Development and Testing of a Microbiological Assay To Detect Residual Effects of Disinfectant on Hard Surfaces

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We describe a glucuronidase bioassay for detecting residual bactericidal activity from the use of disinfectants on hard surfaces; in this assay we used formaldehyde, ethanol, isopropanol, chlorine, and a commercial preparation containing 2-bromo-2-nitro-1,3-propanediol. Chlorine and the commercial preparation showed bactericidal activity (53.5% and 98.2%, respectively) for a week after disinfection.

The use of bacterial bioassays to evaluate the toxicity of chemicals is widespread since they are easy to perform and offer quick results. *Escherichia coli* produces an enzyme, β-D-glucuronidase (BGU), that is capable of hydrolyzing a fluorogenic substrate such as 4-methylumbelliferyl-β-D-glucuronide (MUG) (6). By using this enzyme, it has been possible to design a quick and simple bioassay (fluorogenic surface test) for detecting and quantifying the presence of traces of disinfectant on inert materials that might easily be released in a liquid medium. The premise is that after introducing a previously disinfected carrier into a test tube containing MUG and *E. coli*, there will be no fluorescence only in the absence of active bacteria (and hence an absence of BGU activity as well), indicating the presence of traces of disinfectant at inhibitory concentrations. The tube will show fluorescence if active bacteria are still present. If this fluorescence is lower than that emitted by the control tubes without disinfectant, it will be possible to quantify the residual disinfectant activity in relation to known concentrations of tested disinfectant, since this inhibition is proportional to the concentration of toxic substances (2).

*Escherichia coli* W3110 thy E−, obtain from the Spanish Collection of Culture Types, was grown at 37°C with shaking in 10 ml of Vogel-Bonner minimal medium (VB) supplemented with glucose (20 mg/ml) and thymine (0.05 mg/ml) (both from Sigma) until the late logarithmic phase was reached (at about 18 h according to growth curves). The culture was then centrifuged at 3,030 × g for 5 min to eliminate the remaining glucose, and the precipitate was resuspended in 10 ml of VB supplemented only with thymine (0.05 mg/ml) (VBT). This cell suspension was held at 4°C prior to use.

The following disinfectants were tested: 2% formaldehyde (A. Matachana, S. A., Barcelona, Spain), 20% ethanol and 70% isopropanol (Merck), 2% sodium hypochlorite (Sigma), and CR-36 MURAL (J. Collado, S. L., Madrid, Spain), a commercial preparation composed of 0.1875% 2-bromo-2-nitro-1,3-propanediol, 0.0675% 2,2,4-trichloro-2'-hydroxyphenyl ether, and 1% N-allyl-N,N-dimethylaminomuonphenyl chloride. Each disinfectant was prepared with sterilized and distilled water, except CR-36 MURAL, which was used undiluted. A total of 30 carriers (glass penicylinders 1 cm in diameter and 0.8 cm long), similar to those used in the hard-surface carrier test (5), were then introduced into sterilized shake flasks containing 50 ml of disinfectant. After being shaken to eliminate any bubbles, the shake flasks were left at room temperature for 10 min, and then the carriers were then removed and placed in a petri dish lined with sterilized filter paper for another 30 min until completely dry. Immediately afterwards, five dry carriers were introduced separately into tubes each containing 1.8 ml of VBT supplemented with MUG (0.01 mg/ml) (Sigma) and shaken vigorously for 10 s. An *E. coli* cell suspension with an absorbance of 600 nm of 0.05 ± 0.02 (CS0.05), prepared immediately before each experiment from the suspension conserved at 4°C, was added to each tube (0.2 ml per tube) and incubated in a shaking water bath at 37°C. After 210 min, the fluorescence in each tube was measured with a Perkin-Elmer LS 30 fluorometer with excitation at 340 nm and emission at 445 nm. Other dry carriers were analyzed by an identical procedure 24 h and 7 days after being dried. Controls were prepared simultaneously in the same way but without disinfectant. The number of microorganisms present at the beginning and at the end of each experiment was determined as CFU by counting microorganisms on tryptic soy agar plates (Oxoid).

BGU enzymatic activity was calculated from the amount of methylumbelliferone released per CFU per min by means of a least-squares linear regression; the amount released was determined by using standard curves with methylumbelliferone.

