Spiral gradient endpoint susceptibility testing: a fresh look at a neglected technique

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Received 23 March 2010; returned 17 May 2010; revised 3 June 2010; accepted 4 June 2010

Objectives: Increasing antibiotic resistance and interest in matching antibiotic therapy with pharmacokinetic/pharmacodynamic characteristics of isolates has led to increasing demands for determination of MICs. This can lead to increased costs for the laboratory. The spiral gradient endpoint (SGE) technique, a low-cost method of MIC determination, was developed some years ago. Although the technique showed good correlation with reference methods, it was not widely employed, mainly due to the introduction of alternative methods. We have revisited this technique and evaluated it for the determination of MICs for fastidious organisms.

Methods: The SGE method was first optimized for fastidious organisms using Haemophilus influenzae. Intra-batch and inter-batch reproducibility was determined for H. influenzae, Streptococcus pneumoniae, Moraxella catarrhalis and Neisseria gonorrhoeae. The method was then evaluated by comparison of MICs for clinical isolates of these organisms determined by SGE with those determined with the reference method.

Results: Optimization of the technique resulted in a method with excellent reproducibility for all organisms tested [SD 0.10–0.337; coefficient of variation (CV) 8.59%–18.66%]. These SDs/CVs were lower than those of the reference methods (0.27–2.34; 31.0%–63.8%). There was excellent correlation of the MICs with the reference methods (0.908–0.930) and insignificant differences in numbers of strains in each resistance category, with no tendency for SGE to produce higher or lower MICs than the reference method (P>0.05).

Conclusions: SGE was shown to be reproducible and produced results that correlated well with standard techniques for fastidious organisms. The method offers a rapid, flexible, cost-effective alternative for smaller laboratories and for routine use in developing countries.

Keywords: MICs, Haemophilus, spiral plater, reproducibility, evaluation

Introduction

Tailoring of antibiotic treatment regimens for individual cases with respect to pharmacokinetic and pharmacodynamic properties requires MICs to be determined.1 MIC determination provides evidence of steadily increasing resistance as evidenced by MIC creep.2 This may alert clinicians to rising resistance trends before strains reach resistance breakpoints. A recent conference reiterated the need for good resistance surveillance to reduce mortality and morbidity.3 The cost and labour requirements of current methods potentially reduce the number of MICs determined particularly for surveillance.1,4

The spiral gradient endpoint (SGE) method, an alternative gradient MIC technique,3 which allows greater flexibility of antibiotics, media and incubation conditions, has been neglected due to a complicated MIC calculation and the availability of commercial methods. Recent introduction of software for calculation and improvement in spiral plater design have facilitated SGE MIC determination. We revisited this technique to assess its potential for fastidious organisms. We optimized and evaluated SGE for four organisms in comparison with standard methods.

Methods

Control strains: Haemophilus influenzae ATCC 49247; Streptococcus pneumoniae ATCC 49619; Moraxella catarrhalis ATCC 25238; and Neisseria gonorrhoeae ATCC 49226.

For evaluation, 50 clinical isolates of each organism were used, including three β-lactamase-non-producing ampicillin-resistant (BLNAR) H. influenzae.
Ampicillin was tested against *Haemophilus* for optimization and reproducibility. Penicillin G was used in reproducibility testing for other control organisms. The antibiotics (Sigma-Aldrich, St Louis, MO, USA) for evaluation, stock concentrations and concentration ranges tested, which included the breakpoint and the highest MICs expected, are shown in Table 1.

SGE was performed using a spiral plater (Autoplater4000; Spiral Biotech, Norwood, MA, USA) that produces a continuous logarithmic dilution. Supplemented *Haemophilus* test medium (Oxoid, Basingstoke, UK) was used for *Haemophilus*, GC Sensitivity agar for gonococcus and Mueller–Hinton agar with 5% lysed blood for pneumococcus and *Moraxella*.

After dispensing of stock solution, plates were put aside to allow absorption. Inocula, prepared by direct suspension from an 18 h culture and adjusted spectrophotometrically (OD 600 nm), were then streaked across the gradient from the periphery towards the centre to avoid carry-over of antibiotic from high to low concentration. Plates were incubated in 5% CO₂ at 35°C. Distance from commencement of antibiotic deposition to the endpoint of growth was calculated. Effects of varying agar depth, standing time, incubation time, inoculum density and stock concentration were determined sequentially with the optimal conditions used in subsequent evaluations. Thirty-two repetitions were performed for each variable. Intra-batch reproducibility was evaluated by 48 replicate assays at both concentration ranges and inter-batch reproducibility assessed by repeating eight times at both ranges on six separate days.

Reference MICs were determined for clinical isolates of *H. influenzae* and *S. pneumoniae* by standard broth dilution (SBD) and for other organisms by standard agar dilution (SAD) and compared with SGE MICs. For better comparison, SGE MICs were rounded up to the next 2-fold value of the dilution test.

