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The effects of sub-MIC ciprofloxacin exposure on antibiotic susceptibility and virulence factors in *Pseudomonas aeruginosa* ATCC 9027

Thuc Quyen Huynh [1, 3] and Thi Thu Hoai Nguyen [1, 2, 3, *]

[1] *School of Biotechnology, International University, Vietnam.*
[2] *Research Center for Infectious Diseases, International University, Vietnam.*
[3] *Vietnam National University of Ho Chi Minh City, Vietnam.*

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Abstract

Exposure to antibiotics below their minimum inhibitory concentration (sub-MIC) is known to be a selection pressure for the development of antibiotic resistance. In this work, we aimed to provide more understanding of how antibiotic resistance and cross-resistance developed in *Pseudomonas aeruginosa* under sub-MIC exposure to Ciprofloxacin. Fully susceptible *P. aeruginosa* ATCC 9027 was serially exposed to sub-MIC level of ciprofloxacin for 14 days to generate E1 strain, then cultured in antibiotic-free environment for 10 days to obtain E2 strain. Changes in MIC values, susceptibility profile and virulence (protease, elastin, biofilm formation, motility) of these strains were evaluated compared to the original *P. aeruginosa* ATCC 9027. After antibiotic exposure, *P. aeruginosa* strains developed resistance to the ciprofloxacin with the MIC values increased by 32 times, and the exposed strains only developed resistance to ciprofloxacin and other fluoroquinolones but not to unrelated antibiotics. The resistant phenotype did not revert fully after 10 days in antibiotic-free environment. In addition, there was noticeable changes in the pathogen's virulence factors during antibiotic exposure. In summary, sub-MICs exposure of *P. aeruginosa* provided positive selection for antibiotic resistant phenotype, which did not revert after 10 days in antibiotic- free environment. Exposure to sub-MIC level of antibiotics caused significant changes in virulence and antibiotic susceptibility of the pathogen.

Keywords: Antibiotic; Ciprofloxacin; Drug resistance; *Pseudomonas aeruginosa*; Virulence

1 Introduction

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, asporogenous, and monoflagellated bacterium. It is one of the most common nosocomial infections, with high morbidity and mortality, causing serious opportunistic infections in immunocompromised patients with cancer and patients suffering from severe burns and cystic fibrosis [1]. In clinical setting, *P. aeruginosa* isolates were reported to have resistance to almost all class of commonly used antibiotics including aminoglycosides, cephalosporins, fluoroquinolones, and carbapenems [2,3].

P. aeruginosa presents a serious therapeutic challenge, and prompt initiation of effective antimicrobial therapy is essential to optimize clinical outcome. Unfortunately, selection of the most appropriate antimicrobial therapy is complicated by the great ability of *P. aeruginosa* to develop or acquire resistance to multiple classes of antimicrobials. Its genome in one of the largest with 6.26 Mbp and 5567 genes, with considerable genetic capacity for resistant genes (efflux-pumps, beta-lactamase, etc.) Its large genome, adaptability and high intrinsic antibiotic resistance (contribute to the emergence and spread of antimicrobial resistance strains [4–6].

* Corresponding author: Thi Thu Hoai Nguyen
School of Biotechnology, International University, Vietnam.

Efforts have been focused on evaluating antibiotic resistance and multidrug resistance in clinical and environmental isolates of *P. aeruginosa* [7,8], investigating antibiotic resistance mechanisms (through membrane barrier, drug efflux pump, biofilm, resistant genes and genes transfer) and virulence in clinical *P. aeruginosa*. Some important proteins have been identified from MDR clinical isolates [9–11]. However, no key gene or regulatory protein has been found for the development of resistant phenotypes. The mechanisms underlying the development of resistant traits are still unclear.

As the mechanisms for drug-resistant development in bacteria is still unclear, it is difficult to predict and use antibiotic therapy effectively, especially against bacteria with high adaptability as *P. aeruginosa*. In this research, we explored changes to antibiotic susceptibility and virulence of *P. aeruginosa* when exposed to sub-MIC level of ciprofloxacin (fluoroquinolone), a common antibiotics used in antibiotic therapy. Isolates resistant to ciprofloxacin are also found often in clinical setting [12]. By exposing *P. aeruginosa* to sub-MIC level of antibiotics, we want understand the development of resistance phenotype under antibiotic exposure.

