RESUMEN

El objetivo de este trabajo fue evaluar el recuento total de esporos viables en tiras inoculadas estandarizadas antes de ser sometidas a ciclo de esterilización. Se utilizaron muestras de “Bacterial Spore Sterilization Strip” (R. Biological Lab.) dentro de la fecha de vencimiento, que fueron divididas en: grupo A (B. subtilis) y en grupo B (B. stearothermophylus). Se testearon 24 tiras de cada grupo. Las tiras, en grupos de tres, fueron trituradas y colocadas en agua destilada refrigerada estéril. Luego se homogeneizaron las suspensiones en vortex durante 5 minutos y se transfirieron 10 ml de cada grupo a dos frascos estériles. Las muestras fueron calentadas en baño de agua a 95°C (Grupo A) o 80°C (Grupo B) por 15 minutos y se las enfrió rápidamente en un baño de hielo entre 0° a 4°C durante 15 minutos. Sucesivas diluciones fueron realizadas hasta obtener una alícuota final de 30 a 300 CFU (unidades formadoras de colonias) que fue colocada en placas de Petri con medio de cultivo (agar extracto de soja caseína fundido, adaptado para esporos y enfriado a 45–50°C) y incubadas a 55°C o 37°C. Statistical analysis of the data was performed. El número de esporos presentes a las 48 horas fue mayor que el número presente a las 24 horas, si bien estos resultados no fueron homogéneos en todos los grupos. Los datos fueron analizados estadísticamente. Los datos fueron analizados estadísticamente. El control del recuento de esporos viables pre-esterilización evitaría falsos resultados. La cantidad de esporos viables debe ser menor al 50% ni mayor al 300% del número de spores indicado en el control biológico. Este procedimiento es importante para garantizar la eficacia del control biológico. 

Key words: biological control, biological indicator, spores.

INTRODUCTION

Nickerson and Bluta (1) described a 51% failure rate of dental sterilizers and the actual sterilization process. Evidently, an alarming number of dental office sterilizers fail to kill the spores present in biological indicators (1). The physical, chemical and biological controls monitor the efficacy of the sterilization process. The biological control (BC) or Spore test is the most important of this set of tests. It evaluates the efficacy of the process of sterilization by analyzing the death of bacterial spores in biological controls (2, 3, 4, 5, 6, 7). To date, the use of biological controls is the most reliable method to guarantee the destruction of all forms of microbial life and forms of resistance. Efficacy of the procedure involves the absence of CFU in the samples and the possibility of confirming survival of 10° microorganisms, the level stipulated in quality control regulations for sterilization (3, 8).
These guidelines are in keeping with the regulations of the International Standardization Organization (ISO 1138-3/1995)(9). The test of viable spore counts (TVSC) is essential. Unexpected alterations in manufacture, transport, storage, etc. might cause the germination or death of the spores in the biological control (9, 10).

The present study describes the TVSC Test frequently used in our laboratory as a quality control procedure for the biological controls of the sterilization process. The aim of the present experimental study was to evaluate the amount of viable spores present in standardized biological controls prior to the sterilization process.

**MATERIALS AND METHODS**

**Microbiological study**

**Sampling**

Bacterial spore sterilization strips for biological control of sterilization were tested. Samples of “Bacterial Spore Sterilization Strip” (R Biological Laboratories) inoculated with $2.3 \times 10^6$ *Bacillus subtilis* and $1.6 \times 10^4$ *Bacillus stearothermophilus* were used well before their expiry date. Each strip was placed in a selectively permeable wrapping to allow the entry of the sterilizing agent but not of microorganisms that would contaminate the control and bias the results.

Two groups per microorganism and 16 subgroups corresponding to different incubation times and temperatures were evaluated.

**Sample processing**

Groups of three strips were minced and, maintaining the chain of asepsis, were placed in an Erlenmeyer with 100 ml of sterile chilled distilled water. They were then vortexed for 5 minutes to yield a homogenous suspension. Ten milliliters of the suspension were transferred to two sterile jars with a lid. The jars were heated in a water bath at a plateau temperature of 95°C and 80°C for 15 minutes and cooled rapidly in an ice bath at 0-4°C for 15 minutes. Successive dilutions in sterile distilled water were prepared to yield a final aliquot containing 30-300 CFU for each of the microorganisms in keeping with quality control regulations.

