Disinfectant testing: use of the Bioscreen Microbiological Growth Analyser for laboratory biocide screening

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R. J. LAMBERT, M. D. JOHNSTON AND E.-A. SIMONS. 1998. A new method is described for screening potential biocides based on the traditional suspension test using the Bioscreen optical plate reader. This new method is rapid, reproducible, quantitative and cost effective. Data obtained by this new method are not directly equivalent to the log reduction normally quoted, but give a measurement of the total effect of the biocide on the microbe population, measuring the effect of injury as well as death (non-viability). The method allows for the routine examination of disinfection kinetics, the study of which leads to greater scientific insight into disinfection than that achieved by the standard 5 min, one-point, disinfection tests currently employed.

INTRODUCTION

Laboratory testing of disinfectants is generally carried out by assessing the sensitivity of various micro-organisms to biocidal agents using a microbial suspension test (Croshaw 1981; Bloomfield and Looney 1992; Anon. 1994; Bloomfield et al. 1995; Holah 1995). This method involves adding a test organism to dilutions of the disinfectant under examination. After a 5 min exposure period at 20 °C, aliquots of the solutions are removed and the disinfectant neutralized with a suitable quenching agent. This step is vital in order to eliminate residual bacteriostatic or bactericidal effects during culture of the survivors. A sample of neutralized suspension is transferred to an appropriate agar medium (after serially diluting) to determine the viability (survival) of microbial cells. Biocide effectiveness is determined by comparing the numbers of organisms recorded after treatment to the numbers on untreated (control) plates.

Microbiological research aimed at finding novel biocides often involves the testing of numerous products. The standard testing procedures used are those described above since they ultimately form legislative argument, e.g. European Suspension Test (Anon. 1994). Speculative testing of an experimental disinfectant on five organisms at three different concentrations could entail at least 20 tests with relevant controls. Furthermore, each test procedure may have several examination plates associated with it, leading to a large number of plates which need to be prepared, inoculated, incubated and examined. In cases of complex mixtures, with many more variables involved, one simple, exploratory trial can result in several hundred test combinations with several thousand plates being required.

There are two principal methods of increasing throughput given an available resource: the use of a modelling approach to predict efficacy of disinfectant; and/or the application of a rapid, automated method capable of screening large numbers of test solutions. In the short term, the use of an automated method appeared the better approach. In this paper, the evaluation and use of the Bioscreen Microbiological Growth Analyser (Labsystems Corp., Helsinki, Finland), is described. The ‘Bioscreen’, as an efficient, reproducible and rapid screening method for large numbers of potential biocides.

MATERIALS AND METHODS

Preparation of bacterial suspensions

Organisms used in these experiments were Staphylococcus aureus ATCC 6538, Pseudomonas aeruginosa ATCC 15442, Streptococcus faecium ATCC 10541, Streptococcus faecalis CDC 73–57672, Proteus mirabilis ATCC 14153 and Escherichia coli ATCC 11229.

Bacteria were grown in Tryptone Soya Broth, TSB (Oxoid CM 129) for 24 h at 30 °C. Cultures were centrifuged at 512 g (Sigma model 3K-1) for 10 min and the resulting cell pellets resuspended in 0·1% peptone.
Preparation of test disinfectants

Disinfectants were diluted in sterile distilled water prior to use. Quatdet (SU321), a quaternary ammonium disinfectant, and peracetic acid were obtained from DiverseyLever, Runcorn, UK; Chloros (sodium hypochlorite) was obtained from Ellis & Everard Chemicals Ltd; chlorocresol, phenethyl alcohol, phenol, m-cresol, sodium dodecyl sulphate and hydrogen peroxide were obtained from the Sigma-Aldrich Chemical Company and used without further purification. The pH of the mixtures was controlled by the addition of HCl or NaOH as appropriate.

