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The efficacy and stability of five sanitizing agents challenged with reference microorganisms and clean area isolates

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ABSTRACT

The antimicrobial activity of five sanitizing agents employed in clean areas designated for the pharmaceutical manufacturing of sterile products was tested against nine microorganisms, including four microorganisms from the clean area microbiota. The method consisted of challenging 5 mL of each sanitizing agent - 70% isopropyl alcohol, 0.4% LPH®, 1.16% hydrogen peroxide, 4% hydrogen peroxide, 1% Bioper® and 5% phenol - with 0.1mL each of concentrated suspensions (10^5 – 10^6 CFU/mL) of *Staphylococcus aureus, Candida albicans, Corynebacterium* sp., *Micrococcus luteus, Escherichia coli, Aspergillus niger, Bacillus subtilis, Staphylococcus* sp. and *Bacillus* sp. for 10 minutes, followed by serial dilutions and plating. The results demonstrated that the five agents were effective against *S. aureus, C. albicans, Corynebacterium* sp., and *M. luteus*. The same was true of *E. coli*, except that isopropyl alcohol showed low levels of inactivation. With *A. niger*, isopropyl alcohol, 0.4% LPH® and hydrogen peroxide were more effective and 5% phenol and 1% Bioper® less effective. 1% Bioper® and 4% hydrogen peroxide showed greater inactivation of *Staphylococcus* sp., *Bacillus* sp. and *B. subtilis* than the other agents. Against *S. aureus, C. albicans, Corynebacterium* sp. and *M. luteus*, 5% phenol showed similar activity to other agents, while with *A. niger, B. subtilis, Staphylococcus* sp. and *Bacillus* sp., it was similar to or less active than the other agents. It was demonstrated that two microorganisms from the clean area microbiota, *Staphylococcus* sp. and *Bacillus* sp., were the most difficult to eradicate, requiring more frequent application of hydrogen peroxide and 1% Bioper® than the other strains.

Keywords: Biocides. Biocontrol. Disinfection. Pharmaceutical manufacturing. Sterilization.

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INTRODUCTION

Clean rooms are an essential element in the aseptic production of pharmaceutical products. The design, classification, installation, qualification, and monitoring of clean rooms are intended to attain and maintain an extremely low number of non-viable particles of any kind, organic and inorganic, as well as the total absence of viable particles (microorganisms). Both viable and non-viable particles are potential sources of contamination for parenteral formulations. Thus it is crucial to monitor the sound quality and adequate maintenance of the physical, chemical, and microbiological conditions of the production environment, to demonstrate a continuous state of control.

The microbiological safety of parenteral products is largely determined by the manufacturing process itself, including all auxiliary and complementary operations, by the maintenance of adequately prepared clean rooms and, last but not least, by the behavior of operators. In addition to the use of highly efficient air filters, physical barrier technology, intensive operator training and cleaning techniques, the use of effective sanitizing agents is essential to ensuring the environmental quality of clean rooms.

The production of parenteral formulations requires a high degree of skill and discipline, as it involves human beings who are responsible for maintaining the asepsis of the manufacturing environment, in order to manufacture microbiologically safe products.

The purpose of this study was to assess the efficacy of four sanitizing agents, employed on a daily and weekly basis to clean and sanitize clean room surfaces and equipment, against standard challenge microorganisms and those more frequently found in clean areas intended for pharmaceutical production. A 5% phenol solution was tested against the same microorganisms as an internal control.

MATERIALS AND METHODS

Material

The material employed in this study consisted of sanitizing agents prepared in a laboratory equivalent to the

production area of a pharmaceutical industry. These agents included a 70% solution of isopropyl alcohol, 0.4% LPH® solution, 1.16% and 4% hydrogen peroxide solutions, 1% Bioper® solution, and 5% phenol solution, all prepared in distilled water as instructed by the manufacturer. The standard challenge microorganisms were strains from the American Type Culture Collection (ATCC): *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Candida albicans ATCC 10231, Aspergillus niger* ATCC 16404 and *Escherichia coli* ATCC 8739. Environmental isolates from the pharmaceutical plant included *Bacillus* sp., *Staphylococcus* sp., *Corynebacterium* sp. and *Micrococcus luteus*. All microorganisms were cultured in Soybean Casein and Sabouraud Dextrose culture media, for bacteria and fungi, respectively. 1% Bioper® is a mixture of peracetic acid and hydrogen peroxide and LPH® is a mixture of *o*-phenylphenol and *p*-*tert*-amylphenol.

Method

Precision, standardization and reproducibility must be considered when developing a method to test disinfectants. The scientific value of the method and of the data analysis depend on many factors, such as inoculum size, culture media composition, temperature, concentration of active sanitizing ingredient, etc. All these variables must be reproducible (Cremieux & Fleurette, 1991).

Although official methods exist in the literature, published by AOAC (AOAC, 1995), AFNOR (AFNOR, 1988), DGHM (DGHM, 2003) and so on, the present method was chosen independently of previous publications. It was developed and tested locally by the lab team and met the criteria of precision, standardization and reproducibility referred to above, proving its scientific validity.

Sanitizing agents and microorganisms were prepared and all testing performed under laminar flow conditions, using previously sterilized material and distilled water. Soybean Casein and Sabouraud Dextrose, in agar or broth form (DIFCO), were prepared according to manufacturer's instructions and sterilized by steam at 121ºC for 15 minutes in a standard autoclave cycle.

