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ASSAYING BENEFITS OF POLY[STYRENE-4-(TRIMETHYLAMMONIUM)METHYL TRIIODIDE] IN RESPIRATORY PROTECTION DEVICES

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14. ABSTRACT By analogy to the accepted mechanism for disinfection by iodine in water, a mechanism was earlier proposed to rationalize in-vitro disinfection of bioaerosols after passage through air filter media coated with poly[styrene-4-(trimethylammonium)methyl triiodide] (PSTI) that invoked near-collision transfer of I2 from PSTI onto the negatively charged surface of passing particles. This report describes progress toward two experiments designed to test and refine that model and to evaluate the increment to respiratory protection available by adding PSTI to inert mechanical air filter media. The controlled aerosol test system (CATS) was designed and built to deliver a constant (+/- 10%) challenge of aerosolized respiratory pathogens through samples of air filter media to groups of 5 mice. By varying the challenge concentration and comparing rates of infection in groups behind mechanically equivalent control and PSTI-containing media we expect to measure the effective decrease in penetrating infectivity. The second experiment was accomplished in 20-L bags assembled from commercial food wrapping material, which were filled with a bioaerosol of MS2 coli phage that had been passed through an inert or PSTI-containing air filter medium. After 15 or 30 minutes' incubation the contents of the bag were drawn through an impinger containing dilute thiosulfate solution and plated to determine viable counts. Data noise in the control experiment prevented drawing a firm conclusion but loss of viability in the aerosol phase appeared faster in the treated group at high humidity, suggesting that water is necessary to the antimicrobial action and that PSTI might be more effective in collective than individual protection applications.					
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1.0 Overview

Filtration is the mechanism most commonly used in respiratory protection systems to remove unwanted particles from the air. Antimicrobials have been examined as a way to enhance air purification systems by killing microorganisms in addition to and in lieu of capturing them. Triosyn Corporation was one of many companies who experimented with adding antimicrobial compounds to air purification filters. Triosyn used iodine (I_2) immobilized as a polymeric complex containing triiodide ions (I_3^-) as their antimicrobial and developed iodinated filtration media for both individual and collective protection systems. Iodine has long been known to be an effective, broad-spectrum biocide in solution [1], but its effectiveness in aerosols has been reported only in *in-vitro* experiments. Ratnesar–Shumate, *et al.*, [2] have proposed that the mechanism of displacement of I_2 from the surface-bound I_3^- complex proposed in water by Taylor, *et al.*, [1] applies as well to bioaerosols undergoing near-contact with treated fibers during passage through an air filter medium. However, the time constant for inactivation of microorganisms after this exposure to I_2 in the aerosol state is unknown and interference with the antimicrobial action of I_2 by environmental proteins and by water is possible.

The measurement of antimicrobial efficacy following passage of microbes through an iodinated resin is not entirely straightforward. Typically, biological aerosols are collected in all-glass impingers (AGIs) that contain a liquid medium to trap the microbial agents. One complication with this strategy is that chemical species that off-gas from the filter (*e.g.*, I_2) will also collect in the impinger fluid and may build up to toxic levels [3]. The test system cannot discriminate between potential killing of microbial agents in the aerosol state or in the impinger system. A proposed solution to this problem is the addition of reagents to the AGI solution (*e.g.*, sodium thiosulfate) to inactivate chemical species that build up in the collection fluid. Lee, *et al.*, [3] used this strategy when performing MS2 aerosol challenges of samples of Triosyn's iodinated filtration media. They found that collection in a thiosulfate solution effectively reversed the measured viable removal efficiency (VRE) of the filter, and collection into 5% bovine serum albumin (BSA) decreased the VRE to ~50%. Eninger, *et al.*, [4] also performed tests in which bioaerosols passing through an iodinated filter were collected into gelatin plates and measured no significant reduction in viability due to the iodinated resin. This is presumed to have been a consequence of successful competition for aerosol-bound I_2 by the gelatin matrix. The common feature—relevant primarily in the context of collective respiratory protection—of these two studies is that the iodine–biological particle complex remained in the airstream for only milliseconds before being collected in a neutralizing buffer. The obvious hypothesis arising from them is that the I_2 might inactivate the microorganism given more time to act in the aerosol state. A second question—of equal importance in the context of individual respiratory protection (IRP)—cannot be resolved by *in-vitro* experiments: will the accumulated I_2 afford any increase to the wearer of IRP gear in protection against infection by airborne pathogens?

The questions surrounding the current test systems called for a new approach to evaluate the action of such iodine-containing filtration media. Two approaches were developed to address two specific questions that follow from the discussion above:

- 1) Are microorganisms that pass through an iodinated filter killed in the aerosol state?
- 2) Can I_2 displaced from an iodinated filtration medium cause a greater decrease than traditional filtration media in the frequency of clinical infections?

The first question was addressed by developing a test system in which biological aerosols were first passed through an iodinated air filter medium and then held in a holding chamber at room temperature for various times prior to collection in AGI-30 impingers. The second question will be addressed by performing an animal exposure study in which challenges of a microbial agent will be delivered to the test subjects in parallel experiments through an iodinated medium and through a mechanically matched inert medium. In this design the animal replaces the AGI as the detector, which is expected to provide conclusive evidence that the incorporation of available I₂ at the surface of the air filter fiber does (or does not) convey clinical effectiveness to the medium. The first of these studies and progress toward the second are detailed in this report. A summary of AFRL testing related to the Triosyn projects in previous years is also enclosed.

2.0 Summary of Previous Years of Testing at AFRL

In-vitro results reported by Triosyn Corporation (Williston, Vt.) suggest that air purification products incorporating poly(styrene-4-[trimethylammonium]methyl triiodide) (PSTI) provide a 2-log increase in VRE vs. standard filtration systems. In parallel with developmental activities conducted by Triosyn from 2002–2009 the Air Force Research Laboratory (AFRL) has performed third-party validation testing of their evolutionary prototypes and consistently reproduced their laboratory results [5,6]. AFRL anticipated performing chemical and microbiological challenge testing of three generations of prototype devices targeting an improved IRP canister:

- Bronze—a clip-on device containing a PSTI-coated nonwoven medium that attached to the front face of a commercial off-the-shelf (COTS) canister;
- Silver—a fabricated plastic canister that placed a PSTI-coated nonwoven layer in front of the components of the COTS canister)
- Gold—a redesigned canister in which a PSTI-coated electret medium replaces the high-efficiency particulate air (HEPA) medium used in the COTS canister.

Extensive testing of the bronze prototypes consistently showed their VRE to be 1 or more logs higher than achieved by the COTS canister. (VRE for a nonreactive system is the same as the mechanical particle removal efficiency (PRE).) However, the contribution to PRE by the second filter medium was not determined so the contribution by PSTI to VRE was not dissected out.

Fairly early in the evaluation of the silver canister Triosyn joined with Safe Life Corporation and a corporate decision was made to shift from targeting a replacement IRP device to development of low-pressure-drop (low- ΔP) antimicrobial media *an sich*. Our testing activities shifted first to evaluation of VRE for a “super-HEPA” medium (SHM, Figure 1) prepared by coating a commercial fiberglass HEPA medium with PSTI, which achieved ~6-log VREs and which we showed [7] to achieve VRE two logs higher than its PRE in *in-vitro* experiments that trapped the filter effluent in a phosphate-buffered saline medium, but the filter medium was not backed by carbon and we did not measure I_2 background concentrations in the impinger, so the contribution by PSTI to the *in-vitro* kill was again not unequivocally demonstrated by the experiment. Because this effect was achieved at a ΔP somewhat larger than that of the untreated HEPA medium, development of the SHM was abandoned in favor of electret media, which offer a large decrease in ΔP at a given PRE and are compatible with PSTI.

The first electret we tested was a tailored commercial preparation incorporated into Triosyn’s T5000 N95 filtering facepiece respirator (FFR) (Figure 1). Two of nine T5000 samples tested appeared to be outliers, presumably because of seal leaks in the connection to the test rig; one was rejected on statistical grounds, bringing the average VRE for the eight remaining to 99.99%. However, PRE for the mask was also ~99.9%, so the magnitude of the PSTI coating’s contribution to VRE was again obscured by experimental limitations. A feature common to each of the devices tested was a thin layer of activated carbon located downstream of the coated fibers. Both we and Triosyn measured background concentrations of iodine (off-gassed from the carbon-backed iodinated media) in the impinger medium and found the concentrations to be much too small to produce toxic effects in the impinger.

In collaboration with University of Florida researchers we proposed [2] a mechanism for the transfer of surface-bound I_2 (present as the I_3^- ion of PSTI) from the fiber to negatively charged aerosol particles. The team further determined that the biocidal action of I_2 thus captured is not

