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ORIGINAL ARTICLE

Bacterial vs. fungal spore resistance to peroxygen biocide on inanimate surfaces

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Abstract A sporicidal agent formula based on a mixture of peroxyacetic acid and hydrogen peroxide was assessed for its efficacy on a representative sample of vinyl surface material. Vinyl is the construction material of wall and floor lining in pharmaceutical plants. The experimental manipulations; applied herein, simulated the actual biocidal agent preparation and were carried out using USP purified water, test temperature was 20–25 °C, RH% was 40–60% and pH was 3.08 and 2.86 for 1% and 2% (v/v) respectively. Following the selection of the optimum method of antimicrobial activity neutralization, two disinfectant concentrations were examined for their sporicidal activity. The results of carrier test revealed that the disinfectant concentration (2%) (v/v) was significantly effective as a sporicidal agent after 5 and 10 min for *Aspergillus brasiliensis* and *Bacillus subtilis* subsp. *spizizenii*, respectively, while the concentration of 1% (v/v) did not achieve even one logarithmic reduction after 20 min. The agent was able to achieve more than 100 times reduction from the initial bioburden on the surfaces when used in the concentration of 2% (v/v) after 10 min of contact time. The ideal kinetics of microbial death usually follows 2 parts (by averaging the responses with time for the 3 replicates): initial slow rate of death followed by higher rate. The initial sigmoidal part was only observed with *B. subtilis* upon exposure to 2% (v/v) sporicidal agent. Elimination time for *B. subtilis* spores was 15 min which was about double the time required for eradication of *A. brasiliensis*.

1. Introduction

Microbial contamination costs companies thousands to millions of dollars annually through the equipment damage, production downtime, product contamination, investigations, and energy losses. The scope of the majority of reputable companies; nowadays, is focused on understanding the sources of contaminants, environmental conditions, and facility and equipment designs that can lead to microbial colonization and proliferation. Majority of bacterial species are known to live in the form of vegetative cells; therefore, they require moisture and organic matter for survival and proliferation. However, some bacteria when challenged by unfavorable environmental conditions are capable of producing spores that remain in a dormant state for long periods of time. Once the conditions are again optimum for growth, the spores

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germinate and give rise to new vegetative cells. In terms of environmental control, the presence of molds in manufacturing environments causes great concern because these types of organisms spread very rapidly as they produce spores that are easily transferred from one site to another. Many molds also produce mycotoxins and antibiotics, which can be of concern if these organisms are contaminants of products for human or animal consumption.[1]

In a few bacterial genera, notably *Bacillus*, a unique process takes place in which the vegetative cell undergoes a profound biochemical change to give rise to a structure called a spore or endospore. This process is not part of the reproductive cycle, but the bacterial endospore is highly resistant to adverse environments such as lack of moisture or essential nutrients, toxic chemicals, radiations and high temperatures. Because of their heat resistance all sterilization processes have to be designed to include the bacterial spores in the killing process.[2]

Sporicides are chemical compounds that are capable of destroying all types of organisms, including bacterial spores.[3] Because bacterial spores are more resistant than vegetative cells, a sporicidal agent is usually considered a sterilant. Several types of sporicidal agents are extremely corrosive to stainless steel, plastic, and soft metals and can contribute a health hazard to operators. That is why in sanitization and disinfection program it is preferable to use a sporicidal agent on a less frequent basis in complementation with less aggressive disinfectants such as Isopropyl alcohol 70% or Ethanol 70% and Tego 51 or 2000 1%.[4] Also, the concentration of sporicidal agent must be chosen carefully in order to attain an optimum sporicidal activity in reasonable time. The test protocol used in a disinfectant qualification study varies from one company to another, since the procedures are usually customized to reflect the types of surfaces and application of the chemical products at a particular facility.[1]

However, before conducting any disinfectant efficacy assay a proper preliminary neutralization scheme should be established. Effective neutralization of a chemical biocide is critically important for the quality of the data derived from any assay of biocidal efficacy.[5] Care must be taken to avoid carry-over of active biocide to the recovery media, which may result in biostasis of the test organism. This biostasis would lead to an overestimation of the biocide efficacy. Therefore, the experimental design used to establish the efficacy of biocide neutralization has a major impact on the estimation of antimicrobial efficacy.[6]