Preparation of Microorganisms

The reference microorganisms were acquired from the ATCC in a lyophilized state. The sample was reconstituted and used to inoculate either Soybean Casein Broth, for bacteria, or Sabouraud Dextrose Broth, for yeasts and molds, and incubated for a minimum period of 24 hours at 30ºC-35ºC and not less than 72 hours at 20ºC-25ºC, respectively.

After the isolation and identification of 4 microbial strains during environmental monitoring, a sample was taken from a colony growthed in a Petri dish with a sterile inoculating loop, transferred into sterile Soybean Casein Broth and incubated for at least 24 hours at 30ºC-35ºC. Slants of Soybean Casein agar in assay tubes were then inoculated from the broth cultures and incubated at 30ºC-35ºC for a maximum period of 7 days.

The microorganisms were then harvested from the slants and serially diluted, aliquots of suitable dilutions being transferred to Petri plates for enumeration, using the culture media and incubation conditions described above. The tube containing the dilution corresponding to 10^5-10^6 colony forming units per mL (CFU/mL) was then selected to challenge the sanitizing agents. In cases in which the desired cell density (10^5-10^6) was not attained and the colony-counting plates did not reach 30 and 300 CFU, the next lowest dilution was selected in order to proceed with the experiment.

Preparation of Sanitizing Agents

All solutions were prepared with distilled water, as instructed by the manufacturer, and filtered through a membrane of pore size 0.45mm before use. The sanitizing agents were prepared and maintained at room temperature (20ºC-25ºC) for 4 weeks, in order to check the stability of their antimicrobial activity.

In each test, 5 mL of sanitizing agent was transferred to an empty assay tube and 0.1mL of a previously selected microorganism suspension was added, to yield a final concentration of 10^5 – 10^6 CFU/mL. The contact time was approximately 10 minutes, after which a series of decimal dilutions was prepared in pH 7.2 phosphate buffer and, after shaking, 1 mL of each dilution was transferred to duplicate agar plates, which were incubated under the previously stated culture conditions. This procedure was repeated for each sanitizing agent, testing the agent against each microorganism, every week for a period of four weeks.

The dilutions enabled the residual effects of the sanitizing agents on the microorganisms to be minimized. According to a preliminary series of tests, the addition of polysorbate and lecithin to the plate culture media also reduced these effects.

Soybean Casein Agar plates inoculated with bacteria were incubated at 30ºC-35ºC for not less than 24 hours. Sabouraud Dextrose Agar plates inoculated with fungi were incubated at 20ºC-25ºC for a minimum of 72 hours and a maximum of 7 days. After the experiment, the data obtained were subjected to statistical analysis.

The sanitizing agents were prepared in a quantity sufficient for 4 tests, each test being carried out once a week, in order to monitor the efficacy of the agents over time. They were stored in glass flasks at room temperature (20-25°C) for the four weeks.

RESULTS

The above data were treated statistically by specific methods chosen for the purposes of this study alone. Mean values, medians, standard deviations, minimum and maximum percentages of microorganisms inactivated were calculated for each agent. Also, the Kruskal-Wallis and the Mann-Whitney tests were used in specific situations, as described below.

Statistical Study of Efficacy

The statistical analysis started by calculating the mean values, medians, standard deviations, minimum and

maximum percentages of microorganisms inactivated by each sanitizing agent, as illustrated in Figures 1 to 9.

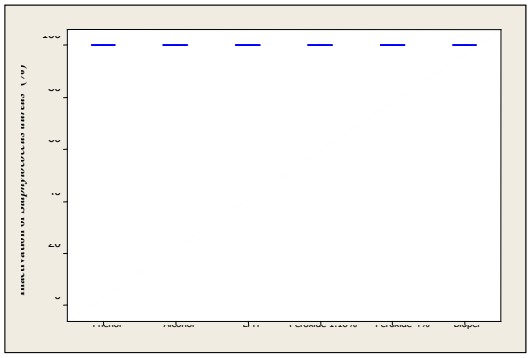

Figure 1. Boxplot of the percentage of *Staphylococcus aureus* inactivated by each sanitizing agent (based on results of the 1st, 2nd, 3rd and 4th tests).

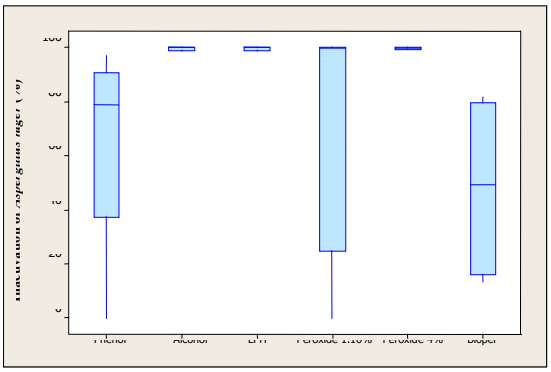

Figure 2. Boxplot of the percentage of *Aspergillus niger* inactivated by each sanitizing agent (based on results of the 1st, 2nd, 3rd and 4th tests).

