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The Effect of Ciprofloxacin Exposure on Ctx-M Gene Pattern of *Escherichia Coli*

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Abstract

Background: An exposure of ciprofloxacin on *Escherichia coli* (*E.coli*) may cause cross-resistance to beta-lactam groups. The researchers obtained that ciprofloxacin, aminoglycoside, cotrimoxazole and second generation of cephalosporins were the risk factors for infection caused by Extended-Spectrum β-Lactamases (ESBL)-producing bacteria. Exposure of ciprofloxacin to *E.coli* causes resistance of cefotaxime and produces ESBL which is genotypically evidenced by the presence of CTX-M.

Objective: To know the effect of ciprofloxacin exposure on CTX-M gene pattern of *E. coli*.

Method: A total of 30 *E.coli* isolates were exposed to ciprofloxacin for 14 days with the Kirby-Bauer antibiotic disc diffusion method. ESBL confirmatory test and Modified Double Disk Susceptibility Test (MDDST) method was used the antibiotic disc and continued electrophoresis using Polymerase Chain Reaction (PCR). The data analysis used the chi-square statistic test with $\alpha = 0.05$.

Results: In the PCR examination, 10.00% of ESBL isolates were obtained to have CTX-M-15 gene, and 33.00% of non-ESBL *E.coli* isolates having CTX-M-15 gene. While 10.00% of *E.coli* ESBL isolates and 53.30% non-ESBL did not have CTX-M-15 genes. There was no significant difference between ciprofloxacin exposure and the presence of CTX-M-15 gene ($p = 0.290$).

Conclusion: Most of the *E.coli* isolates had CTX-M-15 gene after the exposure. There was no difference in ciprofloxacin exposure to the presence of CTX-M-15 genes of *E. coli.*

Keywords: *Ciprofloxacin, Escherichia coli, ESBL, CTX-M genes*

Introduction

Ciprofloxacin is a member of the fluoroquinolone antibiotic class. The mechanism of action of fluoroquinolone class antibiotics is to interfere with the enzymes Gyrase A, B, and Topoisomerase IV, thus inhibiting deoxyribonucleic acid (DNA) replication, and further disrupt protein synthesis and bacterial cell death [1, 2]. *Escherichia coli* (*E.coli*) is a species of the genus Escherichia which is a member of the Enterobacteriaceae family [3]. After the discovery and use of the fluoroquinolone, group began to emerge the fluoroquinolone-resistant *Escherichia coli* strain by expressing a protein that protects the active site of the gyrase enzyme targeted by fluoroquinolone antibiotics [1]. This leads to fewer antibiotic therapy options, prolonged treatment length, high morbidity and mortality, and increased maintenance costs [4, 5].

The use of ciprofloxacin as part of fluoroquinolone is alleged to be a risk factor for urinary tract infection (UTI) caused by *E. coli*, resulting in the enzyme extended strain beta-lactamase (ESBL) [6,8]. In a study conducted on *E. coli* isolated from cats [9]. In a study conducted on E.coli isolated from cats, it was found that the CTX-M

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type ESBL encoding genes were also present in the same plasmid as the Plasmid-Mediated Quinolone-Resistance (qnr) gene [10]. Research conducted in Spain shows that the ESBL enzyme in E.coli was TEM (60.00%), CTX-M (24.00%), and SHV (16.00%). ESBL CTX-M-15 enzyme obtained at E.coli at Dr.Soetomo Teaching Hospital Surabaya for 94.50% in all specimens [11] and 84.00% in pediatric patient feces [12].

The abundance of ciprofloxacin and cefotaxime as an empirical therapy option in UTI should be recognized as potentially causing resistant strains to many antibiotics. This is due to cross-resistance between one antibiotic and another, in this case, ciprofloxacin and cefotaxime because of the similarity in which the gene encodes resistance to both. An exposure of ciprofloxacin to *E.coli* may cause resistance to cefotaxime and produce ESBL which is genotypically attested by the presence of the CTX-M gene. We aimed to know the effect of ciprofloxacin exposure on the pattern of CTX-M gene in *E. coli*.

