔA153-D156}	1	ND	ND	24 (r)	2	6 (i)	
P726	MSHR5864	WT	1	ND	ND	1.5	1.5	0.75	[17]
	MSHR6755	AmrR _{ΔV60-C63}	1.5	ND	ND	0.75	1.5	3	
	MSHR6755	Δ <i>amrR</i>	ND	ND	ND	ND	ND	3	
Bp82 mutants									
N/A	Bp82	WT	0.5	0.75	4	0.094	2	ND	[29]
N/A	Bp82.268	BpeT _{C310R}	2	4	8	0.38	2	ND	[24]
N/A	Bp82.269	BpeT _{L265R}	1	4	8	0.38	2	ND	[24]
N/A	Bp82.270	BpeT _{S280P}	8	>32	ND	ND	2	ND	[24]
N/A	Bp82.284	BpeS _{P29S}	2	>32	ND	ND	2-4	ND	[24]
N/A	Bp82.285	BpeS _{K267T}	8	>32	ND	ND	1-2	ND	[24]
N/A	Bp82.280	Δ <i>AmrAB</i> – <i>OprA</i> Δ <i>BpeR</i>	1	0.75	ND	ND	2	ND	[24,27]
N/A	Bp82.410	Δ <i>AmrR</i>	4	0.75	ND	ND	2	ND	This study
N/A	Bp82.412	AmrR _{S174P}	4	0.75	ND	ND	2	ND	This study

Mutations in resistance-nodulation-division efflux regulatory genes are indicated. Minimum inhibitory concentrations (μg/ml) for wild-type and mutant strains are shown for each antibiotic. Minimum inhibitory concentrations above the intermediate or resistant threshold determined for *B. pseudomallei* are indicated.

[†] Nonsense mutation leading to a premature stop codon.

[‡] Clinical & Laboratory Standards Institute breakpoints for MEM have yet to be determined. Therefore, cutoffs are instead included for the related carbapenem antibiotic, imipenem.

CAZ: Ceftazidime; DOX: Doxycycline; fs: Frameshift mutation; i: Intermediate resistance; MEM: Meropenem; ND: Not determined; r: Resistant; SMX: Sulfamethoxazole; SXT: Trimethoprim-sulfamethoxazole; TMP: Trimethoprim; WT: Wild-type.

Additionally, an *amrR* knockout of the final isolate retrieved from Patient 726 (MSHR6755 Δ*amrR*), which was previously created via allelic exchange [17], was included in the present study to determine the effect of regulator loss on efflux expression profiles.

Burkholderia pseudomallei growth conditions & MIC determination

All clinical strains were grown on Luria–Bertani (LB) agar or in LB broth (Oxoid, South Australia, Australia) at 37°C for 24 h unless otherwise stated. For the select agent-excluded strain Bp82 [29] and its derivatives, media were supplemented with 80 μg/ml adenine (Sigma, MO, USA), and for MIC testing of Bp82-derived strains, Mueller–Hinton agar or broth was supplemented with 40 μg/ml adenine. *Escherichia coli* strains DH5α and RHO3 were used for plasmid DNA manipulation or mobilization, respectively, and were grown according to previously published methods [30,31]. Antimicrobial susceptibility testing was performed using Etests according to the manufacturer's instructions (bioMérieux, New South Wales, Australia). Resistance cutoffs were based on the Clinical and Laboratory Standards Institute guidelines, as follows: CAZ and DOX: S≤4, I=8 and R≥16 μg/ml; SXT: S≤2/38 and R≥4/76 μg/ml; and amoxicillin-clavulanate: S≤8/4, I=16/8 and R≥32/16 μg/ml [32]. The Clinical and Laboratory Standards Institute guidelines do not list MIC values for *B. pseudomallei* toward MEM; thus we categorized decreased MEM susceptibility as MICs ≥3 μg/ml based on prior studies [17,33,34]. All experiments with clinical *B. pseudomallei* isolates were performed in a physical containment level 3 (biosafety level 3) facility

Table 2. Plasmids used in the study.

Plasmid	Description	Ref.
pEXKm5	Km ^r ; allelic-exchange plasmid	[30]
pEXKm5-US-DS- <i>amrR</i>	Km ^r ; pEXKm5 with US-DS- <i>amrR</i>	This study
pEXKm5-US-DS- <i>amrR</i> _{T520C}	Km ^r ; pEXKm5 with US-DS- <i>amrR</i> _{T520C} [†]	This study
pGEM-T Easy	Ap ^r ; PCR amplicon cloning vector	Promega
pGEM-T-US-DS-WT- <i>amrR</i>	Ap ^r ; pGEM-T Easy with US-DS-WT- <i>amrR</i> PCR fragment from Bp82	This study
pGEM-T-US-DS- <i>amrR</i> _{T520C}	Ap ^r ; pGEM-T Easy with US-DS- <i>amrR</i> _{T520C} created via site directed mutagenesis	This study

[†] The T520C mutation corresponds to the S174P mutation present in MSHR0292.

Ap^r: Ampicillin resistant; DS: Downstream; Km^r: Kanamycin resistant; US: Upstream; WT: Wild-type.

according to local regulations, whereas experiments involving strain Bp82 and its derivatives were conducted in the physical containment level 2 laboratory, as Bp82 is excluded from select agent regulations due to an attenuated virulence phenotype conferred by a $\Delta purM$ mutation.

