

# Exhalation of Respiratory Viruses by Breathing, Coughing, and Talking

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There is a lack of quantitative information about the generation of virus aerosols by infected subjects. The exhaled aerosols generated by coughing, talking, and breathing were sampled in 50 subjects using a novel mask, and analyzed using PCR for nine respiratory viruses. The exhaled samples from a subset of 10 subjects who were PCR positive for rhinovirus were also examined by cell culture for this virus. Of the 50 subjects, among the 33 with symptoms of upper respiratory tract infections, 21 had at least one virus detected by PCR, while amongst the 17 asymptomatic subjects, 4 had a virus detected by PCR. Overall, rhinovirus was detected in 19 subjects, influenza in 4 subjects, parainfluenza in 2 subjects, and human metapneumovirus in 1 subject. Two subjects were co-infected. Of the 25 subjects who had virus-positive nasal mucus, the same virus type was detected in 12 breathing samples, 8 talking samples, and in 2 coughing samples. In the subset of exhaled samples from 10 subjects examined by culture, infective rhinovirus was detected in 2. These data provide further evidence that breathing may be a source of respirable particles carrying infectious virus. **J. Med. Virol.** 81:1674–1679, 2009.

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**KEY WORDS:** aerosols; transmission; droplet nuclei

## INTRODUCTION

There is renewed interest in the mechanisms of transmission of human respiratory viral infections. Such interest has been heightened by concerns over emerging zoonotic diseases such as SARS, and new strains of pathogenic avian influenza that threaten to become pandemic in humans. Such infections may be

spread by multiple routes, including direct contact, fomites, and aerosols, although there is uncertainty about the relative importance of each [Goldfrank and Liverman, 2007]. For influenza, recent reports have suggested different mechanisms of transmission, for example, contact and droplets [Brankston et al., 2007], contact and aerosols [Musher, 2003], and mainly aerosols [Tellier, 2006; Atkinson and Wein, 2008] with Atkinson and Wein [2008] concluding that  $\sim 3\mu\text{m}$  particles “inflict most of the damage.”

The respiratory events traditionally associated with generating aerosols of virus are sneezing, coughing, and talking [Xie et al., 2007]. These aerosols include large droplets (diameter,  $150\mu\text{m}$ ), intermediate-sized droplets (diameter  $10\text{--}50\mu\text{m}$ ), and smaller particles (diameter  $<10\mu\text{m}$ ), including droplet nuclei [Blachere et al., 2009]. While larger droplets may travel distances of up to a few meters before settling to the ground, the smaller droplet nuclei from evaporated larger particles may remain airborne for hours and disperse as a cloud [Tang et al., 2006]. Almost all the available data on human-generated aerosols have been determined from studies of aerosols generated by healthy subjects combined with data on virus titer within mucus or respiratory aspirates from infected subjects, and so what is actually generated during viral infections and by what respiratory events this generation occurs, is not well described.

Three recent studies of virus aerosols generated by infected subjects all support the transmission of

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respiratory viruses by small aerosol particles. A novel sampling mask was used to show that breathing alone generated aerosols of rhinovirus and parainfluenza [Huynh et al., 2008], Fabian et al. [2008] collected influenza aerosols generated by breathing onto a Teflon filter and separately showed the majority of exhaled particles were of submicron size, while Blachere et al. [2009] used a cyclone sampler to show that on average 49% of the detectable influenza virus particles collected in the air of a hospital emergency department were in the 1–4  $\mu\text{m}$  respirable fraction. All three studies used polymerase chain reaction (PCR) to detect viruses and it remains to be established whether these PCR assays reflect infectious virus.

In this study, a more refined design of sampling mask was used to demonstrate aerosolization of several types of respiratory viruses during different respiratory events by infected people. Whether the aerosols generated by breathing contained infectious virus and whether viral output differed between air exhaled through the mouth or nose was also investigated in substudies.

## MATERIALS AND METHODS

### Subjects and Sampling

A total of 50 subjects, 33 symptomatic and 17 asymptomatic for colds, were sampled. These consisted of 10 children admitted to the wards of Sydney Children's Hospital Randwick (4–12 years old, 7 male and 3 female) and 40 students at the University of Sydney (21–45, average 26 years old, 16 male and 24 female). Symptom status was classified using a modified common cold questionnaire [Huynh et al., 2008].

