Effect of Methodology on the Tuberculocidal Activity of a Glutaraldehyde-Based Disinfectant

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Although official guidelines recommend a plate counting method for testing the susceptibility of mycobacteria to disinfectants, manufacturers usually prefer to employ the BACTEC procedure. Data showing that the BACTEC method overestimates the activity of a glutaraldehyde-based disinfectant against Mycobacterium tuberculosis in comparison with a conventional plate counting procedure are presented.

Since mycobacteria are known to be more resistant to disinfectants than other bacteria and there are numerous data describing their involvement in nosocomial infections, the interest of microbiologists and hygienists in evaluating the efficacy of disinfectants against mycobacteria has grown (4).

Nevertheless, there is a lack of an accurate standard mycobacterial test. From country to country, there are variations in official test protocols, from the strains (Mycobacterium tuberculosis or Mycobacterium smegmatis) to the methods (suspension or carrier tests) used (8). Besides, most manufacturers avoid following the official guidelines and prefer to study the efficacy of their products by using a posttreatment growth method that measures the release of 14CO2 (BACTEC) instead of a conventional plate counting method. The BACTEC method is preferred because it provides advantages in ease of use and speed, which is interesting when a slow-growing mycobacteria like M. tuberculosis is being tested.

Since several authors have suggested that results obtained with a BACTEC method are not correlated with those obtained when an official procedure is employed, we decided to evaluate the mycobactericidal efficacy of an alkaline glutaraldehyde solution by using these two kinds of methods (5, 6).

The strain selected as a test strain was M. tuberculosis, which was preferred to M. smegmatis, a fast-growing saprophytic mycobacteria, because it is pathogenic, involved in nosocomial infections, and more resistant to disinfectants than M. smegmatis (3, 7, 9). M. tuberculosis H37Ra (ATCC 25177), a clinical strain isolated from human sputum, was grown for 3 weeks on Lowenstein-Jensen medium (Sanofi Diagnostics Pasteur, Viroflay, France) incubated at 37°C. The culture was homogenized in sterile distilled water (10 mg/ml) for 1 min with sterile glass beads. This bacterial suspension was used to prepare additional 10-fold dilutions in sterile distilled water (10^-4 to 10^-10).

The disinfectant used was CIDEX (Johnson and Johnson, Vaucresson, France), a 2% alkaline glutaraldehyde solution which requires activation prior to use. It was prepared immediately before use, according to the manufacturer's recommendations, and then diluted in sterile distilled water to obtain a 0.2% solution. This concentration was selected to reduce the activity of the disinfectant so that surviving bacteria remained and their numbers could be determined by two different methods and then compared.

The filtration method and the BACTEC procedure were carried out with the same bacterium-glutaraldehyde mixture. The assays were repeated threefold. The triplicate experiments involved three different inocula.

The membrane filtration assay consisted first in enumerating the bacteria in the mycobacterial suspension. A 0.2-ml volume of each dilution of the suspension was filtered in triplicate through type HA 0.45-μm-pore-size membranes (Millipore Corp., Bedford, Mass.). Personal unpublished data show that 0.45-μm-pore-size filters trap the same number of mycobacteria that 0.22-μm-pore-size filters do. The membranes were then rinsed with 500 ml of sterile distilled water and then removed aseptically with forceps and placed on a plate containing Middlebrook 7H10 agar (Difco Laboratories, Detroit, Mich.) enriched with oleic acid-albumin-dextrose-catalase (Difco Laboratories). The plates were incubated at 37°C in sealed plastic bags, and colonies were counted after 4 weeks, in order to enumerate the bacteria contained in the mycobacterial suspension. Preliminary tests consisted of determining the volume of distilled water necessary to eliminate the disinfecting solution from the filtration membranes. We therefore used pretreated membranes: 2 ml of a 2% alkaline glutaraldehyde solution was filtered through membranes that were then rinsed with 500 ml of sterile distilled water. These pretreated membranes were then used to enumerate the bacteria contained in the mycobacterial suspension by the previously described procedure. The results obtained with nontreated and pretreated membranes were compared.

The assay was carried out in sterile glass bottles containing 5 ml of a 0.2% alkaline glutaraldehyde solution. The bottle was inoculated with 5 ml of the mycobacterial suspension so that the final concentration of glutaraldehyde was 0.1%. Five minutes exactly after the inoculation, 0.4 ml of the bacterium-glutaraldehyde mixture was filtered in triplicate through a type HA 0.45-μm-pore-size Millipore membrane, which was immediately rinsed with 500 ml of sterile distilled water to stop the activity of the disinfectant. Then, the membranes were placed on a Middlebrook 7H10 agar plate and the plate was incubated at 37°C. Colonies, corresponding to surviving bacteria, were counted after 4 weeks. The numbers of bacteria are the mean of the numbers counted on three plates.

The BACTEC assay consisted of enumerating the bacteria of the same mycobacterial suspension as the one used for the membrane filtration procedure. A 0.2-ml volume of each dilution was used to inoculate BACTEC 12B bottles (Becton Dickinson Europe, Meylan, France). The growth rate of the bacteria was measured with the BACTEC 460 radiometric system. The headspace gas produced was sampled daily for 30 days.

