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Survival of Bacteria on Respirator Filters

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ABSTRACT. The survival and potential growth of two different kinds of bacteria were investigated on NIOSH-certified polypropylene respirator filters. *Bacillus subtilis* var *niger* (*B. subtilis*) spores represented environmentally resistant bacteria, whereas *Pseudomonas fluorescens* (*P. fluorescens*) vegetative cells represented stress-sensitive bacteria. The bacteria were aerosolized and loaded on the respirator filters under three nutritional conditions: water, saliva, and tryptic soy broth (TSB).

The loaded filters were incubated for 0–13 days and analyzed for culturable and total bacteria count. The analysis was optimized through the evaluation of three methods for eluting bacteria from respirator filter samples: low frequency shaking, vortexing, and ultrasonic vibrating. Vortexing was found to be the most effective with the highest total and culturable count and was therefore used for the analysis of bacterial survival and growth.

Neither of the test bacteria was able to grow on the respirator filters even under optimal nutrition and incubation conditions. It therefore appears that reuse of a polypropylene respirator poses minimal risk of bacterial growth, provided the respirator has been carefully handled and stored. The data on respirator survival show that sensitive *P. fluorescens* cells lost their viability in less than three days, whereas resistant *B. subtilis* spores remained viable on the filter for over thirteen days of testing. It appears that resistant bacteria may pose risk of infection to the people near the respirator wearer if the bacteria are reaerosolized from the respirator. However, reaerosolization of a very small fraction of collected bacteria can occur only under extreme conditions of violent sneezing or coughing.

INTRODUCTION

Airborne bacteria may reach sensitive areas of the human body via the respiratory tract and may cause infections, allergies, and toxic reactions. Diseases like tuberculosis, diphtheria, and legionellosis are contracted in this manner (Lacey and Crook 1988; Nevalainen et al. 1993; CDC 1994; Davis et al. 1997; and Fennelly 1997). Several tuberculosis outbreaks led to the pro mulgation of guidelines for preventing the transmission of *Mycobacterium tuberculosis* (Mtb) in health-care facilities (CDC 1994). Bacteria are also known to cause health problems in several other occupational environments, such as waste-water treatment facilities and agricultural settings (Lacey and Dutkiewicz 1994).

The use of respirators is a common control method for preventing the transmission of infec-

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tious airborne droplets emitted by humans during exhalation. These droplets are usually in the size range of 1–5 μm (CDC 1994). The N95 half-mask filtering facepiece respirators, certified by the National Institute for Occupational Safety and Health since 1995, are the most frequently used by health-care workers against the transmission of Mtb (OSHA 1998). These respirators are widely used because they provide a reasonable balance between comfort, cost, practicality, and protection. Recent tests have shown that the filtration efficiency of N95 filters is 99.5% or higher for bacteria of size and shape similar to Mtb (Qian et al. 1998).

In our previous study, it was shown that reaerosolization of bacteria collected on fibrous N95 respirator filters is insignificant at conditions encountered in normal respirator wear (Qian et al. 1997a). Some reaerosolization (< 0.1%) is possible only during violent sneezing or coughing. Another concern related to the risk of respirator reuse is the possibility of microorganism growth on a respirator filter due to improper handling, storage, and reuse. If bacteria grow on a respirator during storage, reaerosolization may occur more readily because many of the new bacteria will grow into the space above the filter fibers and may thus be entrained more easily by the airflow through the filter.

