APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1985, p. 289–293
0099-2240/85/020289-05$02.00/0

Vol. 49, No. 2

Comparison of the Airborne Survival of Calf Rotavirus and Poliovirus Type 1 (Sabin) Aerosolized as a Mixture

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A mixture of a cell culture-adapted strain (C-486) of calf rotavirus and poliovirus type 1 (Sabin) was prepared in tryptose phosphate broth containing 0.1% uranine (physical tracer) and antifoam at a final concentration of 0.001%. By using a six-jet Collison nebulizer, the mixture was aerosolized into a 300-liter stainless-steel rotating (4 rpm) drum. The temperature of the air inside the drum was kept at 20 ± 1°C, and the virus aerosols were held at the following three levels of relative humidity (RH): low (30 ± 5%), medium (50 ± 5%), and high (80 ± 5%). An all-glass impinger, containing 10.0 ml of tryptose phosphate broth with antifoam, was used to collect samples of air from the drum. Both viruses were propagated and quantitated in MA-104 cells. The calf rotavirus was found to survive well at mid-range RH, where 60% of the infectious virus could be detected even after 24 h of virus aerosolization. At the low RH, the half-life of the infectious rotavirus was ca. 14 h. On the other hand, no infectious poliovirus could be recovered from the drum air at the low and medium RH. At the high RH, more than 50% of the infectious rotavirus became undetectable within 90 min of aerosolization. In contrast to this, the half-life of the poliovirus at the high RH was about 10 h. These data, based on the aerosolization of virus mixtures, therefore suggest that there is a pronounced difference in the way RH influences the airborne survival of these two types of viruses held under identical experimental conditions.

Rotaviruses frequently cause outbreaks of acute diarrhea in human and animal populations (7). In spite of this, the modes and vehicles of transmission involved in such outbreaks are poorly understood. However, the seasonal nature of these outbreaks and other epidemiological evidence available suggest that air may play a role in the direct or indirect spread of rotaviruses (8, 9, 13–15, 17).

In an earlier study (23), we had demonstrated that simian rotavirus SA-11 could survive well in the airborne state and that the mid-range (50 ± 5%) relative humidity (RH) was most favorable in this regard. However, Moe and Harper (20) showed that the low (20%) and high (90%) RH levels were more conducive to the airborne survival of the U.K. strain of calf rotavirus. This study was, therefore, initiated to determine if in our experimental setup the effect of various levels of RH would be different on a calf rotavirus as compared to our previously reported findings (23) on the simian rotavirus. Since the influence of RH on airborne polioviruses is relatively well understood from earlier studies (4, 5, 11, 12), we decided to use poliovirus type 1 (Sabin) as a reference standard in this investigation.

MATERIALS AND METHODS

Cells. The MA-104 cell line was used for virus propagation and quantitation. We received a seed culture of these cells through the courtesy of F. W. Doane (University of Toronto, Toronto, Ont., Canada). The procedures for the cultivation, maintenance, and passage of these cells have been described in detail previously (21). Cell monolayers for virus plaque assay were prepared in 12-well plastic plates (Costar, Cambridge, Mass.) by seeding each well with ca. 5×10^4 cells in 2.0 ml of minimal essential medium in Earle base (Autopow; Flow Laboratories Inc., Rockville, Md.) with 5% fetal calf serum (Flow). The plates were sealed individually in plastic bags (Phillips Electronic Ltd., Toronto, Ont., Canada) and incubated at 37°C for ca. 48 h.

Viruses. A cell culture-adapted strain (C-486) of calf rotavirus was kindly provided to us by L. Babiuk (University of Saskatchewan, Saskatoon, Sask., Canada). The Sabin strain of poliovirus type 1 was a gift from D. A. McLeod of the Laboratory Center for Disease Control (Ottawa, Ont., Canada). Both of these viruses were first plaque purified in MA-104 cells and the same cell line was used for the preparation of the virus pools to be used here. The virus-infected monolayers were frozen (−80°C) and thawed three times, and the cell culture harvest was extracted twice with equal volumes of 1,1,2-trichloro-1,2,2-trifluoroethane (Fisher Scientific Co., Pittsburgh, Pa.). The phases were separated by centrifugation at 1,000 × *g* for 15 min. The aqueous (top) phase was removed and 1.0 ml of 10× tryptose phosphate broth (TPB) was added to each 9.0 ml of the virus suspension. It was then passed through a membrane filter (Millipore Corp., Bedford, Mass.) with a pore diameter of 0.45 μm to remove larger particulate matter. To ensure that the calf rotavirus and poliovirus pools contained monodispersed virus particles, they were filtered through polycarbonate membranes (Nuclepore Corp., Pleasanton, Calif.) with pore diameters of 80 and 30 nm, respectively.