Bioscreen Microbiological Growth Analyser

The ‘Bioscreen’ is an automated turbidity reader linked to an integrated PC, manufactured by Labsystems, Helsinki, Finland. The system can measure changes in the turbidity of up to 200 microtitre wells (2 × 100 multiwell plates) and can provide growth curves from each well either directly to the monitor or in the form of a data file suitable for further examination.

Suspension test (traditional plate count method)

Approximately 0.1 ml of a bacterial suspension (approximately 1 × 108 bacteria ml−1) was added to the test disinfectant (10 ml), mixed thoroughly and left at 20 °C (± 1 °C) for a specified contact time (normally 5 min). Following contact, an aliquot (1 ml) was transferred to universal quenching agent, UQA (9 ml of a solution containing 1 g peptone, 5 g Tween-80, 1 g sodium thiosulphate and 0.7 g lecithin l−1 of deionized water, pH 7), for up to 60 min to inactivate the disinfectants. The quenched solutions were serially diluted in 0.1% peptone water and survivors enumerated on Tryptone Soya Agar, TSA, (Oxoid CM131) using 0.1 ml spread plates. The plates were counted after incubation at 30 °C for 48 h.

Suspension test (Bioscreen method)

All procedures involving the manipulation of the Bioscreen plate were carried out in a laminar flow Envair Class II microbiological safety cabinet to avoid contamination. The disinfection contact and quenching stage are identical to the traditional plate count method. However, instead of plating, an aliquot (40 μl) of the quenched test solutions was added to 360 μl of TSB in the microtitre well of the Bioscreen plate. Solutions were thoroughly mixed and a portion (40 μl) discarded. This gave a 10-fold dilution and ensured all sample wells had the same (360 μl) volume. For the control wells, an aliquot (40 μl) from the control quench (cells treated with water only) was added to a solution of broth (360 μl) and thoroughly mixed. An amount (40 μl) was removed from this well and added to another well containing broth (360 μl). This 10-fold dilution was repeated until the required series of controls, 0, 1, 2, 3, 4 and 5 log dilutions was obtained. A set of such controls was prepared for every Bioscreen experiment and organism examined. A minimum of five broth-containing blanks was also included to allow for background corrections. This test plate was placed in the Bioscreen for between 15 and 30 h at 30 °C, with moderate, continuous shaking and with the Bioscreen set to read every well at 10 min intervals at a wavelength of 600 nm.

Results were analysed using the Microsoft Excel spreadsheet.

RESULTS AND DISCUSSION

Log reduction equivalents, log Req

The basis for this rapid method is the direct quantification of the decimal log reduction in microbial numbers following disinfection by reference to a calibration equation. This is achieved by comparing the time for microbial growth to reach a specific optical density (e.g. O.D.600 = 0.20) after disinfection and quenching, to the time required for a specific serial dilution of the initial inoculum to reach the same optical density. As explained below, the decimal log reductions observed by this method are not directly equivalent to those obtained from traditional plate counts. To make this point clear, the terminology of decimal log reduction equivalents, log Req, has been adopted.

Calibration curves

A calibration curve (Fig. 1) was prepared for each experiment and for each organism examined. From the O.D./time curves obtained, the time to reach an optical density of 0.20 A was determined for each serial dilution. Each serial dilution is equal to a log Req. The required linear calibration graph was obtained from a plot of log Req against the time to reach an O.D. of 0.20 A. Table 1 lists the typical calibration data obtained from a range of organisms.

