Assessing the residual antibacterial activity of clinical materials disinfected with glutaraldehyde, o-phthalaldehyde, hydrogen peroxide or 2-bromo-2-nitro-1,3-propanediol by means of a bacterial toxicity assay

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ABSTRACT

This study investigated the use of a rapid bacterial toxicity test for detecting disinfectant residues released by disinfected materials. The test substances included an environmental disinfectant used in hospitals in high-risk areas, such as critical care units or emergency services, and three disinfectants used on clinical devices when a high level of disinfection is required. The test materials were polyurethane, polypropylene, glass, latex and cotton from different instruments and utensils used in hospitals. Of the four test disinfectants, o-phthalaldehyde (OPA) and 2-bromo-2-nitro-1,3-propanediol (BNP) showed the greatest inhibitory activity (as much as 300-fold greater than hydrogen peroxide in the case of OPA) according to the toxicity text. However, with the exception of hydrogen peroxide on latex, it was the most porous test materials, namely latex and cotton, that accumulated the least residue. BNP was the disinfectant that left the least residue on the five test materials, while the greatest residual concentration was left by hydrogen peroxide on latex (as much as 5 μg/cm²). The biotest used in this study permitted the detection of disinfectant residues released by different types of previously disinfected clinical materials, and can be adapted to simulate elution conditions similar to those existing in routine hospital practice.

Keywords Bioassay, 2-bromo-2-nitro-1,3-propanediol, disinfection, glutaraldehyde, hydrogen peroxide, o-phthalaldehyde

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INTRODUCTION

Correct cleaning and disinfection of reusable clinical equipment such as endoscopes, bronchoscopes or anaesthetic instruments is essential if the spread of pathogenic microorganisms is to be avoided. The presence of organisms as diverse as Pseudomonas aeruginosa, Mycobacterium tuberculosis or Helicobacter pylori has been related to outbreaks of nosocomial infection associated with instruments of this type [1–3]. In general, the concentrations at which disinfectants are used on clinical materials are established by susceptibility tests in which a liquid medium is inoculated with a suspension of microorganisms in the presence of serial dilutions of the disinfectant in question. However, disinfectants are less effective on solid surfaces than in suspension tests, as can be seen in the so-called surface tests designed to assess the efficacy of biocides on solid materials [4].

Another problem related to the disinfection of clinical devices, such as endoscopes or bronchoscopes, or materials that come into close contact with patients’ mucous membranes is the possibility that disinfectant residues might cause adverse reactions in patients. Although infrequent, cases of acute colitis have been described that were probably caused by glutaraldehyde (GTA) or hydrogen peroxide (HP) residues [5,6]. Allergic contact dermatitis or occupational asthma related to GTA and other disinfectants have also been reported [7,8]. In addition, most chemicals used to process endoscopes and similar instruments irritate the skin, mucous membranes
and respiratory tract, although these side effects concern mostly the personnel in charge of the sterilisation/disinfection procedures.

Techniques used to detect the presence of chemical residues generally require instruments and methods that are too complicated for routine use in hospitals with limited facilities. Simple analytical techniques for use on medical devices have been developed for only a few substances, such as formaldehyde [9]. Bioassays based on the use of indicator microorganisms have proved to be an efficient screening tool for monitoring different chemicals in water or sediments. Some of these assays have also been used to monitor extraction solvents used in the chemical and biological examination of medical devices [10].

A toxicity test based on the fluorescence produced by the glucuronidase activity of Escherichia coli in the presence of a fluorogenic substrate (4-methylumbelliferyl-β-D-glucuronide) has recently been proposed as a surface toxicity bioassay [11]. The purpose of the present study was to investigate the capacity of this surface biotest to detect disinfectant or chemical residues adhering to different materials associated with clinical instruments such as probes, catheters, ventilation tubes, endoscopes or other instruments that come into close contact with the skin and mucous membranes of patients. To assess this strategy, sample materials from some of these instruments were treated with different disinfectants and chemicals in accordance with hospital protocols. The presence of residues was determined by a toxicity biotest and, where residues were detected, the concentration was calculated by extrapolating the dose–response curves obtained from known concentrations of each of the test disinfectants.