The practice of rotating disinfectants as a means of proactively eradicating a broad spectrum of microorganisms that may be present in a facility is nowadays a common practice in the pharmaceutical industry as well as a regulatory requirement. In the European Commission (EC) Guide to Good Manufacturing Practice,[7] it is stated that "where disinfectants are used, more than one type should be employed. Monitoring should be undertaken regularly in order to detect the development of resistant strains". The practice of rotation of disinfectants is also highlighted in the FDA guide[8] for aseptic processing and in the USP.[9]

A variety of peroxygens possess excellent activity under controlled conditions and are sometimes used in chemical sterilization. Hydrogen peroxide (H_2O_2) and peracetic acid (PAA) are high level disinfectants due to the production of highly reactive hydroxyl radical. They have an additional advantage of producing decomposition products that are nontoxic and biodegradable. Furthermore, the combination of H_2O_2 and PAA has been reported to possess a synergistic effect.[2,10]

When selecting a disinfectant for use in a pharmaceutical manufacturing area, the following factors should be considered: the number and types of microorganisms to be controlled; in addition to its concentration, application method, and contact time. Other important factors include: the nature of the surface material being disinfected; the possible need to maintain a residual bactericidal activity of the disinfectant on the surface; the corrosiveness of the disinfectant to equipment upon frequent application; the safety considerations for operators applying the disinfectant; the compatibility of the disinfectant with cleaning agents and other disinfectants; the disinfection protocol.[11]

Therefore, the aim of the current study has been directed to optimize a practical application protocol of a commercial disinfectant in pharmaceutical plant sanitization program so that it can be used routinely in classified area to control microbial bioburden. This study was performed in complementation with non-sporicidal agents in order to develop an integrated system for sanitization and disinfection program for clean room in pharmaceutical plants especially in the critical processing area that has direct impact on drug quality.

2. Materials and methods

2.1. Preparation of spore suspension

Standardized stable suspensions of test strains were used or prepared as detailed in "Seed-lot culture maintenance techniques". The suspensions used were prepared so that the viable microorganisms used for inoculation are not more than 5 passages removed from the original master seed-lot.[12]

The bacterial test strains *Bacillus subtilis* subsp. *spizizenii* (ATCC 6633) were grown separately in containers containing casein soya bean digest agar; at 30–35 °C for 5–7 days to ensure complete sporulation. The test strain for *Aspergillus brasiliensis* (ATCC 16404) was allowed to grow separately on Sabouraud-dextrose agar; at 20–25 °C for 7 days or until good sporulation is attained, which was ensured by the formation of full spore mat.[13] Mold spores were harvested by washing the agar surface with sterile USP saline TS or a buffer solution containing 0.05% polysorbate 80. A sterile inoculating loop or some sterile glass beads were used to loosen the spores. The hyphae removal from spore suspension was ensured by filtration through glass wool column[14] then washings were combined in a sterile container forming the mold inoculums. To prepare a bacterial spore suspension, the inoculated agar plates were harvested using sterile water then heat shocked for 15 min at 65–70 °C, once the temperature reached 65 °C then the timing was started. The suspension was cooled rapidly in an ice bath (0–4 °C), and the prepared spore suspension was stored under refrigeration. Complete sporulation was confirmed by microscopical examination using the Schaeffer–Fulton staining technique, which stains endospores green and bacterial bodies red.[15] Initial plate count was carried on out to verify the spore population. As soon as the results of serial dilutions could be enumerated, the microbial test suspensions were used.

2.2. Biocidal qualification test

The entire evaluation process of the antimicrobial potency of biocidal agents must ensure that the true disinfectant efficiency is represented without any over or under-estimation in the working environment and conditions.[9] All tests were carried out in triplicates.

