JOURNAL OF MEDICAL MICROBIOLOGY

SHORT COMMUNICATION

Madden *et al.*, *Journal of Medical Microbiology* 2022;71:001593

DOI 10.1099/jmm.0.001593

MICROBIOLOGY SOCIETY

OPEN ACCESS

Rapid fluoroquinolone resistance detection in *Pseudomonas aeruginosa* using mismatch amplification mutation assay-based real-time PCR

Danielle E. Madden[1,2,3], Kate L. McCarthy[4,5], Scott C. Bell[6,7,8], Olusola Olagoke[1,2,3], Timothy Baird[1,2,9], Jane Neill[9], Kay A. Ramsay[8], Timothy J. Kidd[10,11], Adam G. Stewart[5,12], Shradha Subedi[3,12], Keat Choong[3,12], Tamieka A. Fraser[1,2], Derek S. Sarovich[1,2,3]† and Erin P. Price[1,2,3,*,†]

Abstract

Background. Antimicrobial resistance (AMR) is an ever-increasing global health concern. One crucial facet in tackling the AMR epidemic is earlier and more accurate AMR diagnosis, particularly in the dangerous and highly multi-drug-resistant ESKAPE pathogen, *Pseudomonas aeruginosa*.

Objectives. We aimed to develop two SYBR Green-based mismatch amplification mutation assays (SYBR-MAMAs) targeting GyrA T83I (*gyrA*248) and GyrA D87N, D87Y and D87H (*gyrA*259). Together, these variants cause the majority of fluoroquinolone (FQ) AMR in *P. aeruginosa*.

Methods. Following assay validation, the *gyrA*248 and *gyrA*259 SYBR-MAMAs were tested on 84 Australian clinical *P. aeruginosa* isolates, 46 of which demonstrated intermediate/full ciprofloxacin resistance according to antimicrobial susceptibility testing.

Results. Our two SYBR-MAMAs correctly predicted an AMR phenotype in the majority (83%) of isolates with intermediate/full FQ resistance. All FQ-sensitive strains were predicted to have a sensitive phenotype. Whole-genome sequencing confirmed 100% concordance with SYBR-MAMA genotypes.

Conclusions. Our GyrA SYBR-MAMAs provide a rapid and cost-effective method for same-day identification of FQ AMR in *P. aeruginosa*. An additional SYBR-MAMA targeting the GyrB S466Y/S466F variants would increase FQ AMR prediction to 91%. Clinical implementation of our assays will permit more timely treatment alterations in cases where decreased FQ susceptibility is identified, leading to improved patient outcomes and antimicrobial stewardship.

Received 10 March 2022; Accepted 28 July 2022; Published 27 October 2022

Author affiliations: [1]Centre for Bioinnovation, University of the Sunshine Coast, Sippy Downs, Queensland, Australia; [2]Sunshine Coast Health Institute, Birtinya, Queensland, Australia; [3]Infection Research Network Sunshine Coast, Birtinya, Queensland, Australia; [4]Infectious Diseases Unit, Royal Brisbane and Women's Hospital, Herston, Queensland, Australia; [5]University of Queensland Centre for Clinical Research, Herston, Queensland, Australia; [6]Adult Cystic Fibrosis Centre, The Prince Charles Hospital, Chermside, Queensland, Australia; [7]Translational Research Institute, Woolloongabba, Queensland, Australia; [8]Child Health Research Centre, The University of Queensland, South Brisbane, Queensland, Australia; [9]Respiratory Department, Sunshine Coast University Hospital, Birtinya, Queensland, Australia; [10]School of Chemistry and Molecular Biosciences, University of Queensland, St Lucia, Queensland, Australia; [11]Central Microbiology, Pathology Queensland, Royal Brisbane and Women's Hospital, Herston, Queensland, Australia; [12]Infectious Diseases Unit, Sunshine Coast University Hospital, Birtinya, Queensland, Australia.

***Correspondence:** Erin P. Price, eprice@usc.edu.au

Keywords: antibiotic; AMR; ciprofloxacin; fluoroquinolone; *Pseudomonas aeruginosa*; real-time PCR; SYBR-MAMA.

Abbreviations: AMR, antimicrobial-resistant/antimicrobial resistance; BSI, bloodstream infection; CF, cystic fibrosis; CIP, ciprofloxacin; FQ, fluoroquinolone; LEV, levofloxacin; MFX, moxifloxacin; MIC, minimum inhibitory concentration; OFX, ofloxacin; QRDR, quinolone resistance-determining region; SNP, single-nucleotide polymorphism; SYBR-MAMA, SYBR Green-based mismatch amplification mutation assay; WGS, whole-genome sequencing.

Data availability: Illumina data are publicly available via the National Center for Biotechnology Institute (NCBI) Sequence Read Archive database under BioProject PRJNA761496 (https://www.ncbi.nlm.nih.gov/sra?linkname=bioproject_sra_all&from_uid=761496).

†These authors contributed equally to this work

BACKGROUND

The ESKAPE pathogen, *P. aeruginosa*, has a remarkable capacity to develop antimicrobial resistance (AMR) towards all clinically relevant antibiotic classes [1]. This bacterium can cause life-threatening infections, particularly in people with wounds, cancer, or chronic respiratory diseases such as cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD) [2].

Rapid, affordable, accessible, and accurate AMR diagnosis is crucial in the battle against ESKAPE pathogens [1]. However, few diagnostic tests exist [3] for rapidly and inexpensively characterizing AMR-conferring single-nucleotide polymorphisms (SNPs) in *P. aeruginosa*, a striking knowledge gap given that SNPs confer AMR towards anti-pseudomonal drugs such as fluoroquinolones (FQs) [4].

FQs [predominantly ciprofloxacin (CIP)] have proven clinically useful for treating *P. aeruginosa* infections [5]. Yet, upon exposure, *P. aeruginosa* often develops FQ resistance (FQr). Codon-altering mutations within the GyrA quinolone resistance-determining region (QRDR) can confer an intermediate (CIPi) or fully resistant (CIPr) phenotype [6, 7]; in contrast, *gyrB*, *parC*, *parE* and *nfxB* typically require ≥2 mutations to impart CIPi/CIPr [8]. Alteration of treatment, either by shifting to a different antibiotic or increasing CIP dosage, is recommended for patients infected with CIPi/CIPr strains. Importantly, the correct choice of initial antibiotic therapy is known to result in decreased patient mortality [9, 10].

