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High prevalence of atypical virulotype and genetically diverse background among Pseudomonas aeruginosa isolates from a referral hospital in the Brazilian Amazon

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Full Title: High prevalence of atypical virulotype and genetically diverse background among Pseudomonas aeruginosa isolates from a referral hospital in the Brazilian Amazon

Short Title: Virulence, antimicrobial resistance and genotyping of <i>Pseudomonas aeruginosa</i>

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Abstract: *Pseudomonas aeruginosa* is an opportunistic pathogen causing different types of infections, particularly in intensive care unit patients. Characteristics that favor its persistence artificial environments are related to its high adaptability, wide arsenal of virulence factors and resistance to several antimicrobials classes. Among the several virulence determinants, T3SS stands as the most important due to the clinical impact of *exoS* and *exoU* genes in patient's outcome. The molecular characterization of *P. aeruginosa* isolates helps in the comprehension of transmission dynamics and enhance knowledge of virulence and resistance roles in infection process. In the present study, we investigated virulence and resistance properties and the genetic background of *P. aeruginosa* isolated from ICUs patients at a referral hospital in Brazilian Amazon. A total of 54 *P. aeruginosa* isolates were characterized by detecting 19 virulence-related genes, antimicrobial susceptibility testing, molecular detection of β-lactamase-encoding genes and genotyping by MLST and rep-PCR. Our findings showed high prevalence of virulence-related markers, where 53.7% of the isolates presented at least 17 genes among the 19 investigated (P =0.01). The rare *exoS* + /*exoU* + cytotoxic virulotype was detected in 55.6% of isolates. Antimicrobial susceptibility testing revealed percentages of antibiotic resistance above 50% to carbapenems, cephalosporins and fluoroquinolones associated to MDR/XDR isolates. Isolates harboring both *bla* SPM-1 and *bla* OXA genes were also detected. Genotyping methods demonstrated a wide genetic diversity of strains spread among the different intensive care units, circulation of international MDR/XDR high-risk clones (ST111, ST235, ST244 and ST277) and emergence of seven novel MLST lineages. Finally, our findings highlight the spread of strains with high virulence potential and resistance to antimicrobials and may be useful on comprehension of pathogenicity process, treatment guidance and establishment of strategies to control the spread of epidemic *P. aeruginosa* strains.

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1 # High prevalence of atypical virulotype and genetically diverse background among *Pseudomonas aeruginosa* isolates from a referral hospital in the Brazilian Amazon

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30 # Abstract

31 *Pseudomonas aeruginosa* is an opportunistic pathogen causing different types of
32 infections, particularly in intensive care unit patients. Characteristics that favor its
33 persistence artificial environments are related to its high adaptability, wide arsenal of
34 virulence factors and resistance to several antimicrobials classes. Among the several
35 virulence determinants, T3SS stands as the most important due to the clinical impact of
36 *exoS* and *exoU* genes in patient's outcome. The molecular characterization of *P.*
37 *aeruginosa* isolates helps in the comprehension of transmission dynamics and enhance
38 knowledge of virulence and resistance roles in infection process. In the present study, we
39 investigated virulence and resistance properties and the genetic background of *P.*
40 *aeruginosa* isolated from ICUs patients at a referral hospital in Brazilian Amazon. A total
41 of 54 *P. aeruginosa* isolates were characterized by detecting 19 virulence-related genes,
42 antimicrobial susceptibility testing, molecular detection of β-lactamase-encoding genes
43 and genotyping by MLST and rep-PCR. Our findings showed high prevalence of
44 virulence-related markers, where 53.7% of the isolates presented at least 17 genes among
45 the 19 investigated (P =0.01). The rare $exoS^{+}/exoU^{+}$ cytotoxic virulotype was detected in
46 55.6% of isolates. Antimicrobial susceptibility testing revealed percentages of antibiotic
47 resistance above 50% to carbapenems, cephalosporins and fluoroquinolones associated
48 to MDR/XDR isolates. Isolates harboring both $bla_{\text{SPM-1}}$ and bla_{OXA} genes were also
49 detected. Genotyping methods demonstrated a wide genetic diversity of strains spread
50 among the different intensive care units, circulation of international MDR/XDR high-risk
51 clones (ST111, ST235, ST244 and ST277) and emergence of seven novel MLST lineages.
52 Finally, our findings highlight the spread of strains with high virulence potential and
53 resistance to antimicrobials and may be useful on comprehension of pathogenicity

54 process, treatment guidance and establishment of strategies to control the spread of
55 epidemic *P. aeruginosa* strains.

56 Introduction

57 *Pseudomonas aeruginosa* is an opportunistic pathogen, causing a wide spectrum of
58 infections, including acute and chronic respiratory tract infections (RTIs) and
59 bloodstream infections (BSIs) as the main infection sites [1–4]. It is estimated that *P.*
60 *aeruginosa* is responsible to about 10% of all hospital-acquired-infections worldwide and
61 associated to outbreaks in adult, pediatric and neonatal intensive care units (ICUs) due to
62 the spread of multi-drug resistant (MDR) or extensively drug-resistant (XDR) and highly
63 virulent *P. aeruginosa* strains, negatively impacting morbidity, mortality, length of stay
64 and treatment costs of patients [5–7]. *P. aeruginosa* high adaptability, resistance to
65 several antimicrobials classes and secretion of various virulence factors allows its
66 persistence in artificial settings of high selective pressure, such as hospitals [8–10].

67 Pathogenicity in *P. aeruginosa* is multifactorial, relying on the regulation of virulence-
68 related genes and expression of their respective factors, including adhesins, exotoxins,
69 proteases and pigments. Virulence products may be passively secreted from bacterial cell
70 or actively secreted via secretion systems, such as type I secretion system (T1SS), type II
71 secretion system (T2SS) and type III secretion system (T3SS) [11–13]. T3SS is the most
72 important and well characterized virulence determinant in *P. aeruginosa*, which allows
73 the translocation of effector cytotoxic proteins into the host cell via a nano-syringe. So
74 far, only four effector exotoxins encoded by the *exoS, exoU, exoT* and *exoY* genes have
75 been identified, which are variably present and expressed by *P. aeruginosa* strains. ExoS
76 and ExoU are associated to invasive and cytotoxic phenotypes, respectively, and rarely

77 concomitant detected, while ExoT and ExoY demonstrate few cytotoxic effects and are
78 encoded by most of strains [8, 14, 15, 16, 17]. Furthermore, survey of cytotoxic/*exoU*$^{+}$
79 virulotype has been highly recommended due to the impact of this exotoxin in patient's
80 mortality, especially in isolates from high risk settings, such as ICUs [19, 18].

81 The increasing trend of resistance to anti-pseudomonal antibiotics, especially
82 carbapenems, aminoglycosides and fluoroquinolones has contributed to the emergence of
83 *P. aeruginosa* MDR/XDR strains, posing a challenge to infection treatment [20–22].
84 Among the several resistance mechanisms in *P. aeruginosa*, the role of transferable
85 resistance determinants is overwhelming, particularly those encoding classes A and D
86 extended-spectrum β-lactamases (ESBLs) and class B metallo-β-lactamases (MBLs) [23–
87 26].

88 Molecular typing of in-hospital isolates of *P. aeruginosa* contribute to the understanding
89 of transmission and infection dynamics by strains exhibiting high degree of virulence and
90 resistance, allowing the determination of genetic relationships between isolates,
91 outbreaks identification, population structure studies and therapeutic management [27–
92 29]. In spite of the polyclonal population structure of *P.* aeruginosa, several studies have
93 shown a globally spread of strains denominated as high-risk clones (HRC), which are
94 associated with most MDR/XDR isolates and specific virulence markers. Thus,
95 characterization of *P. aeruginosa* isolates must be performed combining clonality
96 evaluation with methods that can identify specific virulence and resistance determinants
97 [19, 23, 31, 32].

98 The molecular characterization of local *P. aeruginosa* isolates is one of the first steps to
99 understand the epidemiology of the pathogen at local and global level, helping to establish

100 surveillance strategies, reduce risk of outbreaks and enhance knowledge of virulence and
101 resistance roles in infection process. Nonetheless, comprehensive epidemiological
102 investigations on molecular level are still scarce, especially ones reporting data from
103 Brazilian territory. In the present study we report in-depth data into the virulence,
104 resistance properties and genetic diversity of in-patient ICU isolates from a referral
105 hospital in the Brazilian Amazon region.

106 Material and Methods

107 Bacterial isolates

108 In this retrospective cross-sectional study, a total of 54 non-repetitive *P. aeruginosa*
109 isolates stored in the Bacteriology and Mycology Section of the Evandro Chagas Institute
110 were evaluated. Isolates were previously recovered of various clinical sources of patients
111 hospitalized in different ICUs at a referral hospital, from January 2010 to June 2013 (S1
112 Table). Identification of isolates was performed on automated VITEK-2 system
113 (bioMérieux, Marcy l'Etoile, France) and complemented by phenotypical and
114 biochemical assays such as observation of colony morphology, Gram stain, oxidase test,
115 absence of carbohydrate fermentation on triple sugar iron agar, oxidative behavior on
116 Hugh Leifson medium and the cytochrome oxidase reaction.

117 DNA extraction and molecular detection of virulence-related
118 genes

119 Genomic DNA was obtained from a single colony of overnight cultures for each *P.*
120 *aeruginosa* isolate using UltraClean^{TM} Microbial DNA Isolation kit (MoBio

121 Laboratories, Carlsbad, CA, USA), following manufacturer's recommendations and
122 quantified using the Picodrop PICO100 spectrophotometer (Picodrop Limited, Hinxton,
123 UK). Adjusted DNA concentrations between 25ng – 50ng/µl were used for all subsequent
124 molecular assays.

125 The detection of 19 virulence-related markers was performed by PCR: adhesion (*algU*
126 and *algD*), T1SS (*aprA*), T2SS (*lasA*, *lasB* and *toxA*), T3SS (*exoS*, *exoU*, *exoT* and *exoY*),
127 oxidative stress (*phzI*, *phzII*, *phzM*, *phzS* and *phzH*) and *quorum sensing* (QS)/regulation
128 (*lasI*, *lasR*, *rhlL* and *rhlR*), using previously described primers and reaction parameters
129 with slightly modifications [33–38]. Each PCR mixture was prepared in a final volume
130 of 25µl, consisted of 1x PCR buffer (Qiagen, Hilden, Germany), 1x Q-solution (Qiagen,
131 Hilden, Germany), 2mM of $MgCl_2$ (Qiagen, Hilden, Germany), 200 µM of each dNTP
132 (Invitrogen™, São Paulo, Brazil), 0,5µM of each primer, 1U of HotStart Taq DNA
133 polymerase and DNA template. Amplifications were performed on Veriti thermocycler
134 (Applied Biosystems, Foster City, CA, USA), under the following conditions for all
135 genes: 95ºC for 15 minutes; followed by 35 cycles at 95ºC for 1 minute, 60ºC for 45
136 seconds, 72ºC for 1 minute and a final extension step at 72ºC for 7 minutes. PCR products
137 were subjected to electrophoresis on 1.5% agarose gel and amplicons visualized under
138 ultraviolet light. *P. aeruginosa* ATCC 27853 and PA14 strains were used as positive
139 control.

