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

K. Nasim,1 S. Elsayed,1,2 J. D. D. Pitout,1,2 J. Conly,1,3 D. L. Church,1,2,3 and D. B. Gregson1,2,3*

Department of Pathology and Laboratory Medicine1 and Department of Medicine,3 University of Calgary, and Calgary Laboratory Services,2 Calgary, Alberta, Canada

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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* spp. 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 cephalosporins 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 on 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 cephamycin 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-

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig1.png}
\caption{M3D assay. AmpC-positive extracts distort the zone around the cefoxitin disk.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig2.png}
\caption{CAM assay. AmpC-positive extracts produce a zone of growth around wells.}
\end{figure}
TABLE 1. Summary results of AmpC detection assays

<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 ceftoxitin conc (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>0</td>
<td>6</td>
</tr>
<tr>
<td>AmpC-negative, ESBL-positive controls</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>AmpC-positive controls</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Cefoxitin-resistant <em>E. coli</em> clinical isolates</td>
<td>55</td>
<td>54</td>
<td>54</td>
<td>6</td>
</tr>
<tr>
<td>Cefoxitin-resistant <em>K. pneumonia</em> clinical isolates</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>E. cloacae</em> clinical isolates&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9</td>
<td>ND</td>
<td>ND</td>
<td>9</td>
</tr>
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<td></td>
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</table>

<sup>a</sup> Susceptible in vitro to extended-spectrum cephalosporins.

<sup>b</sup> ND, not determined.

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 against oxyimino-β-lactams or cephamycins by beta-lactam inhibitors is indirect evidence for their presence, some AmpC enzymes have been shown to be susceptible to inhibition by tazobactam (1, 6). Inhibitors that are active against AmpC enzymes are not readily available. The CAM method with ceftoxitin (4 μg/ml) is simpler to perform and easier to interpret than the M3D method. It allows for testing of up to five isolates and two controls on a single plate and is as sensitive as the M3D method. In addition, clinical isolates with multiple beta-lactamases have been described, resulting in difficulties in interpretation of MIC patterns (13, 33). The CAM method may prove useful in detecting the presence of AmpC-R in isolates that have multiple mechanisms of cephalosporin resistance. The control strain known to be both ESBL and AmpC positive was found to be positive for AmpC by this assay.

The CAM method with 4 μg of ceftoxitin per ml is as sensitive and specific as the M3D method for AmpC detection in *E. coli* and *K. pneumoniae*. The new method is easier to perform and interpret and allows for the testing of multiple isolates on a single plate. Clinical and research laboratories can use this technique to confirm the presence of AmpC-mediated resistance.

REFERENCES


