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Journal of Virological Methods, 18 (1987) 87–106
Elsevier

JVM 00650

Development of methods to study the survival of airborne viruses

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(Accepted 17 June 1987)

Summary

A number of viruses have been shown to be transmitted by the airborne route. It is the ability of these viruses to retain their infectivity for living hosts which play a key role in their aerial dissemination. Data generated by a number of workers on the airborne survival of viruses varies considerably because laboratory techniques have not been standardized. About 5 yr ago we started studies on the airborne survival of a number of animal and human viruses. This paper describes the methodology developed to study the aerobiology of these viruses. These methods should be useful in the aerobiological work of other viruses.

Virus aerosol; Respiratory virus; Enteric virus; Viral aerobiological technique

Introduction

The concept of airborne transmission of microbial agents is an ancient one, but the studies related to airborne transmission of microbes started during the past 5 decades (Ijaz and Sattar, 1987). It is now well established that a variety of infectious agents, including many types of viruses, are able to spread by the airborne route. It is the capacity of these viruses to survive in the airborne state which results in their aerial dissemination. In view of this, a great deal of work has been conducted to determine which factor(s) promote or retard the survival of infectious viral agents in air (Spendlove and Fannin, 1982, Ijaz, 1985). A review of the

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literature revealed a considerable variation in the results of different workers because the laboratory techniques for viral aerobiological work have not been standardized. It has been noted that variations in techniques that may influence the results include: (1) methods of aerosol generation, storage, and sampling procedure, (2) methods of virus assay, (3) differentiation of total, physical and viability decay, (4) methods and extent to which relative humidity and temperature are controlled, and (5) method of data presentation (Harper, 1963).

About 5 yr ago we started studies on the survival of a number of airborne human and animal viruses (Ijaz et al., 1984, 1985 a,b,c; Sattar et al., 1984; Karim et al., 1985). The purpose of this paper is to outline the basic techniques developed to study airborne viruses. These methods should be useful to study the aerobiology of other viruses.

Materials and Methods

Cells

The MA-104 cell line was used throughout this study for the cultivation and quantitation of rota- and polioviruses. A seed culture of these cells was originally received by us through the courtesy of Dr. H. Malherbe (Gull Labs, Salt Lake City, UT). The procedures for the cultivation, maintenance and passage of these cells have been described in detail previously (Ramia and Sattar, 1979). For the growth and quantitation of human coronavirus 229E(HCV/229E), L-132 cells, a continuous line of human lung cells was used throughout this study. A seed culture of these cells was originally obtained from D.A. McLeod, Laboratory Center for Disease Control, Ottawa, Canada. They were routinely propagated as described previously (Kennedy and Johnson-Lussenburg, 1975/76). A-5-HeLa cells were used for the cultivation and quantitation of rhinovirus type 14 (RV-14). These cells were obtained from Dr. K. Lonberg-Holm (E.I. duPont de Nemours and Co., Central Research and Development Department, Wilmington DE). Both the cell growth medium and the split ratio were the same for the A-5-HeLa cells as they were for the L-123 cells.

Viruses

Simian rotavirus SA-11 (strain H-96) was also kindly supplied to us by Dr. Malherbe. A cell culture-adapted human rotavirus (subgroup 2, strain Wa) was a gift sent to us by Dr. R. Wyatt (Wyatt et al., 1980) of the U.S. National Institutes of Health (Bethesda, MD, U.S.A.). Bovine rotavirus (C-486) was obtained from Dr. L.A. Babiuk (University of Saskatchewan, Saskatoon, Saskatchewan). Mouse rotavirus was a gift from Dr. A. Cepica of the Laboratory Center for Disease Control, Ottawa and the Campton U.K. isolate of bovine rotavirus was obtained from Dr. M.A. McCrae (University of Warwick, Coventry, U.K.). The Sabin strain of poliovirus type I was obtained from D.A. McLeod of the Laboratory Center for Disease Control (Ottawa). These viruses were plaque purified in MA-104 cells, and the same cell line was used for preparing the virus pools as described previously (Ijaz et al., 1985a).

Human coronavirus strain 229E was originally obtained from Dr. A.Z. Kapikian of the U.S. National Institutes of Health, Bethesda. The virus was cultivated in L-132 cells and the method for the preparation of the virus pools has been described earlier (Kennedy and Johnson-Lussenburg, 1975/76).

Rhinovirus type 14 as classified by Kapikian et al. (1971) was obtained from Dr. Gwaltney, University of Virginia, U.S.A. The virus was first plaque purified in A-5-HeLa cells containing MEM with 5% heat inactivated fetal calf serum (FCS) (56°C for 30 min) and the same medium was used for further propagation of the virus. The infected monolayers were incubated for 48 h at 33°C. After incubation the virus was released from the cells by freezing and thawing the flasks and contents three times. The virus-containing suspension was transferred to centrifuge tubes and clarified by centrifugation at 3000 rpm for 15 min at 4°C. The RV-14-containing supernatant was resuspended in tryptose phosphate broth (TPB) dispensed into 10 ml aliquots and stored at −80°C.

Virus labelling

Two-day old confluent monolayers of MA-104 cells were incubated for 18 h at 37°C in methionine-free MEM. They were then washed three times with Earle's balanced salt solution (EBSS) and inoculated with the virus at a multiplicity of infection of 5:1. The virus was allowed to adsorb to the cells for 2 h at 37°C. The labelling was carried out using methionine-free MEM with 50 μCi/ml of L-[^{75}Se]selenomethionine purchased from Amersham Corp. (Arlington Heights, IL). The cultures were held at 37°C for 24 h. They were then put through the same procedure as described earlier for the preparation of the virus pools (Ijaz et al., 1985a). The labelled virus was however, concentrated by ultracentrifugation at 100000 × *g* for 120 min. The pellet was resuspended in a 40% (w/v) solution of cesium chloride (CsCl) and layered onto a 55% solution of CsCl and then subjected to isopcynic banding by centrifugation at 100000 × *g* for 20 h. The fractions were collected from the bottom of the centrifuge tube and their refractive index measured. Those fractions with the density of 1.36 – 1.38 g/ml were pooled together and dialyzed against distilled water overnight at 4°C. The dialyzed virus was resuspended in TPB and stored at −80°C.