Burkholderia pseudomallei Bp82 efflux pump regulator mutants

Eight *B. pseudomallei* Bp82 strains with efflux pump regulatory mutations were included, six of which have been previously created [24]: Bp82.268 BpeT_{C310R}, Bp82.269 BpeT_{L265R}, Bp82.270 BpeT_{S280P}, Bp82.284 BpeS_{P29S}, Bp82.285 BpeS_{K267T} and Bp82.280 $\Delta(AmrAB-OprA)$ $\Delta bpeR$. These Bp82 mutants, with the exception of Bp82.280 $\Delta(AmrAB-OprA)$ $\Delta bpeR$, were created via site-directed mutagenesis and knockouts to investigate the mechanisms of SXT resistance in *B. pseudomallei* using previously described methods [24]. Bp82.280 $\Delta(AmrAB-OprA)$ $\Delta bpeR$ was derived from a previously created strain (Bp82.27) as previously described [27,35] leaving an FLP recombinase target (*FRT*) scar in the *bpeR* gene. MIC data for the Bp82 mutants are detailed in Table 1.

Construction of two novel Bp82 *amrR* mutants

It has been recently shown that a T520C point mutation in *amrR* (AmrR_{S174P}) in an Australian clinical *B. pseudomallei* isolate plays an important role in increasing the MIC toward DOX (from 1 to 16 µg/ml) [23]. Two laboratory-generated Bp82 *amrR* mutants (Bp82.410 $\Delta amrR$ and Bp82.412 AmrR_{S174P}) were therefore created in this study to better understand the effect of *amrR* mutations on *AmrAB-OprA* expression. Deletion of *amrR* was achieved using the pEXKm5-based allelic replacement system (Table 2) [30]. Briefly, the upstream sequence (US) and downstream sequence (DS) region of *amrR* was amplified by PCR (using Bp82 genomic DNA as template) using primers *amrR*_UP_F, *amrR*_UP_R, *amrR*_DN_F and *amrR*_DN_R (Table 3). The PCR fragments were purified and assembled into pEXKm5 to create pEXKm5-US-DS-*amrR* (Table 2), using NEBuilder High-Fidelity DNA Assembly system (New England Biolabs, MA, USA). pEXKm5-US-DS-*amrR* was subsequently conjugated into Bp82 using an *E. coli* RHO3 mobilizer strain [30]. Finally, merodiploids were selected for and resolved. The resultant $\Delta amrR$ strain was named Bp82.410. Loss of *amrR* was confirmed by PCR amplification using the primers *amrR*_Fullgene_F and *amrR*_Fullgene_R (Table 3), with the amplified size of WT *amrR* being 1472 base pairs (bp) compared with the *amrR* knockout at 800 bp. For construction of strain Bp82.412 expressing AmrR_{S174P}, the entire *amrR* gene including 400 bp US and 400 bp DS was first ligated into pGEM-T Easy (Promega, WI, USA) (Table 2) to create pGEM-T-US-DS-WT-*amrR* (Table 2) using primers *amrR*_Fullgene_F and *amrR*_Fullgene_R (Table 3). Site-directed mutagenesis was performed using pGEM-T-US-DS-WT-*amrR* together with the *amrR* mutagenic primers *amrR*_T520C_F and *amrR*_T520C_R (Table 3) to create pGEM-T-US-DS-*amrR*_{T520C} (Table 2) following the manufacturer's instructions (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies, CA, USA). The US-DS-*amrR*_{T520C} fragment was subcloned into the *EcoRI* site of pEXKm5 creating pEXKm5-US-DS-*amrR*_{T520C} (Table 2). Mutagenesis was confirmed by dideoxy nucleotide sequencing of the newly introduced *amrR*_{T520C}. The mutagenic plasmid pEXKm5-US-DS-*amrR*_{T520C} was then introduced into Bp82.410 $\Delta amrR$ as described using *E. coli* RHO3 [30], followed by allelic exchange. Merodiploids were selected for and resolved to create Bp82.412 (Bp82 AmrR_{S174P}).

Whole-genome sequencing & accession numbers

Paired-end Illumina whole-genome sequencing (WGS) data for the clinical strains MSHR0052, MSHR0664, MSHR0937, MSHR5651, MSHR5654, MSHR0292, MSHR0293, MSHR6522, MSHR7929, MSHR3763,

Table 3. Primers and probes used for *amrR* knockout, site-directed mutagenesis and detection of efflux pump upregulation.

