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Assessment of biological indicators in the validation of isolator decontamination with hydrogen peroxide

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ABSTRACT

Isolators allow decontamination gases to be employed to create a sterile processing environment. This feature, added to the potential removal of human interference in the process, makes the use of isolators rather advantageous, compared to performing aseptic processes in conventional clean rooms. Decontamination with vaporized hydrogen peroxide (VHP) offers several advantages over other available methods, as it decomposes to water and oxygen and is thus easy to remove after use, is highly compatible with materials usually employed in production areas and it is relatively cheap. The aims of this study were to prove that *Geobacillus stearothermophilus* (ATCC 12980) is more resistant than microorganisms isolated from the normal production area flora and to determine the best material to serve as a support during validation of the decontamination of the inner surfaces of isolators and outer surfaces of materials inside them. *Bacillus* sp., *Micrococcus luteus, Corynebacterium, Staphylococcus* sp. and *Penicillium* sp. were the microorganisms of highest incidence among those identified in the production area. Stainless steel is the best material to be used as a support for the VHP treatment of specimens, as it is inert and the main component of isolators and showed no incompatibility with this sterilizing agent. The results obtained in this phase of the experiment proved that *Geobacillus stearothermophilus* is the most resistant microorganism with which to challenge the effectiveness of hydrogen peroxide, when tested against species of the normal flora. Secondly, the best support material is stainless steel, showing that the commercial bioindicators available on the market with this support material are scientifically proved to be the best choice for this purpose.

Keywords: Biological Indicator. Isolator. Hydrogen Peroxide.

INTRODUCTION

The technology associated with isolators, which are primarily used in the pharmaceutical industry to manufacture sterile products (Coles, 1998) and perform sterility tests (Coles, 1995; USP, 2008), has been gaining increasing prominence in recent decades. Gloveboxes, which were the first isolator prototypes, consisted of sealed boxes with restricted access through gloves and were intended to prevent the process coming into contact with the external environment and to protect the operator from possible exposure to the process (Shipley, 1998). Years later, laminar flow hoods using a HEPA (high efficiency particulate air) air filter became available on the market, raising the level of sterility assurance (Coles, 1998). Conventional barrier systems, such as gloveboxes and laminar flows, provide a certain amount of separation between the operator and the work environment but not complete segregation (Agalloco, 1999). Another disadvantage of conventional barrier systems is that they do not allow the work area to be sterilized but merely sanitized.

Isolators allow sterilizing gases to be used to create a sterile environment. This capacity, together with the possibility of eliminating human interference in the isolated process, means that isolation has a number of clear advantages over aseptic process operations in conventional clean rooms (Agalloco, 1999; Agalloco, 1995). The main advantages of using isolators in the manufacture of sterile products include: 1) increased sterility assurance level (SAL) (Agalloco, 1999; Agalloco, 1995; Dream, 1998), 2) elimination of the need for sterile clothing (Coles, 1998b; Agalloco, 1999; Agalloco, 1995), 3) reduction of environmental monitoring requirements (Coles, 1998b; Agalloco, 1999; Agalloco, 1995), 4) containment of toxic materials (Dream, 1998) and 5) the application and effectiveness of the decontamination process (Coles 1998b; Agalloco, 1999; Agalloco, 1995).

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Decontamination in isolators with vaporized hydrogen peroxide (VHP) has a number of advantages, such as the fact that the degradation products (water and oxygen) can be removed easily after decontamination, the high degree of compatibility of hydrogen peroxide with materials commonly used in production areas and its relatively low cost. VHP has been employed as an alternative to dry heat decontamination, sterilization and depyrogenation of medical devices and other products (Pinto, 1995; Okpara-Hofmann et al., 2005; Otter et al., 2007; Chung et al., 2008). The isolator decontamination process consists of four stages: 1) dehumidification; 2) conditioning; 3) decontamination and 4) aeration (VHP Validation Manual).

The purpose of this study was to prove that *Geobacillus stearothermophilus* (ATCC 12980) is more resistant than any of the microorganisms isolated in the normal production area and to identify the best material to serve as support during validation of the decontamination of the inner surfaces of isolators and outer surfaces of the materials placed inside them.

MATERIALS AND METHODS

Determining the microbial flora in the production area

The viable particles present in the production area, under *as built, at rest* and *operational* conditions, were identified and counted by means of passive and active sampling of the air and the surfaces of walls, equipment and floor (Agalloco, 1996).