2 Material and methods

2.1 Bacterial strain

P. aeruginosa ATCC® 9027 was used in the study. All bacteria samples were stored in Tryptic-Soy broth (TSB, Himedia) with 30% glycerol (TSB: Glycerol 7:3 v/v) until used.

2.2 Strains generation by antibiotic exposure using macro-broth dilution

The development of antibiotic resistance in *P. aeruginosa* ATCC 9027 was performed by macro-dilution method in 24-well plate. *P. aeruginosa* was exposed to sub-MIC concentration of ciprofloxacin (Nam Khoa Biotek Co., Ltd.) for 14 days, then cultured in antibiotic-free environment for 10 days.

The MIC plates with concentration gradients of tested antibiotics was set up daily, containing 1mL of the antibiotics diluted by the standard 2-fold dilution series in Muller-Hinton broth (MHB, Himedia). A frozen stock of *P. aeruginosa* ATCC 9027 was streaked on an LB agar plate, and a single colony was inoculated into LB, grown overnight at 37^0C. This antibiotic-susceptible culture was diluted to an OD620 of 0.08-0.1 and then 1ml culture was inoculated into MIC plates with antibiotic gradients. The plate were incubated at 37^oC for 18-24 hours. The MIC value (the lowest concentration that did not show growth) was be recorded. To propagate, cultures from the highest concentration that showed growth was passaged to fresh MIC plate. Samples at day 14^{th} were collected as exposed-1(E1) for analysis. Then, exposed-2 (E2) was generated by culturing E1 and passaging from control well (without antibiotic) for 10 days. Daily samples were stored in 25% glycerol TSB at -80^0C.

2.3 Antibiotic susceptibility test (AST)

The profile of antibiotic susceptibility will be accessed by using disk diffusion method [13]. Eight types of antibiotics were used, including Cefepime (Cm), Ceftazidime (Cz), Ciprofloxacin (Ci), Gentamicin (Ge), Imipenem (Im), Meropenem (Me), Piperacillin/Tazobactam(Pt) and Tobramycin (Tb) (Nam Khoa Biotek Co., Ldt.). Antibiotic dics with standard concentrations of antibiotics were placed on MHA plates lawned with the bacteria samples. The plates were incubated overnight at 37^oC. Diameter of inhibition zone were compared with the standard inhibition zone of *P. aeruginosa* on CLSI 2020 standard.

2.4 Virulence factor evaluation

2.4.1 Enzymatic activity testing – protease and elastase

The overnight cultures of *P. aeruginosa* ATCC 9027 and exposed strains E1, E2 were evaluated for the following virulence factors expression: protease, elastin, motility and biofilm formation. For protease and elastase, each 2.5uL of overnight bacteria culture (OD620 = 0.08-0.1) was spotted on skim milk agar (Himedia) and elastin agar (Nutrient broth, [14]). The plates are incubated at 37^0C, for 24hr. Enzymatic activity was evaluated by the clear zone developed around each spot after incubation. [15,16]. Each test was done in triplicate.

2.4.2 Swimming and swarming motility testing

The test plate was prepared with M8 solution (Na_2HPO_4, KH_2PO_4, NaCl), $MgSO_4$, Casamino acid, D-Glucose and 0.3% agar powder (swimming) and 0.7% (swarming) [17]. Swimming: A sterile pipette tip was dipped into the overnight culture before it was stabbed into the layer of the plate. Swarming: 2.5ul of bacterial culture was spotted on swarming plates.

The plates were incubated for 24 hours at 37°C, then the result can be observed as the spreading of the bacteria away from the original point.

2.4.3 Microtiter plate assay for biofilm quantification

The *P. aeruginosa* cryopreserved samples were thawed and cultured in LB broth for 24h, at 37^{0}C. In a flat bottom polystyrene 96-well microtiter plate, each well was filled with 100 µL of 5x105 CFU/ mL bacteria sample in LB. The plates were cultured for 24h, at 37°C under static condition. Biofilm formation was analyzed using crystal violet staining method according to a previously described protocol [18]. In short, crystal violet was added into each well of the culture plates and after repetitive cycles of washing, crystal violet stain retained in biofilm was solubilized with 30% acetic acid and measured at OD550nm. Experiments were performed in triplicate.