Subjects provided a sample of nasal mucous and then wore a separate, sterilized collection mask (Fig. 1) for each of the following activities: (a) breathing through nose or mouth (10 min for adults or 5 min for children), (b) talking out loud by reading from a text (10 min for adults or 5 min for children), or (c) 10 simulated coughs (about 1 min). Collection masks were stored separately in sealable plastic bags and the electret collection discs were not touched by subjects or researchers. Used collection masks were transported immediately to the laboratory where a sterile disposable scalpel and pair of tweezers (sterilized with 100% Viraclean, Whiteley Medical, North Sydney, Australia) were used to halve the electret and transfer each half to a separate 600  $\mu\text{l}$  eppendorf tube. Electret and nasal mucous samples were stored at  $-80^{\circ}\text{C}$  until analysis.

For breathing, 35 of the subjects wore the mask and breathed in a manner that felt comfortable, without other instructions. An additional subset of 15 subjects were asked to provide one sample while breathing through their nose and another sample while breathing through their mouth. Mouth breathing was ensured by using a nose plug used commonly for swimming to close the subject's nose.



Fig. 1. The collection mask. The sampling masks used to collect exhaled aerosols are shown. Masks were constructed from parts obtained from disposable continuous positive airway pressure masks with the front elbow removed and a disk of electret held in that location with a washer and four bolts, as shown. The mask is shown as it was worn mounted on a dummy head.

Consent was obtained from all participants or their guardians; this study was approved by the Prince of Wales Hospital and University of Sydney Human Ethics Committees.

### Sampling Mask

The masks were constructed from a continuous positive airway pressure (CPAP) mask (Resmed, Bella Vista, Australia) with the front elbow removed and a disk of electret (ETR115, Japan Vilene, Tokyo, Japan) held in place with a washer and four bolts. After use, the masks were thoroughly washed, dried, and the electret collection filter replaced.

### RNA Extraction and Virus Detection

Virus was eluted from electret by adding 300  $\mu\text{l}$  of 70% ethanol and vortexing (2 min). The eluate was collected by centrifugation for 1 min at 13,000 rpm using specially designed double-ependorf tubes. Total nucleic acid was extracted from 200  $\mu\text{l}$  of eluate containing the virus, by the automated MagNAPure (Roche Applied Sciences, Castle Hill, Australia). Extract was stored as aliquots at  $-20^{\circ}\text{C}$ .

Influenza A, influenza B, parainfluenza 1, 2 and 3, respiratory syncytial virus, and human metapneumovirus were detected using a nested multiplex reverse-transcriptase PCR (RT-PCR) using primers designed previously and in-house [Syrmis et al., 2004; Rohde et al., 2005] (and available from the authors on request). cDNA was synthesized using the one-step RT-PCR kit (Qiagen, Doncaster, Australia) as follows: 8  $\mu\text{l}$  of template was added to 4.4  $\mu\text{l}$  RNase-free water, 4  $\mu\text{l}$  5 $\times$  buffer, 0.8  $\mu\text{l}$  10 mM dNTPs, 0.5  $\mu\text{l}$  of each outer primer,

and 0.8 µl enzyme mix to a final volume of 20 µl. Thermocycler conditions were 50°C for 30 min and then 95°C for 15 min, followed by 35 cycles of 94°C for 45 sec, 57°C for 45 sec, and 72°C for 1 min, with a final extension of 72°C for 7 min.

In the second round PCR, 1 µl of first round product was added to 7 µl RNase-free water, 10 µl Amplitaq Gold (Applied Biosystems, Melbourne, Australia) and 0.5 µM of each inner primer to give a final volume of 20 µl. Cycling conditions were 95°C for 5 min followed by 33 cycles of denaturation, annealing and extension at 94°C for 20 sec, 57°C for 20 sec and 72°C for 20 sec and a final extension of 10 min at 72°C. DNA products were visualized by 2% agarose gel electrophoresis with SYBR<sup>®</sup> Safe staining (Invitrogen, Mount Waverley, Australia). PCR products were confirmed by enzyme-linked amplicon hybridization assay (ELAHA) with specific oligonucleotide probes [Syrmis et al., 2004; Rohde et al., 2005] as described previously [McIver et al., 2005].

