ORIGINAL ARTICLE

Biofilm Formation and Antimicrobial Resistance of Escherichia coli in Vitro Towards Ceftriaxone and Cefotaxime

Jihan Kalishah[1], Dominicus Husada[2], M. Vitanata Arfijanto[3], Agung Dwi Wahyu Widodo[4*]

[1]Faculty of Medicine, Universitas Airlangga Surabaya, Indonesia

[2]Department of Pediatrics, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia – Dr. Soetomo General Hospital Surabaya, Indonesia

[3]Department of Internal Medicine, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia – Dr. Soetomo General Hospital Surabaya, Indonesia

[4]Department of Clinical Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia – Dr. Soetomo General Hospital Surabaya, Indonesia

ABSTRACT

Introduction: Escherichia coli is a Gram-negative bacteria and the most cause of nosocomial infections. Bacteria can form a biofilm that may cause antimicrobe resistance. This biofilm protects the microbes from environmental conditions, making it the most cause of nosocomial diseases, including urinary tract infections and infections resulting from medical instruments. This experiment aims to observe the presence of cefotaxime and ceftriaxone antimicrobial resistance in E. coli as a cause of biofilm formation.

Methods: This study conducted three experiments of E. coli sensitivity test on cefotaxime and ceftriaxone, E. coli resistance test after a 48 hours incubation, and optical density measurement using iMark™ ELISA Microplate Reader.

Results: The results of the sensitivity experiment with antibiotic cefotaxime and ceftriaxone disk showed wild-type E. coli and E. coli ATCC are sensitive. At the same time, after 48 hours of incubation E. coli ATCC remains sharp, and dominant resistance results were observed in wild-type E. coli with three intermediates and one sensitive. The third experiment results were obtained from ELISA then classified into three categories. The results of E. coli ATCC 1 and 2 are mostly high (OD >0.240). The OD in E. coli 1 is mostly high. At the same time, results from E. coli 2 consist of one bacterium in the weak category (OD <0.120), four in the moderate category (OD 1.20[A1] – 0.240), and three in the high category.

Conclusion: It can be concluded that E. coli resistance tests in cefotaxime and ceftriaxone after 48 hours' incubation are mostly resistant in wild-type E. coli but sensitive in E. coli ATCC.

Keywords: Escherichia coli, Resistant, Cefotaxime, Ceftriaxone

Correspondence: Agung Dwi Wahyu Widodo
E-mail: Agungimunologi@gmail.com

Article history: •Received 1 November 2021 •Received in revised form 17 November 2021 •Accepted 1 December 2021 •Available online 31 January 2022

INTRODUCTION

Infection is disturbances caused by bacteria, viruses, fungi, or parasites (WHO, 2017). One of the most common nosocomial infections is Escherichia coli and Proteus mirabilis, causing urinary tract infection (UTI). E. coli had been identified in 90% of cases (Shirtliff & Jeff, 2009). Third-generation cephalosporins had been found as an effective antimicrobe for Gram-negative infections, including those caused by E. coli (Wikaningtyas et al., 2016).

Bacteria invasions have strategies to avoid host immune responses to maintain their existence in the urinary tract. These strategies include the presence of fimbriae, the production of exopolysaccharide capsules, and biofilm formation. The biofilm formation could increase bacterial resistance towards antibiotics (Shirtliff & Jeff, 2009). Besides being the most cause of recurrent UTI, E. coli biofilm is also responsible for persistent contamination in medical instruments (Sharma et al., 2016).

Bacteria also have an antibiotics inactivation mechanism using beta-lactamase as a hydrolysis agent. Several countries in Europe had found an increase in ESBL-producing E. coli (ESBL-EC) prevalence to a percentage of 1.7 – 6.3% in patients with nosocomial UTI in the last decade. The majority of clinical factors related to the colonization and infection of ESBL-EC involve exposure during medical treatments, including inpatients, prolonged stay in medical facilities, hemodialysis usage, and presence of intravascular catheter (Park et al., 2014).

In this study, E. coli had been chosen to be tested in resistance tests towards cephalosporin antibiotics, cefotaxime, and ceftriaxone. As several leading organizations such as the Centers for Disease Control and Prevention (CDC), Infectious Diseases Society of America (IDSA), The World Economic Forum, and World Health Organization (WHO) had stated that antimicrobial resistance is a global health issue, the topic discussed in this study had been being essential substances to be observed (Aslam et al., 2018). This study aims to observe the presence of antimicrobial resistance towards cefotaxime and ceftriaxone in the E. coli planktonic phase. It is hoped that this study could update the medical sciences and make the medical providers more careful in doing medical treatments to the patients.

