Genomic and phenotypic comparison of environmental and patient-derived isolates of *Pseudomonas aeruginosa* suggest that antimicrobial resistance is rare within the environment

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Abstract

Patient-derived isolates of the opportunistic pathogen *Pseudomonas aeruginosa* are frequently resistant to antibiotics due to the presence of sequence variants in resistance-associated genes. However, the frequency of antibiotic resistance and of resistance-associated sequence variants in environmental isolates of *P. aeruginosa* has not been well studied. Antimicrobial susceptibility testing (ciprofloxacin, ceftazidime, meropenem, tobramycin) of environmental (n=50) and cystic fibrosis (n=42) *P. aeruginosa* isolates was carried out. Following whole genome sequencing of all isolates, twenty-five resistance-associated genes were analysed for the presence of likely function-altering sequence variants. Environmental isolates were susceptible to all antibiotics with one exception, whereas patient-derived isolates had significant frequencies of resistance to each antibiotic and a greater number of likely resistance-associated genetic variants. These findings indicate that the natural environment does not act as a reservoir of antibiotic-resistant *P. aeruginosa,* supporting a model in which antibiotic susceptible environmental bacteria infect patients and develop resistance during infection.

Introduction

As an environmental bacterium, *Pseudomonas aeruginosa* has been isolated from many different niches including water sources and domestic and health-care settings (1-4). While rarely causing infections in healthy individuals, this opportunistic pathogen can cause a range of infections in people who are immunocompromised or have predisposing conditions such as cystic fibrosis (CF) (1). For adults with CF, *P. aeruginosa* is the most prevalent bacterium causing respiratory infection and *P. aeruginosa* from these infections are frequently resistant to antibiotics, complicating treatment (5). Resistance is associated with variants in key genes that reduce the intracellular concentrations of antibiotics or the affinities of target proteins for antibiotics, relative to antibiotic-susceptible isolates (6). Infection arises from exposure to

63 environmental sources of *P. aeruginosa* included both the natural and health-care environment
64 (1). Epidemiological studies of isolates obtained from high infection risk areas, such as health-
65 care settings, domestic and community areas show that antibiotic resistant *P. aeruginosa* can
66 be present providing a potential reservoir of infectious resistant bacteria (7-18). Acquisition of
67 *P. aeruginosa* from the natural environment, typically during childhood, is also a major source
68 of infection with a number of studies identifying genotypically indistinguishable strains in the
69 natural environment and the respiratory tract of CF patients (3, 19-22). However antibiotic
70 susceptibility of isolates from natural (non-man-made) environmental sources has had very
71 limited studies and so it is not clear whether the natural environment provides a reservoir of
72 antibiotic-resistant *P. aeruginosa* (7, 23, 24). Using a cohort of clinical isolates as a
73 comparator, here we determined the prevalence of antibiotic resistance in *P. aeruginosa* from
74 the natural environment (river), the domestic and community settings (swimming pool and
75 water tank) by assessing antimicrobial susceptibility profiles and allelic variations in resistance
76 genes of *P. aeruginosa*.
77
78 **Materials and Methods**
79 **Isolate Selection and identification**
80 Fifty environmental *P. aeruginosa* isolates obtained from water sources in Queensland,
81 Australia and 42 clinical *P. aeruginosa* isolates obtained from CF patients residing in Australia
82 and New Zealand were examined (Supplementary Table 1). Environmental isolates included
83 those obtained from rivers (n=36), swimming pools (n=13) and one sample from a domestic
84 water tank. Individual multi-locus sequence types (MLST) were present in singleton isolates,
85 expect for sequence types (ST) -155, -179, -266 and -381 which were represented once each in
86 both the CF and environmental isolate cohorts. Isolate identification and MLST typing were
87 confirmed using molecular techniques (https://pubmlst.org/ paeruginosa/) (25-27).

88 **Antimicrobial Susceptibility Testing**

89 The minimum inhibitory concentration (MIC) of ciprofloxacin, meropenem, tobramycin and
90 ceftazidime was determined for all the 92 isolates using ETEST® strips and methodology. The
91 breakpoint was determined according to the Clinical and Laboratory Standards Institute (CLSI)
92 guidelines (https://clsi.org/).

