SHV-Type Extended-Spectrum Beta-Lactamase Production Is Associated with Reduced Cefepime Susceptibility in Enterobacter cloacae

Dóra Szabó,1,2 Robert A. Bonomo,3 Fernanda Silveira,1 A. William Pasculli,4 Carla Baxter,4 Peter K. Linden,5 Andrea M. Hujer,3 Kristine M. Hujer,3 Kathleen Deeley,1 and David L. Paterson1*

Division of Infectious Diseases, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15213; Institute of Medical Microbiology, Semmelweis University, Budapest, Hungary; Research Service, Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, Ohio 44106; Clinical Microbiology Laboratory, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15213; and Department of Critical Care Medicine, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15213

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Cefepime is a potentially useful antibiotic for treatment of infections with Enterobacter cloacae. However, in our institution the MIC90 for E. cloacae bloodstream isolates is 16 μg/ml. PCR amplification of bla genes revealed that one-third (15/45) of E. cloacae bloodstream isolates produced SHV-type extended-spectrum beta-lactamases (ESBLs) in addition to hyperproduction of AmpC-type beta-lactamases. The majority (11/15) of ESBL producers also produced the TEM-1 beta-lactamase. The SHV types included SHV-2, -5, -7, -12, -14, and -30. All but two of the ESBL-producing E. cloacae isolates, but none of the non-ESBL-producing strains, had MICs of cefepime of ≥2 μg/ml. The MIC90 for cefepime for ESBL-producing strains was 64 μg/ml, while for non-ESBL producers it was 0.5 μg/ml. Using current Clinical and Laboratory Standards Institute breakpoints for cefepime, two thirds (10/15) of ESBL-producing isolates would have been regarded as susceptible to cefepime. Phenotypic ESBL detection methods were generally unreliable with these E. cloacae isolates. Based on these results, pharmacokinetic, pharmacodynamic, and clinical reevaluation of cefepime breakpoints for E. cloacae may be prudent.

Enterobacter cloacae is a leading cause of ventilator-associated pneumonia, bloodstream infections, and urinary tract infections in hospitalized patients. A chromosomal gene in E. cloacae characteristically encodes the AmpC beta-lactamase (9). Mutations that increase the expression of the gene encoding AmpC are responsible for the emergence of resistance of the organism to cephalosporins such as ceftriaxone, ceftaxime, and ceftaxidime during therapy (4, 11). Approximately 30% of Enterobacter cloacae bloodstream isolates was 16 g/ml. For this reason, we sought to investigate the mechanisms of reduced cefepime susceptibility in bloodstream isolates occurring at our institution.

MATERIALS AND METHODS

Bacterial strains. Consecutive bloodstream isolates of E. cloacae from patients at the University of Pittsburgh Medical Center (UPMC) were studied. The isolates were collected from March 2003 through July 2004. The species identification was done by standard biochemical tests. The strains were supplied by the hospital’s clinical microbiology laboratory as part of an Institutional Review Board approved study on mechanisms of antibiotic resistance in hospital pathogens.

Antibiotic susceptibility. The clinical microbiology laboratory at UPMC routinely assesses the MICs of cefepime and other injectable antibiotics commonly used in the treatment of infections with gram-negative bacteria by broth microdilution methods using Clinical and Laboratory Standards Institute (CLSI) criteria, current as of 1 January 2005. Additionally, the MICs of piperacillin-tazobactam, ceftazidime, ceftazidime-clavulanic acid, cefotaxime, cefotaxime-clavulanic acid, cefoxitin, gentamicin, ertapenem, imipenem, meropenem, and ciprofloxacin were determined by Etest for the strains (AB Biodisk, Solna, Sweden). Escherichia coli ATCC 2922 and Klebsiella pneumoniae ATCC 700603 were used as the reference strains for antimicrobial susceptibility testing.

