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Genotypic and Antibiotic Resistance Patterns of blaTEM, blaCTX and blaSHV Producing Klebsiella pneumoniae Isolates in Abdul Moeloek Hospital, Lampung, Indonesia

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Abstract: *The incidence of extended spectrum beta-lactamases (ESBL)-producing strains has been steadly increasing during the past few years, and remains an important cause of treatment failures with cephalosporin. This study intended to investigate the antibiotic resistance patterns and gene types of ESBL-producing Klebsiella pneumoniae isolates in Dr. H. Abdul Moeloek Hospital, Lampung, Indonesia.Klebsiella pneumoniae isolates (N=90) collected from various clinical samples such as pus, sputum, blood, wound swab, urine and body fluids were subjected to usual antibiotics using disc diffusion methods according to NCCLS criteria for resistance analysis.To determine the minimum inhibitory concentration (MIC) and identify the ESBL-producing strains, the isolates were confirmed with automated Vitek-2 system. Betalactamase production was assessed using double disk sinergy test (DDST) methods. While blaTEM, blaSHV, and blaCTX genotypes were determined by polymerase chain reaction (PCR) techniques. The ESBL producing Klebsiella pneumoniae strain was detected in 42 (46.7%) isolates of K. pneumoniae. High resistance of the isolates to the antibiotics was seen consecutively in ampicillin (100%), amoxycillin (78.8%), ceftazidime (57.8%), ceftriaxone (56.7%), cefepime (56.7%), aztreonam 56.7%, cefotaxime (55.6%) and cefazolin (51.5%). Susceptibility of the isolates to amikacin, ertapenem and meropenem is 96.6%, 94.4% and 94.4% respectively. The ESBL genotypes detected in the isolates consecutively are blaSHV (86.7%), blaTEM (60.0%) and blaCTX (45.6%). It is now obvious from this research findings that ESBL producing K. pneumoniae has increased so that therapeutic strategies need to be carefully formulated to control infections by reliable laboratory methods.*

Keywords: *Antibiotic resistance Pattern, Klebsiella pneumoniae, ESBL, blaTEM, blaSHV, blaCTX*

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I. Introduction

Extended spectrum beta-lactamases (ESBL) are enzymes that evolved with the capacity to degrade batalactam antibiotics. They also have extended action against non betalactam antibiotics such as the aminoglicosides, tetracyclines, chloramphenicol and quinolones [1]. Organisms producing ESBL are clinically relevant and remain an importatnt cause of falure of therapy with cephalosporins [2]. ESBL are primarily produced by the Enterobacteriaceae family, in particular Klebsiella pneumoniae and Escherichia coli [3]. Klebsiella pneumoniae is the causative agent of variety of diseases, including urinary tract and soft tissue infections, bacteremia, and pneumoniae. Klebsiella pneumoniae also one of the common causes of nosocomial infections and resistance to many antibiotics, including beta-lactams [4]. The prevalence of ESBL in Klebsiella pneumoniae is increasing worlwide [5].Global data show that the frequency of ESBL producing K. pneumoniae was 44% in South America, 33% in Europe, 22% in Asia and 12% in the United States [6]. The production of ESBL is the main resistance mechanism among bacteria of the Klebsiella genus.They are able to hydrolyze broadspectrum betalactams, such as third and fourth generation cephalosporins, monobactams, but not cephamicins and carbapenems, such as for example temoneira (TEM) enzyme and sulhydryl variable (SHV enzyme). blaTEM and blaSHV type ESBL are most often found in Klebsiella pneumoniae. The proportion of ESBL producers among hospital isolates varies, depending on geographical areas.

ESBL arise mainly due to mutation in beta-lactamases encoded by the blaSHV, blaTEM and blaCTX genes. At the present, more than 300 different ESBL variants have been described (3). Though blaTEM and blaSHV variants are the most common ESBL, during the past decade strains expressing blaCTX-M ESBL have begun to emerge in many countries and are now the most frequent non-TEM , non SHV ESBL type [7].

Previous study in Korea was found the prevalence of blaTEM type ESBL in 64,6%, blaSHV type in 70,7% and blaCTX-M type in 45% of 65 Klebsiella pneumoniae isolates [8].