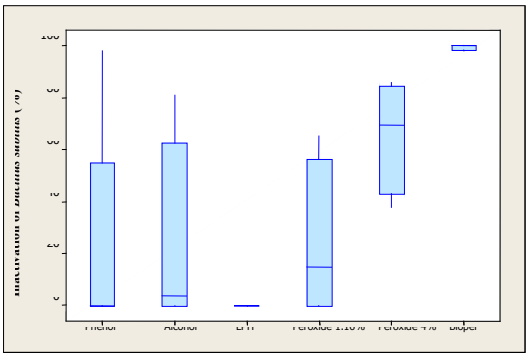

Figure 3. Boxplot of the percentage of *Bacillus subtilis* inactivated by each sanitizing agent (based on results of the 1st, 2nd, 3rd and 4th tests).

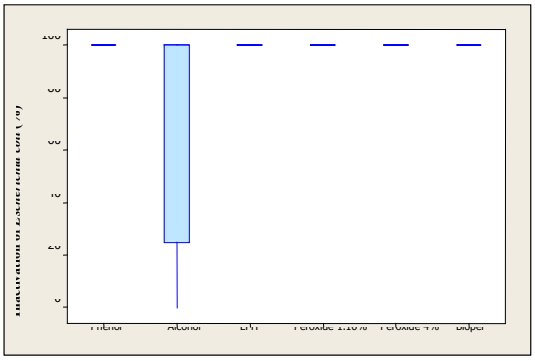

Figure 4. Boxplot of the percentage of *Escherichia coli* inactivated by each sanitizing agent (based on results of the 1st, 2nd, 3rd and 4th tests).

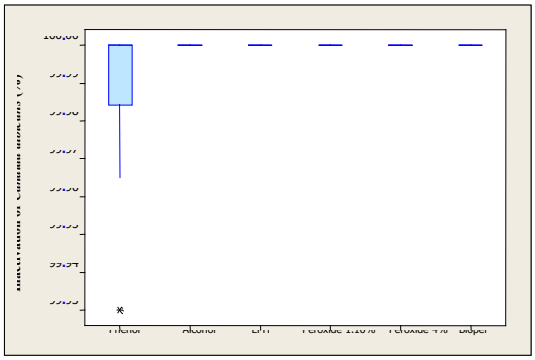

Figure 5. Boxplot on expanded scale of the percentage of *Candida albicans* inactivated by each sanitizing agent (based on results of the 1st, 2nd, 3rd and 4th tests).

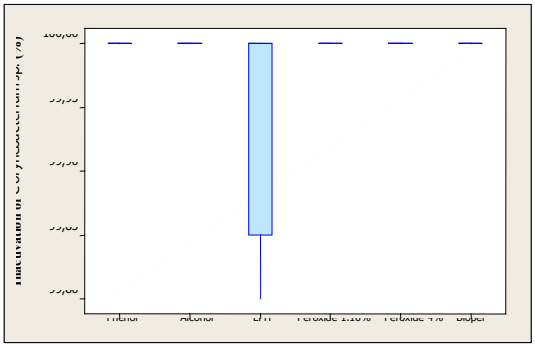

Figure 6. Boxplot on expanded scale of the percentage of *Corynebacterium* sp. inactivated by each sanitizing agent (based on results of the 1st, 2nd, 3rd and 4th tests).

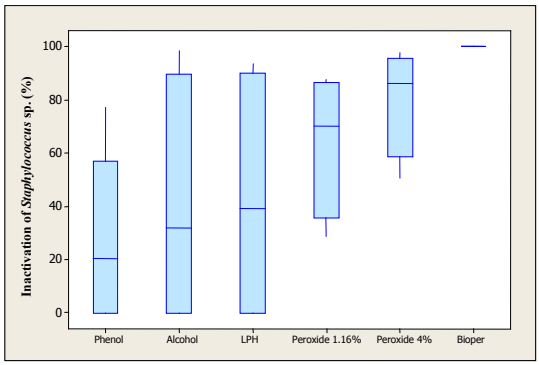

Figure 7. Boxplot of the percentage of *Staphylococcus* sp. inactivated by each sanitizing agent (based on results of the 1st, 2nd, 3rd and 4th tests).

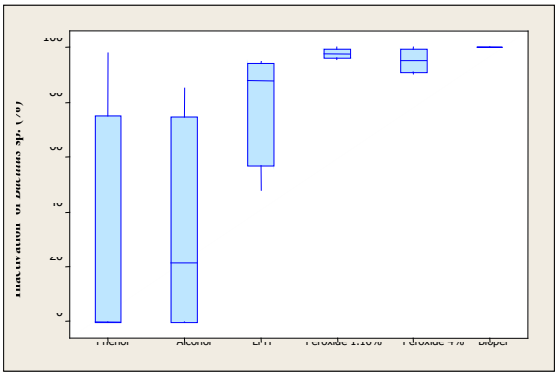

Figure 8. Boxplot of the percentage of Bacillus sp. inactivated by each sanitizing agent (based on results of the 1st, 2nd, 3rd and 4th tests).

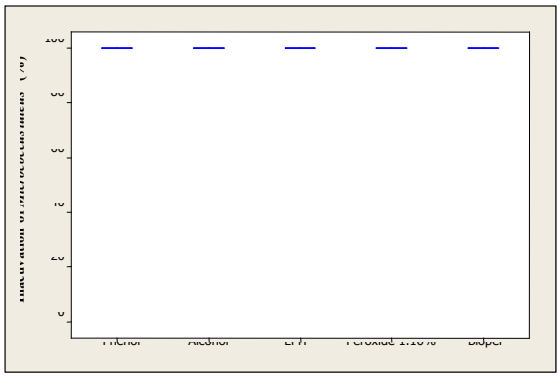

Figure 9. Boxplot of the percentage of *Micrococcus luteus* inactivated by each sanitizing agent (based on results of the 1st, 2nd, 3rd and 4th tests).

