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

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

[1]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; [2]Respiratory Intensive Care Unit, Pneumology Department, Hospital Clínic, Barcelona, Spain; [3]Barcelona Global Health Institute, Department of Clinical Microbiology, Hospital Clínic, University of Barcelona, Barcelona, Spain

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

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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 β-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.[1] *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.[2] 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

clones are associated with certain clonal complexes (CCs) and that their distribution varies depending on the region.[3] However, no previous studies have reported high-risk clones from BE patients.

The most important antipseudomonal agents include quinolones (e.g. ciprofloxacin), β-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 β-lactamases (e.g. ESBLs and carbapenemases); (3) aminoglycoside-modifying enzymes (AMEs); (4) up-regulation 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.[4] 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.[5] 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 non-mucoid 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.[1]

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/).[6]

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 β-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°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 μL including 14 μL of template RNA (extract concentrations adjusted to contain 1 μg of RNA), 1 μL of Reverse Transcription Master Mix, 4 μL of RT buffer 5× (containing dNTPs and Mg^{2+}) and 1 μL 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.[7]

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 μL final volume containing 2.5 μL of diluted (1:20) template cDNA, 1 μL of each primer (corresponding to a final concentration of 0.5 μM), 5 μL of QuantiTect SYBR Green PCR Master Mix (including $MgCl_2$ to reach a final concentration of 2.5 mM) (QIAGEN) and 0.5 μL 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 ΔΔCt 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.[7,9]

Table 1. Primers used in this study

Amplified product	Primer pair	Sequence (5′ to 3′)	Amplicon size (bp)	Annealing temperature (°C)	Reference
gyrA	*gyrA*-F	AGTCCTATCTCGACTACGCGAT	341	55	17
	gyrA-R	AGTCGACGGTTTCCTTTTCCAG			
gyrB	*gyrB*-F	TGCGGTGGAACAGGAGATGGGCAAGTAC	697	55	17
	gyrB-R	CTGGCGGAAGAAGAAGGTCAACAGCAGGGT			
parC	*parC*-F	CGAGCAGGCCTATCTGAACTAT	235	55	17
	parC-R	GAAGGACTTGGGATCGTCCGGA			
parE	*parE*-F	CGGCGTTCGTCTCGGGCGTGGTGAAGGA	592	65	4
	parE-R	TCGAGGGCGTAGTAGATGTCCTTGCCGA			
nalC	*nalC*-F	TCAACCCTAACGAGAAACGCT	814	69	4
	nalC-R	TCCACCTCACCGAACTGC			
nalD	*nalD*-F	GCGGCTAAAATCGGTACACT	789	55	4
	nalD-R	ACGTCCAGGTGGATCTTGG			
mexR	mexR20	CCAGTAAGCGGATAC	1016	51	4
	*mexR*INT	GGATGATGCCGTTCACCTC			
mexT	*mexT*-F	TGCATCACGGGGTGAATAAC	1398	55	4
	mexT-R	GGTAGCGCCAGGAGAAGTG			
mexS	*mexS*-F	ATACAGTCACAACCCATGA	1153	50	4
	mexS-R	TCAACGATCTGTGAATCT			
mexZ	*mexZ*2060	CCAGCAGGAATAGGGCGACCAGGGC	1059	64	4
	*mexZ*1026	CAGCGTGGAGATCGAAGGCAGCCGG			
oprD	*oprD*-F	GGCAGAGATAATTTCAAAACCAA	1384	64	26
	oprD-R	GTTGCCTGTCGGTCGATTAC			
oxa50	*oxa50*-F	AATCCGGCGCTCATCCATC	619	54	32
	oxa50-R	GGTCGGCGACTGAGGCGG			
ges	*ges*-F	GTTTTGCAATGTGCTCAACG	371	55	26
	ges-R	TGCCATAGCAATAGGCGTAG			
imi	*imi*-F	ATAGCCATCCTTGTTTAGCTC	818	55	26
	imi-R	TCTGCGATTACTTTATCCTC			
gim	*gim*-F	TCGACACACCTTGGTCTGAA	477	55	26
	gim-R	AACTTCCAACTTTGCCATGC			
aac(3)-Ia	*aac(3)Ia*-F	CCCTGACCAAGTCCAATCCATGC	435	55	28
	aac(3)Ia-R	GGTGGCGGTACTTGGGTCGATA			
aac(3)-Ic	*aac(3)Ic*-F	CTCTCAAGACGTTGGTGTAATGC	143	55	28
	aac(3)Ic-R	CAGCGATTGCGATGAAGCCAGA			
aac(6″)-Ib	*aac(6″)Ib*-F	GGTATGCCCAGTCGTACGTTGC	281	55	28
	aac(6″)Ib-R	TGGACCATMTGGGGTGGTTACG			
ant(2″)-Ia	*ant(2″)Ia*-F	ATGAGCGAAATCTGCCGCTCTG	150	55	28
	ant(2″)Ia-R	GCCCGCCGAGCATTTCAACTAT			
mcr1	*mcr1*-F	AGTCCGTTTGTTCTTGTGGC	1626	58	29
	mcr1-R	AGAT CCTTGGTCTCGGCTTG			
mexB	*mexB*-F	CAACATCCAGGACCCACTCT	167	60	7
	mexB-R	AGGAAATCTGCACGTTCTGC			
mexD	*mexD*-F	CTACCCTGGTGAAACAGC	250	58	8
	mexD-R	AGCAGGTACATCACCATCA			
mexF	*mexF*-F	TGTACGCGAACGACTTCAAC	163	60	7
	mexF-R	GAGGTGTCGCTGACCTTGAT			
mexY	*mexY*-F	TCAGGCCGACCTTGAAGTAG	159	60	7
	mexY-R	TCTCGGTGTTGATCGTGTTC			
rpsL	*rpsL*-F	TACTTCGAACGACCCTGCTT	163	60	7
	rpsL-R	TTTCCTCGTACATCGGTGGT			