140 Antimicrobial susceptibility assays and definition of resistance 141 phenotypes

142 Antimicrobial susceptibility testing determined the Minimum Inhibitory Concentration
143 (MICs) by the broth microdilution method for six antimicrobial classes: cephalosporins

144 (cefepime – FEP), carbapenems (imipenem – IMP and meropenem – MER), penicilins +
145 beta-lactamase inhibitor (piperacillin+tazobactam – TZP), aminoglycosides (gentamicin
146 – GN), fluoroquinolones (ciprofloxacin – CIP) and polypeptides (polymyxin B – POL).
147 All assays were performed in accordance to the Clinical and Laboratory Standards
148 Institute guidelines and breakpoints [39] and isolates were classified as sensitive (S),
149 intermediate (I) and resistant (R). Reference strains *Escherichia coli* ATCC 25922 and *P.*
150 *aeruginosa* ATCC 27853 were used as quality control. For statistical analysis, isolates
151 exhibiting resistant and intermediate susceptibility phenotypes were grouped as 'non-
152 susceptible'. Isolates were phenotypically classified as MultiS if susceptible to all tested
153 antimicrobial classes; Moderately resistant (ModR) if resistant to $\geq$1 drug in <3
154 antimicrobial classes; MDR if resistant to $\geq$1 drug in $\geq$3 antimicrobial classes and XDR
155 if non-susceptible to 1 agent in all but $\leq$2 tested categories, according to previously
156 described criteria [31, 40].

157 Molecular detection of antimicrobial resistance markers

158 The detection of β-lactamase-encoding genes, including ESBLs (*bla*$_{CTX\text{-}M1}$, *bla*$_{OXA\text{-}2}$,
159 *bla*$_{OXA\text{-}10}$) and carbapenemases (*bla*$_{IMP\text{-}1}$, *bla*$_{IMP\text{-}2}$, *bla*$_{VIM\text{-}1}$, *bla*$_{VIM\text{-}2}$, *bla*$_{SPM\text{-}1}$, *bla*$_{NDM\text{-}1}$ and
160 *bla*$_{KPC\text{-}1}$) was performed by PCR on Veriti thermocycler (Applied Biosystem, Foster City,
161 CA, USA) as previously described [41–47]. PCR products were subjected to
162 electrophoresis on 1.5% agarose gel and amplicons visualized under ultraviolet light.

163 Molecular typing based on MLST

164 MLST genotyping was performed according to protocol described by Curran et al. (2004)
165 [48]. The seven housekeeping genes (*acsA, aroE, guaA, mutL, nuoD, ppsA,* and *trpE*)
166 were amplified by PCR in a Veriti thermocycler (Applied Biosystems, Foster City, CA,

167 USA). Reaction products were bidirectionally sequenced using Big Dye Terminator v3.1
168 chemistry on ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA,
169 USA). Obtained results were compared to available data at PubMLST database
170 (http://pubmlst.org/paeruginosa) to determine allelic profiles and sequence types (STs).
171 Novel alleles and STs were submitted to PubMLST database for validation. PHYLOViZ
172 2.0 platform was used for data management and analysis of clonal complexes (CCs),
173 which were defined by related ST clusters exhibiting variation in a single locus (single
174 locus variants - SLV) or in two loci (double locus variants - DLV) [49].

175 Molecular typing based on rep-PCR

176 The genetic relatedness of isolates was investigated by semi-automated rep-PCR on
177 DiversiLab™ Strain Typing System (bioMérieux, Marcy-L'Étoile, France) using the
178 DiversiLab *P. aeruginosa* kit (Bacterial Barcodes, bioMérieux, Marcy-L'Étoile, France),
179 according to the manufacturer's instructions. Fingerprints were obtained by
180 electrophoresis using microfluidic lab-on-a-chip on Agilent 2100 Bioanalyzer equipment
181 (Agilent Technologies, Palo Alto, CA, USA) and analysis performed with DiversiLab on-
182 line software (v 3.4) applying the Pearson correlation coefficient. Isolates were classified
183 as in the same clonal group (genotypically indistinguishable) if the similarity was ≥97%,
184 and as unique pattern if the similarity was <97%.

185 Statistical analysis

186 The G-test of independence or Fisher's exact test was applied to verify the association
187 between resistance, virulence markers, isolation sites, clonal groups and STs;
188 standardized residuals and adjusted residuals was used as a post-hoc test after a
189 statistically significant G-test of independence. The distribution of virulence-related

190 genes among isolates was verified by the Lilliefors test. Values of $P \leq 0.05$ were
191 considered statistically significant. All analyzes were performed using the statistical
192 software BioEstat® 5.4 [50].

193 Ethics statement

194 No samples were collected for this study. Only stored samples were included without any
195 contact and possibility of identifying the respective patients. Prior sampling and the
196 present study were conducted in accordance with Helsinki Declaration and the Brazilian
197 National Health Council [51] and with approval of the ethics committee at Fundação
198 Santa Casa de Misericórdia do Pará (referral hospital under study) (Nº CAAE
199 0086.0.440.000-10).

200 Results

201 Bacterial isolates and distribution of virulence-related genes

202 This study evaluated 54 *P. aeruginosa* isolates distributed in the adult ICU (AICU, 28/54
203 –51.8%), followed by pediatric ICU (PICU, 14/54 – 26.0%) and neonatal ICU (NICU,
204 12/54 – 22.2%). Regarding clinical origin, isolates were obtained from RTI (23/54 –
205 42.6%), BSI (14/54 – 25.9%) and others various sources (17/54 –31.5%), including
206 catheter (n= 6), rectal swab (n=3), surgical wound (n=3), ocular secretion (n=2), gastric
207 secretion (n=1), urethral secretion (n=1), urine (n=1) (S1 Table).

208 Evaluated isolates harbored at least five virulence-related genes and seven isolates
209 harbored all 19 investigated genes. In addition, a heterogeneous distribution of genes was
210 observed, where 53.7% of the isolates presented at least 17 genes among the 19
211 investigated (P =0.01). The lowest detection frequencies were observed for *lasA* (21/54

212 – 38.9%) and *algU* (25/54 – 46.3%) genes, while *lasB, exoS, rhlL* and *rhlR* genes were
213 detected in all isolates (54/54 – 100%) (S1 Fig and S1 Table). Definition of virulotypes
214 was based on the detection of T3SS *exoS/exoU* genes, where it was observed the presence
215 of invasive/cytotoxic (*exoS*$^{+}$/*exoU*$^{+}$; 30/54 – 55.6%) and invasive (*exoS*$^{+}$/*exoU*$^{-}$; 24/54 –
216 44.4%) virulotypes. *exoS*$^{+}$/*exoU*$^{+}$ virulotype and *phzH* gene were detected with
217 significantly lower frequency at NICU (P =0.0461 and p= 0.0491, respectively), and
218 among isolates from BSI (P =0.0027 and P =0.0244, respectively), as well as *exoY* gene
219 (P =0.0435) (Tables 1 and 2).

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Table 1. Distribution of virulence-related genes according to patient's ICUs.

Related function	Gene	AICU *n* = 28	PICU n = 14	NICU *n* = 12	*P*-value*
Adhesion	*algU*	12	7	6	0.8758
	algD	28	13	12	0.4601
T1SS	*aprA*	21	10	6	0.3204
T2SS	*lasA*	9	7	5	0.5379
	lasB	28	14	12	1.000
	toxA	23	13	11	0.9329
T3SS	$exoS^{+}/exoU^{+}$	17	10	3[a]	0.0461*
	$exoS^{+}/exoU^{-}$	11	4	9	
	exoT	21	12	6	0.1371
	exoY	23	12	6	0.0863
Oxidative stress	*phzI*	27	13	11	0.8346
	phzII	21	11	5	0.0959
	phzM	22	12	7	0.2775
	phzS	20	12	5	0.0581
	phzH	21	12	5[a]	0.0491*
QS/regulation	*lasI*	26	13	12	0.5386
	lasR	26	12	12	0.3334
	rhlL	28	14	12	1.000
	rhlR	28	14	12	1.000

T1SS, type I secretion system; T2SS, type II secretion system; T3SS, type III secretion system; QS, *quorum sensing*; AICU, adult intensive care unit; PICU, pediatric intensive care unit; NICU, neonatal intensive care unit.

* *P* values were calculated using the G-test of independence. [a] Frequency lower than expected at random.

244 **Table 2. Distribution of virulence-related genes according to the *P. aeruginosa***
245 **isolates clinical sources.**

Related function	Gene	RTI $n = 23$	BSI $n = 14$	Other $n = 17$	*P*-valor*
Adhesion	*algU*	12	6	7	0.7617
	algD	23	14	16	0.5003
T1SS	*aprA*	15	7	15	0.0618
T2SS	*lasA*	7	5	9	0.3569
	lasB	23	14	17	1.000
	toxA	21	13	15	0.9132
T3SS	$exoS^{+}/exoU^{+}$	13	3[a]	14	0.0027*
	$exoS^{+}/exoU^{-}$	10	11	3	
	exoT	17	7	15	0.0670
	exoY	19	7[a]	15	0.0435*
Oxidative stress	*phzI*	23	13	15	0.2276
	phzII	17	7	13	0.2489
	phzM	18	8	15	0.1449
	phzS	17	6	14	0.0596
	phzH	17	6[a]	15	0.0244*
QS/regulation	*lasI*	21	13	17	0.3815
	lasR	21	13	16	0.9518
	rhlL	23	14	17	1.000
	rhlR	23	14	17	1.000

246 T1SS, type I secretion system; T2SS, type II secretion system; T3SS, type III secretion system;
247 RTI, respiratory tract infection; BSI, bloodstream infection; Other: catheter (n= 6), rectal swab
248 (n=3), surgical wound (n=3), ocular secretion (n=2), gastric secretion (n=1), urethral secretion
249 (n=1), urine (n=1).

250 * *P* values were calculated using the G-test of independence. [a] Frequency lower than expected at
251 random.

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Antimicrobial susceptibility features

Antimicrobial susceptibility testing revealed that isolates were mainly non-susceptible to carbapenems (IMP and MER; 36/54 – 66.7%), followed by FEP (27/54 – 50.0%), CIP (27/54 – 50.0%), GN (24/54 – 44.4%) and TZP (17/54 – 31.5%). All isolates were susceptible to POL (S1 Table). According to phenotypical classification, most of the isolates were classified as ModR (23/54 – 42.6%), followed by XDR (16/54 – 29.6%), MultiS (9/54 – 16.7%) and MDR (6/54 – 11.1%). MDR/XDR were significantly predominant in the AICU (Table 3) (*P* = 0.0003). There was no significant association between the presence of T3SS virulotypes and antimicrobial resistance (Table 4), where 33.3% (18/30) *exoU*$^{+}$ were classified as MultiS or ModR.

Table 3. Distribution of *P. aeruginosa* isolates according to susceptibility phenotype among the ICUs of in reference hospital.

	MultiS/ModR	**MDR/XDR**	***P*-value***
ICU			
AICU	11	17	0.0003
PICU	9	5	
NICU	12	0	

ICU, intensive care unit; AICU, adult intensive care unit; PICU, pediatric intensive care unit; NICU, neonatal intensive care unit; MultiS, susceptible to all tested antibiotics; ModR, moderately resistant; MDR, multi-drug resistant; XDR, extensively drug-resistant.
* *P* value was calculated using the G-test of independence.