Due to the single-step nature of QRDR mutations in conferring CIPi/CIPr, and their prevalence in clinical isolates [11], we targeted the two most common QRDR SNPs, *gyrA*248 [4] and *gyrA*259 [12], for assay development. These two SNPs occur frequently in *P. aeruginosa* isolates across the globe and demonstrate little, if any, geographical bias [13]. To interrogate these two SNPs, we chose SYBR Green-based mismatch amplification mutation assay (SYBR-MAMA), an inexpensive (~AUD $1–2/assay when run in duplicate), simple, rapid (~1 h turnaround time) and scalable method [14–18] that exploits the differential efficiency of allele-specific amplification for SNP interrogation; this efficiency disparity can be observed in real time by measuring the difference in cycles-to-threshold (ΔC_T) [15, 17, 18].

METHODS

Isolates examined in this study

Eighty-four *P. aeruginosa* isolates from Queensland, Australia, were examined: 42 from sputum derived from adults with CF and chronic *P. aeruginosa* infection admitted to The Prince Charles Hospital between 2017 and 2019 [19]; 35 bloodstream isolates retrieved from adults admitted to several public and private hospitals in Brisbane between 2008 and 2011 [20]; 3 from COPD sputum, collected during in-home community nurse visits in the Sunshine Coast region [19]; 1 from an adult with non-CF bronchiectasis collected in 2017; and 1 from an adult with urinary tract infection collected in 2018, both during admission to the Sunshine Coast University Hospital (Table 1). One ulcer and one ear infection isolate, both from Brisbane, were obtained from the 1000 International *P. aeruginosa* Consortium collection [21]. Strains were isolated from clinical specimens using MacConkey agar (Oxoid, VIC, Australia), incubated at 37◦C for 24 h, and confirmed as *P. aeruginosa* by *ecfX* real-time PCR [22].

Antimicrobial susceptibility testing

Susceptibility towards levofloxacin (LEV; 5 µg), moxifloxacin (MFX; 5 µg) and ofloxacin (OFX; 5 µg) was determined by disc diffusion (Edwards Group, QLD, Australia; Table 2). Minimum inhibitory concentrations (MICs) towards CIP were determined by ETEST (bioMérieux, NSW, Australia). Isolates were classed as CIP sensitive, CIPi, or CIPr using the Clinical and Laboratory Standards Institute (CLSI) M100S-Ed32:2022 guidelines.

DNA extraction and sequencing

Isolates were DNA-extracted using the DNeasy kit (Qiagen, Chadstone Centre, VIC, Australia), followed by Illumina paired-end whole-genome sequencing (WGS) [23]. Quality-filtered reads [24] were assembled with MGAP v1.1 (https://github.com/dsarov/MGAP---Microbial-Genome-Assembler-Pipeline) or SPAdes [25]. AMR prediction was undertaken using a *P. aeruginosa*-specific [26] ARDaP [27] database. Assemblies were deposited in the PubMLST database (https://pubmlst.org/organisms/pseudomonas-aeruginosa). We also tested rapid DNA extraction of 10 representative strains using the 5% chelex-100 rapid heat-soak method [28], followed by a 1:10 dilution in molecular-grade H_2O prior to PCR.

GyrA SYBR-MAMA design

A BLAST database comprising 682 *P. aeruginosa* genomes [13, 29–32] was used to identify conserved regions for oligo design. SYBR-MAMA primers were assessed *in silico* for dimer formation and specificity as described previously [33]. For the *gyrA*248 SYBR-MAMA, gyrA248_T_AMR amplifies the CIPi/CIPr-conferring T83I allele, whereas gyrA248_C_WT amplifies the wild-type allele (Table 3). For the *gyrA*259 SYBR-MAMA, gyrA259_D_AMR amplifies mutant alleles at position 259 (D87N, D87Y, D87H), all of which confer CIPi/CIPr [4, 8], whereas gyrA259_G_WT amplifies the wild-type allele [12]. PCRs consisted of 1× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, NSW, Australia), 0.2 µM primers, 1 µl template, and PCR-grade H_2O, to 5 µl. Thermocycling comprised 95◦C for 2 min, followed by 40 cycles of 95◦C for 5 s and 60◦C for 5 s. The strains used to validate *gyrA* 248 and 259 alleles are listed in Table 2.

Table 1. *Pseudomonas aeruginosa* isolates used in this study, and their DNA gyrase A (GyrA) SYBR-MAMA PCR genotypes, whole-genome sequencing (WGS) results, multilocus sequence types and treatment details

Strain	Disease	MIC (μg ml^{-1})	*gyrA*248 allele (PCR)	*gyrA*259 allele (PCR)	GyrA AMR mutation (WGS)	Other CIP AMR-conferring mutations (WGS)	Sequence type	Treatment at time of collection
CIP resistant								
SCHI0002.S.8	CF	4	WT	WT	None	GyrB (Ser466Phe)	ST4	Day 7 IV CAZ, MEM, TOB
SCHI0003.S.8	CF	2	WT	AMR	D87H	None	ST801	Day 4 IV ATM, MEM, TOB
SCHI0004.S.5	CF	>32	AMR	WT	T83I	None	ST649	Day 14 IV MEM, TOB
SCHI0005.S.9	CF	2	WT	AMR	D87H	None	ST801	Day 10 IV MEM, TOB
SCHI0005.S.10	CF	2	WT	AMR	D87H	None	ST801	Day 10 IV MEM, TOB
SCHI0005.S.11	CF	2	WT	AMR	D87H	None	ST801	Day 10 IV MEM, TOB
SCHI0006.S.10	CF	2	WT	AMR	D87H	None	ST801	None
SCHI0008.S.3	CF	4	AMR	WT	T83I	None	ST649	Day 8 IV ATM, DOX
SCHI0010.S.1	CF	>32	AMR	WT	T83I	None	ST4	Day 7 IV CAZ, TOB
SCHI0016.S.124	UTI	>32	WT	AMR	D87N	MexR (Ile24fs; loss; upregulates MexAB efflux pump)	ST309	Unknown
SCHI0021.S.6	CF	2	WT	AMR	D87H	None	ST801	Day 5 IV CAZ, TOB
SCHI0021.S.8	CF	3	WT	AMR	D87H	None	ST801	Day 13 IV CAZ, TOB
SCHI0021.S.7	CF	>32	WT	AMR	D87H	None	ST801	Day 13 IV CAZ, TOB
SCHI0021.S.11	CF	3	WT	AMR	D87H	None	ST801	Day 1 IV CAZ, TOB
SCHI0021.S.12	CF	3	WT	AMR	D87H	None	ST801	Day 1 IV CAZ, TOB
SCHI0021.S.14	CF	3	WT	AMR	D87H	None	ST801	None
SCHI0025.S.15	CF	8	WT	AMR	D87H	None	ST801	Unknown
SCHI0027.S.1	CF	3	WT	AMR	D87H	None	ST801	Day 1 IV CAZ, TOB
SCHI0027.S.2	CF	2	WT	AMR	D87H	None	ST801	Day 12 IV CAZ, TOB
SCHI0027.S.3	CF	3	WT	AMR	D87H	None	ST801	Day 9 IV CAZ, TOB
SCHI0027.S.5*	CF	2	WT	AMR	D87H	None	ST801	None
SCHI0029.S.1	CF	3	WT	AMR	D87H	None	ST801	Day 1 unknown antibiotic(s)
SCHI0030.S.2	CF	3	WT	AMR	D87H	None	ST801	Day 6 IV ATM, TOB
SCHI0030.S.3	CF	3	WT	AMR	D87H	None	ST801	Day 13 IV ATM, TOB
SCHI0030.S.4*	CF	4	WT	AMR	D87H	None	ST801	None
SCHI0032.S.32	Ulcer	4	WT	AMR	D87N	None	ST143†	Unknown
SCHI0032.S.33	Ear infection	2	WT	AMR	D87Y	None	ST848‡	Unknown
SCHI0033.S.2	BSI	>32	AMR	WT	T83I	ParE (Ala473Val), NalD (Met1del; upregulates MexAB efflux pump)	ST847	Unknown
SCHI0033.S.4	BSI	12	AMR	WT	T83I	None	ST244	Unknown
SCHI0033.S.14	BSI	2	WT	WT	None	None	ST571	Unknown
SCHI0033.S.15	BSI	>32	AMR	WT	T83I	ParC (Ser87Leu)	ST532	Unknown