Method

The population was a clinical isolate of inpatients Dr. Soetomo Teaching Hospital Surabaya who grew *E.coli* at Clinical Microbiology Installation by using the automatic diagnostic method. The sample size used the Central Limit Theorem, i.e., 30 isolates of E.coli sensitive non-ESBL ciprofloxacin from clinical specimens of inpatients in Dr. Soetomo Teaching Hospital. The inclusion criteria of *E. coli* were from clinical specimens of hospitalized patients, calculated colonies of *E. coli* ≥105 CFU/mL if derived from middle transition urine and ≥102 CFU/mL if derived from catheter urine and any amount derived from suprapubic urine, and E.coli was sensitive to ciprofloxacin based on the results of the susceptibility test of the method for diffusion. The exclusion criteria included cefotaxime, ceftazidime and aztreonam resistant isolates tested on double disk synergi test (DDST) basis, and were obtained with more than one type of bacteria in a specimen.

The main subject used in this study was the clinical isolates of E.coli non-ESBL bacteria from clinical specimens of inpatients obtained from the Clinical Microbiology Installation. The additional materials used in this study were Mueller-Hinton agar medium, 5 µg ciprofloxacin antibiotic disc, cefotaxime 30 µg, ceftazidime 30 µg, amoxicillin/clavulanate 20/10 µg, polymerase chain reaction (PCR) mix, agarose, ethidium bromide and Tris Acetate EDTA (TAE).

The researchers conducted a test of ethics (116/ Panke.KKE/II/2017) in Dr. Soetomo Teaching Hospital Surabaya, Indonesia. Data collection with observation and recording of research results to the table that has been provided. Tabulation of observation data was grouped into 2 groups i.e., E.coli ESBL and non-ESBL group. The technique of processing and data analysis were used the chi-square statistical test with SPSS (SPSS, Inc., Chicago, IL).

The procedures started by preparing antibiotic discs (ciprofloxacin, cefotaxime, ceftazidime, aztreonam, amoxicillin), and bacterial isolates by using the Phoenix-BDCLSI M100S 2016 automatic technique, then exposure to ciprofloxacin discs for 22 hours at 37℃. The second-day isolate was suspended in a 0.9% NaCl solution to a turbidity of 0.5 McFarland. On the second day was also prepared exposure discs ciprofloxacin and cefotaxime with isolate sensitivity to cefotaxime as a screening test. If cefotaxime resistant, then it was proceeding with the confirmatory test. If the modified double disk susceptibility test (MDDST) confirmatory test results, it will be stored at room temperature for PCR test. The next step was to read the sensitivity of antibiotics using sensitivity tests. Its classified to be sensitive on ciprofloxacin if the inhibitory zone showed diameter ≥21 mm and resistant if ≤15 mm. Whereas, cefotaxime classified to be sensitive if inhibit zone was ≤26 mm and resistant if the inhibition zone was ≤22 mm. The sensitivity test for ceftazidime said to be sensitive if inhibition zone was ≥21 mm and resistant if the inhibitory zone ≤17 mm. ESBL classified to be positive if resistant to cefotaxime. ESBL confirmation test was positive if found widening of inhibition zone on the side of the disc that faces amoxilin clavulanate. Then, prepared PCR examination with plasmid extraction using commercial kit by PureYieldTM, performed PCR examination by mixing the target DNA of extraction into a 50 µL ependorf tube inserted into a PCR machine then electrophoresis.

Results

Isolate Resistance to CIP and CTX by Kirby-Bauer method

The subjects obtained 5 (13.50%) *E. coli* isolates were resistant to cefotaxime and 3 (8.10%) isolates were resistant to ciprofloxacin sequentially after confirmation of the test by the Kirby-Bauer disc diffusion method

(Table 1). In the post-CIP recurrence of the eighth day, 5 (16.70%) isolates were resistant to cefotaxime. Then, day 12 obtained 4 (13.30%) isolates that became resistant cefotaxime and 1 isolate become sensitive again. On day 14, 4 (13.30%) of cefotaxime resistant isolates were detected and to date, no isolates were resistant to ciprofloxacin (Table 2).