273 **Table 4. Distribution of T3SS virulotypes according to antimicrobial susceptibility**
274 **of *P. aeruginosa* isolates.**

	***exoS*⁺/*exoU*⁺** $n = 30$	***exoS*⁺ /*exoU*⁻** $n = 24$	***P*-value***
Antimicrobial resistance			
FEP	15	12	1.000
IMP	21	15	0.5770
MER	15	11	0.7905
TZP	9	8	1.000
GN	13	11	1.000
CIP	14	13	0.7846
Susceptibility phenotype			
MultiS	3	6	0.2702
ModR	15	8	0.2738
MDR	5	1	0.2100
XDR	7	9	0.3695
MDR+XDR	12	10	1.000

275 FEP, cefepime; IMP, imipenem; MER, meropenem; TZP, piperacillin+tazobactam; GN,
276 gentamicin; CIP, ciprofloxacin; POL, polymyxin B; MultiS, susceptible to all tested antibiotics;
277 ModR, moderately resistant; MDR, multi-drug resistant; XDR, extensively drug-resistant.
278 * *P* values were calculated using the Fisher's exact test.

279 Regarding molecular survey of antimicrobial resistance genes, 20.4% (11/54) of *P.*
280 *aeruginosa* isolates harbored $bla_{\text{CTX-M1}}$, followed by $bla_{\text{SPM-1}}$ (5/54 – 9.2%), $bla_{\text{OXA-2}}$ (3/54
281 – 5.5%) and $bla_{\text{OXA-10}}$ (3/54 – 5.5%). Two isolates harbored both $bla_{\text{SPM-1}}$ and $bla_{\text{OXA-2}}$ and
282 $bla_{\text{SPM-1}}$ and $bla_{\text{OXA-10}}$ genes. One isolate harbored $bla_{\text{OXA-2}}$ gene and one isolate harbored
283 $bla_{\text{OXA-10}}$. Four isolates harboring $bla_{\text{SPM-1}}$ genes and seven isolates harboring $bla_{\text{CTX-M1}}$
284 were classified as XDR. The bla_{IMP}, bla_{VIM}, bla_{NDM} and bla_{KPC} genes were not detected.

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287 # Molecular typing based on MLST

288 MLST genotyping revealed a highly diverse genetic background with the presence of 22
289 different STs, including seven novel STs (ST2524, ST2541, ST2552, ST2554, ST2555,
290 ST 2556 and ST2603) and 15 STs previously reported (ST111, ST170, ST235, ST244,
291 ST277, ST360, ST463, ST500, ST508, ST1076, ST1197, ST1284, ST1655, ST2100 and
292 ST2437). Novel allele 218/*aroE* was associated to ST2603. Genetic relationship analysis
293 demonstrated the presence founders STs of four HRC CC, including CC/ST111 (2/22 –
294 9.0%), CC/ST235 (5/22 – 22.7%), CC/ST244 (9/22 – 40.9%) and CC/ST277 (6/22 –
295 27.2%). Among the seven novel STs, ST 2554 emerged as DLV of CC/ST274, which is
296 a HRC; ST 2555 as SLV of ST 316 and the other five STs (ST2524, ST2541, ST2552,
297 ST 2556 and ST2603) were singletons.

298 Among the isolates, 40.7% (22/54) were associated to HRC and 59.3% (32/54) to non-
299 HRC. HRC were significantly predominant in the AICU (*P* =0.0100) (Table 5), especially
300 ST244 (8/28 – 28.5%). Although no significant association between MLST genotypes
301 and T3SS virulotypes was observed, 50% (11/22) of HRC presented $exoS^{+}/exoU^{+}$
302 virulotype (*P* =0.5820) (Table 5). Non-susceptibility to FEP, IMP, MER, GN, CIP ($P <$
303 0.001) and TZP (*P* =0.0198) was predominant among HRC strains (Table 5). All 16 XDR
304 isolates were associated to the detected HRC ($P < 0.0001$) (Table 5) and only one MDR
305 isolate belonged to non-HCR (ST2524); whereas all ModR isolates were associated to
306 non-HRC ($P < 0.0001$) (Table 5) and only one MultiS isolate belonged to HCR (ST244)
307 (Fig 1). Five isolates harboring bla_{SPM-1}, *bla*OXA$_{-2}$ and bla_{OXA-10} genes belonged to HRC
308 ST277 and one isolate harboring only *bla*OXA$_{-2}$ was associated to non-HRC ST508;

309 while isolates harboring *bla*$_{CTX-M1}$ belonged to HRC ST111, ST244, ST253 (10/11) and
310 to non-HRC ST1284 (1/11).

311 **Fig 1. Minimum spanning tree of the 54 *P. aeruginosa* isolates from the referral**
312 **hospital in the Brazilian Amazon genotyped by MLST.**

313 Each circle represents a different ST and the size of the circle is proportional to the number of
314 isolates related to the respective ST. Each of the 4 susceptibility phenotypes is represented by a
315 different color. Dotted lines represent multilocus variants. The graph shows the percentages of
316 HRC associated with a particular susceptibility phenotype.

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329 **Table 5. Distribution of HRC and non-HRC according to ICUs, T3SS virulotypes**
330 **and antimicrobial susceptibility of *P. aeruginosa* isolates.**

	HRC ***n* = 22**	**non-HRC** ***n* = 32**	***P*-value***
ICU			
AICU	16[a]	12[b]	
PICU	5	9	0.0100
NICU	1[b]	11[a]	
T3SS Virulotype			
exoS+/exoU+	11	19	0.5820
exoS+/exoU-	11	13	
Antimicrobial resistance			
FEP	18	9	0.0002
IMP	20	16	0.0027
MER	17	9	0.0007
TZP	11	6	0.0198
GN	20	4	< 0.0001
CIP	21	6	< 0.0001
Susceptibility phenotype			
MultiS	1	8	0.0676
ModR	0	23	< 0.0001
MultiS+ModR	1	31	< 0.0001
MDR	5	1	0.0706
XDR	16	0	< 0.0001
MDR+XDR	21	1	< 0.0001

331 HRC, high-risk clones; ICU, intensive care unit; AICU, adult intensive care unit; PICU, pediatric
332 intensive care unit; NICU, neonatal intensive care unit; T3SS, type III secretion system; FEP,
333 cefepime; IMP, imipenem; MER, meropenem; TZP, piperacillin+tazobactam; GN, gentamicin;
334 CIP, ciprofloxacin; POL, polymyxin B; MultiS, susceptible to all tested antibiotics; ModR,
335 moderately resistant; MDR, multi-drug resistant; XDR, extensively drug-resistant.
336 * *P* values were calculated using the G-test of independence or Fisher's exact test.
337 [a] Frequency higher than expected at random. [b] Frequency lower than expected at random.

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Molecular typing based on rep-PCR

340 Genotyping by rep-PCR on DiversiLab™ System revealed 36 distinct fingerprint
341 patterns, of which 26 were unique patterns and ten clonal groups comprised of two to
342 eight isolates (Fig 2). Strains associated to clonal groups were found circulating in
343 different ICUs, however, no statistically significant association was observed between
344 clonality and ICUs (P =0.5063) (Table 6). The frequency of virulotypes was similar
345 between the different clonal patterns defined by DiversiLab™ (P =1.000) (Table 6).
346 MDR/XDR isolates were found in higher proportion among clonal groups strains (P
347 =0.0141) (Table 6). Isolates harboring bla_{SPM-1}, blaOXA$_{-2}$ and bla_{OXA-10} genes were
348 related to clonal groups A, B and C/ST277, while bla_{CTX-M1} gene was distributed among
349 clonal groups (D, E, I and H) and unrelated strains (Fig 2). Genetic diversity observed
350 through MLST genotyping presented a good association with fingerprint diversity found
351 by rep-PCR, of which seven clonal groups were associated to HRC, but without reaching
352 significant association (P =0.0573) (Table 6).

353 **Fig 2. Dendrogram of genetic similarity of the 54 *P. aeruginosa* isolates from the**
354 **referral hospital in the Brazilian Amazon genotyped by rep-PCR in the**
355 **DiversiLab™ System.**

356 * Novel STs. ST, sequence type; ICU, intensive care unit; AICU, adult intensive care unit; PICU,
357 pediatric intensive care unit; NICU, neonatal intensive care unit; RTI, respiratory tract infection;
358 BSI, bloodstream infection; T3SS, type III secretion system; MultiS, susceptible to all tested
359 antibiotics; ModR, moderately resistant; MDR, multi-drug resistant; XDR, extensively drug-
360 resistant.

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363 **Table 6. Distribution of rep-PCR patterns according ICUs, T3SS virulotypes and**
364 **antimicrobial susceptibility of *P. aeruginosa* isolates.**

	Clonal Group $n = 28$	**Unique patterns** $n = 26$	***P*-valor***
ICU			
AICU	14	14	
PICU	9	5	0.5063
NICU	5	7	
T3SS Virulotype			
exoS+/exoU+	16	14	1.000
exoS+/exoU-	12	12	
Susceptibility phenotype			
MultiS	3	6	0.2863
ModR	9	14	0.1683
MultiS+ModR	12	20	0.0141
MDR	5	1	0.1938
XDR	11	5	0.1410
MDR+XDR	16	6	0.0141
MLST			
HRC	15	13	0.0573
non-HRC	7	19	

365 AICU, adult intensive care unit; PICU, pediatric intensive care unit; NICU, neonatal intensive
366 care unit; T3SS, type III secretion system; MultiS, susceptible to all tested antibiotics; ModR,
367 moderately resistant; MDR, multi-drug resistant; XDR, extensively drug-resistant; HRC, high-
368 risk clones.
369 * *P* values were calculated using the G-test of independence or Fisher's exact test.

370 Discussion

371 *P. aeruginosa* is currently one of the most relevant opportunistic pathogens causing acute
372 infections, particularly among patients admitted to ICUs [5–6]. The occurrence of
373 infection episodes and mortality in ICUs is significantly higher compared to other hospital
374 wards; this scenario is justified by the complexity of the patients' clinical condition, such

375 as immunosuppression and pre-existing diseases, use of invasive diagnostic and
376 therapeutic maneuvers, as well as, the spread of epidemic and highly virulent strains of
377 *P. aeruginosa* [2, 4, 23, 32]. *P. aeruginosa* isolates included in our study were mainly
378 recovered from RTI (42.6%) and BSI (25.9%) of patients admitted to different ICUs,
379 similarly as previously reported infections frequencies, which varied between 9% to 35%
380 in adult ICUs, reaching 62% in neonatal and pediatric ICUs and with RTI and BSI as
381 major clinical sources [2, 3, 4, 6, 52, 53].

