

LACK OF INFLUENZA TRANSMISSION TO AN INHALING LIFE-LIKE MANIKIN FROM NATURALLY INFLUENZA-INFECTED HUMAN VOLUNTEERS

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SUMMARY

A standard shop display manikin was customised with a life-like inhalation cycle, to simulate a human recipient and exposed to a natural exhalation flows (breathing, talking, coughing) from influenza-infected volunteers. Any aerosolised droplets that were inhaled by the recipient manikin containing influenza were captured onto filters fitted into mouth of the manikin. These were tested for influenza RNA using a sensitive PCR (polymerase chain reaction) assay. No influenza RNA was detected in any other filter sample. This suggests that the viral load in aerosolized droplets produced by these natural human exhalation flows may be significantly less than that found in diagnostic swabs from the same source.

INTRODUCTION

In recent years, discussions over the most clinically significant routes of influenza transmission have been extensive (Tellier et al., 2006; Brankston et al., 2007). Confusion and disagreement surround the definitions of the various transmission routes including ‘close contact’ transmission, ‘airborne’ transmission and ‘droplet’ transmission. However, it is important to note that even in close proximity, multiple transmission routes may all be responsible for disseminating the infection, i.e. person-to-person transmission, and in such situations infection can potentially arise from any of airborne, droplet and direct physical contact transmission routes (Roy and Milton, 2004; Tang et al., 2006; Li et al., 2007).

In the close contact exposure scenario, small droplets generated by an infectious patient can be directly inhaled and deposited in both the upper and the lower airways, whereas large droplets can be also be directly inhaled, but the majority of these will probably deposit in the upper airways only, or directly enter the recipient’s eyes or even the mouth, as a direct droplet infection (as opposed to self-inoculated infection). Long distance airborne transmission has been postulated to be introduced by small droplet nuclei being carried by ambient airflows, where the moisture from small droplets has mostly evaporated away.

The actual clinical and public health implications of these different routes of transmission in everyday situations remains controversial, and many researchers have been focusing on ascertaining human influenza transmissibility during everyday respiratory activities, such as breathing, talking, coughing and sneezing (Fabian et al., 2008; Stelzer-Braid et al., 2009; Lindsley et al., 2010; Milton et al., 2013). These studies have focused on characterising the number, size and content of droplets generated by such activities. Yet this data by itself is not sufficient to determine the true transmissibility potential of any viruses carried in these droplets – they still need to reach a susceptible recipient and be inhaled in a sufficient infectious dose (for that individual) to cause infection and disease.

In this study, a customised, life-like, ‘inhaling’ manikin was used as a model of a ‘recipient’ human to examine the quantity of influenza virus inhaled when exposed to a naturally influenza-infected human volunteer source. This study only examined the inhalation phase of a potential recipient.

METHODOLOGIES

Manikin

For these experiments (performed during the 2010-2011 influenza season in Hong Kong SAR), a customised manikin was used as the model of a human recipient. A normal shop display manikin was obtained and a mouth orifice hollowed out, into which was fitted a mouth-piece connected to a pump through the back of the manikin’s neck (Figure 1A, 1B). This pump maintained a continuous inhalation flow of 12.5 L/min, to simulate the inhalation phase of human respiration. The inhalation rate of the thermal manikin was set to twice that of natural human inhalation to create a similar inhalation flow field when both inhalation and exhalation are present. This also maximized SKC bio-sampler air sampling efficiency.

Aerosol sampling

Once the manikin and the pump were set up, two methods were used to capture aerosolised virus produced by the naturally influenza-infected volunteers: 1) a polytetrafluoroethylene (PTFE) filter inserted into the mouth orifice of the manikin, to trap any aerosolised virus (Figure 1B); 2) and a commercially available air sampler (SKC BioSampler, SKC Inc., Eighty Four, PA USA: <http://www.skcinc.com/prod/225-9594.asp>) that was attached to the mouth orifice through the back of the manikin’s neck (Figure 1C).

Each of these methods was used, separately, with each volunteer to capture aerosolised virus. The two methods were not used in combination with any of the volunteers. The reason for the two sampling methods was to allow the capture of large droplets that travelled ballistically as expelled from the source volunteer, transported by the source exhalation airflow (PTFE filters), as well as any smaller, droplet nuclei that were truly airborne, using the SKC BioSampler.

All the PTFE filters were kept in sealed plastic bags to prevent contamination and installed immediately prior to the experiments. Similarly, the mouth-pieces were sterilised and kept in sealed packets and only installed immediately before each experiment with the human volunteers. To capture the virus using the SKC BioSampler, a tissue culture medium (Medium 199, Life Technologies, Kowloon, Hong Kong SAR) was tested and found to be appropriate for the viral capture media. In addition, a baseline efficiency experiment was also performed to ensure that there was minimal loss of detection sensitivity using the PCR method when detecting for the presence of any viral RNA on the PTFE filter.