Plaque Assay

After collection of the virus-containing sample, the procedure for the quantitation of rotavirus alone or in mixed aerosol with poliovirus type 1 was applied, which was been described in detail previously (Sattar et al., 1984; Ijaz et al., 1985a). Briefly, the cell monolayers were washed with several changes of EBSS to remove as much as possible the FCS that was present in the growth medium. Each well then received 0.1 ml of an appropriately diluted inoculum. For virus adsorption, the inoculated plates were kept for 1 h at 37°C on a pitched orbital shaker (Heidolph, F.R.G.). At the end of the incubation period, each monolayer was overlaid with 2.0 ml of a medium containing MEM, 0.6% Agarose Type 11 (Sigma Chemicals Co., St. Louis, MO) and 5.0 μg/ml of trypsin (ICN Nutritional Biochem., Cleveland, OH). The plates were resealed in plastic bags and incubated at 37°C

for 3 days in a walk-in incubator. The procedure for fixation and staining of the monolayer before counting plaques has been described earlier (Ramia and Sattar, 1979). Plaque assays of HCV 229E were performed by standard method in monolayers of L-132 cells in 75 cm^2 disposable culture flasks (Lux, Scientific Corp.), as previously described (Kennedy and Johnson-Lussenburg, 1975/76).

The procedure for the quantitation of RV-14 is briefly as following. Flasks containing confluent monolayers of A-5-HeLa cells were infected with 0.33 ml of the virus sample to be plaque assayed. The virus was adsorbed for 1 h at 33°C during which time the flasks were gently shaken at 20-min. intervals to distribute the inoculum over the monolayer evenly. At the end of 1-h incubation period, the monolayers were overlaid with a medium containing 25 ml per flask of M-199 (Flow Laboratories): 0.22% $NaHCO_3$, 5-bromodeoxyuridine (BUDR, Calbiochem) 100 μg/ml, DEAE-dextran 50 μg/ml (Sigma), 30 mM $MgCl_2$ (Fisher Certified Reagent) and 0.9% oxoid No. 1 (Oxoid). The flasks were then placed in a CO_2 incubator at 33 °C. The plaques of RV-14 were ready within 72 h of incubation at which time the cells were mixed with 5 ml of a 4% formol saline solution and allowed to stand for 24 h at room temperature to get rid of all infectious virus. The agar overlay was then carefully removed and the remaining fixed monolayer was stained with a 1% solution of crystal violet to enhance the visibility of plaques which were then counted. Virus titers were expressed as plaque forming units per ml (PFU/ml).

Aerosol generation, storage and collection

Virus-containing aerosols were generated using a 6-jet Collison nebulizer as modified from the 3-jet Collison as described by May (1973) and purchased from BGI Inc., Waltham, MA. The property of this nebulizer is that it produces aerosols with an aerodynamic size of less than 5 μm which are of significance for their stability in airborne state and subsequently are capable of being retained in the respiratory tract of the host upon inhalation. After suspending the virus in the appropriate medium it was aerosolized using a 6-jet Collison nebulizer (May, 1973) purchased from BGI Inc., Waltham, MA. A 300-l stainless steel drum (Goldberg et al., 1958; Mitchell et al., 1968) was used for the storage of the aerosols (Fig. 1). The drum was rotated at 4 rpm to reduce the loss of the aerosols by sedimentation (Goldberg et al., 1958). The temperature inside the drum was kept at 20 ±1°C. The drum air containing the virus aerosol was sampled at appropriate intervals using an All Glass Impinger (Tyler and Shipe, 1959). A critical vacuum was maintained in all experiments so that the impinger would operate at its design capacity of 5.6 l/min. After each experiment the drum was thoroughly flushed with room air to remove all the virus and dye left from the experiment.

Relative humidity adjustment and measurements

All relative humidity (RH) measurements were made with the help of a dial type hygrometer (Airguide Instruments Co., Chicago, IL) affixed to the drum. The procedures for achieving the desired RH level in the drum have been described earlier (Sattar et al., 1984; Ijaz et al. 1985a). Experiments were carried out at the