382 Although considered an opportunistic pathogen, the myriad of virulence products of *P.*
383 *aeruginosa* may severally impact patients' clinical condition. The combination of multiple
384 genes and virulence factors tends to affect pathogenesis and determine the outcome of an
385 infectious process, and depending on the location and type of infection, the importance
386 and role of a given factor may be different [10, 38, 54]. The detection of virulence-related
387 markers revealed a high prevalence of genes, with detection of at least five genes per
388 isolate and 17 of the 19 genes investigated in more than 50% of the isolates (*P* =0.01),
389 demonstrating a high virulence potential of our strains (S1 Fig). Although our findings
390 are consistent with several other reports that indicates a high prevalence and conserved
391 nature of the *P. aeruginosa* genome regarding the presence of virulence-related genes,
392 variations in virulence patterns are observed in isolates from different geographic areas
393 and settings, highlighting the need to investigate different markers among distinct *P.*
394 *aeruginosa* populations [1, 13, 23, 35, 55, 56].

395 The *algD* gene was detected in 98.1% of isolates, while absence of *algU* was noted in
396 more than 53.0%. These findings differ from those reported by Hassuna et al (2020) [1]
397 and Fazeli et al (2014) [57], who observed higher frequencies for *algU* and lower
398 frequencies for *algD*, respectively. As both genes encode fundamental proteins for

alginate biosynthesis, functioning as one of its main adhesins and mainly found in the respiratory tract of patients with acute infections and cystic fibrosis (CF), the low frequency of the *algU* may be associated to the high frequency of non-RTI isolates (57.4%) and also indicate deficient alginate production by most of our isolates [10, 58].

The high prevalence of the *aprA* gene is in line with other studies [1, 13, 56, 59], highlighting the importance of zinc-metalloprotease secreted by T1SS, which has functions related to invasion, causing collagen and fibrinogen degradation in synergy with T2SS elastases, and evasion of the immune system through the inactivation of several cytokines, contributing to bacterial survival [60–61]. Three proteases - two elastases (LasA and LasB) and an exotoxin (ExoA) - secreted by *P. aeruginosa* are constituents of T2SS. The detection frequencies of 87.0% for *toxA* and 100.0% for *lasB,* in addition to the absence *lasA* among more than 60% our strains are in agreement with several other studies [1, 13, 56, 57, 62, 63]. Elastases act synergistically and have high elastolytic capacity mainly in blood vessels and lung tissues, while ExoA inhibits host protein synthesis leading to cell death [64–65]. Thus, our results reinforce the hypothesis of a significantly higher prevalence of *lasB* over *lasA* in *P. aeruginosa* clinical isolates [37, 66].

The phenazine compounds encoded by the *phzI, phzII, phzH, phzM* and *phzS* genes have siderophoric activity, cause an increase in oxidative stress and mitochondrial inactivation [10, 67]. Interestingly, Bradbury et al (2010) [13] correlated a high prevalence of these genes to *P. aeruginosa* clinical isolates, disagreeing with the low prevalence reported among our strains, and mainly of *phzH* gene, which absence was significant among isolates from BSI (*P* =0.0244) at NICU (*P* =0.0491) (Tables 1 and 2).

422 The QS genes were detected at frequencies above 92.0%, however, four isolates did not
423 harbor *lasR* gene and three isolates *lasI* gene. The QS system in *P. aeruginosa* is
424 coordinated by the *las* and *rhl* systems that function hierarchically for bacterial survival
425 in the face of environmental changes, biofilm formation and control of several other
426 virulence factors [10, 12]. Although a decrease in the ability to express virulence factors
427 has been reported due to the absence and/or mutations in genes related to such QS, this
428 does not necessarily compromise the ability to cause infection due to regulation mediated
429 by the *rhl* system [12, 68, 69] Our results are in agreement with a study carried out by
430 Senturk et al. (2012) [12] and Ruiz-Roldán et al. (2018) [56]; however, disagreeing with
431 Aboushleib et al. (2015) [69], who reported gene deficiency in more than 60% in clinical
432 isolates.

433 T3SS is the most important virulence marker in *P. aeruginosa*. The *exoS, exoU, exoT* and
434 *exoY* genes encode toxins which are injected directly into the cytosol of the host cells and
435 have functions related to anti-phagocytosis, necrotic and cytotoxic damage by a
436 phospholipase, prevention of healing process and edema formation, respectively [10, 16,
437 54]. T3SS genes are variably present in isolates of *P. aeruginosa*, being the chromosomal
438 genes *exoS, exoT* and *exoY* distributed among almost all strains [16, 18, 23]. T3SS *exoS,*
439 *exoT* and *exoY* genes were detected in 100%, 72.2% and 75.9%, respectively. *exoS* results
440 are in accordance with the hypothesis of a wider distribution of this gene due to its
441 chromosomal nature. Nevertheless, the fact of in nearly 30% of our isolates *exoT* and
442 *exoY* genes were not detected and a significant absence of *exoY* gene among BSI isolates
443 (P =0.0435) (Table 2) demonstrate a contrary trend to the hypothesis of their universal
444 distribution among *P. aeruginosa* strains, as well as, several studies that rarely report
445 absence of these genes [13, 15, 16, 55, 56, 70].

446 The *exoU* gene is found less frequently due to its presence being associated to the PAPI-
447 2 in the accessory genome of *P. aeruginosa*, however, its presence and the secretion of
448 its respective protein are markers of very highly cytotoxic phenotype and associated with
449 several types of acute infections, antimicrobial resistance phenotype, polymicrobial
450 infections and early mortality individuals [18, 31, 71, 72]. Moreover, *exoS/exoU* genes
451 tend to be mutually exclusive, being observed a higher prevalence of the invasive
452 virulotype (*exoS*$^{+}$/*exoU*$^{-}$) among *P. aeruginosa* strains worldwide [16, 19, 23, 59].
453 Interestingly, 55.6% of our isolates presented the invasive/cytotoxic virulotype
454 (*exoS*$^{+}$/*exoU*$^{+}$). Highlighting the rare occurrence of this phenomenon, there are only a few
455 studies reporting a high proportion of this virulotype among *P. aeruginosa* hospital
456 isolates [15, 35, 55]. We also observed a significant absence of *exoS*$^{+}$/*exoU*$^{+}$ virulotype
457 among BSI isolates (p= 0.0027) and in the NICU (p= 0.0461), ward in which occurred
458 the majority of BSI episodes among admitted patients (10/12 – 83.3%) (Tables 1 and 2),
459 contradicting the important association established by several authors between the
460 presence of *exoS*$^{+}$/*exoU*$^{+}$ virulotype among BSI isolates [18, 55, 73, 74]. Despite reports,
461 the concomitant presence of genes still is not well understood, considering: (I) absence
462 of a direct relationship between the genes, where *exoS, exoT* and *exoY* are found in the
463 core genome of *P. aeruginosa*, while *exoU* is found associated to the PAPI-2 in the
464 accessory genome; (II) the impact on bacterial fitness due to the presence of both genes,
465 which would stimulate the loss of one of them; and (III) the production of both toxins
466 could generate a higher immune response, making the pathogen less capable of
467 establishing infection due to its high immunogenicity [13, 16, 19, 23, 71].

468 Our findings revealed that more of 66% of our isolates were resistant to carbapenems,
469 followed by high resistance to cephalosporins, fluoroquinolones and aminoglycosides.

Moreover, 40.7% of our isolates were classified as MDR or XDR, which were significantly predominant in the AICU (*P* =0.0003) (Table 3) and associated to genetically related clonal groups (*P* =0.0141) (Table 6). For instance, our findings agree with recent data reported by Santos et al. (2019) [20], which also showed an increasing trend on resistance carbapenems, aminoglycosides and fluoroquinolones associated to spread of *P. aeruginosa* MDR/XDR strains over 21 years' period in Rio de Janeiro, Brazil. Others studies conducted in Brazil and Latin America also revealed high resistance rates to cephalosporins, fluoroquinolones and aminoglycosides [75–78]. These findings emphasize the growing global trend on antibiotic resistance been reported by several studies and rises concern, especially in Latin America countries, where carbapenems still remains as the main choice for the treatment of infections by MDR *P. aeruginosa* [21, 22, 23, 79, 80]. A reduced number of transmissible resistance markers were detected among our isolates, with *bla*$_{CTX\text{-}M1}$ (20.4% – 11/54) as the most frequent, followed by *bla*$_{SPM\text{-}1}$ (5/54 – 9.2%), *bla*$_{OXA\text{-}2}$ (3/54 – 5.5%) and *bla*$_{OXA\text{-}10}$ (3/54 – 5.5%). Our data demonstrate the predomination and wide spread of SPM-1 MDR/XDR strains in Brazilian hospitals and with the increasing transmission trend of ESBLs CTX-M and OXA variants genes worldwide, as previously reported [20, 23, 45, 76, 81, 82]. Finally, our results highlight the impact of horizontally acquired resistance mechanisms, mainly ESBLs and class B MBL, in the increasing resistance rates and its commonly association to MDR/XDR *P. aeruginosa* strains [18, 21, 23, 79].

The interaction between virulence and antibiotic susceptibility has been subject of several studies, suggesting that these relationships are antagonistic, since the presence of resistance mechanisms can determine a compromising biological cost for bacterial virulence and vice versa. In fact, the acquisition of resistance genes through the

494 incorporation of mobile genetic elements in the bacterial chromosome can lead to the
495 inactivation of genes involved in the production of some virulence factors and decreased
496 cytotoxicity [23, 72, 74, 83]. Nevertheless, other authors suggest that *exoU*$^{+}$ virulotype is
497 associated resistance to fluoroquinolones and carbapenems, pointing out that the
498 relationship between virulence and resistance may also occur in a synergistic sense,
499 especially in high antibiotic pressure settings, such as ICUs [9, 13, 15, 16, 84]. In spite of
500 that, our data do not suggest this association, as the presence of T3SS virulotypes was not
501 related to any antimicrobial class or resistance phenotype (Table 4). Yet, 40% of
502 *exoS*$^{+}$/*exoU*$^{+}$ isolates were associated to MDR/XDR phenotype, rising concern as a high
503 prevalence of *exoS*$^{+}$/*exoU*$^{+}$ could facilitate the virulotype transmission to other
504 MDR/XDR isolates, worsening the clinical scenario in the growing trend dissemination
505 highly virulent MDR/XDR strains.

506 MLST revealed the presence of 22 different STs, including four different HRC (ST111,
507 ST235, ST244 and ST277), seven newly identified STs and a novel allele related to *aroE*
508 gene, demonstrating the high genetic diversity and an absence of relationship among most
509 of our studied *P. aeruginosa* strains. Such findings are also in line with those obtained by
510 rep-PCR genotyping, which revealed the presence of 36 fingerprints, 26 of which are
511 unrelated (Figs 1 and 2). This high genetic diversity may be explained as the study site is
512 a regional reference hospital for several specialties. It is likely that inter-hospital transit
513 patients and/or who spend short periods in the hospital environment, as well as healthcare
514 professionals, carry isolates and insert them in the hospital environment, and
515 consequently causing infections in ICU patients because of numerous risk factors, such
516 as immunosuppression conditions and virulence potential of isolates. Our data also
517 corroborate the hypothesis of epidemic non-clonal population structure of *P. aeruginosa*,

518 indicating the dispersion of global HRC in hospitals settings and the continuous
519 description of new *P. aeruginosa* STs worldwide [15, 23, 55, 56, 76, 85, 86].