Experimental procedures

This study was conducted in a public hospital and a university health clinic during two distinct periods (August to September 2010 and January to February 2011). Patients over 11 years of age with influenza-like illness (ILI: any of cough, fever, sore throat, headache, malaise, myalgia, lethargy) in the previous three days were invited to participate in the study.

A rapid point-of-care test (QuickVue Influenza A+B rapid diagnostic test, Quidel Corp., San Diego, CA, USA: sensitivity: 0.68, specificity: 0.96) (Cheng et al., 2009) was used as instructed by the manufacturer as a screening test, to confirm influenza infection. Patients with positive diagnostic results were then invited for exhale breath sampling experiment. Nasal and throat swabs were collected into universal transport medium (UTM, Copan Diagnostics, Murietta, CA, USA) for diagnostic testing using an in-house influenza reverse transcription polymerase chain reaction (RT-PCR) assay (Chan et al., 2008), to further confirm influenza infection, as well as to establish a baseline viral load. Once the manikin was set up, for each sampling method, the recruited, naturally influenza-infected volunteers were asked to perform various respiratory activities (including breathing, counting, talking and coughing) when facing the customised manikin at a distance of 0.1 m and 0.5 m. After each set of respiratory activities was conducted with the PTFE filter or the SKC BioSampler, the filter or capture media was removed and stored. The PTFE filter was first dipped in 2 mL UTM prior to storage. All specimens were stored at 2 to 8 C° for less than 24 hours before PCR testing to determine the presence and quantity of influenza virus present.

For the PTFE filter samples, briefly, the mouthpiece filter holder was removed carefully and the filter removed. The filter was then soaked in 2 mLs of UTM for 15 minutes, during which there were three episodes of vortexing for 30 seconds to transfer as much virus from the PTFE filter to the UTM for influenza RT-PCR testing. This RT-PCR assay used consensus primers to target the matrix (MP) gene of the virus (Chan et al., 2008). Calibrators were included in each run to allow a standard curve to be plotted to estimate the copy numbers in the samples.

To check the sensitivity of the PTFE capture method, samples of the PTFE filter were inoculated (by droplets of 1, 5, 25 µL volume) with different, known amounts of influenza RNA, then run through the whole extraction and RT-PCR process for influenza RNA detection. Overall, there was relatively little loss of sensitivity with the $\log_{10}(\text{inoculated})$ vs $\log_{10}(\text{detected})$ viral loads being mostly within 10% of each other.

RESULTS AND DISCUSSION

Results were available from 9 volunteers in total (6 female, aged 14-62 years; 3 males 42-47 years), of whom 8 were infected with influenza A and one with influenza B (Table 1). Each of the volunteers either counted (and/or talked) and/or breathed and/or coughed in various combinations for varying durations. Seven of these volunteers were only 0.1 m from the recipient manikin. The last two volunteers who were exposed to the manikin when only the SKC BioSampler was being used (sampling for airborne droplet nuclei), were also exposed from a larger distance of 0.5 m.

Despite the variety of source respiratory activities, the two different sampling methods and exposure distances, no influenza RNA was detected from either the PTFE filter or SKC BioSampler samples from any of the volunteer exposures.

Although one PTFE filter sample did test positive for influenza (viral load = 429,000 RNA copies/mL) after exposure to one human volunteer (diagnostic swab viral load = 4,908,000

RNA copies/mL), the specific respiratory activities associated with this result were not formally documented, so this result was difficult to interpret.

Large droplets may be less likely to transmit influenza

These experiments suggest that influenza virus cannot be detected in the inhaled breath after a source exposure from a minimum distance of 0.1 m or greater. Furthermore, after the participant finished talking or coughing, large droplets were normally visible on the filters (diameters around 1-3 mm) and had not evaporated by the time these filters were immersed in the transport media. Yet, influenza virus RNA was still not detectable even in these samples. The detection of little or no influenza RNA in these experiments was initially surprising, but is compatible with the results of Milton and colleagues, who showed maximum copy numbers of <1000 by day 3 of illness in both coarse (>5 µm) and fine ≤5 µm) aerosol particles (Milton et al., 2013).

Duguid (1946) suggested that large droplets are mostly generated from the anterior mouth. Influenza viruses, however, are rarely found in human saliva (Cowling et al. unpublished data) due to the antiviral substances existing in oral fluids (White et al., 2009). Hence there is a possibility that large droplet transmission of influenza may not be important for transmission. This may also be true from a different angle. Breathing tends not to produce large droplets (Duguid et al., 1946), and breathing is the most common respiratory activity in humans, so this ‘small droplet’ mode of delivery is probably the more important transmission route for human-to-human influenza transmission. Also, although coughing and sneezing do produce larger droplets, very little time is actually spent coughing and sneezing by most people (though admittedly the frequency of coughing and sneezing may increase significantly with some respiratory infections, such as tuberculosis), so these modalities in general, may not be the most important for everyday incidences of influenza transmission. Again, this suggests that influenza viruses may not be common in large particles generated during respiratory activities (Milton et al., 2013).

