Annals of Medicine and Surgery 82 (2022) 104674

Contents lists available at ScienceDirect

Annals of Medicine and Surgery

journal homepage: www.elsevier.com/locate/amsu

Experimental Research

Effect of Ciprofloxacin, Levofloxacin, and Ofloxacin on *Pseudomonas aeruginosa*: A case control study with time kill curve analysis

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ARTICLE INFO

Keywords:
Ciprofloxacin
Levofloxacin
Ofloxacin
Pseudomonas aeruginosa
Time-kill curve

ABSTRACT

Background: Antibiotic resistance is closely related to therapy failure. Most antibiotic resistance is caused by delays in determining antibiotic agents, low administration doses, long periods between doses (inadequate pharmacokinetics) and single drug administration in infections caused by more than one pathogen. Treatment of *Pseudomonas aeruginosa* (*P. aeruginosa*) with ciprofloxacin, levofloxacin, and ofloxacin as monotherapy can lead to drug resistance, although combination therapy also does not provide a better outcome.
Objective: To analyze the time-kill curve for *P. aeruginosa* and Multidrug resistance (MDR) *P. aeruginosa*.
Methods: This research is a case control study using isolates of *P. aeruginosa* ATCC 27853, clinical isolates of *P. aeruginosa* and MDR *P. aeruginosa.* Exposure of ciprofloxacin, levofloxacin, and ofloxacin to isolates with 1MIC, 2MIC, and 4MIC were then cultured at 0, 2, 4, 6, 8, 24 h of testing, then counting the number of colonies that grew and then analyzed by time-kill curve and statistical tests. The statistical test used in this study was the ANOVA and Mann-Whitney test with $p < 0.05$.
Results: Ciprofloxacin and ofloxacin achieved bactericidal activity, especially at a concentration of 4MIC. Levofloxacin ultimately achieved bactericidal activity at all concentrations. Statistical analysis showed there were significant differences in the number of colonies $p < 0.001$ in the second, fourth, sixth, and eighth hour between the three isolates, $p < 0.001$ in the sixth and second 4 h between 1MIC and 4MIC, $p = 0.012$ in the second 4 h between levofloxacin and ofloxacin antibiotics.
Conclusion: Levofloxacin has shown to have better bactericidal activity than ciprofloxacin, and ciprofloxacin has almost the same bactericidal activity as ofloxacin in vitro tests seen from the time-kill curve.

1. Introduction

The increase in antimicrobial resistance reported by the Centers for Disease Control and Prevention in 2019 that *Pseudomonas aeruginosa* (*P. aeruginosa*) caused 2.8 infections and resulted in 35,000 deaths in the United States and 33,000 in Europe each year. It is estimated that by 2050 there will be 10 million cases of mortality and morbidity, and there may be no immediate action taken to prevent this from happening [1]. The association between multiple drug resistance bacteria and clinical outcome is poor, especially in comorbid patients [2].

Today's main problem in treating infectious diseases is the increased bacterial resistance to many antibiotics. Since Alexander Fleming discovered penicillin, antimicrobials have continued to be developed, but the rate of emergence of antibiotic-resistant bacteria is inversely proportional to the development of new antibiotic discoveries [3,4]. In order to solve this resistance problem, researchers are trying hard to find an effective way of treating infectious diseases using readily available antibiotics [2]. Effectiveness means choosing the antibiotic or using the correct dose to reduce the failure to treat infectious diseases using antibiotics. Antibiotic resistance is mainly caused by delays in determining antibiotic agents, low doses, long periods between doses (inadequate pharmacokinetics) and single drug administration in infections caused by more than one pathogen [5]. The choice of antibiotics can be based on epidemiological data of microorganisms and retrospective sensitivity at the site. So microbiological test results must guide the optimal empiric therapy and appropriate therapeutic targets. In addition, the antibiogram is used to guide selecting antibiotics that are expected to be clinically effective. Although empiric therapy with broad-spectrum

