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Development of a Test System To Apply Virus-Containing Particles to Filtering Facepiece Respirators for the Evaluation of Decontamination Procedures[▽]

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A chamber to apply aerosolized virus-containing particles to air-permeable substrates (coupons) was constructed and validated as part of a method to assess the virucidal efficacy of decontamination procedures for filtering facepiece respirators. Coliphage MS2 was used as a surrogate for pathogenic viruses for confirmation of the efficacy of the bioaerosol respirator test system. The distribution of virus applied onto and within the coupons was characterized, and the repeatability of applying a targeted virus load was examined. The average viable virus loaded onto 90 coupons over the course of 5 days was found to be 5.09 ± 0.19 $\log_{10}$ PFU/coupon (relative standard deviation, 4%). To determine the ability to differentiate the effectiveness of disinfecting procedures with different levels of performance, sodium hypochlorite and steam treatments were tested in experiments by varying the dose and time, respectively. The role of protective factors was assessed by aerosolizing the virus with various concentrations of the aerosol-generating medium. A sodium hypochlorite (bleach) concentration of 0.6% and steam treatments of 45 s and longer resulted in log reductions (>4 logs) which reached the detection limits for both levels of protective factors. Organic matter (ATCC medium 271) as a protective factor afforded some protection to the virus in the sodium hypochlorite experiments but was not a factor in the steam experiments. The evaluation of the bioaerosol respirator test system demonstrated a repeatable method for applying a targeted viral load onto respirator coupons and provided insight into the properties of aerosols that are of importance to the development of disinfection assays for air-permeable materials.

The Institute of Medicine reports that a 42-day influenza pandemic outbreak may require over 90 million N95 filtering facepiece respirators (FFRs) to protect workers in the healthcare industry (12). High demand for FFRs may result in local shortages. Decontamination and reuse of FFRs may provide a solution to increased demand in emergency situations (36). There are test methods to assess the efficacy of biological decontamination methods (BDMs) for viruses in suspension and on hard-surface porous or nonporous carriers (4, 5, 30). However, there is no test method to evaluate the effectiveness of BDMs applied to air-permeable materials (APMs), such as disposable FFRs.

Because of the lack of a standardized method for aerosolized application of viruses, current carrier tests are not realistic for aerosols and APMs involving airflow through the test sample. Modifications to current carrier test protocols, specifically modifications to the method of viral challenge application, the use of air-permeable substrates, and airflow through the substrate, are needed for testing FFR decontamination under conditions that better simulate a real-world scenario. Ideally, the test procedure will mimic the airborne transmission of viruses, allow penetration into and through APMs, and provide a way to deposit a reproducible viral challenge on the substrate surface (even for a water-resistant surface, such as some surgical masks and some types of FFRs).

In this work, a bioaerosol respirator test system (BARTS) was developed, characterized, and validated to provide a method to load APMs (FFR sample coupons) with a repeatable targeted load of aerosolized virus-containing particles (VCPs). The physical properties of the bioaerosol generated from the BARTS were examined to better identify the parameters that are important in measuring the virucidal efficacy of BDMs for FFRs contaminated with VCPs. A tryptone-based protective factor medium whose protein concentration was similar to the concentration for the recommended organic challenge of ASTM method E 1053 was examined using two levels (100% and 1%) to determine its effects on the physical properties of the VCPs and on BDMs (5). The particle size distribution of VCPs generated using BARTS and the deposition of the VCPs on the surface of the respirator sample and inside the filter media were determined for each protective factor level. The ability to differentiate the effectiveness of BDMs for various degrees of severity was assessed by performing experiments with a range of sodium hypochlorite concentrations and steam exposure times using both protective factor levels.

MATERIALS AND METHODS

BARTS. A 43-liter (36.5 by 34.5 by 34.5 cm) aerosol testing chamber was constructed from antistatic acrylic panels. As shown in Fig. 1, the chamber is equipped with an aerosol inlet (diameter, 2 cm), a 25-mm-diameter pressure

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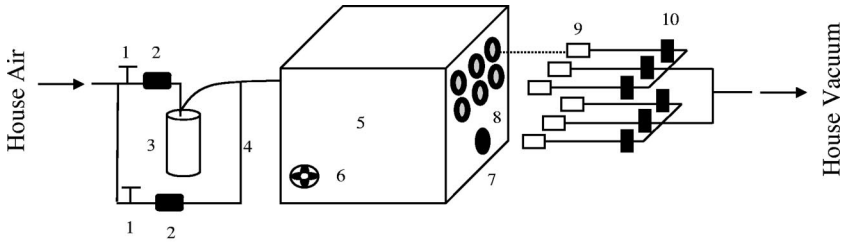

FIG. 1. Schematic diagram of the BARTS.

relief port, and six 25-mm-diameter test specimen holder ports. Coliphage MS2 in suspension was aerosolized using a six-jet Collison nebulizer (BGI Incorporated, Waltham MA). Airflow regulators (Ashcroft, Costa Mesa, CA), one upstream from the six-jet nebulizer and one for the dilution air, were used to control the nebulizer airflow at 10 liters/min (20 lb/in^2) and the dilution airflow at 50 liters/min (13 lb/in^2). A 100-mm (4-in.)-diameter fan inside the chamber was used to mix the bioaerosol. Six test specimen holders (SKC Inc., Eighty Four, PA) (Fig. 2), each containing an FFR coupon, were connected directly to the specimen holder ports for loading with virus. Each specimen holder was connected to a flow meter (Cole-Parmer, Vernon Hills, IL), which controlled and measured the flow rate between the vacuum inlet and each test specimen holder at a calibrated level of 4.0 liters/min for a face velocity of 13 cm/s.

