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SURVIVAL OF MYCOBACTERIA ON HEPA FILTER MATERIAL

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ABSTRACT

Mycobacterium tuberculosis (MTB) is transmitted through the air, and can be captured on ventilation air filters. People handling these filters may be exposed to infectious material. We studied the survival of strains of *Mycobacterium* on high efficiency particulate air (HEPA) filter material. We used a model ventilation system to evaluate survival over time of *Mycobacterium chelonae* and H37Ra (an avirulent stain of MTB) aerosolized and then captured on HEPA filter material. Survival curves for *M. chelonae* incubated at 55% and 75% RH under static conditions were not different with less than 4% survival at 24 hours. H37Ra was subjected to continuous airflow at the design airflow for the filter material following deposition on the HEPA filter material, and less than 0.1% of cells survived to 48 hours (RH not controlled). H37Ra was resistant to immobilized biocide (trimethoxysilylpropyl dimethyloctadecyl ammonium chloride) on HEPA filter material as well as the same biocide in solution. Finally, survival of H37Ra and virulent MTB strain (H37Rv) were not different following deposition onto HEPA filter material from liquid suspension and incubation under static conditions.

INTRODUCTION

Tuberculosis, which results from infection with *Mycobacterium tuberculosis* (MTB), continues to be one of the most important and dangerous of airborne infectious disease, especially in developing countries (Porter & McAdam, 1994). The recent resurgence of the disease in immune-suppressed people, especially those with acquired immune deficiency syndrome (AIDS), and the appearance of multi-drug resistant strains have focused attention on the disease throughout the world. In the United States over the last five years, airborne epidemics have been reported in shelters for the homeless (Porter & Kessler, 1995), prisons (Coninx *et al.*, 1995; Drobniewski, 1995), and in commercial passenger aircraft (CDC, 1995). Nosocomial tuberculosis is particularly of concern both because of the populations of especially susceptible patients, and because of the occupational risk for health care workers (Luby *et al.*, 1994).

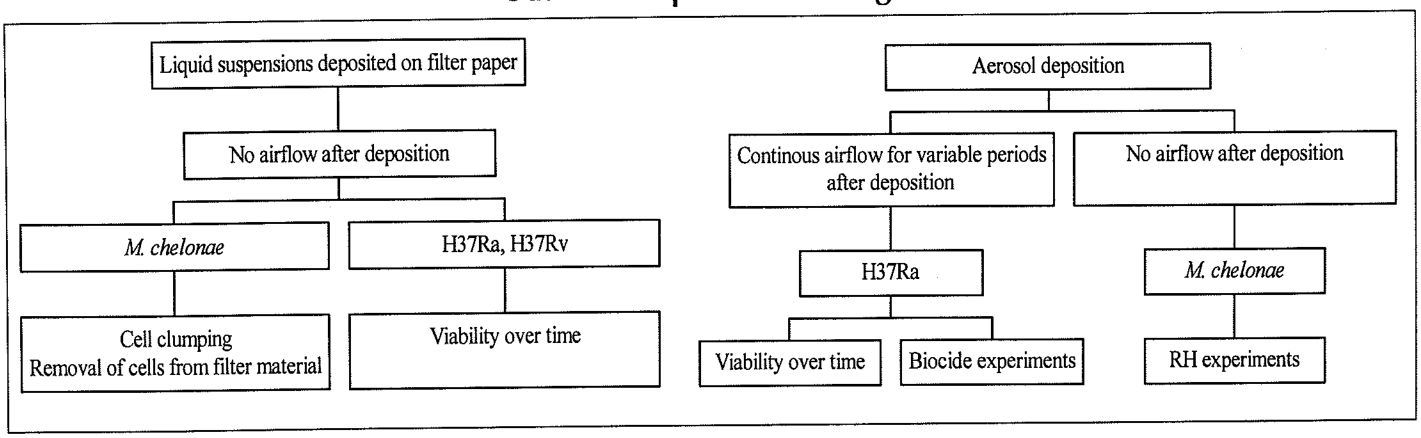
Pulmonary tuberculosis is a true airborne disease, and exposure via aerosols is considered the primary mode of transmission. One commonly-used method to control the spread of tuberculosis in clinical settings is enhanced exhaust ventilation with exhaust air ducts fitted with High Efficiency Particulate Air (HEPA) filters, which trap virtually all MTB cells (Nicas, 1995). The filters must be replaced routinely, a process that might disturb the filter and result in release of deposited cells.

Mycobacterium is relatively tolerant of environmental stress. Culturable cells have been recovered from outdoor air at high altitudes (Imshenetsky *et al.*, 1978), and after heating on slides prepared from sputum smears (Allen, 1981), and have survived on cotton lint for up to 2 months (Hirai, 1991). There is, then, a possibility that viable (and infectious) MTB might be re-aerosolized when a HEPA filter used in a clinical setting is replaced. Anecdotal release of microorganisms including *Mycobacterium* from filter fibers by handing and air movement has been reported (Brosseau, 1997). To assess the risk of such exposure, several factors must be considered.

- the probability of the presence of MTB on a filter, and the estimated deposition density;
- the probability of release of cells from filter fibers;
- the rate of cell death on the filter material

In this study, we evaluated the adherence of *Mycobacterium* to HEPA filter material, and the rate of cell death on the filter material. We used *Mycobacterium chelonae* as a surrogate for MTB to measure the adherence of cells to HEPA filter material during liquid extraction, and to examine the effects of relative humidity on viability (as measured by culturability) under static (no airflow) conditions of cells following deposition from aerosol. We used a model ventilation system to evaluate the survival of H37Ra (an avirulent strain of MTB) aerosolized onto HEPA filter material and subjected to continuous airflow following deposition. We evaluated survival over time of H37Ra deposited on biocide pretreated HEPA filter material. We also evaluated the effect of biocide in solution on survival of H37Ra as well as *Micrococcus roseus* used as a control. Finally, we compared the death rate of H37Ra with that of H37Rv (a virulent strain of MTB) deposited directly from cell suspensions onto HEPA filter material. Our experimental design is outlined in Figure 1.

FIGURE 1
Outline of experimental design.