One ml of the suspension was placed in a Petri dish (15 x 100 mm) with 20 ml of culture medium (soy extract, casein agar adapted for spores, melted and cooled to 45°C, pH 7.3 ± 0.2).

The groups were incubated at 35-37°C or 55-60°C in aerobiosis. The Petri dishes were examined at 24 and 48 hours. The CFU were recorded.

In addition, the strips employed in the present study were exposed to the temperature assessed (80-95°C) and seeded in the same culture medium to analyze the “spore entrapment effect”.

The aim of the present study was to evaluate the amount of viable spores in biological controls prior to sterilization employing microbiological and spore entrapment effect analyses.

**Results**

**Analysis of aliquots**

Statistical analysis of the data was performed employing Poisson’s distribution. Each item was transformed in keeping with the following formula: $x' = \sqrt{x} + 0.5$

**RESULTS**

**Analysis of aliquots**

The final aliquots corresponding to all the microorganisms under study ranged between 30 and 300 CFU. However, the number of CFU at 48 hours was larger than at 24 hours in most of the groups. The values varied in some of the groups. The final mean value for each group was recorded.

Tables I and II show that the percentage recovery of spores and extrapolation to actual practical work were acceptable for groups 1, 2, 3, 6, 7 and 8 of *Bacillus subtilis* that were heated at 80°C and incubated at 37°C but not for groups 4 and 5 treated at the same temperature.

This microorganism failed to grow at 95°C.

For *Bacillus stearothermophilus*, heated at 95°C and incubated at 55°C, the percentage efficacy was evidenced by the percentage recovery of spores in groups 1, 2, 3, 7 and 8 but not in groups 4, 5 and 6 (*B. subtilis* $p< 0.05$ vs subgroups 4 and 5).

(*B. stearothermophylus* $p< 0.05$ vs subgroups 4, 5 and 6).

**Analysis of the percentage recovery**

Spore counts are acceptable when the percentage recovery is within 50-300% of the value indicated on the strip corresponding to the specific strain of the biological indicator employed.
**Bacillus subtilis** heated at 95°C and incubated at 37°C failed to grow. These findings are in keeping with its growth curve. **Bacillus stearothermophilus** inoculated in the appropriate culture medium and incubated in aerobicosis for 24 hours, grew at 55°C but not at 35/37°C. Dark field and phase contrast light microscopy analysis of the morphology of Gram stained microbial colonies was performed. Identification tests were carried out.

The spore entrapment effect was observed in the strips that were evaluated and then seeded.

**DISCUSSION**

Monitoring viable spores pre-sterilization would avoid erroneous results.

Variations in the lethality of the microorganisms in the indicators has been reported in several studies and for different commercial brands. This phenomenon has been observed for different lots of the same species and strain of a single supplier (10, 11). This difference in lethality has been attributed to differences in the carrier material and the “substrate effect” (10). Within this context we evaluated the spore entrapment effect.

The carrier material is an important issue. The values for filter paper were consistently lower than those obtained for fiber-glass inoculated with **Bacillus subtilis** when the indicators were employed to detect superheating. The resistance of these indicators to damp heat was relatively low. **Bacillus stearothermophilus** spores are highly resistant to steam but relatively insensitive to dry heat at high temperatures.

The present study showed that counting viable spores in standardized commercial strips before employing them in sterilization procedures should be a routine quality control step in the process of evaluation of the indicators.

