Fast Antibiotic Susceptibility Testing Utilizing a Unique Spectral Intensity Ratio Analysis via Single Fluorescence Membrane Dye Staining and Flow Cytometry

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Abstract

Selection of antibiotic therapy is crucial for patient care. In the era of increasing antibiotic resistance and shortage of new antimicrobials, rapid Minimum Inhibitory Concentration (MIC) test results will enable the clinician to treat the patient appropriately. Moreover, rapid MIC is crucial for an effective antimicrobial stewardship program. The main limitation of current Antimicrobial Susceptibility Testing (AST) practices is turnaround time, between 5 hours for certain bacteria with a semiautomatic system to 18 hours or more in the routine techniques.

In this study we present a rapid method for AST and MIC determination with a turnaround time of 15 min after 2-4 hours of antibiotics treatment. Our new method is based on single dye staining, which enables rapid detection of the bacteria viability without the time-consuming process of growth curve. The analysis procedure is performed on flow cytometry measurement utilizing the single fluorescent membrane dye staining and unique spectral intensity ratio analysis.

Introduction

The main principal in current standard AST methods is based on bacterial growth observation in different antibiotics concentrations [1]. This allows determining the MIC in which bacteria will not grow. For example, there are the antibiotic disk methods where bacterial growth is detected on an agar plate and the result is determined by measuring the diameter of the inhibition zone or the E-test that enables direct MIC determination by using a calibrated antibiotic strip [1]. These methods, although simple and flexible are prolonged and are not applicable to all antimicrobial agents. More advanced methods are the macro or micro dilution (in tubes or 96 well plates, respectively) however both are time-consuming due to the total time required for the bacterial growth prior to the analysis. Typical times for growth and analysis are 16 to 24 hours for most bacteria and longer for more slow growing ones [1] [2].

Automated AST systems such as BD’s Phoenix and bioMérieux’s VITEK®2 use optical means such as turbidity measurements or fluorescence of bacterial metabolism to determine the susceptibility and MIC. However, the results are obtained within 7 to 16 hours and in some systems require an overnight incubation. Recently, Accelerate Diagnostics Inc. cleared an AST system that obtains the results within 7 hours using bacterial growth morphological changes of the colony forming units.

In this evaluation we present a new concept for the AST determination by following bacterial viability using fluorescence membrane marker (FM1-43) and our new patented spectral intensity ratio analysis method using flow cytometry. This method enables an immediate observation of bacterial viability upon antibiotic exposure, hence a rapid AST that relies on the single bacterial effect and not the bacterial growth. We incorporate the flexibility and accuracy of the macro/micro dilution approach with the sensitivity of optical detection.

FM 1-43 (Synaptogreen) is a styryl dye which changes its fluorescence intensity and spectrum once it is attached to active/inactive bacteria [3]. Figure 1 shows the fluorescence spectrum of active and inactive E. coli stained with FM 1-43 dye. The fluorescence intensity of inactive bacteria is higher than the one of the active bacteria. More importantly the fluorescence spectrum of the active bacteria is shifted toward shorter wavelengths compared to the one of the inactive bacteria. Therefore, by measuring the fluorescence intensities in two selected wavelengths, and their ratio, we can derive the Spectral Intensity Ratio which reflects the spectral shift of the fluorophore. This allows defining inactive and active bacteria populations [3].
Flow cytometry (FCM) is testing a single cell at a time; therefore it enables us to detect the antibiotic effect on a single bacteria level and not as a whole group effect, such as growth curve. By measuring a large number of the bacterial population, in a single test, on the two emission wavelength channels in the FCM, we can determine whether the antimicrobial treatment is successful in its initial stage of changes in the bacteria statehood. Figure 2 is a fluorescence dot-plot that shows the flow cytometric fluorescence measurement of active and inactive bacteria. As can be seen from the figure, using two wavelengths channels (530 nm and 610 nm) enables distinguishing between active (green dots) and inactive bacteria (red dots). Spectral intensity ratio calculation is done by dividing the mean fluorescence value of the 610 nm channel by the mean fluorescence value of the 530 nm channel. Typical values of spectral intensity ratio for active bacteria are between 0.7 and 2, while the values for inactive bacteria are higher (>2.5).
Method

Our method includes bacterial antimicrobial treatment for 2 to 4 hours, bacteria staining with a single fluorescence dye followed by a flow cytometric measurement and mathematical analysis. The following paragraphs describe the method in detail.