MATERIALS AND METHODS

Bacteria and culture methods

Mycobacterium chelonae was isolated from used machining coolant from a manufacturing plant and maintained on R2A agar (DIFCO, Detroit, MI) at room temperature. Avirulent *M. tuberculosis* (H37Ra) and virulent *M. tuberculosis* (H37Rv) were obtained from American Type Culture Collection (ATCC 25177 and ATCC 27294, respectively) as freeze-dried log-phase cultures, and were subcultured on Lowenstein-Jensen agar slants (Remel, Lenexa, KS) for 3-4 weeks at 35°C. *Micrococcus roseus* was also obtained as a log phase freeze-dried culture from ATCC (ATCC 186) and was cultured on R2A at room temperature.

Effect of sonication and vortexing on culturability

M. chelonae cells were harvested from 7-10 day R2A cultures and suspended in phosphate buffered saline (PBS), resulting in a concentration of culturable cells of about 10^6 colony forming units (CFU)/ml. After a 2 minute settling period, the supernatant cell suspensions were subjected to sonication (130 Watt, 47 kHz) using a Bransonic 3200 (Danbury, CT) for 1, 10, or 30 minutes, or vortexed for 1-10 minutes. All treated suspensions, as well as the untreated stock suspension, were serially diluted and a 0.1 ml portion of each dilution was spread-plated onto R2A (30°C incubation). White waxy colonies that appeared after 7-10 days incubation were counted.

Removal of cells from HEPA filter material

A suspension of *M. chelonae* was prepared as described above, sonicated for 5 minutes, and diluted 10-fold with 1.5 ml 0.02% Tween 20 so that there were 10^5 CFU/ml. One hundred µl of this suspension was pulled through a 1 cm^2 piece of HEPA filter material with gentle suction by laboratory vacuum line (maximum 1.33 kPa), thus depositing 10^4 CFU onto the filter. The filter material was then air-dried, immersed in 1.5 ml 0.02% Tween 20, and either sonicated for 30 minutes or vortexed for 10 minutes. The resulting suspension was serially diluted, spread-plated onto R2A agar, and colonies counted after 7-10 days. Total cell counts were determined from each suspension using the AO method described below. The remaining washed HEPA filter material was homogenized in 1.5 ml 0.02% Tween 20 using a tissue grinder and the resulting homogenate was cultured on R2A agar to estimate filter-adherent CFU and also analyzed by AO microscopy to estimate total numbers of adherent cells. Controls for each of these experiments included processing of untreated (neither sonicated nor vortexed) cell suspensions with and without added HEPA material.

Direct microscopic counts

Acridine orange (AO) staining was used for direct microscopic counts (Hobbie *et al.*, 1977; Kepner & Pratt, 1994) in the experiments for determining the percentage of cells released from HEPA filter material by sonication. Briefly, cell suspensions in PBS were fixed by adding formaldehyde to a 1% final concentration and stored at 4°C for later analysis. For counting, 1 ml of each suspension was passed through an Amido-black-stained polycarbonate filter with a pore size of 0.2 μm (Poretics, Livermore, CA). Trapped cells were stained on the filter with 1 ml 0.1% AO (Sigma, St. Louis, MO) in distilled water (DW), washed with 0.02% Tween 20 in DW, air dried on a glass microslide, and mounted in Cargille oil (Cargille Lab. Inc., Cedar Glove, NJ). Fluorescing cells of a size and shape consistent with *Mycobacterium* (1-2 μm diameter rods) were counted at a magnification of 1000x with an Olympus epifluorescence microscope fitted with a 100 W high pressure mercury burner and 490 nanometer blue filter. Cells in 25 randomly chosen fields were counted.