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https://doi.org/10.1016/j.amsu.2022.104674
Received 23 July 2022; Accepted 11 September 2022
Available online 16 September 2022
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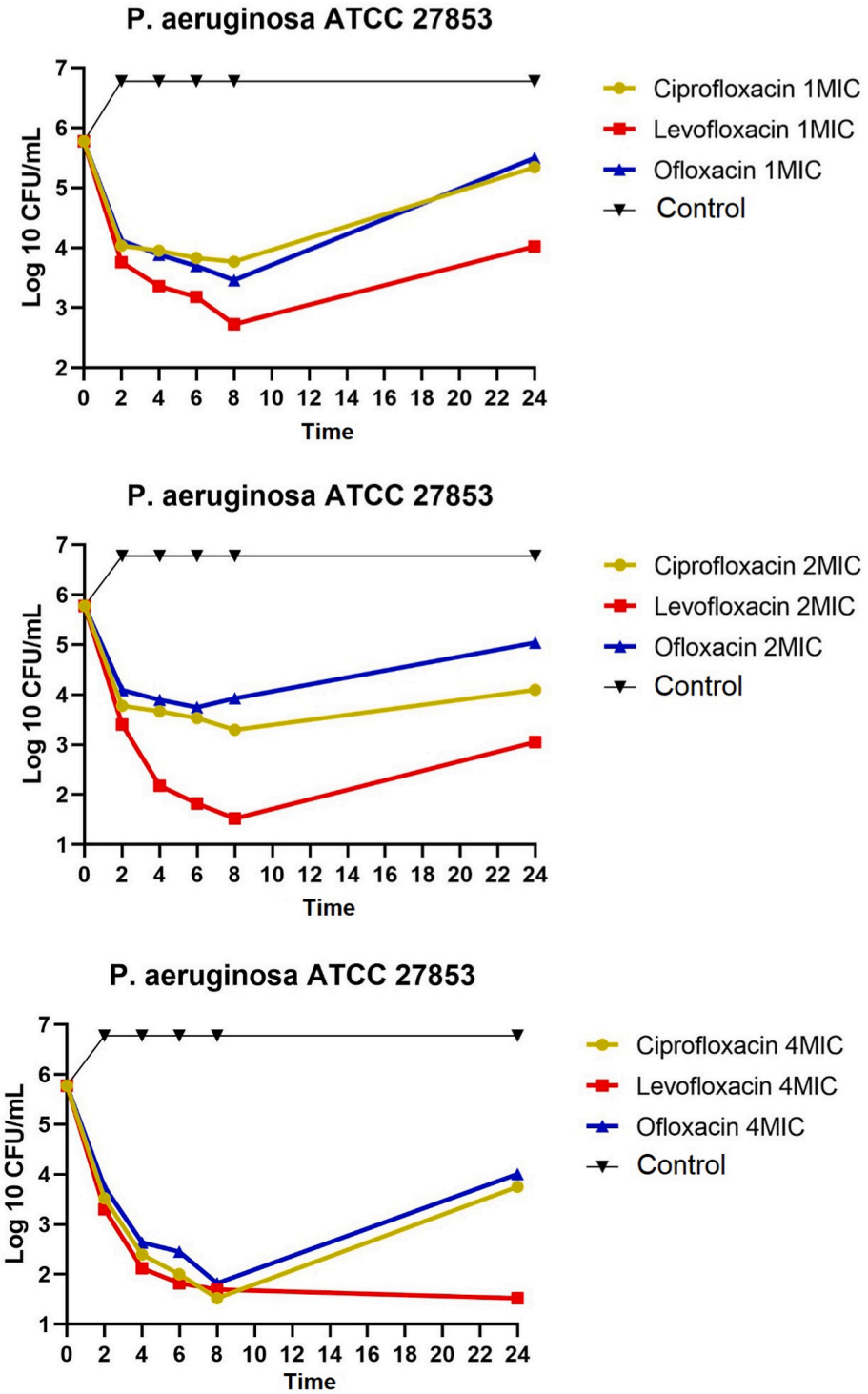

Fig. 1. Graph of time kills *P. aeruginosa* ATCC 27853 post-antibiotic exposure.

antibiotics has a history of therapeutic failure, guidance is still needed to select the best treatment optimally/effectively [6,7].

Treatment of *P. aeruginosa* with ciprofloxacin, levofloxacin, and ofloxacin as monotherapy will lead to drug resistance, although combination therapy does not provide a better outcome. Study at the Ardabil Hospital in Iran, by collecting 84 isolates of *P. aeruginosa* from June 2019 to February 2021, 48.8% (n = 41) were resistant to ciprofloxacin, primarily obtained from sputum samples, 31.7% (n = 13) [8,9]. In a 10-year German study, from January 2004 to August 2014, the response to levofloxacin in sensitivity tests found 30.6% of 168 resistant isolates, showing the poor effectiveness of levofloxacin for community-acquired or nosocomial-acquired pneumonia [10]. Levofloxacin sensitivity test on P. aeruginosa taken from pus and urine isolates in Surabaya from August 2005 to February 2006 showed that 42.76% (n = 145) were resistant to levofloxacin. In a subsequent study at the same place in 2019, it was stated that levofloxacin was still active as a bactericidal for *P. aeruginosa* isolates [11].

In vitro experiments can be based on the minimum inhibitory concentration (MIC) and time-kill curve to see the pharmacokinetic and pharmacodynamic activity of antibiotics. The effect of antibiotics at one

