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Sporicidal efficacy of hydrogen peroxide aerosol

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Because of its high sporicidal activity but low toxicity, hydrogen peroxide (H_2O_2) is a useful agent for gentle antimicrobial treatment of irritable goods. Above all to optimise practical aspects of H_2O_2 application, sporicidal efficacy of aerosols based on 1% H_2O_2 solution was tested. Using commercially available bioindicators with *Bacillus subtilis* spores immobilized on filter paper strips, a 7 h lasting aerosol treatment at room temperature resulted in a reduction of viable count of 2.5 $\log_{10}$ (median, $n = 5$). By combined application of 0.3% sorbitan monolaurate (Span ®20) and 1% H_2O_2 aerosol at room temperature, a reduction of viable count of >6.3 $\log_{10}$ (median, $n = 5$) could be reached within 2 h, already. Following aerosol treatment at 60 °C, all bioindicators tested ($n = 15$) have been found without growth after 10 min, corresponding to a reduction of viable count >6.3 $\log_{10}$. However, this efficacy was possible only if the spores were immobilized on filter paper strips. Changing the carrier materials lower sporicidal activity of H_2O_2 aerosol was found resulting in a reduction of viable count between >5.4 $\log_{10}$ (median, $n = 15$) on polyethylene foil and 0.4 $\log_{10}$ (median, $n = 15$) on glass slides. These differences could not be correlated with surface qualities as porosity or wettability.

1. Introduction

Hydrogen peroxide (H_2O_2) is well known as an effective antimicrobial agent with a broad activity spectrum including bacterial spores and viruses, and a very low toxicity. In the living tissue some protection mechanisms are active to prevent healthy cells from H_2O_2 toxicity (Block 1991; Olson 1998; Hoxey and Thomas 1999). Therefore, besides its traditional use in wound applications, H_2O_2 containing solutions are very useful agents for antimicrobial treatment of irritable goods, which come into close contact with living tissue but cannot be sterilized by conventional methods (Vesley et al. 1991; Rutala and Weber 1996, 1999a,b). A main disadvantage of liquid phase antimicrobial agents is the necessity to soak the products directly into the solution, and to rinse and dry it after treatment. Consequently, several investigations have been undertaken to use H_2O_2 in the vapour phase. In the low-temperature plasma sterilization (LTPS) system, a H_2O_2 gas plasma is created at a temperature around 45 °C under vacuum, using radio frequency or microwave energy to realize the strong electric or magnetic fields required to excite the gas. In the vapour-phase hydrogen peroxide process (VPHP), H_2O_2 is vaporized either in a deep vacuum at 55–60 °C or by heating to 105 °C at atmospheric pressure. Both systems are scheduled for sterilization or rather decontamination of medical devices and aseptic processing equipment (Klapes and Vesley 1990; Parisi and Young 1991; Gruhn et al. 1995; Crow and Smith 1995; Bialasiewicz et al. 1995; Wallenwein 1995; Jahnke and Lauth 1996; Bardat et al. 1996; Graham and Mielnik 1997; Martens et al. 1998; Roberts and Antonoplos 1998; Olson 1998; Hoxey and Thomas 1999; Penna et al. 1999; Mau et al. 2001). Disadvantages of both systems are the large-scale technical equipment necessary to realize sealed deep-vacuum or heated chambers for generating the H_2O_2 gas as well as the limits of the sterilization process. Moreover, deep vacuum, increased temperature, or plasma treatment may be stress factors, which cannot be tolerated by several sterilization goods especially in the field of medical devices. Therefore, the aim of the present study was to test if a H_2O_2 aerosol, which is generated at low temperature and atmospheric pressure, can be used for decontamination purposes.

2. Investigations and results

Soaking of commercially available *Bacillus subtilis* spore strips (BioStrips) directly into 1% H_2O_2 solution for 1 h at room temperature resulted in a reduction of viable count of 1.1 {1.0/1.3} $\log_{10}$ (median {*minimum/maximum*}, $n = 5$). Reduction factors (RF) of 1.9 {1.8/2.2}) $\log_{10}$ (median {*min./max.*}, $n = 5$) and 4.4 {3.4/>6.3} $\log_{10}$ (median {*min./max.*}, $n = 5$) have been found after 3 and 5 h, respectively. All five bioindicators tested have been found without growth after 7 h being equal to a RF > 6.3 $\log_{10}$ because of the mean starting contamination of $N^0_{mean} = 1.8 \times 10^6$ spores per strip ($\log_{10}$ $N^0_{mean} = 6.26 \pm 0.19$, mean ± SD, $n = 5$; Fig. 1). Using the same 1% H_2O_2 solution at room temperature, but as an aerosol, resulted in significantly ($\alpha = 0.05$) lower RF even after 1 h aerosol treatment time (RF = 0.8 {0.7/1.2} $\log_{10}$, median {*min./max.*}, $n = 5$). With a prolongation of the aerosol treatment the difference of reduction factors compared to

