GENOTYPING OF EXTENDED SPECTRUM β-LACTAMASE ESCHERICHIA COLI STRAINS FROM CLINICAL SPECIMENS

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ABSTRACT

The aim of the present study were to determine the incidence and origin of acquisition of ESBL-producing Escherichia coli strains isolated from Dr. Soetomo university hospital and to delineate the clonal diversity and transmission patterns of these strains by comparing results of genome macro restriction analysis – Pulsed Field Gel Electrophoresis (PFGE). The respective values of the genomic typing methods for epidemiologic typing of Escherichia coli were evaluated in more detail. Clinical specimens as the previous prevalence study of ESBL-E. coli and Klebsiella pneumoniae strains during January 2005 till April 2005 have identified 88 ESBL-E. coli strains of 330 E. coli that identified using standard method-NCCLS and DD synergy test. Banding patterns were PFGE (Certified, Bio Red, CHEF DR II) analyzed using manually read by 3 observers; and strains were defined as having PFGE genotype with ≥ 85 % similarity or no more than 2 band differences. Many strains of 88 members of ESBL-E.coli strains were multi various genotype on PFGE typing; on the other, some strains have the clonal genes, that have identical genotype or identical banding pattern. The clonal spreading of ESBL – Escherichia coli strains had detected event among the wards i.e. Surgery, Pediatry, Internal medicine and Obstetric Gynecology. Appropriate conduct on standard precaution procedures needs reinforcement, possible environmental reservoirs need elimination, and antibiotics policies need reconsideration.

Keywords: Escherichia coli, genotyping, Extended Spectrum β-Lactamase, PFGE

INTRODUCTION

Resistance to broad-spectrum cephalosporins has emerged in strains of members of the family Enterobacteriaceae following frequent use of these drugs in the hospital setting. Endemic and epidemic nosocomial infectious caused by extended-spectrum-β-lactamase (ESBL)-producing Escherichia coli and Klebsiella pneumoniae cells represent a persistent problem in many parts of the world, especially in intensive care units (ICU). Epidemic strains of cephalosporin-resistant Escherichia coli and K. pneumoniae have been associated with increased morbidity and mortality in hospitalized patients. Since 1983, nosocomial outbreaks of ESBL-producing K. pneumoniae infections in Europe, the United States, and South America were described (Gruteke 2003).

Nosocomial outbreaks in reference centers due to multi resistant Klebsiella pneumoniae isolates have been described frequently. Appropriate isolation measures need to be taken, hand hygiene procedures may need reinforcement, possible environmental reservoirs need elimination, and antibiotics policies may need reconsideration. Screening in search of colonized patients needs to be instituted, and antibiotics policies may need reconsideration. Screening in search of colonized patients needs to be instituted (Gruteke 2003).

Extended-spectrum β-lactamase (ESBL)-producing Enterobacteria (ESBLE) have emerged at the end of the 1980s within hospitals, causing outbreaks and/or hyper endemic situations in many centers. Several recent data have suggested that ESBLE are currently emerging within the community (Arpin et al. 2005).

Understanding the epidemiology of ESBL-producing Escherichia coli and K. pneumoniae requires the use of accurate epidemiological markers able to differentiate between the spread of resistance plasmids and strain dissemination. Pulsed-field gel electrophoresis (PFGE) analysis of genome macrorestriction fragments was shown to be a more discriminating typing technique (Gori et al. 1996).
However, PFGE is a technically demanding and time-consuming technique that requires specific equipment. PCR-based typing techniques, such as randomly amplified polymorphic DNA (RAPD) analysis, are faster and easier to perform. In recent studies, RAPD analysis has been successfully used to type a diversity of microorganisms. On the other hand PFGE genotype analysis have higher level in discriminatory or more sensitive in differentiation of genotype till subtype than RAPD, because of more DNA banding result from the whole of DNA genome (Gori et al. 1996).