Passive air samples were obtained by exposing settle plates containing Tryptic Soy Agar (soybean-casein digest agar) for 4 hours. The plates were incubated at 20-25°C for 2 days and at 30-35°C for a further 3 days. A model SAS Super 90 air sampler from PBI was used for active air sampling (USP, 2008).

Samples were taken from equipment, wall and floor surfaces with Rodac® contact plates and incubated as previously described (USP, 2008).

Identifying the most suitable material support for the biological indicator - calculating the D value

The first step was the preparation of 24 2x2-cm test coupons of each of the materials routinely used in the production area and inside the isolators; the coupons were cut and cleaned with detergent and 70% isopropanol. Table 1 shows the materials used in this study. After cleaning, 20 coupons from each type of material were inoculated with 10^6 CFU (viable spores) of *Geobacillus stearothermophilus*. The four remaining coupons were used as positive (contaminated with the spores but not exposed to the decontamination agent) and negative controls (not contaminated with the spores but exposed to the decontamination agent).

The isolator area was first subjected to dehumidification and conditioning. After 2 hours of the decontamination cycle, the coupons were exposed to the sterilizing agent, vaporized hydrogen peroxide (VAP), each pair of duplicate coupons being exposed for a different time (0, 1, 2, 3, 4, 5, 6 or 8 minutes). After the time of exposure had elapsed, each coupon was transferred by sterile tweezers into a tube containing Tryptic Soy Broth (TSB, soybean-casein digest medium), labeled with the time of the test, in duplicate. There were also negative controls (coupons exposed to H_2O_2 but not inoculated with spores), which were transferred into separate tubes (in duplicate), with the aim of testing the coupons for viability after being exposed to the VHP and during their transfer into the TSB and also to demonstrate that the combination of the support plus possible VHP residue would not impair the capacity of the medium to promote growth. While the test was being carried out, VHP concentration was monitored with a Draeger Polytron. Table 2 shows all parameters of the decontamination cycle.

Table 1: Description of the various types of material commonly found inside isolators and their uses.

Item	Description / Packaging
Silicone	Silicone ring used as a glass/window seal in isolators
Hypalon	Material used in gloves and long-sleeved gloves in isolators
Glass	Material used in isolator windows
Cloropel-Kevlar-Cloropel (CKC)	Material used in half-suits
Teflon	Ring for sanitary connections
Polypropylene	Material used in RTPs (Rapid Transfer Ports)
Stainless Steel	Material used in isolators and in supports for (commercial) test microorganisms
Latex	Gloves (an alternative to hypalon)
Nylon	Autoclavable bags
Polyvinyl Chloride (PVC)	Half-suits and flexible tubes

Table 2: VHP cycle parameters used in the experiment

Cycle Parameters	Program
Isolator volume (cubic feet)	20
Dehumidification air flow rate (cubic feet /min)	18
Conditioning air flow rate (cubic feet /min)	18
Sterilization air flow rate (cubic feet /min)	15
Aeration air flow rate (cubic feet /min)	18
Dehumidification target (mg/L)*	4.6
Injection rate in conditioning phase (g/min)	4.9
Injection rate in sterilization phase (g/min)	2.8
Dehumidification time (hours:min)	0:15
Conditioning time (hours:min)	0:02
Sterilization time (hours:min)**	5:00
Aeration time (hours:min)	1:00

*Initial (= atmospheric) humidity can cause the hydrogen peroxide to break down into water and oxygen, so dehumidification is necessary to reduce the water content to £ 4.6 mg/L.
**Enough time to allow the complete testing of all coupons.

After cycle completion, the positive controls (coupons inoculated with the spore and not exposed to VHP) were transferred into tubes of TSB in the laminar

flow hood. All tubes were incubated at 55-60 °C for 7 days and then observed in order to determine whether there was any microbial growth (Denyer & Baird, 1990). The experimental D-value on each material was calculated by applying the Stumbo-Murphy-Cochran formula for each type of material evaluated:

$$D = \frac{T}{L - \log\left(\ln\left(N/q\right)\right)}$$

where: ln = natural log, N = number of replicate units tested, q = number of sterile units, L = log (initial number of organisms per unit), T = exposure time (min) and D is the estimated time (in minutes) for 90% reduction.

