# Resistance mechanisms and molecular epidemiology of *Pseudomonas aeruginosa* strains from patients with bronchiectasis

Roberto Cabrera<sup>1,2</sup>†, Laia Fernández-Barat<sup>1,2</sup>\*†, Nil Vázquez<sup>1,2</sup>, Victoria Alcaraz-Serrano<sup>1,2</sup>, Leticia Bueno-Freire<sup>1,2</sup>, Rosanel Amaro<sup>1,2</sup>, Rubén López-Aladid<sup>1,2</sup>, Patricia Oscanoa<sup>1,2</sup>, Laura Muñoz<sup>3</sup>, Jordi Vila<sup>3</sup> and Antoni Torres<sup>1,2</sup>

<sup>1</sup>Hospital Clínic, Cellex Laboratory, CIBERES (Center for net Biomedical Research Respiratory diseases, 06/06/0028) - Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), School of Medicine, University of Barcelona, Spain; <sup>2</sup>Respiratory Intensive Care Unit, Pneumology Department, Hospital Clínic, Barcelona, Spain; <sup>3</sup>Barcelona Global Health Institute, Department of Clinical Microbiology, Hospital Clínic, University of Barcelona, Barcelona, Spain

> \*Corresponding author. E-mail: lfernan1@clinic.cat †Contributed equally.

Received 12 July 2021; accepted 14 February 2022

**Background:** Non-cystic fibrosis bronchiectasis (BE) is a chronic structural lung condition that facilitates chronic colonization by different microorganisms and courses with recurrent respiratory infections and frequent exacerbations. One of the main pathogens involved in BE is *Pseudomonas aeruginosa*.

**Objectives:** To determine the molecular mechanisms of resistance and the molecular epidemiology of *P. aeruginosa* strains isolated from patients with BE.

**Methods:** A total of 43 strains of *P. aeruginosa* were isolated from the sputum of BE patients. Susceptibility to the following antimicrobials was analysed: ciprofloxacin, meropenem, imipenem, amikacin, tobramycin, aztreonam, piperacillin/tazobactam, ceftazidime, ceftazidime/avibactam, ceftolozane/tazobactam, cefepime and colistin. The resistance mechanisms present in each strain were assessed by PCR, sequencing and quantitative RT-PCR. Molecular epidemiology was determined by MLST. Phylogenetic analysis was carried out using the eBURST algorithm.

**Results:** High levels of resistance to ciprofloxacin (44.19%) were found. Mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes were detected in ciprofloxacin-resistant *P. aeruginosa* strains. The number of mutated QRDR genes was related to increased MIC. Different  $\beta$ -lactamases were detected:  $bla_{OXA50}$ ,  $bla_{GES-2}$ ,  $bla_{IMI-2}$  and  $bla_{GIM-1}$ . The *aac(3)-Ia*, *aac(3)-Ic*, *aac(6")-Ib* and *ant(2")-Ia* genes were associated with aminoglycoside-resistant strains. The gene expression analysis showed overproduction of the MexAB-OprM efflux system (46.5%) over the other efflux system. The most frequently detected clones were ST619, ST676, ST532 and ST109.

**Conclusions:** Resistance to first-line antimicrobials recommended in BE guidelines could threaten the treatment of BE and the eradication of *P. aeruginosa*, contributing to chronic infection.

# Introduction

Non-cystic fibrosis bronchiectasis (BE) is a persistent and progressive respiratory disease characterized by irreversible dilation of one or both bronchi. The dilation is a result of a destructive process in the bronchial walls, with damage to the epithelial lining due to the recurrent bacterial infections and continuous inflammation. The symptoms of this disease include sputum production, constant cough, dyspnoea and periodic exacerbations that result in decreased lung function and a worse quality of life.<sup>1</sup> *Pseudomonas aeruginosa* is a Gram-negative opportunistic microorganism that causes severe healthcare infections globally, such as sepsis, urinary tract infections, surgical site infections and respiratory tract infections. This microorganism is one of the most frequent pathogens in BE and chronic respiratory infections.<sup>2</sup> Unfortunately, *P. aeruginosa* diagnosis and eradication therapy have a high rate of failure. Thus, BE patients colonized by *P. aeruginosa* receive frequent antimicrobial agents, favouring the emergence and spread of MDR/XDR *P. aeruginosa* strains and challenging the efficacy of antimicrobial agents. The extensive dissemination of MDR/XDR strains and high-risk clones worldwide adds further concern. Previous studies found that the high-risk

© The Author(s) 2022. Published by Oxford University Press on behalf of British Society for Antimicrobial Chemotherapy. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (https:// creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com 1600 clones are associated with certain clonal complexes (CCs) and that their distribution varies depending on the region.<sup>3</sup> However, no previous studies have reported high-risk clones from BE patients.

The most important antipseudomonal agents include auinolones (e.g. ciprofloxacin),  $\beta$ -lactams (e.g. cefepime, ceftazidime, piperacillin/tazobactam, imipenem and meropenem) and aminoglycosides (e.g. amikacin and tobramycin). A wide range of mechanisms of resistance have been described for the different antimicrobial types: (1) acquisition of mutations in QRDRs; (2) production of  $\beta$ -lactamases (e.g. ESBLs and carbapenemases); (3) aminoglycoside-modifying enzymes (AMEs); (4) upregulation of efflux systems such as MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY with specific exportable substrates including quinolones, cephalosporins, carbapenems and aminoglycosides; and (5) loss or decreased production of the OprD protein used as an entrance channel by carbapenems.<sup>4</sup> Recent information shows that resistance to antimicrobial agents is increasing, even to first-line antimicrobial agents, which may lead to therapeutic failure and chronic infection.<sup>5</sup> The objective of our study was to determine the molecular mechanisms of resistance and the molecular epidemiology of P. aeruginosa strains isolated from patients with BE.

# Materials and methods

Forty-three clinical P. aeruginosa strains were isolated from sputum samples of different consecutive patients with chronic BE during their stable phase, in a prospective observational study carried out in the Hospital Clínic of Barcelona (Spain). This prospective observational study (NCT04803695) was conducted at the pulmonology service of a tertiary care hospital and at the CELLEX research laboratories of the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) in Barcelona, Spain. Thirty-eight patients were included from June 2017 to February 2020 and followed up for 1 year prospectively. One strain was isolated per patient but in five patients with chronic P. aeruginosa infection we isolated two different P. aeruginosa morphotypes (one mucoid and one nonmucoid for one patient, and one small and one large colony for each of the other four: strains 17, 18, 20, 21, 22, 23, 26, 27, 29 and 30). However, they were different in resistance pattern and/or mechanisms of resistance. A visit was performed every 3 months during the stable phase. During each visit: (1) one sputum sample was obtained; and (2) lung function was assessed with an EasyOne World Spirometer (NDD Medical Technologies, Zurich, Switzerland) and classified according to the American Thoracic Society/European Respiratory Society Guidelines.

# Antimicrobial susceptibility testing

The strains from sputum were cultured at 37°C for 24 h and were prepared in 0.9% NaCl at a density adjusted to a 0.5 McFarland (Becton Dickinson, Germany) turbidity standard. Antibiotic susceptibility testing was performed using the Kirby–Bauer method and Etest in accordance with the instructions of the manufacturers (bioMérieux and Liofilchem). MICs were determined by the standard agar dilution method with Mueller–Hinton II agar (Becton Dickinson). Colistin susceptibility was tested by broth microdilution method using MICRONAUT plates (MERLIN Diagnostika GmbH, Bornheim, Germany). The ATCC 27853 strain was used as a control. The following antibiotics were tested: aztreonam, ciprofloxacin, meropenem, imipenem, amikacin, tobramycin, piperacillin/ tazobactam, ceftazidime, ceftazidime/avibactam, ceftolozane/tazobactam, cefepime and colistin. Replicates of each susceptibility test were performed. All results were interpreted in accordance with EUCAST guidelines v9.0 (http://www.eucast.org/clinical\_breakpoints/).<sup>6</sup>

# Mechanisms of resistance

Using PCR and sequencing, we tested the main mechanisms of resistance to ciprofloxacin (mutations in the QRDR), amikacin and tobramycin (the presence of AMEs), aztreonam, meropenem, imipenem, piperacillin/tazobactam, ceftazidime, ceftazidime/avibactam, ceftolozane/tazobactam and cefepime (production of  $\beta$ -lactamases) and colistin (*mcr* genes). Mutations in *oprD* and post-transcriptional regulator genes (*nalC*, *nalD*, *mexR*, *nfxB*, *mexT*, *mexS* and mexZ) were also determined by PCR and sequencing. Gene expression analysis was conducted by quantitative RT-PCR (RT-qPCR). The primers and conditions are shown in Table 1. The PCR products were sequenced by Sanger methods (GENEWIZ, Germany), and were analysed by alignment with the template sequence in GenBank.

#### RNA extraction and reverse transcription

Strains were grown in 10 mL of LB broth at 37°C for 18–24 h up to the late exponential phase and collected by centrifugation. Total RNA extraction was carried out using the QIAGEN RNeasy purification kit. After checking the RNA extraction quality on a 1% agarose gel and measuring the RNA content (Nanodrop, Thermo Fisher Scientific, France), RNA extracts were stored at  $-20^{\circ}$ C until further use. Prior to cDNA synthesis, genomic DNA (gDNA) was removed from 1 µg of total RNA using the gDNA wipeout buffer included in the QuantiTect Reverse Transcription kit (QIAGEN). The reverse transcription was performed in a volume of 20  $\mu$ L including 14  $\mu$ L of template RNA (extract concentrations adjusted to contain 1 µg of RNA),  $1 \mu L$  of Reverse Transcription Master Mix,  $4 \mu L$  of RT buffer  $5 \times$  (containing dNTPs and Mg<sup>2+</sup>) and 1  $\mu$ L of RT primer mix. Reverse transcription was performed in a Veriti PCR Thermal Cycler (Applied Biosystems, France) for 30 min at 42°C followed by a 3 min incubation at 95°C to inactivate the reverse transcriptase. All reactions including RNA handling were carried out on ice. The rpsL gene was used as reference to normalize the relative amount of mRNA.

#### **Real-time PCR assay**

This work was focused on the expression of the four major P. aeruginosa efflux pump genes (mexB, mexD, mexF and mexY). Normalization of expression results was carried out using *rpsL* (reference gene to normalize the relative amount of mRNA) and using the PA01 strain as a control. A LightCycler 96 (Roche Diagnostics, Meylan, France) was used for all quantitative PCRs. All PCR amplification reactions were performed in 96-well plates in a 10  $\mu$ L final volume containing 2.5  $\mu$ L of diluted (1:20) template cDNA,  $1 \,\mu L$  of each primer (corresponding to a final concentration of  $0.5 \mu$ M),  $5 \mu$ L of QuantiTect SYBR Green PCR Master Mix (including MqCl<sub>2</sub> to reach a final concentration of 2.5 mM) (QIAGEN) and 0.5  $\mu L$  of RNase/DNase free water (QIAGEN). The cycling program was set as follows: (1) activation: 1 cycle at 95°C for 15 min; (2) amplification: 45 cycles including a 15 s denaturation at 95°C, a 25 s annealing at 60°C and a 15 s elongation at 72°C; and (3) melting curve: 1 cycle including 5 s at 95°C, 1 min at 65°C and a final increase at 97°C with a transition rate of 0.11°C/s. Each reaction was carried out in duplicate and the experiment was repeated on two different sets of RNA extracts (biological replicate).4,7,8

#### **Evaluation of real-time PCR results**

Using the  $\Delta\Delta$ Ct method, overexpression of *mexB*, *mexD*, *mexF* and *mexY* was considered when the corresponding mRNA level was at least 2-fold higher than that of ATCC PA01 (the *rpsL* gene was used as reference to normalize the relative amount of mRNA), negative if less than 1-fold higher and borderline if between 1- and 2-fold higher.<sup>7,9</sup>