2.5 Statistical analysis

The obtained data were analyzed using MS Excel (Microsoft) and student t-test ($p < 0.05$)

3 Results and discussion

3.1 Sub-MIC level of ciprofloxacin exposure increased MIC of *P. aeruginosa* ATCC 9027

After 14 day exposure of ciprofloxacin, *P. aeruginosa* developed resistance to ciprofloxacin with MIC increased 32 fold, from 0.5 µg/mL to 16.0 µg/mL. The MIC value increased two-fold every one to three days and stood stable at 16 µg/mL from day 11. Ci-exposed 1 (Ci-e1) was collected after 14 days of ciprofloxacin exposure. After culturing Ci-e1 in antibiotic free environment for 10 days, Ci-exposed 2 (Ci-e2) was collected with MIC value dropped 4 fold, to 4 µg/mL. Ci-e1 strain was verified by 16S sequencing.

P. aeruginosa ATCC 9027 developed antibiotic resistance to ciprofloxacin when exposed to sub-MIC level of the antibiotic, and the resistance ability dropped slightly when it was removed from antibiotic stress. Some level resistance was reverted in E2 strain might suggest changes at protein expression level and not at genetic level. However, previous investigation on *S.aureus* had shown that even in when there were some reversion of MIC values, there might still be some underlying genetic changes [19]. Thus, exposure to sub-MIC level can create positive selection pressure that drive the development of resistant phenotype, consistent with previous findings: when treating *P. aeruginosa* to sub-MIC level of antibiotics, 15 mutations occurred in 20 days of exposure, indicating positive selection [20].

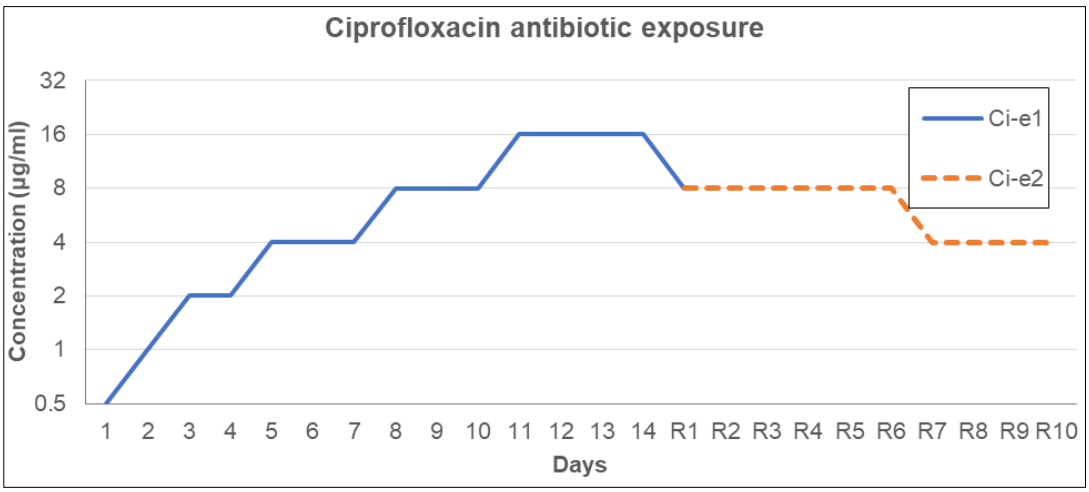

Figure 1 Ciprofloxacin MIC values of *P. aeruginosa* during sub-MIC exposure. Ci-e1 was obtained after 14 days of culturing *P. aeruginosa* ATCC 9027 in sub-MIC ciprofloxacin. Ci-e2 was obtained after culturing Ci-e1 in antibiotic-free environment for 10 days. R1-R10 noted the days cultured in antibiotic-free medium

When the antibiotic susceptibility was evaluated, both Ci-E1 strain and Ci-E2 strain developed resistance to ciprofloxacin (table 1), consistent with the MIC value recorded. However, the resistance to Ciprofloxacin did not affect *P. aeruginosa*'s susceptibility towards other antibiotics. There were no cross resistance to antibiotics in other classes. Previously, increased efflux pump expressions were often implicated in multi-drug resistance strains. There is evidence for protein expression regulation during drug resistance development through *nfxB*, a repressor for the transcription

of *mexCD-oprJ*, a multidrug efflux pump [9]. MexCD-OprJ upregulation correlated with an increased resistance to ciprofloxacin, cefepime, and chloramphenicol in most of the clinical strains, concomitant with a higher susceptibility to ticarcillin, aztreonam, imipenem, and aminoglycosides [11]. Thus, the development of ciprofloxacin development in these strains more likely involve specific changes regarding the action of ciprofloxacin.