Examination of Figures 1, 5, 6, and 9 reveals that *S. aureus*, *Corynebacterium* sp., *M. luteus* and *C. albicans* were highly sensitive to the sanitizing agents tested. After the challenge, the percentage of microbial inactivation was about 100% for these organisms.

A. niger was very sensitive to the following sanitizing agents: 70% isopropyl alcohol, 0.4% LPH®, 4% hydrogen peroxide. The average percent inactivation of this mold by 5% phenol was 80%. 1% Bioper® and 1.16% hydrogen peroxide were less effective against *A. niger*, according to results shown in Figure 2.

1% Bioper®, followed by 4% hydrogen peroxide, was the most effective agent against *B. subtilis* and *Staphylococcus* sp.. The remaining chemical agents were not very effective at inactivating these bacteria, as can be seen in Figures 3 and 7.

All of the sanitizing agents inactivated *E. coli* efficiently, except 70% isopropyl alcohol. After the challenge procedures, the percentage of this microorganism eliminated was about 75% by 70% isopropyl alcohol and 100% by the other agents (Figure 4).

Bacillus sp. was sensitive to 1% Bioper®, 1.16% Hydrogen Peroxide and 4% hydrogen peroxide, followed by 0.4% LPH®, as indicated in Figure 8. 70% isopropyl alcohol and 5% phenol showed little efficacy in the elimination of this microorganism.

Owing to the small number of observations (n=4) for each chemical agent, and the lack of homogeneity of the agent variances, the Kruskal-Wallis non-parametric test was used to detect differences between the percentages of microorganisms inactivated by different agents. When the Kruskal-Wallis test indicated significant differences in percentages of inactivated microorganisms among various agents, the Mann-Whitney test was employed to identify the differences between two agents.

The boxplots of the antimicrobial activity of the various agents against *A. niger*, *B. subtilis*, *Staphylococcus* sp. and *Bacillus* sp. demonstrate how difficult it is to eliminate them.

The results varied widely and highly significant differences in the numbers of inactivated microorganisms were found for *A. niger*, *B. subtilis*, *Staphylococcus* sp. and *Bacillus* sp., as can be seen in Table 1. The Kruskal-Wallis tests were applied to these microorganisms whenever there were 3 or more agents to compare, and the Mann-Whitney test was used when 2 agents were being compared. The results are shown in Table 2.

Table 1. Results of Kruskal-Wallis tests for each microorganism, to detect differences in efficacy between sanitizing agents.

Microorganism	Degrees of freedom	Adjusted H	Significance level (p)
Staphylococcus aureus	5	0.00	1.000 NS
Aspergillus niger	5	22.15	0.000 **
Bacillus subtilis	5	17.30	0.004 **
Escherichia coli	5	3.27	0.659 NS
Candida albicans	5	5.45	0.364 NS
Corynebacterium sp.	5	3.53	0.619 NS
Staphylococcus sp.	5	16.72	0.005 **
Bacillus sp.	5	23.61	0.000 **
Micrococcus luteus	4	3.21	0.668 NS

NS: No significant difference, **: significant difference at 1%.

Analysis of the above results led us to the following observations concerning each microorganism:

• *Aspergillus niger*: no significant differences were found among the very high percentages of microorganisms inactivated by 70% isopropyl alcohol, 0.4% LPH®, 1.16% hydrogen peroxide and 4% hydrogen peroxide. There was no difference in the percentages of *A. niger* inactivated by 5% phenol and 1% Bioper® (approximately 50%), but these percentages differed significantly from those observed with 70% isopropyl alcohol, 0.4% LPH®, 1.16% hydrogen peroxide and 4% hydrogen peroxide (100% or almost 100% inactivation).

• *Bacillus subtilis*: no significant differences were observed among the percentages of microorganisms inactivated by 5% phenol, 70% isopropyl alcohol and 1.16% hydrogen peroxide. Significant differences were found between the percentages of microorganisms inactivated by 4% hydrogen peroxide and 1% Bioper®, as well as among the percentages inactivated by 70% isopropyl alcohol, 5% phenol, 1.16% hydrogen peroxide and 4% hydrogen peroxide (the latter proved the most effective).

• *Staphylococcus* sp.: no significant differences were found among the percentages of microorganisms inactivated by 5% phenol, 70% isopropyl alcohol, 0.4% LPH®, 1.16% hydrogen peroxide and 4% hydrogen peroxide. Significant differences were found in the percentages of microorganisms inactivated by 4% hydrogen peroxide and 1% Bioper®.

• *Bacillus* sp.: significant differences were found among the percentages of microorganisms inactivated by 1% Bioper®, 1.16% hydrogen peroxide and 4% hydrogen peroxide, even though all three agents achieved high percentages of inactivation. 1% Bioper® was the only agent that inactivated 100% of the organisms in every test.

Significant differences were also found when 5% phenol and 70% isopropyl alcohol were compared with 0.4% LPH®. The latter showed a high inactivation rate (80%), while the other two agents showed rates around 32% and 33%.