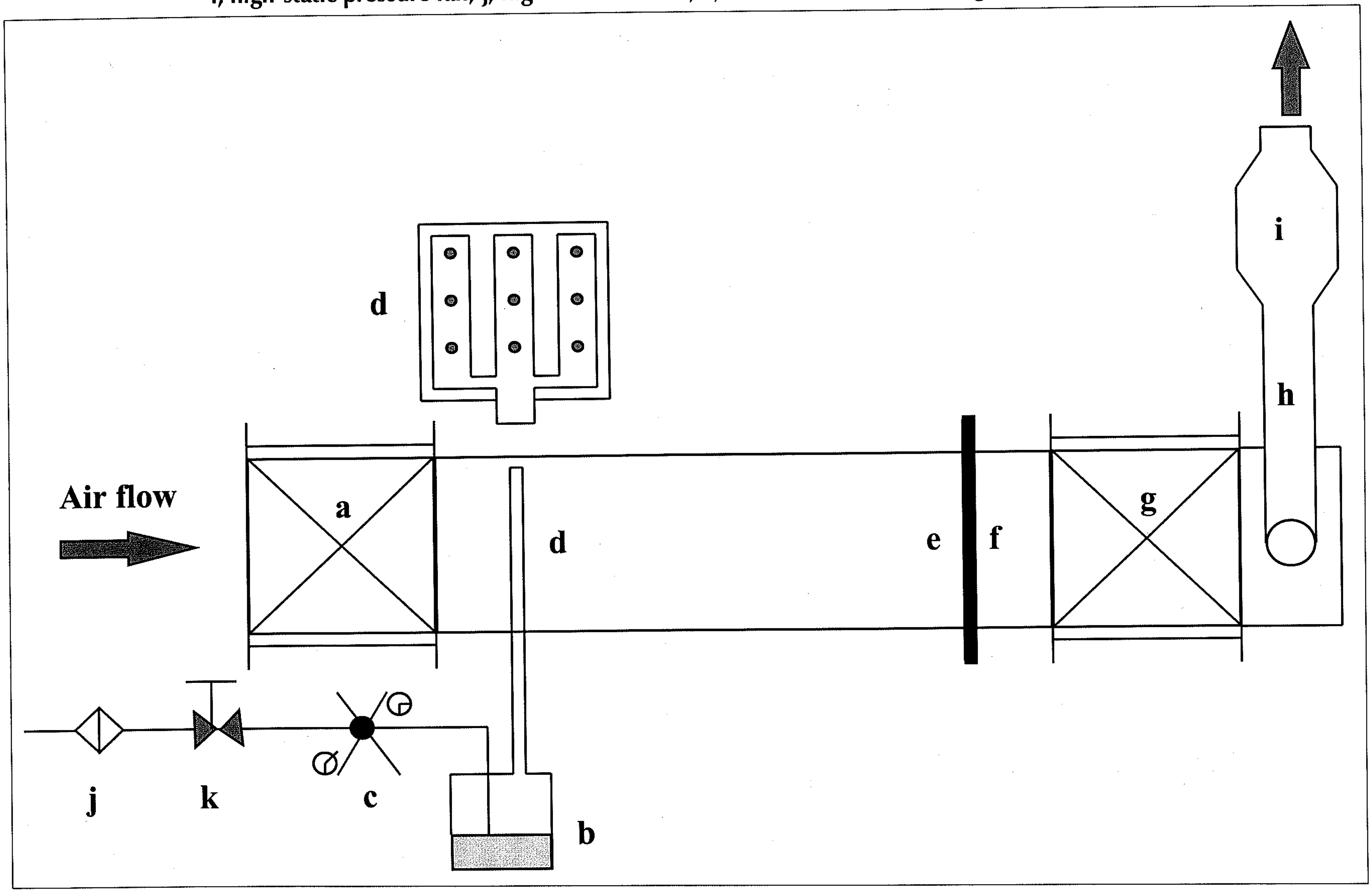
The model ventilation system

The experimental ventilation system apparatus was designed specifically for this project and is shown schematically in Figure 2 with the salient parts labeled. Air enters a 165 mm square duct through the inlet HEPA filter (a). The HEPA filter prevents entry of stray microorganisms and also prevents back-flow of aerosolized microorganisms should an upset condition occur. The test aerosol, generated with a Collison nebulizer (b) (BGI, Waltham, MA) (May, 1973) using filtered air at a gage pressure (c) of 137 kPa is discharged into the main air stream through a distribution manifold (d) having nine uniformly spaced, downstream-facing, 4.88 mm diameter ports to provide a uniform aerosol distribution. The aerosol stream flows through a flat piece of HEPA filter material (e) 381 mm downstream of the manifold. The filter holder assembly consists of two stainless steel frames, 241x241 mm, with a center opening 165 mm square (equal to the dimensions of the duct). The downstream square has a stainless steel support screen (f) against which is placed a cellulose support pad, and on which the flat test filter is placed. The whole assembly is held together by 12 countersunk machine screws and wing-nuts which tightly compress the filter assembly. This filter assembly is gasketed on the outer surfaces and fits between two flanges of upstream and downstream parts of the experimental ventilation duct, and is held together with 8 high-torque clamps. When the two duct halves are separated, the filter assembly is held to the downstream duct by the wing-nuts, allowing the sample plugs to be removed from the HEPA filter paper with minimal manipulation of the filter assembly. The exiting air stream is filtered by a second HEPA filter (g) to ensure thorough decontamination and then flows into the exit duct (h). A variable-speed, high-static pressure fan (i), located at the extreme downstream end of the apparatus, provides the desired airflow rate, which is monitored by a sharp-edged orifice flow meter calibrated with a spirometer. Airflow through the system was maintained throughout these experiments at 1.35 L/s, resulting in a superficial velocity through the test filter of 49.5 mm/s (5 fpm, the design flow rate for this filter). Each section is gasketed to prevent leaks. All parts are constructed from stainless steel, so that they can be easily cleaned and decontaminated in an autoclave.

An autoclavable high-efficiency filter (Gelman Telflo PTFE Membrane Filter [2 μm pore diameter]) (j) is used to clean the pressurized air going to the Collison nebulizer. The assembled system was leak tested using standard ASME N510 in-place filter test methods (ASME, 1989). Penetration of a cold dioctylphthalate challenge aerosol (Air Techniques TDA-4A, Baltimore, MD) as determined using a forward-scattering light photometer (Air Techniques TDA-2-E) was less than 0.01% for each filter. To provide an additional level of protection, the entire

FIGURE 2

Schematic diagram of the aerosol chamber. a, HEPA filter; b, Collison nebulizer; c, pressure regulator; d, manifold; e, test filter and cellulose backing support; f, stainless steel screen; g, HEPA filter; h, exit duct; i, high-static pressure fan; j, high efficient filter; k, air valve. The unit of length is centimeter.



apparatus is housed within a four-foot wide Class II, Type A, NSF 29-certified biological safety cabinet. An aerosol generated in the Collison nebulizer from an aqueous solution of methylene blue was collected on the test filter to verify deposition uniformity. Based on visual observation, the deposition was found to be uniform.

Culturability of aerosol-deposited cells under static conditions

Cell suspensions of *M. chelonae* for aerosolization were prepared as described above, sonicated for 5 minutes, and fetal calf serum (FCS) was added to a final concentration of 10% to simulate salivary protein concentrations (about 3.5 mg/ml [Theunissen *et al.*, 1993]). The Collison nebulizer was run for one hour during which time about 3×10^6 CFU were aerosolized into the experimental ventilation system and pulled through the HEPA filter at about 1.35 L/s. The filter was then divided into two pieces and incubated in static chambers at room temperature with relative humidity controlled at 55% and 75% using pools of saturated $Ca(NO_3)_2 \cdot 4H_2O$ and NaCl (respectively) in equilibrium with the chamber atmosphere. A sharpened cork-borer was used to collect two 0.65 cm^2 samples of each filter after 0, 1, 2, 4, 8, 12, 24, 48, 72 hours. The samples were homogenized and the resulting homogenate (including both suspended and adherent cells) was processed by culture as described above. Experiments were repeated four times.

Culturability of aerosol-deposited cells under dynamic conditions

Aerosols of H37Ra were generated and deposited onto filters in the model ventilation system as described above. The test HEPA filters were left in place with continuous 1.35 L/s airflow. After 0, 1, 2, 4, 8, 12, 24, 36, 48, 72 hours, randomly spaced 0.65 cm^2 plugs were cut from the filter, and processed by homogenization and spread-plated onto Middlebrook medium as described above. The resulting holes in the test filter were patched with slightly oversized disks made from the same roll of HEPA filter material as the test filter, preventing changes in the pattern or rate of airflow. Experiments were repeated three times.

Effect of biocide on culturability of H37Ra

HEPA filter material was treated with a quaternary ammonium compound (trimethoxysilylpropyl dimethyloctadecyl ammonium chloride) following the biocide manufacturer's directions. Briefly, 20x20 cm square pieces of HEPA filter paper were soaked at 37°C for 1 hour in a 1.26% solution (about 1% dry weight) of the biocide as supplied by the manufacturer. The resulting wet HEPA filter material was completely dried in a 70°C oven. The intensity and uniformity of the biocide treatment was verified by staining with analytical grade bromophenol blue as recommended by the manufacturer. The experiments on culturability of aerosol-deposited cells of H37Ra under prolonged exposure to airflow were repeated five times with this treated filter material using 0, 1, 2, and 4 hours of continuous airflow.