The aims of the present study were to determine the incidence and origin of acquisition of ESBL-producing Escherichia coli strains isolated from Dr. Soetomo university hospital and to delineate the clonal diversity and transmission patterns of these strains by comparing results of genome macro restriction analysis (PFGE). The respective values of the genomic typing methods for epidemiologic typing of Escherichia coli were evaluated in more detail using PFGE analysis.

MATERIALS AND METHODS

Antimicrobial susceptibility

Susceptibility to antimicrobial agents was tested by disk diffusion method on Mueller-Hinton Agar as described in the recommendations of the NCCLS 2002. The production of clavulanic acid-susceptible ESBL was detected by using the double-disk synergy test. Additionally, inhibition zone diameters of various β-lactamase, including clinical specimens as the previous prevalence study of ESBL-E. coli and Klebsiella pneumoniae strains during January 2005 till April 2005 have identified 88 ESBL-E. coli strains of 330 E. coli that identified using standard method-NCCLS and DD synergy test.

Pulsed Field Gel Electrophoresis (PFGE)

Each isolate grown over night on blood agar plates, one colony was picked and suspended in 100 µl of EET buffer (100 mM EDTA, 10 mM EGTA, 10 mM Tris-HCL). Bacterial suspensions were embedded in agarose plugs by mixing with equal volumes of 1 % agarose solution; 200- µl plugs were prepared. Plugs were incubated overnight with proteinase K (1 mg/ml) and sodium dodecyl sulfate (1 %) and were subsequently washed with a buffer (10 mM Tris-1mM EDTA) six times for 30 min each time. Subsequently the plugs were stabilized twice, for 30 min each time, in 120 µl of a buffer (SuRe cut H buffer, Boehringer, Mannheim, Germany) and were digested with 40 U of XbaI during overnight incubation at 37°C. Plugs were washed four times, for 30 min each time), with 0,5 x Tris-Borate-EDTA. The DNA present in the agarose plugs was analyzed on a 1 % agarose gel by PFGE (Pulsed Field Certified, Bio-Rad, CHEF DR II) at 14°C and 6 V/cm in 0,5x Tris-Borate-EDTA by using pulse times of 5 to 35 s at an angle of 120° (-60° to + 60°) for 20 h. The agarose gel was stained afterwards in ethidium bromide (5 mg/liter) and photographed under UV illumination. Identical patterns were assigned a letter to designate the type (Grietske 2003). Banding patterns were PFGE (Certified, Bio Red, CHEF DR II) analysed using manually read by 3 observer; and strains were defined as having PFGE genotype with ≥ 85 % similarity or no more than 2 band differences (UPGMA-Dice) (Woodford 2004).

RESULTS

Many strains of 88 members of ESBL-E.coli strains were multi various genotype on PFGE typing; on the other, some strains have the clonal genes, that have identical genotype or identical banding pattern. Among ESBL-E. coli strains, the clonal genes had been identified at strains storage 1610, 1728, and 1730 that have DNA fragment band were the same pattern or 100% identical; the other clonal gene at 1621 and 1624; the number 1938 identical with 1939; strains 1726 identical with 1729; and strains 1852 identical with 1853; on the other hand strain 1871 have 1 band different with strains 1910, that can be categorized as identical or could be an different subtype. (Table 1)

DISCUSSION

This feature indicated that ESBL-E.coli strain number 1621 and 1624 is the endogenous transfer of clone gene in a patient, this event could be suggest a less application of standard precaution. Strain number 1938 with the same genotype, this event at the same with time (month) and in the same specimen (urine), but different ward, may indicate a clonal spreading in the hospital. The clonal spreading also detect in the other ward i.e. surgery, as number 1871 and 1910; and in other ward in the hospital as 1610, 1728 with 1730; wards of 1852 with 1853.
Figure 1. PFGE Genotyping analysis of ESBL producing *Escherichia coli* clinical strains.