BARTS was housed inside a Safeaire chemical fume hood (Fisher Hamilton, Pittsburgh, PA). A chemical fume hood is appropriate when biosafety level 1 agents are used. The production of bioaerosols containing potentially hazardous agents would require use of a biosafety cabinet.

N95 respirator. The N95 respirator used for this study is a National Institute for Occupational Safety and Health (NIOSH)-approved FFR commonly utilized by healthcare workers for protection against particulate hazards. NIOSH-certified N95 respirators are 95% efficient or better at filtering particles with an aerodynamic mass median diameter of around 300 nm. This model of FFR is comprised of three layers. The exact composition of the various layers in an FFR is often considered proprietary information. However, most FFRs share common traits regarding the types of materials used and the roles of the various layers. The innermost layer, which directly contacts the user's face, is designed to be soft and is often fluid resistant to prevent perspiration or saliva from interfering with or exiting the facemask (12). The second layer consists of a nonwoven filter medium designed to capture particulates. Typical filter media used in FFRs today are made from electret-treated spun-bonded polypropylene. The outermost layer is designed using fabric materials to impart stability during handling and contact and to prevent larger particles and debris from reaching the inner layers.

Media, virus, and host cells. American Type Culture Collection (ATCC) medium 271 (www.atcc.org) was used for growth of *Escherichia coli* and preparation, storage, recovery, and assay of MS2. The aerosol-generating medium was comprised of 100% or 1% ATCC medium 271 (deionized water was used as the diluent for 1% ATCC medium 271). When used as the aerosol-generating solution, 100% ATCC medium 271 was referred to as high-protective-factor (HPF) medium and 1% ATCC medium 271 was referred to as low-protective-factor (LPF) medium. The two levels of the protective factor were chosen to assist in characterization of the properties of the generated bioaerosol and not necessarily to replicate the exact organic challenge that one may expect in an FFR reuse situation. ATCC medium 271 was amended with 2.5 g of agar (catalog no. A7002; Sigma) to obtain the soft agar medium used in the plaque assays.

Coliphage MS2 (ATCC 15597-B1) was replicated using *E. coli* ATCC 15597 as the host. A 5-h culture of *E. coli* (1 ml) was used to inoculate 30 ml of ATCC medium 271. After 2.5 h, 1.5 ml of an MS2 stock was added to the flask and incubated overnight. The sample was centrifuged at 7,100 $\times$ *g* for 30 min at 4°C (IEC Multi RF; Thermo Electron Corporation), and the supernatant was filtered through a 0.22-μm-pore-size filter (Fisherbrand). The virus suspension (10^{11} PFU/ml) was stored at 4°C in 50-ml conical tubes (Falcon, Becton Dickinson) for use in experiments. MS2 was selected for the study based on its moderate resistance to disinfectants, survivability, ease of preparation and assay, and nonpathogenicity (18, 38).

Virus aerosol generation and loading onto respirator coupons. Circular coupons (5 cm^2) were cut from respirators using a single model of a NIOSH-approved N95 FFR. The respirator coupons were not sterilized prior to loading but were handled with sterile forceps to minimize contamination with biological or chemical matter. Six respirator coupons (excised from at least two different respirators) were placed into separate test specimen holders and attached to the six BARTS sample ports. A quantified titer (10^5 to 10^8 PFU/ml depending on the aerosol medium and viral loading target level) was suspended in LPF or HPF medium. The virus challenge solution (23 ml) was added to the nebulizer glass jar. The compressed air valves were opened to the nebulizer (20 lb/in^2, 10 liters/min) and the dilution air (13 lb/in^2, 50 liters/min). After 5 min to allow the aerosol concentration to stabilize within the chamber, the vacuum was initiated and regulated at a value of 4 liters/min for each sample port. The aerosol was passed through the coupons for 30 min.

Virus recovery. Three procedures, vortexing (Vortex Genie 2; Scientific Industries, Bohemia, NY), sonication (model 8510; Bransonic, Danbury, CT), and shaking (Gyromax Steadyshake 757; Amerex Instruments, Inc., Lafayette, CA), were tested in triplicate to evaluate MS2 recovery efficiency. MS2 in HPF me-

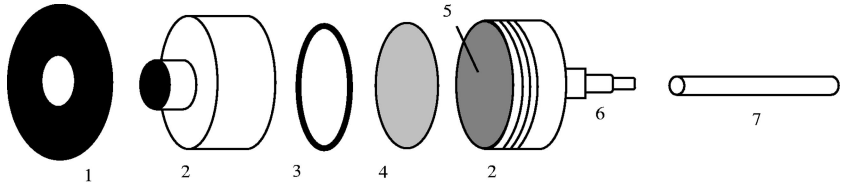

FIG. 2. Schematic diagram of the test specimen holder assembly.

dium (10 μl of a 10^5-PFU/ml solution) was applied to coupons using a micropipette and allowed to dry for 10 min. The coupons were resuspended in 10 ml ATCC medium 271 in 50-ml conical tubes or 50-ml flasks (with shaking). Triplicate sets were subjected to 2 min of vortexing at the highest setting or to shaking at 200 rpm for 15 min or were placed in a sonicating bath for 10 min. Upon completion of the recovery procedure, the coupons were discarded, and the supernatant was assayed for active MS2 as previously described. The virus recovery methods were not tested using the LPF medium as this experiment preceded inclusion of an LPF level in the overall study. Upon review of the literature and consideration of the results of the HPF recovery experiments, the vortex method was chosen for recovery of virus from coupons for both levels of protective factor.