Available at https://e-journal.unair.ac.id/CIMRJ ; DOI: 10.20473/cimrj.v3i1.32549

This work is licensed under a Creative Commons Attribution-ShareAlike 4.0 International License.

METHODS

This research is a valid experimental study conducted in the Microbiology Laboratory of Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, from February to April 2019. This study uses a pretest-posttest-only design with a sample size calculated with Federer's formula, 16 times replications. Two kinds of treatments were given, so the sample size is 32. Consecutive sampling was used.

Materials used in this study include E. coli ATCC 35218, wild-type E. coli (non-specified), which have been prepared by the microbiology laboratory of the medical faculty of Airlangga University, Eosin Methylene Blue (EMB) agar, MacConkey agar, Mueller Hinton agar, crystal violet or gentian violet, cefotaxime disk (30g) and ceftriaxone disk (30g) antibiotics, and broth solution.

Three experiments were conducted to prove that biofilm formation causes an antimicrobial resistance of E. coli towards cefotaxime and ceftriaxone. The first experiment was done to ensure that the E. coli used are sensitive towards cefotaxime and ceftriaxone antibiotics. Clinical isolates of E. coli were taken from EMB, and MacConkey agar was then tested using the antibiotic disks.

The second experiment was done to do a resistance test during the E. coli planktonic phase after an incubation of 2 x 24 hours. Six tubes each for E. coli ATCC and wild-type E. coli were prepared on the rack. Bacterial suspensions were then made by putting 4 ml broth solution in each box using a pipette and mixing it with bacteria taken from the agar using one, then putting it in the vortex mixer until it is mixed turbid. Bacteria from the tubes were then taken using a micropipette, 1 ml from each box, then transferred to the box thereafter in sequence on the six boxes so that the dilution occurs up to 5 times put on the microplate much as 150 microliters. Then, 16 times replication was done on the E. coli ATCC and wild-type E. coli. The microplates were closed tightly using aluminum foil and incubated under the temperature of 37 Celcius degrees for 2 x 24 hours. The supernatant from the microplates was then taken and smeared onto the Mueller Hinton agar then cefotaxime and ceftriaxone antibiotics were put in it. Another 24 hours incubation was done then the diameter on the agar was measured using a caliper. In cefotaxime 30 g, a sensitive result was concluded if the diameter found was more than or equal to 26 mm. It is classified as intermediate if it is 23 – 25 mm, and resistant if less than or equal 22 mm. In ceftriaxone 30g, a sensitive result was concluded if the diameter found is more than or equal to 23 mm, intermediate if it is 20 – 22 mm, and resistant if the diameter located is less than or equal to 19 mm.

The third experiment was done to measure the optical density. Microplates whose supernatant had been previously taken were stained with crystal violet 1%. It was then observed using iMark™ ELISA Microplate Reader with wavelength 490 nm to measure its optical density.

The 16 times replications of E. coli ATCC and wild-type E. coli are classified into two groups, each consisting of 8 replicas under the name of the alphabet from A to H. Each group consists of cefotaxime-tested (CTX) and ceftriaxone-tested (CRO) replicates.

RESULTS

The results of this experiment are diameter found in E. coli ATCC before the 2 x 24 hours incubation in cefotaxime and ceftriaxone antibiotics were 31 mm, while the diameter found in wild-type E. coli were 26 mm-wide. According to CLSI standards, it can be concluded that E. coli ATCC and wild-type E. coli used in this study are sensitive towards cefotaxime and ceftriaxone antibiotics.

Sensitivity experiment results were obtained from both groups of E. coli ATCC 1 and E. coli ATCC 2, while various results were obtained from both groups of wild-type E. coli 1 and 2. In wild-type E. coli 1 tested with cefotaxime (CTX), the results were one sensitive, four resistants, and three intermediates. In contrast, in the one tested with ceftriaxone (CRO), none of them were sensitive with one intermediate and seven resistants. Different results were obtained from wild-type E. coli 2, with all the groups being resistant to cefotaxime (CTX) and ceftriaxone (CRO) (Table 2).