ESBL Confirmation Test from CTX resistant E.coli

On the day 4, there was no isolate changed to ESBL. While on day 8, was found 10.00% of the isolates into ESBL. Additional of new three ESBL isolates were obtained on day 6, 7, and 8. On day 12 the ESBL isolate did not increase with total 4 (13.30%) isolates until day 14. There were 5 isolates of E.coli exposed to CIP (1357PS, 1564US, 1590SS, 2015SS, and 2707PS) resistant to cefotaxime but after confirmation testing, there were only 4 isolates (1357PS, 1564US, 2015SS, and 2707PS) confirmed ESBL. Isolate 1590SS was not confirmed as ESBL but the next day becomes sensitive to cefotaxime. In the E.coli isolate (2056US) of the cefotaxime-exposed group that was resistant to cefotaxime but after a confirmatory test, it was not proven to be ESBL. Its becomes sensitive to cefotaxime by the next day. However, in both groups, no isolates were obtained to be resistant to ciprofloxacin (Table 3).

PCX gene CTX-M-15 examination results in E.coli exposed to ciprofloxacin

A total of 30 isolates of E.coli exposed to ciprofloxacin, there were 13 (43.30%) had CTX-M-15, 1 (3.30%) ESXL isolates which did not have the CTX-M-15 and 10 (33.30%) genes isolated with the gene CTX-M-15 but phenotypically were not ESBL. All of 30 clinical isolates of E.coli non-ESBL and ciprofloxacin-sensitive exposed to ciprofloxacin for 14 days, was found 4 isolates became ESBL (Table 4). All of these isolates were then examined using PCR. Statistical results showed there is no significant differences the presence of CTX-M-15 genes after exposure to ciprofloxacin ($p = 0.290$).

Table 1. Resistance of isolates to CIP and CTX by Kirby-Bauer method

Sensitivity	Phoenix		Kirby-Bauer	
	CTX	CIP	CTX (%)	CIP (%)
Sensitivity	37	37	86.50	91.00
Resistency	0	0	13.50	8.10

CIP : Ciprofloxacin

CTX: Cefotaxime

Table 2. *Escherichia coli* resistance to CIP and CTX post recurrent exposure of CIP method Kirby Bauer

Antibiotic	Exposure to day-to-							
	4		8		12		14	
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
CTX	100.00	0.00	83.30	16.70	86.70	13.30	86.70	13.30
CIP	100.00	0.00	100.00	0.00s	100.00	0.00	100.00	0.00

CIP: Ciprofloxacin CTX: Cefotaxime S: Sensitivity R: Resistance

Table 3. ESBL confirmation test results from CTX resistant E.coli

Exposure to Antibiotics	Exposure to day-to-							
	4		8		12		14	
	Non ESBL (%)	ESBL (%)	Non ESBL (%)	ESBL (%)	Non ESBL (%)	ESBL (%)	Non ESBL (%)	ESBL (%)
CIP	100.00	0.00	100.00	0.00	100.00	0.00	26.00	0.00

ESBL: Extended-Spectrum β-Lactamases

CTX: Cefotaxime

Table 4. Polymerase Chain Reaction (PCR) results of CTX-M-15 gene on E.coli exposed to ciprofloxacine

Phenotype	Genotype CTX-M-15		p
	Positive n = 13	Negative n = 17	
ESBL	10.00	3.30	0.290
Non ESBL	33.33	53.30	

