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Gas–plasma sterilization: relative efficacy of the hydrogen peroxide phase compared with that of the plasma phase

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Abstract

Recent modifications specified in the European Pharmacopoeia IIIrd Edition (1 January 1997) have led us to investigate the sensitivity of *Bacillus stearothermophilus* ATCC 7953, a sporulated bacterial species now used instead of *Bacillus subtilis* var *niger* ATCC 9372 as a reference in peroxide sterilization. Inactivation kinetic studies have shown that this choice is well founded and also indicate that inherent efficacy of the STERRAD® 100 gas–plasma process is above all due to hydrogen peroxide. The ionization obtained during the plasma phase is validated in its role of a residue detoxifying process, without any sporicidal effect. © 1998 Elsevier Science B.V.

Keywords: *Bacillus pumilus*; *Bacillus stearothermophilus*; *Bacillus subtilis*; Biological indicators; Hydrogen peroxide; Plasma

1. Introduction

Among new low-temperature sterilization methods, the STERRAD® 100 gas–plasma technique employing hydrogen peroxide was presented a few years ago (Addy, 1991; Holler et al., 1993) and has been successfully marketed in several European countries, Japan and the United States. This reliable, simple-to-use technology has made it possible to use a sterilization method which is universally acknowledged as being effective, and provides hospitals with new applications. This system has three main advantages: (i) heat-sensitive equipment can be sterilized; (ii) no toxic residues are generated; and (iii) sterilization cycles are relatively rapid. The equipment sterilized is once again available, without a desorption phase being necessary, within about 1 h.

There have been few works concerning microbicidal mechanisms of these procedures. A certain number of publications are in fact contradictory and certain authors do not consider the method

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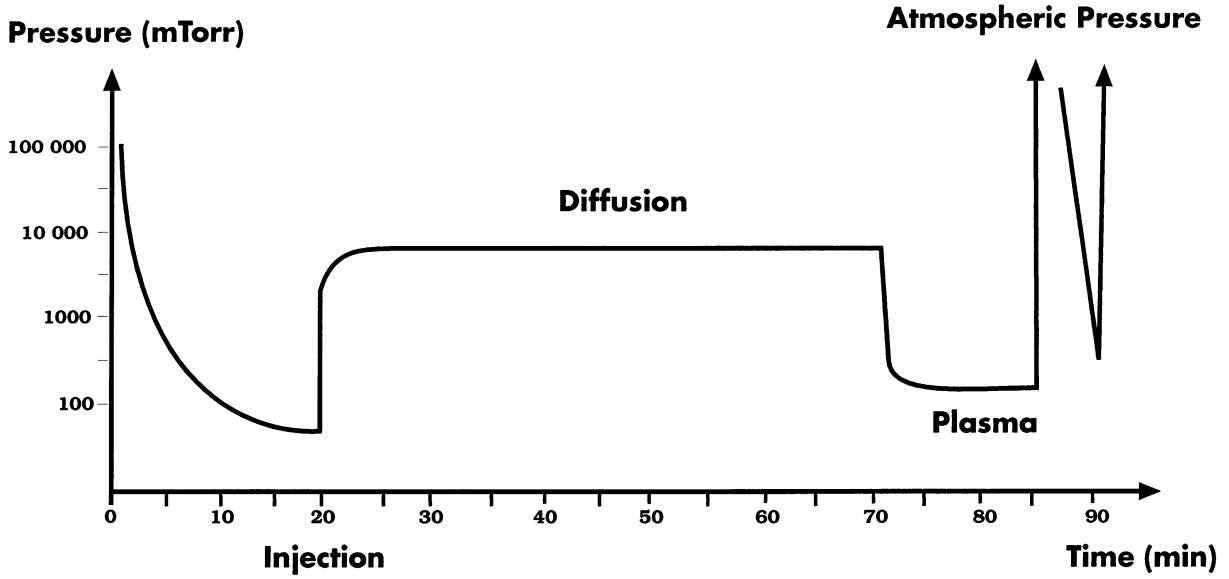