Continued

Table 1. Continued

Strain	Disease	MIC (μg ml^{-1})	*gyrA*248 allele (PCR)	*gyrA*259 allele (PCR)	GyrA AMR mutation (WGS)	Other CIP AMR-conferring mutations (WGS)	Sequence type	Treatment at time of collection
SCHI0033.S.19	BSI	>32	AMR	WT	T83I	ParC (Ser87Leu)	ST235	Unknown
SCHI0033.S.26	BSI	3	WT	WT	None	None	ST147	Unknown
CIP intermediate								
SCHI0002.S.9	CF	1.5	WT	WT	None	None	ST683	Day 7 IV CAZ, MEM, TOB
SCHI0002.S.12	CF	1	WT	WT	None	None	ST683	Day 37 IV CAZ, MEM, TOB
SCHI0003.S.1	CF	1.5	AMR	WT	T83I	None	ST649	Day 4 IV ATM, MEM, TOB
SCHI0003.S.3	CF	1.5	AMR	WT	T83I	None	ST649	Day 4 IV ATM, MEM, TOB
SCHI0008.S.2	CF	1	AMR	WT	T83I	None	ST649	Day 8 IV ATM, DOX
SCHI0008.S.4	CF	1	AMR	WT	T83I	None	ST649	Day 8 IV ATM, DOX
SCHI0013.S.2	CF	0.75	WT	WT	None	GyrB (Ser466Tyr), ParE (Ala473Val)	ST775	Day 4 FOF, MEM
SCHI0013.S.12	CF	1	WT	WT	None	GyrB (Ser466Tyr), ParE (Ala473Val)	ST775	Day 4 FOF, MEM
SCHI0016.S.57	BE	1.5	WT	WT	None	GyrB (Ser466Phe), MexR (Gln25stop; upregulates MexAB efflux pump)	ST2601	>2 weeks MEM, GEN, CRO, FEP
SCHI0021.S.5	CF	1.5	WT	AMR	D87H	None	ST801	Day 5 IV CAZ, TOB
SCHI0021.S.10	CF	1	WT	AMR	D87H	None	ST801	Day 1 IV CAZ, TOB
SCHI0025.S.9	CF	1	AMR	WT	T83I	None	ST649	Unknown
SCHI0033.S.29	BSI	0.75	AMR	WT	T83I	None	ST244	Unknown
CIP sensitive								
SCHI0005.S.8	CF	0.5	WT	WT	None	ParE (Ala473Val), AmgS (Val121Gly; upregulates MexXY efflux pump)	ST262	Day 10 IV MEM, TOB
SCHI0020.S.4	CF	0.5	WT	WT	None	ParE (Ala473Val), NfxB (large deletion, upregulates MexCD efflux pump)	ST3828§	Day 1 IV CAZ, TOB
SCHI0020.S.5	CF	0.5	WT	WT	None	ParE (Ala473Val), NfxB (large deletion, upregulates MexCD efflux pump)	ST3828§	None
SCHI0020.S.6	CF	0.5	WT	WT	None	ParE (Ala473Val), NfxB (loss, upregulates MexCD efflux pump)	ST3828§	Day 1 IV CAZ, TOB
SCHI0027.S.4*	CF	0.25	WT	WT	None	None	ST801	None
SCHI0030.S.1	CF	0.25	WT	WT	None	None	ST275	None
SCHI0030.S.5*	CF	0.25	WT	WT	None	None	ST275	None
SCHI0033.S.1*	BSI	0.125	WT	WT	None	None	ST3864§	Unknown
SCHI0033.S.3	BSI	0.19	WT	WT	None	None	ST3829	Unknown
SCHI0033.S.5	BSI	0.19	WT	WT	None	None	ST274	Unknown
SCHI0033.S.7	BSI	0.094	WT	WT	None	None	ST252	Unknown
SCHI0033.S.8	BSI	0.25	WT	WT	None	None	ST649	Unknown
SCHI0033.S.9	BSI	0.25	WT	WT	None	None	ST708	Unknown