Table 2. Results of Kruskal-Wallis and Mann-Whitney tests for each microorganism, to evaluate differences in efficacy between sanitizing agents.

Microorganism	Test	Agents compared	Degrees of freedom	Adjusted H or W	Significance level (p)
Aspergillus niger	Kruskal-Wallis	70% Isopropyl Alcohol, 0.4% HPL®, 1.16% Hydrogen Peroxide, 4% Hydrogen Peroxide	3	1.06	0.786 NS
Aspergillus niger	Mann-Whitney	5% Phenol vs 1% Bioper®	-	257.0	0.615 NS
Aspergillus niger	Mann-Whitney	(70% Isopropyl Alcohol, 0.4% LPH®, 1.16% Hydrogen Peroxide and 4% Hydrogen Peroxide) versus (5% Phenol and 1% Bioper®)	-	497.0	0.000 **
Bacillus subtilis	Kruskal-Wallis	5% Phenol, 70% Isopropyl Alcohol, 1.16% Hydrogen Peroxide	2	0.07	0.963 NS
Bacillus subtilis	Mann-Whitney	(5% Phenol, 70% Isopropyl Alcohol, 1.16% Hydrogen Peroxide) vs 0.4% LPH®	-	361.0	0.352 NS
Bacillus subtilis	Mann-Whitney	(5% Phenol, 70% Isopropyl Alcohol, 1.16% Hydrogen Peroxide) vs 4% Hydrogen Peroxide	-	318.0	0.039 **
Bacillus subtilis	Mann-Whitney	4% Hydrogen Peroxide vs 1% Bioper®		10.0	0.027 **
Staphylococcus sp.	Kruskal-Wallis	5% Phenol, 70% Isopropyl Alcohol, 0.4% LPH®, 1.16% Hydrogen Peroxide, 4% Hydrogen Peroxide	4	7.82	0.098 NS
Staphylococcus sp.	Mann-Whitney	4% Hydrogen Peroxide vs 1% Bioper®	-	10.0	0.027 **
Bacillus sp.	Kruskal-Wallis	0.4% LPH®, 1.16% Hydrogen Peroxide, 4% Hydrogen Peroxide, 1% Bioper®	3	10.66	0.014 **
Bacillus sp.	Kruskal-Wallis	0.4% LPH®, 1.16% Hydrogen Peroxide, 4% Hydrogen Peroxide	2	5.35	0.069 NS
Bacillus sp.	Kruskal-Wallis	1.16% Hydrogen Peroxide, 4% Hydrogen Peroxide, 1% Bioper®	2	6.12	0.047 **
Bacillus sp.	Mann-Whitney	1.16% Hydrogen Peroxide vs. 4% Hydrogen Peroxide	-	20	0.665 NS
Bacillus sp.	Mann-Whitney	(1.16% Hydrogen Peroxide and 4% Hydrogen Peroxide) vs. 1% Bioper®	-	38.5	0.027 **
Bacillus sp.	Mann-Whitney	5% Phenol vs 70% Isopropyl Alcohol	-	166.0	0.877 NS
Bacillus sp.	Mann-Whitney	(5% Phenol and 70% Isopropyl Alcohol) vs 0.4% LPH®	-	223.0	0.031 **

NS = Not significant; ** = Significant difference at 1% level; H = Kruskal-Wallis statistic; W = Mann-Whitney (Wilcoxon) statistic

Results of efficacy over time

The results are summarized in Tables 3 to 7. The data demonstrated that the activity of 70% isopropyl alcohol against *A. niger* can fall over time. However, its activity remained steady against the other microorganisms. It was found that the activity of 0.4% LPH® remained steady over the four-week period, for all the microorganisms tested. The data demonstrated that 1.16% hydrogen peroxide lost its activity against *Bacillus* sp. over the four weeks, though its activity remained steady against the other microorganisms. The antimicrobial activity of hydrogen peroxide at 4% remained stable over time, for all the microorganisms tested, while that of 1% Bioper® decreased over time, with respect to *B. subtilis*.

Table 3. Log reductions obtained with 70% Isopropyl Alcohol

Microorganism	Log reduction/week				Observations
	1st	2nd	3rd	4th	
Staphylococcus aureus	3	4	4	5	Totally effective in the 4 weeks.
Aspergillus niger	3	2	1	2	Partially effective in the 4 weeks.
Bacillus subtilis	0	0	1	0	No efficacy in the 1st and 4th weeks. Little efficacy in the 2nd and 3rd weeks.
Escherichia coli	0	5	4	5	No efficacy in the 1st week. Totally efficient in the other weeks.
Corynebacterium sp.	3	5	4	5	Totally effective in the 4 weeks.
Candida albicans	3	5	4	5	Totally effective in the 4 weeks.
Staphylococcus sp.	0	1	0	1	No efficacy in the 1st and 3rd weeks. No significant efficacy in the 2nd and 4th weeks.
Bacillus sp.	0	1	0	0	No significant efficacy in the 1st week. Partially efficient in the 2nd week. No efficacy in the remaining weeks.
Micrococcus luteus	4	4	4	5	Totally effective in the 4 weeks.