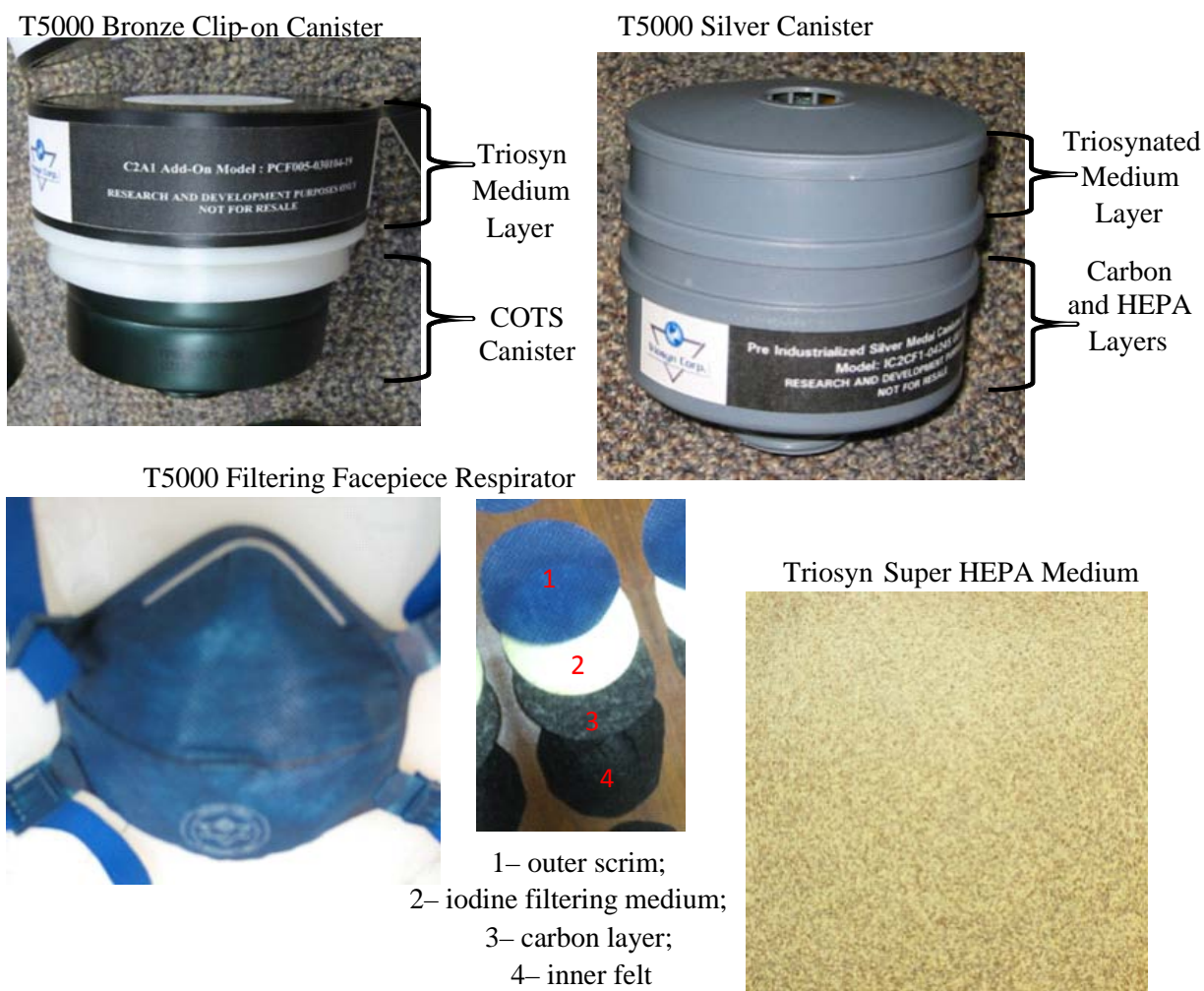


Figure 1. Triosyn Iodinated Products Tested by AFRL

immediate, by quenching experiments in which the penetrating aerosol was captured first in an impinger charged with a dilute solution of sodium thiosulfate (I_2 was neutralized and the observed biocidal effect was negligible) and subsequently in a medium containing 5% bovine serum albumin (BSA), which competed for loosely bonded (and possibly free) I_2 and decreased the measured kill to ~50% [3].

A path to determining the mode of action is to test iodinated and non-iodinated filtration media that have identical filtration properties. Triosyn produced a “Super High Efficiency Particulate Air (HEPA) Medium” (SHM) by incorporating PSTI into a standard HEPA medium. AFRL demonstrated that the SHM (no backing carbon layer) did provide a 2-log increase in VRE in comparison to standard HEPA media when the aerosols were collected in phosphate-buffered saline [7]. However, when sodium thiosulfate was added to the collection media, the SHM performed the same as standard HEPA media. These results are in line with what was described by Lee, *et al.*, [3] and, because the iodine background was not measured, do not discriminate between impinger poisoning and aerosol capture of I_2 as the cause for the increase in VRE. It is also possible, as mentioned in the overview, that the mechanism of aerosol kill may take more

time than is allowed for in the current test systems. The equivocal nature of the data led AFRL to develop both the mouse exposure study and the aerosol holding study.

In addition to the uncertainty of the added protective capability afforded by PSTI (and any other reactive antimicrobial treatment applied to air filter media), this program spawned questions about the adequacy of present-generation air filtration technology to deal with a hypothetical viral or prion weapon delivered in a narrow particle size distribution (PSD) centered near the most-penetrating particle size (MPPS) of fiber filters at ~200 nm. We have reproduced [8] Triosyn's demonstration that MS2 viral aerosols easily penetrate HEPA filters in quantities that deliver a dose at the median infective dose (MID₅₀) in seconds, but the lab-scale test is run in a closed, controlled environment and it is not certain that the aerosols created in lab tests are a credible threat that could be duplicated in the field. Accordingly, AFRL organized and led a subject matter expert (SME) panel to identify and address key questions about HEPA filtration systems. The panel concluded [9] that additional study was needed to provide better answers, noting that such critical parameters as PSD, bioaerosol concentration, duration of the attack, and removal and deactivation processes are environmental factors and engineering challenges that will affect net viable penetration and exposure behind the air filter. In open-air scenarios HEPA protection appears to be fully adequate; however, credible scenarios might be possible in confined spaces.

The SME panel report points up profound distinctions between requirements of an air filtration system in the contexts of IRP and collective protection (CP)[9]:

- Protective technologies for IRP must act within a few milliseconds—the time of travel of an inspired particle from the exit face of the mask to a respiratory mucosal surface and the fluids bathing it. They must function for only a few hours, the physiological limit of the wearer. And the quality of the face-to-mask seal is a paramount consideration.
- In the CP environment the time for protective action can be as much as tens of minutes, function must be maintained continuously for many months, and factors unrelated to the filter medium and seals can compromise the integrity of the protected airspace.

We were thus faced with two questions requiring a definitive experiment to discriminate what increment to protection against airborne infection is afforded by PSTI as a reactive constituent of an air filtration system:

- For IRP, is the rate of infections behind a PSTI-treated air filter medium any lower than behind a mechanically equivalent unreactive filter medium—and if so, by how much? To address this question we have designed and undertaken an animal-exposure study to count the frequency of infections caused in these two circumstances at a range of challenge concentrations delivered to the filter at constant temperature and relative humidity. Progress to date is described in Section 3.
- Whereas environmental intrusions and degradation of performance with time are outside the scope of this effort, the enormously longer time window for beneficial disinfection in CP environments calls for measurement of the loss of viability of airborne infective particles with time. Initial experiments in which the time between filtration and collection in a quenching medium was varied are described in Section 4.

3.0 Mouse Exposure Study

3.1 *Development and Validation of the Animal Exposure System*

Introduction—Reviews of experimental inhalation exposure systems have been reported by Drew and Laskin, [10] MacFarland, [11] Cheng and Moss, [12] Jaeger, *et al.*, [13] Wong [14] and others. Their summaries will not be repeated here. An important early exposure system was the Henderson apparatus [15], a system that made the innovation of exposing only the nose and mouth of the animal (in Henderson’s experiments, guinea pigs and rabbits) to infectious aerosols, rather than the animal’s whole body. Henderson measured the coefficient of variation (CV) of colony-forming units collected from the aerosol cloud and found that within nine runs using aerosolized *Bacillus subtilis* and 15 runs using *Chromobacterium prodigiosum*, the mean CV was 5.73% and no measured CV was above 15%. Henderson remarked that this was very consistent and a number of this magnitude will be used as a goal subsequently. Later, highly sophisticated nose-only exposure systems were created for the study of tobacco smoke. [16]

Experiments exposing animals to aerosols are common, as are experiments passing bioaerosols through filters; however, experiments using animal models to study the infectivity of filtered aerosols are not. Studies have been done on the reduction in infection that occurs when filters are added to the cages of pigs [17–19] and chickens [20,21] in an agricultural setting, but these studies examined casual transmission between animals and did not expose the animals to a measured dose of aerosol. No reports of controlled exposures of an animal model of human respiration to an infectious bioaerosol penetrating a filter were found in the literature. This is likely because the behavior of an aerosol—biological or not—passing through and behind a simple fiber filter is predictable from first principles and therefore such determinations provide no justification for animal experiments. Exposure of a bioaerosol to the PSTI–iodine chemistry described in section 2 introduces the complications of time- and environment-dependent effects on the viability of microbial components of the aerosol. Because these effects can affect the claimed antimicrobial capability of the PSTI component of the fiber, measurement of the protective impact exerted by PSTI can be accomplished only with a biological indicator. It was thus necessary to develop and characterize a system to perform such an animal exposure experiment.

Wong [14] states that variability in four key factors can adversely affect an animal inhalation study:

1. animal environment,
2. exposure atmosphere,
3. inhaled dose,
4. individual response of the animal.

Factor 4 is outside the scope of this work, as is a major component of factor 1, the housing environment of the animals. The remainder of factor 1 is the exposure environment. A nose-only system referred to as a “mouse tree” and described in a US Patent [22] and separately [13] was, among other reasons, chosen to prevent cutaneous and enteric infections to the mice. This system uses sealed restraining tubes to contain the mice and, because the device holds the mouse’s nose in an air stream at slight positive pressure, the source of each mouse’s breathing air is controlled.

The inhaled dose received by the animal can be calculated at steady state [14] as

$$D = CV_m Ft,$$

wherein

- C is the concentration of test material in the animal's breathing air,
- V_m is the minute volume of the animal (breathing rate [in min^{-1}] times tidal volume),
- F is the fraction of material deposited, and
- t is the duration of exposure in minutes.

In the steady-state assumption, these values are all constant, but in reality they may vary in time. In a bioaerosol test, C will have units of viable microbes (plaque-forming units (PFU) or colony-forming units (CFU)) per volume of air. The fractional deposition depends on the particle size distribution (PSD), and the sites in the animal's respiratory tract on which the particles deposit will affect infectivity. Even particles that are not infectious can cause irritation or other effects that may affect the infectivity of the bioaerosol. Therefore variation in these values—or in the PSD—introduces error in the dose; the exposure atmosphere (factor 2) strongly affects the inhaled dose. Breathing rates of animals in laboratory conditions can vary wildly from their textbook values. Real-time measurements of respiration have been made on large animals, but there are no instances in the literature of real-time respiration measurements on mice. However, the experiment proposed to examine the effects of the iodinated resin compares dose-dependent responses to deduce the relative reduction in the dose provided as a result of the iodinated resin. It is less important that the values of these parameters be exactly known than that they remain constant for the duration of the experiments. Thus, keeping a consistent exposure atmosphere largely depends on keeping the PSD and the concentration of viable microbes consistent.

