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Original Article

Viable influenza A virus in airborne particles expelled during coughs versus exhalations

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Background To prepare for a possible influenza pandemic, a better understanding of the potential for the airborne transmission of influenza from person to person is needed.

Objectives The objective of this study was to directly compare the generation of aerosol particles containing viable influenza virus during coughs and exhalations.

Methods Sixty-one adult volunteer outpatients with influenza-like symptoms were asked to cough and exhale three times into a spirometer. Aerosol particles produced during coughing and exhalation were collected into liquid media using aerosol samplers. The samples were tested for the presence of viable influenza virus using a viral replication assay (VRA).

Results Fifty-three test subjects tested positive for influenza A virus. Of these, 28 (53%) produced aerosol particles containing viable influenza A virus during coughing, and 22 (42%) produced aerosols with viable virus during exhalation. Thirteen subjects had both cough aerosol and exhalation aerosol samples that contained viable virus, 15 had positive cough aerosol samples but negative exhalation samples, and 9 had positive exhalation samples but negative cough samples.

Conclusions Viable influenza A virus was detected more often in cough aerosol particles than in exhalation aerosol particles, but the difference was not large. Because individuals breathe much more often than they cough, these results suggest that breathing may generate more airborne infectious material than coughing over time. However, both respiratory activities could be important in airborne influenza transmission. Our results are also consistent with the theory that much of the aerosol containing viable influenza originates deep in the lungs.

Keywords Aerosols, air microbiology, airborne transmission, cough, infectious disease transmission, influenza.

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Introduction

During an influenza pandemic, measures to stop the transmission of influenza virus will be a critical part of the public health response. Although influenza is known to be transmitted through respiratory secretions containing the virus, infectious material can be passed from person to person in many different ways. The relative importance of the different pathways is uncertain and probably varies depending upon the setting, the severity of the illness, the characteristics of the viral strain, environmental conditions, and other factors.[1] In order to choose the appropriate interventions to block the spread of the virus, it is necessary to understand which routes of transmission occur and when they are likely to be important.

The role of airborne transmission in the spread of influenza has been a question of particular concern to the public health community while planning for a possible pandemic.[2,3] If patients can readily infect others via aerosols (small airborne particles) produced during coughing, speaking, sneezing, and breathing, then interventions such as patient isolation and cohorting, increased air ventilation and filtration, air disinfection, and the use of respirators or other personal protective equipment may help to protect healthcare workers and other patients from the illness. On the other hand, such interventions can be costly and time-consuming

and would place additional burdens on healthcare systems when they are already under considerable strain during a pandemic. Because of these issues, organizations such as the Institute of Medicine and the World Health Organization have called for more research to provide a better understanding of influenza transmission, especially airborne transmission.[3,4]

Several reports have provided support for the idea that airborne influenza transmission can occur.[5–8] Influenza virus RNA has been detected in respirable airborne particles collected in healthcare facilities and other locations.[9–16] Influenza virus RNA also has been found in aerosol particles collected directly from infected patients while they were coughing and breathing.[17–23] Six studies have demonstrated that influenza patients expel airborne particles containing viable virus.[13,18,19,21,24,25] Pantelic *et al.* found that subjects with influenza emitted up to 1000 viable influenza virions over 30 minutes during normal tidal breathing.[25] Lindsley *et al.* detected viable influenza A virus in airborne particles produced during coughing by 7 of 17 influenza patients (41%).[24] However, even with these reports, the likelihood of airborne transmission is still unclear, in part because many questions remain about the production of aerosols carrying infectious influenza during respiratory activities. For example, no studies have compared the production of virus-laden airborne particles between different types of respiratory activities, such as coughing and exhalation. This is an important question, because the airflow dynamics of coughs and exhalations are very different. Coughing produces a high-velocity jet that can propel a plume of aerosol particles long distances, which disperses the airborne particles widely.[26] Exhalations have much lower velocities and are likely to produce higher particle concentrations in the immediate vicinity of a patient and lower concentrations further away. Exhalations are also more common than coughs, which could affect the amount of infectious aerosol that is generated. These differences could have a significant impact on disease transmission and on the choice of interventions.