Continued

Table 1. Continued

Strain	Disease	MIC (μg ml^{-1})	*gyrA*248 allele (PCR)	*gyrA*259 allele (PCR)	GyrA AMR mutation (WGS)	Other CIP AMR-conferring mutations (WGS)	Sequence type	Treatment at time of collection
SCHI0033.S.10*	BSI	0.19	WT	WT	None	None	ST244	Unknown
SCHI0033.S.11*	BSI	0.5	WT	WT	None	None	ST3865§	Unknown
SCHI0033.S.12*	BSI	0.125	WT	WT	None	None	ST865	Unknown
SCHI0033.S.13A*	BSI	0.5	WT	WT	None	None	ST395	Unknown
SCHI0033.S.13B*	BSI	0.19	WT	WT	None	None	ST395	Unknown
SCHI0033.S.16	BSI	0.5	WT	WT	None	NfxB (Phe126fs; upregulates MexCD efflux pump)	ST909	Unknown
SCHI0033.S.17	BSI	0.25	WT	WT	None	None	ST274	Unknown
SCHI0033.S.18	BSI	0.19	WT	WT	None	None	ST348	Unknown
SCHI0033.S.20	BSI	0.19	WT	WT	None	None	ST274	Unknown
SCHI0033.S.23	BSI	0.5	WT	WT	None	None	ST3843§	Unknown
SCHI0033.S.24	BSI	0.5	WT	WT	None	None	ST471	Unknown
SCHI0033.S.25	BSI	0.38	WT	WT	None	None	ST815	Unknown
SCHI0033.S.27	BSI	0.25	WT	WT	None	None	ST395	Unknown
SCHI0033.S.28	BSI	0.38	WT	WT	None	None	ST3804	Unknown
SCHI0033.S.30	BSI	0.19	WT	WT	None	None	ST782	Unknown
SCHI0033.S.32	BSI	0.38	WT	WT	None	None	ST298	Unknown
SCHI0033.S.33	BSI	0.5	WT	WT	None	MexR (Arg91fs; upregulates MexAB efflux pump	ST17	Unknown
SCHI0033.S.35	BSI	0.38	WT	WT	None	None	ST348	Unknown
SCHI0033.S.36	BSI	0.5	WT	WT	None	None	ST3830§	Unknown
SCHI0033.S.37	BSI	0.25	WT	WT	None	None	ST298	Unknown
SCHI0033.S.38	BSI	0.25	WT	WT	None	None	ST235	Unknown
SCHI0033.S.39	BSI	0.19	WT	WT	None	None	ST1189	Unknown
SCHI0038.S.3	COPD	0.19	WT	WT	None	None	ST888	None
SCHI0039.S.1	COPD	0.125	WT	WT	None	None	ST3134	None
SCHI0050.S.1	COPD	0.125	WT	WT	None	ParC (Gln405Arg)	ST3323	Day 3 oral CIP
PAO1	Wound	ND	WT	WT	None	None	ST549	Unknown

*Whole-genome sequence data assembled with SPAdes [25] rather than MGAP.
†SCHI0032.S.32 submitted to PubMLST under isolate name AUS205 (ID:1023) [40]
‡SCHI0032.S.33 submitted to PubMLST under the isolate name AUS134 (ID:952) [40]
§Novel multilocus sequence type (ST) identified in this study
AMR, antimicrobial-resistant; ATM, aztreonam; BE, bronchiectasis; BSI, bloodstream infection; CAZ, ceftazidime; CF, cystic fibrosis; CIP, ciprofloxacin; COPD, chronic obstructive pulmonary disease; CRO, ceftriaxone; DOX, doxycycline; FEP, cefepime; FOF, fosfomycin; GEN, gentamicin; IV, intravenous; MEM, meropenem; MIC, minimum inhibitory concentration (based on ETEST); SYBR-MAMA, SYBR Green-based mismatch amplification mutation assay; TOB, tobramycin; UTI, urinary tract infection; WGS, whole-genome sequencing; WT, wild-type.

GyrA T83I and GyrA D87H/N/Y prevalence in global *P. aeruginosa* isolates

We analysed a global collection (n=283) of genome-sequenced isolates [30–32, 34–36] with corresponding CIP phenotype data to determine the geographical prevalence of these two GyrA AMR variants.

RESULTS AND DISCUSSION

SYBR-MAMAs were screened across the 85 genome-sequenced *P. aeruginosa* isolates, comprising 40 CIP-sensitive (including PAO1), 13 CIPi, and 33 CIPr strains (Table 1). Of these, only one, SCHI0050.S.1, was derived from a participant receiving FQ (CIP) treatment,

Table 2. *Pseudomonas aeruginosa* isolates used for *gyrA*248 and *gyrA*259 SYBR-MAMA assay validation, and associated fluoroquinolone-class antibiotic disc diffusion data

gyrA phenotype	Isolate ID	CIP	LEV	OFX	MFX	WT to AMR (codon change)
AMR (mutant)	SCHI0003.S.3	I	I	R	R	*gyrA*248 T→C (T83I)
	SCHI0008.S.4	I	I	R	R	
	SCHI0010.S.1	R	R	R	R	
	SCHI0025.S.9	I	R	R	R	
	SCHI0016.S.124	R	R	R	R	*gyrA*259 G→A (D87N)
	SCHI0032.S.32	R	R	R	R	
	SCHI0003.S.8	R	I	R	R	*gyrA*259 G→C (D87H)
	SCHI0005.S.10	R	I	I	R	
	SCHI0025.S.15	R	R	R	R	
	SCHI0029.S.1	R	R	R	R	
	SCHI0032.S.33	R	I	R	R	*gyrA*259 G→T (D87Y)
WT (control)	SCHI0038.S.3	S	S	S	S	No mutation at *gyrA*248 or *gyrA*259
	SCHI0039.S.1	S	S	S	S	
	SCHI0050.S.1	S	S	S	S	
	PAO1	S	S	S	S	

AMR, antimicrobial-resistant; CIP, ciprofloxacin; I, intermediate; LEV, levofloxacin; MFX, moxifloxacin; OFX, ofloxacin; R, resistant; S, sensitive; WT, wild-type.

and this isolate was CIP-sensitive (Table 1). Unexpectedly, none of the 46 CIPi/CIPr strains were from participants known to be receiving contemporaneous FQ antibiotics (Table 1), although we were unable to investigate historical FQ exposure due to ethical limitations on participant data collection. It is therefore possible that some of our participants have previously received FQ antibiotics in the weeks or months prior to our sample collection. Alternatively, given that most of our participants were hospitalized, another possibility is that the CIPi/CIPr strains were nosocomially acquired, either from other admitted patients who had been or were being treated with FQs, or from the hospital environment.