The aerosol source is another important potential source of variation. Wong [14] states that it is “notoriously more difficult to maintain a stable concentration” of aerosols. However, Collison nebulizers have been used to aerosolize a steady concentration of viruses and bacteria for bioaerosol experiments. [2–4,23,24] A steady Collison output depends on a steady feed pressure; variations in pressure can alter the PSD. [25] In practice, evaporation of the nebulization liquid over time will cause the aerosol concentration to increase with time. Also, because Collison nebulizers are recirculating systems that impose large shear forces, microbes in suspension accumulate metabolic damage as a run proceeds. [24]

Research has shown that infectivity of a bioaerosol in an animal model can be strongly affected by humidity, as can the survivability of the microbe in the aerosol. [26,27] The effects of humidity and temperature depend on the type of microbe along with the makeup of its cell wall and can vary between strains of the same species. For some microbes, an unfavorable humidity can cause more than 99% of microbes in an aerosol to lose viability. [28] For the purpose of this project, moderate loss of viability within the system can be tolerated as long as the concentration of viable microbes is consistent and the desired concentrations of viable microbes are attainable. Therefore, it is important to keep the loss of viable particles due to humidity and temperature constant by keeping those factors consistent. Another factor that can affect PSD in this system is the loading on the filter. The ΔP of an intact filter and its particle removal efficiency (PRE) typically increase with loading of particles on its surface. [29] However, if the cumulative

loading during the period of experimentation is low enough, changes in PRE and ΔP are negligible.

Accordingly we undertook the design and engineering of an aerosol delivery system—based on the Collison nebulizer—to provide, at selectable concentrations, a verifiably consistent respiratory challenge of bioaerosol particles that can be delivered to multiple groups of mice in separate experiments through treated and untreated control air filters. Consistency is achieved (and verified) by maintaining consistency of the PSD and of the count of viable microbes in the aerosol.

The capacity of the mouse tree to deliver infectious aerosol to mice was not tested in this validation. Animal testing with this system is enabled by the research effort that developed, built and characterized the mechanical performance of this apparatus. The mouse tree itself has already been verified in the literature so it is not necessary to repeat that process. [13]

Instrument description—The apparatus (illustrated in Figures 2 and 3 and referred to as the Controlled Aerosol Test System, or CATS) will enable experiments using a common laboratory mouse, *Mus musculus*, and treated and mechanically equivalent untreated filter media to measure infection rates as a function of pathogen challenge delivered to each filter. The CATS generates a stream of biological aerosol at a range of constant concentrations, passes the aerosol through a filter, and delivers the penetrating particles to a mouse model of human respiration. The system maintains nearly constant temperature and humidity. Accommodation of the mouse model was a necessary aspect of the design and construction processes. This system will be used in the infectivity studies described in section 3.2. The characteristics of the biosafety level 3 (BSL-3) facility in which the system will be located in at the University of Nebraska Medical Center (UNMC) impose some extra design constraints, which are noted below.



Figure 2. Photograph of CATS Unit. (Not pictured: control panel and impinger hook-up.)

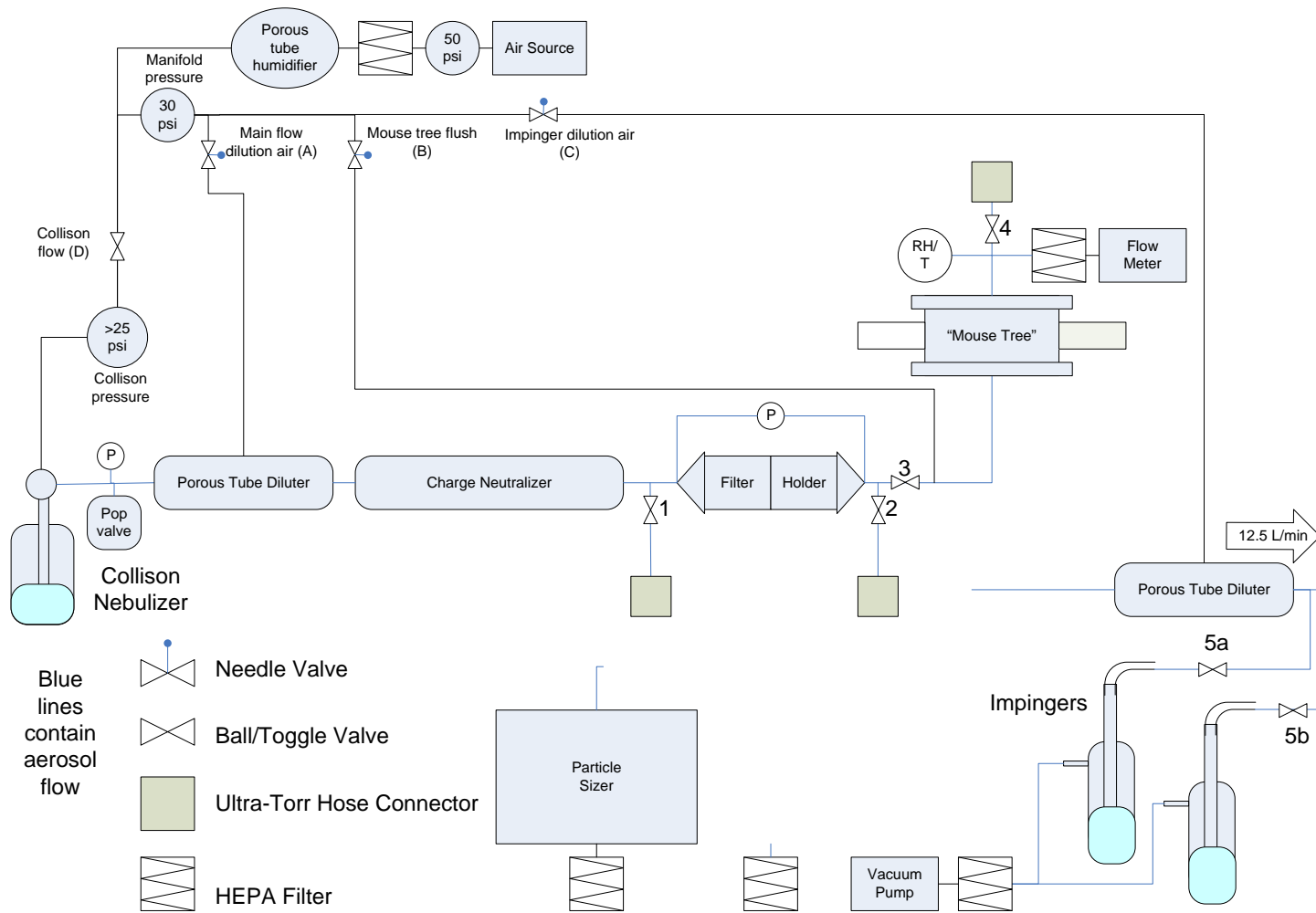


Figure 3. Process-Flow Diagram of CATS

In the flow diagram of the system (Figure 3) the flowrate from needle valve C is adjusted to deliver 12.5 L/min of total air to the impingers; this value may vary while flow is removed upstream for other instruments. Only one impinger will be in use at any given time.

The following components are included in the experimental filtration system:

- **Collison nebulizer** (BGI Inc, Waltham, Mass.)—the Collison generates the bioaerosol. A three-jet nebulizer would produce excessive flow, so a single-jet nebulizer is used with make-up air supplying the rest of the flow. There is significant variation among different models of Collisons. [30] This variation is not enough to change an experimental protocol, but it is wise to use only one model of Collison in any series of experiments.
- **Charge neutralizer** (TSI, Inc., Shoreview, Minn.; Model #3012A)—this component is necessary to neutralize the electrical charge put on the particles by the nebulization process. The 3012A charge neutralizer uses a 370-MBq ^{85}Kr beta-emitting source. It can be used with flows as high as 50 L/min.
- **Sample holder** (Triosyn Corp, Williston, Vt.)—a custom-built holder comprising an inner and outer sleeve is used to hold a 47-mm diameter disc (40 mm exposed) of filter medium compressed (by bolts) around the edges between elastomeric annular seals. With the supplied reducers, the holder accommodates 32-mm (25-mm exposed) media coupons.
- **Mouse tree** (CH Technologies Westwood, N.J.)—a Jaeger-NYU Modular Nose-Only Directed-Flow Rodent Inhalation Exposure Unit [22], herein referred to as a *mouse tree*, will be used to expose the mice to the aerosolized microbe. Each mouse is placed in a polycarbonate holder and constrained with a sealed restraint inserted in the rear opening of the holder, so that the tip of the mouse's nose projects out the end. The holder inserts securely into a socket on the mouse tree. Vents inside the body of the tree blow an airstream containing the filtered aerosol at the nares of the mice as their only source of breathing air. Exhaled air and excess flow are drawn away from the mouse. [13] The mouse tree can hold up to 12 mice at one time. It is reported that each mouse has a mean tidal volume of 0.18 mL and breathes 255 times a minute. [31] However, in a laboratory situation, the minute volume of an animal may vary widely from values recorded in less stressful situations, and flows from 1.5 to 10 times the total minute volume of the exposed animals have been recommended for nose-only systems. [14]
- **Particle sizers**—two different instruments are used to measure PSDs. For particles 1 μm or larger, such as most bacteria, an aerodynamic particle sizer (APS) is used. For particles smaller than 1 μm , such as viruses, a scanning mobility particle sizer (SMPS) is used.
 - The APS (TSI, Inc., Model 3321) operates by measuring the time of flight of particles accelerated through a nozzle. The acceleration is measured by parallel lasers. Particles from 0.5 μm to 20 μm can be sized by the APS. The APS samples at a flowrate of 5 L/min and can sample continuously.
 - TSI's SMPS consists of a Model 3080 electrostatic classifier with a 3081 long differential mobility analyzer and a 3785 condensation particle counter. The 3080 and 3081 separate particles based on their electrical mobility. The separated

particles pass into the 3785, which “grows” the particles by condensation of water vapor and counts the resulting droplets optically. Particles as small as 10 nm and as large as 1 μm can be sized with the SMPS. The length of the particle sizer’s sampling interval depends on the total concentration of particles in the air—fewer particles require more sampling time. The particle range depends on the sampling rate. At 0.6 L/min, particles from 10 to 410 nm in size can be measured, and other size ranges require a sampling rate of about the same magnitude.

