ORIGINAL ARTICLE

Bali Medical Journal (*Bali MedJ*) 2024, Volume 13, Number 1: 811-815
P-ISSN.2089-1180, E-ISSN: 2302-2914

Comparison of generation time between *Escherichia coli* non-extended spectrum beta-lactamase (non-ESBL) and ESBL on ciprofloxacin and tetracycline induction: Experimental research

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Received: 2024-10-23
Accepted: 2024-01-17
Published: 2024-02-14

ABSTRACT

Background: *Escherichia coli* (*E. coli*) is the leading cause of sepsis in the world, a country with low-middle income and the fourth largest population globally. First-generation antibiotics are still common, especially fluoroquinolones and tetracyclines, which often experience resistance in *E. coli*. This study aimed to analyze comparison of generation time of *E. coli* on ciprofloxacin and tetracycline.

Methods: This study was experimental with a posttest-only control group design. The subjects of this study were *E. coli* non-extended spectrum beta-lactamase (non-ESBL) and ESBL, which have ciprofloxacin of 0.25 µg/mL and tetracycline of 4 µg/mL exposed. Colony counting was performed every hour for twelve hours. The statistical test used was the independent *t*-test with $p < 0.05$.

Result: *E. coli* non-ESBL on ciprofloxacin induction showed a generation time of 52.76 min and tetracycline of 53.66 min. However, *E* ESBL on ciprofloxacin induction showed a generation time of 53.96 min and tetracycline of 55.40 min. The comparison of *E. coli* non-ESBL exposed to ciprofloxacin and tetracycline showed no significant difference ($t = 1.364$; 95% CI = -0.565 – 2.352; $p = 0.202$) and *E. coli* ESBL also ($t = 1.469$; CI 95% = -0.748 – 3.645; $p = 0.173$).

Conclusion: No significant comparison exists between generation time in *E. coli* non-ESBL and ESBL on ciprofloxacin and tetracycline induction.

Keywords: Antibiotic resistance, *Escherichia coli*, infection disease, generation time.
Cite This Article: Mulyawan, B., Widodo, A.D.W., Endraswari, P.D. 2024. Comparison of generation time between *Escherichia coli* non- extended spectrum beta-lactamase (non-ESBL) and ESBL on ciprofloxacin and tetracycline induction: Experimental research. *Bali Medical Journal* 13(1): 811-815. DOI: 10.15562/bmj.v13i1.4890

INTRODUCTION

Escherichia coli (*E. coli*) is a member of the Enterobacteriaceae family, which ranks first in causing sepsis and is one of the most frequent causes of bacterial sepsis.[1] *E. coli* is an opportunistic pathogenic bacterium that shows increased resistance to various antibiotics and is one of the causes of the emergence of extended-spectrum beta-lactamase (ESBL).[2] A systematic review and meta-analysis showed that infection with multi-resistance *E. coli* is associated with a significant increase in mortality.[3] The World Health Organization (WHO) states that the multi-resistance pathway of this bacterium is one of the critical criteria in the list of priority pathogens for antimicrobial research and development.[4]

ESBL still becomes a health problem that needs attention because its prevalence tends to increase, resulting in high morbidity and mortality. Epidemiologically, the prevalence of ESBL spread in various countries varies. The prevalence of ESBL produced by *E0. coli* in Latin America is 42.7%, North America of 5.8%, Europe of 2-31%, and Asia of 4.8-12%.[5] Surveillance conducted in Indonesia in July-December 2012 showed the presence of *E. coli* ESBL of 52%.[6] A study in Nigeria reported that among all ESBL-producing isolates, 35% were from the community and 65% from the hospital. ESBL isolates show high resistance to gentamicin, levofloxacin, ceftriaxone, cefuroxime, ciprofloxacin, tetracycline and Augmentin (a combination of amoxicillin and clavulanic acid).[7] These ESBL-producing bacteria can inactivate most beta-lactam class of antibiotics such as penicillin, cephalosporin and monobactam. They can cause cross-resistance with several other antibiotics, such as the fluoroquinolone, aminoglycoside, tetracycline and trimethoprim-sulfamethoxazole groups.[8]