Dispersion of exhaled aerosols with distance may reduce the likelihood of transmission

These negative results may also be due to low virus concentration at a distance from the source caused by a dispersion effect. This is perhaps one of the more important differences between our experiment and other studies with successful virus recovery (Fabian et al., 2008; Milton et al., 2013), i.e. that we did not attempt to capture the whole exhaled breath volume (regardless of modality, i.e. breathing, talking, coughing, etc.) from the sources. Assuming spreading angle of respiratory activities is about 40° (Gupta et al., 2009; 2010) as shown in Figure 2. The concentration differences between source and different distances can be calculated as:

$$\delta_x = \frac{D_0^2}{(D_0 + 2x \tan(\frac{1}{2}\alpha))^2} \quad (1)$$

Given $\alpha=40^\circ$ and $D_0=1.24$ cm (Gupta et al., 2009), the particles or droplets concentration is only 0.03% at 1 m distance, 2.1% at 0.1 m distance ($x = 0.1$ m) and 6.5% at distance of 0.05 m ($x = 0.05$ m) compared with concentration at the source. Hence if the airflow dynamics of the exhaled puff from the source and the zone of inhalation of the recipient are not considered, the final numbers of droplets and any virus that they might be carrying that actually arrive at the inhalation zone of the recipient may be considerably lower than that which left the source. Despite the sensitivity of RNA detection by the PCR method, this

dispersal and dilution (with ambient air) effect may combine to make it difficult to detect any influenza RNA at the manikin's face, even via an inhalation route. The relatively short exposure durations of the recipient to the source (about 15 minutes in these experiments) may have also reduced the amount of detectable influenza RNA in the recipient manikin.

Possible limitations of the sampling and detection methods

In these experiments, a pump is used to extract air through filters, which will increase the evaporation rate of droplets deposited on the filter. Together with the accompanying shear stresses applied to the influenza virus (which is a relatively labile, lipid-enveloped RNA virus), this would also decrease the virus survival rate on the filter, though this should not significantly affect the PCR detection sensitivity of viral RNA. However, the SKC BioSampler also has limitations in that its collection efficiency decreases significantly with increasing particle diameter from about 100% with 4 μm particles to about 30% with 9 μm particles (Kesavan et al., 2010). This may underestimate the viral loads detected in larger particles.

With the PTFE filter capture and detection methods, any loss of sensitivity was relatively limited, with the $\log_{10}(\text{inoculated})$ vs $\log_{10}(\text{detected})$ viral loads being mostly within 10% of each other, according to the baseline experiments.

Comparison with other similar, recent studies

Two other studies by Bischoff and colleagues have estimated approximate viral concentrations at certain distances. A study on the potential transocular transmission of influenza suggested that exposure to aerosolised influenza at a distance of up to 1 foot, would be sufficient to inoculate (i.e. infect) most exposed human subjects via the ocular route (Bischoff et al., 2011). However, this exposure may bear little resemblance to a natural exposure with wild-type seasonal influenza virus, as it consistent of a 20 minute exposure to a mechanical aerosol generator emitting a mono-dispersed aerosol (of approximately 4.9 μm diameter) of the live attenuated vaccine ('Flumist') strain of influenza. The virus concentrations of this artificially generated aerosol and the naturally generated aerosols are difficult to compare.

More recently, Bischoff et al. (2013) subsequently attempted to define concentration contours around patients infected with influenza, using Andersen samplers to sample air at head level distances of ≤ 0.305 m (1 ft), 0.914 m (3 ft) and 1.829 m (6 ft) away the heads of influenza infected patients. The upper limits of the viral concentration measured at 0.305 m and 0.914 m were roughly similar at approximately 400-600 and 350-600 influenza virus RNA copies per 10-L human respiratory minute volume". Although this unit is difficult to compare to the results of these experiments it does seem to agree with the implications of Milton et al. (2013) - that the airborne viral load exhaled by infected patients/ volunteers is not particularly high, and together with the dispersion calculations above, may well result in very little virus actually reaching and depositing within the breathing zone of a susceptible recipient up to 1 m away.

CONCLUSIONS

Further experiments are required to confirm these surprising results. Yet, if confirmed, this suggests that influenza may not be particularly transmissible by the aerosol route in most circumstances. However, this does not exclude the possible transmission of the virus in situations with longer exposure/ contact periods, or in super-spreader individuals who may well shed higher levels of virus in aerosolised form.

