Phenotypic and molecular detection of ^{BLA}CTX-M gene extended-spectrum beta-lactamases in *escherichia coli* and *klebsiella pneumoniae* of north sumatera isolates

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Abstract. The application of antibiotics expanded-spectrum third-generation cephalosporin for the treatment of infectious diseases in hospitals is known contribute to increasing resistance due to the presence of the ^{bla}CTX-M gene in the bacteria producing ESBLs. This study was aimed to detect ESBLs, isolate phenotype and ^{bla}CTX-M genes on *Escherichia coli* and *Klebsiella pneumoniae* collected from H. Adam Malik Central Hospital. Phenotypes of the bacterial were detection using Vitek two compact, while the ^{bla}CTX-M genes were detection using polymerase chain reaction technique. The results showed that 85 (100%) isolates were ESBLs consisted of 41(48%) of *Escherichia coli*, and 44 (52%) of *Klebsiella pneumoniae*, respectively. ^{bla}CTX-M genes were detection in 62 (72.94%) of the isolates which 31 (36.47%) were *Escherichia coli*, and 31 (36.47%) of the isolates were *Klebsiella pneumoniae*, respectively. This study indicates the high prevalence of ^{bla}CTX-M genes in *Escherichia coli* and *Klebsiella pneumoniea* causing bacterial antibiotic resistance.

Keywords: antibiotic resistance, ESBLs, infections, molecular detection, polymerase chain reaction

1. Introduction

The presence of ^{bla}CTX-M gene in bacteria causes resistance to the broad spectrum of cephalosporin groups. ESBLs are enzymes derived from mutated beta-lactamase. This mutation leads to an increase in the enzymatic activity of beta-lactamase to penicillin, cephalosporins, and aztreonam. This resistance mechanism is widespread in the world, with reports of clinical isolates produce these beta-lactamase are from Europe, Africa, Asia, South America and North America [1,2]. ESBLs are most commonly produced by Enterobacteriaceae especially *Escherichia coli* and *Klebsiella pneumoniae* [3,4].

The use of beta-lactam group antibiotics, especially the third generation cephalosporin widely for the treatment of infections in hospitals, is a risk factor for infected by ESBLs producing bacteria

[5]. In addition to being resistant to beta-lactam antibiotics, ESBL-producing bacteria also often exhibit resistance to the use of quinolones. Excessive use of antibiotics, patients with severe disease, LOS (Length of Stay) and treated with invasive medical devices for a long time is also a high risk for being infected by ESBLs producing bacteria. Research on ESBLs is highly developed, coming from more than 30 different countries and reflects the distribution of ESBLs around the World[4]. Infection due to ESBL-producing bacteria are increasingly, and it is very difficult to treated since the choice of antibiotics becomes very limited and the emergence of new mutants [6], consequently this causes antibiotic failure against infectious bacteria [14,15].

Escherichia coli and *Klebsiella pneumoniae* are the primary causes of hospital-related infections and infections in the population (community) [6]. The worldwide increase in ESBL prevalence provides the basis for the importance of detecting ESBL phenotype and ^{bla}CTX-M genotypes in ESBLs-producing bacteria indispensable for improved therapeutic management and control of the bacterial transmission. This study aimed to detect ESBLs, and ^{bla}CTX-M gene in *Escherichia coli* and *Klebsiella pneumoniae* isolates from North Sumatra.

2. Methods

2.1 Bacterial isolates and phenotypic characterization

Sampling is done by purposive sampling based on the data of Germs Pattern of Haji Adam Malik Hospital Medan of the first semester of 2015. Bacterial isolates were collected from routine examination at Diagnostic Laboratory Installation, Microbiology Clinical Unit of Haji Adam Malik Hospital Medan. The phenotypic characterization was done using Vitek two Compact which included antimicrobial susceptibility test and ESBLs phenotyping.

2.2 DNA isolation

Isolates with ESBLs phenotype of *Escherichia coli* and *Klebsiella pneumonia* were subculture on Mac-Conkey agar and incubated 24 hours at 37℃. DNA isolation was conducted by the freeze-thaw cycling method, by inserting a bacterial colony into 20 µL aqua-bidest in a microtube and the vortex. The bacterial solution was freezer at -20℃ for 10 minutes, followed by heating it at 90℃ for 10 minutes using the dry block, freeze-thaw cycling done as six times. The freeze-thawed solution was spin at 13,000 rpm for 5 minutes. The supernatant was separated from cell, and subjected to DNA purification check.