2.2.1. Preliminary neutralization validation study

This study was carried out according to the principle stated by Sutton et al.[6] and performed as described by Eissa et al.[16] The aim of this study was to ensure that the assumed contact time is valid, i.e. the neutralizing agent can efficiently stop the action of the tested sanitizer and that it has no inhibitory or toxic effect on used microorganisms. Herein, two comparisons among three populations are performed; the first comparison is Neutralizer Efficacy (NE) which can be determined by evaluating the survivors in the neutralizing broth in the presence and the absence of the biocide. The second comparison is Neutralizer Toxicity (NT); this aspect of neutralization was determined by comparing the number of survivors in the neutralizing medium without the biocide to that in the viability (growth) control medium.[6,16]

2.2.1.1. Test group. Using neutralizing broth as diluent; a 1:100 dilution of the test solution was made at the working concentration, then 1 ml of this dilution is transferred to each duplicate petri dish. The *neutralizer exposed group* was prepared in parallel as detailed under the test group but using sterile saline or buffer instead of the test solution. The *viability control group* was prepared using saline or buffer without test solutions or neutralizing broth. Inoculums of the used microorganisms ranging from 30 to 100 CFU were added to each duplicate of 90 mm sterile petri plates. About 20 ml of the molten suitable medium at 45 °C was added; allowed to solidify, then incubated at a suitable temperature of 30–35 °C for 3 days; for bacteria, and 20–25 °C for at least 5 days; for mold. Duplicate plate counts were recorded and used as a positive control. Negative control for each media with the same volume of diluents or neutralizers added was prepared to ensure the sterility of all used materials. Recovery ratio was calculated as follows: *NE*: ratio of the microbial count recovered from neutralizer–disinfectant combination to that recovered from the neutralizer in the absence of biocide (as control), *NT*: ratio of the growth without the biocide in the neutralizer to the growth in a rich medium (as control).

2.2.2. Effectiveness as antimicrobial agent

The test was adopted from USP,[9] and carried out as detailed in the study by Eissa et al.[4] The peroxygen biocidal agent stock solution (denoted by: Codas) composition was as follows; hydrogen peroxide 27%, peracetic acid 4.5% and inert ingredients 68.5%. This stock solution was diluted to the working concentration by purified water USP in sterile containers prior to the test. Temperature during the test was 20–25 °C, RH was 40–60% and pH was 3.08 and 2.86 for 1% and 2% (v/v) respectively. Conditions that simulate actual biocidal agent preparation and testing were ensured.[9,10] Coupon surfaces were made from vinyl material which is used as covering material for wall and floor in clean areas of pharmaceutical facility. They were cut into small parts about 2 × 2 inches. Each of the test organisms was examined with one of the Codas solutions (1% and 2%). Codas was excluded in the control study.

One of the suspensions settled on the surface of one of the tested materials (inoculums level was adjusted so that microbial count ranges within 10^5–10^6 CFUs). The prepared Codas (1%) was added gently on the inoculated coupon surface and allowed to be in contact with the surface for 1, 5, 10, 15 and 20 min. After contact time the liquid was drawn up, mixed thoroughly and transferred to a sterile tube containing 19.8 ml of the suitable neutralizing broth. The mixture was homogenized, and then 1 ml of broth was transferred to a second sterile tube containing 9 ml of neutralizing broth followed by vortex mixing. Broth aliquots were transferred from second dilution tubes to plates of 9 mm sterile petri dishes, molten agar medium (at 45 °C) was added to each plate and swirled gently. The process was repeated to prepare agar plates using aliquots from the first broth.

The count for each readable dilution was read then multiplied by the reciprocal of the dilution factor. The base line inoculum was determined for each organism and disinfectant, showing at least 10^4 CFUs, then $\log_{10}$ reduction of microorganisms exposed to disinfectants for each contact time was calculated. Graphical interpretation of results was interpreted using Microsoft Office Excel 2007 to demonstrate the kinetics of microbial death.

2.2.3. Acceptance criteria

1. NE and NT results were considered acceptable if $\geqslant 0.75$ recovery was achieved.[6]
2. An agent was considered effective as antimicrobial if the inoculum is reduced by at least $2\log_{10}$ for bacterial spores and $3\log_{10}$ for fungi on sample surface coupon.[9]
3. Significant reduction in microbial count compared to the initial one (greater than a 0.3log reduction); which is defined as Normal Plating Variability[17] was chosen as true criteria of biocidal effect and compared with data obtained from statistical analysis calculated by GraphPad Prism version 5.00.288 for Windows. Any complex calculations and data interpretations were performed using Microsoft Office Excel 2007.

3. Results

Fluid Thioglycolate Medium (FTM) had passed both NT and NE in disinfectant validation preliminary study with both *A. brasiliensis* (ATCC 16404) and *B. subtilis* subsp. *spizizenii* (ATCC 6633). Results are illustrated in Tables 2 and 3.

