Author’s response to reviews

Title: Determination of decimal reduction time (D value) of sanitary agents in hospital usage.

Authors:

Priscila G Mazzola (priscila_mazzola@hotmail.com)
Dr Thereza CV Penna (tcvpenna@usp.br)
Alzira M Martins (alzira_martins@edwards.com)

Version: 3 Date: 1 Jul 2003

PDF covering letter
Dear Emma,

We attached the revised manuscript entitled: “Determination of Decimal Reduction Time of Chemical Agents Used in Hospital Disinfection” by the authors: Priscila Gava Mazzola, Thereza Christina Vessoni Penna*, Alzira M. da S. Martins, in accordance with the suggestions and comments required by the reviewers.

I am sorry for our delay.

We very much appreciated every suggestion and comment that help us to improve the manuscript.

Thank you very much for your help and attention and understanding.

Looking forward for your appreciation and reply,

Sincerely yours,

Thereza Christina Vessoni Penna

---

**Reviewer’s Report**

Reviewer: Waldemar Gottardi

1. Some concentrations of disinfectants are demonstrated repeatedly in a wrong way.
   A: The concentrations are expressed in: perceptual (%) and mg/L (ppm). See Table 1. p. 19-20.

2. Decimal numbers should be presented consistently (at maximum one decimal in time statements).
   A: Your suggestion was followed. See Table 1. p. 19-20.

3. The definition of sterilization is wrong.
   A: All definitions were rewritten in the item: Background, pages 3, (lines: 12-30); page 4, (lines 33-35); page 8 (lines 1-34).

4. At which temperature and in which medium were the microorganisms cultured? Were they washed?
   A: Methods, Culturing, page 5, lines 19-35; and Assay for D-value, page 6 (lines 23-38) and page 7 (lines 1-14).

5. The authors describe that they transferred 1mL of a 24h suspension of bacteria to 100mL of sanitizer solution, So, how many CFU were exposed to the disinfectants at time zero?
   A: Assay, pages 6-7: “The determination of the D-value for the test disinfectant consisted in the transference of 1.0 mL of a 24 h suspension of the test bacterial
strain into 100 mL of the disinfectant solution that is kept in constant agitation at a
controlled temperature room (at 25°C ± 1.0 °C), at time zero. The initial
concentration of bacteria (N₀) exposed to the disinfectant at time zero was around
10⁴ to 10⁵ CFU/mL (colony forming units/mL).”

6. Which dilutions in what did they apply before plating?
7. A: Assay: page 7, lines 4-9:
At regular intervals (1 min for vegetative forms and 5 min for sporeforms), a
sample of the 1.0 mL mixture was transferred to 8 mL of TSB containing 1 mL of a
inactivating agent at 1% concentration to guarantee a complete inactivation of the
disinfectant without interfering with survivor growth. Using TSA pour plates, the
survivors were evaluated by dilution (1:10, 1:100, 1:10³, 1:10⁴, 1:10⁵) in saline
solution.

8. pH and temperature of the disinfecting solutions during the tests?
A: Assay, temperature of assays: (at 25°C ± 1.0 °C), and pH-values of every
disinfectant solution are shown in Table 1, pages: 19-20.

9. Add more details about intervals of exposure for different bacteria to each
disinfectant solution were:
A: At regular intervals (1 min for vegetative forms and 5 min for sporeforms), a
sample of the 1.0 mL mixture was transferred to 8 mL of TSB containing 1 mL of a
inactivating agent at 1% concentration to guarantee a complete inactivation of the
disinfectant without interfering with survivor growth.

10. Did they perform negative controls and what would be more important –
positive growth controls for the used bacteria?
A: Assay page 7, lines 9-11,
“A negative control was made in a tube of 9 mL TSB plus 1 mL of an inactivating
agent. A positive control was made adding 0.1 mL organism suspension in 10 mL TSB
in a tube.”

11. What do the authors mean with reduction of ... log 10 cycles?
A: Analysis of the Results:Page 7

Analysis of the Results

Determination of D-Value. The decimal reduction time (D-value), the interval of
time required to reduce one decimal logarithm of the initial bacterial population, at a
specified disinfectant concentration (at constant temperature of 25°C), was determined
from the negative reciprocal of the slopes of the regression lines, using the linear
portions of the survivor curves (log₁₀ CFU/mL versus time of exposure to the
disinfectant solution, at constant temperature (7) (Figure 1).