Microorganisms may grow in the presence of sufficient moisture and nutrients (Madigan et al. 1997a). Pasanen et al. (1993, 1994) have shown that the storage of respirator filters in humid environments can cause rapid fungal growth on respirator filters made of cellulose because the fungi are able to digest cellulose. However, only a few groups of bacteria are capable of decomposing polymeric substances (Madigan et al. 1997b), which are typically used in filter materials today. Therefore, bacteria cannot generally use modern filter materials as nutrient. This was confirmed by Maus et al. (1997), who demonstrated that the viability of *Micrococcus luteus* and *Escherichia coli* declined within 1 h after collection on ventilation filter media when no nutrients were present and the test was performed at low relative humidity (30–60%). Much less is known about bacterial behavior on respirator filters under realistic use conditions. When the respirator is worn for several hours, it may become wetted on the inside from the humidity in the exhalation air, depending upon how well the exhalation valve performs. This aspect was taken into account by Brosseau et al. (1997), who showed that *B. subtilis* spores, as well as *Staphylococcus epidermis* and *Mycobacterium abscessus* vegetative cells, survived on respirator filters when stored at relative humidity of 85% for five days. However, the respirator wear does not only increase the moisture content of the respirator but also affects the nutritional condition in the respirator. Exhalation air generally contains saliva, which consists of various components including cystatins, mucins, histatins, and other molecules that might act as either nutrients or antibacterial agents (Levine 1993). Particles that are collected onto the respirator filters from the air may contain nutrients suitable for bacteria survival and growth. If used respirators are stored in plastic bags, the humidity and nutrients collected on the filter material may favor microbial growth or survival.

In the present study, we investigated the growth and survival of bacteria collected on a respirator filter and stored under conditions favorable for bacteria growth. Two typical environmental bacteria, *B. subtilis* spores and *P. fluorescens* vegetative cells, were selected to represent two extremes in their sensitivity. *B. subtilis* endospores are gram-positive bacteria and are known to be very resistant to many environmental stresses (Sneath 1986), whereas *P. fluorescens* is a gram-negative bacterium and is thus much more sensitive to stresses (Neidhardt et al. 1990). *B. subtilis* has been used as a physical simulant for Mtb in respirator filter testing (Johnson et al. 1994). New (not previously used) polypropylene respirator filters were challenged with *B. subtilis* or *P. fluorescens* under three nutritional conditions: in the absence of any nutrients, in the presence of human saliva, and in the presence of TSB. Human saliva simulates the nutritional conditions during respirator wear, whereas TSB represents ideal nutritional conditions.

To analyze the bacterial concentrations on respirator filters, the first step is to remove them from the filter media. There is little information available on the effectiveness of different methods to remove bacteria from filters (McCullough et al. 1998). One objective of the present study was therefore to develop a procedure for eluting bacteria from a loaded filter. The main objective of this paper is to present the methods developed for determining the survival of bacteria on respirator filters and to analyze the data obtained under different nutritional conditions.

MATERIALS AND METHODS

Microorganisms and their preparation for the experiments

B. subtilis spores were received from the US Army Edgewood Laboratories (Courtesy of Agnes Akiyemi and Dr. Edward Stuebing, Edgewood Research, Development, and Engineering Center; Aberdeen Proving Ground, MD). *P. fluorescens* culture was obtained from the American Type Culture Collection (ATCC 13525, Rockville, MD). Before using them in the experiments, the *B. subtilis* spores were activated at 60°C for 25 min and washed twice with sterile deionized water by centrifugation at 4,800 × g for 15 min (Marathon 6K centrifuge, Fisher Scientific, Pittsburgh, PA). *P. fluorescens* was prepared for dispersion by growing 1 ml of bacteria culture in 100 ml of TSB at 28°C for 18 h (TSB, Becton Dickinson Microbiology System, Cockeysville, MD). The cells were then washed three times in sterile deionized water by centrifugation at 4,800 × g for 15 min.

Immediately before filter loading, 5 ml of the prepared bacterial suspension was mixed with 45 ml of one of the following suspensions: deionized water, autoclaved human saliva, or nutrient broth TSB. The total concentration in each suspension was 10^9–10^{10} No/ml, measured with a hemocytometer as described below.

Filter loading

One type of typical N95 respirator filters, made by a major manufacturer and composed of polypropylene fibers, was tested in this study. Before challenging them with the test bacteria, identical half-mask filtering facepiece respirators were randomly selected from their supply box and circular filter samples of 40 mm diameter each were cut out of the respirators. Prior to testing, the filter samples were preconditioned as required for certification testing (42CFR, part 84): 37°C and 85% relative humidity. The preconditioning chamber was saturated with a potassium chloride salt solution to maintain the relative humidity at 85% (Greenspan 1977).