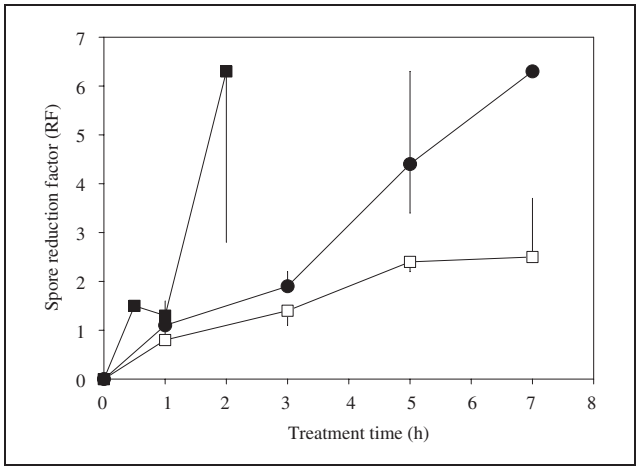

Fig. 1: Median spore reduction factors (RF) after treatment of BioStrips by 1% H_2O_2 solution (filled circles), by 1% H_2O_2 aerosol (open squares) and by 1% H_2O_2 aerosol containing 0.3% sorbitan monolaurate (filled squares) at room temperature (n = 5 each; error bars: spread between minimum and maximum RF values).

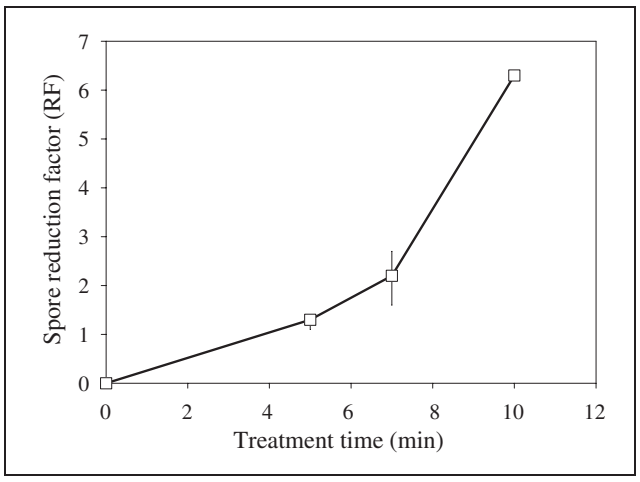

Fig. 2: Median spore reduction factors (RF) after treatment of BioStrips by 1% H_2O_2 solution at 60 °C (n = 5 each, exception: n = 15 at 10 min treatment time; error bars: spread between minimum and maximum RF values).

the direct soaking into H_2O_2 solution increased. After 7 h treatment time the reduction of viable count was not higher than 2.5 {2.4/3.7} $\log_{10}$ (median {*min./max.*}, n = 5; Fig. 1). The sporicidal efficacy has been intensified substantially by addition of 0.3% sorbitan monolaurate (Span ®20) to the 1% H_2O_2 solution. Applying this mixture as an aerosol at room temperature, four out of five BioStrips have been found sterile after 2 h treatment time (RF > 6.3 {2.8/> 6.3} $\log_{10}$, median {*min./max.*}, n = 5; Fig. 1). A 3 h lasting aerosol treatment with 0.3% sorbitan monolaurate, only, did not result in any sporicidal effect (data not shown).