#### Table 1. Primers used in this study

gyrAgyrA FAGTCCTATETCGACTACGCGAT3415517gyrBgyrB FCTGGCGGAAAGAGGAGGGCAAGTAC697557gyrB RCTGGCGGAAAGAGGGCAAGTAC697557gwrB RCCGGGCGACTGCGAACGAGGAGGCAAGTAC235557gwrC RGAAGGACTTGGGATCGGCGAGGGA992656gwrC RCGGGCGTCGTCGTCGGGAGGGGAGGAGGA992656malCmalC FTCAACGAGGGAGAGGCG814696malCmalC FTCAACGAGGGAGACTC789556malDmalD FGCGGCCTAAGGGGAACTCG789556masRmasR2DCCAGTAAGCGGAAACTCG1016516masRmasR2DCCAGTAAGCGGAAACTCG7866masRmasR2DCCAGTAAGCGGAAACCGT1388556masRmasR2DCCAGTAAGCGGAACCCATGGA1153506masSmasSFATACGGTCAAACCCATGGA1153506masZmasZ102CCCAGCTGGAGGAAGAACCCA138466gesges-RGTCCCTGCCAGGGGAAGGA138467gesges-RGTCCCTGCCCAGGGCAAGGC138467gesges-RGTCCCTGCCCAGGGCAAGGCA371557gesges-RGTCCCTGCCCAGGGCAAGGCAGA737gesges-RGTCCCTGCCCCAGGGCAAGCCCAGA7757gesges-RGCCCACCTGCCCCCAGGGCAAGCCCAGGCAGA77	Amplified product	Primer pair	Sequence (5' to 3')	Amplicon size (bp)	Annealing temperature (°C)	Reference
Byrk         ATCGACCOTTICCAG         Factor           gyrk         gyrk P         GGGGGGAACAGGAGGTGGGCACAGCAGC         697         55         17           gyrk P         GGGGGGAACAGGAGGTGGGCAACAGGAGGT         697         55         17           gyrk P         CTGGGGGAACAGGAGGTCATCTA         235         55         17           gyrk P         CGGGGCACGCTCGTCTGGAACGA         592         65         4           port P         CGGGCGTCTGCTCGGCGGAACGA         592         65         4           nall P         TGCACCTCACCGAGAAACGCT         814         69         4           nall P         TGCACCTCACCGAGGGAAACGCT         814         69         4           nall P         GGGGCTAACGAGAAACGCT         814         69         4           nall P         GGGGCTAAACGAGAACGCT         814         69         4           nall R         GGTACTCACCTCC         814         69         4           mexi R         TGCAACTCACGGGGGAAAGGG         1398         55         4           mexi R         GGTAGCACAGGAAATAGGGCGACAGGC         159         64         4           mexi R         TGCAACGACATGAAGGCGGACAGGC         159         54         4           gges R         GTTGCGC	avrA	avrA-F	AGTCCTATCTCGACTACGCGAT	341	55	17
gyr8gyr8-FTCCGOTGGAACAGGAATGGCCAATGAC GYACGGGTG6975517gyr6-Fgyr6-FCGAGGAAGAGCTAACGGAATGCCAATGAC CGAGGAAGAGCTAACGCAAGGCT2255527parcparc-FCGAGGACGAGCTATGCGATGCCCGA77parc-FCGAGGACGAGCTAACGAACGACGAACGAC CaatGAACGACGAACGAC592657naliCnaliC-FTCCAACGTAACGAAAGACGCT814697naliDnaliD-FTCCAACTCAACGAAAACGCT789557naliDnaliD-FTCCAACTCAACGAAAAGCGT789557makRmakR20CCAATAAGGGAATAAC1398557makRmakR20CCAATAAGGGAATAAC1398557makRmakR20CCAATAAGGGAATAAC1398557makRmakR20CCAATAAGGGAATAACTC1398557makRmakR20CCAACTAAGGGAATAACTC1383506makRmakS7TCCAACGCCGAGGAAATAGCGG1059644makRmakS7GGGCAGGAATAGCGGGACCAGGGC1059642opdD-FGGCGCAGCGAATAGGGCGACCAGGGC1059557gesges-RGTTCCCTGCCAATGAGCGGA371557gesges-RGTTCCCTGCCAATGAGCGGA371557ginngin-RAACTCTCGCCCCTGTGAA477557ginngin-RAACTCTCAACTTGCCGATG143553ginngin-RAACTCTCAACTTGCCGATG14353	99	gvrA-R	AGTCGACGGTTTCCTTTTCCAG			
gyr# R         CTG6GG6AAGAAGCAGTGCAACACCAGGGGT           porC         porC-F         CGAAGGACCTGCGCATACTGAACGA         235         55         7           porE         porC-F         CGAAGGACTTGCGCATGCTGAACGA         592         65         4           porE         porE-F         CGAAGGACTTGCGCAGTGGAAGAACGCT         814         69         4           naliC         naliC-F         TCAACCCTAACGGAAAACGCT         814         69         4           naliD         naliD-R         CCGGCTAAAATGCGTACACT         789         55         4           naliD-R         ACGTCCAGGTGAACTC         789         55         4           mexR         MacGTCCAGGTGAACTC         1398         55         4           mexR         TGCATCACGGGTAACT         1398         55         4           mexR         TGCATCACGGGTAACTCACACCCAGGAGAATGT         1398         55         4           mexR         GGTAGCGCCAGGAGAATATCACACCCAGT         153         50         4           mexS         mexTCACACACCCAGGACACAGCGC         1059         64         4           mexS         GTAGCGCCAGGAACAACGGGACA         1384         64         36           mexS         GTAGCAGCGCGGGACACACGGGC         105         55	avrB	avrB-F	TGCGGTGGAACAGGAGATGGGCAAGTAC	697	55	17
parC         parC-F         CGAGCAGCCTACTICATICACATAT         235         55         17           parE         parG-R         GAAGGACTIGTGCGGA         235         55         17           parE         parG-R         GGAGCATIGTGCGACGGA         592         65         1           parE-R         TCGAGCGCGATAGTACATGCCTTCGCGGA         592         65         1           nall         nall-F         TCGACCTACGGAACGCCT         789         55         1           nall         nall-R         ACGTCCACGGATACCT         789         55         1           mexR         mexRR         GGAGCTCAACGGATAACGCT         789         55         1           mexR         GGTACCCCCAGGAGAAGGATGC         1016         51         1         1           mexR         GGTACCCCCCAGGAGAAGGAGTG         mexF         TCAACGGACACAGGCCCAGGGACAGGCG         1059         64         1           mexS         mexS F         ATACGGCAGACAGGCCAGGGACAGGGG         1059         64         2         2           oprD-R         GTCCCCTGGGAGACACGGCG         1059         64         2         2         2           oprD-R         GTCCCCTGGGAGACAGCACGGGG         1284         65         2         2         2	9).0	avrB-R	CTGGCGGAAGAAGAAGGTCAACAGCAGGGT			
porC-R         GAAGGACTTGGGATCGTCCGGA         Sec           parF         parF-F         CGGAGGACTTGGGATCGTCCGGA         592         65         4           parF-F         CGGAGGCAGTGAFACATTCTCTCCGCA         814         69         4           nalC         nalC+F         TCCAAGCCAGAGAACTGC         814         69         4           nalD-R         TCCAAGCCAGTCGAGTCTGG         814         69         4           nalD-R         ACGTCCAGTGGGATCTGG         8         7         4           mexR         mexRX         CCAGIAMAGGGAIXAC         1016         51         4           mexR         mexRX         GGAGGAACACCCATGGAATCAGG         1338         50         4           mex7         F         FGCAICACCGAGGGAGAACGAGGGC         1059         64         2           mex7         mex5-F         ATACAGTCACACCAACGGC         1153         50         4           mex7         mex52060         CCAGCGGACAACGGAGGGGGCACAGGGC         1059         64         2           oprD-R         GTTGCCTGCGATTACT         619         54         12           oac50         oxa50-R         GGTGGCGGATACGTCGCGG         2         2           ges         ge-F         GTTGCGCGCGATTACGCG	parC	parC-F	CGAGCAGGCCTATCTGAACTAT	235	55	17
porfporfRCGGCGTTGGTCGGGGCGTGGTAAAGGA592654nalCnalC-RTCCAGGGCGTAGTAAGTGCGTTGCGCA814694nalDnalD-RTCCACCTCACCGAAGTGC814694nalDnalD-RCCGGTCAAATGCGTACACAT789554nalDnalD-RCCGGTGAAATGCGTACACAT789554mexRmexR20CCAGTAAGCGGATAC1016514mexRmexF.FTGCATCACGGGGGTAATAAC1198504mexRmexS-FTCAACGTCGCAAAGCCATGA1153504mexRmexS-FTCAACGTCGCAAAGCCATGA1153504mexRmexS-FTCAACGTCGCAAGGCAACCCAGGGC1059644mexSmexS-FATACAGTCGCAAAGCCAACGCAGGGC1059644mexSmexS-FATACAGTCGCAAAGCCAAGCGGC1059644mexSmexS-FATACAGTCGCAAGCGCAGGGC1059644mal2mexS-FATACGTGCAAGCGCAAGCCCGG1059644mexSmexS-FATACGCGGGCCATCAC61952mal2mexS-FATACGCGGGCCATCACC619552mal2mexS-FATACGCGGGCGCATCACC619552mal3miFATACGCGGGCGCATCACC619552mal3miFATACGCCGGCGCATCACC619552mal3miFTTGCAATGGGGGGGTAAG1136mal3miFTCGAACCAT		parC-R	GAAGGACTTGGGATCGTCCGGA			
parts         parts         TCGAGGGCGTAGTAGATGAGTGTCCTTGCCGA         Dist         Dist           nalC         nalC+F         TCCACCCTAACGAGAAACGCT         814         69         *           nalD         nalD+F         TCGACCCTAACGAGAAACGCT         814         69         *           nalD         nalD+F         GCGCGCTAAAACGGTCACACT         789         55         *           nalD-F         GCGCGCAGAACCGCTCACCT         1016         51         *           mexRR         mexRRD         CCCAGTAAGCGGGTACC         1016         51         *           mexRR         TGGTCAGGGGGTAGACC         1016         51         *         *           mexRR         TGGTCAGGGGTAGACCCTTCAGCT         1398         50         *         *           mexRR         GGTAGCCCAGGAGATACCCTGA         1153         50         *         *           mexS         mexSPR         CCAGCAGGAGATATTCAGGGGTAGACAGGGCG         153         50         *           mexSC         mexSPR         CCAGCGGGACACCAGGCC         153         50         *         *           gorD-F         GGTCAGCGACTAGGGCGTACCCAGC         153         55         7         *         *           gorDD-F         GGCTCAGCGACTCAGGGTAG	parF	pare R parF-F	CGCCGTTCGTCTCGGCCGTGGTGAAGGA	592	65	4
