New Method for Laboratory Detection of AmpC β-Lactamases in *Escherichia coli* and *Klebsiella pneumoniae*

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A new cefoxitin-agar medium (CAM)-based assay was compared to the previously published modified three-dimensional (M3D) assay for the detection of AmpC production in *Escherichia coli* and *Klebsiella pneumoniae*. Clinical isolates of cefoxitin-resistant *E. coli* (*n* = 5) and *K. pneumoniae* (*n* = 7) and multiple control strains with and without AmpC enzymes were tested by both methods. The CAM method with 4 μg of cefoxitin/ml was equivalent to the M3D method for detecting AmpC production in *E. coli* and *K. pneumoniae*. This new method is easier to perform and interpret and allows for testing of multiple isolates on a single plate.

AmpC-mediated beta-lactam resistance (AmpC-R) in *Escherichia coli* and *Klebsiella pneumoniae* is an emerging problem (28). High-level AmpC production is typically associated with in vitro resistance to all beta-lactam antibiotics except for carbapenems and cefepime. In addition, treatment failures with broad-spectrum cephapolorins have been documented (26, 36). These enzymes are not affected by available beta-lactamase inhibitors and, in association with the loss of outer membrane porins (OMP), can produce resistance to carbapenems (7, 8, 31). Genes for these beta-lactamases are found on the chromosomes of some members of the family *Enterobacteriaceae* (2, 19, 20, 25). Plasmid-mediated AmpC-R has arisen through the transfer of chromosomal genes for the inducible AmpC beta-lactamases onto plasmids (5, 9, 30, 34). Plasmids with these genes can spread among other members of the family *Enterobacteriaceae* (22, 35, 38), have been documented in many countries (4, 12, 14, 21), and can cause nosocomial outbreaks (7, 22).

Detecting AmpC-R is a challenge for laboratories (32). There is no National Committee for Clinical Laboratory Standards (NCCLS) guideline for its detection. We wished to address this issue by studying the use a cefoxitin agar medium (CAM), in comparison with a modified three-dimensional AmpC assay (M3D) for the detection of AmpC-R in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*.

Isolates of *E. coli* containing MIR-1, FOX-1, and MOX-1; one *K. pneumoniae* strain known to be both AmpC and extended-spectrum-beta-lactamase (ESBL) positive; and *E. coli* ATCC 25922 were used as controls. Clinical isolates of *E. coli*, *K. pneumoniae*, and *Enterobacter cloacae* encountered at Calgary Laboratory Services and selected for study included 6 cefoxitin-sensitive ESBL-negative *E. coli* isolates, 8 AmpC-negative and ESBL-positive *E. coli* isolates, 55 cefoxitin-resistant and ESBL-negative *E. coli* isolates, 6 cefoxitin-resistant and ESBL-negative *K. pneumoniae* isolates, and 9 *E. cloacae* isolates showing in vitro susceptibility to extended-spectrum cephalosporins. Clinical isolates were identified using the Vitek automated microbial identification system (bioMérieux Inc., Durham, N.C.). Cefoxitin resistance was detected using the disk diffusion technique, and ESBL production was determined using double-disk diffusion methods, according to NCCLS guidelines (23, 24). Cefoxitin MICs were determined by the E-test method (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations.

The M3D assay was performed as described by Coudron et al. (13) and was used as the “gold standard” for detecting AmpC-R (Fig. 1). For the CAM assay, crude enzyme extracts were prepared by freezing and thawing cell pellets from centrifuged tryptic soy broth cultures as described previously (13). Mueller-Hinton agar with cefoxitin concentrations of 2, 4, 8, and 16 μg/ml was used. Plates were inoculated with *E. coli* ATCC 25922 to cover the entire surface. Circular wells with diameters of 5 mm were made in the agar and filled with 30 μl of extract from individual strains. Positive-control (*E. coli* with MOX-1) and negative-control (*E. coli* ATCC 11775) strains were included on each plate. Plates were incubated overnight aerobically at 35°C. A zone of growth around the periphery of a well was considered a positive CAM assay and evidence for the presence of an AmpC enzyme (Fig. 2).

