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Development of an improved methodology to detect infectious airborne influenza virus using the NIOSH bioaerosol sampler

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Abstract

A unique two-stage cyclone bioaerosol sampler has been developed at NIOSH that can separate aerosols into three size fractions. The ability of this sampler to collect infectious airborne viruses from a calm-air chamber loaded with influenza A virus was tested. The sampler's efficiency at collecting aerosolized viral particles from a calm-air chamber is essentially the same as that from the high performance SKC BioSampler that collects un-fractionated particles directly into a liquid media (2.4×10^4 total viral particles per liter of sampled air (TVP/L) *versus* 2.6×10^4 TVP/L, respectively, after 15 min) and the efficiency is relatively constant over collection times of 15, 30 and 60 min. Approximately 34% of the aerosolized infectious virus collected after 15 min with the NIOSH bioaerosol sampler remained infectious, and infectious virus was found in all three size fractions. After 60 min of sampling, the infectious virus/liter air found in the NIOSH bioaerosol sampler was 15% of that found in the SKC BioSampler. This preservation of infectivity by the NIOSH bioaerosol sampler was maintained even when the initial infectivity prior to aerosolization was as low as 0.06%. The utility of the NIOSH bioaerosol sampler was further extended by incorporating an enhanced infectivity detection methodology developed in our laboratory, the viral replication assay, which amplified the infectious virus making it more readily detectable.

Introduction

Concern regarding human exposure to bioaerosols has led to the development of a variety of air sampling devices. However, research addressing the efficacy of current samplers to detect infectious airborne viruses is sparse and points to the need for a more efficient sampler.[1] A two-stage cyclone bioaerosol sampler has been developed by the National Institute for Occupational Safety and Health (NIOSH).[2] The NIOSH bioaerosol sampler is unique in that it size-fractionates aerosols and collects them in disposable centrifuge tubes, facilitating direct sample processing. As ambient air is drawn into an inlet at 3.5 L min^{-1}, the first stage of the NIOSH bioaerosol sampler deposits aerosol particles that are >4 μm on the wall of a 15 ml centrifuge tube. In the second stage, 1 to 4 μm particles are deposited on the wall of a 1.5 ml microcentrifuge tube, and particles that are <1 μm are collected on a 37 mm polytetrafluoroethylene (PTFE) filter. The first stage of the sampler collects the non-

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respirable size fraction, while the second stage and filter collect the respirable fraction.[3] The sampler is lightweight and can be used either as a stationary sampler (such as on a tripod in a hospital room), or as a personal breathing zone air sampler that can be worn on the clothing of workers in occupational environments.

In a previous study, collection of aerosolized influenza virus in a calm-air settling chamber was characterized using an earlier version of the NIOSH bioaerosol sampler.[4] Analysis by quantitative polymerase chain reaction (qPCR) demonstrated that the sampler effectively captured and separated viral-laden particles based on their aerodynamic size. In later studies, improved NIOSH bioaerosol samplers (enlargements of the inlet and outlet ports, the addition of a 15 ml collection tube, and enlargement of the second stage outlet to reduce particle loss due to turbulence) were used as personal and stationary samplers in a hospital emergency department and an urgent care clinic during the influenza season. Analysis of the collected air samples showed that airborne influenza virus RNA could be found in both facilities and that 42–53% of the detectable viral RNA was found in the respirable fraction of the aerosol.[5,6] Collectively, these studies support the potential for airborne transmission of influenza. However, the infectivity of the captured viral aerosols was not addressed.

Numerous reports have shown that the viability of airborne viruses is dependent on the virus type, environmental conditions, and on the methods of collection and handling of bioaerosol samples.[7] For example, the survival of airborne influenza was shown to greatly depend on the relative humidity, as well as on ambient air temperature and ultraviolet radiation levels.[8] Collecting influenza, measles, and mumps virus into a viral maintenance fluid instead of distilled water resulted in the increased recovery of infectious virus from a bubbling air sampler.[9] Furthermore, airborne bacteriophages have been shown to retain infectivity longer after collection when refrigerated than when stored at room temperature.[10]

In this study, we have examined the ability of the NIOSH bioaerosol sampler to collect infectious airborne influenza A virus and compare it to the SKC BioSampler. An enhanced infectivity detection methodology has also been developed in our laboratory[11] and this method is used to demonstrate the potential utility of the NIOSH bioaerosol sampler for collecting real-world environmental samples containing infectious viral particles.

Materials and methods

Cell culture

Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were propagated and maintained in 75-cm^2 Corning CellBind Surface flasks (Corning Inc. Life Sciences, Lowell, MA). Complete growth medium for MDCK cells consisted of Eagle's minimal essential medium (EMEM) (ATCC) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc, Logan, Utah), 0.4 units/ml penicillin G (Invitrogen, Carlsbad, CA), and 0.4 μg ml^{-1} streptomycin (Invitrogen). All incubations were performed at 35 °C in a humidified 5% CO_2 incubator. Cells were grown until ~90% confluent.

Virus

Influenza strain A/WS/33 (H1N1, ATCC VR-825, Lot#: 58023547 @1.58×10^8 chicken embryo infectious dose 50% endpoint ($CEID_{50}$)/ml and Lot#: 58772128 @2.8×10^6 $CEID_{50}$/ml) was purchased from ATCC.

Bioaerosol samplers

Bioaerosol samplers developed by NIOSH were used to collect influenza virus-containing aerosols generated in the laboratory.[2,5] For comparison, SKC BioSamplers (SKC Inc, Eighty Four, PA) were used. The SKC BioSampler contained 15 ml of Hank's Balanced Salt Solution (HBSS) supplemented with 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO), 100 units/ml penicillin G and 100 μg ml^{-1} streptomycin in the 20 ml collection vessel.

Calm-air chamber aerosolization and collection of influenza virus

Aerosol collection experiments were conducted in a calm-air settling chamber as shown in Fig. 2.[12] For each experiment, one vial (1 ml) of influenza A/WS/33 (ATCC) was diluted in 30 ml or 60 ml of supplemented HBSS. The test aerosol was generated using an Aeroneb micropump nebulizer (Aerogen, Galway, Ireland) and combined in a mixing chamber with 30 L min^{-1} of filtered conditioned air to achieve a 20% relative humidity (RH). An RH of 20% was chosen because it is similar to levels measured in a US healthcare facility during influenza season.[5] The aerosol flowed through a dispersion nozzle into the top center of a 40 L calm-air chamber and down to the bottom of the chamber where the NIOSH bioaerosol samplers and a sample inlet for the SKC BioSamplers were located.

