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Viable Influenza A Virus in Airborne Particles from Human Coughs

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Patients with influenza release aerosol particles containing the virus into their environment. However, the importance of airborne transmission in the spread of influenza is unclear, in part because of a lack of information about the infectivity of the airborne virus. The purpose of this study was to determine the amount of viable influenza A virus that was expelled by patients in aerosol particles while coughing. Sixty-four symptomatic adult volunteer outpatients were asked to cough 6 times into a cough aerosol collection system. Seventeen of these participants tested positive for influenza A virus by viral plaque assay (VPA) with confirmation by viral replication assay (VRA). Viable influenza A virus was detected in the cough aerosol particles from 7 of these 17 test subjects (41%). Viable influenza A virus was found in the smallest particle size fraction (0.3 μm to 8 μm), with a mean of 142 plaque-forming units (SD 215) expelled during the 6 coughs in particles of this size. These results suggest that a significant proportion of patients with influenza A release small airborne particles containing viable virus into the environment. Although the amounts of influenza A detected in cough aerosol particles during our experiments were relatively low, larger quantities could be expelled by influenza patients during a pandemic when illnesses would be more severe. Our findings support the idea that airborne infectious particles could play an important role in the spread of influenza.

Keywords aerosols, air microbiology, airborne transmission, cough, infectious disease transmission, influenza

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INTRODUCTION

Influenza is of great concern to the occupational health community because of the annual burden of seasonal outbreaks and the potential for newly emerging strains to cause a severe global pandemic. During a pandemic, health care workers would be at tremendous risk for exposure to the virus as they care for a surge of infected patients. To implement effective infection control measures in health care facilities, the pathways by which the disease spreads from person to person need to be identified so that transmission can be interrupted. Although influenza is known to infect people through contact with respiratory secretions containing the virus, it is possible to transfer this infectious material between people in many different ways, and the importance of different routes of infection is not yet known.[1]

One possible mode of influenza transmission that is of particular concern is infection by the inhalation of airborne particles that are produced by infected patients as they cough, sneeze, speak, and breathe. If influenza is disseminated to a significant degree by airborne transmission, this would suggest that interventions in health care facilities such as improved ventilation and filtration, air disinfection, patient isolation and cohorting, and the use of respirators or other personal protective equipment might be required to reduce its spread, especially during a pandemic. However, the role of aerosol particles in the dispersion of influenza has not been clearly determined. Some investigators have concluded that transmission by airborne particles accounts for a significant

fraction of influenza cases,[2–4] while others believe that it rarely, if ever, occurs.[5]

The likelihood of someone becoming infected with influenza by inhaling virus-laden aerosol particles depends in part upon the amount of aerosolized virus to which the person is exposed and the size of the particles carrying the virus.[6] Several studies have shown that airborne influenza viral RNA can be detected in a variety of health care facilities.[7–13] Airborne particles containing influenza RNA were found up to 1.8 m from hospitalized patients with influenza [10] in one study, and up to 3.7 m away from patients at home in another study.[11] Two studies measured airborne influenza in a hospital emergency department and an urgent care outpatient clinic, and determined that about half of the influenza viral RNA was contained in airborne particles less than 4 μm in aerodynamic diameter.[12,13] Aerosol particles in this size range (called the respirable size fraction) are of particular concern because they can remain airborne for an extended time and are easily inhaled, and because they can reach the deepest regions of the lungs.

Airborne particles containing influenza virus have also been detected in samples collected directly from influenza patients.[14–19] Multiple respiratory viruses, including influenza, were found in particles $\leq$5 μm that were collected during breathing (80% of samples) and coughing (82%) from 12 adults and 41 children with symptomatic respiratory infections.[18] In another study, influenza patients were found to exhale about 19 viral copies/minute in particles $<$5 μm.[17] During the 2009 H1N1 influenza pandemic, influenza virus RNA was detected in the cough aerosol particles from 81% of influenza patients in an outpatient clinic, and 65% of the influenza RNA was found in respirable particles.[16]

Although these studies have demonstrated that patients do expel influenza virus in airborne particles, a frequent criticism has been that the potential for the virus in the aerosols to infect someone is unknown, because PCR-based methods cannot distinguish between viable and non-viable virus. Only four studies have reported detecting viable influenza virus in airborne particles, and these results were limited. Lindsley et al.[16] were able to culture influenza virus from aerosol samples collected from 2 out of 20 influenza patients during voluntary coughs. Similarly, Milton et al.[17] detected viable influenza in the fine particle fraction ($<$5 μm) from 2 of their 37 patients. Lednicky and Loeb [11] detected viable airborne influenza virus in particles from 1 μm to $<$0.25 μm up to 3.7 m from their patients. Hatagishi et al.[19] detected viable influenza in droplets and aerosol particles collected on dry gelatin filters from 3 of 56 influenza patients. These studies demonstrate that at least some influenza patients do eject aerosol particles containing viable influenza virus into the environment, but because of the limited sample numbers, many questions remain. It is not clear what fraction of influenza patients release infectious virus in aerosols, how much infectious virus is released, or how much this varies depending upon the stage or severity of the illness or the strain of the virus.