The experimental setup for loading the respirator filters with test bacteria is schematically shown in Figure 1. This is a modification of the setup previously used for studying particle reentrainment from respirator filters (Qian et al. 1997b). The test bacteria were aerosolized with the nutrient at a flow rate of 6 L/min by a three-nozzle Collison nebulizer (BGI, Inc., Waltman, MA). The aerosol flow was dried with clean dry air of 30 L/min and then entered the loading chamber, where the test filter was mounted in an open-face filter holder (Gelman Sciences, Ann Arbor, MI). The airflow rate through the respirator filter with effective filtration area of 10.8 cm^2 was 5 L/min during loading, corresponding to a filtration velocity of 7.8 cm/s. This simulates the average air velocity through a half-mask respirator filter used under medium work load conditions. The stability of the airborne bacteria concentration in the loading chamber was monitored with a real-time aerosol size spectrometer which sampled upstream of the respirator filter (Aerosizer, Amherst Process Instruments, Inc., Hadley, MA). The Aerosizer showed an average airborne concentration of 110 bacteria/cm^3 and less than 7% variation in aerosol concentration during loading, as defined by the coefficient of variation. The loading time for each filter sample was 10 min.

The loaded filters were incubated at 85% relative humidity and a temperature of 37°C for *B.*

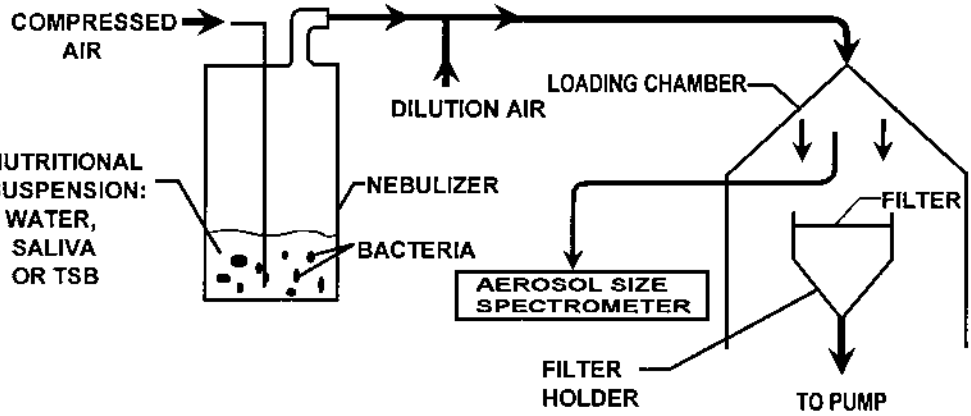

FIGURE 1. Experimental setup for respirator filter loading.

subtilis (Sneath et al. 1984) or at 28°C for *P. fluorescens* (Palleroni et al. 1984). After 0, 3, 6, 9, or 13 days of incubation, filters were randomly selected for bacterial analyses.

Elution and analysis of bacteria

To analyze the total and culturable counts of bacteria on the filter media after incubation, the first step was to remove the bacteria from the filters. Each test filter was submerged for 30 min in a plastic tube filled with 50 ml of 0.1 N potassium phosphate buffer solution and 0.02% Tween-80. In order to have the largest possible number of bacteria available for analysis, several techniques were compared with each other for eluting *B. subtilis* spores from the filters immediately after loading: low frequency shaking, vortexing, and ultrasonic vibrating. Low frequency shaking was performed with a water shaker (Gyrotory, Model G76, New Brunswick Scientific Co., Inc., Edison, NJ), vortexing with a vortex touch mixer (Model 231, Fisher Scientific, Pittsburgh, PA), and ultrasonic vibrating with an ultrasonic bath (Branson Cleaning Equipment Company, Shelton, CT). The resulting suspensions were analyzed for total and culturable bacteria count. It cannot be assumed, however, that these counts represent the "true" bacterial counts on the filter, because a portion of the bacteria are likely to remain on the filter even when using the most effective elution method. Furthermore, the most energetic methods are likely to reduce the culturability of bacteria or may cause deaggregation of bacterial clusters (McCullough et al. 1998). Therefore, the elution method that resulted in the highest total and culturable counts was then utilized in the microbial survival and growth study.