### Statistical analysis

Differences between means were determined by ANOVA (SPSS 16.0), homogeneity of variance using the Levene statistic, and post-hoc tests were used to perform multiple comparisons of pairs of means. Differences in proportions of strains in susceptibility categories were determined by Chi-squared test. Pearson correlation coefficients were calculated to measure overall association between log2 dilution MIC values determined by Chi-squared test. Pearson correlation coefficients were calculated to measure overall association between log2 dilution MIC values.

Differences in antibiotic stock concentration were also found to cause significant variation (P<0.001). Best correlation was obtained at lower stock concentrations.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Drug</th>
<th>concentration (mg/L)</th>
<th>MIC (mg/L)</th>
<th>SGE stock concentration (mg/L)</th>
<th>assay range (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pneumoniae</em></td>
<td>penicillin G</td>
<td>0.03–16</td>
<td>700</td>
<td>10000</td>
<td>0.0179–68.37</td>
</tr>
<tr>
<td></td>
<td>cephalosporin</td>
<td>0.075–16</td>
<td>2400</td>
<td>8000</td>
<td>0.061–54.20</td>
</tr>
<tr>
<td></td>
<td>erythromycin</td>
<td>0.015–32</td>
<td>1300</td>
<td>14000</td>
<td>0.033–95.72</td>
</tr>
<tr>
<td></td>
<td>vancomycin</td>
<td>0.015–16</td>
<td>1300</td>
<td>14000</td>
<td>0.033–95.72</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>ampicillin</td>
<td>0.03–64</td>
<td>400</td>
<td>10000</td>
<td>0.01–68.37</td>
</tr>
<tr>
<td></td>
<td>tetracycline</td>
<td>0.03–128</td>
<td>200</td>
<td>5000</td>
<td>0.005–34.19</td>
</tr>
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<td></td>
<td>chloramphenicol</td>
<td>0.03–16</td>
<td>1200</td>
<td>—</td>
<td>0.03–8.130</td>
</tr>
<tr>
<td></td>
<td>cefuroxime</td>
<td>0.03–16</td>
<td>400</td>
<td>10000</td>
<td>0.01–68.37</td>
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<tr>
<td><em>N. gonorrhoeae</em></td>
<td>penicillin G</td>
<td>0.06–32</td>
<td>200</td>
<td>8000</td>
<td>0.005–54.20</td>
</tr>
<tr>
<td></td>
<td>cefotaxime</td>
<td>0.06–16</td>
<td>600</td>
<td>5000</td>
<td>0.015–34.19</td>
</tr>
<tr>
<td><em>M. catarrhalis</em></td>
<td>penicillin G</td>
<td>0.15–16</td>
<td>600</td>
<td>5000</td>
<td>0.015–34.19</td>
</tr>
<tr>
<td></td>
<td>erythromycin</td>
<td>0.03–16</td>
<td>600</td>
<td>5000</td>
<td>0.015–34.19</td>
</tr>
</tbody>
</table>

### Results

#### Optimization

Data were normally distributed for all parameters (P>0.05). There were no significant differences in MICs associated with agar volume or standing times, which were set at 20 mL and 1 h, respectively. However, variation in incubation time led to differences in MICs (P<0.001) with significantly lower values at shorter incubation times (post-hoc test P<0.05). The expected MIC of 4 mg/L was obtained after 20–24 h of incubation. Inoculum density variation significantly affected MIC values (P<0.01), but the density of 10⁹ cfu/mL used in the evaluation gave an MIC closest to the reference method. Differences in antibiotic stock concentration were also found to cause significant variation (P<0.001). Best correlation was obtained at lower stock concentrations.

#### Reproducibility

For *H. influenzae*, *S. pneumoniae* and *N. gonorrhoeae* the overall standard deviations (SDs) and coefficients of variation (CVs) obtained for both intra-batch (0.041–0.327, 6.22%–14.56%) and inter-batch (0.078–0.4, 8.49%–18.56%) testing indicated good reproducibility, superior to that of the conventional dilution method (SD 0.274–2.338; CV 31%–64%), especially for *H. influenzae*. Problems were encountered with *M. catarrhalis* as suspensions tended to clump when the cell density was high. This resulted in higher CV and SD for SGE, though lower than for SAD.

#### Evaluation

Generally the MIC was calculated from the low-range plate, which included the susceptible category breakpoints. High-range plates were used if growth covered the entire low-range gradient. MICs determined by SBD/SAD and SGE were consistently within the acceptable reference range.

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**Table 1.** Antimicrobial agents and concentration ranges for reference dilution tests and the SGE technique.
H. influenzae
For H. influenzae, 54% of MICs of all antibiotics were the same by both methods, with 96% of SGE values within one dilution of the SBD method. Agreement was lowest for tetracycline (94%) and highest for cefuroxime (98%). Susceptibility category changes were observed for 6% of strains, all being minor errors involving a difference of one dilution. All discrepancies occurred at breakpoints and were attributable to clustering of MIC values near the breakpoint between intermediately and highly resistant. Numbers in each susceptibility category determined by SBD and SGE did not differ significantly ($P=0.191$) and there was no trend for MICs by SGE to be higher or lower ($P=0.674$). There was good correlation between MICs for the two methods ($r^2=0.914$). The regression coefficient for SGE MIC is 0.954 (SE=0.020, $P<0.001$) (Figure 1a).