Plaque assay. A single-agar-layer method was used to enumerate the viruses (1, 35). Eight milliliters of ATCC soft agar medium 271 was placed into glass culture tubes and incubated at 47°C in a water bath. Log-phase *E. coli* (0.5 ml) and 1 ml of the MS2 suspension were added to the culture tubes. The soft agar containing *E. coli* and MS2 was poured into an empty petri plate and mixed by swirling. The plates were allowed to harden at room temperature and placed in an incubator at 37°C and 30% relative humidity overnight. Viruses on the plates were counted on the following day, and the data for the plates containing 30 to 300 PFU were recorded.

Effect of charge neutralization of MS2 aerosol. The use of a particle neutralizer (TSI model 3012) was assessed to determine the effect of particle charge on loading coupons with the MS2 challenge. Neutralizers use a radioactive source to ionize the surrounding atmosphere to form positive and negative ions. Particles carrying charges capture ions with the opposite polarity. The particles reach charge equilibrium, and the aerosol has a bipolar distribution. Virus was loaded onto the coupons under two conditions, with and without neutralization, in quadruplicate for 10, 20, and 30 min using the loading procedures described above. After exposure to MS2, the coupons were removed from the filter holders, and the virus was recovered and assayed as described above.

Validation studies. (i) Repeatability. Six coupons were loaded simultaneously with MS2 (a targeted load of 5 log PFU/coupon) during three runs per day for 5 days. MS2 was recovered and quantitated for all 90 coupons as described above.

(ii) Particle size and desiccation determination. MS2 was aerosolized in water and LPF and HPF suspension media as previously described using BARTS. Particle size and concentration were measured at the filter sample holder-aerosol chamber interface using an aerodynamic particle sizer (APS) (model 3321; TSI Inc.) and a scanning mobility particle sizer (SMPS) (model 3080; TSI Inc.), including a condensation particle counter (model 3025A; TSI Inc.). The APS measured particle sizes ranging from 500 to 20,000 nm, and the SPMS measured particle sizes ranging from 20 to 900 nm. The full-scale size distribution (20 to 20,000 nm), which expresses the concentration of particles as a function of particle diameter, was obtained by combining the data from the SMPS and the APS. Matching of the SMPS and APS data was performed by calculating the ratio from the overlap data for sizes between 500 and 900 nm. The APS data were then multiplied by the ratio to obtain the best fit to a lognormal distribution of the SMPS data. The chamber was purged of particles between loading procedures.

To characterize the extent of evaporation of water from particles generated by BARTS, the mass median equilibrium diameter of a completely desiccated particle was calculated for LPF and HPF media using the equation previously described by Nicas et al.: $d_{eq} = (C_{nonvolatile/\rho nonvolatile})^{1/3} \times d_0$, where d_{eq} is the equilibrium particle diameter, d_0 is the initial diameter, $C_{nonvolatile}$ is the mass concentration (in g/liter) of the solutes in the medium (20 g/liter for HPF medium and 0.2 g/liter for LPF medium), and $\rho_{nonvolatile}$ is the dry density of the solutes (often estimated to be the density of water or 1,000 g/liter) (23). The output distribution of the BGI nebulizer in the BARTS has a mass median diameter of 2.5 μm and was used for d_0 in the above equation (10). The calculated particle size was compared to the measured values for LPF and HPF media.

(iii) Virus distribution on or within the respirator coupon. Eight respirator coupons (aerosol-exposed area, 3.1 cm^2) for each suspension medium (LPF or HPF medium) were exposed to MS2 as described above. Using a cork borer, four coupons for each suspension medium type were separated into two samples, inner circles with an area of 1.5 cm^2 and outer rings with an area of 1.6 cm^2. The MS2 deposited on quadruplicate samples of the inner circles and outer rings were enumerated as described above. The three layers of the other four coupons were separated, and the virus was enumerated to test viral penetration through the respirator coupon.

(iv) Decontamination treatment for viruses. (a) Sodium hypochlorite. The decontamination efficacy of sodium hypochlorite (Clorox; Environmental Protection Agency EPA registration no. 5813-50) at concentrations of 0.0006, 0.006, 0.06, and 0.6% was tested in triplicate. Respirator coupons were used as air-permeable inanimate carriers and exposed to MS2 (approximately 6 logs/coupon) as described above for the BARTS methods. After the exposure to MS2, one coupon was placed directly into 10 ml of ATCC medium 271 as a loading level control, one coupon was submerged in water (dipping control), and one coupon was placed in sodium hypochlorite at each of the different concentrations in a 50-ml conical tube. After 10 min of treatment, the coupons were carefully removed from the sodium hypochlorite solutions and air dried for 2 min. Each coupon was then placed in 10 ml of ATCC medium 271. The tubes were capped and vortexed for 45 s. The active MS2 remaining on the coupon was enumerated by performing a plaque assay as described above. The process was repeated for triplicate sample sets for each suspension medium.