Fig. 1. STERRAD® 100 sterilization cycle.

as being a sterilization process (Nelson and Berger, 1989; Lundholm and Nyström, 1994; Peeters and Borchers, 1995; Darbord, 1996; Alfa, 1996; Alfa et al., 1996)

The recently added specification in the European Pharmacopoeia (1997), stating that *Bacillus stearothermophilus* ATCC 7953 must be used as a biological indicator where peroxide sterilization is used, has led us to consider that it is of value to investigate the resistance of other sporulated bacterial species which are recognized biological indicators in other official reference texts (*Bacillus subtilis* var *niger* ATCC 9372 for sterilization by dry heat and ethylene oxide and *Bacillus pumilus* ATCC 27142 for sterilization by irradiation); we also wanted to determine the respective roles of hydrogen peroxide and electromagnetically induced ionization in the efficacy of the plasma method.

2. Methods

2.1. Sterilizer

STERRAD® 100 apparatus, supplied by Johnson and Johnson Medical SA (France) and manufactured by ASP Inc. (Irvine, CA, USA) was used. The reference cycle (Fig. 1) for this type of apparatus comprises (following application of high vacuum) a hydrogen peroxide diffusion phase in a chamber with a capacity of about 175 l (1810 mg doses containing 58% hydrogen peroxide) in order to obtain a final concentration of about 6 mg/l, followed by a plasma phase lasting 15 min (300 W) induced by an electromagnetic wave generator (13.56 MHz, 10 eV). The chamber temperature is 45 ± 2°C. In order to investigate the effects at sub-lethal doses, we used cartridges containing less hydrogen peroxide (Table 1) as well as specially designed software which made it possible to vary the duration of the sterilization phases. All sterilization phases were performed on Johnson and Johnson Medical France's premises in Chatenay Malabry, under the supervision of the company's managerial staff.

2.2. Preparation of the biological indicators

We used spores of three bacterial species described in the European Pharmacopoeia (1997) that is to say *B. stearothermophilus* ATCC 7953, *B. subtilis* var *niger* ATCC 9372, *B. pumilus* ATCC 27142.

Table 1
Hydrogen peroxide weight and concentrations for the injections, and final concentrations in the chamber during the experiments

Types	Total weight (mg/well)	Hydrogen peroxide (%/well)	Final concentration in the chamber (mg/l)
Full cycle	1810	58	6
T	1788	0 (water)	0
A	530	2.90	0.09
B	1260	2.48	0.18
C	2180	2.92	0.36

Spores were obtained by cultivating these bacteria in a specific medium (tryptone 3 g/yeast extract 2.5 g/$MnSO_4$ 0.05 g/$CaCl_2$ 0.10 g/distilled water 500 ml/pH within a range of 6.8–7.2). Following an incubation period of 20 days at a temperature of 37°C for *B. pumilus* ATCC 27142 and *B. subtilis* ATCC 9372, and 45°C for *B. stearothermophilus* ATCC 7953, cultures were centrifuged and decanted several times, then each sediment was collected, placed in suspension (absolute ethanol) and stored at a temperature of +4°C for 1 week. Spores which could be revived were counted by plating of suitable dilutions and the characteristics of each species were verified (API 50CH, API 20 E). The suspensions (50 μl) were then applied to the three types of carriers which are more often used in sterilizations: paper strips (grade 740 E, Schleicher and Schuell) in reference to the Pharmacopeiae, polyethylene (Tyvek®) strips, recommended by the manufacturer of STERRAD®, (ASP), or polystyrene tubes (standard type for haemolysis). Polystyrene tubes were used in this study to compare a total hydrophobic surface with the others strips. Once dry (48 h at 30°C), the spores which could be revived were counted on 20 strips or tubes, using the above-mentioned technique (No.) and the others were packed in pouches consisting of a plastic and a polyethylene (Tyvek®) side (ref. TX 76004-0130, ASP), before sterilization.