S. pneumoniae
For S. pneumoniae, 98% of SGE MICs were within one dilution of the SAD method. Agreement ranged from 100% (vancomycin) to 94% (erythromycin). Thirteen minor errors involved changes at the breakpoint between susceptible and intermediate categories. Regression analysis yielded a correlation coefficient of 0.922 (Figure 1b). There was no significant difference in numbers in each susceptibility category ($P=0.856$), and no trend to higher or lower values ($P=0.674$). CLSI non-meningitis breakpoints

![Figure 1. Log$_2$ reference method MIC versus log$_2$ SGE MIC.](image-url)
were used to categorize penicillin susceptibility as most strains were respiratory isolates.\textsuperscript{6}

\textbf{M. catarrhalis}

For \textit{M. catarrhalis}, 96% of SGE MICs were within ±1 dilution of the SAD value. Erythromycin MICs had better agreement (96%) than penicillin (84%), but there were no differences in numbers in susceptibility categories. There was significant association \((r^2 = 0.930)\) between the values obtained by the two methods (Figure 1c), with no trend for lower or higher MIC results \((P = 0.286)\).

\textbf{N. gonorrhoeae}

For \textit{N. gonorrhoeae}, 97.5% of SGE MICs were within ±1 dilution of the SAD value. Minor errors representing a difference of one dilution were observed for two strains. Regression analysis yielded an \(r^2\) value of 0.908 (Figure 1d). Categories of susceptibility assigned by the two methods were not significantly different \((P = 0.937)\).

\section*{Discussion}

SGE showed excellent intra-batch and inter-batch reproducibility and very good correlation, with CVs superior to those of standard methods. Optimization revealed that inoculum density was the most important parameter affecting test results. For \textit{M. catarrhalis}, clumping problems occurred with the high inoculum, probably contributing to the higher SD and CV (23.4%). Direct suspension was found to give better reproducibility than an overnight suspension. Our validation results are comparable to those of an earlier study\textsuperscript{7} with the exception of the requirement for 24 h incubation, which may reflect slower growth of fastidious organisms.

A previous evaluation for non-fastidious organisms reported good correlation (0.86–0.96) and percentage agreement (46%–88% within one dilution).\textsuperscript{5} In comparison, in our evaluation for fastidious organisms, there was 95%–100% agreement within one dilution and good susceptibility category agreement between tests for all organisms and antibiotics tested.

Correlations between SGE and dilution methods were comparable to those reported in the evaluation of the Etest for \textit{H. influenzae}. Etest identification of BLNAR \textit{H. influenzae} can be problematic, but these were correctly categorized by SGE.\textsuperscript{7}

Etest correlates well with SBD for pneumococci MICs, but there are problems especially with categorization of penicillin highly resistant strains as intermediate.\textsuperscript{8}

Although monitoring of gonococcal susceptibility is essential, Etest MICs may be substantially lower or higher than SAD determinations.\textsuperscript{5} Whilst other classes of antibiotics need to be investigated for SGE, the correlation with SAD (0.908) for those tested was comparable to the Etest evaluations.

Although SBD is labour intensive and its inherent errors may lead to ambiguous results, recent inclusion of panels for streptococci and \textit{Haemophilus} has allowed use of automated instruments. However, commercially prepared panels are expensive, with high initial investment for the instrument. The range of antibiotics is quite inflexible in standard panels. Incubation times are longer negating the time-saving advantage of automation.\textsuperscript{9}

Etest strips are relatively costly, especially if several drugs require testing. SGE may offer an economic alternative for developing countries and smaller laboratories performing few MICs. Whilst SGE requires the purchase of a spiral plater, this technique offers the advantage of extreme flexibility of antimicrobial agents, allowing rarely used drugs to be tested. Rapidly available results can lead to timely changes in antimicrobial therapy and shortened hospital stays.\textsuperscript{10} Lack of precision of disc diffusion means that pneumococcal isolates appearing oxacillin resistant require confirmation by MIC. Reduced costs of SGE allow MIC determination for all invasive pneumococci, reducing the time for recognition of penicillin-resistant strains to 1 day. They also allow for surveillance of more antibiotic MICs to monitor both overall resistance and MIC creep. SGE provides an exact MIC value that more easily demonstrates increases in average MIC that may not be apparent with large increments in dilution methods.

This study has shown that SGE is a precise and accurate method of MIC determination for fastidious organisms. It is easy to perform, requires minimal equipment and offers an alternative to gradient methods.

\section*{Acknowledgements}

We wish to thank the Department of Clinical Pathology, Queen Elizabeth Hospital and the Department of Health, Hong Kong for providing the clinical isolates used in this study. We are grateful to Dr Tony Chan for his advice on statistical analysis. We thank Spiral Biotech for technical advice and provision of SGE software.

\section*{Funding}

This study was supported by a Post-Graduate Research Grant of the School of Nursing, The Hong Kong Polytechnic University.

\section*{Transparency declarations}

None to declare.

\section*{References}


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