Table 1. The clinical feature of these patients were

<table>
<thead>
<tr>
<th>Storage No.</th>
<th>Specimens</th>
<th>Wards</th>
</tr>
</thead>
<tbody>
<tr>
<td>1621 *</td>
<td>Feces</td>
<td>ICU-GBPT</td>
</tr>
<tr>
<td>1624 *</td>
<td>Sputum</td>
<td>ICU-GBPT</td>
</tr>
<tr>
<td>1938 **</td>
<td>Urine</td>
<td>Obstetry-Gynecology</td>
</tr>
<tr>
<td>1939 **</td>
<td>Urine</td>
<td>Internal Medicine</td>
</tr>
<tr>
<td>1726 ***</td>
<td>Urine</td>
<td>Urology</td>
</tr>
<tr>
<td>1729 ****</td>
<td>Feces</td>
<td>Pediatric</td>
</tr>
<tr>
<td>1610 *****</td>
<td>Sputum</td>
<td>ICU-GBPT</td>
</tr>
<tr>
<td>1728 ****</td>
<td>Feces</td>
<td>Pediatric</td>
</tr>
<tr>
<td>1730 *****</td>
<td>Feces</td>
<td>Pediatric</td>
</tr>
<tr>
<td>1871 *****</td>
<td>Urine</td>
<td>Surgery</td>
</tr>
<tr>
<td>1910 *****</td>
<td>Pus</td>
<td>Surgery</td>
</tr>
<tr>
<td>1852 ******</td>
<td>Blood</td>
<td>Internal Medicine</td>
</tr>
<tr>
<td>1853 ******</td>
<td>Blood</td>
<td>Intensive Care Room</td>
</tr>
</tbody>
</table>

Number of * = The strains on the same genotype  
Number 1621 and 1624, the specimen from the same patient.
In ESBL- \textit{Klebsiella pneumoniae} strains, there is also a clonal gene spreading in the hospital that be transferred among different wards and different specimen, these is suspected because of at close time. ESBL genes are usually carried by plasmids, and some of them are located within transposable element that facilitate their spread between DNA replicons and bacterial strains of even different species, outbreaks have been attributed to the dissemination of plasmids among strains of members of the \textit{Enterobacteriaceae}. In other cases, spread of a given ESBL in a single environment due to appearance of the same gene within unrelated plasmids. These plasmids have genes responsible for resistance to other antibiotics, and this has resulted in the growth multidrug-resistant organisms. ESBL-producing strains can be maintained over prolonged hospitals and can cause clonal outbreaks. They can be transferred between different wards between different hospitals or health care instructions and even, with the case of international traveling countries. Multiple identification of some ESBL variants in distant geographical result more likely from convergent evolutionary events (Gniadkowski 1998).

Isolated since the mid-1980s, extended-spectrum \(\beta\)-lactamases (ESBLs) are usually encoded by plasmid and confer resistance to penicillins, cephalosporins (cephamycins), and monobactams. \(\beta\)-lactamase inhibitor (clavulanic acid, sulbactam, and tazobactam) block the activity of ESBLs, and this often causes organisms to appear susceptible to some \(\beta\)-lactamase-inhibitor combinations. ESBLs are spectrum penicillinases, such as TEM-1 or \(-2\) or SHV-1, and ESBL activity is determined of several positions, i.e., 104, 164, 237, 238, and 240, within their amino acid sequences (Fieet et al. 2000).

Another group of \(\beta\)-lactamases demonstrating inhibitor resistance (IR) activity has been isolated 1900s. These enzymes confer resistance to penicillins and their combinations with \(\beta\)-lactamases. The majority of IR \(\beta\)-lactamases known to date are derivatives of TEM-1 and \(-2\) penicillin and mutations several amino acid positions of these, i.e., 69, 130, 244, 275, and 276, were play a role in determining IR activity. A combination of ESBL- and IR-specific lactamases results in the formation of a s-called complex mutant enzyme. Two natural variant TEM-50/CMT-1 and SHV-10, have been studied to date and were found to express either both of moderate level or only one of these (Fieet et al. 2000).