Table 1 Antibiotic susceptibility profile of *Pseudomonas aeruginosa* ATCC 9027 and exposed strains Ci-e1, Ci-e2. Ciprofloxacin (Ci), Cefepime (Cm), Ceftazidime (Cz), Gentamicin (Ge), Imipenem (Im), Meropenem (Mer), Piper/Tazobactam (Pt), Tobramycin (Tb). Susceptibility was evaluated according to CLSI 2020 Performance Standards for Antimicrobial Susceptibility Testing (R=Resistance, S=Susceptible, I=Intermediate)

	ATCC 9027	**Ci-e1**	**Ci-e2**
Ci	S	R	R
Cz	S	S	S
Cm	S	S	S
Me	S	S	S
Im	S	S	S
Pt	S	S	S
Tb	S	S	S
Ge	S	S	S

3.2 Altered morphology and virulence factors indicate adaptation and trade-off evolution

Colony morphology characterization often complements conventional microbial identification to detect intra-strain diversity [21]. Colony morphology of initial and exposed strains was observed and evaluated according to previous guidelines according Sousa et al. There were alteration in colony margin and texture (table 2) after sub-MIC exposure to ciprofloxacin. In addition, there were colonies with mixed textured in Ci-e1. Some morphological changes in Ci-e1 did not recovered in Ci-e2 strain. Alterations in colony morphological traits suggest an adaptation to environmental stress, such as antibiotic stress [22].

Table 2 Characteristic of single colony morphologies of *P. aeruginosa,* ciprofloxacin-exposed strains culturing on TSA [22]

	PA ATCC 9027	**Ci-e1**	**Ci-e2**
Form	Circular	Circular	Circular
Margin	Irregular	Entire	Irregular
Texture	Rough	Smooth/Rough	Rough
Size	Large	Small	Small
Color	Yellow	Yellow	Yellow

Similar to colony morphology, treated strains exhibited some changes in virulence factors, as antibiotic resistance phenotype developed, and these changes persisted or did not fully recovered when Ci-e1 was cultured in antibiotic-free environment (figure 2, 3). Biofilm is an important virulence factor and mechanism to adapt to environmental stresses, such as antibiotic stress. Interestingly, drug resistant clinical isolates often has increased biofilm formation [9,23,24]. Biofilms facilitate horizontal transmission due the high microbial density of the populations, thus facilitate the spread of resistance genes and virulence factors, especially under antibiotic selective pressure [23]. Observed decreased biofilm formation in Ci-e1 (figure 3C) did not follow the trend in clinical isolates. In many cases, antibiotic resistance often constitute a fitness cost [25]. Decreased extracellular enzyme activity in Ci exposed strains (table 3) also indicated possible evolutionary trade-offs between developing resistance and maintaining virulence.

In fact, studies on clinical isolates and in-vitro induced resistant strains had shown that some mutations that promote resistance phenotype does not affect growth and virulence while others incurs a fitness cost [23]. Analysis of clinical isolates showed that adaptation to antibiotic stress might affect virulence, enzyme production biofilm formation, motility, etc. differently, depending on the set of mutation the specific strains acquired. Specifically, mutation that modifies the target proteins or enzyme expression often does not reduce the pathogen virulence. Mutations that overexpress the MDR determinants MexABOprM and MexCDOprJ decreased the production of proteases and other virulence of the *P. aeruginosa* mutants [26]. Overproduction of either MexCD-OprJ or MexEF-OprN was associated with a reduction in the transcription of the type III secretion system (TTSS) regulon, leading to lower extracellular enzyme activity [7].