A comparison of infectious particle production during coughing and exhaling also would provide clues as to the sites of origin of influenza-laden particles from within the respiratory tract. Humans produce more aerosol particles when they cough vs. when they exhale.[27,28] Most of the aerosol particles produced during normal breathing are thought to originate deep in the respiratory tract, while coughing may produce aerosol both from the lower airways and also from the upper airways.[29–31] Thus, if coughing produces much more infectious aerosol than exhaling, this would suggest that much of the virus in cough-generated particles may be coming from the upper airways. Conversely, if the production of infectious aerosol particles during coughing and exhaling is similar, then that would suggest that much of the virus-laden aerosol is originating in the bronchioles and alveoli.

The purpose of this study was to directly compare the production of aerosol particles containing viable influenza virus by infected people during coughs and exhalations. Greater knowledge about the generation of infectious aerosol particles during different respiratory maneuvers will help to better understand the likelihood and dynamics of the possible modes of influenza transmission in different scenarios and will assist in the selection and evaluation of interventions to prevent the spread of disease.

Methods

All procedures involving human subjects were reviewed and approved by the National Institute for Occupational Safety and Health (NIOSH) and West Virginia University (WVU) Institutional Review Boards. Written informed consent was obtained from all study participants.

Aerosol particle collection system

Cough- and exhalation-generated aerosols were collected using an aerosol particle collection system (Figure 1) similar to that described previously.[24] An ultrasonic spirometer (Easy One, NDD Medical Technologies) measured the volume and flow rate of each cough, and a modified 10-liter piston-style mechanical spirometer (SensorMedics model 762609) served as an accumulation chamber for the cough and exhalation aerosols. Aerosol particles were collected using an SKC BioSampler with a 5-ml vessel (#225-9593, SKC) containing 5 ml of viral transport media (VTM) consisting of Hank's balanced salt solution (HBSS;

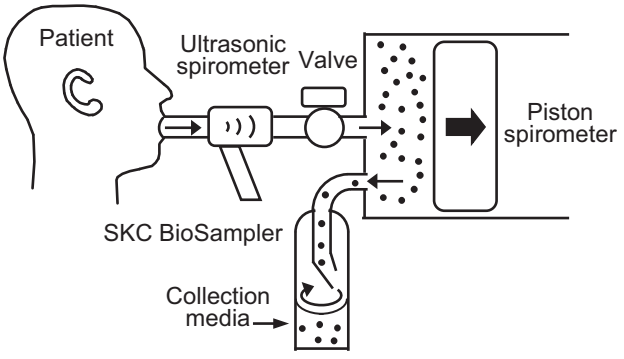

Figure 1. Collection system for airborne particles produced by subjects during coughing and exhalation. Before each respiratory activity, the piston spirometer was purged and partially filled with 4 liters of dry filtered air. The subject then sealed their mouth around the mouthpiece and coughed or exhaled as instructed. The cough or exhalation traveled through the ultrasonic spirometer, which measured the volume and flow rate, and then into the piston spirometer. When the subject was finished, the valve was closed and the SKC BioSampler was used to collect the aerosol particles produced by the subject.

Invitrogen) supplemented with 0·1% bovine serum albumin (BSA; Sigma-Aldrich), 100 units/ml penicillin G, and 100 units/ml streptomycin (Invitrogen). The particle collection efficiency for the SKC BioSampler (i.e., the percentage of particles of a given size that are collected by the sampler) is approximately 10% for particles with aerodynamic diameters of 0·1 μm; 50% for 0·3 μm particles; 96% for 1 μm particles; 100% for 2 μm particles; and 50% for 8 μm particles.[32–34] Particles larger than 10–15 μm are expected to be removed by the sampler elbow and not collected.