Poliovirus antiserum. Rabbit antiserum against the poliovirus was kindly provided to us by P. Payment (Institute Armand-Frappier, Ville de Laval, Que., Canada). This antiserum, when added to a poliovirus-rotavirus mixture at a final concentration of 1:1,000, completely inhibited poliovirus plaques without affecting the number or appearance of the rotavirus plaques.

Plaque assay. The cell monolayers in 12-well plates were washed with several changes of Earle balanced salt solution to remove as much as possible of the fetal calf serum present in the growth medium. Each well was then inoculated with 0.1 ml of an appropriately diluted virus suspension. Virus

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TABLE 1. Effect of uranine and Antifoam A on infectivity of calf rotavirus (strain C-486) and poliovirus type 1 (Sabin)[a]

Suspending medium	Virus PFU/ml ($\times 10^7$)	
	Rotavirus	Poliovirus
TPB	1.4	5.0
TPB with uranine	1.3	4.5
TPB with antifoam	1.4	4.8
TPB with uranine and antifoam	1.5	4.7

[a] Uranine and antifoam were added to TPB to give final concentrations of 0.01% and 0.001%, respectively. The virus and suspending medium under test were mixed together, and the suspension was held for 30 min at room temperature. The samples were plaque assayed in MA-104 cells.

adsorption was carried out by holding the inoculated cultures for 1 h at 37°C. At the end of the incubation period, each culture for poliovirus plaque assay was overlaid with 2.0 ml of a medium containing minimal essential medium and 0.6% agarose type II (Sigma Chemical Co., St. Louis, Mo.). Along with the above two ingredients, the overlay for the calf rotavirus assay also contained 5.0 μg of trypsin (ICN Nutritional Biochemicals, Cleveland, Ohio) per ml. The plates were resealed in plastic bags and incubated at 37°C. The plaques of the poliovirus were ready in 48 h, whereas those of the rotavirus required 72 h. The procedures for fixing and staining the monolayers before determining the number of virus PFU have been described elsewhere (21).

Aerosol generation, storage, and collection. In this study, TPB was chosen as aerosol generation and collection fluid because it not only contained an organic and inorganic load but also was harmless to polio- and rotaviruses (22). Aerosolization was carried out with the help of a modified Collison nebulizer (16) with six jets (BGI Inc., Waltham, Mass.). A 300-liter stainless-steel drum (10, 19) was used for storage of the aerosols. The drum was rotated at 4 rpm to reduce the loss of the aerosols by sedimentation (10). The temperature of the air inside the drum was maintained at 20 ± 1°C. Drum air containing the virus aerosols was sampled at appropriate intervals, using an all-glass impinger (25). A critical vacuum was maintained in all experiments so that the impinger would operate at its design capacity of 5.6 liters/min.

RH adjustment and measurements. The effect of the following three levels of RH on airborne virus survival was tested in this study: low (30 ± 5%), medium (50 ± 5%), and high (80 ± 5%). All RH measurements were made with the help of a dial-type hygrometer (Airguide Instruments Co., Chicago, Ill.) affixed to the drum. The procedures for achieving the desired RH levels of the air in the drum were as given before (23). Briefly, for the experiments at the low RH, the drum was first filled with air passed through a Drierite cylinder (Hammond Drierite Co., Xenia, Ohio). Distilled water was sprayed into the drum to raise the RH to the desired level before conducting experiments at the medium and high RH levels.

Determination of physical decay of aerosols. Uranine (sodium fluorescein), purchased from Fisher Scientific Co. (Fair Lawn, N.J.), was used as a physical tracer (2) in this study. Reference solutions for the standardization of the dye were prepared in TPB, and an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.) was used for measuring the concentrations of the dye in all the samples. The excitation and emission wavelengths used were 493.5 and 512 nm, respectively.