Picornaviruses were detected using a monoplex RT-PCR [Hakonarson et al., 1999]. cDNA was synthesized by adding 8 µl of template to 5.4 µl RNase-free water, 4 µl 5× buffer, 0.8 µl 10 mM dNTPs, 0.5 µl of each forward and reverse primer, and 0.8 µl one-step enzyme mix (Qiagen). Cycling conditions were 50°C for 30 min, 95°C for 15 min followed by 50 cycles of denaturation, annealing and extension at 94°C for 20 sec, 60°C for 30 sec and 72°C for 30 sec, respectively, with a final extension at 72°C for 7 min. DNA products were visualized by 2% agarose gel electrophoresis with SYBR<sup>®</sup> Safe staining (Invitrogen). PCR products were purified with the Wizard<sup>®</sup> SV Gel and PCR Clean-Up System according to the manufacturer's instructions (Promega, Sydney, Australia), sequenced using the PCR primers as described previously [Woon et al., 2008] and compared to sequences in the National Centre for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/>) for identification.

### Virus Culture and TCID<sub>50</sub> Assays

In the subset of subjects who were PCR positive for rhinovirus, 10 were chosen at random in an attempt to culture infectious rhinovirus from the other half of their stored electret sample. For this, HeLa cells were cultured in Eagle's minimum essential medium (EMEM) (Sigma, Castle Hill, Australia) supplemented with 10% fetal calf serum (FCS) (Invitrogen), penicillin (100 U/ml) (Invitrogen), streptomycin (100 µg/ml) (Invitrogen), and L-glutamine (292 µg/ml) (Invitrogen). The HeLa cells were maintained in T-75 cm<sup>2</sup> ventilated culture flasks (Greiner Bio-One, Frickenhausen, Germany) in 5% CO<sub>2</sub>/95% air. For the TCID<sub>50</sub> assays 10<sup>4</sup> cells were added to each well of a 48-well plate (Greiner Bio-One) and incubated for 1 day at 37°C at 5% CO<sub>2</sub>/95% air. To remove virus from the stored electret, 300 µl of EMEM containing 2% FCS and supplements was added to the electret in specially designed double-ependorf tubes and vortexed (2 min). Tubes were

centrifuged at 13,000 rpm for 1 min, the eluate collected in a fresh ependorf tube and the process repeated until 1.5 ml of liquid was collected. Fresh media (1.5 ml) with 2% FCS and supplements were added to the nasal mucous samples and mixed by vortexing. Serial dilutions of the electret eluate and nasal mucous solution were made and added in duplicate to the inoculated 48-well plates, and incubated at 37°C with 5% CO<sub>2</sub>/95% air for 1 hr. The inoculation solution was then replaced with fresh media (750 µl) containing 2% FCS and supplements and incubated at 37°C at 5% CO<sub>2</sub>/95% air for up to 7 days, until cytopathic effects (CPE) were visible in the positive control dilutions. Viral concentrations were determined as the lowest concentration that produced CPE in 50% of the wells.

## RESULTS

### PCR Detection of Respiratory Viruses From Electret

Table I shows the number of subjects in different groups determined by the detection of different viruses by PCR, the route of aerosol generation and whether the subjects had symptoms of a cold or not. Twenty-five subjects overall were virus positive by PCR, 23 of these were positive for a single virus, either picornavirus (human rhinovirus (HRV) or enterovirus), influenza A or B, parainfluenza 3, and human metapneumovirus, and two additional subjects were infected with two viruses. Of the 25 virus-positive subjects, 21 had symptoms consistent with the common cold. In the 19 subjects positive for a picornavirus, sequencing confirmed that the virus type detected in nasal mucous samples was the same as the virus type detected in exhaled samples. The types of picornaviruses detected were Entero 68 (n = 7), Antwerp HRV (n = 6), HRV B (n = 3), and other HRVs (n = 3). In Table I, for simplicity, these are all labeled as HRV, as Entero 68 is also identified as human rhinovirus 87 [Ishiko et al., 2002] and they are all phylogenetically very similar [Laine et al., 2005]. Testing for respiratory syncytial virus and parainfluenza 1 and 2 was negative in all subjects.

Of the 25 subjects with positive nasal mucous, 12 subjects had positive samples from breathing through the mouth or nose, 9 subjects had positive samples from talking, while 2 had positive samples from coughing. One subject had rhinovirus and influenza in both nasal mucous and nasal-breathing samples and the other had influenza detected in the mouth-breathing sample only and rhinovirus in the nasal mucous only. Ten subjects had virus detected in only their nasal mucous samples and not in any other samples.

