RESEARCH ARTICLE

Gene Families of AmpC-producing Enterobacteriaceae Present in the Intensive Care Unit of Cipto Mangunkusumo Hospital Jakarta

Lucky Hartati Moehario[1,*], Thomas Robertus[1], Anis Karuniawati[2], Rudyanto Sedono[3], Delly Chipta Lestari[2], Andi Yasmon[2]

[1]Department of Microbiology, Faculty of Medicine and Health Science, Atma Jaya Catholic University of Indonesia, Jl. Pluit Raya No. 2, Jakarta, Indonesia
[2]Department of Microbiology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 4, Jakarta, Indonesia
[3]Intensive Care Unit, Cipto Mangunkusumo Hospital, Jl. Diponegoro No. 71, Jakarta, Indonesia

*Corresponding author. E-mail: luckyhmoehario@gmail.com

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Abstract

BACKGROUND: Antibiotic resistance has become a worldwide problem. Among Asia countries, Indonesia has high prevalence of multi-drug resistant organisms mainly due to Gram-negative bacilli Enterobacteriaceae. This study aimed to find out whether gene family of AmpC and AmpC/ESBL-producing Enterobacteriaceae were present in the Intensive Care Unit (ICU) of Cipto Mangunkusumo Hospital, Jakarta, Indonesia.

METHODS: Specimens were obtained from several body sites of adult patients with infection hospitalised in ICU of Cipto Mangunkusumo Hospital. VITEK®2 was used to identify the microorganisms. Antibiotic susceptibility tests were conducted using VITEK®2 and disc diffusion technique according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Double disc synergy (DDS) test method was employed to detect AmpC activity. Gene families of *ampC* were identified using multiplex polymerase chain reaction (PCR).

RESULTS: Forty five isolates were identified as putative AmpC, extended-spectrum β-lactamases (ESBL) and AmpC/ESBL-producing Enterobacteriaceae. *Klebsiella pneumoniae* (n=32) were predominant, followed by *Escherichia coli* (n=6), *Enterobacter cloacae* (n=5) and *Enterobacter aerogenes* (n=2). AmpC activity was detected in 9 isolates, in which 4 isolates were AmpC producing and 5 isolates were AmpC/ESBL. *In vitro*, AmpC-producing Enterobacteriaceae showed good susceptibility to many antibiotic tested, while those of AmpC/ESBL-producing only to Amikacin. The gene families of *ampC* were DHA, EBC and CIT identified from 6 isolates.

CONCLUSION: DHA, EBC and CIT gene families were identified from AmpC and AmpC/ESBL-producing Enterobacteriaceae in the ICU of Cipto Mangunkusumo Hospital. While the AmpC-producing was still susceptible to almost all antibiotics tested, the AmpC/ESBL-producing showed resistant except for Amikacin.

KEYWORDS: Enterobacteriaceae, β-lactamases, AmpC, ESBL

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Introduction

Antimicrobial resistance has become a serious problem worldwide, especially in Asia.(1) Indonesia is one of the Asian countries where the prevalence of multi drug-resistant organisms (MDRO) is very high. The high resistance rates is mainly found in Gram-negative bacteria such as *Pseudomonas aeruginosa*, Enterobacteriaceae and *Acinetobacter baumannii*. The same situation applies in animal farms.(2) The resistance rates of *Klebsiella pneumoniae*, *Klebsiella ozaenae* and *Escherichia coli* isolated from Intensive Care Unit (ICU) of Fatmawati Hospital were 75.7%, 81.5% and 46.2% to Ceftriaxone; 67.9%, 100% and 46.2% to Cefotaxime; 73%, 85.7% and 38.5% to Ceftazidime.(3) In the ICU of Cipto

Mangunkusumo Hospita, Jakarta, however, the prevalence of carbapenem resistant *i.e*., Enterobacteriaceae 27.6%, *P. aeruginosa* 21.9% and *A. baumannii* 50.5%.(4)

