Occurrence of Extended-Spectrum and AmpC Beta-Lactamases in Bloodstream Isolates of *Klebsiella pneumoniae*: Isolates Harbor Plasmid-Mediated FOX-5 and ACT-1 AmpC Beta-Lactamases

Philip E. Coudron,1* Nancy D. Hanson,2 and Michael W. Climo3

Pathology and Laboratory Medicine Service/1131 and Department of Medicine;3 McGuire Veterans Affairs Medical Center, Richmond, Virginia 23249-0001, and Department of Medical Microbiology and Immunology, Creighton University School of Medicine, Omaha, Nebraska 681783

Received 12 August 2002/Returned for modification 4 October 2002/Accepted 12 November 2002

We tested 190 *Klebsiella pneumoniae* bloodstream isolates recovered from 189 patients in 30 U.S. hospitals in 23 states to determine the occurrence of extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase producers. Based on growth inhibition by clavulanic acid by disk and MIC test methods, 18 (9.5%) of the isolates produced ESBLs. Although the disk diffusion method with standard breakpoints identified 28 cefotixin-nonsusceptible isolates, only 5 (18%) of these were confirmed as AmpC producers. Of two AmpC confirmatory tests, the three-dimensional extract test was easier to perform than was the double-disk approximation test using a novel inhibitor, Syn2190. Three of the five AmpC producers carried the bla*FOX-5* gene, while the other two isolates harbored the bla*ACT-1* gene. All AmpC genes were transferable. In vitro susceptibility testing with standard inocula showed that all five AmpC-producing strains were susceptible to ceftepime, imipenem, and ertapenem but that with a high inoculum, more of these strains were susceptible to the carbapenems than to ceftepime. All but 1 of 14 screen-positive AmpC nonproducers (and ESBL nonproducers) were susceptible to ceftriaxone and ceftepime at the standard inoculum as were 6 of 6 isolates that were randomly selected and tested with a high inoculum. These results indicate that (i) a significant number of *K. pneumoniae* bloodstream isolates harbor ESBL or AmpC β-lactamases, (ii) confirmatory tests are necessary to identify true AmpC producers, and (iii) in vitro, carbapenems are active against AmpC-producing strains of *K. pneumoniae*.

A recent study of clinical outcomes underscored the need for clinical microbiology laboratories to identify and report *Klebsiella pneumoniae* isolates that harbor extended-spectrum β-lactamases (ESBLs), even when cephalosporin MICs are in the susceptible range (17). Four (100%) of four patients and 15 (54%) of 28 patients experienced clinical failure when the MICs of the cephalosporins used for treatment were in the intermediate and susceptible ranges, respectively. Therefore, it is important to identify ESBL producers and report these organisms as resistant to aztreonam and all cephalosporins, with the exception of cephalexin, regardless of the MIC. However, in a 1998 survey that included 369 clinical microbiology laboratories in the United States, only 117 (32%) performed tests to identify ESBL producers, and of 112 laboratories that reported their methods, only 83% used adequate ESBL screening methods (4). The laboratories that commented on the interpretation of test results reported that only 70 and 53% of ESBL producers were resistant to all extended-spectrum cephalosporins and aztreonam, respectively. The National Committee for Clinical Laboratory Standards (NCCLS) issued the first guidelines for interpreting test results of ESBL producers in 1994 (14).

Efforts to detect other β-lactamases, such as AmpC enzymes, in *klebsiellae* and *Escherichia coli* isolates are largely nonexistent. Undoubtedly, this is due in part to the lack of standard guidelines for detecting AmpC-producing isolates. Expression of AmpC β-lactamases can be generated by chromosomal or plasmid genes (19). Plasmid-mediated AmpC β-lactamases are thought to have originated from the chromosomess of several *Enterobacteriaceae* species and are rarely inducible (19). Unlike ESBLs, plasmid-encoded cephalosporinases are active against cephamycins and are not inhibited by clavulanic acid. The number of infections caused by AmpC-producing organisms is increasing (2, 7, 9, 26). Distinguishing between AmpC- and ESBL-producing organisms has epidemiological significance and may have therapeutic importance as well.