The procedure described above was repeated (second run), but this time with a 10^2 CFU inoculum on each coupon of the various types of material, since several layers of microorganisms are formed when 10^6 CFU are used and this may lead to doubtful results.

It was observed visually, in all runs, that the nylon coupons disintegrated upon exposure to VHP. Additionally, it was also noted that rubber latex absorbed a great amount of VHP, as revealed by the fact that the positive controls did not show any growth. Therefore, these materials must not be used inside isolators that have VHP as the decontamination agent.

Identifying the most resistant microorganism for use as a biological indicator - calculating the D value

In the third run, thirty coupons of stainless steel were inoculated with suspensions containing 10^2 CFU of microorganisms isolated from the production area and *Geobacillus stearothermophilus* (ATCC 12980), following the same procedures as described above. Of these, 4 coupons were used as positive and negative controls. The coupons were placed in the isolator and subjected to the dehumidification and conditioning phases. Each coupon was exposed to VHP for 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24-minute periods and transferred to tubes containing TSB. The VHP concentration was again monitored during the cycle. The tubes were incubated at 30-35°C for bacteria, 20-25°C for fungi and 55-60°C for *Geobacillus stearothermophilus* (ATCC 12980) (Denyer & Baird, 1990). After the 7-day incubation period, the tubes were observed for the presence of any microbial growth, and the experimental D-value for each microorganism was calculated by means of the Stumbo-Murphy-Cochran formula (Sirch, 1998).

RESULTS

The most frequent microorganisms in the production area were *Bacillus* sp., *Micrococcus luteus*, *Corynebacterium* sp. and *Staphylococcus* sp. Although *Penicillium* sp. was not one of the most frequent, it was included in the study to serve as a representative mold. Figure 2 shows the incidence of each of the main microorganisms isolated in the production area, as a percentage of total sampling sites in each clean area class (FDA, 2003).

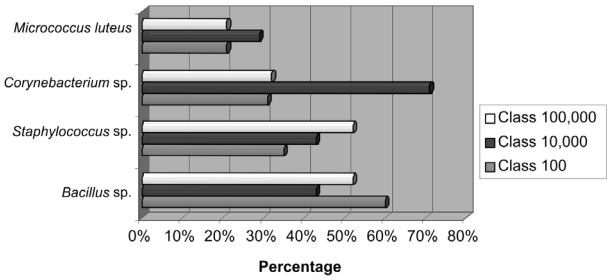

Figure 2. Incidence of the main microorganisms isolated from the production area based on percent sampling sites in each clean area class.

The D-values, calculated for each of the materials in the isolators, inoculated with 10^6 CFU (1st cycle) and 10^2 CFU (2nd cycle) of *Geobacillus stearothermophilus* (ATCC 12980), are shown in Table 3.

Table 3: Experimental D-values calculated for various types of material inside isolators inoculated with *Geobacillus stearothermophilus* (ATCC 12980).

Material tested	Experimental D value (minutes) 1st cycle	Experimental D value (minutes) 2nd cycle
PVC	> 1.28	0.06
Stainless Steel	> 1.28	0.06
CKC	> 1.28	0.06
Teflon	> 1.28	0.06
Polypropylene	> 1.28	0.06
Latex	> 1.28'	0.06
Silicone	> 1.28	0.06
Hypalon	> 1.28	0.06
Glass	> 1.28	0.06
Nylon	> 1.28	> 2.75
Aluminum bag	> 1.28	0.06

Table 4 shows the results for the inoculum size and D-value calculated for each microorganism inoculated on the stainless steel coupons. Monitoring of the VHP concentration yielded the results shown in Table 5.

Table 4: Size of inoculum and D-value calculated for each microorganism inoculated on the stainless steel coupons.

Microorganism	Inoculum size (CFU/unit)	Experimental D value (minutes) 3rd cycle
Bacillus sp.	1.9×10^2	0.05
Micrococcus luteus	1.7×10^2	0.05
Corynebacterium sp.	0.9×10^2	0.08
Staphylococcus sp.	1.1×10^2	0.05
Penicillium sp.	1.8×10^2	0.05
Geobacillus stearothermophilus	1.5×10^2	2.99

Table 5: VHP concentration measured during the decontamination cycle to determine the most suitable support and the most resistant microorganism for use as a biological indicator.

