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Research paper

Epidemiology and antibiotic resistance trends in clinical isolates of *Pseudomonas aeruginosa* from Rio de janeiro - Brazil: Importance of mutational mechanisms over the years (1995–2015)

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A B S T R A C T

Pseudomonas aeruginosa is a major health concern globally and treating infections caused by MDR-isolates unarguably a humongous challenge that remains an unmet need in modern medicine. To determine patterns and mechanisms of antimicrobial resistance and its spread over the years in Rio de Janeiro, Brazil, 88 *P. aeruginosa* isolates were selected from 1995 to 2015. Phenotypic and genotypic characterization of antimicrobial resistance was evaluated and isolates were submitted to clonality by PFGE and MLST. PFGE analysis showed a great variability of clonal groups mainly over the past 10 years of this study. STs predominant in the early years (ST804, ST1860, ST487 and ST1602) associated to multidrug resistance (MDR) phenotype were replaced by ST277, ST244, ST1945, ST1791 with extensive drug resistance (XDR) in last years, with significant increase in resistance to carbapenems, fluoroquinolones and aminoglycosides. Colistin resistance was detected in 3.5%. The main mechanisms of antimicrobial resistance were mutational mechanisms (mutations in *oprD, mexT* and *gyrA* genes). We found the ESBL genes bla_{TEM} ($n = 2$), bla_{SHV} ($n = 3$) and bla_{CTX} ($n = 1$).The carbapenemases genes was present in ST277 (bla_{SPM}, $n = 3$), ST1560 (bla_{KPC}, n = 3) and ST1944 (bla_{KPC}, $n = 2$). The 16S RNA methylase gene (*rmt*D) was found in five isolates belonged to ST277. In conclusion, molecular epidemiological investigation reveals an increase of antimicrobial resistance in *P. aeruginosa* over 21 years in Rio de Janeiro with higher population structure and occurrence of high risk clone in the last years. The mutational mechanisms of resistance were present in all XDR isolates.

1. Introduction

Pseudomonas aeruginosa is a major health concern globally and treating infections caused by MDR-isolates unarguably a humongous challenge that remains an unmet need in modern medicine. Furthermore, *P. aeruginosa* ranking first as a cause of nosocomial pneumonia in Brazilian hospitals (Dortet et al., 2012). The success of these infections is linked to a complex interaction between pathogenicity, epidemicity and resistance to antimicrobials and, more recently, a regulation and association between mechanisms of resistance and virulence (Balasubramanian et al., 2013).

The emergence of phenotypes multidrug resistant (MDR - nonsusceptibility to at least one agent in three or more antimicrobial categorie), extensively-drug resistant (XDR - nonsusceptibility to at least one agent in all but two or fewer categories) and Pan-drug resistant (PDR - nonsusceptibility to all antimicrobial agents in all categories) in *P. aeruginosa* increased mortality and morbidity (Magiorakos et al., 2012; Lodise et al., 2007). Usually, the emergence of MDR and XDR occurs through the accumulation of mutational resistance mechanisms in porins (e.g., *oprD*), chromosomal beta-lactamase (e.g., *AmpC*), efflux pumps (e.g., MexEF-OprN) and the acquisition of mobile genetic elements containing resistance determinants, such as carbapenemases, aminoglycoside modifying enzymes, 16S sRNA methylases and plasmid-mediated quinolone resistance (PMQR) (Meletis and Bagkeri, 2013; Diene and Rolain, 2014; Puzari and Chetia, 2017).

Despite of the epidemiology of *P. aeruginosa* infections shows a polyclonal population, MDR and XDR phenotypes are often associated with few high-risk clones (Woodford et al., 2011). The high-risk clones ST111, ST235, ST244 have been found in many countries around the world (Oliver et al., 2015). In this study, we intend to determine the variations in the resistance patterns and clonal diversity of *P. aeruginosa* recovered in Rio de Janeiro over 21 years.

