The environmental deposition of influenza virus from patients infected with influenza A(H1N1)pdm09: Implications for infection prevention and control

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Abstract

In a multi-center, prospective, observational study over two influenza seasons, we sought to quantify and correlate the amount of virus recovered from the nares of infected subjects with that recovered from their immediate environment in community and hospital settings. We recorded the symptoms of adults and children with A(H1N1)pdm09 infection, took nasal swabs, and sampled touched surfaces and room air. Forty-two infected subjects were followed up. The mean duration of virus shedding was 6.2 days by PCR (Polymerase Chain Reaction) and 4.2 days by culture. Surface swabs were collected from 39 settings; 16 (41%) subject locations were contaminated with virus. Overall, 33 of the 671 (4.9%) surface swabs were PCR positive for influenza, of which two (0.3%) yielded viable virus. On illness Day 3, subjects yielding positive surface samples had significantly higher nasal viral loads (geometric mean ratio 25.7; 95% CI 1.75, 376.0, p=0.021) and a positive correlation (r= 0.47, p= 0.006) was observed between subject nasal viral loads and viral loads recovered from the surfaces around them. Room air was sampled in the vicinity of 12 subjects, and PCR positive samples were obtained for five (42%) samples. Influenza virus shed by infected subjects did not detectably contaminate the vast majority of surfaces sampled. We question

the relative importance of the indirect contact transmission of influenza via surfaces, though our data support the existence of super-spreaders via this route. The air sampling results add to the accumulating evidence that supports the potential for droplet nuclei (aerosol) transmission of influenza.

Introduction

Little is definitively known about the modes of influenza transmission and their relative importance, and important health policy and infection control issues remain unresolved. The World Health Organization, the Centers for Disease Control and Prevention (CDC), the European Centre for Disease Prevention and Control and the U.S. Institute of Medicine have each prioritized improving the understanding of influenza transmission as a critical component for pandemic preparedness and response.[1-3]

Influenza transmission begins with the production of virus containing particles by actions such as coughing and sneezing, which generate an 'expiratory spray' containing particles varying in size from <1 to 1000 μm. The majority are small and have a geometric mean diameter of 13.5 μm during coughing.[4] Large droplets (typical size >20 μm) deposit on mucous surfaces of the upper respiratory tract (URT), such as the mouth and nose; they can be inhaled, but are too large to reach the lungs. Droplet nuclei (frequently called aerosols; typically ≤5 μm) are inhaled and can reach the lower respiratory tract (LRT).[5] Contact

transmission involves the transfer of particles to the mucous membranes either directly, e.g., via kissing, or indirectly via hands or fomites.

Laboratory studies have confirmed the ability of human influenza virus to survive in these environments,[6-8] but few studies have attempted to investigate its presence, quantity and viability around infected patients. In previous research, viral shedding has mostly been determined by the measurement of the virus that is recoverable from the nasopharynx, i.e., via a deliberately performed invasive technique. Such 'viral shedding' studies in fact measure the virus shed from infected cells into the nasopharynx but do not actually measure the amount of virus deposited into the environment (on surfaces or in the air); therefore, they imply but do not define environmental contamination and the actual hazard posed to others. In this study, we describe viral shedding and its relationship to symptom duration, illness severity and the amount of virus recovered from the immediate environment.

Methods

We conducted a multi-center, prospective, observational cohort study over two influenza seasons, comprising the second and third waves of the 2009/10 pandemic in England [September 2009 - January 2010 (Year 1) & December 2010 – January 2011 (Year 2)]. An accredited UK Research Ethics Committee approved the study.

Participants

Adults and children (<16 years) in hospitals and in the community were recruited from three sites in the UK. Written informed consent was obtained at enrolment for all participants.

Screening and eligibility criteria

We approached patients who had an influenza-like illness (ILI) defined as: fever (or recent history of fever) and any one of cough, sore throat, runny nose, fatigue or headache OR any two of cough, sore throat, runny nose, fatigue or headache. A rapid antigen test (Quidel QuickVue®) was used to assist with the early diagnosis of cases (but positivity was not an inclusion criterion). We excluded cases where illness had been present for >48 hours (community cases) or >96 hours (hospital cases).