520 Contrary to the predominance in other studies, HRC were associated with only 40% of
521 our isolates [18, 23]. However, such strains demonstrate significantly association
522 MDR/XDR phenotypes, carriage of resistance markers and persistence in ICUs (Table 5).
523 ST111 has been detected in all continents [23, 31] and in our study was associated to
524 clonal group I, composed by two MDR/XDR from different periods, of which only one
525 harboring was *bla*$_{CTX-M1}$ gene, suggesting the latter acquisition of this marker (Fig 2).
526 ST235 is the most widely spread *P. aeruginosa* HRC associated MDR/XDR isolates and
527 carriage various resistance markers [23, 87]. Of the five ST235 isolates were MDR/XDR,
528 of which three comprised the clonal group D, dispersed in different ICUs and carrying
529 the gene *bla*$_{CTX-M1}$ gene, and two unrelated isolates, of which one also harboring *bla*$_{CTX-}$
530 $_{M1}$ gene (Fig 2). Kos et al. (2015) [88] has also detected the presence of CTX-M β-
531 lactamase in Brazilian ST235 isolates. ST244 also has a worldwide distribution, being
532 associated to carriage of different resistance markers, but not always related to
533 MDR/XDR isolates [23, 88, 89, 90]. High frequencies were also reported by Brazilian
534 studies evaluating clinical and environmental isolates [20, 76, 91]. ST244 was the most
535 prevalent HRC among our strains, being predominantly detected in AICU and mostly
536 related to MDR/XDR isolates, except for one isolate presenting MultiS phenotype. Two
537 of nine isolates comprised clonal group H/ST244, where *bla*$_{CTX-M1}$ was shared by both
538 isolates and two comprised clonal group I/ST244; the other five isolates were genetically
539 unrelated and three harbored *bla*$_{CTX-M1}$ gene (Fig 2). Endemic clone in Brazil, ST277 is
540 frequently associated to MDR/XDR isolates and dissemination of *bla*$_{SPM-1}$ gene [20, 23,
541 92]. Interestingly, isolates comprising clonal group A/ST277 shared *bla*$_{SPM-1}$, but

harbored distinct *bla*$_{\text{OXA}}$ genes; one isolate of clonal group B/ST277 harbored both *bla*$_{\text{SPM-1}}$ and *bla*$_{\text{OXA-10}}$ genes, while the other harbored only *bla*$_{\text{OXA-10}}$ gene; isolates comprising clonal group C/ST277 shared *bla*$_{\text{SPM-1}}$, but only one isolate harbored *bla*$_{\text{OXA-2}}$ (Fig 2). To our knowledge this is first report in Brazil of *P. aeruginosa* ST277 harboring both *bla*$_{\text{SPM-1}}$ and *bla*$_{\text{OXA}}$ variants genes.

Most of our isolates were identified as non-HCR, which among them non-resistant phenotypes (MultiS and ModR) were significantly predominant (P <0.0001) (Table 5), demonstrating that susceptible isolates are associated to higher clonal diversity [31]. Clonal group E/ST1284 comprised three isolates, which curiously, the only isolate harboring *bla*$_{\text{CTX-M1}}$ was negative for *exoU* gene, suggesting a reduction in virulence due to acquisition of a resistance marker (Fig 2). ST508 isolate was the only non-HRC harboring a resistance gene (*bla*OXA$_{\text{-2}}$) (Fig 2). Novel ST2554 was identified as DLV of HRC ST274, belonging to CC274. High-risk CC/ST274 strains have been recently detected in Indonesian ICUs, but are still predominant among CF patients, demonstrating high occurrence of mutation in genes related to antibiotic resistance [93, 94, 95]. ST2554 isolate presented a ModR phenotype and *exoS*$^{+}$/*exoU*$^{+}$ virulotype, also indicating acquisition of PAPI-2 containing *exoU* gene during divergence from ST274 (Fig 2). In spite of the non-detection of ST274 among our isolates, it may not rule out the presence of this genotype in the hospital, as we observe a more direct genetic relationship between both strains.

Certain clonal strains are expected to be linked to T3SS virulotypes. Recent phylogenetic analysis by Sawa et al. 2020 [19] attributed *exoU*+ virulotype to ST253, possibly explaining the highest virulence potential of ST235 observed clinical studies [18, 23]. Also, Sanchez-Diener et al. (2017) [72] on animal model based study showed that lower

566 virulence was linked to XDR profiles, which are typically found among HRC. However,
567 different virulence potentials were observed among HRC, being higher for ST111 and
568 ST235 and lower for ST175. Our MLST lineages were not significantly associated to
569 specific T3SS virulotypes (*P* =0.5820) (Table 5), however, we observed the presence of
570 *exoS*$^+$/*exoU*$^+$ virulotype among the four detected HRC. Moreover, we observed isolates
571 HRC ST244 and non-HRC ST508 harboring *exoS*$^+$/*exoU*$^+$ virulotype, while they were
572 expected to be linked the invasive *exoS*$^+$ virulotype as recently reported [19]. Therefore,
573 our findings highlight the importance of virulence genotyping and suggest the local
574 acquisition of PAPI-2 containing *exoU* gene by HRC, which could worsen the local
575 clinical scenario as most of HCR were also MDR/XDR.

576 The elevated virulence and resistance potential presented by our isolates might negatively
577 affect patient's outcomes, however the present study did not perform associations to
578 clinical and epidemiological data due to unavailability of patients records. Moreover, we
579 did not perform phenotypic tests evaluating secretion/expression of virulence factors such
580 as elastases, proteases, T3SS exotoxins, QS molecules and the sequencing of the
581 virulence genes, thus preventing a better establishment of genotype-phenotype
582 relationships. Finally, the non-characterization of hospital environment isolates may have
583 underestimated the genetic background in the study site, also impairing analysis of
584 transmission from in-hospital environmental reservoirs. Studies comprising a larger
585 number of clinical and environmental isolates from the study site and from other hospitals
586 are highly recommended in order to better establish *P. aeruginosa* transmission in the
587 region, as well as, relationships between of virulence, antibiotic susceptibility and genetic
588 diversity.

589

Conclusions

To conclude, this is the first study deeply exploring virulence, antibiotic resistance and genetic background of *P. aeruginosa* isolates from ICUs at specialized hospital in the Brazilian Amazon region. Herein, we reported a high prevalence of virulence-related markers, particularly of the rare *exoS*$^{+}$/*exoU*$^{+}$ virulotype, which may be associated to a high virulence and cytotoxic potential and transmission of genetic elements containing *exoU* gene among local isolates. It was also observed high percentages of resistance to different classes of antibiotics, particularly to carbapenems, in addition to MDR/XDR isolates harboring resistance genetic elements, alerting to a possible dissemination of these mechanisms and of isolates acting as resistance reservoirs. The importance genotyping by rep-PCR and MLST techniques in the surveillance of in-hospital isolates was noteworthy, which demonstrated circulation of MDR/XDR HRC clones, emergence of novel genetic lineages and a wide genetic diversity of strains circulating in different ICUs. Finally, our findings may be useful on comprehension of complex mechanisms of pathogenicity process, treatment guidance and establishment of strategies to control the spread of epidemic *P. aeruginosa* strains with a high lethality potential.

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The present study will be presented by Yan Corrêa Rodrigues to obtain his Ph.D. within the doctoral programme ‘Parasitic biology in Amazon Region’ at the State University of Pará, Brazil.

612 # References

613 1. Hassuna NA, Mandour SA, Mohamed ES. Virulence Constitution of Multi-Drug-
614 Resistant *Pseudomonas aeruginosa* in Upper Egypt. Infect Drug Resist. 2020;13:587-
615 95.
616 2. Braga IA, Campos PA, Gontijo-Filho PP, Ribas RM. Multi-hospital point prevalence
617 study of healthcare-associated infections in 28 adult intensive care units in Brazil. J
618 Hosp Infect. 2018;99(3):318-24.
619 3. Berezin EN, Solorzano F, Latin America Working Group on Bacterial R. Gram-
620 negative infections in pediatric and neonatal intensive care units of Latin America. J
621 Infect Dev Ctries. 2014;8(8):942-53.
622 4. Luna CM, Rodriguez-Noriega E, Bavestrello L, Guzman-Blanco M. Gram-negative
623 infections in adult intensive care units of latin america and the Caribbean. Crit Care
624 Res Pract. 2014;2014:480463.
625 5. Haque M, Sartelli M, McKimm J, Abu Bakar M. Health care-associated infections -
626 an overview. Infect Drug Resist. 2018;11:2321-33.
627 6. Russotto V, Cortegiani A, Graziano G, Saporito L, Raineri SM, Mammina C, et al.
628 Bloodstream infections in intensive care unit patients: distribution and antibiotic
629 resistance of bacteria. Infect Drug Resist. 2015;8:287-96.
630 7. Suarez C, Pena C, Tubau F, Gavalda L, Manzur A, Dominguez MA, et al. Clinical
631 impact of imipenem-resistant *Pseudomonas aeruginosa* bloodstream infections. J
632 Infect. 2009;58(4):285-90.
633 8. Rodrigues YC, Sarges ESNF, Conceição ML, Matos ECO, Gomes NJP, Quaresma
634 AJG, Lima KVB. Infecções por *Pseudomonas aeruginosa*: aspectos clínicos,

635 microbiológicos e moleculares. In: Salgado YCS, organizer. Patologia das doenças.
636 4th ed. Ponta Grossa (PR): Atena Editora; 2018. pp. 53-69. Portuguese.

637 9. Cho HH, Kwon KC, Kim S, Koo SH. Correlation between virulence genotype and
638 fluoroquinolone resistance in carbapenem-resistant *Pseudomonas aeruginosa*. Ann
639 Lab Med. 2014;34(4):286-92.

640 10. Gellatly SL, Hancock RE. *Pseudomonas aeruginosa*: new insights into pathogenesis
641 and host defenses. Pathog Dis. 2013;67(3):159-73.

642 11. Jabalameli F, Mirsalehian A, Khoramian B, Aligholi M, Khoramrooz SS, Asadollahi
643 P, et al. Evaluation of biofilm production and characterization of genes encoding type
644 III secretion system among *Pseudomonas aeruginosa* isolated from burn patients.
645 Burns. 2012;38(8):1192-7.

646 12. Senturk S, Ulusoy S, Bosgelmez-Tinaz G, Yagci A. Quorum sensing and virulence
647 of *Pseudomonas aeruginosa* during urinary tract infections. J Infect Dev Ctries.
648 2012;6(6):501-7.

649 13. Bradbury RS, Roddam LF, Merritt A, Reid DW, Champion AC. Virulence gene
650 distribution in clinical, nosocomial and environmental isolates of *Pseudomonas*
651 *aeruginosa*. J Med Microbiol. 2010;59(Pt 8):881-90.

652 14. Lombardi C, Tolchard J, Bouillot S, Signor L, Gebus C, Liebl D, et al. Structural and
653 Functional Characterization of the Type Three Secretion System (T3SS) Needle of
654 *Pseudomonas aeruginosa*. Front Microbiol. 2019;10:573.

655 15. Horna G, Amaro C, Palacios A, Guerra H, Ruiz J. High frequency of the
656 exoU+/exoS+ genotype associated with multidrug-resistant "high-risk clones" of
657 *Pseudomonas aeruginosa* clinical isolates from Peruvian hospitals. Sci Rep.
658 2019;9(1):10874.