Primers/Probe	Sequence (5'-3')
Deletion of <i>amrR</i>	
<i>amrR</i> _UP_F	CCTGTTATCCCTACCCGGGCTCTGCCCTTCCTCGTAGG
<i>amrR</i> _UP_R	CAGTGCGCGTGGGGGGGTGCTCCCTTA
<i>amrR</i> _DN_F	CACCCCCCAGCGCACTGAACGCGCA
<i>amrR</i> _DN_R	GGGATAACAGGGTAATCCCGCGGGCGGAAGAAACGGAAC
Amplification of <i>amrR</i> + 400 bp US & DS	
<i>amrR</i> _Fullgene_F	TCCTGCCCTTCCTCGTAG
<i>amrR</i> _Fullgene_R	GCGGGCGGAAGAAACGGA
Site directed mutagenesis of <i>amrR</i> T520C	
<i>amrR</i> _T520C_F	TGGTCCAGATCATCGGGCCGAAGATGCCTTC
<i>amrR</i> _T520C_R	GAAGGCATCTTCGGCCCGATGATCTGGACCA
Triplex efflux assay	
<i>amrB</i> _For_1	TGTTGCGCATGGGTGATCTCC
<i>amrB</i> _Rev_1	GACCGATTCTCGACGACCT
<i>amrB</i> _BHQ_Probe_1	FAM-TGTTCATCATGCTGGGCGGCATC-BHQ1
<i>bpeB</i> _For_1	GCCGTCGATCCAGATCACC
<i>bpeB</i> _Rev_1	TGCTCGATCACCTGCGTG
<i>bpeB</i> _BHQ_Probe_1	JOE-TTCGGCGAAGACCGTCAAGACAC-BHQ1
<i>bpeF</i> _For_1	TGACGTATCTGCGCAACTACG
<i>bpeF</i> _Rev_1	TGCGGATCGAGCCACAC
<i>bpeF</i> _BHQ_Probe_1	Quasar 670-CTCATCAACGTGAAGGATCGCTGTAC-BHQ3

BHQ: Black hole quencher; DS: Downstream; US: Upstream.

MSHR4083, MSHR5864, and MSHR6755 were previously generated to approximately 80–90× coverage using the HiSeq2000 or HiSeq2500 platforms (Macrogen, Inc., Geumcheon-gu, Seoul, Republic of Korea). These reads have been deposited into the Sequence Read Archive database under accession numbers SRR5818275, SRR5927082, SRR2886988, SRR3381886, SRR3404570, SRR4254580, SRR4254579, SRR5949104, SRR6075126, SRR2887021, SRR2887030, SRR6075121 and SRR6075122, respectively. Illumina reads for MSHR9766 and MSHR9872 were generated on the NextSeq platform as part of the current study (Macrogen, Inc.), and have been deposited under accession numbers SRR6384102 and SRR6384101, respectively.

Genomic analyses

A comparative genomics approach has been previously employed to identify genetic variants in seven cases [16,17,22,23]. This same approach was also employed to identify variants between isolates obtained from a new melioidosis case, P1048, which arose during our study. Reference-based assembly of the initial P1048 isolate, MSHR9766, was performed with MGAP v1.0, using the closed genome of Australian strain MSHR1153 [36] for scaffolding. The MSHR9766 assembly was error corrected by self-read mapping to correct for a small number of single-nucleotide polymorphism (SNP) and insertion–deletion (indel) errors. The corrected assembly was subsequently used as a reference to identify genetic variants (SNPs, indels, copy number variants) between the P1048 strains, and to rule out gene loss in the latter strain. Read mapping was carried out using BWA [37], SAMTools [37], GATK [38] and SnpEff [39], which are wrapped in the SPANDx pipeline v3.2.1 [40]. Default SPANDx settings were used, with the flag for indel detection (-i) enabled. A second analysis using the Thai clinical strain *B. pseudomallei* K96243 [41] as the reference genome was performed to enable accurate variant annotation.

RNA extractions & cDNA synthesis

To determine expression levels of the three RND efflux pumps, *B. pseudomallei* clinical strains and mutants were grown to mid-log phase (OD₆₀₀ = 0.8–1; incubation time (5–8 h) varied depending on the growth rate of each *B. pseudomallei* strain) at 37°C in 5ml LB broth for RNA extraction unless otherwise stated. For two *B. pseudomallei* isogenic pairs (MSHR5864 and MSHR6755; MSHR3763 and MSHR4083) and an *AmrR* knockout (MSHR6755

$\Delta amrR$), LB was supplemented with 0.25 $\mu\text{g/ml}$ MEM (Sigma-Aldrich, New South Wales, Australia); all other strains were grown in the absence of antibiotics. For the clinical *B. pseudomallei* pairs, biological replicates of total RNA were extracted using TRIzol according to the manufacturer's instructions (Thermo Fisher Scientific, Victoria, Australia). RNA samples were treated with TURBO DNase according to the manufacturer's instructions (Thermo Fisher Scientific) followed by cDNA conversion using the QuantiTect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen, Victoria, Australia). Eradication of contaminating DNA prior to cDNA synthesis was confirmed with real-time PCR on the RNA extracts without the inclusion of reverse transcriptase to ensure no amplification. For the Bp82 mutants, biological triplicates of total RNA were extracted using the RNeasy Protect Bacteria kit according to the manufacturer's instructions (Qiagen), followed by DNase treatment (Fermentas, MA, USA) and cDNA conversion using the SuperScript III First-Strand Synthesis SuperMix all according to the manufacturer's instructions (Thermo Fisher Scientific).