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Table 1. No influenza RNA was detected using either sampling method (PTFE filters or the SKC BioSampler), with any of the respiratory activities, despite high titres of influenza RNA being detected in the diagnostic nasal swab.

Subject code no.	Flu' A or B)	Age (yrs)	Sex (M/F)	Days post onset of illness	Air sampling method	Test distance (m)	Patient 'source' activities	Flu' RNA detected in filter/sampler (cop/mL)	Flu' RNA copies/mL in source diagnostic swab
00302	A	47	M	3	PTFE filter/ SKC BioSampler	0.1	Count 1-20 Cough 10 times	None	95044000
01402	A	42	M	3	PTFE filter/ SKC BioSampler	0.1	Count 1-100 Cough 10 times	None	139000
01702	A	14	F	2	PTFE filter/ SKC BioSampler	0.1	Breath 1 min Count 1-20 Cough 20 times	None	167000
02602	A	17	F	3	PTFE filter/ SKC BioSampler	0.1	Talk 10 min Count 1-100 Cough 20 times	None	419000
02702	A	22	F	2	PTFE filter/ SKC BioSampler	0.1	Talk 10 min Count 1-100 Cough 20 times	None	8673000
03802	A	49	F	3	PTFE filter/ SKC BioSampler	0.1	Talk 10 min Count 1 to 100 Cough 20 times	None	7397000
04102	A	57	F	2	PTFE filter/ SKC BioSampler	0.1	Talk 10 min Count 1 to 100 Cough 20 times	None	3007000
05602	A	62	F	2	SKC BioSampler	0.1, 0.5	Talk 10 min Count 1 to 100 Cough 20 times	None	538000
00203	B	not given	M	3	SKC BioSampler	0.1, 0.5	Talk 10 min Count 1 to 100 Cough 20 times	None	3703000

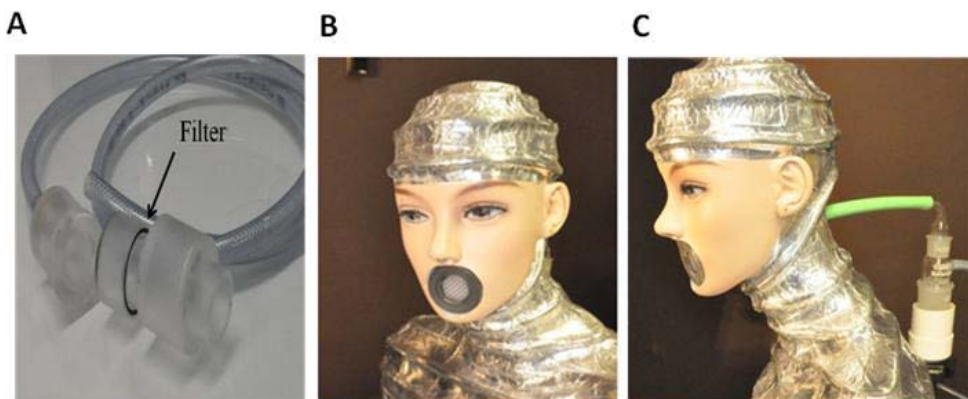


Figure1. Airborne sampling experimental set-up. **A.** Design of the mouth-piece. **B.** Installation of airborne sampler in a thermal manikin. **C.** Connection method of SKC bio-sampler to the mouth-piece

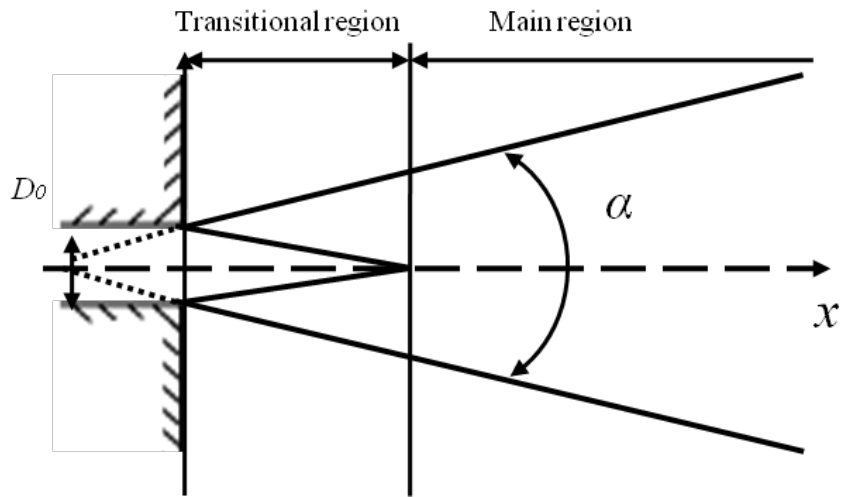


Figure 2. Idealized respiratory jet from an individual with a round mouth opening area (diameter D_0) and a spreading angle of α .