ESBL: Extended-Spectrum β-Lactamases

CTX: Cefotaxime

Discussion

There was no significant difference between ciprofloxacin exposure and the presence of CTX-M-15 genes. It was possible that there were other genes that encode ceftazidime (CAZ) resistance [20]. This statement can be denied considering that in isolation E.coli was sensitive to CAZ or ESBL screening and confirmation methods were less accurate [19]. The sensitivity of ESBL confirmation test with modified DDST method according to Clinical and Laboratory Standards Institute (CLSI) was 65.4% (21). This was because some ESBLs could not reach the concentrations that detected by the antibiotic disc diffusion test, thereby thwarting the therapy [19]. The presence of porcine was quite instrumental in making antibiotics capable to killing bacteria. In small amounts of porins, beta-lactam antibiotics could not be able to enter the cells and phenotypically become a resistant bacteria. However, if the number of porins is large, there is an antifungal that not hydrolyzed (beta-lactamase) which is able to kill bacteria. In this condition, bacteria was sensitive to beta-lactam [22]. This causes *E. coli* strains that genetically have CTX-M-15 genes but were phenotypically not ESBL. However, referring to several bands in the electrophoresis results, this was because the primers used were less specific for the CTX-M-15 gene, so bands from other genes become emerging. Each class of antibiotics has its own mechanism in inhibiting and killing bacterial cells. Such mechanisms of damaging antibiotic molecules, altering antibiotic action targets, and decreasing intracellular antibiotic concentrations [13]. In these three mechanisms, repeated exposure to antibiotics will make bacteria remains alive. This was due to the mechanism of cross-resistance among antibiotic classes [14, 15]. In this study, the bacteria did not die or were outside the inhibition zone due to antibiotic concentrations.

The main mechanism of fluoroquinolone class antibiotic resistance through GyrA and GyrB gyrase encoding mutations was contained in chromosomes. In addition to the main mechanism, there was an additional mechanism, namely Plasmid-Mediated Quinolone Resistance (PMQR), in which all the genes were located in the plasmid. In certain plasmids (Inc-H, IncF, IncP) it containing the gene encoding of PMQR, there was also a CTX-M-15 gene encoding one of the ESBL enzymes. Repeated exposure of ciprofloxacin to *E. coli* may make it an effort to adapt by transferring plasmids Inc-H, Inc-F, and Inc-P which makes *E. coli* increase with ESBL-encoding genes to become dominant. The result showed that 4 isolates of *E. coli* exposed to ciprofloxacin were changed to ESBL while at exposure cefotaxime none became ESBL. All of *E. coli* ESBL was still sensitive to ciprofloxacin. PMQR was not the primary mechanism of bacterial resistance to quinolones, but the presence of an improved MIC-enhanced PMQR gene that does not always reach the limits of the resistance criterion (biological resistance) [8]. *E.coli* strain with PMQR were more sensitive to ciprofloxacin [16] but not enough to kill the bacteria, or there was a non-dominant resistance strain. The number of resistant strains between 1-1.000 for each 109 bacteria [17].

The result of this research showed that the isolate of non-ESBL phenotype but on PCR examination was obtained CTX-M-15. There were 23.66% isolates become sensitive to ceftazidime and non-ESBL and 2.17% of K. pneumonia with CTX-M-Group IV genes are both sensitive ceftazidime of 93 E.coli isolates. CTX-M group IV refers to CTX-M-9, 13, 14, 16, 17, 18, 19, 21, and 27 as well as Toho-2 [18]. Another study stated that 38 isolates of E.coli non-ESBL (52.60%) had ESBL-encoding genes [19].

Conclusion

Msost of the E.coli isolates (43.00%) had CTX-M-15 gene after exposure to ciprofloxacin. There was no difference between the presence of CTX-M-15 gene after ciprofloxacin exposure that caused by screening method, ESBL confirmation was less accurate, or the presence of porins. The recommendation may be that the exposure

time of ciprofloxacin is extended until proven for certain time required by the bacteria to be resistant to cefotaxime and ciprofloxacin and further studies are conducted to determine the pattern of gene encoding resistance to other antibiotics (aminoglycosides, tetracyclines, and heavy metals) in isolates exposed to it.

Ethical Clearance: This research is in accordance with ethical clearance, has not been published before and is not being considered for publication elsewhere.

Conflict of Interest: There is no conflict of interest in this study.

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