(b) Microwave-generated steam. Steam treatment times of 0, 15, 30, 45, 60, and 75 s were tested in triplicate for efficacy against MS2. Steam sterilization chambers were constructed from plastic pipette tip boxes. Nine holes that were approximately 4 mm in diameter were drilled in the top of each pipette tip box lid for ventilation. The base of the pipette tip box was filled with 50 ml of room temperature water. Respirator coupons were loaded with MS2 as described above. After MS2 loading, the coupons were placed on a rack inside the steam sterilization chamber approximately 2.5 cm above the water level. The vented pipette tip box lid was placed over the base, and the chamber was heated in a microwave oven on high for the appropriate treatment time. The power output (rated, 1,100 W; measured, 750 W) of the commercially available microwave oven used (model R305KS; Sharp Electronics, Mawwah, NJ) was validated previously by Viscusi et al. (36). After treatment, the coupons were carefully removed from the chamber and placed in 10 ml of ATCC medium 271 in conical tubes. The tubes were capped and vortexed for 45 s. The coupons were removed and discarded, and each suspension was assayed by performing a plaque assay.

(v) Toxicity controls. To determine whether the residual sodium hypochlorite on treated coupons had been neutralized by ATCC medium 271, three coupons that received no MS2 challenge were treated with 0.6% sodium hypochlorite, allowed to dry, and vortexed in ATCC medium 271. Suspensions of MS2 were prepared by placing 3 ml of the coupon recovery medium and 3 ml of fresh ATCC medium 271 into separate 10-ml conical tubes. A known titer of MS2 (100 μl) was added to all conical tubes, and plaque assays were performed.

Data analysis. Unless otherwise specified, the MS2 recovered from experimental and control coupons for all experiments was quantified by calculating the $\log_{10}$ of the viable MS2 titer recovered from each coupon, and the averages and standard deviations were calculated.

The loading of MS2 onto FFR coupons was further assessed by calculating the relative standard deviation (RSD) for all 90 coupon samples. An analysis of variance was performed using EXCEL (Microsoft Office 2003) for comparison of the variability among the sample ports.

The dispersion of MS2 on the outer surface of an FFR coupon was determined by enumerating the viable MS2 for both the outer ring and the inner circle and expressing the results in $\log_{10}$ PFU/cm^2. The three-layer deposition of MS2 was expressed as a percentage of the viable MS2 particles per layer compared to the total number of MS2 particles loaded for the full coupon.

The antimicrobial activities of bleach and steam treatments of FFR coupons were determined by calculating $\log_{10} N/X$, where N is the titer of viable MS2 recovered from untreated samples and X is the titer of viable MS2 recovered from the treated coupons. A two-tailed t test (Student test) was performed by using EXCEL (Microsoft Office 2003) for each decontamination treatment, where the log reduction (LR) was greater than 1 (for at least one sample set) and less than the limit of detection (for both sample sets).

Where applicable, in statistical analyses the P values were compared at the 95% confidence level.

RESULTS

Preliminary experiments to optimize VCP application onto coupons and recovery from coupons were performed before validation and characterization of BARTS. The difference in the numbers of VCPs loaded onto respirator coupons with and without charge neutralization was examined. Figure 3 shows the control and charge-neutralized VCP loading onto FFR samples at three different time points. The levels of active MS2 recovered from the coupons treated with the control and neutralized aerosol were similar for all time points (10, 20, and 30

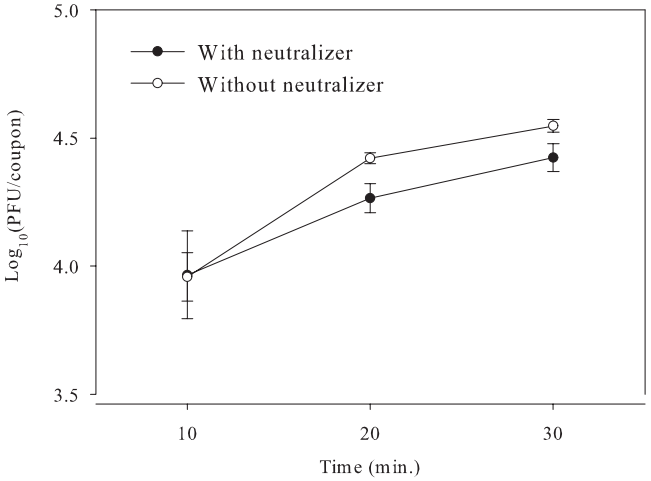

FIG. 3. Effects of a neutralizer on the efficiency of MS2 application to FFR coupons. The symbols indicate the averages and the error bars indicate the standard deviations for four coupons loaded in two experimental runs.

