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Molecular Characterization of Extended-Spectrum β-Lactamases-Producing *Lebsiellapneumoniae* Isolated from Clinical Specimens at a Tertiary-Referral Hospital in Denpasar, Bali, Indonesia

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Almost no study has been performed in Bali to characterize genotype of ESBL-producing *Klebsiellapneumoniae*. The aim of this study was to determine genotype of ESBL produced by *K.pneumoniae* isolated from clinical specimens in Sanglah General Hospital, Bali, Indonesia. Ninety-seven non-duplicative ESBL-producing *K.pneumonia* isolates were examined for 16s rRNA, and then with Duplex PCR to detect bla_{SHV} and bla_{CTX-M} genes, and with Uniplex PCR for bla_{TEM} gene. Half of the isolates (50.5%) had coexistences of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes. Presence of two genes, bla_{TEM} and bla_{SHV}; bla_{TEM} and bla_{CTX-M}; and bla_{SHV} and bla_{CTX-M}, were 12.4%; 15.5%; and 8.2%, respectively. This study showed the widespread of TEM-, SHV-, and CTX-M-type ESBLs in Bali. Prudent use of antibiotics can save our planet from multidrug resistance microorganisms (MDROs) threat.

Keywords: Extended Spectrum β-Lactamase, *Klebsiellapneumoniae*, PCR, Sanglah General Hospital Denpasar.

1. INTRODUCTION

Multidrug resistance microorganisms (MDROs) tend to increase worldwide recently. This condition is not only important for hospital setting, but also can put our planet in danger. The misuses of broad-spectrum antibiotics agents can promote higher prevalence of MDROs. One of MDROs, which is dangerous for critically ill patients in hospital, is Extended-spectrum β-lactamases (ESBL) produced by-*Enterobacteriaceae*.[1] Nowadays, ESBLs are more prevalent produced by *Klebsiella pneumoniae*.[1,2] The isolates have been linked to healthcare associatedinfections (HAIs), which are usually difficult to be treated. ESBLs are β-lactamases that hydrolyze penicillins and oxymino—cephalosporins, including monobactam, but are inhibited by β-lactamases inhibitor. These enzymes are resulted from point mutations of TEM or SHV genes,[3] however, recently CTX-M type ESBLs have been reported to increase rapidly.

Several studies reported the CTX-M enzymes spread mostly in Asia, South America, Europe and Africa.[4,5] Data of ESBL-producing *K.pneumoniae* prevalence in Indonesia is limited. Previous study reported that CTX-M-15 type ESBL was the most prevalent ESBL produced by *K.pneumoniae* clinical isolates from Surabaya, Indonesia[6].The prevalences of ESBL-producing *K.pneumoniae*at the tertiary-referral hospital, Sanglah General Hospital, in Bali have shown increasing based on phenotypic test. The prevalence was about as high as 35% of all *K.pneumoniae* isolated in January-June 2013 (*unpublished data*); however, almost no study has been conducted in Bali to determine molecular characterization of ESBL-producing *K.pneumoniae*. Therefore, the aim of this study is to investigate molecular characterization of clinical isolates of ESBL-producing *K.pneumoniae* at Sanglah General Hospital, Denpasar, Bali Indonesia.

2. EXPERIMENTAL DETAILS

This study has obtained Ethical Clearance Approval from Ethic Commission, Research and Development Unit of Faculty of Medicine/Sanglah General Hospital, Denpasar. Bali, Indonesia.

2.1. Bacterial Isolates and Phenotypic Characterization

Ninety-seven non-duplicative clinical isolates of *K.pneumoniae* were included in this study. ESBL producing *K.pneumoniae* were

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Table I. Primer sets used for bla_{TEM}, bla_{SHV}, and bla_{CTX-M} detection.

PCR target	Primer sequence (5'-3')	Size (bp)	Ref.
16S rRNA	AGA GTT TGA TCM TGG CTC AG ACG GHT ACC TTG TTA CGA CTT	~1,500	[9]
bla_{TEM}	ATG AGT ATT CAA CAT TTC CG CCA ATG CTT AAT CAG TGA GG	858	[8]
bla_{SHV}	ATG CGT TAT ATT CGC CTG TG AGC GTT GCC AGT GCT CGA TC	862	[8]
bla_{CTX-M}	SCS ATG TGC AGY ACC AGT AA ACC AGA AYV AGC GGB GC	585	[8]

isolated from blood (36 isolates), urine (28 isolates), pus/ wound (18) sputum (10 isolates) and CSF (5 isolates). Phenotype of ESBL isolates was determined by using Disk Diffusion Synergy Test (DDST) as describe previously with slight modification.[2,7] The distance between Amoxicillin-Clavulanic Acid (AMC) disk to Cefotaxime (CTX), Ceftazidime (CAZ), Cefepime (FEP), and Aztreonam (ATM) disks was 25 mm center-to-center. *K.pneumoniae* ATCC BAA-1706 was used as the negative control isolate in this study.