The major mechanism of resistance of Gram-negative bacteria is from the production of β-lactamases, such as AmpC β-lactamases (AmpC) and extended spectrum β-lactamases (ESBL).(5) The AmpC producers confer resistance to penicillins, cephamycins (*i.e.*, Cefoxitin, Cefotetan), oxyimino-cephalosporins (*i.e*., Ceftazidime, Cefotaxime, Ceftriaxone) and monobactams (*i.e*., Aztreonam). This type of microorganism shows resistant to antibiotic combination of β-lactam and β-lactamase inhibitors *i.e*., Amoxicillin/Clavulanic acid. The AmpC β-lactamases are chromosomal-mediated, and many Gram-Negative bacteria such as *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., *Morganella morganii*, *Aeromonas* spp. and *Hafnia alvei* have been known to produce AmpC β-lactamases.(6,7,8) The widely use of β-lactam antibiotics and β-lactamase inhibitors induces a high level expression of chromosomal-mediated AmpC and causes the resistant to among other the 3rd generation of cephalosporin and carbapenem. In hospitals where 3rd generation cephalosporins are being used repeatedly for a long period of time, the problem of antibiotic resistance arise. Enterobacter aerogenes and Enterobacter cloacae which are initially susceptible to 3rd generation cephalosporins have become resistant upon therapy.(8)

Apart from the chromosomal gene, the AmpC is also encoded by the plasmid. Gram-negative bacteria such as *Klebsiella oxytoca*, *Proteus mirabilis*, *Citrobacter freundii* and *Enterobacter aerogenes* are among those with plasmid-mediated AmpC.(8,9,10,11) Differ from chromosomal-mediated AmpC, plasmid-mediated AmpC are expressed significantly, and are typically associated with broad multidrug resistance.(10,11) The microorganisms that are over expressing AmpC can become resistant to carbapenem when there is disturbance of the outer membrane permeability.(6) At present, 6 types of plasmid-mediated *ampC* gene family has been found, namely DHA (firstly isolated at Dhahran Hospitals in Saudi Arabia), EBC (isolated from *Enterobacter cloacae*), CIT (firstly isolated from *Citrobacter freundii*), Ambler class C (ACC), FOX (Active on Cefoxitin), and MOX (Active on Moxalactam).

In the past decades, the prevalence of AmpC-producing Enterobacteriaceae had increased, and resulted in a serious threat, among other these bacteria are the major cause of hospital-acquired infections.(12,13) However, the information regarding AmpC-producing Enterobacteriaceae originated from hospitalised patients in Indonesia is not available thus far. This study aimed to explore the presence of AmpC-β-lactamase producing Enterobacteriaceae gene family in the ICU of Cipto Mangunkusumo Hospital, Jakarta, and also their antibiotic susceptibility.

Methods

This was a descriptive and cross sectional study. Clinical specimens *i.e.*, blood, lower respiratory tract secretions, urine, wound swabs, pus and soft tissues obtained from adult patients with infection in the ICU of Cipto Mangunkusumo Hospital within 6 months from April to September 2015 were subjected for the investigation. Bacterial cultivation workup was performed in the Clinical Microbiology laboratory of the Department of Microbiology, Faculty of Medicine, Universitas Indonesia. All patients that agreed to give their specimens were enrolled in this study, and signed informed consent.

Bacterial Isolates

Bacterial identification was conducted using VITEK®2 (Bio-Mérieux, Craponne, France). Anti-biotic susceptibility tests were conducted using VITEK®2 for Ampicillin, Gentamicin, To-bramycin, Amikacin, Ampicillin/Sulbactam, Piperacillin/Tazobactam, Cefoxitin, Cefotaxime, Ceftazidime, Cefepime, Meropenem, Ciprofloxacin, Levofloxacin, Fosfomycin, Tetracycline, Cotrimoxazole. As for Ceftriaxone, Cefpodoxime, and Aztreonam, disc diffusion technique was employed according to Clinical and Laboratory Standards Institute (CLSI) 2014 guidelines.(14) The antibiotic activity was classified as good if the susceptibility of all isolates to the antibiotic was 80% or greater. Isolates which less susceptible or resistant to one or more of 3rd generation cephalosporins (*i.e*., Cefpodoxime, Ceftazidime, Cefotaxime, Ceftriaxone) and/or Aztreonam and/or resistance to Cefoxitin were considered as putative AmpC β-lactamases, ESBL or AmpC/ESBL producers, and were included in this study. These isolates underwent confirmation test *i.e*., double disc synergy test (DDS) for AmpC β-lactamases and ESBL and subjected to multiplex polymerase chain reaction (PCR) for the detection of ampC gene families.