The aerosol concentration and size distribution in the chamber air was measured using a Model 3321 Aerodynamic Particle Sizer (APS; TSI, Shoreview, MN) with a 100 : 1 aerosol dilutor (Model 3302A, TSI). The APS drew air at 5 L min^{-1} through a vertical probe at the same height as the sampler inlets. From three to five NIOSH bioaerosol samplers were positioned inside the calm-air chamber on the bottom plate. The NIOSH bioaerosol samplers were connected to Model 224-PCXR4 personal air sampling pumps (SKC, Eighty Four, PA, USA) and operated for 15, 30 or 60 min at 3.5 L/min. The flow rates through the NIOSH bioaerosol samplers were set before each experiment. The BioSamplers were outside the chamber, and drew aerosol samples through a vertical probe in the center of the bottom plate of the chamber. Each BioSampler was connected to the sampling port for 15 min and then removed, and the next BioSampler was then attached to the port. A ball valve in the sampling port was closed while changing BioSamplers. The SKC BioSamplers were connected to a central vacuum line and operated at 12.5 L/min.

At the start of each experiment, the Aeroneb nebulizer was operated for 10 min to stabilize the aerosol concentration in the chamber. During this time, the APS was in operation and a vacuum purge was used to draw the aerosol into the chamber, but the samplers were not on. After 10 min, the vacuum purge was turned off and the NIOSH bioaerosol samplers and SKC BioSampler were switched on. The nebulizer was continuously operated during each experiment to provide a constant loading of aerosols in the chamber at about 0.1 ml of fluid/min. After the aerosol collection was completed, the nebulizer, the pumps and the vacuum

line were turned off. The exterior of the samplers were wiped to remove the deposited particles. The NIOSH bioaerosol samplers were disassembled and the collected aerosol fractions in each tube and on the filter were suspended in 1 ml of supplemented HBSS.

Viral RNA isolation and cDNA transcription

Viral RNA was isolated directly from aerosol samples using the MagMax™-96 Viral RNA Isolation Kit (Applied Biosystems/Ambion, Austin, TX). Briefly, following resuspension of collected aerosolized virus from the NIOSH bioaerosol sampler in 1 ml of supplemented HBSS, 500 μl of Lysis/Binding Solution Concentrate (Ambion) was added to 500 μl of each sample and stored at −20 °C. To process virus collected with the SKC BioSampler, 500 μl of Lysis/Binding Solution Concentrate was added directly to 500 μl of each sample and stored at −20 °C. Upon thawing, 500 μl of isopropanol was added to each sample to complete the Lysis/Binding Solution preparation and viral RNA was extracted according to the manufacturer's instructions. The final eluted total-RNA volume was 32 μl. RNA was immediately transcribed into cDNA using High Capacity RNA to cDNA Master Mix (Applied Biosystems, Foster City, CA). The final cDNA volume was 40 μl.

Real-time qPCR analysis

To detect influenza virus, real-time qPCR analysis was performed using the following matrix1 gene primers[13] (corresponding to the 33 amino acids at the n-terminus): Forward 5′-AGATGAGTCTTCTAACCGAGGTCG-3′, Reverse 5′-TGCAAAAACATCTTCAAGTCTCTG-3′and probe: 6FAM-TCAGGCCCCCTCAAAGCC-MGBNFQ. All primers and probes were synthesized by Applied Biosystems and used at a final concentration of 0.8 μM and 0.2 μM, respectively. The qPCR (45 cycles) was performed with the Applied Biosystems 7500 Fast Real-Time PCR System as follows: 20 s at 95 ′C (initial denaturation), 3 s at 95 ′C (amplification), and 30 s at 60 ′C (extension). To determine the relative viral genome copy, a standard curve was generated from 10-fold serial dilutions of the influenza M1 matrix gene and analyzed concurrently with all qPCR reactions. A negative control without template was also included in all real-time PCR reactions. All reactions were run in duplicate and averaged.

Viral plaque assay (VPA)

MDCK cells were detached with 0.25% Trypsin-EDTA (Invitrogen), washed and re-suspended in complete EMEM at a density of 1.0×10^6 cells/ml. Next, 2 ml of the cell suspension was added to each well of a 6-well CoStar tissue culture plate (Corning) and incubated overnight. Confluent cell monolayers were washed twice with 2 ml of phosphate-buffered saline (PBS), inoculated with 800 μl of each collected aerosol sample diluted with supplemented HBSS, and incubated for 45 min. Inoculated cells were then washed once with 2 ml of PBS, overlaid with Dulbecco's modified Eagle's medium (DMEM)/F12 (ATCC) supplemented with 100 units/ml penicillin G, 100 μg ml^{-1} streptomycin, 2mM L-glutamine, 0.2% BSA, 10 mM HEPES (Invitrogen), 0.22% sodium bicarbonate (Invitrogen), 0.01% DEAE-dextran (MP BioMedicals, LLC, Solon, OH), 2 μg ml^{-1} N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (Sigma-Aldrich) and 0.6% agarose (Oxoid Ltd., Hampshire, England). The cells were incubated for 60 h, fixed with 2 ml of 10% formalin for 15 min, and the agarose overlay was removed by washing with tap water. Plaques were

stained with 2 ml of 1% crystal violet/0.19% methanol for 15 min, rinsed with tap water, dried, counted, and the plaque forming units (PFU) were calculated.