The aim of the present study was to characterize clinical isolates of Klebsiella pneumoniae from various clinical specimens in Dr. H. Abdul Moeloek Hospital Lampung and determine the susceptibility patterns to antibiotics, evaluate the prevalence of ESBL and identify the genes type involved in the resistance.

II. Materials And Methods

2.1 Bacterial strains

The study was carried out 90 isolates from various clinical specimen. Consecutive, non repeated Klebsiella pneumoniae isolates obtained from pus (46,15%), sputum (15,38%), blood (15,38%), wound swab (10,98%), urine (8,79%) and bodyfluids (3,29%).The isolates were identified on the basis of conventional microbiological procedure at Microbiology Laboratory of Dr. H. Abdul Moeloek Hospital Lampung. Identification of isolates were done using automated Vitek 2 system too.

2.2 Antibiotic Susceptibility testing

Antibiotic susceptibility was performed by the Disk Diffusion- Kirby Bauer methods on Mueller Hinton agar. Inhibition zone were interpreted as Sensitive(S), Intermediate(I) and Resistant(R) by reference to Clinical and Laboratory Standard Instituted (CLSI) recommendation.

2.3 Automated Susceptibility testing using Vitek 2 compact system

MIC (minimal inhibitory concentrations) of 15 antimicrobial agents: amoxicillin, ampicillin, ampicillin-sulbactam, piperacillin-tazobactam, cefazolin, cefotaxime, ceftazidime, ceftriaxon, cefepime, aztreonam, ertapenem, meropenem, amikacin, gentamicin, and ciprofloxacin was determined. MIC values were interpreted as S, I and R by reference to CLSI breakpoints.

2.4 ESBLs Production Test

ESBL production also performed with Automated Vitek 2 system to all strains with reduced susceptibility of third generation cephalosporins. A confirmatory test for phenotypic detection of ESBLs were done with Double Disk Sinergy Test (DDST) methods according to the method described by Jarlier et al. [9].

2.5 Genotype Detection of ESBL genes

All the isolates were further analyzed by PCR to detect betalactamase genes. Total DNA extraction was performed using Presto^{TM} mini gDNA Bacteria Kit and KAPA Tag Extra HotStar Readymix 200 rxn from bacterial samples. blaTEM, blaSHV and blaCTX genes were detected by PCR. PCR were carried out using thermal cycler. The PCR mix was prepared in a volume of 20 µl containing 1 µl DNA template, 2 ml of 10 X PCR buffer, 1,5 mM MgCl2, 0,6 µl 2 mM dNTPs, 1 U taq polymerase, I µl of 10 pmol of each three primers, volume made up to 20 µl with distilled water. The primer sequences and cycling conditions used and product size for three different PCR (Table 1.)

Table 1. PCR conditions for amplification of blaTEM, blaCTx and blaCTX

Gene detected	Denaturation Time/temp	Annealing Time/temp	Extension Time/temp	No. of cycles	product
blaTEM	94°C – 1 mnt	58ºC – 1 mnt	72ºC – 1 mnt	30	1100 bp
blaCTX	94°C – 1 mnt	58ºC – 1 mnt	72ºC – 1 mnt	30	930 bp
blaSHV	94°C – 1 mnt	58ºC – 1 mnt	72ºC – 1 mnt	30	544 bp

PCR was performed using 2 sets of primers, each targeting different regions and was detect blaTEM, blaSHV and blaCTX encoding genes. The specific primers (Table 2.) were use in this study [10].

Table 2. Primers used for amplification of blaTEM, blaSHV and blaCTX

SI.No	Gene detected	Primer
1.	blaTEM	F5' ATAAAATTCTTGAAGACGAAA 3' R5' GACAGTTACCAATGCTTAATCA 3'
2.	blaSHV	F'5' GGGTAATTCTTATTTGTCGC 3' R5' TTAGCGTTGCCAGTGCTC 3'
3.	blaCTX-M	F 5' TTTGCGATGCAGTACCAGTAA 3' R5' CGTATATCGTTGGTGGTGCCATA 3'

The PCR products were separated by gel electrophoresis on 1% agarose gel.

2.6 Control strains

For Susceptibility test, phenotypic methods and for uniplex PCR, two ATCC strains (American Type Culture Collection, USA) have been used: Klebsiella pneumoniae ATCC 108833 as negative control and ATCC 700603 as ESBL positive control.