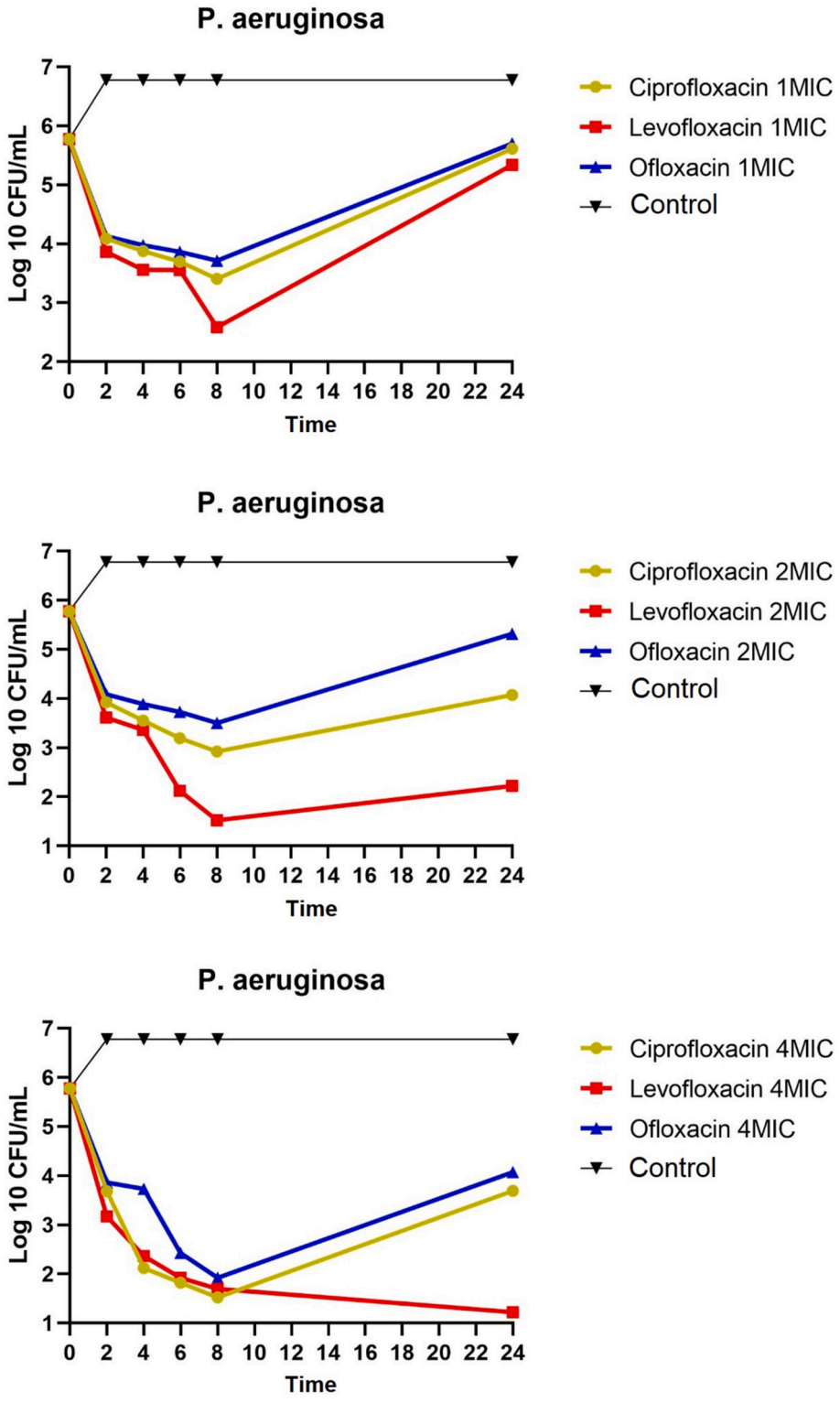

Fig. 2. Graph of *P. aeruginosa* kills time post-antibiotic exposure.

time occurring during the incubation period with the same concentration of the antibiotic agent was related to the MIC. MIC does not provide information on the rate of bacterial growth over time or the effect of antibiotics. At the same MIC value, the MIC cannot produce a combination of activity of growth rate and death of microorganisms. So it is more appropriate to use the time-kill curve method to observe antibiotic activity after microorganisms are exposed to antibiotics. The information obtained is more detailed and dynamic because this method shows antibiotic activity that depends on the amount of concentration and time [12,13].

Based on previous data regarding resistance to ciprofloxacin, levofloxacin and ofloxacin against *P. aeruginosa*, these three antibiotics are still often used as therapy, so this study wanted to see a comparison of the potency of these antibiotics in vitro with time-kill curve analysis of bacterial isolates *P. aeruginosa*.