The total bacteria count was obtained by counting the bacteria in the suspension with a hemocytometer (Petroff-Hausser counter, Hausser Scientific Partnership, Horsman, PA) using a phase contrast microscope at a magnification of 400 × (Labophot-2, Nikon Corp., Tokyo, Japan).

The total bacteria concentration (total count) was determined as follows:

Total bacteria concentration on filter,

$$\text{No/cm}^2 = \frac{C}{A}\left(\frac{V_1}{V_{\text{cell}}}\right), \quad (\mathbf{1})$$

where C is the average bacteria count in seven hemocytometer cells, V_1 is the volume of elution solution for each filter sample (50 ml), V_{cell} is the volume of each hemocytometer cell $(8 \cdot 10^{-7}$ ml), and A is the effective filtration area of each filter sample (10.8 cm^2).

The culturable bacteria count was obtained by cultivating two replicates of three consecutive dilutions of the elution suspension on Tryptic Soy Agar (TSA, Becton, Dickinson Microbiology Systems, Cockeysville, MD). The agar plates with *B. subtilis* were incubated for 18 h at 37°C, the plates with *P. fluorescens* for 40 h at 28°C. The culturable bacteria concentration (culturable count) was calculated as follows:

Culturable bacteria concentration on filter,

$$\text{cfu/cm}^2 = \frac{N}{10^{-n}A}\frac{V_2}{V_2}, \quad (2)$$

where N is the average number of colony forming units on the agar plates, n is the dilution factor (typically 1–3), and V_2 is the volume of diluted solution cultivated on each agar plate (0.2 ml). V_1 and A are the same as defined in Equation (1). The fraction of culturable bacteria is

Fraction of culturable bacteria,

$$\% = \frac{\text{Culturable Count}}{\text{Total Count}} 100\%. \quad (3)$$

Statistical analysis

Each experimental set was repeated three times, and the results are reported as means and standard deviations. The statistical analysis were performed with log-transformed data because the concentrations were log-normally distributed. The general linear model followed by the Scheffe's test was used for the data analysis (Statistical Analysis System, SAS Institute, Inc., Gary, NC). When analyzing the survival of bacteria on filters, the concentration of culturable bacteria was the dependent variable, the total bacteria concentration was a covariate, and the nutritional condition and the storage time were independent variables.

RESULTS AND DISCUSSION

Figure 2 shows the total and culturable count of *B. subtilis* spores eluted from respirator filters as a function of elution time. It is seen that the total bacteria concentrations were below $4.0 \cdot 10^6$ No/cm^2 for low frequency shaking and ultra-

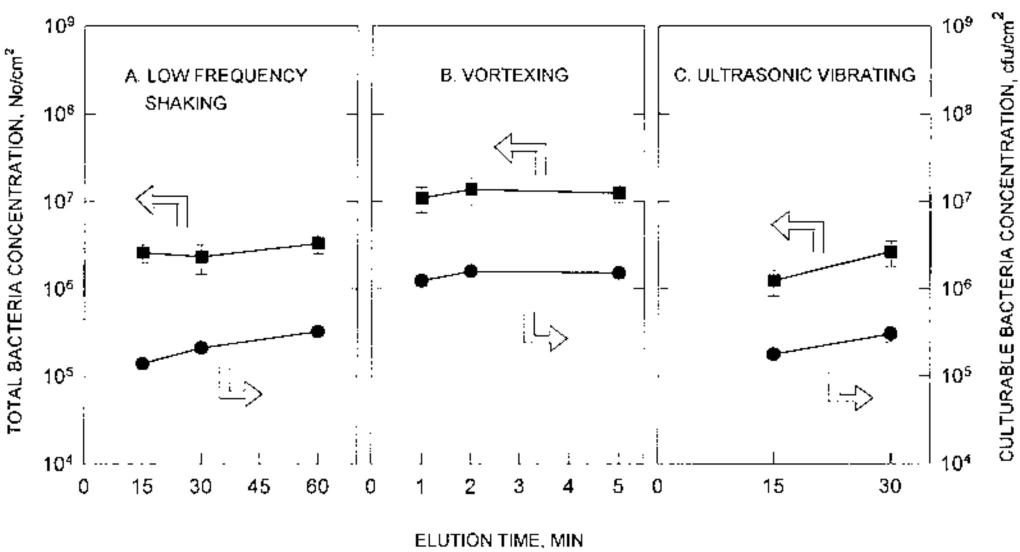

FIGURE 2. Comparison of three methods for eluting *B. subtilis* spores from polypropylene respirator filters. Error bars indicate the standard deviation from the means of three repeats. ■ is the total bacteria concentration; ● is the culturable bacteria concentration.