III. Results

A total of 90 Klebsiella pneumonia isolates were included in this study, the majority of isolates (42 strains: 46,15%) were from pus samples. The antibiotics susceptibility test show that all of isolates were resistant 100% to ampicillin followed by amoxicillin (75,8%), ceftazidime (57,1%), ceftriaxone (56,0%), cefepime (56,0%), aztreonam (56,0%), cefotaxime(54,8%) and cefazolin (53,8%). The most efficient antibiotics were amikacin (96,7% as susceptibility rate), followed by meropenem and ertapenem (94,5% each other) (Table 3). Of the 90 isolates screened for ESBL production, 42 isolates (46.7%) were positive ESBL producers (Fig 1). There were different about susceptibility rates between ESBL producers with ESBL non producers. ESBL producers strain were more resistant than ESBL strains non producers(Table 4). According to the PCR, the genotypes blaTEM, blaSHV and blaCTX were distributed as follow: blaSHV 78 (86,7%), blaTEM 54 (60,0%) and blaCTX 41 (45,6%) (Fig 2and 3).

Table 3. Antibiotic susceptibility pattern of Klebsiella pneumonia (n=90)

Antibiotics	Susceptibility rate (%)		
	R	I	S
AMC	71 (78.8%)	3 (3.3%)	8 (8.8%)
AMP	90 (100%)	0	0
SAM	41 (45.6%)	12 (13.3%)	37 (41.2%)
TZP	8 (8.88%)	18 (20.0%0	64 (71.2%)
KZ	49 (51.5%)	0	41 (45.6%)
CTX	50 (55.6%)	0	40 (43.4%)
CAZ	52 (57.8%)	0	38 (42.3%)
CRO	51 (56.7%)	0	39 (43.4%)
FEP	51 (56.7%)	0	39 (43.4%)
AZT	51 (56.7%)	0	39 (43.4%)
ERT	5 (5.6%)	0	85 (94.4%)
MEM	5 (5.6%)	0	85 (94.4%)
AK	1 (1.11%)	0	87 (96.6%)
CN	29 (32.3%)	0	59 (65.5%)
CIP	26 (28.8%0	0	60 (66.7%)

AMC: amoxicillin, AMP: ampicillin, SAM: ampicillin-sulbactam, TZP:piperacillin-tazobactam, KZ:cefazolin, CTX: cefotaxime, CAZ: ceftazidime, CRO:ceftriaxone, FEP:cefepime, AZT:aztreonam, ERT:ertapenem, MEM:meropenem, AK: amikacin, CN: gentamicin, CIP: ciprofloxacin

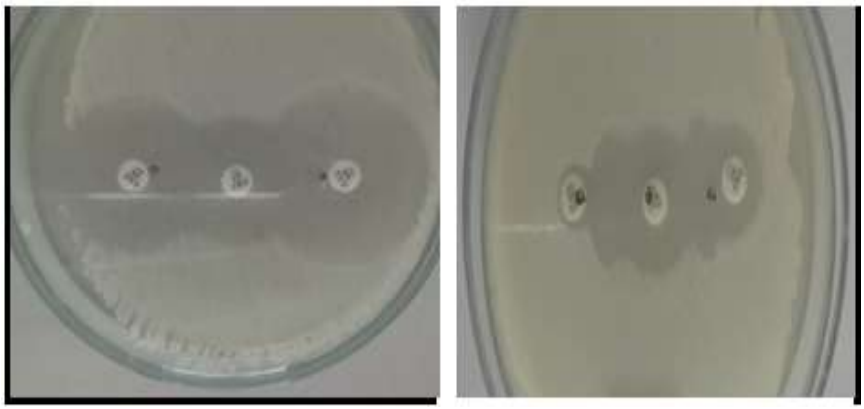

Fig 1. ESBL with negative (A) and positive producers (B)

Both blaTEM and blaCTX genes were present in only 3 isolates (3,33%), blaTEM and blaSHV in 19 isolates (21,1%), blaCTX and blaSHV in 6 isolates (6,66%). For more two ESBL genes were present in 31 isolates (34,4%). The antibiotic susceptibility pattern show that all of ESBL type genes were higher resistant to ampicillin and amoxicillin. The group with three ESBL type genes were higher resistant to all antibiotics (Table 5).