**MATERIALS AND METHODS**

**Preparation of disinfectants and carriers**

The test disinfectants—all in aqueous solution—were GTA, o-phthalaldehyde (OPA), HP and 2-bromo-2-nitro-1,3-propanediol (BNP) (Sigma-Aldrich, Madrid, Spain). Surface biotests were conducted with solutions prepared according to the dilutions recommended for clinical use (i.e., GTA 2% v/v, OPA 0.5% v/v, HP 3% v/v, and BNP 0.1% v/v). The materials used as carriers in the surface tests were polyurethane penicylinders and polypropylene penicylinders from catheters made by B. Braun Melsungen (Melsungen, Germany), glass penicylinders (Albus, Cordoba, Spain), latex rubber and 100% cotton material from hospital cloths. The materials were cut into fragments of the same size with a scalpel under sterile conditions. The measurements for calculating the surface area of the carriers were taken with an electronic digital calliper (Comecta, Barcelona, Spain).

**Procedure for the surface biotest**

Surface tests were performed as described previously [11]. In brief, a fluorogenic bioassay involving the β-glucuronidase-mediated conversion of 4-methylumbelliferyl-β-D-glucuronide to 4-methylumbelliferone was used to evaluate the toxic effect of the four disinfectants on E. coli. The assay is based on the premise that there will be no fluorescence if a disinfected carrier with residual traces of disinfectant at an inhibitory concentration is introduced into a test tube containing methylumbelliferyl-β-D-glucuronide and E. coli. If the tube shows a lower level of fluorescence than that emitted by control tubes without disinfectant, it is possible to quantify the disinfectant in relation to known concentrations of the test substance in question.

Before the surface biotest was conducted, Eppendorf tubes containing 1 mL of sterile nutrient broth supplemented with NaCl (1 g/L) and thymine (0.4 mg/L) were inoculated with E. coli strain W3110 thy {−} F{−} (Spanish Collection of Culture Types, Valencia, Spain). Following incubation for 18 h at 37 °C, 0.5 mL of sterile glycerine 50% v/v was added to each tube; the tubes were then stored at –20 °C until use. Immediately before use in the test, the tubes were thawed at 22 °C for 10 min and then centrifuged for 5 min at 3300 g. The precipitate was resuspended in 5 mL of Vogel-Bonner minimal medium (0.8 mM MgSO4·7H2O, 10 mM citric acid monohydrate, 57.4 mM K2HPO4, 3 mM NaNH4HPO4•4H2O) and held at 4 °C. This bacterial suspension was used directly in the assays. The viable count (1.3–2 × 10⁸ CFU/mL) was verified at the beginning of each experiment by spreading ten-fold dilutions in phosphate buffer on tryptic soy agar plates (Oxoid, Basingstoke, UK) and incubating at 37 °C for 24 h. Plate counts were performed in duplicate.

For the surface biotest, the carriers were dipped into a sterile Erlenmeyer flask containing 50 mL of diluted disinfectant and left at 22 °C for 10 min. The carriers were then removed and placed on a piece of sterile filter paper in a Petri dish. Once dry (30 min at 22 °C was sufficient), they were introduced separately into tubes containing 2 mL of VBMT medium (Vogel-Bonner minimal medium supplemented with methylumbelliferyl-β-D-glucuronide 0.01 mg/L and thymine 0.05 mg/L, both from Sigma-Aldrich) and shaken vigorously for 10 s. An aliquot (1.8 mL) was then added to a 100 × 16 mm tube with a screw cap containing 0.2 mL of bacterial suspension, and incubated in a shaking water bath at 37 °C for 210 min. After incubation, the fluorescence of each tube was measured in relative fluorescence units (RFUs) with a model LS 30 fluorometer (Perkin Elmer, Beaconsfield, UK), with excitation at 440 nm and emission at 485 nm, followed by subtraction of the fluorescence values of the corresponding control tubes. Controls were prepared simultaneously in sterile distilled water without disinfectant.

