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METHOD FOR MEASURING THE SIZE DISTRIBUTION OF AIRBORNE RHINOVIRUS

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ABSTRACT
About 50% of viral-induced respiratory illnesses are caused by the human rhinovirus (HRV). Measurements of the concentrations and sizes of bioaerosols are critical for research on building characteristics, aerosol transport, and mitigation measures. We developed a quantitative reverse transcription-coupled polymerase chain reaction (RT-PCR) assay for HRV and verified that this assay detects HRV in nasal lavage samples. A quantitation standard was used to determine a detection limit of 5 fg of HRV RNA with a linear range over 1000-fold. To measure the size distribution of HRV aerosols, volunteers with a head cold spent two hours in a ventilated research chamber. Airborne particles from the chamber were collected using an Andersen Six-Stage Cascade Impactor. Each stage of the impactor was analyzed by quantitative RT-PCR for HRV. For the first two volunteers with confirmed HRV infection, but with mild symptoms, we were unable to detect HRV on any stage of the impactor.

INDEX TERMS
Bioaerosols, Infectious disease, Measurement methods, Quantitative RT-PCR, Rhinovirus

INTRODUCTION
Indoor airborne bioaerosols contribute to a broad range of health effects including communicable respiratory illness. The majority of the US population has one or more respiratory illnesses annually (Turner, 1997). The important bioaerosols include human-produced droplet nuclei containing infectious virus and bacteria, non-infectious bacteria and fungi (e.g., molds), and allergens from plants, dust mites, insects (e.g., cockroach), and animals (e.g., pets). Measurements of the airborne concentrations of these bioaerosols are critical for research on the relationship of exposures to health effects, for studies of the influence of building characteristics on exposures, and for evaluations of exposure mitigation measures. Only very limited data exist regarding the size distribution of human-produced droplet nuclei and no data are available on droplet nuclei containing human rhinovirus (HRV).

There are enormous limitations in the sampling and analysis methods used traditionally for many of these bioaerosols. Airborne virus measurement has required very large samples to overcome sensitivity limitations and culturing within the tissue or cells of a host (e.g. human tissue); hence, these measurements are extremely rare. Polymerase chain reaction (PCR) is extremely sensitive, and the use of primers with DNA sequences unique to the target of interest make it also very specific. Recent development of a real-time PCR system makes quantitation of the target sequence faster and easier. The use of PCR for applied health research began primarily with the analysis of body fluids or nasal washings to diagnose disease. For example, Johnston *et al*. (1995) used PCR to detect common respiratory viruses,

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including rhinovirus, in nasal aspirates and found that asthma exacerbation in children (9-11 years) was associated with a respiratory infection about 80% of the time. PCR has also been used recently (Aintablian *et al*., 1998, Sawyer *et al*., 1994, Kashima *et al*., 1991, and Sawyer *et al.*, 1995) for the non-quantitative or semi-quantitative detection of selected viruses and bacteria in air samples (e.g., Varicella-zoster virus, Respiratory Syncytial virus, M. Tuberculosis bacteria).

The goals of this study are to develop a quantitative reverse transcription-coupled PCR (RT-PCR) assay for human rhinovirus (HRV), to detect airborne HRV in bioaerosols, and to use this assay to learn about the aerodynamic size distribution of airborne particles with rhinovirus. Most of these particles are expected to be droplet nuclei containing rhinovirus that are produced by infected people during coughing and sneezing; however, some of these droplet nuclei may attach to other airborne particles. Dick *et al*. (1987) demonstrated that rhinovirus infections are transmitted, in part, through inhalation of virus. A review of ten epidemiologic studies found that building characteristics, such as ventilation rates, are significantly associated with the prevalences of respiratory illnesses experienced by building occupants, with most adjusted relative risks or odds ratios between 1.3 and 2.0 (Fisk 2000). The principal explanation for these findings is that building characteristics influence exposures to droplet nuclei with viable virus. The aerodynamic size of these droplet nuclei determine their range of transport indoors, rates of loss from indoor air via deposition, location of deposition in the respiratory system, and the efficacy of control measures such as filtration. Very few data however, are published on the size distribution of human-produced droplet nuclei (Brosseau *et al*., 1994).