Viral replication assay (VRA)

To increase the sensitivity of detecting viable virus, a modified tissue culture infectious dose endpoint 50% ($TCID_{50}$) assay, the VRA, was developed.[11] With the VRA, an aerosol sample containing a mixture of infectious and non-infectious virus is first incubated with MDCK cells to amplify the number of infectious virus present in the sample. Non-infectious virus is then washed from the cells, and the infected cells are lysed to release the amplified infectious viral particles which are subsequently detected by qPCR. We have previously shown that in the VRA, infectious virus in an aerosol sample was amplified 4.6×10^5 fold.[11] Briefly, prior to viral treatment, MDCK cells were trypsinized, washed and re-suspended in DMEM (Invitrogen) supplemented with 1% BSA (Invitrogen), 25 mM HEPES (Invitrogen), 2 μg ml^{-1} L-10 tosylamido-2-phenylethyl chloromethyl ketone (TPCK) Trypsin (Sigma, St. Louis, MO), 0.2 units/ml penicillin, and 0.2 μg ml^{-1} streptomycin. Cells (200 μl) were plated in CoStar flat bottom 96-well plate 4 replicates) at a density of 5.0×10^4 cells per well (Corning) and incubated overnight. Plated cells were next treated with five 50 μl serial dilutions of each viral aerosol sample for 45 min. Infected cells were then washed with PBS, overlaid with 200 μl supplemented DMEM without agarose. Following 20 h of incubation, the cells were washed with PBS, 63 μl of Lysis/Binding Solution Concentrate was added to each well, and the plate was shaken at maximum speed for one minute on a Titer Plate Shaker. The 96-well plate containing the cellular lysate was stored at −20 °C until RNA extraction. Upon thawing, 77 μl of isopropanol was added to each sample well to complete the Lysis/Binding Solution preparation, and total RNA was extracted according to the manufacturer's instructions. The final eluted total RNA volume was 32 μl. RNA was immediately transcribed into cDNA (40 μl) using High Capacity RNA to cDNA Master Mix.

Calculations

For each aerosol sampler in each experiment, the total viral particles collected (TVP), which represents both infectious and non-infectious virus, were determined by qPCR analysis of the collected sample. The total viral particles/liter of air sampled (TVP/L) was then calculated by dividing the TVP by the sampler flow rate and collection time. Similarly, the amount of infectious virus in each sampler was determined using VPA, and the number of PFU/liter of air sampled (PFU/L) was found by dividing the PFU by the sampler flow rate and collection time.

The aerosol particle concentration in the calm-air chamber and the amount of infectious and total virus particles in the nebulized solutions varied among the experiments, which introduced variations in the amount of infectious and total influenza particles collected by the samplers. To control for this, we normalized the total influenza particles and amount of infectious virus collected by the samplers as follows:

First, the aerosol particle concentration (total number of aerosol particles/liter of air) during the particle collection by each sampler during each experiment was determined based on the aerosol concentration data from the APS. Then, these concentrations for each sampler were

divided by the overall average aerosol concentration for all of the samplers during all of the experiments to give an aerosol concentration normalization factor, $N_{aerosol}$.

Next, the concentrations of total influenza virus in the nebulizer solution for each experiment (as measured by qPCR) were divided by the overall average concentration of total virus for all of the experiments to give a total influenza concentration normalization factor, $N_{total\ virus}$.

In a similar fashion, the concentrations of infectious influenza virus in the nebulizer solution for each experiment (as measured by VPA) were divided by the overall average concentration of infectious virus to give an infectious influenza concentration normalization factor, $N_{infectious\ virus}$.

Finally, these factors were used to calculate the normalized TVP, PFU, TVP/L and PFU/L collected by each sampler in each experiment:

$$\text{normalized TVP} = \frac{\text{experimental TVP}}{N_{aerosol} \times N_{total\ virus}}$$

$$\text{normalized TVP/L} = \frac{\text{experimental TVP/L}}{N_{aerosol} \times N_{total\ virus}}$$

$$\text{normalized PFU} = \frac{\text{experimental PFU}}{N_{aerosol} \times N_{infectious\ virus}}$$

$$\text{normalized PFU/L} = \frac{\text{experimental PFU/L}}{N_{aerosol} \times N_{infectious\ virus}}$$

Results

Assessment of airborne influenza virus collection by the NIOSH bioaerosol sampler

The NIOSH bioaerosol sampler (Fig. 1) was assessed for its ability to collect aerosolized influenza virus in a calm-air chamber (Fig. 2). The normalized TVP collected in the sampler tubes and filter increased with time from 1.3×10^6 TVP after 15 min to 2.2×10^6 TVP after 30 min and 1.6×10^7 after 60 min (Table 1). The normalized TVP/liter of air for the NIOSH bioaerosol sampler was constant up to 30 min of collection, with 2.4×10^4 TVP/L found after 15 min of sampling and 2.1×10^4 TVP/L found after 30 min. After 60 min of sampling, the normalized TVP/L increased to 7.6×10^4 TVP/L (Table 1). For all experiments using the NIOSH bioaerosol samplers combined, the overall average collection was 3.5×10^4 TVP/L, which was comparable to the overall average of 2.6×10^4 TVP/L for the SKC BioSamplers.

Assessment of viral infectivity following collection by the NIOSH sampler

Prolonged collection time has been shown to result in decreased viral recovery.[10] Therefore, to determine whether the NIOSH sampler is able to collect infectious virus over an extended sampling period, collected samples were assayed for infective virus by the VPA. In Fig. 3, the number of PFU/liter of air that was detected in the NIOSH bioaerosol sampler is shown relative to the PFU/L found using the SKC BioSampler, which has been reported to preserve essentially all influenza virus infectivity during collection.[1] The NIOSH bioaerosol sampler contained 34% of the PFU/L in the SKC BioSampler after 15 min, 28% after 30 min, and 15% after 60 min. Infectious virus was found in all three size fractions of the NIOSH bioaerosol sampler.

Distribution of collected airborne influenza virus in different sampling stages

The particle sizes and concentrations of aerosols loaded in the calm-air chamber were monitored by an aerodynamic particle sizer (APS) for each experiment. A typical mass-based aerosol particle size distribution in the calm-air chamber is shown in Fig. 4. The average distribution of collected infectious and total viral particles in the collection tubes and backup filter of the NIOSH bioaerosol samplers is shown in Table 2. Eighty-six percent of infectious particles (mean of the samples collected at three collection times) were contained in aerosols with diameters ≤4 μm. When the experimental results are averaged, the distribution of the infectious viral particles in the two stages and on the backup filter was similar regardless of collection times, though the distribution of the collected total viral particles appeared to vary with collection times. When compared to the 15 min collection time, the fraction of the total viral particles collected after 60 min decreased from 21% to 3% in the Stage 1 tubes and increased from 37% to 63% on the backup filter (Table 2). However, within the same experiment, samplers with different collection times showed much less variability when comparing the distribution of infectious and total viral particles. This can be seen, for example, by comparing experiment #1 and experiment #2 where collection occurred for 30 and 60 min in each experiment, and with experiment #3 where collection occurred for 15 and 30 min (Table 3). These results indicate that the NIOSH bioaerosol samplers performed consistently over time when sampling the same aerosol cloud.