Increasing the temperature slightly up to 60 °C, 15 BioStrips have been found completely free of viable spores after 10 min treatment with 1% H_2O_2 aerosol without any chemical additives (RF > 6.3 $\log_{10}$; Fig. 2). This efficacy could be realized only with commercially available spore test strips, where cellulose-based filter paper serves as spore carrier material. Using other carrier materials, the best sporicidal efficacy was reached on polyethylene, where 9 among 15 spore strips have been found without growth under the same treatment conditions. Because of a starting contamination of $N^0_{mean} = 2.5 \times 10^5$ spores per strip, this corresponded to a median RF > 5.4 $\log_{10}$ (Table 1). Using other materials, the number of sterile test strips as well as the median RF decreased in the following sequence: ceramics > polystyrene > aluminium foil > paper > glass. A slight exception was paper, where five among 15 strips where found without growth, but because of the high number of surviving spores on the non-sterile strips the median RF was lower compared to aluminium foil where only one sterile strip has been found after aerosol treatment (Table 1). This graduation in sporicidal efficacy on different materials could not be correlated with special surface qualities of the spore carrier materials. Based upon scanning electron microscope (SEM) pictures (10000fold magnitude), smooth and non-structured surfaces of the carriers made from polyethylene, polystyrene, aluminium foil, and glass could be assumed. Ceramics showed a porous surface whereas on the BioStrips and the paper strips a layered fibrous surface was seen. On spore-inoculated surfaces, 1–2 µm long rod-shaped *B. subtilis* spores were visible distinctly, which partially laid one upon the other. On paper strips the spores were localized partially inside caverns formed by paper fibres (scanning electron microscopic pictures are not shown).

As another surface characteristic the wettability by 1% H_2O_2 solution has been estimated using contact angle measurements. The best wettability was found on ceramics demonstrated by low contact angles between 33.1° and 36.9°. On BioStrips a contact angle measurement was not possible because of the absorbent character of the filter paper being used as spore carrier material. Contact angles on glass and polystyrene have been measured in the same range between 57.2° and 67.2°. On paper, aluminium foil and polyethylene the highest contact angles between 79.9° and 88.2° were measured, whereas the lowest wettability was found on polyethylene (Table. 2).

Table 1: Results of treatment of different *Bacillus subtilis* spore carriers by 1% H_2O_2 aerosol for 10 min at 60 °C

Spore carrier material	$\log_{10}$ spore count per carrier before treatment $\log_{10} N^0_{mean}$ (mean, n = 5)	Number of sterile (n_0) related to the whole number of equally treated spore carriers (n) n_0/n	Spore reduction factors $RF = \log_{10} N^0_{mean} - \log_{10} N^t_{single}$		
			Median	Minimum	Maximum
BioStrip	6.3	15/15	>6.3	>6.3	>6.3
Polyethylene	5.4	9/15	>5.4	1.4	>5.4
Ceramics	5.4	6/15	3.5	2.3	>5.4
Polystyrene	5.3	1/15	2.2	1.6	>5.3
Aluminium foil	5.4	1/15	1.9	0.3	>5.4
Paper	4.8	5/15	1.2	0.6	>4.8
Glass	5.4	1/15	0.4	0.1	>5.4

(n = 15 each)

Table 2: Contact angles of 1% H_2O_2 solution on different materials; results from three single measurements

Material	1.	2.	3.
Ceramics	33.1°	33.3°	36.9°
Glass	57.2°	59.3°	62.8°
Polystyrene	64.3°	65.3°	67.2°
Paper	79.9°	82.7°	84.8°
Aluminium foil	84.3°	87.0°	87.0°
Polyethylene	86.7°	88.2°	88.2°

3. Discussion

In a variety of medical speciality areas an increasing array of technologically advanced reusable instruments are used which are physically complex, fragile and expensive, and which cannot be sterilized by heat or high-energy radiation (Bond et al. 1991). For antimicrobial treatment of such devices, gases or vapours are often suitable as they can penetrate into niches inaccessible to liquids, and are quickly eliminated from an enclosure by displacement. The preferred use of H_2O_2 as chemical antimicrobial agent is due to its microbicidal effectiveness as well as to its safety and breakdown in innocuous products (water and oxygen). Even if it is well known that H_2O_2 just as other chemicals is far more efficient as a sterilant in the aqueous phase (Olson 1998), decontamination systems based on gas or vapour phase H_2O_2 are well established in the meantime. The aim of the study presented here was to test the antimicrobial efficacy of fogging with a H_2O_2 aerosol, because only few data is published on the use of H_2O_2 in the vapour phase (Sokolowa et al. 1977; Kramer et al. 1987; Hoxey and Thomas 1999).