Study Procedures

Where possible, subjects were followed up on a daily basis for a maximum of 12 days. A symptom diary was completed at each visit.

A nasal swab, performed by a nurse or physician, was taken by rotating a swab round one anterior nare three times. Surface swabs were taken in hospital rooms and in the subjects' own homes from pre-defined surfaces (Table A, supplementary data). The swabs were moistened with viral transport medium (VTM – Remel M4RT®) and then rubbed across an area of approximately 100 cm^2 in three different directions while applying even pressure.

Nasal and surface swabs were placed into VTM and kept on ice for no longer than three hours before being frozen at -70^{0}C.

Air particles were collected using a National Institute for Occupational Safety and Health two-stage cyclone bioaerosol sampler that separates particles into 3 size fractions [<1 μm (stage 1), 1-4 μm (stage 2) and >4 μm (stage 3)] and has been validated for use with influenza.[9-10] Sampling was usually performed on only one day. The flow rate through each sampler was set to 3.5 L/min with a flow calibrator (Model 4143, TSI). Samplers were mounted on tripods at a height of 150 cm, placed approximately 2 m from the subject and run for 1-3 hours. After sampling, VTM was added to both stage 1 and 2 tubes and the filter paper from stage 3 was immersed in a 15-ml tube containing VTM. The samples were stored at –70°C.

Further details are provided in Supplementary Table A.

Virological Assessments

Polymerase Chain Reaction (PCR): A novel influenza A(H1N1) pentaplex assay was devised to detect the virus genome in the samples. The assay was designed to detect A(H1N1)pdm09, seasonal H1 and H3 influenza A, influenza B and an internal control bacteriophage MS2. It is highly sensitive and has a very wide dynamic range (10 logs) and can reliably detect as few as 3.85 genome equivalents per PCR reaction. The performance of the assay has been assessed on multiple occasions by Health Protection Agency/Public Health England external quality assessment panels. Viral load data for A(H1N1)pdm09 were generated using a PCR assay and a plasmid containing the hemagglutinin (HA) gene target

to create a standard curve, such that the concentration of the genome present in each sample could be estimated.

Culture: An immunofluorescence (IF) assay was used to detect the influenza A/B nucleoprotein to demonstrate the presence of live replicating virus.[11]

Further methodological detail is provided in the Supplementary Data section.

The following sample processing rules were instituted to limit the analysis of likely negative samples:

1. Nasal swabs from day 4 onwards were not tested if days 1-3 were all PCR negative.
2. Culture was only performed on PCR positive samples.
3. Environmental swabs were not processed if nasal swabs taken on the three previous days from a case were PCR negative

Outcome Measures

Virus shedding (nasal swab):

A positive nasal swab was defined as a sample in which a cycle threshold (Ct) value of <35 (2342 copies/ml) for ≥1 triplicate of a sample was obtained (a Ct value of 35 is a log up from the identified limit of detection of the assay). Unexplainable results and any single triplicates separated by >48 hours from other positive samples were disregarded. Viral loads represent the geometric mean (GM) value of the triplicate assay. A value of half of the lower limit of detection was imputed for undetectable values. The duration of virus shedding was defined as the time between symptom onset and the last day that a positive specimen was

obtained. Because subjects were seldom recruited on the day that symptoms began, an assumption was made that they were shedding virus from the first day of symptoms to the last positive specimen day.

Subject symptoms:

Daily symptom scores were categorized into the i) URT score; ii) the LRT score; iii) the systemic score; and iv) the total symptom score (sum of URT, LRT and systemic scores plus a score for diarrhea and/or vomiting). Individual symptoms were given a severity score of 0-3. A similar index has previously been used to assess respiratory tract illnesses of viral etiology.[12]

Environmental deposition (surfaces and air samples):

A positive surface swab or air sample was defined as a sample in which a Ct value of <35 for ≥1 triplicate of a sample was obtained. *Post-hoc,* a Ct value of <40 (122 copies/ml) for ≥1 triplicate of a sample, if it was obtained from a subject with a positive nose swab taken on the same day (to help confirm plausibility), was defined as positive. Reducing the threshold of detection to limit false negative results was considered to be reasonable based on our own data that showed false positives to be unlikely; only 1 swab (Ct = 39.5) was excluded because of a negative nose swab on the same day.