- **Impingers** (Ace Glass Inc., Vineland, N.J.)—aerosol samples are collected in all-glass AGI-4 impingers before and after filtration. AGI-4 impingers are designed to be used at a 12.5-L/min flowrate. [23] Make-up air is supplied to increase the total flow to this value. The efficiency of an impinger decreases with time, due to evaporation and aerosolization of the sampling liquid. [23] Therefore it is necessary to have two impingers in the system so that the operator can switch to a fresh impinger after a time and replace the used impinger base with a fresh one. More-expensive collection devices, such as the SKC BioSampler have not been consistently reported to perform significantly better than the all-glass impingers. [23] The Biosampler causes slightly less reaerosolization, but has increased internal losses. [32,33]
- **Porous tube diluters** (Mott Corporation, Farmington, Conn.; model #7610105-020)—diluters are used to deliver humidified make-up air into the airstream to adjust the flowrate both after the nebulizer and before the impingers.
- **Air compressor**—for the development effort the lab air line was filtered through a DFC-21 HEPA particle trap (Porous Media, St Paul, Minn.) to feed the nebulizer and porous tube diluters. The animal studies will require a source of breathable air free of both particles and toxic gases and vapors, to be provided onsite.
- **Vacuum pump**—this is a generic component, used only to draw air through the impingers, so it is not a potential source of contamination.

There are also less significant components: pressure gauges (Dwyer Magnehelic) to measure ΔP across the filter and the gauge pressure at the mouse tree; a 1-psi pop valve to limit back pressure on the nebulizer; a HEPA filter on the exhaust air; and a flow meter (TSI Model #4143D) at the end of the system.

Tubing used to connect components containing aerosol flow is ½-inch stainless steel. All curves in the tubing containing the main aerosol flow are gradual and smooth, with an inner curvature radius greater than 1 inch. All valves carrying aerosol flow are ½-inch stainless ball valves. Flows of clean air are controlled by ¼-inch needle valves, backed by rotameters to verify the flowrate.

The experimental filtration system design includes a dimensional constraint: all components carrying aerosol must fit inside a biological safety cabinet. The cabinet in which the system will finally reside at UNMC is a SterilGARD III Advanced Animal Transfer Station (Baker Company, SG603-ATS), which has interior dimensions of 27 in H \times 20 in D \times 68 in W. The largest sash opening allowed when an infectious agent is in the hood is 8 inches, which limits the reach of the operator(s). The convenience of the operator was considered in the design.

System validation—A series of quality control exercises were conducted to characterize first the performance of the components within the CATS, then subsystems of the CATS and finally to compare the mechanical performance of filter media in the CATS to that in the aerosol lab. Demonstrating representative behavior of the filter media and consistency of performance (CVs for delivery <20%; no detectable leaks) by the CATS were necessary conditions prior to starting animal studies.

Materials and methods

- **Preliminary checks**—Before each aerosolization, the system was leak tested by replacing the Collison nebulizer with a plug and pressurizing the system to a few inches of water, then observing it for an hour. Correlation of ports was performed using 250-nm and 1- μ m PSL beads. An arbitrary volume of beads was dispersed in deionized water as the nebulization liquid for the Collison nebulizer. The makeup flow was adjusted to deliver a total flow of 5.3 L/min, and the system was allowed to equilibrate. No filter was in the filter holder during this test. Each of the sampling ports (1, 2, and 4) and the ports on the impinger hook-up (5a and 5b) were sampled repeatedly with the particle sizer. No extra dilution air was used for the impinger hook-up in this test: valve C was closed.
- **Consistency tests** – To test the consistency of the challenge delivered, a bioaerosol challenge was created and flowed through the system. A bacterial spore, *Bacillus atrophaeus*, was grown in tryptic soy broth (TSB), and MS2 coli phage virus was grown in *E. coli* according to standard protocols [34,35]. These microbe stocks were individually diluted in deionized water and delivered into the Collison nebulizer. Air to the Collison was turned on and the make-up flow was adjusted to deliver a total flow of 5.3 L/min. (This creates a face velocity of 7.08 cm/s through the filter medium. At the 85-L/min flow rate used by NIOSH for testing FFRs, this scales to an FFR with a 200-cm² surface area, which is reasonable.) The system was allowed to equilibrate for 15 minutes before 5-minute impinger samples were taken at sampling ports 1 and 2, and particle size measurements were taken upstream periodically. (Downstream particle counts were found to be too close to the instrument error to be useful.) ΔP was observed throughout the experiment.

After the experiment, the impinger solutions were serially diluted and plated in triplicate, and incubated overnight at 37 °C. Plaques or colonies were counted the next day. The remaining liquid in the Collison nebulizer was also plated in triplicate and counted. For *B. atrophaeus*, the nebulizer liquid was plated with a spiral plater (Microbiology International, Frederick, Md.) onto tryptic soy agar (TSA) plates. For MS2, a single-layer plaque assay was performed according to standard protocols [35].

Results

- **Preliminary checks**—The system was leak checked and found to hold 3 inches of H₂O for an hour. Counts of PSL beads at the ports were consistent within 10% for both 250-nm and 1- μ m beads. The CV of 1- μ m beads counted at port C was just under 10%, probably because the path through the mouse tree is somewhat tortuous. CVs for relative humidity (RH), temperature (T), and the exhaust flow rate were lower than 2% for observations during a 90-minute period.

- **Consistency tests**—The first series of tests nebulized dispersions of MS2 virus with titers ranging from 10^8 to 10^{12} PFU/mL. For each test in this series, a 47-mm circular swatch cut from a T-5000 FFR was used as the filtration medium. Pairs of particle sizer measurements upstream of the filter concurrent with an impinger collection downstream alternated with downstream sampling into impingers: thus, there were two upstream PSD measurements and a downstream viable count during the first, third and fifth 5-minute sampling period, and upstream collections into impingers during the second, fourth and sixth periods, resulting in a total of six values for PSD and three each for viable counts before and after the filter. Total sampling time was a bit more than 30 minutes because switching between impingers was labor intensive. ΔP was not observed to change noticeably over the course of the run. Plated viable MS2 counts were not measurable, probably due to contamination in the laboratory workspace.

Averaged values of RH, T, and the count median diameter are presented in Table 1, plus geometric standard deviations (GSDs) and total particle counts (TPCs) measured in these experiments. Table 2 contains the coefficients of variation (CVs) of the median, GSD, and TPC measured within the experiment. The PSD was observed to be approximately log-normal. The PSD varied very little over the 30 minutes observed, as reflected in the very low CVs—all 6% or less. The statistical quantities of the PSD were not observed to trend upwards or downwards in time during the 30 minutes of observation.

Table 1. Average Relative Humidity, Temperature, and Particle Size Distribution Statistical Quantities during Experiments

Experiment ID	RH (%)	T (°C)	Median particle size (nm)	GSD ^a	Total Particle Count (#x10 ⁶ /mL)
20090724	65	No data	81.41	1.70	2.65
20090728	61	22.7	74.22	1.69	4.51
20090730	64	22.4	75.72	1.68	4.16
20090811	55	27.0	75.62	1.72	5.13
20090812	53	26.5	76.53	1.70	4.51
20090813	56	26.0	77.96	1.70	4.11
minimum	53	22.4	74.22	1.68	2.65
maximum	65	27.0	81.41	1.72	5.13
average	59	24.9	76.91	1.70	4.18

^a Geometric standard deviation

A second series of tests nebulized dispersions of *B. atrophaeus* spores with titers of 1×10^7 to 8×10^7 CFU/mL. Circular swatches were cut from T-5000 FFRs and from 3M N95 FFRs; one disc from a 3M N95 was exposed to vapors of 2-propanol (IPA) in an effort to remove its

Table 2. Coefficients of Variation of PSD Statistical Quantities during Experiments

Experiment ID	CV of		
	Median size	GSD ^a	TPC ^b
20090724	1.23%	0.19%	4.66%
20090728	3.09%	1.02%	5.21%
20090730	1.20%	0.21%	3.31%
20090811	1.93%	0.56%	3.16%
20090812	0.49%	0.08%	6.00%
20090813	0.37%	0.29%	5.15%
minimum	0.37%	0.08%	3.16%
maximum	3.09%	1.02%	6.00%
average	1.38%	0.39%	4.58%

^a Geometric standard deviation. ^b Total particle count

electric charge and enhance penetration. Although the expected viable counts were measured upstream, no viable microbes were detected downstream—even of the IPA-treated N95 medium—because the challenge concentrations were not large enough to overcome the high filtration efficiency of these filters at particle sizes near and above 1 μm . The impingers sampled at a rate of 2.5 L/min during the first three runs and at 1.5 L/min during the final three. Because the APS draws much more air than the SMPS, it was impossible to operate an impinger and the APS simultaneously; therefore, particle size measurements were taken before and after impinger measurements, resulting in seven particle size measurements and three viable counts at each sampling point per run. The total length of a run was about 40 minutes. No change in ΔP was observed over the course of any run in this series.

Table 3 identifies the filter used in each test, lists the averaged values of RH and T, and reports values of count median diameter, geometric standard deviation (GSD), and total particle count (TPC) measured within these experiments, plus the concentration of viable microbes remaining in the Collison medium after a run and the average airborne viable concentration. Table 4 contains the CVs of the median size, GSD, TPC, and upstream viable concentration measured within the experiment. The PSD was observed to be bimodal, particles in the peak near 1 μm containing bacteria, and the approximately log-normal pattern of smaller particles presumed to contain only dissolved solids from the aerosolization medium. The low CVs in Table 4 for the PSD—all less than 10%—witness that the PSD varied only slightly during the 40 minutes of observation. The statistical quantities of both the PSD and the more-variable upstream viable concentration showed no discernible trend upward or downward in time during the 40-minute observation period.

