Title

Humidity Reduces Rapid and Distant Airborne Travel of Viable Viral Particles in Classroom Settings

Authors

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Abstract

The transmission of airborne pathogens via aerosols is considered to be the main route through which a number of known and emerging respiratory diseases infect their hosts. It is therefore essential to quantify airborne transmission in closed spaces and determine what recommendations should be implemented to minimize the exposure to the pathogen in built environments. We have developed a method to detect viable virus particles from aerosols by using an aerosolized bacteriophage Phi6 in combination with its host *Pseudomonas phaseolicola*, which when seeded on agar plates acts as a virus detector that can be placed at a range of distances away from the aerosol-generating source. Based on this method we present two striking results: (1) We consistently detected viable phage particles at distances of 18 feet away from the source within 15-minutes of exposure in a classroom equipped with a state of the art HVAC system. (2) Increasing the relative humidity beyond 40% at a maintained temperature of (22.8 ± 0.2) ℃ significantly reduces the risk of transmission. Our method can be used to quantify the exposure to pathogens at various distances from the source for different amounts of time, data which can be used to set safety standards for room capacity and the efficacy of interventions which aim to reduce pathogen levels in closed spaces of specified size and intended use.

Summary

We present a method to experimentally determine the exposure to airborne pathogens in closed spaces.

Introduction

Airborne transmission of human pathogens has been the driver of major outbreaks of known and novel respiratory diseases [1]. It is now known that a number of contagious diseases can spread through a process of aerosolization, where an infected individual generates virus-carrying particles that can remain suspended in the air for a length of time [2,3]. While airborne transmission has been found to rarely occur outdoors [4], indoor spaces may facilitate transmission via aerosols even under the conditions of social distancing [5]. Experiments have shown that SARS-CoV-2 remains infectious for long periods of time when suspended in aerosols [6]. As the population readies to return to using shared spaces (classrooms, offices, etc.) following a major pandemic, it is crucial to determine the distances and timescales over which a pathogen can spread indoors and ask what types of shared space usage recommendations can be implemented to prevent current or future outbreaks, especially when vaccination rates are low or when vaccines are not readily available.

To parameterize viral dispersal in the built environment, we developed a method to extract viable virus particles from aerosols by using a bacterium *Pseudomonas phaseolicola* genetically modified to produce LacZ-α, which serves as the host for *LacZ-*β-marked bacteriophage, Phi6. Phage Phi6 is a known proxy for SARS-CoV-2 due

to its similar shape, size and physiology [7] and is used as a surrogate for human enveloped viruses [8,9]. When plated on agar containing X-Gal, infected hosts produce easily identifiable blue plaques (Fig 1C, inset). Agar plates overlaid with soft agar containing the host and exposed for varying durations act as virus detectors, which we place at a range of distances from the aerosol-generating source of phage Phi6 (Fig 1B). Here, we report two major results: (1) aerosolized viral particles that we studied can travel distances of 18 feet in under 15 minutes in a closed room equipped with an HVAC system operating above an industry recommended level [10], and (2) the exposure to the aerosolized virus decreases with the increase in relative humidity. Specifically, we find that the exposure is significantly reduced when the relative humidity is above 40% at a room temperature of 22.8 (± 0.2) ℃. These results may be useful in determining the types of interventions capable of reducing or eliminating the exposure to airborne pathogens in shared closed spaces.

Materials and Methods

Preparation of Viral Surrogate and Host

A single colony of LacZ-α producing *Pseudomonas phaseolicola* was added to 10 mL of Luria-Bertani broth (LB) and incubated for 18 h with rotary shaking (220 rpm) at 25℃ [11,12]. 500 µL of the overnight culture was added to 50 mL of LB supplemented with 200 µg/mL ampicillin and incubated for 18 h with rotary shaking (200 rpm) at 25℃ to provide overnight culture for soft agar overlay plates that served as detectors for our experiments. To produce Phi6 lysate, 5 mL of stationary-phase culture was added to 200 mL fresh LB, along with 10 µL frozen phage stock when the culture reached exponential phase. Following 18 h incubation with shaking, phages were isolated by filtration through 0.22-µm filters (Durapore; Millipore, Bedford, MA). Phage particles per mL were quantified via serial dilution and determination of titers according to standard methods (Supplemental Information) [13].

Generation of Aerosols

To generate aerosolized viral droplets we introduced 10 mL of diluted phage lysate into a medical grade nebulizer (Uni-HEART™ Lo-Flo Continuous Nebulizer, Westmed Inc), which continually generates aerosols of 2-3 µm mass median aerodynamic diameter (MMAD). Aerosols of that size are known to deposit in the nose, lungs and bronchi in adults upon inhaling [14]. Generation of aerosols in this size range has been associated with talking and coughing [15]. The inherent variability of aerosol sizes generated by the nebulizer might effectively capture some of the variability in aerosols that are also produced by other common actions such as breathing, sneezing, exhaling, and softly talking [15].

We diluted phage lysates to concentrations of approximately 10^8 phage particles per mL, and estimated titers used in each experiment by spot plating 10 µL aliquots at different dilutions on replicate plates. The titers we report in SI Data Tables 1 & 2 are averages over 3-5 replicates. These titers led to consistently countable numbers of plaques across all distances, exposure times and external conditions. Estimates show that an individual carrying influenza virus can exhale on the order of thousands of viral particles in a single breath [16]. Tidal volume estimates (volume of air exhaled) are around 500 mL [17]. Minute ventilation estimates (volume of air exhaled per minute) of a person at rest are in the range of 20 L [18], which suggests a total of 10^6 - 10^8 viral particles exhaled per hour. Total phage released in our experiments was at the higher end of this range.