2.3 Amplification and detection of CTX-M gene using PCR method

CTX-M gene amplification was performed by PCR technique using specific primer: ATG-TGC-AGY-ACC-AGT-AAR-GTK-ATG-GC forward and TGG-GTR-AAR-TAR-GTS-ACC-AGA-AYC-AGC-GG reverse [7]. Amplify the in PCR solution of 12.5µl master mix green go-Taq, 1µL of forward and reverse primer each, 8.5µL nuclease-free water, and 2µL bacterial DNA. Thermocycling reaction was conducted for denaturation at 94^{o}C for 2 minutes, extended denaturation at 94^{o}C for 1 minutes, annealing for 52^{o}C for 30 sec, extension at 72^{o}C at 45 sec, and extended extension at 72^{o}C for 5 minutes, this reaction is 30 cycles. PCR product was visualized in mini gel electrophoresis and documented in UV Reader/Gel Documentation System.

3. Results and Discussion

3.1 Phenotypic characterization

Phenotypic characterization showed that all samples (n = 85) contained ESBLs. The ESBLs phenotype was determined from the results of the antimicrobial susceptibility test according to 2015 Clinical Laboratory Susceptibility Institute standard using Vitek 2 compact. The percentage of antimicrobial susceptibility is presented in Table 1.

Table 1. Percentage of Antimicrobial Susceptibility Test (AST)

Antimicrobial Agents		Antimicrobial Sensitivity (%)	
Group	Type of Antibiotics	Sensitive	Resistant
Penicillins	Amoxicillin	0	100
	Ampicillin	0	100
Cephalosporins	Cefotaxime	0	100
	Ceftriaxone	0	100
	Ceftazidime	0	100
	Cefepime	0	100
Monobactams	Aztereonam	0	100
Beta-lactamase Inhibitor	Amoxicillin/Clavulanic Acid	27	73.0
	Piperacillin/Tazobactam	43.5	56.4
	Cefoperazone/Sulbactam	63.5	36.4
Aminoglicosides	Gentamycin	35.3	64.7
	Amikacin	100	0
Carbapenem	Imipenem	85.8	14.2
	Meropenem	85.8	14.2
Fluoroquinolone	Ciprofloxacin	0	100
	Levofloxacin	25.8	74.2
Fosfomycin	Fosfomycin	7.0	93.0
Folate Pathway Inhibitor	Cotrimoxazole	0	100
Tigecil	Tigecycline	0	100

It seemed that all bacterial isolates were resistant to beta-lactam group antibiotics such as penicillin, cephalosporins, and monobactams, which indicated are carrying ESBLs. A number of isolates resistant to beta-lactamase inhibitors such as amoxicillin/ clavulanic, piperacillin/tazobactam, and cefoperazone/sulbactam acid were lower with the percentage of antimicrobial resistant of 73.0, of 56.4, and of 36.4%, respectively. Interestingly, all isolates were resistant to other classes of antibiotics such as tigecycline, cotrimoxazole, and ciprofloxacin. Saverin *et al*. [17] study in Surabaya, Indonesia, showed that ^{bla}CTX-M gene prevalence was found very high. 65 out of 73 isolates of ESBLs *Escherichia coli* bore ^{bla}CTX-M gene [17]. Another study reported that as much as 50.5% of ESBLs *Klebsiella pneumoniae* isolates in Tertiary-Referral Hospital, Bali, Indonesia was detected to have the ^{bla}CTX-M gene. This study showed the widespread of ^{bla}CTX-M ESBLs in Indonesia [18].

The antibiotic application can be pressure for bacterial cell. However, mutations allow them to survive. These bacteria pass through this trait to their offspring, leading to the evolution of resistance. Increased resistance due to mutation may facilitate by high bacterial cell number with short generation time[8]. Phenotypic resistance is expression by the presence of ESBL genes such as ^{bla}CTX-M. ESBL-producing bacteria are also often resistant to aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and sulfamethoxazole-trimethoprime [12].

3.2 Amplification of ^{bla}CTX-M gene

Amplification of ^{bla}CTX-M gene resulted in a 539 bp amplicon in both *Escherichia coli* and *Klebsiella pneumoniae* isolates (Figure 1) with the percentage of 72.94%.

