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Original Article

EVALUATION OF THE EFFICACY OF A HYDROGEN PEROXIDE DISINFECTANT

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ABSTRACT

Objective: To evaluate the efficacy of a disinfectant based on hydrogen peroxide.

Methods: The method used to assess the efficacy of the disinfectant was the agar plate technique. With this procedure, it was possible to determine the percentage of inhibition of the high-level disinfectant of STERIS against four microorganisms, i.e., *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* (Beta-Hemolytic 227), *Salmonella choleraesuis* (Kuznedorf CMDM 074), and *Bacillus subtilis* (ATCC 6633). The effectiveness of five disinfectant concentrations (0.02%, 0.04%, 0.08%, 1%, and 2%) was determined and evaluated in three different times 5, 10, and 15 min, for vegetative strains and 3, 6, and 9 h for the sporulated strain.

Results: According to the experimental test, the reduction of the microbial population was, on average, 100% for the disinfectant concentrations of 0.08%, 1%, and 2%.

Conclusion: The results obtained demonstrated that the high-level disinfectant of STERIS based on hydrogen peroxide is 100% effective when the concentration recommended by the commercial house (2%) is used in the shortest time exposure to disinfectant. The minimum level of effectiveness was 0.08%; however, if lower concentrations are used, destruction of the microorganisms is not guaranteed.

Keywords: Bactericidal activity, Effectiveness, Antiseptic, The percentage inhibition

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INTRODUCTION

In all fields of research, in which chemical products must be used, a disinfectant is required to reliably prevent microbial contamination and keep surfaces and work equipment clean at the same time. Cleaning and disinfection, together with sterilization, constitute the primary and most effective elements to break the epidemiological chain of infection. Hospital infections are a topic of relevant interest due to their frequency, severity, and economic repercussions.

The disinfection of instruments and surfaces of workstations, precisely in the laboratory where scientists analyze numerous biological samples, require the use of disinfectants to avoid possible contamination [1].

Hospital-acquired infection (HAI) is one of the critical concerns because it takes a heavy toll on patients and their families, as it causes illness, prolongs hospital stays, reduces the quality of life, increases the potential of disabilities, and increases the resistance of the microbes to antimicrobials as well as leading to excess costs and sometimes death of the patient. Education and training of healthcare workers about standard infection control and strict adherence by healthcare staff to aseptic practice can reduce the extent of risks of HAI. Rational use of disinfectants leads to a substantial reduction in HAI and requirement of disinfectants [2].

Hospital care poses the highest hygiene requirements because medical devices and surgical instruments, such as implants, have direct contact with body fluids. Poor hygiene of these instruments can cause irreversible infections in immunosuppressed patients or death. For this reason, it is necessary to use a disinfectant that is effective not only in eliminating the microorganisms quickly and reliably but also avoiding harm to the patient's health and eliminating unpleasant odors [3]. For the development of this research, the high-level disinfectant of STERIS, with a hydrogen peroxide base, was evaluated in *in vitro* tests that guarantee its use in instruments and medical devices in the hospital sector.

MATERIALS AND METHODS

Study population

High-level disinfectant of STERIS, based on hydrogen peroxide, was used in the disinfection of equipment and devices for hospital purposes.

Study design

This research is descriptive analytic research with quantitative methods.

Sampling

The study was carried out facing strains of the following microorganisms (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella choleraesuis*, *Bacillus subtilis*) at different disinfectant concentrations. The strains were provided by the Pontificia Universidad Javeriana, Bogotá, Colombia. Different concentrations of the disinfectant were evaluated against diverse microbial strains. Two repetitions were made for each microorganism with the three levels of the disinfectant. The contact times of the disinfectant versus microorganism was 5, 10, and 15 min for vegetative bacteria and a period of 3, 6, and 9 h for the sporulated strain. Using different times helped us to determine if the recommended time established by the disinfectant house production is appropriate or if it is necessary to increase the exposure time when applying the disinfectant on equipment [4].

Study variables

Dependent variables

Percentage of microbial inhibition of each concentration of the evaluated disinfectant will be calculated using the average inhibition of the replicas of each microorganism assessed.

Independent variables

The different concentrations at which the disinfectant agent was evaluated (recommended level, half, and double). 2. The different exposure times for the three bacterial strains 5 min, 10 min, and 15 min; 3, 6 and 9 h for the sporulated strain.

Controls

To evaluate disinfectants, methods such as phenolic coefficient, plate count, tube dilution, and determination of the minimum inhibitory concentration (mic), among others are used. This work used as a positive control (mic) a 90% alcohol suspension to confront the microorganisms at the same times to verify the inhibition. As a negative control, this work used a suspension of 0.85% saline solution to confront the microorganisms at the same time to verify the growth.