However, another multidrug-resistant *P. aeruginosa* clinical isolates from Cystic Fibrosis (CF) patient with enhanced virulence, high levels of beta-lactamase activity coupled with upregulation of QS-regulated virulence genes, upregulation of the MexAB-OprM efflux pump in relation to resistance to beta-lactam antibiotics (Tomás et al., 2010). Thus, while some antibiotic resistance came with virulence trade-offs, other factors are involved in modulating the virulence and antibiotic resistance of *P. aeruginosa*. Further investigation of other virulence factors will better help elucidate the connection between virulence and antibiotic resistance of individual antibiotic.

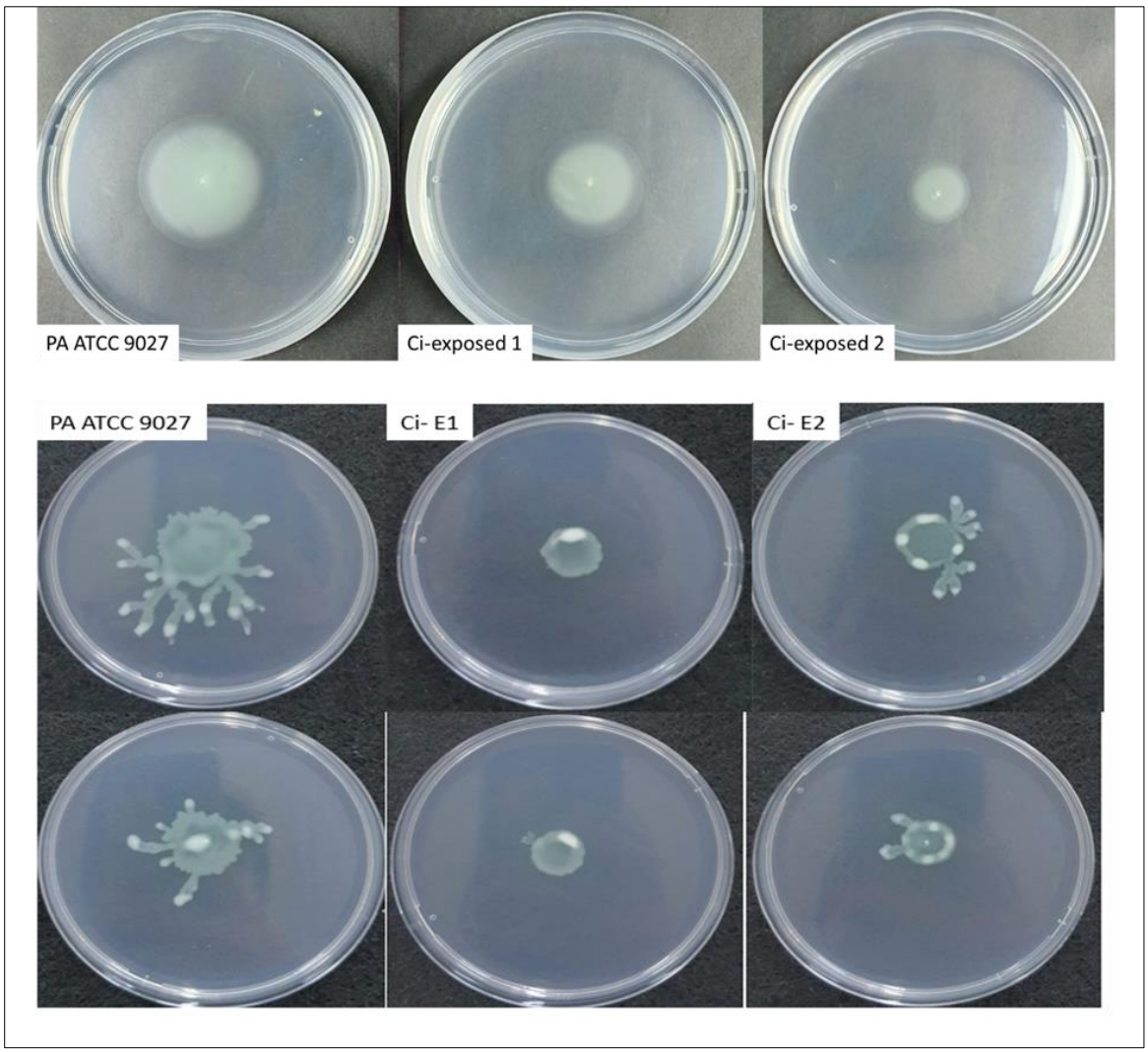

Figure 2 (A) Swimming and (B) Swarming motility of *P. aeruginosa* on 0.7% agar plate, after incubation at 37^{o}C for 24h