Statistical methods

A descriptive analysis of the data is presented. Student’s t-tests were used to compare mean values; Pearson's correlation tests were used to determine associations between

continuous variables; and Odds Ratios (ORs) and 95% confidence intervals (CIs) represent associations between variables with binary categorical outcome measures. Chi-squared tests were used to test the significance of ORs. Differences in viral loads were measured using GMs and compared using GM ratios and paired t-tests. P values ≤0.05 were considered to be significant. All statistical analyses were conducted in Stata version 11 (Statacorp, Inc.).

Results

Demography, treatment and outcomes

One-hundred-two subjects with ILI were studied, 49 (48%) of whom had a confirmed influenza infection; 44 (90%) had A(H1N1)pdm09 (age range 0 to 58); and 5 (10%) had influenza B (age range 5 to 66) (Figure 1). Two patients with A(H1N1)pdm09 were excluded from analysis because they were recruited outside of the specified timeframe. Therefore, data from 42 patients with confirmed A(H1N1)pdm09 cases are presented in this paper. The demography of these subjects is shown in Table 1.

Symptoms

The most frequently reported symptoms were cough (93%), sore throat (88%) and rhinorrhea (86%). During follow up, the symptom scores were highest on Day 3 of symptomatic illness and declined thereafter (Figure 2).

Virus Shedding

PCR:

The mean duration of shedding was 6.2 days from symptom onset (range 2-15 days, interquartile range 5-7). There was no significant difference between adults and children [mean difference = 0.29 (95% CI: -1.33, 1.90), p= 0.720] (Figure 3A).

Viral loads varied widely, ranging from 2033 – 24,521,397 copies/ml, and declined over time. No significant differences were observed in the GM viral loads between adults vs. children and community vs. hospital cases on illness Days 3, 4 and 5 (Table B, supplementary data).

Culture:

Twenty-four of 39 cases (62%) were culture positive for A(H1N1)pdm09 (insufficient sample was available for culture in three cases). The mean duration of shedding by culture was 4.6 days (range 3-10 days, interquartile range 4-5). Ten of 39 (26%) subjects shed live virus for at least five days from the onset of illness (Figure 3B).

Environmental Deposition

Surfaces:

Multiple surface swabs were collected in 39 separate locations (houses and hospital rooms) inhabited by subjects, some of whom lived together; 16 premises (41%) were contaminated with virus. A mean of 16 swabs (range 6-42) were taken from each location, ≥2 positive samples were obtained from 8 of the 16 premises. In total, 671 collected swabs were tested and influenza A(H1N1)pdm09 was detected by PCR in 33 (4.9%). Seventeen surface samples (selected on the basis of PCR results with low Ct values) were examined for viable

virus, and two surfaces (a games console and a kettle handle) were positive, representing 11.7% of the samples cultured and 0.3% of the total samples taken) (Table C, supplementary data).

Air:

Samples were collected from the immediate environment of 12 subjects (Season 1 = 5, Season 2 = 7). Subjects were targeted on the basis of a positive rapid test, early in the course of illness, convenience and special interest (HDU cases); six were in the hospital, nine were adults and eight were rapid antigen test positive. These samples were positive by PCR for five subjects (42%) (Table 2). Virus was detected in all of the particle size fractions collected; 7/26 (27%) of the collections for particles <1 μm were positive; 9/27 (33%) of 1-4-μm particles were positive and 7/27 (26%) of >4-μm particles were positive. No significant associations were found with respect to the sampling distance or time and the finding of positive samples. Viral loads ranged between 238 and 24,231 copies/mL. No air samples were positively cultured.

<u>The relationship between symptoms, virus shedding and virus deposition</u>

Symptoms and viral load:

Poor correlations were observed between the total symptom scores and log transformed nasal viral loads on illness Day 3 (day of maximum symptoms) (r= -0.063; p= 0.751) and Day 4 (r= -0.07; p= 0.69).