2.3. Viewing of the surviving spores

Following preparation of the biological indicators, or after treatment, we counted on each carrier the units forming colonies (UFC/carrier) as follows:

2.3.1. Analysis of strips (for survival curves)

Each strip was placed in a tube containing 5 ml of Soybeam casein broth and 5 g of sterile glass beads. Ultra-sound was then applied to the tubes. (Sonoclean, Bandel in electronic RK 102 H) for 3 min, then mechanical stirring was performed (Vortex®).

The suspension obtained in this manner was diluted from 10^{-1} to 10^{-4} (using a peptone aqueous solution, 2 g/l in saline) and counted by plating on Soybeam casein agar medium and incubation at 37°C for *B. subtilis* var *niger* ATCC 9372 and *B. pumilus* ATCC 27142, or 45°C for *B. stearothermophilus* ATCC 7953, during 72 h, as described in the European Pharmacopoeia (1997).

2.3.2. Analysis of tubes (for survival curves)

We added 5 ml of broth to each tube before applying ultra-sound for 3 min; each suspension was then used in the same manner as that used for the strips.

2.3.3. Analysis of strips and tubes (for negative fraction)

For determination of negative fraction (Caputo et al., 1979), we performed sterility test using soybean casein broth and incubation during 7 days (6 mg/l cycle only).

3. Results

3.1. Determination of hydrogen peroxide concentration and diffusion time

It is not possible to study the plasma phase kinetics when standard concentrations (6 mg/l) are used. So, we firstly determined the hydrogen peroxide diffusion parameters (concentration and diffusion time) during the initial phase of the cycle, to choose experimental conditions which enable that at least 10^5 spores remained viable on each carrier, and ensured that the kinetics of the plasma phase could be investigated: Fig. 2 shows the results obtained in the inactivation of the three strains of *Bacillus* with a 30 min-diffusion time. Fig. 3 shows the kinetics of inactivation of the most resistant strain (*B. stearothermophilus* ATCC 7953), and Table 2 shows the decimal reduction times (*D* values) which represent the exposure making it possible to reduce each type of biological indicator by one logarithmic unit on a hydrogen peroxide-related basis only. Combined, these results confirm that hydrogen peroxide in the STERRAD® 100 cycle is extremely sporicidal (Rogovski et al., 1996) and exhibits a consistent dose-effect kinetic profile. *B. pumilus* ATCC 27142 was nearly resistant as *B. stearothermophilus* ATCC 7953 and the carriers did not seem to influence the results. We selected, for the following phases of our study, type C cassettes, for a final concentration in the chamber of 0.36 mg/l of hydrogen peroxide, and a 30 min-diffusion time.

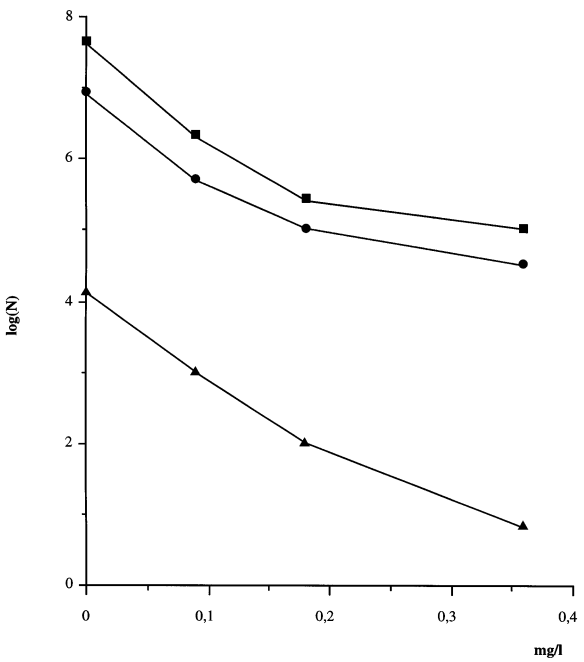

Fig. 2. Inactivation of spores under low concentrations of hydrogen peroxide (45°C). Ordinate: logarithm UFC/carrier ($n = 5$), abscissa: H_2O_2 concentration in the chamber. ■ *Bacillus stearothermophilus* ATCC 7953, ● *Bacillus pumilus* ATCC 27 142, ▲ *Bacillus subtilis* ATCC 9372.