noiC         noiC+         TCAACCCTAACGAGAAACCGCT         814         69         4           noiD         noiD+R         TCCACCTAACGGAACTGC         789         55         4           noiD         noiD+R         GCCAGTAAACCGGACTACCT         789         55         4           mexR         mexRPUT         GGATGATGGCGTGAACTCGGACTACCT         1016         51         4           mexR         mexRPUT         GGATGATGCCACGAGAAATCG         1016         51         4           mexR         mexT-F         TGCACCCAGGAAATACC         1988         55         4           mexS         mexF-F         TGCACGGGGGCACCAGGGC         1059         64         4           mexS         mexS1026         CCAGGGGGACCAGGGGC         1059         64         2           oprD         oprD-R         GTTGCCTGGGACTGCAGGGC         1059         64         2           oprD         GGCGAGAGATAATTGAAACCAA         1384         64         2         2           oprD-R         GTTGCCTGTGCGGTCGACTGAGGC         371         55         2         2           oprD-R         GTTGCCTGTGCGGTCGACGCAGGCGGTGA         371         55         2         2           oprD-R         GTTGCCTGTGCGGTCGAGCGAGGCGGTG	p 0.12	parE-R	TCGAGGGCGTAGTAGATGTCCTTGCCGA	552		
noliC.RTCCACCTCACCGAACTGCTCCACCTCACCGGAACTGCnalDnalD-FGCCGCTCAAATCGGTCACT799554nalDnalD-FGCCGCTCAAGTGGATCACT1016514mexRmexRD0CCCAGTAAGCGGATAC1016514mexTmexRINTGGTACCCCAGGAGAATC1398554mexTmexT-FTGCATCACGGGGGTGAATAC1398504mexSmexF ATCAACGATCTGTGAATC15566mexZmexZ050CCACGCAGGAATGGAGGCCACCAGGCC1059644opD-FGGTCACGTGGAGATTGAAGGCACCCGG1059644opD-RGTTCCCTGTCGGTGGTCATCCATC6195432opD-RGTTCCCTGCGGTGGTCATCCATC6195432opd-RGTTCCGCTGGGTGCTCAGGCGG3715524imiimi-RATCCGCGCCTCAGCGGATAC6195432oggsges-RGCTCCATACCAATAGCGCGTAA3715524imiimi-RATCCGCCCTCAGCGGATGC3715524imiimi-RACTCCAATCCAGCTGGTGTAATCC8185524ooc(3)-laooc(3)lc-FCCTCTCACAACTCCATGCTGGTGTAATCC5528ooc(3)-laooc(3)lc-FCCTCTCACGACTGTGCTGTAATCC2815528ooc(3)-laooc(3)lc-FCCTCTCACGACGTGGCGTAATCC2815528ooc(3)-laooc(3)lc-FCCTCTCACGACTCCATCGTGTGCTGAATCC2815528ooc(3)-laooc(3)lc-FCCTCCCACTGC	nalC	nalC-F	ΤΓΑΑΓΓΓΤΑΑΓΓΑΑΑΑΑΓΓ	814	69	4
nalb         nalb-F         GGGGCTAAAATGGTACACT         789         55         4           nalb-F         GGGGCTAAAATGGTGCACTT         789         55         4           mex/R         mex/R00         CCAGTAAGGTGGATCT         1016         51         4           mex/T         mex/TCAGTGCGGGGGATAC         1398         55         4           mex/T         mex/TCAGTGCGGGGGGGATAC         1398         55         4           mex/T         mex/TCAGTGCGGGGGGGATAC         1398         50         4           mex/T         mex/TCAGGGGGGGATAC         1398         50         4           mex/T         mex/TCAGGGGGGATAC         1398         50         4           mex/T         mex/TCAGGGGGGATAC         1398         50         4           mex/T         mex/TCAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG		nalC-R		011		
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	nalD	nalD-F	GCGGCTAAAATCGGTACACT	789	55	4
mexR         mexR20         CCAGTAAGCGGATAC         1016         51         4           mexR1         mexR1.F         TGCATCAGCGGGTACCCT         1016         51         4           mexT4         GGTAGCGCCAGGAGAAGTG         1398         55         4           mexT4         GGTAGCGCCAGGAGAAGTG         1398         50         4           mexT2         mexSF         ATACAGTCAGAACCCAFGA         1153         50         4           mexZ1026         CCAGCGTGGAGATGAAGGGCGCCCGG         1059         64         4           oprD         oprD F         GGCAGAGAATAGTCAAACCCAA         1384         64         26           oprD R         GTTGCCTGCGGCTACAGGGGG         1059         64         4           oxo50 R         GTTGCCTGCGGCTACAGGCGG         100         27         26           ges R         GTTTCCAGGCGCTAGCGG         371         55         26           imi i         imi-F         ATACCGCGCGTAGA         17         55         26           gim ges R         TCTCGCATACTGCTGCCGGTGAGG         371         55         26           oac(3)L-F         CTCTGCGCACTAGCGATAGCGCGTAG         27         26         27           gim gim -F         TCGCGATACTTGCTGCCCCTGGAGGTAG </td <td>halb</td> <td>nalD-R</td> <td></td> <td>, 05</td> <td>55</td> <td></td>	halb	nalD-R		, 05	55	
Imenal         Imenal of Control Contrel Contecon Control Control Contecon Control Control Control Con	meyP	mexP20		1016	51	4
mexT         mexT+F         TGCATACGGGGGTGAATAAC         1398         55         4           mexT-R         GGTACGGCGGGTGAATAAC         1398         50         4           mexS-R         TCAACGATCGTGGAATAAC         1153         50         4           mexS-R         TCAACGATCGTGGAAATAGGCCACCAGGC         1059         64         4           mexZ1026         CCACGCGGGAGAATAGGCCCACCAGGC         1059         64         4           oprD         oprD-R         GGTCGGCAATAGGCCCGC         619         54         26           ox350         ox350-R         GGTCGCGCACTGAGGCGG         371         55         26           ges         ges-F         GTTTTCCAATGCTCATCCATC         619         54         26           ges         ges-F         GTTTTCCAATGCATAGCGCTACCA         318         55         26           imi         imi-F         ATACCGCATCCTTGTTTAGCTC         818         55         26           gim         gim-R         TCCGCAAGCTTGGTGTAA         477         55         26           gim-R         AACTCCCACCATGCAAGCCATGC         328         55         28           aac(3)L-R         GCTGGCGCAATCTGGTGGTGAAGC         435         55         28           aac	mexit	mexPINT	GENTENTECCETTCACCTC	1010	51	
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	movT	maxT-E		1208	55	4
mex5         mex5+F         ATACAGTCACAACCCATGA         1153         50         4           mex5-R         TCAACGATCACAACCCATGA         1153         50         4           mex2         mex20260         CCCAGCGAGGAATAGGACCCAGGC         1059         64         4           opr0         opr0-F         GGCAGGAGTAATTCAAACCAA         1384         64         2           oxs50         oxs50-F         AATCCGGCGCTCATCCATC         619         54         22           ges         ges-F         GTTTGCCTGCGCGATACC         619         55         26           ges         ges-F         GTTCCATGCACTCACGG 371         55         26           ges         ges-F         TCGCATAGCACTCAGGCGCG         7         7           gim         jm-F         TCGCATAGCACTCCTGTTTACCTC         818         55         26           ges         ges-R         TCGCCATAGCACTCCCACGC         818         55         26           gim         jm-F         TCGCATAGCACTCCTGTTACCTC         818         55         26           ges         ges-R         TCGCCATAGCACTCCTGTCACCACGC         83         35         28           gim         gim-F         TCGCACACTCTGGCTGTGTACGTC         35         28     <	mexi	mexT D		1550	66	
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	mays	max <sup>c</sup> E		1150	FO	4
IMEX-R         INCLACINE CONCAGEGA           mex2         mex21026         CCAGCCAGGAGATCGAAGGCCAGCGG           mex1026         CCAGCCAGGAGATCGAAGGCCAGCCGG         Immedia           opr0-R         GGTCGCGGGCACTCAAGGCCAG         1384         64         26           opr0-R         GGTCGCGGCCCATCCAATC         619         54         32           oxo50         oxo50-F         AATCCGGCGCTCATCCATC         619         54         32           ges         ges-R         GGTTGGCGGACTGAAGGCGAG         71         55         26           ges         ges-R         TGCCATAGCATTAGGCTTAAC         818         55         26           gim-R         ATAGCCATCCTTGTTAGCT         818         55         26           gim-R         TCGGCACACTTGGTGAA         477         55         26           gim-R         CACCCTTGCATGACCATGCCATGC         33         55         28           oac(3)I-R         GGTGGCGGACTTGGAGGTGAT         33         35         28           oac(3)I-F         CCTCTAAGACAGTCGTGGTCATAC         35         28           oac(3)I-F         GGTAGCCGAGTGGTGGATAG         36         36         36           oac(6')I-B         GGTAGCCGAGTGGTGGATAG         37         36         <	THEX5	mexS-F		1155	50	