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2. Materials and methods

2.1. Clinical data collection

The Laboratório de Pesquisa em Infecção Hospitalar at Instituto Oswaldo Cruz (LAPIH-IOC/Fiocruz), contributes to the Nosocomial Infections Monitoring Network associated to the Health Ministry of Brazil and routinely received clinical isolates from different Brazillian states for confirmation of multidrug profile. During the period of 21 years (1995–2015), LAPIH received a total of 615 *P. aeruginosa* isolates from 10 hospitals from Rio de Janeiro. For the present study, a total of 88 nonduplicate isolates (14,3%) were rondom selected and distributed over four periods. The 1st period (1995–1999 years) with 22 isolates; 2nd period (2000–2005 years) with 11 isolates; 3rd period (2006–2010 years) with 39 isolates; 4th period (2011–2015 years) with 16 isolates. Isolates included the following: bloodstream (31.8%), lower respiratory system (18.2%), secretions (12.5%), urine (7,9%), catheter (6.8%), surveillance swabs (5.7%) and cerebrospinal fluid (1%). The isolates were identified by conventional biochemical tests.

2.2. Phenotypic detection of antibiotic resistance

The antimicrobial susceptibility test was performed by disk diffusion method and minimum inhibitory concentrations (MICs) following the CLSI 2015 criteria. Six antimicrobial classes were included in the analysis: carbapenems (imipenem - 10 μg, meropenem −10 μg and doripenem - 10 μg), cephalosporins (ceftazidime – 30 μg and cefepime −30 μg), penicillins + beta-lactamase inhibitors (ticarcillin + clavulanic acid - 75/10 μg and Piperacillin + Tazobactam - 100/10 μg), monobactam (aztreonam – 30 μg), aminoglycosides (amikacin - 30 μg and gentamicin - 10 μg), fluoroquinolones (ciprofloxacin – 5 μg and levofloxacin - 5 μg). The MIC of imipenem was determined by Etest® (Biomérieux, Marcy-l'Eloile - França) and for colistin by broth microdilution method (CLSI, 2018). *P. aeruginosa* ATCC27853 and *Escherichia coli* ATCC25922 strains were used as control. For phenotypic detection of carbapenemase-producing carbapenem-resistant isolates, the CarbaNP test was performed according to CLSI 2015. Detection of metallo-β-lactamases producing was performed using CarbaNP with addition of EDTA as described by Dortet et al. (2012).

2.3. Molecular detection of antibiotic resistance

The presence of resistance acquired genes: Extended spectrum beta-lactamases (ESBL) (bla_{GES}, bla_{CTX-M}, bla_{PER}, bla_{VEB}, bla_{TEM}, bla_{SHV}) (Mohammad et al., 2014; Hasman et al., 2005; Mulvey et al., 2003; Umadevi et al., 2011), carbapenemases genes (bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{SPM}, bla_{KPC}) (Monteiro et al., 2012; Gales et al., 2003; Juan et al., 2008), and RNA methylase genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA*) (Berçot et al., 2011) were investigated by PCR. For sequencing bla_{KPC} gene was used the primers: F- ATGTCACTGTATCGCCSTC and R-TTACTGCCCSKTGACGCC (1011 pb) Chromosomal mutations in *oprD, gyrA* and *mexT* genes (positive regulator of the MexEF-OprN efflux pump) were investigated by PCR and sequencing in selected isolates according to the clonal and resistance profiles. For *oprD* were used the primers: 603F – GGGGTTCATCGAAGACAGCA, 2321R – GAGGAGTCA GCAGGCAATG (1718 pb) for amplification and sequencing and internal primers for sequencing: 1054F- GCCGACCACCGTCAAATCG and 1891R- CGTTGCCGCCAAGAAGAAA). For *mexT* were used the primers F – CGATCGCCTGGACACGCACC and R – GCGCGCGATCGATTTTCCCG (1311 pb) and for *gyrA,* primers described by Giraud et al. (1999).

DNA was extracted from fresh bacterial colonies using an AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen Scientific, Union city, CA, USA). The PCR mixture was prepared in a final volume of 25 μL, containing 2 μL of DNA, 9 μL of MilliQ water, 0.75 μL (20 pmoles) of each primer and 12.5 μL of the ReadyMix ™ Reaction JumpStart ™ Mix REDTaq® (Sigma). Reaction conditions for *oprD* and *mexT* were: 94 °C for 5 min; 30 cycles at 94 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min; followed by 72 °C for 10 min. The amino acid sequences were compared to the amino acid sequence of the *P. aeruginosa* PAO1 strain (Genebank accession number: GCA_000006765.1) using the software Geneious R9. The PROVEAN software was used to predict whether a mutation was neutral or deleterious to protein (http://provean.jcvi.org/index.php).