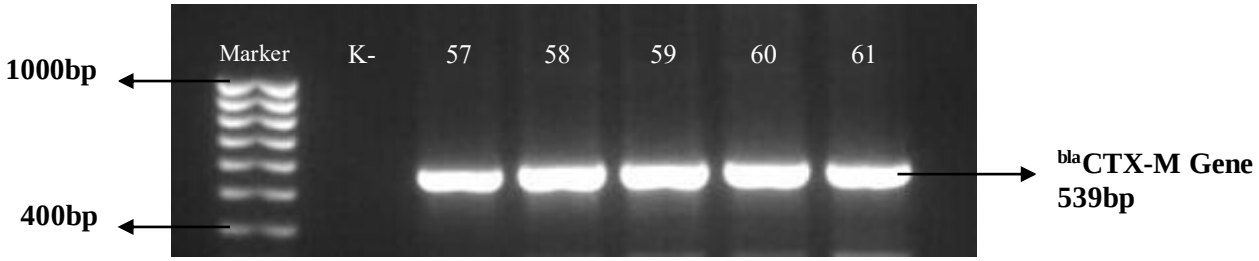

Figure 1. PCR profiles of molecular detection blaCTX-M gene of ESBLs
K- (Non-ESBLs), 57 (*E. coli* 11926), 58 (*K. pneumoniae*11688),
59 (*E. coli* 11779), 60 (*K. pneumoniae* 12006), 61 (*K. pneumoniae* 11901)

The primer used in this study amplified blaCTX-M gene in bacterial isolates resulted in the 539bp amplicon. It is primer could be used to amplify blaCTX-M gene. Hout et al. [7] using this primer showed that 50% of clinical isolates of the Cambodia Surgical Center collection was detected. Resistance to beta-lactam antibiotics may be due to inactivation of antibiotics by beta-lactamase enzyme, target modification of the penicillin binding protein (PBP), and failure penetration of drug disorders to PBP [15].

The presence of the blaCTX-M gene in bacteria is responsible for hydrolyzing antibiotics cefotaxime. Resistance may extend to other types of cephalosporin antibiotics [9]. Reported that unlike other countries, blaCTX-M genes were predominant in Europe. Several studies showed that ESBL types produce by *Escherichia coli* and *Klebsiella pneumoniae*. It was also reported that the ESBL prevalence increased with the emergence of blaCTX-M type genes [10]. Some researchers suggested that blaCTX-M gene found more common in ESBL isolates compared to that of SHV and TEM [16].

Today, the emergence of new variants on ESBLs producing microorganisms, especially blaCTX-M suggested the co-resistance involvement of other drug classes during endemics. The presence of the co-resistance is due to the different transmission of resistant gene types in the same clones [11]. Some studies showed that the blaCTX-M genotype is usually found in large plasmids often carrying other resistance genes of other antimicrobial agents including aminoglycosides, fluoroquinolones, chloramphenicol, tetracyclines mainly OXA and TEM [12].

It was find that 48% of *Escherichia coli* and 52% of *Klebsiella pneumoniae* isolates showed to have ESBLs, in which 36.47% of both bacteria was bearing blaCTX-M gene (Table 2). The other 27% of ESBL-producing bacteria might bearing another gene of beta-lactam resistance.

Table 2. Percentage of ESBL phenotype and blaCTX-M gene

Bacterial species	ESBLs phenotype	blaCTX-M gene
Escherichia coli	41 (48%)	31 (36.47%)
Klebsiella pneumoniae	44 (52%)	31 (36.47%)
Total :	85 (100%)	62 (72.94%)

The ability of ESBL-producing bacteria to hydrolyze beta-lactam antibiotics most of the due to some mutations in several genes such as TEM and SHV. Such mutations generally occur in the active site of the enzyme causing enzyme activity increases [6]. High prevalence of blaCTX-M genes in *Escherichia coli* and *Klebsiella pneumoniae* bacteria may increase the risk of transfer of this resistance gene to an-ESBLs bacteria. This study was the first study in North Sumatra revealing the existence of blaCTX-M in ESBL *Escherichia coli* and *Klebsiella penumoniae*, therefore need for the hospital awareness to avoid cross infection through health facilities and paramedics.

4. Conclusions

This study showed that prevalence of ESBLs *Escherichia coli* and *Klebsiella pneumoniae* is quite high in North Sumatra, Indonesia. All isolates were ESBLs bearing. The molecular detection of the ^{bla}CTX-M gene showed that 72.94% of the isolates possessed the gene. However, the remains isolates of ESBLs may bear other genes but ^{bla}CTX-M gene.

Acknowledgments

The author gratefully acknowledges to Clinical Microbiology Unit of Diagnostic Laboratory Installation of H. Adam Malik Hospital Medan for providing bacterial isolates.

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