Statistical model

The data obtained during the investigation were statistically analyzed by the Mann–Whitney, and Kruskal–Wallys test with a confidence level of 75%. In this way, the effectiveness of the different concentrations of the disinfectant was determined, compared with the microorganisms mentioned in the technical sheet.

Microorganisms

The microorganisms evaluated were *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* beta-hemolytic, and *Salmonella choleraesuis,* which were preserved in Petri dishes at 5 °C and made a microscopic identification using the Gram stain. The microorganisms were provided from the Pontificia Universidad Javeriana Bogotá, Colombia.

Disinfectant

It was supplied by STERIS, the manufacturer, and prepared according to its recommendations.

Culture media

Brain heart infusion (BHI) broth was used to inoculate the microorganism and reproduce it; BHI agar was used as a culture medium for sowing and recovery of *Salmonella choleraesuis*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*.

Inoculation preparation

(preparation of microorganisms for testing): The bacterial suspension was standardized following the CLSI guidelines and was grown in BHI broth, for 18–24 h; the suspensions of the microorganisms were prepared in saline solution at 0.85% (w/v) in a tube of 13x100 mm, whose final concentration should be 6 x10^8cells/ml. In a 16x150 mm tube with BHI broth, the suspension of microorganisms prepared in saline solution was inoculated. The inoculum was incubated at 37 °C for 24 h. At the end of the incubation time, the purity of the strains was verified using Gram staining, and a count was carried out to determine the exact value of each of the numbers, ensuring that they were at 10^8. In the case of *Bacillus subtillis*, it was incubated at a temperature of 37 °C for 72 h. From this inoculum, the tests for the evaluation of the disinfectant were carried out [5].

Preparation of disinfectant concentrations

Disinfectant concentrations were prepared at 0.02%, 0.04%, 0.08%, 1%, and 2% to perform *in vitro* tests for the effectiveness against the bacterial strains.

Disinfectant evaluation

Efficacy of the disinfectant was evaluated at different concentrations and different times against the established microorganisms, according to [6].

Five levels of the disinfectant were previously prepared at the recommended dosage by the commercial house, at the half of the recommended dose and twice the recommended dose.

2 ml of each disinfectant concentration was evaluated in test tubes.

0.2 ml of the suspensions with the established microorganisms were inoculated into each tubes with the disinfectant concentrations.

Tubes were then shaken for 5, 10, and 15 min.

Subsequently, the suspension with the disinfectant and the microorganisms were dispensed in Petri plates with BHI agar for 48 h, and a plate count was carried out to verify the inhibition percentages from the initial count [6, 7].

Reading and interpretation

After the incubation time, the samples were read, and the plate count was made.

Control

For the negative control, we expected to have grown around 10^8CFU, and the positive control boxes should show growth inhibition of 100% [6, 8].

The results obtained through this experimental test were examined using a descriptive analysis through tables and graphs, where the growth of each microorganism is observed after being exposed to each of the concentrations and evaluation time of the disinfectant. In this way, the best disinfectant concentration that inhibits the growth of each microorganism was established.

For this purpose, the following hypotheses were tested:

Null hypothesis (Ho)

The percentage of inhibition of the evaluated concentrations (0.02%, 0.04%, 0.08%, 1%, and 2%) of the disinfectant is ≥ than 75%.

Alternate hypothesis (Hi)

The percentage of inhibition of the concentrations evaluated (0.02%, 0.04%, 0.08%, 1%, and 2%) of the disinfectant is<75%.

Decision

For all probability values equal to or less than 0.05, the alternative hypothesis is accepted; therefore, the null hypothesis is rejected. Conclusively, the percentage of inhibition is not the same in the three evaluated concentrations of the disinfectant, at least one is different.

RESULTS

Inhibition percentage

Table 1: Percentage of inhibition of each microorganism against different concentration of the disinfectant

Concentration	*Staphilococcus aureus*			*Pseudomonas aeruginosa*			*Salmonella chlorerasuis*			*Bacillus subtillis*		
	5 Min	10 Min	15 Min	5 Min	10 Min	15 Min	5 Min	10 Min	15 Min	3 h	6 h	9 h
0.02%	0	0	25	0	0	28.12	0	0	42.15	50	50	87.25
0.04%	0	43.75	100	87.5	100	100	0	62.5	100	68.75	96	100
0.08%	100	100	100	100	100	100	100	100	100	100	100	100
1%	100	100	100	100	100	100	100	100	100	100	100	100
2%	100	100	100	100	100	100	100	100	100	100	100	100