Confirmation Tests for AmpC β-lactamases and ESBL Using DDS Test

DDS test method using Total ESBL+AmpC kit 98019 (ROSCO Diagnostica, Taastrup, Denmark) was employed to detect AmpC and ESBL activity. The assay was performed

according to recommendations in the user manual. Antibiotic discs used were Cefotaxime + Cloxacillin (CTXCX), Cefotaxime + Clavulanate (CTXC), Cefotaxime + Clavulanate + Cloxacillin (CTXCC), Ceftazidime + Cloxacillin (CAZCX), Ceftazidime + Clavulanate (CAZC) and Ceftazidime + Clavulanate + Cloxacillin (CAZCC). Isolates were confirmed as AmpC-producing when the inhibition zone produced around CTXC/CTXCC and/or CAZC/CAZCC discs was differ ≥ 5 mm. An ESBL-producing was confirmed if the inhibition zone produced around the CTXCX/CTXCC and/or CAZCX/CAZCC discs was ≥ 5 mm. If the inhibition zones produced around CTXCX/CTXCC and the CTXC/CTXCC discs were differ ≥ 5 mm, and/or the CAZCX/CAZCC and CAZC/CAZCC discs were ≥ 5 mm then they were confirmed as producing both AmpC/ESBL enzymes. *Escherichia coli* ATCC 25922 was used as quality control strain.

The proportion of AmpC and AmpC/ESBL-producing Enterobacteriaceae was determined by dividing the number of those obtained from DDS test with all putative isolates which were less susceptible or resistant to one or more of 3rd generation cephalosporins and/or Aztreonam and/or resistance to Cefoxitin.

Multiplex PCR for the Identification of AmpC Gene Families

The DNA extraction was performed from fresh culture using boiling techniques. Five to 10 colonies of overnight bacterial culture were suspended into 500 µL of sterile phosphate buffer saline (PBS), mixed on a vortex mixer, and spin at 12,000 rpm for 2 min (2 times). After the supernatant was decanted, the pellet was resuspended in 200 µL of TE buffer and the mixture was briefly mixed on a vortex mixer. The cells were lysed by heating at 90°C for 20 min and cellular debris was removed by centrifugation at 12,000 rpm for 10 min. A 100 µL aliquot of the supernatant was transferred to a sterile tube and stored at -20°C until PCR testing. Multiplex PCR (Philisa® ampC ID Kit, Streck, Inc., La Vista, USA) was conducted to detect 6 different families of plasmid-mediated *ampC* genes *i.e.*, ACC, CIT, DHA, EBC, FOX and MOX, and primers were used as published earlier.(7) The assay was performed according to recommendations in the user manual.

Ethical Clearance

This study has passed ethical evaluations by the Faculty of Medicine, Universitas Indonesia No. 51/UN2.F1/ETIK/2015 and Cipto Mangunkusumo Hospital Ethics Committee No. LB.02.01/X.2/105/2015.

Results

A total of 370 clinical specimens were collected from patients with infections in the ICU of Cipto Mangunkusumo Hospital from April 2015 to September 2015. After the cultivation and bacterial identification processes 63 isolates were identified as Enterobacteriaceae, and tested further for antibiotic susceptibility. The results showed 45 isolates composed of *K. pneumoniae*, *E. coli*, *E. cloacae* and *E. aerogenes* with decreased susceptibility to one or more of the 3rd generation cephalosporins and/or Aztreonam and/or resistance to Cefoxitin. Among those 4 species, *Klebsiella pneumoniae* was the most prominent (71.1%) and sputum was the main source of isolates (75.6%) (Table 1).

The DDS test was employed to these 45 isolates and the results were shown in Table 1, as follow: Four isolates were the AmpC-producing Enterobacteriaceae *i.e.*, *K. pneumoniae* (n=1), *E. cloacae* (n=2), and *E. aerogenes* (n=1). Thirty three isolates were the ESBL-producers in which *K. pneumoniae* was the most prominent among other Enterobactericeae. Five isolates were the AmpC/

Table 1. Species bacteria isolated, type of specimen and AmpC-producing Enterobacteriaceae determined by DDS test taken from patients in the ICU of Cipto Mangunkusumo Hospital.

Bacteria	Specimen Types (%)						Double Disc Synergy Test			
	Sputum	Wound Swab	Blood	Urine	Abscess Aspirate	Total, n (%)	AmpC	ESBL	AmpC ESBL	Non-producing
K . pneumoniae	27	3	2	0	0	32 (71.1)	1	27	2	2
E . coli	2	1	0	2	1	6 (13.4)	0	5	1	0
E . cloacae	4	1	0	0	0	5 (11.1)	2	0	2	1
E . aerogenes	1	0	1	0	0	2 (4.4)	1	1	0	0
Total, n (%)	34 (75.6)	5 (11.1)	3 (6.7)	2 (4.4)	1 (2.2)	45 (100)	4	33	5	3

ESBL producers *i.e.*, *K. pneumoniae* (n=2), *E. cloacae* (n=2) and *E. coli* (n=1). Three isolates were non-producing β-lactamases. In brief, out of 45 isolates of the putative AmpC β-lactamases and/or ESBL producers, only 9 (20%) isolates were confirmed by DDS test as the AmpC and AmpC/ESBL producers.