Table 4. Association between ESBL producing with Antibiotic susceptibility Pattern to Cephalosporin

Antibiotics	ESBL(n=42)		Non ESBL(n=48)		p-value*
	R	S	R	S	
KZ	37	5	12	36	0,000
CTX	35	7	15	33	0,000
CAZ	40	2	12	36	0,000
CRO	40	2	11	37	0,000

*chi-square test; significant if p value p ≤ 0,05

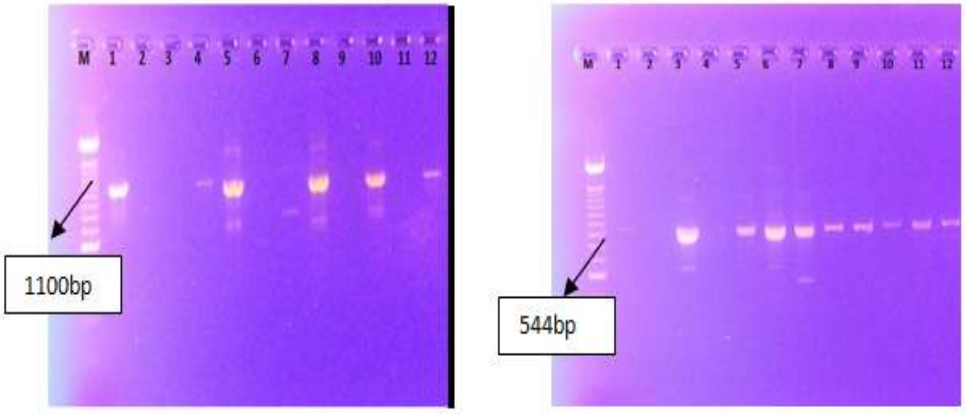

Fig 2. Agarose gel of PCR products following amplification of blaTEM genes (positive line 1,4,5,8,10,12 and blaSHV genes (positive line 1,3, 5-12)

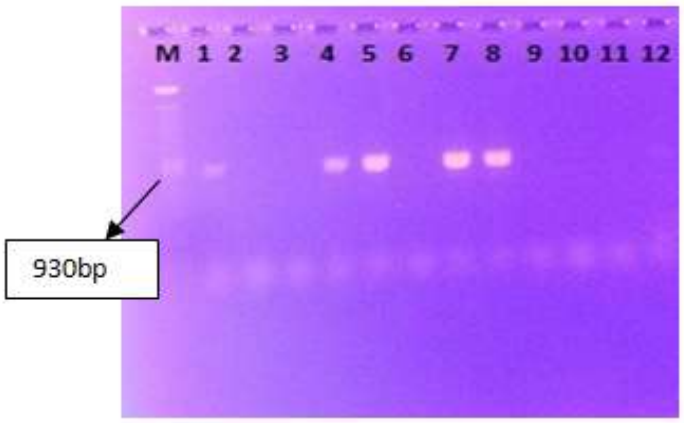

Fig 3. PCR products following amplification of blaCTX genes (positive line 1, 4,5,7,8)

Table 5. ESBL positive strains: resistance to potentially active drugs according to gene type

Antibiotics	TEM (n=54)	CTX (n=41)	SHV (n=78)	TEM + CTX (n=3)	TEM + SHV (n=19)	CTX + SHV (n=5)	TEM + SHV + CTX (n=31)
% Resistant							
AMC	85,1	87,8	76,9	100	78,9	100	90,3
AMP	98,1	100	100	100	84,2	100	100
SAM	50,0	73,1	42,3	33,3	5,26	66,6	80,6
TZP	9,25	19,5	10,2	0	0	50,0	16,12
KZ	62,9	78,0	52,5	66,6	36,8	66,6	80,6
CTX	57,4	65,8	52,5	100	31,5	66,6	67,7
CAZ	70,3	78,0	53,8	100	52,6	66,6	80,6
CRO	70,3	78,0	55,12	100	52,6	66,6	80,6
FEP	70,3	78,0	55,12	100	52,6	66,6	80,6

AZT	70,3	78,0	55,12	100	52,6	66,6	80,6
ERT	7,4	9,7	6,41	0	0	0	12,9
MEM	7,4	9,7	3,84	0	0	0	12,9
AK	0	0	1,28	0	0	0	0
CN	40,7	33,3	33,3	33,3	5,26	50,0	64,5
CIP	37,0	33,3	33,3	33,3	10,52	50,0	54,8

Statistically, the isolate with ESBL blaCTX type genes more significantly associated with resistant to antibiotics than isolates with ESBL blaSHV type genes (Table 6).