Sample collection procedure

Potential test subjects presenting with influenza-like symptoms at an outpatient clinic were recruited after they had been seen by their healthcare provider. Potential participants were excluded from the study if they reported respiratory illnesses such as severe asthma, chronic obstructive pulmonary disease, or tuberculosis; serious illnesses such as diabetes or heart disease; pregnancy; or any condition that would make it difficult or uncomfortable to inhale deeply and cough and exhale forcefully. After the study was explained to the test subject and informed consent was obtained, two nasopharyngeal swabs and an oropharyngeal swab were taken from the subject and placed in 3 ml of VTM (these will be referred to as NOP swabs). The subject's oral temperature was measured and a brief health questionnaire was administered. The subject was then asked to sit in front of the aerosol collection system. The subject was instructed to inhale as deeply as possible, seal their mouth around the mouthpiece, and cough into the machine using as much of the air in their lungs as possible. After each cough, the cough-generated aerosol was collected using the aerosol sampler. This procedure was repeated for a total of three coughs from each subject. Next, the subject was asked to repeat the procedure but to exhale as much and as rapidly as possible rather than coughing. This was also repeated three times, and the exhalation-generated aerosol was collected after each exhalation using the aerosol sampler. The order of the coughing and exhalation was alternated so that odd-numbered subjects were asked to cough three times followed by three exhalations, while even-numbered subjects were asked to exhale three times followed by three coughs. To prevent cross-contamination, the collection system was purged three times with clean dry air after each cough or exhalation, and a new mouthpiece was used for each subject. After the coughs or exhalations were completed, the VTM were removed from the SKC BioSampler and placed in a storage tube. All samples in VTM were kept on ice until the end of the day and then transported to the laboratory and stored at −80°C until analysis. Each subject was only asked to perform one test session.

Viral replication assay (VRA)

In previous studies of cough and exhalation aerosols by our group and others, the largest problem has been detecting the small amounts of viable virus present in these aerosol samples.[13,18,19,21,24] In this study, a viral replication assay (VRA) was used to determine whether viable influenza virus was present in the samples that were collected.[35] The VRA is more sensitive and easier to use with small sample quantities than a traditional viral plaque assay or tissue culture infectious dose assay.[35] In experiments with aerosols containing viable influenza virus, the VRA amplified the amount of infectious virus in the samples by a factor of $4{\cdot}6 \times 10^5$.[35]

Detection of viable influenza virus in NOP swab samples

For the NOP swab samples, Madin Darby canine kidney (MDCK) cells (CCL-34) were plated at a density of $5{\cdot}0 \times 10^4$ per well in a 96-well plate (CoStar 96-well tissue culture plate, Corning). Triplicate wells were treated with 100 μl of each sample for 45 minutes. The wells were washed by adding 100 μl of phosphate-buffered saline (PBS) ml to the inoculum and removing the resulting supernatant. The cells were overlaid with 100 μl supplemented Dulbecco's modified Eagle's medium (DMEM)/F12 and incubated for 20 hours at 35°C in a humidified 5% CO_2 incubator to allow for viral replication. Total RNA was isolated from the cells and supernatant with the MagMax™-96 Total RNA Isolation Kit (Ambion) and transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). A 5 μl cDNA volume was analyzed using quantitative PCR (qPCR) with a custom primer/probe set specific for the matrix (M1) gene or the H3 hemagglutinin gene of influenza A virus. Details about the primers and probes are provided in the online supporting information.

Detection of viable influenza virus in cough and exhalation aerosol samples

For the cough and exhalation aerosol samples, a 6-well formatted VRA assay was used to increase the sensitivity for detecting influenza virus in the aerosol samples. MDCK cells plated at a density of $1{\cdot}5 \times 10^6$ per well (CoStar 6-well tissue culture plate, Corning) were incubated at 35°C in a humidified 5% CO_2 incubator overnight. For each sample, duplicate wells with confluent cellular monolayers were next washed two times with 2 ml PBS (Invitrogen) and treated with a 1·2 ml sample volume for 45 minutes. The wells were washed by adding 1·2 ml of PBS to the inoculum and removing the resulting supernatant. One ml of supplemented Dulbecco's modified Eagle's medium (DMEM)/F12 containing 100 units/ml penicillin G/100 μg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 0·2% BSA

(Sigma-Aldrich), 10 mM HEPES (Invitrogen), 0·22% sodium bicarbonate (Invitrogen), 0·01% DEAE-dextran (MP BioMedicals, LLC, Solon, OH), and 2 μg/ml N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (Sigma-Aldrich) was added to each well. Treated MDCK cells were subsequently incubated for 20 hours at 35°C in a humidified 5% CO_2 incubator to allow for viral replication. The treated cellular monolayer was lysed with 1 ml of MagMax™ Lysis/Binding Solution Concentrate (Ambion) and the lysate was pooled with the reserved culture supernatant (final volume of ~ 2 ml) and stored at −80°C until total RNA was isolated.