Viral load and surface deposition of virus:

On illness Day 3, a significantly higher GM viral load was observed in those who had surface positive swabs compared to those who did not (GM ratio 25.7; 95% CI 1.75, 376.0, p=0.021) (Table 3). A positive correlation (r= 0.47, p= 0.006) was observed between the log transformed subject nasal viral loads and viral loads recovered from surfaces on illness Day 3 (r= 0.38, p= 0.03).

Symptoms and surface deposition of virus:

There were statistically significant findings for URT symptoms and similar but non-significant trends for LRT symptoms, which suggest that patients with higher symptom scores reflected in the likelihood of there being influenza positive surface swabs taken from the environment (Table 3).

Symptoms, viral load and virus deposition in air:

There were no differences in the GM viral loads on illness Day 4 or in the LRT and URT scores on illness Days 3 and 4 between those with positive and negative air samples (Table D, supplementary data).

Discussion

This is the first study to examine the relationship between influenza virus shedding from the nose with virus deposition in the air and on surfaces from the patient's immediate environment, in both inpatient and home settings. As such, it offers important information for infection prevention and control practices.

Our findings on the duration of virus shedding are broadly in agreement with published findings concerning seasonal influenza and A(H1N1)pdm09,[13-16] although we did not identify a significant correlation between symptoms and viral load where others have done so. Virus was detected on surfaces in the near environment of 38% of subjects. Overall, however, virus was infrequently isolated by PCR from surface swabs (4.9%), and on only two occasions was live (infectious) virus recovered. These data suggest that although environmental contamination occurs, it is not usually extensive or heavy.

To our knowledge, there are no data concerning the infectious dose for indirect contact routes; however, we believe that the following data helps justify our conclusion. The ratio of the tissue culture infectious dose 50 (TCID50) to the number of virions (and therefore to the number of genome copies) for influenza A has been estimated by various authors.[17-19] Assuming that the 1 TCID50 is equal to 400 genome copies/mL, then an infectious aerosol dose (calculated to be 0.6 to 3 TCID50)[20] would be 240–1200 copies/mL and an infectious intranasal dose (100-1000 TCID50)[21-23] would be 40,000-400,000 copies/ml. The difference in the infectious dose between aerosol and direct nasal inoculation is at least 2 logs. It is likely that the infectious dose for aerosol transmission is also significantly less than that needed for indirect contact transmission. The copy number range for environmental swabs was 100 – 43000, with a median of 1200 copies/ml. We argue that while 38% of subjects contaminate their surroundings, the amount of virus recovered from the vast majority of environmental swabs does not represent an infectious dose.

Our data show similarities to findings from a randomized trial that investigated hand hygiene and surface contamination in Thailand.[24,25] However, our results contrast with those of a study that detected influenza virus on over 50% of all swabs taken from a number of surfaces in the home and in child care centers.[26] Differences between studies may be influenced by the strain of influenza virus; the subjects involved; swabbing and detection methods, including the timing of swabbing and the surfaces selected; environmental conditions; and the proportions of subjects taking antiviral drugs. Indeed we have demonstrated some of these points in the current study. Both swabbing and laboratory processing were more selective in Year 2, as we attempted to target surface samples that had a greater chance of positivity; the swab positive rates (1.4 vs. 10.7%) reflect this. For example, 4 out of 9 chosen surfaces in Year 1 (bedside table, dining table, patient table and windowsill) were not items that could be picked up or grasped by the hand, and in many instances, they were made of wood, a material that does not support virus survival[27] (see supplementary data.)

Two randomized clinical trials (RCTs) supporting the indirect contact route of transmission have shown significant effects of hand hygiene on the incidence of laboratory confirmed influenza and absenteeism due to ILI in school children[28,29]. Other RCTs report negative findings,[30-32] and a systematic review and meta-analysis found no significant effect of hand hygiene on the reduction of laboratory confirmed influenza infections.[33] Notwithstanding any positive trial results (which may reflect a reduction in transmission that involves hand to hand or hand to face touching but not fomites), the indirect contact route transmission pathway seems to be implausible. How likely is it that an infectious dose of virus can persist while passing along a transmission chain? Researchers studying rhinovirus transmission

concluded that an infective dose of virus is unlikely to reach the end of a transmission chain.[34] Based on our data, we question how frequently an infectious dose of influenza virus persists while passing from infected secretions, to touched surfaces (our data), to hands, and finally, to mucous membranes to initiate infection in a second person. We argue that this may be rarer than is often reflected in infection control guidance for influenza where hand hygiene is often centrally emphasized.

