

THE PREVALENCE OF EXTENDED SPECTRUM β -LACTAMASE (ESBL) PRODUCING GUT BACTERIAL FLORA AMONG PATIENTS IN DR. SOETOMO HOSPITAL AND PRIMARY HEALTH CENTER IN SURABAYA

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ABSTRACT

The extended-spectrum β -lactamase (ESBL) producer bacteria until now are mostly identified in hospital environment. The aim of this study was to analyze the prevalence of ESBL-producing gut flora and distribution of ESBL encoding genes between hospitalized patients in Tropical Wards of Dr. Soetomo Hospital and patients from a primary health center (PHC) as community environment in Surabaya. Thirty rectal swab samples from patients of Dr. Soetomo Hospital and of PHC (60 samples in total) were collected for this study. Samples were screened in MacConkey agar supplemented with 2 mg/L of cefotaxim, incubated at 37°C for 24 hours. Furthermore, the growing colony were confirmed with Disk Diffusion Synergy test (DDST) for diagnosis of ESBL producer. The identified ESBL producers were then identified the bacteria species by biochemical method. ESBL gene were detected by PCR with specific primers. The results showed that there was no difference in the positive number of ESBL-producing gut bacterial flora between patients of Dr. Soetomo Hospital, 25/30 (83.3%) and PHC, 11/30 (36.7%) ($p=1$). The pattern of ESBL gene distributions among samples from the hospital showed that SHV was 12%, TEM was 36%, and CTX-M was 80%, and from PHC were SHV 18.2%, TEM 27.3% and CTX-M 81.8%. Statistical analysis showed that the pattern was not significantly different among hospitals and PHC samples as shown by SHV gene ($p=0.631$), TEM ($p=0.715$), and CTX-M ($p=1$). From each ESBL gene, the dominant genes that found producing ESBL were the CTX-M genes followed by TEM and SHV genes. The prevalence of ESBL producers in intestinal flora of both the hospital (83.3%) and the PHC (36.7%) was very high. There was no significant difference between the prevalence of ESBL producer in gut flora of hospitalized patients compared to PHC. We also found other patterns of ESBL gene combinations in the hospital. The patterns are SHV+CTX-M genes, TEM+CTX-M, SHV+TEM+CTX-M genes, and in PHC, the combination pattern are SHV+CTX-M, TEM+CTX-M.

Keywords: Gut flora; ESBL; Dr. Soetomo Hospital; Primary Health Center (PHC)

ABSTRAK

Bakteri penghasil spektrum β -laktamase (ESBL) yang meluas sebagian besar diidentifikasi di lingkungan rumah sakit. Penelitian ini bertujuan untuk menganalisis perbandingan prevalensi dan distribusi gen ESBL pada bakteri flora usus pada pasien di RSUD Dr. Soetomo ruang tropik dan Puskesmas "X" di Surabaya. Sampel penelitian sebanyak 60 sampel terdiri dari 30 sampel pasien rumah sakit dan 30 sampel pasien puskesmas diambil dari hasil swab rektum pada masing-masing lokasi. Sampel ditanam di media selektif MacConkey dengan penambahan sefotaksim 2 mg/L, dan diinkubasi selama 24 jam pada suhu 37°C. Koloni yang tumbuh didiagnosis dengan pemeriksaan biokimiawi, kemudian dilanjutkan dengan deteksi fenotipik dengan uji Disk Diffusion Synergy test (DDST) dan genotipik menggunakan PCR untuk mendeteksi gen ESBL. Hasil: Hasil pemeriksaan menunjukkan tidak terdapat perbedaan jumlah positif bakteri ESBL flora usus antara pasien rumah sakit Dr. Soetomo, yaitu 25/30 (83,3%) dan pasien puskesmas, yaitu 11/30 (36,7%) ($p=1$). Pola distribusi gen ESBL pada rumah sakit ditemukan gen SHV (12%), TEM (36%), dan CTX-M (80%), dan di puskesmas ditemukan gen SHV (18,2%), TEM (27,3%) dan CTX-M (81,8%). Hasil analisis menunjukkan bahwa tidak terdapat perbedaan pola prevalensi dan distribusi gen pada rumah sakit dan puskesmas gen SHV ($P=0,631$), TEM ($P=0,715$), dan gen CTX-M ($P=1$). Dari gen ESBL yang diteliti (gen SHV, TEM, dan CTX-M) ditemukan bahwa, gen CTX-M adalah gen yang dominan menghasilkan bakteri ESBL, diikuti gen TEM dan SHV. Kesimpulan: Prevalensi gen ESBL pada bakteri usus baik di rumah sakit maupun Puskesmas sangat tinggi. Tidak ada perbedaan prevalensi di antara gen ESBL flora usus pasien rumah sakit di banding Puskesmas. Ditemukan pola kombinasi gen ESBL pada rumah sakit, kombinasi gen SHV+CTX-M, TEM+CTX-M, SHV+TEM+CTX-M dan puskesmas, kombinasi gen SHV+CTX-M, gen TEM+CTX-M.