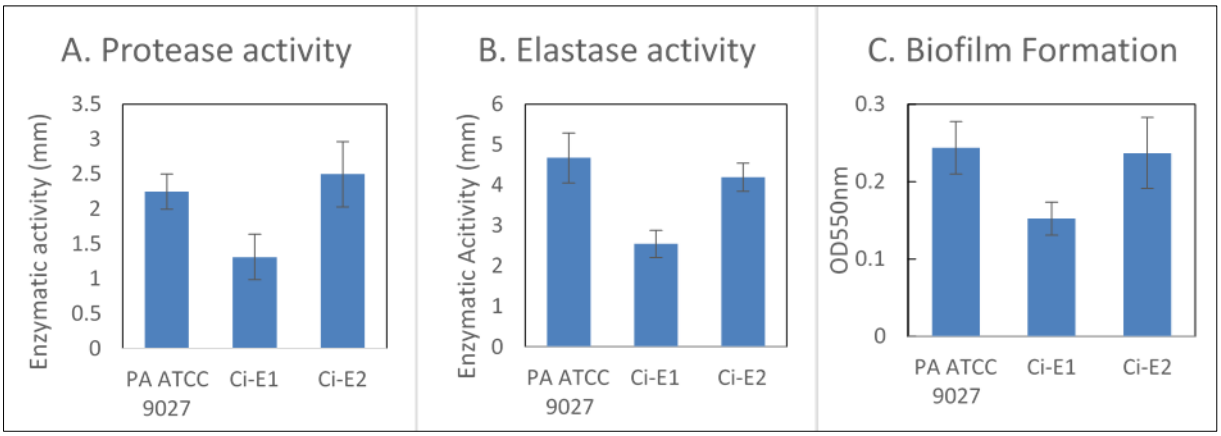

Figure 3 Virulence alteration in *P. aeruginosa* ATCC 9027, after 14-day exposure to sub-MIC level of Ciprofloxacin (Ci-E1), and after subsequent 10-day antibiotic free culture (Ci-E2). (A)Protease activity, (B) Elastase activity, (C) Biofilm formation. Enzymatic activities were significantly reduced in Ci-E1 ($p<0.05$).

Table 3 Alterations of virulence factors of *P. aeruginosa* over exposure to Ciprofloxacin. Enzymatic activity (EA) of protease and elastase is graded: (–) when no visible halo is present, (+) when EA value is limited to 1-2 mm and (++) when EA value is equal or more than 2 mm

	PA ATCC 9027	Ci-E1	Ci-E2
Biofilm	+++	++	+++
Protease	+++	+	+++
Motility	+++	+	++
Elastin	+++	+	++
Swimming	+++	+	+
Swarming	+++	+	++

3.3 Development of antibiotic resistance under sub-MIC antibiotic exposure

Because of its large genome, low permeable outer-membrane, with large number of efflux pumps, *P. aeruginosa* has high adaptability and high intrinsic antibiotic resistance [6]. The development of antibiotic resistant might involve both gene regulation and genetic modifications. Previous study in *P. aeruginosa* PAO1 indicated that the development of in-vitro induced ciprofloxacin resistance involved a combination of both genomic mutations and modulation of one or more pre-existing cellular pathways [27]. In *P. aeruginosa*, there was evidence for protein expression regulation during drug resistance development through nfxB, a repressor for the transcription of *mexCD-oprJ*, a multidrug efflux pump [9]. MexCD-OprJ upregulation correlated with an increased resistance to ciprofloxacin, cefepime, and chloramphenicol in most of the clinical strains, concomitant with a higher susceptibility to ticarcillin, aztreonam, imipenem, and aminoglycosides [11].