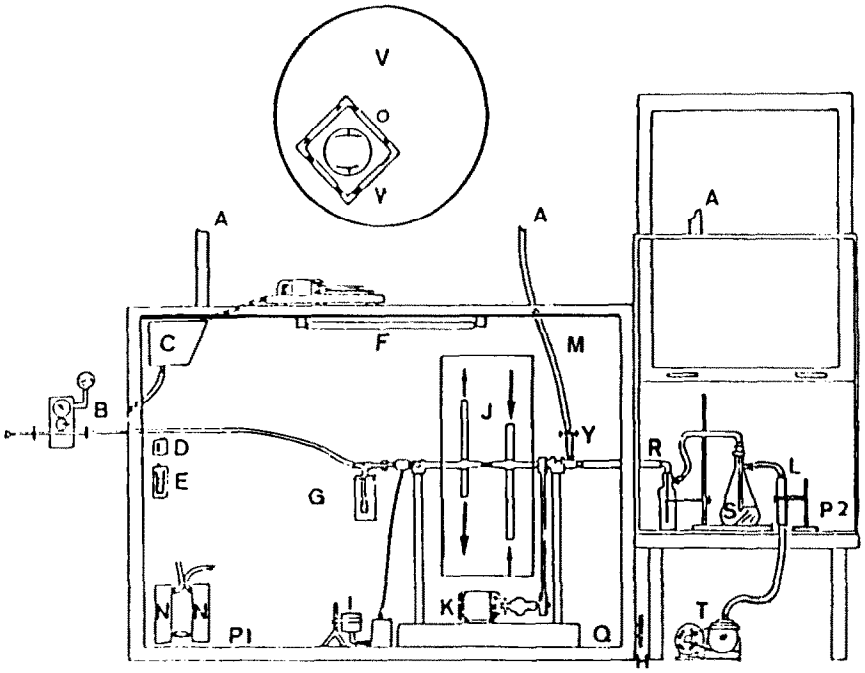

Fig. 1. Schematic diagram of the equipment used for the generation, storage and collection of virus aerosols. A = exhausted air to HEPA filter in ceiling; B = pressure regulator for compressed air being supplied to the nebulizer; C = refrigeration unit; D = thermostat; E = thermometer; F = ultraviolet light; G = Collison nebulizer; H = air inlet filter; I = Drierite tank; J = rotating drum; K = motor; L = Drierite container; M = air overflow from rotating drum; N = Drierite; P^1 = insulated containment cabinet; P^2 = fume hood; Q = cabinet air inlet filter; R = all-glass impinger; S = liquid disinfectant; T = vacuum pump; V = side view of rotating drum; W = porthole with inserted hygrometer; Y = clamp to close circuit.

following three RH levels: low (30±5%), medium (50±5%) and high (80±5%). For the experiments at low RH, the drum was first filled with air passed through a Drierite cylinder (Hammond Drierite Co., Xenia, OH). When experiments were carried out at medium and high RH level, distilled water was sprayed into the drum to raise the RH to the desired level and then the virus aerosolization was carried out.

Determination of physical decay of the viral aerosols

The ideal tracer would be radiolabelled virus which permit each virion to act as a self-tracer. The use of radiolabelled virus is not only expensive but could be hazardous. So the feasibility of using fluorescent dyes was considered and thus a comparative study was made with radiolabelled virus. Either Rhodamine B (Songer, 1967; Elazhary and Derbyshire, 1977) or uranine (Beard and Easterday, 1965; Ijaz et al., 1985a) were used as the physical tracer in this study. Reference solutions for the standardization of the dye were prepared in TPB. An Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Springs, MD) was used

for measuring the dye concentration in the samples. The excitation and emission wavelengths used were 546 and 590 nm for rhodamine and for uranine 493.5 and 512.0 nm, respectively. All the readings were taken at room temperature. In order to determine the suitability of the dye as a physical tracer, initial comparative tests were run using ^{75}Se-labelled virus. The quantity of the radio-labelled virus was measured using a scintillation counter (Model LS 250, Beckman Instrument Inc., CA).

Determination of size of the viral aerosols

The distribution of virus aerosol in the drum air was determined at three RH levels using an Anderson sampler (Anderson, 1958). The same sampler was used throughout the experiments. This sampler is designed in such a way that it separates the aerosols according to their aerodynamic size. As recommended by Anderson (1958), each Petri dish contained 27 ml of collecting medium. The collecting medium in the Petri dishes was modified from Guerin and Mitchell (1964). A 10% solution of gelatin (Fisher Scientific Ltd., Canada) was prepared in sterile distilled water and heated at 100°C for about 30 min. Pre-sterilized TPB was kept at 37°C and gelatin solution was added to give a final concentration of 3%. These plates were placed at 4°C until used. Before each sampling, the Petri dishes containing the TPB and gelatin to a final concentration of 3% were loaded onto the stages of the sampler and the sampler was operated for 1 min to draw 28 l/min of drum air onto the surface of the medium (Anderson, 1958).

Test procedure

When ^{75}Se-labelled virus was to be nebulized, it was suspended in TPB alone. However, the spray fluid for the unlabelled virus also contained 2.5 mg/ml of Rhodamine and Antifoam C (Sigma) at a final concentration of 1%. The Collison nebulizer, with 15 ml of the appropriate spray fluid, was attached to the inlet of the rotating drum and aerosolization was carried out at a pressure of 1.8 kg/cm^2. The first air sample from the drum was collected after a 15 min period of aerosol stabilization. An impinger, containing 10.0 ml of either MEM or TPB with 1% antifoam as aerosol collection fluid, was operated for 1 min. to draw 5.6 l of the drum air. Using the same procedure, additional samples of air were collected 0.5, 1, 2, 4, 8, 24, 48 and 72 h after virus aerosolization. The fluid from the impinger was divided into two portions. One of these was used for estimating the amount of the physical tracer and the other for the determination of the virus by plaque assay. The extent of biological decay of the virus was calculated using the following formula:

$$\% \text{ Virus survival} = (D_0/D_t) \times (V_t/V_0) \times 100.$$

When D_0 and D_t are the dye concentration at times 0 and t h, respectively, similarly V_0 and V_t are the virus titers at 0 and t h, respectively. It should be noted that time 0 is taken at the end of the 15 min equilibration period in the drum. The difference between the input virus concentration and the 15 min sample concen-

tration expressed as a percent represents the 'initial loss' due to the process of generating the virus cloud. Survival of the aerosolized virus was tested at three levels of RH: low (30 ± 5%), medium (50 ± 5%) and high (80 ± 5%).

The survival of the aerosolized virus under various experimental conditions was plotted against time using an exponential model:

$$\% \text{ Virus survival} = A \times \exp(-B \times \text{Time})$$

and the coefficients A and B were estimated by least squares curve fitting. At least three experiments at each RH and temperature were carried out and the half-lives of the viruses were calculated by regression analysis (Lawless, 1982).

Results

In order to be able to compare the data generated on the airborne survival of the various types of viruses tested, it was essential to keep the variations in materials and techniques to a minimum. In view of this, during the initial stages of this study, a number of materials and techniques were tested for their suitability in working with all the viruses selected here.