Triplex assay design & quantitative PCR conditions

For the quantification of AmrAB–OprA, BpeAB–OprB and BpeEF–OprC expression, a triplex assay was designed to detect the RND transporter genes (*amrB*, *bpeB* and *bpeF*) of these efflux pumps. Primers and probes were designed using Primer Express software v3.0.1 (Applied Biosystems, Scoresby, VIC, Australia) and checked for specificity and binding efficiency using MegaBLAST. NetPrimer was used to guide oligo design to avoid primer dimer artefacts, with a within-assay cross- and self-dimer (ΔG) cutoff of -10 or higher deemed acceptable. Each assay was first performed and optimized in singleplex format (testing a range of primer [0.2 μM , 0.25 μM , 0.3 μM , 0.35 μM and 0.4 μM] and probe [0.15 μM , 0.2 μM , 0.25 μM and 0.3 μM] concentration combinations), with probes initially being labeled with the FAM dye, before converting the assays to a triplex-compatible format using three spectrally distinct dyes: 5'-FAM-*amrB*-BHQ-1-3', 5'-JOE-*bpeB*-BHQ-1-3' and 5'-Quasar670-*bpeF*-BHQ-1-3'. For the triplex format, the optimal primer and probe concentrations were as follows: for *amrB* and *bpeB*, 0.35 μM of each primer was used (*amrB*-For.1, *amrB*-Rev.1, *bpeB*-For.1, *bpeB*-Rev.1) with 0.2 μM of *bpeF* primers, together with 0.25 μM of each Black Hole Quencher probe (*amrB*-BHQ-Probe.1, *bpeB*-BHQ-Probe.1 and *bpeF*-BHQ-Probe.1) (LGC Biosearch Technologies, USA) and 1 $\times$ TaqMan Environmental PCR Master Mix (Applied Biosystems) to a final volume of 5 μl , including the addition of 1 μl of template.

Two conserved genes were used as controls for normalizing efflux pump gene expression. For all isolates, the TaqMan MGB probe-based *mmsA* (BPSS0619; also known as 266152) assay, which targets methylmalonate-semialdehyde dehydrogenase was used as previously described [42]. The *mmsA* PCRs together with the triplex assays were carried out using either 384-well optical plates on the QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific), or in 96-well optical plates using the CFX96 Touch Real-Time PCR detection system (Bio-Rad, CA, USA). The LoD and LoQ for the *mmsA* assay have previously been determined as 4 fg, or 0.5 genomic equivalents (GEs), which is the equivalent of a single PCR template [42], making this assay attractive for low-concentration target quantification. For efflux pump gene normalization in eight Bp82 mutants, a 23S rDNA assay was also used as described elsewhere [27]. For 23S qPCRs, 0.2 μM of each primer was used with 2 $\times$ SYBR Select Mastermix (Thermo Fisher Scientific). The following conditions were used for thermocycling: enzyme activation for 2 min at 50°C, initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing for 1 min at 60°C. The auto setting was used on each instrument when determining the threshold for each of the three assays in the triplex format.

Triplex assay performance

The performance of the triplex qPCR assay was tested across several criteria to determine the LoQ, LoD and linearity (efficiency) following previously published methods [42,43]. Briefly, genomic DNA from *B. pseudomallei* MSHR4420 was used as template due to this sample being reasonably concentrated (286 ng/ μl according to NanoDrop 2000 [Thermo Fisher Scientific] spectrophotometric analysis), of high quality and from a recent extraction. To establish the LoQ, LoD and linearity, 1:10 serial dilutions of *B. pseudomallei* MSHR4420 ranging from 40 to 4 $\times 10^{-6}$ ng across eight replicates at each concentration were used as PCR template. GEs were calculated using an average molecular weight of 660 g/mol/bp and a 7.2 Mbp genome size.

Triplex qPCR assay use & statistics

The triplex qPCR assay was performed on the 15 clinical strains, and eight Bp82 laboratory-generated mutants that contained regulator mutations. Average relative expression values (calculated as both times [x] change and log₂ fold

Figure 1. qPCR amplification plots of the *B. pseudomallei* efflux pump triplex assay.

change in expression) were calculated based on biological replicates or triplicates. The relative expression data were analyzed by two-way analysis of variance and Tukey's multiple-comparison test using GraphPad Prism (GraphPad Software, Inc., CA, USA). P-values of <0.05 were considered significant.

Results

LoQ & LoD, & linearity of the triplex qPCR assay

Following the design of probe-based assays targeting *amrB*, *bpeB* and *bpeF*, the lower LoQ and LoD was first calculated for each assay in the singleplex format, and subsequently in the triplex format. LoD was defined as the lowest analyte concentration at which detection is feasible, and LoQ was defined as the lowest concentration of analyte that can be determined with an acceptable level of precision and accuracy [43]. Based on these definitions, the LoQ was determined as the lowest amount of DNA where eight out of eight replicates amplified with a C_T standard deviation (σ) of <0.8, and with good efficiency (R^2 values >0.98), and LoD was defined as the concentration where at least two out of eight replicates amplified, irrespective of σ or efficiency. The lower LoQ in the singleplex format for all three assays was $\geq 4 \times 10^{-4}$ ng (≥ 400 fg, 52 GEs), and the LoD was $\geq 4 \times 10^{-6}$ ng (≥ 4 fg, 0.5 GEs), $\geq 4 \times 10^{-5}$ ng (≥ 40 fg, 5 GEs) and $\geq 4 \times 10^{-5}$ ng (≥ 40 fg, 5 GEs) for *amrB*, *bpeF* and *bpeB*, respectively. In the triplex format, for all targets, the LoQ was $\geq 4 \times 10^{-3}$ ng (≥ 4 pg, 515 GEs), and the LoD was $\geq 4 \times 10^{-5}$ ng (≥ 40 fg, 5 GEs) (Figure 1).