Estimation of disinfection log reduction

This rapid method, as it implicitly uses log Req, does not require enumeration of the initial inoculum level of organisms, unlike the traditional methods which require knowledge of the initial inoculum level (Bloomfield 1995). As an example, Fig. 2 shows the effect of phenol (0.7%) on Staph. aureus with respect to contact time. By inspection and comparison with the corresponding control growth response curves (Fig. 1), qualitative log Req can be gauged, e.g. an 18 min contact time is approximately equivalent to a 3 log Req. By
Fig. 1 Typical calibration graph for *Staphylococcus aureus*. (●), log $R_{eq} = 0$; (■), log $R_{eq} = 1$; (▲), log $R_{eq} = 2$; (△), log $R_{eq} = 3$; (○), log $R_{eq} = 4$; (■), log $R_{eq} = 5$; (□), log $R_{eq} = 6$

Table 1 Typical calibration data for a range of organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>Slope $\times 100$</th>
<th>Intercept</th>
<th>$r_{eq}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em>, ATCC 6358</td>
<td>7.88</td>
<td>-0.744</td>
<td>0.999</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em>, ATCC 15442</td>
<td>7.6</td>
<td>-0.873</td>
<td>0.999</td>
</tr>
<tr>
<td><em>Escherichia coli</em>, ATCC 11229</td>
<td>9.961</td>
<td>-0.557</td>
<td>0.999</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em>, CDC 73-57672</td>
<td>9.75</td>
<td>-0.571</td>
<td>0.997</td>
</tr>
<tr>
<td><em>Streptococcus faecium</em>, ATCC 10541</td>
<td>9.332</td>
<td>-0.625</td>
<td>0.998</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em>, ATCC 14153</td>
<td>10.2</td>
<td>-0.857</td>
<td>0.999</td>
</tr>
</tbody>
</table>

using the equation obtained from the calibration curves, log $R_{eq}$ can be quantitatively assessed.

**Reproducibility and reliability**

A series of 5 min tests using hypochlorite and quaternary ammonium based disinfectants against *Pseudomonas aeruginosa* was examined (Table 2). The results indicate the reproducibility of the Bioscreen method and that the data and errors observed are comparable with similar data obtained from the traditional suspension test methodology. This also suggests that the errors inherent in the European Suspension Test are not due to counting errors but are more likely due to the initial biocide testing stage. Previous workers have highlighted the need for coherence in the test populations (Bloomfield et al. 1995).

Injury, log $R_{eq}$ and log $R$

The log $R_{eq}$ results from the Bioscreen are higher than the log $R$ obtained from plates, apparently suggesting that the biocides were more effective when examined using the Bioscreen than by plates, despite the fact that the biocide testing for both methods was identical. It is hypothesized that the reason for this was the presence of injured organisms capable of repair and subsequent growth. After a disinfection test there are three possible microbial states: dead, uninjured and injured. When plated out, the uninjured microbes will grow up into normal sized colonies; the injured organisms grow after repair and have smaller colony sizes after a given time of incubation relative to the uninjured population. Thus, the plate count will give the total number of microbes living/viable after the disinfection which includes the numbers of injured organisms.

After the biocide test, each well of the Bioscreen plate will contain all microbe states: injured, uninjured and dead. The least injured cell will divide earlier than a more severely injured cell as less time is needed for repair. The turbidity in the well will then be due principally to the uninjured or least injured organisms dividing and multiplying. Thus, the Bioscreen method will not take account of the more severely injured cells, unlike a plate.

For the screening of potential biocides this is a very useful property because a biocide which caused serious injury but did not cause death would be highlighted by the Bioscreen but ignored by the plate method. Such information could direct further research. The method also gives the ability to quantify the level of injury by comparing a Bioscreen log $R_{eq}$ to a plate log $R$. The difference between them is the level of viable injury in terms of log numbers of microbes. Figure 3 shows the correlation between log $R_{eq}$ and log $R$ for the phenol and phenethyl alcohol (PeA) disinfection of *Staph. aureus* at various concentrations and contact times. With PeA,
Fig. 2 Disinfection of *Staphylococcus aureus* with phenol—effect of contact time. Disinfection contact times (●), 3 min; (■), 6 min; (▲), 9 min; (★★), 12 min; (△), 18 min; (●), 24 min; (○), 30 min; (■), 36 min; (■), 48 min; (○), 60 min

