Rapid Ertapenem Susceptibility Testing and \textit{Klebsiella pneumoniae} Carbapenemase (KPC) Phenotype Detection in \textit{Klebsiella pneumoniae} Using Automated Microscopy of Immobilized Live Bacterial Cells

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Running Head: Automated microscopy for rapid ertapenem resistance detection

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We evaluated detection of ertapenem (ETP) resistance and *Klebsiella pneumoniae* carbapenemase (KPC) in 47 *Klebsiella pneumoniae* isolates using a novel, automated microscopy system. Automated microscopy correctly classified 22/23 isolates as ETP resistant and 24/24 as ETP susceptible, and correctly classified 21/21 isolates as KPC-positive and 26/26 as KPC-negative.
Carbapenem-resistant *Enterobacteriaceae* (CRE) are emerging as a global threat; the plasmid-borne carbapenemase gene *bla*KPC (KPC) is the predominant mechanism conferring carbapenem resistance in North America (1-5). This resistance gene has been reported in most species of *Enterobacteriaceae*, but it is most commonly found in *K. pneumoniae*. Timely detection of carbapenem resistance is critical for prompt optimization of antimicrobial therapy, but the sensitivity of antimicrobial susceptibility testing methods for CRE detection are variable and turnaround time can be slow (6-10). It has been demonstrated that *in vitro* detection of KPC expression can be difficult, varying by bacterial species and level of enzyme expression.

The objective of our study was to evaluate automated microscopy for detection of ertapenem (ETP) resistance in *K. pneumoniae* and the ability to attribute the mechanism of this resistance to the KPC enzyme. Forty-six *K. pneumoniae* isolates recovered from clinical specimens obtained at Barnes-Jewish Hospital (St. Louis, MO) were tested, in addition to one KPC-negative, ESBL-positive, *K. pneumoniae* quality control strain, ATCC® 700603 (Table 1). The ertapenem and meropenem (MEM) susceptibility profile of the isolates was determined by disk diffusion according to CLSI standards (11) and isolates were characterized for *bla*KPC using a laboratory-developed real-time PCR assay (6).

For automated microscopy, bacterial suspensions were centrifuged (12,000 × *g* for 4 min), washed in 1 mM L-histidine buffer at a pH of 7.2, and resuspended in a low ionic strength electrokinetic buffer containing 10 mM L-DOPA and 1 mM L-histidine at a pH of 7.0 (reagents from Sigma-Aldrich). This created an inoculum of approximately 1 × 10^6 CFU/mL for testing and then automated microscopy was performed. Bacterial inocula were pipetted into independent flowcells of a multichannel disposable fluidic cassette (Accelerate Diagnostics Inc., Tucson, AZ). Bacteria were immobilized on the transparent lower surface of each flowcell using electrokinetic concentration (Fig. 1). Mixtures of antibiotics in Mueller-Hinton broth (Becton Dickinson)
containing 0.85% noble agar (Affymetrix, Santa Clara, CA) were introduced into each flowcell channel. Dark-field images of each flowcell channel were taken at 10 min intervals during a fixed 3 h antibiotic exposure period. An offline image analyzer tracked each immobilized bacterial progenitor cell as it replicated into a clone of daughter cells throughout a series of time-lapse images for each flowcell. The analyzer computed a growth probability score for each growing clone derived from coefficients of a cubic polynomial \( f(x) = ax^3 + bx^2 + cx + d \) fitted to the computed log of relative clone mass (integrated pixel intensity) vs. time. The growth probability score transformed each clone's growth data into a numerical score ranging between 0 and 1 that represented the probability of the clone continuing to grow (> 0.8), to arrest (0.2-0.8), or to lyse (< 0.2). The slope of a straight line fit by linear regression was used to calculate the division rates for individual clones. The median clonal division rate (div/h) weighted for number of growing clones and growth probability was used to calculate a “resistance score” for each test condition.