The lower efficacy of the ultrasound-generated aerosol compared to the direct soaking of test items into the solution has been confirmed using a water-based H_2O_2 solution at room temperature and atmospheric pressure. Synergistic activities of H_2O_2 with chemical agents are known in principle (Bayliss and Waites 1976; Waites et al. 1979; Stevenson and Shafer 1983; Kramer et al. 1987; Block 1991). Our intention to combine sorbitan monolaurate (Span ®20) with H_2O_2 was based on former experience with inclusion compounds of H_2O_2 and surfactants in urea, which showed higher antimicrobial activity but lower toxicity compared to pure H_2O_2 solutions (Jülich et al. 1999). One of the most effective inclusion compound contained H_2O_2 and sorbitan monolaurate in the same ratio as used in the aerosol application demonstrated in the study presented here. The use of a mixture of 1% H_2O_2 and 0.3% sorbitan monolaurate (Span ®20) for aerosol generation resulted in a substantial improvement of the sporicidal efficacy of H_2O_2 aerosol, which exceeded the effects of direct soaking into pure H_2O_2 solution at room temperature. Consequently, by this synergistic combination of two chemical agents, an antimicrobially effective aerosol treatment of surfaces and devices with relatively low stress caused by moisture or heat will be possible even if a treatment time as long as 2 h was still necessary to realize a useful RF of at least 6 $\log_{10}$.

Ultraviolet radiation or ultrasonic energy are physical agents which have been reported in the literature to act synergistically with H_2O_2 (Stevenson and Shafer 1983; Bayliss and Waites 1979, 1980; Kramer et al. 1987; Waites et al. 1988; Block 1991; Russell 1999b). Naturally, also an influence of temperature on the antimicrobial activity of H_2O_2 is well known. Several authors have confirmed the general statement that the antimicrobial efficacy increases with increasing temperature (Wallhäußer 1995). Most of these studies have been performed with bacterial spores, using H_2O_2 solutions 1–30% at temperatures between 10 and 80 °C (Swartling and Lindgren 1968; Toledo et al. 1973; Sokolowa et al. 1977; Stevenson and Shafer 1983; Block 1991; Wilkins et al. 1994; Hoxey and Thomas 1999). On the other hand, also inverse temperature effects have been found with H_2O_2 aerosol or vapour-phase H_2O_2 generated by a VPHP surface decontamination system. This was explained by more rapid decomposition of H_2O_2 at the higher temperatures under such application conditions (Sokolowa et al. 1977; Kramer et al. 1987; Klapes and Vesley 1990; Hoxey and Thomas 1999). From our point of view this explanation is not satisfactory, especially since several authors emphasize the stability of H_2O_2 at higher temperatures up to 130 °C (Stevenson and Shafer 1983; Olson 1998). Moreover, our own investigations could not confirm such an inverse temperature effect of H_2O_2 aerosol. We found a substantial increase in sporicidal efficacy if the aerosol treatment has been performed at 60 °C. This confirms a report of Hecker et al. (1997) who stated a steep reduction of the VPHP performance time if the temperature was increased from 20 °C to >50 °C. In our study, the slight temperature rise up to 60 °C resulted in a reduction of viable count on the BioStrips of at least 6.3 $\log_{10}$ within 10 min already. This temperature should be acceptable for the majority of heat sensitive products.

Consequently, the use of H_2O_2 aerosol is a very effective, fast, gentle as well as easy to realize possibility for antimicrobial treatment of several irritable goods. Moreover, the vapour application at 60 °C causes very low moistening of the test objects, which could get back dry from the aerosol treatment chamber.

Surprisingly, this intensive sporicidal efficacy of 1% H_2O_2 aerosol at 60 °C was strongly dependent on the material that was used as spore carrier. On one hand, 15 commercially available BioStrips could be found without growth after 10 min treatment time only (RF $>$ 6.3 $\log_{10}$). On the other hand, on glass slides a RF of only 0.4 $\log_{10}$ was found, i.e. nearly no sporicidal effect occurred under exactly the same treatment conditions.