Test procedures. The virus suspension to be sprayed contained 1 mg of uranine per ml and Antifoam A (Sigma) at a final concentration of 0.001%. A Collison nebulizer, with 14 ml of the virus-containing 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 ml of collection fluid (TPB with Antifoam A at a final concentration of 0.001%), was operated for 2.0 min to draw 11.2 liters of the drum air. Using the same procedure, we collected additional samples of the air at 2, 4, 8, and 24 h after virus aerosolization. The same nebulizer and impinger were used throughout this study to avoid variations between samples and experiments.

The fluid from the impinger was divided into two portions. One of these was used for estimating the amount of physical tracer and the other was titrated to determine the number of virus PFU. The extent of biological decay of the viruses was calculated as described before (23).

RESULTS

Effect of uranine and Antifoam A on viability of the viruses. In this study uranine and Antifoam A were to be added to the virus mixture to be sprayed. It was, therefore, necessary to determine if these additives could in any way affect the viability of either the poliovirus or the rotavirus. Separate suspensions of these viruses were prepared in the spray fluid with or without the two additives. After an incubation of 30 min at 23°C, they were plaque assayed. The results of these experiments are summarized in Table 1. Neither uranine nor the antifoam, singly or in combination, proved to be deleterious to the viability of the two viruses used in this study. As a result, in all subsequent experiments they were routinely added to the spray fluid. The antifoam was also regularly used to suppress frothing in the impinger fluid.

Effect of nebulization on dye concentration in the spray fluid. Passage of relatively dry air through the spray fluid during the process of nebulization could result in the accelerated loss of moisture, thereby leading to an increase in the concentration of the dye in the spray fluid remaining in the nebulizer (2). The other, but perhaps less likely, possibility is the enhanced loss of the dye from the nebulizer due to the phenomenon described by Blanchard and Syzdek (3). Any such changes in dye concentration could influence the proper determination of the rates of biological decay of the microorganism under test. We therefore measured the concentration of uranine in the spray fluid before and after the operation of the Collison nebulizer for up to a period of 10 min. There was no detectable difference in the concentration of the dye in the spray fluid as a result of the process of nebulization (Table 4).

Effect of the process of nebulization on viability of the viruses in the spray fluid. During the operation of the

TABLE 2. Effect of nebulization on viability of calf rotavirus (strain C-486) in the spray fluid[a]

Expt	Virus PFU/ml ($\times 10^7$)		% PFU recovered[b]
	Before nebulization	After nebulization	
1	1.3	1.34	103
2	3.3	3.3	100
3	1.0	1.3	130
4	4.0	3.7	92.5

[a] The virus was added to TPB with 0.1% uranine and 0.001% antifoam. The suspension was placed in a six-jet Collison nebulizer, which was operated for 10 min at a pressure of 1.8 kg/cm^2.

[b] Mean ± standard deviation: 106.4 ± 16.4

TABLE 3. Effect of nebulization on viability of poliovirus type 1 (Sabin) in the spray fluid[a]

Expt	Virus PFU/ml ($\times 10^7$)		% PFU recovered[b]
	Before nebulization	After nebulization	
1	7	7	100
2	1	0.95	95
3	1.5	1.7	113
4	1.3	1.3	100

[a] The virus was added to TPB with 0.1% uranine and 0.001% antifoam. The suspension was placed in a six-jet Collison nebulizer, which was operated for 10 min at a pressure 1.8 kg/cm^2.
[b] Mean ± standard deviation, 102 ± 7.70.

TABLE 4. Effect of nebulization on concentration of uranine (physical tracer) in the spray fluid[a]

Expt	Dye (mg/ml)		% Dye recovered[b]
	Before nebulization	After nebulization	
1	1.0	1.0	100
2	1.0	0.99	99
3	1.0	1.01	101
4	1.0	1.0	100

[a] TPB containing 1 mg of uranine per ml was placed in a six-jet Collison nebulizer, which was operated for 10 min at 1.8 kg/cm^2.
[b] Mean ± standard deviation; 100 ± 0.8.

Collison nebulizer, virus present in the spray fluid is subjected to strong shearing forces. This could lead to virus inactivation. To test this, the spray fluid containing the two viruses was titrated for plaques before and after operating the nebulizer for 10 min. There was no significant loss in the plaque titer of the viruses in the spray fluid as a result of the process of nebulization (Tables 2 and 3).

