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Running head: ESBL producing E. coli isolated from Indonesia
SUMMARY

The purpose of this study was to investigate extended-spectrum beta-lactamase (ESBL)-producing *E. coli* isolated from pediatric (0-3 year old) diarrhea patients, as well as bacterial antibiotic susceptibilities, ESBL typing, multilocus sequence typing (MLST) typing and diarrheagenic *E. coli* (DEC)-typing in Surabaya, Indonesia, where this kind of survey is rarely done. ESBL-producing *E. coli* were detected in 18.8% of strains. Many ESBL-producing *E. coli* had significantly less susceptibility to gentamicin (p<0.0001), quinolones (nalidixic acid: NA, p=0.004 and ciprofloxacin: CPFX, p<0.0001) than ESBL- non-producers. In ESBL-producing *E. coli*, 84.0% of strains were CTX-M-15 typed with or without combination typing. MLSTs showed sequence type (ST) 617 in 24.0% of ESBL-producers and all had the CTX-M-15 type gene and we had 3 DEC-related genes: 2 enteroaggregative *E. coli* and 1 enteropathogenic *E. coli*. In conclusion, CTX-M-15 typed ESBL-producing *E. coli* ST617 are spreading in the developing countries.
INTRODUCTION

*Escherichia coli* can cause diarrhea or food poisoning, which is called as diarrheagenic *E. coli* (DEC) (1, 2). DEC are classified into 5 categories on the basis of their specific virulence properties: Shiga-toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC) (3). In children under 5 years old in UNHCR refugee camps, especially in developing countries, diarrhea is the 3rd most frequently seen disease in Asia and the 4th commonest in Africa (4). Pathogenic microorganisms isolated from diarrhea patients are bacterial in about 80% of cases, with *E. coli* and *Shigella* being characteristic examples (5). Recently extended spectrum beta-lactamase (ESBL) antibiotic resistant strains have been increasingly reported from fecal samples, especially in *E. coli*, and the ESBL ratio in all *E. coli* has increased to 8-10% in European, Asian and North American countries (6, 7, 8). ESBL is a class A beta-lactamase with a plasmid-related resistance mechanism, categorized into TEM and SHV types derived from narrow matrix-specific enzyme (9,10) and a CTX-M type derived from beta-lactamase presented on the *Kluyvera* chromosome (11). Reportedly ESBL-producing strains have been recently changing from TEM or SHV type to CTX-M (12). The current data need to be compared with previous reports in order to grasp the cloning changes and the spread of antibiotic resistances.

Moreover, it is essential to study bacterial gene sequences using multilocus sequence typing (MLST) to understand the epidemiological features of resistant strains. MLST is an analysis of housekeeping gene DNA playing a main role in bacterial
survival by a software comparison of sequence type (ST) with known registered data (www.mlst.net).

In this study, we examined recent DEC isolated from pediatric diarrhea patients in Indonesia for antibiotic susceptibilities, ESBL production, cloning type using MLST analyses and detection of DEC-related genes such as eaeA of ETEC/EPEC, stx1 and stx2 of ETEC, aggR of EAEC, est and elt of ETEC, and invE of EIEC.
MATERIALS AND METHODS

DEC Strains

A total of 133 *E. coli* strains were isolated from stools of pediatric (0-3 year old) diarrhea patients in 2012 in Soetomo General Hospital, Surabaya, Indonesia. The ethics committee of the Institute of Tropical Disease, Airlangga University, approved this study. Verbal informed consent to using the clinical samples for research purposes was obtained before sample collection from the parents of all participants.

Antibiotic susceptibility tests

All stool specimens were cultured on MacConkey agar (Oxoid) for the selection of *E. coli* isolates, and were incubated for 24 h at 37°C. Stool specimens contaminated by other pathogenic bacteria (*Vibrio, Shigella* and *Salmonella* species) as determined by standard biochemical methods were excluded retrospectively. Antibiotic susceptibility tests were performed according to Clinical and Laboratory Standards Institute (CLSI) recommendations for ampicillin (ABPC), sulbactam/ampicillin (ABPC/SBT), piperacillin (PIPC), cefotaxime, cefidiuzime (CAZ), cefepime (CFPM), cefpodoxime (CPDX), imipenem (IPM), aztreonum (AZT), gentamicin (GM), amikacin (AMK), fosfomycin (FOM), tetracycline (TC), chloramphenicol (CP), nalidixic acid (NA), ciprofloxacin (CPFX) and sulfamethoxazole-trimethoprim (S-T) [13]. We used *E. coli* ATCC 25922 as a quality control.
ESBL screening

ESBL screening was initially tested with the CLSI confirmatory test using both cefotaxime (CTX) (30 mg) and CAZ (30 mg) disks alone and in combination with clavulanic acid (CA) (10 mg) (Eiken chemical, co. ltd., Tokyo, Japan). The test was considered positive when an increase in the growth-inhibitory zone around either the CTX or the CAZ disk with CA was 5 mm or greater of the diameter around the disk containing CTX or CAZ alone (13).