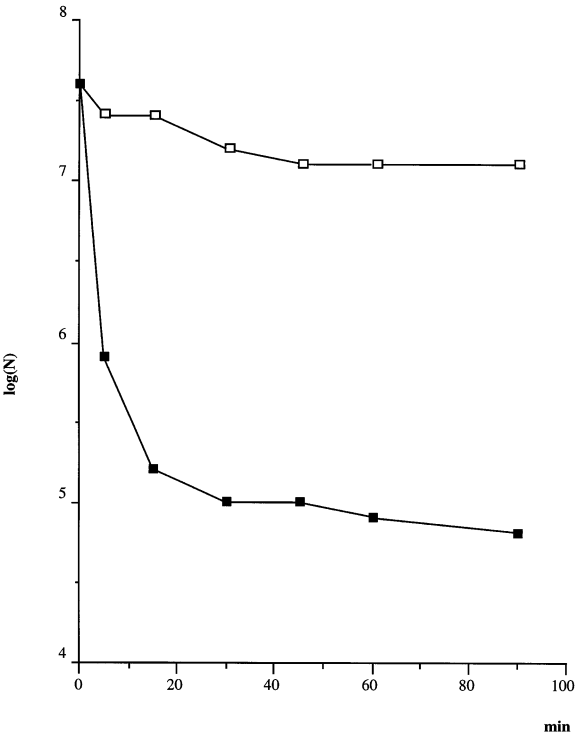

Fig. 3. Inactivation of Bacillus stearothermophilus ATCC 7053 (45°C) by hydrogen peroxide. Ordinate: logarithm UFC/carrier ($n = 10–30$), abscissa: exposure time. ■ 0.36 mg/l of hydrogen peroxide in the chamber, □ injection of 1.730 mg of water in the chamber (blank).

3.2. Studies of the plasma-phase

Fig. 4 shows the inactivation kinetics seen with the three *Bacillus* obtained by the plasma phase.

Table 2
Determinations of the *D* values with different types of spores or carriers

Species	Carriers	Hydrogen peroxide (mg/l)	Average *D* value (min)	Range of *D* value (min)
Bacillus stearothermophilus ATCC 7953	Paper (strips)	0	>50	—
		0.09	>50	—
		0.18	20	16–28
		0.36	10	8–14
		6	5	3.4–7
	Polyethylene (strips)	0	>50	—
		0.09	>50	—
		0.18	16	12–24
		0.36	9	8–14
		6	5	4–6.2
Bacillus pumilus ATCC 27 142	Paper (strips)	0	>50	—
		0.36	10	8–13.2
		6	4	3.1–6.1
	Polystyrene (tubes)	0	>50	—
		0.09	>50	—
		0.18	22	15–25
		0.36	10	8–13
		6	4	3.2–6
Bacillus subtilis var *niger* ATCC 9372	Paper (strips)	0	>50	—
		0.36	3.9	2.4–5
		6	2	—
	Polyethylene (strips)	0	>50	—
		0.36	10	8–13
		6	2.4	2–3.2

For the determination of *D* values, microbial destruction data were analyzed relative to the survival curve by direct enumeration method, except for the 6 mg/l cycle (fraction negative methods according to the Spearman–Karber procedure).

We didn't observe a time-effect kinetic profile. The slight increase of the survival spores, observed with *B. stearothermophilus* ATCC 7953 may be induced by mechanical or thermical action of the electromagnetic rays.