Physical tracers and antifoams

Since fluorescent dyes were considered for use as physical tracers, it was considered essential to determine that their presence in the spray fluid would not be deleterious to the infectivity of the viruses and their quantitation. A great deal of froth was generated when TPB was used as the virus spray and aerosol collection fluid. It was, therefore, necessary to suppress this frothing by the use of an antifoam which would not inactivate the viruses or in some way interfere with virus plaque assay.

A known amount of rotavirus SA-11, which was the virus used in the initial

TABLE I

Effect of the physical traces and antifoams on the infectivity of simian rotavirus SA-11.

Suspending medium	Virus PFU/ml × 10^7
Tryptose phosphate broth (TPB)	1.70
TPB with Antifoam C	0.30
TPB with Rhodamine B	0.40
TPB with Rhodamine B and Antifoam C	0.40
TPB	6.50
TPB with uranine	6.52
TPB with Antifoam A	6.49
TPB with uranine and Antifoam A	6.51

The virus was added the TPB alone or TPB containing the additive(s) under test. After an incubation of 30 min at room temperature (22°C) the samples were diluted in EBSS and plaque assayed.

TABLE 2

Effect of uranine and Antifoam A on the infectivity of corona, polio and rotaviruses.

Viruses	Virus PFU/ml × 10^7			
	TPB alone	TPB with uranine	TPB with Antifoam A	TPB with uranine and Antifoam A
Human coronavirus 229E	2.10	2.13	2.18	2.22
Poliovirus type 1 (Sabin)	5.00	4.50	4.80	4.70
Human rotavirus (Wa)	6.00	6.10	5.80	5.90
Bovine rotavirus (C-486)	1.40	1.30	1.40	1.50
Bovine rotavirus (UK)	70.00	69.50	71.00	70.50
Mouse rotavirus	45.00	46.00	45.00	45.50
Rhinovirus (RV-14)	1.8	2.4	2.3	2.5

The virus under test was added to TPB alone or TPB containing the additive(s) under test. After incubation of 30 min at room temperature (22°C), the samples were plaque assayed.

phases of this study, was suspended in TPB with and without the dyes and the antifoams. The suspensions were held at room temperature for 30 min before being plaque assayed. The results of these tests are presented in Table 1. Rhodamine B and Antifoam C, alone or in combination with each other, reduced the virus plaque titer by at least 4-fold. On the other hand, neither uranine nor Antofoam A, separately or as a mixture, were found to affect the infectivity of the virus.

Subsequent tests showed (Table 2) that uranine and Antifoam A were equally harmless to the infectivity of the corona-, polio-, rhino- and other rotaviruses used in this study. In view of this, these two substances were routinely added to the virus spray fluid. Antifoam A was also regularly incorporated in the aerosol collection fluid.

TABLE 3

Effect of nebulization on the viability of corona, polio, rhino and rotaviruses.

Viruses	Virus PFU/ml × 10^7		
	Before nebulization	After nebulization	% PFU recovered
Human coronavirus 229E	1.45 ± 0.57	1.41 ± 0.58	98.90 ± 3.90
Poliovirus type 1 (sabin)	5.00 ± 0.92	5.10 ± 1.00	101.0 ± 2.40
Human rotavirus (Wa)	4.45 ± 2.00	5.00 ± 2.60	111.8 ± 14.9
Bovine rotavirus (C-486)	2.40 ± 1.47	2.41 ± 1.26	106.4 ± 16.4
Simian rotavirus SA-11	3.45 ± 1.56	3.50 ± 1.6	101.45 ± 5.34
Bovine rotavirus (UK)	30.0[a]	31.0	103.00
Mouse rotavirus	63.0[a]	62.5	99.21
Rhinovirus (RV-14)	1.39 ± 0.52	1.48 ± 54.0	104.5 ± 13.3

A 10 ml volume of TPB containing the virus under test was added to the Collison nebulizer which was then operated for 10 min. The virus was plaque assayed to determine its titer in the suspending medium before and after the nebulizer was operated. At least 4 experiments were performed and the figures represent the mean ± S.D.

[a] No S.D. available because only one experiment was carried.

TABLE 4

Effect of nebulization on the concentration of the physical tracers in the spray fluid.

Type of dye	Dye concentration in mg/ml		
	Before nebulization	After nebulization	% Dye recovered
Rhodamine B	2.50 ± 0.02	2.49 ± 0.03	100.17 ± 0.67
Uranine	1.00 ± 0.01	0.99 ± 0.03	99.950 ± 0.10

A 10 ml volume of TPB containing either of these two dyes was added to the Collison nebulizer which was then operated for 10 min. The concentration of the dyes was measured in an Aminco Bowman spectrofluorometer. At least four experiments were performed with each dye under test and the figures are expressed as mean ± S.D.

Effect of the process of nebulization on the viability of the viruses

During the operation of the Collison nebulizer, virus particles present in the spray fluid are subjected to strong shearing forces. This could lead to the inactivation of the virus being aerosolized. In order to test this, the titer of the virus in the spray fluid was measured before and after operating the nebulizer for 10 min. There was no significant loss in the viability of the corona-, polio, rhino- and rotaviruses tested (Table 3). These findings established the fact that the Collison nebulizer could be safely used for the aerosolization of all the viruses selected for this study.