Kata kunci: Flora usus; ESBL; Rumah Sakit Dr. Soetomo; Puskesmas X

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INTRODUCTION

The effect of antibiotic therapy on clinical infection cases may have an impact on commensal bacteria present in humans, such as intestinal flora. The effect of antibiotic use may lead to increased populations of resistant bacteria to antibiotic in human intestine. The pattern of bacterial populations in the body depends on exposure to the type of antibiotic used, antibiotic action, and the level of resistance present in the community (Jernberg et al 2010).

Nowadays, the most important of bacterial resistance to antibiotics is Extended-spectrum β -lactamase (ESBL) bacteria. ESBL is a β -lactamase enzyme that may induce bacterial resistance to penicillin, 1, 2 and 3 cephalosporins generation, and aztreonam (but not to cephamine and carbapenem) by hydrolyzing antibiotics, which can be inhibited by β -lactamase inhibitors, such as clavulanic acid (Peterson & Bonomo 2005). ESBL is produced by gram-negative bacteria, one of which is the Enterobacteriaceae family which is one of the normal flora of the intestine. These bacteria have been reported to be resistant to β -lactam antibiotics. The bacteria Enterobacteriaceae is one of the main causes of bacterial infections in hospitals, site of treatments, or in the community (Shakil & Khan 2010).

During 2008 in Thailand, the bacteria of Enterobacteriaceae was identified in about 60% of the stools of healthy individuals in the community (Sasaki et al 2010). In 2002, ESBL-producing bacteria from rectal swabs were found in 0.9% among community health centers (Severin 2010). The spread of microorganism resistance easily occurs among strains and even among species, since ESBL encoding gene is located in the plasmid, which facilitates the transfer of bacterial ESBL genes (Colodner & Raz 2005). There are three types of the ESBL gene group: sulfhydryl variable (SHV), type of Temoneira enzyme (TEM), and cefotaximase-munich (CTX-M) (Peterson & Bonomo 2005, Pitout & Kevin 2008). In the UK, there are two studies on the wide-spread of ESBL-producing bacteria. The first study, from York, bacterial member of Enterobacteriaceae was found to produce CTX-M genes in community and hospitalized patients. A second study was conducted by Woodford et al. who examined 291 samples and found *Escherichia coli* that produced the CTX-M gene (Kumar and Babu, 2013). A research in Indonesia found the prevalence of CTX-M gene was about 94.5% (Severin 2010).

In hospitals and primary health centers, the spread of ESBL-producing bacteria poses a major challenge in reducing the spread of multi-resistant bacteria. This study discloses important information about increasing

of ESBL-producing gut flora in both hospital and primary health center (PHC) to explore the spread of ESBL producing bacterial and planning the program for controlling bacterial resistance. This study aimed to determine the pattern of ESBL gene distribution in Tropical Wards of Dr. Soetomo Hospital and in PHC in Surabaya.

MATERIALS AND METHODS

The study was conducted from April to July 2017 at Department of Clinical Microbiology, Dr. Soetomo Hospital, Surabaya, and Institute of Tropical Disease, Universitas Airlangga. All rectal swab samples were collected from patients in tropical ward of Dr. Soetomo Hospital and PHC. Total sampel was 60 rectal swab samples consisted of 30 samples from the tropical ward of Dr. Soetomo Hospital taken with consecutive sampling technique and met the inclusion and exclusion criteria; and 30 samples from the PHC taken with systematic random sampling technique. Samples from the PHC was randomly selected from the samples of previous study (Dewanti et al 2016). The samples were grown in MacConkey selective media supplemented with cefotaxime of 2 mg/L, incubated for 18-24 hours at 37°C (Sasaki et al 2010). The bacteria which were able to grow on that medium was suspected as ESBL producing bacteria.

