Colistin stability and MIC testing in agar dilution in comparison to E-test, micro- and macrobroth

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Introduction
The emergence and spread of multidrug resistant Gram-negative bacteria has led to the reintroduction of old antibiotics, such as colistin, into clinical practice. However, its unique physical properties challenge antibiotic susceptibility testing (AST) with no standard method defined to date¹,². In addition, heteroresistant cell populations further complicate colistin AST. Here, we compared microbroth, macrobroth, E-test and agar dilution methods for colistin MIC testing.

Material and Methods
Colistin susceptibility testing was performed using colistin sulphate salt (Lot#SLBD8306V, Sigma) and a set of six strains (four clinical isolates and two controls) (Table). Agar dilutions were performed twice, whereas other methods were repeated at three different time-points. MIC cut-offs were set according to the EUCAST, since there are no colistin breakpoints for Enterobacteriaceae in CLSI guidelines¹,². Additionally, the shelf life of colistin agar plates was tested over one week, and MIC reproducibility and distribution of colistin in agar was evaluated by testing in triplicate at each time point. The obtained MICs were read independently by two researchers. To investigate solubility and distribution of colistin in agar plates, strains were spotted on different regions of the plate (Figure A). To control the material influence, we tested microbroth in both 96-well polystyrene plate and in 16-well glass bottom plates. For heteroresistance detection, growth of CS-1 was monitored for 24h at 37°C using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific) with readings every 15 minutes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Isolation site</th>
<th>Agar (µg/ml)</th>
<th>Macrobroth (µg/ml)</th>
<th>Microbroth polyethylene plate (µg/ml)</th>
<th>Microbroth glass-bottom plate (µg/ml)</th>
<th>E-test (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-1</td>
<td>Klebsiella pneumoniae</td>
<td>Bronchial-aspirate</td>
<td>0.25-0.5</td>
<td>0.25-32</td>
<td>0.5-4</td>
<td>0.5-16</td>
<td>0.125-0.25</td>
</tr>
<tr>
<td>CS-2</td>
<td>Klebsiella pneumoniae</td>
<td>Blood</td>
<td>0.25-0.5</td>
<td>0.5-32</td>
<td>0.5-64</td>
<td>0.25-4</td>
<td>0.125-0.25</td>
</tr>
<tr>
<td>CR-1</td>
<td>Klebsiella pneumoniae</td>
<td>Infection site</td>
<td>128-256</td>
<td>32-64</td>
<td>64</td>
<td>64</td>
<td>4-8</td>
</tr>
<tr>
<td>CR-2</td>
<td>Klebsiella pneumoniae</td>
<td>Infection site</td>
<td>128</td>
<td>32-64</td>
<td>64</td>
<td>32</td>
<td>4-8</td>
</tr>
<tr>
<td>ATCC25922</td>
<td>Escherichia coli</td>
<td>Not applicable</td>
<td>0.25</td>
<td>0.25-2</td>
<td>0.5-4</td>
<td>0.25</td>
<td>0.125</td>
</tr>
<tr>
<td>ATCC27883</td>
<td>Pseudomonas aeruginosa</td>
<td>Not applicable</td>
<td>2</td>
<td>0.5-1</td>
<td>1-2</td>
<td>0.5</td>
<td>0.5-1</td>
</tr>
</tbody>
</table>

Table. Overview of the strains used in the study and the obtained MIC values.

Results and Discussion
MIC readings were highly comparable between the investigators with Cohen’s kappa coefficient of 0.948. The summary of the obtained MICs can be found in the table. The overview of the mean values of logMICs of all tested methods is presented in figure B. For agar dilutions we obtained very reproducible results irrespective of the spot position and the batch. We also found that colistin is stable in agar plates for over one week (Figure C). On investigating heteroresistance, the growth of CS-1 was detected even in medium supplemented with 8 µg/ml of colistin (Figure D).

Figure A: Strain distribution scheme for agar dilution, 0 – blank, 1 – ATCC25922, 2 – ATCC27883; B: Overview of the mean values of logMICs obtained with all tested methods; C: Influence of agar plate storage on logMIC values; D: Growth assays utilizing CS-1 without colistin (MHB) and with colistin (COL4 – 4 µg/ml, COL8 – 8 µg/ml).

Both broth dilution methods showed variability that might be explained by the presence of heteroresistant subpopulations, which we confirmed for the CS-1 strain. MIC values for CR strains obtained with E-test were significantly lower than with the other methods (e.g. 16-folds compared to the agar dilution). Such high discrepancies were not observed for colistin sensitive strains. In conclusion, we found that agar dilution performed very well in our hands and provided the most reliable results of all methods tested.

References
(2) CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 26th ed. CLSI supplement M100S. Wayne, PA: Clinical and Laboratory Standards Institute, 2016

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