Analytical isoelectric focusing. Analytical isoelectric focusing was performed on isolates with cefepime MICs of 0.5 μg/ml or more, as described previously (19). After removal of whole cells and debris by centrifugation, the supernatant was used to determine the isoelectric point (pI). Electrophoresis was performed using precast polyacrylamide gels, pH 3 to 10 (Bio-Rad, Hercules, CA). Enzyme activity was detected by placing filter paper soaked in nitrocefin (500 μg/ml) (Becton Dickinson, Sparks, MD) over the focused gel. Standards from Bio-Rad (Bio-Rad, Hercules, CA) were used, with the following isoelectric points: 4.45, 4.65, 4.75, 5.1, 6.0, 6.5, 6.8, 7.0, 7.1, 7.5, 7.8, 8.00, 8.20, and 9.6.

Plasmid profiles. The plasmid DNA of the extended-spectrum beta-lactamase (ESBL)-producing clinical isolates was extracted with a plasmid extraction kit (Wizard Plus Miniprep DNA purification system; Promega, Madison, WI), according to the manufacturer’s instructions. Plasmid DNA electrophoresis was performed with 0.8% agarose gel and visualized with ethidium bromide under UV light. λ HindIII (Promega, Madison, WI) was used as a molecular size marker.

* Corresponding author. Mailing address: UPMC Division of Infectious Diseases, Suite 3A Falk Medical Building, 3601 5th Avenue, Pittsburgh, PA 15213. Phone: (412) 648-6478. Fax: (412) 648-6399. E-mail: patersond@dom.pitt.edu.
antagonist test (16). Negative cefoxitin-cefotaxime antagonist test; partially derepressed AmpC mutants.

Sequencing reactions were performed with corresponding primers specific for the sequences available in the GenBank database. The amplified products were sequenced using ABI 4500 Sequence analysis.

**PFGE**. Genomic DNA was isolated and digested with XbaI (New England Biolabs, Beverly, Mass.). Pulsed-field gel electrophoresis (PFGE) was performed with the CHEF III system (Bio-Rad, Hercules, CA) with the following run parameters: block I, with a switch time of 3 to 65 s and a run time of 17 h, and block II, with a switch time of 15 to 30 s and a run time of 6 h. Dendrograms were created with BioNumerics (Bio-Rad, Hercules CA) by using the Dice coefficient, unweighted-pair group method with arithmetic means, and a position tolerance of 1.3%. Relatedness of the isolates was determined by the criteria of Tenover et al. (24).

Detection of ESBL genes by PCR. Detection of genes encoding ESBLs was attempted for all isolates with cefepime MICs of 0.5 μg/ml or more. A single colony of each test isolate was resuspended in 400 μl water and boiled for 15 min. The resulting supernatant was used as a bacterial template DNA in PCR assays.

The primers for detection of the bla<sub>TEM</sub> and bla<sub>SHV</sub> genes were as follows: 5′-ATGATGTATACAGATTCCGTTG-3′ and 5′-TTTACGAATTGCTATGCATGTTG-3′ for bla<sub>TEM</sub> (6), 5′-ATGATGTATACAGATTCCGTTG-3′ and 5′-TTTACGAATTGCTATGCATGTTG-3′ for bla<sub>SHV</sub> (6), and 5′-CGGTTGCG ATGTTGCAG-3′ and 5′-ACCGGCGATGTTGCAG-3′ for bla<sub>CTX-M</sub> (2). PCR reactions were performed with RedTaq DNA polymerase (Sigma, St Louis, MO), according to the instructions of the manufacturer, in the presence of 2 μl of the template DNA preparation in a total volume of 50 μl. The DNA amplification programs consisted of an initial denaturation step (96°C, 5 min) followed by 30 cycles of denaturation (96°C, 30 s), annealing (annealing temperature designed for each primer set, 30 s), and extension (72°C, 1 min), and a final extension of 5 min at 72°C. Ten microliters of reaction mixture containing the PCR product was analyzed by electrophoresis in 0.8% (wt/vol) agarose (1% agarose, Bio-Rad, Hercules, CA).

