Original Article

Antibiotic Susceptibilities and Genetic Characteristics of Extended-Spectrum Beta-Lactamase-Producing Escherichia coli Isolates from Stools of Pediatric Diarrhea Patients in Surabaya, Indonesia

Eddy Bagus Wasito1,10, Katsumi Shigemura2,3,4,8, Kayo Osawa3,4, Alpha Fardah5, Akiho Kanaida6, Dadik Raharjo2,10, K. Kuntaman1,13, Usman Hadi6, Sugeng Harjono3,10, Subijanto Marto Sudarmo5, Tatsuya Nakamura7, Keigo Shibayama8, Masato Fujisawa7, and Toshiro Shirakawa3,10

2Department of Urology, 4Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe 650-0017; 6Division of Infectious Diseases, Department of International Health, Kobe University Graduate School of Health Science, Kobe 650-0017; 7Department of Clinical Laboratory, Kobe University Hospital, Kobe 650-0017; 8Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo 208-0011, Japan; 1Department of Microbiology, Faculty of Medicine, 3Department of Internal Medicine, Faculty of Medicine, Airlangga University/Dr. Soetomo Hospital, Surabaya; 5Indonesia-Japan Collaborative Research Center for Emerging and Re-emerging Infectious Diseases, Institute of Tropical Disease, 7Department of Pediatrics, Faculty of Medicine; and 10Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia

SUMMARY: The purpose of this study was to investigate extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli isolates from pediatric (aged 0 to 3 years) diarrhea patients in Surabaya, Indonesia, where this kind of survey is rare; our study included assessment of their antibiotic susceptibilities, as well as ESBL typing, multilocus sequence typing (MLST), and diarrheagenic E. coli (DEC)-tying. ESBL-producing E. coli were detected in 18.8% of all the samples. Many ESBL-producing E. coli had significantly lower susceptibility to gentamicin (p < 0.0001) and the quinolones nalidixic acid (p = 0.004) and ciprofloxacin (p < 0.0001) than non-producers. In ESBL-producing E. coli, 84.0% of strains expressed CTX-M-15 alone or in combination with other ESBL types. MLST revealed that 24.0% of ESBL-producers had sequence type 617, all of which expressed the CTX-M-15 gene; we also detected expression of 3 DEC-related genes: 2 enteroaggregative E. coli genes and 1 enteropathogenic E. coli gene. In conclusion, CTX-M-15-type ESBL-producing E. coli ST617 appear to have spread to Indonesia.

INTRODUCTION

Some strains of Escherichia coli, called diarrheagenic E. coli (DEC), can cause diarrhea or food poisoning (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), enterohemorrhagic E. coli (EHEC), and enteroinvasive E. coli (EIEC) (3). In refugee camps run by the United Nations High Commissioner for Refugees, diarrhea is the 3rd commonest disease in Asia and the 4th commonest in Africa in children under 5 years old (4). Pathogenic microorganisms isolated from diarrhea patients are bacterial in approximately 80% of cases, with E. coli and Shigella being characteristic examples (5). Recently, extended-spectrum beta-lactamase (ESBL)-producing antibiotic-resistant strains, especially E. coli strains, have been increasingly reported in fecal samples, and the proportion of ESBL-producing strains in all E. coli has increased to 8–10% in European, Asian, and North American countries (6–8). ESBLs are class A beta-lactamases with a plasmid-related resistance mechanism; they are categorized into TEM- and SHV-types derived from the natural narrow-spectrum enzymes (9,10) and a CTX-M-type derived from beta-lactamase on the Kluyvera chromosone (11). Reportedly, ESBL-producing strains, which were TEM- or SHV-type, are increasingly CTX-M-type (12). The current data must be compared with previous reports in order to gain a full understanding of the genetic changes in the strains and the spread of antibiotic resistance.

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

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

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

Antibiotic susceptibility tests: All stool specimens were cultured on MacConkey agar (Oxoid, Basingstoke, UK) for 24 h at 37°C for the selection of E. coli isolates. Stool specimens contaminated by other pathogenic bacteria (Vibrio, Shigella, and Salmonella species), as determined by standard biochemical methods, were retrospectively excluded. Antibiotic susceptibility tests were performed according to the Clinical and Laboratory Standards Institute (CLSI) recommendations for ampicillin (ABPC), sulbactam/ampicillin (ABPC/SBT), piperacillin (PIP), cefotaxime (CTX), ceftazidime (CAZ), cefepime (CFPM), cefpodoxime (CPDX), imipenem (IPM), aztreonam (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 performed with the CLSI confirmatory test, using both cefotaxime (30 mg) and CAZ (30 mg) disks alone and in combination with clavulanic acid (CA) (10 mg) (Eiken Chemical, Tokyo, Japan). The test was considered positive when the diameter of the growth-inhibitory zone around either the cefotaxime or the CAZ disk in combination with CA increased by ≥5 mm compared to the growth-inhibitory zone around the disk containing CTX or CAZ alone (13).

DNA isolation: The DNA template was obtained using the illustra™ bacterial genomic Prep Mini Spin Kit (GE Healthcare Japan, Tokyo, Japan).

CTX-M, TEM, and SHV typing: We used a method described in detail in our previous study (14). In brief, we determined the ST with regard to blactx_m, blatem, and blasma using strains with confirmed production of ESBLs. PCR was carried out using Takara Ex Taq (Takara Bio, Shiga, Japan) to identify blactx_m, blatem, and blasma. Positive strains were determined by PCR using CTX-M, TEM, and SHV group-specific primers (15), as follows: A (5'-SCSATGTCAGYACCCCAAGTAA-3') and B (5'-CCGCRATATGRTGGTGGTGTG-3') for blactx_m, C (5'-TCGGGAAATGTCGGC-GG-3') and D (5'-TGCTTTACGTTAGAGGACC-3') for blatem, and OS-5 (5'-TTATCCTCCGTGTTAGCACC-3') and OS-6 (5'-GATTTGCTGATTCCGCTGG-3') for blasma.