Effect of prolonged storage on viability

To determine whether prolonged storage of collected virus in the sampler's collection tubes and filter, which may be unavoidable in some field studies, affected recovery and infectivity of the virus, a NIOSH bioaerosol sampler was placed at room temperature for 24 h before extracting the collected virus. The TVP detected from that stored sampler and the infectivity of the collected virus were compared with another sampler that was immediately processed after collection. The loss in infectivity of virus collected in the >4 μm, 1–4 μm, and <1 μm fractions was 74%, 57%, and 71%, respectively, resulting in a 60% overall loss in infectivity (data not shown). In contrast, the loss in TVP detected in the >4 μm, 1–4 μm, and <1 μm fractions was 15%, 17%, and 32%, respectively, resulting in a 20% overall loss in TVP (data not shown).

Enhanced infectivity detection assay

The utility of the NIOSH sampler was extended by incorporating an enhanced infectivity detection methodology, the VRA. In the VRA, collected samples are first infected into MDCK cells and allowed to replicate in order to increase the ability to detect low levels of infectious virus in an aerosol sample. Calm-air chamber experiments that began with very low initial infectious virus (0.13–0.14%) were chosen for further analysis by the VPA as this may reflect the extent of infectivity expected in real-world environmental samples. Table 4 shows that when the nebulizer was loaded with 2.8×10^7 TVP/ml, 6.4×10^2 infectious virus were detected by the VPA in the collected sample. With the VRA, 2.1×10^{10} matrix gene transcripts were detected from this sample. The viral suspension loaded into the nebulizer was then diluted 332-fold to 1.3×10^2 PFU/ml to determine whether the sensitivity of detecting infectious virus by the VRA exceeded that of the VPA. As shown in Table 4, no infectious virus was detected with the VPA. In contrast, 4.1×10^6 matrix gene transcripts were detected with the VRA.

Influenza virus concentration in the nebulizer

The initial and final concentration of virus in the nebulizer solution was measured during eight experiments. The average total virus concentration in the nebulizer fluid was 4.2×10^7 viral particles/ml (SD 1.1×10^9) at the start of the experiments and 4.3×10^7 viral particles/ml (SD 4.1×10^7) at the end. The average concentration of infectious virus was initially 6.7×10^4 PFU/ml (SD 3.2×10^4) and was 6.5×10^4 PFU/ml (SD 2.6×10^4) at the end. Thus, the total viral particle number and the amount of infectious influenza virus in the nebulizer solution stayed the same over the course of the experiments.

Discussion

Concerns regarding the vulnerability of large populations to viruses that exacerbate respiratory infections such as pandemic strains of influenza A have prompted a number of studies into the modes of transmission.[14–21] Aerosol samplers vary greatly in their efficiency to collect airborne particles, fractionate them by size, and maintain the infectivity of collected viruses. Recently, the efficiencies of four commercial air samplers were compared.[1] In that study, collection of total viral particles by the SKC Bio-Sampler (which collects particles directly into a liquid media) was superior to 37 mm cassette samplers containing either a Teflon filter (74% recovery) or a gelatin filter (63% recovery), and the CCI sampler that contains a polyurethane foam filter (32% recovery). Of the four samplers tested, the SKC BioSampler maintained essentially 100% of the viability of the collected virus, whereas recoveries of viable virus from samplers with a gelatin filter, Teflon filter, or polyurethane filter were only 10%, 7%, and 22%, respectively. In our study, the ability of the NIOSH bioaerosol sampler to collect aerosolized particles containing influenza virus was essentially the same as that of the SKC BioSampler and was relatively constant over the collection times of 15, 30, and 60 min. The NIOSH bioaerosol sampler maintained an average of 26% of the virus infectivity compared to the SKC BioSampler. These results are higher than those reported[1] for three other samplers, although it should be noted that a direct comparison cannot be made since Fabian *et al.*[1] used a different viral strain, different aerosolization and collection media, and performed their experiments at 50 to 55% relative

humidity *versus* 20% for our experiments. The amount of infectious virus recovered from the NIOSH bioaerosol sampler is significantly lower than seen with the SKC BioSampler and does show a clear decline after 60 min of collection, suggesting that the dry collected virus gradually loses infectivity due to desiccation or degradation. This indicates that collection times should be kept to a minimum, and that the potential advantages of the NIOSH bioaerosol sampler need to be weighed against this drawback. Prolonged collection times with the SKC BioSampler result in significant loss of the collection media due to evaporation (26% loss of collection media in 15 min; data not shown) and thus should be avoided as well.

In this study, the infectivity of virus (assessed by the VPA) in the initial suspension used to generate the aerosolized particles was only 0.06–1.15% (average 0.53%) of the total viral particles. Viral stocks with similarly low (0.3–0.5%) infectivity were used by Fabian *et al.*[1] Conceivably the viability is actually much higher as viability assessed by the chicken embryo infectious dose endpoint 50% ($CEID_{50}$) from the manufacturer (ATCC) is 10–50× higher. This discrepancy may simply reflect a lack of sensitivity of the VPA for determining infectivity or the initial infectivity of the stock may have degraded with storage, and emphasizes the need to increase the sensitivity of the infectivity assessments for future studies. To address this, we have combined the use of the NIOSH bioaerosol sampler with our VRA methodology which greatly facilitated our ability to detect infectious virus even when the initial infectivity in the nebulizer was only 0.13–0.14%. Dilution of the viral suspension loaded into the nebulizer from 3.8×10^4 PFU/ml to 1.3×10^2 PFU/ml (283-fold dilution), resulted in no PFU recovered in the collected samples, however, matrix gene transcripts arising from infectious virus in the samples were detected by the VRA.

A previous study[1] on collection of infectious airborne virus has suggested that samplers containing collection medium can better preserve the infectivity of collected virus compared to those without collection medium. We found that the addition of supplemented HBSS to the collection tubes did not improve infectivity (data not shown), probably because the virus is deposited at the top of the tube and above the media rather than into the media. Adding more media, however, would negatively alter the aerodynamics of collection. In another approach, the top portion of the Stage 1 collection tubes were coated with 2% agar or 0.1% mucin, or supplemented HBSS-soaked electrets filters were inserted near the top. However, neither infectivity nor the total amount of recovered viral particles were increased over that found when uncoated tubes were used (data not shown).

