A rapid and simple method to detect ESBL in Enterobacter cloacae based on MIC of cefepime

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ABSTRACT

Introduction: The aim of this study was to identify a rapid and simple phenotypic method for extended-spectrum β-lactamase (ESBL) detection in Enterobacter cloacae. Methods: A total of 79 consecutive, non-repeated samples of E. cloacae were evaluated. Four phenotypic methods were applied for ESBL detection, results were compared to multiplex polymerase chain reaction (PCR) as the gold standard reference method: 1) ceftazidime and cefotaxime disks with and without clavulanate, both with boronic acid added; 2) disk approximation using cefepime and amoxicillin/clavulanate; 3) ESBL screening by minimum inhibitory concentration (MIC) ≥ 16µg/mL and 4) by MIC ≥ 2µg/mL for cefepime. Results: Method 4 showed the best combination of sensitivity (100%) and specificity (94%). Conclusions: MIC ≥ 2µg/mL for cefepime would be very useful for the phenotypic detection of ESBL in samples of E. cloacae.

Keywords: Enterobacter cloacae. ESBL. Phenotypic detection.

Enterobacter spp. are important opportunistic pathogens known to cause hospital acquired infections. A major cause of resistance to third-generation cephalosporins by Enterobacter spp. is the expression of AmpC-type β-lactamase. In addition, resistance in many clinical samples is due to the presence of conjugative plasmids encoding the gene extended-spectrum β-lactamase (ESBL)(1).

Until the late 1990s, most ESBLs detected belonged to either the Temoniera (TEM) or sulhydryl variable (SHV) types. More recently, the cefotaximases (CTX-M) type has rapidly spread and has been detected in samples from various countries(2). The presence and types of ESBLs in Brazil, however, has not been well studied. We previously reported that the frequency of ESBL in Enterobacter spp. isolates was higher than was observed in both Escherichia coli and Klebsiella spp(3).

The standard tests for ESBL detection were developed by the Clinical and Laboratory Standards Institute (CLSI), and are based on the ability of clavulanic acid (CA) to inhibit the enzyme. However, this test is complicated by the presence of AmpC in Enterobacter spp. isolates, because CA also causes induction of AmpC(1). To overcome β-lactamase interference in ESBL detection, the use of AmpC inhibitors has been proposed. However, the proposed methods have proved difficult to implement in routine laboratory testing. The aim of the present study was to identify the best and simplest phenotypic method for ESBL detection in clinical samples of Enterobacter cloacae.

This prospective study was conducted over a period of two years (2009-2011) in a 240-bed tertiary care hospital in Brazil. We used a total of 79 consecutive, non-repeated samples of E. cloacae recovered from clinical samples, including urine (44%), blood (21%), secretions (19%), and colonization swabs (16%). E. cloacae isolates were obtained primarily from patients in a 24-bed adult intensive care unit. All samples, belonging to 55 clones, were typed by the enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) molecular technique(4). Isolates were judged to belong to the same genetic clone if the Dice correlation coefficient was 0.93 or greater. In this study we examined all independent isolates, because samples judged to belong to the same clone by ERIC-PCR may have different sensitivity profiles and show different phenotypic test results. Additionally, there is no standard procedure for selecting which isolates could be considered representative of their clonal groups for further analysis (Figure 1).

The identification and tests for antimicrobial sensitivity of isolates were carried out using an automated system (BD Phoenix™, Becton, Dickinson and Company, Sparks, MD, USA). Minimum inhibitory concentrations (MIC) of ceftazidime, ceftriaxone, and cefepime were assessed by the agar dilution method(5) in Mueller Hinton Agar (Becton, Dickinson and Company) plates. Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used as controls.

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FIGURE 1 - Dendrogram plot obtained by the enterobacterial repetitive intergenic consensus-polymersase chain reaction (ERIC-PCR) analysis of the ESBL isolates. Hospital wards: ICU: intensive care unit; 1, 3, 5, and 8: clinical and surgical patients, differing only in the number of patients/room; 2: SUS (Brazilian National Unified Health System) patients; 4: emergency care; 6: maternity; 9: surgical center. Nas/Or swab: nasal or oral swab; MIC: minimum inhibitory concentration; CRO: ceftriaxone; CAZ: ceftazidime; FEP: cefepime; ESBL: extended spectrum β-lactamase; CABA: ceftazidime and cefotaxime disks with and without clavulanic acid + boronic acid; DDac: disk approximation using cefepime and amoxicillin/CA; CA: clavulanic acid; CTX-M: ceftaximases; TEM: Temonieri; NEG: negative; POS: positive.
For detection and typing of ESBL, we performed multiplex PCR with primers for identification of the predominant ESBL types (TEM, SHV, and CTX-M), as described(6). We performed four assays of phenotypic detection for ESBL activity. The first was ceftazidime and cefotaxime disks, with and without CA (10µg/disk) added of 20µL of boronic acid (BA) solution at a concentration of 20g/L (Sigma, St. Louis, MO, USA), in which an increase ≥ 5mm in the diameter of the halo surrounding the disk containing the drug + CA + BA compared to the disk containing only the drug + BA was considered a positive result for ESBL(7) (CABA method). The second assay was disk approximation using cefepime and amoxicillin/CA positioned at a distance of 20 mm center to center, in which the presence of a ghost zone (synergism) between the disks was considered a positive result for ESBL activity(9) (DDAc method). The final two tests were a cefepime 16 (CPM16 method) and cefepime 2 (CPM2 method) assay, in which MICs for cefepime ≥ 16µg/mL and ≥ 2µg/mL, respectively, were considered positive tests for ESBL activity(5)(9).