However, the broad spectrum of spore reduction factors found on the different materials could not be correlated with different characteristics of surface topography estimated by SEM. Because it was assumed that the "active units" of the H_2O_2 aerosol are tiny droplets of H_2O_2 solution which precipitate on the spore contaminated test material surfaces, a different ability to spread onto the materials might influence the sporicidal efficacy. Therefore, the wettability of the different materials has been tested by contact angle measurements. On the BioStrips, the test solution has been fully and immediately absorbed by the filter paper material. This might be an explanation of the high sporicidal efficacy found with the BioStrips. On the other materials, a correlation between the spore reduction factors and the contact angles was not possible. Polyethylene has been found to have the lowest wettability of all materials tested, demonstrated by contact angles of nearly 90°, although with spore strips made of this material the second highest reduction factors have been found. On glass slides nearly no sporicidal effect occurred despite the wettability was much better than that of polyethylene.

Effects of carrier properties on the spore resistance have been reported for steam and dry heat as well as for formaldehyde and ethylene oxide gas sterilization (Doyle and

Ernst 1968; Spicher and Borchers 1987; Weiss 1993; Spicher et al. 1993, 1996; Russell 1999a). Several authors pointed out that cellulose-based materials cannot be processed by VPHP or LTP procedures, respectively, because of the high H_2O_2 absorption of this material (Block 1991; Mecke 1992; Rutala and Weber 1996; Hecker et al. 1997; Hoxey and Thomas 1999; Rutala and Weber 1999b; Mau et al. 2001). However, this restriction seems to be mainly caused by the danger of remaining H_2O_2-residues after treatment but not by a limited antimicrobial efficacy, e.g. because of H_2O_2 decomposition on cellulose materials. From our point of view, the H_2O_2 absorption may be a reason for the high sporicidal activity found in our study. This is confirmed by the fact, that also on polyethylene carriers high reduction factors have been found. This material is also reported to sorb low levels of H_2O_2 (Olson 1998). Nevertheless, a satisfactory explanation of the different sporicidal efficacy of identical H_2O_2-aerosol treatments on different carrier materials cannot be given up to now.

Summarizing all, the treatment by H_2O_2 aerosol seems to be a useful alternative for decontamination of irritable goods, which cannot be sterilized by conventional procedures. By addition of a surfactant or treatment at moderately increased temperature spore reduction factors of at least 6 $\log_{10}$ could be proved experimentally. However, it must be stated that the antimicrobial efficacy of H_2O_2 aerosol seems to depend strongly on the material treated. Therefore, processing parameters of H_2O_2-based sterilization procedures should not be estimated using commercially available bioindicators only. After all, it is another argument for the necessity to adapt an individual sterilization procedure to any individual product. This should be true especially in the case of irritable goods, which can be treated by very gentle procedures only.

4. Experimental

4.1. Experimental set-up for aerosol treatment

An experimental set-up for aerosol generation and treatment of test strips was specially designed and constructed (Institute of Nonthermal Plasma-Physics, Greifswald, Germany). The aerosol was generated using an ultrasound nebulizer (U-3002-S, Schulte-Elektronik GmbH, Olsberg, Germany) with an aerosol tube (Respiflo fold tube, Kendall – Medizinische Erzeugnisse GmbH, Neustadt, Germany), and discharged through a 100 cm long pipe into a closed but not sealed chamber (23.5 × 16.5 × 9.5 cm). Pipe and chamber were to be heated as well as heat insulated. On the bottom of the chamber holding devices for spore test strips were localized. Temperature and relative humidity inside the chamber could be monitored by a commercially available digital thermo hygrometer. Because this type of ultrasound nebulizer is normally used for inhalation therapy, the size of aerosol droplets should be in the range of 5 µm.

4.2. Chemical agents

H_2O_2 test solutions 1% were prepared from a 30% stock solution (Ph. Eur., stabilized, Roth GmbH, Karlsruhe, Germany) by dilution with distilled water. Addition of 0.3% sorbitan monolaurate (Span®20) was done based on a 10% stock suspension of the surfactant (Span®20 solution, 10% in water, Fluka).

4.3. Microbiological tests

Commercially available bio-indicators for sterilization monitoring (BAG BioStrips, 32 × 8 mm, Biologische Analysensysteme Gmbh, Lich, Germany; *Bacillus subtilis* var. *niger* ATCC 9372) made from cellulose-based filter paper were used. Additionally, self-prepared spore carriers have been used based on strips (32 × 8 mm) made of wood-free drawing paper (190 $g \cdot m^{-2}$), aluminium foil, polystyrene, and polyethylene foil, ceramic strips (51 × 5 mm) and neutral glass slides (round, 13 mm diameter). To inoculate the carriers, 100 µL each of a use-dilution *B. subtilis var. niger* ATCC 9372 spore suspension (approximately 10^7 CFU $\cdot$ mL^{-1}) have been spread homogeneously over the whole area of one side of test carrier, and dried for at least 4 h at room temperature inside a biosafety cabinet.