**TABLE 1. Effect of five disinfectants on the BGU activity of E. coli W3110 in VBT-MUG**

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>MICa (ppm)</th>
<th>EC50 ± SDb (ppm)</th>
</tr>
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<tbody>
<tr>
<td>Chlorine</td>
<td>1.23</td>
<td>0.039 ± 0.012</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>6.2</td>
<td>0.468 ± 0.094</td>
</tr>
<tr>
<td>CR-36</td>
<td>333</td>
<td>11.6 ± 3.1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>100,000</td>
<td>26,245 ± 1,384</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>50,000</td>
<td>30,474 ± 2,011</td>
</tr>
</tbody>
</table>

a MIC of tested substances for *E. coli* BGU activity (inhibition of 100% BGU activity).

b Concentration of disinfectant required for suppression of *E. coli* BGU activity by 50%.

The MIC and EC50 for CR-36 could be 0.62 and 0.021 ppm, respectively, if only the bactericidal component (bronopol) of this compound is considered (see the text).
(Sigma) dissolved in VBT. The inhibition of *E. coli* BGU activity produced by traces of disinfectant on carriers was calculated as described previously (3). The results were expressed as percent inhibition with respect to the control without disinfectant (negative values of percent inhibition indicate stimulation of BGU activity). The horizontal lines are means, and the error bars indicate standard deviation for percent inhibition.

The mean concentration of each disinfectant released by the carriers was calculated as follows. A series of tubes containing VBT-MUG (1.8 ml) and a CS₀.₀₅ (0.2 ml) was prepared, with each tube containing a known concentration of disinfectant. The tubes were incubated, and their fluorescence was measured as described above. The percent inhibition was calculated as described above, and a dose-response curve was obtained with the log₁₀ concentration on the x axis and the corresponding percent inhibition on the y axis. By using the
TABLE 2. Effect of test disinfectant adhering to the surface of glass carriers on the BGU activity of *E. coli* as a function of the time since their immersion in the tested solutions

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>0 h</th>
<th>24 h</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR-36</td>
<td>100 (0.0000)</td>
<td>100 (0.0000)</td>
<td>98.2 ± 1.2 (0.0003)</td>
</tr>
<tr>
<td>Chlorine</td>
<td>100 (0.0000)</td>
<td>88.7 ± 9.8 (0.0074)</td>
<td>53.5 ± 16.6 (0.0287)</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>5.3 ± 2.88 (NS)</td>
<td>0 (NS)</td>
<td>0 (NS)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0 (NS)</td>
<td>0 (NS)</td>
<td>0 (NS)</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>0 (NS)</td>
<td>0 (NS)</td>
<td>0 (NS)</td>
</tr>
</tbody>
</table>

* a Effect is expressed as percent inhibition of BGU activity calculated from the mean EA per minute and per CFU in samples, in relation to the controls without disinfectant, ± standard deviation for the values included in the mean.
* b NS, not significant statistically (P > 0.05).

The regression line of these dose-response curves and knowing the mean of the percent inhibition obtained for the carriers submerge in each disinfectant, the mean concentration of disinfectant released by the carriers was calculated by regressing the percent inhibition corresponding to the log10 known concentrations of disinfectant. Simultaneously, the antibacterial efficacy of the tubes containing a known concentration of disinfectant was determined as the MIC by means of a Student t test with the arithmetic mean of the values of the percent inhibition. The 50% effective concentrations EC50 were calculated from the aforementioned dose-response curves, as the concentration of substances in ppm required for an inhibition of 50% (2).

The EC50s and MICs of the five disinfectants tested refer to the BGU activity in relation to the controls without disinfectant and are shown in Table 1. Chlorine, formaldehyde, and CR-36 showed a high toxicity, while ethanol and isopropanol showed a lower toxicity. All experiments were carried out in hermetically closed test tubes to avoid losing disinfectant through volatilization during the assays. According to the CFU counts performed at the end of the experiments, no bacterial growth was observed in the concentration corresponding to the MIC of each disinfectant.

As indicated in Table 1, CR-36 seemed at first to be less active than chlorine and formaldehyde. Nevertheless, taking into account that the bactericidal component of CR-36, 2-bromo-2-nitro-1,3-propanediol (bronopol) accounts for only 0.1875% of its composition, the MICs and EC50s could be 0.62 and 0.011 ppm, respectively (instead of 333 and 11.6 ppm), if only the concentration of bronopol is considered. In this study, no attempt was made to check if the inhibition caused by CR-36 was produced only by bronopol, and the good absorption of CR-36 by materials probably contributed to these results.

The curves showing percent inhibition of BGU activity plotted against log10 concentrations of the tested disinfectants are given in Fig. 1. An increase in the concentration of chlorine, formaldehyde, and CR-36 led to a gradual inhibition of BGU activity. However, the other two substances tested, ethanol and isopropanol, stimulated BGU activity at low concentrations (lower percent inhibition than that for the controls). In both cases, this effect was not consistent with an increase in the CFU observed at the end of the test or with the stimulation of BGU activity (BGU activity per cell), and for this reason it was probably caused by an increase in cellular permeability, similar to the effect observed (3) for dimethyl sulfoxide in relation to BGU activity. This stimulation of BGU activity at subtoxic concentrations could be related to a lower impermeability of the substratum or of its enzymatic cleavage products in a process similar to that described by Van Poucke and Nelis (8) for toluene.