659 16. Sawa T, Shimizu M, Moriyama K, Wiener-Kronish JP. Association between
660 *Pseudomonas aeruginosa* type III secretion, antibiotic resistance, and clinical
661 outcome: a review. Crit Care. 2014;18(6):668.
662 17. Hauser AR. The type III secretion system of *Pseudomonas aeruginosa*: infection by
663 injection. Nat Rev Microbiol. 2009;7(9):654-65.
664 18. Pena C, Cabot G, Gomez-Zorrilla S, Zamorano L, Ocampo-Sosa A, Murillas J, et al.
665 Influence of virulence genotype and resistance profile in the mortality of
666 *Pseudomonas aeruginosa* bloodstream infections. Clin Infect Dis. 2015;60(4):539-
667 48.
668 19. Sawa T, Momiyama K, Mihara T, Kainuma A, Kinoshita M, Moriyama K. Molecular
669 epidemiology of clinically high-risk *Pseudomonas aeruginosa* strains: Practical
670 overview. Microbiol Immunol. 2020;64(5):331-44.
671 20. de Oliveira Santos IC, Pereira de Andrade NF, da Conceicao Neto OC, da Costa BS,
672 de Andrade Marques E, Rocha-de-Souza CM, et al. Epidemiology and antibiotic
673 resistance trends in clinical isolates of *Pseudomonas aeruginosa* from Rio de janeiro
674 - Brazil: Importance of mutational mechanisms over the years (1995-2015). Infect
675 Genet Evol. 2019;73:411-5.
676 21. Matos ECO, Andriolo RB, Rodrigues YC, Lima PDL, Carneiro I, Lima KVB.
677 Mortality in patients with multidrug-resistant *Pseudomonas aeruginosa* infections: a
678 meta-analysis. Rev Soc Bras Med Trop. 2018;51(4):415-20.
679 22. Hong DJ, Bae IK, Jang IH, Jeong SH, Kang HK, Lee K. Epidemiology and
680 Characteristics of Metallo-beta-Lactamase-Producing *Pseudomonas aeruginosa*.
681 Infect Chemother. 2015;47(2):81-97.

682 23. Oliver A, Mulet X, Lopez-Causape C, Juan C. The increasing threat of *Pseudomonas*
683 *aeruginosa* high-risk clones. Drug Resist Updat. 2015;21-22:41-59.
684 24. Yezli S, Shibl AM, Memish ZA. The molecular basis of beta-lactamase production in
685 Gram-negative bacteria from Saudi Arabia. J Med Microbiol. 2015;64(Pt 2):127-36.
686 25. Martins AF, Zavascki AP, Gaspareto PB, Barth AL. Dissemination of *Pseudomonas*
687 *aeruginosa* producing SPM-1-like and IMP-1-like metallo-beta-lactamases in
688 hospitals from southern Brazil. Infection. 2007;35(6):457-60.
689 26. Livermore DM. Multiple mechanisms of antimicrobial resistance in *Pseudomonas*
690 *aeruginosa*: our worst nightmare? Clin Infect Dis. 2002;34(5):634-40.
691 27. Liu H, Kong W, Yang W, Chen G, Liang H, Zhang Y. Multilocus sequence typing
692 and variations in the oprD gene of *Pseudomonas aeruginosa* isolated from a hospital
693 in China. Infect Drug Resist. 2018;11:45-54
694 28. Maâtallah M, Bakhrouf A, Habeeb MA, Turlej-Rogacka A, Iversen A, Pourcel C, et
695 al. Four genotyping schemes for phylogenetic analysis of *Pseudomonas aeruginosa*:
696 comparison of their congruence with multi-locus sequence typing. PLoS One. 2013
697 ;8(12):e82069. Erratum in: PLoS One. 2015;10(9):e0137931.
698 29. Achtman M. Evolution, population structure, and phylogeography of genetically
699 monomorphic bacterial pathogens. Annu Rev Microbiol. 2008;62:53-70.
700 30. Feil EJ, Enright MC. Analyses of clonality and the evolution of bacterial pathogens.
701 Curr Opin Microbiol. 2004;7(3):308-13.
702 31. Mulet X, Cabot G, Ocampo-Sosa AA, Dominguez MA, Zamorano L, Juan C, et al.
703 Biological markers of *Pseudomonas aeruginosa* epidemic high-risk clones.
704 Antimicrob Agents Chemother. 2013;57(11):5527-35.

705 32. Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the
706 role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol
707 Rev. 2011;35(5):736-55.
708 33. Wozniak DJ, Ohman DE. Transcriptional analysis of the *Pseudomonas aeruginosa*
709 genes algR, algB, and algD reveals a hierarchy of alginate gene expression which is
710 modulated by algT. J Bacteriol. 1994;176(19):6007-14.
711 34. Schurr MJ, Martin DW, Mudd MH, Deretic V. Gene cluster controlling conversion
712 to alginate-overproducing phenotype in *Pseudomonas aeruginosa*: functional
713 analysis in a heterologous host and role in the instability of mucoidy. J Bacteriol.
714 1994;176(11):3375-82.
715 35. Finnan S, Morrissey JP, O'Gara F, Boyd EF. Genome diversity of *Pseudomonas*
716 *aeruginosa* isolates from cystic fibrosis patients and the hospital environment. J Clin
717 Microbiol. 2004;42(12):5783-92.
718 36. Wolska K, Szweda P. Genetic features of clinical *Pseudomonas aeruginosa* strains.
719 Pol J Microbiol. 2009;58(3):255-60.
720 37. Zhu H, Conibear TC, Bandara R, Aliwarga Y, Stapleton F, Willcox MD. Type III
721 secretion system-associated toxins, proteases, serotypes, and antibiotic resistance of
722 *Pseudomonas aeruginosa* isolates associated with keratitis. Curr Eye Res.
723 2006;31(4):297-306.
724 38. Sabharwal N, Dhall S, Chhibber S, Harjai K. Molecular detection of virulence genes
725 as markers in *Pseudomonas aeruginosa* isolated from urinary tract infections. Int J
726 Mol Epidemiol Genet. 2014;5(3):125-34.

727 39. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial
728 Susceptibility Testing: Approved Twenty-: Document M100-S28. Wayne, PA, USA:
729 CLSI, 2018.
730 40. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al.
731 Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an
732 international expert proposal for interim standard definitions for acquired resistance.
733 Clin Microbiol Infect. 2012;18(3):268-81.
734 41. Bonnet R, Sampaio JL, Labia R, De Champs C, Sirot D, Chanal C, et al. A novel
735 CTX-M beta-lactamase (CTX-M-8) in cefotaxime-resistant Enterobacteriaceae
736 isolated in Brazil. Antimicrob Agents Chemother. 2000;44(7):1936-42.
737 42. Bert F, Branger C, Lambert-Zechovsky N. Identification of PSE and OXA beta-
738 lactamase genes in *Pseudomonas aeruginosa* using PCR-restriction fragment length
739 polymorphism. J Antimicrob Chemother. 2002;50(1):11-8.
740 43. Gonçalves DCPS, Lima ABM, Leão LSNO, Carmo Filho JR, Pimenta FC, Vieira
741 JOG. Detecção de metalo-beta-lactamase em *Pseudomonas aeruginosa* isoladas de
742 pacientes hospitalizados em Goiânia, Estado de Goiás. Rev Soc Bras Med Trop. 2009;
743 42:411-414. Portuguese.
744 44. Poirel L, Naas T, Nicolas D, Collet L, Bellais S, Cavallo JD, et al. Characterization
745 of VIM-2, a carbapenem-hydrolyzing metallo-beta-lactamase and its plasmid- and
746 integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France.
747 Antimicrob Agents Chemother. 2000;44(4):891-7.
748 45. Gales AC, Menezes LC, Silbert S, Sader HS. Dissemination in distinct Brazilian
749 regions of an epidemic carbapenem-resistant *Pseudomonas aeruginosa* producing
750 SPM metallo-beta-lactamase. J Antimicrob Chemother. 2003;52(4):699-702.

751 46. Nordmann P, Poirel L, Carrer A, Toleman MA, Walsh TR. How to detect NDM-1
752 producers. J Clin Microbiol. 2011;49(2):718-21.
753 47. Lomaestro BM, Tobin EH, Shang W, Gootz T. The spread of Klebsiella pneumoniae
754 carbapenemase-producing K. pneumoniae to upstate New York. Clin Infect Dis.
755 2006;43(3):e26-8.
756 48. Curran B, Jonas D, Grundmann H, Pitt T, Dowson CG. Development of a multilocus
757 sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. J
758 Clin Microbiol. 2004;42(12):5644-9.
759 49. Francisco AP, Vaz C, Monteiro PT, Melo-Cristino J, Ramirez M, Carrico JA.
760 PHYLOViZ: phylogenetic inference and data visualization for sequence based typing
761 methods. BMC Bioinformatics. 2012;13:87.
762 50. Ayres M, Ayres Júnior M, Ayres DL, Santos AS. BioEstat 5.0. Aplicações estatísticas
763 nas áreas das Ciências Bio-Médicas. Belém (PA): Instituto de Desenvolvimento
764 Sustentável Mamirauá; 2008. Portuguese.
765 51. Brasil, Conselho Nacional de Saúde. Resolução Nº 466, de 12 de dezembro de 2012.
766 Brasília. Avaliable from: http://conselho.saude.gov.br/resolucoes/2012/Reso466.pdf.
767 Acesso em 14.May.2020.
768 52. Matos EC, Matos HJ, Conceicao ML, Rodrigues YC, Carneiro IC, Lima KV. Clinical
769 and microbiological features of infections caused by *Pseudomonas aeruginosa* in
770 patients hospitalized in intensive care units. Rev Soc Bras Med Trop. 2016;49(3):305-
771 11.
772 53. Royer S, Faria AL, Seki LM, Chagas TP, Campos PA, Batistao DW, et al. Spread of
773 multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* clones in

774 patients with ventilator-associated pneumonia in an adult intensive care unit at a
775 university hospital. Braz J Infect Dis. 2015;19(4):350-7.
776 54. Kipnis E, Sawa T, Wiener-Kronish J. Targeting mechanisms of *Pseudomonas*
777 *aeruginosa* pathogenesis. Med Mal Infect. 2006;36(2):78-91.
778 55. Morales-Espinosa R, Delgado G, Espinosa LF, Isselo D, Méndez JL, Rodriguez C,
779 Miranda G, Cravioto A. Fingerprint Analysis and Identification of Strains ST309 as
780 a Potential High Risk Clone in a *Pseudomonas aeruginosa* Population Isolated from
781 Children with Bacteremia in Mexico City. Front Microbiol. 2017;8:313.
782 56. Ruiz-Roldan L, Belles A, Bueno J, Azcona-Gutierrez JM, Rojo-Bezares B, Torres C,
783 et al. *Pseudomonas aeruginosa* Isolates from Spanish Children: Occurrence in Faecal
784 Samples, Antimicrobial Resistance, Virulence, and Molecular Typing. Biomed Res
785 Int. 2018;2018:8060178.
786 57. Fazeli N, Momtaz H. Virulence Gene Profiles of Multidrug-Resistant *Pseudomonas*
787 *aeruginosa* Isolated From Iranian Hospital Infections. Iran Red Crescent Med J.
788 2014;16(10):e15722.
789 58. Stacey SD, Pritchett CL. *Pseudomonas aeruginosa* AlgU Contributes to
790 Posttranscriptional Activity by Increasing rsmA Expression in a mucA22 Strain. J
791 Bacteriol. 2016;198(13):1812-26.
792 59. Lomholt JA, Poulsen K, Kilian M. Epidemic population structure of *Pseudomonas*
793 *aeruginosa*: evidence for a clone that is pathogenic to the eye and that has a distinct
794 combination of virulence factors. Infect Immun. 2001;69(10):6284-95.
795 60. Ballok AE, O'Toole GA. Pouring salt on a wound: *Pseudomonas aeruginosa*
796 virulence factors alter Na+ and Cl- flux in the lung. J Bacteriol. 2013;195(18):4013-
797 9.