![Image of disinfection data](image)

**Table 2** Comparison of Bioscreen and plate methods

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Concentration (%)</th>
<th>log R&lt;sub&gt;eq&lt;/sub&gt;*</th>
<th>log R (plates)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloros</td>
<td>0.028</td>
<td>&gt;5.5</td>
<td>5.48 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>&gt;5.5</td>
<td>5.48 ± 0.12</td>
</tr>
<tr>
<td>Quatdet</td>
<td>0.01</td>
<td>3.56 ± 0.28</td>
<td>2.82 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>0.014</td>
<td>5.48 ± 0.03</td>
<td>4.4 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>0.016</td>
<td>&gt;5.5</td>
<td>5.28 ± 0.26</td>
</tr>
</tbody>
</table>

*Each test performed five times.

![Graph showing log R vs log R<sub>eq</sub>](image)

**Table 2** Comparison of Bioscreen and plate methods

A linear relationship was observed with log R<sub>eq</sub> > log R. We can define log R<sub>eq</sub> as log R = m log R<sub>eq</sub>, where 1 - m is the proportion of the log of injured organisms. For PeA, log R = 0.75 log R<sub>eq</sub>, i.e. one quarter of the log R<sub>eq</sub> can be attributed to injured but viable cells.

A linear relationship between log R and log R<sub>eq</sub> suggests a constant proportion of injured cells throughout the disinfection process, i.e. a time-independent population distribution of injury. The limit is when log R = log R<sub>eq</sub>, i.e. m = 1 (no injured organisms); at this point, the two disinfection methodologies are in agreement.

If the plot of log R vs log R<sub>eq</sub> is not linear, then the proportion of injury is not constant. The phenol disinfection of *Staph. aureus* shows that as higher log R values are recorded, the proportion of injured microbes drops, with m approaching 1. This suggests that the value of injury (m) is related to the numbers being killed. A plot of log R against m gave an approximate straight line with positive slope.

Skytta and Mattila-Sandholm (1991) have studied the use of the Bioscreen as a replacement for Agar Diffusion studies. Their method was based on the assumption that the Bioscreen data could be related directly to colony forming units, (cfu). Our results clearly show that for biocide tests of the type described here, this assumption may not be valid.

**Disinfection rates**

The European Suspension Test requires a 5 min contact time for a disinfectant to achieve five log R with five different organisms (Anon. 1994). A serious problem with this method is the exclusion of any real regard for the disinfectant concentration coefficient (Hugo and Denyer 1987). This coefficient describes the reduction of biocidal activity on dilution. Small dilution errors in the preparation of solutions for the
EST test can have serious effects on the simple Pass/Fail criteria. As disinfection, unlike preservation, is a rate process, it may be more worthwhile to develop tests which examine the whole process. As the Bioscreen method is a rapid technique, disinfection rates can be studied more easily.

The rate of disinfection of Staph. aureus by phenol can be visualized in Fig. 2. By repeating such tests for various concentrations of phenol, the rate parameters can be obtained. However, as log \( R_{eq} \) is not the same as log \( R \), differences in the rate data obtained between the Bioscreen method and the traditional plate method were expected, e.g. with the dilution coefficient. However, in general, the constants obtained from the Bioscreen method (data not given) are similar to those published (Hugo and Denyer 1987; Reichart 1994), which suggests the applicability of normal disinfection kinetics to log \( R_{eq} \). In many cases, log-linear plots are obtained, e.g. with hydrogen peroxide, but in some cases, such as with SDS, non-linearity (tailing) was observed.

It is worth noting that important features observed with log \( R \) vs time plots such as lags and other non-linearities (tailing) are missed by the simple 5 min testing regimes.

In conclusion, this method for examining biocides is rapid, reproducible and quantitative. It can give insight into the disinfection process and can be used to gauge the level of injury following a disinfection test.

REFERENCES


