Prevalence of CTX-M Extended Spectrum β-lactamase-producing Enterobacteriaceae at a Private Tertiary Hospital in Southern Philippines

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The emergence of extended spectrum β-lactamase (ESBL)-producing Enterobacteriaceae is one of the growing healthcare concerns worldwide. ESBLs are plasmid encoded enzymes that confer resistance to broad-spectrum cephalosporins and monobactams. Plasmids that carry genes that code for ESBLs often carry other resistance determinants and because of these, infections caused by ESBL-producing Enterobacteriaceae are difficult to treat contributing to the problem of nosocomial infection. To evaluate the prevalence of ESBL-producing Enterobacteriaceae at Mindanao Sanitarium and Hospital in southern Philippines, \textit{Escherichia coli}, \textit{Klebsiella} species, and \textit{Enterobacter} species isolated from clinical samples, were screened and confirmed for the presence of ESBLs. Specific primers for CTX-M, TEM and SHV ESBL enzymes were used. From a total of 583 isolates collected from September 2005 to September 2008, thirty (5.1\%) were confirmed as ESBL-producers; the majority (60\%) of which produce CTX-M type ESBLs. Most (89\%) ESBL producers co-express resistance to quinolones, 61\% are susceptible to aminoglycosides and all remained susceptible to carbapenems.

Key Words: CTX-M β-lactamases, Enterobacteriaceae, ESBL, prevalence, resistance

INTRODUCTION

Extended spectrum β-lactamases (ESBLs) are plasmid encoded enzymes that confer resistance to broad-spectrum cephalosporins and monobactams (Bradford 2001). They are commonly produced by \textit{Escherichia coli} and \textit{Klebsiella} species, but they are also found in other bacteria (Paterson & Bomono 2005). The presence of ESBLs poses as a threat in clinical settings because the ESBL genes are located in plasmids which can be easily transferred to non-ESBL isolates. Moreover, plasmids that carry genes that code for ESBLs also commonly contain other genes that encode for mechanisms of resistance to many other antibiotics (e.g. quinolones, aminoglycosides, chloramphenicol). Because of this, infections caused by ESBL-producing Enterobacteriaceae are difficult to treat, contributing significantly to increased nosocomial infection (Paterson & Bomono 2005).

The prevalence of ESBL-producing Enterobacteriaceae is high in Asia particularly in China and India (Hawkey 2008), and the predominant type of ESBLs vary from country to country and between regions (Hirakata et al. 2005; Hawkey 2008). Studies have shown that TEM and SHV variants are now being replaced by CTX-M types as the most common type of ESBL (Hirakata et al. 2005; Hawkey 2008).
The emergence of ESBL-producing Enterobacteriaceae is becoming a common problem in the Philippines due to increased cephalosporin use (Villanueva et al. 2003), but data on the prevalence of ESBLs are scarce in this country since most laboratories do not detect their presence. Few preliminary studies have been conducted in this field, and most of them are based only on phenotypic resistance to indicator antibiotics (third-generation cephalosporins and monobactams) and synergy observed between the indicator antibiotic and a β-lactamase inhibitor (e.g. clavulanic acid) (Villanueva et al. 2003; Bomasang & Mendoza 2003). In order to contribute further to understanding the prevalence of ESBL-producing Enterobacteriaceae in the Philippines, the present study (1) determined the prevalence of ESBL-producing Enterobacteriaceae, specifically Escherichia coli, Klebsiella species and Enterobacter species from various clinical samples, and (2) determined the type of β-lactamase using primers specific for blaCTX-M, blaTEM and blaSHV coding for the respective enzymes.