The results of the fluorogenic test to detect the presence of disinfectant on the carriers are shown in Table 2. Only the carriers submerged in CR-36 and 2% chlorine solution showed residual inhibition (P < 0.05) of BGU activity. For CR-36, this inhibition persisted for at least a week, practically without losing capacity, whereas the inhibitory activity of 2% chlorine solution dropped to approximately half its capacity (53.5%) after the 7-day period. As for the other three disinfectants, only the 2% formaldehyde showed some inhibition at time zero, although the differences were not statistically significant in relation to the controls without disinfectant. When determining the number of CFU in relation to the inoculum at the end of each test, in all cases it was found that the total or partial inhibition of fluorescence coincided with a decrease in the number of microorganisms. This suggests that the decrease in BGU activity is due to a decrease in the number of bacteria and not to the specific inhibition of BGU as is produced by some inhibitors of BGU (3) that are capable of inhibiting this enzyme but are nontoxic for bacteria. As shown in Fig. 1 and Table 2, the standard deviation for the percent inhibition values of chlorine was much greater than that observed for the other four disinfectants. In a study on the effect of chlorine on *E. coli* BGU activity, Tryland et al. (7) suggested that the alteration of cellular permeability brought about by low concentrations of chlorine could favor EA by facilitating the transport of enzymes or substrates in the presence of decrease in the number of CFU. In the test under discussion the number of CFU per milliliter was between 2.3 and 4.5 times smaller than expected for the observed BGU activity.

To calculate the average amount of chlorine and CR-36 released by the carriers (Table 3), it was necessary to prepare a series of doubled dilutions in VBT-MUG from the test tubes containing the previously disinfected carrier before adding the
bacterial inoculum, so as to obtain a percent inhibition less than 100%. The residual inhibitory activity of CR-36 and chlorine remained high even after 1 week (Table 2) and succeeded in inactivating relatively large bacterial inocula (between $1.4 \times 10^6$ and $8.1 \times 10^6$ CFU of *E. coli* per ml present in the 2 ml of medium used in each experiment). Unlike the 2% chlorine solution, CR-36 is a commercial preparation specially made for disinfecting surfaces. As mentioned above, its bactericidal effect on the carriers remained practically stable for at least 1 week (Table 2) whereas chlorine lost nearly half its effect after the same period. The persistence of these disinfectants, together with their low toxicity for humans when used at the recommended concentrations, should be taken into account when a prolonged biocide activity may be desired. On the other hand, ethanol and isopropanol, given their volatility and, as a consequence, their limited persistence on surfaces, did not show any residual inhibitory activity (Table 2) and therefore should not be used when a prolonged effect is desired.

Formaldehyde is a well-known disinfectant, although nowadays its use as a surface disinfectant is limited due to its high toxicity (4). Formaldehyde gas is used for sterilizing surgical material; in this case, its residual presence at concentrations higher than 5 mg/cm$^2$ is not recommended (1). On the other hand, the fluorogenic bioassay under discussion makes it possible to detect concentrations of formaldehyde greater than 0.08 mg/ml, as shown in Fig. 1. Taking into account that the total surface area of the carriers used in the tests is 4.7 cm$^2$, the average amount of disinfectant released by each carrier was calculated from the equation of the straight line obtained with formaldehyde (Fig. 1) and the percent inhibition (5.3%) for this substance (Table 2). The estimated average amount of residual formaldehyde present on the carriers (Table 3) was 0.035 mg/cm$^2$. It is important to take into account that the carriers used in the tests described above were submerged in a liquid solution of formaldehyde, and so the results are not comparable to those obtained by methods in which formaldehyde gas is used in an autoclave.

The application of the method described in this paper is now being investigated with clinical materials such as carriers (probes, catheters, or ventilation tubes) and other substances used in clinical sterilization. The preliminary results suggest that it is also usually possible to detect residues of compounds such as glutaraldehyde or formaldehyde at greater concentrations than those detected on glass carriers. Hence, this method could be used to control residual disinfectants or sterilants on materials on which they might cause health problems. An environmental application of this method could also be the assessment of the permanency of pesticides or other substances that stick to surfaces. In addition, it has the advantage of being simple, quick, and cheap. The development of methods for other enzymatic fluorogenic substrates could widen the scope of this bioassay in relation to its application to microorganisms other than *E. coli*.

**REFERENCES**