In the disinfectant validation study, Codas 2% biocidal agents were able to significantly reduce the microbial count after 5 min for *A. brasiliensis* which was not recovered after 10 min. For *B. subtilis* subsp. *spizizenii*, the reduction was observed after 10 min and the recovery could not be detected after 15 min. However, Codas 1% even after 20 min did not achieve good microbial reduction of spores for both microorganisms which were not significant from each other using One-way ANOVA followed by Tukey's Multiple Comparison Test. These results are illustrated in Fig. 1.

In addition, our results revealed that *B. subtilis* spores showed relatively greater tolerance to the used disinfectant

Table 1 Comparison between *Bacillus subtilis* and *Aspergillus brasiliensis* spores in the kinetics of death on vinyl surface using two criteria for comparison (Harmonized Chapter USP <61> and Tukey's Multiple Comparison Test).

Point of comparison	Harmonized Chapter USP <61> 0.3 log	Tukey's Multiple Comparison Test	Time required to achieve target LR[a]
1. Effect of Codas 1% *Bacillus subtilis* LR[a] from initial count	Significant after 20 min	Not significant even after 20 min	> 20 min
2. Effect of Codas 1% *Aspergillus brasiliensis* LR[a] from initial count	Significant after 15 min	Not significant even after 20 min	> 20 min
3. Effect of Codas 2% *Bacillus subtilis* LR[a] from initial count	Significant after 5 min	Significant after 10 min	< 10 min
4. Effect of Codas 2% *Aspergillus brasiliensis* LR[a] from initial count	Significant after 1 min	Significant after 1 min	< 5 min
5. Time of significant difference of Codas 1% and 2% in *B. subtilis* LR[a]	10 min	10 min	NA
6. Time of significant difference of Codas 1% and 2% in *A. brasiliensis* LR[a]	1 min	5 min	NA

[a] Logarithmic reduction obtained by calculating $\log_{10}$ of initial inoculum count minus $\log_{10}$ of the remaining bioburden after specific contact time with biocidal agent.

while *A. brasiliensis* showed greater sensitivity to doubling the concentration of biocidal agent (Fig. 1). Furthermore, the kinetics of logarithmic death (logarithmic reduction (LR) vs. time in minutes) of spores (the mean sporicidal effect from the replicates) for *B. subtilis* was composed of 2 parts initial slow rate of death followed by second more rapid decline in spore count, in case of Codas 2%. The initial shoulder was almost not observed with *A. brasiliensis* in any of the two Codas concentrations. The same observation was recorded for *B. subtilis* in case of 1% concentration Fig. 1.

Table 1 demonstrates that there was a difference in the time elapsed for considering significant logarithmic reduction (LR) when comparing Normal Plating Variability criterion with Tukey's Multiple Comparison Test regardless of whether the required $\log_{10}$ was achieved or not for both microorganisms. It should be noted that both criteria were in agreement with the following: (1) *A. brasiliensis* spores showed significant reduction after one minute exposure to Codas 2% (v/v) i.e. the killing rate was obvious and fast after 1 min contact with disinfectant and (2) The tested biocidal agent at 2% (v/v) showed significant effect in LR from 1% (v/v) at 10 min exposure in case of *B. subtilis* spores. This means that even after 5 min exposure to biocidal agent, there was no significant difference between the rate of killing at both concentrations 1% and 2% (v/v).

4. Discussion

Spore forming organisms belonging to the genera *Bacillus* are common isolates from the pharmaceutical environments, including air and surfaces, often as a result of feet/wheel contamination. Unlike bacterial spores, fungal spores are part of the normal life cycle of fungi, and, therefore, they are less resistant to chemicals and adverse environmental conditions.[1] In the current work, we observed that *A. brasiliensis* spores were more sensitive to doubling the disinfectant concentration by about 29 times while *B. subtilis* subsp. *spizizenii* was much less sensitive; as the increase in the rate constant was less than 3 times i.e. about 10 times less than *A. brasiliensis*. They were microorganisms of choice in this study because of their chemical resistance; besides, the fungal spores which were found to be highly spreadable in a variety of locations. On the other hand, most of the protocols designed for the evaluation of the microbiocidal properties are based on a minimum requirement of 2-log reduction in spore-forming bacteria and a minimum of 3-log reduction in fungi USP.[9] Furthermore, effective neutralization of a chemical biocide is critically important for the quality of the data derived from any assay of biocidal efficacy.[5] Complete neutralization of disinfectants is important for the accuracy of a biocidal assay as microbicidal activity is commonly measured as survivors count with time, where the inhibition of the microbial growth by traces of residual biocide would lead to exaggerated measures of microbicidal activity.[18–20]