To test the general effectiveness of the biocide in solution (not bound to HEPA filter material), the biocide, as supplied, was diluted with PBS to prepare solutions containing 0%, 2%, 20%, and 100% biocide. Approximately 10^6 CFU of H37Ra and *Micrococcus roseus* were suspended in 5 ml of each of these solutions. *Micrococcus roseus* was used as a control because *Micrococcus sp.* has been commonly used in disinfectant studies (Nicoletti *et al.*, 1993) and by the biocide manufacturer to demonstrate the efficacy of their biocide. After 0, 1, 2, and 4 hours, 100 μl portions were removed from the suspensions and spread-plated in duplicate on Middlebrook agar (H37Ra) or R2A (*M. roseus*) to determine survival rate. Zero % biocide at time=0 was set to 100%. These experiments were repeated twice with H37Ra and three times with *M. roseus*.

Culturability of H37Ra and H37Rv on HEPA filter material under static conditions

H37Ra and H37Rv cell suspensions were prepared in PBS with fetal calf serum as described above except that cell suspensions were not subject to sonication due to biosafety concerns. One hundred μl of prepared cell suspension was pulled through each of ten 1 cm^2 pieces of HEPA filter material with gentle suction using a laboratory vacuum line (maximum 1.33 kPa). A total of 10^4 CFU were thus deposited on 1 cm^2 piece of filter material. After incubation for 0, 1, 2, 4, 8, 24, 48, 72 hours at room temperature under static conditions, a piece of HEPA filter material was randomly selected and homogenized by tissue grinder. The resulting homogenates was serially diluted and 0.1 ml portions were spread-plated onto Middlebrook agar and colonies were counted after 2-3 weeks of incubation at 37°C. These experiments were repeated three times.

Data analysis

Intercepts of the linear regression models were set to 100 percent at zero time. Exponential survival curves were fitted to our data. Wilcoxon Signed Rank test was used to test the effect of relative humidity and biocide on *Mycobacterium* survival using exponential survival curves of three and five replicates respectively. Data were manipulated and graphs prepared in Microsoft Excel 5.0. Statistical analyses were performed using STATA (Stata Corp., College Station, TX) and included the Scheffe method for multiple comparisons, plus the Wilcoxon Rank Sum and Signed Rank tests.

RESULTS

Effect of sonication on culturability of *M. chelonae* and release from HEPA filter material

Neither vortexing nor sonication significantly affected recoveries of *M. chelonae* from suspensions (Scheffe, $p>0.966$, $p>0.956$ respectively). Both methods were subsequently used to assure uniformly mixed suspensions as well as in experiments related to removal of cells from filter material. Ten minutes of vortexing released a maximum of 3.4% of culturable cells initially applied to filter material from liquid suspensions. Sonication for 30 minutes released a maximum of 3.4% of culturable cells and 9% of stainable cells. Because of these low recoveries from the filter fibers, all following experiments included homogenized filter material in cultural analyses.

Effect of RH on survival of *M. chelonae* under static conditions

Cell densities of *M. chelonae* deposited on HEPA filter material from aerosol varied from 3.2×10^3 to 7.7×10^4 (CFU/cm^2) at time=0 in four replicates. Recovered average CFU from two 100 µl samples from 1.5 ml homogenate of two 0.65 cm^2 HEPA filter fragments among four replicates ranged from 279 CFU to 6637 CFU at time=0, and from 5 CFU to 0 CFU at time=24 hours. There was no apparent relationship between the slope of survival curves and initial cell concentrations based on descriptive statistics. Survival curves for *M. chelonae* deposited on HEPA filter material from aerosol and incubated under static conditions at 75% and 55% RH were not statistically different (Wilcoxon Signed Rank, $p=0.47$), although a higher percentage of cells was recovered after 8, 12, and 24 hours at 55% than at 75% RH (Table 1, Figure 3). Colonies were recovered after 48 hours only at 75% RH, and no colonies were recovered after 72 hours either at 55% or at 75% RH.