$\begin{tabular}{ c c c c c c c c c c c c c c c c c c c$	7	111ex3-R		1050	<i>C1</i>	4
$\begin{tabular}{ c c c c c c c c c c c c c c c c c c c$	mexz	mex22060		1059	64	
op/D+         OGLARASTINATI (CAAAACCAA         1384         64         42           op/D-R         GTTOCTCTGGGTGGATTAC         332         332         332           oxa50-R         GGTCGGGGGCTACCATCC         619         54         32           ges         ges-F         GTTTGCAATGTGCTCAACG         371         55         76           imi         imi-F         ATAGCCATCGGCTAC         818         55         76           gim         gim-F         TCGCATACCTTGTTTACCTC         818         55         76           gim-R         ACTCTGCCATTGCTTTATCCTC         818         55         76           gim-R         ACTCTGCACTTGTTTATCCTC         313         55         78           aac(3)-Ia         aac(3)Ia-F         CCCTGACAACCTGGTGCAATGCATGC         435         55         78           aac(3)-Ia         aac(3)Ia-R         GGTGGCGTACTTGCGATGCATGC         143         55         28           aac(6'')Ib-R         GGTAGCCAATGCCGTGGTACTTGC         150         55         28           aac(6'')Ib-R         GGTAGCCGATCGATGCGTTACG         281         55         28           aac(6'')Ib-R         TGGCATGCCATGCGTGTACTG         150         55         28           aac(6'')Ib-R		mex21026		1207	<i>c</i> /	26
oppL-kof IndeC Its ICG IGG IGA IACoxa50xox50-FAATCC GGC GCC ICC ICC CTC6195432gesges-FGTTTIG CATG IGC ICA AGG GGG3715526imiimi-FATAGC CATAG CAATAG GGG TAG75526gimgim-FTCG CAATAC CTTIG TTAGCT C8185526gimgim-RAACTT CCAACTTIG GT CTGAA4775526aac(3)-Iaaac(3)Ia-FCCCT GAC CAAGTIC CATC C4355528aac(3)-Iaaac(3)Ia-FCCCT GAC CAAGT CCATG C4355528aac(3)-ICaac(3)Ia-FCCT CAACAC CTTIG GG TG AAT ICC AT ICC AT ICC AGA ICC AT ICC AGA ICC AT ICC AT ICC AGA	oprD	oprD-F	GGCAGAGATAATTTCAAAACCAA	1384	64	
$\begin{array}{c c c c c c c } \begin{tabular}{ c c c c c c c c c c c c c c c c c c c$		oprD-R	GIIGUUIGIUGGIUGAIIAU	610	F.(	32
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	oxa50	oxa50-F	AATCCGGCGCTCATCCATC	619	54	52
gesges-FGTTTGCAATGGCTCAACG37155		oxa50-R	GGTCGGCGACTGAGGCGG			26
$\begin{tabular}{ c c c c c c c c c c c c c c c c c c c$	ges	ges-F	GTTTTGCAATGTGCTCAACG	371	55	20
imi imi-F ATAGCATCCTTGTTTAGCTC 818 55 49 imi-R TCTGCGATTACTTTATCCTC gim gim-F TCGACACACCTTGGTCTGAA 477 55 26 gim-R AACTTCCAACTTGCCATGC 435 55 28 aac(3)I-aR GGTGGCGTACTTGGGTCGATA aac(3)I-cF CTCTCAAGACGTTGGTGATAGC 143 55 28 aac(3)I-cF CTCTCAAGACGTTGGTGATAGC 143 55 28 aac(6'')-Ib aac(6'')Ib-F GGTATGCCCAGTCGTGGCGTACTGCG 281 55 28 aac(6'')-Ib aac(6'')Ib-R TGGACCATMTGGGGTGGTTACG ant(2'')-Ia ant(2'')Ia-F ATGAGCGAAATCTGCCGCTGC 150 55 28 ant(2'')-Ia ant(2'')Ia-R GCCCGCGGGGATATC mex1 mcr1 A AGAT CCTTGGTGTCGCGCTGG 150 55 28 mcr1-R AGAT CCTTGGGCCGTGTCGGCCGGCGTG mexB MexB-F CAACATCCAGGACTTCAACTAT mexF AGGAAATCTGCCGGTTGTTCTGCGCCTG mexD-R AGCAGTCGCGGGACATCCAGCC mexF-R GGAGGTACTGCAGCTCG mexF-R GAGGTGCGCGGACGCCCACTCA mexF-R GAGGTGCGCGGACGCCCACTCA mexY MexY-F TCAGGCCGAACGACTCAAC mexY MexY-F TCAGGCCGACGCCTGGTGTC mexF TCCAGGCGGACGTGGTGACGTGC mexF-R GAGGTGCGCGGACGTCGACGTCG mexY MexY-F TCAGGCCGACGCCTGGTGT mexY MexY-F TCAGGCCGACCCTGGTGT mexF TCCAGGCGGACGTGGACCTCACCTG mexY MexY-F TCAGGCCGACCCTGGTGT mexY MexY-F TCCGGCGGTGGTGGTGCC mexF-R GAGGTGTGCGCTGACCTTGAC mexY MexY-F TCCGGCGACGTTGAGT mexY MexY-F TCCGGCGGTGGTGACCTGGTC mexF-R GAGGTGCGCGGACCCTGCTT 163 60 7 mexY-R TCCGGGTGACCTTGAGT mexY MexY-F TCCGGCGACGCTTGAGT mexY-R TCCGGGTGGTGACCTTGAT mexY-R TCCGGGTGAACCGGTCGTG mexF-R GAGGTGTGCGCGGACCCACCTGCT 163 70 7 mexY-R TCCGGCGACGCACCTTGAGT mexF-R TCCGGCGAACGACTTCAAC 163 60 7 mexY-R TCCGGCGACCCTGCACCTTGAGT mexF-R TCCGGGGACGCCCGGTGGTGCCCGACCTTGAGT mexF-R TCCGGCGAACGACTTCAAC 163 60 7 mexY-R TCCGGCGACCCTGCACCTTGAGT mexF-R TCCGGCGACCCTGCTTGAGT mexF-R TCCGGCGACCCTGCTTGAGT mexF-R TCCGGCGAACGACTTCAAC 163 60 7 mexF-R TCCGGCGAACGACTTCAAC 163 60 7 mexF-R TCCGGCGAACGACTTCAAC 163 60 7 mexF-R TCCGGCGAACGACTTCAAC 163 60 7 mexF-R TCCGGCGACCCTTGAACTGGTC mexF-R TCCGGCGACCCTGGTT 16AC 164 60 7 mexF-R TCCGGCGACCCTGCTTGAACTCAC 163 60 7 mexF-R TCCGGCGACCCTGCTTGAAC 163 60 7 mexF-R TCCGGCGACCCTGCTTGAAC 163 60 7 mexF-R TCCGGCGACCCTGCTTGAAC 163 60 7 mexF-R TCCGGCGACCCTGCTTGACCTGCTTGAC 164 7 mexF-R TCCGGCGACCCTGCTTGA		ges-R	TGCCATAGCAATAGGCGTAG			26
imi-R         TCTGCGATTACTTTATCCTC           gim         gim-F         TCGGACACACCTTGGTCGAA         477         55         26           gim-R         AACTTCCAACTTTGCCATGC         435         55         28           aac(3)-la         aac(3)la-F         CCCTGACACACTTGGGTGATGC         435         55         28           aac(3)-la         aac(3)lc-F         CTCTCAAGACGTTGGTGAATGC         143         55         28           aac(3)-la         aac(3)lc-R         CAGCGATTACGGATGACCAGAC         28         28           aac(3)-la         aac(3)lc-R         CAGCGATTGGGTGAATGGC         28         28           aac(6'')lb-R         GGTAGCCAGTGGAGCGACCAGAC         281         55         28           aac(6'')lb-R         GGTAGCCAGTGGAGCGACCTGTG         281         55         28           aat(2'')-la         aat(2'')la-R         GGCCGCCAGCAGTTCAACTAT         55         28           mer1         mcr1-F         AGTCCGTTGGTCGGCGTG         150         55         28           mer2         mcr1-R         AGACT CCTTGGTCGGCTTG         1626         58         29           mer4         mer4-F         CAACATCCAGGACCACTTCT         1626         58         29           mer4         mer4-F	imi	imi-F	ATAGCCATCCTTGTTTAGCTC	818	55	20
gimgim-FTCGACACACCTTGGTCTGAA4775528gim-RAACTTCCAACTTGCCATGC3538aac(3)Ia-RGGTGGCGGTACTTGGGTCGATA35528aac(3)Ic-RGGTGGCGGTACTTGGGTGGATA35528aac(3)Ic-RCAGCGATTGCGATGAGGGGGGTACG1435528aac(6")Ib-FGGTAGCCCAGTCGACGTGGC 32815528aac(6")Ib-RTGGACCATMTGGGGTGGTACG1505528aac(6")Ib-RGCCGCCGAGCATTTCAACTAT5528aar(2")I-Iaant(2")Ia-FATGAGCGAAATCGCCGCTCTG1505528ant(2")I-Iaant(2")Ia-FAGGCGGTGATCGGTG16265829mcr1mcr1-FAGCCGCTGAGCATTTGGGCT167607mexBmcr1-RAGCCCTTGGTCTGGCTTG167607mexBmexB-RAGGAAATCTGCACGTTCTGC163607mexFmexD-FCTACCCTGGTGAAACAGC250588mexFTGACGCGACCTACACTCAACCACTCA163607mexYmexY-FTCAGCGGACCTGACTTGAT163607mexY-RTCCGGTGTTGATGAGTGTG163607mexY-RTCCGGTGTTGATGAGTGT163607mexY-RTCCGGTGTTGATGAGTGT163607mexY-RTCCGGTGTTGATGGTGGTTGATCGTGTT163607mexY-RTCCGGTGGTTGATGGTGGTGTGT163607mexY-RTCCGGTGGTTGATGGTGGTTG163607mexY-RT		imi-R	TCTGCGATTACTTTATCCTC			20
$\begin{array}{c c c c c c } gim_R & AACTTICCAACTTIGCATGC \\ gac(3)-Ia & aac(3)Ia-F & CCCTGACCAAGTCCAATCCATGC & 435 & 55 & 28 \\ aac(3)Ia-R & GGTGGCGGTACTTGGGTGGAAA \\ aac(3)Ic-R & CAGCGATTGGCGATGAAGCCAGA \\ aac(6'')Ib-R & CAGCGATTGCGATGAAGCCAGA \\ aac(6'')Ib-R & GGTAGCCAAGTCGTACGTTGC & 281 & 55 & 28 \\ aac(6'')Ib-R & TGGACCATMTGGGGTGGTTACG \\ ant(2'')Ia-R & GCCCGCGAACATTCGCCGCTCTG & 150 & 55 & 28 \\ ant(2'')Ia-R & GCCCGCCGAGCATTCAACTAT \\ mcr1 & mcr1-F & AGTCGTTTGTTCTGGCC & 1626 & 58 & 29 \\ mcr1-R & AGGA CCTTGGTCGCGGCTTG & 167 & 60 & 7 \\ mexB & mexB-F & CAACATCCAGGACCCACTCT & 167 & 60 & 7 \\ mexB-R & AGGAAATCTGCACGTTCGC \\ mexD & mexD-F & CTACCTGGTGGAAACAGC & 250 & 58 & 8 \\ mexD-R & AGGAAATCTGCACGTTCACTA \\ mexF & MexF-F & TGTACGGAAACAGCC & 250 & 58 & 8 \\ mexF & GAGCGTCCGTGGTCGCTGC & 163 & 60 & 7 \\ mexF & mexF-F & TGTACGGAACACTCACATCA & 163 & 60 & 7 \\ mexF & mexF-F & TGTACGGAACGACTTCAACT & 167 & 60 & 7 \\ mexF & mexF-F & TGTACGCGAACGACTTCAACT & 163 & 60 & 7 \\ mexF & mexF-F & TGTACGGAACGACTCACATCA & 163 & 60 & 7 \\ mexF-R & GAGGTGCTGCGCTGGACTTCAACT & 167 & 7 \\ mexF-R & GAGGTGCCGACCTTGAT & 7 \\ mexF-R & TCTCGGTGTGGTGGAACGAGG & 159 & 60 & 7 \\ mexF-R & TCTGGGTGGTGGTGGTGGTGGT & 7 \\ mexF-R & TCTCGGTGTGATCGTGC & 7 \\ mexF-R & TCTCGGTGTGATCGTGC & 7 \\ mexF-R & TCTCGGTGTGGTGGTGGTTC & 7 \\ mexF-R & TCTCGGTGTGGTGGTGGTGGT & 7 \\ mexF-R & TCTCGGTGTGGTGGTGGTGGT & 7 \\ mexF-R & TCTCGGTGTGGTGGTGGTGGTGGTGGT & 7 \\ mexF-R & TCTCGGTGTGGTGGTGGTGGT & 7 \\ mexF-R & TCTCGGTGTGGTGGTGGTGGT & 7 \\ mexF-R & TCTCGGTGTGGTGGTGGTGGT & 7 \\ mexF-R & TCTCGGTGTGGTGGTGGTGGTGGTGGT & 7 \\ mexF-R & TCTCGGTGTGGTGGTGGTGGTGGTGGTGGT & 7 \\ mexF-R & TCTCGGTGTGTGTGGTGGTGGTGGTGGT & 7 \\ mexF-R & TCTCGGTGTGTGTGGTGGTGGTGGTGGTGGTGGTGGTGGT$	gim	gim-F	TCGACACACCTTGGTCTGAA	477	55	26