Antibiotic resistant bacteria thrive because of the selective pressure from antibiotics. While high level of antibiotics (more than minimum inhibitory concentration) can kill bacteria or select for resistant strains, low level of antibiotics (sub-MIC, below minimal inhibitory concentration) has been shown to contribute to the evolution of highly resistant bacteria by providing positive selection pressure for resistant genes [28]. Long-term exposure to Streptomycin at sub-MIC level creates several small-effect resistance mutations that combined to high-level resistance *Salmonella Enterica* [28]. Sub-MIC level of antibiotics may drive a number of phenotypic and genotypic changes (Bulgakova, Vinogradova, Orlova, Kozhevin, & Polin, 2014) and thus contribute to the development and spread of antimicrobial resistance bacteria. Sub-MIC antibiotics may be present in the environment and in human body and tissues (especially during antibiotic therapy, due to low bioavailability or inadequate dosage) [12], and is contributing the current development and spread of antimicrobial resistance bacteria, especially *P. aeruginosa* in clinical settings.

From above results, the effect of sub-MIC antibiotic exposure lasted beyond the initial 14 days. Ci-E2 showed no or only partial recovery in some of its virulence factors, corresponding to the reduced MIC value from E1 to E2. Even when E1 strains were cultured in antibiotic-free environment for 10 days, the reduced virulence induced by the antibiotic might still continue (table 3). These strains seemed have response memory, which is when a gene regulatory network continues to persist after the removal of its external inducer. Previously, bacteria exposed to one stressors over time can adapt better against other stressors [29]. Development and persistence of MDR, then, is the accumulation of small changes in gene regulation changes and genetic mutations in response to environmental (antibiotic) stress. Continuous exposure to a series antibiotics might train and prepare the bacteria to better adapt to antibiotic stress, giving rise to MDR strains. In fact, previous research where *P. aeruginosa* was exposed to 2 antibiotics in succession showed accumulations of mutations that contribute to the development of MDR pathogen [20]. Specific sequence of antibiotic treatments might have a higher chance of accumulate mutations and inducing MDR than others.

4 Conclusion

Sub-MIC Ciprofloxacin can provide selective pressure to induce the development of antibiotic resistance *P.aeruginosa*. The development of ciprofloxacin accompanied complex changes in morphology and virulence factors that likely involve changes at protein expression level and genomic level. These changes can persist even after 10 days in antibiotic-free environment, increasing the likelihood of developing multidrug resistance, especially during antimicrobial therapy.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

References

[1] Wu M, Li XF. Chapter 87: *Klebsiella pneumonia* and *Pseudomonas* aeruginosa. In Tang Y-W, Sussman M, Liu DY, Poxton I, Schwartzman J, eds. Molecular medical microbiology. 2nd ed. Amsterdam: Academic Press, 2015. p. 1547-1564.

[2] Hancock REW, Speert DP. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. Drug Resist Updat Rev Comment Antimicrob Anticancer Chemother. 2000; 3: 247–55.

[3] Poole K. Pseudomonas Aeruginosa: Resistance to the Max. Front Microbiol. 2011; 2: 65.

[4] Lambert PA. Mechanisms of antibiotic resistance in Pseudomonas aeruginosa. J R Soc Med 2002; 95(41): 22–6.

[5] Meletis G, Bagkeri M. *Pseudomonas aeruginosa*: Multi-Drug-Resistance Development and Treatment Options. Infect Control. 2013. [online].

[6] Gellatly SL, Hancock REW. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. Pathog Dis. 2013; 67: 159–73.

[7] Linares JF, López JA, Camafeita E, Albar JP, Rojo F, Martínez JL. Overexpression of the multidrug efflux pumps MexCD-OprJ and MexEF-OprN is associated with a reduction of type III secretion in Pseudomonas aeruginosa. J Bacteriol. 2005; 187: 1384–91.

[8] Tomás M, Doumith M, Warner M, Turton JF, Beceiro A, Bou G, et al. Efflux pumps, OprD porin, AmpC beta-lactamase, and multiresistance in Pseudomonas aeruginosa isolates from cystic fibrosis patients. Antimicrob Agents Chemother. 2010; 54: 2219–24.

[9] Purssell A, Poole K. Functional characterization of the NfxB repressor of the mexCD-oprJ multidrug efflux operon of Pseudomonas aeruginosa. Microbiol Read Engl. 2013; 159: 2058–73.