The *gyrA*248 SYBR-MAMA robustly discriminated GyrA T83I from wild-type strains, with matched alleles consistently amplifying earlier than mismatched counterparts (T83I ΔC_T=4.0±0.03 vs wild-type ΔC_T=7.6±0.1 [Fig. 1a, b]). Four tested GyrA T83I strains also demonstrated intermediate or full resistance towards LEV, MFX and OFX (Table 2), confirming the importance of this variant in broader FQr. T83I is considered to be the most common GyrA variant in CIPi/CIPr strains [4, 11, 37]; for example, two Japanese studies reported 82% (60/73) [38] and 75% (112/150) [39] T83I prevalence among CIPi/CIPr isolates, and a Vietnamese study reported 54% (76/141) prevalence [11]. In our dataset, T83I was detected in 28% (13/46) CIPi/CIPr strains and 0% (0/38) CIP-sensitive strains (Table 1), suggesting that T83I is an important, but not dominant, cause of CIPi/CIPr in Australian isolates, although testing across a broader isolate collection is required to confirm this observation.

Like *gyrA*248, there was clear discrimination between AMR and wild-type genotypes for the *gyrA*259 SYBR-MAMA, with AMR alleles amplifying earlier in AMR-encoding strains (D87Y ΔC_T=14.7 [Fig. 2a]; D87N ΔC_T=9.6±0.04 [Fig. 2b]; D87H ΔC_T=13.5±0.2 [Fig. 2c]), and vice versa for wild-type strains (ΔC_T=10.8; Fig. 2d). *gyrA*259 AMR was detected in 54% (25/46) CIPi/CIPr and 0% (0/38) CIP-sensitive strains, suggesting that this SNP is the most common cause of CIPr in Australian isolates.

Table 3. SYBR Green-based mismatch amplification mutation assay (SYBR-MAMA) primers designed in this study

gyrA SNP	GyrA AMR variant	Primer name	Sequence (5'–3')*	Optimized concentration (µm)
***gyrA*248**	T83I	gyrA248_T_AMR	ACGATGGTGTCGTAGACCG**t**Ga	0.30
		gyrA248_C_WT	CGATGGTGTCGTAGACCG**t**Gg	0.30
		gyrA248_F	TGTGGTCGGCGACGTGATC	0.20
***gyrA*259**	D87N, D87Y, D87H	gyrA259_D_AMR	CATGCGCACGATGGTG**a**d	0.20
		gyrA259_G_WT	GCCATaCGCACGATGGTG**a**c	0.20
		gyrA259_F	AGCTGGGCAACGACTGGAA	0.20

*Bold nucleotides indicate the SNP; underlined nucleotides indicate deliberately incorporated antepenultimate/penultimate mismatches to enhance allele specificity.
AMR, antimicrobial resistant (allele); SNP, single-nucleotide polymorphism; WT, wild-type (allele)

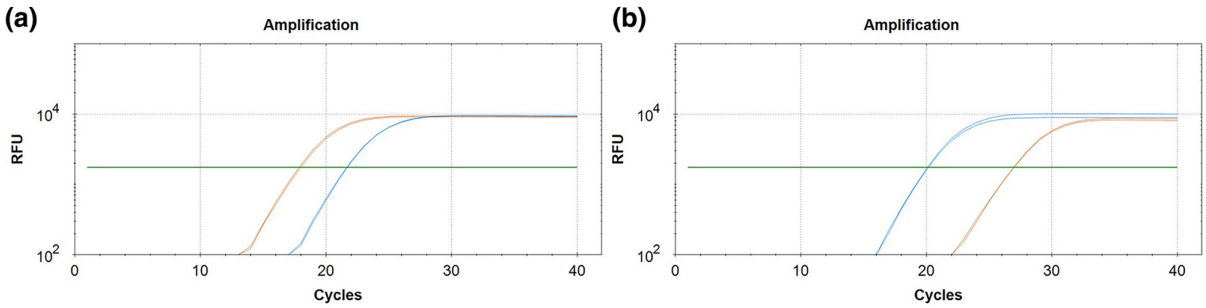

Fig. 1. SYBR Green-based mismatch amplification mutation assay (SYBR-MAMA) interrogation of the *gyrA*248 (T→C) biallelic single-nucleotide polymorphism, resulting in a threonine to isoleucine substitution at position 83 (T83I) in certain fluoroquinolone (FQ)-resistant *P. aeruginosa* isolates. *gyrA*248 SYBR-MAMA performance in: (a) SCHI0010.S.1 (FQ-resistant, encodes T83I); (b) SCHI0038.S.3 (FQ-sensitive wild-type isolate). Orange, antimicrobial-resistant allele; blue, wild-type allele. No-template controls did not amplify. All samples were run in duplicate. RFU, relative fluorescence units.

The degenerate nature of our *gyrA*259 AMR primer has the advantage of enabling all four nucleotide variants to be detected using just two reactions; it does not require each variant to be tested individually. As our assay cannot discriminate the three *gyrA*259 AMR variants from each other, we instead used WGS to determine their prevalence. D87H was most common, accounting for 88% (22/25) of the *gyrA*259 AMR strains in our database (Table 1). This high prevalence is in sharp contrast to what is observed elsewhere; when we assessed two global datasets (total *n*=656 strains [13, 29]), we found a complete absence of the D87H variant in this large international collection. Upon further examination, we found that the basis for this geographicl difference is the monopoly of the D87H variant in CF strains belonging to ST801 (AUST-06), a genotype that is almost exclusively found in people with CF from Queensland, Australia [40]. Indeed, 22/23 (96%) ST801 strains in our dataset encoded D87H, suggesting that this antimicrobial-resistant variant has become fixed in this lineage. This finding is alarming, as it indicates that FQ antibiotics are