Four asymptomatic subjects had rhinovirus detected in their nasal mucous, coughing, and breathing samples. Sequencing revealed that these patients were all infected with a virus most closely related to a human enterovirus 68 isolate (GenBank accession no. EF107098) in the NCBI database. All these subjects also had self-reported asthma.

TABLE I. Results of PCR Detection of Virus in Exhaled Bioaerosols and Nasal Mucus

| Number of subjects | Breathing through mouth and/or nose |            | Talking | Coughing | Nasal mucus | Symptomatic or asymptomatic |
|--------------------|-------------------------------------|------------|---------|----------|-------------|-----------------------------|
| 1                  | HRV                                 |            |         |          | HRV         | Asymptomatic                |
| 3                  | HRV                                 |            | HRV     |          | HRV         | Asymptomatic                |
| 13                 | None                                |            | None    | None     | None        | Asymptomatic                |
| 1                  | HRV                                 |            | HRV     |          | HRV         | Symptomatic                 |
| 1                  | HRV                                 |            |         |          | HRV         | Symptomatic                 |
| 1                  |                                     |            | HRV     |          | HRV         | Symptomatic                 |
| 1                  |                                     |            |         | HRV      | HRV         | Symptomatic                 |
| 1                  |                                     |            |         |          | HRV         | Symptomatic                 |
| 1                  | Flu                                 |            |         |          | Flu         | Symptomatic                 |
| 12                 | None                                |            | None    | None     | None        | Symptomatic                 |
|                    | Nose only                           | Mouth only |         |          |             |                             |
| 6                  |                                     |            |         |          | HRV         | Symptomatic                 |
| 2                  | HRV                                 |            | HRV     |          | HRV         | Symptomatic                 |
| 1                  | HRV and flu                         |            | Flu     |          | HRV and flu | Symptomatic                 |
| 1                  | Flu                                 |            |         |          | HRV         | Symptomatic                 |
| 1                  |                                     |            |         |          | Flu         | Symptomatic                 |
| 1                  |                                     |            |         | HPMV     | Flu         | Symptomatic                 |
| 1                  |                                     |            | Para 3  |          | HPMV        | Symptomatic                 |
| 1                  |                                     |            |         |          | Para 3      | Symptomatic                 |
| 1                  |                                     |            |         |          | Para 3      | Symptomatic                 |

The columns show the number of subjects in the different groups, the different groups of respiratory events, whether the subjects were symptomatic or asymptomatic for a cold and the type of virus found in each subject group. The respiratory events were breathing through the nose and/or mouth in 35 subjects, or the nose and mouth separately in a subset of 15 subjects; talking, coughing, or in the nasal mucus sample. The type of virus is abbreviated as HRV: human rhinovirus, Flu: influenza A or B, Para 3: parainfluenza virus 3 and HPMV: human metapneumovirus. None indicates the PCR assays were all negative. No samples were positive for either respiratory syncytial virus or parainfluenza 1 and 2.

**Nose Breathing Versus Mouth Breathing**

In the subgroup where separate nasal and oral breathing samples were collected (see Table I), four subjects had virus detected in their mucous and nose-breathing samples but not their mouth-breathing samples; while one had influenza detected in their mouth-breathing sample and rhinovirus in the mucous sample.

**Culture of Live Virus From Electret**

Two out of 10 subjects positive for rhinovirus by PCR were also positive in HeLa cell culture. The viruses were not sequenced. One subject who had nasal-breathing samples taken on two consecutive days gave TCID<sub>50</sub>/ml values of 2.0 × 10<sup>3</sup> and 2.0 × 10<sup>2</sup>. The coughing sample from another subject had a TCID<sub>50</sub>/ml value of 2.0 × 10<sup>2</sup>.

**DISCUSSION**

The results demonstrate that of the subjects who reported symptoms of a cold, about two-thirds (21/33) had a virus detected by PCR in their mucus and about one-third (12/33) of all subjects with a cold had the same type of virus detected in samples collected during breathing. These results confirm our original findings of viruses exhaled by breathing alone [Huynh et al., 2008], and also confirm the findings of Fabian et al. [2008] that aerosols of influenza are produced by breathing.