Molecular typing using pulsed-field gel electrophoresis revealed multiple different clones of ESBL-producing strains. Although within five of the eight participating hospitals it was possible to demonstrate a few related clones, most isolates were not related (Villegas 2004).

Most ESBLs are derivate of TEM or SHV enzymes. There are now >90 TEM-type \(\beta\)-lactamase and > 25 SHV-type enzymes (for amino acid sequences fore TEM, SHV, and OXA extended-spectrum and inhibitor-resistant \(\beta\)-lactamase. With both of these groups of enzymes a few point mutations at selected loci within the gene give rise to the extended-spectrum phenotype. TEM-and SHV-type ESBLs are most often found in \textit{E. coli} and \textit{K. pneumoniae}; however, they have also been found in \textit{Proteus spp.}, \textit{Providence spp.}, and other genera of \textit{Enterobacteriaceae} (Bradford 2001).

TEM-1 is the most commonly encountered \(\beta\)-lactamase in gram-negative bacteria. Up to 90 % of ampicillin resistance in \textit{E. coli} is due to the production of TEM-1. This also responsible for the ampicillin and penicillin resistance that is seen in \textit{H. influenzae} and \textit{N. gonorrhoeae} in increasing numbers. TEM-1 is able to hydrolyse penicillins and early cephalosporins such as cephalothin and cephapirin. TEM-2, the first derivative of TEM-1, had a single amino acid substitution from the original \(\beta\)-lactamase. This cause a shift in the isoelectric point from a pI of 5.4 to 5.6, but it did not change the substrate profile. TEM-3, originally reported in 1989, was the first TEM-type \(\beta\)-lactamase that displayed the ESBL phenotype. In the years since that first report, over 90 additional TEM, SHV and OXA extended-spectrum and inhibitor-resistant \(\beta\)-lactamase. Some of these \(\beta\)-lactamases are inhibitor resistant enzymes, but the majority of the new derivatives are ESBLs (Bradford 2001).

It has been suggested that the naturally occurring TEM-type ESBLs are the result of fluctuating selective pressure from several \(\beta\)-lactams within a given institution rather than selection with a single agent. Although TEM-type-\(\beta\)-lactamase are most often found in \textit{E. coli} and \textit{K. pneumoniae}, they are also found in other species of gram-negative bacteria with increasing frequency (Bradford 2001).

In the early 1990s \(\beta\)-lactamase that were resistant to inhibition by clavulanic acid were discovered. Nucleotide sequencing revealed that these enzymes were variants of the TEM-1 or TEM-2 \(\beta\)-lactamase. These enzymes were at first give the designation \textit{IRT} for inhibitor-resistant TEM-\(\beta\)-lactamase; however, all have subsequently been renamed with numerical TEM designations. There are at least 19 distinct inhibitor resistant Tem- \(\beta\)-lactamase (for amino acid sequences for TEM, SHV and OXA extended-spectrum and inhibitor resistance \(\beta\)-lactamase. Inhibitor-resistant TEM- \(\beta\)-lactamase have been found mainly in clinical isolates of \textit{E. coli}, but also some strains of \textit{K. pneumoniae}, \textit{Klebsiella oxytoca}, \textit{P. mirabilis}, and
**Citrobacter freundii.** Although the inhibitor-resistant TEM variants are resistant to inhibitors combinations of amoxicillin-clavulanate, ticarcillin-clavulanate, and ampicillin-sulbactam, they remain susceptible to inhibition by tazobactam and subsequently the combination of piperacillin and tazobactam (Bradford 2001).

Point mutation leads to the inhibitor-resistant phenotype occur at a few specific amino acid residues within the structural gene for the TEM enzyme, Met 69, Arg-244, Arg-276. The sites of these amino acid substitutions are distinct from those that lead to the ESBL phenotype. Laboratory mutants that the IRT and the ESBL phenotype have been constructed (Bradford 2001).