2.2. PCR Amplification for 16s rRNA, Uniplex PCR for bla_{TEM}, and Duplex PCR for bla_{SHV} and bla_{CTX-M} Amplification

Bacterial genomic DNA was isolated from colonies by using Roche High Pure PCR Template Isolation Kit (Roche Life Science, Indianapolis, USA) based on manufacturer's instruction. DNA was eluted with 50 μ_l of elution buffer. PCR was conducted using Go Taq® Green Master Mix (Promega, Madison, USA). Uniplex PCR to detect 16S rRNA gene was performed. It was followed by uniplex PCR to detect blaTEM gene and duplex PCR to detect bla_{SHV} and bla_{CTX-M}. Lists of primer sets used in this study were shown in Table I.

Protocols of PCR were described in previous study with modification for duplex PCR.[8] PCR cycle was initiated with pre denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 30 sec and extension at 72 °C for 1 min; and final extension at 72 °C for 4 min (iCycler, Biorad thermal cycler). Duplex PCR was performed with two primer sets of bla_{SHV} (0.2 mM final concentration of each primer) and bla_{CTX-M} (0.3 mM final concentration). Amplicons were electrophoresed in 0.8% agarose gel in TBE buffer, at 60 volt for 35 min. DNA was visualized with ethidium bromide and captured (Gel Doc, Biorad).

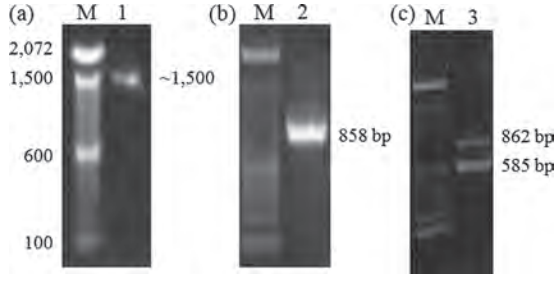

Fig. 1. 16SrRNA gene (~1500 bp) detected by using Uniplex-PCR (a). bla_{TEM} detected by using Uniplex PCR (lane 2) (b), and bla_{SHV} *and* bla_{CTX-M} genes simultaneously detected by using Duplex-PCR (lane 3) (c) from *K.pneumoniae* isolate no. 0177.

Table II. bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes detected by using Uniplex- and Duplex-PCR from 97 ESBL-producing *K.pneumoniae* isolates.

No.	Identified genes	No. (%)
1.	bla_{TEM}	7 (7.2)
2.	bla_{SHV}	3 (3.1)
3.	bla_{CTX-M}	3 (3.1)
4.	bla_{TEM} and bla_{SHV}	12 (12.4)
5.	bla_{TEM} and bla_{CTX-M}	15 (15.5)
6.	bla_{SHV} and bla_{CTX-M}	8 (8.2)
7.	bla_{TEM}, bla_{SHV}, and bla_{CTX-M}	49 (50.5)
	Total	97 (100.0)

3. RESULTS AND DISCUSSION

All 97 clinical isolates were positive tested for the presence of 16s rRNA gene (Fig. 1(a)) and all isolates were *K.pneumoniae.* Gene of blaTEM was detected using Uniplex-PCR, while blaSHV and bla CTX-M genes by Duplex-PCR (Figs. 1(b) and 1(c)).

Amplicons were electrophoresed in 0.8% agarose gel and visualized with ethidium bromide. (M = marker, 100 bp DNA Ladder, Invitrogen). It was found that most of isolates had coexistences of more than one gene. As shown in Table II, 49 (50.5%) ESBL-producing *K.pneumoniae* were positive harboring bla_{TEM}, bla_{SHV}, and bla_{CTX-M}. Coexistences of two genes were also observed in this study. Twelve (12.4%) of isolates had bla_{TEM} and bla_{SHV}, 15 (15.5%) had bla_{TEM} and bla_{CTX-M} and 8 (8.2%) had bla_{SHV} and bla_{CTX-M}

This study showed that *K.pneumoniae* that produce ESBL mostly possessed multiple genes, which were also reported in previous study conducted by Goyal et al.[10] This group found that 57.1% of ESBL-producing *K.pneumoniae* had multiple genes of ESBL.[10] Several studies also showed that *K.pneumoniae* isolates had more than one type of ESBLs, although it was not as high as that of this study.[6,11] Higher prevalence of ESBLs detected in this study might be caused by misuse of antibiotics,[2] or better screening method of MDROs in the hospital laboratory. Severin[6] reported that most of ESBL type found in Surabaya is CTX-M ESBL.[6] Other studies in Singapore, Vietnam and Thailand also showed prominent spreading of CTX-M ESBL type.[4,12,13] In this study, CTX-M type prevalence was also found to be high but still SHV type was predominantly found among the isolates, suggesting there might be spreading of both SHV and CTX-M in Denpasar, Bali.

4. CONCLUSION

This study showed ESBL produced by *K.pneumonia* isolated from clinical specimens in Bali are mostly encoded by bla_{TEM}, bla_{SHV}, and bla_{CTX-M}. The molecular characterization of these types ESBL is noteworthy for clinical microbiologist to perform early and prompt laboratory detection, and also to plan infection prevention and control program in preventing spread of multidrug resistance organisms. It can act as an alarm for the clinicians in management of patients with serious *K.pneumoniae* infections, especially to use antibiotics prudently. At the end, we can save our planet from spreading of multidrug resistant microorganism (MDROs). Further investigation of molecular epidemiology of hospital- or community-ESBLs with bigger number of samples and involving several health care enter would be promising to get good database for ESBLs-producing microorganisms in Bali.

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