aac(3)-Iaaac(3)Ia-FCCCTGACCAAGTCCAATCCATGC4355528aac(3)Ia-RGGTGGCGGTACTTGGGTGAATGC1435528aac(3)I-Caac(3)Ic-FCTCTCAAGACGTTGGTGAAAGCCAGA1435528aac(6")-Ibaac(6")Ib-FGGTATGCCCAGTGGTGACGTTGC2815528aac(6")-Ibaac(6")Ib-RGGACGATMTGGGGTGGTAACG2815528aart(2")I-aant(2")Ia-FATGAGCGAAATCTGCCGCTCG1505528ant(2")I-aRGCCCGCCGAGCATTTCAACTAT77mcr1mcr1-FAGTCCGTTTGTCTTGTGGC16265829mcr1-RAGAC CCTTGGTCCGGCTTG167607mexBmexB-RAGGAAATCTGCACGTTCTGC77mexDmexD-FCTACCCTGGTGAAACAGC250588mexFmexF-FTGTACGCGAACGACTCAAC163607mexYmexY-FTCCAGGCCGACCTGCATCA163607mexY-RTCTCGGTGTGAACAGGTCCTGCTT163607mexL-RtTCCGGTGTGAACGACCTGCTT163607mexY-RTCTCGGTGTGAACGACCTGCTT163607mexY-RtTCCGGTGTGACGTGTC163607mexY-RtTCCGGTGTGACGTGCTC163607mexY-RtTCCGGTGTGACGTGTT163607mexY-RtTCCGGTGTGACGTGCTT163607mexY-RtTCCGGTGTGACGTGTT163607mexY-RtTCCGGTGTGACCGGTGGTT <t< td=""><td></td><td>gim-R</td><td>AACTTCCAACTTTGCCATGC</td><td></td><td></td><td>20</td></t<>		gim-R	AACTTCCAACTTTGCCATGC			20
aac(3)Ia-R GGTGGCGGTACTTGGGTCGATA aac(3)I-F CTCTCAAGACGTTGGTGAATGC 143 55 28 aac(3)Ic-R CAGCGATTGCGATGAAGCCAGA aac(6")Ib-R GGTATGCCCAGTCGTACGTTGC 281 55 28 aac(6")Ib-R TGGACCATMTGGGGTGGTACG aat(2")Ia-R ATGAGCGAAATCTGCCGCTCTG 150 55 28 ant(2")Ia-R ATGAGCGAAATCTGCCGCTCTG 55 29 mcr1-R AGGACATTTCAACTAT mcr1 mcr1-F AGGCCGATGCTGGCGTTGG 1626 58 29 mcr1-R AGGAAATCTGCGGCTTG 1626 58 29 mcr1-R AGGAAATCTGCGGCTTG 1626 58 29 mcr1-R AGGAAATCTGCGGCTTG 1627 60 7 mexB mexB-F CAACATCCAGGACCCACTCT 167 60 7 mexD mexD-F CTACCCTGGTGAACAGC 250 58 8 mexD-R AGGAAATCTGCACGTTCTGC 58 mexF TGTACCGGTGGAACAGC 250 58 8 mexD-R AGCAGGTACATCACCATCA 163 60 7 mexF mexF-F TGTACGCGAACGACTTCAAC 163 60 7 mexY mexF-F TGTACGCGAACGACTTCAACTAT mexY TCAGGCGGACCTTGAATGAGT 7 mexY TCAGGCGGACCTTGAT 7 mexY TCAGGCGGACCTTGAATGAGT 7 mexY-R TCAGGCGGACCTTGAT 7 mexY-R TCAGGCGACCTTGAT 7 mexY-R TCAGGCGACCTTGAT 7 mexY-R TCCGGTGTGAAGTAGT 7 mexY-R TCCGGTGTGAAGTAGT 7 mexY-R TCCGGTGTGAAGTAGT 7 mexY-R TCCGGTGTGAACTGGTGT 7 mexY-R TCCGGTGTGAACTGGTGT 7 mexY-R TCCGGTGTGAACTGGTGT 7 TCCGGTGTGACGTGACTTGAT 7 TCCCGTACCTGGTGTGACTTGAT 7 TCCCGTACCTGGTGTGACTTGAT 7 TCCCGTGTGTGACGTGACTTGAACTACCACTGA 7 TCCCGTGTGTGACGTGACTTGAT 7 TCCCGTGTGTGACGTGACTTGAT 7 TCCCGTGTGTGACTTGAT 7 TCCCGTGTGTGACTTGAC 7 TCCCGTGTGTGACTTGAT 7 TCCCGTGTGTGACTTGAT 7 TCCCGTGTGTGTGT 7 TCCCGTGTGTGTGT 7 TCCCGTGTGTGTGT 7 TCCCGTGCTGTGACTTGAT 7 TCCCGTGTGTGTGT 7 TCCCGTGTGTGTGT 7 TCCCGTGTGTGTGT 7 TCCCGTGTGTGACTTGAT 7 TCCCGTGCTGACTTGAT 7 TCCCGTGTGTGTGT 7 TCCCGTGCTGACTTGAT 7 TCCCGTGTGTGT 7 TCCCGTGCTGACTTGAT 7 TCCCGTGTGTGTGT 7 TCCCGTGCTGTGCT 7 TCCCGTGCTGACTTGAT 7 TCCCGTGCTGTGCT 7 TCCCGTGCTGTGCT 7 TCCCGTGCTGTGCT 7 TCCCGTGCTGACTTGGTG 7 TCCCGTGCTGTGCT 7 TCCCGTGCTGTGCTGT 7 TCCCGTGCTGTGCT 7 TCCCGTGCTGTGCTGT 7 TCCCGTGCTGTGCT 7 TCCCGTGCTGTGCTGT 7 TCCCGTGCTGTGCTGT 7 TCCCGTGCTGTGCTGTGCT 7 TCCCGTGCTGTGCTGT 7 TCCCG	aac(3)-Ia	aac(3)Ia-F	CCCTGACCAAGTCCAATCCATGC	435	55	28
aac(3)-Icaac(3)Ic-FCTCTCAAGACGTTGGTGAATGC1435528aac(3)Ic-RCAGCGATTGCGATGAAGCCAGAaac(6'')Ib-FGGTATGCCCAGTCGTACGTTGC2815528aac(6'')Ib-RTGGACCATMTGGGGTGGTTACG150552828ant(2'')-Iaant(2'')Ia-FATGAGCGAAATCTGCCGCTCTG1505528ant(2'')-Iaant(2'')Ia-RGCCCGCCGAGCATTTCAACTAT16265829mcr1mcr1-FAGACTCCTGGTCTGGGCTTG16265829mcr1-RAGAT CCTTGGTCTCGGCTTG167607mexBmexB-RAGGAAATCTGCACGATTCTGC7mexDmexD-FCTACCCTGGTGAAACAGC250588mexFmexF-FTGTACGCGAACGACTTCAACATCA7mexYmexY-FTCAGGCCGACCATTGAATG7mexYrmexY-FTCCGGTGTTGATGATCG163607rpsL-RrpsL-RTACTTCGAACGACCCTGCTT163607		aac(3)Ia-R	GGTGGCGGTACTTGGGTCGATA			
$\begin{array}{ c c c c } aac(3)Ic-R & CAGCGATTGCGATGAAGCCAGA \\ \hline aac(6'')Ib-F & GGTATGCCCAGTCGTACGTTGC & 281 & 55 & 28 \\ \hline aac(6'')Ib-R & TGGACCATMTGGGGTGGTTACG \\ \hline TGGACCATMTGGGGTGGTTACG & 150 & 55 & 28 \\ \hline ant(2'')Ia-R & GCCCGCCGAGCATTTCAACTAT & 1626 & 58 & 29 \\ \hline ant(2'')Ia-R & AGGCGTTGGTTCGGCTTG & 1626 & 58 & 29 \\ \hline mcr1 & Mcr1-F & AGTCCGTTGGTCTGGGCTTG & 167 & 60 & 7 \\ \hline mcr8 & mcr4 & AGACT CCTTGGTCTGGCTTG & 167 & 60 & 7 \\ \hline mcr8 & mcr4 & AGGAAATCTGCACGTTCTG & 163 & 60 & 7 \\ \hline mcr8 & mcr0 & mcr0 & 100 & 100 & 100 & 100 & 100 \\ \hline mcr0 & mcr0 & mcr0 & 100 & 100 & 100 & 100 & 100 \\ \hline mcr0 & mcr0 & mcr0 & 100 & 100 & 100 & 100 & 100 \\ \hline mcr0 & mcr0 & mcr0 & 100 & 100 & 100 & 100 & 100 & 100 & 100 \\ \hline mcr0 & mcr0 & mcr0 & 100 &$	aac(3)-Ic	aac(3)Ic-F	CTCTCAAGACGTTGGTGTAATGC	143	55	28
aac(6")-Ibaac(6")Ib-FGGTATGCCCAGTCGTACGTTGC2815528aac(6")Ib-RTGGACCATMTGGGGTGGTTACG1505528ant(2")-Iaant(2")Ia-FATGAGCGAAATCTGCCGCTCTG1505528ant(2")Ia-RGCCCGCCGAGCATTTCAACTAT777mcr1mcr1-FAGTCCGTTGGTCTCGGCTTG16265829mcr1-RAGAT CCTTGGTCTCGGCTTG167607mexBmexB-FCAACATCCAGGACCCACTCT167607mexDmexD-FCTACCCTGGTGAAACAGC250588mexFmexF-FTGTACGCGAACGACTTCAAC163607mexYmexY-FTCAGGCCGACCTTGATGTTC159607mexY-rpsL-FTACTTCGAACGACCCTGCTT163607rpsL-RTrpsL-RTACTTCGAACGACCTGCTT163607		aac(3)Ic-R	CAGCGATTGCGATGAAGCCAGA			
aac (6")Ib-RTGGACCATMTGGGGTGGTTACGant (2")-Iaant (2")Ia-FATGAGCGAAATCTGCCGCTCTG1505528ant (2")Ia-RGCCCGCCGAGCATTTCAACTAT16265829mcr1mcr1-FAGTCCTTTGTTCTTGTGGC16265829mcr1-RAGAT CCTTGGTCTCGGCTTG167607mexBmexB-FCAACATCCAGGACCCACTCT167607mexDmexD-FCTACCCTGGTGAAACAGC250588mexF-RAGGAGTACATCACCATCA163607mexYmexF-FTGTACGCGAACGACTTCAAC163607mexYmexY-FTCCAGGCCGACCTTGAT163607rpsL-RfrpsL-FTACTTCGAACGACCCTGCTT163607rpsL-RtrpsL-FTACTTCGTACAGCACTTCACT163607	aac(6″)-Ib	<i>aac(6″)Ib-</i> F	GGTATGCCCAGTCGTACGTTGC	281	55	28
ant(2")-Iaant(2")Ia-FATGAGCGAAATCTGCCGCTTG1505528ant(2")Ia-RGCCCGCCGAGCATTTCAACTATT<		aac(6″)Ib-R	TGGACCATMTGGGGTGGTTACG			
ant(2")Ia-RGCCCGCCGAGCATTTCAACTATmcr1-FAGTCCGTTTGTTCTTGTGC16265829mcr1-RAGAT CCTTGGTCTCGGCTTG167607mexBmexB-FCAACATCCAGGACCCACTCT167607mexDmexD-FCTACCCTGGTGAAACAGC250588mexF-RAGCAGGTACATCACCATCA163607mexYmexY-FTGTACGCGAACGACTTCAAC163607mexYmexY-FTCCAGGCCGACCTTGAAGTAG159607mexY-RTCTCGGTGTTGATCGTGTTC163607mexY-RTTTCCTCGTACATCACCATCT163607mexY-RTTTCCTCGTACATCGGTGTT163607	ant(2")-Ia	ant(2")Ia-F	ATGAGCGAAATCTGCCGCTCTG	150	55	28
mcr1mcr1-FAGTCCGTTTGTTCTTGTGGC16265829mcr1-RAGAT CCTTGGTCTGGCTTGmexB-RAGACATCCAGGACCCACTCT167607mexB-RAGGAAATCTGCACGTTCTGC16760788mexD-RAGCAGGTACATCACCATCA2505888mexF-RAGCAGGTACATCACCATCA1636077mexYmexY-FTGTACGCGAACGACTTCAAC163607mexYmexY-FTCAGGCCGACCTTGAAGTAG159607rpsLrpsL-FTACTTCGAACGACCCTGCTT163607rpsL-RTTTCCTCGTACATCGGTGGT163607		ant(2")Ia-R	GCCCGCCGAGCATTTCAACTAT			
mcr1-RAGAT CCTTGGTCTCGGCTTGmexBmexB-FCAACATCCAGGACCCACTCT167607mexB-RAGGAAATCTGCACGTTCTGCmexD-FCTACCCTGGTGAAACAGC250588mexD-RAGCAGGTACATCACCATCAnexF-RGAGGTGTCGCTGACCATCAC163607mexFmexF-FTGTACGCGAACGACTTCAAC163607mexYmexY-FTCAGGCCGACCTTGAAGTAG159607mexY-RTCTCGGTGTTGATCGTTC163607rpsLrpsL-FTACTTCGAACGACCTGCTT163607	mcr1	mcr1-F	AGTCCGTTTGTTCTTGTGGC	1626	58	29
mexBmexB-FCAACATCCAGGACCCACTCT167607mexB-RAGGAAATCTGCACGTTCTGCmexD-FCTACCCTGGTGAAACAGC250588mexD-RAGCAGGTACATCACCATCA163607mexFmexF-FTGTACGCGAACGACTTCAAC163607mexYmexY-FTCAGGCCGACCTTGAAGTAG159607mexY-RTCTCGGTGTTGATCGTGTTC163607rpsLrpsL-FTACTTCGAACGACCTGCTT163607		mcr1-R	AGAT CCTTGGTCTCGGCTTG			
mexB-RAGGAAATCTGCACGTTCTGCmexD-FCTACCCTGGTGAAACAGC250588mexD-RAGCAGGTACATCACCATCA163607mexF-RGAGGTGTCGCTGACCTTGAT159607mexY-RTCTCGGTGTTGATCGTGTC163607mexY-RTCTCGGTGTTGATCGTGTTC163607rpsLrpsL-FTACTTCGAACGACCTTGCT163607	mexB	mexB-F	CAACATCCAGGACCCACTCT	167	60	7
mexDmexD-FCTACCCTGGTGAAACAGC250588mexD-RAGCAGGTACATCACCATCA163607mexFmexF-FTGTACGCGAACGACTTCAAC163607mexYmexY-FGAGGTGTCGCTGACCTTGAAGTAG159607mexY-RTCTCGGTGTTGATCGTGTTC163607rpsLrpsL-FTACTTCGAACGACCTGCTT163607		mexB-R	AGGAAATCTGCACGTTCTGC			
mexD-RAGCAGGTACATCACCATCAmexF-FTGTACGCGAACGACTTCAAC163607mexF-RGAGGTGTCGCTGACCTTGAT159607mexY-RTCTCGGTGTTGATCGTGTTC163607rpsLrpsL-FTACTTCGAACGACCTGCTT163607rpsL-RTTTCCTCGTACATCGGTGGT163607	mexD	mexD-F	CTACCCTGGTGAAACAGC	250	58	8
mexFmexF-FTGTACGCGAACGACTTCAAC163607mexF-RGAGGTGTCGCTGACCTTGAT159607mexY-RTCTCGGTGTTGATCGTGTTC163607rpsLrpsL-FTACTTCGAACGACCTGCTT163607rpsL-RTTTCCTCGTACATCGGTGGT163607		mexD-R	AGCAGGTACATCACCATCA			
mexF-RGAGGTGTCGCTGACCTTGATmexYmexY-FTCAGGCCGACCTTGAAGTAG159607mexY-RTCTCGGTGTTGATCGTGTTC163607rpsLrpsL-FTACTTCGAACGACCCTGCTT163607rpsL-RTTTCCTCGTACATCGGTGGT163607	mexF	mexF-F	TGTACGCGAACGACTTCAAC	163	60	7
mexYmexY-FTCAGGCCGACCTTGAAGTAG159607mexY-RTCTCGGTGTTGATCGTGTTCrpsLrpsL-FTACTTCGAACGACCCTGCTT163607rpsL-RTTTCCTCGTACATCGGTGGT		mexF-R	GAGGTGTCGCTGACCTTGAT			
mexY-RTCTCGGTGTTGATCGTGTTCrpsL-FTACTTCGAACGACCCTGCTT163607rpsL-RTTTCCTCGTACATCGGTGGT	mexY	mexY-F	TCAGGCCGACCTTGAAGTAG	159	60	7
rpsL rpsL-F TACTTCGAACGACCCTGCTT 163 60 <sup>7</sup> rpsL-R TTTCCTCGTACATCGGTGGT		mexY-R	TCTCGGTGTTGATCGTGTTC			
rpsL-R TTTCCTCGTACATCGGTGGT	rpsL	rpsL-F	TACTTCGAACGACCCTGCTT	163	60	7
		rpsL-R	TTTCCTCGTACATCGGTGGT			