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**TABLE I: Spore count per strip in Groups A and B.**

<table>
<thead>
<tr>
<th>Group A</th>
<th>N</th>
<th>Spore count per strip B subtilis*</th>
<th>Group B</th>
<th>N</th>
<th>Spore count per strip B stearothermophilus*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 control</td>
<td>3</td>
<td>2.3 x 10⁶</td>
<td>1 control</td>
<td>3</td>
<td>1.6 x 10⁴</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1.4 x 10⁶</td>
<td>2</td>
<td>3</td>
<td>1.7 x 10⁴</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>2.1 x 10⁶</td>
<td>3</td>
<td>3</td>
<td>1.6 x 10⁴</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1.0 x 10⁶</td>
<td>4</td>
<td>3</td>
<td>1.1 x 10⁴</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>1.1 x 10⁶</td>
<td>5</td>
<td>3</td>
<td>1.5 x 10⁴</td>
</tr>
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<td>6</td>
<td>3</td>
<td>1.7 x 10⁶</td>
<td>6</td>
<td>3</td>
<td>1.0 x 10⁴</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>1.1 x 10⁶</td>
<td>7</td>
<td>3</td>
<td>1.8 x 10⁴</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>2.2 x 10⁶</td>
<td>8</td>
<td>3</td>
<td>1.6 x 10⁴</td>
</tr>
</tbody>
</table>

*B subtilis H: Heating at 80°C / I: Incubation at 37°C  
B stearothermophilus H: Heating at 95°C / I: Incubation at 55°C

* Spore count per strip: D x 10² x CFU / n° of B.I.

**TABLE II: Microbiological evaluation of Groups.**

<table>
<thead>
<tr>
<th>Group A</th>
<th>N</th>
<th>% spore viability B subtilis</th>
<th>Group B</th>
<th>N</th>
<th>% spore viability B stearothermophilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 control</td>
<td>3</td>
<td>50-300%*</td>
<td>1 control</td>
<td>3</td>
<td>50-300%**</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>50-300%</td>
<td>2</td>
<td>3</td>
<td>50-300%</td>
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<tr>
<td>3</td>
<td>3</td>
<td>50-300%</td>
<td>3</td>
<td>3</td>
<td>50-300%</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>&lt;50%</td>
<td>4</td>
<td>3</td>
<td>&lt;50%</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>&lt;50%</td>
<td>5</td>
<td>3</td>
<td>50-300%</td>
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<tr>
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<td>50-300%</td>
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<td>&lt;50%</td>
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<tr>
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<td>3</td>
<td>50-300%</td>
<td>8</td>
<td>3</td>
<td>50-300%</td>
</tr>
</tbody>
</table>

Each item was transformed according to the following formula: x' = √(x + 0.5)

*B subtilis* p< 0.05 vs subgroups 4 and 5.  
*B stearothermophilus** p< 0.05 vs subgroups 4 and 6.
sterility chain. The use of strips without the adequate quantity of spores may bias the results.

In a previous study by our laboratory the strips were used close to their expiry date and the number of viable spores failed to reach the values informed by the manufacturer. As suggested by other authors, certain factors may alter the resistance of the inoculated microorganisms such as the expiry date, inadequate transport conditions, storage at temperatures in excess of 24°C, relative humidity above 70%, carrier and packaging instability, etc. (1, 12, 13, 14).

Spichery et al. demonstrated the convenience of using Bacillus subtilis and Bacillus stearothermophilus as bioindicators of the efficacy of the sterilization process (15).

Sapia (16) reported that the inoculated carriers should contain 10^4-10^9 bacterial spores for vapor sterilization and that the carriers should be made of medium speed filter paper, grades 740E or 591 A of Scheicher and Schulle (10, 16).

Shintani and Akersi (14) studied the factors involved in the preparation of bioindicators that affect the decimal reduction time. They demonstrated that the combined effect of salt in suspension during drying and entrapment of microorganisms in the cotton carrier increase the resistance values of the microorganisms. This effect also depends on the culture medium (10).

In our study, the incubation temperature was a condition of selectivity that influences the survival of the microorganisms in the biological controls. The microorganisms must have the same morphological and biochemical characteristics as the colonies of the strains employed in biological controls.

A faulty incubation may lead to an erroneous result (16, 17).

CONCLUSIONS

The present data would allow us to draw the following conclusions:

1. Monitoring the number of viable spores prior to sterilization would guarantee the accuracy of the results.
2. The number of spores in the biological control must be 50%-300% of the spore number informed by the supplier.
3. This test is essential to evaluate the efficacy of a biological control.

ACKNOWLEDGMENTS

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