The NIOSH bioaerosol sampler can be used for collection of bioaerosols in an outside environment although the presence of other microorganisms, dust, or pollen may alter the aerodynamics of fractionation or provide an additional vehicle hitchhiking mode of transport of transmission for the virus. In an earlier study, we co-aerosolized influenza A and *Aspergillus versicolor* fungal spores and found that the NIOSH bioaerosol sampler efficiently separated the two, but there was a shift in the overall deposition of virus to the Stage 1 collection tube and fewer viral particles were found on the backup filter.[4] Humidity is also likely to influence recovery of infectious virus, as indicated in a study that showed the stability of influenza A is minimal at 50% relative humidity (RH), high at 60–80% RH, and maximal at 20–40% RH.[22] A later study had similar results, finding that transmission of

influenza does not occur at 80% RH, is low at 50% RH, high at 65% RH, and maximal at 20% and 35% RH.[23] Moreover, the RH has been shown to affect the stability of other viruses including Semliki forest virus,[24] HIV,[25] and respiratory syncytial virus.[26] For this study, we maintained the RH at 20%. Future work will include an investigation into the role of humidity on infectious recovery with the NIOSH sampler.

An important distinction between the NIOSH bioaerosol sampler and the SKC BioSampler is the ability to fractionate aerosols and identify which fractions contain infectious virus. Coughing, sneezing and talking generate airborne particles ranging in size from a few millimetres to <1 μm. Knowing whether infectious influenza is present in the respirable fraction would help provide a better assessment for risk of infection. Such information would dictate the personal protective equipment and guidelines for preventing personal exposure such as the type of particle mask or respirator to use, enable appropriate adjustments to air handling systems, and determine which personal protective measures need to be taken while conducting potentially aerosol-generating medical procedures during influenza outbreaks. Although the SKC BioSampler is significantly better at preserving viral infectivity and has a higher sample flow rate, the NIOSH bioaerosol sampler can be used with personal sampling pumps and worn as a personal sampler. Because it does not require aqueous buffers for collection of airborne virus, the collection time for the NIOSH bioaerosol sampler is not limited by water evaporation from the media, and the sampler is not sensitive to bouncing or tilting.

Conclusions

The use of the NIOSH bioaerosol sampler for the collection and detection of aerosols holds much promise. The collection efficiency of total aerosol viral particles is essentially the same as that of the SKC BioSampler. For the 15, 30, and 60 min collection times, on average, 26% of the aerosolized infectious virus collected with the NIOSH bioaerosol sampler remained infectious as compared with that collected over 15 min from the SKC BioSampler. Infectious virus was found in all three size fractions, although infectivity did decline with longer collection times. The demonstrated ability of the NIOSH bioaerosol sampler to collect infectious surrogate virus, influenza A, leads us to be cautiously optimistic for its potential use in the detection of other viruses and microorganisms.

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Environmental impact

Coughing, sneezing, and breathing generate a range of airborne particles that may contain infectious influenza virus, and respirable particles <10 μm are, arguably, the most problematic as they can remain airborne for hours. The use of the NIOSH aerosol sampler for investigation of environmental samples for influenza virus and potentially other viruses and microorganisms holds much promise. In this study, we show that the NIOSH air sampler can size fractionate collected aerosols and retain infectivity of aerosolized influenza to provide better assessments for risk of infection and precautionary guidelines for prevention.