Data from table 1 correspond to the average of the replicates and show the inhibition rates over time of the strains used against the different concentrations of disinfectant

Table 2: Comparison of the behavior of the strains in connection with the concentrations of the disinfectant

Microorganism	Inhibition 0.02%			Inhibition 0.04%			Inhibition 0.08%			Inhibition 1%			Inhibition 2%		
Exposition time in minutes	5	10	15	5	10	15	5	10	15	5	10	15	5	10	15
Staphilococcus aureus	0	0	25	0	44	100	100	100	100	100	100	100	100	100	100
Pseudomonas aeruginosa	0	0	28	88	100	100	100	100	100	100	100	100	100	100	100
Salmonella chlorerasuis	0	0	42	0	63	100	100	100	100	100	100	100	100	100	100
Exposition time in minutes	3	6	9	3	6	9	3	6	9	3	6	9	3	6	9
Bacillus subtillis	50	50	100	69	96	100	100	100	100	100	100	100	100	100	100

Comparison of the percentages of inhibition of the different strains concerning the concentrations of disinfectant can be observed in table 2

Evaluation of the efficacy of the disinfectant against micro-organisms

The table 1, show the effectiveness of the high-level hydrogen peroxide-based disinfectant of STERIS in the presence of *Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella choleraesuis, and Bacillus subtillis.*

DISCUSSION

For the disinfectant concentration of 0.02%, there is no statistically significant evidence that the percentage inhibition of the disinfectant against microorganisms (*Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Salmonella choleraesuis*), for the three exposure times (5, 10, and 15 min) is greater than 75% because of $P>0.05$. However, for the concentration of 0.04%, there is statistically significant evidence that the percentage of inhibition of the disinfectant before microorganisms is higher than 75%. $P<0.05$ at 15 min of exposure, except for *Pseudomonas aeruginosa*, presents a percentage of inhibition higher than 75% at 5 and 10 min. For the concentrations of 0.08, 1%, and 2%, the average inhibition was 100%; therefore, the disinfectant was 100% at 5, 10, and 15 min of exposure. In the case of *Bacillus subtillis,* at 0.02% disinfectant concentration, there is no statistically significant evidence that the percentage inhibition of the disinfectant at the three exposure times (3, 6, and 9 h) is greater than 75% because $P>0.05$. However, for the concentration of 0.04%, there is statistically significant evidence that the percentage of inhibition of the disinfectant against microorganisms is higher than 75% because $P<0.05$ for 6 and 9 h of exposure. For the concentrations of 0.08, 1% and 2%, the average was 100; therefore, the disinfectant is effective at that concentration in 100% at 3, 6, and 9 h of exposure. In all cases, the control showed the expected results with a 100% inhibition of microorganisms.

The tests carried out to determine the efficacy of the high-level hydrogen peroxide-based disinfectant of STERIS in the presence of *Staphylococcus aureus* are shown in table 1. The disinfectant concentration of 0.02%, with an exposure time contact of 5, 10, and 15 min, demonstrated no bactericidal action above the minimum acceptance criterion established 75%, for this microorganism.

At 0.04% disinfectant concentration, an increase in the percentage inhibition was observed, which does not meet the minimum acceptance criterion of 75% in an exposure time of 5 min. However, when testing the concentration of 0.08%, a 100% inhibition percentage was reached for the three established times. After 10 min of exposure at the 0.02% concentration, *Staphylococcus aureus* did not show an increase in the percentage of inhibition; comparatively, when testing the concentration of 0.04%, an inhibition percentage of 43.75% was obtained, and, at a level of 0.08%, total inhibition of growth was evidenced. After 15 min of contact with the disinfectant, an increase of 100% in the percentage of inhibition was observed with the concentration of 0.04%, in the same way with a level of 0.08%.

As shown in table 1, the bacterial population is reduced in its totality, reaching a 100% inhibition percentage, after 5 min of treatment with the high-level hydrogen peroxide-based disinfectant of STERIS, when they were used in half the concentration (1%) and the concentration recommended by the commercial house (2%). These data show us that the partial or destruction of *Staphylococcus aureus* when put in contact with the disinfectant under study is determined by the concentration of the disinfectant and by the time of exposure. The reason why *Staphylococcus aureus* is not inhibited at concentrations of 0.02% and 0.04% with the high-level disinfectant of hydrogen peroxide-based STERIS is because this microorganism produces the extracellular enzyme catalase. This enzyme breaks down the hydrogen peroxide in water and molecular oxygen when this compound is in small amounts, according to [9] (Linley *et al.*, 2012) in more significant quantities, under experimental conditions *Staphylococcus aureus* is inhibited by the accumulation of hydrogen peroxide in the medium.