Classroom Experiments

We arranged the placement of the source of aerosols and the detectors in such a way to consider the risks of asymptomatic spreading of airborne viruses in a classroom (Fig 1A), resembling a situation where mask wearing is not enforced. The detectors -- LB plates overlaid with host strain -- were located 3, 6, 12 and 18 feet away from the source to study the impact of physical distancing on transmission in closed spaces. We placed two sets of detectors that are laterally 3 feet apart (Fig 1B) while being approximately the same distance away

from the source. Single detectors within each set were exposed to the aerosolized phage for progressively longer durations of time for up to one hour (i.e., 15, 30, 45, & 60 minutes). Such placement of the detectors provides a replicate measurement within the same experiment. We repeated our experiments over multiple days in two different rooms at The New School, located on different floors and whose climate is controlled by a separate, independent HVAC system. The results that we report here are consistent among the two rooms despite the variation generated by changes in external conditions, differences in room size, room layouts, and other sources (Supplemental Information & Fig. S2).

Results & Discussion

Aerosolized phage can spread over large distances

We consistently observed plaque-forming units (PFUs) on plates at distances of up to 18 feet away from the source and exposed for a duration of 15 minutes from the start of aerosol generation (Fig 1C). While it remains unclear if such doses would result in a successful infection of the human host (for influenza even a few PFUs can lead to a new infection [19]), our experiments show that viable aerosolized viral particles can travel relatively large distances in a short amount of time. We performed our experiments in two replicate classrooms at The New School in New York City, both of which are equipped with a state of the art HVAC system, with filtration corresponding to two rows of filters (pre-filter M8 and final filter M14), which continuously operates at about 15 room air changes per hour (private correspondence). Yet, we found that the exposure to aerosolized phage particles at 18 feet away from the source shows on average a modest 1.6x reduction when compared to the exposure observed at 6 feet over the same durations (Fig. 1C). Therefore, we consider it likely that the airflows present inside the room facilitate the spread of aerosolized phage to multiple locations within. While it is possible that the HVAC system filters out some of the phage from the environment, it most likely does so at timescales that are longer than the time it takes the aerosolized phage to spread throughout the room.

Humidity impacts the spread of aerosolized phage

We further found experimental evidence that the humidity of the surrounding air impacts the spread and therefore the exposure to aerosolized phage. Fig. 1C constitutes the data we collected across two rooms and a range of external air conditions. Further inspection revealed that air temperature was consistently maintained by the HVAC system in the range 22.5 - 23 ℃, across all experiments, with temperatures fluctuating on average below 1% (Fig. S3 & S4). However, we noticed an apparent difference in the data when we aligned the PFU counts with relative humidity recordings. Circles in Fig 1C that are dispersed across a range of PFUs show normalized counts collected at relative humidity below 40%, while triangles, which are located in a narrow band at very low PFU counts, correspond to data collected at relative humidity higher than 40%.

Figure 2 panels A-D show the rate at which PFUs are generated per 15-minute exposure time as a function of relative humidity. For plates that were exposed for longer than 15 minutes, we consider the average rate of PFU formation in a 15-minute time window. Each panel in Fig. 2 corresponds to mean rates of deposition in a single experiment collected at a set distance away from the source. The two sets of data points in each panel correspond to data collected in two different rooms. At all distances, we find that increasing the relative humidity above 40% results in a substantial decrease in exposure rates. These results are consistent among the two classrooms in which we conducted our experiments. Experimental evidence suggests similar dependence on humidity for transmission of influenza [20–22].

Discussion

We speculated that the reduction in exposure at higher relative humidity is a result of larger rates of surface deposition due to coalescing of the phage-carrying aerosols with moisture suspended in the air [2] . We conducted additional experiments by placing portable battery-operated personal humidifiers at one of the two

replicate locations at different distances away from the source, and compared the exposure in the same room, under the same environmental conditions, of a set of detectors with and without a personal humidifier located in their vicinity (Fig. S5). Our preliminary data suggests that where levels of humidity higher than 40% cannot be achieved or controlled in the whole room, a relatively inexpensive personal humidifier might provide an individualized layer of protection across a variety of shared spaces (Fig. S5). Experiments to further quantify the reduction in exposure are currently underway.

Observed plaques in our experiments show a range of sizes. Insertion of a genetic marker to phage Phi6 is known to induce mutations that may subsequently resolve in smaller plaque sizes [13]. In our experiments we also noticed that plates located closer to the source (3 feet and 6 feet away) are more likely to contain larger plaques while the plates further away are more likely to contain smaller plaques, which might indicate that larger plaques may have been initially formed from larger aerosol particles that carry multiple phage. Since we cannot distinguish between a plaque generated by a single phage or multiple phage, we count each plaque as a single infection event. The true integrated exposure to aerosolized phage might be higher than our plates suggest.

We also note that breathing is an active process that involves sampling the air from a volume whereas our plates detect the rates of aerosol deposition on vertical surfaces. Establishing a mapping between the counts of PFUs on plates and dosage through inhalation can be achieved by using an air sampler in parallel to the static plates and is left for future work.

Finally, we wish to comment on current recommendations that involve the use of HVAC systems coupled with social distancing in closed spaces [10,23]. In the case of asymptomatic transmission, we find exposure may occur at any conceivable distance, regardless of the length of time a person may be exposed. Air flows in a closed room might be influenced by multiple factors, including opening and closing the doors, windows, and migration of the people in and outside of the room, and are therefore dependent on the specifics of the space and are coupled to the activity of persons occupying that space. Those types of considerations should be included when specific use recommendations are being made. Such efforts are already underway [24,25] .

The disparity between the conditions within which people of different socio-economic status live and work is a major driver of the covid pandemic [26,27]. Many office buildings and private institutions may have the required funds to implement a range of exposure-reducing interventions, while public institutions or shared-housing complexes may not. Here, we developed a method to measure the transmission of airborne pathogens in closed spaces, which is portable (can be produced in a wet lab and ported to site), relatively inexpensive, and can be deployed to a variety of spaces with different configurations and intended use. This method, supplemented with epidemiological modeling, could form a basis for inferring data-driven recommendations on room capacity and risk-reducing interventions that can be specific to the space and its intended occupants.