93 **Whole genome sequencing and bioinformatic analysis**

94 DNA was extracted from each strain using the MoBio UltraClean® Microbial DNA isolation
95 kit in accordance with the manufacturer's instructions. Isolates were sequenced to ~50-fold
96 (50x) coverage using illumina MiSeq, 2x150, or 2x300 basepair (bp) paired-end reads. Raw
97 sequence reads were trimmed using Trimmomatic version 0.36 (28). Draft genomes were
98 assembled using SPAdes version 3.12.0 as described previously (29).

99 **Resistance Gene Analysis**

100 Genome assemblies were used for the comparison of 25 well characterised genes associated
101 with antibiotic resistance, with reference sequences sourced from
102 https://www.pseudomonas.com (Supplementary Table 2). Protein Variation Effect Analyzer
103 (PROVEAN) version 1.1 was utilised to identify and analyse genetic variants within the chosen
104 genes (30). Genetic variants (amino acid substitutions) were categorised in accordance to
105 PROVEAN scores, as predicted function-altering variants (PROVEAN score: $\leq$ -2.5) or
106 variants not predicted to affect function (PROVEAN score: > -2.5). Additionally, each genome
107 was manually screened using tblastn for premature stop codons and deletions which were also
108 classified as function-altering variants (31).
109 ResFinder version 3.1 was used with a prebuilt ResFinder database, including all relevant
110 antibiotics, to determine whether resistance could be affected by acquired genes
111 (https://cge.cbs.dtu.dk/services/ResFinder/).

112 **Statistical analysis**

SPSS version 25 was used for statistical analysis. Pearson's chi-square test was used to examine the association between niche and phenotype (susceptible or resistant) and niche and genotype. When more than 20% of the expected values were less than five, Fisher's exact test were used. A *P*-value of <0.05 was considered significant.

Results and Discussion

A total of 50 isolates of *P. aeruginosa* from the natural environment and 42 isolates from patients with CF were included in this study. The isolates are genetically diverse and broadly representative of the *P. aeruginosa* species (Supplementary Figure S1).

Environmental isolates are antibiotic susceptible

All 50 environmental isolates were susceptible to ciprofloxacin, meropenem and tobramycin and only one of these isolates was resistant to ceftazidime (Figure 1, Supplementary Table 3). Conversely, a non-susceptible (intermediate or resistant) phenotype was observed for 15 of the 42 CF isolates. Specifically, 10%, 17% 19% and 21% of the CF isolates were non-susceptible to tobramycin, meropenem, ceftazidime and ciprofloxacin, respectively (Figure 1, Supplementary Table 4). Overall, the frequency of resistant isolates was significantly less in the environmental cohort than the CF cohort (ceftazidime *P*=0.010, ciprofloxacin *P*=0.001, meropenem *P*=0.003, tobramycin *P*=0.040). Due to limited availability of clinical information we were unable to characterise CF infections as either early/transient or chronic to assess whether resistance is more prevalent in chronically infected patients. However, when we categorised the CF isolates according to patient age (adult ≥18 years; adult n=29, paediatric n=13), isolates from two paediatric patients (15.4%) demonstrated antibiotic resistance compared with isolates from 13 adults (44.8%) (*P*=0.066) (Figure 1). Overall our findings are in agreement with a model of bacterial adaptation during infection leading to increased resistance over time (32). Our results demonstrate that environmental isolates are susceptible

to clinically relevant antibiotics and that the environments we tested are not a reservoir for antibiotic resistant isolates.

Environmental isolates have fewer predicted function-altering variants in resistance-associated genes

We analysed 25 well characterised resistance-associated genes for the presence of likely function-altering variants that could influence antibiotic susceptibility (Supplementary Table 2). The environmental isolates had strikingly fewer predicted function-altering variants than the CF isolates. A total of 75 predicted function-altering variants were identified within 18 of 25 resistance genes analysed for the 50 environmental isolates. No predicted function-altering variants were identified in the remaining seven genes (Supplementary Table 3). In this cohort, the predicted function-altering variants are generally not sufficient to confer antibiotic resistance as all except one isolate was fully susceptible to the antibiotics tested. In contrast, 110 predicted function-altering variants affecting all 25 resistance-associated genes were identified in the 42 CF isolates. Function-altering variants not analysed by PROVEAN, including premature stop-codons and deletions, were absent in the environmental cohort but were present in nine CF isolates (premature stop-codons n=8, deletions n=2) (Supplementary Table 4). Overall, variants in the environmental cohort had a significantly higher PROVEAN score than those in the CF cohort ($P<0.001$), indicating the presence of fewer amino acid substitutions that may affect function of resistance-associated proteins (Supplementary Figure 2).