Calculation of the Confidence Level
The confidence level and the final number (N₉) of the surviving population per mL solution was calculated to be equivalent to a 6log₁₀ and
12log₁₀ reduction in viable bioburden, considering, respectively, low level
disinfection and high level disinfection (4).

12. Did they use spore suspensions?
A: Culturing, page 5, lines:34-35,
The spore suspensions of B. subtilis and B. stearothermophilus were used for
the tests of the D-value determination.
13. Define biological indicators
A: Page 4; lines 28-38: “The selection of test microorganism(s) should be related to the use of the disinfectant, defined by the hospital program, and to the bioburden present in the specific environment or on the material. The selected bacteria should be associated with outbreaks of infections in healthcare environment. The effectiveness of a chemical agent can be related to the resistance of a specific microbiological species that can be used as a biological indicator (BI), and can be defined in terms of decimal reduction time (D-value), the exposure time required, under a defined set of conditions, to cause a one log10 or 90% reduction in bioburden, (7, 8). Any reduction in BI bioburden levels, as related to the extent of exposure to the test disinfectant, depends upon the disinfection procedure, the chemical agent used and the application of this disinfectant to a particular environment or material.

14. D values of 8.3 and 5.9 min do not mean that these strains are “resistant” to chlorexidine.
A: By the definition of D value:
The effectiveness of a chemical agent can be related to the resistance of a specific microbiological species that can be used as a biological indicator (BI), and can be defined in terms of decimal reduction time (D-value), the exposure time required, under a defined set of conditions, to cause a one log10 or 90% reduction in bioburden, (7, 8: ISOs: 14937 and 11134).
The resistance is given by the resistance to continue alive with all vital metabolism working. Therefore, D-value is a measure of the resistance to an environmental stress, independent on the concentration of the disinfectant but is related to the time of exposure under stress. It is in accordance with international directives, as ISO 14937 and 11134, the European and USA Pharmacopeias.

15. What is inactive activity?
A: It was erased from the manuscript.

16. In the table, what does t mean?
A: The headlines of Table 1 were completed, and the legend in page 20 gives the meanings:

**Legend:**
1 Survivor Curve: log Nf = log No-1/D x t; No= bioburden; Nf= survival population; D-value= decimal reduction time; (-1/D) = slope.
2 t= n x D, where: t=total exposure time (min); n = log10 reduced cycles
3 t= n*D and n= 6-log10, t= the exposure time for a 6-log10 reduction in the bioburden (No) with a defined D-value
4 t= n*D and n= 12-log10, t= the exposure time for a 12-log10 reduction in the bioburden (No) with a defined D-value

17. How many times were the experiments repeated?
A: Assays:

18. Chemical agents concentrations:
A: The concentrations were expressed in perceptual (%), and mg/L (ppm), as shown in Table 1.
19. Was the CFU count at time zero virtually as low as 1.5 to 2 log10? How many repetitions experiments?
A: Assays:
✓ page 7, lines 2-7; “The initial concentration of bacteria (N₀) exposed to the disinfectant at time zero was around 10⁴ to 10⁵ CFU/mL (colony forming units/mL). At regular intervals (1 min for vegetative forms and 5 min for sporeforms), a sample of the 1.0 mL mixture was transferred to 8 mL of TSB containing 1 mL of an inactivating agent at 1% concentration to guarantee a complete inactivation of the disinfectant without interfering with survivor growth.”
✓ page 7, lines: 12-14: “The method for each disinfectant and test bacterium was repeated at least four times, when bacterial cultures were exposed to four different samples of diluted disinfectant, before the determination of D-values by the survivor curves.”

20. Obviously the 6 and 12 log reductions have been calculated and not determined experimentally. This should be stated clearly.
A: It was explained:
Objectives: Page 5, lines 6-8: “The confidence levels were set for 6 to 12 log₁₀ reduction of the initial population of bacterium in order to a predicted probability of a surviving microorganism of 10⁻¹ or better (ISO 14937).

**PAGE 7: Calculation of the Confidence Level**
The confidence level and the final number (N_F) of the surviving population per mL solution was calculated to be equivalent to a 6 log₁₀ and 12 log₁₀ reduction in viable bioburden, considering, respectively, low level disinfection and high level disinfection (1, 4, 9).