Ciprofloxacin is a member of the third-generation quinolone, which is recognized to have excellent activity against Gram-negative bacteria.[9] A study in Iran showed that 45% of *E. coli* isolates were resistant to ciprofloxacin.[10] Another study showed that ciprofloxacin doses of 500 mg and 750 mg significantly reduced the number of *E. coli* bacteria colonies in the first 2 hours.[11] Meanwhile, tetracycline is a broad-spectrum antibiotic that can prevent bacterial growth by inhibiting protein biosynthesis.[12] The prevalence of

tetracycline resistance from *E. coli* isolates increased by 0.45% annually from 1950 to 2001.[13] In Indonesia, tetracycline is the most widely available antibiotic in health care units, especially in community health center "Puskesmas", which has led to increased tetracycline resistance.[14]

Ciprofloxacin and tetracycline are essential drugs used in Indonesia. These selected drugs are most needed for health services, including diagnosis, prophylaxis, therapy and rehabilitation, which are available in health facilities according to function and level and included in the first-line (access) antibiotic group. These two antibiotics have the most patient benefit-risk ratio, guaranteed quality, stability and bioavailability, the highest benefit-cost ratio based on direct and indirect costs, and easy to obtain known drugs.[15]

The prevalence of *E. coli* non-ESBL and ESBL in several countries is still high. Data showing the frequent used of ciprofloxacin and tetracycline has motivated the researchers to examine the effect of exposure to these two antibiotics on the growth of *E. coli* non-ESBL and ESBL resistant by observing the generation time. Factors influencing bacterial growth include the availability of nutrients, humidity, temperature, pH, oxygen and osmotic pressure.[16] Previous studies have shown that in addition to antibiotic resistance, bacteria can survive with antibiotics as a carbon source.[17] Therefore, this study aimed to compare the generation time of *E. coli* non-ESBL and ESBL exposed to ciprofloxacin and tetracycline.

METHODS

This study used an experimental study with a posttest-only control group design. Subjects in this study consisted of *E. coli* non-ESBL and ESBL. This study measured the time generation of *E. coli* on antibiotic drugs exposed. The antibiotic drugs used consisted of ciprofloxacin and tetracycline. Ciprofloxacin is a member of the third-generation quinolone, which is recognized to have excellent activity against Gram-negative bacteria.[9] Tetracycline is a broad-spectrum antibiotic that can prevent bacterial growth by inhibiting protein biosynthesis.[12] The minimum inhibitory concentration (MIC) used in this study was 0.25 µg/mL (ciprofloxacin) and 4 µg/mL (tetracycline) based on Clinical and Laboratory Standards Institute 2022.[18] This study was conducted from January to December 2022.

The procedure in this study included that each *E. coli* non-ESBL and ESBL isolate was put into Mueller Hinton Broth (MHB). Each subject was exposed to ciprofloxacin and tetracycline according to the MIC dose at an aerobic incubation temperature of 35-37°C. Generation time measurement at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 hours. Exposure of *E. coli* isolates with antibiotics was repeated 6 times, and the results used were the average of the 6 results. Generation time calculations were assisted by the BD Phoenix automated system (BD Biosciences, USA).

Measurement data were collected and analyzed using statistical product and service solution (SPSS) software version 21.0 (IBM Corp., Armonk, NY, USA). The statistical analysis used in this study was an independent *t*-test in which a normality test carried out the measurement data. The results of the analysis were declared significant if $p < 0.05$.

RESULT

Ciprofloxacin

The exponential phase of the growth curve of *E. coli* non-ESBL upon exposure to ciprofloxacin lasted for five hours, occurring from hour zero to hour five (Figure 1A). The average value of colonies at the beginning of the exponential phase was 7.0×10^3 CFU/mL and the average value of colonies at the end of the exponential phase was 3.6×10^5 CFU/mL. Meanwhile, *E. coli* ESBL found conditions similar to the growth of *E. coli* non-ESBL (Figure 1B). The average value of colonies at the beginning of the exponential phase was 9.0×10^3 CFU/mL and the average value of colonies at the end of the exponential phase was 4.2×10^5 CFU/mL. On ciprofloxacin, the mean total generation time value was 53.36 ± 1.60 min with a median of 53.14 (51.80 – 54.07) min. Meanwhile, the minimum generation time in ciprofloxacin is 51.51 min and the maximum is 56.37 min. Based on ESBL, the mean generation time value in E. coli ESBL was 53.95 ± 2.00 min and non-ESBL was 52.76 ± 0.90 min ($t = 1.322$; 95% CI = -0.814 – 3.191; $p = 0.216$; Table 1).