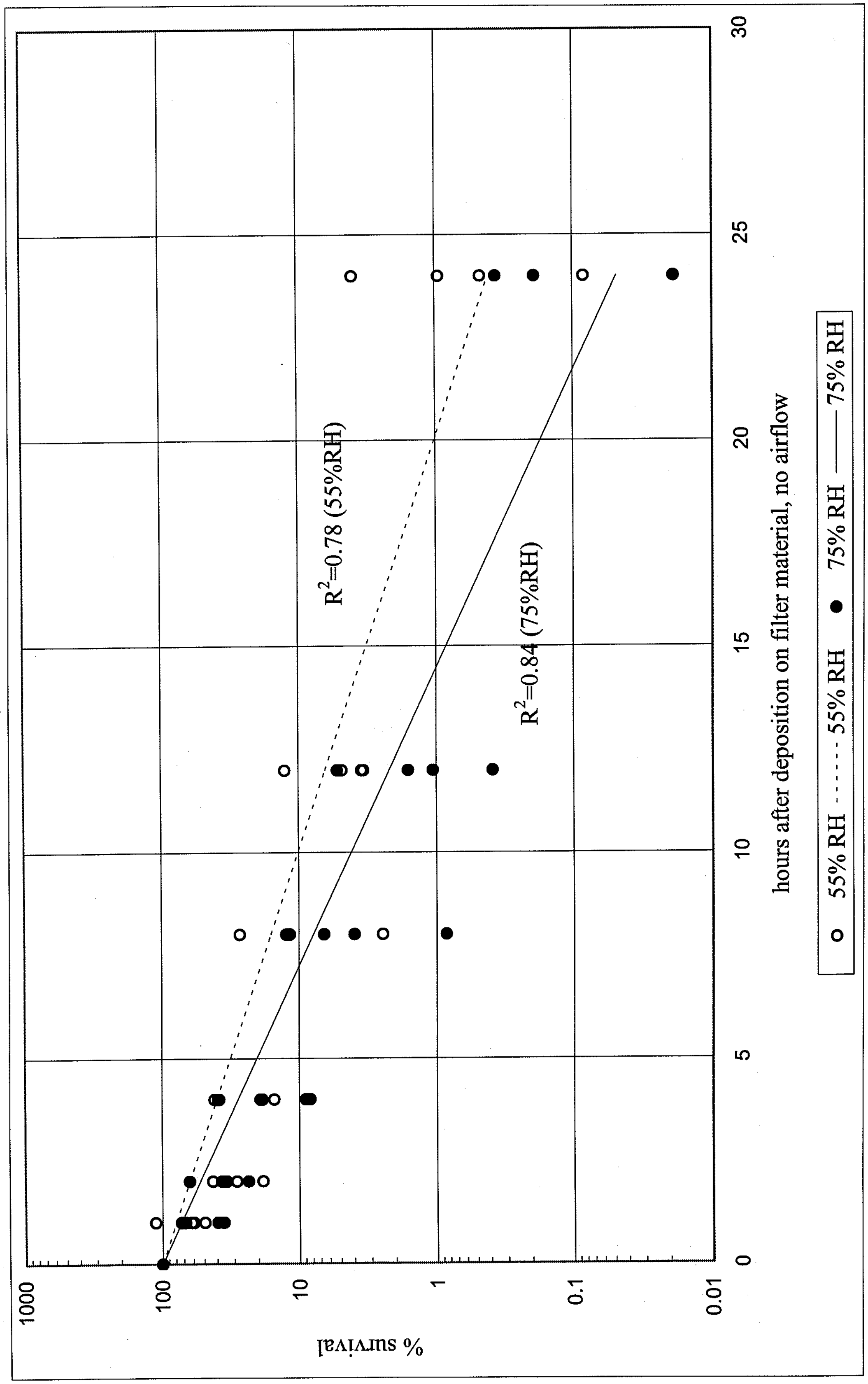
TABLE 1

Estimated parameters of the culturability of *Mycobacterium* on HEPA filter material.

	N	Conditions	RH	Death rate constant[a] Mean ± SE
M. chelonae	4	Aerosol deposition, no airflow	55%	-0.23 ± 0.01
M. chelonae	4	Aerosol deposition, no airflow	75%	-0.32 ± 0.02
H37Ra	3	Aerosol deposition, Continuous airflow	~50%	-0.23 ± 0.03
H37Rv	3	Liquid deposition, no airflow	~25-45%	-0.07 ± 0.01
H37Ra	3	Liquid deposition, no airflow	~25-45%	-0.07 ± 0.01

[a] The fitted equation was $V = 100 \cdot e^{at}$, where a is the death rate constant, V is the viability percentage, and t is time after deposition onto HEPA filter material. The intercept for regression line was set at 100%.

FIGURE 3
Effects of relative humidity on survival of *M. chelonae*.



Survival of H37Ra under continuous airflow conditions

Cell densities of H37Ra deposited on HEPA filter material from aerosol varied from 2.2×10^4 to 7.5×10^4 (CFU/cm^2) at time=0 in three replicates. Recovered average CFU from two 100 µl samples from 1.5 ml homogenate of two 0.65 cm^2 HEPA filter fragments among four replicates ranged from 1940 CFU to 6540 CFU at time=0, and from 3 CFU to 50 CFU at time=24 hours. Nine data points were missing in these experiments. Survival curves of replicates did not show any particular trend related to cell densities on HEPA filter material at zero time based on descriptive statistics. The exponential survival curve representing three replicate experiments is presented in Figure 4.

Effect of biocide on H37Ra and *Micrococcus roseus*

Survival curves for H37Ra deposited from aerosol onto HEPA filter material with and without biocide are presented in Figure 5. Cell densities of H37Ra deposited on HEPA filter material from aerosol varied from 2.0×10^4 to 7.2×10^4 (CFU/cm^2) at time=0 in three replicates. Recovered average CFU from two 100 µl samples from 1.5 ml homogenate of two 0.65 cm^2 HEPA filter fragments among five replicates ranged from 1730 CFU to 6280 CFU at time=0, and from 320 CFU to 820 CFU at time=4 hours. Two data points (at 2 hours) were missing in these experiments. The survival curves under the two conditions were not different (Wilcoxon Signed Rank test, $p=0.75$). Recoveries of H37Ra and *M. roseus* from the biocide solutions are presented in Table 2. About 6×10^3 CFU per ml of H37Ra colonies were recovered from 0% biocide solution at time=0 while cell lawn (more than 10^5 CFU per ml) of *M. roseus* were recovered from 0% biocide solution at time=0. Recovered average CFU from two 100 µl samples ranges from 5 ml PBS solutions ranged from 272 CFU to 1192 CFU at time=0, and from 18 CFU to 599 CFU after 4 hours. H37Ra colonies were recovered from all concentrations of biocide in solution whereas no *M. roseus* colonies were recovered from any biocide-containing solution.

TABLE 2
Mean % recoveries of culturable *M. roseus* and H37Ra.
Recoveries from 0% biocide solution at time=0 are taken as 100%.

		% biocide solution in PBS			
	Time (hours)	0%	2%	20%	100%
M. roseus (N=3)	0	100[a]	0	0	0
	1	~100	0	0	0
	2	~100	0	0	0
	4	~100	0	0	0
H37Ra (N=2)	0	100	197	132	45
	1	105	144	81	40
	2	52	113	29	<1
	4	99	55	14	3

[a] Recovered CFU from two 100 µl samples from 0% biocide containing solution at time=0 for each replicate are taken as 100%.

FIGURE 4
Survival over time of H37Ra.