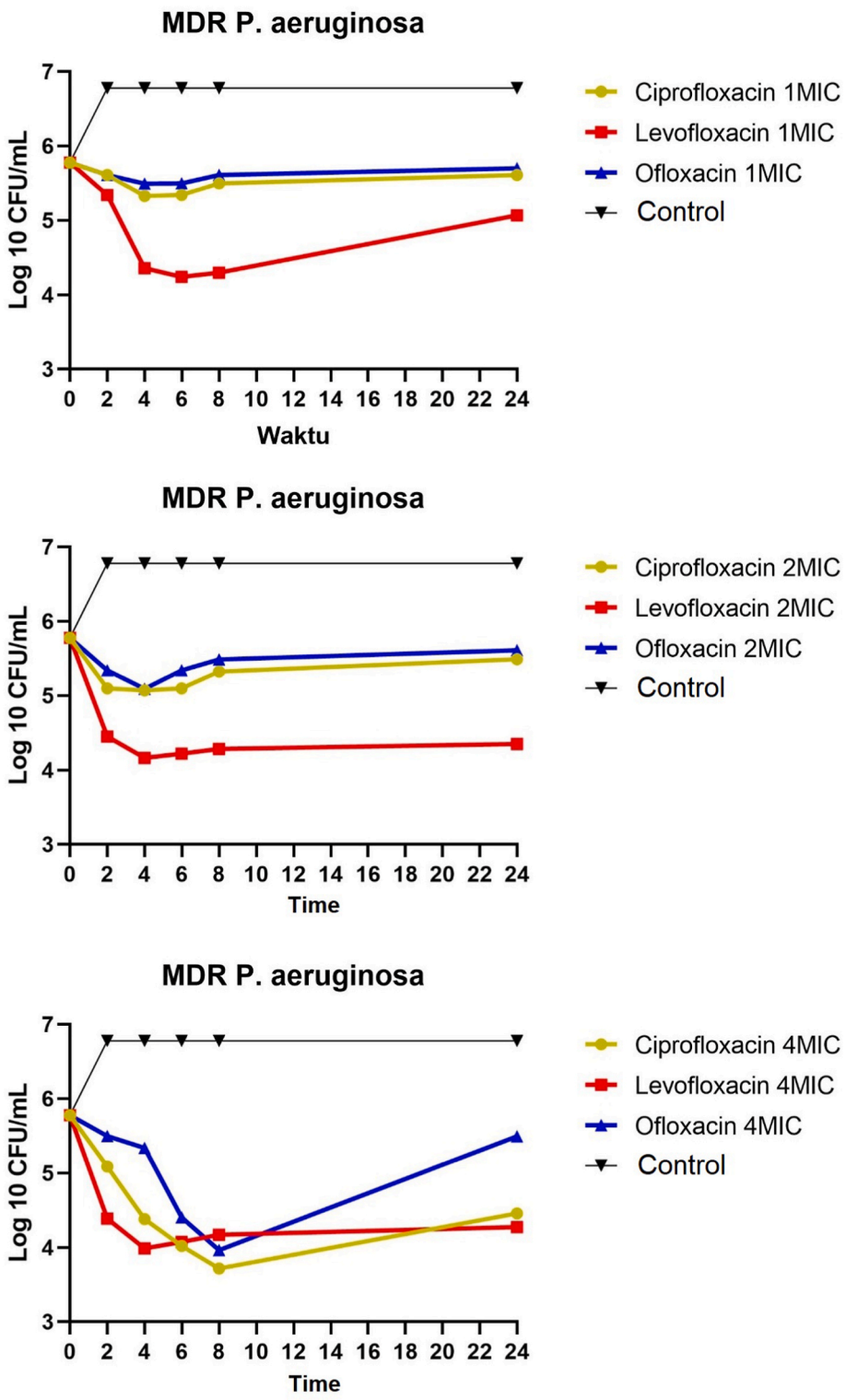

Fig. 3. Graph of *P. aeruginosa* MDR kills time post-antibiotic exposure.

2. Method

This study uses a case-control study with a posttest control group design. Subjects in this study consisted of *P. aeruginosa*, *P. aeruginosa* American type culture collection (ATTC) 27853, and multidrug resistance P. aeruginosa. The antibiotics used included ciprofloxacin (Graha farma ltd, Surakarta, Indonesia), levofloxacin (generic manufacturer), and ofloxacin (generic manufacturer). This study was carried out from June 2021–May 2022. In this study, each subject was divided into 3 MICs, such as 1 MIC, 2 MIC, and 4 MIC where the details of each MIC are as follows ciprofloxacin 0.25 p/mL (1 MIC), 0.5 p/mL (2 MIC), and 1 p/mL (4 MIC); levofloxacin at 2 p/mL (1 MIC), 4 p/mL (2 MIC), and 8 p/mL (4 MIC); ofloxacin at 4 p/mL (1 MIC), 8 p/mL (2 MIC), and 16 p/mL (4 MIC).

The procedure in this study included that each isolate of *P. aeruginosa* was given a MIC, and time-kill was assessed several times, such as 0, 2, 4,

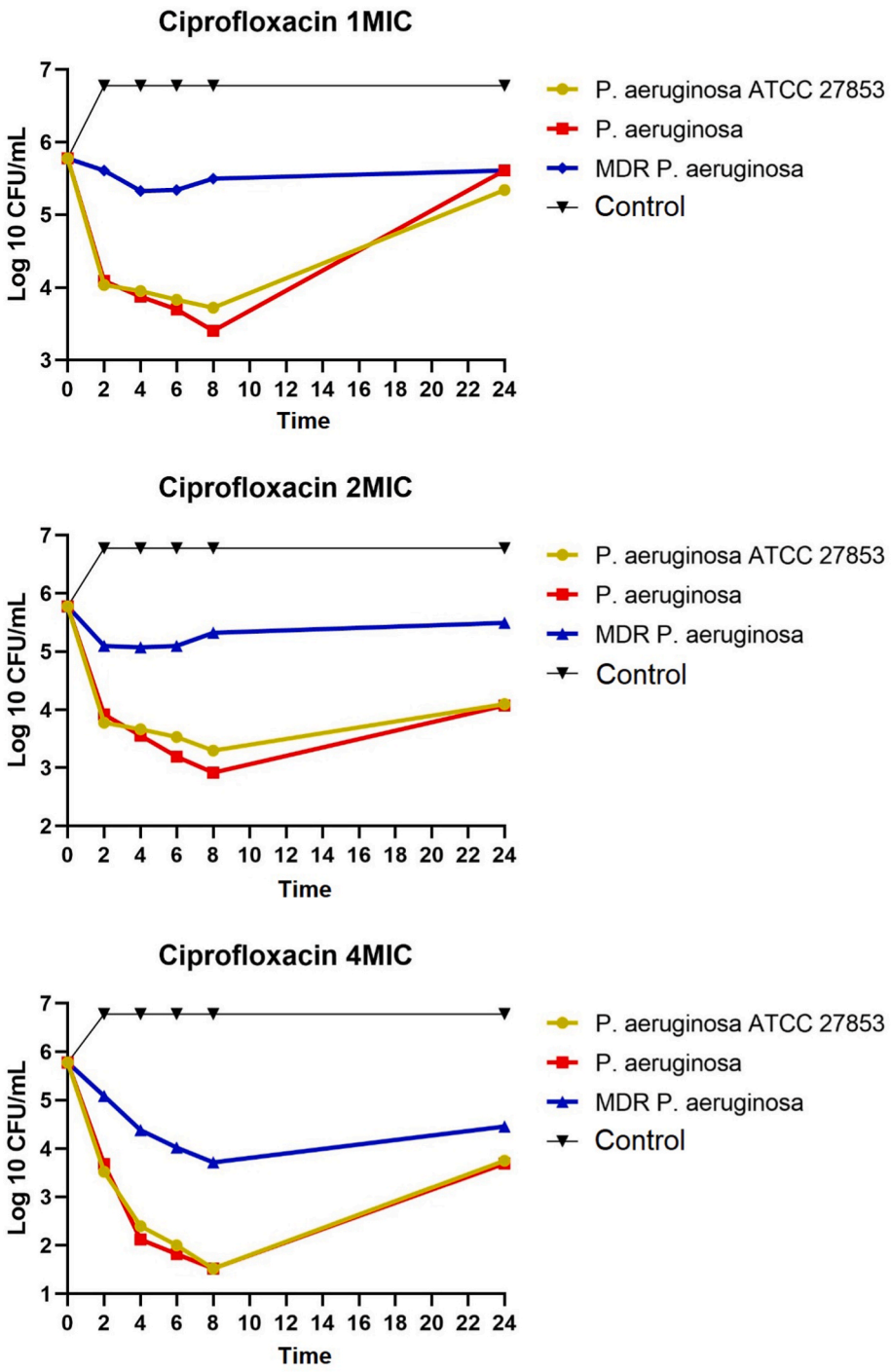

Fig. 4. Graphical kill time of ciprofloxacin in *P. aeruginosa*.