The SHV-1-β-lactamase is most common found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species. In many strains of *K. pneumoniae*, bla SHV-1 or a related gene is integrated into the bacterial chromosome. Although it has been hypothesized that the gene encoding SHV-1 may exist as part of a transposable element, it has never been proven. Unlike the TEM-type β-lactamase, there are relatively few derivatives of SHV-1. The majority of SHV variants possessing an ESBL phenotype are characterized by the substitution of a serine for glycine at position 238. A number of variants related to SHV-5 also have a substitutions mirror those seen in TEM-type ESBLs. The serine residue at position 238 is critical for the efficient hydrolysis of cefotaxime, and the lysine residue is critical for the efficient hydrolysis of cefotaxime (Bradford 2001).

In recent years a new family of plasmid-mediated ESBLs, called CTX-M, which preferentially hydrolyze cefotaxime has arisen. They have mainly been found in strains of Salmonella enteric serovar Typhimurium and *E. coli*, but have also been described in other species of *Enterobacteriaceae* (Bradford 2001).

A phylogenetic study of the CTX-M family of β-lactamase showed four major types: the CTX-M-1 type, including CTX-M-1 and CTX-M-3; the CTX-M-2 type, include CTX-M-2, CTX-M-4, CTX-M-5, CTX-M-6, CTX-M-7, and Toho-1, Toho-2; and CTX-M-8, the latter two groups containing only one member to date (Bradford 2001).

Kinetic studies have shown that the CTX-M-type β-lactamase hydrolyze cephalothin or cephaloridine better benzylpenicillin and they preferentially hydrolyze cefotaxime over cefazidime. In addition to the rapid hydrolysis of cefotaxime, another unique feature of these enzymes is that they are better inhibited by the β-lactamase inhibitor tazobactam than by sulbactam and clavulanate (Bradford 2001).

The OXA-type enzyme is another growing family of ESBLs. These -β-lactamase differ from the TEM and SHV enzymes in that they belong to molekuler class D and functional group. The OXA-type-β-lactamase confers resistance to ampicillin and cephalexin and is characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid (Bradford 2001).

CTX-M enzymes predominantly hydrolyse cefotaxime and most are only weakly active against ceftazidime, although some such as CTX-M 15 also have strong activity against ceftazidime. CTX-M enzymes have been the predominant ESBLs in Argentina for > 10 years, but have a growing distribution and prevalence in many other parts of the world, including Eurepo. In the UK, CTX-M-type β-lactamase were first detected in 2000-2001, with a CTX-M-9-producing isolates Birmingham; and four unrelated CTX-M-15-producing isolates of *Escherichia coli* from three widely scattered hospitals (Bradford 2001).

Most producers of TEM-and SHV type ESBLs have been nosocomial isolates, predominantly *Klebsiella*, although recent data suggest a significant prevalence in *Enterobacter spp.*, where detection is complicated by the co-presence of AmpC. However, during 2003, the Health Protection Agency’s Antibiotic Resistance Monitoring and Reference Laboratory (ARMRL) began to receive isolates of *E. coli* for confirmation of ESBL production, with a phenotype implying a CTX-M-type -β-lactamase. Of particular concern was that many isolates were reported to be from patients attending general practice, with limited or no history of recent hospital contact. The emergence of ESBL producing *E.coli* in the community is also being seen concurrently elsewhere in Europe and in Canada (Bradford 2001).

The recognition of CTX-M-producing community strains is a cause of concern in many other countries besides the UK. In the current analysis, almost one quarter of UK isolates were derived from sample received from the community.

**CONCLUSION**

The clonal spreading of ESBL – *Escherichia coli* strains had detected event among the wards (i.e. Surgery, Pediatric, Internal Medicine and Obstetric Gynecology) in Dr. Soetomo Hospital from January until April 2005, based on PFGE genotype analysis. Appropriate isolation measures need to be taken, hand hygiene procedures
need reinforcement, possible environmental reservoirs need elimination, and antibiotics policies need reconsideration, screening in search of colonized patients may need to be instituted.

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