Table 1. The Diameter of E. coli ATCC and Wild-type E. coli after 2 x 24 hours Incubation

Replication	E.coli ATCC				Wild-type E.coli			
	1		2		1		2	
	CTX	CRO	CTX	CRO	CTX	CRO	CTX	CRO
A	30	27	26	27	17	17	20	19
B	27	27	28	27	20	19	18	17
C	28	27	30	27	20	18	20	17
D	28	27	31	30	21	18	19	19
E	27	27	27	29	17	18	17	16
F	27	27	31	23	25	22	17	16
G	30	28	27	27	16	16	16	16
H	28	29	28	29	14	14	17	16
Mean	28	27	21	27	18	20	18	17
Min Value	27	27	21	27	14	14	16	16
Max Value	30	29	31	30	25	22	20	19

Tabel 2. Interpretation of Diameter of E. coli ATCC and Wild-type E. coli after 2 x 24 hours Incubation

Replication	E.coli ATCC				Wild-type E.coli			
	1		2		1		2	
	CTX	CRO	CTX	CRO	CTX	CRO	CTX	CRO
A	S	S	S	S	R	R	R	R
B	S	S	S	S	I	R	R	R
C	S	S	S	S	I	R	R	R
D	S	S	S	S	I	R	R	R
E	S	S	S	S	R	R	R	R
F	S	S	S	S	S	I	R	R
G	S	S	S	S	R	R	R	R
H	S	S	S	S	R	R	R	R

Note:
E.coli ATCC: E.coli ATCC 35218
Wild-type E.coli: Unspecified E.coli (free-type)
S: Sensitive
Sensitive CTX: ≥ 26 mm
Sensitive CRO: ≥ 23 mm
I: Intermediate
Intermediate CTX: 23 - 25 mm
Intermediate CRO: 20 - 22 mm
R: Resistant
Resistant CTX: ≤ 22 mm
Resistant CRO: ≤ 19 mm

Optical Density (OD) measurement obtained from ELISA Microplate Reader is classified as in the Biofilm Formation by Sayal (Sayal et al., 2016). In E. coli ATCC 1 and 2, the majority of the replications are in the high category (OD > 0.240), and so in wild-type E.coli 1. Different results were

obtained from wild-type E. coli 2 with one replication in weak category (OD < 0.120), 4 in moderate category (OD 0.120 – 0.240), and 3 in high category (OD > 0.240) (Table 3). The representation in the scatter diagram can be seen in Figure 1.

Table 3 The Optical Density of E.coli ATCC and Wild-type E.coli after 2x24 hours Incubation

Replication	E.coli ATCC		Wild-type E.coli	
	1	2	1	2
A	Moderate	High	Moderate	Moderate
B	High	High	Moderate	High
C	High	High	High	Weak
D	High	High	High	High
E	High	High	High	High
F	High	High	High	Moderate
G	High	Moderate	High	Moderate
H	High	High	High	Moderate

Note:
High: OD > 0.240
Moderate: OD 0.12 - 0.24
Weak: OD < 0.120

Note:
E.coli ATCC 1: E.coli ATCC group 1
E.coli ATCC 2: E.coli ATCC group 2
E.coli 1: Wild-type E.coli group 1
E.coli 2: Wild-type E.coli group 2

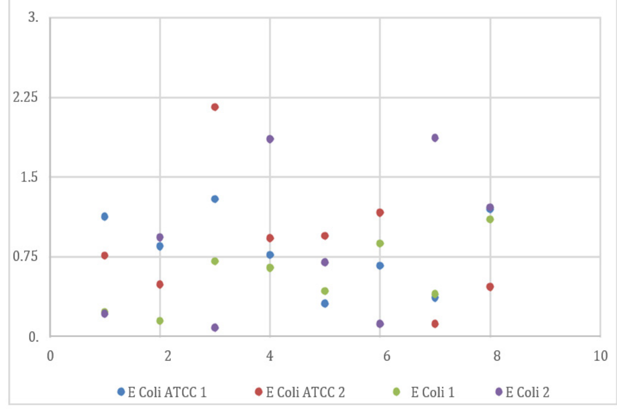

Figure 1 Scatter Diagram of Optical Density of E. coli ATCC and Wild-type E. coli after 2 x 24 hours Incubation

DISCUSSION

Disk diffusion or Kirby-Bauer test is one of the classical microbiology techniques and is still commonly used because it is convenient, efficient, and low-cost. This method might be the most used technique to determine antimicrobial resistance worldwide (Sandle, 2016). In this study, cefotaxime and ceftriaxone disk were only used as a standard to measure the resistance diameter of E. coli before and after incubation. Although in another study, Valsan had proven that this method could be used in more complicated experiments as in Enterobacter isolate with the 12-disk procedure (Valsan et al., 2013). This procedure can also be used in detecting phenotypes of beta-lactamases such as ESBLs, AmpCs, K1 beta-lactamase, and carbapenemase altogether using antibiotic disk combination in a single 150-mm and Mueller Hinton agar. However, this 12-disk procedure cannot detect the existence of ESBL in three isolates and can only interpret the presence of an AmpC producer. Another method of Vitek can also see ESBL existence and carbapenemase in which 12-disk procedure can only interpret it as a carbapenemase producer. It can be concluded that the disk diffusion method has its advantages and disadvantages in detecting resistances (Valsan et al., 2013).