Effect of nebulization on the concentration of the dye in the spray fluid

In an earlier study, it was suggested that the passage of dry air through a dye-containing spray fluid during the process of nebulization could result in the rapid evaporation of water and thus lead to an increase in the concentration of the dye in the spray fluid (Beard and Easterday, 1965). In order to test this, the concentration of the dye in the spray fluid (TPB) was measured before and after the operation of the nebulizer for 10 min. As can be seen from the data presented in

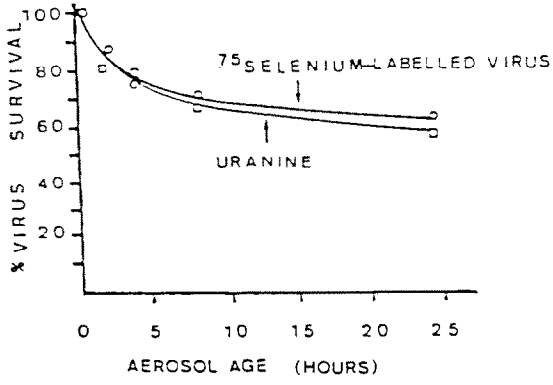

Fig. 2. Comparison of the rates of biological decay of airborne Simian rotavirus SA-11 at the medium relative humidity level (20 ± 1°C) as determined with the use of two different physical tracers.

Table 4, there was no detectable change observed in the concentration of uranine or rhodamine B in the spray fluid as a result of the process of nebulization.

Suitability of uranine as a physical tracer

Before any experiments on the airborne survival of the viruses could be carried out, it was considered imperative to determine the suitability of uranine as a physical tracer. In order to do this, uranine and Antifoam A were added to TPB containing ^{75}Se-labelled rotavirus SA-11. This mixture was aerosolized into the rotating drum (50 ± 5%, RH; 20 ± 1°C) and samples of the air from the drum were collected at appropriate intervals and assayed for the dye, radioactivity and infectious virus. The results are presented in Fig. 2. The rates of biological decay of the virus calculated using the two different physical tracers were found to be almost the same. These data clearly demonstrated the fact that a fluorescent dye could be not only relatively simple and safe, but also highly reliable as a physical tracer.

Determination of the extent of virus inactivation during the period of aerosol stabilization

Apart from the physical loss of the virus, the extent of virus inactivation immediately after its aerosolization depends largely upon the RH and temperature of the aerosol-receiving air. In aerobiology, such a reduction in virus infectivity is generally referred to as the 'initial loss'. That proportion of the airborne virus which survives the initial loss requires a certain amount of time for its uniform distribution in the air inside the rotating drum. In view of this, the first air sample from the drum was always collected 15 min after the termination of virus aerosolization. This period of aerosol stabilization was, therefore, considered essential to allow for: (a) the initial loss in virus infectivity to occur, (b) the physical loss of larger

TABLE 5

Percent recovery of the viruses after aerosolization and equilibration of the aerosol cloud at 20 ± 1°C.

Viruses	Relative humidity		
	High (80 ± 5%)	Mid (50 ± 5%)	Low (30 ± 5%)
Human coronavirus 229E	55.0 ± 3.5	90.9 ± 1.6	87.0 ± 2.5
Poliovirus type 1 (Sabin)	104.1 ± 7.8	0	0
Rotavirus (Wa)	34.3 ± 3.8	86.8 ± 4.7	45.8 ± 3.7
Bovine rotavirus (C-486)	27.3 ± 1.8	70.0 ± 1.5	36.5 ± 5.2
Simian rotavirus SA-11	24.7 ± 4.7	78.0 ± 2.3	42.1 ± 2.6
Bovine rotavirus (UK)	29.7	73.4	38.5
Mouse rotavirus	20.4	90.6	49.0
Rhinovirus (RV-14)	100.0 ± 2.1	1.22 ± 0.78	0

In all experiments a 15–min period was allowed for aerosol stabilization and the first air sample was collected at the end of this period. To determine the extent of virus survival during this period, the titer of the virus in the air sample was compared with the amount of virus aerosolized. At least 3 separate experiments were conducted with all viruses except bovine rotavirus (UK) and mouse rotaviruses. The results are presented as the mean ± S.D.

particles to take place and (c) the homogeneous distribution of the airborne particles in the air inside the drum.

To determine the initial loss in virus infectivity, the amount of virus sprayed into the drum air under different conditions of RH and temperature was compared with the amount recovered in the first air sample. The results of these experiments are presented in Table 5.

At the low and medium RH levels, with an air temperature of 20 ± 1°C, the initial loss in the infectivity of the rhino and poliovirus was so pronounced that no infectious virus could be detected in the air samples collected at the end of the aerosol stabilization period. In contrast to this, there was no detectable initial loss in the infectivity of the aerosolized poliovirus when the RH of the air inside the drum was kept at the high level with the air temperature at 20 ± 1°C.

At 20 ± 1°C, the initial loss in the infectivity of the five isolates of rotaviruses tested was the highest at 80 ± 5% RH and they appeared to survive best during the aerosolized stabilization period when the RH was at the medium level. Although lowering of the air temperature to 6 ± 1°C showed an increase in the overall capacity of the human rotavirus to survive during the stabilization period, the initial loss in the virus titer was still the lowest at the medium RH level and greatest at the high RH level (Table 6).

At a higher air temperature, the general pattern in the initial loss of the coronavirus was very similar to that seen with the rotaviruses. However, when the air temperature was reduced to 6 ± 1°C, not only was there a general reduction in the initial loss, but the virus appeared to survive equally well at all 3 RH levels (Table 6).

Determination of the particle size distribution of viral aerosols produced by the collison nebulizer and held at the 3 RH levels (20 + 1°C)

These experiments were performed to determine the size distribution of the virus-containing particles within an aerosol produced by the Collison nebulizer and stored in the rotating drum at the 3 levels of RH (20 ± 1°C). Rotavirus SA-11 was

TABLE 6

Percent recovery of the viruses after aerosolization and equilibration of the aerosol cloud at 6 ± 1°C.