MATERIALS AND METHODS

Sample collection and detection of ESBL-producing Enterobacteriaceae

Sample collection was conducted at Mindanao Sanitarium and Hospital, a 120-bed tertiary-care hospital in Iligan City, southern Philippines, from September 2005 to September 2008. Clinical samples (e.g. sputum, urine, blood) from admitted and out patients were processed for bacteriological culture and antimicrobial susceptibility using the Kirby-Bauer disk diffusion according to the criteria of the Clinical Laboratory Standards Institute (CLSI 2009). Bacterial isolates, identified by API 20E as Klebsiella species, Escherichia coli and Enterobacter species showing resistance or reduced susceptibility to any third-generation cephalosporins were considered possible ESBL producers (CLSI 2009). Those isolates were further screened for the presence of ESBLs using five indicator antibiotic disks (ceftazidime, ceftriaxone, cefotaxime, cefpodoxime, aztreonam) placed 20-25mm apart from the disk containing the β-lactamase inhibitor (e.g. amoxicillin/clavulanic acid) as suggested by Thomson & Sanders (1992). After incubation, an enhanced zone of inhibition between any one of the five indicator antibiotic disks and the β-lactamase inhibitor is a presumptive evidence for the presence of an ESBL. Isolates showing enhanced zone of inhibition in any of the five indicator antibiotic disks were confirmed using the double disk diffusion test using the ceftazidime disk, with and without clavulanic acid. A ≥ 5mm increase in the zone of inhibition for the ceftazidime/clavulanic acid disk versus the zone for the disk containing ceftazidime alone indicates that the isolate is a confirmed ESBL-producer (CLSI 2009). Escherichia coli ATCC 25922 was used as non ESBL producing strain.

PCR with CTX-M, SHV and TEM primers

Once confirmed ESBL-producer by the phenotypic methods, the type of ESBL gene present in the isolate was determined using three primers specific for CTX-M, SHV and TEM-type ESBLs (Table 1). The CTX-M primers will allow amplification of group 1 and 2 CTX-M ESBLs only. DNA extraction was performed by boiling a colony in 2 mL sterile distilled water at 100°C for 10 min. The supernatant from this suspension was used as template for PCR. PCR was carried out for each primer pairs in a 100 µL reaction mixture each containing 50 µL previously boiled DNA template, 50 µM dNTPs, 20 pmol of each primers, 1.5 mM MgCl₂, and 2.0 units of Taq polymerase. Amplification of DNA was performed in a programmable thermal cycler (Perkin Elmer Cetus DNA Thermal Cycler) with the following cycle parameters: initial cycle at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min, extension at 72°C for 7 min. Escherichia coli ATCC 25922 served as negative control. PCR products were run in 1.5 % agarose gel electrophoresis at 100 volts for 3 h or until the DNA ladder had reached ¾ of the gel length. A 1 kb ladder (Intron biotechnology) was used as molecular weight marker. The primers, dNTPs, and Taq polymerase used in this study were purchased from Eurogentec Singapore, Intron biotechnology, and New England Biolabs, respectively.

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Expected Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX-M Forward</td>
<td>5’CGCTTTTGGCATGTCGAG3’</td>
<td>550 base pairs</td>
<td>Bonnet et al. 2000</td>
</tr>
<tr>
<td>CTX-M Reverse</td>
<td>5’ACCGCGATATCGTTGGT3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHV Forward</td>
<td>5’ACTGAGGAGCGGCCTCC3’</td>
<td>297 base pairs</td>
<td>Gniadkowski et al. 1998</td>
</tr>
<tr>
<td>SHV Reverse</td>
<td>5’ATCCCCAGATAAACACACC3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM Forward</td>
<td>5’ATGAGTATCACATATTTCCG3’</td>
<td>351 base pairs</td>
<td>Rasheed et al. 2000</td>
</tr>
<tr>
<td>TEM Reverse</td>
<td>5’TACTGTCATGCCCCATCC3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

ESBL prevalence and Infection rate
A total of 583 Enterobacteriaceae were evaluated, and thirty (5.1%) were confirmed as ESBL producers by phenotypic methods (Table 2). An increase in prevalence was observed from 2005 to 2008. Of the confirmed ESBL-producers, 19 (63%) were identified as Escherichia coli, six (20%) Enterobacter species (one Enterobacter cloacae, three Enterobacter aerogenes, two Enterobacter gergoviae), and five (17%) Klebsiella species (one Klebsiella ozanae and four Klebsiella pneumoniae). Most (56.7%) of the confirmed ESBL-producers were obtained from urine.

Table 2. ESBL-producing Enterobacteriaceae collected from September 2005 to September 2008 at Mindanao Sanitarium and Hospital from admitted and out patients.

<table>
<thead>
<tr>
<th>Year</th>
<th>Total Number of isolates</th>
<th>ESBL-producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>59</td>
<td>1 (1.69%)</td>
</tr>
<tr>
<td>2006</td>
<td>167</td>
<td>3 (1.79%)</td>
</tr>
<tr>
<td>2007</td>
<td>181</td>
<td>13 (7.18%)</td>
</tr>
<tr>
<td>2008</td>
<td>176</td>
<td>13 (7.38%)</td>
</tr>
<tr>
<td>Total</td>
<td>583</td>
<td>30 (5.1%)</td>
</tr>
</tbody>
</table>

The infection rate caused by ESBL-producing Enterobacteriaceae range from 1.8% to 8.2% and was highest in 2007 (Table 3). Review of antibiotic susceptibility results showed that 89% of the isolates were resistant to quinolones (e.g. ciprofloxacin, levofloxacin, moxifloxacin), 61% are susceptible to aminoglycosides (gentamicin, amikacin, netilmicin) and all isolates remained susceptible to carbapenems (imipenem and meropenem).