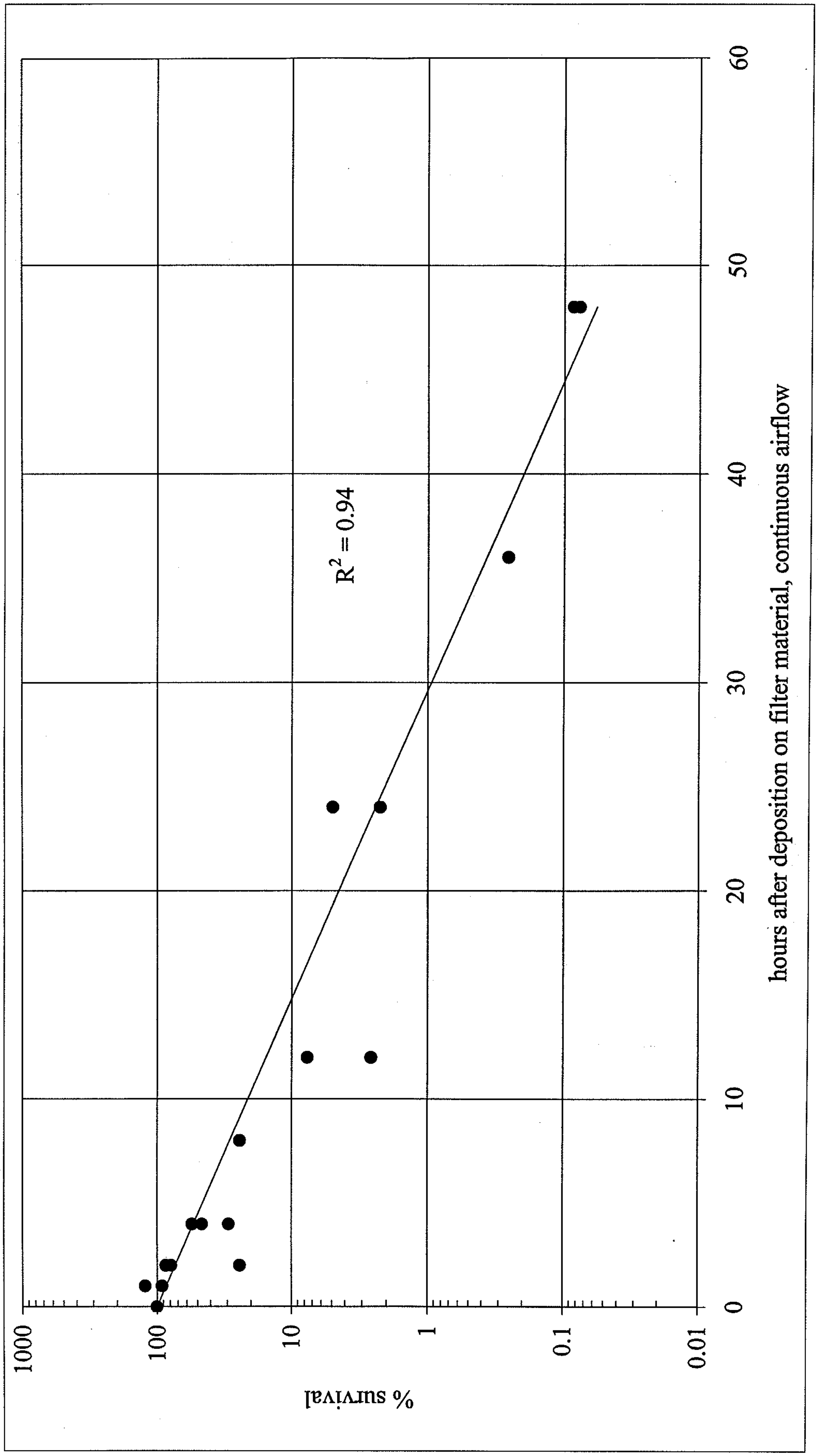
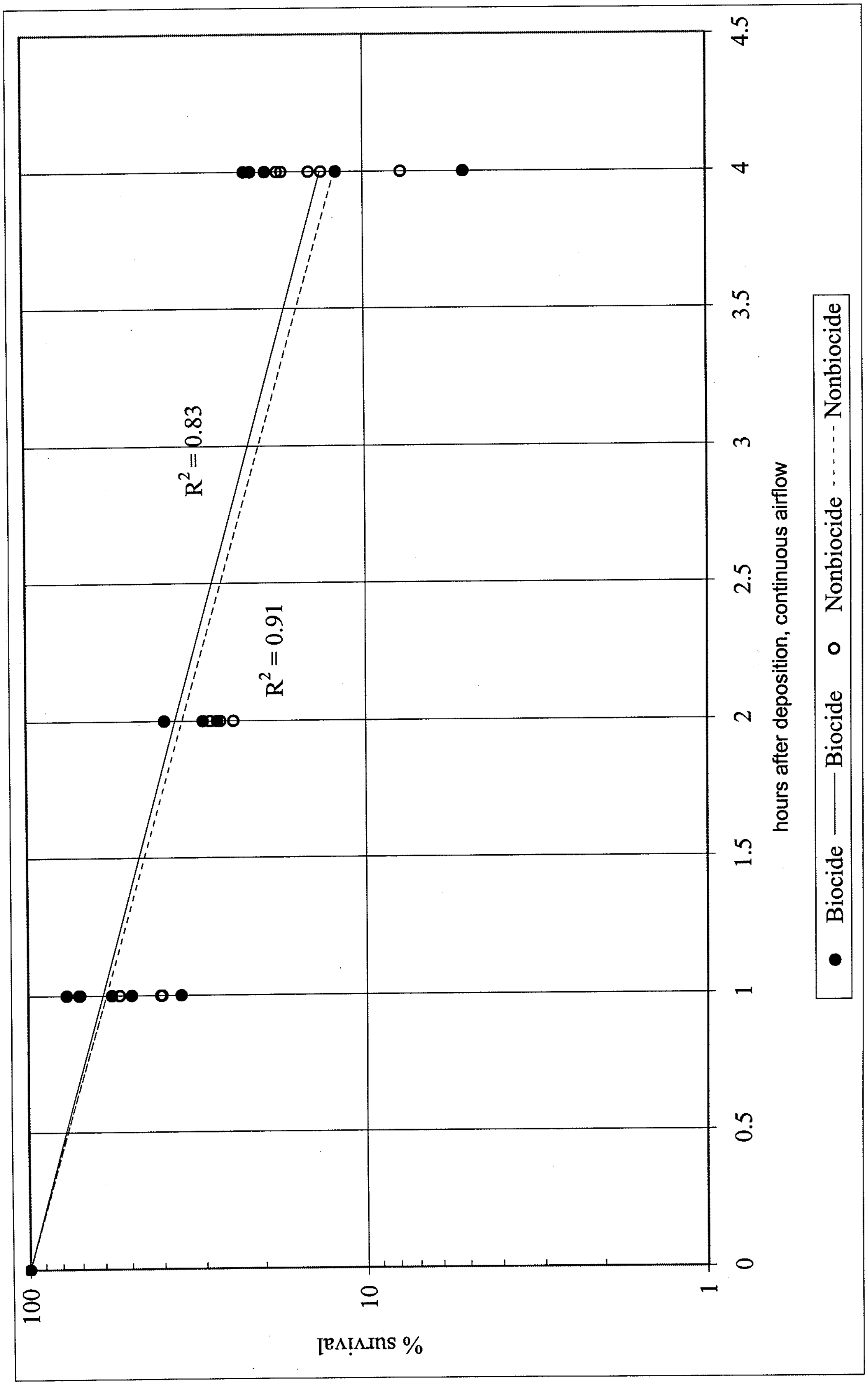


FIGURE 5

Effect of immobilized biocide on survival of H37Ra.