6, 8, and 24 h. Exposure to P. aeruginosa isolates with antibiotics was repeated 6 times, and the results were the average of these 6 results. Before the research, the isolate was confirmed to be not a fungal or sterile isolate, and *P. aeruginosa* isolate indicated to be sensitive to antibiotics manually. Measurement data were collected and analyzed using statistical product and service solution (SPSS) software version 21.0 (IBM Corp., Armonk, NY, USA). In addition, the analysis was also assisted by GraphPad Prism software version 8.0 (GraphPad Software, Inc., San Diego, CA). The statistical analysis used in this study was the ANOVA test, where the analysis results were declared significant if $p < 0.05$.

3. Result

The decrease in the number of *P. aeruginosa* ATCC 27853 colonies was effective 8 h after antibiotic exposure, whereas at 8 h, the bacterial count decreased at 1MIC, 2MIC, and 4MIC. The lowest decrease in the number of *P. aeruginosa* ATCC 27853 colonies was found at 8 h post-exposure to ciprofloxacin (4MIC) and levofloxacin (2MIC) which were 33.33 Log CFU/mL each (Fig. 1). The same condition was found in *P. aeruginosa* (Fig. 2). Different conditions were found in MDR *P. aeruginosa* where the lowest decrease in the number of colonies was different for each antibiotic use. When using ciprofloxacin on MDR *P. aeruginosa*, the lowest colony reduction was found as follows: 1MIC at

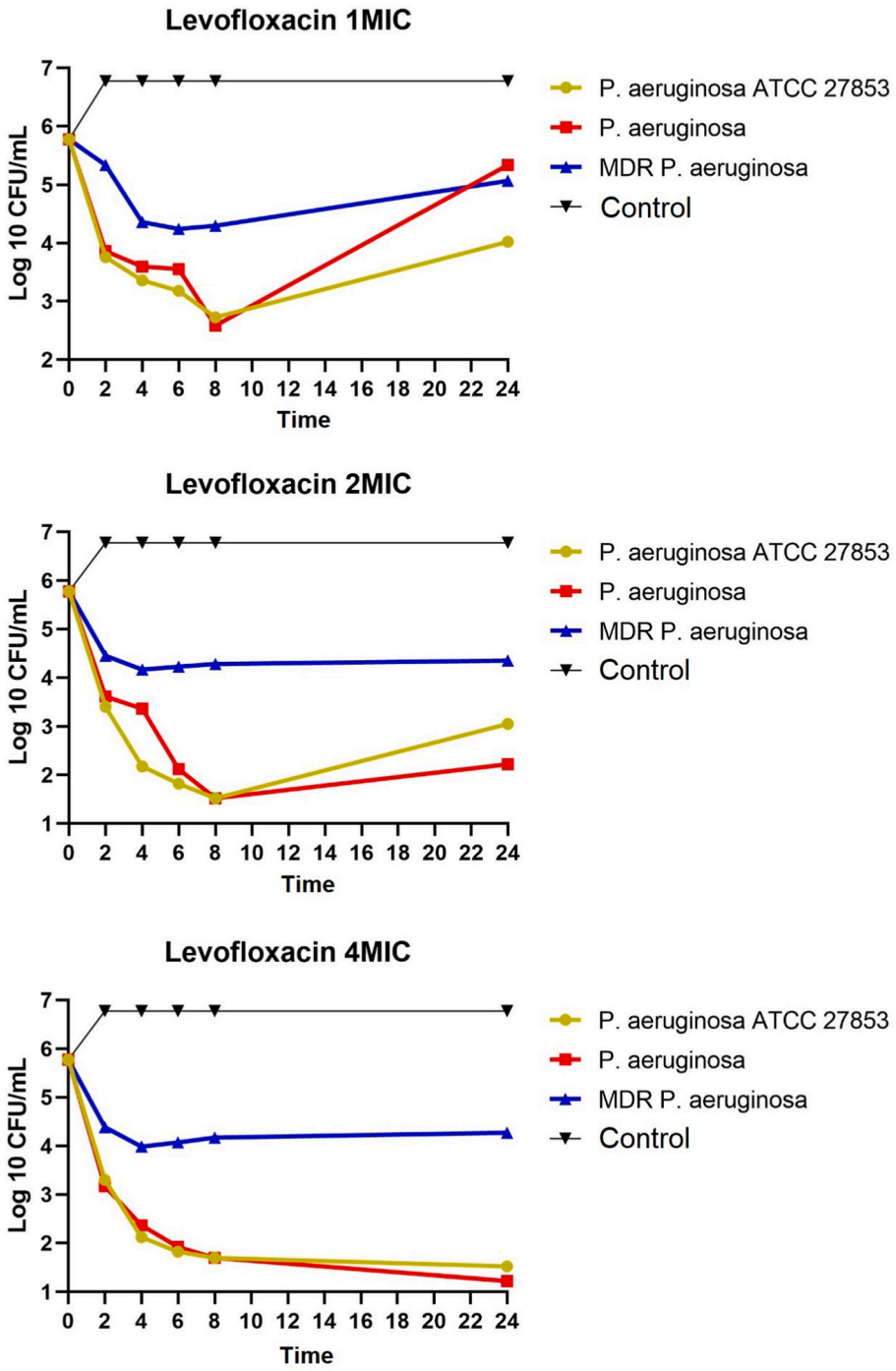

Fig. 5. Graphical kill time of levofloxacin in *P. aeruginosa*.