Statistical analysis

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.[9]

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).[10,11]

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 Clínic 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 ± SD forced expiratory volume in 1 s (FEV_1) at inclusion of 58.92% ± 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

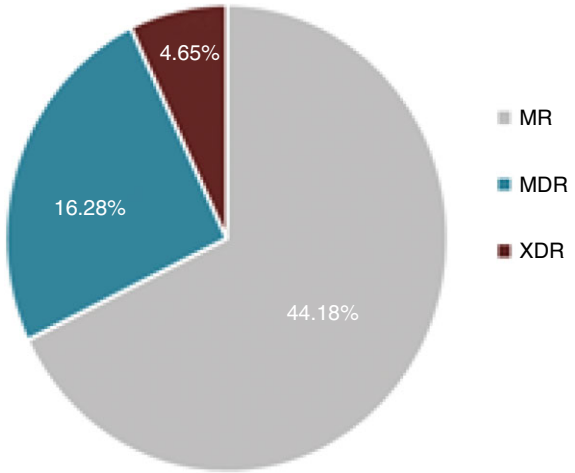


Figure 2. Antimicrobial profile of all *P. aeruginosa* strains analysed. MR, moderately drug resistant; XDR, isolates resistant to all the antimicrobial agents except ≤2.

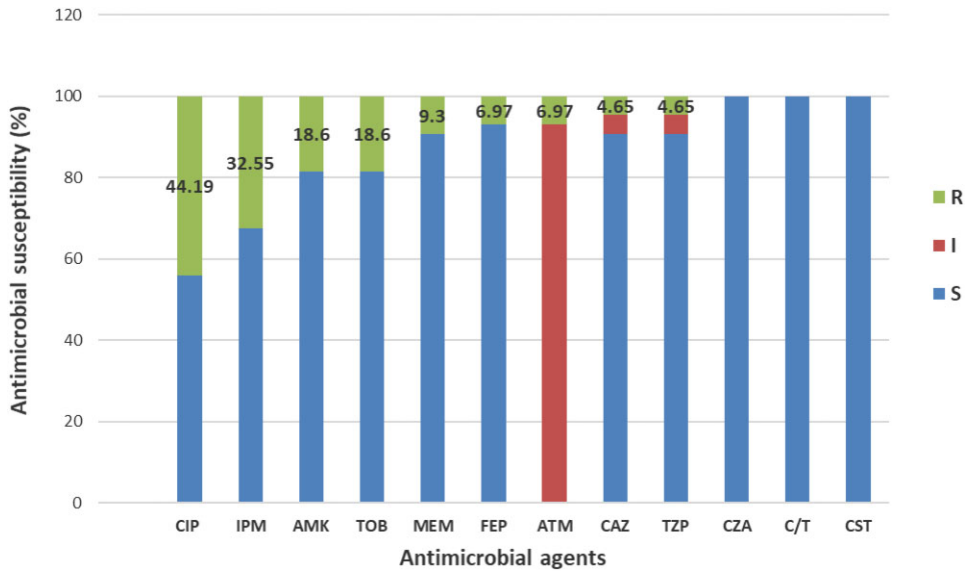


Figure 1. Antimicrobial susceptibility of all *P. aeruginosa* strains analysed by Etest. CIP, ciprofloxacin; IPM, imipenem; AMK, amikacin; TOB, tobramycin; ATM, aztreonam; MEM, meropenem; CAZ, ceftazidime; TZP, piperacillin/tazobactam; CST, colistin; CZA, ceftazidime/avibactam; C/T, ceftolozane/tazobactam;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

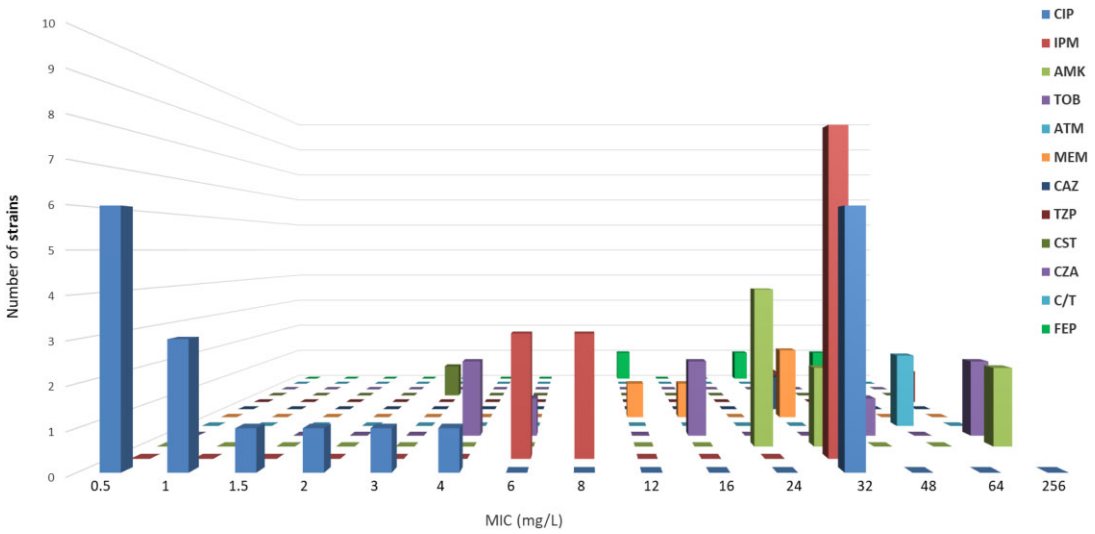


Figure 3. Number of resistant *P. aeruginosa* strains with each MIC value. CIP, ciprofloxacin; IPM, imipenem; AMK, amikacin; TOB, tobramycin; ATM, aztreonam; MEM, meropenem; CAZ, ceftazidime; TZP, piperacillin/tazobactam; CST, colistin; CZA, ceftazidime/avibactam; C/T, ceftolozane/tazobactam; FEP, cefepime.