Differences in the expression of each gene of interest were tested using the single sample *t*-test versus cut-off values of 0.5 for underexpression and 2 for overexpression.<sup>9</sup>

### Molecular typing

Molecular epidemiology was analysed by MLST (https://pubmlst.org/ paeruginosa/). Allelic profiles of seven *P. aeruginosa* housekeeping genes (*acsA, aroE, guaA, mutL, nuoD, ppsA* and *trpE*) were analysed by PCR and confirmed in 2% agarose gel. Next, PCR products were sequenced by GENEWIZ. Phylogenetic analysis was carried out using the eBURST algorithm (http://www.phyloviz.net/goeburst).<sup>10,11</sup>

#### Ethics

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki (current version, Fortaleza, Brazil, October 2013) and its later amendments and it was conducted in accordance with the requirements of the 2007 Spanish Biomedical Research Act or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. Hospital Clinic ethical committee reference number: HCB/ 2018/0236.

# Results

#### Antimicrobial susceptibility

A total of 43 strains of *P. aeruginosa* were isolated from the sputum of 38 BE patients during their stable phase with mean  $\pm$  SD forced expiratory volume in 1 s (FEV<sub>1</sub>) at inclusion of 58.92%  $\pm$  19.26%. Overall, 7 strains were obtained from BE patients with intermittent *P. aeruginosa* colonization versus 36 from patients with chronic *P. aeruginosa* colonization. *P. aeruginosa* isolates

were resistant to ciprofloxacin (44.19%), imipenem (32.55%), amikacin (18.6%), tobramycin (18.6%), meropenem (9.3%), cefepime (6.97%), aztreonam (6.97%), piperacillin/tazobactam (4.65%) and ceftazidime (4.65%). The strains showed three different antimicrobial profiles: moderately resistant (MR; 44.18%), MDR (16.28%) and XDR (4.65%) (Figures 1 and 2). Ciprofloxacin and imipenem had the highest MICs (Figure 3). All strains showed resistance to at least one antimicrobial agent.

#### Mechanisms of resistance

Ciprofloxacin-resistant *P. aeruginosa* strains contained mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes. The most frequent mutations







**Figure 1.** Antimicrobial susceptibility of all *P. aeruginosa* strains analysed by Etest. CIP, ciprofloxacin; IPM, imipenem; AMK, amikacin; TOB, tobramycin; ATM, aztreonam; MEM, meropenem; CAZ, ceftazidime; TZP, piperacillin/tazobactam; CST, colistin; CZA, ceftazidime/avibactam; C/T, ceftolozane/ tazobactam; FEP, cefepime; R, resistant; I, intermediate; S susceptible.



**Figure 3.** Number of resistant *P. aeruginosa* strains with each MIC value. CIP, ciprofloxacin; IPM, imipenem; AMK, amikacin; TOB, tobramycin; ATM, aztreonam; MEM, meropenem; CAZ, ceftazidime; TZP, piperacillin/tazobactam; CST, colistin; CZA, ceftazidime/avibactam; C/T, ceftolozane/tazobactam; FEP, cefepime.

were T83I in GvrA (21.05%), S466F in GvrB (21.05%), S87W in ParC (21.05%) and D539E in ParE (36.84%). A large number of mutated genes in the QRDR were associated with increased MIC (Table 2). Several β-lactamases were detected; sequencing showed allelic variants bla<sub>GES-2</sub> (44.18%), bla<sub>IMI-2</sub> (11.62%), bla<sub>GIM-1</sub> (2.32%) and  $bla_{0XA50}$  (97.67%), an intrinsic  $\beta$ -lactamase in *P. aeruginosa*. Allelic variants of OXA-50 were determined. OXA-396 and OXA-1034 being the most frequent. The variants were widely distributed among the different clones, and no specific correlation with clones was found. The aac(3)-Ia (41.6%), aac(3)-Ic (25%), aac(6")-Ib (8.33%) and ant(2")-Ia (25%) genes were associated with aminoglycoside-resistant strains. The mcr-1 gene was detected in one strain and confirmed by sequencing, although not associated with resistance (Table 3). OprD absence and different mutation patterns found in the oprD gene were associated with resistance to carbapenems. Five different mutation patterns (MP1 to MP5) were detected. In 9.3% of strains, the OprD porin was inactivated (Table 3).

#### Gene expression analysis

Gene expression analysis showed overexpression in the MexAB-OprM efflux system. The *mexB* gene was expressed at significantly higher levels (46.5%; *P* < 0.001 by *t*-test) than the *mexD*, *mexF* and *mexY* genes. Although there is no evidence that the amino acid changes listed in post-transcriptional regulators are involved in the overexpression, these results are consistent with the high number of mutations in post-transcriptional regulatory genes associated with *mexB* overexpression (*nalC*: G71E, S209R, A186T, A145V; *nalD*: L33Q, A211T, L17Q, L33P, V28A; *mexR*: L13G, M14W, V126E, A12T, D8K, P11S, A103G, A103T, A12R, V132A). Interplay between *mexB* and *mexF* was observed in two strains. Interplay between *mexD* and *mexY* was observed in one strain. Expression of the MexCD-OprJ operon was considerably lower (Figure 4).

 Table 2.
 Relationship between the number of mutated genes and ciprofloxacin MIC

QRDR mutations	Number of strains	MIC (mg/L)	Mean MIC (mg/L)
One mutation			
qyrB	1	1.5	0.75
parC	1	0.5	
parE	2	0.5-0.5	
Two mutations			
gyrB+parC	1	0.5	9.06
gyrB + parE	2	0.5-32	
parC + parE	2	0.5-2	
gyrA + parE	2	1.0-4	
gyrA + parC	1	32	
Three mutations			
gyrA + gyrB + parE	2	1-32	20
gyrA + parC + parE	2	32-32	
gyrB + parC + parE	1	3	
Four mutations			
gyrA + gyrB + parC + parE	1	32	32

#### Molecular epidemiology

A wide variety of clones were found but the Hamming distance showed high genetic proximity between them (Figure 5). Twenty-seven STs were identified in our strains. The most frequent clones detected were ST619 (11.4%), ST676 (9.09%), ST532 (9.09%) and ST109 (6.8%), followed by ST1811, ST1251, ST1095 and ST389 (4.65%) and ST181, ST1213, ST155, ST1885, ST308, ST594, ST1568, ST898, ST1720, ST17, ST671, ST447, ST699, ST667, ST377, ST2910, ST2314, ST927 and ST207 (2.32%). The four most frequent clones were distributed in four Table 3. Resistance patterns and mechanisms of resistance found in *P. aeruginosa* strains

		QRDR mutations				Resistance genes			
Strain	Resistance pattern	gyrA	gyrB	parC	parE	β-lactamases	aminoglycosides colistin		
1	CIP-ATM-CST		N366D	K66Q,K69M		bla <sub>0XA50(0XA-395)</sub>	mcr-1		
2 3	CIP-ATM CIP-ATM-IPM-TOB	L41W <b>T83I</b>	N366D	S87W	K380Q R378G,Y536T, 4537P D539F	bla <sub>OXA50(OXA-396)</sub> bla <sub>OXA50(OXA-1034)</sub> ,bla <sub>GES-2</sub>	aac(6″)-Ib		
4 5	CIP-ATM-AMK ATM-MEM			K46E	10571,0555E	bla <sub>OXA50(OXA-395)</sub> bla <sub>OXA50(OXA-1034)</sub> ,bla <sub>IMI-2</sub> ,	aac(3)-Ia		
6	CIP-TZP-AMK-ATM-CAZ-MEM-IPM	N57Q,D58R, W59L N60F	S466F		S373I,N374Y, A375D R378H	bla <sub>GIM-1</sub> bla <sub>OXA50(OXA-396)</sub> ,bla <sub>GES-2</sub>	aac(3)-Ia		
7 8 9 10 11	ATM-IPM AMK-ATM-TOB ATM-MEM ATM ATM					bla <sub>OXA50(OXA-396)</sub> ,bla <sub>GES-2</sub> bla <sub>OXA50(OXA-1032)</sub> bla <sub>OXA50(OXA-905)</sub> ,bla <sub>GES-2</sub> bla <sub>OXA50(OXA-395)</sub> bla <sub>OXA50(OXA-396)</sub>	aac(3)-Ia		
12 13	ATM CIP-ATM		Q443H		G376A,	bla <sub>OXA50(OXA-395)</sub> bla <sub>OXA50(OXA-395)</sub>			
14 15	ATM ATM-MEM				K370H,D339L	bla <sub>OXA50(OXA-1034)</sub> bla <sub>OXA50(OXA-396)</sub> ,bla <sub>GES-2</sub>			
16	CIP-TZP-ATM-CAZ-MEM-IPM-TOB	T83I	Q443H	D35W, <b>S87W</b>	D539E	bla <sub>OXA50(OXA-396)</sub> ,bla <sub>GES-2</sub>	ant(2")-Ia		
17 18 19	AMK-ATM-TOB AMK-ATM-TOB-IPM CIP-ATM		S466F	I33N	Y536T,A537P,	bla <sub>OXA50(OXA-1032)</sub> ,bla <sub>GES-2</sub> bla <sub>OXA50</sub> (OXA-905),bla <sub>GES-2</sub> bla <sub>OXA50(OXA-395)</sub>	ant(2")-Ia ant(2")-Ia		
20 21 22 23 24	ATM ATM ATM ATM CIP-ATM-AMK-MEM-IPM		N366D,		D539E	bla <sub>OXA50</sub> (OXA-396) bla <sub>OXA50</sub> (OXA-1034) bla <sub>OXA50</sub> (OXA-395) bla <sub>OXA50</sub> (OXA-396),bla <sub>GES-2</sub> , bla <sub>OXA50</sub> (OXA-396),bla <sub>GES-2</sub> ,	aac(3)-Ia		
25 26 27	CIP-ATM-MEM ATM ATM-MEM-IPM		3400	K69M	R379Q,D539E	bla <sub>IMI-2</sub> bla <sub>OXA50(OXA-396)</sub> ,bla <sub>GES-2</sub> bla <sub>OXA50(OXA-1032)</sub> bla <sub>OXA50(OXA-905)</sub> ,bla <sub>GES-2</sub> , bla <sub>IMI-2</sub>			
28 29	CIP-ATM-MEM-IPM CIP-ATM-AMK-MEM-IPM	T83I		K46E, <b>S87W</b> K120Q	R378H,D539E A375Y,R378G	bla <sub>OXA50(OXA-395)</sub> ,bla <sub>GES-2</sub> bla <sub>OXA50(OXA-396)</sub> ,bla <sub>GES-2</sub> , bla <sub>IMI-2</sub>			
30 31 32 33	AMK-ATM ATM CIP-TZP-ATM-CAZ-MEM-IPM-TOB CIP-ATM-IPM-TOB	T83V			A375Y,G376A	bla <sub>OXA50(OXA-1034)</sub> bla <sub>OXA50(OXA-395)</sub> bla <sub>OXA50(OXA-1034)</sub> ,bla <sub>GES-2</sub> bla <sub>OXA50(OXA-1956)</sub> ,bla <sub>GES-2</sub>	аас(3)-Іс аас(3)-Іс аас(3)-Іс		
34 35	CIP-ATM-TZP-TOB-MEM-IPM CIP-ATM		S466F		R378Q,D539E A368L,E369D, S373I,N374Y	bla <sub>OXA50(OXA-396)</sub> ,bla <sub>GES-2</sub> bla <sub>OXA50(OXA-1032)</sub>	aac(3)-Ia		
36 37 38	ATM-CAZ ATM CIP-ATM				D539E	bla <sub>OXA50(OXA-905)</sub> ,bla <sub>GES-2</sub> bla <sub>OXA50(OXA-395)</sub> bla <sub>OXA50(OXA-396)</sub>			
39 40 41	AIM CIP-ATM ATM-MEM-IPM	D87G			R378G	bla <sub>OXA50(OXA-1034)</sub> bla <sub>OXA50(OXA-395)</sub> bla <sub>OXA50(OXA-1034)</sub> ,bla <sub>GES-2</sub> ,			
42 43	ATM-MEM-IPM CIP-ATM	T83I		M34Y,D35G, <b>S87W</b>		bla <sub>OXA50(OXA-396)</sub> ,bla <sub>GES-2</sub> bla <sub>OXA50(OXA-396)</sub>			

Bold signifies the most frequent mutation related to antimicrobial resistance.