4 h (212,333.33 Log CFU/mL), 2MIC at 4 h (118,000 Log CFU/mL), while at 4MIC (5216.67 Log CFU)./mL) at the 8th hour. When using Levofloxacin on MDR *P. aeruginosa*, the lowest colony reduction was found at 1MIC at 6 h (315,000 Log CFU/mL), at 2MIC at 4 h (123.816.67 Log CFU/mL), and at 4MIC at 8 h too (9250 Log CFU/mL; Fig. 3).

Ciprofloxacin effectively reduced *P. aeruginosa* and *P. aeruginosa* ATCC colonies at 8 h of use (Fig. 4). Similar conditions were found with levofloxacin (Fig. 5) and ofloxacin (Fig. 6). Significant differences between antibiotics were only found in Levofloxacin vs Ofloxacin ($z = 2519$; $p = 0.012$; Table 1). There were some significant differences between antibiotics and *P. aeruginosa* species, among others, at 2 h post-antibiotics there was a significant difference between *P. aeruginosa* ATCC vs. MDR *P. aeruginosa* ($z = 3580$; $p < 0.001$) and *P. aeruginosa* vs. MDR *P. aeruginosa* ($z = 3.582$; $p < 0.001$); At 4 h post-antibiotics there was a significant difference between *P. aeruginosa* ATCC vs MDR *P. aeruginosa* (95% CI = 1.071–2.374; $p < 0.001$) and *P. aeruginosa* vs. MDR *P. aeruginosa* (95% CI = 0.764–2.067; $p < 0.001$); At 6 h post-antibiotics there was a significant difference between *P. aeruginosa* ATCC vs. MDR *P. aeruginosa* (95% CI = 1.027–2.557; $p < 0.001$) and *P. aeruginosa* vs. MDR *P. aeruginosa* (95% CI = 1.002–2.532; $p < 0.001$); and 8 h post-antibiotics, there was a significant difference between *P. aeruginosa* ATCC vs. MDR *P. aeruginosa* (z = 3490; p < 0.001) and *P. aeruginosa* vs. MDR *P. aeruginosa* ($z = 3.536$; $p < 0.001$; Table 2).

The effectiveness of the antibiotic dose against *P. aeruginosa* was found within a few hours of its use. Significant dose comparisons were obtained several hours after antibiotic administration, namely 6 h at