Sample preparation - Macro dilution method

Thirty clinical isolates of *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Proteus mirabilis* and *Citrobacter freundii*, purchased from JMI Labs (IA, USA), were tested for antimicrobial susceptibility with gentamicin, ampicillin and ciprofloxacin (Sigma-Aldrich, USA). A total of 90 isolates-antibiotics combinations were tested.

a) All strains were cultured onto 5% sheep blood agar (Hylabs, Israel), following overnight incubation, colonies were harvested and suspended in Phosphate Buffer Saline (PBS, Sigma-Aldrich). Each bacterial solution was then adjusted to 0.5 McFarland standard (~1.5x10⁸ CFU/ml).

b) The adjusted bacterial suspensions were diluted 1:100 with Cation-Adjusted Mueller Hinton Broth (CAMHB, Hylabs Israel) to a concentration of ~1x10⁶ CFU/ml.

c) Antimicrobial stock solutions were prepared in CAMHB according to CLSI recommendations starting at concentration which was 2 fold higher from the highest concentration of each bacterium/antimicrobial combination [2].

d) For each bacterium/antimicrobial combination a set of tubes was prepared. Excluding the first tube, 1 ml of CAMHB medium was added.

e) To each first tube of the set 2 ml from the antimicrobial stock solution (c) was dispensed.

f) From the first tube two-fold serial dilutions were prepared by transferring 1 ml of the solution to the next tube until the lowest required concentration by CLSI for each bacterium/antimicrobial combination was reached. One ml was discarded from the last tube in each set of tubes.

g) For each serial dilution set, two controls were added. As a negative control, a tube with 2 ml of CAMHB was used. As a positive control, a tube with bacterial inoculum without an antimicrobial agent was used.

h) To each tube in the set, 1 ml of bacterial solution (b) was added which resulted in a final inoculum concentration of about 5x10⁵ CFU/mL.

i) All tubes were incubated at 37°C.

j) 2-4 hours from incubation initiation, 200 µl from each tube were transferred into a fresh tube, dyed with 2 µl of Synaptogreen or FM 1-43 (Sigma-Aldrich; Molecular Probes, respectively) and measured by flow cytometer. The rest of the sample was further incubated for 16-20 hours and then was visually evaluated for growth.

Data interpretation

In order to quantify the influence of a certain antimicrobial treatment we define the spectral intensity ratio as follows:

\[
\text{Spectral Intensity Ratio} = \frac{I_{\lambda=610}}{I_{\lambda=530}}
\]

Where (I) is the mean value of the scatter plot at each wavelength. Low spectral intensity ratio values correspond to active bacterium population, while high values show a larger inactive bacterial population. The main advantage of using a single dye and the above spectral intensity ratio is the elimination of the result dependency on the dye concentration and optical efficiency.
The MIC is calculated by plotting the spectral intensity ratio as a function of the antimicrobial concentration and approximating it to a step function in the form of

\[ y(x) = a \cdot \text{erf}\left(\frac{b\pi(x - c)}{2}\right) \]

Where \( a \), \( b \), and \( c \) are parameters and \( \text{erf} \) is the error function. The MIC is the value of the parameter \( c \).

**Results**

Ninety samples (combination of 30 strains and 3 antibiotics) were analyzed in triplicates. Each sample was measured by the flow cytometer and the MIC was calculated using the spectral intensity ratio calculation and step function estimation. Figure 3 shows fluorescence scatter plots (typical flow cytometric measurements) of *E. coli* treated with gentamicin. As can be seen from the figure, as long as the antibiotics concentration is below the MIC, the majority of the bacterium population is active (green dots in the scatter plot and Figure 4). Once the antibiotic concentration reaches the MIC, the inactive bacterium population increases significantly (red dots in the scatter plot and Figure 4). The increase in the inactive bacterial population increase the spectral intensity ratio as can be seen in Figure 5. The spectral intensity ratio as a function of antibiotic concentration is approximated to a step function (solid line in Figure 5) and the MIC is determined to be 2mg/ml.