sonic vibrating, whereas the amount obtained by vortexing was above $1.0 \cdot 10^7$ No/cm^2. The culturable bacteria count exhibits the same trend as the total bacteria count: below $4.0 \cdot 10^5$ cfu/cm^2 for low frequency shaking and ultrasonic vibrating, above $1.0 \cdot 10^6$ cfu/cm^2 for vortexing. The vortexing resulted in statistically significantly higher counts for both total and culturable bacteria $(p < 0.05)$. Figure 2(B) indicates that the number of recovered bacteria reached its maximum value after 2 min of vortexing. An additional 3 min of vortexing did not increase the number of bacteria recovered.

The differences among these elution methods can be related to the differences in their frequencies and amplitudes, as shown in Table 1. It appeared that with its low frequency of 2.3 Hz at an amplitude of 30 mm, the shaker could not provide enough energy to detach the bacteria from the filter fiber. Ultrasonic vibrations failed to elute the bacteria from the filter fibers any better. It appeared that the ultrasonic waves of very small amplitude ($\ll$ 1 mm) and very large frequency (42,000 Hz) failed to overcome the inertia of the bacteria and fibers and get them into sufficient vibration for effectively separating them from each other. Vortexing, at a frequency of 47 Hz and an amplitude of 5 mm, provided an effective relative motion between the bacteria and filter fibers to remove the bacteria from the filters more effectively than low frequency shaking or ultrasonic vibrating. Based on these results, vortexing for 2 min was selected as the elution method throughout the rest of our experiments.

The results on the survival of *B. subtilis* spores on a polypropylene respirator filter after storage under three nutritional conditions are presented in Figure 3. The fraction of culturable bacteria in the original nebulizer suspension before aerosolization was 72%. This fraction was about 25% when analyzed from the respirator filters immediately after loading. During the 13-day storage of the respirator filters, the total bacteria counts stayed approximately constant between $1.9 \cdot 10^7$ No/cm^2 and $2.5 \cdot 10^7$ No/cm^2, while the culturable bacteria counts decreased to different levels for each of the three nutritional conditions: $2.0 \cdot 10^5$ cfu/cm^2 without any nutrients, $1.2 \cdot 10^6$ cfu/cm^2 with saliva, and $3.1 \cdot 10^5$ cfu/cm^2 with TSB. The fraction of culturable *B. subtilis* spores on the respirator filter ranged from 1.5% to 6% after 13 days of incubation. Statistical analysis showed that *B. subtilis* spores survived better with saliva and nutrient broth than without any nutrients $(p < 0.05)$.

TABLE 1. Frequencies and amplitudes of the elution equipment.

	Frequency, Hz	Peak Amplitude, mm
Low Frequency Shaker[a]	2.3	30
Vortex[b]	47	5
Ultrasonic[c]	42×10^3	$\ll 1$

[a]Measured by authors.
[b]Provided by Fisher Scientific.
[c]Typical for ultrasonic baths.

The survival of *P. fluorescens* cells on the polypropylene respirator filter after storage is presented in Figure 4. Before generation, the initial culturable fraction of bacteria was 95% in the nebulizer liquid, but decreased to about 0.1% immediately after loading on the filters. The total bacteria count on the respirator filters remained approximately between $7.0 \cdot 10^6$ No/cm^2 and $5.0 \cdot 10^7$ No/cm^2 for each nutritional condition during six days of incubation. The culturable count of *P. fluorescens* decreased from about 10^4 cfu/cm^2 to values below the detection limit of 8 cfu/cm^2 during the first three days for each nutritional condition. Therefore, the test was discontinued after six days.