[10] Arevalo-Ferro C, Hentzer M, Reil G, Görg A, Kjelleberg S, Givskov M, et al. Identification of quorum-sensing regulated proteins in the opportunistic pathogen Pseudomonas aeruginosa by proteomics. Environ Microbiol. 2003; 5: 1350–69.

[11] Jeannot K, Elsen S, Köhler T, Attree I, van Delden C, Plésiat P. Resistance and virulence of Pseudomonas aeruginosa clinical strains overproducing the MexCD-OprJ efflux pump. Antimicrob Agents Chemother. 2008; 52: 2455–62.

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

[13] Jorgensen JH, Turnidge JD. Susceptibility test methods: dilution and disk diffusion methods,. In Murray PR, Baron EJ, Jorgensen JH, Landry ML, and Pfaller MA, eds, Manual of clinical microbiology, 9th ed. Washington,D.C.: ASM Press; 2007. p. 1152–1172

[14] Rust L, Messing CR, Iglewski BH. Elastase assays. Methods Enzymol. 1994; 235: 554–62.

[15] Younis G, Awad A, Mona M, Fatma S. Extracellular Enzymes and Toxins of Pseudomonas aeruginosa Strains Isolated from Clinically Diseased Egyptian Cows. Adv Anim Vet Sci. 2015; 3: 522–6.

[16] Sokol PA, Ohman DE, Iglewski BH. A More Sensitive Plate Assay for Detection of Protease Production by Pseudomonas aeruginosa. J Clin Microbiol. 1979; 9: 538–40.

[17] Filloux A, Hachani A, Bleves S. The bacterial type VI secretion machine: yet another player for protein transport across membranes. Microbiol Read Engl. 2008; 154: 1570–83.

[18] Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001; 9: 34–9.

[19] Thai VC, Lim TK, Le KPU, Lin Q, Nguyen TTH. ITRAQ-based proteome analysis of fluoroquinolone-resistant Staphylococcus aureus. J Glob Antimicrob Resist. 2017; 8: 82–9.

[20] Yen P, Papin JA. History of antibiotic adaptation influences microbial evolutionary dynamics during subsequent treatment. PLOS Biol. 2017; 15: e2001586.

[21] Qamer S, Sandoe JAT, Kerr KG. Use of colony morphology to distinguish different enterococcal strains and species in mixed culture from clinical specimens. J Clin Microbiol. 2003; 41: 2644–6.

[22] Sousa AM, Machado I, Nicolau A, Pereira MO. Improvements on colony morphology identification towards bacterial profiling. J Microbiol Methods. 2013; 95: 327–35.

[23] Beceiro A, Tomás M, Bou G. Antimicrobial Resistance and Virulence: a Successful or Deleterious Association in the Bacterial World? Clin Microbiol Rev. 2013; 26: 185–230.

[24] Li S, Jia X, Li C, Zou H, Liu H, Guo Y, et al. Carbapenem-resistant and cephalosporin-susceptible Pseudomonas aeruginosa: a notable phenotype in patients with bacteremia. Infect Drug Resist. 2018; 11: 1225–35.

[25] Geisinger E, Isberg RR. Interplay Between Antibiotic Resistance and Virulence During Disease Promoted by Multidrug-Resistant Bacteria. J Infect Dis. 2017; 215: S9–17.

[26] Sánchez P, Linares JF, Ruiz-Díez B, Campanario E, Navas A, Baquero F, et al. Fitness of in vitro selected Pseudomonas aeruginosa nalB and nfxB multidrug resistant mutants. J Antimicrob Chemother. 2002; 50: 657–64.

[27] Su H-C, Ramkissoon K, Doolittle J, Clark M, Khatun J, Secrest A, et al. The Development of Ciprofloxacin Resistance in Pseudomonas aeruginosa Involves Multiple Response Stages and Multiple Proteins. Antimicrob Agents Chemother. 2010; 54: 4626–35.

[28] Wistrand-Yuen E, Knopp M, Hjort K, Koskiniemi S, Berg OG, Andersson DI. Evolution of high-level resistance during low-level antibiotic exposure. Nat Commun. 2018; 9: 1599.

[29] Lagator M, Colegrave N, Neve P. Selection history and epistatic interactions impact dynamics of adaptation to novel environmental stresses. Proc R Soc B Biol Sci. 2014; 281: 20141679.