To account for the larger sample volume in the 6-well formatted VRA, total RNA was isolated using a modified MagMax™-96 Total RNA Isolation Kit (Ambion) protocol. A 1 ml volume of molecular-grade 2-propanol (Sigma) was mixed by inversion into each thawed, pooled sample followed by the addition of 20 μl prepared Bead Mix (Thermo Scientific). Samples were then gently shaken for 5 minutes and magnetically captured. The supernatant was discarded and the resulting RNA-bound bead pellet was resuspended in 150 μl Wash Solution 1 and transferred to a 96-well processing plate. The manufacturer's instructions were followed for the remainder of the total RNA isolation procedure. Total RNA was eluted with 30 μl of elution buffer and transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). A 5 μl cDNA volume was analyzed for the M1 gene using qPCR.

Data analysis

During the qPCR assay portion of the VRA, the samples were subjected to 45 PCR amplification cycles. The limit of quantitation (LOQ) of the qPCR assay was 15 viral copies per PCR tube, which corresponded to a threshold cycle (C_t) value of 34·1. The limit of detection (LOD) based on qPCR only was 10 viral copies per tube, which corresponded to a threshold cycle (C_t) value of 35·8. In cases where a PCR product was detected but the C_t value was higher than the C_t value for the LOQ, then the PCR product was evaluated by agarose gel electrophoresis to verify that the PCR product was the correct size (101 base pairs for the M1 matrix gene). Sample volumes of 10 μl were loaded into a 4·5% agarose gel (Nusieve GTG Agarose, Lonza) along with 10 μl of a 100-bp DNA ladder (N3231L, New England Biolabs). Electrophoresis was carried out in 1X TAE at 90 volts for approximately 90 minutes. DNA was visualized by ethidium bromide staining. The LOD with this additional step was as little as 1 viral copy per reaction tube. For additional verification, DNA sequence analysis was performed on randomly chosen cough and exhale samples by a commercial laboratory (Genewiz, Inc.) using pre-defined Sanger DNA sequencing.

Because of the low concentration of influenza virus in the cough and exhalation aerosol samples, in many cases the amount of virus detected using the qPCR assay was below the limit of quantitation for the assay. For this reason, the results are reported here only as positive or negative for influenza A. To reduce the possibility of false-positive results, only test subjects who had NOP swabs that were positive for influenza by the M1 and H3 gene assays were considered to be confirmed to have an influenza infection and were used in the data analysis.

When analyzing the experimental data, a sample was considered to be positive for influenza if a PCR product was detected in one or more of the qPCRs and the product was confirmed to be the correct size by gel electrophoresis. For example, each cough or exhalation aerosol sample was tested by inoculating and incubating two culture wells of MDCK cells, isolating and reverse transcribing the RNA produced by the cells in each well, and conducting duplicate qPCR assays for each well. Because of the low amounts of viable influenza found in the cough and exhalation aerosols, many of the qPCRs had C_t values that were close to the maximum limit of 45 cycles for the qPCR assay. For this reason, if any one of the four qPCRs yielded a PCR product of the correct size, then that sample was considered influenza positive even if no PCR product was detected in the other three reactions. The full results from the qPCR assays are presented in the supporting information with the online version of this article.

Statistical analyses included comparison of proportion of positive coughs and exhalations using McNemar's test for paired dichotomous data.[36] The chi-square test was performed to test for differences in positive cough and exhalation proportions between the two orders of testing (cough then exhalation vs. exhalation then cough). All tests were two-tailed and performed using a 0·05 significance level.

Results

For this study, 61 adult volunteer subjects were recruited from college students presenting with influenza-like symptoms at WVU Medicine Student Health Services in Morgantown, West Virginia, USA, during January and February in 2015. A summary of the demographic information, oral temperatures, cough volume, cough peak flow rate, and symptoms reported by the test subjects in which viable influenza A virus was detected is shown in Table 1.

Nasopharyngeal and oropharyngeal (NOP) swabs were tested for viable influenza A virus using the viral replication assay (VRA) with qPCR assays for the M1 matrix gene. Fifty-three NOP swab samples (87%) were positive for viable influenza A. The H3-type hemagglutinin gene was detected in all 53 samples, consistent with the prevalence of H3N2 influenza A in the United States during the 2014-2015 influenza season. Only test subjects with influenza-positive NOP swabs were considered to be confirmed to be infected with influenza and were used in the data analysis.