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Supplement:
Methods text.
Fig S1. Plots of plaque counts at all distances
Fig S2. Floor plans
Fig S3 & S4. Temperature and humidity recordings
Fig S5. Experiments with personal humidifiers
Table 1 & 2: Data tables

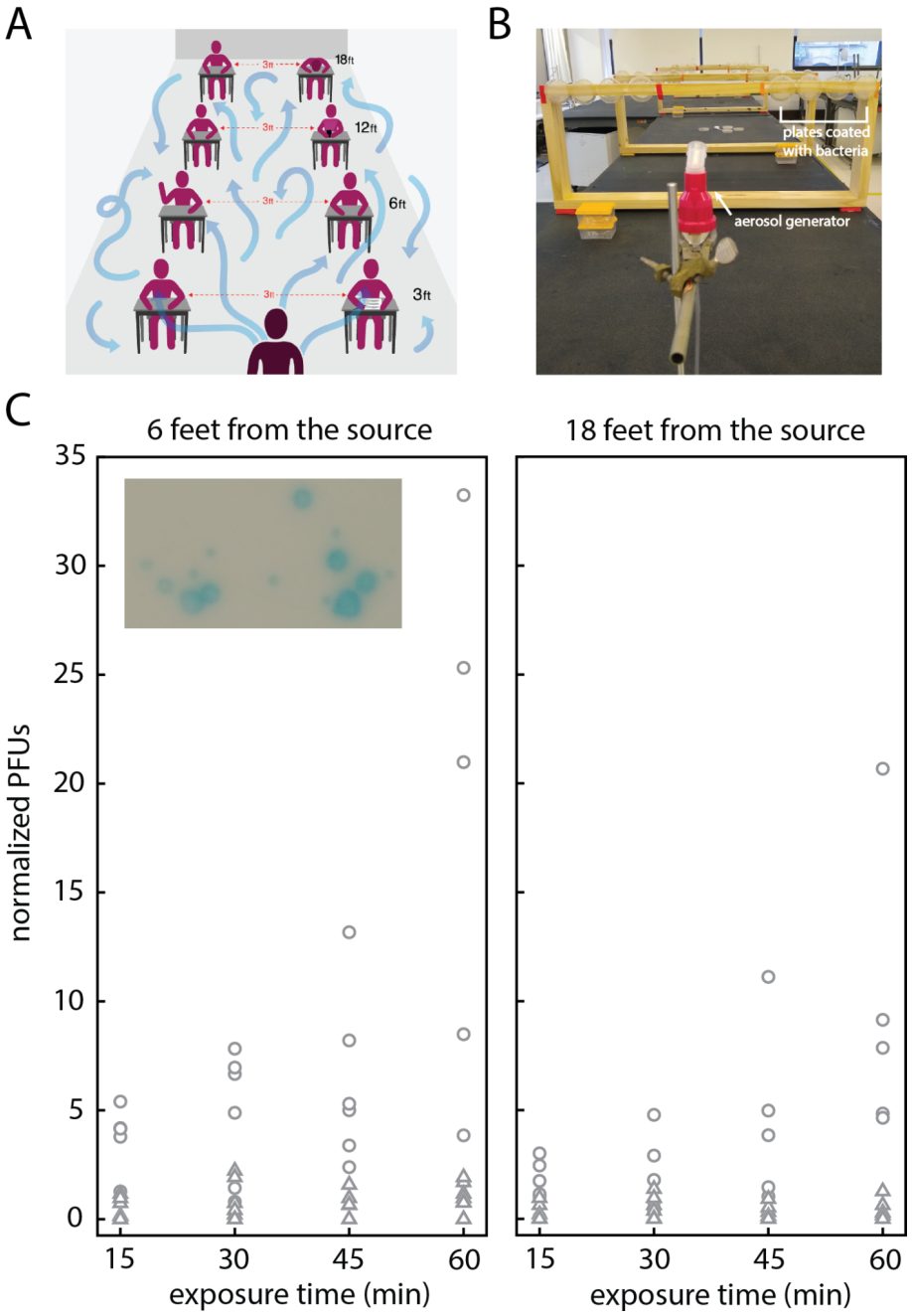

Figure 1: Experiments to determine the spreading of viable aerosolized pathogens in closed spaces. (A) Graphical representation of a typical classroom setting where social distancing is enforced. Blue arrows depict the routes by which aerosolized particles might travel due to diffusion and airflows. (B) Layout of the experiment. Detectors are placed at specified distances away from the aerosol-generating device (located in the front of the photo). At each location we placed 4 detectors which we expose for increasing durations of time: 15, 30, 45 and 60 minutes total. (C) Plaque counts as a function of exposure time for detectors placed 6 feet away (left panel) and 18 feet away from the source (right panel). Data points correspond to data collected in 2 different rooms, in a total of 7 independent experiments. Circles correspond to data collected at relative humidities below 40%, triangles correspond to data points collected at relative humidities above 40%. Counts are normalized to 4×10^7 total phage released. Data at all distances is shown in Fig. S1. Inset - blue plaque morphology shows distinct size differences.