The decreases in the culturable fraction of bacteria after loading indicate stress on the bacteria population. As expected, *B. subtilis* spores demonstrated less loss of culturability during aerosolization and longer survival on respirator filters than *P. fluorescens* cells because of the hardy nature of the *B. subtilis* endospores

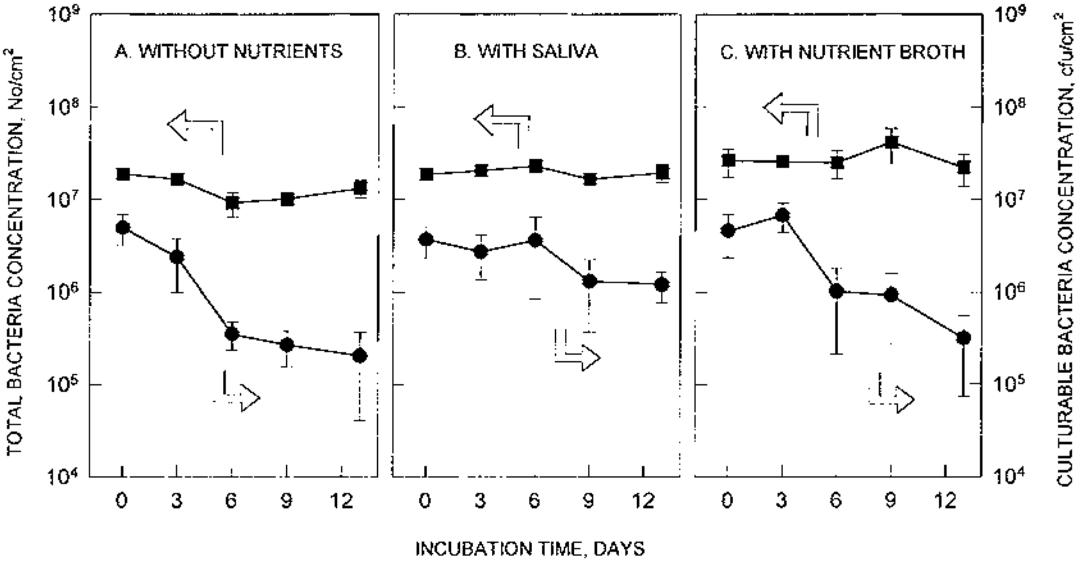

FIGURE 3. Survival of *B. subtilis* spores on polypropylene respirator filters during two weeks following loading under different nutritional conditions. Error bars indicate the standard deviation from the means of three repeats. ■ is the total bacteria concentration; ● is the culturable bacteria concentration.