were T83I in GyrA (21.05%), S466F in GyrB (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_{GES-2} (44.18%), bla_{IMI-2} (11.62%), bla_{GIM-1} (2.32%) and bla_{OXA50} (97.67%), an intrinsic β-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			
gyrB	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		$bla_{OXA50(OXA-395)}$		*mcr-1*
2	CIP-ATM	L41W	N366D		K380Q	$bla_{OXA50(OXA-396)}$		
3	CIP-ATM-IPM-TOB	**T83I**		**S87W**	R378G,Y536T, A537P,D539E	$bla_{OXA50(OXA-1034)}$,bla_{GES-2}	*aac(6″)-Ib*	
4	CIP-ATM-AMK			K46E		$bla_{OXA50(OXA-395)}$	*aac(3)-Ia*	
5	ATM-MEM					$bla_{OXA50(OXA-1034)}$,bla_{IMI-2}, bla_{GIM-1}		
6	CIP-TZP-AMK-ATM-CAZ-MEM-IPM	N57Q,D58R, W59L,N60E	**S466F**		S373I,N374Y, A375D,R378H	$bla_{OXA50(OXA-396)}$,bla_{GES-2}	*aac(3)-Ia*	
7	ATM-IPM					$bla_{OXA50(OXA-396)}$,bla_{GES-2}		
8	AMK-ATM-TOB					$bla_{OXA50(OXA-1032)}$	*aac(3)-Ia*	
9	ATM-MEM					$bla_{OXA50(OXA-905)}$,bla_{GES-2}		
10	ATM					$bla_{OXA50(OXA-395)}$		
11	ATM					$bla_{OXA50(OXA-396)}$		
12	ATM					$bla_{OXA50(OXA-1034)}$		
13	CIP-ATM		Q443H		G376A, R378H,D539E	$bla_{OXA50(OXA-395)}$		
14	ATM					$bla_{OXA50(OXA-1034)}$		
15	ATM-MEM					$bla_{OXA50(OXA-396)}$,bla_{GES-2}		
16	CIP-TZP-ATM-CAZ-MEM-IPM-TOB	**T83I**	Q443H	D35W, **S87W**	D539E	$bla_{OXA50(OXA-396)}$,bla_{GES-2}	*ant(2″)-Ia*	
17	AMK-ATM-TOB					$bla_{OXA50(OXA-1032)}$,bla_{GES-2}	*ant(2″)-Ia*	
18	AMK-ATM-TOB-IPM					$bla_{OXA50\ (OXA-905)}$,bla_{GES-2}	*ant(2″)-Ia*	
19	CIP-ATM		**S466F**	I33N	Y536T,A537P, D539E	$bla_{OXA50(OXA-395)}$		
20	ATM					$bla_{OXA50(OXA-396)}$		
21	ATM					$bla_{OXA50(OXA-1034)}$		
22	ATM					$bla_{OXA50(OXA-395)}$		
23	ATM					$bla_{OXA50(OXA-1034)}$		
24	CIP-ATM-AMK-MEM-IPM		N366D, **S466F**			$bla_{OXA50(OXA-396)}$,bla_{GES-2}, bla_{IMI-2}	*aac(3)-Ia*	
25	CIP-ATM-MEM			K69M	R379Q,D539E	$bla_{OXA50(OXA-396)}$,bla_{GES-2}		
26	ATM					$bla_{OXA50(OXA-1032)}$		
27	ATM-MEM-IPM					$bla_{OXA50(OXA-905)}$,bla_{GES-2}, bla_{IMI-2}		
28	CIP-ATM-MEM-IPM	**T83I**		K46E,**S87W**	R378H,D539E	$bla_{OXA50(OXA-395)}$,bla_{GES-2}		
29	CIP-ATM-AMK-MEM-IPM			K120Q	A375Y,R378G	$bla_{OXA50(OXA-396)}$,bla_{GES-2}, bla_{IMI-2}		
30	AMK-ATM					$bla_{OXA50(OXA-1034)}$	*aac(3)-Ic*	
31	ATM					$bla_{OXA50(OXA-395)}$		
32	CIP-TZP-ATM-CAZ-MEM-IPM-TOB					$bla_{OXA50(OXA-1034)}$,bla_{GES-2}	*aac(3)-Ic*	
33	CIP-ATM-IPM-TOB	**T83V**			A375Y,G376A	$bla_{OXA50(OXA-396)}$,bla_{GES-2}	*aac(3)-Ic*	
34	CIP-ATM-TZP-TOB-MEM-IPM				R378Q,D539E	$bla_{OXA50(OXA-396)}$,bla_{GES-2}	*aac(3)-Ia*	
35	CIP-ATM		**S466F**		A368L,E369D, S373I,N374Y	$bla_{OXA50(OXA-1032)}$		
36	ATM-CAZ					$bla_{OXA50(OXA-905)}$,bla_{GES-2}		
37	ATM					$bla_{OXA50(OXA-395)}$		
38	CIP-ATM				D539E	$bla_{OXA50(OXA-396)}$		
39	ATM					$bla_{OXA50(OXA-1034)}$		
40	CIP-ATM	**D87G**			R378G	$bla_{OXA50(OXA-395)}$		
41	ATM-MEM-IPM					$bla_{OXA50(OXA-1034)}$,bla_{GES-2}, bla_{IMI-2}		
42	ATM-MEM-IPM					$bla_{OXA50(OXA-396)}$,bla_{GES-2}		
43	CIP-ATM	**T83I**		M34Y,D35G, **S87W**		$bla_{OXA50(OXA-396)}$		