Table 1. Demographic and medical information for study participants confirmed to be infected with influenza. Information for all of the patients is included in the online supporting information

# Of subjects	53	
Gender	30 Male, 23 Female	
	Mean	SD
Age (years)	21·0	3·4
Height (cm)	172	10
Weight (kg)	76·6	20·0
Temperature (°C)	37·4	0·7
# of days of symptoms	2·2	2·1
Cough volume (liters)	2·7	1·1
Peak flow rate during coughs (liters/second)	7·5	2·2
Exhalation volume (liters)	3·5	1·0
Peak flow rate during exhalation (liters/second)	4·8	2·1
Number of subjects reporting		
Fever/chills	43	
Headache	40	
Fatigue	43	
Cough	44	
Sore throat	41	
Sinus congestion	32	
Runny nose	37	
Sneezing	28	
Muscle aches	43	
Took medication for symptoms	27 yes, 26 no	
Received influenza vaccine within last 6 months	6 yes, 43 no, 4 unsure	

Viable influenza A virus was found in cough aerosol samples from 28 of 53 subjects and in exhalation aerosol samples from 22 of 52 subjects confirmed to have influenza (one exhalation aerosol sample was lost before analysis). The difference in the number of influenza-positive coughs vs. influenza-positive exhalations was not statistically significant ($P = 0{\cdot}2207$). 37 subjects had influenza-positive NOP swabs and influenza-positive cough or exhalation aerosols, while for 15 subjects, influenza was detected in the NOP swabs but not in the cough or exhalation aerosols. Thirteen subjects had both cough aerosol and exhalation aerosol samples that contained viable influenza A virus, 15 had positive cough aerosol samples but negative exhalation samples, 9 had positive exhalation samples but negative cough samples, and 15 had negative cough and exhalation samples. The order in which the experiment was performed (coughs followed by exhalations, or exhalations followed by coughs) did not have a significant effect on the results ($P = 0{\cdot}2499$). The influenza results for all test subjects are shown in Table 2.

To confirm that the qPCRs in the VRA were amplifying influenza virus, the size of the PCR products were verified by agarose gel electrophoresis. An example electrophoretic gel is

Table 2. Presence or absence of viable influenza A virus in NOP swabs, cough aerosol particles, and exhalation aerosol particles for each patient. H3 and M1 indicate the influenza A gene that was targeted in the PCR portion of the VRA

Patient ID	NOP swab (M1)	NOP swab (H3)	Cough (M1)	Exhalation (M1)
Subjects confirmed to have influenza				
FC134	+	+	–	–
FC135	+	+	–	–
FC136	+	+	+	+
FC137	+	+	+	–
FC138	+	+	+	+
FC139	+	+	–	+
FC140	+	+	+	+
FC141	+	+	–	–
FC142	+	+	–	–
FC143	+	+	+	+
FC144	+	+	–	–
FC145	+	+	+	–
FC146	+	+	–	+
FC150	+	+	+	+
FC151	+	+	–	+
FC152	+	+	+	–
FC153	+	+	–	+
FC154	+	+	+	–
FC155	+	+	+	–
FC157	+	+	+	–
FC158	+	+	+	–
FC159	+	+	+	+
FC160	+	+	–	–
FC161	+	+	–	+
FC162	+	+	–	–
FC163	+	+	–	–
FC164	+	+	+	+
FC165	+	+	–	+
FC166	+	+	+	–
FC167	+	+	+	+
FC168	+	+	+	–
FC171	+	+	–	–
FC172	+	+	–	+
FC173	+	+	–	–
FC174	+	+	–	–
FC175	+	+	–	+
FC176	+	+	+	–
FC177	+	+	–	–
FC178	+	+	+	+
FC179	+	+	+	+
FC180	+	+	+	–
FC181	+	+	+	–
FC182	+	+	–	–
FC183	+	+	–	–
FC184	+	+	+	+
FC185	+	+	+	–
FC186	+	+	+	–
FC187	+	+	–	Lost
FC188	+	+	+	+
FC190	+	+	–	+
FC191	+	+	–	–

Table 2. (*Continued*)