4. Discussion

Our studies clearly demonstrate that *B. stearothermophilus* ATCC 7953 and *B. pumilus* ATCC 27142 are the most resistant species when a sterilization by STERRAD® 100 is applied. Spores of *B. stearothermophilus* ATCC 7953 on a paper strip constitute the best biological indicator for peroxide sterilizations, confirming the indications of the European Pharmacopoeia (1997). The use of *B. pumilus* ATCC 27142 could also be recommended. A number of authors (Mecke, 1992; Geiss et al., 1994; Bialasiewicz et al., 1995) have specified that this micro-organism should be used because commercial preparations exist: they are biological indicator for the monitoring of ionizing radiation sterilizations, and contain higher concentrations of spores (10^7–10^8) than commercial preparations of *B. stearothermophilus*

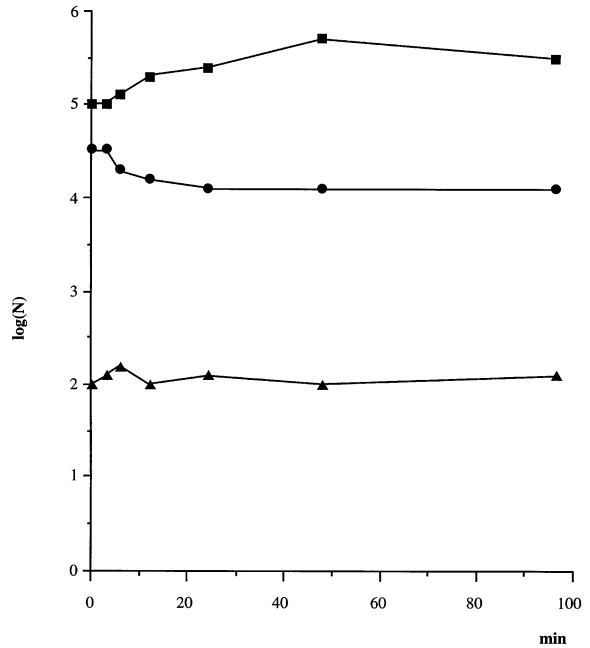

Fig. 4. Effect of electromagnetic activation ('plasma') after partial inactivation by hydrogen peroxide (30 min, 0.36 mg/l in the chamber). Ordinate: logarithm UFC/carrier ($n = 10–30$), abscissa: exposure time. ■ *Bacillus stearothermophilus* ATCC 7953, ● *Bacillus pumilus* ATCC 27 142, ▲ *Bacillus subtilis* var *niger* ATCC 9372.

(10^5–10^6). The use of *B. subtilis* var *niger* ATCC 9372 is however probably not advisable because it is more sensitive and far more variable results are obtained. This species was in fact recommended for vapor phase hydrogen peroxide sterilization (Rickloff and Graham, 1989) which has, as far as we know, been discarded as being too low-perfomance.

The STERRAD® 100 plasma phase appears to be non-sporicidal, in any case under our study conditions, whatever the exposure duration, the species or the carrier. This is a new finding, as only complete cycles, (Nelson and Berger, 1989; Mecke, 1992; Geiss et al., 1994; Kyi et al., 1995; Peeters and Borchers, 1995; Alfa et al., 1996), divided cycle fractions employing a hydrogen peroxide diffusion phase to plasma phase ratio that is proportional (Addy, 1991; Borneff et al., 1995) or variable diffusion phases followed by a complete 15 min-plasma phase (Jacobs and Szu Min Lin, 1996) have been described in the literature for the validation of this type of sterilization. Following in-depth investigation of inactivation kinetics as shown in Fig. 3 combined with the Fig. 4, it can be observed that this process involves several steps: During the 30-min diffusion phase, the slope of the curve decreases, indicating that efficacy decreases with time, most likely by binding or consumption of the gas. Then, sporicidal efficiency ceases suddenly right at the beginning of the plasma phase and this can be explained by the destruction of the molecular form of hydrogen peroxide. However, this observation should not suggest that the plasma phase in a full cycle is of less value: it provides destruction of peroxide radicals and of all toxic residues which could soil medical devices following sterilization.

Sterilization using hydrogen peroxide and ionization techniques (STERRAD® 100 gas-plasma) is an effective and safe process which has become more and more widely used over the past 6 years. It was thus shown that the sterilizing effect is hydrogen peroxide-related, while the ionization process in the plasma phase plays above all a detoxifying role. This finding makes it possible to monitor the efficacy of sterilization cycles by using better defined biological indicators. This new kinetic approach could provide optimization of the process, enabling investigation of new applications.

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