The M3D assay was negative with all AmpC-negative controls and positive with all known AmpC-positive controls and the nine *E. cloacae* clinical isolates (AmpC noninduced). Fifty-four of 55 *E. coli* strains and 1 of 6 *Klebsiella pneumoniae* strains were positive by the M3D method. The results of CAM with 4 μg of cefoxitin per ml were 100% concordant with those of the M3D method (Table 1). At higher and lower cefoxitin concentrations, the CAM method did not correlate as well with the M3D method. Cefoxitin MICs ranged between 16 and ≥256 μg/ml. No correlation was found between MICs and zone sizes by the CAM method (data not shown).

*K. pneumoniae*, *E. coli*, *Salmonella* spp., and *Proteus mirabilis* lack inducible AmpC enzymes (28). *E. coli* does carry an *ampC* gene but lacks the regulatory gene (*ampR*), leading to negligible enzyme production (18). In clinical isolates of *E. coli*, cephamycin resistance can be due to promoter or attenuator
gene mutations (11, 15), the acquisition of plasmids with \textit{ampC} genes (3, 12, 14, 16, 27, 29, 34, 35, 37, 39), and OMP changes (10). In our clinical strains of \textit{E. coli}, only 1 of 55 showed no evidence of AmpC-R. In \textit{Klebsiella} spp., interruption of a porin gene by insertion sequences has been described as a common type of mutation that causes increased cefoxitin resistance (17). In our study, only one of six cefoxitin-resistant \textit{K. pneumoniae} strains was positive for AmpC enzymes by both the M3D and CAM methods, implying that cefamycin resistance in these isolates is due to OMP changes.

The CAM method is potentially useful for differentiating AmpC-R from other resistance mechanisms. In \textit{Klebsiella} and \textit{Salmonella} spp., both of which lack a chromosomal \textit{ampC} gene, a positive test suggests plasmid-mediated AmpC resis-

FIG. 1. M3D assay. AmpC-positive extracts distort the zone around the cefoxitin disk.

![M3D assay](image1)

FIG. 2. CAM assay. AmpC-positive extracts produce a zone of growth around wells.

![CAM assay](image2)
tance. In *E. coli*, once enzyme-mediated AmpC resistance has been detected, its plasmidic basis can be confirmed by transferring the plasmid to recipient bacterial strains via transconjugation or by detection of specific genes known to be transferred on plasmids (27).

Currently, detection of AmpC enzymes is a problem for clinical laboratories. Although lack of inhibition of activity on plasmids (27).

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>No. of strains</th>
<th>No. of strains positive by μ3D assay</th>
<th>No. of strains positive by CAM assay with the following cefoxitin concentration (μg/ml):</th>
<th>Cefoxitin MIC (μg/ml):</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmpC-negative, ESBL-negative controls</td>
<td>6</td>
<td>0</td>
<td>6 0 0 0 0 0</td>
<td>&lt;8</td>
</tr>
<tr>
<td>AmpC-negative, ESBL-positive controls</td>
<td>8</td>
<td>0</td>
<td>6 0 0 0 0 0</td>
<td>≥16</td>
</tr>
<tr>
<td>AmpC-positive controls</td>
<td>6</td>
<td>0</td>
<td>6 0 0 6 6 4</td>
<td>32–256</td>
</tr>
<tr>
<td>Cefoxitin-resistant <em>E. coli</em> clinical isolates</td>
<td>55</td>
<td>54</td>
<td>55 54 50 27</td>
<td>32–256</td>
</tr>
<tr>
<td>Cefoxitin-resistant <em>K. pneumoniae</em> clinical isolates</td>
<td>6</td>
<td>1</td>
<td>1 1 1 1 1 1</td>
<td>16–256</td>
</tr>
<tr>
<td><em>E. cloacae</em> clinical isolates*</td>
<td>9</td>
<td>NDb</td>
<td>9 9 4 4 0 0</td>
<td>128–256</td>
</tr>
</tbody>
</table>

* a Susceptible in vitro to extended-spectrum cephalosporins.

b ND, not determined.

REFERENCES