Patient ID	NOP swab (M1)	NOP swab (H3)	Cough (M1)	Exhalation (M1)
FC192	+	+	+	+
FC193	+	+	+	–
Positive	53	53	28	22
Negative	0	0	25	30
Total	53	53	53	52
Subjects not confirmed to have influenza				
FC133	–	+	+	–
FC147	–	–	+	–
FC148	–	–	–	–
FC149	–	–	–	–
FC156	–	–	+	–
FC169	–	–	–	–
FC170	–	+	–	–
FC189	–	–	–	+

shown in Figure 2. A total of 484 qPCRs were performed to analyze the cough and exhalation aerosol samples from the 61 subjects. Of these, a matrix gene PCR product was detected in 89 reactions. In 79 reactions (89%), gel electrophoresis of the PCR product produced a 101-base pair band, indicating the presence of influenza A. In the remaining 192 PCRs, a PCR product was not detected and no 101-base pair bands were observed. The PCR products from 12 H3 gene analyses of the NOP swabs also were tested and found to be of the correct size (150 base pairs). As additional verification, the VRA M1 gene PCR products from 7 cough aerosol samples and 9 exhalation aerosol samples were sent to a commercial laboratory for sequence analysis. All 7 of the cough aerosol PCR products and 7 of the 9 exhalation aerosol PCR products were confirmed to match the matrix gene segment M1 from influenza A. Two of the exhalation aerosol PCR products could not be sequenced.

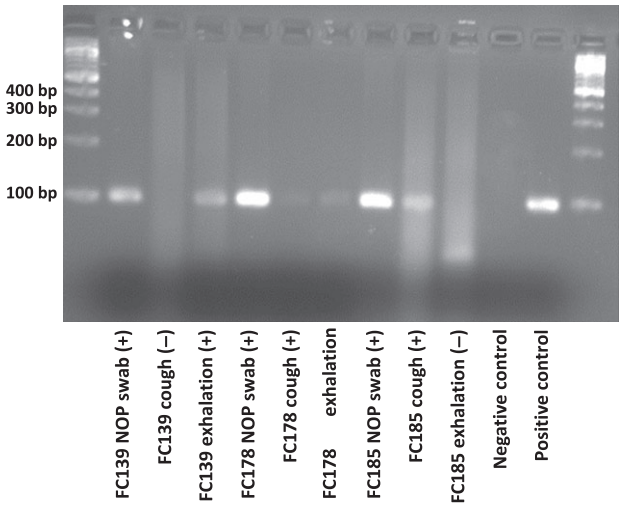

Figure 2. Electrophoretic gel used to determine the presence or absence of a 101-base pair PCR product corresponding to the influenza A M1 matrix gene. The PCR products for the NOP swabs, cough aerosols, and exhalation aerosols for three test subjects are shown. (+) indicates the sample is positive for influenza A. (–) indicates the sample is negative. The PCR products for the cough and exhalation samples for subject FC178 were confirmed to be from the influenza A M1 matrix gene by DNA sequence analysis. The negative control contained all PCR reagents, primers, and probe but no template. The positive control contained 10^4 M1 copies and was run in parallel with the experimental samples.

Discussion

Humans infected with influenza virus have been shown to expel small airborne particles containing viable virus into the environment when they cough or exhale, which suggests that the potential exists for the airborne transmission of influenza.[13,18,19,21,24] However, it is not clear how often airborne transmission actually occurs or what factors affect the likelihood of transmission by the airborne route, in part because many questions remain about the processes involved in infectious aerosol production and the dynamics of these aerosols in the environment. Consistent with previous studies, our results show that aerosol particles containing viable influenza virus are produced by infected individuals both during coughing and during exhalation. Viable virus was detected more often in cough aerosol samples (53% of influenza-positive subjects) compared to exhalation aerosols (42% of influenza-positive subjects). However, this difference is not substantial and was not statistically significant. As people breathe constantly but cough sporadically, this suggests that patients infected with influenza may release more virus into the air over time in small airborne particles by breathing compared to coughing. On the other hand, as coughing involves much higher air velocities than breathing, coughing may spread the virus further in a given location. Thus, both mechanisms for producing infectious aerosols may be important depending upon such factors as the distance from a patient, the timescale, the infectious dose, and the air flow within a room.