Table 3. Filter used, Temperature, Relative Humidity, Average PSD Statistical Quantities and Viable Concentrations Measured during Experiments

Experiment ID	Filter	RH (%)	T (°C)	Median Particle Size (µm)	GSD ^a	Total Particle Count (#/mL)	Viable Counts Measured	
							Collison Medium (x10 ⁶ CFU/mL)	Upstream (CFU/mL air)
20090819	T-5000	58	23.6	1.08	1.33	123	26.3	1.16
20090820	T-5000	57	24.0	1.10	1.21	59.2	14.0	0.357
20090901	3M N95	50	23.0	0.95	1.33	254	2.80	0.152
20090908	3M N95	47	22.3	0.95	1.34	528	5.57	0.255
20090909	3M N95	47	23.0	0.95	1.34	1210	42.0	0.599
20090910	IPA 3M N95	45	23.2	0.96	1.34	1170	18.0	0.622
minimum		45	22.3	0.95	1.21	59.2	2.80	0.152
maximum		58	24.0	1.10	1.34	1210	42.0	1.16
average		51	23.2	1.00	1.31	558	18.1	0.525

^aGeometric standard deviation

Table 4. CVs of PSD Statistical Quantities and Upstream Airborne Viable Concentration during Experiments

Experiment ID	Coefficient of Variation of			
	Median size	GSD ^a	TPC ^b	Upstream viable
20090819	0.69%	0.42%	5.21%	19.21%
20090820	0.60%	0.58%	7.42%	24.02%
20090901	0.70%	0.15%	6.81%	18.23%
20090908	0.42%	0.22%	3.84%	25.54%
20090909	0.91%	0.19%	9.12%	14.17%
20090910	0.30%	0.14%	3.52%	5.00%
minimum	0.30%	0.14%	3.52%	5.00%
maximum	0.91%	0.58%	9.12%	25.54%
average	0.60%	0.28%	5.99%	17.69%

^aGeometric standard deviation. ^bTotal particle count

Summary—This component of the experiment has been successfully accomplished—the PSD holds steady for a period of time long enough to deliver the doses needed to perform the animal exposure trials. In the bacterial spore tests, it is likely that inexperience with the plating method contributed to the variability observed. The variability in the concentration of colony-forming units is worse than that observed by Henderson [15], but not by much.

It is unfortunate that no viable penetration was measured through the filters, but this inconvenience does not invalidate the performance of the CATS. If the aerosol challenge is consistent and the filtration is consistent, one can reason that the downstream viable population will be as well. The data support a conclusion that the CATS satisfied the key conditions—PSDs remained acceptably constant and ΔP did not change discriminably—so the PRE can be presumed not to have changed during these experiments, in which dissolved solids were not

added and thus did not load up the filter medium. Our experiences with dissolved solids are discussed in section 3.2, and we close this section with the projection that substitution of a viral mouse pathogen of diameter less than 200 nm will decrease the PRE of the media to be tested sufficiently that they will pass infective quantities of particles from aerosols nebulized from attainable titers in the Collison nebulizer’s reservoir.

3.2 *Mouse exposure experiment*

Introduction—The rationale for using a live-animal detection experiment to measure attenuation of infectivity of an aerosolized pathogen is detailed in chapter 2. For these experiments the CATS will be moved to UNMC and installed in the BSL-3 cabinet it was designed to fit inside. The apparatus will challenge mice with a range of concentrations of infectious aerosols that have passed through either of two Triosyn filtration media, which are mechanically identical except that the fibers of one contain PSTI. A shift in the nebulized median infective dose between the PSTI-treated and untreated media will show the PSTI to be effective at preventing infection, and the amount of the shift (the *effectiveness factor*, Ef_0 in Figure 4) will describe the amount by which protection is enhanced. The microorganism originally selected to be the challenge was *Francisella tularensis* (*Ft*), which was reported [36] to be a small coccobacillus (~0.2 x 0.7 μm diameter) that should penetrate an air filter. A threat agent, *Ft* has a median infectious dose (MID₅₀) of ~ 10 microorganisms. Human pathogenicity of the *Ft* live vaccine strain (LVS) is much milder and LVS produces symptoms of infection in mice, so this organism appeared at the outset to provide an ideal platform to evaluate the effect of PSTI in an antimicrobial air filtration medium.

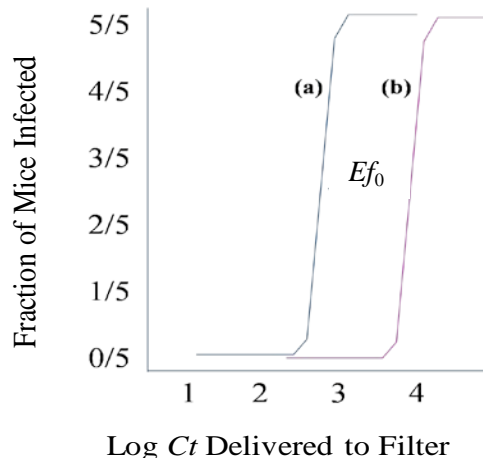


Figure 4. Hypothetical plot showing fraction of mice infected vs. dose delivered to a) an inert filter medium and b) an effective antimicrobial medium. The shift between the two *S* curves— Ef_0 —is the increment of protection added by the treatment.

The reality of *Ft* has proved to be quite another thing. Experiments described below detail a path of discovery of complications—most importantly that the diameter reported for *Ft* is about one-third of the diameter of any *Francisella* spp. particles we have been able to acquire and to grow. This process and a recent redesign of the experiment to substitute an H1N1 influenza A virus for *Ft* will delay the completion of the study until 2010. Progress to date is detailed below.

Test plan for *Francisella*—The original test plan was organized around a schedule (Table 5) for delivering to mice in groups of five ($n = 5$) a range of doses of *Ft* at nominally constant environmental conditions after penetrating an air filter in two experimental situations: a conventional air filter medium is used in the sample holder (described on p. 10) during exposure of the control group of mice, and a mechanically equivalent medium that contains PSTI is used for the treated series of exposures. The availability of 12 ports in the mouse tree allows us to deliver three different doses to sets of mice during a 60-minute run for each aerosolized challenge: while one group of five mice is exposed for the full hour, a second group will be installed on the mouse tree, exposed for 30 minutes, removed, and a third group installed, exposed for 15 minutes and removed. At constant aerosol particle concentration C ($C = C_0 \times \text{VRE}$; C_0 is the aerosol concentration delivered by the Collison. VRE is the viable removal efficiency of the filter = PRE for the control medium), the three durations of exposure (15, 30, and 60 minutes) produce three doses ($Ct = \text{concentration} \times \text{time}$) in the ratio 1:2:4 from each experimental run. Success requires that we deliver concentrations through both the untreated and treated filter that produce responses ranging from 0% infected to 100% infected, to generate plots of the sort cartooned in Figure 4.

Table 5. Experimental Conditions to Measure Threshold Infectivity of *Ft* in CD1 Mice

Filter Type (P95 or N95 Flat Sheets)	Challenge Concentration (PFU/L of air)	Number of Mice	Exposure time	Cage Group	
Filter medium (The same set of experiments will performed using both control and PSTI- containing filter media)	1×10^3	5	15	A1	
		5	30	A2	
		5	60	A3	
	1×10^5	5	15	B1	
		5	30	B2	
		5	60	B3	
	1×10^7	5	15	C1	
		5	30	C2	
		5	60	C3	
	3×10^3	5	15	D1	
		5	30	D2	
		5	60	D3	
	3×10^5	5	15	E1	
		5	30	E2	
		5	60	E3	
	3×10^7	5	15	F1	
		5	30	F2	
		5	60	F3	
	Naïve Controls [§]	-	10		M,N
	Total Mice	-	190		

[§] Also a source of replacements for animals in groups A1–F3 that die prior to exposure.

To minimize variability in size and age and to exclude effects of sex we chose female CD1 mice 5–7 weeks old and weighing 18–22 grams at the time of exposure for this study. To limit confounding effects from stress to the mice during exposure, each animal will be acclimated to the processes and to the team during the week preceding exposure: the mouse will be inserted into the restraint for an hour twice on each of the seven days prior to her exposure trial. Procedures for generating the aerosol for the exposure trials will be the same as used during shakedown and evaluation of the CATS, except that a titered dispersion of *Ft* LVS will be introduced into the Collison in lieu of the beads, MS2 phage or *B. atrophaeus* spores used in section 3.1. Each mouse will be exposed to the bio-aerosol once, as scheduled in Table 6. Immediately following exposure, her nose will be wiped, and she will be extracted from the holder and housed with her exposure group for a week, during which time she will be observed for symptoms (fever, respiratory). After a 7-day incubation period, each mouse will be euthanized in a CO₂ chamber and necropsied, and homogenized tissue plates will be assessed. During aerosolization, both before and after the 60-minute exposure sequence, aerosol samples will be collected in AGI-30 impingers upstream and downstream of the filter to quantify the challenge and penetration concentration of the aerosol. Post aerosolization the dispersion of *Ft* LVS remaining in the impinger reservoir will be assayed by viable plating.

Table 6. Schedule of H1N1 Titers and Filter Media to be Used during Mouse Exposure Trials

Week	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6	
	PSTI [§]	Titer, [¶] TCID ₅₀ / mL	PSTI	Titer, TCID ₅₀ / mL	PSTI	Titer, TCID ₅₀ / mL	PSTI	Titer, TCID ₅₀ /mL	PSTI	Titer, TCID ₅₀ / mL	PSTI	Titer, TCID ₅₀ / mL
1	+	1x10 ⁷	+	1x10 ⁸	-	1x10 ⁵	-	1x10 ⁶	+	3x10 ⁷	-	3x10 ⁶
2	+	3x10 ⁸	+	3x10 ⁶	-	1x10 ⁷	-	3x10 ⁵	+	1x10 ⁷	-	1x10 ⁵

[§] + indicates the medium containing poly(styrene-4-[trimethylammonium]methyl triiodide) will be used; - indicates the inert control.