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			R82L				2.07	0.84	1.17	1.48
2	T103S,K115T,F170L,A397T	G71E, S209R		L13G, M14W				Q101A	0.36	0	0.72	0.31
3	MP1	G71E, A186T						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	L33Q						4.61	0.01	1.18	0.53
6	_	G71E, S209R		V126E			V73A		6.57	0.19	0.18	0.05
7	NM								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	L13W				Q101A, E124S	11.56	0.92	2.79	1.02
10	MP1	G71E, S209R					H8M	Q101A	3.75	0.32	1.99	0.93
11	MP4	G71E, S209R, A145V	L17Q	V126E	R21H, D56G, T140P				1.44	0.3	1.17	0.69
12	MP5	G71E, S209R, A145V	L33P	V126E	R21H, D56G				9.46	0.37	0.53	0.38
13	MP1	G71E, S209R		A12T					4.25	0.04	0.08	0.09
14	MP3	G71E, S209R, A145V		V126E, D8K	R21H, D56G		T119P	Q101A, L105R	6.17	0.05	2.15	0.89
15	MP3	G71E, S209R, P210L		P11S					0.57	1.06	1.08	1.08
16	MP1	G71E, A186T						Q101A,G162E	0.18	0.33	0.68	0.45
17	MP1	G71E, S209R	A211T						4.28	0.1	0.7	0.43
18	MP2	G71E, S209R	A211T						1.17	0.04	0.16	0.12
19	MP2	G71E, S209R		V126E, A103G				L105R	0.28	0.88	1.42	0.71
20	MP1	G71E, S209R			R21H, D56G			I75Y, L76F	3.72	0.01	1.41	0.89
21	MP1	G71E, S209R			R21H, D56G, R63W				4.10	0.02	1.32	0.77
22	MP3	G71E, S209R, A145V	V28A	V126E			K229N	R71A, I75T	25.75	0.2	0.29	0.2
23	MP3	G71E, S209R, A145V		V126E				Q70P	0.32	1.53	1.19	1.28
24	MP1	G71E, S209R							22.04	0.12	0.17	0.04
25	MP4	G71E, S209R			T39P		G331A	Q101A	1.74	0.04	0.79	0.4
26	MP1	G71E			R82L	S295I			4.33	0.48	0.75	0.57
27	MP1	G71E			R82L				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							I75N, 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. MP3 = 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