Table 6. Association between ESBL genotype and antibiotic susceptibility profile

Antibiotics	**Genotypes**								
	blaTEM (n=54)			**blaCTX** (n=41)			**blaSHV** (n=78)		
	R	S	p-value*	R	S	p-value*	R	S	p-value*
KZ	35	19	0,022	32	9	0,000	41	37	0,361
CTX	31	23	0,772	28	13	0,026	41	37	0,145
CAZ	38	16	0,005	32	9	0,000	44	34	0,503
CRO	38	16	0,002	32	9	0,000	43	35	0,453

*Chi-square test, significancy if p value p ≤ 0,05

IV. Discussion

In our study, from susceptibility test, the highest resistant were found to ampicillin (100%), followed by amoxicillin (78.8%) and cephalosporin group (55.6% to 57.8%). The most efficient antibiotics were amikacin (96.6%), meropenem (94.45%0 and ertapenem (94.4%). This finding is similar to result previously study described by Kaftandzieva et al. [11]. This finding may be due to uncontrolled consumption of antibiotics, consequences of easy access to inefficient and cheap for ampicillin and amoxicillin antibiotics. The isolate were found 46.7% ESBL producers. This data similar with the results of the Regional Resistance Survellance program susceptibility rates from 12 Asia-Pasific countries (APAC) in 2011 (APAC rate 47%). Indonesia is the highest rate of the prevalence of ESBL production in Klebsiella pneumoniae [12]. The comparison between ESBL producing strains and non ESBL, showed that ESBL producers were significantly more resistant to penicillins, cephalosporin than non ESBL producers. In this study, the genotype blaSHV was predominant in ESBL and non ESBL isolates (86.7%). This finding not similar with previously study. The blaCTX enzymes was the dominant ESBL type in Southeast Asia region [10, 12]. Other study was found that blaSHV type were the predominant ESBL type in Klebsiella pneumoniae (51.5%) than blaTEM type and blaCTX type although blaCTX type of isolates show significancy associated with antibiotics resistant from all group [13]. The presence of more than one gene type in some of the isolates like blaTEM + blaCTX, blaTEM + blaSHV and blaSHV + blaCTX means that the ESBL producing strains may be related to complex antimicrobial resistance. In this study, combination blaTEM + blaSHV were the common combination type of the isolates (21.2%). All of the isolates mostly sensitive of betalactamase inhibitor such as ampicillin-sulbactam and piperacillin tazobactam (5.26% and 0% respectively). This finding similar with results by Kaftandzieva et al. [11].

Regardless of it all, the emergence of ESBL producing organisms seems to be the result of complex interactions between the type of ESBL, the genetic background of the strain and selective pressures existing in ecologic niches. Heavy antibiotic use (especially the third generation cephalosporins) is one of the selective pressures and a risk factor for acquisition of ESBL producing. Therefore, clinicians should be familiar with the clinical importance of these enzymes and potential strategis for dealing with them. The correct detection of ESBL producing is a challenge for the laboratories, requiring not only phenotypic test but also genotypic test for all ESBL type genes.

V. Conclusions

Our findings suggest that all of isolates Klebsiella pneumoniae were resistant 100% to ampicillin. Cephalosporin group were intermediate susceptibility rate (51.5% to 57.85%). The most efficient antibiotics were amikacin (96.7%) and followed by meropenem and ertapenem (94.4%). The presence of blaSHV as the predominant genotype in Klebsiella pneumonia (86.7%) than blaTEM and blaCTX. The isolate with ESBL producers and ESBL type genes more resistant than non producers and non ESBL type genes. The results of this study describe the genetic characteristics and molecular epidemiology of ESBL among Klebsiella pneumoniae at Dr. H. Abdul Moeloek Hospital Lampung, Indonesia.

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