**Sequence analysis.** The amplified products were sequenced using ABI 4500 and ABI 3100 genetic analyzers according to the manufacturer's instructions. Sequencing reactions were performed with corresponding primers specific for the bla<sub>TEM</sub> and bla<sub>SHV</sub> genes used for the previous amplification. Sequence analysis was performed using Lasergene DNASTAR sequencing analysis software (DNASTar, Madison, WI). Each sequence of bla<sub>TEM</sub> and bla<sub>SHV</sub> genes was identified by comparison with known ESBL sequences available in the GenBank and EMBL databases by multiple-sequence alignment using the BLAST program.

**Phenotypic detection of beta-lactamase production.** Disk diffusion testing was performed using ceftazidime, cefotaxime, and cefepime alone (30 μg each; Remel, Lenexa, KS), and in combination with clavulanic acid: ceftazidime-clavulanic acid, cefotaxime-clavulanic acid, and cefepime-clavulanic acid, and cefepime-clavulanic acid.

Classification of <i>E. cloacae</i> strains as having inducible, partially derepressed, or derepressed AmpC production was determined by the methods of Sanders et al. (22), in which the cefoxitin-cefoxime antagonist test was performed (16, 22). The <i>E. cloacae</i> isolates were categorized as follows: derepressed AmpC mutants had a cefoxitin MIC of =32 μg/ml, a cefoxime MIC of =16 μg/ml, and a negative cefoxitin-cefoxime antagonist test; partially derepressed AmpC mutants had the same characteristics as derepressed AmpC mutants but with a positive cefoxitin-cefoxime antagonist test; and inducible AmpC-producing strains had a cefoxime MIC of =8 μg/ml and a positive cefoxitin-cefoxime antagonist test (16).

**RESULTS**

**PFGE.** There was no relatedness between 41/45 isolates. All 30 isolates which were found not to be ESBL producers were unrelated. Two of 15 ESBL producers (ES1 and ES22) were closely related, and two additional ESBL-producing strains (ES31 and ES43) were possibly related (data not shown).

**Antibiotic susceptibility testing of ESBL-producing <i>E. cloacae</i> strains.** The antibiotic susceptibilities of the non-ESBL- and ESBL-producing <i>E. cloacae</i> strains are seen in Tables 1 and 2, respectively. Forty percent (18/45) of isolates had cefepime MICs of 0.5 μg/ml or more. The distribution of the cefepime MICs is shown in Fig. 1. The MIC<sub>90</sub> for cefepime was 0.125 μg/ml. The MIC<sub>90</sub> for cefepime-clavulanic acid was 0.125–1 μg/ml or more. The distribution of the cefepime MICs is shown in Fig. 1. The MIC<sub>90</sub> for cefepime was 0.125 μg/ml. The MIC<sub>90</sub> for cefepime-clavulanic acid was 0.125–1 μg/ml or more.

**Plasmid profile analysis.** Selected ESBL-producing <i>E. cloacae</i> strains isolated during this period were analyzed for their plasmid content (Fig. 2; Table 3). All ESBL-producing <i>E. cloacae</i> isolates harbored plasmids, although there was diversity in the sizes of these plasmids (Fig. 2).

**Antibiotic susceptibility testing of ESBL-producing strains.** Antibiotic susceptibility testing results are shown in Table 2. The cefepime MIC distributions of ESBL-producing isolates were 0.5 μg/ml (one isolate), 1 μg/ml (one isolate), 2 μg/ml (three isolates), 4 μg/ml (two isolates), 6 μg/ml (one isolate), 8 μg/ml (two isolates), 12 μg/ml (one isolate), 16 μg/ml (two isolates), 64 μg/ml (one isolate), and >256 μg/ml (one isolate).