Interference between the two viruses. The poliovirus could readily grow and form countable plaques in MA-104 cells. As has been shown earlier with rotavirus SA-11 (21), strain C-486 of calf rotavirus could form plaques in MA-104 cells only in the presence of trypsin in the overlay medium. Therefore, when the virus mixtures were to be titrated to determine the number of poliovirus PFU, overlay medium without trypsin could be used. This completely suppressed plaque formation by the calf rotavirus. However, for titration of the rotavirus contained in the virus mixtures it was necessary to neutralize the poliovirus first and then perform the plaque assay with a trypsin-containing overlay.

Since mixtures of these two separate types of viruses were to be used in this study, it was considered important to find out if they interfered with each other in any way, thereby influencing the plaque titers. This was tested by preparing mixtures with known amounts of these viruses and then titrating them by plaque assay. The results of these tests are presented in Table 5. There was no detectable interference between these viruses as seen by both the quantity and the quality of plaques produced by them.

Virus survival during aerosol generation and stabilization. A certain proportion of the infectious virus particles surviving the process of nebulization may become inactivated immediately after aerosolization; the extent of such loss of virus infectivity depends on the nature of the spray fluid as well as the temperature and RH of the air (5, 24). To determine these losses at the three RH levels, the amounts of infectious virus recovered in the first (15 min) air sample from the drum were compared with those in the spray fluid. The results of these experiments are summarized in Table 6.

At the low, medium, and high levels of RH the amounts of aerosolized infectious rotavirus that could be recovered were 36.5 ± 5.2, 70.0 ± 1.5, and 27.3 ± 1.8%, respectively. No infectious poliovirus could be detected in the air samples at either the low or the medium RH level. In contrast to this, there appeared to be no immediate loss of poliovirus infectivity at the high RH level.

Effect of RH on virus survival. To determine the effect of RH on virus survival during aging of the aerosols, the amount of infectious virus recovered in the first air sample was normalized to 100%. At least three experiments were performed at each one of the three RH levels. The results of these experiments are given in Fig. 1.

The rotavirus was found to survive best at mid-range RH where its half-life was >56 h. Nearly 60% of the infectious virus could be recovered even after an aerosol age of 24 h. The half-life of this virus at the low RH was ca. 14 h, with almost 45% of the infectious virus surviving in the drum air 24 h after aerosolization. In contrast to this, the high RH was found to be the least favorable to the airborne survival of this virus as more than 50% of the infectious virus became undetectable in the air within 90 min of virus aerosolization. At this RH, the air sample drawn 24 h after virus aerosolization contained only 1% of the infectious virus.

The behavior of the poliovirus was found to be markedly different from that of the rotavirus. No infectious poliovirus could be detected in any of the samples of the drum air when the virus aerosols were held at either the medium or the low RH range. On the other hand, at the high RH, the half-life of the poliovirus was found to be nearly 10 h; about 20% of the infectious virus was present in the drum air even after 24 h of aerosol age.

DISCUSSION

The findings of this study confirm and extend our initial observations (23) with regard to the influence of RH on the airborne survival of rotaviruses. The apparent discrepancy in our results versus those of Moe and Harper (20) may be due to the following factors.

(i) In our study, the first air sample from the drum was collected after an aerosol stabilization time of 15 min, whereas in their investigation air sampling began almost immediately after virus aerosolization. We considered the aerosol stabilization period essential because it allowed the larger particles to settle and at the same time permitted a more homogeneous distribution of the smaller particles in the air inside the drum.

(ii) We sampled the air from the drum for a period of 24 h as opposed to only 2 h in the study by Moe and Harper (20). The longer sampling period was more appropriate based on our earlier observations with simian rotavirus SA-11 (23).

(iii) The lack of agreement in the results of these two studies may also be due to basic differences in the behavior

TABLE 5. Calf rotavirus (strain C-486) and poliovirus type 1 (Sabin) titration from mixtures[a]

Virus	Virus PFU/ml ($\times 10^2$)	
	Control	Mixture
Rotavirus	1.2	1.3
Poliovirus	1.6	1.6

[a] TPB was used as virus-suspending medium.