The purpose of this study was to examine the production of infectious aerosols by patients with influenza when they cough. A better understanding of the amount of infectious airborne material released by patients and the size of the particles carrying the virus will assist in assessing the possible role of airborne virus-laden particles in the spread of influenza and in determining the appropriate precautions to prevent transmission of the virus.

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.

Cough-generated aerosols were collected using a cough aerosol particle collection system (Figure 1) similar to that described previously.[16] An ultrasonic spirometer (Easy One, NDD Medical Technologies, Andover, MA) measured the volume and flow rate of each cough, and a modified 10 liter piston-style mechanical spirometer (model 762609, SensorMedics, Yorba Linda, CA) served as an accumulation chamber for the cough aerosol. A stainless steel tray was attached to the piston of the mechanical spirometer to collect any large ballistic cough spray droplets that impacted the piston. Cough aerosol particles were collected using an SKC BioSampler with a 5 ml vessel (#225-9593, SKC, Eighty Four, PA) containing 5 ml of viral transport media (VTM) consisting of Hank's Balanced Salt Solution (HBSS, Invitrogen, Life Technologies, Grand Island, NY) supplemented with 0.1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO), 100 units/ml penicillin G and 100 units/ml streptomycin (Invitrogen). The SKC BioSampler collects particles with aerodynamic diameters of approximately 0.3 μm to 8 μm into the collection media, although the upper cut-off diameter is not sharp.[20–22] The SKC BioSampler has an elbow at its inlet. Based on studies of particle collection in bends,[23] particles approximately 10 to 15 μm and larger in diameter would be expected to accumulate in the elbow.

The test procedure was as follows: After the study was explained to the test subject and informed consent was obtained, two nasopharyngeal swabs were taken from the subject (for participants in 2014, an oropharyngeal swab was also collected and combined with the nasopharyngeal swabs) and placed in 3 ml of VTM. 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 cough aerosol collection system. The subject was instructed to inhale as deeply as possible, seal his or her mouth around the mouthpiece, and cough into the machine using as much of the air in his or her lungs as possible. After each cough, the cough-generated aerosol was collected using the aerosol sampler. This procedure was repeated for a total of six coughs from each subject.

After all coughs were completed, the spray droplet tray was removed from the spirometer and swabbed with 5 ml of

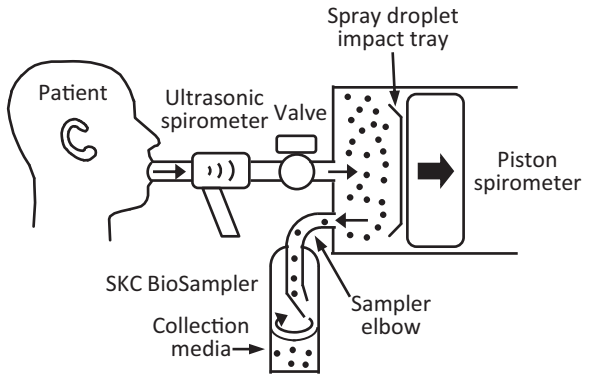

FIGURE 1. Cough aerosol particle collection system. Before each cough, the piston spirometer was purged and partially filled with 4 L of dry filtered air. When the patient coughed into the mouthpiece, the cough flowed through an ultrasonic spirometer which measured the cough volume and flow rate. The cough then flowed through a valve and into the piston spirometer, displacing the piston to the right. The droplet tray collected any large drops that impacted it. When the subject finished coughing, the valve was closed and the SKC BioSampler was turned on. The cough aerosol was pulled out of the spirometer and collected by the aerosol sampler. Airborne droplets larger than about 10–15 μm collected in the sampler elbow, while smaller particles were collected in the sampler collection media. As the aerosol sampler drew air, the piston moved to the left until no air remained in the spirometer.

VTM to recover any virus in droplets that had impacted on the tray. The elbow was removed from the SKC BioSampler and swabbed with 3 ml of VTM to remove any virus that had impacted in the elbow during collection. The VTM collection media was removed from the SKC BioSampler and placed in a storage tube. All samples in VTM were placed on ice until the end of the day and then transported to the laboratory and stored at -80°C until analysis.