The antibiotic susceptibilities of <i>Enterobacteriaceae</i> were determined by the criteria of Tenover et al. (24). Relatedness of the isolates was determined by the criteria of Tenover et al. (24).
susceptible to numerous antibiotics: 14/15 ESBL-producing isolates were nonsusceptible to ceftazidime, 8/15 were nonsusceptible to piperacillin-tazobactam, 12/15 were nonsusceptible to gentamicin, 8/15 were nonsusceptible to ciprofloxacin, and 2/15 were nonsusceptible to ertapenem. All 15 isolates were susceptible to imipenem and meropenem.

**Phenotypic detection of ESBL production.** Applying CLSI ESBL screening disk diffusion criteria to *E. cloacae* showed that 23 isolates had a “positive” result, but 9 of these isolates were non-ESBL producers (Table 4). Confirmatory results were positive for 7/15 ESBL-producing strains but for no non-ESBL-producing isolate. Applying CLSI ESBL screening MIC criteria gave similar results (Table 5), although confirmatory tests were positive for just 2/15 ESBL-producing strains. The conventional double disk diffusion tests utilizing ceftazidime and cefotaxime disks were rarely positive (Table 4).

Use of cefepime susceptibility results as a marker for ESBL production appeared more useful (Table 6). All 13 isolates with cefepime MICs of 2 μg/ml or more were ESBL producers, and 15/18 (83%) of isolates with cefepime MICs of >0.25 μg/ml were ESBL producers. A confirmatory test with cefepime-clavulanic acid was only 73% sensitive using disk diffusion and 53% sensitive using ≥3 MIC reductions (Table 6).

**DISCUSSION**

There are now numerous reports of *E. cloacae*-producing ESBLs (1, 3, 7, 8, 10, 14, 16, 17), including several from the
United States (5, 13, 21). The earliest reports of ESBL-producing *E. cloacae* strains from the United States were of TEM-12 and TEM-26 producers from Boston in 1988 (21) and SHV-3 producers from Boston in the 1990s (5). In a subsequent report, Levison and colleagues found SHV-7- and SHV-12-producing *E. cloacae* in three different hospitals in Philadelphia in 2000 and 2001 (13). In Pittsburgh, we have subsequently found SHV-2, -5, -7, -12, -14, and -30 in *E. cloacae* strains. In a 1-year period, 33% (15/45) of *E. cloacae* bloodstream isolates were found to be ESBL producers.

**TABLE 3. Characterization of the ESBL-producing *E. cloacae* isolates**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Beta-lactamase characterization</th>
<th>Genotypic evaluation</th>
<th>pI</th>
<th>Plasmid profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES1</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-7 Present</td>
<td>5.4+7.6+9.0</td>
<td>P1</td>
</tr>
<tr>
<td>ES6</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-7 Present</td>
<td>5.4+7.6+9.0</td>
<td>P2</td>
</tr>
<tr>
<td>ES7</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-5 Absent</td>
<td>8.2+9.0</td>
<td>P3</td>
</tr>
<tr>
<td>ES11</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-14 Present</td>
<td>5.4+7.0+9.0</td>
<td>P4</td>
</tr>
<tr>
<td>ES15</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-14 Absent</td>
<td>7.0+9.0</td>
<td>P5</td>
</tr>
<tr>
<td>ES18</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-7 Present</td>
<td>5.4+7.6+9.0</td>
<td>P6</td>
</tr>
<tr>
<td>ES20</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-2 Absent</td>
<td>7.6+9.0</td>
<td>P7</td>
</tr>
<tr>
<td>ES22</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-7 Present</td>
<td>5.4+7.6+9.0</td>
<td>P1</td>
</tr>
<tr>
<td>ES24</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-7, SHV-30 Present</td>
<td>5.4+7.0+7.6+9.0</td>
<td>P8</td>
</tr>
<tr>
<td>ES31</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-12 Present</td>
<td>5.4+8.2+9.0</td>
<td>P9</td>
</tr>
<tr>
<td>ES37</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-7 Present</td>
<td>5.4+7.6+9.0</td>
<td>P10</td>
</tr>
<tr>
<td>ES40</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-7 Present</td>
<td>5.4+7.6+9.0</td>
<td>P11</td>
</tr>
<tr>
<td>ES43</td>
<td>Partially derepressed + ESBL</td>
<td>SHV-12 Present</td>
<td>5.4+8.2+9.0</td>
<td>P9</td>
</tr>
<tr>
<td>ES44</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-7 Present</td>
<td>5.4+7.6+9.0</td>
<td>P10</td>
</tr>
<tr>
<td>ES45</td>
<td>Derepressed AmpC + ESBL</td>
<td>SHV-14 Absent</td>
<td>7.0+9.0</td>
<td>P12</td>
</tr>
</tbody>
</table>