DNA isolation

The DNA template was obtained using the Illustra™ bacterial genomic Prep Mimi Spin kit (GE Healthcare Japan, Tokyo, Japan).

Detection of CTX-M, TEM and SHV typing

The method was described in detail in our previous study (14). In brief, we determined the ST of the $bla_{CTX-M}$, $bla_{TEM}$, and $bla_{SHV}$ using strains with confirmed production of ESBL. PCR was carried out using TaKaRa Ex Taq (TaKaRa Bio Inc, Shiga, Japan) to identify $bla_{CTX-M}$, $bla_{TEM}$, and $bla_{SHV}$. The $bla_{CTX-M}$, $bla_{TEM}$, and $bla_{SHV}$-positive strains were determined by PCR using CTX-M, TEM, and SHV group-specific primers (15) as shown as followed: A (5’-SCSATGTGCAGYACCAGTAA-3’) and B (5’-CCGCRATATGRTTGGTGTTG-3’) for $bla_{CTX-M}$, C (5’-TCGGGGAATGTGCAGCACC-3’) and D (5’-TGCTTAATCAGTGAGGCCACC-3’) for $bla_{TEM}$, and OS-5 (5’-TTATCTCCCTGTAGCCACC-3’) and OS-6 (5’-GATTGCTGATTTGCCTCGG-3’) for $bla_{SHV}$. The PCR procedures consisted of denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec,
annealing at 55°C for 30 sec, and elongation at 72°C for 1 min and elongation at 72°C for 10 min as described previously (14). PCR products were purified using a QIAquick PCR Purification Kit (Quiagen, Hilden, Germany). DNA sequencing was performed at Eurofins Genomics, Inc. (Tokyo, Japan), using the purified PCR products.

**Multilocus sequence typing: MLST**

MLST was performed as previously described using 25 ESBL-producing strains (14) to compare STs with the ST131 clone of this *E. coli* serotype, which is the most prevalent worldwide (14). PCR was performed using 7 primer sets targeting 7 housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) following the recommended procedure at the *E. coli* MLST web site (http://mlst.ucc.ie/dbs/Ecoli).

**Detection of Diarrheagenic *Escherichia coli* (DEC)-related genes**

The method was according to our previous study (16) and we used Cica Geneus Pathogenesis Gene Detection PCR Kit (for DEC) (Kanto Chemical Co., Inc.) including the primers targeted for *eaeA* of ETEC/EPEC, *stx1* and *stx2* of ETEC, *aggR* of EAEC, *est* and *elt* of ETEC, and *invE* of EIEC. The PCR mixture consisted of DNA template, Apta Taq DNA Master, PCR supplement, and primer mixture 1 (*eaeA*, *stx1* and *stx2*), primer mixture 2 (*est* and *elt*), or primer mixture 3 (*aggR*, *invE* and 16SrDNA for internal control). The PCR procedure performed by 30 cycles of denaturation at 94°C for 15 sec and annealing at 60°C for 30 sec.

**Statistical analysis**

We determined significant differences using the $\chi^2$ test or Fisher’s exact test.
Differences were considered to be statistically significant at $P < 0.05$. 
RESULTS

ESBL screenings

ESBL-producing *E. coli* were detected in 18.8 % (25/133 strains) using the disc method (Table 1).

Comparison of antibiotic susceptibilities of ESBL producers and non-producers

We compared the susceptibilities of non-producers (n=108) and ESBL producers (n=25) to 13 representative antimicrobial agents (Table 2). ESBL producers had significantly lower antibiotic susceptibilities as follows: ABPC (p=0.0359), PIPC (p <0.0001), ABPC/SBT (p=0.0025), cefotaxime (p <0.0001), CAZ (p <0.0001), CPDX (p<0.0001), CFPM (p<0.0001), AZT (p<0.0001), GM (p<0.0001), N/A (p=0.004), CPFX (p<0.0001) and S-T (p<0.0001) but not in IPM (p=1.000), AMK (p=1.000), FOM (p=1.000), TC (p=0.431) and CP (p=0.137). All 134 strains were susceptible to IMP and AMK (Table 1).