Table 1 shows the results of the percentage of inhibition of the disinfectant evaluated against *Pseudomonas aeruginosa*. These results indicate that at 5 min with a concentration of 0.02%, there was no inhibition of the microorganism. When the microorganisms were subjected to a concentration of 0.04%, a decrease in the viability of the microorganism was observed as a function of the time of exposure to the disinfectant because a value of 88% was obtained after 5 min, thereby complying with the minimum acceptance criterion of 75%. The disinfectant is 100% effective when 0.08%, 1%, and 2% concentrations were used. For a contact time with the disinfectant for 10 min at a concentration of 0.02%, there was no evidence of inhibition, while, at the concentrations of 0.04%, 0.08%, 1%, and 2%, the percentage of inhibition was 100%. At 15 min with a concentration of 0.02%, there was a small amount of inhibition (table 1), whereas, with the concentrations of 0.04%, 0.08%, 1%, and 2%, the inhibition was absolute.

These results show that, for this strain, the concentrations are recommended at 0.08%, 1%, and 2% because they presented a significant percentage inhibition at 5, 10, and 15 min even in the shortest time, while a concentration of 0.02% and 0.04% is not recommended for use because the destruction of the microorganism is not guaranteed in any of the three exposure times. In table 1, the total reduction of microbial growth is observed, when a concentration of 1% and the recommended concentration of 2% is used, for a contact time of 5 min. These results indicate that the high-level disinfectant of STERIS, based on hydrogen peroxide, has a bactericidal effect for this microorganism using the recommended concentration. The results obtained indicate that *Pseudomonas aeruginosa* does not show resistance when it is subjected to a concentration of disinfectant based on 2% hydrogen peroxide, which complies with the guidelines established by the commercial house. According to the consulted bibliography, this microorganism is sensitive to hydrogen peroxide, an active component of the disinfectant under study because this generates a disturbance of the components of the cell membrane. A disturbance is also generated in chemiosmosis, which is the diffusion of ions across a permeable membrane, causing an alteration in the transport membrane and further causing damage to a cell wall [6,10]. *Pseudomonas aeruginosa* can present resistance by several mechanisms such as the variation in the composition of lipopolysaccharide (LPS) and the content of cations such as magnesium, which produces stable bonds between molecules of LPS and as a complement to this mechanism. This bacterium presents small porins that prevent the passage through the diffusion of certain antimicrobial substances [11]. Also, *Pseudomonas aeruginosa* can form glycocalyx, which is a polysaccharide or glycoprotein that covers the cell wall of this microorganism, thus forming a barrier against disinfectants [6, 12].

The results of the percentage inhibition of the high-level hydrogen peroxide-based disinfectant of STERIS against *Salmonella choleraesuis* is shown in table 1, where the data demonstrates that at

a concentration of 0.02%, in contact time between 5 and 10 min, the disinfectant has no inhibitory effect on this microorganism. Although a concentration of 0.04% shows an increase in the percentage of inhibition, it does not meet the minimum acceptance criterion of 75% for an interval between 5 and 10 min because an inhibition percentage between 0 and 63% was obtained. However, a total reduction of 100% was measured, when concentrations of 0.08%, 1%, and 2% were used. At 15 min of contact between the microorganism and the disinfectant, an increase in the percentage of inhibition of 42.15% was observed with a concentration of 0.02% (table 1). When using a concentration of 0.04% and a concentration of 0.08%, a 100% inhibition percentage was obtained. Table 1, shows the average of inhibition of the disinfectant against *Salmonella choleraesuis*, expressed as a percentage, in which it is observed that the bacterial population is reduced in its totality, reaching a level of 100% inhibition when a concentration of 1% is used, half of the recommendation and a concentration of 2% recommended by the commercial house with the minimum time of exposure. The low percentages of inhibition obtained when the levels of 0.02% and 0.04% were tested can be explained because this microorganism has several mechanisms that can elude the action of antimicrobial agents; for example, they can modify the cell membrane, making it less permeable to antimicrobials. They also have specific enzymes that modify or inactivate antimicrobials. Further, the resistance may be due to the action of a flow pump or modifications of the cell wall [3,7,8,10].