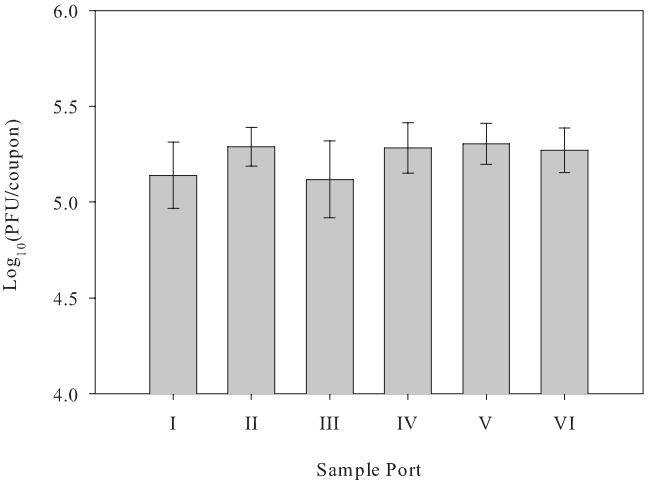

FIG. 4. Repeatability of the application of MS2 using BARTS. MS2 was loaded onto 90 FFR coupons over a 5-day period. Six coupons were loaded simultaneously during three runs per day. The bars indicate the average for each filter sample port (ports I to VI).

min). Marginal but statistically significant ($P < 0.003$) increases in the number of MS2 particles extracted from coupons loaded using the control aerosol were seen for the 20- and 30-min time points. The optimal method for MS2 recovery from coupons was examined by comparing the efficiencies of sonication, vortexing, and shaking. The three methods exhibited similar efficiencies and repeatabilities for extraction of MS2 from coupons (Table 1); however, vortexing was chosen as the recovery method for all other tests.

BARTS was validated and characterized to assess its applicability for evaluating decontamination efficacies of BDMs for FFRs. Figure 4 shows the repeatability of application of MS2 onto respirator coupon samples using BARTS. ThIs figure shows the average $\log_{10}$ PFU/coupon for each filter sample port (ports I to IV)as well as the standard deviations of the means. The difference among the ports was not significant ($P = 0.68$). Moreover, the average amount of viable virus loaded onto the 90 coupons was found to be 5.09 ± 0.19 $\log_{10}$ PFU/coupon, indicating that there was sufficient repeatability (RSD, 4%).

The distribution of MS2 among the three layers of the respirator coupon and deposited over the entire surface of the outer layer of the coupon was determined for both LPF and HPF aerosol media. Figure 5 shows the data for deposition of MS2 on the three layers of an N95 FFR coupon. Virus aerosolized from LPF medium was deposited predominately on the middle layer, and significant deposition also occurred on the outer layer. Virus in the HPF medium was entrapped on the outer layer, and significant deposition also occurred in the middle layer. With neither medium was there significant accumulation of virus on the inner layer compared to the middle and outer layers. The amounts loaded onto the outer layer of the coupon for the inner circle and outer ring were 4.4 ± 0.26 and 4.7 ± 0.06 $\log_{10}$ PFU/cm^2 (averages $\pm$ standard deviations) ($P = 0.11$), respectively, for LPF medium and 5.5 ± 0.16 and 5.3 ± 0.20 $\log_{10}$ PFU/cm^2 ($P = 0.03$), respectively, for HPF medium. Similar concentrations of viable virus were recovered from the inner circle and outer ring of the outer layer for each medium type (5.5 $\log_{10}$ PFU/cm^2 versus 5.3 $\log_{10}$ PFU/cm^2 for HPF medium and 4.4 $\log_{10}$ PFU/cm^2 versus 4.7 $\log_{10}$ PFU/cm^2 for LPF medium), indicating that there was uniform deposition on the outer layer.

The particle mass distributions for sizes from 20 to 20,000 nm for water and each aerosol medium type are shown in Fig. 6. The mass median diameters were also calculated for water, LPF medium, and HPF medium and were found to be 58, 141,

TABLE 1. Comparison of MS2 recovery methods

Extraction method	Log recovery	SD
Sonication	5.69	0.04
Vortexing	5.87	0.11
Shaking	5.82	0.15

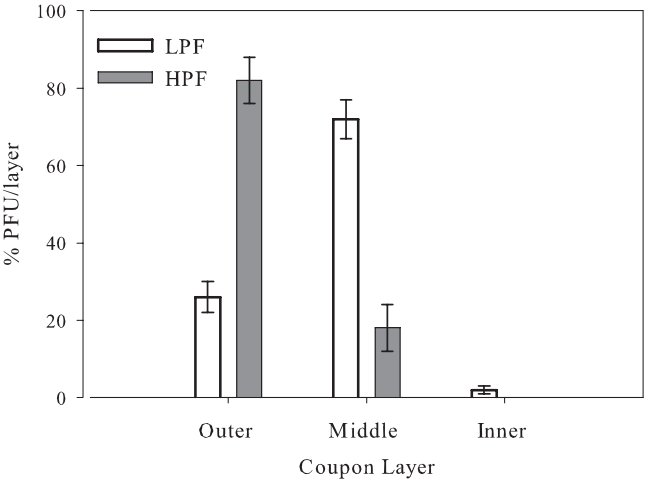

FIG. 5. Percentage of MS2 deposited on each coupon layer for LPF and HPF suspension media. The error bars indicate standard deviations of four samples.