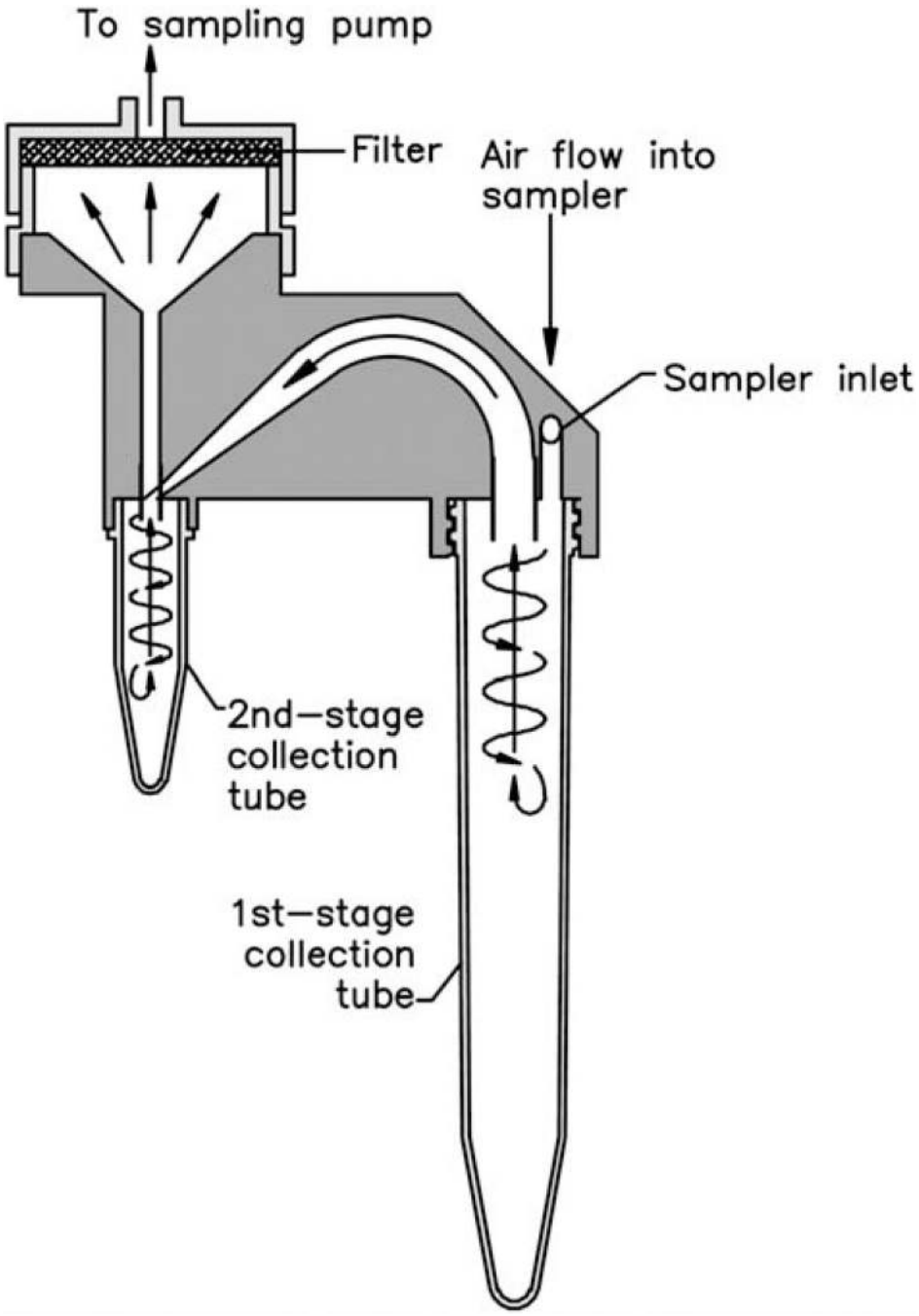

Fig. 1. Description of the NIOSH Bioaerosol Sampler

Ambient air is drawn into the sampler's inlet at 3.5 L min^{-1} and initially enters the 1st stage 15 ml polypropylene tube, where aerosol particles >4 μm are deposited on the wall of the tube. The air then enters the 2nd stage 1.5 ml polypropylene tube where 1 to 4 μm particles are deposited. After the air exits the 2nd stage, particles <1 μm are collected on a 37 mm PTFE filter.

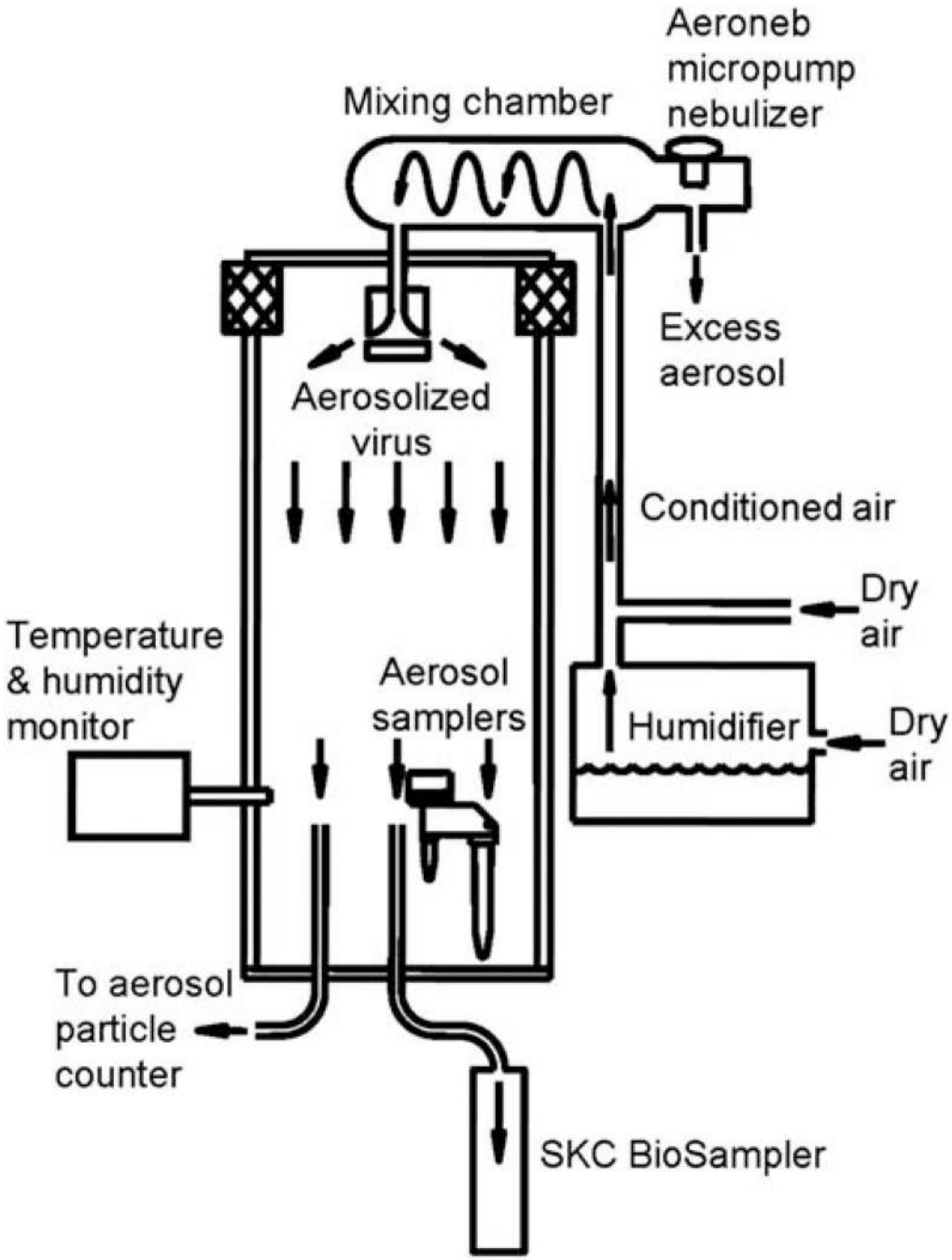

Fig. 2. Description of the Calm-air Chamber

Influenza virus is loaded into a nebulizer, mixed with dry air, and the aerosolized particles are dispersed into an air chamber. NIOSH bioaerosol samplers are placed into the bottom of the chamber and an SKC BioSampler is placed outside the chamber.