	Porin	MexAB-OprM			MexCD-OprJ MexEF-OprN			MexXY Gene expression				
Strains	OprD	nalC	nalD	mexR	nfxB	mexT	mexS	mexZ	DDCT-mexB	DDCT-mexD	DDCT-mexF	DDCT-mexY
1	MP1	G71E S209R			R821				2.07	0.84	1.17	1.48
2	T1035 K115T E170L A397T	G71E S209R		113G M14W				Q101A	0.36	0	0.72	0.31
3	MP1	G71E, A186T		2100, 111111				E74S	0.51	0.29	0.33	0.37
4	MP2	G71E S209R							29.11	0.24	0.27	0.1
5	MP4	G71E S209R	1330						4.61	0.01	1.18	0.53
6		G71E S209R	2000	V126E			V73A		6.57	0.19	0.18	0.05
7	NM	OTTE, OLOUN		TLOL					1.06	0.95	1 39	1 78
8	MP5	G71E S209R A145V		V126E					3.56	1.07	1.69	1 39
9	MP1	G71E	A211T	113W				01014 E124S	11 56	0.92	2 79	1.00
10	MP1	G71E S209R		LIGHT			H8M	01014	3 75	0.32	1 00	0.93
11	MP4	G71E S209R A145V	1 170	V126E	R21H D56G T140P		TIOM	alont	1.44	0.32	1.55	0.55
12	MP5	G71E S209R A145V	133P	V126E	R21H D56G				9.46	0.37	0.53	0.39
13	MP1	G71E S209R	2001	A12T	112111, 0000				4.25	0.04	0.08	0.09
14	MP3	G71E S209R A145V		V126E D8K	R21H D56G		T119P	0101A   105R	6.17	0.05	2.15	0.99
15	MP3	G71E S209R P210I		P11S	112111, 0000		11101	GIOTA, ETOOR	0.57	1.05	1.09	1.09
15	MP1	G71E A186T		1110				Q101A G162E	0.18	0.33	0.68	0.45
17	MP1	G71E S209R	A211T					aron, oroll	4.78	0.0	0.7	0.43
10	MP2	G71E S209R	A211T						1.17	0.04	0.16	0.13
10	MP2	G71E S209R	ALL I	V126E A103G				L 105R	0.79	0.04	1.42	0.71
20	MP1	G71E S209R		V 1202, A1000	R21H D56G			175Y 1 76F	3 72	0.05	1.41	0.99
20	MP1	G71E S209R			R21H D56G R63W			1101, 2101	4.10	0.02	1 32	0.33
22	MP3	G71E S209R A145V	V28A	V126E	11211, 2000, 10011		K229N	R71A 175T	25.75	0.02	0.29	0.77
23	MP3	G71E S209R A145V	120/1	V126E			THE LOT	Q70P	0.32	1.53	1.19	1.28
24	MP1	G71E S209R						a.r.o.	22.04	0.12	0.17	0.04
24	MP4	G71E S209R			T39P		G331A	01014	1.74	0.04	0.79	0.4
25	MP1	G71E			R821	S2951	000174	alon	4.33	0.48	0.75	0.57
27	MP1	G71E			R82L	02001			30.18	0.3	0.47	0.13
28	MP2	G71E, A186T					R332P	G162E	0.66	0.63	0.64	0.01
29	MP2	G71E			R82L			A33P	0.28	1.44	0.69	0.73
30	MP2	G71E			R82L			Q101A	0.24	0.87	0.7	0.31
31	T103S.K115T.F170L	G71E							0.20	0.75	0.74	0.75
32	MP1	G71E, A186T							31.73	0.29	1.79	0.34
33	V129A							175N. C30R	0.51	0.44	0.87	0.44
34		G71E, S209R, A145V		V126E, A103T				Q62S	0.85	1.02	1.71	0.83
35	K2E.K398Q.							Q101A	0.41	2.04	1.71	2.64
36	MP1	G71E, S209R		A12R					1.19	1.09	1.15	0.66
37	MP2	G71E, S209R		V126E, V132A					0.10	1.25	1.74	1.19
38		G71E, S209R, A145V		V126E	R21H. D56G				0.45	0.79	0.75	0.43
39	MP4	G71E, S209R, A145V		V126E	R21H, D56G			E124S	28.47	1	0.55	0.24
40	A416G,H418A,D423Q								0.52	0.39	1.44	1.2
41		G71E, S209R		V126E					32.59	0.03	0.63	0.54
42	MP1	G71E, A186T							45.03	0.51	0.55	0.31
43	MP1	G71E, A186T						G162E	1.04	0.5	1.04	0.65

**Figure 4.** Mutations detected in OprD, regulators of efflux systems and gene expression heat map for efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY. MP, mutation pattern; NM, no mutation; \_, absence. MP1=D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, A281G, K296Q, Q301E, R310G, V359L, 372(V-DSSSSYAGL-)383. MP2=K2E, D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, A281G, K296Q, Q301E, R310G, V359L, 372(V-DSSSSYAGL-)383. MP2=K2E, T103S, K115T, F170L, E185Q, P186G, V189T, R310E, A315G. MP4=S57E, S59R, V127L, E185Q, P186G, V189T, E202Q, I210A, E230K, S240T, N262T, T276A, A281G, K296Q, Q301E, R310E, A315G, L347M, 372(V-DSSSSYAGL-)383. MP5=T103S, K115T, F170L, E185Q, P186G, V189T, R310E, A315G.

different CCs: CC175, CC676, CC532 and CC253 (Figures 5 and 6). The XDR profile was associated with the most frequently found clones, ST619 and ST532, while the MDR profile had a broader distribution, being found in ST619, ST676, ST308, ST17, ST155, ST667 and ST699. Resistance to ciprofloxacin was widely extended and found in 13 different clones: ST619, ST676, ST109, ST308, ST17, ST155, ST181, ST377, ST667, ST671, ST1213, ST1568 and ST1720. Resistance to the other antimicrobial agents was distributed in all clones except resistance to piperacillin/tazobactam (ST619 and ST532), ceftazidime (ST619 and ST532) and colistin (ST181) (Figure 6).

# Discussion

Several papers have focused on *Pseudomonas* resistance in BE. However, to the best of our knowledge, this is the first study reporting the mechanisms of resistance combined with the ST and CCs in *P. aeruginosa* from BE patients. We found a high prevalence of ciprofloxacin-resistant strains (ciprofloxacin being the first-line treatment for *P. aeruginosa* eradication)<sup>12</sup> and an association between a higher number of mutations in the QRDR and a higher ciprofloxacin MIC. Finally, we identified two new emerging high-risk clones in BE.

Although some studies have reported high rates of MDR in strains of *P. aeruginosa* from BE patients, for instance during exacerbations, their associated mechanisms of resistance have not

been analysed previously.<sup>13</sup> Mensa *et al.*<sup>14</sup> found a similar average resistance (20%) to that found herein (15%) towards most antipseudomonal antibiotics in Spain. Consistent with our results, they found that colistin and ceftolozane/tazobactam showed activity close to 95%. However, they included *P. aeruginosa* strains from other types of infection and excluded those from BE patients.

We found a higher incidence of antimicrobial resistance [ciprofloxacin (44.19% versus 38.4%), tobramycin (18.6% versus 16.3%), amikacin (18.6% versus 4%) and imipenem (32.55% versus 15.6%)] compared with that found by Barrio-Tofiño *et al.*,<sup>3,15</sup> who also described mechanisms of resistance and molecular epidemiology, but like others did not exclusively use respiratory samples, nor were they exclusively from patients with BE.

Several reports have indicated that mutations in *gyrA* (75%) and *parC* (98%) genes are the primary target for quinolone resistance in *P. aeruginosa*.<sup>16</sup> In our study, the most frequent mutations were T83I in GyrA (21.05%) and S87W in ParC (21.05%). We identified two other amino acid changes in GyrA (T83V and D87G) that could be characteristic of *P. aeruginosa* strains from BE patients since different amino acid changes have been described in other respiratory infections such as in positions 83 (T83I) in GyrA and 87 (S87L or S87T) in ParC.<sup>4,17</sup>

Despite not being the main QRDR target, mutations in the gyrB (3%–29%) and *parE* (2%–7%) genes are still important, since those amino acid changes that we described in GyrB (S466F)



Figure 5. Genetic distance among the different STs. Hierarchical clustering of all STs found in *P. aeruginosa* strains, including alleles for the different housekeeping genes and CCs.

were previously reported to greatly increase the ciprofloxacin MIC.<sup>4,16</sup> We found ParE amino acid substitution that differed from those previously reported in the literature (D419N, E459D, A473V and S457R), D539E being the most frequent in our strains. In addition, we found that a greater number of different mutated genes in the QRDR were associated with an increased MIC, as reported in Table 2.<sup>4,16</sup>

Different  $\beta$ -lactamases were detected [ $bla_{OXA50}$ , MBL (GIM-1) and serine carbapenemases (GES-2 and IMI-2)]. bla<sub>OXA50</sub> plays an important role in our strains since the classic β-lactamase inhibitors show weak activity against *bla<sub>OXA50</sub>*.<sup>18,19</sup> MBLs were barely found in our strains. Nevertheless, we found one strain with a GIM-1 instead of VIM and IMP, which are the most prevalent types in *P. aeruginosa*.<sup>19,20</sup> Although the worldwide prevalence of GES-type serine carbapenemase is rather low,<sup>19,20</sup> almost half of our strains carried the GES carbapenemase, being characteristic of strains from Spain.<sup>14</sup> This incidence of GES-2 explains the aztreonam resistance found in our strains since other authors have reported that GES is active against aztreonam.<sup>21</sup> We also highlight the presence of IMI-2 in our strains, a carbapenemase of chromosomal origin that is present at low levels in *P. aeruginosa*.<sup>19,22,23</sup> However, an IMI of plasmid origin has recently been described in Escherichia coli, which could facilitate gene transfer exchange between different species.<sup>24</sup> Our strains could carry this plasmid.

Previous studies have reported that the loss or mutation of OprD is associated with non-susceptibility to imipenem. In contrast, the mechanism leading to meropenem resistance is multifactorial (OprD inactivation plus hyperexpression of MexAB-OprM).<sup>4,14,25,26</sup> We described five different mutation patterns and also OprD absence in strains resistant to imipenem (Figure 4), and multifactorial resistance mechanisms [overexpression of MexAB-OprM and serine carbapenemases (Table 3)] in strains resistant to meropenem. However, it is difficult to establish clear causality since each strain combines multiple resistance mechanisms.

The most commonly described AMEs in *P. aeruginosa* are the acetyltransferases AAC(3') and AAC(6') (conferring resistance to both tobramycin and amikacin in the first case and to both or only tobramycin in the second case) and the nucleotidyltransferase ANT(2')-I (conferring resistance to gentamicin and tobramycin).<sup>18,27,28</sup> We detected the presence of these AMEs in our aminoglycoside-resistant strains, the most frequent being AAC(3')-Ia (Table 2). AMEs have high clinical impact since, like  $\beta$ -lactamases with a higher hydrolytic profile, class B  $\beta$ -lactamases (MBLs) and ESBLs, they are usually associated with transferable genetic elements (plasmids or transposons).<sup>14</sup>

Our study confirms that ceftazidime/avibactam, ceftolozane/ tazobactam and colistin are an ultimate line of attack against MDR Gram-negative pathogens in chronic respiratory diseases. However, the recent emergence of plasmid-mediated *mcr-1* colistin resistance is a challenge to public global health since it increases the potential dissemination of the *mcr-1* gene.<sup>29</sup> In a previous study of samples from ICU patients with different sources of infection, 10% of colistin-resistant isolates were



**Figure 6.** Minimum spanning tree of the 43 *P. aeruginosa* strains based on the MLST allelic profile and main CCs. Each circle represents a clone. The size of the circle corresponds to the number of isolates ascribed to that particular clone and each different colour inside the circle represents a different antimicrobial profile associated with each clone.

positive for the *mcr-1* gene. We detected the *mcr-1* gene in only one *P. aeruginosa* strain but it was not associated with resistance.<sup>30</sup>

In our study, MexAB-OprM, a pump with a wide substrate profile, was the pump with the highest prevalence and overexpression. Our finding coincides with that of Serra *et al.*<sup>7</sup> and others<sup>4,31</sup> who also found a high prevalence and overexpression of *mexB* and *mexY* genes in their clinical *P. aeruginosa* strains. We only found one strain with overexpression of MexXY associated with intrinsic resistance to aminoglycosides. The simultaneous overexpression of MexB and MexF (observed in two strains) and the low level of expression of MexCD-OprJ (<5%) are consistent with previous studies (Figure 4).<sup>7,9,25,32</sup>

High-risk P. aeruginosa clones associated with MDR/XDR strains (e.g. ST175, ST111 and ST235) are widely disseminated around the world.<sup>33,34</sup> However, in our study these clones were not identified except for ST235 and ST308. Therefore, our strains presented different clonal distribution compared with previous studies of P. aeruginosa strains from other infections and samples. A multicentre study of P. aeruginosa bacteraemia in Spain revealed that 90% of XDR isolates belonged to the aforementioned high-risk clones.<sup>3,32,35</sup> Although we found that 21% of isolates had the MDR/XDR resistance profile, similar to the  ${\sim}30\%$ recently described (Figure 2),<sup>3,18,26</sup> our study included two emerging high-risk clones among the most frequent of our P. aeruginosa strains, ST619 and ST532, which were also associated with the MDR/XDR phenotype and had not been described before in P. aer*uginosa* strains from BE.<sup>11,36</sup> The high frequency of these emerging high-risk clones in BE patients is a matter of concern since

it favours the spread of resistance. Here we stress that ST619 is found within the same CC (CC175) as ST175, a clone with a high prevalence in Spain. So this CC is even more important in the dissemination of MDR/XDR strains. Our findings are quite different from previous studies, as besides the new emerging highrisk clones, we did not find the ST179 reported previously as being associated with other MDR *P. aeruginosa* causing chronic respiratory infections in Spanish hospitals.<sup>26,37,38</sup> In addition, we barely (2.3%) found ST308, which is associated with MDR/XDR strains producing carbapenemases, also described by Ruiz *et al.*<sup>26</sup>

This study has some limitations. First, the number of strains was low because our strains came exclusively from BE patients. Other studies with more strains describe the mechanisms of resistance and epidemiology but in strains from different infections. Second, we did not assess the virulence of our *P. aeruginosa* strains. Previous studies have shown the association between some type III secretion system (TTSS) genotypes and antibiotic resistance patterns. Despite its aforementioned limitations this study provides novel information about resistance to first-line treatment, essentially analysis of antibiotic resistance genes and antimicrobial resistance associated with clonal distribution in *P. aeruginosa* strains from BE, with potential clinical implications.