METHODS

Quantitative RT-PCR Assay: Precautions were taken to minimize contamination during sampling, RNA extraction, and reaction mix preparation (Kwok and Higuchi, 1989). A negative control was included in each set of samples processed. Before reverse transcription, RNA secondary structure was removed by heating the sample RNA with the forward primer HRV01F (Table 1) for 4 minutes at 72°C, then immediately chilling the sample on an ice bath. The RNA/primer mixture was reverse transcribed using 200 units of Moloney-murine leukemia virus reverse transcriptase (M-MLV RT) and 30 units of a ribonuclease inhibitor for 1 hour at 40°C. The resulting cDNA was stored at -80°C or amplified immediately.

Table 1. HRV RT-PCR Primer sequences.

Primer	*Sequence*	*Position*
HRV01F	5’-GAAACACGGACACCCAAAGTAG	531-552
HRV01D	5’-TAGCCTGCGTGGCTGCC	349-366

The quantitation of the 203 base pair (bp) HRV PCR product generated by primers listed in Table 1 was performed using a rapid air microcapillary cycler with real-time product detection by fluorescence (LightCycler, Roche Molecular Biochemicals). cDNA was amplified using the LightCycler-FastStart DNA Master Mix reagent (Roche Molecular Biochemicals) with a final concentration of 12.5 pmol each primer and 3 mM $MgCl_2$. Thermal cycling conditions are given in Table 2. A serial dilution of the external quantitation standard (HRV QS) is included with each group of samples that are amplified.

Table 2. Thermal Cycling Conditions for Quantitative HRV RT-PCR Assay.

Segment	*Step*	*Conditions*		*Cycles*
		+Temp.°C	*Time (sec.)*	
denaturation	1	95	10 min.	1
initiation	1	95	10	4
	2	73	10	
quantitation	1	95	5	35
	2	69	15	
	3	72	10	
	4	84	5*	
melting	1	70	15**	1
+(0.1°C/sec.)	2	95	1	

+All temperature transition rates are 20°C/sec. unless indicated.
*single fluorescence reading
**continuous fluorescence acquisition

Quantitation Standard(QS): The HRV QS containing the 203 bp HRV target sequence was obtained by cloning a 425 bp fragment of the 5'untranslated region HRV genome into a bacterial cell line. Briefly, the 425 bp HRV fragment was ligated into a pGEM vector (obtained from Promega) creating a pHRV vector. Competent *E. coli* cells (JM109) were transformed with the pHRV vector. Using the proper selection media, the transformed bacteria were grown, colonies were selected and screened for the presence of the pHRV vector. Large quantities of the pHRV vector were isolated and purified. After linearizing the pHRV vector, the RNA HRV QS standard was produced by *invitro* translation. A fluorescent assay was used to accurately determine the concentration of the HRV QS.

Bioaerosol Sampling: Adult volunteers exhibiting symptoms of a common head cold were recruited from employees according to Human Use Protocols. Volunteers spent 2 hours in a $27m^3$ research chamber while aerosol samples were collected. The research chamber environment had an average temperature of 23°C and a relative humidity of 35% and was supplied with outside air. A small floor fan was set at low speed to provide mixing, while the sampler was placed on a 3 ft. high computer table about 3 ft. from the subject. Chamber air was sampled with a six-stage viable cascade impactor (ThermoAndersen) with 50% size cuts of 7, 4.7, 3.3, 2.1, 1.1 and 0.65 microns. A 45 mm teflon-coated glass fiber filter (Gelman Pallflex, T60A20) was used as a final stage (to collect particles with sizes <0.65 microns). The impactor was prepared for sampling by exposing each side of every stage to a germicidal light for 30 minutes before use. A sterile glass petri dish containing 10 ml of sterile mineral oil was placed into each stage. A sampling rate of 28.3 lpm was maintained with a Gilian AirCon2 high volume pump and was calibrated with a dry gas meter (Rockwell International). A tape recorder was used to provide information on the periods of coughing and sneezing. After the sampling period, a nasal lavage was obtained from each nare of the volunteer. The lavage was stored at -20°C until analysis. The impactor and filter were stored at 4°C until analysis.

HRV RNA Isolation: RNA was isolated from lavage samples using Tri Reagent LS (Sigma Chemical Company) according to the manufacture's directions. The isolated RNA was resuspended in ribonuclease-free H_2O and then reverse transcribed immediately, as described

below. RNA was isolated from viable HRV type 89 (American Type Culture Collection) for use as a positive control. To recover HRV from each stage of the impactor, the petri dishes were rinsed 4 times with 3 ml chloroform each and transferred to a 50 ml polypropylene sterile centrifuge tube. The dishes were scraped each time with a sterile policeman to facilitate the transfer and resuspension of the particles. Each dish was washed one final time with 3 ml of RNA lysis buffer (Chomczynski and Sacchi, 1987). The two phases were mixed together by shaking and vortexing, then separated by centrifugation at 2,000g for 2 minutes at 15°C. The HRV RNA in the lysis buffer was recovered by alcohol precipitation as described by Chomczynski and Sacchi (1987). RNA was resuspended in ribonuclease-free H_2O and then immediately reverse transcribed.