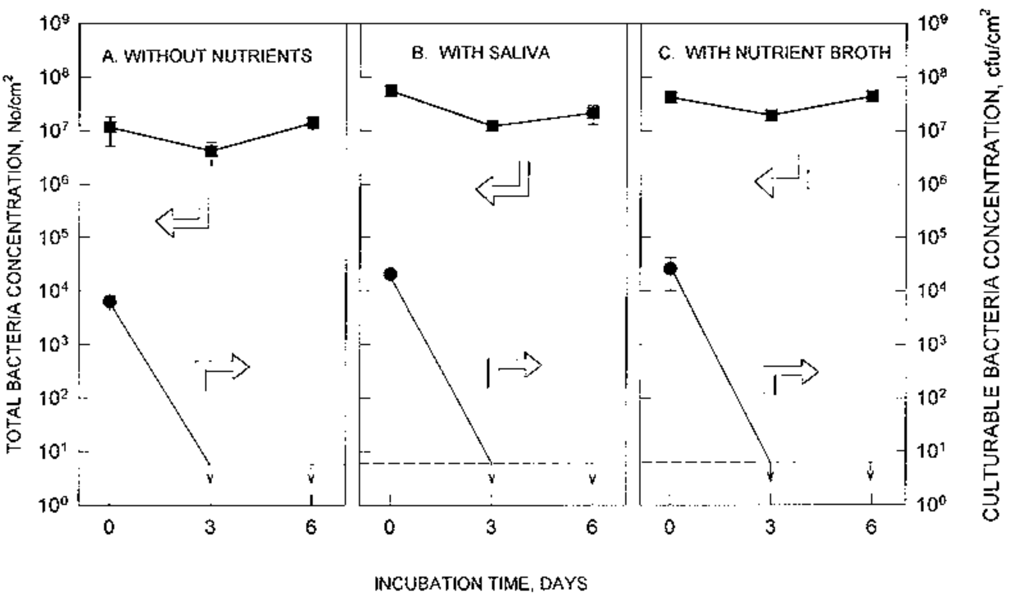

FIGURE 4. Survival of *P. fluorescens* cells on polypropylene respirator filters during one week following loading under different nutritional conditions. Error bars indicate the standard deviation from the means of three repeats. ■ is the total bacteria concentration; ● is the culturable bacteria concentration.

versus that of the gram-negative *P. fluorescens* microorganism. This finding supports the results of Brosseau et al. (1998), who concluded that *B. subtilis* spores survive better on respirator filters than vegetative cells of *S. epidermis* and *M. abscessus*. In this study, we stored the respirators with *B. subtilis* or *P. fluorescens* deposited on their surfaces under temperature and relative humidity conditions optimal for bacteria growth. Therefore, our tests represent worst-case conditions for respirator storage. We also added nutrients onto the filter simulating real-use conditions when bacteria are collected on a respirator together with other airborne materials which can act as bacterial nutrients.

Under the experimental conditions of these tests, the Collison nebulizer aerosolized about 2 ml of suspension in 10 min (Ulevicius et al. 1997). Assuming a mean droplet diameter of 3 μm (Ferron et al. 1997) and taking into account the flow rate through the filter and the area of the filter, we estimated that these droplets form a 0.28 mm thick nutrient layer on the filter fibers. In addition, each 1 μm diameter bacterium is estimated to be coated by an approximately 1 μm thick layer of nutrient medium. This can be considered to simulate an extreme amount of nutritional loading for respirator filters. The condition of using saliva as nutrient can be considered close to that of actual respirator wear, while TSB was selected to simulate optimal nutritional conditions for bacteria growth.

The data suggest that the amount of nutritional supply is insufficient to support bacterial growth even for the resistant bacteria *B. subtilis* spores. However, *B. subtilis* survived over 13 days on the filter and the survival was better with saliva and TSB than without any nutrients. In a recent study (Reponen et al. 1998) under similar experimental conditions, but using *Mycobacterium smegmatis* as a surrogate for Mtb, no bacteria growth was found during respirator storage. The decrease in the culturability of *M. smegmatis* during incubation showed a trend similar to that of *B. subtilis* and *P. fluorescens*. This suggests that our findings may be applicable for many other bacteria.

To cause airborne infections, bacteria must stay alive on the respirator filter and must have the opportunity to become airborne again. In this study, we have shown that *B. subtilis* and *P. fluorescens* are unable to grow on a polypropylene respirator filter even under optimal nutrition and incubation conditions. This study also demonstrates that sensitive *P. fluorescens* cells cannot survive for more than three days on the respirator filter, while resistant *B. subtilis* spores can remain viable on filters for more than 13 days. However the culturable fraction of *B. subtilis* spores remained below 25% when analyzed from the loaded filters. Qian et al. (1997a) reported that reaerosolization of bacteria from the fibrous filters of N95 filtering facepiece respirators is insignificant under conditions encountered in normal respirator wear. Some reaerosolization ($< 0.1\%$) is possible only during violent sneezing or coughing.

CONCLUSIONS

The study concludes that neither spore-forming bacteria (*B. subtilis*) nor vegetative bacteria (*P. fluorescens*) were found to grow on common polypropylene respirator filters, even under optimal nutrition and incubation conditions. Taking into account the low rate of reaerosolization and the low fraction of viable bacteria, it appears that reused respirators may pose minimal risk of reaerosolization of environmental sensitive bacteria, provided that the respirators are carefully handled and stored. Resistant bacteria may pose some risk, but only during violent sneezing and coughing.

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