The frequency of function-altering variants within each gene was determined for both cohorts of isolates (Table 1). Statistical differences were noted for 6 genes in the CF cohort with a greater number of function-altering variants being present in *amgS* and *fusA*1 (tobramycin resistance), *gyrA* and *gyrB* (ciprofloxacin resistance) and *mexZ* (broad-spectrum resistance) genes for isolates in the CF cohort, and in *ampE* (β-lactam resistance) for the natural

environmental isolates (Table 1). Specific variants present in these genes, such as T83I (*gyrA*), and R504C (*ftsI*) previously associated with resistance were only present in the CF isolate cohort only (33, 34). However, a strong association between individual gene variants and resistance phenotype was not observed (Supplementary Table 4) due to the low numbers of isolates with variants for each gene combined with the multifactorial nature of antibiotic resistance in *P. aeruginosa*.

ResFinder analysis assessing the presence of horizontally acquired resistance genes identified a gene *crpP* associated with fluoroquinolone resistance (35). However there was no correlation between ciprofloxacin resistance and the presence of *crpP* for the isolates in this study (Supplementary Table 3 and 4). No other horizontally-transferred resistance genes were identified.

In conclusion, our findings show that isolates from *P. aeruginosa* from natural environments have low frequencies of antibiotic resistance, and of genetic variants associated with resistance, compared to isolates from patients with CF. These findings indicate that the natural environment is unlikely to act as a reservoir of antibiotic-resistant *P. aeruginosa*. They are consistent with a model in which patients are infected by antibiotic-sensitive *P. aeruginosa* from the environment which then evolves to become antibiotic-resistant during infection (32).

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201 K.A.R, S.J.T-W, W.M.P, B.B, D.W.R and I.L.L have nothing to declare. S.C.B is a Member
202 of Advisory Board, Member of Writing group, Site Principle Investigator, author on several
203 Rempex sponsored studies, a member of advisory boards for Vertex, Abbvie and Galapagos
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207

208 Table 1: Comparison of predicted function altering variants identified in the CF and

209 environmental isolates (n = 92).

Gene	Predicted function altering variants[$]		*P*-value†
	Environment (n=50)	CF (n=42)	
amgR	0	1	0.457*
amgS	0	5	0.017*
ampC	15	17	0.293
ampD	9	5	0.418
ampDh2	1	3	0.328*
ampDh3	6	6	0.746
ampE	14	4	0.026
ampG	0	1	0.457*
ampR	7	3	0.336*
aph3''IIb	19	19	0.204
ftsI	1	5	0.089*
*fusA*1	0	16	<0.001
galU	0	1	0.457*
gyrA	1	10	0.001
gyrB	0	6	0.007*
mexR	1	1	1.00*
mexZ	1	14	<0.001
mpl	1	4	0.174*
nalC	1	3	0.328*
nalD	0	3	0.091*
nfxB	3	5	0.462*
nuoG	3	2	1.00*
oprD	41	36	0.631
parC	1	1	1.00*
parE	2	3	0.657*

210 [$]PROVEAN predicted function-altering variants, premature stop codons and deletions.