2.4. Pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) analysis

Molecular typing was performed by pulsed-field gel electrophoresis (PFGE). Genomic DNA was fragmented by restriction endonuclease *SpeI* (Thermo Scientific) (Rojo-Bezares et al., 2011). The PFGE results were visually assessed, and a dendrogram was generated by unweighted pair group method using arithmetic average (UPGMA) clustering in BioNumerics (versão 6.6, Applied Maths, KortrijK, Belgium). The isolates were grouped into clonal groups if the Dice coefficient was > 85%, while patterns with no differences in fragments were considered to be of the same Pulsotype (Tenover et al., 1997).

Multilocus Sequence Typing (MLST) analysis was performed on the representative isolates of most frequent PFGE clonal groups (> 3 isolates) and for isolates carrying carbapenemase genes. Standard DNA amplification and sequencing of seven housekeeping genes (*acsA*, *aroE*, *guaA, mutL, nuoD, ppsA* and *trpE)* were performed as described previously (Curran et al., 2004*)*. The allele sequences and sequence types (ST) were identified at https://pubmlst.org/paeruginosa.

3. Results

3.1. Antimicrobial susceptibility tests

The disk diffusion test showed > 60% of isolates were non-susceptible to doripenem (73.8%), amikacin (71.5%), meropenem (66%), gentamicin (66%), cefepime (64.7%), imipenem (63.6%), ciprofloxacin (62.5%), levofloxacin (62.5%), ceftazidime (60.3%), Aztreonam (56.9%) and Piperacillin / Tazobactam (55.7%). The lowest susceptibility rates were observed for ticarcillin / clavulanic acid (6,8%). MIC for imipenem ranged from 0.5 to ≥32 μg / mL (MIC_{50} of 2 μg / mL and MIC_{90} of ≥32 μg / mL). MIC for colistin ranged 0,5 to ≥256 μg / mL (MIC_{50} of 0.5 μg / mL and MIC_{90} of 2 μg / mL) and resistance was observed in 3 isolates (3.5%) from 4th period. The phenotypes observed was Resistant (35,3%), MDR (31,8%), XDR (28,4%) and susceptible (4,5%). Over the four studied periods, the rates of antimicrobial resistance increased for all antimicrobials, mainly for carbapenems, aminoglycosides and fluoroquinolones. The XDR rate and MIC for imipenem also incresead over the years (Table 1).

3.2. Distribution of resistance determinants

Among the acquired resistance genes surveyed, the ESBL genes were found in 6 isolates: bla_{TEM} was detected in 2 isolates from 3rd and 4th periods, bla_{SHV} in 3 isolates from 2nd period (n = 2) and 3rd period (n = 1) and bla_{CTX-M} was detected in 1 isolate from 4th period. Among the carbapenemase genes tested, 8 isolates were positives. The bla_{SPM} gene was detected in 3 isolates from 2007, 2008 and 2010 (3rd period) and the bla_{KPC-2} was detected in 5 isolates from 2015 (4th period). Imipenem hydrolysis was detectable in these 8 isolates producing carbapenemase by Carba NP test. The *rmtD* gene was found in 5 aminoglycoside-resistant strains from 3rd period. Two aminoglycoside-resistant strains were also SPM-positive. From all isolates, 24 were analysed for *oprD* gene mutations (15 resistant to imipenem, IPM-R, and 9 susceptible, IPM−S). Two IPM-R isolates (MIC ≥32 μg/mL) and one IPM-S isolate (MIC = 1,5 μg/mL) had the OprD amino acid sequence identical to *P. aeruginosa* PAO1 (wild type-WT). Six IPM-S isolates from 1th period and 1 IPM-S isolate from 2nd period showed silent mutations in its *oprD* sequence. IPM-R isolates had *oprD* gene inactivating

Table 1
Resistance profile of *P. aeruginosa* isolates over the years.

