An *in vitro* model to evaluate virus aerosol characteristics using a GFP-expressing adenovirus

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Assessment of virus aerosol characteristics is important in designing methods for controlling infectious virus aerosols. The factors relevant to aerosol characteristics include aerosol particle size, concentration, infectivity and virulence. To investigate these characteristics and their changes in different environmental conditions, a laboratory model is required. In this study, replication-deficient recombinant adenovirus (RDRADS) encoding green fluorescent protein (GFP) was used as a model virus. Model virus aerosols were generated using a TK-3 aerosol generator attached to a chamber which could simulate different environmental conditions. Virus aerosol specimens were collected with an FA-1 six-stage impact sampler. The relative genome copy number of viruses in the aerosol was determined by real-time fluorescence quantitative PCR. The number of virus-infected PK15 (pig kidney) cells was determined by counting cells with green fluorescence under a fluorescence microscope at 48 h post-inoculation. Fifteen experiments in different conditions were performed. We found that the viral DNA was present in stages 4–6 of the sampler, with the peak value at stage 5, corresponding to aerosols with a particle size of 0.65–3.3 µm. PK15 cells with green fluorescence showed the same size distribution range at temperatures >29 °C and above, where no green fluorescent cells were found, while the genome copy number assayed by real-time PCR remained unchanged. In the presence of high concentrations of particulate matter created by burning biomass, the peak value of virus genome copy number and green fluorescent cell counts shifted to stage 4 of the sampler, corresponding to aerosols with a particle size of 2.1–3.3 µm. The results provide evidence that viruses are present in the atmosphere as aerosols, which are much larger than their own particle size, and that the viruses in the aerosols are affected by atmospheric conditions. Our laboratory model was shown to be feasible for investigating the relationship between the characteristics of viruses and atmospheric conditions.

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INTRODUCTION

Viral transmission by aerosols has attracted public attention, after the spread of SARS coronavirus across five continents. Aerosolization is potentially an important long-distance dispersal mechanism and may account for the observed widespread distributions of some viruses. Virus-containing aerosols with particles less than 2.0 µm in size can remain airborne for long periods and can be carried distances of up to thousands of kilometres from the source by wind. When a virus is encased in an aerosol, its infectivity is enhanced compared to an isolated airborne

virus, due to shielding from drying, temperature and sunlight (Tyrrell, 1967). Due to such encasement, virus aerosols less than 2.0 µm in size have higher infectivity than those of the virus itself (Couch *et al.*, 1965). It has been reported that different environmental conditions, including relative humidity (RH), temperature (Lowen *et al.*, 2007), solar UV radiation (Sagripanti & Lytle, 2007), wind speed (Brownstein *et al.*, 2006) and the suspending medium (Agranovski *et al.*, 2005), influence the characteristics of airborne viruses, including aerodynamic diameter, concentration and infectivity. These characteristics influence the stability and transmission of virus aerosols in the atmosphere. Aerosols in the 2 µm size range can reach the pulmonary alveoli and cause disease of the cardiovascular

Abbreviations: GFP, green fluorescent protein; RDRADS, replication-deficient recombinant adenovirus; RH, relative humidity.

and respiratory systems through circulation of the blood (Stuart, 1973).

The above facts highlight the importance of studying virus aerosols and their characteristics in order to develop efficient preventive procedures. The natural virus aerosol concentration in the atmosphere is not sufficient for assaying these indices quantitatively, particularly for testing viral infectivity. We need to develop laboratory models to enable us to evaluate characteristics of virus aerosols with accuracy and convenience. After essential data have been collected, they may then be verified in field experiments. The current study was initiated to evaluate the feasibility of a model for reliable and efficient monitoring of the infectivity, genome copy number and size distribution of virus aerosols, with the aim of investigating the effects of environmental changes on virus infection and identifying factors relevant to the spread of viruses.