Next, the linearity of these assays was determined in the triplex and singleplex format. Efficiency (linearity) was measured by the rate at which a PCR amplicon is generated [44], where the maximum quantitative accuracy occurs in assays that function at or near 100% efficiency. In the triplex format, the standard curves for *amrB*, *bpeF* and *bpeB* all had an R^2 value of >0.95 and showed good amplification efficiencies ($\sim 90\%$ for all three targets) (Supplementary Figure 1). In singleplex format, the R^2 values were >0.98, with an efficiency of >98% for all three assays (Supplementary Figure 2).

Figure 2. Efflux pump expression in four melioidosis cases harboring *B. pseudomallei* isolates that encode efflux pump regulator mutations.

The conserved genes *mmsA* & 23S rDNA are suitable housekeeping genes for normalized expression analysis in *B. pseudomallei*

To determine the performance of the conserved gene *mmsA* as an expression normalization control for all clinical isogenic and Bp82 pairs, we compared normalized efflux pump expression against both 23S and *mmsA* in the eight Bp82 mutants. The normalized fold change of the triplex efflux assay for the eight Bp82 pairs was consistent when either 23S or *mmsA* was used for normalization (Supplementary Table 1), suggesting that *mmsA* expression, at least across the conditions that were tested in this study, is uniform. These results support the use of *mmsA* as a single-copy normalization control gene in *B. pseudomallei*.

Comparative genomic analysis identifies a novel *amrR* mutation in the latter P1048 isolate

SPANDx analysis of MSHR9766 and MSHR9872 was carried out to identify the genetic basis for decreased MEM susceptibility in the latter isolate. Two missense mutations were detected in MSHR9872: a mutation in *AmrR* (AmrR_{G50E}), and a mutation in the isoleucine tRNA synthetase gene, *ileS* (*BPSL0906*; IleS_{H505G}), the product of which catalyzes the aminoacylation of Ile-tRNA. In addition, we detected a frameshift mutation in MSHR9872 within *BPSS2161* (BPSS2161_{A28fs}), which encodes for a propanoate metabolism protein belonging to the MmgE-PrpD family. No other mutations (i.e., gene acquisition or loss, gene copy number variation) were identified between this pair.

Efflux pump upregulation in eight clinical strains with regulatory mutations

The triplex qPCR assay was tested on eight clinical *B. pseudomallei* isolates that encompassed *amrR*, *bpeR* or *bpeT* mutants, including MSHR0052 AmrR_{E190*}, which lacks an isogenic pair. None of the clinical strains tested in our study encoded *bpeS* mutations. The triplex assay showed increased efflux expression in at least one of the RND efflux pumps in those isolates containing *amrR*, *bpeR* or *bpeT* regulatory mutations (Figure 2), consistent with their role in locally coordinating efflux pump expression. The latter strain from P215, MSHR0937, which encodes a mutation within the *BpeAB–OprB* regulator, *bpeR* (BpeR_{D176A}), had a corresponding increase in the expression of *bpeB* (15×; 3.8-fold; Figure 2A) compared with its WT isogenic pair, MSHR0664. Similarly, the latter strain isolated from Patient CF6, MSHR5654, which encodes a mutation within the *BpeEF–OprC* regulator, *bpeT* (BpeT_{T314fs}), showed significant upregulation of *bpeF* (9.5×; 3.2-fold) when compared with its WT isogenic pair, MSHR5651 (Figure 2B).

Figure 3. Efflux pump expression in *B. pseudomallei*, with the latter isolate of each pair containing *amrR* point mutations or deletions that confer decreased meropenem (MEM) susceptibility.

Six latter strains, all of which exhibited decreased susceptibility toward MEM (3–8 µg/ml; Table 1), encoded mutations within the *AmrAB–OprA* regulator, *amrR*. When these six strains were tested with the triplex qPCR assay, only three (MSHR9872 *AmrR*_{G50E}, MSHR0292 *AmrR*_{S174P} and MSHR0052 *AmrR*_{E190*}) showed a significant increase in *amrB* expression, which ranged from 5× (2.3-fold; MSHR0292 *AmrR*_{S174P}) to 27× (4.7-fold; MSHR0052 *AmrR*_{E190*}) (Figure 2C & D). The remaining three *amrR* mutant strains, MSHR7929 *AmrR*_{E30D}, MSHR4083 *AmrR*_{ΔA153-D156} and MSHR6755 *AmrR*_{ΔV60-C63}, showed no significant increase in *amrB* expression (Figure 3A). Importantly, the amplification of the triplex assay for all the isogenic strains was within the LoQ of the triplex assay, ruling this factor out as a cause for the lack of differential expression.

Induction of *AmrAB–OprA* in *B. pseudomallei* *amrR* mutants exhibiting decreased MEM susceptibilities

To better understand the lack of differential expression of *AmrAB–OprA* in some strains with altered *amrR*, the triplex qPCR assay was tested on RNA extracted from MSHR4083, MSHR6755 and MSHR6755 *ΔamrR* grown in the presence of a subinhibitory (0.25 µg/ml) concentration of MEM. For MSHR6755 *AmrR*_{ΔV60-C63}, *amrB* was dramatically upregulated (24×; 4.6-fold) in the presence of MEM (Figure 3C). Additionally, the triplex assay revealed a subtle (2×; ~0.9-fold) increase in the expression of *bpeB* and *bpeF* in this strain when grown in the presence of MEM (Figure 3C). In MSHR6755 *ΔamrR*, MEM induction also resulted in 12× (3.5-fold) *amrB* upregulation (Figure 3C). Similarly, MEM induction led to 21× (4.2-fold) upregulation of *amrB* in MSHR4083 *AmrR*_{ΔA153-D156} (Figure 3B). Unlike MSHR6755 *AmrR*_{ΔV60-C63}, MEM did not induce *bpeB* and *bpeF* upregulation in MSHR6755 *ΔamrR* or MSHR4083 *AmrR*_{ΔA153-D156}.