		Characteristics of isolates over the study periods			
		1st period (n = 22)	2nd period (n = 11)	3rd period (n = 39)	4th period (n = 16)
% non-susceptibility	AMI	4,5	27,3	28,2	58,8
	GEN	4,5	54,5	33,3	58,8
	IPM	4,5	18,2	35,9	94,1
	MER	4,5	0	35,9	94,1
	DOR	0	0	30,8	70,6
	CAZ	45,5	9,1	30,8	76,5
	FEP	36,4	0	23,1	88,2
	CIP	4,5	45,5	38,5	70,6
	LEV	4,5	45,5	38,5	70,6
	TIM	90,9	100	89,7	100
	TZP	50	36,4	51,3	88,2
	ATM	59,1	45,5	43,9	94,1
	COL	0	0	0	18.7
AR classification	MDR	54,4	36,4	23	18,75
	XDR	0	18,2	25,6	81,25
MIC	IPM MIC_{50}	1,5 μg/mL	2,0 μg/mL	≥ 32 μg/mL	≥ 32 μg/mL
	Colistin MIC_{50}	0,5 μg/mL	1 μg/mL	0,5 μg/mL	1 μg/mL
Resistance genes (n)	bla_{SHV}	–	2	1	–
	bla_{TEM}	–	–	1	1
	bla_{CTX}	–	–	–	1
	bla_{SPM}	–	–	3	–
	bla_{KPC}	–	–	–	5
	*rmt*D	–	–	5	–
Mutations	*opr*D (n = 24)	PNU(n = 6) Del (n = 1) Ins (n = 1)	PNU(n = 1) WT (n = 1)	WT (n = 2) Del (n = 7) Ins (n = 3)	Del (n = 2)
	*mex*T (n = 27)	nfxC(n = 2)	nfxC(n = 6)	nfxC(n = 9) PNU(n = 1)	nfxC(n = 9)
	*gyr*A (n = 27)	WT (n = 1)	T83I(n = 2) WT (n = 1)	T83I(n = 9) WT (n = 2)	T83I(n = 10) WT (n = 2)

AMI – Amikacin; GEN – Gentamicin; IPM – Imipenem; MER – Meropenem; DOR – Doripenem; CAZ – Ceftazidime; FEP – Cefepime; CIP – Ciprofloxacin; LEV – Levofloxacin; TIM - Ticarcillin/Clavulanic acid; TZP - Piperacillin/Tazobactam; ATM – Aztreonam; MDR - Multidrug resistance; XDR - Extensively drug-resistant; COL – Colistin.
WT – Wild Type; PNU - single nucleotide polymorphisms; Del – Deletion; Ins – Insertion.

mutations: a frameshift produced by deletion (n = 8; 33%), followed by insertions (n = 4; 17%). Two IPM-S isolates also showed *oprD* deletion mutations (8%). Frameshift in *OprD* gene was predominant in MDR (45,83%) and XDR (37,5%) phenotypes. *mexT* gene mutations were investigated in 27 IMP-R and/or fluoroquinolones resistants (FLQ-R) isolates. Twenty-six isolates showed deletion of 8 nucleotides in *mexT* corresponding to the *nfxC* mutant present at all study periods. Quinolone resistance determining region (QRDR) of *gyrA* gene was evaluated in all ciprofloxacin-resistant isolates (n = 27). Twenty-one isolates from all periods showed a single Thr83Ile mutation (Table 1).

3.3. PFGE and MLST

Among all *P. aeruginosa* isolates, 7 were non-typable. The others were typed in 33 clonal groups by PFGE. Eleven clonal groups were represented by > 2 isolates being the most prevalent the clonal groups U/ST804 and J/ST277 found in 11 isolates each one. Most isolates belonging to these 11 clonal groups presented XDR or MDR phenotype (Fig. 1).