	Relative humidity		
Viruses	High (80 ± 5%)	Mid (50 ± 5%)	Low (30 ± 5%)
Human coronavirus 229E	104.8 ± 5.1	96.5 ± 3.0	91.0 ± 2.6
Human rotavirus (Wa)	43.8 ± 3.3	95.8 ± 2.6	57.2 ± 3.8
Rhinovirus (RV-14)	100.0 ± 2.7	7.1 ± 4.4	1.0 ± 1.40

After allowing a period of 15 min for aerosol stabilization, the first air sample was collected. To determine the extent of virus survival during this period, the titer of the virus in the air sample was compared with the amount of virus aerosolized. At least 3 separate experiments were conducted and the results are presented as the mean ± S.D.

suspended in TPB containing the dye and the antifoam. The virus suspension was sprayed into the drum and the viral aerosols held at the desired RH level.

At 0.25, 2, 8 and 24 h after aerosolization, samples of the air from the drum were collected with the help of an Anderson sampler (Anderson, 1958) according to the procedure described in Materials and Methods.

The gelatin-containing medium from each of the six plates in the sampler was collected separately after incubating the plates for 1 h at 37°C. These samples were then assayed for the dye as well as the infectious virus.

TABLE 7

Size distribution of dye and simian rotavirus SA-11 containing airborne particles at RH 30 ± 5% (20 ± 1°C).

Aerosol age (h)	Stage No.[a]	% Recovery		Cumulative % recovery from last three stages[a]	
		Dye	Virus	Dye	Virus
0.25	1	0.00	0.00		
	2	0.50	0.30		
	3	7.00	7.00		
	4	19.00	22.00		
	5	51.00	50.30	91.0	92.4
	6	18.00	20.4		
2	1	0.00	0.00		
	2	2.50	2.30		
	3	5.00	6.00		
	4	30.50	30.50		
	5	49.00	49.00	92.5	91.7
	6	13.00	12.20		
8	1	0.00	0.00		
	2	0.70	0.30		
	3	1.30	1.70		
	4	35.00	35.50		
	5	49.00	48.00	98.0	98.0
	6	14.00	14.50		
24	1	0.00	0.00		
	2	0.30	0.30		
	3	1.70	2.00		
	4	33.00	32.80		
	5	59.00	58.50	98.0	97.7
	6	6.00	6.50		

A 10 ml volume of TPB containing the virus and uranine (1 mg/ml) was added to the Collison nebulizer and aerosolized into the rotating drum. After 15 min of cloud stabilization, a 1-min air sample (28 l) was drawn with the help of the Anderson sampler. Samples from each of these stages of the sampler were divided into two portions. The virus was quantitated by plaque assay and the concentration of the dye was measured by spectrophotofluorometry.

[a] Stages No. 4, 5 and 6 are designed to recover particles with diameters of 2.0 – 3.3 μm, 1.0 – 2.0 μm and <1.0 μm, respectively.

TABLE 8

Size distribution of dye and simian rotavirus SA-11 containing airborne particles at RH 50 ± 5% (20 ± 1°C).

Aerosol age (H)	Stage No.[a]	% Recovery		Cumulative % recovery from last three stages[a]	
		Dye	Virus	Dye	Virus
0.25	1	0.50	1.00		
	2	4.30	3.50		
	3	6.00	6.00		
	4	46.00	45.00		
	5	38.00	35.00	89.4	89.5
	6	5.40	9.5		
2	1	0.50	0.10		
	2	2.50	1.80		
	3	7.00	8.00		
	4	32.30	32.00		
	5	52.00	52.00	89.8	90.0
	6	5.50	6.00		
8	1	0.70	0.00		
	2	0.90	1.00		
	3	6.00	6.00		
	4	37.40	35.00		
	5	48.90	50.00	92.4	93.0
	6	6.10	8.00		
24	1	0.00	0.00		
	2	0.00	0.00		
	3	2.40	3.00		
	4	29.40	29.00		
	5	62.00	61.00	97.6	97.0
	6	6.20	7.00		

A 10 ml volume of TPB containing the virus and uranine (1 mg/ml) was added to the Collison nebulizer and aerosolized into the rotating drum. After 15 min of cloud stabilization, a 1-min air sample (28 l) was drawn with the help of the Anderson sampler. Samples from each of the stages of the sampler were divided into two portions. The virus was quantitated by plaque assay and the concentration of the dye was measured by spectrophotofluorometry.

[a] Stages No. 4, 5 and 6 are designed to recover particles with diameters of 2.0 – 3.3 μm, 1.0 – 2.0 μm and <1.0 μm, respectively.

The results of these experiments are shown in Tables 7–9. Irrespective of the RH level, more than 87% of the infectious virus, as well as the dye recovered from the drum air, were collected in stages 4, 5 and 6 of the sampler. These 3 stages of the Anderson sampler are designed to recover airborne particles in the size range of smaller than 1.0 – 3.3 μm in diameter (Anderson, 1958). Such particles can remain airborne for prolonged periods and, upon inhalation, they also have the potential for retention in the respiratory tract.

TABLE 9

Size distribution of Dye and simian rotavirus SA-11 containing airborne particles at RH 80 ± 5% (20 ± 1°C).

Aerosol age (h)	Stage No.[a]	% Recovery		Cumulative % recovery from last three stages[a]	
		Dye	Virus	Dye	Virus
0.25	1	0.45	0.50		
	2	0.49	0.40		
	3	0.90	1.00		
	4	28.00	30.10		
	5	49.25	48.00	98.25	98.10
	6	21.00	20.00		
2	1	0.40	0.50		
	2	2.40	2.00		
	3	5.00	4.00		
	4	52.00	60.00		
	5	36.20	31.00	92.20	93.50
	6	4.00	2.50		
8	1	0.40	0.70		
	2	1.00	1.30		
	3	11.00	10.00		
	4	46.00	51.00		
	5	39.00	36.00	87.60	88.00
	6	2.60	1.00		

A 10 ml volume of TPB containing the virus and uranine (1 mg/ml) was added to the Collison nebulizer and aerosolized into the rotating drum. After 15 minutes of cloud stabilization, a 1-min air sample (20 l) was drawn with the help of the Anderson sampler. Samples from each of the stages of the sampler were divided into two portions. The virus was quantitated by plaque assay and the concentration of the dye was measured by spectrophotofluorometry.