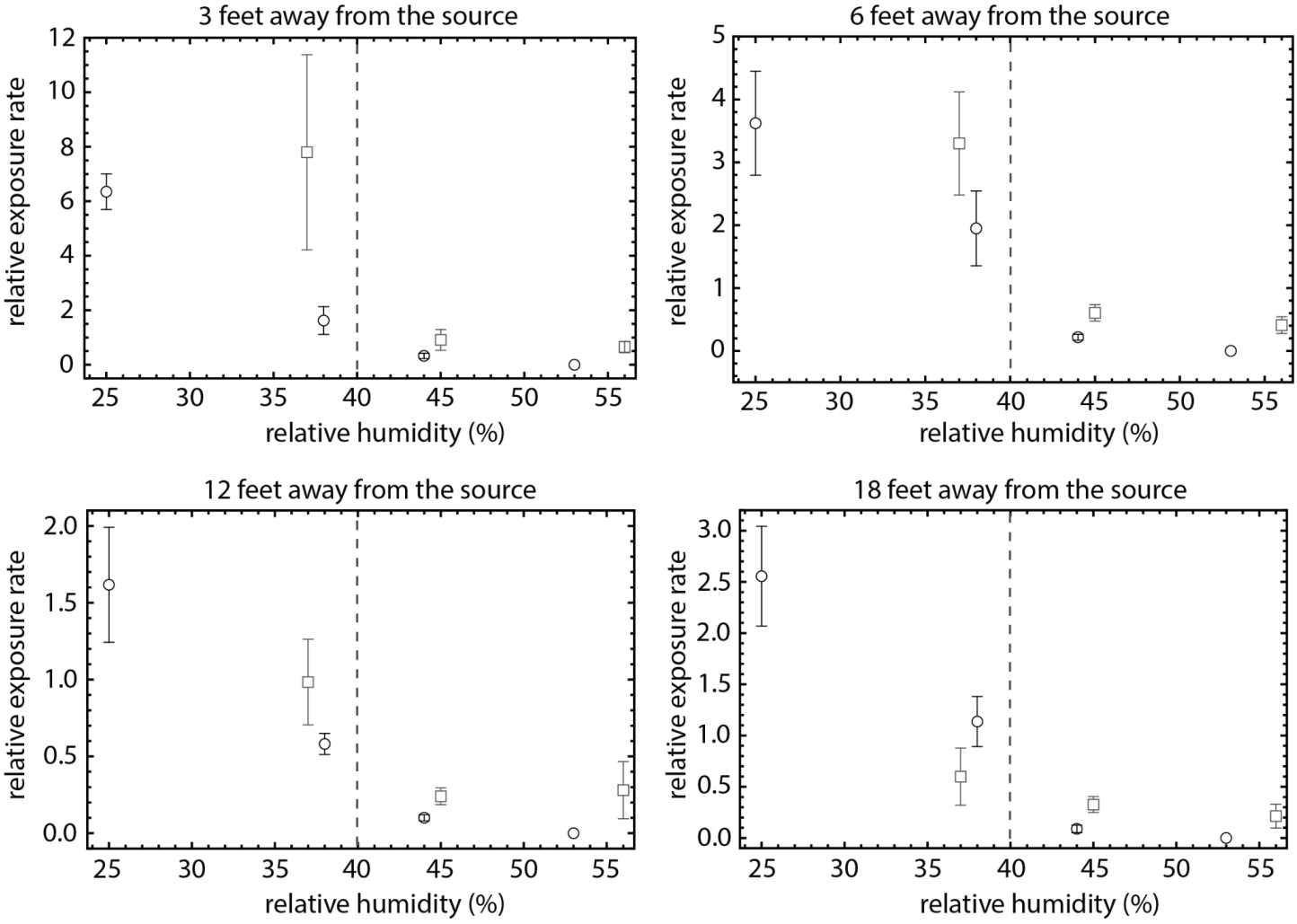

Figure 2. Exposure to aerosolized pathogens decreases with the increase in relative humidity. Panels correspond to exposure at predetermined distances away from the source. Points are averages of PFUs normalized to 4×10^7 total phage released and expressed as a rate per 15-minute exposure for a particular experiment, as a function of humidity, in two classrooms at The New School (rectangles -- Room 300, circles - Room 400). Error bars are S.E. Dashed vertical line at 40% relative humidity visually separates regions of high exposure (humidity below 40%) and low exposure (humidity above 40%).

Supplementary Information

Humidity Reduces Rapid and Distant Airborne Travel of Viable Viral Particles in Classroom Settings

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Methods

Agar Preparation

LB agar and broth were prepared according to the manufacturer's instructions (Fisher Scientific). LB agar plates were supplemented with 200 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal) and 200 µg/mL Ampicillin (Fisher Scientific). LB broth was also supplemented with 200 µg/mL Ampicillin (Fisher Scientific). Plates were poured the night before and kept in the dark to cool. Top agar was prepared using LB broth supplemented with agar (7g per litre). 3 mL of molten top agar was aliquoted into glass tubes and maintained at 48℃ in a heating block. 200 µL of an overnight culture of *Pseudomonas phaseolicola* was added, the contents vortexed gently and poured on top of an LB agar plate. For each batch of *P. phaseolicola* seeded plates, 2 plates were placed in the incubator as controls to assay for contamination.

Determination of titers

Lysate was produced as previously described and stored in glass bottles at 4℃ until use. Before each experiment, the titer was determined using a spot method. We confirmed that the results obtained by using the spot method were comparable to the ones using the agar plate method. Furthermore, we found the spot method provided a measure of sensitivity of the plate to phage landing on its surface. Lysate was serially diluted from 10^{-1} to 10^{-6} in LB. 10 µl of each dilution was spotted onto a seeded plate. The spotted plates were incubated for two days at 25℃. The titer was determined using the formula

Number of plaques * (1,000 µL/mL) / 10 µL / dilution factor = PFU/mL

Choice and Calibration of Nebulizers

Several brands of commercially available nebulizers were examined for their capability to resemble what is known about simulating breathing and coughing and we chose Uni-HEART™ Lo-Flo Continuous Nebulizers from Westmed Inc. owing to their ability to generate aerosols in the range of 2 to 3 µm. We noticed a variability in the rate of aerosolization between individual nebulizer units. We labeled all of our nebulizer units and paired them with dedicated compressors. To calibrate the rate of nebulization, we ran a number of nebulizers in parallel for one hour and periodically monitored their operation. For these runs we used 10 mL of buffer (PBS) and measured the remaining volume after one hour. For our experiments we chose the nebulizers which contained similar volumes of liquid after calibration. We note the rate of nebulization depends on the media used. The nebulizers we used for the experiments expelled 5.6 ± 0.8 mL of lysate in one hour.