Viable influenza virus was detected in the cough aerosol, exhalation aerosol, or both from 37 of 53 influenza-positive test subjects (70%), which suggests that this is a common phenomenon. It should be noted that the aerosol collection system used in these experiments does not capture particles larger than 10–15 μm in the collection media, and thus collects only small particles capable of airborne transmission and not the "large droplets" often referenced in droplet disease transmission. Viable influenza was detected in both the cough and exhalation aerosols for 35% of these subjects (13/37), while it was only detected in the cough aerosol for 41% (15/37) and only in the exhalation aerosol for 24% (9/37). These results are consistent with somewhat more

infectious aerosol being released during coughing than breathing, although they probably also reflect the fact that the airborne viable virus concentrations are quite low and are difficult to detect.

Two patients had influenza-positive cough aerosols but negative NOP swabs, while one had a positive exhalation aerosol but a negative NOP swab. One possible explanation is that, because some patients did not tolerate the nasopharyngeal swabs well, the sample obtained may not have been sufficient for detection of influenza. Alternatively, Milton *et al.* [19] reported that the amount of influenza RNA detected in NOP swabs was only weakly correlated with the amount detected in exhaled breath; thus, it may be that these three patients had sufficient influenza virus in their lower respiratory tract to produce infectious aerosol particles but insufficient virus in their nasopharyngeal region to be detected. Finally, the possibility of a false-positive cough or breath sample or a false-negative NOP swab result cannot be excluded. For consistency and to reduce the possibility of false-positive results, only patients with positive NOP swabs were considered to be confirmed to be infected with influenza and included in our analysis.

Because of the low concentrations of airborne viable influenza virus in the cough and exhalation aerosol samples, we were not able to quantify the amount of airborne viable virus present in the original samples in our experiments. However, this should not be interpreted to mean that the risk of infection is low. Our samples were collected from only three coughs and three exhalations, while a person infected with influenza would be expected to cough dozens of times and breathe hundreds of times per hour and thus could still release a considerable amount of airborne infectious material over the course of a day. In addition, the infectious dose for airborne influenza is very low; one study found that inhalation of small aerosol particles containing only 0·7 to 3·5 plaque-forming units (PFU) of influenza was sufficient to cause seroconversion in 50% of the human subjects tested.[37]

The fact that the number of aerosol samples with viable influenza was not significantly greater for coughing than for exhalation is consistent with the theory that a substantial portion of the influenza-laden aerosol produced by infected people originates in the deepest parts of the lungs rather than in the upper airways and oropharyngeal region. Smaller aerosol particles have been proposed to be produced in the alveolar and bronchial regions during both breathing and coughing by the formation and rupture of menisci as airways contract and expand. Larger particles are thought to be created by shear forces acting on fluid-covered upper airways, where air velocities are much higher than in the deeper regions. This phenomenon is thought to occur primarily during coughing because the air flow rates are much higher than during breathing.[29–31] Since, in this theory, deep lung particle generation occurs during both breathing and coughing while upper airway particle generation occurs only during coughing, then the modest increase in the number of positive samples seen during coughing compared to exhalation in our experiments supports the idea that much of the infectious aerosol is originating in the deep lung regions.

The ability of our system to collect cough and exhalation aerosols separately was useful for the present study, but it also significantly limited the study because of the small amount of aerosol that was collected. By comparison, the system used by Milton *et al.* collects aerosols produced by natural coughs and exhalations over a 30-minute period, and that group has reported greater success in detecting and quantifying airborne influenza virus.[19,25,38] Thus, our results suggest that future work studying infectious aerosol production and the presence of infectious aerosols in the environment should collect sample volumes that are as large as practically possible, which would likely entail using high sample flow rates and long sample times. Unfortunately, however, maintaining high flow rates and long sample times while attempting to collect airborne viruses and maintain their viability is very challenging, especially when the viruses are contained in submicrometer aerosol particles.

Finally, it is important to acknowledge the limitations of our experiments. First, the single most difficult aspect of studying the production of aerosols containing viable influenza virus during respiratory activities is the low concentration of such viruses in the air and the difficulty in collecting enough material and maintaining viability to detect the viable virus. To maximize the sensitivity of our assays and reduce the possibility of false-negative results as much as possible, the evaluation criteria for our results were designed to provide the greatest likelihood of detecting small amounts of viable influenza virus, with steps then taken to minimize the possibility of false-positive results. However, we recognize that the possibility of some false-positive outcomes cannot be ruled out in our analysis.