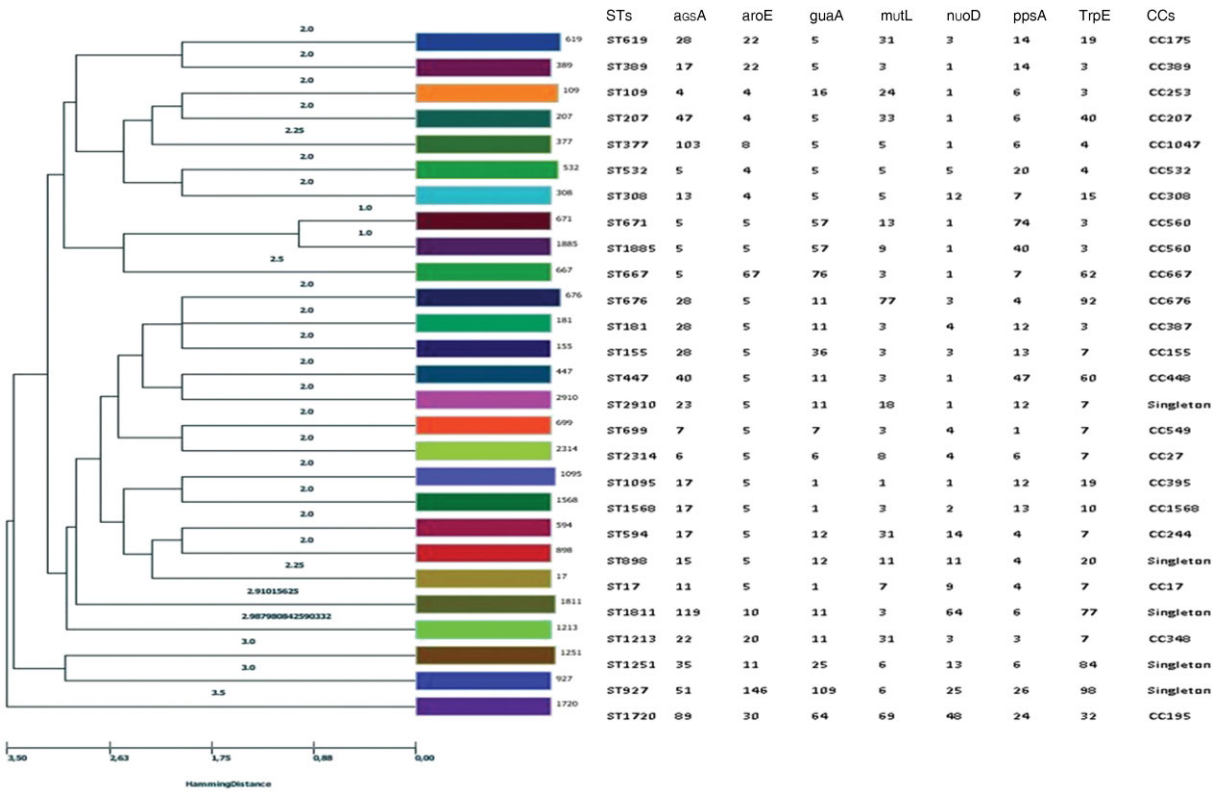
Several papers have focused on *Pseudomonas* resistance in BE. However, to the best of our knowledge, this is the first study reporting the mechanisms of resistance combined with the ST and CCs in *P. aeruginosa* from BE patients. We found a high prevalence of ciprofloxacin-resistant strains (ciprofloxacin being the first-line treatment for *P. aeruginosa* eradication)[12] 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.[13] Mensa *et al.*[14] 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.*,[3,15] 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*.[16] 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.[4,17]

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)



STs	agsA	aroE	guaA	mutL	nuoD	ppsA	TrpE	CCs
ST619	28	22	5	31	3	14	19	CC175
ST389	17	22	5	3	1	14	3	CC389
ST109	4	4	16	24	1	6	3	CC253
ST207	47	4	5	33	1	6	40	CC207
ST377	103	8	5	5	1	6	4	CC1047
ST532	5	4	5	5	5	20	4	CC532
ST308	13	4	5	5	12	7	15	CC308
ST671	5	5	57	13	1	74	3	CC560
ST1885	5	5	57	9	1	40	3	CC560
ST667	5	67	76	3	1	7	62	CC667
ST676	28	5	11	77	3	4	92	CC676
ST181	28	5	11	3	4	12	3	CC387
ST155	28	5	36	3	3	13	7	CC155
ST447	40	5	11	3	1	47	60	CC448
ST2910	23	5	11	18	1	12	7	Singleton
ST699	7	5	7	3	4	1	7	CC549
ST2314	6	5	6	8	4	6	7	CC27
ST1095	17	5	1	1	1	12	19	CC395
ST1568	17	5	1	3	2	13	10	CC1568
ST594	17	5	12	31	14	4	7	CC244
ST898	15	5	12	11	11	4	20	Singleton
ST17	11	5	1	7	9	4	7	CC17
ST1811	119	10	11	3	64	6	77	Singleton
ST1213	22	20	11	31	3	3	7	CC348
ST1251	35	11	25	6	13	6	84	Singleton
ST927	51	146	109	6	25	26	98	Singleton
ST1720	89	30	64	69	48	24	32	CC195

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.[4,16] 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.[4,16]

Different β-lactamases were detected [bla_{OXA50}, MBL (GIM-1) and serine carbapenemases (GES-2 and IMI-2)]. bla_{OXA50} plays an important role in our strains since the classic β-lactamase inhibitors show weak activity against bla_{OXA50}.[18,19] 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*.[19,20] Although the worldwide prevalence of GES-type serine carbapenemase is rather low,[19,20] almost half of our strains carried the GES carbapenemase, being characteristic of strains from Spain.[14] This incidence of GES-2 explains the aztreonam resistance found in our strains since other authors have reported that GES is active against aztreonam.[21] 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*.[19,22,23] However, an IMI of plasmid origin has recently been described in *Escherichia coli*, which could facilitate gene transfer exchange between different species.[24] 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).[4,14,25,26] 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).[18,27,28] 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 β-lactamases with a higher hydrolytic profile, class B β-lactamases (MBLs) and ESBLs, they are usually associated with transferable genetic elements (plasmids or transposons).[14]