Increased efflux expression in laboratory-generated efflux regulator mutants

The triplex qPCR assay was tested on eight laboratory-generated Bp82 mutants containing mutations affecting the *bpeT*, *bpeS* or *amrR* efflux pump regulators (Figure 4). The three *bpeT* missense mutants, Bp82.268 *BpeT*_{C310R}, Bp82.269 *BpeT*_{L265R} and Bp82.270 *BpeT*_{S280P}, exhibited *bpeF* upregulation of 28× (4.8-fold), 38× (5.2-fold) and 73× (6.1-fold), respectively, but no significant upregulation of the other two efflux pumps. The two *bpeS* missense mutants, Bp82.284 *BpeS*_{P29S} and Bp82.285 *BpeS*_{K267T}, exhibited upregulation of *bpeF* of 89× (6.5-fold) and 145× (7.2-fold), respectively. Upregulation of *amrB* was confirmed in the two *amrR* mutants Bp82.412 *AmrR*_{S174P} (6×; 2.6-fold) and Bp82.410 *ΔamrR* (7×; 2.8-fold), with no demonstrable differential expression of *bpeB* and *bpeF*. Last, in Bp82.280 *Δ(AmrAB–OprA) ΔbpeR*, the triplex qPCR assay showed no *amrB* amplification due to deletion of this locus, but 5× (2.3-fold) upregulation of *bpeB* due to deletion of *bpeR*. In all instances where

Figure 4. Efflux pump expression in eight laboratory-generated *B. pseudomallei* Bp82-derived mutants containing regulator mutations relative to WT Bp82.

amplicons were produced, triplex assay amplification for the isogenic Bp82 mutants was within the LoQ of the triplex assay.

Discussion

Despite dramatic improvements in survival rates over the past 30 years, melioidosis continues to have a stubbornly high fatality rate of 10–40% of treated cases in the hyperendemic regions of northern Australia and northeast Thailand, respectively, [45]. It is becoming clearer that at least some of these cases can be linked to the development of previously unrecognized resistance or decreased susceptibility toward clinically relevant antibiotics [17,22,46,47]. Of most concern, antibiotic resistance in *B. pseudomallei* has now been documented toward almost all antibiotics [17]. Identification of an increasing number of novel acquired antibiotic resistance mechanisms in clinical *B. pseudomallei* strains, together with the broadening global presence of melioidosis-endemic regions, has increased the urgency for methods that can provide rapid detection of emerging antibiotic resistance.

In *B. pseudomallei*, antibiotic resistance determination currently relies on culture-based diagnostics including inhibition zone measurement (e.g., Etests) or serial dilutions of the antibiotic in culture media (e.g., broth or agar microdilutions) [48]. Despite culture-based methods being accurate, they have lengthy turn-around times, and do not determine the genetic basis of resistance, both of which are important considerations when determining optimal therapeutic strategies. Nucleic acid-based detection methods such as real-time PCR have the potential to circumvent many shortcomings of traditional culture-based methods, offering more rapid turn-around times and determination of the underlying resistance mechanism, which is essential for identifying potential cross-resistance mechanisms [46,49] or stepwise progression toward high-level resistance development [23,50,51]. Information generated from such assays has the potential to inform optimal treatment strategies for melioidosis patients in near real time and reduce the number of cases where undiagnosed antibiotic resistance emerges during treatment [52].

RND efflux pump upregulation is common in *B. pseudomallei* strains that exhibit decreased susceptibility toward clinically relevant antibiotics. This mechanism can either by itself, or in concert with additional mutations, cause resistance toward DOX, SXT and MEM [17,22–24], three invaluable antibiotics in melioidosis treatment regimens. Therefore, the main goal of this study was to design, optimize and validate a triplex qPCR probe-based assay targeting the AmrAB–OprA, BpeAB–OprB and BpeEF–OprC RND efflux pumps in *B. pseudomallei* [53]. Several nucleic acid-based assays have been designed for the identification of antibiotic resistance in *B. pseudomallei* [26,46,47,54,55]; however, all are in singleplex format, making them unattractive for characterizing more than a handful of antibiotic

resistance-conferring mutations. In contrast, probe-based PCR methods can simultaneously identify multiple targets in a single assay while being efficient, affordable, sensitive and specific.