[a] Stages No. 4, 5 and 6 are designed to recover particles with diameters of 2.0 – 3.3 μm, 1.0 – 2.0 μm and <1.0 μm, respectively.

Virus mixtures

Because a considerable amount of information is available regarding the airborne survival of polioviruses (Harper, 1961; 1963; De Jong, 1970; De Jong et al., 1973; Ijaz et al., 1984; 1985a), the Sabin strain of poliovirus type 1 was used as a reference standard in our experiments dealing with the aerobiology of rota- and coronaviruses. Poliovirus was mixed with either a rotavirus or coronavirus and the mixture aerosolized. This allowed us to compare the rate of biological decay of both the viruses in the mixture under identical experimental conditions.

The air samples collected in these experiments contained poliovirus with either rota- or coronaviruses. Therefore, it was necessary to develop procedures so that the quantitation of one type of virus in the sample would not interfere with the plaque forming ability of the other.

Poliovirus could readily grow and form countable plaques in MA-104 cells. Rotaviruses are also able to form plaques in MA-104 cells but only in the presence

TABLE 10

Plaque assays to detect interference between viruses in the mixtures used for aerosolization.

Virus	PFU/ml × 10^3			
	Without poliovirus	Mixed with poliovirus	Poliovirus alone	Poliovirus in mixture
Human cornavirus 229E	3.10	3.50	0.20	0.20
Human rotavirus (Wa)	5.00	5.40	0.90	0.87
Bovine rotavirus (C-486)	1.20	1.30	0.16	0.16

TPB was used as the virus suspending medium. Suspensions of one virus and virus mixtures were held at room temperature (22 ± 1°C) for 15–20 min before plaque assay.

of trypsin in the overlay medium (Ramia and Sattar, 1979). Therefore, when the virus mixtures containing polio- and rotaviruses were to be titrated to determine the number of poliovirus PFU, an overlay medium without trypsin was used. This completely suppressed plaque formation by rotaviruses. However, for titration of the rotavirus contained in the mixtures, it was necessary to neutralize the poliovirus with the specific hyperimmune serum (1:1000) first and then perform the plaque assay with a trypsin-containing overlay.

Poliovirus could also form countable plaques in L-132 cells and in mixtures containing this virus and coronavirus, hyperimmune antiserum was used to neutralize poliovirus before the plaque assay of coronavirus. To determine the number of poliovirus PFU in such mixtures, no special pretreatment of the samples was required since poliovirus could form plaques in 48 h at 37°C and 6 days of incubation at 33°C was necessary for the development of countable plaques by the coronavirus.

To determine whether there was any interference between the viruses, mixtures containing the viruses were plaque assayed and the titers compared with appropriate controls. The results of these tests are presented in Table 10. There was no detectable interference between these viruses as seen by both the quantity and the quality of the plaques produced by them.

Comparison of the survival of airborne human rotavirus and poliovirus 1 (Sabin)

Because much information is available regarding the airborne stability of poliovirus (De Jong 1970; De Jong et al., 1973), this virus was used as a reference standard in our experiment. This allowed us to compare the rate of biological decay of both HR and polio 1 under identical experimental condition. The pattern of biological decay of both polio 1 and HR in mixed aerosols at 20 ± 1°C and at three different RH levels are shown in Fig. 3. At least three experiments were performed at each of the three RH levels. The survival pattern of HR was found to be similar to other rotaviruses (Ijaz et al., 1984, 1985a,b). In contrast to this, the behavior of polio 1 was found to be markedly different from that of HR studied under the same experimental conditions and similar to RV-14 (Karim et al., 1985). No infectious poliovirus could be recovered in any of the samples of drum air at

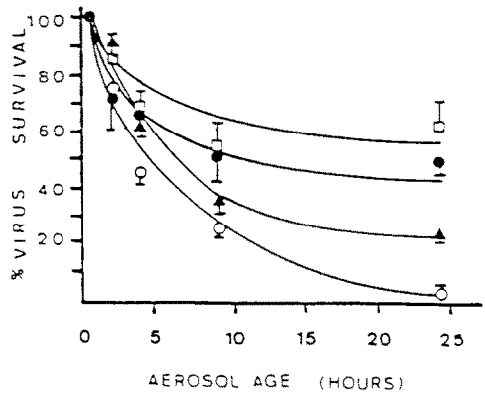

Fig. 3. The effect of three different levels of relative humidity (20 ± 1°C) on the airborne survival of human rotavirus (Wa) and poliovirus type 1 (Sabin) aerosolized as a mixture. Rotavirus RH 30 ± 5% (●); Rotavirus RH 50 ± 5% (□); Rotavirus RH 80 ± 5% (○); Poliovirus RH 80 ± 5% (▲).

medium or low RH range. On the other hand, polio 1 was found to survive very well at high RH, having a half-life of approximately 10 h; about 22% of the infectious virus was present in the drum air even after 24 h of aerosol age.

Discussion

Dissemination of viruses through airborne route is dependent upon the survival characteristics of the virus-containing aerosol under various environmental conditions (Spendlove and Fannin, 1982). Ideally these characteristics would be best studied under field conditions but due to the potential problems encountered in a field situation, survival of airborne viruses is studied in the laboratory under controlled experimental set up. A number of factors have been shown to influence the viruses in the airborne state, e.g., temperature, RH and suspending medium (Ijaz and Sattar, 1987). TPB was selected as aerosol generation and collection fluid in our studies because earlier work carried out in our laboratory has shown it to be harmless to these viruses. Also, it would simulate more closely the natural situation where these viruses would become airborne from body fluids. It has been shown by a number of workers that viruses aerosolized from body fluids do survive better under their optimum conditions of survival (Donaldson, 1973; Elazhary and Derbyshire, 1979a,b; Ijaz and Sattar, 1987).