21. Discuss the results fit to the present guidelines at least in their country.
A: Every disinfectant was related to the Brazilian policies.
Results and Discussion, pages 7-14.

22. The results should be separated from the discussion, and the discussion should deal concisely with the results of this study.
A: For every disinfectant, the results were commented and related to Brazilian directives, as an individual discussion. The conclusions are concisely with the results and individual discussion for each disinfectant. Conclusions: Page 15.

Thank you for your observations, suggestions and comments.
Look forward to hearing from you
Sincerely yours
Thereza Christina Vessoni Penna
1. How many spores forming organisms are used in the test? How many spores as opposed to vegetative cells are present in the test suspensions.

A: Methods, Culturing, page 5

**Culturing** (14) Working cultures were kept on Tryptic Soy Agar (TSA, Difco, Detroit, Michigan, USA) at 4°C with weekly transfers. The 24 h cultures, grown on TSA at 22°C for *S. marcescens*, and at 35–37°C for *E. cloacae, A. calcoaceticus, E. coli, S. aureus*, were harvested into Tryptic Soy Broth (TSB, Difco), centrifuged (1000xg/15 min/4°C) and suspended in saline solution (0.9% NaCl). Bacterial viability was estimated on TSA pour plates by confirming populations $10^6$ CFU/mL. Spore cultures, were developed for six days in a sporulation medium [g/L: D-glucose, 2.5 (Sigma, St. Louis, Missouri, USA); L-glutamic acid, 0.4 (Sigma); yeast extract, 4.0 (Difco); peptone, 5.0 (Difco); sodium chloride, 0.01; manganese sulfate, 0.01; bacteriological agar, 20.0 (Difco)] at 37°C for *B. subtilis*, and at 62°C for *B. stearothermophilus*, were harvested, centrifuged (4X at 1935xg/30 min), and kept suspended in chilled 0.02 M calcium acetate solution (pH=9.7) at 4°C (15). The viability of heat-shocked (80°C/10 min for *B. subtilis* and 100°C/20 min for *B. stearothermophilus*) spores was obtained through TSA pour plates by confirming populations $10^6$ spores/mL. The spore suspensions of *B. subtilis* and *B. stearothermophilus* were used for the tests of the D-value determination.

2. Define sterilization, disinfection consistently with international guidelines.
3. Define sanitizer.
4. Define cleansing program. This should be considered a separate process, from disinfection/sterilization.
5. Clarify the use of sanitizing as opposed to disinfection.

A: Background: Page 3: lines 11-36:

Antimicrobial agents applied to a material under set conditions can be classified in accordance with the level of decontamination provided (3, 4) as: (i) antiseptics - chemical agents that inhibit or kill microbial growth and are nontoxic when applied to living tissues, compounds used for handwashing or for treating surface wounds. Under certain circumstances, some antiseptics are also effective disinfectants; (ii) disinfectants - chemical and/or physical agents used to destroy or irreversibly inactivate many or all of the pathogenic microorganisms but not necessarily spores and not all viruses (5). Under set conditions the disinfectants may exhibit sterilizing activity. (iii) sanitizers - chemical agents used to reduce, but not necessarily eliminate, bacteria from the inanimate material to levels considered acceptable as determined by public health codes or regulations. The main difference between a sanitizer and a disinfectant is that, at a specified dilution, the disinfectant must have a higher kill capability for pathogenic bacteria compared to that of a sanitizer (6). (iv) sterileants are high level disinfectants that, under appropriate circumstances, provide sterilization by complete killing or remove all life forms from inanimate objects and surfaces, to specified sterility levels, including the inactivation or removal of spores and viruses. Sterilization is associated with the total absence of viable microorganisms that refers to an absolute condition and assures the greatest safety margin than any other antimicrobial method (7, 8). Depending on the antimicrobial effectiveness expected from chemical agents under set conditions, disinfection can be classified (9) in health care centers as either “high level” (sterilization activity), “intermediate level” (inactivation of *Mycobacterium tuberculosis* and the more resistant types of viruses, such as the ones without a protein membranes in their structure (5) or “low level” (reduction of bioburden). Disinfection at intermediate and low levels are not effective against spores (1).

6. Microbiological culturing methods should be provided more details.

A: Method, pages 5, 6, 7, with details as required.

7. How were at least 12 logs of bacteria cultured and tested? This indeed challenging for range biocides tested. 12 log reduction is not sufficient to
indicate sterilization process. Refer to ISO 14937 for further information in sterilization process.