The bla_{SPM} and *rmt*D genes were detected in isolates belonging to J/ST277 and bla_{KPC} was found in clonal groups Q/ST1560 (n = 3) and clonal group P/ST1944 (n = 2). The bla_{TEM} was present in one isolate of D/ST244 and one of clonal group Gg/ST1117. The bla_{SHV} was detected in three different clonal groups: V, X and Y. The $bla_{CTX\text{-}M\text{-}like}$ was detected in an isolate belonging to clonal group D/ST244. Colistin-resistant isolates belonged to two prevalent clonal groups (D/ST244 and F/ST1791) and one isolate to clone R.

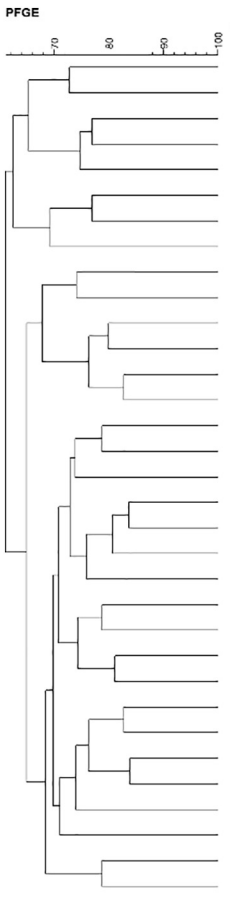
Nine clonal groups were found among 19 typable isolates from the 1th period (1995–1999). The clonal group U/ST804 was the most prevalent (n = 9) followed by I/ST487 (n = 3), Eight clonal groups were found among 11 isolates from 2nd period (2000–2005). Twenty-three clonal groups were found among 36 typable isolates from the 3nd period (2006–2010). Some clonal groups were previously detected in the first two periods such as Gg/ST1117 (n = 1), I/ST487 (n = 1), O/ST1860 (n = 1) and U/ST804 (n = 2). Furthermore, MLST analysis identified 2 high risk clones: J/ST277 (n = 8) and D/ST244 (n = 3). Finally, seven clonal groups were found among 15 typable isolates from the last period (2011–2015). The high risk clones (J/ST277 and D/ST244) were found in 3 and 2 isolates respectively (Fig. 1).

4. Discussion

The prevalence of *P. aeruginosa* with MDR and XDR phenotypes has increased over the years, which further limits therapeutic options (Morales et al., 2012; Oliver et al., 2015). These phenotypes have been associated with high morbidity, mortality and cost for several hospitals worldwide (Lautenbach et al., 2010). The prevalence and emergence of carbapenemases-producing *P. aeruginosa* have also contributed to increasing MDR and XDR phenotypes (Barrio-Tofiño et al., 2017).

Our data show progressive changes from MDR phenotypes into XDR in *P. aeruginosa* isolates recovered from Rio de Janeiro, Brazil over 21 years. Furthermore, phenotypes are associated with acquired carbapenemase (bla_{SPM} and bla_{KPC}) and RNA methylase genes, and mainly with chromosomal gene mutations (*oprD, mexT* and *gyrA*).

MDR *P. aeruginosa* harboring $bla_{KPC\text{-}2}$ has increased worldwide in the last years, including in Brazil. KPC-producing *P. aeruginosa* have been described in Brazil since 2010 in Recife, Paraná (2010, 2011 and 2012), São Paulo (2012) and Minas Gerais (2014), associated to



PFGE	ST	1995-1999 (n)	2000-2005 (n)	2006-2010 (n)	2011-2015 (n)	No isolates
Gg	1117		R (2)	R (1)		3
Cc				R (1)		1
G			R (1)	XDR (1)		2
Bb				R (1)		1
Ee				MDR (1)		1
A				R (2)		2
Aa				S (1); MDR (1)		2
X				MDR (1)		1
C				R (1)		1
R				R (1)	XDR (1)	2
Y			MDR (1)	R (1)		2
Q	1560				XDR (3)	3
Dd			R (1)			1
Z		MDR (1)				1
N				R (1)		1
H		R (1)		R (1)		2
E	1945		R (1)		XDR (2)	3
D	244			R (1); MDR (1); XDR (1)	MDR (1); XDR (1)	5
J	277			R (1); MDR (1); XDR (6)	MDR (1); XDR (2)	11
I	487	S (2); R (1)		R (1)		4
T		R (1)				1
F	1791			XDR (1)	XDR (2)	3
M				S (1)		1
O	1860	R (1)	XDR (2)	MDR (1)		4
Ff				R (1)		1
S		R (1)				1
U	804	R (2); MDR (7)		R (2)		11
V			MDR (1)			1
W	1602	MDR (1)	MDR (2)			3
P	1944				MDR (1); XDR (1)	2
B				R (1)		1
L				MDR (2)		2
K		MDR (1)				1