[¶]Number of TCID₅₀ units of H1N1 virus per mL of liquid in the nebulizer.

Preliminary experiments with *F. tularensis*—*Ft* is known to be a fastidious microorganism so preservation of viability during and after aerosolization will require delivery of these bacteria in an aerosolization medium containing carbohydrate or protein species. Samples of control HEPA media were challenged in a rig fashioned after our Collison-based system at UNMC with *Ft* LVS dispersed in aqueous media containing either 0.1% raffinose or 2% peptone. Both additives accumulated on the filter medium, causing ΔP to increase drastically in less than 15 minutes. In addition, viability of bioaerosols so generated cultured out at ~4% that of the organism delivered into the nebulizer’s reservoir and—the *coup de grâce*—the diameters of (spherical) *Ft* LVS grown either in a liquid culture medium or on chocolate agar plates, of *F. philomiragia*, which we had acquired as a technical training aid and grown in the same two media, and of radiation-killed *Ft* were consistently measured to be 750~800 nm.

The avalanche of incompatibilities made it clear that the purpose of the exercise—to isolate and measure the increment to IRP afforded by the presence of PSTI at the surface of air filter fibers—could not be realized with an organism that is too large to penetrate the filters in quantity and that cannot be delivered at a constant rate for 90 minutes. Accordingly we shifted our attention to

selecting a virus to replace *Ft* as our probe for this exercise. During the course of separate activities underway with influenza A H1N1-PR8 at both AFRL and UNMC, we had accumulated experience from which we were able to determine that the size (80~120 nm) of this entity is compatible with the experimental design and aims. H1N1 is an enveloped virus, so stability during aerosolization and transport through the CATS to the mouse or impinger was a question, but it is pathogenic to mice, a BSL-2 species (requiring BSL-3 containment as an aerosol), and of intrinsic interest both generically and in the context of the flu panic underway. A convenient tissue culture assay is available [37] that uses Madin–Darby canine kidney (MDCK) cells to measure a median tissue culture infectious dose (TCID₅₀). Finally, the availability of a human vaccine attenuates the slight risk of working with a moderate human pathogen, so it was decided to conduct a test of the viability of H1N1-PR8 dispersions in DI water in the nebulizer reservoir and in aerosols caught in AGI-30 impingers. Loss of viability measured at UNMC was minimal so we revised the test plan to substitute H1N1 for *Ft* and have secured approval for animal use.

Revised test plan—The sense of the experimental matrix mapped in Table 5 will be retained, with direct substitution of H1N1 for *Ft*. The MID₅₀ of H1N1 in CD1 mice is not recorded and the calculation of *C* in Table 5 has not been adjusted for actual PSD (which will determine the fraction of the bioaerosol captured by the filter), so the sequence of exposure experiments is scheduled in Table 6, with an anticipated element of flexibility that will allow us to adjust concentrations and possibly the sequence during the second week of exposure trials to accommodate results during week 1.

As an example of the flexibility we consider necessary, consider the possibility that both values of fractions infected from days 1 and 2 are 0/5. Insufficient virus reached the mice, so a larger titer would be delivered on day 5. Conversely, if both values are 5/5, the delivered dose is larger than the MID₅₀ and a smaller dose (lower titer) should be delivered on day 5 to approach the median infective level.

It is also clear that the flat wings of the *S* curves idealized in Figure 4 contain no useful information. The target of the adjustments in the preceding paragraph is to find the region of partial response. Therefore we will adjust the titers listed for the later exposure trials to increase data density in the *S* region of each of the curves as soon as it is located in each experimental series. The value of *C* (in units of TCID₅₀/L of air behind the filter) will be measured in the impingers downstream of the filter immediately before and after each run, and the average of the two values will be plotted against the fraction infected during the trial to estimate the MID₅₀ for this mouse–virus pair. The value of the aerosol concentration upstream of the filter, also measured immediately before and after the trial, will be plotted against the fraction infected during the trial to recreate Figure 4 with data, and the enhancement factor Ef_0 measured as illustrated in Figure 4.

4.0 Effect of PSTI on Rate of Decay of Viability of MS2 Aerosols

Introduction—The subscript 0 in the notation Ef_0 is used in section 3.2 to label the enhancement factor caused by PSTI at the surface of fibers in an air filter on viable bioaerosol particles penetrating it and immediately encountering a wet environment—either respiratory mucosa or collection medium in an AGI-30 impinger). In the experiments in Section 3 PRE is unchanged by the incorporation of PSTI, so any difference between VRE and PRE will be attributed to the effect of PSTI. As implied earlier, the time for action of captured I_2 is very short, a few milliseconds, so it is possible that Ef_0 will be measured to be 1 (*i.e.*, no effect from PSTI on this time scale) after the mouse trials.

Although a filter medium with $Ef_0 = 1$ would afford no advantage over ambient technology to the wearer of an IRP device, the five-log increase in the time scale applicable in collective protection environments will allow many or all chemical interactions of a microbe with adhering I_2 equivalents to proceed to their thermodynamic end state, which might include addition to unsaturated membrane fatty acids, oxidation of amino acid residues containing reduced-sulfur groups (cysteine, cystine, methionine), and nuclear substitution of –I on phenylalanine, tyrosine, tryptophan or histidine—any of which conversions can impair viability or host recognition. To distinguish the significance of time we designate the time-dependent enhancement factor Ef_t , which includes Ef_0 , the enhancement factor at $t \sim 0$.

To assess the possibility that passage through a filter medium surfaced with PSTI can effect useful acceleration of the loss of viability by airborne pathogens, we developed a system in which viable MS2 aerosols were passed through filtration media and then trapped and held in a reservoir for an arbitrary duration of time before collection in a quenching medium for viable enumeration (Figure 5). The viability of ordinary bioaerosols decays spontaneously over time, and the process can be accelerated by hostile environmental conditions. Both of the preceding will be characteristic of individual microbes, so the results from any experiment will be applicable to only the microbe tested; however, trends will be generic.

Environmental control is thus a necessary condition for designing these experiments. If measurable acceleration is observed for a single type of virus at constant environmental conditions, a systematic evaluation of the effects of T and RH should eventually be conducted for a series of microbes to assay the windows of utility of the technology. Paired measurements using reactive and inert filters in otherwise identical experiments are needed to tease out the value of Ef_t at different times—the target of this exploration.

Because there is no direct precedent for the experiment, it was also necessary to validate the performance of the test system to ensure that the results were accurate and representative of the phenomenon we wish to test. Accordingly, control experiments were conducted to ensure 1) that filling of collection bags is measurable and reproducible, and 2) that viable MS2 coli phage penetrates the filter medium (Triosyn T5000) selected for testing to provide measurable concentrations of viable particles as decay proceeds over the time window of the experiment.

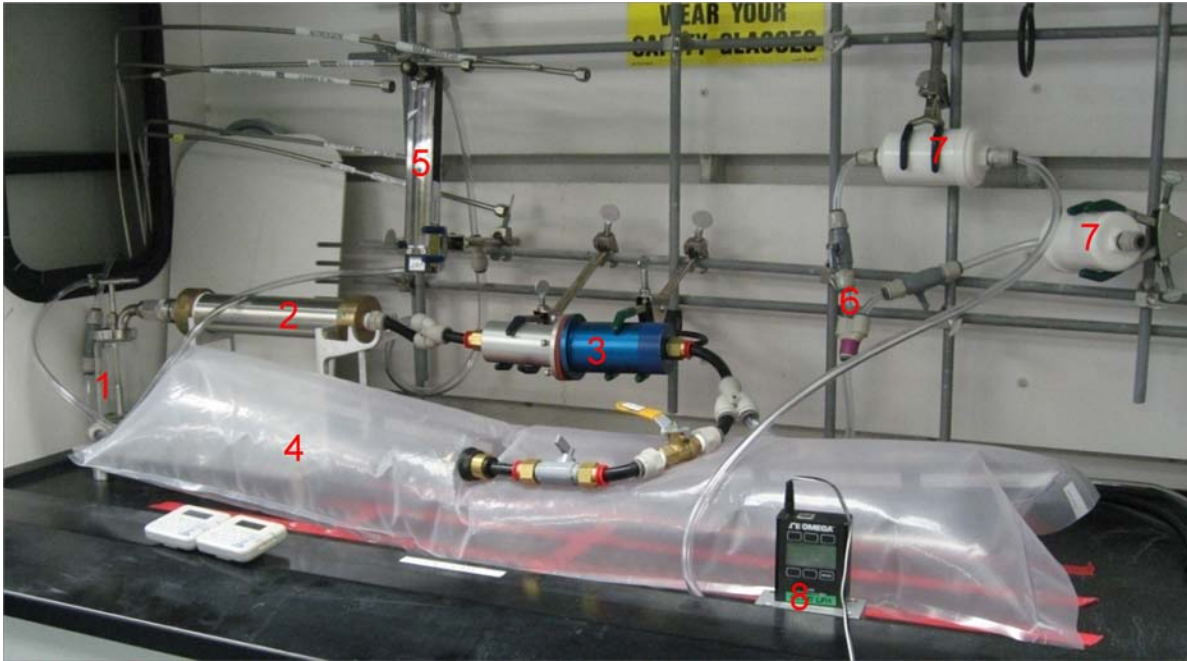


Figure 5. Test apparatus for collecting and holding biological aerosols following filtration. (1 Collison nebulizer, 2 Diffusion dryer, 3 Sample holder, 4 Food Saver® heat-seal bag, 5 Rotameter, 6 Overflow valves, 7 HEPA filters, 8 Volumetric flow meter)

Materials and Methods

Test and control filter media—For this exercise discs of 47-mm diameter were cut with a circular punch from two models of air purification media containing PSTI: the Triosyn T5000 FFR is a commercially available unit fabricated in multiple layers (Figure 6), of which one is a filtering medium containing PSTI. The FFR also has a carbon fabric downstream of the filter to prevent iodine off-gassing. The second treated medium used was Triosyn’s SHM, a commercial fiberglass HEPA medium impregnated with fused PSTI dust. Discs of the inert control medium were punched from a 3M 1860s FFR.