TABLE 6. Percent virus survival after aerosol generation and stabilization at three different levels of RH (20 ± 1°C)[a]

Expt	% Virus survival at RH of:					
	30 ± 5%		50 ± 5%		80 ± 5%	
	Rotavirus	Poliovirus	Rotavirus	Poliovirus	Rotavirus	Poliovirus
1	30.5	0	70.0	0	25.5	92.0
2	39.5	0	68.5	0	20.0	110.0
3	39.5	0	71.5	0	27.5	100.0
Mean ± SD	36.5 ± 5.2		70.0 ± 1.5		27.3 ± 1.8	100.7 ± 9.0

[a] The virus under test was suspended in TPB containing uranine and antifoam and aerosolized into a rotating drum. With an all-glass impinger, air samples from the drum were collected after an aerosol stabilization period of 15 min. The samples were plaques assayed in MA-104 cells.

of the two strains of calf rotavirus. However, the results of the experiments with simian rotavirus (23) and our strain of calf rotavirus strongly suggest that different types of rotaviruses may behave similarly in this regard.

Although the spray fluid was also different in these two studies, our tests with rotavirus SA-11 (manuscript in preparation) have shown that the airborne survival of this virus is not significantly altered when minimal essential medium is used as the spray fluid instead of TPB.

A number of investigators have studied the influence of various environmental factors on the airborne survival of polioviruses (4, 5, 11, 12). It was for this reason that we chose to include poliovirus type 1 as a test standard in this study, and as far as we are aware from the published literature, this is the only study in which two different types of mammalian viruses were aerosolized as a mixture. This permitted us to compare the influence of RH on two distinct types of viruses under identical experimental conditions.

Our findings on the influence of various levels of RH on the airborne survival of the poliovirus are in agreement with those of earlier reports (4, 5, 11, 12). Therefore, it may be concluded that the experimental setup in this study was in no way introducing a negative or positive bias toward the calf rotavirus.

The capacity of aerosolized rotaviruses to survive better at medium and low RH levels, as seen here and in the earlier study (23), is different from that of other nonenveloped viruses (1, 4–6, 11), but similar to that of many types of enveloped viruses (11, 18, 19).

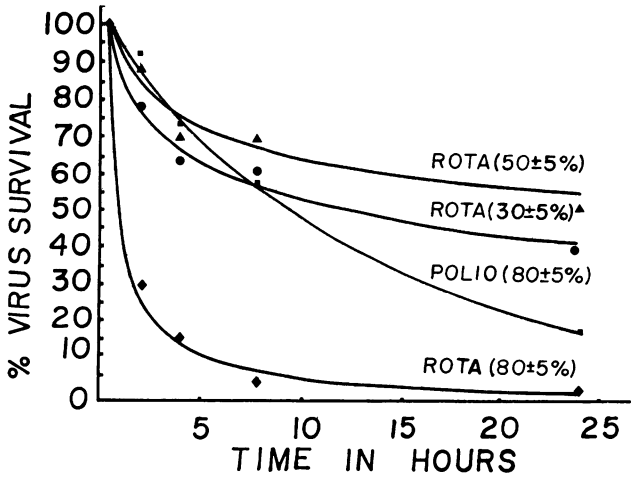

FIG. 1. Effect of three different levels of RH (20 ± 1°C) on airborne survival of calf rotavirus and poliovirus type 1 (Sabin).

Rhodamine B was used as a physical tracer in the aerobiological studies with simian rotavirus SA-11 (23). This tracer, in combination with Antifoam C, could partially inactivate the simian rotavirus. As opposed to this, uranine was found to be completely innocuous to both the calf rota- and polioviruses used in this study. It was at the same time readily soluble in TPB and its concentration could be easily measured by using standard techniques.

The experiments under way are aimed at studying the airborne survival of laboratory-adapted and field strains of human rotaviruses. Such studies, taken together with the data presented here, should lead to a better understanding of the epidemiology of outbreaks of rotavirus diarrhea in human and animal populations.

ACKNOWLEDGMENTS

This study was supported by grants from the World Health Organization and Health and Welfare Canada.

We are grateful to Anton Brunner for excellent technical assistance and Lina Beaulieu for competent secretarial services. The help of Hanne White, Christina Chudzio, and Linda Therrien is also appreciated. We thank D. Kennedy and J. C. N. Westwood for helpful and stimulating discussions and A. J. Springthorpe for the computer program used to analyze the data in this study. Complimentary supplies of gentamicin sulfate (Cidomycin) were provided to us by Roussel Canada Inc.

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