Viral plaque assays (VPA) were performed as described previously.[24] Briefly, confluent monolayers of Madin Darby canine kidney (MDCK) cells (CCL-34) were treated with 0.8 ml of the clinical samples for 45 min, washed, and overlaid with an agarose medium solution. The cells were then incubated at 35°C for 48 hr. Plaques were stained with crystal violet and visually enumerated, and the numbers of plaque forming units (PFU) were calculated. The MDCK cells were a kind gift from Dr. Daniel Perez (University of Maryland, College Park, MD).

To confirm that the samples with positive plaque assay results contained viable influenza A virus, a viral replication assay (VRA) was used as described previously.[24] For the 2011 samples, the VRA was performed on RNA isolated from the MDCK cell monolayer only (not the cell supernatant), while for the 2014 samples the VRA was performed on RNA isolated from both the cell monolayer and the cell supernatant.

For all of the 2011 samples and the 2014 nasopharyngeal swab samples, MDCK cells in a 96-well plate were treated with 50 μl of each sample for 45 min and incubated for 20 hr at 35°C in a humidified 5% CO_2 incubator. Total RNA was isolated with the MagMax-96 Total RNA Isolation Kit (Ambion, Grand Island, NY) and transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY). A 5 μl cDNA volume was analyzed using quantitative PCR (qPCR) with a custom primer/probe set specific for the matrix (M1) gene of influenza A virus.

For the 2014 cough aerosol samples, a six-well formatted VRA protocol was used to increase the sensitivity of the assay. MDCK cells plated at a density of 2.0×10^6 per well (CoStar 6-well tissue culture plate, Corning, Tewksbury, MA) were incubated at 35°C in a humidified 5% CO_2 incubator overnight. Confluent cellular monolayers were next washed two times with 2 ml 1X PBS (Invitrogen) and treated with a 1200 μl sample volume. Following 45 min of adsorption, the sample inoculum was removed and 1 ml of supplemented Dulbecco's modified Eagle's medium (DMEM)/F12 containing 100 units/ml penicillin G/100 μg/ml streptomycin (Invitrogen, Gibco, Grand Island, NY), 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 at 35°C in a humidified 5% CO_2 incubator. Following 20 hr of incubation, the treated cellular monolayer was lysed with 1 ml of MagMax Lysis/Binding Solution Concentrate (Ambion), pooled with the reserved culture supernatant (final volume of $\sim$2 ml) and stored at -80°C until total RNA was isolated.

To account for the larger sample volume in the six-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 30 μl prepared Bead Mix (Thermo Scientific, Waltham, MA). Samples were then gently shaken for 5 min and magnetically captured. Supernatant was discarded; 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 10.9 μl cDNA volume was analyzed using qPCR.

The amounts of influenza A virus detected in the swab samples of subjects with influenza-positive and influenza-negative cough aerosols were compared using a two-tailed Student's T-test with a significance level of $p \leq 0.05$. In some of the research papers cited here, the quantity of viable influenza A was presented in units of Median Tissue Culture Infective Dose, or $TCID_{50}$. To allow comparison with our data, these results were converted to PFU by multiplying by 0.7.[25]

TABLE I. Demographic and Medical Information for Study Participants Who Were Influenza-Positive

Influenza A detected in cough aerosol particles?	**Yes**		**No**	
# of subjects	7		10	
Gender	5 male, 2 female		7 male, 3 female	
	Mean	SD	Mean	SD
Age (years)	19	1.2	23	5.9
Height (cm)	172	10	177	12
Weight (kg)	74	21	86	28
Temperature (°C)	37.9	0.84	37.4	0.47
# of days of symptoms	2.0	2.0	1.9	0.9
Volume of each cough (liters)	2.3	0.5	2.3	1.2
Peak flow rate during coughs (liters/second)	6.9	1.5	6.9	2.9
Number of subjects reporting:				
Fever/chills	7		10	
Headache	6		8	
Fatigue	6		8	
Cough	7		9	
Sore throat	3		7	
Sinus congestion	3		3	
Runny nose	6		9	
Sneezing	3		6	
Muscle aches	5		9	
Received influenza vaccine within last 6 months	0		1	

Notes: A subject was considered influenza-positive if at least one of their samples (swab, sampler elbow, or tray) produced both a positive VPA and a positive VRA. Information for all of the patients is included in the online supplemental information.