ESBL confirmation was performed using Double Disk Synergy Test (DDST). The antibiotic disks used for DDST included Cefotaxime (CTX), Ceftazidime (CAZ), Ceftriaxone (CRO), and Aztreonam (ATM) discs. Each disc contained 30 μ g disks, and Amoxicillin-clavulanic acid (AMC) (30/10 μ g disk). The results were positive if an inhibitory zone or synergy occurs between one disc and AMC disk (Dalela 2012, Dhara et al 2012). The species were identified using colonial morphology on Mac-Conkey selective agar plates, followed by bio-chemical examination, other tests such as triple sugar iron (TSI), indole, methyl red (MR), voges proskauer (VP), citrate, and motility test (Tille 2014). Genotypic identification by using PCR amplification, with the primers were SHV-F, 5'-GGTTATGCGTTATATTCGCC-3', SHV-R, 5'-TTAGGTTGCCAGTGCT-3' with amplicon size of 867 bp, PCR conditions of 1 cycle of 5 min at 96°C; 35 cycles of 1min at 96°C (denaturation), 1min at 60°C, 1min at 72 °C (annealing); 1 cycle of 10 min at 72 °C (extension). TEM-F primers, 5'-ATGAGTATTCAACA TTTCCG-3', TEM-R, 5'-CTGACAGTTACCAATGCT TA-3' with amplicon size of 867 bp, PCR conditions of 1 cycle of 5 min at 96°C; 35 cycles of 1min at 96°C (denaturation); 1min at 60°C (annealing); 1min at 72 °C; 1 cycle of 10 min at 72°C (extension). CTX-MF 5'-

ATGTGCAGYACCAGTAARGT-3 primer and CTX-M-R 5' TGGGTRAARTARCTSACCAGA 3' with amplicon size of 593 bp, PCR conditions of 1 cycle of 7 min at 94°C; 35 cycles of 50 sec at 94 °C (denaturation), 40 sec at 52°C (annealing), 1 min at 72°C; 1 cycle of 10min at 72°C (extension) (Ferreira et al 2011). Data were statistically analyzed by Fisher's Exact test with SPSS version 23 program.

RESULTS

Characteristic of the subjects showed that the highest percentage was females as many as 25/30 subjects (83.3%) from the hospital, and 19/30 samples (63.3%) from PHC. There was no significant difference in sex distribution between samples from hospital and PHC ($p=0.143$). Based on age characteristics, we took all age groups categories obtained from sampling sites. The highest percentage from the hospital was productive age (15-60 years) those were 20/30 patients (66.7%) from the hospital, whereas, the highest percentage from the PHC, was younger age (<15 years) those were 14/30 patients (53.3%). There was no significant difference in age distribution between samples from hospital and PHC ($p=0.108$).

Data on the use of antibiotic in hospital showed that 21 out of 30 patients (70%) used antibiotics, while in PHC there was no patient used antibiotics (0%). There was a significant difference in antibiotic usage patterns between the hospital and PHC ($p=0.000$). The prevalence of ESBL producing gut flora (after confirmed by DDST) in the hospital and PHC was 25/30 (83.3%) and 11/30 (36.7%) respectively. Genotyping ESBL encoding genes by using PCR found 22 samples from the hospital and 10 samples from PHC containing ESBL

encoding genes. There was no significant differences on the distributions of ESBL encoding gene between the hospital and PHC samples ($p=1$). ESBL encoding genes were identified in samples from the hospital and 1 samples from PHC (Tabel 1).

DISCUSSION

General characteristics by age and sex indicate no risk of ESBL gene in the feces, which is usually reported as rare. Other studies found that the distribution of ESBL was equally distributed in all age groups (Nakayama et al 2012, Yuwono 2011). There was a significant difference in antibiotic usage patterns between hospitals and PHC. In this study the use or not using of antibiotics showed differences in the prevalence and distribution genes. Although there was no antibiotic use in PHC, the frequency of ESBL resistance was high. It cannot be explained the role of antibiotic use against the prevalence and distribution of ESBL genes in this study. There may be other contributing factors, such as the spread of ESBL-producing bacteria, between PHC patients and hospital patients, PHC patients and the community, between individuals in the community, or the food chain cycles in the environment. Hilty et al (2012) found a dynamic spread in patient families, patients with patients and hospitals with communities. Another study conducted by Imasari et al (2017) showed the ESBL gene have spread among animals (dairy cows) (72%) and humans (79.1%) (people around farms), this spread may occur through direct contact or indirectly. The results of this study were similar to those of Sarassari (2017) that found 98% of ESBL positive intestinal flora in medical student community with no antibiotic use.