Table 4. Log reduction obtained with 0.4% LPH®

Microorganism	Log reduction/week				Observations
	1st	2nd	3rd	4th	
Staphylococcus aureus	5	5	4	5	Totally effective in the 4 weeks.
Aspergillus niger	4	3	2	3	Partially effective in the 3rd week.
Bacillus subtilis	0	0	0	0	No efficacy in the 4 weeks.
Escherichia coli	5	4	5	5	Totally effective in the 4 weeks.
Corynebacterium sp.	5	4	4	3	Totally effective in the 4 weeks.
Candida albicans	5	3	4	5	Totally effective in the 4 weeks.
Staphylococcus sp.	0	1	0	0	No efficacy in the 1st and 4th weeks. Partially efficient in the 2nd and 3rd weeks.
Bacillus sp.	0	2	1	2	No significant efficacy in the 1st week. Very efficient in the 2nd week. Partially efficient in the remaining weeks
Micrococcus luteus	4	4	4	5	Totally effective in the 4 weeks.

Table 5. Log reduction obtained with 1.16% hydrogen peroxide

Microorganism	Log reduction/week				Observations
	1st	2nd	3rd	4th	
Staphylococcus aureus	4	5	5	5	Totally effective in the 4 weeks.
Aspergillus niger	0	3	4	3	No efficacy in the 1st week. Very efficient in the 2nd and 4th weeks. Totally efficient in the 3rd week.
Bacillus subtilis	0	0	0	0	No significant efficacy in the 1st and 4th weeks. No efficacy in the 2nd and 3rd weeks.
Escherichia coli	5	5	4	5	Totally effective in the 4 weeks.
Corynebacterium sp.	5	4	4	4	Totally effective in the 4 weeks.
Candida albicans	4	4	4	5	Totally effective in the 4 weeks.
Staphylococcus sp.	0	0	1	1	Partially efficient in the 1st and 2nd weeks. Very efficient in the remaining weeks.
Bacillus sp.	3	1	1	1	Very efficient in the 1st week. Partially efficient in the 2nd week. No significant efficacy in the remaining weeks.

Table 6. Log reduction obtained with 4% hydrogen peroxide

Microorganism	Log reduction/week				Observations
	1st	2nd	3rd	4th	
Staphylococcus aureus	5	4	5	5	Totally effective in the 4 weeks.
Aspergillus niger	2	2	4	4	Efficient in the 1st and 2nd weeks. Totally efficient in the 3rd and 4th weeks.
Bacillus subtilis	1	1	1	1	Partially effective in the 4 weeks.
Escherichia coli	5	4	5	4	Totally effective in the 4 weeks.
Corynebacterium sp.	4	3	4	4	Totally effective in the 4 weeks.
Candida albicans	4	3	4	4	Totally effective in the 4 weeks.
Staphylococcus sp.	0	2	1	1	Partially effective in the 4 weeks.
Bacillus sp.	2	1	3	2	More efficient in the 1st and 4th weeks. Partially efficient in the 2nd week.
Micrococcus luteus	4	4	4	5	Totally effective in the 4 weeks.

Table 7. Log reduction obtained with 1% Bioper®

Microorganism	Log reduction/week				Observations
	1st	2nd	3rd	4th	
Staphylococcus aureus	4	5	5	5	Very efficient.
Aspergillus niger	1	1	0	0	Little efficiency throughout tests.
Bacillus subtilis	5	4	5	2	Little efficiency in the last week.
Escherichia coli	5	5	5	5	Very efficient.
Corynebacterium sp.	4	4	5	5	Very efficient.
Candida albicans	4	5	5	5	Very efficient.
Staphylococcus sp.	4	4	5	5	Very efficient.
Micrococcus luteus	4	5	5	5	Very efficient.
Bacillus sp.	1	4	4	4	Little efficiency in the 1st week, but very efficient in the remainder.

DISCUSSION

According to the results reported here, in some situations the sanitizing agents did not inactivate all the inoculum of certain microorganisms within the period of time tested. In these cases, the size of the inoculum must be taken into consideration. At times, the probability of not eliminating the inoculum is proportional to its size (Anderson, 1990; Bagge-Raun, 2003; Bawden, 1982). However, the inoculum size employed in control tests should be large enough to permit the reduction in the number of microbial cells to be quantified (Krainiak, 1998).

Kang states that the level of lethality of an antimicrobial also depends on the level of stress microorganisms are subjected to. Injured are more susceptible than uninjured microorganisms to the lethality of a treatment (Kang et al., 2001). Furthermore, microorganisms can be stressed (metabolically injured) not only by chemical substances, such as antimicrobial agents, but also by physical processes, such as heat and UV radiation (PDA, 1986).

In a study of the sporulation and growth of the anaerobic bacterium *Clostridium welchii* (*perfringens*), Collee and collaborators (Collee et al., 1961) found that an apparent decrease of 50% in the number of cells occurred in the first hours of incubation in broth, followed by a significant increase in that number by 6 hours. They called this growth response the Phoenix Phenomenon, which seems to have three phases. The first, or injury, phase is characterized by a decrease in the microbial population. The second, or repair, phase involves an increase in the number of survivors from the first phase. The third, or growth, phase involves a large rise in the microbial population (Khadre & Yousef, 2001). It is thus possible to explain cases in which the chemical agent does not completely eliminate microorganisms, showing bacteriostatic or fungistatic activity, while the organisms eventually adapt and develop resistance to the previously adverse conditions.