#### Conclusions

The high level of resistance to first-line recommended antimicrobial agents for *P. aeruginosa* eradication in BE, the combination of multiple resistance mechanisms found in each strain and the identification of two emerging high-risk clones, not described before in BE, threatens the treatment and eradication of *P. aeruginosa* in BE patients. In view of our results and although there are still therapeutic options for *P. aeruginosa* in BE such as colistin, new antipseudomonal therapies are urgently needed. Other IV antimicrobial agents such as ceftolozane/tazobactam, not currently included in BE guidelines, could become therapeutic candidates for BE patients with MDR *P. aeruginosa*. Secondly, since diagnostic accuracy is a key aspect for the adequacy of antimicrobial treatment, further investigations are needed to determine whether improvements in microbial diagnostics could positively influence *Pseudomonas* eradication rates and decrease the emergence of new resistant strains as well as the spread of current ones.

# Acknowledgements

We thank Dr Joaquim Ruiz, Dr Elisenda Bañon and Mireya Fuentes for their professional advice.

# Funding

This study was funded by ISCIII-FEDER with the FIS (PI1800145) to A.T./ L.F.B., intramural CIBERES (ES18PI01) to A.T./L.F.B., CIBER de enfermedades respiratorias -CIBERES (CB 06/06/0028, an initiative of ISCIII), SEPAR 2016 (Grant: 208) and SEPAR 2018 (Grant: 628) to L.F.B., PFIS-FSE to R.L.A. (FI19/00090) and SGR-Generalitat de Catalunya, IDIBAPS and ICREA Academy Award to A.T.

# **Transparency declarations**

A.T. has received grants from Medimmune, Cubist, Bayer, Theravance and Polyphor and personal fees as an Advisory Board member from Bayer, Roche, The Medicines Company and Curetis. He has received personal speaker's bureau fees from GSK, Pfizer, AstraZeneca and the Biotest Advisory Board, unconnected to the study submitted here. All other authors: none to declare.

#### Author contributions

R.C. assessed the mechanisms of resistance, conducted the MLST including the analysis of gene sequences, and performed the gene expression analysis and the phylogenetic analysis. R.C., L.F.B. and N.V. participated in the study of antimicrobial susceptibility. R.C., L.F.B. and A.T. participated in the protocol development, study design and study management. R.C. and L.B.F. participated in data interpretation and writing of the manuscript. L.F.B., R.A., L.B., V.A.S. and P.O. participated in the recruitment of patients. R.C., N.V., L.F.B., L.M. and J.V. participated in the identification of microorganisms. L.F.B., N.V., R.L.A., V.A., L.B.F., R.A. and A.T. obtained the respiratory specimens and critically reviewed the manuscript. All authors participated in data collection and reviewed the manuscript.

# Data availability

All data generated or analysed during this study are included in this published article.

## References

**1** Fernández-Barat L, Alcaraz-Serrano V, Amaro R *et al. Pseudomonas aeruginosa* in bronchiectasis. *Semin Respir Crit Care Med* 2021; **42**: 587–94.

**2** McDonnell MJ, Jary HR, Perry A *et al*. Non cystic fibrosis bronchiectasis: a longitudinal retrospective observational cohort study of *Pseudomonas* persistence and resistance. *Respir Med* 2015; **109**: 716–26.

**3** Del Barrio-Tofinō E, Zamorano L, Cortes-Lara S *et al.* Spanish nationwide survey on *Pseudomonas aeruginosa* antimicrobial resistance mechanisms and epidemiology. *J Antimicrob Chemother* 2019; **74**: 1825–35.

**4** Solé M, Fàbrega A, Cobos-Trigueros N *et al. In vivo* evolution of resistance of *Pseudomonas aeruginosa* strains isolated from patients admitted to an intensive care unit: mechanisms of resistance and antimicrobial exposure. *J Antimicrob Chemother* 2015; **7**: 3004–13.

**5** Treepong P, Kos VN, Guyeux C *et al.* Global emergence of the widespread *Pseudomonas aeruginosa* ST235 clone. *Clin Microbiol Infect* 2018; **24**: 258-66.

**6** EUCAST. Breakpoint tables for interpretation of MICs and zone diameters. http://www.eucast.org/clinical\_breakpoints/.

**7** Serra C, Bouharkat B, Touil-Meddah AT *et al.* MexXY multidrug efflux system is more frequently overexpressed in ciprofloxacin resistant French clinical isolates compared to hospital environment ones. *Front Microbiol* 2019; **10**: 366.

**8** Al Rashed N, Joji RM, Saeed NK *et al*. Detection of overexpression of efflux pump expression in fluoroquinolone-resistant *Pseudomonas aeruginosa* isolates. *Int J Appl Basic Med Res* 2020; **10**: 37.

**9** Tomás M, Doumith M, Warner M et al. Efflux pumps, OprD porin, AmpC  $\beta$ -lactamase, and multiresistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob Agents Chemother* 2010; **54**: 2219–24.

**10** Carriço JA, Crochemore M, Francisco AP *et al.* Fast phylogenetic inference from typing data. *Algorithms Mol Biol* 2018; **13**: 4.

**11** Feil EJ, Li BC, Aanensen DM *et al.* eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* 2004; **186**: 1518–30.

**12** Polverino E, Goeminne PC, McDonnell MJ *et al*. European Respiratory Society guidelines for the management of adult bronchiectasis. *Eur Respir J* 2017; **50**: 1700629.

**13** Menéndez R, Méndez R, Polverino E *et al.* Risk factors for multidrug-resistant pathogens in bronchiectasis exacerbations. *BMC Infect Dis* 2017; **17**: 659.

**14** Mensa J, Barberán J, Soriano A *et al.* Antibiotic selection in the treatment of acute invasive infections by *Pseudomonas aeruginosa*: Guidelines by the Spanish Society of Chemotherapy. *Rev Esp Quimioter* 2018; **31**: 78–100.

**15** Kaehne A, Milan SJ, Felix LM *et al.* Head-to-head trials of antibiotics for bronchiectasis. *Cochrane Database Syst Rev* 2018: CD012590.

**16** 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.

**17** Akasaka T, Tanaka M, Yamaguchi A *et al*. 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**: 2263–8.

**18** Horcajada JP, Montero M, Oliver A *et al*. Epidemiology and treatment of multidrug-resistant and extensively drug-resistant *Pseudomonas aeruginosa* infections. *Clin Microbiol Rev* 2019; **32**: e00031-19.

**19** Nicolau CJ, Oliver A. Carbapenemases in *Pseudomonas* spp. *Enferm Infecc Microbiol Clin* 2010; **28** Suppl 1: 19–28.

**20** Botelho J, Grosso F, Peixe L. Unravelling the genome of a *Pseudomonas aeruginosa* isolate belonging to the high-risk clone ST235 reveals an integrative conjugative element housing a *bla*<sub>GES-6</sub> carbapenemase. *J Antimicrob Chemother* 2018; **73**: 77–83.

**21** Poirel L, Brinas L, Fortineau N *et al.* Integron-encoded GES-type extended-spectrum  $\beta$ -lactamase with increased activity toward aztreonam in *Pseudomonas aeruginosa. Antimicrob Agents Chemother* 2005; **49**: 3593–7.

**22** Queenan AM, Bush K. Carbapenemases: the versatile  $\beta$ -lactamases. Clin Microbiol Rev 2007; **20**: 440–58.

**23** Walther-Rasmussen J, Høiby N. Class A carbapenemases. *J Antimicrob Chemother* 2007; **60**: 470–82.

**24** Rojo-Bezares B, Martín C, López M *et al.* First detection of  $bla_{IMI-2}$  gene in a clinical *Escherichia coli* strain. *Antimicrob Agents Chemother* 2012; **56**: 1146–7.

**25** Riera E, Cabot G, Mulet X *et al. Pseudomonas aeruginosa* carbapenem resistance mechanisms in Spain: impact on the activity of imipenem, meropenem and doripenem. *J Antimicrob Chemother* 2011; **66**: 2022–7.

**26** Horna G, Amaro C, Palacios A *et al.* High frequency of the exoU+/ exoS+ genotype associated with multidrug-resistant 'high-risk clones' of *Pseudomonas aeruginosa* clinical isolates from Peruvian hospitals. *Sci Rep* 2019; **9**: 10874.

**27** Poole K. *Pseudomonas aeruginosa*: resistance to the max. *Front Microbiol* 2011; **2**: 65.

**28** Castanheira M, Deshpande LM, Woosley LN *et al.* Activity of plazomicin compared with other aminoglycosides against isolates from European and adjacent countries, including Enterobacteriaceae molecularly characterized for aminoglycoside-modifying enzymes and other resistance mechanisms. *J Antimicrob Chemother* 2018; **73**: 3346–54.

**29** Ye H, Li Y, Li Z *et al*. Diversified *mcr*-1-harbouring plasmid reservoirs confer resistance to colistin in human gut microbiota. *mBio* 2016; **7**: e00177.

**30** Abd El-Baky RM, Masoud SM, Mohamed DS *et al.* Prevalence and some possible mechanisms of colistin resistance among multidrug-resistant and extensively drug-resistant *Pseudomonas aeruginosa. Infect Drug Resist* 2020; **13**: 323–32.

**31** Vatcheva-Dobrevska R, Mulet X, Ivanov I *et al.* Molecular epidemiology and multidrug resistance mechanisms of *Pseudomonas aeruginosa* isolates from Bulgarian hospitals. *Microb Drug Resist* 2013; **19**: 355–61.

**32** El Zowalaty ME, Al Thani AA, Webster TJ *et al. Pseudomonas aeruginosa*: arsenal of resistance mechanisms, decades of changing resistance profiles, and future antimicrobial therapies. *Future Microbiol* 2015; **10**: 1683–706.

**33** Oliver A, Mulet X, López-Causapé C *et al.* The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist Updat* 2015; **10**: 41–59.

**34** García-Castillo M, Del Campo R, Morosini MI *et al*. Wide dispersion of ST175 clone despite high genetic diversity of carbapenemnonsusceptible *Pseudomonas aeruginosa* clinical strains in 16 Spanish hospitals. *J Clin Microbiol* 2011; **49**: 2905–10.

**35** Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev* 2009; **22**: 582–610.

**36** Founou RC, Founou LL, Allam M *et al.* First report of a clinical multidrug-resistant *Pseudomonas aeruginosa* ST532 isolate harbouring a ciprofloxacin-modifying enzyme (CrpP) in South Africa. *J Glob Antimicrob Resist* 2020; **22**: 145–6.

**37** Juan C, Zamorano L, Mena A *et al*. Metallo-β-lactamase-producing *Pseudomonas putida* as a reservoir of multidrug resistance elements that can be transferred to successful *Pseudomonas aeruginosa* clones. *J Antimicrob Chemother* 2010; **65**: 474–8.

**38** Gomila M, Del Carmen Gallegos M, Fernández-Baca V *et al.* Genetic diversity of clinical *Pseudomonas aeruginosa* isolates in a public hospital in Spain. *BMC Microbiol* 2013; **13**: 138.