METHODS

Test viruses and cells. In this study, for sensitivity, specificity and safety of experiments, replication-deficient recombinant adenovirus (RDRADS) encoding green fluorescent protein (GFP) was used as a model virus. All experiments with infectious RDRADS encoding GFP were performed in a Biosafety level II laboratory and were conducted under appropriate conditions. A seed stock of the virus was obtained from the Experimental Medical Research Center of Guangzhou Medical College. The viruses are replication deficient and can be propagated only in their packing cells. This feature enabled us to assay infected cells with accuracy by counting cells with green fluorescence under a fluorescence microscope. The virus-containing material was obtained by culturing with HEK293 (human embryonic kidney) cells and subsequent triple freezing of the infected cell culture in Dulbecco's-modified Eagle medium (DMEM) maintenance medium. Prior to testing, the medium containing the viruses was kept at -70°C . For finding sensitive cell lines, 100 μl samples of tenfold serial dilutions of the viral suspension were inoculated onto a confluent monolayer of BHK21 (baby hamster kidney) and PK15 (pig kidney) cells in 96-well plates and incubated for 90 min. After the viruses were removed, inoculated cell cultures were maintained in DMEM supplemented with 2% inactivated fetal bovine serum (Hyclone, SH), 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} . Inoculated cell cultures were examined after 12, 24, 48 and 72 h of incubation under a fluorescence microscope.

Experimental setup. A 2000-L test chamber for virus sampling was provided by the State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, CAS, People's Republic of China. The air temperature in the chamber can be adjusted by electric heaters controlled by a temperature sensor, and RH by a blanket covered with glycerol at different concentrations on the bottom of the chamber. Another purpose of the blanket is to absorb large virus containing fluids, to prevent collection of possible sediment when sampling.

An FA-1 six-stage impactor was placed in the chamber. This impactor is a design modelled on the Andersen six-stage impactor, which collected the viruses aerosolized from the air into cell culture plates placed in the test chamber. The impactor is based on the principle of air accelerating through an entry plate with critically sized orifices and inertially impacting onto a surface below. The sizes of the aerosol particles sampled from the first stage to the sixth stage in the FA-1 six-stage impactor were $>7.0\text{ }\mu\text{m}$, $4.7\text{--}7.0\text{ }\mu\text{m}$, $3.3\text{--}4.7\text{ }\mu\text{m}$, $2.1\text{--}3.3\text{ }\mu\text{m}$,

$1.1\text{--}2.1\text{ }\mu\text{m}$ and $0.65\text{--}1.1\text{ }\mu\text{m}$, respectively. A $100\times 20\text{ mm}$ cell culture plate with 2 ml DMEM containing 2% inactivated bovine serum, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} was used as the impaction surface for each stage.

A TK-3 aerosol generator attached to the chamber aerosolized viruses during experiments. The viral particles were aerosolized from a 2 ml initially prepared suspension. After 30 min, a stable virus aerosol had been formed; and the lid of the FA-1 impactor was then opened and the impactor was operated to collect the virus aerosol in parallel at 28.3 l min^{-1} for 5 min.

Virological analytical procedures. The samples collected by impactor onto the cell culture medium were analysed for genome copy number by real-time PCR and for infectivity by green fluorescence of PK15 cells.

For analysing gene copy number, DNA from 500 μl of the fluid samples was extracted by using the QIAamp DNA Blood Mini kit (Qiagen), as instructed by the manufacturer. The primers used for real-time fluorescence quantitative PCR were based on PEGFP-C₂ gene sequences obtained from GenBank (accession number CVU 57606). The forward primer 5'-tgccatcctggtcgagctg-3' (nt 650–669) and the reverse primer 5'-ccgtaggtcagggtgggtcac-3' (nt 796–815) were used. The primers represent a 166 nt amplicon. A GenBank basic local alignment search tool (BLAST) search (<http://www.ncbi.nlm.nih.gov/BLAST>) revealed no homology between the forward primer or reverse primer in the gene assay and other human or viral sequences.

For the calibration curve used for the quantitative analysis of the virus gene copy, a tube with sufficient amount of original virus prepared from packing cells was used as the main stock. The standard working stock was a 10-fold dilution with cell culture medium. This working stock was then subjected to serial fourfold dilution until the required concentration was reached. DNA was then extracted as described above.

PCR was performed in a standard programme. Briefly, 4 μl RDRADS DNA was amplified in a 25 μl reaction containing 12.5 μl 2 $\times$ Master mix, 1 μl primers, 2.5 μl reference dye and 5 μl RNase-free water. The thermocycler conditions used were as follows: 95°C for 5 min; and 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s.