In a subset of samples from breathing, live virus was also detected, supporting the hypothesis that such aerosols are capable of transmitting infections. This indicates that the detection of virus by PCR, at least in

some cases, also reflects infective virus and not simply viral RNA released into the mucous. Why infective rhinovirus was not detected in the other PCR-positive samples is not known; possibilities include real differences in PCR and infectious particles between samples, differences only in sensitivity between the culture and PCR systems, and reductions of virus titer on freezing and storage. No attempt was made to culture influenza virus and whether it is infectious in aerosols collected by this method remains to be established. It has been presumed that N95 and surgical masks when used for prophylactic protection may become contaminated with infectious virus; while we are not aware of any direct supporting data for this, the collection of infective virus by masks made of the same filter materials, would support this presumption.

Rhinovirus-positive PCR results were obtained more frequently with samples collected from exhalation through the nose than through the mouth. However, the number of samples here is too small to generalize and further research is required to determine whether this reflects factors relating to the site of infections or to mechanisms of viral shedding. In the other samples collected without distinguishing between nose and mouth breathing, several subjects complained of stuffy noses and mouth breathing would have been more likely; however, this was not recorded formally. It is noteworthy that rhinovirus was detected in the exhaled breath of four asymptomatic controls, all of whom had self-reported asthma. The persistence of rhinovirus infections in the lungs of asthmatics has been demonstrated recently [Wos et al., 2008] and this may suggest that mouth breathing by such subjects might generate

positive aerosols. Further studies of this aspect are required.

There are numerous limitations to the study: (a) the majority of the study population were university students who were well enough to attend classes and thus may have had more mild infections or have passed their peak of viral shedding; (b) the nasal mucus control sample harvested by blowing the nose may have underestimated the numbers of virus-positive subjects compared to nasal pharyngeal aspirates. However, performing this more invasive sampling procedure would have limited recruitment in this setting; (c) sampling time was short; the only other study to measure exhaled influenza used 20 min [Fabian et al., 2008], whereas the protocol sampled for 5 or 10 min. (d) The proportion of each sample analyzed for a virus may have also limited our detection; Fabian et al. [2008] used the entire filter in their PCR whereas our study used approximately 2.6% of the total sample per multiplexed PCR reaction. Recent data obtained by us this year [Ng, unpublished data] from sampling children in hospitals, show a much higher rate of virus-positive samples and a higher concordance between the different breathing activities than found in this population.

This study differed from an earlier study which used an occlusive, surgical-style mask to hold the electret filter [Huynh et al., 2008]. In the current study, a rigid mask was used that sealed to the face and maintained the sampling filter ~2 cm away from the mouth and nose. This eliminated any particle loss from leakage around the edges of the mask and also ensured that there was no possibility of contamination of the filter by direct contact with the skin.

Various estimates of the size range of particles generated by coughing and talking have been described, see reviews by Nicas et al. [2005] and Li et al. [2007] with recent data [Chao et al., 2009; Morawska et al., 2009]. Smaller particles, as generated by breathing have been described in normal subjects [Edwards et al., 2004; Clarke, 2005] and in infected subjects [Fabian et al., 2008], although their origins remain unclear [Fiegel et al., 2006]. The size of aerosols collected onto the electret filter membrane in our mask positioned close to the nose or mouth was not measured. They probably range from droplets generated by coughing and bubbling mucus to smaller undesiccated droplets associated with breathing. A recent study, which measured the size of influenza-positive aerosols in a room [Blachere et al., 2009], showed these varied widely with the occasion, with approximately half in the 1–4  $\mu\text{m}$  size fraction.

The current study, and that of Fabian et al. [2008], have implications for strategies to control transmission of influenza. These data suggest that viruses may be transmitted by somebody quietly breathing through their nose, as well as by visibly coughing or talking; the activities associated previously with virus aerosolization. Such small particles may not be filtered efficiently by surgical masks and may remain airborne for hours.

Further studies quantifying virus aerosolization are required to better understand patterns of transmission. These include studies conducted during the clinical course of infections and within family groups to identify those who are asymptomatic, and those shedding high titers of virus [Atkinson and Wein, 2008]. It was shown that collecting exhaled aerosols is technically simple to perform and has the potential to change perspectives and therefore improve strategies for the prevention of transmission of these viral respiratory diseases.

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