RESULTS

Adult volunteer subjects were recruited from patients presenting with influenza-like symptoms at the WELL-WVU student health clinic in Morgantown, West Virginia, in early 2011 and early 2014 during the local influenza season. Sixty-four symptomatic test subjects were recruited for the study (32 in 2011 and 32 in 2014). For the subjects tested in 2011, nasopharyngeal swabs were collected from 20 participants, while nasopharyngeal and oropharyngeal swabs were collected from all participants in 2014. Cough aerosol samples were collected from all participants in both years of the study.

Seventeen participants had positive viral plaque assays for which the presence of viable influenza A was confirmed using the viral replication assay. For 15 of the participants, viable influenza A virus was cultured from their swab samples. One participant was not swabbed but had viable influenza A virus in his cough aerosol, while one participant had viable influenza A virus confirmed in his cough aerosol but not in the swab sample. A summary of the demographic information, oral temperatures, cough volume, cough peak flow rate, and symptoms reported by the test subjects confirmed to have influenza is shown in Table I. The numbers of influenza A plaque-forming units (PFU) detected and the results of the VRA are shown in Table II for the nasopharyngeal and oropharyngeal swabs, the aerosol sampler collection media, the sampler elbow, and the spray droplet impact tray for influenza-positive test subjects. The results for all test subjects are provided as an online supplement.

Viable influenza A was identified in coughs from 7 of 17 of the subjects confirmed to have influenza. Viable influenza A was found in the sampler elbow and droplet tray from only one subject, but was detected in 7 of the aerosol sampler media specimens, which contained cough aerosol particles from 0.3 μm to 8 μm in diameter. The amount of influenza A detected in the influenza-positive media samples ranged from 5 to 538 PFUs, with a mean of 142 (SD 215).

The amount of viable influenza A found in swab samples from patients with influenza-positive cough aerosols (mean 6,946,422; SD 15,735,275) was higher than the amount found in swabs from patients with influenza-negative cough aerosols (mean 177,571; SD 275,035), but the difference was not statistically significant either if each year is examined separately (2011, $p = 0.3092$; 2014, $p = 0.2562$) or when data from both years are combined ($p = 0.1852$). No viable

TABLE II. Viral Plaque Assay (VPA) and Viral Replication Assay (VRA) Results for Influenza-Positive Test Subjects

			Naso- and Oropharyngeal Swabs		Aerosol Sampler Media		Sampler Elbow		Droplet Tray	
	Year	Subject #	VPA (PFU)	VRA (# of copies)	VPA (PFU)	VRA (# of copies)	VPA (PFU)	VRA (# of copies)	VPA (PFU)	VRA (# of copies)
Subjects with viable influenza A in swab samples but with no viable virus in cough aerosol	2011	FC76	3.38×10^5	2.41×10^7	0	0	0	0	0	0
		FC80	2.13×10^5	1.17×10^8	0	0	0	0	0	0
		FC81	1.21×10^5	1.47×10^8	0	0	0	0	0	0
		FC82	9.00×10^5	1.66×10^8	0	0	4	0	0	0
		FC97	2.11×10^4	8.01×10^6	0	0	0	0	0	0
	2014	FC102	2.63×10^4	5.93×10^6	0	0	0	n/t	0	n/t
		FC105	114	9.62×10^4	0	0	0	n/t	0	n/t
		FC106	6.86×10^4	2.85×10^7	0	674	0	n/t	0	n/t
		FC113	8.03×10^4	3.09×10^7	5	0	0	n/t	0	n/t
		FC121	8.10×10^3	2.67×10^6	0	0	0	n/t	0	n/t
Subjects with viable influenza A virus in cough aerosol	2011	FC84	25	8.16×10^7	6	2.91×10^6	0	0	0	0
		FC88	n/a	n/a	356	1.99×10^6	0	2.85×10^5	0	0
		FC95	5.13×10^4	5.52×10^{10}	538	4.40×10^8	26	1.76×10^6	6	1.47×10^6
	2014	FC100	6	0	5	1.49×10^3	0	n/t	0	n/t
		FC104	2.55×10^6	2.92×10^8	25	1.30×10^4	0	n/t	0	n/t
		FC109	3.90×10^7	4.40×10^7	50	1.26×10^4	0	n/t	0	n/t
		FC123	7.73×10^4	1.61×10^7	13	4.14×10^3	0	n/t	0	n/t

Notes: For the aerosol sampler collection media, sampler elbow, and droplet tray, the results are the totals found from six voluntary coughs collected over about 20 min. N/a indicates that swabs were not collected from that patient. N/t indicates that a VRA was not performed. The results for all of the patients are included in the online supplemental information.

influenza A virus was found in cough aerosol samples from patients without viable influenza A virus in their swabs.