Antibiotic susceptibility tests were carried out as mentioned in the methods for the 42 AmpC, ESBL, and AmpC/ESBL confirmed isolates. Three isolates of the non-producing β-lactamases were not tested. As shown in Tables 2, the AmpC-producing isolates (4 isolates) showed ≥75% susceptibility to Gentamicin, Tobramycin, Amikacin, Cefepime, Meropenem, Ciprofloxacin, Levofloxacin, Tetracycline and Cotrimoxazole. All 5 isolates of the AmpC/ESBL-producing isolates showed resistant to all antibiotics but to Amikacin.

Gene family of AmpC-producing Enterobacteriaceae was determined by multiplex PCR on all of 45 isolates (Figure 1). The results showed that *ampC* genes were detected in 6 isolates consisted of 4 species *i.e.*, *K. pneumoniae*, *E. cloacae*, *E. aerogenes* and *E. coli*. The ampC gene families, *i.e.*, DHA, EBC and CIT were identified. DHA gene family was identified from 2 isolates of *K. pneumoniae* and 1 isolate of *E. cloacae*. EBC was from 1 isolate each of *E. cloacae* and of *E. aerogenes*, and lastly CIT was from 1 isolate of *E. coli* (Table 3).

Discussion

This study demonstrates that gene families of AmpC-producing Enterobacteriaceae are present in the ICU of Cipto Mangunkusumo Hospital, Jakarta. The distribution of the total proportion of AmpC-producing Enterobacteriaceae was 20%, consisting of 9 isolates out of 45 putative putative AmpC β-lactamases and/or ESBL producers. The result was quite similar with previous studies carried in India, Singapore and South Korea.(8,9,12,15) Our study showed that among 9 isolates of the AmpC producers, 5 of them produced AmpC and ESBL. Unlike the AmpC/ESBL producers that showed resistant to all antibiotics except for Amikacin, the AmpC-producing isolates were susceptible to many antibiotics such as Gentamicin, Tobramycin, Amikacin, Cefepime, Meropenem, Ciprofloxacin, Levofloxacin, Tetracycline, and Cotrimoxazole. In this study, 1 of the AmpC-producing isolate and 3 AmpC/ESBL-producing isolates showed

Table 2. Antimicrobial susceptibility patterns of the AmpC, ESBL and both AmpC/ESBL producing Enterobacteriaceae.

Antibiotic	AmpC (%) (n=4)	ESBL (%) (n=33)	AmpC/ESBL (%) (n=5)
Beta-lactams			
Ampicillin	0	0	0
Ampicillin/sulbactam	0	0	0
Piperacillin/tazobactam	25	42	20
Cefoxitin	0	58	0
Cefotaxime	25	0	0
Ceftriaxone	25	0	0
Cefpodoxime	25	0	0
Ceftazidime	25	0	0
Cefepime	75	0	20
Meropenem	75	73	40
Aztreonam	50	9	0
Quinolones			
Ciprofloxacin	75	9	0
Levofloxacin	75	15	20
Aminoglycosides			
Gentamicin	100	24	40
Tobramycin	75	6	20
Amikacin	100	76	100
Others			
Tetracycline	75	21	0
Trimethoprim/sulphamethoxazole	75	33	20
Fosfomycin	50	88	40