In view of the various degrees of compliance by laboratories in identifying ESBL-producing organisms, together with the potential impact of β-lactamase-producing organisms on patient outcomes, we designed a study to determine the occurrence of ESBLs and AmpC enzymes in clinical strains of *K. pneumoniae*. Organisms were collected from patients living in a broad range of geographical regions of the United States, and only bloodstream isolates were tested. In addition, the effect of inoculum size is a factor in treatment of serious infections caused by β-lactamase producers. Therefore, in this study, MIC-based susceptibility tests of selective agents against all AmpC-producing isolates were performed using standard and high inocula.

(This work was presented, in part, at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy, December 2001, Chicago, Ill. [abstr. C2-2232].)
TABLE 1. Primers used for amplification and sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequencea</th>
<th>Amplicon length (bp)b</th>
<th>Positionc</th>
<th>Accession no.c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification</td>
<td>NH AmpCF</td>
<td>ATTCGTTAATCCTGTTGCATCTGCCACC</td>
<td>396</td>
<td>383–406</td>
<td>AF411149</td>
</tr>
<tr>
<td></td>
<td>NH AmpCR</td>
<td>CATGACCAAGTTGCGCATATGCTG</td>
<td>778</td>
<td>778–755</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FOX-5</td>
<td>CACCAGGAAATCATTACCG</td>
<td>1,184</td>
<td>683–698</td>
<td>X77455</td>
</tr>
<tr>
<td></td>
<td>FOX D1R</td>
<td>GCTTGAACACTCGAC</td>
<td>1,866</td>
<td>1866–1851</td>
<td></td>
</tr>
<tr>
<td>Sequencing</td>
<td>ACT-1</td>
<td>CTIGAATGCATGATTACCG</td>
<td>NA</td>
<td>3–20</td>
<td>U58495</td>
</tr>
<tr>
<td></td>
<td>PC ACT-1F</td>
<td>ATGCCATTGCTGCTGCCG</td>
<td>317</td>
<td>317–333</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PC ACT-2I</td>
<td>TATGACCGGGCTGATACCG</td>
<td>598</td>
<td>598–615</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PC ACT-1R</td>
<td>GCAATTGTTCATACCGG</td>
<td>1,183</td>
<td>1183–1166</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PFC Fox5F</td>
<td>CGCCCTTGAACATGCGCTG</td>
<td>NA</td>
<td>129–111</td>
<td>AY007369</td>
</tr>
<tr>
<td></td>
<td>PFC Fox5R</td>
<td>CGCTATGTTGCGCCTG</td>
<td>1,029</td>
<td>1029–1047</td>
<td></td>
</tr>
</tbody>
</table>

a All primer sequences are written in the 5'-to-3' direction.

b Expected size of the amplification product.

c Nucleotide position of the primer in the sequence.

d GenBank accession number of a sequence containing the corresponding primer sequence.

e NA, not applicable.

MATERIALS AND METHODS

Bacterial isolates. A total of 190 K. pneumoniae isolates were recovered from the bloodstream of 189 patients who lived in the northeastern (78 isolates), southeastern (59 isolates), northwestern (30 isolates), and southwestern (23 isolates) quadrants of the United States (SCOPE National Surveillance Program, 1995 to 1999). All isolates were initially identified by the API 20E System (bioMérieux, Hazelwood, Mo.). Some isolates were also tested for motility and assimilation of ethanolamine, histamine, and melezitose (Sigma Chemical Co., St. Louis, Mo.). The latter tests were used by Westbrook et al. (25) to distinguish K. pneumoniae from K. planticola and K. terrigena.

Tests for ESBL- and AmpC-producing isolates. Isolates were screened for ESBL production by the double-disk potentiation method (10). Mueller-Hinton agar (Becton Dickinson, Sparks, Md.) was inoculated, and disks (Becton Dickinson) containing the standard 30 μg of aztreonam, ceftazidime, or ceftriaxone or 10 μg of cefepime were placed 15 mm (edge to edge) from an amoxicillin-clavulanic acid disk (20 and 10 μg, respectively) (5). After incubation, an enhanced zone of inhibition between any one of the β-lactam disks and the clavulanic acid disk was interpreted as presumptive evidence for the presence of an ESBL. Isolates were also tested by the NCCLS confirmatory method using standard ceftazidime and cefotaxime disks with and without clavulanic acid (10 μg) (15). The control strains, E. coli ATCC 25922 and K. pneumoniae ATCC 700603, demonstrated the expected zone patterns (15). AmpC β-lactamases were screened by the standard disk diffusion test using 30-μg cefoxitin disks. Isolates with zone diameters of less than 18 mm were selected for additional testing.