Survival of H37Ra and H37Rv on HEPA filter material

Survival curves for H37Ra and H37Rv deposited directly from liquid suspension onto HEPA filter material and incubated under static conditions were not significantly different (Wilcoxon Rank Sum test, $p=0.80$). Colony counts for both strains appeared to increase but were highly variable during the first several hours after deposition (Table 3). Initial die-off of H37Ra with liquid deposition and static incubation was similar to that following aerosol deposition, but percent survival after 24, 48, and 72 hours was much higher (Figure 4, Table 3).

TABLE 3

Recovered CFU of H37Ra and H37Rv at predetermined time. (liquid deposition, no airflow).

		Time (hour) 0	1	2	4	8	24	48	72
H37Ra	Replicate 1	3830[a]	12840	860	210	2015	935	0.5	45
		(100[b])	(335.2)	(22.5)	(5.5)	(52.6)	(24.4)	(0.0)	(1.2)
	Replicate 2	5415	20100	9410	1180	550	435	135	115
		(100)	(140.5)	(257.5)	(21.8)	(10.2)	(8.0)	(2.5)	(2.1)
	Replicate 3	8350	1710	565	415	2210	10	0.5	0.1
		(100)	(20.5)	(6.8)	(5.0)	(26.5)	(0.1)	(0.0)	(0.0)
	Mean	100	165.4	95.6	10.8	29.8	10.8	0.8	1.1
	SE	0	158.8	140.4	9.6	21.4	12.4	1.4	1.1
H37Rv	Replicate 1	2470	3470	6360	2265	355	260	130	m[c]
		(100)	(140.5)	(257.5)	(91.7)	(14.4)	(10.5)	(5.3)	
	Replicate 2	9480	9800	8450	7950	2730	3300	510	180
		(100)	(103.4)	(89.1)	(8.4)	(28.8)	(34.8)	(5.4)	(1.9)
	Replicate 3	19200	2390	445	1025	645	1115	45	10
		(100)	(12.5)	(2.3)	(5.3)	(3.4)	(5.8)	(0.2)	(0.1)
	Mean	100	85.5	116.3	35.1	15.5	17.0	3.6	1
	SE	0	65.9	129.8	49.0	12.7	15.6	3.0	1.3

[a] Recovered average CFU from two 100 µl samples from a 1.5 ml homogenate of two 0.65 cm^2 HEPA filter fragments sampled at the indicated time.

[b] Percentage recoveries. Recoveries from time=0 sample for each replicate are taken as 100%.

[c] missing data

DISCUSSION

To facilitate development of experimental protocols we used avirulent strains of *Mycobacterium* and performed only a limited series of experiments on the virulent agent. *M. chelonae*, an environmental isolate, was chosen because it grows rapidly and its use enabled us to perform necessary control experiments quickly. H37Ra is a strain of *Mycobacterium tuberculosis* that is considered to differ from the virulent strain only in the virulence factor. It should be noted that we used only one isolate of each of these organisms, and the differences we report may be isolate-specific rather than characteristic of the strain or species.

Exponential decay of non-replicating microbial populations is a well-recognized phenomenon. The exponential decay model assumes that all individuals of the microbial population are of similar sensitivity, and probability itself determines the actual time of death of any given individual (Stanier *et al.*, 1976). It was observed, however, that actual decrements in percent survival were greater during the initial few hours of the experiments than is accurately described by exponential decay. Our data suggest that initial die-off of cells for the *Mycobacterium* strains deposited onto filter material from aerosol is rapid within the range of conditions provided, and that only a small percentage of cells survive beyond 24 hours. These survival characteristics suggest different sensitivities within the cell population. Power and non-linear survival models were also used to describe these survival characteristics. However, neither of them fit our data statistically better than the exponential survival curves, and some data sets were not large enough to fit complex nonlinear survival curves.

Dynamic experiments were designed to simulate conditions in ventilation systems where more or less continuous airflow exists. We did not compare static and dynamic conditions within species or strains. Nevertheless, the fact that survival curves were not significantly different for *M. chelonae* (no airflow) and H37Ra (with continuous airflow) under similar RH conditions suggests that the presence of airflow did not effect survival under the conditions we provided.

Survival curves for the virulent and avirulent strains of MTB did not differ following deposition onto filter medium from solution and maintenance under static conditions. H37Ra cells died more slowly following liquid deposition than following aerosol deposition (Table 1). Aerosol generation, dispersion, and impaction onto HEPA filter material may have increased susceptibility of the *Mycobacterium* cells to subsequent environmental damage compared to deposition from liquid (Cox, 1989). Confirmation of a more rapid death for H37Rv following aerosolization and under continuous airflow would require aerosolization of virulent agents and was not done for these studies.

Few *Mycobacterium* cells were released from filter material even with vortexing or sonication. Because the focus of these experiments was the assessment of viability rather than release, we included homogenized filter material with its adherent cells in all experiments. Adhesion of biological particles to filter fibers is well recognized (Nicas, 1995; Walters *et al.*, 1994). We did not test cell release from dry filter material. However, a recent study indicated that re-aerosolization of *Bacillus spp.* deposited on the fibrous filter of an N95 respirator by high velocity air (300 cm/sec) simulating cough and sneezing was very limited (Qian *et al.*, 1997). These data suggest that release of *Mycobacterium* cells from dry HEPA filter material under field condition would also be limited. However, experiments with dry HEPA filter material are necessary for confirmation.

Initial cell densities as high as about 10^4 CFU/cm^2 were maintained in our experiments to recover enough cells to fit survival curves over prolonged time periods. These initial cell densities are likely to be much higher than would occur in real situations. The range of cell densities on HEPA filters in hospital settings is unknown. However, mathematical approximation from several reported outbreaks have produced source strength estimation for TB patients ranging from about 1 infective MTB per hour for treated patients in hospital, to 13 infective MTB per hour for a highly infectious office worker, to 250 infectious MTB per hour for bronchoscopy patients (Nardell, 1994). Thus, cell densities as high as 10^4 CFU/cm^2 on filters are very unlikely even when extremely infectious patients are undergoing bronchoscopy. If high cell densities affected our survival curves, the effect would most likely have been to protect bacteria by minimizing exposure to hostile environmental conditions.