Plate placement

The plates and nebulizer were placed on benches that are 37 inches from the floor. Plates were mounted to custom made wooden stands with double-sided mounting tape. The center of the plates was at a height of 17.5 inches (room 300) and 19 inches (room 400) above the bench owing to differences in the height of the stands used in each room. The nebulizer was held aloft using a retort stand and clamp such that the mouth opening of the nebulizer was in line with the center of the agar plates. Air ducts are located to the sides of the bench as shown in Fig. S2. Duct openings are located at a height of 6.75 ft above the surface of the bench and 29 in beneath the ceiling. The ceiling height in both rooms is 12 ft. Air returns are located on the wall adjacent to the doors, approximately in line with the orientation of the benches on which experiments were done. In room 300 the nebulizer was at the opposite end of the air return and in room 400 it was below and next to the air return (see Fig. S2), which allowed us to test two orientations of the experiment with respect to the overall direction of the airflow.

Experimental setup

Each plate was labelled with the date, room number, distance, exposure time and location of the plate with respect to the nebulizer. While the plates were being set up, each nebulizer was started without liquid to allow residual alcohol to evaporate. The humidity and temperature was also monitored for a half hour to establish a baseline before the experiments began using Govee Model:H5074 devices (accuracy ± 0.2℃ and ± 2% RH). Figures S3 and S4 show temperature and humidity charts for each individual experiment.

To start the experiment we added 10 mL of phage lysate to the nebulizer, removed the lids from the plates and started the compressor. At 15, 30, 45 and 60 minute marks an experimenter entered the room, covered and removed a set of plates from the room. After all the plates were removed, the compressor was turned off.

The plates were incubated for 2-4 days at 25℃. Plaques were counted after two days of incubation after which they were moved to the bench. We did not observe additional plaques forming by the 4th day. After the experiment, the nebulizer was detached from the compressor and the remaining liquid removed using a thin tube attached to a syringe. The liquid was removed to a 15 mL conical and the volume recorded. The extracted liquid was kept at room temperature for several days to monitor for contamination while the plates from the experiment were incubating. After each use, the nebulizers were washed in deionized water, the water removed and the unit sprayed with 70% ethanol. The remaining alcohol was removed with a syringe and allowed to dry out before the next experiment. The nebulizers were replaced after 5-10 uses, after they started to leak or if they showed signs of contamination.

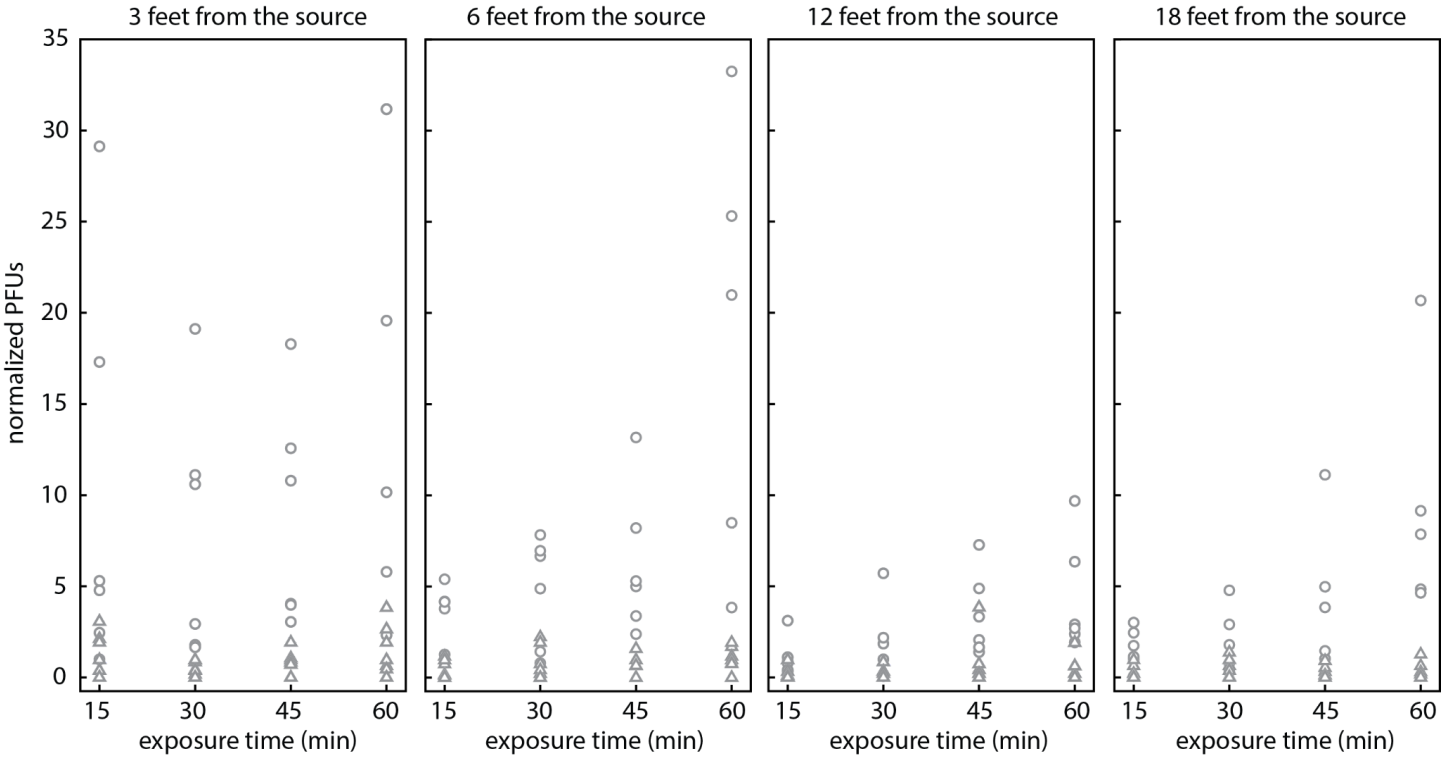

Figure S1: Plaque counts as a function of exposure time for detectors placed at all distances away from the source. Circles - data collected at relative humidities below 40%, triangles - data collected at relative humidities above 40%. Counts are normalized to 4×10^7 total phage released.