The concentration of the disinfectant released from each type of carrier was calculated from the inhibition of E. coli β-glucuronidase activity. The mean of the fluorescence emission in RFUs of five replicates (with disinfectant) and the mean of the corresponding controls (without disinfectant) were used, and the results of the test were expressed as
the percentage inhibition vs. controls (% inhibition: \( \frac{RFU_{\text{disinfectant}} - RFU_{\text{control}}}{RFU_{\text{control}}} \times 100 \)).

Simultaneously, a series of tubes of VBMT medium (1.8 mL) containing serial dilutions of known disinfectant concentrations were prepared for each test substance, except for three tubes that were used as controls. Following inoculation with 0.2 mL of bacterial suspension, the tubes were incubated at 37°C for 210 min, after which the fluorescence was measured as described above. A dose–response curve was obtained for each disinfectant from the percentage of inhibition vs. controls and the log\(_{10}\) of the corresponding disinfectant concentrations. The dose–response curves for known disinfectant concentrations were obtained and the assays to determine the residual inhibition associated with each type of carrier were carried out simultaneously for the five tests conducted on each individual test material. The average concentration of disinfectant released by each type of carrier was calculated by regressing the percentage of inhibition, corresponding to the log\(_{10}\) of known disinfectant concentrations, using the regression line of the dose–response curve. The antibacterial efficacy against \( E. coli \) W3110 of each disinfectant was defined as the lowest disinfectant concentration with the capacity to inhibit glucuronidase activity.

RESULTS

The antibacterial activities of the four disinfectants, as determined with the fluorescence bioassay based on the glucuronidase activity of \( E. coli \), are shown in Fig. 1. HP had the least inhibitory effect on glucuronidase activity, with a concentration of >300 mg/L being required to inhibit glucuronidase activity by 50%. In contrast, OPA and BNP produced 50% inhibition at concentrations of c. 1 mg/L, while the corresponding concentration of GTA was c. 20 mg/L.

According to the results of the toxicity bioassay, OPA and BNP were also the two substances that showed the greatest inhibitory effect (p < 0.05) on the glucuronidase activity of \( E. coli \) at the lowest concentrations. Higher concentrations of HP (up to 300-fold and 60-fold greater, respectively) and GTA (up to 20-fold and four-fold greater, respectively) were required to produce the same effect as BNP or OPA. The lowest concentrations at which the four test disinfectants were capable of inhibiting the glucuronidase activity of \( E. coli \) are shown in Table 1.

In the tests conducted to estimate the quantity of disinfectant released by each of the test materials, some residual inhibitory activity was detected with all five of the materials. Only OPA 0.5% v/v on latex and cotton, and HP 3% v/v on polypropylene and cotton, failed to show significant levels of inhibition (Table 2). The highest inhibitory activity was detected on glass disinfected with OPA 0.5% v/v, and polyurethane and latex disinfected with HP 3% v/v.

DISCUSSION

This study examined the residual toxic activity on five clinical materials of four disinfectants used commonly in hospitals. Although safety limits for the residual presence of BNP, GTA, HP or OPA on disinfected clinical materials have not been established, toxicity data are available [12].

In the hospital environment, use of BNP is confined to surface disinfection in high-risk areas. Bearing in mind its low working concentration (0.1% v/v), its exclusive use as a disinfectant of inert surfaces, its low toxicity [12] and the low residual levels of BNP detected in the fluorescence bioassay (0.002–0.226 μg/cm²), its use is unlikely to pose a health risk for patients or medical staff exposed to surfaces disinfected with this substance.

GTA, OPA and HP are used for the high-level disinfection of clinical devices and other hospital materials—e.g., probes, catheters or endoscopes—that come into contact with the mucous membranes of patients. GTA is probably the substance that has been associated most often with undesirable effects in patients. However, the individuals with the highest risk of exposure to this substance in the hospital environment are the personnel who use it for the sterilisation of medical equipment or as a laboratory reagent, to the extent that discontinuation of its use has been proposed [13]. As shown in Table 2, the least porous materials disinfected with GTA—polyurethane, polypropylene and glass—had greater residual inhibitory activity, which would correspond to a greater concentration of released disinfectant. In theory, 0.25 mg of disinfectant could be released after vigorous vortexing for 10 s of a 100-cm polyurethane catheter treated previously with GTA 2% v/v (Table 2). For a patient with a body weight of 60 kg, this would mean an intake of 0.002 mg/kg body weight, which is far below the established toxicity level [12]. The residual quantities released by the other test materials would be similar or even less.