211 †Chi-square value; [*]Fisher's Exact Test used in place of chi-square test

212 *P*-value <0.05 is considered significant

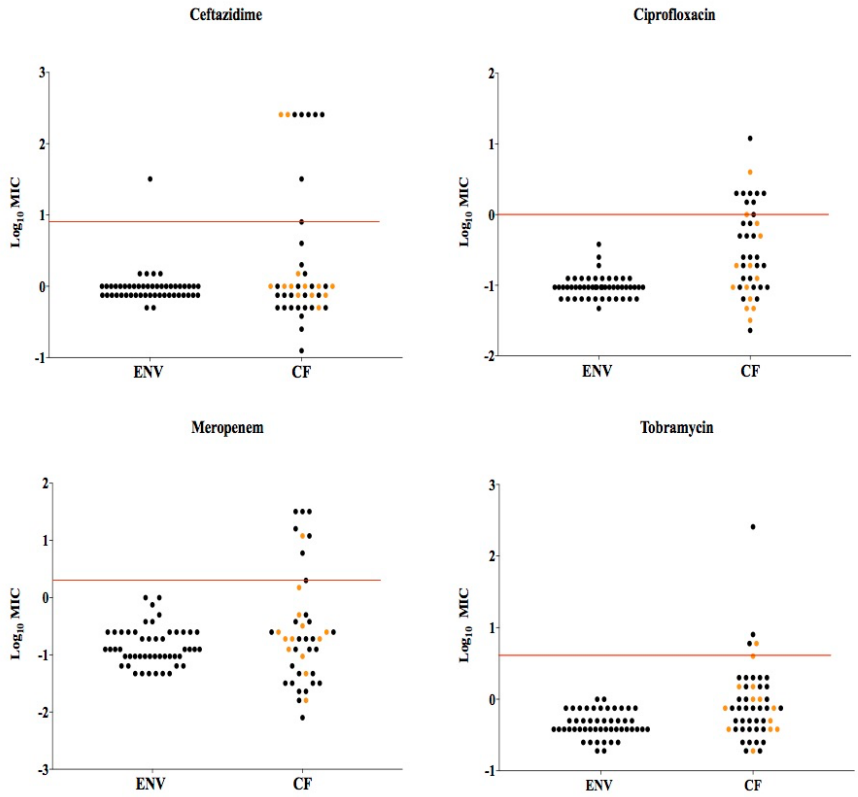

Figure 1: Distribution of antimicrobial susceptibility results of environmental (ENV; n=50) and cystic fibrosis (CF; n=42) isolates for ciprofloxacin, ceftazidime, meropenem and tobramycin. The horizontal red lines represent the antibiotic breakpoint as defined by the CLSI. The CF isolates have been further differentiated into samples collected from adult (black circles) and paediatric (orange circles) patients. The MIC results have been log transformed (log_{10}) for ease of graphical representation.

References:

1. Gellatly SL, Hancock RE. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog Dis. 2013;67(3):159-173. doi: 10.1111/2049-632x.12033

2. Decraene V, Ghebrehewet S, Dardamissis E, Huyton R, Mortimer K, Wilkinson D, Shokrollahi K, Singleton S, Patel B, Turton J, Hoffman P, Puleston R. An outbreak of multidrug-resistant *Pseudomonas aeruginosa* in a burns service in the North of England: challenges of infection prevention and control in a complex setting. J Hosp Infect. 2018. doi: 10.1016/j.jhin.2018.07.012

3. Kidd TJ, Ritchie SR, Ramsay KA, Grimwood K, Bell SC, Rainey PB. *Pseudomonas aeruginosa* exhibits frequent recombination, but only a limited association between genotype and ecological setting. PLoS One. 2012;7(9):e44199. doi: 10.1371/journal.pone.0044199

4. Purdy-Gibson ME, France M, Hundley TC, Eid N, Remold SK. *Pseudomonas aeruginosa* in CF and non-CF homes is found predominantly in drains. J Cyst Fibros. 2015;14(3):341-346. doi: 10.1016/j.jcf.2014.10.008

5. Elborn JS. Cystic fibrosis. Lancet. 2016;388(10059):2519-2531. doi: 10.1016/s0140-6736(16)00576-6

6. Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev. 2009;22(4):582-610. doi: 10.1128/cmr.00040-09

7. Bhuiya M, Sarkar MKI, Sohag MH, Ali H, Roy CK, Akther L, Sarker AF. Enumerating antibiotic susceptibility patterns of *Pseudomonas aeruginosa* isolated from different sources in Dhaka City. Open Microbiol J. 2018;12:172-180. doi: 10.2174/1874285801812010172

8. Devarajan N, Kohler T, Sivalingam P, van Delden C, Mulaji CK, Mpiana PT, Ibelings BW, Pote J. Antibiotic resistant Pseudomonas spp. in the aquatic environment: A prevalence