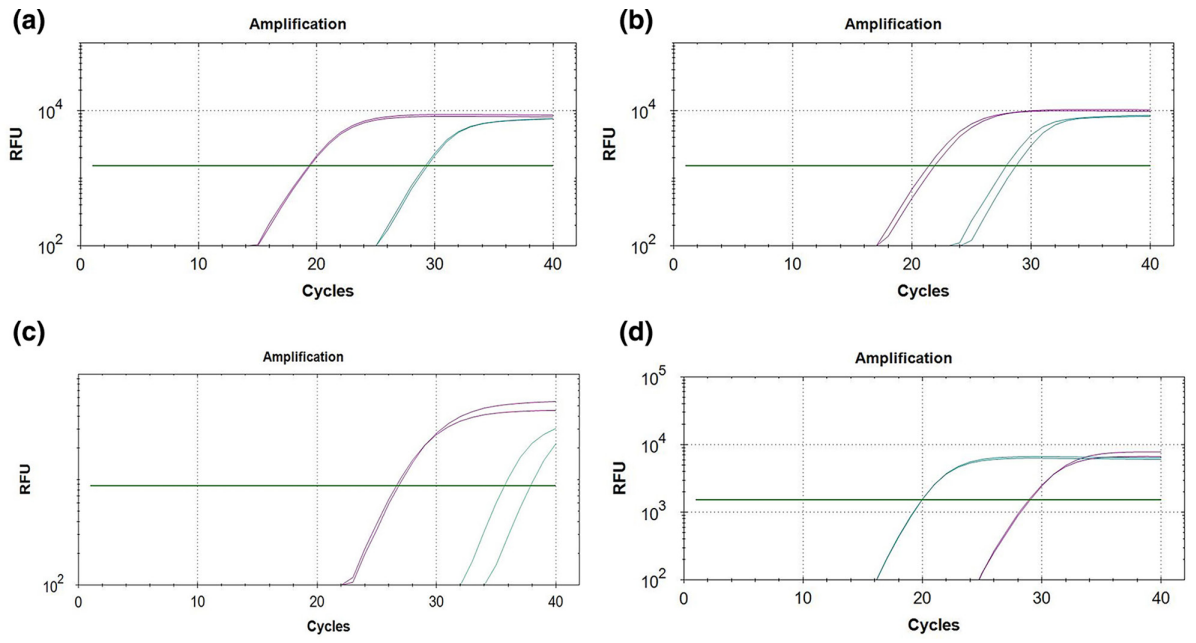

Fig. 2. SYBR Green-based mismatch amplification mutation assay (SYBR-MAMA) interrogation of the *gyrA*259 (G→A/C/T) tetra-allelic single-nucleotide polymorphism, resulting in aspartate to asparagine (D87N), histidine (D87H), or tyrosine (D87Y) substitutions, respectively, at position 87 in certain fluoroquinolone (FQ)-resistant *Pseudomonas aeruginosa* isolates. *gyrA*259 SYBR-MAMA performance in: (a) SCHI0005.S.10 (intermediate resistance towards FQs, encodes D87H); (b) SCHI0032.S.32 (FQ-resistant, encodes D87N); (c) SCHI0032.S.33 (FQ-resistant, encodes D87Y); and (d) SCHI0038.S.3 (FQ-sensitive wild-type isolate). Purple, antimicrobial-resistant allele; aqua, wild-type allele. No-template controls did not amplify. All samples were run in duplicate. RFU, relative fluorescence units.

ineffective in ST801 eradication. Identification of this lineage, especially in naïve international CF populations, should prompt CIP antimicrobial susceptibility testing as a priority due to the high risk of CIPi/CIPr.

Another discordant finding was the prevalence of dual *gyrA*248 and *gyrA*259 antimicrobial-resistant variants between our dataset and others. Dual *gyrA*248 and *gyrA*259 antimicrobial-resistant variants appear at a prevalence of 3 [29] to 14% [13] of *gyrA* QRDR-mutated strains, whereas we did not observe a strain encoding both of these antimicrobial-resistant variants in our dataset. Although the basis of this discordance is not known, we cannot rule out biases in our dataset as the cause of this discrepancy, and acknowledge our relatively small isolate number (n=84), assessment of only clinical isolates, and inclusion of multiple strains from single patients as possible reasons for these observed differences in dual *gyrA* mutation prevalence.

Like T83I, seven tested strains encoding D87Y, D87N and D87H all exhibited intermediate or full LEV, MFX and OFX resistance (Table 2). The *gyrA* mutations had a larger impact on MFX and OFX (causing AMR) compared with CIP and LEV (causing intermediate resistance) (Table 2). This observation supports the hypothesis that additional mutation(s) are sometimes required to confer AMR towards CIP and LEV [12].

It has been previously reported that, in isolation, the GyrA T83I and GyrA D87N/Y/H variants give rise to different MICs towards CIP (1 μg ml^{-1} and 0.25–0.5 μg ml^{-1}, respectively) [8]. These findings imply that strains encoding the GyrA T83I variant are more likely to have a higher CIP MIC compared with their GyrA D87N/Y/H counterparts. However, we did not observe such clear delineation in CIP MICs between strains encoding these two SNPs; strains encoding only T83I but no other CIPi/CIPr mutations (n=10) exhibited CIP MICs between 0.75 and >32 μg ml^{-1}, and strains encoding D87H/N/Y but no other CIPi/CIPr mutations exhibited CIP MICs between 1 and 8 μg ml^{-1} (n=24) (Table 1). One possibility for this difference is that our study examined clinical strains, which may encode enigmatic mutations that contribute towards the observed CIPi and CIPr phenotypes. Further studies are needed to determine the basis of this observation. Nevertheless, our results confirm the utility of our two *gyrA* SNP assays for rapidly testing for CIPi and CIPr strains. We recommend that the detection of strains encoding AMR alleles at either of these SNPs should prompt a discontinuation or avoidance of FQ therapy (pending antimicrobial susceptibility testing results) due to a higher potential for treatment failure.

We tested our two SYBR-MAMAs against chelex-extracted heat-soak DNA to determine their performance using a simple and rapid (~20 min) extraction method. In all instances, isolates yielded excellent, early amplification, and genotyped as expected. Although not tested in this study, further time savings could be made by performing SYBR-MAMAs using colony PCR [41]; however, in our experience, this approach requires a high degree of skill due to the inhibitory nature of total cellular extracts in PCR, and typically results in a low proportion of PCR failures, even with skilled operators. This issue is particularly acute when using low (e.g. 5 μl) reaction volumes, as used in our study to minimize costs. Therefore, we recommend that chelex extractions followed by 1:10 dilution be performed where rapid DNA extraction is desired to ensure 100% amplification success.

Of the eight CIPi/CIPr strains that did not encode *gyrA*248 or *gyrA*259 AMR variants, three had GyrB Ser466Phe or Ser466Tyr missense mutations with or without other FQr-conferring mutations (ParE Ala473Val, MexR Gln25Stop), whereas four had no known FQr determinants (Table 1). Although these isolates were CIPr, the MIC for these strains was relatively low, ranging from 1 to 3 μg ml^{-1}, suggesting that this resistance phenotype may be due to efflux pump upregulation rather than genomic mutation. Interestingly, even WGS was unable to identify CIPr causing mutations, yielding no additional information in comparison to the SYBR-MAMA assays.