Table 1. Distribution of ESBL (+) and genotyping of ESBL gene by PCR

	Hospital		PHC		p
	ESBL (+) n (%)	ESBL (-) n (%)	ESBL (+) n (%)	ESBL (-) n (%)	
Distribution of ESBL (+)					
ESBL	22 (73.3)	5 (16.7)	10 (33.3)	19 (63.3)	p= 1
ESBL Unidentified gene*	3 (10)	0	1 (3.4)	0	
Total	25 (83.3)	5 (16.7)	11 (36.7)	19 (63.3)	
ESBL gene					
SHV	3 (12)	22 (88)	2 (18.2)	9 (81.8)	p= 0.637
TEM	9 (36)	16 (64)	3 (27.3)	8 (72.7)	p= 0.703
CTX-M	20 (80)	5 (20)	9 (81.8)	2 (18.2)	p= 1
Unidentified*	3 (10)	0	1 (3.4)	0	
Total**	35	43	14	19	

Note: *was not match with the used primer, would be the other type of ESBL gene

**Total number of identified-genes were more than ESBL isolates due to gene combinations in certain ESBL-producing bacteria.

Table 2. Combination of ESBL genes in ESBL-producing bacteria

Combination of ESBL gene	Hospital n (%)	PHC n (%)
SHV + TEM	0	0
SHV + CTX-M	2 (8)	2 (18,2)
TEM + CTX-M	6 (24)	2 (18,2)
SHV + TEM + CTX-M	1 (4)	0
Total	9	4

Table 3. Identifications of species and distribution pattern of ESBL-producing gut bacteria

Gut bacteria	Hospital	Primary Health Centre	p
	n (%) = 25	n (%) = 11	
<i>Escherichia coli</i>	20 (80)	8 (72.7)	p= 0.002
<i>Klebsiella pneumoniae</i>	2 (8)	3 (27.3)	
<i>Pseudomonas aeruginosa</i>	2 (8)	0	
<i>Enterobacter aerogenes</i>	1 (4)	0	
Total	25	11	

In this study, the CTX-M gene pattern was more dominant than the TEM and SHV genes. In the hospital, CTX-M gene was found in 20/25 (80%), while in the PHC the CTX-M gene was 9/11 (81.8%) (Table 1). The results of this study were similar to that of a study conducted by Severin (2010) in *Escherichia coli* who found that CTX-M gene (94.5%) was higher than SHV (65.3%) genes and TEM-type ESBLs were not detected in any of the isolates. Feces may play a role in the spread of Enterobacteriaceae bacteria in hospitals and the environment. The prevalence of ESBL-producing bacteria has been reported in Asia. In Indian and Chinese populations, ESBL distribution in the feces may be around 10% (Hawkey 2008). In Lebanese infant stools CTX-M genes was found in 47.2%, TEM was 20.7%, and SHV was 1.9%. The study also found a CTX-M gene (54.7%) with two or three types of genes (blaCTX-M-9, blaCTX-M-15, and blaCTX-M-2) (Moustafa et al 2015).

Examination of healthy people in Thailand showed that 65.7% of feces detected containing ESBL-producing Enterobacteriaceae carried the CTX-M gene, among them CTX-M-9 60.6% and CTX-M-1 gene was 38.7% (Luvsanharav et al 2012). The transmission of ESBL encoding Enterobacteriaceae in 112 healthy children, found 3 (2.6%) *Escherichia coli* producing ESBL (CTX-M-1, TEM-52 and SHV-12) (Guimaraes et al 2009). Asymptomatic colonization of the intestine with ESBL-producing Enterobacteriaceae is considered a prerequisite for infection. This increase is related to ESBL dominance with CTX-M type enzyme. The ESBL coding gene is easy to move, just like ISEcp1 (Kumar & Babuz, 2013). ISEcp1 insertion is a transposable

element that may combine and mobilize fragmentation of DNA flanking through a final transposition mechanism, which may experimentally mediate the capture of blaCTX-M genes from the *Kluyvera* spp chromosome and may facilitate inter-replication in host bacteria such as *Escherichia coli* (Bonnet 2004, Rossolini et al 2008).