The PCR protocol consisted of denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min and elongation at 72°C for 10 min, as previously described (14). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). DNA sequencing was performed at Eurofins Genomics (Tokyo, Japan), using the purified PCR products.

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 (adh, fumC, gyrB, ics, mdh, purA, and recA) following the recommended procedure at the E. coli MLST website (http://mlst.ucc.ie/dbs/Ecoli).

Detection of DEC-related genes: We used the method described in our previous study (16); we used Cica-Genes™ Pathogenesis Gene Detection PCR Kit (for DEC) (Kanto Chemical, Tokyo, Japan) with the primers for eaeA (ETEC/EPEC), stxl and stx2 (ETEC), aggR (EAE), est and elt (ETEC), and invE (EIEC). The PCR mixture consisted of DNA template, AptaTaq DNA Master, PCR supplement, and primer mixture 1 (eaeA, stxl, and stx2), primer mixture 2 (est and elt), or primer mixture 3 (aggR, invE, and 16S rDNA as an internal control). The PCR procedure included 30 cycles of denaturation at 94°C for 15 s and annealing at 60°C for 30 s.

Statistical analysis: We determined significant differences using the χ² 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 disk 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 than non-producers, as follows: to ABPC (p=0.0359), PIP (p=0.0001), ABPC/SBT (p=0.0025), CTX (p=0.0001), CAZ (p=0.0001), CPDX (p<0.0001), CFPM (p=0.0001), AZT (p=0.0001), GM (p<0.0001), NA (p=0.004), CPFX (p<0.0001), and S-T (p<0.0001), but not to IPM (p=1.000), AMK (p=1.000), FOM (p=1.000), TC (p=0.431), and CP (p=0.137). All 133 strains were susceptible to IPM and AMK (Table 3).

<table>
<thead>
<tr>
<th>Number of strains</th>
<th>CAZ resistance</th>
<th>CTX resistance</th>
<th>CPDX resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>18/25 (72%)</td>
<td>positive (+)</td>
<td>24/25 (96%)</td>
<td>25/25 (100%)</td>
</tr>
</tbody>
</table>

Table 1. ESBL confirmation test
Molecular typing of ESBL-producing E. coli and antibiotic susceptibilities: Of the 25 strains of ESBL-producing E. coli, CTX-M-15-types accounted for 84.0% (21/25 strains); CTX-M-15 alone was expressed in 56% (14/25 strains), CTX-M-15 in combination with TEM-1 in 24.0% (6/25 strains), and CTX-M-15 in combination with SHV in 4.0% (1/25 strains). CTX-M-15 expression, alone or in combination, tended to be associated with resistance to such antibiotics as GM and CPFX (Table 3).

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

DEC-related genes: Among the 25 ESBL-producing E. coli tested, we found only 2 strains that expressed DEC-related genes: 1 strain (ST2142) expressed aggR (EAEC) and 1 strain (ST29) expressed eaeA (EPEC). As shown in Table 2, ST2142 strain with the combination type of CTX-M-14 and TEM-1 expressed aggR, suggesting a diagnosis of EAEC. A TEM-1-type strain expressed eaeA, suggesting a diagnosis of EPEC (Table 2).

DISCUSSION

ESBL-producing Enterobacteriaceae have reportedly spread over the last decade, especially strains of Klebsiella pneumoniae and E. coli (9,17). The former strains are most commonly found in Europe and the United States, while the latter are commonly found in both Asian and Western countries (18–24).

In particular, the isolation of ESBL-producing E. coli has rapidly increased in Asian countries, where the studies from Korea and Japan report that ratios have reached almost 30% of all E. coli isolates. However, studies from developing nations are lacking; in particular, to our knowledge, no studies have been performed in eastern Indonesia for more than 10 years, as the most recent report is from 2005. The current study, which assessed specimens gathered in 2012, showed that the proportion of ESBL producers among all E. coli isolates was similar, but the ESBL typing and antibiotic susceptibilities were different, compared with the strains in the 2005 report (25). Current antibiotic susceptibilities were not apparently worse than previously described, suggesting that the risks associated with ESBL-producing E. coli have not changed in eastern Indonesia between 2005 and 2012; this information is critical in both the medical and governmental settings.

ESBL-producing E. coli have shown good susceptibilities only to carbapenems and AMK (26,27); this is largely in agreement with our data showing good susceptibilities of ESBL producers to carbapenems (IPM), AMK, and FOM. Taken together with our antibiotic susceptibility results for strains with ST617, the data indicate that these 3 antibiotics have good activity against ESBL producers, even in strains with ST617, which is associated with high resistance potential.

ESBL gene typing is also important for monitoring the epidemiological spread of the bacteria. The CTX-M-15-type 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 comprised 84% of the strains, including some strains with a combination CTX-M-15 and TEM-1 or SHV-7. Further long-term observations will be necessary to monitor the epidemiological aspects of this bacterial feature.

Various types of ESBL genes have been identified,
but their impacts on antibiotic susceptibility are not fully understood. TEM-, SHV-, and CTX-M-type enzymes are the most representative ESBL gene types (32–35). Of these 3 subtypes, TEM- and SHV-type strains have amino acid mutations in the natural narrow-spectrum TEM-1, TEM-2, or SHV-1 beta-lactamase genes. The CTX-M enzymes originally demonstrated expanded-spectrum activity (10,36). The CTX-M-type beta-lactamas...