Tetracycline

The exponential phase of the growth curve of *E. coli* non-ESBL to tetracycline exposure lasted for five hours from hour zero to hour five (Figure 2A). The average value of colonies at the beginning of the exponential phase was 1.0×10^4 CFU/mL and the average value of colonies at the end of the exponential phase was 4.8×10^5 CFU/mL. On the growth curve of *E. coli* ESBL to tetracycline exposure, results were similar to those of *E. coli* non-ESBL (Figure 2B). The average value of colonies at the beginning of the exponential phase was 1.2×10^4 CFU/mL and the average value of colonies at the end of the exponential phase was 5.1×10^5 CFU/mL. The mean of total generation time in the tetracycline group was 54.53 ± 1.56 min with a median of 54.77 (52.78 – 55.46) min. In the generation time in tetracycline, the minimum value was 52.57 min and the maximum value was 57.22 min. In the tetracycline group, the mean generation time value in *E. coli* ESBL was 55.40 ± 1.34 min and non-ESBL was 53.66 ± 1.32 min ($t = 2.264$; 95% CI = 0.027 – 3.458; $p = 0.047$; Table 1).

Comparison of generation time between Escherichia coli on antibiotic drug expose

Comparison of the generation time of *E. coli* non-ESBL exposed to ciprofloxacin and tetracycline showed no significant comparison ($t = 1.364$; 95% CI = -0.565 – 2.352; $p = 0.202$). Meanwhile, *E. coli* ESBL showed no significant comparison between the generation time of ciprofloxacin and tetracycline exposure ($t = 1.469$; 95% CI = -0.748 – 3.645; $p = 0.173$). Comparative description of the analysis of the generation time of *E. coli* on ciprofloxacin and tetracycline induction (Figure 3). No significant comparison exists of generation time in E. coli between ciprofloxacin and tetracycline ($t = -1.808$; 95% CI = -2.514 – 0.172; $p = 0.084$)

DISCUSSION

E. coli ranks first as a cause of sepsis.[1] *E. coli* is an opportunistic pathogenic bacterium which is one of the causes of ESBL emergence.[2] A study showed that

infection with multi-resistant strains of *E. coli* was associated with a significant increase in mortality and was even recommended by WHO as a target for antimicrobial development.[3] Surveillance in Indonesia showed the prevalence of *E. coli* ESBL reaches 31.4-52%.[6]

Ciprofloxacin (fluoroquinolone) and tetracycline are the most widely used antimicrobials globally, including in Indonesia.[19] These two antibiotics are essential national drugs in the first-line antibiotic group. The high use of ciprofloxacin and tetracycline is known to increase the prevalence of bacterial resistance to these two types of antibiotics.[20] The use of ciprofloxacin and tetracycline as first-line antibiotics with a high prevalence of resistance can result in high bacterial exposure to these antibiotics at concentrations lower than the MIC.[21]

The use of substandard and counterfeit (fake) antibiotics in society increases the risk of bacterial exposure to antibiotics with subinhibitory concentrations. Substandard antibiotics are antibiotics that do not meet established quality test standards. Counterfeit antibiotics are substandard antibiotics with discrepancies between the labels listed and the ingredients contained in the drug; for example, the content is lower, higher, does not match the type or does not exist.[22] Systematic reviews estimate the prevalence of substandard antibiotics worldwide to be around 17-25%, with the highest prevalence in developing countries (low- and middle-income countries, LMIC).[23,24]

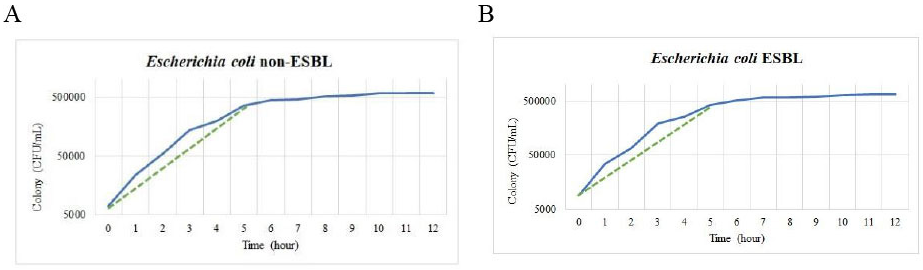

Figure 1. Generation time curve of *E. coli* non-ESBL and ESBL on ciprofloxacin exposure.