**FIG. 2.** Plasmids from representative ESBL-producing *E. cloacae* strains. Lanes 1, 9, and 17 contain λ HindIII size markers. Lanes 2 to 8 contain ESBL-producing *E. cloacae* strains ES1, ES6, ES7, ES11, ES15, ES18, and ES22, respectively. Lanes 10 to 16 contain ESBL-producing *E. cloacae* strains ES24, ES31, ES37, ES40, ES43, ES44, and ES45, respectively.
fortunately, there are no surveillance data from the United States in order to determine whether this percentage is high or whether it is within the range found in similar hospitals. In a recently published study from Korea, 43% of \textit{E. cloacae} blood culture isolates were found to be ESBL producers (16). Wild-type \textit{E. cloacae} strains obtained prior to the commercial release of cefepime had cefepime MICs of 0.06 to 0.5 g/ml. It is uncertain whether any of the strains tested in these studies produced ESBLs. In our center, the MIC$_{90}$ for cefepime for ESBL-producing strains was 64 g/ml, while for non-ESBL producers it was 0.5 g/ml. Studies examining outer membrane proteins, efflux pumps, and the expression levels and hydrolytic activities of SHV-type ESBLs against cefepime are currently under way. Thus, at the present time, we are unable to conclusively state that the production of ESBLs is responsible for the elevated cefepime MICs that we observed. The clinical implications of this elevation in cefepime MIC are under evaluation, since some patients in our series failed cefepime therapy. Assessments of the pharmacokinetics and pharmacodynamics of cefepime presented at CLSI Antimicrobial Susceptibility Testing subcommittee meetings would suggest that cefepime may not be effective for treating serious infections when dosed at 1 g every 12 h for an organism with a MIC of 8 g/ml or higher. Clinical data from patients with serious infections with ESBL-producing \textit{Klebsiella} spp. or \textit{E. coli} would support the concept that cefepime activity may be compromised against some ESBL-producing organisms with MICs in the current susceptible range (18, 27). Thus, it would appear prudent for cefepime breakpoints to be reconsidered.

Given that we have evaluated just 15 ESBL-producing isolates and 30 non-ESBL-producing isolates, we cannot categorically comment on ESBL detection methods for \textit{E. cloacae}. Conventional methods using cefotaxime or ceftazidime are likely to be unreliable. However, a cefepime MIC of $\geq$2 g/ml appears to be a consistently robust marker of ESBL production. While tests incorporating cefepime plus clavulanic acid may detect some ESBL producers with lower MICs, the tests were not more than 75% sensitive in our hands.

Why would SHV-type ESBLs evolve in an \textit{E. cloacae} host? In our isolates, the SHV-type ESBLs are present in the company of a derepressed AmpC. Increased production of the AmpC beta-lactamase confers resistance to oxyiminocephalos-