Given the large number of mutations that can cause RND efflux pump upregulation [17,22,23,28,56], we opted for a multiplex probe-based assay format that identifies increased gene expression in favor of designing individual assays targeting each regulatory mutation, the latter of which would be both impractical (due to the large number of assays required) and prone to false negatives (due to the high likelihood of missing novel variants). In addition, detecting RNA expression ensures that efflux pump upregulation is identified without requiring a thorough understanding of the underlying regulatory networks. We first optimized the three highly specific efflux pump qPCR assays in both the singleplex and triplex formats, with all assays demonstrating high robustness and sensitivity levels, similar to the highly robust *B. pseudomallei* *mmsA* probe-based assay [42]. Following optimization, we tested the triplex qPCR assay on 16 clinical and laboratory-generated *B. pseudomallei* mutants that had alterations in *amrR*, *bpeR*, *bpeT* or *bpeS* and which exhibited resistance toward DOX or SXT or had decreased MEM susceptibilities. We demonstrated that the triplex qPCR assay accurately quantified upregulation of efflux in all 16 regulatory mutants. Our findings confirm the value of the triplex qPCR assay for the detection of RND efflux pump upregulation.

The mechanisms conferring DOX and SXT resistance, and decreased MEM susceptibility, have recently been unraveled in *B. pseudomallei*. Importantly, all resistant strains analyzed to date have upregulated efflux pump expression [17,22–24]. Our study confirmed that upregulation of *amrB*, *bpeB* or *bpeF* was always associated with decreased MEM susceptibility. Thus, in a clinical setting, the triplex assay can be used for the accurate detection of *B. pseudomallei* isolates exhibiting decreased MEM susceptibility without the need for MIC determination. In contrast, additional mutations outside of efflux pump regulatory regions, which affect either the methyltransferase, *BPSL3085* or the tetrahydrofolate synthesis pathway-linked enzyme pterin reductase, *folM/ptrI*, are required for the shift toward high-level DOX and SXT resistance [23,24]. The two-step nature of these mechanisms means that the triplex qPCR assay alone cannot be used to definitively determine if a strain is resistant to DOX or SXT. However, the assay can be used to rule out clinically significant antibiotic resistance, as a strain with little or no efflux activity is highly likely to remain sensitive to these drugs. Importantly, the ability to rapidly screen isolates and detect precursor mutations to clinically relevant DOX and SXT resistance provides a unique opportunity for health practitioners to make informed decisions regarding the suitability of current treatment, and to be aware of the risk of future resistance emergence toward these antibiotics. If increased efflux pump activity is detected in any *B. pseudomallei* strain, we strongly recommend that isolates be subjected to further MIC testing and/or the patient's treatment regimen altered to avoid selecting for resistance emergence and subsequent treatment failure. In cases where efflux pump upregulation is detected, a regimen using CAZ in the acute phase followed by a prolonged course of SXT would be appropriate, provided that the patient is frequently monitored for SXT resistance emergence. Imipenem (acute phase) or amoxicillin-clavulanate (eradication phase) could also serve as suitable alternatives in cases where efflux pump upregulation is detected or SXT resistance develops.

It has been previously shown that MEM induces the expression of AmrAB–OprA in *amrR*-mutated *B. pseudomallei* strains, leading to decreased MEM susceptibility [17]. In the current study, the triplex qPCR assay confirmed that some, but not all, *amrR* mutants required MEM for the induction of AmrAB–OprA. In contrast, the two tested WT strains, MSHR5864 and MSHR3763, did not show an increase in the expression of *amrB* in the presence of MEM. Efflux pump expression was inducible in strains encoding in-frame, four-residue *amrR* deletions (MSHR4083 AmrR $_{\Delta A153-D156}$ and MSHR6755 AmrR $_{\Delta V60-C63}$), and when *amrR* was completely lost (MSHR6755 $\Delta amrR$); however, induction was not required for two strains encoding either a point mutation (MSHR0292 AmrR $_{S174P}$) or a premature stop codon (MSHR0052 AmrR $_{E190*}$). The reason for this difference is not yet fully understood. One possibility is that RND efflux pump regulatory genes are capable of binding to substrates of the efflux systems that they regulate, and in doing so, dissociate from their promoter regions, leading to efflux pump upregulation [57,58]. This hypothesis may explain the molecular mechanism in the strains that encode in-frame *amrR* deletions due to altered MEM affinity; however, it cannot explain why MEM would be required for induction in strains lacking *amrR* nor why WT strains do not increase efflux pump expression when exposed to MEM. Although not examined in this study, it is possible that a second, as-yet-undiscovered regulator may also be involved in AmrAB–OprA regulation. Alternately, certain mutations or methylation patterns may reverse the function of AmrR, resulting in activation rather than repression of the operon. Further work using methods such as transcriptomics (RNA-seq) or bisulfite sequencing (methyl-seq) would be needed to confirm this hypothesis. Based on our results, we recommend that a subinhibitory concentration of MEM (i.e., 0.25 $\mu\text{g/ml}$) is sufficient to induce RND efflux pump upregulation in strains with decreased MEM susceptibility, while not impacting on RND efflux pump expression in WT strains.

Although not investigated here, MEM may not be required for induction if RNA is extracted directly from clinical samples due to the preservation of native expression/methylation profiles, or even the presence of MEM *in vivo* upon sample collection.