Table 1 shows that, based on these results, we can say that, when a 0.02% disinfectant concentration is used for *Bacillus subtilis* microorganisms, a 50% reduction in the population is achieved for a contact time between 3 and 6 h, and a decrease of 87% for 9 h. When analyzing the data using a concentration of 0.04%, an increase in the inhibition percentage of 68% was achieved at 3 h, 96% at 6 h, and 100% for 9 h. The disinfectant is 100% effective when a concentration of 0.08% is used, for the three frames times established in the test. From the analysis of the results obtained (table 1) with half the concentration (1%) and the recommended concentration (2%), we can say that the high-level hydrogen peroxide-based disinfectant of STERIS has a high sporicidal power at 3 h of exposure because we achieved an absolute decrease of bacterial population for this microorganism. For this particular microorganism, different contact times with the disinfectant were established; in this case, it was 3, 6, and 9 h because this species has the capacity to form spores as resistance structures, and, by increasing the exposure time compared with the different concentrations, the disinfectant can exert its action against the spores, preventing the formation of the cortex between the internal and external membrane before the spore matures [6]. The resistance of *Bacillus subtilis* to disinfectants is attributed to the fact that the sporulated microorganisms form a barrier to the entry of antimicrobial agents because the membranes that surround the nucleus of the endospore act as an additional factor when limiting the penetration of the chemical agent [9]. When evaluating a disinfectant against a sporulated microorganism such as *Bacillus subtilis*, it is necessary to increase the exposure time for many reasons; for example, the spores have a core with a high content of calcium dipicolinate; in addition, the nucleus is partially dehydrated. This characteristic increases the thermoresistance of the spore, and, at the same time, it confers resistance to chemical substances such as hydrogen peroxide. Also, from the low water content of the spore, the pH of the core cytoplasm contains high levels of specific core proteins termed “small acid-soluble spore proteins” (SASPs). These proteins bind tightly to the DNA in the spore’s nucleus and protect it from potential damage from UV radiation, desiccation, and chemical agents [3, 8, 12].

The results obtained in this study, for each microorganism, show that the inhibitory effect of the disinfectant is directly influenced by the exposure time; for this case, it is recommended to use the high-level hydrogen peroxide-based disinfectant, STERIS, at a concentration of 2%, with a contact time of 5 min, as established by the producer. Several studies [10,13-15], in which the microorganisms *Bacillus subtilis, Pseudomonas aeruginosa*, and *Staphylococcus aureus* were used as a control, showed that hydrogen peroxide is 100% effective against these microorganisms at a concentration of 3% with a time of 5 min contact for vegetative bacteria and more than 2 h for the sporulated microorganism. The same effect was achieved in the present study, compared with all the strains tested, with the same time but at a lower concentration of the product at 2%.

Refer to table 2 to examine the vegetative bacteria (*Pseudomonas aeruginosa*, *Salmonella chole raesuis*, and *Staphylococcus aureus*). The most significant reduction of microbial growth occurred in the strain of *Pseudomonas aeruginosa*, which reached a percentage of inhibition of 88% when subjected to a disinfectant concentration of 0.04% with an exposure time of 5 min. These results are according to [13], which revealed that *Pseudomonas aeruginosa* could exhibit inhibition with different concentrations of this disinfectant. While the smallest reduction was observed with the strain of *Staphylococcus aureus*, in which, at 10 min of exposure, the population decreases to 44%. On the other hand, the sporulated *Bacillus subtilis* strain showed a 69% decrease in growth for the first 3 h of exposure to the disinfectant, at a concentration of 0.04%. The reduction of the population on average was 100% for the levels of 0.08%, 1%, and 2%.

Based on the above, we can say that the high-level disinfectant of STERIS based on hydrogen peroxide is 100% effective when using the concentration recommended by the commercial house (2%) in the shortest time of exposure. Likewise, we can establish that the minimum inhibitory concentration, i.e., the lowest level of the disinfectant capable of inhibiting *in vitro* the visible growth of microorganisms, was 0.08% because, with this value and in the shortest time of contact with the disinfectant evaluated, they achieved satisfactory results.

CONCLUSION

For the *in vitro* microbiological assays that tested the evaluation of three concentrations of the disinfectant, in this study satisfactory results were obtained. When facing the microbial strains against the high-level hydrogen peroxide-based disinfectant of STERIS at a 2% concentration, recommended by the commercial house, it was proved that it exerts a total inhibition of microbial growth in a time of exposure of 5 min, proposed by the commercial house for cell vegetative and 6 h for the sporulated strain. It is worth mentioning that, in this study, the specific microorganisms were not used in the technical data sheet, i.e., the same ATCC, but studies carried out by [13], used *Pseudomonas aeruginosa* ATCC 9027 [13]; also used was *Bacillus subtilis* ATCC 6633, the same strains used for this study; further, 100% satisfactory results were reported for each microorganism when they used products with a concentration of hydrogen peroxide higher than 3%.

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AUTHOR CONTRIBUTIONS

All authors contributed equally. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

Declared none

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