DISCUSSION

Determining the amount of viable airborne influenza virus that is expelled into the environment by influenza patients is a critical part of the process to determine the likelihood that influenza could be transmitted from person to person by infectious airborne particles. However, very little information is currently available about the quantity of viable virus that is coughed, sneezed, or exhaled into the air by people with influenza.

Airborne influenza A virus was detected in the cough aerosol particles from 41% of the influenza-positive patients in our study, which suggests that this is a common phenomenon among people with influenza. The largest amount of viable influenza A was detected in the media of the aerosol sampler, which collects the smallest particles (0.3 to 8 μm). These results are consistent with previous studies.[11,16,17] Since the collection efficiencies of the sampler elbow and droplet tray have not been measured, it is not clear if the differences in the amount of virus detected occurred because no viable virus was present in the larger particles, virus collected on the dry surfaces of the elbow and tray but then lost its viability, or if these particles were not collected. However, the fact that viable influenza A was detected in the smallest particles is quite important, because the smallest aerosol particles are also the ones that remain airborne the longest (an 8 μm particle takes about 13 min to fall 1.5 m in still air, and the settling time rapidly increases as particle size decreases).[26] Small particles also are easiest to inhale, and can deposit most deeply in the respiratory tract. Thus, airborne influenza A virus was detected in the small particles that are most likely to cause airborne transmission.

The amount of viable influenza A virus detected in the small airborne cough particles was relatively low, with a mean of 142 PFU in six coughs. However, the dose of influenza required for inoculation by the aerosol route is also quite low; one study found that inhalation of an aerosol containing approximately 0.7–3.5 PFU of influenza was sufficient to cause seroconversion in 50% of the human subjects tested.[27] In contrast, the infectious dose by intranasal inoculation in two other studies was 89–224 PFU, which is considerably higher.[28,29] Aerosol inoculation also is reported to result in more severe symptoms, presumably because aerosol particles are able to deposit deeper in the respiratory tract than droplets administered by intranasal inoculation.[30]

The amount of influenza A virus detected in cough aerosols in this study was probably much lower than would be seen in a severe influenza pandemic. First, studies of nasopharyngeal swabs from patients with naturally occurring influenza infections have shown that influenza viral shedding typically peaks on or around the first day of acute respiratory illness.[31–33] Patients in our study typically presented at the clinic the day after their symptoms developed, which was after the expected peak in nasopharyngeal viral shedding. The amount of influenza A virus in nasopharyngeal swabs is only weakly correlated with the amount in cough aerosols, but both are reported to decline rapidly over time.[17,19]

In addition, the test subjects recruited for this study were young, otherwise healthy ambulatory outpatients. Patients who are more severely ill cough more and have higher viral loads, which would likely increase the amount of virus in the cough-generated aerosols.[34] This would be especially important during a pandemic, when much larger numbers of severely ill patients would be expected. Finally, our informal observations indicated that the sickest and most symptomatic patients were least likely to volunteer for the study. All of these limitations suggest that the amount of airborne influenza virus that would be expelled by patients during a pandemic would be significantly higher than was observed here.

Finally, some important limitations of this study must be acknowledged. First, because of the small sample size, these results need to be interpreted with caution. More studies with larger numbers of patients and with a broader range of medical conditions will be needed to fully understand the amount of viable airborne virus that is projected into the environment by patients. Second, our experiments focused on viable virus released during coughing. However, this does not mean that infectious virus is not released during other respiratory maneuvers such as breathing and speaking. Third, we asked the study participants to inhale deeply and cough using as much of the air in their lungs as possible. This produced a deeper and more forceful cough than would be seen during natural coughs, which may have increased aerosol production. Lastly, although our results show that patients with influenza A expel small airborne particles carrying viable influenza A virus into the environment, they do not show that transmission actually occurs by the airborne route, nor do they indicate what the relative risk is that the virus could be spread by the airborne pathway compared to other means. More work will be needed to determine the likelihood of airborne transmission in real-world environments and the necessity of interventions to prevent it.

CONCLUSION

The purpose of this study was to gain insight into production of potentially infectious aerosol particles by influenza patients during coughing. Our results suggest that a significant proportion of patients with influenza A release airborne particles containing viable virus into the environment. Although the amount of airborne influenza A virus found in our study was low, the infectious dose for aerosol transmission is also reported to be low, and higher emission levels could be seen from patients in a severe pandemic. Our findings support the idea that airborne transmission could play an important role in the spread of influenza, but more information is needed to determine the relative importance of this pathway.

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SUPPLEMENTAL MATERIAL

Supplemental data for this article can be accessed at tandfonline.com/uoeh. AIHA and ACGIH members may also access supplementary material at http://oeh.tandfonline.com/.

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