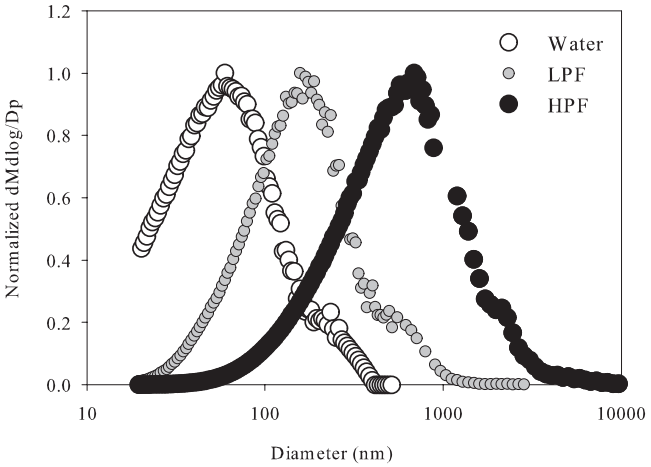

FIG. 6. Size distribution of particles generated from the nebulization of suspension media: combined SMPS and APS size distributions for particles as measured at the sample ports for water, LPF medium, and HPF medium. The data were normalized to the peak bin value of the scan for each medium type.

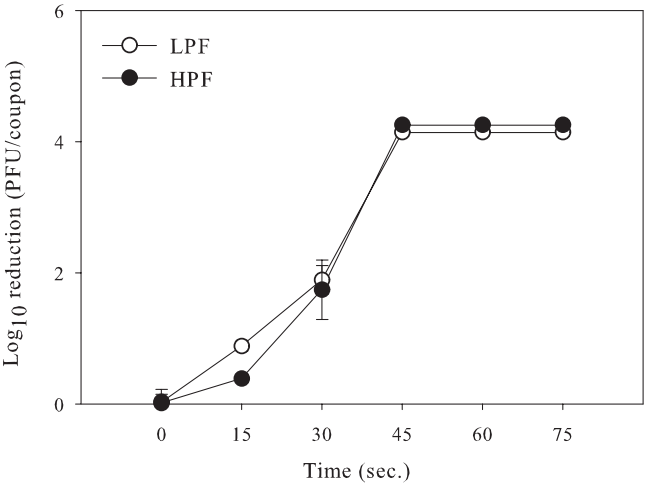

FIG. 8. Steam treatment time-dependent reduction of MS2 aerosolized in LPF and HPF media. The error bars indicate the standard deviations of three samples. Note that steam treatments of 45 s and longer for both LPF and HPF media resulted in LRs which reached the detection limits.

and 492 nm, respectively, and the corresponding mass mean diameters were 72, 187, and 629 nm. In count mode, very few particles larger than 1,000 nm were observed. The count median diameter for water was 27 nm, compared to 30 nm for LPF suspension medium and 56 nm for HPF suspension medium. The count mean diameters were determined for water, LPF medium, and HPF medium and were found to be 29, 32, and 62 nm, respectively. Under constant flow conditions, an increase in particle number and an increase in particle size coincided with the increase in the level of protective factor in the suspension medium. Addition of MS2 to the suspension medium had no effect on the particle size (data not shown).

The efficacy of sodium hypochlorite decontamination for FFR samples loaded with MS2 is shown in Fig. 7. The LR of MS2 resuspended in HPF and LPF media increased with increasing concentrations of sodium hypochlorite. However, the increase in the MS2 LR for all sodium hypochlorite concentrations was greater for the LPF medium set than for the HPF medium set ($P < 0.02$), except when both sets reached detection limits (0.6% sodium hypochlorite). The recovery buffer presumably neutralized the experimental samples for sodium hypochlorite treatments, as indicated by the toxicity tests (LR, 0.03 ± 0.11). Plaque assays examining the same sample (0.6%) of recovery medium on consecutive days confirmed that the recovery solution was adequate for neutralizing the active components in bleach as quantities remained constant over an approximately 24-h period (data not shown).

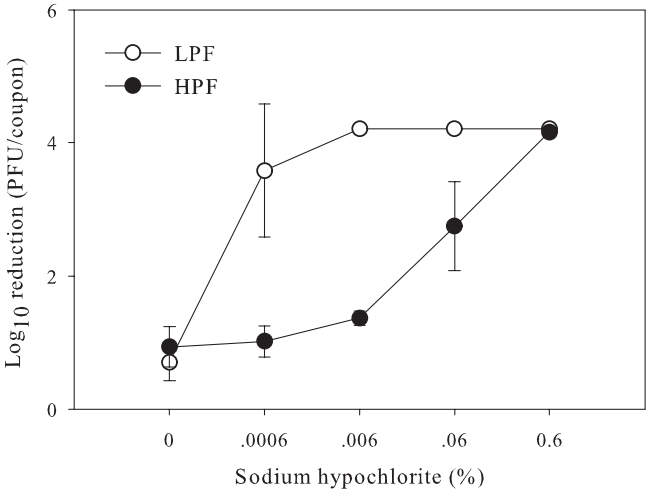

FIG. 7. Sodium hypochlorite concentration-dependent reduction of MS2 aerosolized in LPF and HPF media. The error bars indicate the standard deviations of three samples. Note that bleach concentrations of 0.006%, 0.06%, and of 0.6% for LPF medium and 0.6% for HPF medium resulted in LRs which reached the detection limits.

Figure 8 shows the decontamination efficacy of steam treatment for different exposure times. For steam sterilization the sample sets for HPF and LPF media had similar LRs for all treatment times. As expected, a longer steam treatment time resulted in greater efficacy. Within the limits of detection, the protective factor had little effect on steam treatment. Statistical significance for the steam treatment was not calculated as only a single time point fell within the parameters outlined above.