This study found a combined phenomenon of ESBL gene in some strains of bacteria. The most combination and the most dominant genes were found in hospital isolates, ie TEM + CTX-M genes of 6 (24%), whereas in PHC, the TEM + CTX-M genes as many as 2 (18,2%) (Table 2). In this study only one strain carrying the TEM+SHV+CTX-M (4%) genes was identified in the hospital. Results of outpatient and inpatient study in Japan showed a combination pattern of TEM/SHV + CTX-M for three bacterial agents, such as *E. coli* 120 (53.8%), *K. pneumoniae* 24 (77.4%) and *Proteus mirabilis* 8 (66.7%) (Chong et al 2013). Transmission of ESBL producing Enterobacteriaceae in 112 healthy children showed 3 (2.6%) *E. coli* producing ESBL (CTX-M-1, TEM-52 and SHV-12) (Guimaraes et al 2009). Conjugative plasmids play an important role in increasing the spread of ESBL through the transfer of resistance genes to other bacteria in the hospital as well as the environment.

ESBL is mostly encoded by plasmids, some of which are known to be large (up to 100 kb or more). Large plasmids are also found to encode the CTX-M gene, which is an IncFII plasmid type that may insert or encode other antibiotic resistance genes that may be transferred from strain to strain and between bacterial species (Jarlier et al 1988, Rao 2012). The plasmid type

of IncFII is the most common replica and detected in 20 (58.8%) (10 *E. coli* and 10 *K. pneumoniae*) of 34 isolates (Rakotonirina et al 2013). The CTX-M gene may carry more than one replicon and may associate with the OXA, TEM and aac- (6) genes in the IncFII plasmid (Carattolli 2009).

The research has found that the predominant ESBL-producing bacteria were *E. coli* (80%) and *K. pneumoniae* (8%) from the hospital and *E. coli* (72.7%) and *K. pneumoniae* (27.3%) from the PHC. Analysis of bacterial distribution showed significant difference between the distribution in the hospital and PHC. Other bacterias were also found in this study, such as *Enterobacter aerogenes* and *Pseudomonas aeruginosa* (Table 3). A research in Surabaya (2017) found ESBL-producing gut flora in Tropic infection patients, which were *E. coli* (66%), *K. pneumoniae* (6.6%), and *Enterobacter aerogenes* (3.3%), and in community were *E. coli* (71%), *K. pneumoniae* (10%), *Enterobacter aerogenes* (0%) from rectal swab patients. (Sarassari 2017). The results of this study were an improvement from a previous one conducted by Kuntaman et al (2016) in Dr. Soetomo Hospital, Surabaya, on the prevalence of ESBL-producing bacteria where *E. coli* (62%) and *K. pneumoniae* (45.1%).

Other studies have shown that *Escherichia coli*, which is an intestinal flora, was resistant to cefotaxime in hospitalized patients (at least 5 days of hospitalization), but on the first day of hospitalization. The bacteria are thought to come from the community (Amrin 2001). Cross-transmission may occur in an environment such as water, soil, and plants, where the exchange of genetic material between bacteria and/or environmental origin occurs. Other studies have demonstrated poor access to drinking water, poverty and high population density as a very efficient risk factor for the spread of ESBL-producing bacteria, such as disease through fecal-oral transmission. Aquatic ecosystems, such as water or waste pollution (Woerther et al 2013) and well water (Amaya et al 2012), allow the spread of ESBL strains from animals to humans through the food industry (Kluytmans et al 2013), and pets (Poirel et al 2013).

CONCLUSION

The ESBL carrier rate of patients in hospital and PHC was high and not significantly different, respectively were 83.3% and 36.7%. The ESBL gene was dominated by CTX-M, then, at a lower level, TEM and SHV. This findings indicated that there has been a uniform distribution between hospital and PHC. It cannot be explained here whether ESBL-producing bacteria moved from hospital to PHC or from PHC to hospital.

We found other patterns of ESBL gene combinations in the hospital of SHV+CTX-M genes, TEM+CTX-M, SHV+TEM+CTX-M genes and PHC, the combination pattern of SHV+CTX-M, TEM+CTX-M. Appropriate infection control practices are essential to prevent the spread and outbreak of ESBL-producing bacteria. There is no doubt that ESBL will become increasingly complex and diverse and its detection becomes increasingly challenging for clinical microbiology laboratories. It is recommended to undergo studies with larger quantities of samples and in various PHC and to find out the cause of increase of ESBL prevalence in PHC.

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