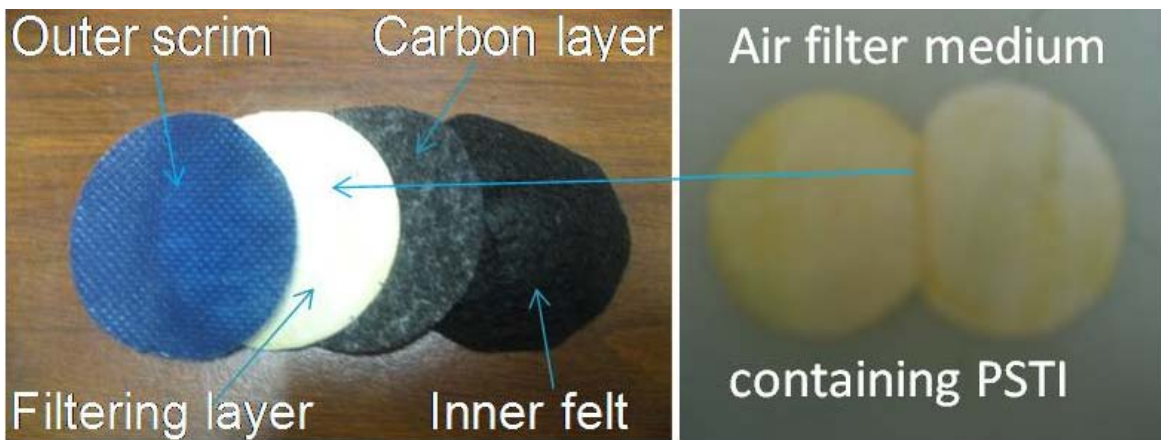


Figure 6. Layers of the Triosyn T5000 FFR

Test rig design—Aerosols containing MS2 coli phage were generated using a Collison nebulizer (BGI Inc., Waltham, Mass.) (labeled **1** in Figure 5) and passed in turn through a conductive electrical tubing connector (used for all connections because it has a low potential for attracting aerosol particles), a diffusion dryer (TSI Inc, Shoreview, Minn.) (**2**), which dries the aerosol to ~20% RH, and a 47-mm disc of filter medium contained in a custom fabricated holder (Triosyn Corp., Williston, Vt.) (**3**). To increase the airflow to the rated face velocity of the filter medium, HEPA-filtered dilution air is added through a “T” fitting at the base of the Rotameter (034-62G, Cole-Parmer, Vernon Hills, Ill.) (**5**) inserted between the diffusion drier and filter holder to monitor the total airflow rate. The holder is connected through threaded fittings at each end, which allows a convenient exchange of holders and challenges to several samples during a nebulizer run. The airstream exits the filter to a “Y” connector, from which an open leg leads to a second Y junction (**6**) that feeds two HEPA filters (**7**); of these one HEPA is vented to the hood as a guard against overpressurization, and the other feeds into a digital flow meter (FMA-1601A, Omega Engineering, Stamford, Conn.) (**8**). The second leg from the initial Y is controlled by a ball valve (yellow handle in the center foreground of Figure 5). When this valve is open the flow resistance of the HEPA canisters (**7**) diverts the flow into a 3-foot section of Foodsaver® heat-sealed bag (prepared from T150-00011-002 11-inch roll stock, Jarden Consumer Solutions, Rye, N.Y.) (**4**) through a port installed in the side. Closing the ball valve to terminate filling the bag directs the flow to the HEPAs (**7**). The filling capacity of the bag was 20 L.

Test rig validation—A round coupon 47 mm in diameter was cut from a Triosyn T5000 FFR and sealed into the filter holder, which was connected into the test system as shown in Figure 5. A 30-mL volume of a suspension of MS2 coli phage diluted to 10^{10} PFU per mL in DI water, was added to the reservoir of the single-jet Collison nebulizer, which was then pressurized to 20 psi. The airstream exiting the filter holder (**3**) was diverted to the digital flow meter (**8**) while the flow rate was adjusted to 5.4 LPM by adding dilution air between the diffusion dryer and sample holder. The system was allowed to run for 15 minutes, whereupon the ball valve was closed to divert the filtered aerosol into the heat-sealed bags for 2.5 minutes. After loading, the bag was sealed by closing the ball valve, disconnected from the rig and carried to the collection room, where the entire content of the bag was drawn by a vacuum pump into an AGI-30 impinger containing 20 mL of 1X PBS/1% sodium thiosulfate. These steps were repeated seven times. The filter holder was then replaced with a filter holder containing no filtration medium and one 3-foot bag was filled and collected into the impinger by the same procedures. Viable MS2 in the impingers was quantified using a standard single-layer plaque assay [35].

The test rig validation was repeated at a higher flow rate, with the following changes: A three-jet Collison nebulizer was used to generate the challenge aerosol, which also produced more water vapor. The airflow was increased to 11.5 LPM and the bag fill time was lowered to 1.3 minutes. RH in the bag was measured using a standard humidity probe.

Aerosol Decay Studies—MS2 aerosols were delivered through a 47-mm disc cut from a T5000 FFR under the high-flowrate condition described above. Nine bags were filled with aerosols, of which three bags each were incubated for 0, 15, and 30 minutes prior to being emptied into separate AGI-30 impingers for plating and viable enumeration. The filter element was removed from test rig and one more bag was collected and immediately sampled into an impinger for plating to quantify the challenge. The same steps were repeated using a disc cut from a 3M 1860s

FFR (no antimicrobial component) and again with a disc cut from Triosyn SHM. Viable MS2 in each impinger was quantified using a standard single-layer plaque assay [35].

A second set of determinations was performed as above, except that the diffusion dryer (2) was not piped in (to elevate the RH) and only six bags were filled for delay times of 0 and 15 minutes before evacuation into the impingers.

Results and discussion—The initial validation test of the experimental rig at the low flow rate (5.3 LPM) showed (Figure 4) that each bag delivered an equivalent amount of virus into its impinger. However, the amount of virus in the bag was only 10^1 PFU/L of air, which is too low to make a measurement of decay rate over time. *In-vitro* data suggest that PSTI-containing air filter media cause a 2-log decrease in culturable penetrating counts, so a bare minimum of three logs of virus in each bag would be needed at $t = 0$ to retain a countable population of infective MS2 at $t = 30$ minutes. Accordingly, we tripled the rate of delivery and increased the flowrate through the test rig to 11.3 LPM. The validation test (Figure 8) at this flow rate again showed acceptably uniform loading of virus into and back out of the bags, and the 1-log plus decrease in PRE of the diffusionally dominated MS2-containing particles predicted to be caused by a bit more than doubling the flowrate [9] is clearly evident by comparing the respective amounts of viables penetrating in Figures 7 and 8. These two effects combined to raise the viable particle count in the bags to the threshold value, $\sim 10^3$ PFU/ L of air. Note that, although this running condition is able to support measurements of waning viability during a 30-minute experiment, the flowrate exceeds the rated face velocity for the filter, and shortening the residence time might decrease the effectiveness of the postulated [2] particle–PSTI interaction to deliver I_2 to the passing aerosol. However, a qualitative answer to the basic question will test the validity of the proposed mechanism, and a detectably positive outcome would provide impetus to conduct a more-extensive investigation to quantify the effect in a three-dimensional data space (t , T, RH) for a number of pathogens. We concluded that the experiment initially proposed would remain instructive under these slightly nonstandard conditions and determined to proceed with the measurements of viability vs time.

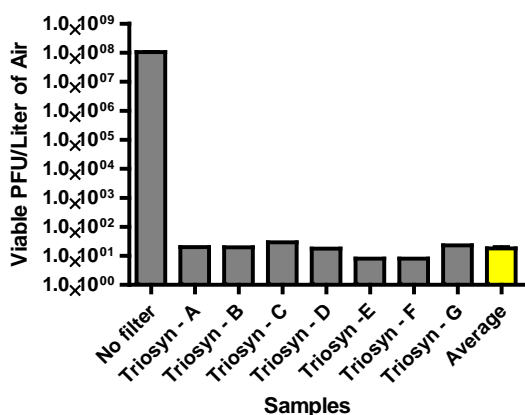


Figure 7. Recovery of viable MS2 Aerosols delivered at 5.3 LPM into Heat-Sealed Bags (single-Jet Collison, dryer installed)

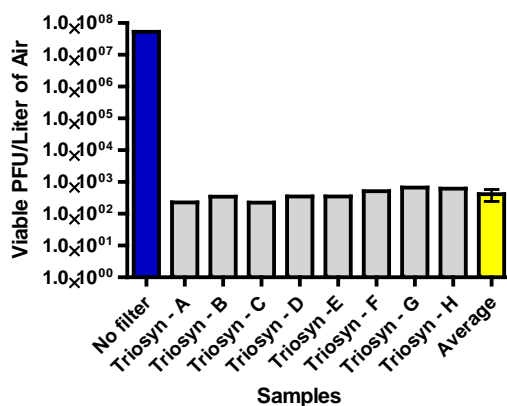


Figure 8. Recovery of viable MS2 Aerosols delivered at 11.3 LPM into Heat-Sealed Bags (three-Jet Collison, dryer installed)

At the lower humidity condition (50% RH) viability of MS2 declines rapidly with time. Figure 9 and Table 7 show consistent but slight enhancement of the rate of loss of viability after penetration of all three of the media containing PSTI. At 15 minutes spontaneous decay of viability was nearly 80% and did not increase over the next 15 minutes. The T5000 FFR and SHM achieved an average decay rate 10% higher, but the result for our small data set was not statistically significant ($P > .05$). Passage through any of these filters selects strongly for viral singlets that are bare or nearly so, and it is known that non-enveloped viruses like MS2 are not stable at low humidity conditions so the indeterminacy of the result is not surprising. That all four plots are flat from 15 to 30 minutes suggests that the decay might be complete before the 15-minute collection, and that the observed differences result from variations in the time from collection in the bag to trapping in the impinger. Repeating this experiment using less-efficient mechanical media at design face velocity and with shorter delays before capture in the impingers might provide intermediate values of spontaneous and augmented decay that can be interpreted. These results do not rule out the possibility that capture of I_2 from PSTI but neither do they provide any evidence for it.