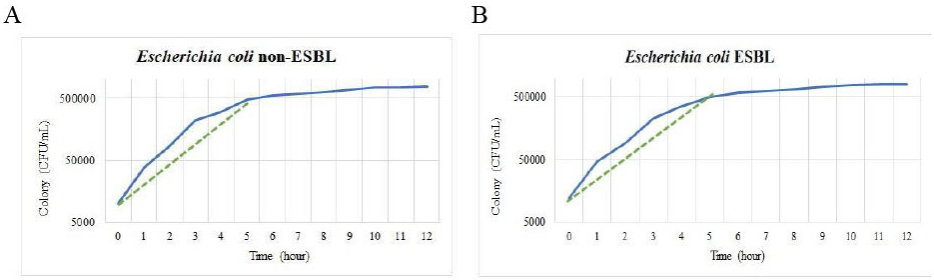

Figure 2. Generation time curve of *E. coli* non-ESBL and ESBL on tetracycline exposure.

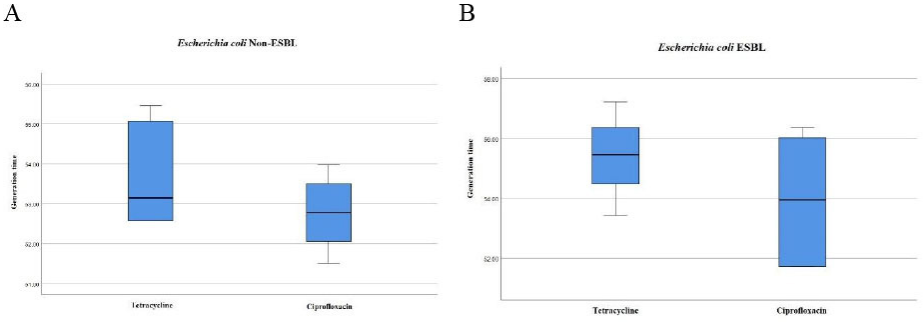

Figure 3. Generation time curve of *E. coli* on ciprofloxacin and tetracycline exposure.

Ciprofloxacin works by inhibiting DNA gyrase and DNA topoisomerase IV enzymes. Ciprofloxacin binds to the DNA-enzyme complex and inhibits DNA replication, and its activity tends to be dose-dependent and bactericidal.[25] Bacterial resistance to ciprofloxacin can occur through several mechanisms, with mutations in the gyrA gene being the mechanism that most contributes to the increase in MIC that occurs.[26,27] Bacteria that were resistant to ciprofloxacin showed mutations in genes encoding global transcriptional regulators, central metabolic pathways, and stress responses.[27,28]

The study showed that the average generation time when exposed to ciprofloxacin at concentrations below the MIC was higher than that of *E. coli* found in the literature.[29] Exposure to ciprofloxacin at subinhibitory concentrations triggers an SOS response in *E. coli* bacteria, causing mutagenic modification, inhibiting cell division, and causing bacterial filamentation.[30,31] Exposure to ciprofloxacin at subinhibitory concentrations is also known to induce biofilm formation in various bacteria, including *E. coli*.[32]

Tetracycline binds to the 30S subunit of the bacterial ribosome and inhibits protein synthesis. Tetracyclines are bacteriostatic, partly due to their reversible binding to the bacterial ribosome. Most resistance to tetracycline is caused by the expression

Table 1. Comparison of generation time between *E. coli* ESBL and non-ESBL

Antibiotic	ESBL		Non-ESBL		*t*	*p*-value
	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)		
Tetracycline	55.40 ± 1.34	55.46 (54.21 – 56.58)	53.66 ± 1.32	53.14 (52.57 – 55.16)	2.264	0.047*
Ciprofloxacin	53.95 ± 2.00	53.94 (51.72 – 56.11)	52.76 ± 0.90	52.78 (51.92 – 53.61)	1.322	0.216

SD: Standard deviation; IQR: Interquartile range; E. coli=Escherichia coli; ESBL=extended spectrum beta-lactamase; non-ESBL=non-extended spectrum beta-lactamase
**Significant <0.05*

of the efflux pump encoded by a gene in the plasmid.[12] *E. coli* was resistant to tetracycline changes in global metabolism and transcription, including changes in the tricarboxylic acid (TCA) cycle and redox homeostasis, which aims to support the work of the efflux pump.[33]