It is well established that the capacity of viruses to survive in the airborne state is modulated by a number of environmental factors. Of these, RH and air temperature have been found to be of particular significance. In this section, an attempt has been made to point out the reasons for the selection of the various methods and experimental conditions used in this investigation. The significance of the findings of this study has also been discussed here in relation to what is known about the epidemiology of rota- and coronavirus infections.

The high, medium and low levels of RH used in this study were chosen to represent the seasonal variations of RH levels in both indoor and outdoor environments. The experiments conducted at the medium level RH also stimulated the conditions generally present in climatically controlled buildings such as modern hospitals and laboratories.

The air temperature of 20 ± 1°C was selected to exemplify the conditions generally encountered indoors in many temperate regions. This temperature was, however, somewhat lower than what is generally regarded as room temperature that could be achieved and maintained accurately for the duration of a given experiment. This was also the limiting factor in the selection of the lower (6 ± 1°C) air temperature.

In order to determine the viability of viruses in airborne state, it is important to account for physical loss by deposition, settling and dilution in the drum. This is achieved by using a tracer which is added in the virus suspension to be aerosolized. A number of tracers have been used by different workers (Songer, 1967; De Jong et al., 1973; Trouwborst and De Jong, 1973; Donaldson and Ferris, 1976; Elazhary and Derbyshire, 1977; Sattar et al., 1984; Ijaz et al., 1985a). However, the best tracer would be radiolabelled virus, which would permit each virion to serve as a self-tracer. The use of radiolabelled virus is not only expensive but can also be hazardous (May, 1973). The suitability of uranine as a physical tracer was compared directly by spraying with it rotavirus which had been labelled with ^{75}Se. This isotope was selected because of its relatively short half-life (about 120 days) so that the equipment used in these experiments would not continue to pose a radiation hazard for extended periods of time. Since the half-life of both dyes and radioisotopes were fairly comparable we decided to include one of the dyes in subsequent experiments. Other workers have also shown the dye to be a reasonable tracer (Spendlove and Fannin, 1982).

As has been mentioned earlier, poliovirus was used as a reference virus in this study to ensure that the results generated with rota-, corona- and rhinoviruses could be compared with those of a virus whose airborne survival characteristics were better known. Here it should also be pointed out that, as far as we are aware, this is the first study where two different vertebrate viruses were aerosolized as a mixture in order to compare their airborne survival under identical experimental conditions.

Our findings on the influence of various levels of RH on the airborne survival of poliovirus are in agreement with those of earlier reports (Hemmes et al., 1960; Harper, 1961, 1963; De Jong, 1970; De Jong et al., 1973). This provides additional support to confirm that the experimental set-up in this study was not introducing any bias in the results of the airborne survival experiments on the rota-, rhino- or coronaviruses.

The particle size of virus-containing aerosol is important in determining their physical stability, dispersion, deposition and retention in the respiratory tract (Knight, 1973). It was therefore, essential to demonstrate that the differences seen in the survival of the airborne rota- and coronavirus at the 3 different levels of RH were not due to variations in the size of the aerosols. The experiments using the

Anderson sampler (Anderson, 1958) clearly demonstrated that there were no major differences in the size of the aerosols being generated and aged at the 3 RH levels. The data generated in these experiments also showed that the aerosolized particles containing the dye and the virus under test belonged in the same range. However, a slight shift in the recovery of particles from stages 4 to 5 and 6 was observed in the experiments carried out at medium and low RH compared with high RH. These results correlate with the results of earlier workers (Couch et al., 1965). This could be due to the fact that the rate of evaporation is generally higher at low and medium than at high RH, which is further enhanced by high temperature (Elazhary and Derbyshire, 1977). It has been shown that biological aerosols are hygroscopic in nature, i.e., under different environmental conditions of RH and temperature they lose or gain moisture (Knight, 1973). Thus, smaller virus-containing aerosols although containing less virus would disseminate widely, and upon inhalation in the respiratory tract of the host will have better chances of being retained due to their hygroscopicity.

The results of the present study together with our earlier work (Sattar et al., 1983, 1984; Ijaz et al., 1984, 1985a,b) revealed that the capacity of rotavirus to survive in the airborne state is influence by RH. In this regard, the RH in the mid-range was found to be more favorable compared to the other 2 RH levels tested. In contrast to this, other non-enveloped viruses such as polio (Harper, 1961); adeno (Miller and Artenstein, 1967); reo (Adams et al., 1982) and rhinoviruses (Karim et al., 1985) have been found to survive better at high RH levels. Therefore the behavior of rotaviruses appears to resemble that of enveloped viruses (Harper, 1961). In contrast to this, work carried out with other enveloped (HCV/229E) and non-enveloped (polio and rhino) viruses under the same experimental set up correlate with our and other workers findings (Harper, 1961; Songer, 1967; Elazhary and Derbyshire, 1979a,b; Sattar et al., 1984; Ijaz et al., 1984, 1985a,b,c; Karim et al., 1985).

The methods developed to study the airborne survival of these viruses should be useful to examine the aerobiology of other viruses, which would help us in understanding the genesis of outbreaks due to these viruses and in instituting proper measures for their prevention and control.

Acknowledgements

We express our appreciation to Irene Kosokowsky (VIDO) for her skillful typing of this manuscript. This work was supported by grants from the World Health Organization (SAS) and Health and Welfare Canada (CMJL).

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