Ten µg/mL of ETP (Merck, Whitehouse Station, NJ) was used for susceptibility testing and KPC detection used four flowcell channels per sample. The first KPC channel contained 16 µg/mL of MEM (Sigma-Aldrich), the second contained 32 µg/mL of MEM, the third contained 16 µg/mL of MEM plus 32 µg/mL 3-nitrophenylboronic acid (NPBA, Sigma-Aldrich) (19), and the fourth contained 32 µg/mL of MEM plus 32 µg/mL NPBA. NPBA was included as it is an inhibitor of the KPC enzyme (12). Interpretation algorithms in the image analyzer computed the difference between resistance scores in MEM alone and MEM+NPBA to obtain an “inhibition score,” weighted by the number of growing clones in each condition. The operator interpreting the ETP susceptibility and KPC status of the isolate was blinded to the phenotypic data and the bla\textsubscript{KPC} PCR result.
The automated microscopy system classified 22/23 *Klebsiella pneumoniae* isolates as ETP-resistant and 24/24 as ETP-susceptible using a resistance score cutoff of 1.4 (Fig. 2) and therefore achieved 96% sensitivity (CI 76-100%) and 100% specificity (CI 83-100%) for detection of ETP resistance. There was one instance of susceptibility reported by automated microscopy for an isolate that tested as resistant by disk diffusion (KP-505: 13 mm ETP zone diameter, microscopy score 0.93). Growth controls had an average ± standard deviation of 55 ± 27 growing clones per field of view, which was therefore the same number of viable clones in all channels. The average resistance score ± standard deviation for ETP-susceptible isolates was 0.46 ± 0.41 (range: 0.00 to 1.25) and for ETP-resistant isolates was 1.89 ± 0.24 (range: 0.93 to 2.17). Images of ETP-resistant isolate wu33 and ETP-susceptible isolate wu9 at 0, 90 and 180 min after exposure to 10 µg/mL ETP are shown in Fig. 3. Upon exposure to 10 µg/mL ETP, ETP-resistant strains showed robust growth with minimal lysis, with most clones growing in a spheroidal clone morphology. In contrast, ETP-susceptible strains exhibited rapid growth arrest and lysis (Fig. 3).

The microscopy system classified 21/21 isolates as KPC-positive and 26/26 strains as KPC-negative as compared to PCR results. Upon exposure to MEM alone, spheroidal clone morphology was observed for KPC-positive clones with minimal lysis (Fig. 4). Rapid growth arrest and lysis was observed for KPC-positive isolates in the presence of MEM+NPBA, as well as for all KPC-negative isolates upon exposure to MEM and MEM+NPBA. The microscopy method achieved 100% sensitivity (CI 81-100%) and 100% specificity (CI 83-100%) for KPC characterization of the isolates. The mean inhibition score ± standard deviation for KPC-positive isolates was 0.54 ± 0.19 (range: 0.19 to 0.91) and for KPC-negative isolates was -0.75 ± 0.38 (range: -1.00 to -0.08). Images of KPC-positive strain wu15 at 0, 90 and 180 min are shown in
Fig. 4. All 21 KPC-positive strains were classified as ETP-resistant by microscopy analysis and by disk diffusion.

Of note, two strains evaluated in this study (KP-456 and KP-505) were negative for KPC by PCR, but resistant to both ETP and MEM using disk diffusion. The microscopy analysis correctly classified both strains as KPC-negative. Microscopy analysis correctly classified KP-456 as ETP-resistant, but incorrectly classified KP-505 as ETP-susceptible (Fig. 2). Additional susceptibility testing of isolate KP-505 demonstrated that the isolate was susceptible to piperacillin-tazobactam, ticarcillin-clavulanic acid, cefotetan, amikacin, and tobramycin, and resistant to gentamicin; this profile is different than the KPC-producing strains in this study. The KPC-producing strains were typically gentamicin-susceptible and resistant to piperacillin-tazobactam, ticarcillin-clavulanic acid, amikacin, and tobramycin. Together, these data suggest that in isolate KP-505, carbapenem resistance could be attributed to an ESBL-producing strain with a porin or efflux mutation(s). Visual examination of the time-lapse images for isolate KPC-505 revealed that the microscopy error was due to technical limitations, including loss of focus in two of the six fields of view and very low clone counts in the remaining four fields of view, which weighted the scoring algorithm towards a score in the susceptible range. Improvements in instrument focus, setting strict requirements for the number of clones analyzed prior to reporting, and algorithm optimization could help to reduce or eliminate these sources of error in future analyses. For isolate KP-456, additional susceptibility testing results revealed cefepime susceptibility and cefotetan resistance, suggesting that in this isolate, an acquired AmpC with porin and/or efflux mutations could be plausible explanations for the observed carbapenem resistance in the absence of KPC.