There are some recognized limitations to our study. First, there are greater difficulties associated with handling RNA compared with DNA due to the high lability of bacterial mRNA if not protected appropriately [59]. This requirement limits the application of our assay to freshly collected specimens, appropriately preserved material, or viable cultures. Second, we only assessed the use of our triplex assay on RNA extracted from subcultured isolates, and our TRIzol-based RNA extraction method is relatively slow (~4 h from pelleted culture to purified and quality-controlled RNA). Additionally, the assays presented in this study are not able to identify all antibiotic resistance mechanisms in *B. pseudomallei* and are not meant to be an exhaustive detection method. These factors pose a significant barrier to the rapid diagnosis of RND efflux pump upregulation. An assessment of rapid RNA extraction methods from bacterial cultures, or ideally, directly from clinical specimens [60,61], is needed to achieve same-day identification of strains with increased RND efflux expression. Finally, the complex nature of antibiotic resistance mechanisms may act as a barrier to the uptake of molecular antibiotic resistance assays in clinical laboratories due to an unfamiliarity with molecular techniques or difficulties in communicating results among healthcare professionals. The latter issue can be overcome with the availability of report templates, such as the *Mycobacterium tuberculosis* template for antibiotic resistance determination in tuberculosis patients based on WGS data, which communicates molecular results from antibiotic resistance strains to health practitioners in a clear and unambiguous manner, and without the need for specialized molecular knowledge [62].

Conclusion

Burkholderia pseudomallei is an important mammalian pathogen that is endemic in an increasingly recognized number of tropical and subtropical regions. As new endemic regions continue to be unveiled, and as better recognition of this neglected pathogen filters through to the clinical setting, the number of melioidosis cases reported globally is expected to increase substantially. Due to the high mortality rate of this disease even with antibiotic treatment, patient management should include regular screening of clinical isolates for the emerging development of antibiotic resistance to ensure optimal treatment strategies are being implemented, and to minimize the possibility of unintended treatment failure. With this goal in mind, we developed and optimized a triplex qPCR assay that simultaneously detects the expression of AmrAB–OprA, BpeAB–OprB and BpeEF–OprC in *B. pseudomallei* isolates. The triplex assay was tested on a panel of genetically characterized *B. pseudomallei* isolates of both clinical and laboratory origin, with known mutations leading to SXT or DOX resistance, or decreased MEM susceptibilities. In all *B. pseudomallei* strains that contained regulatory mutations, upregulation of at least one efflux pump was accurately detected. Rapid and facile detection of efflux upregulation is a crucial component of detecting emerging drug resistance in *B. pseudomallei* and will aid in prompt and effective administration of individualized treatment regimens for melioidosis patients.

Future perspective

In just the past decade, WGS has greatly accelerated the identification and functional characterization of antibiotic resistance mechanisms in *B. pseudomallei*. This rapid knowledge expansion has made it feasible to use molecular (DNA and RNA) approaches to accurately predict how this pathogen evolves resistance to clinically relevant antibiotics without the need for culture-based MIC testing. In the coming decade, molecular diagnostics will be used alongside, and in many cases replace existing culture-based methods for detecting *B. pseudomallei* resistance emergence in melioidosis patients, with potential application in the bedside diagnostic setting. WGS is already beginning to take a more prominent role in the microbiology clinic. Computational tools have recently been developed that can interpret the MIC profile of clinical isolates from WGS data and generate easily decipherable reports for the clinician. Nevertheless, rapid assays capable of detecting a wide range of resistance mechanisms will still be faster and cheaper than WGS long into the future, and remain an effective way of detecting antibiotic resistance, especially in resource-poor or diagnostically challenging settings. We foresee that molecular diagnostics will become routine in most clinical laboratories in the next decade, and the continued development of DNA- and RNA-based assays capable of rapidly detecting antibiotic resistance will be crucial for the move away from empiric to personalized medicine.

Executive summary

Background & problem

- *Burkholderia pseudomallei* is the causative agent of the tropical disease melioidosis, a high-fatality disease if left untreated.
- Intrinsic resistance to the antibiotics used in melioidosis treatment is rare, but resistance can develop during infection, increasing the risk of mortality.
- Antibiotic resistance emergence is problematic due to the relatively small number of effective drugs and the prolonged nature of treatment necessary to eradicate the infection.
- Efflux pump upregulation is critical for the development of antibiotic resistance to many clinically relevant antibiotics. This mechanism can either by itself, or in concert with additional mutations, cause resistance toward doxycycline (DOX), meropenem (MEM) and trimethoprim-sulfamethoxazole (SXT).
- Current diagnostic methodology relies on culture-based methods, which can be costly and time consuming.

Outcomes

- We developed a triplex reverse-transcriptase quantitative PCR assay to detect upregulation of the three clinically relevant RND efflux pumps in *B. pseudomallei*.
- To validate assay performance, we used a range of antibiotic-resistant clinical strains and genetically modified mutants with known efflux pump regulatory mutations. We demonstrated that our assay was both sensitive and specific, and could detect efflux pump upregulation in all strains demonstrating resistance to DOX, MEM and SXT.

Discussion & conclusion

- Melioidosis has a stubbornly high fatality rate, which is increasingly being attributed to the development of antibiotic resistance during infection.
- RNA-based detection of efflux pump upregulation avoids the requirement for development of individual assays for each regulatory mutation, instead directly detecting the functional consequences of genetic mutation via altered gene expression.
- The triplex assay can be used to detect decreased susceptibility to MEM, and precursor mutations that lead to DOX and SXT resistance.
- This assay provides a rapid and cost-effective alternative to conventional minimum inhibitory concentration testing to regularly monitor a patient's infection for the development of antibiotic resistance, offering the potential to respond to resistance emergence in near real time.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/fmb-2018-0155

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