If the amount of virus released is key to transmission, then individuals who release the most virus, so called 'super-producers' or 'super-spreaders', are likely to be better transmitters than others.[35] The amount of virus released by individuals is governed by a number of factors, of which the viral load and symptoms are most important. The findings from our study support the concept that super-spreaders of influenza infection via the indirect contact route might exist. On illness Day 3, nasal viral loads were significantly higher in those with positive surface swabs compared to those with negative surface swabs, and a significant correlation between nasal and surface viral loads was found. Furthermore, the symptom scores were generally higher (significantly so for the URT scores) in those with positive surface swabs. These findings suggest that individuals who release the most virus into the environment are likely to be responsible for the most surface contamination and therefore for the majority of transmissions that occur via the indirect contact route. In practical terms, we suggest that the intuitive feeling that patients with high viral loads and strong symptoms have the characteristics that makes them the most likely individuals to contaminate surfaces is indeed correct.

Influenza virus has been detected (by PCR) in air samples taken from medical facilities[9,36,37] and from the directly exhaled breath and coughs of infected patients.[38-40] Our study demonstrates that samples of air collected from around infected subjects contain influenza virus. All of the particle sizes collected contained virus that was detectable by PCR, notably including the <1 μm and 1-4 μm fraction sizes, which are respirable (they can reach the distal airways of the respiratory tract),[5] and health attendants require respirators, not surgical masks, to avoid exposure. Different sampling times did not always run concurrently, which may explain why longer sampling times did not always result in more virus being collected, as specific 'shedding events' would not have been captured equally.

Attempts can be made to understand whether the PCR copy number found in the air samples could represent an infectious dose. Again assuming that 1 TCID50 is equal to 400 genome copies/mL, then an infectious aerosol dose (calculated to be 0.6 to 3 TCID50)[30] would be 240–1200 copies/mL. Our samplers, operating at 3.5 L/min, commonly collected these amounts. By way of comparison, an adult human typically inhales 6 L/min. If the virus collected is infectious, then the majority of positive samples collected during this study could contain infectious doses of influenza. Although we were unable to culture virus from any air samples, the detection of live virus in air samples is known to be methodologically challenging; the difficulties include virus fragility, especially its susceptibility to desiccation, and the fact that the concentration of virus being sampled in the air is low. Thus, failure to identify live virus in air samples does not necessarily exclude its presence.

There are a number of limitations to this study. First, the difficulty in recruiting subjects early in the course of their illness meant that the data on the initial days of illness were not

collected. The first few days of illness are usually times of peak symptoms and viral shedding (and by inference, environmental deposition). Due to logistic constraints, most analyses could only be attempted on illness days 3 and 4. Second, the comparison of symptom data between adults and children is imperfect. The data collection method was the same, and while this some allows for a comparison, it is clear that its interpretation must be guarded, as responses to the symptom diary card in children and adults may be different. A specific problem arises when parents estimate symptoms on the behalf of younger children. Third, the majority of subjects from around whom air samples were obtained (including all of those with positive samples) were selected on the basis of a positive rapid antigen test. This may have biased the air sampled group somewhat and led to a high proportion of positive air samples, as a positive rapid antigen test has been associated with higher nasal viral loads.[41] Finally, no measurements or estimates of room air flow patterns or ventilation were made when collecting samples. Such parameters are likely to have an influence on the ability to detect virus in the air.

Detecting virus, particularly live virus in the environment, is challenging; accessing the subject early in the course of illness, executing optimal sampling while preserving virus viability, and performing sensitive detection tests in the laboratory are all key factors and present logistical challenges. While based on limited data, these findings are of sufficient importance to justify further efforts to reproduce them, including further attempts to detect live virus, as they have potentially important implications for infection control strategies.

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Figure Legends and Tables

Figure 1: Participant flow diagram.

Note: 'Others' consisted of influenza-like illness with no confirmed viral etiology or confirmed infections with rhinovirus or respiratory syncytial virus (RSV). Two influenza A(H1N1)pdm09 cases were excluded on the basis of being recruited >5 days after symptom onset.