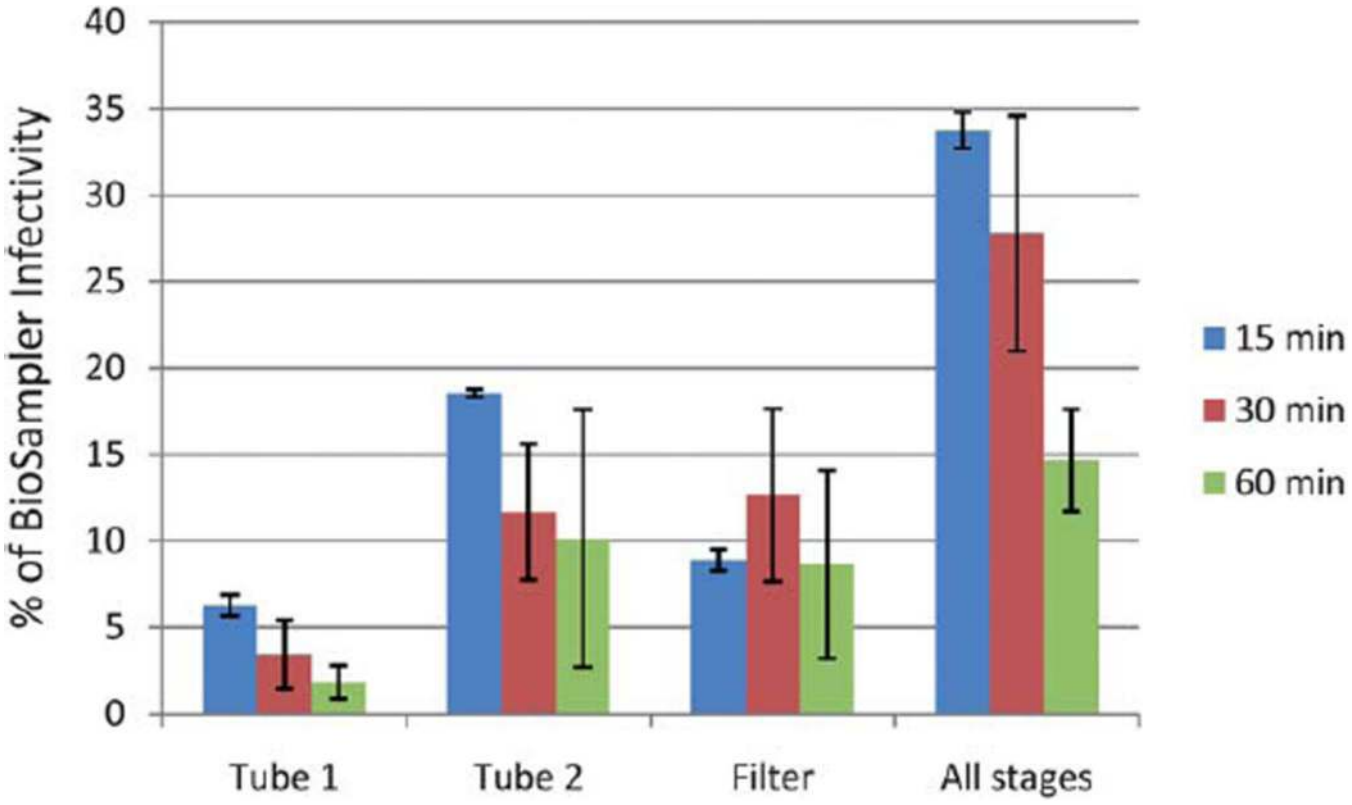

Fig. 3. Infectious influenza virus detected in the NIOSH bioaerosol sampler

The amount of infectious influenza virus detected in each stage per liter of air collected is shown here relative to the amount detected in the SKC BioSampler. After 15, 30 and 60 min, the infectious virus/liter air found in the NIOSH bioaerosol sampler was 34%, 28% and 15% of that found in the SKC BioSampler.