240 study under tropical and temperate climate conditions. Water Res. 2017;115:256-265. doi:
241 10.1016/j.watres.2017.02.058
242 9. Ebrahimpour M, Nikokar I, Ghasemi Y, Sedigh Ebrahim-Saraie H, Araghian A,
243 Farahbakhsh M, Ghassabi F. Antibiotic resistance and frequency of class 1 integrons among
244 *Pseudomonas aeruginosa* isolates obtained from wastewaters of a burn center in Northern Iran.
245 Ann Ig. 2018;30(2):112-119. doi: 10.7416/ai.2018.2202
246 10. Kummerer K. Antibiotics in the aquatic environment--a review--part II. Chemosphere.
247 2009;75(4):435-441. doi: 10.1016/j.chemosphere.2008.12.006
248 11. Odumosu BT, Ajetunmobi O, Dada-Adegbola H, Odutayo I. Antibiotic susceptibility
249 pattern and analysis of plasmid profiles of *Pseudomonas aeruginosa* from human, animal and
250 plant sources. Springerplus. 2016;5(1):1381. doi: 10.1186/s40064-016-3073-9
251 12. Olga P, Apostolos V, Alexis G, George V, Athena M. Antibiotic resistance profiles of
252 *Pseudomonas aeruginosa* isolated from various Greek aquatic environments. FEMS Microbiol
253 Ecol. 2016;92(5):fiw042. doi: 10.1093/femsec/fiw042
254 13. Ruiz L, Dominguez MA, Ruiz N, Vinas M. Relationship between clinical and
255 environmental isolates of *Pseudomonas aeruginosa* in a hospital setting. Arch Med Res.
256 2004;35(3):251-257. doi: 10.1016/j.arcmed.2004.02.005
257 14. Spindler A, Otton LM, Fuentefria DB, Corcao G. Beta-lactams resistance and presence
258 of class 1 integron in Pseudomonas spp. isolated from untreated hospital effluents in Brazil.
259 Antonie Van Leeuwenhoek. 2012;102(1):73-81. doi: 10.1007/s10482-012-9714-2
260 15. Tumeo E, Gbaguidi-Haore H, Patry I, Bertrand X, Thouverez M, Talon D. Are
261 antibiotic-resistant *Pseudomonas aeruginosa* isolated from hospitalised patients recovered in
262 the hospital effluents? Int J Hyg Environ Health. 2008;211(1-2):200-204. doi:
263 10.1016/j.ijheh.2007.02.010

264 16. Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM-1 positive
265 bacteria in the New Delhi environment and its implications for human health: an environmental
266 point prevalence study. Lancet Infect Dis. 2011;11(5):355-362. doi: 10.1016/s1473-
267 3099(11)70059-7
268 17. Schiavano GF, Carloni E, Andreoni F, Magi S, Chironna M, Brandi G, Amagliani G.
269 Prevalence and antibiotic resistance of *Pseudomonas aeruginosa* in water samples in central
270 Italy and molecular characterization of *oprD* in imipenem resistant isolates. PLoS One.
271 2017;12(12):e0189172. doi: 10.1371/journal.pone.0189172
272 18. Gad GF, El-Domany RA, Zaki S, Ashour HM. Characterization of *Pseudomonas*
273 *aeruginosa* isolated from clinical and environmental samples in Minia, Egypt: prevalence,
274 antibiogram and resistance mechanisms. J Antimicrob Chemother. 2007;60(5):1010-1017. doi:
275 10.1093/jac/dkm348
276 19. Kidd TJ, Ramsay KA, Vidmar S, Carlin JB, Bell SC, Wainwright CE, Grimwood K.
277 *Pseudomonas aeruginosa* genotypes acquired by children with cystic fibrosis by age 5-years.
278 J Cyst Fibros. 2015;14(3):361-369. doi: 10.1016/j.jcf.2014.12.007
279 20. Ranganathan SC, Skoric B, Ramsay KA, Carzino R, Gibson AM, Hart E, Harrison J,
280 Bell SC, Kidd TJ. Geographical differences in first acquisition of *Pseudomonas aeruginosa* in
281 cystic fibrosis. Ann Am Thorac Soc. 2013;10(2):108-114. doi: 10.1513/AnnalsATS.201209-
282 077OC
283 21. Rosenfeld M, Emerson J, McNamara S, Thompson V, Ramsey BW, Morgan W, Gibson
284 RL. Risk factors for age at initial *Pseudomonas acquisition* in the cystic fibrosis epic
285 observational cohort. J Cyst Fibros. 2012;11(5):446-453. doi: 10.1016/j.jcf.2012.04.003
286 22. Romling U, Wingender J, Muller H, Tummler B. A major *Pseudomonas aeruginosa*
287 clone common to patients and aquatic habitats. Appl Environ Microbiol. 1994;60(6):1734-
288 1738. doi:

289 23. Kittinger C, Lipp M, Baumert R, Folli B, Koraimann G, Toplitsch D, Liebmann A,
290 Grisold AJ, Farnleitner AH, Kirschner A, Zarfel G. Antibiotic Resistance Patterns of
291 Pseudomonas spp. Isolated from the River Danube. Front Microbiol. 2016;7:586. doi:
292 10.3389/fmicb.2016.00586
293 24. Liew SM, Rajasekaram G, Puthucheary SA, Chua KH. Antimicrobial susceptibility and
294 virulence genes of clinical and environmental isolates of *Pseudomonas aeruginosa*. PeerJ.
295 2019;7:e6217. doi: 10.7717/peerj.6217
296 25. Anuj SN, Whiley DM, Kidd TJ, Bell SC, Wainwright CE, Nissen MD, Sloots TP.
297 Identification of *Pseudomonas aeruginosa* by a duplex real-time polymerase chain reaction
298 assay targeting the *ecfX* and the *gyrB* genes. Diagn Microbiol Infect Dis. 2009;63(2):127-131.
299 doi: 10.1016/j.diagmicrobio.2008.09.018
300 26. Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb
301 software, the PubMLST.org website and their applications. Wellcome Open Res. 2018;3:124.
302 doi: 10.12688/wellcomeopenres.14826.1
303 27. Matsuda K, Tsuji H, Asahara T, Kado Y, Nomoto K. Sensitive quantitative detection
304 of commensal bacteria by rRNA-targeted reverse transcription-PCR. Appl Environ Microbiol.
305 2007;73(1):32-39. doi: 10.1128/aem.01224-06
306 28. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina
307 sequence data. Bioinformatics. 2014;30(15):2114-2120. doi: 10.1093/bioinformatics/btu170
308 29. Freschi L, Jeukens J, Kukavica-Ibrulj I, Boyle B, Dupont MJ, Laroche J, Larose S,
309 Maaroufi H, Fothergill JL, Moore M, Winsor GL, Aaron SD, Barbeau J, Bell SC, Burns JL,
310 Camara M, Cantin A, Charette SJ, Dewar K, Deziel E, Grimwood K, Hancock RE, Harrison
311 JJ, Heeb S, Jelsbak L, Jia B, Kenna DT, Kidd TJ, Klockgether J, Lam JS, Lamont IL, Lewenza
312 S, Loman N, Malouin F, Manos J, McArthur AG, McKeown J, Milot J, Naghra H, Nguyen D,
313 Pereira SK, Perron GG, Pirnay JP, Rainey PB, Rousseau S, Santos PM, Stephenson A, Taylor

314 V, Turton JF, Waglechner N, Williams P, Thrane SW, Wright GD, Brinkman FS, Tucker NP,
315 Tummler B, Winstanley C, Levesque RC. Clinical utilization of genomics data produced by
316 the international *Pseudomonas aeruginosa* consortium. Front Microbiol. 2015;6:1036. doi:
317 10.3389/fmicb.2015.01036
318 30. Choi Y, Sims GE, Murphy S, Miller JR, Chan AP. Predicting the functional effect of
319 amino acid substitutions and indels. PLoS One. 2012;7(10):e46688. doi:
320 10.1371/journal.pone.0046688
321 31. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL.
322 BLAST+: architecture and applications. BMC Bioinformatics. 2009;10:421. doi:
323 10.1186/1471-2105-10-421
324 32. Sousa AM, Pereira MO. *Pseudomonas aeruginosa* diversification during infection
325 development in cystic fibrosis lungs-a review. Pathogens. 2014;3(3):680-703. doi:
326 10.3390/pathogens3030680
327 33. Lopez-Causape C, Cabot G, Del Barrio-Tofino E, Oliver A. The versatile mutational
328 resistome of *Pseudomonas aeruginosa*. Front Microbiol. 2018;9:685. doi:
329 10.3389/fmicb.2018.00685
330 34. Rehman A, Patrick WM, Lamont IL. Mechanisms of ciprofloxacin resistance in
331 *Pseudomonas aeruginosa*: new approaches to an old problem. J Med Microbiol. 2019;68(1):1-
332 10. doi: 10.1099/jmm.0.000873
333 35. Chavez-Jacobo VM, Hernandez-Ramirez KC, Romo-Rodriguez P, Perez-Gallardo RV,
334 Campos-Garcia J, Gutierrez-Corona JF, Garcia-Merinos JP, Meza-Carmen V, Silva-Sanchez
335 J, Ramirez-Diaz MI. CrpP Is a Novel Ciprofloxacin-Modifying Enzyme Encoded by the
336 *Pseudomonas aeruginosa* pUM505 Plasmid. Antimicrob Agents Chemother. 2018;62(6). doi:
337 10.1128/aac.02629-17