Polymerase chain reaction with CTX-M, SHV and TEM primers
Eighteen (60%) of the 30 ESBL-producing isolates carried the CTX-M gene, one isolate each (3.3%) carried the SHV-type gene, and the TEM-type gene. One isolate carried two ESBL genes: CTX-M and TEM. Eleven isolates (36.7%) did not yield products with any of the three β-lactamase gene primers.

Table 3. Infection rate caused by ESBL-producing Enterobacteriaceae at Mindanao Sanitarium and Hospital.

<table>
<thead>
<tr>
<th>Year</th>
<th>Total Number of Patients</th>
<th>No. of Infected Patients</th>
<th>% Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>54</td>
<td>1</td>
<td>1.85%</td>
</tr>
<tr>
<td>2006</td>
<td>151</td>
<td>3</td>
<td>1.99%</td>
</tr>
<tr>
<td>2007</td>
<td>159</td>
<td>13</td>
<td>8.18%</td>
</tr>
<tr>
<td>2008</td>
<td>149</td>
<td>11</td>
<td>7.38%</td>
</tr>
<tr>
<td>Total</td>
<td>513</td>
<td>28</td>
<td>5.46%</td>
</tr>
</tbody>
</table>

DISCUSSION

Although we found only 5.1% ESBL-producing Enterobacteriaceae isolates, we report for the first time in the Philippines a high (60%) prevalence of CTX-M type ESBLs. Our results are in accordance with the already widespread CTX-M type ESBLs in many Asian countries (Hawkey 2008). In contrast to the study conducted by Cabrera & Rodriguez (2009) employing molecular methods such as PCR and sequencing at the Philippine General Hospital, they found that SHV-12 is the predominant ESBL type produced by species of Enterobacteriaceae. This result is not surprising since the dominant type of ESBLs may vary from country to country and between regions (Hirakata et al. 2005; Hawkey 2008).

CTX-M enzymes have been divided into six groups based on their amino acid sequence identities (Rossolini et al. 2008), and since the CTX-M primers used in this study can amplify groups 1 and 2 only, the prevalence of CTX-M type ESBLs may be higher. The fact that eleven ESBL-producing strains did not yield products with any of the three β-lactamase gene primers suggests that those isolates may possess other CTX-M type ESBLs (groups 3-6), other resistance mechanism, or it may be due to the possible existence of undescribed ESBLs given their recent rapid expansion (Paterson & Bonomo 2005; Hirakata et al. 2005; Hawkey 2008; Rossolini et al. 2008).

ESBLs are difficult to detect because they have different levels of activity against various cephalosporins. For example, TEM & SHV-type ESBLs present resistance to ceftazidime but variable to cefotaxime; CTX-M ESBLs display resistance to ceftazidime but variable to ceftazidime (Rossolini et al. 2008). In a study conducted by Pitout and colleagues (2004), only 14% of ESBL-producing strains will be detected if only ceftazidime is used and since most of the isolates in this study produce CTX-M type ESBLs, some of the ESBL-producers may have gone undetected because only ceftazidime was in routine use for susceptibility testing in our hospital.

This study has shown a high frequency of isolates that are both ESBL-producers and quinolone resistant. This high association may be attributed to the presence of a transferrable plasmid or transposon carrying both resistance
mechanisms (Jacoby & Martinez 1998). It is also possible that quinolone use (Lautenbach et al 2001) or use of other antibiotics might have selected ESBL-producing strains since genes coding for ESBLs carry multiple resistance determinants (Paterson & Bonomo 2005).

In conclusion, this study found that most ESBL-producing Enterobacteriaceae isolates at Mindanao Sanitarium and Hospital carry the CTX-M type β-lactamase. We are unaware of this because most laboratories do not detect their presence and they are likely to be underestimated especially whether cefotaxime is not included in ESBL detection. Continuing surveillance is necessary to verify whether the CTX-M-type ESBLs will continue to prevail over the TEM- and SHV-type ESBLs, which are still widespread in some areas.

ACKNOWLEDGMENTS

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