DISCUSSION

Current disinfectant testing methods, the most common of which are suspension tests and carrier tests, are not adequate for assessing the antimicrobial activity of decontamination treatments of APMs exposed to a bioaerosol. Suspension tests are convenient for initial screening, but inanimate carrier tests are more appropriate for evaluating surface disinfectants. Carrier tests, which are commonly performed by dipping or spiking the viral challenge onto the substrate, do not account for the physical characteristics of aerosols, such as particle size, composition, velocity, desiccation, and subsequent substrate penetration (8, 21, 30). It has been shown previously that the testing method has an effect on the activities of disinfectants (9, 24, 38). Woolwine and Gerberding showed that inactivation of

MS2 in suspension tests and carrier tests using glass slides, ceramic tile, and pigskin as the carrier substrates resulted in different decontamination efficacies for the same chemical treatment under identical conditions (38). Thus, it is not only the testing method (e.g., carrier or suspension test) which alters the activities of the decontaminant, but also the carrier substrate (e.g., porous, nonporous, or air permeable). The method of application of the viral challenge may have similar effects on activities of BDMs. By passing a bioaerosol through an FFR sample coupon, BARTS provides a more realistic assessment of the decontamination efficiency for APMs than other carrier or suspension tests.

The complexity of testing methods for assessment of the virucidal activities of decontamination procedures results in little or no standardization, procedural deficiencies, and a lack of guidelines for the established protocols (8, 30, 38). Developing a test method using bioaerosols adds to the complexity of decontamination studies. The preliminary experiments addressed two issues associated with developing a bioaerosol decontamination test method, particle charge neutralization and virus recovery from the substrate. The use of a neutralizer is common in many aerosol applications, such as filtration testing, where charge neutralization presents a more challenging set of conditions. However, many of the experimental bioaerosol-generating systems developed for other research applications have not included a neutralizer (11, 15, 17, 37). Aerosol charge may affect the culturability and viability of microorganisms and may cause a loss of microbes, which may stick to the walls of the test system through electrostatic interactions (20, 33). Although it is possible that the neutralizer may have had a positive impact on viability or the wall effect, the total number of viable viruses recovered from the coupon was lower (Fig. 3); thus, the neutralizer was deemed unnecessary for this study. Using the BARTS for other applications, such as bioaerosol filtration testing of APMs containing integrated antimicrobial technologies, may require modification to include a neutralizer to accommodate filtration testing recommendations.

The elution and recovery of virus from a substrate is a problem for all carrier tests, and the use of an aerosol and an APM increases the difficulty of the procedure. Although the recovery methods were comparable in terms of MS2 recovery from FFR coupons, vortexing proved to be the most time efficient. The recovery methods tested in this study were also previously examined for elution of bacteria from FFR samples, and vortexing not only was more time efficient but provided the most energy and relative motion to separate the bacteria from respirator fibers (37).

It has been documented that at typical levels of room relative humidity and room temperature water from aerosol particles evaporates almost immediately upon generation to form droplet nuclei (29, 32). A study examining the size distribution of droplet nuclei generated from coughs of human subjects at 35% relative humidity found particle sizes ranging from 0.58 to 5.42 μm, with 82% of the particles in the 0.74- to 2.12-μm range (39). Particles that are smaller than 3 μm essentially remain airborne because of low settling velocities (32). One would expect that ultrafine and submicron VCPs would be readily trapped on FFRs through inhalation in a high-risk exposure setting, such as a hospital during a pandemic. Also, in the case of influenza virus, transmission is greater at lower relative humidity, which further suggests that studying particles in the ranges used in this study is important (19). The particle size determination data for both LPF and HPF media demonstrate that the size distribution is comparable to that in the system developed by Hogan et al., with the majority of particles in the submicron and ultrafine range (17). The mass median diameters of the VCPs at room relative humidity (20 to 25%), as measured at the sample collection ports of the aerosol chamber, were approximately 141 nm and 492 nm for LPF and HPF media, respectively. These values are similar to the calculated desiccated particle sizes (calculated using the equation given above) for LPF and HPF media (146 nm and 678 nm), indicating that there is formation of droplet nuclei.

The constituents of the aerosol medium provide the protective factor and contribute to the particle size (17). Protective factors, used to mimic the organic and inorganic molecules that are often associated with the biological contaminant in real-world decontamination scenarios, may provide protection to the virus in the VCPs by neutralizing the antimicrobial compound or by acting as a physical barrier. The aerosol media, LPF medium and HPF medium, which were chosen to provide two distinct levels of protective factor in the decontamination test, provided different particle size distributions (Fig. 6). The two particle size distributions in this study resulted in different deposition patterns inside and on the surface of the respirator coupons. For example, compared to viruses recovered from experiments using LPF medium, viruses recovered from experiments using HPF medium were more likely to be found on the outer layer (Fig. 5). This may be important in cases where the decontamination treatment (for example, UV light) may not have access to the viral challenge if the viral particles are deposited on an internal layer of an FFR. It may be possible to expose multiple layers of an APM to a viral challenge using typical application methods used in carrier tests, such as spiking or dipping; however, other properties of aerosols, such as protective factor concentration, would be neglected.