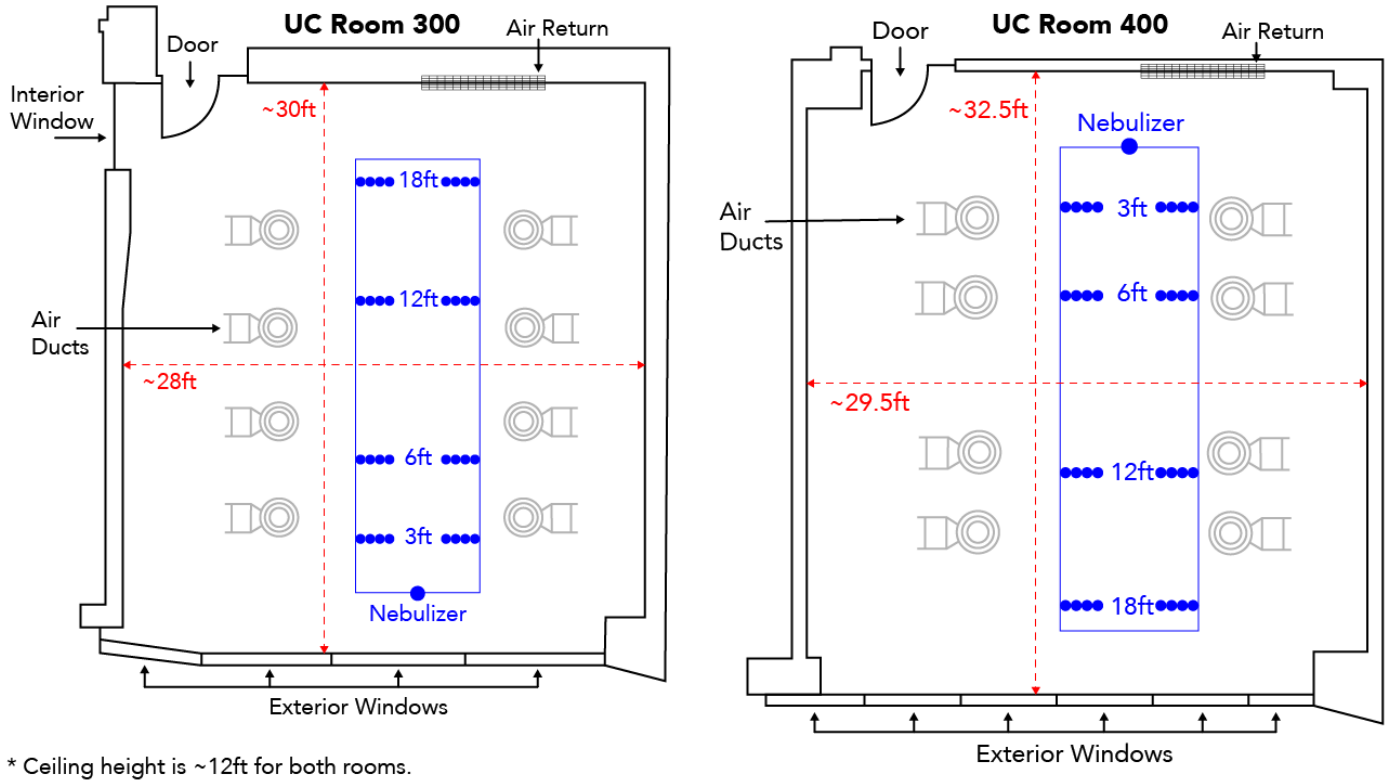

Figure S2: Floor plans of rooms 300 & 400 with approximate dimension, placement of air ducts, air return, and their relation to the experimental setup.