MICs were determined for all ESBL and AmpC producers by the microdilution method, using Mueller-Hinton broth and a standard inoculum of 5 × 10⁶ CFU/ml (13). The effect of a high inoculum on the MICs for AmpC producers was tested by agar dilution using the standard inoculum and a 100-fold-higher inoculum of 10⁸ and 10⁹ CFU/spot, respectively (13). The antibiotic powders were provided by the manufacturers. Control strains included E. coli ATCC 25922, E. coli ATCC 35218, and Pseudomonas aeruginosa ATCC 27853; MIC results were within expected ranges (15).

β-Lactamase testing. Cell extracts from isolates that showed enhanced inhibition by the double-disk test or that showed decreased cefoxitin zone diameters were examined using isoelectric focusing (IEF) (5, 12). IEF patterns of enzymes that demonstrated a loss of nitrocefin hydrolysis in the presence of clavulanic acid or cloxacillin were presumptively interpreted to represent the ESBL or AmpC enzyme, respectively. Control strains for IEF harbored the TEM-1, TEM-2, TEM-3, SHV-2, SHV-3, SHV-4, SHV-5, and ACT-1 enzymes. AmpC β-lactamase activity was also examined in some isolates by the modified three-dimensional extract method (6, 23). Induction of AmpC β-lactamase was demonstrated by the disk approximation method using cefazidime and cefotaxim disks.

The ability of Syn2190, a 1,5-dihydroxy-4-pyridone monobactam, to detect group 1 β-lactamase activity was tested by the double-disk potentiation method. A stock solution of Syn2190 (NAEJA Pharmaceutical Inc., Edmonton, Alberta, Canada) was prepared, as instructed by the manufacturer, by initially mixing equimolar amounts of powder of sodium carbonate and inhibitor and then adding water to the mixture. Mueller-Hinton agar was inoculated with the test organism, and a disk containing 200 μg of Syn2190 was placed at approximately 3 mm distant from a standard cefoxitin disk.

Molecular analysis. Pulsed-field gel electrophoresis (PFGE) was performed on ESBL- and AmpC-producing isolates. Genomic DNA was isolated and prepared as described previously, except that lysozyme was used in the lysis step in place of lysostaphin (1). Following restriction endonuclease digestion with Apel (New England Biolabs, Beverly, Mass.), PFGE was performed on a CHEF-DRIII instrument (Bio-Rad, Hercules, Calif.) under the following conditions: 0.5 M Tris-borate-EDTA buffer-1% agarose at 4°C and 200 V for 20 h, with the switch interval ramped from 1 to 20 s. Conjugations were performed by filter mating using the recipient E. coli strain (26R793) (6). The plasmid-mediated AmpC genes blacXT-1 and blacFOX-5 were identified by sequencing the PCR-amplified products directly. PCR amplification was performed in a final volume of 50 μl containing 1.5 mM MgCl₂, 0.5 μM primer, and 2 μl of template (Table 1) (9). The 396-bp blacXT-1 gene fragment was amplified using 28 cycles of denaturation (95°C for 30 s), annealing (55°C for 30 s), and extension (72°C for 2 min). The full-length amplification products of blacXT-1 and blacFOX-5 were generated using similar cycling parameters, with the denaturation and annealing steps modified to 40 s and 47°C, respectively, for blacXT-1 and a 51°C annealing temperature used to generate the 1184-bp blacFOX-5 amplification product. Amplification products were generated and sequenced twice using an ABI 377 automatic sequencer with dideoxy chain termination chemistry. Sequence alignments and analyses were performed on-line using the BLAST program (www.ncbi.nlm.nih.gov).

RESULTS

β-Lactamase producers. Based on phenotypic results (see below), 18 (9.5%) and 5 (2.6%) of 190 K. pneumoniae isolates harbored ESBL-like and AmpC enzymes, respectively. K. pneumoniae strains containing these enzymes were detected in 7 of 30 hospitals within the SCOPE network, and the majority of these hospitals were located in the eastern part of the United States. Of the 23 isolates, 2 (MCV-21 and MCV-23) were recovered from the same patient 6 weeks apart and were considered different strains based on PFGE patterns (Table 2).
All 23 isolates demonstrated unique PFGE patterns, although four isolates (MCV-5, MCV-9, MCV-57, and MCV-62W) were closely related (Tables 2 and 3).