Table 1: Demographic characteristics of the participants, treatments, and outcomes.

Setting:	**AC**	**CC**	**AH**	**CH**	**Total n (%)**
Enrolled	13	11	14	4	42
Female sex	8	3	9	4	24 (57%)
Median age (yrs) **Range**	29 21-58	4 2-12	28 19-57	2.5 0-15	22 0 - 58
Ethnic group - **White** - **Black** - **Asian**	 10 1 2	 8 0 1	 8 1 5	 4 0 0	 30 (71%) 2 (5%) 8 (19%)

- **Other**	0	2	0	0	2 (5%)
Mean time from symptom start to enrolment (days)	2.2	1.5	2.4	2.8	2.1 (Range 0-4)
Mean duration of follow up (days)	5.6	8.3	5.9	7.5	6.6 (Range 1-12)
Rapid antigen test positive	3/13 (23%)	7/9 (78%)	5/14 (36%)	1/4 (25%)	16 (40%)
Antiviral Treatment Any / Within 48 hours	0/0	4/3	13/8	3/1	20/12 (48/29%)
High Dependency Care / Died during follow up	0/0	0/0	1/0	1/0	2/0

Note: AC = Adult Community, CC = Child Community, AH – Adult Hospital, CH = Child Hospital

Figure 2: The mean symptom scores of A(H1N1)pdm09 cases over time.

Footnote: Data only shown where ≥3 observations were available

Figure 3A+B: The percentage of subjects who shed virus against time.

3A: Shedding by PCR

3B: Shedding by culture

Table 2: Positive air sample results by location, age, particle size, and virus copies/mL.

	Adult	Adult	Child	Child	Adult
Subject setting (+ infected others)	Hospital bed in side room	Hospital bed in side room	Playing in living room (6-year-old infected child also present)	Cot on neonatal unit (2 infected neonates also present on ward)	Bedroom
Illness Day of sampling	4	3	3	5	4
Nasal Viral Load (copies/ml)	173,000	8,250,000	24,520,000	18,480,000	4000
Any surface swabs positive	No	Yes (Day 4)	No	No	Yes (Day 3)

Room Temperature (^{0}C)	21.6			23.3				18.0				24.0		17.0
Room Humidity (relative %)	50			50				60				40		44
Duration of sampling (hours)	1	3		1			2	1		3		3		3
Approximate distance from subject (m)	1-2	≥2	1-2	≥2	1-2	≥2	≥2	1-2	≥2	1-2	≥2	1-2	≥2	≥2
Particle size detected in (µm)	<1 1068		<1 238 1-4		1-4			<1 13199 1-4	<1 5156 1-4	1-4	<1 2149 1-4	<1 2577 1-4	<1 1287 1-4	1-4

PCR			258		603			5179	7107	24231	5166	3527	3889	5388
copies/ml			>4					>4	>4	>4	>4	>4	>4	
			511					8210	4028	5603	4889	3639	2245	

Table 3: Viral loads and symptom scores compared between those with positive and those with negative surface swabs.

Illness day	GM nasal VL surface positive (95% CI)	GM nasal VL surface negative (95% CI)	GM ratio	P value
Day 3	464225.5 (79759.9, 2701927.0)	18072.4 (1573.2, 207613.6)	25.7 (1.75, 376.0)	**0.021**
Day 4	77514.8 (7301.8, 822885.5)	118788.1 (19080.1, 739547.4)	0.7 (0.0, 10.5)	0.753
Illness day	**Mean URT score surface positive**	**Mean URT score surface negative**	**Mean difference**	**P value**
Day 3	8.5	3.9	-4.6	**0.002**
Day 4	6.6	3.6	-3.0	**0.009**
Illness day	**Mean LRT score surface**	**Mean LRT score**	**Mean difference**	**P value**

	positive	surface negative		
Day 3	4.2	3.2	-1.0	0.140
Day 4	3.8	2.7	-1.1	0.051

Note: GM = Geometric Mean, VL = Viral Load, URT = Upper Respiratory Tract, LRT = Lower Respiratory Tract