![Figure 3: Two wavelength fluorescence scatter plot, Green – active *E. coli* population, Red – inactive *E. coli* population.](image)

*Figure 3: Two wavelength fluorescence scatter plot, Green – active *E. coli* population, Red – inactive *E. coli* population.*

![Figure 4: Active and inactive *E. coli* populations as a function of antibiotic concentration, Green – active, Red – inactive.](image)

*Figure 4: Active and inactive *E. coli* populations as a function of antibiotic concentration, Green – active, Red – inactive.*
Figures 6 and 7 are an example of a resistant strain, C. freundii, which is resistant to gentamicin. As can be seen from Figure 6 the flow cytometry fluorescence scatter plot remains practically the same even at high antibiotic concentrations. This is immediately reflected in the spectral intensity ratio (Figure 7), where the value remains almost constant.
The following tables summarize the performance of the method. The spectral intensity ratio method has a 97.8% (88/90) essential agreement (the MIC result is within ±1 antibiotic dilution from the reference method), and 92.2% categorical agreement [4]. The only errors were minor errors [4].

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>No. of Strains</th>
<th>Reference Method Susceptibilities</th>
<th>Essential Agreement</th>
<th>Categorical Agreement</th>
<th>Minor Error</th>
<th>Major Error</th>
<th>Very Major Error</th>
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<tr>
<td></td>
<td></td>
<td>S  I  R</td>
<td>No.  %</td>
<td>No.  %</td>
<td>No.  %</td>
<td>No.  %</td>
<td>No.  %</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>30</td>
<td>16 5 9</td>
<td>30 100.00%</td>
<td>27 90.00%</td>
<td>3 10.00%</td>
<td>0 0.00%</td>
<td>0 0.00%</td>
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<tr>
<td>Gentamicin</td>
<td>30</td>
<td>21 4 5</td>
<td>30 100.00%</td>
<td>27 90.00%</td>
<td>3 10.00%</td>
<td>0 0.00%</td>
<td>0 0.00%</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>30</td>
<td>19 2 9</td>
<td>28 93.30%</td>
<td>29 96.70%</td>
<td>1 3.30%</td>
<td>0 0.00%</td>
<td>0 0.00%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>97.80%</td>
<td>92.20%</td>
<td>7.80%</td>
<td>0.00%</td>
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Table 1: Results analysis

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<th>Antimicrobial Agent</th>
<th>Range [μg/ml]</th>
<th>Essential Agreement [%]</th>
<th>&lt;=-5</th>
<th>-4</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>&gt;=5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0.125-32</td>
<td>100.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>16.70%</td>
<td>76.70%</td>
<td>6.70%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>100.00%</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.125-32</td>
<td>100.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>23.30%</td>
<td>66.70%</td>
<td>10.00%</td>
<td>0.00%</td>
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<tr>
<td>Ciprofloxacin</td>
<td>0.0078-32</td>
<td>93.30%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>3.30%</td>
<td>3.30%</td>
<td>46.70%</td>
<td>43.30%</td>
<td>3.30%</td>
<td>0.00%</td>
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</table>

Table 2: MIC Agreement

**Conclusion**

This study shows that antimicrobial susceptibility testing can be conducted using a single dye stain and a flow cytometry measurement. By defining the spectral intensity ratio parameter and plotting it as a function of the antibiotic concentration, it is possible to determine the MICs and whether it is susceptible, intermediate or resistant to a certain antibiotic. Spectral intensity ratio method is done within 15 min following 2-4 hours of antibiotic treatment.

**Bibliography**


[2] “Performance standards for antimicrobial susceptibility testing”, M100-S17, v. 27 no. 1, CLSI