For assaying virus infectivity, 1500 μl of the fluid samples was added to 80% confluent PK15 cell monolayers in 24-well plates. The plates were rocked every 10–15 min during a 90 min period. Then the inocula were replaced with DMEM containing 2% inactivated fetal bovine serum, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} , and the cells were incubated at 37°C for 48 h in a humidified incubator with a 5% CO₂ atmosphere. Virus infectivity was quantified by counting cells with green fluorescence under a fluorescence microscope.

RESULTS AND DISCUSSION

The test virus was selected for two reasons. Firstly, due to replication deficiency, communicability had been removed, which ensured the safety of experiments, and cells could be observed in a wide time window because there was no secondary infection between cells by replicated virus. Secondly, because the virus carries a GFP gene that can be expressed in host cells, we could determine the infectivity of the virus with accuracy and convenience: there was no need to use specific procedures to detect the virus.

Two strains of cells were used in our experiments. To select the best strain, BHK21 and PK15 cells were inoculated with

tenfold serial dilutions of the virus and observed by fluorescence microscopy at 12, 24, 48 and 72 h after inoculation. Green fluorescence was detectable in both BHK21 and PK15 cells, indicating that they can be infected by the virus and support GFP expression. There were significant differences between BHK21 and PK15 cells in the brightness of fluorescence and the number of cells that showed fluorescence. The PK15 cell strain was more sensitive to the virus infection. In repeated experiments, fluorescent cell counts were found to increase with time up to 48 h, then remained unchanged up to 72 h, providing a relatively stable time window for assessment (Fig. 1). At 48 h post-inoculation, the cells were collected and examined by transmission electron microscopy, which revealed typical viral particles with crystal lattice structures in the cytoplasm and nucleus of PK15 cells. The results indicated that virus aerosols collected from the air in the chamber were typical of RDRADS carrying GFP.

Titration of the virus was performed in PK15 cells infected with the virus by counting cells with green fluorescence. The results showed that the lowest value that could be observed was at 10^5 -fold dilution of the virus working stock, which was 3 log decades lower than that for BHK21 cells.

Fifteen experimental runs with different environmental conditions were undertaken and the results are shown in Table 1. The original experiment design included a series of stringent condition controls. In practice, the chamber conditions of temperature and humidity could not be adjusted to the precise values required. And more importantly, as the virus aerosol was a secondary aerosol formed in the chamber by mixing virus and natural aerosol, the physicochemical features of the aerosol could not be kept the same between experiments, due to environmental changes. Although there were deficiencies in the control of conditions, the data nevertheless show some interesting phenomena.

One striking finding is that in the usual experimental conditions ($T < 29^\circ\text{C}$), both virus genome and virus-infected cells were found in the samples of stages 4–6 of the

sampler (size range $0.65\text{--}3.3\text{ }\mu\text{m}$). The relationship between virus aerosol particle size and viral infectivity has been the subject of investigation. It was reported that when the particle size was $1\text{--}3\text{ }\mu\text{m}$, a single cell produced infection, while it required about 800 cells if the particle size was $12\text{ }\mu\text{m}$ (Krueger *et al.*, 1974). Ijaz *et al.* (1987) demonstrated that 87% of aerosolized viruses have a particle size smaller than $2.1\text{ }\mu\text{m}$. Our study showed the majority of particles ranged from 0.65 to $3.3\text{ }\mu\text{m}$, with the peak at around $1.0\text{ }\mu\text{m}$. Those viral aerosol particles are much larger than the particle size of the virus itself. The particle size distributions suggest that the ‘carrier-to-virus’ particle size ratio is $\sim 10^1$, as viral aerosols usually travel as clumps attached to other particles (Otten & Burge, 1999). Thus our artificial model virus aerosol has features favourable for spreading and infectivity, consistent with the characteristics of adenovirus, which is among the most prevalent virus of infection in human and animals.

In three of the experiments, the temperature within the chamber was above 29°C (29.3 , 33 and 34°C). At 48 h post-inoculation, no green fluorescence was evident in the PK15 cells at 33°C or 34°C . At 29.3°C , PK15 cells with green fluorescence were not found initially, but after prolonged incubation for 5 days, some green fluorescence was observed. This implied that the infectivity of the virus was inhibited at about 29°C and above. Also, the C_t values of real-time PCR for viral DNA showed a virus genome distribution shift, with a tendency for increased numbers in larger particle sizes, which was not found in experiments at lower chamber temperatures. The results indicate that high air temperatures can affect viral infectivity and aerosol diameter. Others in our laboratory also noticed this phenomenon with the temperature slightly higher (30°C , data not shown). The influences of relative humidity on aerosol diameter were not taken into account. At higher air temperatures, the aerodynamic diameter of virus particles was larger than at lower air temperatures. The low incidence of viral infectious disease at high air temperatures could be related to such effects.