Decontamination time (hours)	*VHP concentration (ppm) 1st cycle	*VHP concentration (ppm) 2nd cycle	*VHP concentration (ppm) 3rd cycle
0	30	20	30
1	1465	1465	1460
2	1655	1670	1680
3	1710	1705	1710
4	1700	1660	1690
5	1620	1605	1610

*Concentration measured with a Draeger Polytron monitor

DISCUSSION

The biological indicator (BI) support has been shown to influence greatly the D-value. In addition, the nonwoven, high density, polyethylene pouch typically used to package BIs increases the resistance of the BI unit. Unpackaged BIs demonstrate a considerably lower resistance (Reich & Caputo, 2004). The FDA has suggested, in its draft aseptic processing guideline, that when evaluating the efficacy of an isolator decontamination procedure, an appropriate, quantified BI challenge should be placed on various materials (FDA, 2003), as was carried out in this experiment.

Despite the fact that materials containing cellulose were not tested in this experiment, it is known that cellulose absorbs moisture and thus should be avoided, as there is a risk that the sterilizing agent will be absorbed, giving rise to doubtful results. The use of materials containing nylon should also be avoided as it was found to decompose in the presence of hydrogen peroxide during the experiment. Such materials should be replaced by substances compatible with the VHP treatment. The same applies to materials containing rubber latex, as this was observed to absorb hydrogen peroxide, thus potentially affecting the distribution of the sterilizing agent.

The most suitable material to be used as a support in VHP treatment is stainless steel, because it is both inert and the main component used in isolators. Other types of material can be used as supports, as was observed in this experiment. However, because stainless-steel supports are readily available and can easily be treated, this is the preferred material for this application.

Several commercial BIs were tested in a calibrated VHP biological indicator evaluator resistometer (BIER) unit (Khorzad et al., 2003). A wide range of VHP resistance values have been reported among commercial BIs, with large disparities among the manufacturers' resistance claims (Reich & Caputo, 2004). The results obtained in the present phase of the experiment show that *Geobacillus stearothermophilus* (ATCC 12980) is the microorganism that is most resistant to the action of hydrogen peroxide and therefore the most suitable for use as a biological monitor.

CONCLUSION

The use of a biological indicator consisting of Geobacillus stearothermophilus (ATCC 12980) as a challenge microorganism on a stainless steel support is scientifically justified.

RESUMO

Avaliação de indicadores biológicos na validação de processos de esterilização de isoladores por peróxidos de hidrogênio

Os isoladores permitem a aplicação de descontaminação por gases, resultando em ambiente estéril. Esta característica, adicionada a possibilidade de não interferência humana no processo, torna o emprego dos isoladores consideravelmente vantajosa quando comparada com a performance dos processos em salas limpas convencionais. A descontaminação empregando peróxido de hidrogênio é vantajosa em relação a outros métodos disponíveis uma vez que é de fácil remoção após aplicação; sendo água e oxigênio seus produtos de degradação, apresenta boa compatibilidade com materiais usualmente empregados nas áreas produtivas; e seu custo é relativamente baixo. O propósito deste estudo foi demonstrar que o *Geobacillus stearothermophilus* **(ATCC 12980) é o microrganismo mais resistente quando comparado com microrganismos isolados presentes em áreas produtivas, assim como avaliar o melhor material a servir de suporte durante a validação do processo de descontaminação das superfícies internas do isolador e externas de materiais presentes.** *Bacillus* **sp.,** *Micrococcus luteus***,** *Corynebacterium* **sp.,** *Staphylococcus* **sp. e** *Penicilium* **sp. foram os microrganismos que apresentaram maior incidência na área produtiva. Aço inoxidável é o material mais adequado a ser usado como suporte para o emprego do peróxido de hidrogênio, por ser inerte e o principal componente dos isoladores e por não demonstrar incompatibilidade com o agente esterilizante. Os resultados obtidos nesta etapa do estudo demonstraram que o** *Geobacillus stearothermophilus* **é o microrganismo mais resistente para ser utilizado na avaliação da eficácia do peróxido de hidrogênio quando comparado com aqueles microrganismos encontrados na flora normal. Adicionalmente, o melhor suporte é o aço inoxidável, significando que os indicadores biológicos comercialmente disponíveis neste material são a melhor opção para este propósito.**

Palavras-chave: Indicador biológico. Isolador. Peróxido de hidrogênio.

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