Fig. 1. Prevalence of *Pseudomonas aeruginosa* clones over time.
S – susceptible; R – resistant; MDR - Multidrug resistance; XDR - Extensively drug-resistant.

different STs (Jácome et al., 2012; Carrara et al., 2015; Rizek et al., 2014a, 2014b; Paula-Petroli et al., 2018; de Oliveira Santos et al., 2018). Here, we found 5 KPC-producing *P. aeruginosa* from the 4nd period (2015) belonging to 2 different clonal groups.

SPM-1 is particularly widespread in Brazil. It was described in 2002 in São Paulo from an isolate only susceptible to polymyxins and this phenotype remains to the present day in different Brazillian states (Gales et al., 2003). We only detected the presence of bla_{SPM} in three isolates from the 3nd period. However, previous studies showed the presence of this gene since 1999 in Rio de Janeiro (Pellegrino et al., 2002; Carvalho et al., 2006).

Polymyxins (polymyxin B and colistin) are the therapeutical choice for MDR and XDR *P. aeruginosa* infections. Resistance to these antimicrobials in *P. aruginosa* is not common in Brazil. Rossi et al. (2017) described the resistance to polymyxin in 0,9% *Pseudomonas* spp. isolates from 2010 to 2014 in nine hospitals from São Paulo, Brazil. A recent study have desribed a polymyxin-resistant *P. aeruginosa* ST245 in São Paulo, Brazil (Orsi et al., 2019). In the present study, we found three colistin-resistant isolates (3,5%) from 2011 ($n = 2$) and 2014 ($n = 1$) associated to three different clonal groups but we draw attention to the presence of this resistance in a isolate belonged to the high-risk clone ST244.

Despite a great clonal diversity, we showed the ST277 as one the most frequent clone in *P. aeruginosa* isolates from the 3nd and the 4nd periods. Our data suggest that ST277 clone continues to be endemic in Rio de Janeiro and are in accordance with previous Brazilian publications (Cacci et al., 2016). The clone ST277 has also been described as responsible for bla_{SPM} dissemination within the Brazilian territory and later detected in Switzerland and the United Kingdom (Hopkins et al., 2016; Salabi et al., 2010). Furthermore, 16 s RNA methylase - RmtD, conferring high levels of aminoglycoside resistance has been described in this clone (Doi et al., 2007).

Although we found high-risk clones (ST277 and ST244) prevalent in recent years other less frequent STs were also associated with XDR phenotypes, like ST1791, ST1995, ST1117, drawing attention to the emergence and spreading of new high-risk clones and the ability of STs to emerge as MDR or XDR (Silva et al., 2017). This can be explained by the high genetic recombination in this species, guaranteeing an unrelated population structure and alert to the important participation of selective pressure by the use of antimicrobials in increasing resistance in *P. aeruginosa* (Oliver et al., 2015). Corroborating for this hypotesis, the mutational resistance mechanisms, such as *oprD* alteration, efflux pump overexpression and *gyr*A alteration, were predominant in XDR strains, including epidemic clones ST277 and ST244.

5. Conclusions

Hence, we show the change in *P. aeruginosa* epidemiology over 21 years in Rio de Janeiro, with increased resistance (including colistin-resistance) and the emergence of high-risk clones in the last years, as well as a polyclonal population. The main resistance mechanisms were chromosomal mutations such as mutation in *oprD*, efflux pumps and *gyrA*, emphasizing the continuous need for surveillance strategies and control measures of *P. aeruginosa* infection independent of the production of carbapenemase, since other resistance mechanisms are contributing to the MDR or XDR phenotype found in the different institutions of Rio de Janeiro. Our results help to provide an understanding of the evolution and spread of MDR and XDR-*P. aeruginosa* isolates in Brazil.

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