Biocide treatment of HEPA filter material before cell deposition had little effect on the survival of H37Ra (Figure 5). H37Ra was also resistant to biocide in solution. Although increasing concentrations of biocide increased the rate of cell death, some culturable cells remained even after four hours in undiluted biocide. We calculated that cells deposited on treated filter material were exposed to a biocide concentration approximately equivalent to the 2% solution, although actual exposure was probably much lower because only a part of each cell would be likely to contact the filter material. Recoveries greater than 100% of CFU concentrations at time=0 and biocide%=0 were observed in 2% and 20% biocide solutions (Table 2). These increases may represent growth of MTB in the biocide solutions, repair or activation of dormant cells, or may simply represent variability in the number of CFU in suspensions made from the individual solutions.

Variability among survival curves for individual experiments with each strain was large. Some of this variability may be due to changes in the stored inoculum, or the variable numbers of cells initially deposited on the filter material, or clumping of cells. *Mycobacterium* suspended from agar culture tends to form clumps, making the production of single-cell aerosols a significant challenge. We used sonication and vortexing to reduce the size of these clumps. Possible effects of these treatments on *Mycobacterium* are 1) to kill cells (leading to lower colony recoveries); 2) to break the clumps (leading to higher colony recoveries); 3) a combination of these effects; 4) no effect. At least for *M. chelonae*, both sonication and vortexing appeared to slightly (but not significantly) increase recoveries. Although it was not possible to determine whether this slight increase was due to breaking clumps alone, or to a combination of breaking clumps and killing cells, the data did support the decision to use these methods to suspend cells and to remove cells from filter material. We did not treat H37Rv and H37Ra suspensions for liquid deposition with sonication due to biosafety concerns. Both sonication treatment and aerosol generation by high pressure might help to achieve more uniform cell suspension by breaking clumps. The presence of clumps may in part explain the higher variability in recoveries for the first few hours following liquid deposition of H37Ra and H37Rv.

Finally, we deposited *Mycobacterium* cells on new HEPA filter material throughout our experiments. Soiled HEPA filters loaded with various dusts, debris, other organic and inorganic materials may provide additional protection to *Mycobacterium* cells. The risks associated with handling of used HEPA filters loaded with typical hospital dust need to be further determined.

CONCLUSION

The studies described here were initiated to evaluate the potential for exposure to virulent *M. tuberculosis* after collection on HEPA filters in ventilation systems. Overall, the data indicate that the potential for exposure to viable cells during changing of filters is minimal. Our data indicate that 1) *Mycobacterium* cells are difficult to remove from clean, new, wet HEPA filter fibers, 2) few culturable cells, less than 0.1%, remain after 48 hours (i.e. one weekend), and 3) exposure to re-aerosolized viable cells from disturbed HEPA filter material is unlikely.

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REFERENCES

Allen, B. W. 1981. Survival of tubercle bacilli in heat-fixed sputum smears. J. Clin. Path. 34:719-22.

ASME 1989. N510. Testing of nuclear air treatment systems. The American Society of Mechanical Engineers. NYC.

Brosseau, L. M., McCullough, N. V. & Vesley, D. 1997. Bacterial survival on respirator filters and surgical masks. J. Am. Biolog. Safety Assoc. 2:32-43.

CDC 1995. Exposure of passengers and flight crew to *Mycobacterium tuberculosis* on commercial aircraft, 1992-1995. MMWR 44:137-140.

Coninx, R., Eshaya-Chauvin, B. & Reyes, H. 1995. Tuberculosis in prisons [letter; comment]. Lancet 346:1238-9.

Cox, C. S. 1989. Airborne bacteria and viruses. Sci. Prog. Oxf. 73:469-500.

Drobniewski, F. 1995. Tuberculosis in prisons—forgotten plague [see comments]. Lancet 346:948-9.

Hirai, Y. 1991. Survival of bacteria under dry conditions; from a viewpoint of nosocomial infection. J. Hosp. Infect. 19:191-200.

Hobbie, J. E., Daley, R. J. & Jasper, S. 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-8.

Imshenetsky, A. A., Lysenko, S. V. & Kazakov, G. A. 1978. Upper boundary of the biosphere. Appl. Environ. Microbiol. 35:1-5.

Kepner, R. L., Jr. & Pratt, J. R. 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol. Rev. 58:603-15.

Luby, S., Carmichael, S., Shaw, G., Horan, J., Gamble, W., Jr. & Jones, J. 1994. A nosocomial outbreak of *Mycobacterium tuberculosis*. J. Family Practice 39:21-5.

May, K. R. 1973. The Collison nebulizer: description, performance, and application. J. Aerosol Sci. 4:235.

Nardell, E. A. 1994. Transmission and safety issues. In *Tuberculosis: Current Concepts and Treatment*, pp. 53-70. Edited by L. N. Friedman: CRC Press.

Nicas, M. 1995. Respiratory protection and the risk of *Mycobacterium tuberculosis* infection. Am. J. Ind. Med. 27:317-333.

Nicoletti, G., Boghossian, V., Gurevitch, F., Borland, R. & Morgenroth, P. 1993. The antimicrobial activity *in vitro* of chlorhexidine, a mixture of isothiazolinones ('Kathon' CG) and cetyl trimethyl ammonium bromide (CTAB). J. Hosp. Infect. 23:87-111.

Porter, J. & Kessler, C. 1995. Tuberculosis in refugees: a neglected dimension of the 'global epidemic of tuberculosis'. Trans. Royal Soc. Tropical Med. Hyg. 89:241-2.

Porter, J. D. & McAdam, K. P. 1994. The re-emergence of tuberculosis. Ann. Rev. Public Health 15:303-23.

Qian, Y., Willeke, K., Grinshpun, S. A. & Donnelly, J. 1997. Performance of N95 respirators: Reaerosolization of bacteria and solid particles. Am. Ind. Hyg. Assoc. J. 58:867-880.

Stanier, R. Y., Ingraham, J. L., Wheelis, M. L. & Painter, P. R. 1976. The microbial world. Fifth edn. Englewood Cliffs, New Jersey: Prentice-Hall.

Theunissen, H. J., Lemmens-den Toom, N. A., Burggraaf, A., Stolz, E. & Michel, M. F. 1993. Influence of temperature and relative humidity on the survival of *Chlamydia pneumoniae* in aerosols. Appl. Environ. Microbiol. 59:2589-93.

Walters, M., Milton, D., Larsson, L. & Ford, T. 1994. Airborne environmental endotoxin: a cross-validation of sampling and analysis techniques. Appl. Environ. Microbiol. 60:996-1005.