Spores can survive for long periods of time in the presence of sanitizers, occasionally demanding an increase in the time of contact or concentration of the agent (Khadre & Yousef, 2001). However, microorganisms originating in the microbiota did not reveal any resistance. The microbial contamination of the clean area is highly susceptible to sanitizers, as sources of carbon and energy are scarce and, furthermore, viable particles are submitted to the antimicrobial activity of sanitizing cleaners; in other words, microorganisms isolated from the microbiota are stressed. *B. subtilis* and *A. niger*, as well as the *Bacillus* sp. and *Staphylococcus* sp. derived from the clean area microbiota, demonstrated a certain amount of resistance to the agents tested (Figures 2,3 and 8).

Kelsey and collaborators (Kelsey et al., 1974) have tested sporicidal chemical agents with *B. subtilis*, obtaining marginal results. These authors stated that experiments employing sporicides can produce good results, but these are often due to insufficient attention to the neutralization of sanitizing agent residual action. The neutralizing agent may inhibit growth and must be tested with the microorganisms employed in the challenge and with each concentration of the chemical agent tested. When the incubation conditions for microorganisms recovery are improved or prolonged, the microorganism shows growth, indicating greater resistance. Some sanitizing agents commonly used as sporicides are inefficient when tested under better incubation conditions for the microorganisms. According to Kelsey and collaborators (Kelsey et al., 1974), glutaraldehyde did not prove effective as a sporicide. Iodine derivatives were less effective than hypochlorites and 5% phenol derivatives showed low sporicidal activity (Kelsey et al., 1974).

The contact time and concentration of the agent also influence the level of inactivation of microorganisms. A shortened period of contact must be compensated by an increase in the concentration (Cremieux & Fleurette, 1991). Thus, when the concentration of hydrogen peroxide was raised to 4%, considered sporicidal, the antimicrobial activity also increased against *B. subtilis*, according to Figure 3. As for *Bacillus* sp., the activity of hydrogen peroxide at 1.16 and 4% was similar, showing that this isolate had lower resistance than than *B. subtilis*, which belongs to the same genus. This result coincides with Akers' statement (Akers & Agalloco, 2001), already noted, that microbiota isolates are easily eliminated.

However, increasing the concentration cannot be applied to alcohol, as studies employing a variety of methods reveal important peculiarities about alcohol as a germicide. It is one of the few chemical agents that are more efficient in a diluted form. The fact that 70% ethanol is more toxic to bacteria than other concentrations is due to the biochemical disorder provoked in the microbial cell by this mixture, which increases the bactericidal power of this agent when in contact with microorganisms.

The 10-minute contact time employed proved to be ineffective in destroying a high percentage of *Staphylococcus* sp. Hydrogen peroxide and 1% Bioper® were the most effective agents against this microorganism.

The example above and the varying levels of antimicrobial activity of sanitizing agents challenged with a wide range of microorganisms found in clean rooms has induced manufacturers to rotate application of the agents (Akers & Agalloco, 2001). The response of microorganisms to biocides is variable. Resistance can be inherent to a microorganism or acquired as a consequence of mutation or the transference of genetic material. Gram-negatives are usually more resistant than Gram-positive bacteria, since their cell wall has a more complex structure, less permeable to these agents (Fernandez et al., 2002).

According to Fernandez and collaborators (Fernandez et al., 2002), some results of low or practically no inactivation of *Bacillus* sp. may be explained by variations in the structure of its wall, as it can be Gram-variable.

However, development of resistance following the action of the agent is improbable. In contrast to antibiotics, which are selectively active against certain microbial species, disinfectants are generally toxic to all types of cells. The effectiveness of a particular agent is determined to a large extent by the conditions under which it is applied, including such factors as concentration, exposure time, pH, temperature, the nature of the test organism and presence of organic material (Cremieux & Fleurette, 1991). The greatest potential risk is that the agent does not eliminate all the microorganisms following application, which may lead to an increase in the microbial population. Despite this, the practice of rotating disinfectants has persisted for years (Akers & Agalloco, 2001; Athayde, 1999).

The quality of the water and cleanliness of the labware employed to prepare working dilutions of the agent and exposure of the latter to light and heat deserve special attention during its preparation, as they directly affect product stability. Exposure to light and heat can alter the efficacy and color of the product (Athayde, 1999). The material of which the flask used to prepare working dilutions of the agent is made must be compatible with the agent it contains. Glass and stainless steel recipients are the most appropriate, as plastic has several incompatibility problems.

The constitution of the containers in which the agents are maintained can also have an influence on the effect of preservatives, which may be absorbed, lowering the effective antimicrobial activity (PDA, 1990). Furthermore, low-density polyethylene offers no protection against the effects of light (Janik et al., 1977) and absorbs chlorinated phenols, as do polypropylene containers (Kelsey et al., 1974). On the other hand, polyurethane can reduce the activity of phenolics and quaternary ammonium compounds (Cowen & Steiger, 1997).

To ensure its full efficacy, a sanitizing agent must be free from adulteration arising from environmental contaminants. These could provoke molecular reactions and consequent degradation by oxidation and hydrolysis of the agent. Such factors can influence the potency of the agent since some microorganisms are able to survive under adverse conditions, by means of adapting themselves or synthesizing enzymes capable of converting agents into inactive compounds (Burlin, 2000).