798 61. Laarman AJ, Bardoel BW, Ruyken M, Fernie J, Milder FJ, van Strijp JA, et al.
799 *Pseudomonas aeruginosa* alkaline protease blocks complement activation via the
800 classical and lectin pathways. J Immunol. 2012;188(1):386-93.
801 62. Mitov I, Strateva T, Markova B. Prevalence of virulence genes among bulgarian
802 nosocomial and cystic fibrosis isolates of *Pseudomonas aeruginosa*. Braz J Microbiol.
803 2010;41(3):588-95.
804 63. Najafi K, Kafil HS, Shokrian S, Azimi S, Asgharzadeh M, Yousefi M, et al. Virulence
805 genes and antibiotic resistance profile of *Pseudomonas aeruginosa* isolates in
806 Northwest of Iran. Journal of Pure and Applied Microbiology. 2015:(9):383- 389.
807 64. Matsumoto K. Role of bacterial proteases in pseudomonal and serratial keratitis. Biol
808 Chem. 2004;385(11):1007-16.
809 65. Du X, Youle RJ, FitzGerald DJ, Pastan I. Pseudomonas exotoxin A-mediated
810 apoptosis is Bak dependent and preceded by the degradation of Mcl-1. Mol Cell Biol.
811 2010;30(14):3444-52.
812 66. Finlayson EA, Brown PD. Comparison of antibiotic resistance and virulence factors
813 in pigmented and non-pigmented *Pseudomonas aeruginosa*. West Indian Med J.
814 2011;60(1):24-32.
815 67. Lau GW, Hassett DJ, Ran H, Kong F. The role of pyocyanin in *Pseudomonas*
816 *aeruginosa* infection. Trends Mol Med. 2004;10(12):599-606.
817 68. Dekimpe V, Deziel E. Revisiting the quorum-sensing hierarchy in *Pseudomonas*
818 *aeruginosa*: the transcriptional regulator RhlR regulates LasR-specific factors.
819 Microbiology. 2009;155(Pt 3):712-23.

820 69. Aboushleib HM, Omar HM, Abozahra R, Elsheredy A, Baraka K. Correlation of
821 quorum sensing and virulence factors in *Pseudomonas aeruginosa* isolates in Egypt.
822 J Infect Dev Ctries. 2015;9(10):1091-9.
823 70. Hu P, Chen J, Chen Y, Zhou T, Xu X, Pei X. Molecular epidemiology, resistance, and
824 virulence properties of *Pseudomonas aeruginosa* cross-colonization clonal isolates in
825 the non-outbreak setting. Infect Genet Evol. 2017;55:288-96.
826 71. Gomez-Zorrilla S, Juan C, Cabot G, Camoez M, Tubau F, Oliver A, et al. Impact of
827 multidrug resistance on the pathogenicity of *Pseudomonas aeruginosa*: in vitro and
828 in vivo studies. Int J Antimicrob Agents. 2016;47(5):368-74.
829 72. Sánchez-Diener I, Zamorano L, López-Causapé C, Cabot G, Mulet X, Peña C, et al.
830 Interplay among Resistance Profiles, High-Risk Clones, and Virulence in the
831 Caenorhabditis elegans *Pseudomonas aeruginosa* Infection Model. Antimicrob
832 Agents Chemother. 2017;61(12):e01586-17.
833 73. Pobiega M, Chmielarczyk A, Kozioł J, Pomorska-Wesolowska M, Ziolkowski G,
834 Romaniszyn D, et al. Virulence factors genes and drug resistance in *Pseudomonas*
835 *aeruginosa* strains derived from different forms of community and healthcare
836 associated infections. Postępy Higieny i Medycyny Doświadczalnej. 2018;72:751-9.
837 74. Recio R, Sanchez-Diener I, Viedma E, Melendez-Carmona MA, Villa J, Orellana
838 MA, et al. Pathogenic characteristics of *Pseudomonas aeruginosa* bacteraemia
839 isolates in a high-endemicity setting for ST175 and ST235 high-risk clones. Eur J Clin
840 Microbiol Infect Dis. 2020;39(4):671-8.
841 75. de Almeida Silva KCF, Calomino MA, Deutsch G, de Castilho SR, de Paula GR,
842 Esper LMR, et al. Molecular characterization of multidrug-resistant (MDR)
843 *Pseudomonas aeruginosa* isolated in a burn center. Burns. 2017;43(1):137-43.

844 76. Cacci LC, Chuster SG, Martins N, Carmo PR, Girão VB, Nouér SA, et al.
845 Mechanisms of carbapenem resistance in endemic *Pseudomonas aeruginosa* isolates
846 after an SPM-1 metallo-β-lactamase producing strain subsided in an intensive care
847 unit of a teaching hospital in Brazil. Mem Inst Oswaldo Cruz. 2016;111(9):551-8..
848 77. Sader HS, Jones RN, Gales AC, Silva JB, Pignatari AC; SENTRY Participants Group
849 (Latin America). SENTRY antimicrobial surveillance program report: Latin
850 American and Brazilian results for 1997 through 2001. Braz J Infect Dis. 2004
851 Feb;8(1):25-79.
852 78. Pellegrino FL, Teixeira LM, Carvalho Md Mda G, Aranha Nouer S, Pinto De Oliveira
853 M, Mello Sampaio JL, et al. Occurrence of a multidrug-resistant *Pseudomonas*
854 *aeruginosa* clone in different hospitals in Rio de Janeiro, Brazil. J Clin Microbiol.
855 2002;40(7):2420-4.
856 79. Labarca JA, Salles MJ, Seas C, Guzman-Blanco M. Carbapenem resistance in
857 *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in the nosocomial setting in
858 Latin America. Crit Rev Microbiol. 2016;42(2):276-92.
859 80. Hermes DM, Pormann Pitt C, Lutz L, Teixeira AB, Ribeiro VB, Netto B, et al.
860 Evaluation of heteroresistance to polymyxin B among carbapenem-susceptible and -
861 resistant *Pseudomonas aeruginosa*. J Med Microbiol. 2013;62(Pt 8):1184-1189.
862 81. Poirel L, Bonnin RA, Nordmann P. Genetic support and diversity of acquired
863 extended-spectrum beta-lactamases in Gram-negative rods. Infect Genet Evol.
864 2012;12(5):883-93.
865 82. Bradford PA. Extended-spectrum beta-lactamases in the 21st century:
866 characterization, epidemiology, and detection of this important resistance threat. Clin
867 Microbiol Rev. 2001;14(4):933-51.

868 83. Cabot G, Lopez-Causape C, Ocampo-Sosa AA, Sommer LM, Dominguez MA,
869 Zamorano L, et al. Deciphering the Resistome of the Widespread *Pseudomonas*
870 *aeruginosa* Sequence Type 175 International High-Risk Clone through Whole-
871 Genome Sequencing. Antimicrob Agents Chemother. 2016;60(12):7415-23.
872 84. Garey KW, Vo QP, Larocco MT, Gentry LO, Tam VH. Prevalence of type III
873 secretion protein exoenzymes and antimicrobial susceptibility patterns from
874 bloodstream isolates of patients with *Pseudomonas aeruginosa* bacteremia. J
875 Chemother. 2008;20(6):714-20.
876 85. Cho HH, Kwon KC, Sung JY, Koo SH. Prevalence and genetic analysis of multidrug-
877 resistant *Pseudomonas aeruginosa* ST235 isolated from a hospital in Korea, 2008-
878 2012. Ann Clin Lab Sci. 2013;43(4):414-9.
879 86. Aguilar-Rodea P, Zuniga G, Rodriguez-Espino BA, Olivares Cervantes AL, Gamino
880 Arroyo AE, Moreno-Espinosa S, et al. Identification of extensive drug resistant
881 *Pseudomonas aeruginosa* strains: New clone ST1725 and high-risk clone ST233.
882 PLoS One. 2017;12(3):e0172882.
883 87. Treepong P, Kos VN, Guyeux C, Blanc DS, Bertrand X, Valot B, et al. Global
884 emergence of the widespread *Pseudomonas aeruginosa* ST235 clone. Clin Microbiol
885 Infect. 2018;24(3):258-66.
886 88. Kos VN, Deraspe M, McLaughlin RE, Whiteaker JD, Roy PH, Alm RA, et al. The
887 resistome of *Pseudomonas aeruginosa* in relationship to phenotypic susceptibility.
888 Antimicrob Agents Chemother. 2015;59(1):427-36.
889 89. Del Barrio-Tofino E, Lopez-Causape C, Cabot G, Rivera A, Benito N, Segura C, et
890 al. Erratum for del Barrio-Tofino et al., "Genomics and Susceptibility Profiles of

891 Extensively Drug-Resistant *Pseudomonas aeruginosa* Isolates from Spain". Antimicrob Agents Chemother. 2018;62(1).

90. Chen Y, Sun M, Wang M, Lu Y, Yan Z. Dissemination of IMP-6-producing *Pseudomonas aeruginosa* ST244 in multiple cities in China. Eur J Clin Microbiol Infect Dis. 2014;33(7):1181-7.
91. Miranda CC, de Filippis I, Pinto LH, Coelho-Souza T, Bianco K, Cacci LC, et al. Genotypic characteristics of multidrug-resistant *Pseudomonas aeruginosa* from hospital wastewater treatment plant in Rio de Janeiro, Brazil. J Appl Microbiol. 2015;118(6):1276-86.
92. Silveira MC, Rocha-de-Souza CM, Albano RM, de Oliveira Santos IC, Carvalho-Assef APD. Exploring the success of Brazilian endemic clone *Pseudomonas aeruginosa* ST277 and its association with the CRISPR-Cas system type I-C. BMC Genomics. 2020;21(1):255.
93. Pelegrin AC, Saharman YR, Griffon A, Palmieri M, Mirande C, Karuniawati A, et al. High-Risk International Clones of Carbapenem-Nonsusceptible *Pseudomonas aeruginosa* Endemic to Indonesian Intensive Care Units: Impact of a Multifaceted Infection Control Intervention Analyzed at the Genomic Level. mBio. 2019;10(6).
94. Lopez-Causape C, Sommer LM, Cabot G, Rubio R, Ocampo-Sosa AA, Johansen HK, et al. Evolution of the *Pseudomonas aeruginosa* mutational resistome in an international Cystic Fibrosis clone. Sci Rep. 2017;7(1):5555.
95. Parkins MD, Somayaji R, Waters VJ. Epidemiology, Biology, and Impact of Clonal *Pseudomonas aeruginosa* Infections in Cystic Fibrosis. Clin Microbiol Rev. 2018;31(4).