Finally, we determined the discriminatory power of the two *gyrA* SNPs across a global collection (n=283) of genome-sequenced isolates [30–32, 34–36] with corresponding CIP phenotype data. Based on just these two SNPs, CIP non-susceptibility was predicted with 91% accuracy. Notably, 9% (8/87) CIP-sensitive strains possessed one of these two SNPs, despite being phenotypically sensitive to CIP. QRDR mutations have previously been reported in CIP-sensitive strains [32]; however, this phenomenon is rare, and the cause is not yet known, although it has been proposed that reversion to susceptibility can occur infrequently due to fitness cost [42]. Alternatively, errors in antimicrobial susceptibility reporting, metadata collation, sample mix-ups, or WGS processing may account for inconsistencies between CIPr genotypes vs phenotypes.

CONCLUSIONS

Our two *gyrA*248 and *gyrA*259 SYBR-MAMAs detected FQ non-susceptibility in 83% Australian CIPi/CIPr strains. Importantly, all FQ-sensitive strains yielded wild-type genotypes for both assays, demonstrating 100% specificity. Our two *gyrA* SYBR-MAMAs thus provide a same-day, inexpensive, simple, and accurate tool for detecting the two most prevalent causes of FQ non-susceptibility in *P. aeruginosa*. Implementation of these assays in the diagnostic laboratory would enable routine surveillance of CIPi and CIPr strains, leading to quicker alterations to antimicrobial treatment, a decrease in inappropriate antibiotic therapy administration, enhanced antimicrobial stewardship measures and, ultimately, improved patient outcomes [9, 10]. An additional SYBR-MAMA targeting GyrB Ser466Phe and Ser466Tyr would increase detection of FQ

non-susceptibility to 91% without loss of specificity. Conversion of our assays to single-tube Melt-MAMA or agarose MAMA [14] formats would decrease assay costs, further increasing their accessibility in low-resource laboratories.

Funding information
This work was supported by Advance Queensland (awards AQIRF0362018 and AQRF13016-17RD2), an Australian Government Research Training Program Scholarship, the Wishlist Sunshine Coast Health Foundation (award 2019-14), and the National Health and Medical Research Council (award 455919). The funders had no role in study design, data acquisition, analysis, interpretation, writing or submission of the manuscript.

Acknowledgements
We thank Patrick Harris (UQ Centre for Clinical Research) for helpful discussions.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Ethical approval for collection and analysis of clinical samples was obtained from The Prince Charles Hospital (TPCH) Human Research Ethics Committee (HREC), project IDs HREC/13/QPCH/127 [19], HREC/2019/QPCH/48013 [19] and HREC/18/QPCH/110 [23], and the Royal Brisbane and Women's Hospital HREC, project ID DA: jl [20]. Site-specific approvals were subsequently obtained for sample collection across several public and private hospitals in Southeast Queensland, Australia. All participants provided written consent, except for the bloodstream, urinary tract infection and non-CF bronchiectasis isolates, where a waiver of informed consent was granted due to the low to negligible risk associated with these studies [20, 23].

References

1. Breidenstein EBM, de la Fuente-Núñez C, Hancock REW. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol* 2011;19:419–426.
2. Botelho J, Grosso F, Peixe L. Antibiotic resistance in *Pseudomonas aeruginosa* - mechanisms, epidemiology and evolution. *Drug Resist Updat* 2019;44:100640.
3. Schwartz T, Volkmann H, Kirchen S, Kohnen W, Schön-Hölz K, *et al*. Real-time PCR detection of *Pseudomonas aeruginosa* in clinical and municipal wastewater and genotyping of the ciprofloxacin-resistant isolates. *FEMS Microbiol Ecol* 2006;57:158–167.
4. Higgins PG, Fluit AC, Milatovic D, Verhoef J, Schmitz FJ. Mutations in GyrA, ParC, MexR and NfxB in clinical isolates of *Pseudomonas aeruginosa*. *Int J Antimicrob Agents* 2003;21:409–413.
5. Poole K. *Pseudomonas aeruginosa*: resistance to the max. *Front Microbiol* 2011;2:65.
6. Lee JK, Lee YS, Park YK, Kim BS. Alterations in the GyrA and GyrB subunits of topoisomerase II and the ParC and ParE subunits of topoisomerase IV in ciprofloxacin-resistant clinical isolates of *Pseudomonas aeruginosa*. *Int J Antimicrob Agents* 2005;25:290–295.
7. Yonezawa M, Takahata M, Matsubara N, Watanabe Y, Narita H. DNA gyrase gyrA mutations in quinolone-resistant clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1995;39:1970–1972.
8. Rehman A, Jeukens J, Levesque RC, Lamont IL. Gene-gene interactions dictate ciprofloxacin resistance in *Pseudomonas aeruginosa* and facilitate prediction of resistance phenotype from genome sequence data. *Antimicrob Agents Chemother* 2021;65:e0269620.
9. Micek ST, Lloyd AE, Ritchie DJ, Reichley RM, Fraser VJ, *et al*. *Pseudomonas aeruginosa* bloodstream infection: importance of appropriate initial antimicrobial treatment. *Antimicrob Agents Chemother* 2005;49:1306–1311.
10. Darie AM, Khanna N, Jahn K, Osthoff M, Bassetti S, *et al*. Fast multiplex bacterial PCR of bronchoalveolar lavage for antibiotic stewardship in hospitalised patients with pneumonia at risk of Gram-negative bacterial infection (Flagship II): a multicentre, randomised controlled trial. *Lancet Respir Med* 2022; :S2213-2600(22)00086-8.
11. Nguyen KV, Nguyen TV, Nguyen HTT, Le DV. Mutations in the *gyrA*, *parC*, and *mexR* genes provide functional insights into the fluoroquinolone-resistant *Pseudomonas aeruginosa* isolated in Vietnam. *Infect Drug Resist* 2018;11:275–282.
12. Rehman A, Patrick WM, Lamont IL. Mechanisms of ciprofloxacin resistance in *Pseudomonas aeruginosa*: new approaches to an old problem. *J Med Microbiol* 2019;68:1–10.
13. Kos VN, Déraspe M, McLaughlin RE, Whiteaker JD, Roy PH, *et al*. The resistome of *Pseudomonas aeruginosa* in relationship to phenotypic susceptibility. *Antimicrob Agents Chemother* 2015;59:427–436.
14. Birdsell DN, Pearson T, Price EP, Hornstra HM, Nera RD, *et al*. Melt analysis of mismatch amplification mutation assays (Melt-MAMA): a functional study of a cost-effective SNP genotyping assay in bacterial models. *PLoS One* 2012;7:e32866.
15. Germer S, Higuchi R. Single-tube genotyping without oligonucleotide probes. *Genome Res* 1999;9:72–78.
16. Donà V, Smid JH, Kasraian S, Egli-Gany D, Dost F, *et al*. Mismatch amplification mutation assay-based real-time PCR for rapid detection of *Neisseria gonorrhoeae* and antimicrobial resistance determinants in clinical specimens. *J Clin Microbiol* 2018;56:e00365–00318.
17. Sarovich DS, Price EP, Von Schulze AT, Cook JM, Mayo M, *et al*. Characterization of ceftazidime resistance mechanisms in clinical isolates of *Burkholderia pseudomallei* from Australia. *PLoS One* 2012;7:e30789.
18. Germer S, Holland MJ, Higuchi R. High-throughput SNP allele-frequency determination in pooled DNA samples by kinetic PCR. *Genome Res* 2000;10:258–266.
19. Webb KA, Olagoke O, Baird T, Neill J, Pham A, *et al*. Genomic diversity and antimicrobial resistance of *Prevotella* species isolated from chronic lung disease airways. *Microb Genom* 2022;8:000754.
20. McCarthy KL, Paterson DL. Long-term mortality following *Pseudomonas aeruginosa* bloodstream infection. *J Hosp Infect* 2017;95:292–299.
21. Freschi L, Jeukens J, Kukavica-Ibrulj I, Boyle B, Dupont M-J, *et al*. Clinical utilization of genomics data produced by the international *Pseudomonas aeruginosa* consortium. *Front Microbiol* 2015;6:1036.
22. Anuj SN, Whiley DM, Kidd TJ, Bell SC, Wainwright CE, *et al*. Identification of *Pseudomonas aeruginosa* by a duplex real-time polymerase chain reaction assay targeting the ecfX and the gyrB genes. *Diagn Microbiol Infect Dis* 2009;63:127–131.
23. Stewart AG, Price EP, Schabacker K, Birikmen M, Harris PNA, *et al*. Molecular epidemiology of third-generation-cephalosporin-resistant Enterobacteriaceae in southeast Queensland, Australia. *Antimicrob Agents Chemother* 2021;65:e00130–21.
24. Price EP, Viberg LT, Kidd TJ, Currie BJ, *et al*. Transcriptomic analysis of longitudinal Burkholderia pseudomallei infecting the cystic fibrosis lung. *Microb Genom* 2018;4:e000194.
25. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012;19:455–477.
26. Madden DE, Baird T, Bell SC, McCarthy KL. Keeping up with the pathogens: Improved antimicrobial resistance detection and prediction in *Pseudomonas aeruginosa*. *MedRxiv* 2022.