Gene typing of ESBL-producing *E. coli* and antibiotic susceptibilities

In 25 strains of ESBL-producing *E. coli*, CTX-M-15 types accounted for 84.0 % (21/25 strains), of which single typing with CTX-M-15 accounted for 56 % (14/25 strains), combination typing with CTX-M-15 and TEM-1 accounted for 24.0 % (6/25 strains), and the combination of CTX-M-15 and SHV accounted for 4.0 % (1/25 strains). Regarding the antibiotic susceptibilities, CTX-M-15 typing alone or in combination tended to be associated with resistance to antibiotics such as GM and ciprofloxacin (Table 3).
MLST

MLST typing showed that ST617 accounted for 24.0% (6/25 strains) of ESBL producers and all had the CTX-M-15 gene type. The other 19 strains had STs or were non-typable (Table 2). Regarding MLST typing and antibiotic resistance, ST617 showed a high level of resistance to the 17 antibiotics we tested except for CFPM, imipenem, GM, AMK and FOM (data not shown).

DEC-related genes

In 25 ESBL-producing *E. coli* tested, we had only 2 DEC-related genes-positive strains; one was detected of *aggR* of EAEC (ST2142) and the other one was detected of *eaeA* of EPEC (ST29). As shown in Table 2 regarding the relationship between ESBL typing, MLST and the presence of DEC-related genes, ST2142 strain in the combination type of CTX-M-14 and TEM-1 had detection of *aggR*, suggesting the diagnosis of EAEC. TEM-1 typed-strain had detection of *eaeA*, suggesting the diagnosis of EPEC (Table 2).
DISCUSSION

ESBL-producing Enterobacteriaceae have been reported to be spreading over the last decade, especially strains of *Klebsiella pneumoniae* and *E. coli* (9, 17). The former strains are most commonly seen in European countries or the United States while the latter are commonly seen in both Asian and Western countries (18-24).

In particular, the isolation of ESBL-producing *E. coli* has rapidly increased in Asian countries, where studies from Korea and Japan report that ratios have reached almost 30% of all *E. coli* isolates. However, studies from developing nations are lacking and in particular to our knowledge no study from east Indonesia has been done for almost 10 years; the most recent previous report is from 2005. The current 2012 study showed that the ratio of ESBL production among all *E. coli* isolates was similar but ESBL typing and antibiotic susceptibilities were different compared with the 2005 report (25). Fortunately, current antibiotic susceptibilities were not apparently worse than previously, suggesting that possibly the situation with ESBL-producing *E. coli* did not change in eastern Indonesia from 2005 to 2012, information which is needed in both the medical and the governmental setting.

In general, ESBL-producing *E. coli* showed good susceptibilities only to carbapenems and AMK (26, 27), partly in agreement with our data showing good susceptibilities to carbapenems (imipenem), AMK and FOM. Taken together with our antibiotic susceptibility results for strains with ST617 shown above, the data indicate that these 3 kinds of antibiotics still have good activity against ESBL producers even in strains with ST617, which has been shown to have high resistance potential.

ESBL gene typing is also important for monitoring the epidemiological spread
of the bacteria. The CTX-M-15 typed ESBL-producing *E. coli* ST131 clone has spread across many regions of the world (28-31). Our data showed that the CTX-M-15 type accounted for of the strains and some strains were a combination of this type with other cloning types such as TEM-1 or SHV-7. Further studies with long term observation will be necessary to monitor the epidemiological aspects of this kind of bacterial feature.

Various types of ESBL genes have been identified but their differences in antibiotic susceptibility based on ESBL gene typing have not been fully understood. TEM, SHV and CTX-M-type enzymes are the most representative ESBL gene types (32-35). Of these 3 subtypes, the TEM and SHV types show amino acid mutations from the natural narrow-spectrum TEM-1, TEM-2, or SHV-1 beta-lactamase genes. The CTX-M enzymes originally demonstrate expanded-spectrum activity (10, 36). In terms of the relationship between antibiotic resistance and CTX-M-type, beta-lactamases were previously more active to cefotaxime compared with CAZ (36) but recently some CTX-M-type beta-lactamases have begun to show enhanced activity against CAZ, especially CTX-M-15, -16, -19, and -27 (37). From the study of Asian country, CTX-M-15 was more frequent in ST131 than in non-ST131 (38). Our data showed that CTX-M-15 typed strains and the strains with the TEM-1 gene type had comparatively higher rates of antibiotic resistance to GM or CPFX than ESBL with non-CTX-M-15 genes. Even though direct comparison is not easy to be performed, because of the small sample numbers, our data appear consistent with other studies from developed country such as France or Asian country (Korea). Moreover, we need MLST analyses to achieve more detailed bacterial typing by DNA sequence. ESBL ST617 has also become worldwide (28, 29, 39, 40) for instance, Belgium, Sweden, Nigeria and France. Our MLST data showed that even though 24% of the ESBL strains were non-typable, ST617
was seen most often in our ESBL isolates like other studies aforementioned, and this ST type showed higher resistance to most of the antibiotics tested in this study, except imipenem, AMK and FOM as mentioned above. These data could be informative for selecting antibiotics for treatment, from both the clinical and the molecular biology aspects. In addition, our data of DEC-related genes detection, we had only 2 DEC-related genes (aggR: EAEC (CTX-M-14+TEM-1: ST2142) and eaeA: EPEC (TEM-1: ST29). In this situation, we had no conclusive statement from this small number of positive data and further study with more number of isolated is our future task.