915 # Supporting information

916 **S1 Table. Origin, resistance, genotyping and virulence data of the 54 *P. aeruginosa***
917 **isolates from the referral hospital under study.**

918 * Novel ST

919 [a] Novel *aroE* allele

920 ICU, intensive care unit; AICU, adult intensive care unit; PICU, pediatric intensive care unit; NICU,
921 neonatal intensive care unit; RTI, respiratory tract infection; BSI, bloodstream infection; FEP, cefepime;
922 IMP, imipenem; MER, meropenem; TZP, piperacillin+tazobactam; GN, gentamicin; CIP, ciprofloxacin;
923 POL, polymyxin B; MultiS, susceptible to all tested antibiotics; ModR, moderately resistant; MDR, multi-
924 drug resistant; XDR, extensively drug-resistant; ST, sequence type.

925 **S1 Fig. Prevalence of virulence-associated genes in the 54 *P. aeruginosa* isolates from**
926 **the referral hospital under study.**

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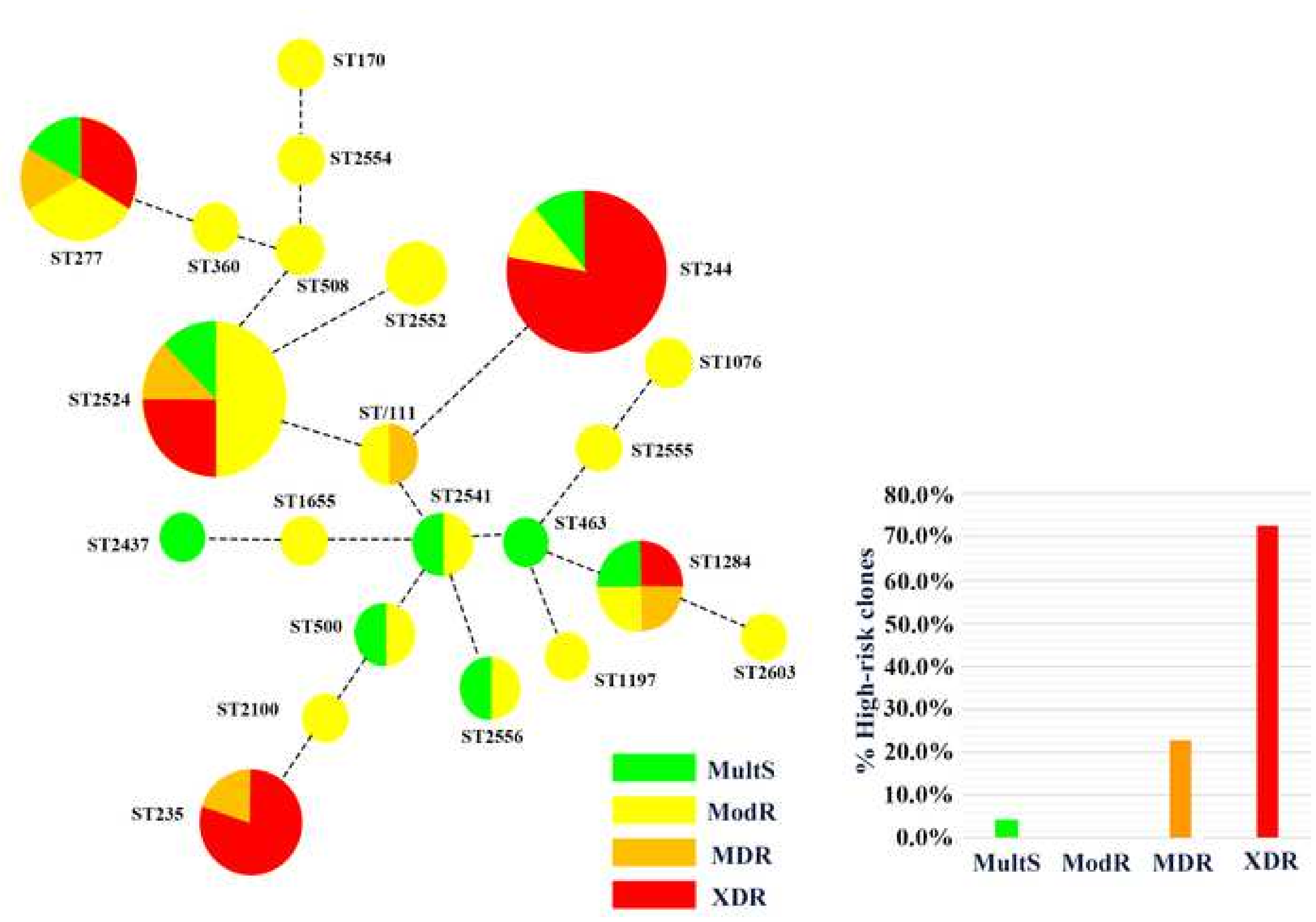

Figure 2_Dendrogram

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Id	Clonal group / Pattern	ST	Year	ICU	Clinical source	T3SS virulotype	Susceptibility phenotype	β-lactamase gene
SC060	A	277	2011	PICU	RTI	$exoS^{+}/exoU^{+}$	MDR	bla_{SPM-1}, bla_{OXA-10}
SC083	A	277	2011	AICU	RTI	$exoS^{+}/exoU^{-}$	XDR	bla_{SPM-1}, bla_{OXA-2}
SC007	B	277	2010	PICU	Other	$exoS^{+}/exoU^{+}$	XDR	bla_{SPM-1}, bla_{OXA-10}
SC016	B	277	2010	AICU	RTI	$exoS^{+}/exoU^{+}$	MDR	bla_{OXA-10}
SC015		1197	2010	AICU	Other	$exoS^{+}/exoU^{+}$	ModR	
SC115	C	277	2012	PICU	BSI	$exoS^{+}/exoU^{-}$	XDR	bla_{SPM-1}, bla_{OXA-2}
SC139	C	277	2012	PICU	RTI	$exoS^{+}/exoU^{-}$	XDR	bla_{SPM-1}
SC047		500	2011	NICU	BSI	$exoS^{+}/exoU^{-}$	MultS	
SC059		500	2011	NICU	BSI	$exoS^{+}/exoU^{-}$	ModR	
SC061		170	2011	AICU	RTI	$exoS^{+}/exoU^{-}$	ModR	
SC103		2554*	2012	AICU	BSI	$exoS^{+}/exoU^{+}$	ModR	
SC101		1655	2011	AICU	RTI	$exoS^{+}/exoU^{+}$	ModR	
SC037	D	235	2010	AICU	Other	$exoS^{+}/exoU^{-}$	XDR	bla_{CTX-M2}
SC033	D	235	2010	AICU	RTI	$exoS^{+}/exoU^{+}$	XDR	bla_{CTX-M2}
SC099	D	235	2011	PICU	Other	$exoS^{+}/exoU^{+}$	XDR	bla_{CTX-M2}
SC008		235	2010	AICU	Other	$exoS^{+}/exoU^{+}$	MDR	bla_{CTX-M2}
SC129		235	2012	AICU	RTI	$exoS^{+}/exoU^{+}$	XDR	
SC145	E	1284	2012	PICU	Other	$exoS^{+}/exoU^{+}$	ModR	
SC187	E	1284	2013	PICU	RTI	$exoS^{+}/exoU^{+}$	ModR	
SC162	E	1284	2013	PICU	Other	$exoS^{+}/exoU^{-}$	ModR	bla_{CTX-M2}
SC080	F	2524*	2011	NICU	BSI	$exoS^{+}/exoU^{-}$	ModR	
SC082	F	2524*	2011	NICU	BSI	$exoS^{+}/exoU^{-}$	ModR	
SC079	F	2524*	2011	NICU	BSI	$exoS^{+}/exoU^{-}$	MultS	
SC081	F	2524*	2011	NICU	BSI	$exoS^{+}/exoU^{-}$	MultS	
SC084	F	2524*	2011	NICU	Other	$exoS^{+}/exoU^{+}$	ModR	
SC086	F	2524*	2011	AICU	Other	$exoS^{+}/exoU^{+}$	MDR	
SC090	F	2524*	2011	PICU	RTI	$exoS^{+}/exoU^{+}$	ModR	
SC097	F	2524*	2011	AICU	RTI	$exoS^{+}/exoU^{+}$	ModR	
SC116		1076	2012	PICU	RTI	$exoS^{+}/exoU^{+}$	ModR	
SC128		463	2012	PICU	BSI	$exoS^{+}/exoU^{+}$	MultS	
SC169		244	2013	NICU	BSI	$exoS^{+}/exoU^{-}$	MultS	
SC032	G	244	2010	AICU	Other	$exoS^{+}/exoU^{+}$	XDR	
SC065	G	244	2011	AICU	Other	$exoS^{+}/exoU^{-}$	XDR	
SC026	H	244	2010	AICU	Other	$exoS^{+}/exoU^{+}$	MDR	bla_{CTX-M2}
SC029	H	244	2010	AICU	BSI	$exoS^{+}/exoU^{-}$	XDR	bla_{CTX-M2}
SC058		244	2011	AICU	RTI	$exoS^{+}/exoU^{-}$	XDR	bla_{CTX-M2}
SC048		244	2011	AICU	RTI	$exoS^{+}/exoU^{-}$	XDR	
SC069		244	2011	AICU	RTI	$exoS^{+}/exoU^{-}$	XDR	bla_{CTX-M2}
SC094		244	2011	AICU	Other	$exoS^{+}/exoU^{+}$	XDR	bla_{CTX-M2}
SC092	I	111	2011	AICU	Other	$exoS^{+}/exoU^{+}$	XDR	
SC158	I	111	2013	AICU	RTI	$exoS^{+}/exoU^{-}$	MDR	bla_{CTX-M2}
SC156		2100	2013	PICU	Other	$exoS^{+}/exoU^{+}$	ModR	
SC157		2603*	2013	PICU	Other	$exoS^{+}/exoU^{+}$	ModR	
SC150		508	2013	NICU	BSI	$exoS^{+}/exoU^{+}$	ModR	bla_{OXA-2}
SC136		2556*	2012	NICU	BSI	$exoS^{+}/exoU^{-}$	MultS	
SC159		2556*	2013	NICU	BSI	$exoS^{+}/exoU^{-}$	ModR	
SC010	J	2552*	2010	AICU	RTI	$exoS^{+}/exoU^{+}$	ModR	
SC012	J	2552*	2010	AICU	RTI	$exoS^{+}/exoU^{+}$	MultS	
SC119		2437	2012	AICU	RTI	$exoS^{+}/exoU^{+}$	MultS	
SC163		1284	2013	AICU	RTI	$exoS^{+}/exoU^{+}$	ModR	
SC009		2541*	2010	AICU	RTI	$exoS^{+}/exoU^{-}$	ModR	
SC004		2541*	2010	AICU	RTI	$exoS^{+}/exoU^{-}$	MultS	
SC095		360	2012	PICU	RTI	$exoS^{+}/exoU^{-}$	ModR	
SC126		2555*	2012	NICU	Other	$exoS^{+}/exoU^{+}$	ModR	

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