The use of microtiter in this study not only can be used as a media for the biofilm growth of Escherichia coli. Djordjevic had used this microtiter media to do a standardization test on polyvinyl chloride (PVC) microtiter plate in comparing several strains of L. Monocytogenes on biofilm formation (Djordjevic et al., 2002). Microtiter plate biofilm test reportedly could give researchers more significant differences in biofilm production observation compared to the microscopic biofilm test.

Stepanovic reported that inoculum in his Staphylococcus sp. experiment was made from bacteria cultivated on agar (Stepanovic et al., 2007). Wells of the microtiter plate were filled with 180 ml Tryptic Soy Broth (TSB) added with 1% glucose for 20 ml of bacterial suspension were then added to each well. The addition of glucose is used to prove whether it affects the speed of biofilm growth or not. The inoculated plates were closed and incubated in aerobic conditions for 24 hours and 30 minutes with a temperature of 35 – 37 Celcius degree and static position.

Those procedures are different from what had been done in this study. In this study, wells of the microtiter were filled with 150 ml bacterial suspension that had been previously diluted using the broth and not further added with any other solutions. The conditions of the incubation are also different. Incubation in this study was done for 2 x 24 hours at 37 Celcius degrees. A resistance test was then conducted on the microtiter plate to observe any differences between antimicrobial resistance towards cefotaxime and ceftriaxone. There is no significant difference with incubation time given the different treatments.

After staining the plate with crystal violet and incubating it for 24 hours to measure optical density, the plate was then flushed and closed immediately with aluminum foil without previously heated. This plate was then read on ELISA Microplate Reader to measure its optical density. Crystal violet is suitable for biofilm size measurement but not for estimating its activity.

In a liquid environment, bacteria experience a hydrodynamic force, especially when approaching the surface (Beloin et al., 2008). It can be inferred that, although in this study the materials were made in the form of suspension, the bacteria can still survive because gram-negative bacteria, including E. coli and Salmonella sp, have a flagellum that gives it active motility to swim against early adhesion towards the abiotic surface of the liquid or semi-liquid media (Beloin et al., 2008). Phytochemistry and electrostatic interactions between the bacteria protective mechanism and the substrate have a high impact on bacteria detachment from the surface to further merged into a planktonic phase, whether it is because of a light sliding force or because of the bacteria motility. This bacterial detachment is also affected by environmental conditions, including pH, ionic media force, temperature, or even the characteristics of the surface itself (Beloin et al., 2008).

The 2 x 24 hours incubation in a temperature of 37 Celcius degree in this study highly affects the biofilm layers' condition and forces E. coli to decide whether it would stay still or detach and go into the planktonic phase to find a more favorable state. However, Dunne reported that E. coli has flagellar motility that helps it neutralize the hydrodynamic and electrostatic force near the surface so that the biofilm would not be easily detached (Dunne, 2002). Bacteria would

then form a matrix as a specific characteristic of a biofilm. It provides absorption of diffusion barriers and toxic molecules such as anti-microbes, hydroxyl radicals, and superoxide anions. It can also limit removals of enzymes, nutrition, or even signal molecules. Local accumulations of the substances create a more favorable microenvironment in the biofilm that leads to persistent infection (Beloin et al., 2008).

Although further studies are still needed to observe biofilm and antimicrobial resistance mechanisms, this study has been sufficiently explained some differences. The results of this study stated that wild-type E. coli has a dominancy of resistance towards cefotaxime and ceftriaxone, while E.coli ATCC remains sensitive towards both antibiotics. It can be concluded that the biofilm layer in an E. coli infection blocks the cephalosporin mechanisms towards E. coli. Another mechanism supporting cephalosporins resistance involves reducing Penicillin-binding proteins (PBP) affinity and or acquisitions of a non-sensitive beta-lactam PBP (Munita et al., 2016).

E. coli is one bacteria that has a protective mechanism towards anti microbes by producing beta-lactamase. This product can be vastly produced and determined by the DNA chromosomes or plasmid. Resistance level facilitated by beta-lactamase is associated with the number of enzymes produced, with or without induction in the enzyme's location and kinetic activity. The location of the enzymes is in the extracellular space for Gram-positive organisms and in periplasm for Gram-negative organisms such as Enterobacteria (Munita et al., 2016).

There is a significant correlation between porin and cephalosporin resistance in bacteria. It shows that the outer membrane is the only barrier for drugs to come in. Although in Pseudomonas aeruginosa, there is an additional inhibiting factor between the outer membrane and PBP (Livermore, 1987).