RESULTS

The specificity of the HRV RT-PCR assay is determined by primer sequence. Primers HRV01F and HRF01D (see Table 1) were slightly modified from primer ncr2 and probe Rh of Andeweg *et al.*, (1999). The primers are located in regions of high genetic homology of the 5'untranslated region of the HRV genome as indicated by a genetic analysis and sequence alignment of 25 HRV strains (Andeweg *et al.*, 1999). As a result, this assay detects more than 90% of HRV strains and will not detect the genetically similar enterovirus family; however, several enterovirus-like HRV strains (87,14,37,72) are not detected. Computer-aided analysis of several different HRV strain sequences obtained from GenBank was used to confirm this observation.

Before beginning chamber studies, the HRV RT-PCR assay was tested on the nasal lavages of 6 volunteers with head cold symptoms and 1 volunteer with allergy symptoms. Four of the 6 head cold specimens were found to be HRV positive while the specimen from the allergy sufferer was negative and served as a negative control. Melting curve analysis at the end of the amplification serves to identify each product formed during PCR (see Figure 1). The melting curve is unique to each product and is dependent on length and nucleotide sequence of the DNA. RNA from HRV type 89 was used to confirm a positive lavage sample (5R) and the HRV QS standard. All have a melting temperature of 88°C. A negative sample (2R) shows non-specific product formation at lower temperatures.

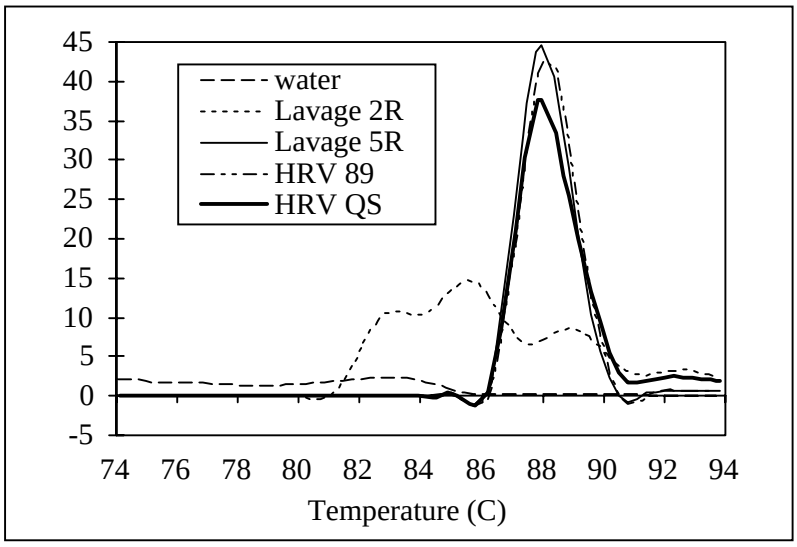

Figure 1. Melting Curve Analysis of HRV PCR Products.

During PCR there is an exponential accumulation of product; this phase of the reaction is used for quantitation. The concentrations of unknown samples are determined from the first cycle of the PCR run in which the signal can be distinguished from the background. By extrapolating this cycle number from a standard curve generated from a dilution series of the HRV QS (Figure 2), the concentration of an unknown sample may be determined. This remains true as long as the standard and unknown are amplified with the same efficiency as is true for this assay. A detection limit of 5 fg of HRV QS was observed. At this point non-specific product formation competes with HRV product accumulation and although detection is still possible at this level, quantitation is no longer valid. The standard curve is linear over a 1000-fold range and the assay has a variance of 8% at the 5000 fg level and 20% at the 50 fg level (n=3).