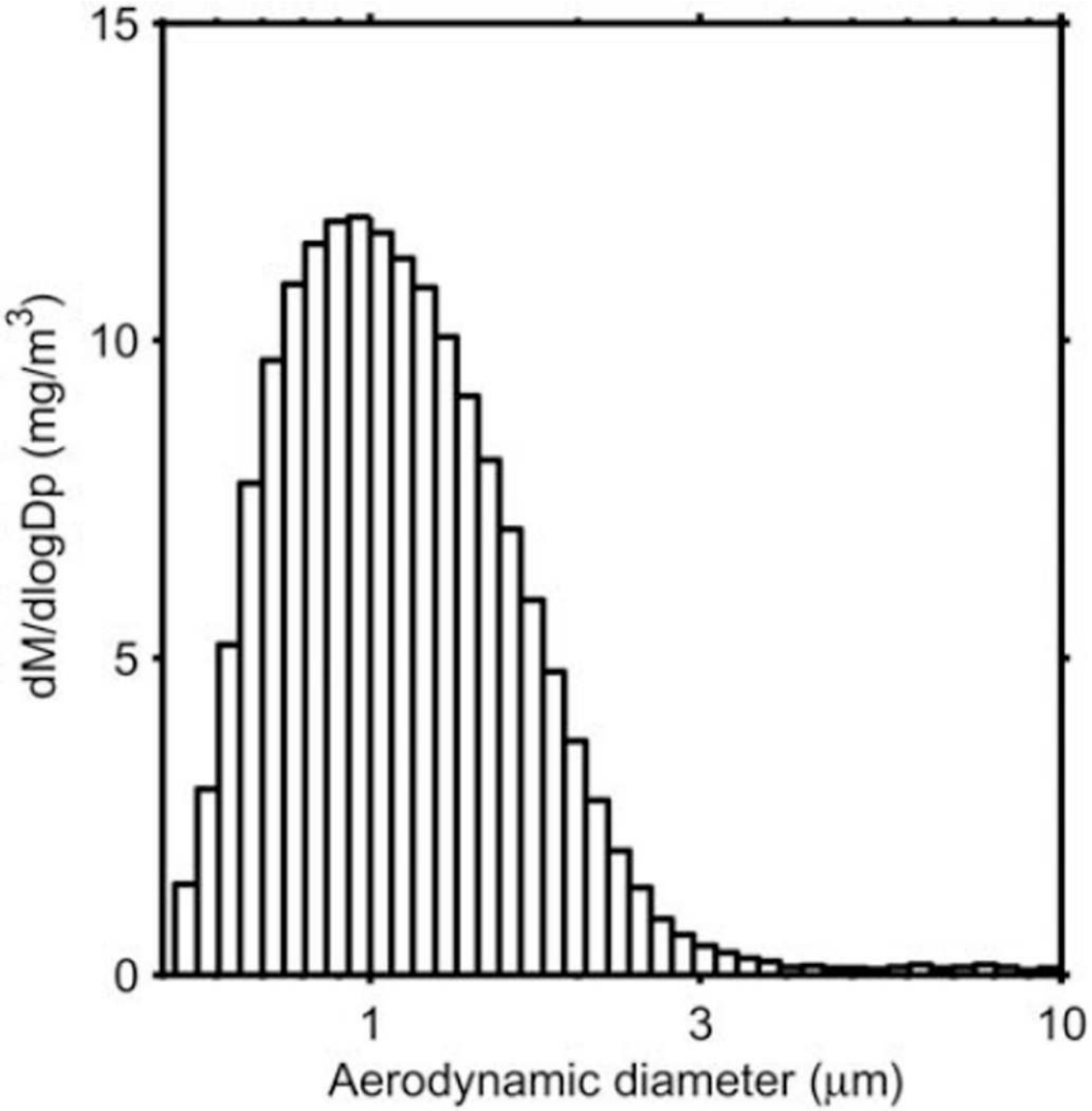

Fig. 4. Size distribution of influenza-laden aerosol particles in the calm-air chamber

The plot shows the estimated mass of aerosol particles *vs.* particle aerodynamic diameter for a typical experiment as measured by an Aerodynamic Particle Sizer. If the viral particles are evenly distributed in the nebulized droplets, the mass is proportional to the viral content. Over the range of the APS, the test aerosol containing HBSS and influenza virus had an average concentration of 1.33×10^6 particles/cm^2, with a count median aerodynamic diameter of 0.8 μm and a geometric standard deviation of 1.26. The HBSS-only aerosol had

an average concentration of 1.20×10^6 particles/cm^2, with a count median diameter of 0.7 μm and a geometric standard deviation of 1.23.

Table 1

Total influenza viral particles collected by NIOSH bioaerosol samplers and SKC BioSamplers in a calm-air chamber[a,b]

Number of NIOSH bioaerosol samplers	Collection time (min)	Normalized TVP	Normalized TVP/L of air collected
2	15	1.3×10^6 (2.2×10^5)	2.4×10^4 (4.1×10^3)
16	30	2.2×10^6(1.4×10^6)	2.1×10^4 (1.3×10^4)
6	60	1.6×10^7(1.6×10^7)	7.6×10^4 (7.7×10^4)
All 24 samplers combined	–	–	3.5×10^4 (4.5×10^4)
Number of SKC BioSamplers	Collection Time (min)	Normalized TVP	Normalized TVP/L of Air Collected
18	15	4.8×10^6 (5.2×10^6)	2.6×10^4 (2.8×10^4)

[a]Abbreviations: TVP, Total Viral Particles; Standard Deviation shown in parentheses.

[b]Number of samplers per experiment varied from one to four.

Table 2

Average distribution of collected infectious and total influenza virus in the NIOSH samplers[a]

		[b]Distribution of collected infectious virus (%)			[b]Distribution of collected total virus (%)		
Number of NIOSH samplers	**Collection time (min)**	**>4 μm**	**1–4 μm**	**<1 μm**	**>4 μm**	**1–4 μm**	**<1 μm**
2	15	19 (1)	55 (2)	26 (1)	21 (3)	42 (2)	37 (1)
16	30	12 (5)	43 (11)	45 (12)	11 (7)	31 (12)	58 (13)
6	60	11 (6)	48 (9)	41 (11)	3 (2)	34 (5)	63 (7)

[a]Average of all samplers used in all experiments for each collection time.

[b]Standard Deviation shown in parentheses.

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Table 3

Distribution of collected infectious and total influenza virus in three individual experiments[a]

Experiment #1	Particle size	30 min (2 samplers)		60 min (3 samplers)	
		% Infectious	% of Total	% Infectious	% of Total
	>4 μm	$_{8}$[b]	4 (0.4)	14 (7)	3 (0.3)
	1–4 μm	$_{57}$[b]	39 (10)	52 (6)	36 (3)
	<1 μm	$_{35}$[b]	57 (10)	34 (8)	60 (3)
Experiment #2	Particle size	30 min (2 samplers)		60 min (2 samplers)	
		% Infectious	% of Total	% Infectious	% of Total
	>4 μm	9 (4)	7 (10)	9 (6)	1 (0.1)
	1–4 μm	38 (3)	23 (4)	40 (11)	29 (4)
	<1 μm	53 (1)	69 (14)	51(18)	71 (4)
Experiment #3	Particle size	15 min (2 samplers)		30 min (2 samplers)	
		% Infectious	% Total	% Infectious	% Total
	>4 μm	19 (1)	21 (2)	22 (5)	25 (1)
	1–4 μm	55 (2)	42 (2)	51 (8)	40 (9)
	<1 μm	26 (1)	37 (1)	27 (3)	35 (9)

[a] Standard deviation shown in parentheses.

[b] PFU could not be determined for one sampler.

Table 4

Increased utility of the NIOSH sampler when coupled to an enhanced viability detection assay[a,b]

Particle size	PFU/ml Initially in nebulizer	TVP/ml Initially in nebulizer	PFU/TVP Initial % infectious	PFU Collected	TVP Collected	PFU/TVP Collected % infectious	Matrix transcripts post VRA
>4 μm				5.1×10^1	7.0×10^4	0.07	1.9×10^9
1–4 μm				1.5×10^2	2.4×10^5	0.06	1.0×10^{10}
<1 μm				4.4×10^2	4.7×10^5	0.09	8.4×10^9
Total	3.8×10^4	2.8×10^7	0.14	6.4×10^2	7.8×10^5	0.08	2.1×10^{10}
>4 μm				bd	bd	N/A	1.7×10^5
1–4 μm				bd	1.5×10^1	N/A	3.7×10^5
<1 μm				bd	4.9×10^1	N/A	2.6×10^5
Total	1.3×10^2	1.1×10^5	0.13	N/A	6.4×10^1	N/A	4.1×10^6

[a]The concentration of strain A/WS/33 in the nebulizer prior to aerosolization is shown. TVP initially in the nebulizer or collected was determined by quantification of the matrix gene by qPCR. PFU initially in the nebulizer or collected was determined by the standard plaque assay. Matrix transcripts in the collected samples following amplification *via* the Viral Replication Assay (VRA) were determined by qPCR.

[b]Abbreviation: bd, below detection: NA, not applicable.