This study shows that the presence of biofilm could be a supporting factor in antimicrobial resistance. Limitations of this study include the different locations between the ELISA microplate reader[A1] and the laboratory facilities where the experiment was being held so that the E. coli in the microplates should be transferred manually. The instruments used in this study are mostly too conventional compared to other experiments with more sophisticated instruments. Weaknesses of this study include the limited ability of this study that can only prove antimicrobial resistance mechanism through a biofilm formation and only probabilities of E. coli ability to produce beta-lactamase.

CONCLUSION

Escherichia coli ATCC and wild-type Escherichia coli are sensitive towards cefotaxime and ceftriaxone antibiotics. However, after 48 hours of incubation in liquid, the antibiotics test showed domination of resistant wild-type E. coli, while the E. coli ATCC remains sensitive. Although further studies on bacterial mechanisms in forming biofilm are still needed and studies on additional factors resulting in antimicrobial resistance, clinicians should be much more aware of patients‘ management, including targetted antibiotic administration and following updates whether on international medical standards or local hospital regulations. These are needed to reduce nosocomial infections because biofilm can be quickly grown in any place.

ACKNOWLEDGEMENT

Polite praises should be given to those who had been involved and participated in this study, including Agung Wahyu Widodo, MD, Ph.D. and Dominicus Husada, MD, Ph.D. as supervisor of this study, also M. Vitanata Arifijanto, MD as a revisor, and all the staff in the department of clinical microbiology of Faculty of Medicine, Universitas Airlangga.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

REFERENCES

Aslam B, Wang W, Arshad M, Kurshid M, Muzammil S, Rasool M, et al. 2018. Antibiotic resistance: a rundown of a global crisis. Infect Drug Resist 11:1645–1658.

Beloin C, Roux A, Ghigo JM. 2008. Escherichia coli biofilms. Curr Top Microbiol Immunol 322:249–289.

Djordjevic D, Wiedmann M, Mclandsborough LA. 2002. Microtiter Plate Assay for Assessment of Listeria monocytogenes Biofilm Formation. Appl Enviromental Microbiol 68(6):2950–2958.

Dunne WMJ. 2002. Bacterial adhesion: seen any good biofilms lately? Clin Microbiol Rev 15(2):155–166.

Livermore DM YYJ. 1987. Beta-lactamase lability and inducer power of newer beta-lactam antibiotics in relation to their activity against beta-lactamase-inducibility mutants of Pseudomonas aeruginosa. J Infect Dis 155(4):775–782.

Munita J, Arias C. 2016. Mechanism of Antibiotic Resistance. Microbiol Spectr 4(2).

Park K, Sup W, Suk E, Won S, Hur J, Keun Y, et al. 2014. International Journal of Infectious Diseases Factors associated with ciprofloxacin- and cefotaxime-resistant Escherichia coli in women with acute pyelonephritis in the emergency department. Int J Infect Dis 23:8–13.

Sandle T. 2016. Antibiotics and preservatives. Pharm Microbiol 171–183.

Sayal P, Devi P, Singh K. 2016. Bacterial Colonization and Biofilm Formation among Diabetic Patients : A Therapeutic Challenge. Int J Curr Microbiol Appl Sci 5(9):174–181.

Sharma G, Sharma S, Sharma P, Chandola D, Dang S, Gupta S, et al. 2016. Escherichia coli biofilm: development and therapeutic strategies. J Appl Microbiol 121(2):309–319.

Shirtliff ME, Jeff GL. 2009. The role of biofilms in device-related infections, vol. 3, The Springer Series on Biofilms. Los Angeles, Springer, p219.

Stepanovic S, Vukovic D, Hola V, Bonaventura GDi, Djukic S, Cirkovic I, et al. 2007. Quantification of biofilm in microtiter plates : overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. Acta Pathol Microbiol Immunol Scand 115(8):891–899.

Valsan C, Chinnan JP, Sathiavathy K. 2013. Phenotypic detection of β-lactamases in enterobacteriaceae using a 12-disk procedure. J Acad Clin Microbiol 15(1):7–10.

Wikaningtyas P, Sukandar EY, Retnoningrum DS, Parwati IA. 2016. Multicenter surveillance and reporting of antimicrobial resistance: focus on ceftriaxone-resistant escherichia coli in the year 2012 and 2013. Int J Pharm Clin Res 8(7):685–689.

World Health Organization. 2017. Infectious diseases, health topics. Accessed 29 September 2019. Available from: https://www.who.int/topics/infectious_diseases/en/.