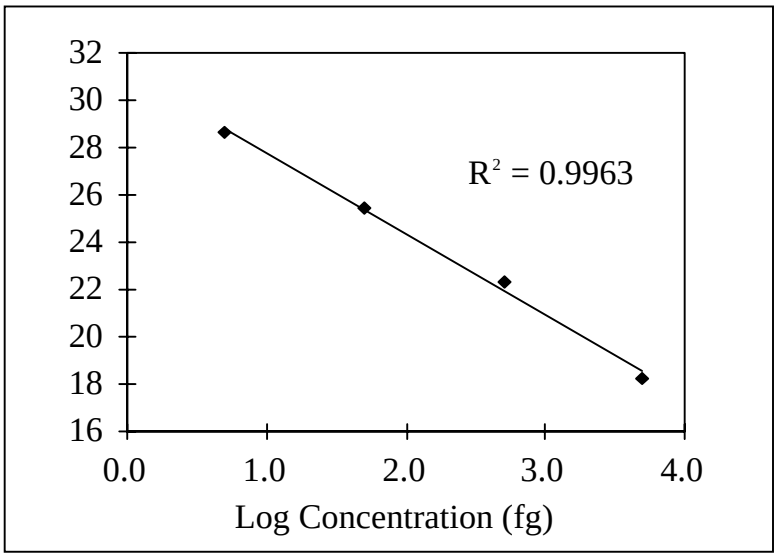

Figure 2. Standard Curve for Quantitation of HRV.

The HRV RNA quantitation standard was used to develop a sample extraction method. Filters and petri dishes were spiked with various amounts of the HRV QS and then subjected to 4 hours of sampling using the six-stage impactor. As a control, the HRV QS was isolated directly from the lysis buffer without any sampling or sample extraction. Recovery of 10 pg by the alcohol precipitation method from the impactor was 60% with a variance of 30% (n=3). In comparison, precipitation directly from the lysis buffer gave a recovery of 65%. Stages were extracted a second time to determine how much of the HRV QS remained. Only 1% of the initial amount spiked into the mineral oil was detected.

To date, we have recruited 4 volunteers exhibiting head cold symptoms to participate in the chamber experiment. Two of the volunteers displayed very strong cold symptoms, but were negative for HRV. The other two volunteers, who displayed only light symptoms (some coughing and no sneezing), were positive for HRV. However, no HRV could be detected on any stage of the impactor.

DISCUSSION

The most common bioaerosol measurement approach is the impaction of particles on culture media, followed by growth of these organisms. HRV identification by viral culture is difficult and can take up to 3 weeks. Using a PCR based assay for the detection of HRV eliminates the

need to recover viable organisms, increases sensitivity three-fold over viral culture systems, and reduces the assay time to two hours (Johnston *et.al.*, 1995). Existing HRV PCR assays are developed for clinical diagnostic purposes and are qualitative or semi-quantitative in nature. The HRV RT-PCR assay we describe here is validated as a strictly quantitative method with a detection limit of 5 fg and is capable of detecting over 90% of the more than 125 characterized HRV strains.

The HRV genome consists of 7,102 nucleotide bases, therefore 1 fg of HRV RNA corresponds to about 280 HRV organisms. Our calculations show that HRV viral production in the nasal mucosa of infected individuals is great enough to suggest the feasibility of detecting HRV in aerosol samples. We used the HRV QS to estimate the viral load in one nare of an individual to approximately $1x10^7$ HRV genome equivalents. Taking into account the assay sensitivity and sampling and recovery efficiencies, we need to collect $3x10^3$ HRV organisms or 0.03% of the viral load that an individual might produce.

One of the keys to successful analysis of HRV in aerosol samples is the improvement of the sample recovery process. Initial experiments with the HRV QS show that RNA is not degraded during the sampling process with the six-stage impactor; however, half of the RNA is lost during the alcohol precipitation step. We also examined RNA recovery from a commercially available solid-phase substrate, but recovery of the HRV QS at the 10 pg level was found to be only 10%. This method was not pursued. Currently, very little is known about viral production during an HRV-induced cold. Conflicting reports of virus shedding vary from 1 day before symptoms occur to between 2 days and 3 weeks after the onset of cold symptoms (Turner, 1997). One of our volunteers participated in the chamber experiment after experiencing cold symptoms for 4 days. Although this nasal lavage was weakly positive, it is unknown if viral shedding would have been greater at the onset of symptoms. It is also difficult to find volunteers that have not taken an antihistamine to suppress many of the cold symptoms that would normally produce an aerosol.

CONCLUSION

We developed a quantitative RT-PCR assay specific for HRV that can quantify the virus to 5 fg. We also can recover HRV RNA from previously spiked multistage impactors. This HRV RT-PCR assay is validated and is ready to be used in the detection of HRV in aerosols.

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