**ESBL-like phenotypes.** All ESBL-like producers showed enhanced inhibition to one or more extended-spectrum β-lactams by the disk potentiation method and demonstrated a 5-mm or greater increase in the zone diameter of ceftazidime plus inhibitor with respect to ceftazidime alone. The MICs of selected antimicrobial agents for these isolates are shown in Table 2. The MIC ratio of ceftazidime to ceftazidime plus clavulanate was greater than 8 for all ESBL producers. Although the ceftazidime MIC for the MCV-109 isolate was only 1.0 μg/ml, the MICs of cefotaxime and cefotaxime plus inhibitor were 4.0 and 0.03 μg/ml, respectively. All ESBL producers were susceptible to imipenem and the new carbapenem, ertapenem.

**AmpC producers.** Although 28 (15%) of the 190 isolates demonstrated cefoxitin zone diameters less than 18 mm, only 5 isolates were positive by the three-dimensional test and demonstrated clavulanil- and not clavulanate-inhibited bands by IEF (Fig. 1). These five AmpC-producing isolates demonstrated other clavulanate-inhibited bands, indicating the presence of additional β-lactamases (Table 3). All five strains tested positive with the Syn2190 inhibitor and transferred the gene conferring cefoxitin resistance to the recipient *E. coli* strain 26R793. The cefoxitin MIC for each of five transconjugants was 16- to 64-fold greater than the cefoxitin MIC for the recipient strain. Induction of AmpC in MCV-4 and MCV-80G was demonstrated by a flattening of the inhibition zone near the cefotaxime disk (Fig. 2). PCR and sequence data for MCV-4 and MCV-80G indicated that these strains carried bla<sub>ACT-1</sub> while strains MCV-63, MCV-29, and MCV-30W carried bla<sub>FOX-5</sub> (3, 20).

The effects of standard and high inocula were determined for the five AmpC-producers by MIC tests with cefotaxime, cefepime, imipenem, and ertapenem (Table 4). The MIC ratio for high inoculum to standard inoculum for MCV-4, MCV-80G, MCV-63, MCV-29, and MCV-30W showed the following fold increases: for cefotaxime, 16, 32, 8, and 8; for cefepime, 4, 128, 128, 128, and 64; for imipenem, 16, 32, 4, and 8; and for ertapenem, 2, 32, 2, and 4.

**DISCUSSION**

The phenotypic data generated in this study indicate that in the United States a significantly large number of *K. pneumoniae*...
moniae bloodstream isolates harbor ESBLs. The 9.5% occurrence rate found in this study was lower than the 19% rate reported earlier (5). However, isolates in the earlier study were collected from one facility and were recovered from consecutive routine cultures, whereas in the present study the isolates were recovered from patients in 30 hospitals in 23 states and represented nonrepeat isolates recovered from the bloodstream only. These data, together with reports of therapeutic failure of extended-spectrum cephalosporins for bloodstream infections due to ESBL-producing strains of *K. pneumoniae* and *E. coli*, support the current NCCLS recommendation that microbiology laboratories implement procedures to detect these organisms accurately (15, 17, 26).

The 2.6% occurrence of AmpC producers found in this study was slightly higher than the 1.1% occurrence reported in an earlier study of 371 consecutive, nonrepeat *K. pneumoniae* isolates that were recovered from routine cultures at the McGuire Veterans Affairs Medical Center (6). Plasmid-mediated *bla*ACT-1 and *bla*FOX-5 genes, which are thought to have originated from *Enterobacter* and *Aeromonas* spp., respectively, were recently found in *K. pneumoniae* strains from New York City (3, 20). Three of the five AmpC producers in this study were recovered from patients in a New York City hospital; two of these isolates harbored ACT-1 and one harbored FOX-5 (Table 3). The other two isolates harbored FOX-5 and were found in a Washington, D.C., hospital. All five AmpC producers demonstrated unique PFGE patterns, suggesting plasmid-mediated spread of β-lactamase genes. Only three inducible

![Flow chart of results for isolates that are positive by the cefoxitin screen test. CLA, clavulanic acid; CRO, ceftriaxone; FEP, cefepime.](http://jcm.asm.org/)

FIG. 1. Flow chart of results for isolates that are positive by the cefoxitin screen test. CLA, clavulanic acid; CRO, ceftriaxone; FEP, cefepime.
plasmid-mediated AmpC enzymes have been reported in strains of *K. pneumoniae*. These include ACT-1, DHA-2, and, most recently, DHA-1 (8, 21; E. S. Moland, T. J. Cleary, M. D. Reishig, Y. Jiang, P. E. Coudron, T. J. Lockhart, N. D. Hanson, and K. S. Thomson, Program Abstr. 41st Intersci. Conf. Anti-

microb. Agents Chemother., abstr. C1-1489, 2001). Because few laboratories test for the production of AmpC β-lactamases and fewer still test for induction, the true occurrence of these enzymes in strains of *K. pneumoniae* and *E. coli* remains unknown, as does their impact on therapeutic issues and clinical outcome.