- **A:** Objective: Page 5, lines 6-8; “The confidence levels were set for 6 to 12 log_{10} reduction of the initial population of bacterium in order to a predicted probability of a surviving microorganism of 10^{-1} or better.”
- **A:** Analysis of the results, page 7, lines

  “Calculation of the Confidence Level. The confidence level and the final number (N_f) of the surviving population per mL solution was calculated to be equivalent to a 6log_{10} and 12log_{10} reduction in viable bioburden, considering, respectively, low level disinfection and high level disinfection (1, 4, 9).”
- **A:** Results and Discussion, page 8, lines 20-36:

  “The overkill approach to disinfectant agent exposure is based on the premise that the extent of treatment will inactivate the initial bioburden (> 10^4 CFU/mL) and provide an additional safety factor [7, 8]. Confirmed the inactivation of a bioburden (N_o= initial population of bacterium) (N_o), the extent of the treatment for the disinfection procedure is determined by extrapolation to a predicted probability (safety factor) of a surviving microorganism of 10^{-6} or better (7, 8), with the D-value determined from the inactivation kinetics curve given by the equation: t = D*(log N_o-log N_f) = D*n (1), where D=D-value (min) at specified conditions, N_o = bioburden of the chosen bacterium as the BI; N_f = surviving population after an exposure time, t (min), to the selected disinfectant. Taking into consideration the following populations of the chosen bacterium: (i) N_o = 10^6 and N_f = 10^9 in the BI; (ii) N_o = 10^6 and N_f = 10^6 in the BI, the exposure time to the selected disinfectant set conditions, respectively, for 6 decimal logarithm (6log_{10}) reduction and for an overkill of 12 decimal logarithm (12log_{10}) of reduction in the bacterium population were calculated by the equation (1).”

8. Some actives tested are known to be non sporocidal and therefore can not be even considered as sterilizing. A six log reduction can indicate the effectiveness of a disinfection process, but the test methods employed do not consider surface disinfection, as suspension tests.

9. Chlorexidine, as other liquid biocides, the activity will vary depending on the formulation.

  **A:** Chlorhexidine can be used to show that we considered your suggestion and rewrote the item Results and Discussion as suggested. Pages 9 and 10:

  **Chlorhexidine**

  Chlorhexidine at 0.4% concentration in water or 70% alcohol is widely applied as a skin low level disinfectant, becoming a leading hospital antiseptic in recent years due to its confirmed bactericidal (no sporocidal) effect and no toxic side effects [11, 20, 21]. Chlorhexidine at 0.2% concentration is a well-known antimicrobial in the dental profession and 2% chlorexidine solution has been applied to a disinfection of orthodontic prosthesis after mechanical cleansing.

  The vegetative strains which showed the best resistance to the solution of 0.4% chlorhexidine were *E. cloacae* (D=8.3 min) and *S.aureus* (D=5.9 min); the more sensitive ones were *A. calcoaceticus* (D=4.1 min), *S. marcescens* (D=4.0 min) and *E.coli* (D=3.0 min). A time interval of 3 to 4 minutes was enough to reduce 90% of the population to *E. coli, S. marcescens* and *A. calcoaceticus*; a 3log_{10} reduction for these species varied between 9 to 12 minutes.

  The spore strains exposed to 2% chlorhexidine showed close D-values among themselves D=9.1 min for *B. stearothermophilus* and D=6.7 min for *B. subtilis* and similar values showed by *E. cloacae* and *S. aureus*, respectively, when exposed to 0.4%.

  In Brazil, 0.4%, 2% and 4% chlorexidine solutions (11) are well known as a widely used disinfectants in hospitals. Within its principal applications, chlorhexidine is recommended for the sanitizing the hands and forearms of the surgical team and the patient’s skin (pre-operative and invasive procedures) as well as in the bathing of newborns. It was reported that a preparation with 0.5% chlorhexidine in 70% alcohol applied to the babies’ skin before invasive procedures provided a significant delay in the colonization of catheters (20).