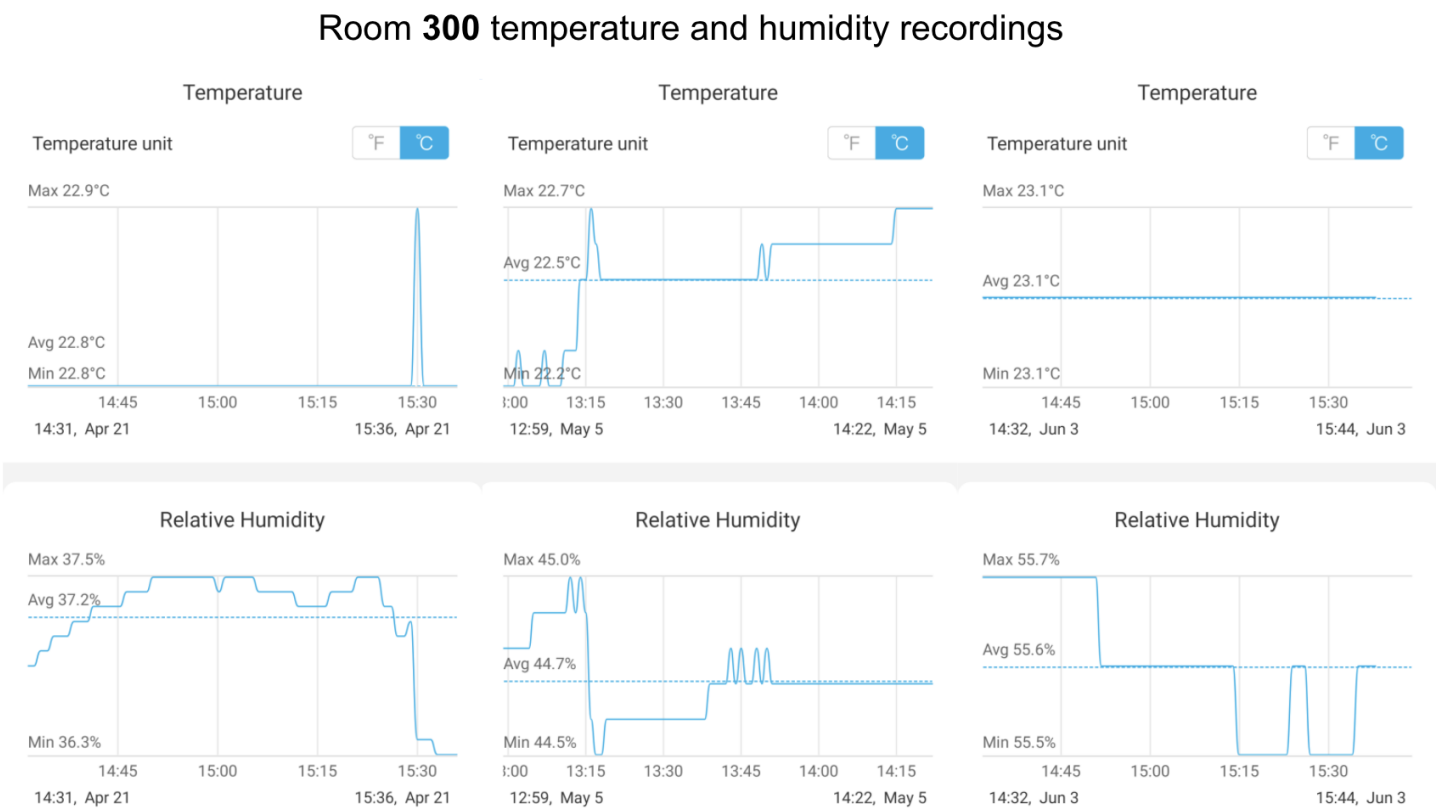

Figure S3: Temperature and humidity data for Room 300

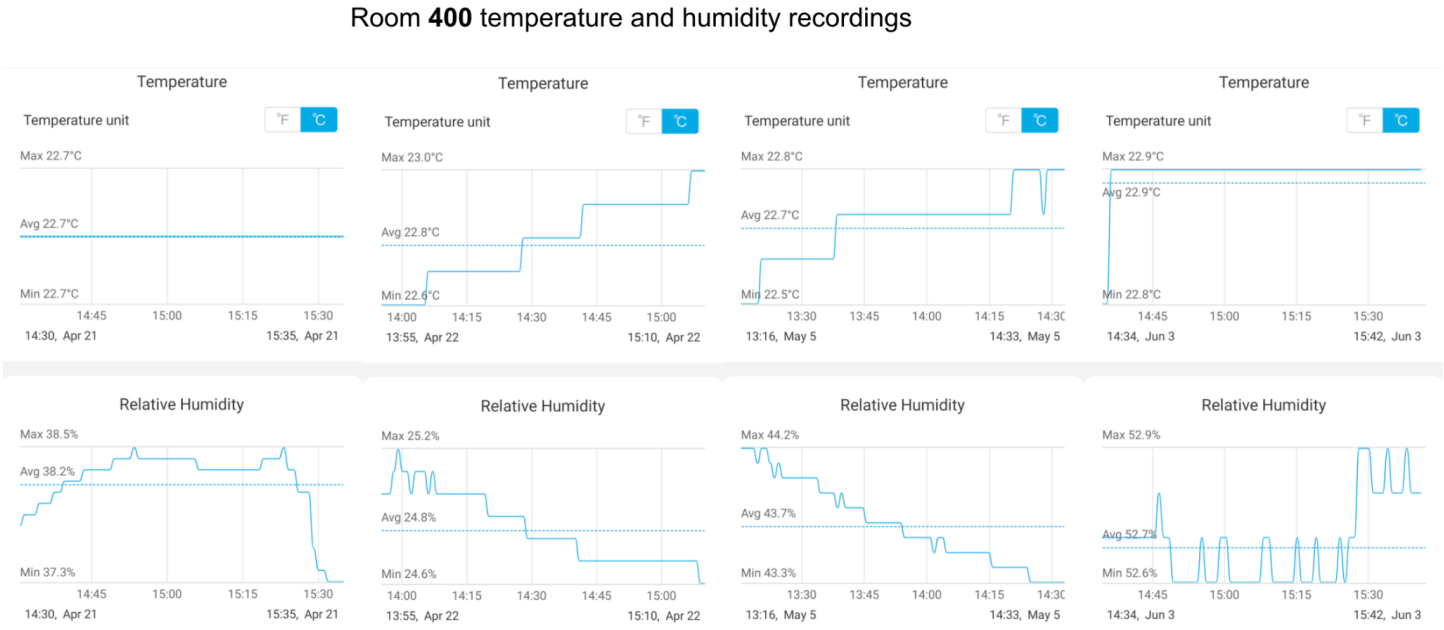

Figure S4: Temperature and humidity data for Room 400

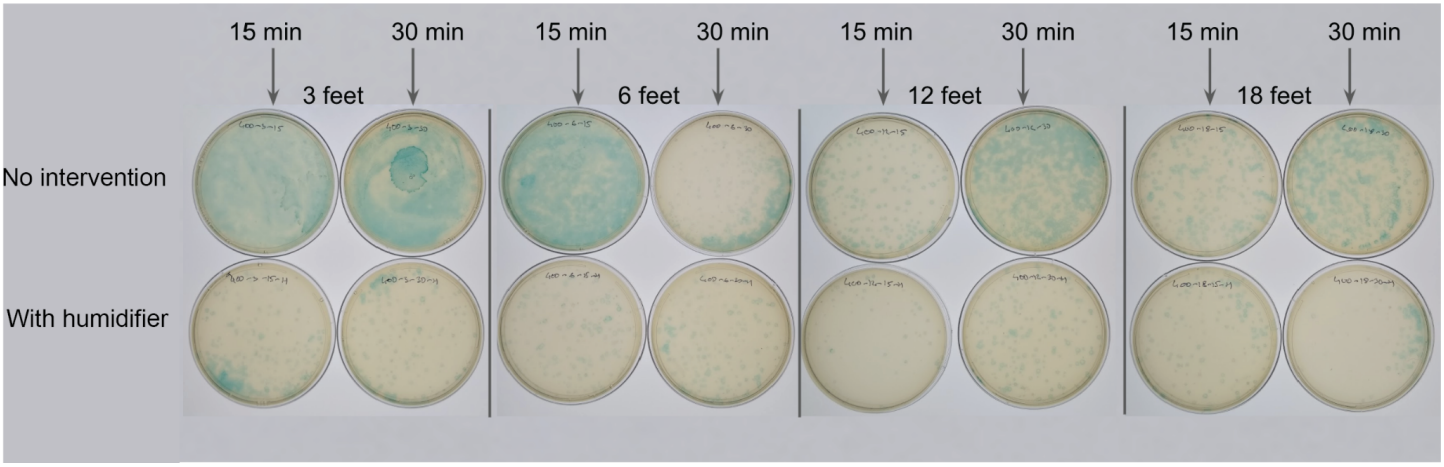

Figure S5: Experiments with personal humidifiers. We tested a number of personal humidifiers which we placed in front of one set of plates, while the other set at the same distance from the source had no intervention. We arranged the humidifiers in a zig-zag pattern to control for port-starboard preference. Plates with the humidifier show reduction of plaque counts when compared to the plates without intervention within the same experiment.

Table 1: Data collected in **Room 300**; plate counts are PFUs (letter C next to an entry means that plate had a contaminant)

Date: April 21st, 2021	Titer $0.9x10^8$ PFU/mL		Volume nebulized: 5.6 mL		Relative Humidity: 37.2%	Average Temp.: 22.8°C	Total phage $5x10^8$	Controls: 0, 0
Replicates	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
Distance/Time	**3 feet**		**6 feet**		**12 feet**		**18 feet**	
15 min	218	367	16	68	14	11	22	1
30 min	37	140	18	84	1	1	10	4
45 min	51	136	166	63	26	42	3	13
60 min	73	128	107C	319	80	122	99	1

Date: May 5th 3rd, 2021	Titer $0.9x10^8$ PFU/mL		Volume nebulized: 4.2 mL		Relative Humidity: 44.7%	Average Temp.: 22.5°C	Total phage $4x10^8$	Controls: 0, 0
Replicates	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
Distance/Time	**3 feet**		**6 feet**		**12 feet**		**18 feet**	
15 min	20	29	11	7	3	1	6	2
30 min	4	8	7	21	3	8	6	13
45 min	10	9	6	15	4	7	3	5
60 min	6	25	16	11	2	18	6	12

Date: June 3rd, 2021	Titer $0.8x10^7$ PFU/mL		Volume nebulized: 5.2 mL		Relative Humidity: 55.6%	Average Temp.: 23.1°C	Total phage $4x10^7$	Controls: 0, 0
Replicates	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
Distance/Time	**3 feet**		**6 feet**		**12 feet**		**18 feet**	