Currently, no standard guidelines exist for detecting AmpC-producing organisms. With the disk diffusion screen test, we identified 28 isolates as possible AmpC producers (cefoxitin zone diameter less than 18 mm). However, 23 (82%) of these isolates were negative for AmpC by the three-dimensional test and IEF. The presence of decreased cefoxitin zone diameters is a poor indicator of AmpC producers, and the question of using this agent to treat serious infections due to these organisms remains unresolved (Table 3) (6, 19).

Unfortunately, few laboratories perform confirmatory tests for AmpC production, and some physicians may assume that carbapenems are the drugs of choice for treating all infections due to *Enterobacteriaceae* isolates that demonstrate nonsusceptibility to cefoxitin. Because a relatively large number (82%) of screen-positive isolates in our study did not harbor AmpC β-lactamases, we performed additional susceptibility testing for some of these isolates. Of 23 screen-positive, AmpC nonproducers, 9 produced ESBLs and are included in Table 2 (Fig. 1). Of the remaining 14 ESBL nonproducers, 13 (93%) demonstrated ceftriaxone and cefepime MICs of less than 1.0 and 0.5 µg/ml, respectively, using standard inoculum (data not shown). In addition, six of six isolates selected at random from the above isolates showed no inoculum effects with either of these cephalosporins (data not shown). These results suggest that carbapenems may be more effective than cefepime for treating serious infections that involve large numbers of AmpC-producing organisms.

We conclude that a significant number of bloodstream isolates of *K. pneumoniae* harbor plasmid-mediated ESBLs and AmpC β-lactamases. Based on in vitro susceptibility test results using standard and high inocula, we found that imipenem and ertapenem are active against AmpC producers whereas extended-spectrum cephalosporins are active against screen-positive, AmpC nonproducers. This suggests that it is important to distinguish between cefoxitin-resistant AmpC producers and cefoxitin-resistant AmpC nonproducers.

<table>
<thead>
<tr>
<th>Strain</th>
<th>CRO MIC (µg/ml)</th>
<th>FEP MIC (µg/ml)</th>
<th>IMP MIC (µg/ml)</th>
<th>ERT MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁶ CFU/spot</td>
<td>10⁷ CFU/spot</td>
<td>10⁶ CFU/spot</td>
<td>10⁷ CFU/spot</td>
</tr>
<tr>
<td>MCV-4</td>
<td>16</td>
<td>256</td>
<td>0.13</td>
<td>0.5</td>
</tr>
<tr>
<td>MCV-80G</td>
<td>8</td>
<td>256</td>
<td>0.13</td>
<td>16</td>
</tr>
<tr>
<td>MCV-63</td>
<td>8</td>
<td>64</td>
<td>0.5</td>
<td>64</td>
</tr>
<tr>
<td>MCV-29</td>
<td>8</td>
<td>64</td>
<td>0.5</td>
<td>64</td>
</tr>
<tr>
<td>MCV-30W</td>
<td>8</td>
<td>64</td>
<td>1</td>
<td>64</td>
</tr>
</tbody>
</table>

*All isolates were nonsusceptible based on the cefoxitin disk diffusion screening test.*

*Abbreviations: CRO, ceftriaxone; FEP, cefepime; IMP, imipenem; ERT, ertapenem.*

**FIG. 2.** Blunting of the zone of inhibition around the ceftazidime disk is due to the induction of AmpC by cefoxitin.
the clinical significance of these data, it is imperative that the clinical laboratory be able to identify these organisms.

ACKNOWLEDGMENTS

We are grateful to Merck and Co. for financial support and to NAEJA Pharmaceutical, Inc., for providing Syn2190 powder.

We thank Dick Wenzel, who provided the SCOPE isolates used in this study, and Sandy Tallent and Ellen Smith Moland for their help with the PFGE and enzyme induction tests.

REFERENCES