The FFR decontamination test method using BARTS was able to differentiate between BDMs with various degrees of expected efficacy. The experiments included both a chemical treatment (sodium hypochlorite) and a physical treatment (steam) and two distinct protective factor challenges. For the chemical treatment, the BARTS test method was able to discern the efficacy of decontamination for the various concentrations of sodium hypochlorite, as well as the effect of the protective factor. The physical decontamination treatment test detected a difference in efficacy for the different steam treatment times, yet demonstrated no effect for the protective factor. As expected, for both the physical and chemical treatments, greater LRs coincided with the potency of the treatment. Yet within the detection limits, the protective factor influenced only the chemical test. Protective factors in the HPF medium deposited on the carrier substrate (in this case the FFR) may act to neutralize the chemical disinfectant or inhibit the diffusion of the chemical treatment to the MS2, whereas in the steam treatment the constituents of the protective factor have little effect on the rate of heat conduction.

The primary purpose of this work was to develop and validate a test system to apply VCPs to APMs for use in disinfection assays. Sodium hypochlorite disinfection has been well

characterized (7, 13, 22, 25–28, 38). Steam treatment is also a well-known method for inactivating viruses on surfaces (6, 14, 16, 26, 31). However, typical steam sterilization requires the use of an autoclave which utilizes pressurized steam at 121°C, which was shown previously to have a deleterious effect on FFR performance (36). In this work, a commercial microwave oven was used to generate a less rigorous ambient pressure steam treatment. Initial attempts to study microwave irradiation for FFR decontamination resulted in melted coupons (data not shown). Other studies involving microwave decontamination found that water was needed for sterilization (26). The use of microwave irradiation or heating to generate steam for decontamination or sterilization is not new and has benefits. Microwave energy was used to heat water to sterilize surfaces for use with NASA's mammalian cell bioreactor, where the thermal impact on the system was minimized through the rapid and selective properties of microwave heating (6). Microwave-generated steam has also been shown to decontaminate medical waste and respirators (14, 16).

One concern with using a commercially available microwave oven to generate steam is repeatability. The data in Fig. 8 suggest that the microwave oven and steam chamber used in this study were able to produce repeatable results. Steam treatment for 45 s or longer resulted in LRs which reached the detection limits for all 18 of the coupons tested (LPF and HPF media). For treatment times of 15 and 30 s the RSD ranged from 9 to 26%, which is similar to the results for other decontamination methods described previously, as discussed further below. Although promising, the data presented in this study obtained using a single microwave oven and one model of FFR do not indicate that the use of a commercial microwave oven to generate steam should be adopted as a means of sterilization without further study. The use of microwave ovens for sterilization of medical devices has not been cleared by the Food and Drug Administration (26). Furthermore, additional studies are needed to separate the effects of microwave heating, microwave irradiation, and steam on the efficacy of decontamination.

In germicide testing, assessment of repeatability and reproducibility is of great importance for validation of a standard method. ASTM International and AOAC International require evaluation of the precision of a test method within a laboratory (repeatability) and between laboratories (reproducibility) before a test method becomes a standard method (2, 3). However, there is no recommendation for how precise a test method must be to be accepted as repeatable or reproducible. Tilt and Hamilton performed a literature review to survey the repeatability and reproducibility of germicide tests and found that for germicides with LRs of >2 the RSDs ranged from 3 to 36% (34). The precision of BARTS as a means to expose FFR to a consistent viral challenge provided an RSD of 4% (Fig. 4), which corresponds to the lower limit of typical RSDs in germicide testing. The variability of the loading process is the cumulative variation of all the steps in the procedure, including the day-to-day production of the virus aerosol solution, the virus recovery step, and the plaque assays. However, loading the viral challenge is only part of the complete test method. A better estimate of the repeatability of the entire method can be found in Fig. 7 and 8. For the triplicate samples generated for sodium hypochlorite and steam decontamination test methods that resulted in LRs of >2, the RSDs were found to range from 2 to 32%. These data were generated in a series of experiments and do not incorporate other sources of error, such as day-to-day differences. However, for three independent experiments performed in triplicate on separate days using 0.006% sodium hypochlorite (the only conditions that resulted in LRs of >2 without complete reduction for all tests), the RSD was 27%, which falls within the range (3% to 36%) described previously. Further work is needed to address interlaboratory reproducibility, including assessment of the repeatability and reproducibility of experiments with higher LRs.

The limit of detection for the decontamination experiments reported in this study allowed demonstration of an approximately 4-log reduction in the viable virus level for both the bleach and steam treatments. It may be possible to demonstrate a greater LR using BARTS as the method for virus application with modifications to the experimental parameters. For example, an increase in the initial titer of the virus in the nebulization medium, in the virus loading time, or in the airflow through the coupon would result in an increase in the number of virus particles loaded onto the substrate and allow reporting of greater LRs. Using a different nebulization medium, virus enumeration method, or virus may also impact the limits of detection.

BARTS proved to be valuable for achieving a targeted viral load on the substrate and for assessing properties important in bioaerosol testing methods. Further studies are needed to develop a decontamination test method for APMs exposed to bioaerosols, specifically VCPs. A comparison of the efficacy of decontamination procedures for virus applied using standard techniques (pipetting or dipping) to the efficacy for VCPs applied as droplet nuclei should further characterize the effects of bioaerosols. Many of the standard suspension tests and carrier tests use similar media amended with defined challenge proteins, such as tryptone or bovine serum albumin. Standardization of aerosol medium may be possible as well. The requirements for the protective factor have to be determined using real-world scenarios. Consideration must also be given to other FFR types, as well as other APMs. Evaluation of other APMs may require addition of filters and particle-detecting instruments placed downstream of the test substrate to enhance characterization of the APM performance.

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