Four experiments were performed with a high concentration of particulate matter (PM 2.5) created by burning

Fig. 1. Expression of GFP in BHK21 and PK15 cells infected with 10^3 dilutions of the virus at different times post-inoculation.

Table 1. Results of real-time fluorescence quantitative PCR and virus infectivity in PK15 cells

T, air temperature in chamber; RH, relatively humidity in chamber; AV, mean of green fluorescence in five visual fields; C_t, mean result ($\pm$ SD) from duplicate cycle threshold of real-time fluorescence quantitative PCR.

Sampling date*	Simulated conditions		Virus aerosol particle size (μ m)						
	RH (%)	T (°C)		>7.0	4.7–7.0	3.3–4.7	2.1–3.3	1.1–2.1	0.65–1.1
1	38.5	20.6	AV	0	0	0	22 $\pm$ 9.23	62 $\pm$ 15.93	44 $\pm$ 8.90
			C _t	30.55 $\pm$ 0.03	30.65 $\pm$ 0.52	30.21 $\pm$ 0.14	27.59 $\pm$ 0.06	27.75 $\pm$ 0.13	27.12 $\pm$ 0.78
	99	21.1	AV	0	0	0	0	12 $\pm$ 3.78	71 $\pm$ 18.8
			C _t	30.17 $\pm$ 0.10	30.33 $\pm$ 0.06	31 $\pm$ 0.02	30.76 $\pm$ 0.15	29.4 $\pm$ 0.071	25.44 $\pm$ 0.11
2	97	24.6	AV	0	0	0	8 $\pm$ 2.41	28 $\pm$ 7.92	23 $\pm$ 6.76
			C _t	27.65 $\pm$ 0.99	30.13 $\pm$ 0.62	29.42 $\pm$ 0.06	27.83 $\pm$ 0.00	26.28 $\pm$ 0.18	28.75 $\pm$ 0.47
	93.3	24.7	AV	0	0	0	8 $\pm$ 2.07	50 $\pm$ 3.56	25 $\pm$ 10.70
			C _t	29.81 $\pm$ 1.48	30.14 $\pm$ 1.20	31.39 $\pm$ 0.16	29.49 $\pm$ 1.87	27.33 $\pm$ 0.37	28.24 $\pm$ 1.12
	89.5	29.3	AV	0	0	0	0	0	0
			C _t	30.8 $\pm$ 0.37	31.69 $\pm$ 1.00	31.57 $\pm$ 0.04	30.72 $\pm$ 0.38	28.91 $\pm$ 0.18	27.33 $\pm$ 0.05
3	80	33	AV	0	0	0	0	0	0
			C _t	26.87 $\pm$ 1.87	29.86 $\pm$ 0.01	30.04 $\pm$ 0.35	29.69 $\pm$ 0.18	27.25 $\pm$ 0.08	26.84 $\pm$ 0.04
	65	34	AV	0	0	0	0	0	0
			C _t	28.57 $\pm$ 0.37	29.85 $\pm$ 0.95	27.61 $\pm$ 1.28	30.03 $\pm$ 0.11	27.72 $\pm$ 0.84	29.79 $\pm$ 0.80
4	50	21	AV	0	0	0	8 $\pm$ 2.74	2 $\pm$ 1.87	5 $\pm$ 2.12
			C _t	29.71 $\pm$ 0.05	29.61 $\pm$ 0.21	28.89 $\pm$ 0.31	25.53 $\pm$ 0.15	23.87 $\pm$ 0.17	24.89 $\pm$ 0.12
	50	21	AV	0	0	0	2 $\pm$ 1.41	8 $\pm$ 2.74	4 $\pm$ 2.55
			C _t	29.71 $\pm$ 0.36	30.24 $\pm$ 0.18	30.08 $\pm$ 0.21	28.53 $\pm$ 0.21	26.51 $\pm$ 0.10	27.76 $\pm$ 0.60
	32	22	AV	0	0	0	2 $\pm$ 1.58	6 $\pm$ 1.58	3 $\pm$ 1.00
			C _t	32.95 $\pm$ 0.06	32.64 $\pm$ 0.27	29.75 $\pm$ 0.11	32.75 $\pm$ 0.35	27.83 $\pm$ 0.13	28.8 $\pm$ 0.015
	32	22	AV	0	0	0	3 $\pm$ 1.22	7 $\pm$ 2.23	2 $\pm$ 0.71
			C _t	31.29 $\pm$ 0.88	30.61 $\pm$ 0.21	30.18 $\pm$ 0.25	26.66 $\pm$ 0.26	25.9 $\pm$ 0.24	26.79 $\pm$ 0.09
5†	75	21	AV	0	0	1 $\pm$ 0.94	16 $\pm$ 4.45	0	0
			C _t	30.58 $\pm$ 0.50	29.27 $\pm$ 0.16	29.97 $\pm$ 0.06	24.71 $\pm$ 0.17	23.84 $\pm$ 0.08	25.32 $\pm$ 0.11
	75	21	AV	0	0	0	0	0	0
			C _t	31.18 $\pm$ 0.22	31.53 $\pm$ 0.07	30.97 $\pm$ 0.11	26.18 $\pm$ 0.39	30.26 $\pm$ 0.86	24.98 $\pm$ 0.28
6‡	75	21	AV	0	0	0	0	0	0
			C _t	32.22 $\pm$ 0.25	31.16 $\pm$ 0.29	31.11 $\pm$ 0.06	32.15 $\pm$ 0.09	27.6 $\pm$ 0.52	25.82 $\pm$ 0.24
	75	21	AV	0	0	2 $\pm$ 0.71	5 $\pm$ 1.00	0	0
			C _t	31.98 $\pm$ 1.20	32.44 $\pm$ 0.19	32.59 $\pm$ 2.46	28.49 $\pm$ 0.10	25.46 $\pm$ 0.04	25.33 $\pm$ 0.01