Second, our test subjects were asked to inhale as deeply as possible and then cough or exhale using as much of the air in their lungs as possible. Most natural coughs and normal tidal breathing use smaller fractions of the total lung capacity, which may reduce aerosol generation. On the other hand, natural coughs are stimulated by a need to clear secretions from the airways, and thus, natural coughs may produce more aerosol particles than forced coughs. It is also possible that the ratio of the amount influenza-laden aerosol particles produced during natural coughing to that produced during natural breathing may be different than the ratio we found when comparing forced coughs to forced exhalations.

Third, the particle collection efficiency of the SKC BioSampler decreases from about 96% for 1 μm aerosol particles to about 50% for 0·3 μm particles and 10% for

0·1 μm particles.[32–34] Thus, many of the smallest particles carrying influenza virus may not have been collected in our experiments. As noted earlier, particles larger than 10–15 μm were not collected and thus their potential contribution to disease transmission is not known. In addition, some cough aerosol particles may have deposited inside the system before they could be collected. In our previous study using the cough aerosol collection system,[24] swab samples from the face of the spirometer piston and the BioSampler elbow found little influenza, suggesting that particle losses in these locations were minimal. However, other parts of the system, such as the mouthpiece, were not tested.

Last, influenza viral shedding peaks around the first day of acute respiratory illness and then declines rapidly.[19,21,39–41] In our study, patients presented at the clinic an average of two days after their symptoms developed (Table 1), well after the expected maximum in viral shedding. In addition, our test subjects were college-aged ambulatory outpatients with no other reported respiratory illnesses or significant health conditions. Patients who are more severely ill would generally be expected to have higher viral loads and may be more likely to produce cough and exhalation aerosols containing infectious influenza virus, especially in the early stages of illness.[40,42] Patients who are younger or older, immunocompromised, or have underlying pulmonary illness such as asthma or chronic obstructive pulmonary disease also may have very different infectious aerosol generation patterns. This could be an important factor during an influenza pandemic, when healthcare facilities would be expected to receive large numbers of severely ill patients.

Conclusions

The purpose of this study was to directly compare the expulsion of aerosol particles containing potentially infectious influenza virus during coughing and exhalation. Our results confirm that the production of aerosols containing viable influenza virus is common among infected people. Viable virus was detected more often in cough aerosols than in exhalation aerosols, but the difference was not large. As individuals breathe more often than they cough, these results suggest that breathing may generate more airborne infectious material than coughing over time. However, both respiratory activities could be important in airborne influenza transmission. Our results are also consistent with the theory that much of the aerosol containing viable influenza originates deep in the lungs, although more direct investigation would be needed to verify this.

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AUTHOR BIOGRAPHIES

William G. Lindsley, designed and constructed the aerosol collection system, coordinated the study, analyzed the data, and was the primary author of the manuscript.

Francoise M. Blachere, developed the viral replication assay and adapted it to this work, developed the qPCR assay for the H3 analysis, analyzed the samples, assisted in the data analysis, and contributed to the writing of the manuscript.

Donald H. Beezhold, helped develop the concept for the study, provided input to the methodology and analysis, and contributed to the writing of the manuscript.

Robert E. Thewlis, assisted with the development of the clinical methodology, coordinated the study with the clinic staff, provided logistical support, and recruited subjects for the study.

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William T. Goldsmith, developed the equipment and methodology to collect the cough and exhalation airflow data, analyzed the data, and collected samples in the clinic.

Cynthia M. McMillen, conducted the assays for the H3 analysis, assisted with the sample processing and analysis, and analyzed the H3 data.

Michael E. Andrew, performed the statistical analysis of the data, assisted in the interpretation, and contributed to the writing of the manuscript.

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John D. Noti, helped develop the viral replication assay and adapt it to this work, provided input and advice on the sample processing and data analysis, supervised the overall research effort, and contributed to the writing of the manuscript.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. For the direct PCR analysis of the nasopharyngeal and oropharyngeal (NOP) swabs, viral RNA was extracted directly from 50 μl of each NOP sample.