We would like to emphasize the limitations of this study. First, the number of *E. coli* isolates may not be enough to draw for definitive conclusions. Also, we only used stool samples for this study. Third, the small number of ESBL-producers limited our statistical analyses. These limitations will be overcome in our future studies.

In conclusion, CTX-M-15 typed ESBL-producing *E. coli* ST617 with higher antibiotic resistance potential is definitively spreading to developing countries such as Indonesia. At this point imipenem, AMK and FOM still showed good susceptibilities even in ST 617 strains. Further investigation is necessary to monitor the diffusion of antibiotic-resistant *E. coli* strains in Indonesia.
Acknowledgements and funding

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Conflict of interest

None to declare
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prospective observational multicentre cohort study looking at epidemiology, microbiology and risk factors. BMC Infect Dis. 2014;14:528.


Table 1. ESBL tests and confirmation tests (n=25)

<table>
<thead>
<tr>
<th></th>
<th>CAZ resistance</th>
<th>Difference of inhibition zone* between CAZ and CAZ/CVA</th>
<th>CTX resistance</th>
<th>Difference of inhibition zone* between CTX and CTX/CVA</th>
<th>CPDX resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of strains</td>
<td>18/25 (72 %)</td>
<td>positive (+)</td>
<td>24/25 (96 %)</td>
<td>positive (+)</td>
<td>25/25 (100 %)</td>
</tr>
</tbody>
</table>

*+: 5mm or more in diameter of difference of inhibition zone; -: less than 5mm in diameter of difference of inhibition zone

CAZ: ceftadizime, CAZ/CVA: ceftadizime/clavulanie acid; CTX: cefotaxime; CPDX: cefpodoxime
<table>
<thead>
<tr>
<th>Type of ESBLs</th>
<th>Antibiotic susceptibilities (%)</th>
<th>ST</th>
<th>DEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GM</td>
<td>AMK</td>
<td>S-T</td>
</tr>
<tr>
<td>CTX-M-15 (n=14, 56%)</td>
<td>78.6</td>
<td>100</td>
<td>14.3</td>
</tr>
<tr>
<td>CTX-M-15 + TEM-1 (n=6, 24%)</td>
<td>33.3</td>
<td>100</td>
<td>16.7</td>
</tr>
<tr>
<td>CTX-M-15 + SHV-7 (n=1, 4%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CTX-M-14 + TEM-1 (n=2, 8%)</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>CTX-M-1 (n=1, 4%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>TEM-1 (n=1, 4%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Total (n=25, 100%)</td>
<td>72.0</td>
<td>100</td>
<td>20.0</td>
</tr>
</tbody>
</table>

GM: gentamicin; AMK: amikacin; S-T: sulfamethoxazole-trimethoprim; CPFX: ciprofloxacin; TC: tetracycline
Table 3. Antibiotic susceptibilities (%) of ESBL producing and non-producing *E. coli*.

<table>
<thead>
<tr>
<th></th>
<th>ABPC</th>
<th>ABPC/ SBT</th>
<th>PIPC</th>
<th>CTX</th>
<th>CAZ</th>
<th>CFPM</th>
<th>CPDX</th>
<th>IPM</th>
<th>AZT</th>
<th>GM</th>
<th>AMK</th>
<th>FOM</th>
<th>TC</th>
<th>CP</th>
<th>NA</th>
<th>CPFX</th>
<th>S-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-ESBL</td>
<td>19.4</td>
<td>75</td>
<td>52.8</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>98.1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>96</td>
<td>28</td>
<td>44</td>
<td>76.9</td>
<td>97.2</td>
</tr>
<tr>
<td>ESBL</td>
<td>0</td>
<td>44</td>
<td>0</td>
<td>0</td>
<td>28</td>
<td>64</td>
<td>4</td>
<td>100</td>
<td>24</td>
<td>72</td>
<td>100</td>
<td>96</td>
<td>28</td>
<td>32</td>
<td>48</td>
<td>44</td>
<td>20</td>
</tr>
</tbody>
</table>

ABPC: ampicillin; ABPC/SBT: ampicillin/sulbactam; CTX: cefotaxime; CAZ: ceftadizime; CFPM: cefepime; CAZ/CVA: ceftadizime/clavulanic acid; CPDX: cefpodoxime; IPM: imipenem; AZT: aztreonam; GM: gentamicin; AMK: amikacin; FOM: fosfomycin; TC: tetracycline; CP: chloramphenicol; NA: nalidixic acid; CPFX: ciprofloxacin; S-T: sulfamethoxazole-trimethoprim