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and the radioactivity present was automatically measured and displayed as a growth index, from which the enumeration of bacteria was deduced.

After 5 min of contact, 0.2 ml of the bacterium-glutaraldehyde mixture previously described and used for the membrane filtration procedure was added to 35 ml of distilled water, so that the disinfectant was diluted enough to be ineffective (unpublished results). The suspension was centrifuged (3,000 rpm, 30 min), the supernatant was collected, and the bacteria were resuspended in 2 ml of distilled water. This new suspension was used to prepare additional 10-fold dilutions (10^1 to 10^6). A 0.2-ml volume of each new dilution was inoculated in a BACTEC 12B bottle, and the radioactivity was measured daily and converted into a growth index, which indicated the number of surviving bacteria.

Each of the three achieved a 1-log reduction in the number of bacteria with both the membrane filtration procedure and the BACTEC method. These results were compared by a paired Student t test.

For the membrane filtration method, the enumeration of the mycobacterial suspension with nontreated membranes was no different from that obtained with pretreated membranes which were rinsed with 500 ml of distilled water (1.65 × 10^7 UFC/ml versus 2.5 × 10^7 UFC/ml), indicating that 500 ml of distilled water is sufficient to eliminate the disinfecting solution from the membranes.

The enumeration of the bacteria in the same mycobacterial suspension by the BACTEC method resulted in 5 × 10^6 UFC/ml.

The reduction in viability, expressed as a log reduction, could be calculated for each method for each of the three assays (Table 1).

The statistical analysis (paired Student’s t test) showed that these data are significantly different (α = 5%), indicating that the BACTEC method overestimates the efficacy of the alkaline glutaraldehyde solution against M. tuberculosis H37Ra in comparison to the membrane filtration procedure (P < 0.05).

In several countries, the principle of quantitative suspension tests has been adopted for the official evaluation of the activity of disinfectants against mycobacteria. Whereas the BACTEC method is taken into account by none of the official guidelines, in the United States (Association of Official Analytical Chemists) as well as Europe (Association Française de Normalisation in France and Deutsch Gesellschaft für Hygiene und Mikrobiologie in Germany), some manufacturers choose not to evaluate the efficacy of their products by following an official procedure and prefer to use the BACTEC method (1, 2). Some reasons are well known: the implementation of the BACTEC method is simple, results are obtained in a short time period (about 2 weeks), and the BACTEC procedure enables the reduction of the cost of such studies, relative to that of an official method.

Some published data seem to show that results obtained with these two types of methods are different. Chantefort and Hocqueloux (5) noticed a difference when the minimal bactericidal concentrations of a disinfectant on M. tuberculosis were compared. He concluded that the BACTEC method was more favorable to the product, but the results he compared had been obtained with two different reference strains (ATCC 25177 and IP 2283604). Our results are in accordance with those described by Chantefort and Hocqueloux since the log reduction of bacteria after 5 min of contact with the disinfectant is greater with the BACTEC method than with a conventional plate procedure. Our results are all the more significant since comparative tests were carried out at the same time, using the same bacterium-disinfectant mixture.

Broadley et al. determined the antimycobacterial activity of a peroxygen disinfectant and showed that results obtained with a BACTEC method were different from those obtained with a plate method (6). They suggested that the disinfectant could damage without inactivating mycobacterial cells, thereby prolonging the lag phase or reducing the growth rate.

We can also make the assumption that, when the BACTEC method is used, some mycobacteria are killed or lost during the centrifugation so that the number of bacteria killed after exposure to the disinfectant seems to be falsely enlarged.

In conclusion, our results show that data obtained with the BACTEC method are not comparable to those obtained with a membrane filtration assay, when the activity of disinfectants against mycobacteria is evaluated. This must be kept in mind by people, and especially hygienists, who are responsible for selecting hospital disinfectants and thus have to compare the efficacies of several products, since the BACTEC method overestimates the activity of disinfectants against M. tuberculosis relative to the activity of a plate counting method based on official test procedures.

### TABLE 1. Numbers of surviving bacteria and log reductions of numbers of bacteria after 5 min of exposure to a glutaraldehyde-based disinfectant, evaluated by a membrane filtration procedure and a BACTEC method

<table>
<thead>
<tr>
<th>Assay</th>
<th>Membrane filtration</th>
<th>BACTEC</th>
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<tbody>
<tr>
<td></td>
<td>Surviving bacteria (UFC/ml)</td>
<td>Log reduction in no. of bacteria</td>
</tr>
<tr>
<td>1</td>
<td>1.5 × 10^5</td>
<td>2.04</td>
</tr>
<tr>
<td>2</td>
<td>3.5 × 10^5</td>
<td>1.67</td>
</tr>
<tr>
<td>3</td>
<td>7 × 10^4</td>
<td>2.37</td>
</tr>
</tbody>
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### REFERENCES