The KPC type of all strains was determined (Table 1) as previously described (13). In addition, Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR was used to assess the
genetic relatedness of the KPC-producing strains. PCR products were resolved using DiversiLab DNA chips (bioMérieux, Durham, NC) on the Agilent 2100 system (Agilent Technologies, Santa Clara, CA). DiversiLab software was used to compare banding patterns and determine the similarity of the isolates (similarity index, SI) whereby identical isolates have an SI of 85% or greater (14-17). Molecular typing of the specific KPC variants contained in the isolates identified 13 blaKPC-2- and 8 blaKPC-3-containing strains (Table 1). With ERIC-PCR, the isolates clustered into 4 major strain types: the first cluster with 5 isolates, the second with 3 isolates, the third with 12 isolates, and the fourth with 1 isolate.

State-of-the-art automated antimicrobial susceptibility testing systems vary in their ability to detect carbapenemase production (8, 18). Reliable detection of carbapenem resistance is important to guide selection of appropriate therapy. Automated microscopy, as evaluated in this study, detected ETP resistance and showed promising phenotypic results that were consistent with KPC expression, using data acquired in 3 h. Using ETP as an indicator, the automated microscopy method evaluated in this study was 96% sensitive and 100% specific for detection of carbapenem resistance. This rapid result could be important for guiding antimicrobial therapy in critically ill patients and quickly initiating appropriate infection control measures.
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REFERENCES


### TABLE 1 Characterization of bacterial strains.

<table>
<thead>
<tr>
<th>Carbapenem resistance</th>
<th>No. of isolates</th>
<th>Automated Microscopy, No. of isolates</th>
<th>ETP resistance</th>
<th>Zone Diameter Mean (Range), mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KPC detection</td>
<td>ETP (S ≥ 22)</td>
<td>MEM (S ≥ 23)</td>
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<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
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<tr>
<td>KPC-positive</td>
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<td>0</td>
<td>21</td>
</tr>
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<td>13</td>
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</tr>
<tr>
<td>KPC-3</td>
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<td>8</td>
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<td>8</td>
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<tr>
<td>Ertapenem resistant</td>
<td>2</td>
<td>0</td>
<td>26</td>
<td>1</td>
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<tr>
<td>Ertapenem susceptible</td>
<td>24</td>
<td>0</td>
<td>24</td>
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</tr>
</tbody>
</table>

*S = CLSI susceptible breakpoint
FIG 1 Bacterial cell immobilization by electrokinetic concentration. Sample suspension flows in, then stops (A). Electric field forces bacterial cells to the positively-charged poly-L-lysine (PLL) capture coating (B). PLL binds cells upon contact, and immobilizes them after the electric field turns off. ITO=indium tin oxide.
FIG 2 Scatter plot of the automated microscopy resistance score vs. ertapenem disk diffusion reference. Vertical dashed lines indicate CLSI 2013 breakpoints for susceptible and resistant, horizontal dashed line indicates microscopy score interpretation criterion value of 1.4. KPC-positive and -negative strains are indicated by white and black circles, respectively. Strains KP-456 and KP-505 were KPC-negative by PCR but resistant by disk diffusion for ertapenem and meropenem.
FIG 3  Ertapenem-resistant (ETP-R) (wu33, ETP disk zone=8 mm) and susceptible (ETP-S) (wu9, ETP disk zone=35 mm) *K. pneumoniae* strains. Rows show clone images at 90 min intervals after exposure to growth medium alone (Growth Control), or 10 µg/mL ertapenem (ETP-R and ETP-S). Images are zoomed in and contrast enhanced. The scale bar in the lower right is 20 µm.
FIG 4 KPC-positive *K. pneumoniae* strain wu15 (disk diffusion zone sizes: ETP/MEM 8 mm/12 mm). Rows show clone images at 90 min intervals after exposure to growth medium alone (top row), 16 µg/mL meropenem (MEM) (middle row) and 16 µg/mL meropenem + 32 µg/mL of the inhibitor 3-nitrophenylboronic acid (MEM+NPBA) (bottom row). Images are zoomed in and contrast enhanced. The scale bar in the lower right is 20 µm.