To estimate the spore count on a carrier it was transferred into a sterilized test tube (10 × 100 mm) containing 5 ml tryptic soy broth (BAG Biologische Analysensystem GmbH, Lich, Germany) as well as 3 sterile glass spheres (5 mm diameter) and agitated for 30 min on a shaking machine THYS 2 (MLW Labortechnik Ilmenau, Germany) with the maximum frequency of 6 s^{-1}. Afterwards, another 5 ml tryptic soy broth were added and agitated for another 30 min on the shaking machine. The spore count in the resulting tryptic soy broth-based spore suspension was estimated by the viable spread plate technique. Samples of 100 µL each, of graduated 1 : 10 dilutions of the spore suspension have been spread onto tryptic soy agar plates (BAG Biologische Analysensystem GmbH, Lich, Germany). After overnight incubation at 37 °C the number of colonies per plate were counted. In the case of sterile agar plates the incubation was continued up to 7 d. The spore count was calculated in relation to the respective dilution factor, to estimate the number of colony forming units (CFU) per carrier.

To get *B. subtilis var. niger* ATCC 9372 spore suspensions for carrier inoculation, BioStrips were incubated for 48 h in sterile culture vessels containing 10.0 mL tryptic soy broth (BAG Biologische Analysensystem GmbH, Lich, Germany) at 37 °C. From a resulting *Bacillus subtilis* bacterial suspension a sporulation culture has been grown over 7 days at 37 °C on wheat grits agar (Hallmann 1953). The harvested crop has been suspended in 10 mL sodium chloride solution 0.9%. For washing purposes, the homogenised suspension was centrifuged three times (4500 min^{-1}), and in between the spore pellets were resuspended in 10 mL fresh sodium chloride solution 0.9%. The population of the final 10 mL stock spore suspension was quantified by the viable spread plate technique as described above. Final use-dilution *B. subtilis* spore suspensions for carrier contamination have been prepared by making dilutions in sodium chloride solution 0.9% based on the stock suspension (approximately 10^9 CFU $\cdot$ mL^{-1}).

Estimation of spore count on the test strips was performed immediately after the end of treatment by H_2O_2 containing solutions or aerosols, respectively, in all experiments. Consequently, because of the addition of 5 ml of tryptic soy broth without delay before starting the agitation of the strips, a neutralization of possible H_2O_2 residues on the test strips was guaranteed by dilution.

4.4. Material surface characterization

A scanning electron microscope Leo 440 (Leo Elektronenmikroskopie GmbH, Oberkochen, Germany) based upon gold sputtered pieces (Sputter coater Cressington 108 auto, Elektronen-Optik Service GmbH, Dortmund, Germany) with 10000 fold magnification was used to characterise the material surfaces.

To evaluate surface wettability, the contact angle of H_2O_2 solution was evaluated optically. A goniometer was used with a camera, a sample holder and an external lighting system to illuminate the sample. For each measurement, a 10 µl droplet of 1% H_2O_2 solution was applied to the test surface, and a picture was taken within the next 10 s. From the developed photos contact angles were estimated geometrically (Hesby et al. 1997; Grainger and Healy 1999).

4.5. Statistics and data presentation

The sporicidal efficacy of the different aerosol treatments on the different carrier materials has been quantified by calculation of reduction factors (RF) for each single spore carrier. For this purpose the difference between the $\log_{10}$ of the mean spore count on a special carrier material before treatment ($\log_{10} N^0_{mean}$, mean ± SD, n = 5) and the $\log_{10}$ of the spore count (CFU) on one single carrier of the same material after antimicrobial treatment ($\log_{10} N^t_{single}$) was calculated:

$$RF = \log_{10} N^0_{mean} - \log_{10} N^t_{single} \quad (1)$$

In the case of a sterile spore carrier after antimicrobial treatment, the resulting RF was given as $>\log_{10} N^0_{mean}$. To summarize RF values, median with minimum and maximum was given, because a Gaussian distribution of the results could not be assumed. For statistical comparison of the $\log_{10}$ RF values of different treatments, the Wilcoxon rank correlation test for non-related samples was used.

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