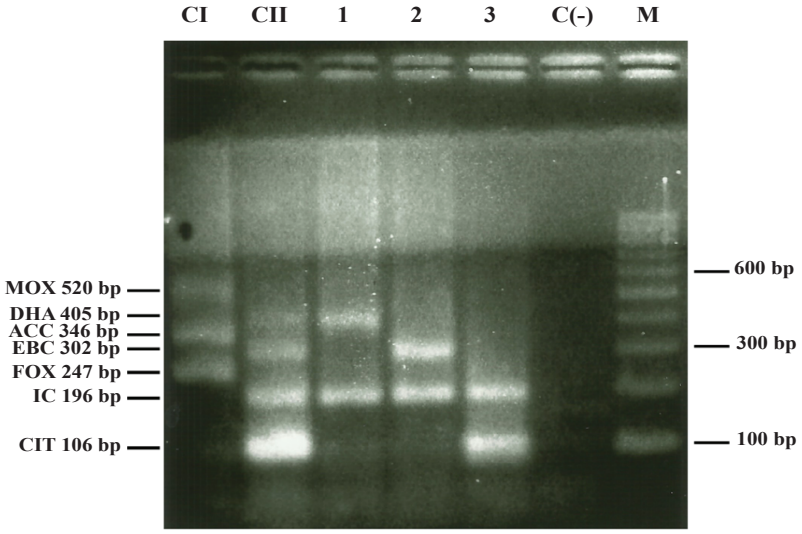

Figure 1. Multiplex PCR for *ampC* gene family. Lane C I: Positive control I: MOX (520 bp), ACC (346 bp), FOX (247 bp). Lane C II: Positive control II: DHA (405 bp), EBC (302 bp), CIT (160 bp). Lane C (-): Negative control. Lane 1, 2, 3: Samples with DHA positive, EBC positive and CIT positive respectively. Lane M: Standard DNA ladder for molecular size. MOX, DHA, ACC, EBC, FOX, and CIT are *ampC* gene gamily (DHA: firstly isolated in Dhahran Hospitals Saudi Arabia, EBC: isolated from *Enterobacter cloacae*, CIT: firstly isolated from *Citrobacter freundii*, ACC: Ambler class C, FOX: Active on Cefoxitin, and MOX: Active on Moxalactam, IC: internal control).

decreased susceptibility to Meropenem. This results might suggest that these microbes carry another resistance mechanism, such as porin loss.(6) Porins play a critical role in the penetration of antibiotics into the cells, and the loss of porins can reduce susceptibility to cephalosporins and carbapenem. Porins loss are associated with boosting the resistance to carbapenem by means of ESBL and AmpC β-lactamases.(16)

Up until today, studies about molecular epidemiology of resistance pathogens in Indonesia were still limited. To our knowledge, our study was the first investigation about the gene family of AmpC-producing Enterobacteriaceae in Indonesia. Using the multiplex PCR, DHA, EBC and CIT gene families of the AmpC-producing were identified. The DHA gene family seemed to be more common than EBC and CIT. Our results were in agreement with other studies carried in India that found the CIT and DHA gene families.

Table 3. Detection of gene family of AmpC-producing Enterobacteriaceae from 6 isolates phenotypically confirmed by DDS test.

Gene Family	DDS Test	Enterobacteriaceae Species
DHA	AmpC/ESBL	*Enterobacter cloacae*
	AmpC/ESBL	*Klebsiella pneumoniae*
	AmpC	*Klebsiella pneumoniae*
EBC	AmpC	*Enterobacter cloacae*
	AmpC	*Enterobacter aerogenes*
CIT	AmpC/ESBL	*Escherichia coli*
Not detected	AmpC/ESBL	*Klebsiella pneumoniae*
Not detected	AmpC/ESBL	*Enterobacter cloacae*
Not detected	AmpC	*Enterobacter cloacae*

(9,10,11) In Singapore, the presence of CIT gene family was reported while the EBC and DHA gene families have been found in Malaysia.(10,17) Other reports from Thailand (18) and South Korea (12) showed the presence of CIT, MOX, DHA and CIT respectively.

Hansen, *et al*., 2012 identified DHA gene family of AmpC-producing *K. pneumoniae*, however, the β-lactamase was not detected.(19) This condition could have occurred since DHA genes are inducible by β-lactam antibiotics, instead of expressed naturally.(6) Therefore the use of β-lactam antibiotics must be prudent to avoid an expression or even over expression of the *ampC* plasmid-mediated gene. In this study, *ampC* genes were only detected in 6 isolates out of 9 confirmed AmpC and AmpC/ESBL producers. This result could be due to the fact that of *ampC* gene family continue to expand, while the primers used in this study were only designated to these 6 *ampC* gene families. In addition, they also exist as varian *i.e*., the varian for the DHA are DHA-1 and DHA-2, the CIT are CMY-2 and CMY-4, the EBC are ACT-1 and MIR-1 and many more. There were some constraints in the present study, among other β-lactamase standard microorganisms for the DDS test was not available. Number of AmpC and AmpC/ESBL isolates positive from the DDS test was too low, which then might not show the presence of all ampC gene family has ever reported by PCR.

Conclusion

This study showed the AmpC and AmpC/ESBL-producing Enterobacteriaceae were simultaneously co-exist in the ICU of Cipto Mangunkusumo Hospital. Three *ampC* gene families, DHA, EBC and CIT were identified. All of the

AmpC/ESBL-producing isolates showed resistance to almost all antibiotic tested except for Amikacin. Therefore, the use of antibiotics for the treatment of the patients with infection in the ICU must be prudent to prevent the increase and the spread of the multi-drug resistant bacteria.

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