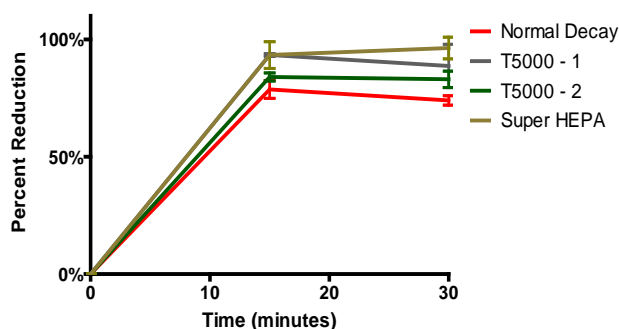


Figure 9. Loss of Viability of MS2 after Penetration through Filter Media (~50% RH)

Table 7. Average Fractional Loss of MS2 Viability after Penetration through PSTI-treated and Inert Air Filter Media at ~50% RH, T ~22 °C.

Hold time (min)	3M1860s	T5000		Super HEPA
		1	2	
0	[0%] [§]			
15	79%	93%	84%	93%
30	74%	89%	83%	96%

[§]Attenuation is scaled from value at $t = 0$.

The experiment was repeated in a high-humidity environment (~90% RH) to encourage the MS2 to remain viable over the 15-minute incubation period. In this series of experiments average spontaneous decay of viability was only ~10% (inert-medium control experiments) (Figure 10, Table 8) but the data variability for the control tests was high as seen in the error bar for the control value. Passage of MS2 through the T5000 medium resulted in a fairly reproducible 35% decay over the 15-minute incubation period, but the result was not statistically significant ($P > .05$) when compared to the highly variable control data.

The SHM—which was not backed by carbon felt—caused an 88% reduction in viability during the 15-minute incubation period, which difference was statistically significant ($P < .05$) from both the T5000 and control data. This shows that iodine from the SHM is effective against the aerosol on a time scale in minutes, but at least two factors confound the interpretation of the result. 1) Lee, *et al.*, [3] measured I_2 concentrations that were toxic to MS2 in water downstream of PSTI-containing media absent the backing felt. The quenching medium used in this experiment eliminates the possibility of interference from toxicity of free (and bound) I_2 in the

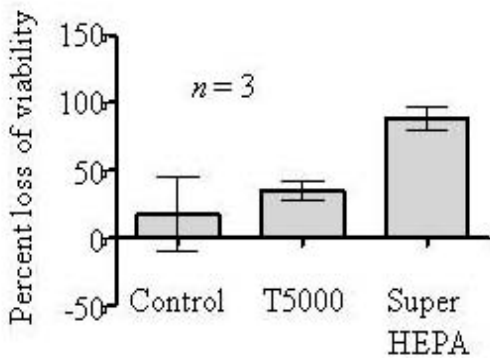


Figure 10. Average Decay of MS2 Following Penetration through Filter Media (~90% RH)

Table 8. Loss of Viability by MS2 15 Minutes after Penetration through PSTI-treated and Control Filter Media (90% RH)

Hold time, minutes	3M1860s	T-5000	Super HEPA
15	10%	35%	88%

impinger. The source of I_2 causing toxicity during incubation of the aerosol is, however, uncertain because data noise made the results from the T5000 experiment equivocal. The concentration of I_2 in the gas phase behind other bare PSTI-containing media is on the order of a part per million, which value is small enough that random-collision processes should not contribute significantly to poisoning of free aerosol particles; however, an attractive interaction between microbe and PSTI, which we proposed [2] to rationalize the apparent capture of I_2 from PSTI by passing bioaerosols, could also greatly enhance the cross section for interaction of I_2 with a passing aerosol particle. 2) The same rationalization proposed an uncharacterized distance-dependent factor for probability of capture of I_2 during passage by any fiber. The distance between fibers in the T5000 medium—functionally an N99 electret—is much greater than in the SHM, which is a fiberglass HEPA medium, so the obvious extension is that capture of I_2 in the narrower spaces of the SHM should be much more efficient than in the wide open spaces of the electret, and if the attenuation observed for the T5000 is genuine, the set of results would be precisely consistent with the mechanism proposed earlier.

Summary—This study was designed to look for and quantify antimicrobial activity transferred to microbes penetrating air filter fibers containing PSTI during incubations lasting for minutes before quenching of all iodine chemistry and viable enumeration. MS2 was selected as the test microbe, and its sensitivity to a relatively low-humidity (50% RH) environment is such that in the initial experiment at 50% RH the spontaneous rate of loss of viability was fast enough that none remained after the first 15-minute time interval, so Figure 9 shows only the variability in measurement of a complete kill. Raising the RH to 90% in a second set of incubations extended the airborne lifetime of MS2 and allowed the measurement of viable counts after 15 minutes of incubation. This experiment proved almost equally disappointing because data from the inert medium used as the untreated control were so noisy that statistical significance of the partial kill measured for the T5000 medium was not significant at 95% confidence level. Augmentation of kill by the SHM at the high-humidity condition was found to be statistically significant and was certainly caused by PSTI, but interpretation of the result was confounded by the absence of the carbon backing, which allows during incubation involvement by free iodine that escaped the filter.

The 80% increase in kill rate is one order of magnitude within the accuracy of our results. That this is a log less than the VREs consistently observed in previous tests suggests that water is an important factor in the disinfection process, and might be taken as a hopeful sign for the mouse study if the killing action is established almost immediately upon contact with water. The results of the exposure study will resolve this question. Less accessible at present are the relationships to kill rate of concentration of available I_2 after passage through an adsorbent layer, and mean minimum distance of approach to fibers, the respective contributions of which to the data in Figure 10 we can still only speculate about.

The possibility that free I_2 participates in the gas phase raises the possibility of a practical limit on the use of the SuperHEPA medium, because iodine is a substance regulated by OSHA in the form of both a Threshold Limit Value (TLV), which limits the average air concentration in occupied spaces, and a Short-Term Exposure Limit (STEL), which value limits short-term excursions of concentration. If effective kills were observed only at air concentrations near or higher than the TLV application in occupied spaces would likely be prohibited.

Some of the questions above will be answered by the results of the mouse experiment, but several follow-on studies are in order, most urgently to increase the n of the high-humidity incubation experiment to establish that the attenuation of viability by the T5000 medium is or is not real. A follow-on study in which MS2 is co-aerosolized with various concentrations of iodine from 0.1~10 ppm is equally important to test the hypothesis that an attractive interaction between I_2 and the surface of microbes can cause attenuation of viability and, if this is the case, to identify the threshold concentration to provide biocidal activity, first at the single T, RH condition tested and subsequently to map the threshold concentration over the range of values of T and RH of interest to individual and collective protection.

5.0 Project Summary

Test systems and experimental designs used in past studies evaluating the effectiveness of PSTI-containing antimicrobial media products have yielded equivocal data from which it has not been possible to evaluate the benefit—if any—of such media for respiratory protection of humans in infectious environments. *In-vitro* data from two laboratories comparing inert to PSTI-treated media have consistently demonstrated a 2-log decrease in viable counts of several microbes after passage through PSTI-containing products and immediate collection in a nonreducing aqueous medium. However, some of these experiments involved an extra mechanical layer that was not controlled for and others did not measure iodine loading in the impingers, both factors that could contribute to the observed decreases. Lee, *et al.*, [3] found that addition of a strong reducing agent to the impinger medium reverses the added 2-log kill and that addition of 5% serum albumin into the collection medium introduces a competition for available I_2 that reverses roughly half of the kill, from which the kinetics of the killing process was seen to be an important factor that had until then been neglected.

In the context of individual respiratory protection the impinger experiment is accurately representative of the time scale of an exposure event, but for collective protection the mean time from filter to respiratory mucosa will be in minutes, which allows that amount of time for I_2 bound to the surface of a microbe to act on or migrate to sensitive sites. Accordingly we developed a simple test system to elucidate the mechanism of action of PSTI-coated fibers on a penetrating virus in contained “free” air and found that at 50% RH and 20 °C spontaneous loss of viability of MS2 is so fast that no viability remained after 15 minutes. Repeating the experiment at 90% RH extended the lifetime of MS2 to a few hours and allowed observation of a measurable increase in die rate after passage through a coupon cut from a Triosyn T5000 mask and faster loss of viability after passage through a coupon of SHM. The control data were very noisy and the concentration of I_2 was not measured in the impinger used in the SHM experiments, so some uncertainty attaches to these results, but the data are consistent with our published hypothesis that captured I_2 acts to neutralize a fraction of viable microbes penetrating a PSTI-coated medium and further suggest that water is a major factor in the neutralization process. Additional experiments are planned to address both uncertainties and clarify the aerosol-phase processes.

For IP applications the only recourse to answer the question about the protectiveness of PSTI-coated media is to generate *in vivo* data using an animal model behind treated and control filters. These data are expected to provide a conclusive measure of the effect of PSTI on the rate of clinical infections caused by incrementally increased viral challenge concentrations filtered through mechanically equivalent treated and control media. In preparation for this experiment a test device was built and validated to deliver a constant concentration of viral particles at a constant rate to mice in groups of five, and a test plan has been approved to challenge the device with a fine-particle bioaerosol. The test validation demonstrated that the CATS is a robust device that is capable of delivering a filtered aerosol challenge to the mice. As of the date of this report, the mouse challenge has yet to be performed owing to unresolved details, both technical and operational. We expect these data to be collected in 2010, after which expect to have a reliable measure of the usefulness of PSTI-treated media in both IP and CP applications.

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