The results indicate that the sanitizing agents tested maintained their antimicrobial activity for 4 weeks, with few occurrences of reduction of activity over time. Isopropyl alcohol suffered a loss of efficacy against *A. niger* in the last week and similar observations were recorded for 1.16% hydrogen peroxide and *Bacillus* sp. and 1% Bioper® against *B. subtilis*. Agent volatility may explain such results, especially for isopropyl alcohol. In this specific case, two or three applications of isopropyl alcohol with natural drying can be recommended (Fernandez et al., 2002).

Although the agents were tested for 4 weeks and their stability demonstrated for at least 3 weeks, it is stated in the literature that some diluted sanitizing agents, such as phenolic compounds at 0.5% and 1%, can exhibit continued efficacy up to 90 days. Quaternary ammonium compounds may be effective up to 1 month, while chlorine derivatives and oxidizing agents exhibit good activity for a week or less (PDA, 2001).

It must be emphasized that to maintain adequate cleanness and disinfection of clean areas, the maximum expected bioburden must be taken into account; typically, this should not exceed a range of 10^3 to 10^4 CFU/cm^2 . Where

the bioburden is greater, the limited ability of sanitizing agents to eliminate microorganisms may endanger process safety (Fernandez et al., 2002).

The results reflect just a part of a whole clean room validation in a pharmaceutical company. Much work was done before and after the sanitizing agent validation, involving the manufacturing area itself (eg. design qualification) and the laboratory (eg. methods validation and testing the sanitizing agents on some sample surfaces in the area). Nevertheless, even when the validation is finished, keeping the area clean is a challenge and requires the dedication of specialists to analyze the data gathered from the area on a routine basis.

The results demonstrated that the five sanitizing agents were effective against the following microorganisms: *S. aureus*, *C. albicans*, *Corynebacterium* sp. and *M. luteus*. Isopropyl alcohol exhibited similar levels of activity, except against *E. coli*. Isopropyl alcohol, 0.4% LPH®, and hydrogen peroxide were more effective against *A. niger*, while 5% phenol and 1% Bioper® exhibited marginal efficacy. 1% Bioper® and hydrogen peroxide at 4% showed higher activity against *Staphylococcus* sp., *Bacillus* sp. and *B. subtilis* than the other agents tested. Employed as a standard, 5% phenol exhibited similar high levels of efficacy against *S. aureus*, *C. albicans*, *Corynebacterium* sp., and *M. luteus*. However, its antimicrobial activity against *A. niger*, *B. subtilis*, *Staphylococcus* sp. and *Bacilus* sp. was similar or inferior to that of other agents. It was demonstrated that two of the microorganisms originating in the clean areas, *Staphylococcus* sp. and *Bacillus* sp., were the hardest to eliminate, requiring the use of hydrogen peroxide and 1% Bioper® to destroy them. All the agents tested were stable for a four-week period. A slight decrease in the antimicrobial activity was found in the last week with 70% isopropyl alcohol against *A. niger*, 1.16% hydrogen peroxide against *Bacillus* sp. and 1% Bioper® against *B. subtilis*. This could be explained in terms of the stability of the sanitizing agents, but further studies must be performed to investigate this hypothesis.

RESUMO

Eficácia e estabilidade de cinco agentes sanitizantes avaliados frente a microrganismos de referência e isolados de áreas limpas

O objetivo deste estudo é avaliar a atividade antimicrobiana de cinco agentes sanitizantes empregados em áreas limpas construídas para a fabricação de produtos farmacêuticos estéreis contra nove microrganismos, incluindo quatro microrganismos oriundos da área limpa. A metodologia constituiu em desafiar 5 mL de cada agente sanitizante, álcool isopropílico 70%, LPH® 0,400%, peróxido de hidrogênio 1,160% e 4%, Bioper® 1% e fenol 5% com 0,1 mL de suspensão concentrada (10^5 – 10^6 UFC/mL) de *Staphylococcus aureus, Candida albicans, Corynebacterium* sp., *Micrococcus luteus, Escherichia coli, Aspergillus niger, Bacillus subtilis, Staphylococcus* sp. e *Bacillus* sp. individualmente por 10 minutos, seguido de diluições seriadas e plaqueamento. Os resultados demonstraram que os cinco agentes sanitizantes foram efetivos contra *S. aureus, C. albicans, Corynebacterium* sp., e *M. luteus*. Os mesmos resultados foram observados com *E. coli*, exceto para o álcool isopropílico, que demonstrou baixos níveis de inativação. Contra *A. niger*, álcool isopropílico, 0.4% LPH® e peróxido de hidrogênio foram mais efetivos e fenol e Bioper® menos efetivos. Bioper® e peróxido de hidrogênio 4% demonstraram altos níveis de inativação de *Staphylococcus* sp., *Bacillus* sp. e *B. subtilis* quando comparados com outros agentes. Fenol demonstrou atividade antimicrobiana similar aos outros agentes contra *S. aureus, C. albicans, Corynebacterium* sp. e *M. luteus*. Contra *A. niger, B. subtilis, Staphylococcus* sp. e *Bacillus* sp., a atividade antimicrobiana do fenol foi similar ou inferior a dos outros agentes. Foi demonstrado que os microrganismos isolados da área limpa, *Staphylococcus* sp. e *Bacillus* sp., foram os que apresentaram maior dificuldade para inativar, sendo necessária a aplicação de peróxido de hidrogênio e Bioper®, com maior frequência.

Palavras-chave: Biocidas. Biocontrole. Desinfecção. Produção farmacêutica. Esterilização.

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