NT study performed for the FTM neutralizers used in this study revealed that it was non-toxic and could be used in the validation program. The scheme followed was using FTM as a primary neutralizer. This choice was supported by previous work,[6] where NIH Thioglycolate (close in composition to FTM) was reported to be non toxic or of low toxicity against microorganisms. However, the combination of microorganism, neutralizer and disinfectant is unique and thus the success of one combination with one microorganism does not mean that same combination with other microorganisms will do accordingly.[6] According to USP[17] of 0.3 log plating variability, no difference was observed between both microbial spores in the NT study with FTM.

Results of NE reflected the greater impact of Codas 2% over 1% in the recovery which is in agreement with the observations reported by Eissa et al.,[16] which demonstrated that the role of the chemical neutralization process in protecting microorganisms is affected by two main factors; the first of which includes nature of disinfectant, mechanism of biocidal action, chemical reactivity and concentration exponent. The second one is concerned with the nature of microorganism and its sensitivity to the outcome of neutralizer-biocide combination. The study also recovered *B. subtilis* from peroxygen compound. However; based on USP[16] for Normal Plating Variability limits, variations between control and test groups in both NE and NT studies were considered within ordinary limit of variability and there was no significant difference between them.

Surface challenge tests[9] are customized procedures based on the Association of Official Analytical Chemists (AOAC)

Table 2 Neutralizer toxicity screening study for used disinfectants against *Bacillus subtilis* and *Aspergillus brasiliensis*.

Microorganism in spore form	Neutralizer	Test plate count (CFU)	Control plate count (CFU)	Recovery ratio
Bacillus subtilis (ATCC 6633)	FTM[a]	83	87	0.95
Aspergillus brasiliensis (ATCC 16404)	FTM[a]	53	49	1.08

[a] Fluid Thioglycolate Medium.

Table 3 Neutralizer efficiency screening study for used disinfectants against *Bacillus subtilis* and *Aspergillus brasiliensis*.

Microorganisms in spore form	Disinfectant[a]	Neutralizer	Test plate count (CFU)	Control plate count (CFU)	Recovery ratio
Bacillus subtilis (ATCC 6633)	Codas[c] 1%	FTM[b]	80	88	0.91
Aspergillus brasiliensis (ATCC 16404)		FTM[b]	28	30	0.93
Bacillus subtilis (ATCC 6633)	Codas[c] 2%	FTM[b]	58	76	0.76
Aspergillus brasiliensis (ATCC 16404)		FTM[b]	46	52	0.88

[a] Disinfectant to the final neutralizing diluent ratio (v:v) 1:100.
[b] Fluid Thioglycolate Medium.
[c] Commercial sporicidal agent based on mixture peroxyacetic acid + hydrogen peroxide.

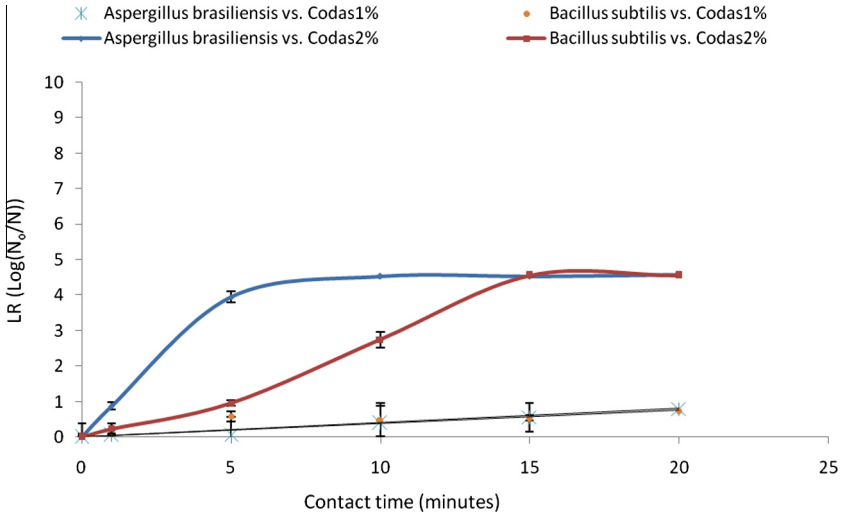

Figure 1 Kinetics of microbial death for *Bacillus subtilis* subsp. *spizizenii* and *Aspergillus brasiliensis* in Codas 1% and 2% (v/v). Results represent mean ± S.D. No S.D. bars in plateau as no CFUs were recovered from agar plates.