The study showed that the average generation time when exposed to tetracycline with concentrations below the MIC was higher than that of *E. coli* found in the literature.[29] Previous study showed that exposure to tetracycline 0.015 µg/mL and 0.03 µg/mL resulted in a higher final density of bacteria than the control. This effect was not found at all concentrations of the tetracycline subinhibitory used in the study because a concentration of 0.3 times the MIC (0.12 µg/mL) reduced the density of *E. coli* bacteria to 86% of the control group.[34] Tetracycline with a lower concentration of MIC induced biofilm formation in *E. coli* as a ribosome response to translational disturbances produced by these antibiotics.[35] Another study found that exposure to subinhibitory concentrations of tetracycline to *E. coli* tends to reduce the bacterial metabolic rate, although the mechanism by which this occurs was not fully understood.[36]

Previous studies have shown that ciprofloxacin and tetracycline with subinhibitory concentrations trigger a similar subcellular response from *E. coli* bacteria.[32] However, the two antibiotics have different mechanisms of action in inhibiting bacterial growth.[32] These responses can be specific to the given concentration of antibiotics.[30,32] They are using ciprofloxacin and tetracycline as first-line empiric therapy in populations with a high prevalence of resistance results in bacteria exposure to subinhibitory concentrations. Consumption of sub-standard and counterfeiting antibiotics also increases the risk. This exposure has the risk of causing the selection of resistant strains, increasing genotypic and phenotypic diversity, and changes in the physiology of bacterial cells caused by the role of antibiotics as signaling molecules.[3]

This study showed that the ability of bactericidal antibiotics is sometimes better than bacteriostatic groups. This is determined by the minimum inhibitory and bactericidal concentrations.[37] A study of the successful treatment of patients with severe infections in the bactericidal (beta-lactam, glycopeptide, ciprofloxacin and aminoglycoside) and bacteriostatic (tigecycline, linezolid, macrolides, sulfonamides, tetracycline and streptogramin) antibiotics did not show a cure rate and no difference in mortality between the two groups.[38]

The division of antibiotics into bacteriostatic and bactericidal groups is based on laboratory findings. These definitions and limitations cannot be used in clinical settings. The use of low-dose bactericidal antibiotics can show bacteriostatic effects. Conversely, high doses of bacteriostatic can kill microorganisms, as in the bactericidal group. Factors influencing these results include strains, drug concentrations, and changes like stored isolates.

There are several limitations in this study. The resistance of the study isolates to ciprofloxacin and tetracycline was determined using phenotypic methods, and genotypic information was unavailable. Another limitation of this study was using one concentration for each antibiotic tested. The use of multiple concentrations below the MIC is essential for tetracycline antibiotics, which show different effects at various concentrations. We suggest that a future study use various concentrations of antibiotics such as ciprofloxacin and tetracycline, which they have needed to monitor efficacy for bacteria (resistance).

CONCLUSION

The average generation time of *E. coli* non-ESBL and ESBL to ciprofloxacin exposure is 52.76 and 53.96 min, respectively. The average generation time of *E. coli* non-ESBL and ESBL to tetracycline exposure is 53.66 and 55.40 min, respectively. There is no comparison between the generation time of *E. coli* non-ESBL exposed to ciprofloxacin and tetracycline. There is no comparison between the generation time of *E. coli* ESBLs exposed to ciprofloxacin and tetracycline.

ACKNOWLEDGEMENT

We would like to thank our assistance, "Fis Citra Ariyanto".

CONFLICT OF INTEREST

All authors declare no conflict of interest.

ETHICAL STATEMENT

We have conducted an ethical approval based on the Declaration of Helsinki with registration research at the Health Research Ethics Committee in Dr. Soetomo General Academic Hospital, Surabaya, Indonesia (1751/120/2/X/2022).

FUNDING

None.

AUTHORS' CONTRIBUTION

BM: Data collection, data analysis, literature search, study design, experimental studies, statistical analysis, and manuscript preparation; ADWC: conceptualization, literature search, experimental studies, supervision, visualization, statistical analysis, data analysis, manuscript preparation, manuscript editing, and guarantor; APDE: Conceptualization, supervision, experimental studies, data analysis, statistical analysis, and manuscript review.

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