27. Madden DE, Webb JR, Steinig EJ, Currie BJ, Price EP, *et al*. Taking the next-gen step: Comprehensive antimicrobial resistance detection from *Burkholderia pseudomallei*. *EBioMedicine* 2021;63:103152.
28. de Lamballerie X, Zandotti C, Vignoli C, Bollet C, de Micco P. A one-step microbial DNA extraction method using "Chelex 100" suitable for gene amplification. *Res Microbiol* 1992;143:785–790.
29. van Belkum A, Soriaga LB, LaFave MC, Akella S, Veyrieras J-B, *et al*. Phylogenetic distribution of CRISPR-cas systems in antibiotic-resistant *Pseudomonas aeruginosa*. *mBio* 2015;6:e01796–15.
30. Cabot G, López-Causapé C, Ocampo-Sosa AA, Sommer LM, Domínguez MÁ, *et al*. Deciphering the resistome of the widespread *Pseudomonas aeruginosa* sequence type 175 international high-risk clone through whole-genome sequencing. *Antimicrob Agents Chemother* 2016;60:7415–7423.
31. Del Barrio-Tofiño E, López-Causapé C, Cabot G, Rivera A, Benito N, *et al*. Genomics and susceptibility profiles of extensively drug-resistant *Pseudomonas aeruginosa* isolates from Spain. *Antimicrob Agents Chemother* 2017;61:1–13.
32. Sherrard LJ, Tai AS, Wee BA, Ramsay KA, Kidd TJ, *et al*. Within-host whole genome analysis of an antibiotic resistant *Pseudomonas aeruginosa* strain sub-type in cystic fibrosis. *PLoS One* 2017;12:1–15.
33. Fraser TA, Bell MG, Harris PNA, Bell SC, Bergh H, *et al*. Quantitative real-time PCR assay for the rapid identification of the intrinsically multidrug-resistant bacterial pathogen *Stenotrophomonas maltophilia*. *Microb Genom* 2019;5:e000307.
34. Tsang KK, Maguire F, Zubyk HL, Chou S, Edalatmand A, *et al*. Identifying novel β-lactamase substrate activity through in silico prediction of antimicrobial resistance. *Microb Genom* 2021;7:000500.
35. Buhl M, Kästle C, Geyer A, Autenrieth IB, Peter S, *et al*. Molecular evolution of extensively drug-resistant (XDR) *Pseudomonas aeruginosa* strains from patients and hospital environment in a prolonged outbreak. *Front Microbiol* 2019;10:1742.
36. Ramanathan B, Jindal HM, Le CF, Gudimella R, Anwar A, *et al*. Next generation sequencing reveals the antibiotic resistant variants in the genome of *Pseudomonas aeruginosa*. *PLoS One* 2017;12:e0182524.
37. Farahi RM, Ali AA, Gharavi S. Characterization of *gyra* and *parc* mutations in ciprofloxacin-resistant *Pseudomonas aeruginosa* isolates from tehran hospitals in iran. *Iran J Microbiol* 2018;10:242–249.
38. Takenouchi T, Sakagawa E, Sugawara M. Detection of *gyrA* mutations among 335 *Pseudomonas aeruginosa* strains isolated in Japan and their susceptibilities to fluoroquinolones. *Antimicrob Agents Chemother* 1999;43:406–409.
39. Akasaka T, Tanaka M, Yamaguchi A, Sato K. Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: role of target enzyme in mechanism of fluoroquinolone resistance. *Antimicrob Agents Chemother* 2001;45:2263–2268.
40. Kidd TJ, Ritchie SR, Ramsay KA, Grimwood K, Bell SC, *et al*. *Pseudomonas aeruginosa* exhibits frequent recombination, but only a limited association between genotype and ecological setting. *PLoS One* 2012;7:e44199.
41. Bergkessel M, Guthrie C. Colony PCR. *Methods Enzymol* 2013;529:299–309.
42. Levin BR, Perrot V, Walker N. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics* 2000;154:985–997.