method for germicidal spray products and designed to evaluate the effectiveness of a disinfectant against standard strains and environmental isolates when applied to representative surfaces found in a manufacturing facility. This test has been reported to be the preferred disinfectant qualification method by the regulatory agencies. It is quantitative and demonstrates log reduction of the test organism upon exposure to the selected disinfectant concentration as it is used by a company during a cleaning procedure. In order not to deliberately contaminate the manufacturing areas, surface challenge tests are performed in a laboratory setting and using representative surfaces (referred to as coupons) that are scaled down to a size of about 2×2 in Ref.[1].

According to USP[9] the plots of the log of the number of microorganisms per ml surviving in a disinfectant solution indicate that first-order kinetics can be applied as a gross approximation to the reduction in microbial count with respect to time. In practice, the plots show a more sigmoid curve with a slower initial reduction in numbers followed by an increasing rate with respect to time. The rate constant, K, for the disinfection process can be calculated by the formula: $(1/t)(\log N_O/N)$ in which t is the time, in minutes, for the microbial count to be reduced from N_O to N; N_O is the initial number of organisms, in CFU per ml; and N is the final number, in CFU per ml, of organisms. This could be observed with *B. subtilis* subsp. *spizizenii* with Codas 2% only, while *A. brasiliensis* with Codas 2% did not show initial slow rate probably due to an insufficient time interval to give a notable rate to be observed. Both organisms with Codas 1% did not show initial shoulder (initial slow rate) because the slopes of both were much less than that of 2% so the initial shoulder could not be distinguished from the rest of the curve.

Upon the use of USP[17] Normal Plating Variability criterion for true difference, it gave either earlier point of difference (as compared with One-way ANOVA using Tukey's Multiple Comparison Test at $P < 0.05$), or significant point of difference when no difference was detected using statistical analysis. In such instances graphical presentation for the kinetics of microbial death will facilitate the detection of behavior and performance difference. As demonstrated in the graphical

presentation of the kinetics of microbial spore death, it could be observed that the slopes of Codas 1% for both *B. subtilis* subsp. *spizizenii* and *A. brasiliensis* spores are about 1/20 and 1/10 of that for Codas 2% *A. brasiliensis* and *B. subtilis* subsp. *spizizenii*, respectively. This is an indication that the effect of dilution of disinfectant did not produce equivalent effects on both organisms. Additionally, requalification of disinfectants and cleaning procedures is not required unless a significant change has been made to the sanitization protocol. The regulatory expectation is that facilities, systems, equipment, programs, and processes (including cleaning and disinfection) should be periodically reviewed to confirm that they remain valid and in a state of control.[21]

Microbial contamination control is not simply a task. It is a continuous effort involving all parts of the facility, all aspects of the process, and company personnel. It is indeed a true continuous improvement activity, one that requires the support of company management and must be embraced by manufacturing operators involved in the production of pharmaceutical drug products.

5. Conclusion

The method used for the assessment of sporicidal activity described is simple, not expensive and could be of help in designing disinfection program parameters. In such program, using a proper effective amount of sporicidal agent ensures a maximum achievement in biocidal effect, however, the contact time should not compromise the operator's health and safety, in addition to the usage life span for installation of the plant. It is advisable to use simple and cost effective method of corrosiveness testing, in case of aggressive disinfectant in the future (especially sporicidal ones) and those obtained from suppliers claiming to include anticorrosive substances in their own formula of biocidal agent. Each health care company or facility can perform in-house corrosiveness testing by ''weight difference method'' using its specific material coupons to detect actual wearing of these materials using biocidal agent as a part of sanitization and disinfection qualification program.

6. Conflict of interest

Authors declare that there is no conflict of interest among them.

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