  The 2% and 4% chlorhexidine used for surgical hand scrub products were verified to achieve a 3log_{10} reduction in microorganisms from baseline population count, and the 2% solution caused less irritation to hands than the 4% preparation (20), considering 20 seconds of hands friction (11, 21).
For total immersion of invasive medical devices, a total exposure time for: (i) 6 to 12 log_{10} reductions of vegetative bioburden with 0.4% chlorhexidine solution, varied between 49.8 min and 99.6 min in relation to E. cloacae resistance, considering low disinfection level; and (ii) 12 log_{10} of sporulating strains (n=12) varied between 80.4 min (1 h 20 min) and 109.2 min (1 h 49 min), considering high level disinfection with 2% chlorhexidine solution.

The 2% chlorhexidine in 70% alcohol will be tested in our laboratory to determine the D-values of the test strains of bacteria, as well as in the hand asepsis and the immersion of various medical devices at a hospital setting in the city of São Paulo.”

10. If specific products containing chlorhexidine, formaldehyde and glutaraldehyde are used these should be listed.
11. Further information should be provided on the neutralization techniques employed.

A: “Method, Chemical Agents, page 6:

“Chemical Agents.

Chlorhexidine digluconate (biguanide, 1,6-dichorophenyldiguanido hexane; 40% w/v; Zeneca Farmacêutica, SP, Br); and sodium dichloroisocyanurate (NaDCC, sodium salt 50% w/w in tablets, Johnson and Johnson, J&J, SP, Br), glutaraldehyde (1,5-pentanediol; 2.0% w/v with sodium bicarbonate, pH = 8.3, Aster Produtos Médicos, SP, Br), formaldehyde (monaldehyde; 37% w/v, Aster Produtos Médicos, SP, Br); sodium hypochlorite (10% w/v, Aster Produtos Médicos, SP, Br); hydrogen peroxide (40%, Laborosa Farmacêutica, SP, Br); a mixture of peracetic acid (4.5% v/v, PAA) and hydrogen peroxide (2.2% v/v, H_{2}O_{2}) plus acetic acid, 10 mg/L (Minncare®, pH=1.3, Minntech Corporation, Minneapolis, MN, USA) were used. The chemical solutions were prepared in sterile water for injection (WFI) to obtain: 0.4% and 2.0% v/v Chlorhexidine (pH=6.2); 2.0% w/v glutaraldehyde (pH=7.4); 0.1% and 0.2% v/v sodium dichloroisocyanurate (pH=7.0); 0.025%, 0.05% and 0.1% sodium hypochlorite (pH=7.0); 0.5% v/v formaldehyde (pH=6.5); 1.0% v/v Minncare® (0.45% of PAA + 2.2% of H_{2}O_{2}, pH between 2.0 and 2.3) and 1.5% v/v and 26.5% hydrogen peroxide (pH=3.3). The concentration of total available chlorine and hydrogen peroxide was determined by the iodometric method [16]. The dilute solutions, prepared with chlorine-free glassware, were filtered through a 0.22 µm membrane (Millipore, Bedford, MA, USA) for daily use. The solutions of the test chemical agents are approved for hospital disinfection use by the Brazilian Ministry of Health [11].

The agents [17] used to inactivate the test disinfectants in solutions at 1% concentration, were respectively, polysorbate 80 (Tween 80) to quench chlorhexidine, glycine to bind glutaraldehyde and formaldehyde, catalase to degrade peracetic acid plus hydrogen peroxide; sodium thiosulphate to degrade sodium dichloroisocyanurate and hypochlorite. The efficacy of the disinfectant inactivating agents were confirmed by: (i) not having an inhibitory effect on the previous bioburden; (ii) completely eliminate the activity of the disinfectant; (iii) and when combined with the disinfectant, the resultant product was non-toxic to the test bacterium.

The manuscript title as well the objective, method, results, discussion and conclusion were about the “The effectiveness of chemical agents used in hospital disinfection programs over the bacteria isolated in outbreaks of infections in nurseries. The classification of the activity spectrum for each chemical agent according to D-values (contact time of the bacteria in the disinfectant solution necessary for the reduction of one log_{10} cycle of the bioburden) for test bacteria at set conditions outlines its possible utility in infection control programs in the health care environment (3, 8, 10). However, there is no disinfectant that can serve all situations and meet all needs, for different conditions of usage in health care routine (3, 5, 6, 8, 10)." For a better understanding of a disinfectant’s effectiveness and standardization of use in hospital disinfection programs, the test bacteria in solution were considered standard biological indicators (BI).”

Thank you very much for your comments and suggestions that helped us to improve the manuscript.
Sincerely yours,
Thereza Christina Vessoni Penna