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.[29] In a previous study of samples from ICU patients with different sources of infection, 10% of colistin-resistant isolates were

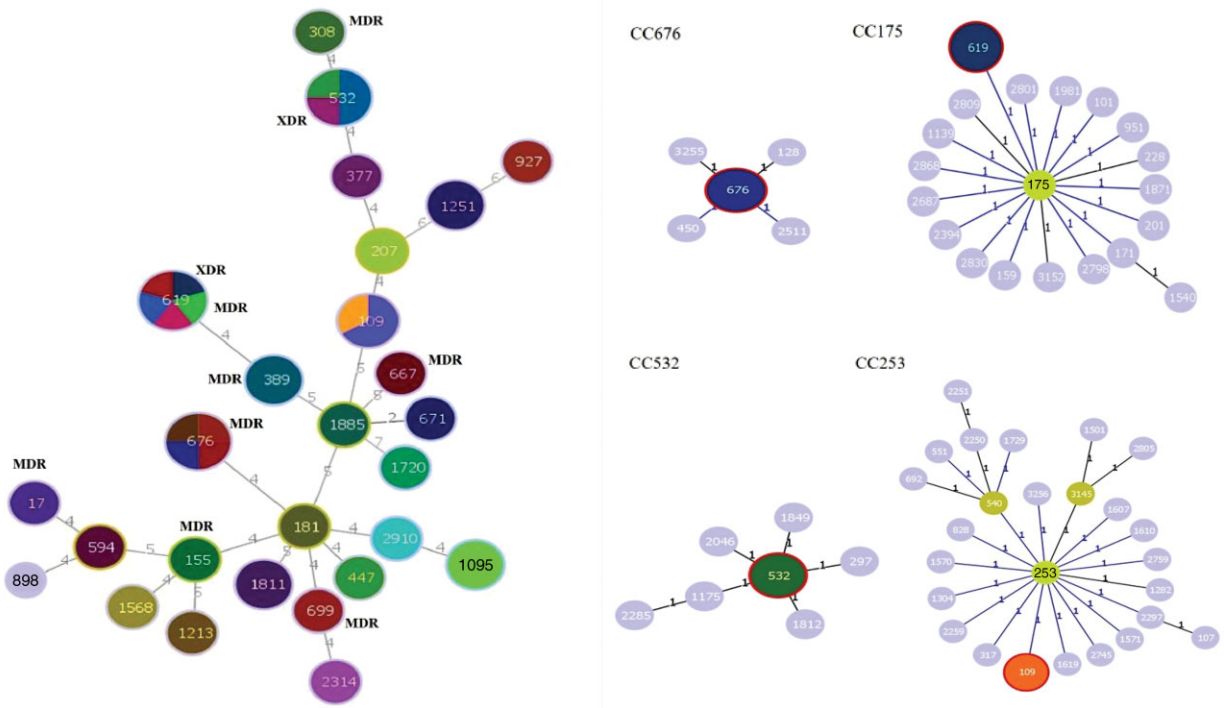


Figure 6. Minimum spanning tree of the 43 *P. aeruginosa* strains based on the MLST allelic profile and main CCs. Each circle represents a clone. The size of the circle corresponds to the number of isolates ascribed to that particular clone and each different colour inside the circle represents a different 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.[30]

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.*[7] and others[4,31] 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).[7,9,25,32]

High-risk *P. aeruginosa* clones associated with MDR/XDR strains (e.g. ST175, ST111 and ST235) are widely disseminated around the world.[33,34] 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.[3,32,35] Although we found that 21% of isolates had the MDR/XDR resistance profile, similar to the ~30% recently described (Figure 2),[3,18,26] 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. aeruginosa* strains from BE.[11,36] 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 high-risk clones, we did not find the ST179 reported previously as being associated with other MDR *P. aeruginosa* causing chronic respiratory infections in Spanish hospitals.[26,37,38] In addition, we barely (2.3%) found ST308, which is associated with MDR/XDR strains producing carbapenemases, also described by Ruiz *et al.*[26]

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.

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Transparency declarations

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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.

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