\begin{table}
\caption{Distribution of the results of different disk diffusion techniques}
\begin{tabular}{llllll}
\hline
Test and drug & Test result & Category (no. of isolates) & Sensitivity & Specificity \\
& & ESBL & Non-ESBL & ESBL & Non-ESBL \\
\hline
CLSI ESBL initial screen$^a$ & Ceftazidime & Positive & 14 & 9 & 0.93 & 0.70 \\
& & Negative & 1 & 21 & & \\
& Cefotaxime & Positive & 14 & 9 & 0.93 & 0.70 \\
& & Negative & 1 & 21 & & \\
\hline
CLSI ESBL phenotypic confirmatory test$^b$ & Ceftazidime & Positive & 7 & 0 & 0.47 & 1 \\
& & Negative & 8 & 30 & & \\
& Cefotaxime & Positive & 5 & 0 & 0.33 & 1 \\
& & Negative & 10 & 30 & & \\
\hline
Double disk diffusion test (20 mm) & Ceftazidime & Positive & 6 & 0 & 0.40 & 1 \\
& & Negative & 9 & 30 & & \\
& Cefotaxime & Positive & 7 & 0 & 0.47 & 1 \\
& & Negative & 8 & 30 & & \\
\hline
Double disk diffusion test (30 mm) & Ceftazidime & Positive & 1 & 0 & 0.07 & 1 \\
& & Negative & 14 & 30 & & \\
& Cefotaxime & Positive & 7 & 0 & 0.47 & 1 \\
& & Negative & 8 & 30 & & \\
\hline
\end{tabular}
\end{table}

$^a$ The CLSI disk inhibition break points for ESBL initial screen of \textit{E. coli} and \textit{Klebsiella} spp. were $\leq$22 mm for ceftazidime (30 $\mu$g) and $\leq$27 mm for cefotaxime (30 $\mu$g).

$^b$ A $>$5-mm increase in zone diameter in combination with clavulanic acid versus when tested alone.

\begin{table}
\caption{Distribution of the results of MICs obtained by Etest as a means of ESBL detection and phenotypic confirmation}
\begin{tabular}{llllll}
\hline
Test stage and drug & Test result & Category (no. of isolates) & Sensitivity & Specificity \\
& & ESBL & Non-ESBL & ESBL & Non-ESBL \\
\hline
Initial screening$^a$ & Ceftazidime & Positive & 15 & 8 & 1 & 0.73 \\
& & Negative & 0 & 22 & & \\
& Cefotaxime & Positive & 15 & 8 & 1 & 0.73 \\
& & Negative & 0 & 22 & & \\
\hline
Phenotypic confirmation$^b$ & Ceftazidime & Positive & 2 & 0 & 0.13 & 1 \\
& & Negative & 13 & 30 & & \\
& Cefotaxime & Positive & 0 & 0 & 0 & 1 \\
& & Negative & 15 & 30 & & \\
\hline
\end{tabular}
\end{table}

$^a$ The CLSI MIC breakpoints for ESBL screening for \textit{E. coli} and \textit{Klebsiella} spp. were 2 $\mu$g/ml or more for ceftazidime and cefotaxime.

$^b$ A $\geq$3 twofold-concentration decrease in MIC for either antimicrobial agent tested in combination with clavulanic acid versus its MIC tested alone.


References

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**ACKNOWLEDGMENTS**

Table 6. Distribution of the results using cepafmine as an indicator for ESBL production

<table>
<thead>
<tr>
<th>Test and result</th>
<th>Category (no. of isolates)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disk diffusion screening</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>test†</td>
<td>Positive</td>
<td>13</td>
<td>0.87</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Disk diffusion confirmatory test‡</td>
<td>Positive</td>
<td>11</td>
<td>0.73</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Double disk diffusion test (20 mm)</td>
<td>Positive</td>
<td>13</td>
<td>0.87</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Double disk diffusion test (30 mm)</td>
<td>Positive</td>
<td>8</td>
<td>0.53</td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>MIC-based screening test‡</td>
<td>Positive</td>
<td>14</td>
<td>0.93</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>MIC-based confirmatory test‡</td>
<td>Positive</td>
<td>8</td>
<td>0.53</td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

*Based on zone diameter of <27 mm as a screen for potential ESBL production.
† A >5-mm increase in zone diameter of cepafmine in combination with clavulanic acid versus cepafmine tested alone.
‡ Cepafmine breakpoint based on our study was 1 μg/ml.
§ A >3 twofold-concentration decrease in MIC for either antimiicrobial agent tested in combination with clavulanic acid versus its MIC tested alone.