More recently, OPA or HP—as gas plasma—have been suggested as alternatives to GTA 2% v/v for high-level disinfection [14]. Although
OPA may pose occupational hazards similar to those of GTA, the risk is reduced significantly by the low active concentration of OPA [15] and the relatively low vapour pressure of OPA-based commercial products. HP is a natural substance that leaves no potentially hazardous residues at the end of a sterilisation process [16]. Undesirable effects as a consequence of patients coming into contact with materials sterilised with HP have seldom been observed [17]. According to the results of the toxicity bioassay, the highest levels of toxicity (5.184, 3.681 and 1.761 g/cm², respectively) were detected for OPA and HP (Table 2). The porous materials (latex and cotton) were the materials with the lowest residual concentrations of OPA. Polypropylene and cotton had the lowest residual concentrations of HP.

As with GTA, the quantities of HP and OPA released by a 100-cm fragment of any of the test materials would be significantly below the established toxicity level [12]. However, it is important to note that the conditions used for the assay in the present study represent an artificial situation. Actual patient exposure would be difficult to determine, since, in addition to the type of material and disinfectant

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**Table 1.** Lowest concentrations of the four test disinfectants that inhibited the glucuronidase activity of *Escherichia coli*, as measured by the fluorescence assay.

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Concentration (mg/L)</th>
<th>Mean percentage inhibition ± SD</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-bromo-2-nitro-1,3-propanediol</td>
<td>0.001</td>
<td>17.98 ± 6.26</td>
<td>0.0007</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>0.02</td>
<td>9.61 ± 6.35</td>
<td>0.0105</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>0.3</td>
<td>14.85 ± 7.16</td>
<td>0.0103</td>
</tr>
<tr>
<td>ortho-Phthalaldehyde</td>
<td>0.005</td>
<td>11.12 ± 5.38</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

*Effect is expressed as percentage inhibition of glucuronidase activity in relation to controls without disinfectant. Five replicates were assayed with each disinfectant.

*Statistical significance compared to controls (without disinfectants); each parameter was analysed by paired Student’s t-test. p < 0.05 was considered to be statistically significant.

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**Fig. 1.** Effects of glutaraldehyde, ortho-phthalaldehyde, 2-bromo-2-nitro-1,3-propanediol and hydrogen peroxide on *Escherichia coli* β-glucuronidase activity. The symbols represent the individual five replicate tests conducted with each disinfectant.
used, exposure would depend on the precise clinical intervention (e.g., the duration of an endoscopy procedure) and the surface area of the disinfected material or device.

Other studies conducted with ethylene oxide or using cytotoxicity tests have shown differences—which are sometimes quite considerable—in residue concentrations detected on different materials [18,19]. Although the results cannot be compared directly, since they are based on different methods and chemicals, the findings of the present study do not agree with these previous results, insofar as it appeared that porous materials picked up and retained more chemical agents than non-porous materials. This may relate to the fact that the present study measured the concentrations of disinfectant agents released by the test materials, rather than the concentrations of agents adhering to them. Further studies are currently in progress to analyse this difference.

The toxicity test used in this study permitted the detection of very low disinfectant concentrations—as residues present on disinfectant materials—released after shaking in an aqueous medium for only 10 s. It was observed in a previous study using different disinfectants that an increase in the elution time did not produce a significant increase in the elution of disinfectant [11]. However, the toxicity test based on glucuronidase activity is readily adaptable to different circumstances and could be used as a rapid screening method to determine the presence of residual inhibitory activity stemming from the use of disinfectants, and in studies designed to establish the potential role of these residues in the prevention of infections produced by pathogenic microorganisms in hospitals.

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REFERENCES