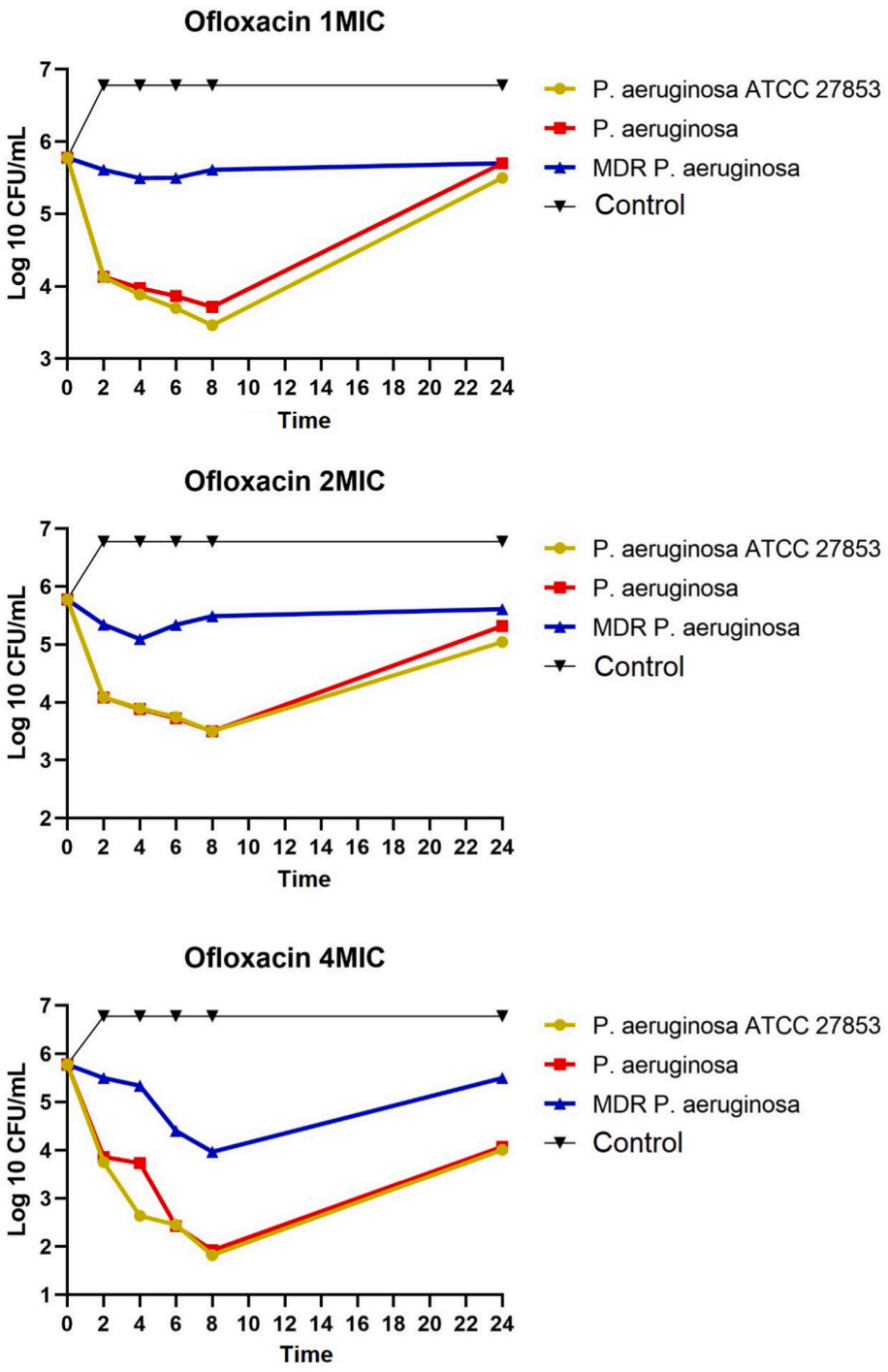

Fig. 6. Graphical kill time of ofloxacin in *P. aeruginosa.*

4MIC vs. 1MIC (95% CI = 0.328–2.337; $p = 0.011$), 24 h at 1MIC vs. 2 MIC ($z = 2.170$; $p = 0.030$), and 24 h hours at 4MIC vs. 1MIC ($z = 2.920$; $p = 0.004$; Table 3).

4. Discussion

The success of antimicrobial therapy is determined by the interaction of drug administration, the state of the host and the causative agent of infection. In the clinic, varying drug doses affect the host response, so to minimize variations in response to dose, drug characteristics, infectious agents, and the host must consider determining the antibiotic and dose to be used. Failure of therapy will trigger the emergence of resistant strains [14]. Fluoroquinolones are antibiotics often used in clinical practice in infections caused by *P. aeruginosa* because fluoroquinolones have a narrow spectrum of activity. Fluoroquinolones are generally well tolerated by the human body [15]. Often found in the use of fluoroquinolones, great potential for the emergence of antibiotic resistance. Levofloxacin susceptible test on *P. aeruginosa* taken from pus and urine isolates in Surabaya from August 2005 to February 2006 showed 42.76% (n = 145) resistance to levofloxacin [11,16].

The time-kill curve test has been widely used to test the concentration level of antimicrobial agents with bactericidal activity. This bactericidal activity can be used in concentration-dependent and time-dependent antimicrobial [17]. The time-kill test was used to see the

Table 1
Analysis of differences in the effectiveness of antibiotics used in *P. aeruginosa*.

Time Kill	Antibiotic		CI 95%	*p*-value
2 h	Ciprofloxacin	Levofloxacin	–	–
	Levofloxacin	Ofloxacin	–	–
	Ofloxacin	Ciprofloxacin	–	–
4 h	Ciprofloxacin	Levofloxacin	−0.400–1.476	0.248
	Levofloxacin	Ofloxacin	−0.161–0.074	0.061
	Ofloxacin	Ciprofloxacin	−0.581–1.295	0.440
6 h	Ciprofloxacin	Levofloxacin	−0.467–1.705	0.251
	Levofloxacin	Ofloxacin	−0.171–2.002	0.095
	Ofloxacin	Ciprofloxacin	−0.789–1.383	0.578
8 h	Ciprofloxacin	Levofloxacin	–	–
	Levofloxacin	Ofloxacin	–	–
	Ofloxacin	Ciprofloxacin	–	–
24 h	Ciprofloxacin	Levofloxacin	–	0.077
	Levofloxacin	Ofloxacin	–	0.012*
	Ofloxacin	Ciprofloxacin	–	0.215

Note: *Significant <0.05; **Significant <0.01.

Table 2
Analysis of the difference in the effectiveness of antibiotics on each type of *P. aeruginosa*.

Time Kill	*P. aeruginosa*		CI 95%	*p*-value
2 h	Regular	ATCC	–	0.310
	ATCC	MDR	–	<0.001**
	MDR	Regular	–	<0.001**
4 h	Regular	ATCC	−0.345–0.958	0.341
	ATCC	MDR	1.071–2.374	<0.001**
	MDR	Regular	0.764–2.067	<0.001**
6 h	Regular	ATCC	−0.740–0.791	0.946
	ATCC	MDR	1.027–2.557	<0.001**
	MDR	Regular	1.002–2.532	<0.001**
8 h	Regular	ATCC	–	0.894
	ATCC	MDR	–	<0.001**
	MDR	Regular	–	<0.001**
24 h	Regular	ATCC	–	–
	ATCC	MDR	–	–
	MDR	Regular	–	–

Note: *Significant <0.05; **significant <0.001.

Table 3
Analysis of different antibiotic doses on *P. aeruginosa*.