*1, 12 April 2007; 2, 15 April 2007; 3, 16 April 2007; 4, 18 April 2007; 5, 19 April 2007; 6, 20 April 2007.

†Burning leaves of *Cinnamomum burmanni* (Nees) Blume.

‡Burning leaves of *Pinus massoniana* Lamb.

leaves of *Cinnamomum burmanni* and *Pinus massoniana* at an RH of 75 % and an air temperature of 21 °C. Green fluorescence could be observed in PK15 cells infected with sample fluids of stages 3 and 4, but not with those from stages 5 and 6 of the sampler. The results indicate that the viral activity of virus aerosol in the 0.65–2.1 μ m size range had declined under these conditions. No obvious virus genome copy number distribution shift was found compared with normal conditions. We are attempting to determine whether the chemical characteristics of PM 2.5 affected viral infectivity of the virus or activity of the PK15 cells.

In most experiments, the genome number of virus determined by using real-time fluorescence quantitative

PCR was equivalent to about a 10⁴-fold dilution. At this concentration, virus kept in the medium should infect cells and be detected readily by fluorescence count, since the lowest concentration of virus that could be detected was about 10⁵-fold dilution. In fact all the experiments showed cell counts of zero for some of the particle size ranges. Several reasons may account for the observed loss of the virus infectivity: declines due to sampling stress and dehydration have been reported (Li *et al.*, 1999), and the virus aerosol may lose some of its activity due to contact with different chemicals present in the aerosol. We believe that the last of these may play an important role in the change of infectivity. Understanding the physicochemical features and behaviour of aerosols may

help us to develop methods to prevent the spread of viruses by air.

Conclusions

Both PCR and cell count assays proved that this model adenovirus mainly existed in the air in the form of a virus aerosol of particle size 0.65–3.3 μm . Temperatures higher than 29 °C caused a dramatic decline in the infectivity of the virus, and may also affect the size distribution. The results provide evidence that viruses are present in the atmosphere as virus aerosols, which are much larger than their own particle size, and that the viruses in the aerosols are affected by atmospheric conditions.

The laboratory model presented here has been shown to be feasible for the detection of a relationship between viral character and environmental conditions. Although RDRADS encoding GFP cannot represent all types of viruses, due to the wide range of viral structures and nucleic acids, the model could be extended by using other typical viruses. The detection of antigens by immunofluorescence in the cells can provide confirmation of infection by the virus as a response to viral infectivity.

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