All the phenotypic tests were performed using fresh cultures. The inoculum was adjusted to the 0.5 McFarland standard, which contains approximately 1 to 2 × 10⁸ colony forming units (CFU)/mL. This adjustment was necessary because an inoculum-dependent effect is observed in β-lactamase-producing samples(10).

A high (56%) frequency of ESBL gene prevalence was detected from the E. cloacae isolates, and all ESBL-positive samples also tested positive in the CPM2 assay. No correlation was identified between clonal genotypes and the absence or presence of ESBL.

Bell et al. and Park et al. proposed screening methods for ESBL in Enterobacter spp. based on MIC > 0.25µg/mL and MIC ≥ 1µg/mL for cefepime, respectively(11)(12). When applying these criteria to our samples, we did not find similar sensitivity and specificity to these reports. However, when we increased the stringency by raising the cutoff MIC value for cefepime to ≥ 2µg/mL, we obtained 100% sensitivity and 94% specificity (Table 1). We therefore conclude that this is the most accurate and easily applicable method for ESBL detection activity in Enterobacter spp.

CPM16 revealed greater (100%) specificity with reduced (80%) sensitivity (Table 1), but use of this method is limited in clinical routines, where the MIC is determined using automated methods. With these methods, 16µg/mL of cefepime is a concentration not routinely included in dilution panels, whereas 2µg/mL of cefepime is frequently found.

Jeong et al. achieved 98.4% sensitivity for ESBL detection in chromosomal AmpC producers using the CABA method(7). This is in contrast with the 62% sensitivity and 91% specificity we attained in this study (Table 1).

Although Tzelepi et al.(8) demonstrated that the DDAc method using cefepime worked well in species that possessed a chromosomal ampC gene(6), in this study we achieved only 49% sensitivity with this method (Table 1). This may have been due to differences in interpretation of the results, which can be subjective. In our case, it was difficult to determine whether the β-lactam inhibitory zones were qualitatively altered.

Interpretation of results from this assay likely requires expertise of the analyst. Furthermore, the ideal distance between disks depends on the sensitivity profile of the bacteria, as has been previously reported(13). A modification to this test was recently suggested; adding phenylboronic acid to the cefepime disk would improve detection of ESBL activity in AmpC producing samples(14).

To consider the accuracy of the methods, the CABA and DDAc methods detected three ESBL-like samples that were negative by PCR (Figure 1), while the CPM2 method detected two. The discrepancy may be due to the primer sequences, which were designed to recognize the most common ESBL families (TEM, CTX-M, and SHV). However, there are other, less prevalent families that were not assessed in this study(13). Therefore it is possible that these were not false-positive results from the phenotypic assays, but false-negative results from the PCR-based assay.

In the present study, nine (20%) samples showed sensitivity to cefepime (MIC between 2µg/mL and 8µg/mL) and were positive to ESBL with both CPM2 and the genotypic method (Table 1). These cases demonstrate the necessity for a routine clinical use of a rapid and simple method to detect ESBL activity in Enterobacter spp. isolates since cefepime may be the drug of choice for treatment of Enterobacter spp. infections(15).

However further clinical studies are needed to evaluate patients treated with cefepime to assess the efficacy of this cephalosporin drug in the treatment of ESBL-producing Enterobacter spp. infections. In addition, the rapid detection of ESBL isolates would enable infection control practitioners to implement precautions, avoiding further spread of this pathogen in the hospital setting.

In conclusion, we demonstrate here that for those laboratories that use dilution methods to determine antimicrobial sensitivity, adopting the CPM2 method would be very useful for the phenotypic detection of ESBL in samples of Enterobacter spp., while for laboratories that use only the disk-diffusion method, the CABA method is highly effective. Our findings also suggest the need for greater surveillance of ESBL in Enterobacter spp. infections for both improved treatment options and to reduce the risk of wider outbreaks.

**TABLE 1 - Sensitivity and specificity of the four different phenotypic methods used for ESBL detection in Enterobacter cloacae isolates.**

<table>
<thead>
<tr>
<th>Methods</th>
<th>CABA</th>
<th>DDAc</th>
<th>CPM16</th>
<th>CPM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (%)</td>
<td>64.0</td>
<td>50.0</td>
<td>80.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>91.0</td>
<td>91.0</td>
<td>100.0</td>
<td>94.0</td>
</tr>
</tbody>
</table>

ESBL: Extended-spectrum β lactamase; CABA: ceftazidime and cefotaxime disks with and without clavulanic acid + boronic acid; DDAc: disk approximation using cefepime and amoxicillin-clavulanic acid; CPM16 and CPM2: MICs for cefepime ≥ 16µg/mL and ≥ 2µg/mL, respectively. MICs: minimum inhibitory concentrations.

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REFERENCES