Time Kill	Antibiotic Dose		CI 95%	*p*-value
2 h	1MIC	2MIC	–	–
	2MIC	4MIC	–	–
	4MIC	1MIC	–	–
4 h	1MIC	2MIC	−0.653–1.206	0.545
	2MIC	4MIC	−0.282–1.577	0.163
	4MIC	1MIC	−0.005–1.854	0.051
6 h	1MIC	2MIC	−0.551–1.458	0.361
	2MIC	4MIC	−0.126–1.883	0.084
	4MIC	1MIC	0.328–2.337	0.011*
8 h	1MIC	2MIC	–	–
	2MIC	4MIC	–	–
	4MIC	1MIC	–	–
24 h	1MIC	2MIC	–	0.030*
	2MIC	4MIC	–	0.185
	4MIC	1MIC	–	0.004*

Note: MIC = Minimum inhibitory concentration; *Significant <0.05; **significant <0.001.

change in the number of colonies at different concentrations in vitro, and the MIC was used to quantify the antibiotic activity against bacteria [18]. Although the time-kill test is clearly defined in the CLSI protocol, the enumeration has not been standardized. For the time-kill test, determine the MIC of the antimicrobial agent, typically 1MIC, 2MIC, and 4MIC. Also, determine the time to see the growth rate of bacteria, usually 4, 8, 24, 48, but in this study, due to using antibiotics that have concentration-dependent bactericidal activity, the closer time points are 0, 2, 4, 6, 8, 24 h [19,20].

With the administration of fluoroquinolones, maximizing the intensity of exposure by maximizing the antibiotic concentration is like giving the total daily dose in single-dose rather than divided doses [14]. Levofloxacin was statistically more bactericidal than ciprofloxacin, and ciprofloxacin was more potent than ofloxacin [21]. Regrowth often occurs in the time-kill test of bacteria with antimicrobials. This phenomenon has several possibilities. The first possibility is due to the presence of a subpopulation or a small number of bacteria that are susceptible to fluoroquinolone exposure, and this subpopulation can survive exposure to antibiotics. This subpopulation is called persisters. It was also explained earlier that as a weakness of the time-kill test, several factors influence the measurement of the lethal effect of antibiotics in vitro studies, including persistence, paradoxical and resistance. Factors from the technique, such as bacterial growth phase and inoculum size, must be calculated [22].

When antibiotic exposure is given, this subpopulation's metabolism is inactive or dormant but returns to being susceptible to antibiotics when the antibiotic concentration is lowered below the MIC, or antibiotic exposure is removed [23]. The second possibility is the occurrence of spontaneous mutations. It is known that the emergence of resistance of susceptible bacterial isolates is obtained through spontaneous mutation or resistance genes from other bacterial isolates with resistance genes [6]. Spontaneous mutations can occur because the fluoroquinolone concentration is given at the MIC concentration. To prevent spontaneous mutations, fluoroquinolones should be given at concentrations above the mutant prevention concentration (MPC) or in the range of MIC and MPC concentrations, called the mutant selection window (MSW). MPC for each drug was different, MPC 3 μg/mL for ciprofloxacin and 9.5 μg/mL for levofloxacin [24].

Fluoroquinolones must be administered in high concentrations to prevent the development of resistance to *P. aeruginosa*; this is consistent with the fact that fluoroquinolones are concentration-dependent bactericidal antibiotics [25]. When the antibiotic concentration was increased but followed the MIC, several colonies would initially die, but there would still be ±1% of the surviving colonies. This subpopulation will undergo spontaneous mutations, and if there are repeated mutations, then this mutant will become a resistant subpopulation. When the concentration is increased beyond the MIC to reach MPC, the mutant will be cleared by the drug concentration and can reduce the number of colonies from the surviving subpopulation to 0%. Above concentrations that exceed the MIC, no more mutants can survive. So, the MPC is the MIC of the susceptible subpopulation, then mutated many times into a mutant subpopulation. The MPC provides a guide to the dosing strategy used as an antimutant, and keeping the drug concentration for therapy above the MPC is hoped to limit mutant regrowth [26].

The structure of this subpopulation of mutants is different from that of susceptible isolates, the mutants will replicate into many, and these resistant mutants become the dominant member of the population [27]. It is thought that drug levels must exceed at least 8–10 times the MIC to prevent the emergence of a resistant population, which can be seen with the use of such drugs as a single daily dose of fluoroquinolones [28]. In clinical practice, in antibiotic therapy, to minimize the increase in the size of the pathogen inoculum, antibiotic therapy in the shortest possible time is required using MSW [29].

5. Conclusion

Levofloxacin showed better bactericidal activity than ciprofloxacin and ofloxacin in vitro tests seen from the time-kill curve. Ciprofloxacin has bactericidal activity not as good as levofloxacin and almost the same as ofloxacin in vitro tests seen from the time-kill curve. Ofloxacin has bactericidal activity not as good as ciprofloxacin and levofloxacin in vitro tests seen from the time-kill curve. Statistically, there was a significant difference in the number of *P. aeruginosa* ATCC 27853,

P. aeruginosa and MDR *P. aeruginosa* starting from the initial exposure to the three antibiotics.

Ethical approval

We have conducted an ethical approval base on the Declaration of Helsinki with registration research at the Health Research Ethics Committee in Dr. Soetomo General Academic Hospital, Surabaya, Indonesia.

Please state any sources of funding for your research

None.

Author contribution

All authors contributed toward data analysis, drafting and revising the paper, gave final approval of the version to be published and agree to be accountable for all aspects of the work.

Registration of research studies

1. Name of the registry: Health Research Ethics Committee in Dr. Soetomo General Academic Hospital, Surabaya, Indonesia.
2. Unique Identifying number or registration ID: 0364/KEPK/I/2022.
3. Hyperlink to your specific registration (must be publicly accessible and will be checked).

Guarantor

Agung Dwi Wahyu Widodo is the person in charge of the publication of our manuscript.

Consent

All participants are required to fill out an informed consent.

Provenance and peer review

Not commissioned, externally peer-reviewed.

Declaration of competing interest

Tiar Sondang Uli Sihotang, Agung Dwi Wahyu Widodo, and Pepy Dwi Endraswarideclare that they have no conflict of interest.

Acknowledgement

We would like thanks to our editor, “Fis Citra Ariyanto”.

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