15 min	2	1	1	0	0	1	1	0
30 min	1	1	2	0	0	0	1	1
45 min	0	2	1	1	4	0	0	0
60 min	1	2	1	2	0	0	0	0

Table 2: Data collected in **Room 400**; plate counts are PFUs (letter C next to an entry means that plate had a contaminant; TNTC - too numerous to count -- in those cases we used 300 as a reference value)

Date: April 21st, 2021	Titer $0.9x10^8$ PFU/mL		Volume nebulized: 6.7 mL		Relative Humidity: 38.2%	Average Temp.: 22.7°C	Total phage $6x10^8$	Controls: 0, 0
Replicates	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
Distance/Time	**3 feet**		**6 feet**		**12 feet**		**18 feet**	
15 min	15	37	63	57	5	12	15	37
30 min	27	25	118	12	28	15	7	27
45 min	60	46	36	51	21	25	22	75
60 min	35	295	58	26	29	36	73	70

Date: April 22nd, 2021	Titer $0.7x10^8$ PFU/mL		Volume nebulized: 5.5 mL		Relative Humidity: 24.8%	Average Temp.: 22.8°C	Total phage $4x10^8$	Controls: 0, 0
Replicates	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
Distance/Time	**3 feet**		**6 feet**		**12 feet**		**18 feet**	
15 min	51	46	40	8	4	30	11	29
30 min	184	102	67	47	21	55	28	46
45 min	121	176	51	79	47	70	37	107
60 min	TNTC	TNTC	320	202	28	26	88	199

Date: May 5th, 2021	Titer $1.5x10^8$ PFU/mL		Volume nebulized: 6.4 mL		Relative Humidity: 43.7%	Average Temp.: 22.7°C	Total phage $10x10^8$	Controls: 0, 0
Replicates	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
Distance/Time	**3 feet**		**6 feet**		**12 feet**		**18 feet**	
15 min	9	9	4	3	2	0	1	0
30 min	4	9	5	10	8	5	9	1
45 min	20	17	38	16	5	5	22	3
60 min	92	11	19	18	15	15	7	4

Date: June 3rd, 2021	Titer $0.8x10^7$ PFU/mL		Volume nebulized: 5.8 mL		Relative Humidity: 52.7%	Average Temp.: 22.9°C	Total phage $5x10^7$	Controls: 0, 0
Replicates	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
Distance/Time	**3 feet**		**6 feet**		**12 feet**		**18 feet**	
15 min	0	0	0	0	0	0	0	0
30 min	0	0	0	0	0	0	0	0
45 min	0	0	0	0	0	0C	0	0
60 min	0C	0	0	0	0	0	0	0