AEROSOL STABILITY STUDIES WITH THE JAPAN 305/57 STRAIN OF INFLUENZA VIRUS

by

K. Harold Malone, Jr.

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SIGNED: K. Harold Malone Jr.

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Robert J. Janssen
ROBERT J. JANSSEN
Associate Professor of Microbiology

July 8, 1968
Date

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ABSTRACT

Experiments were conducted to determine the effects of relative humidity upon the survival of the Japan 305/57 strain of influenza virus, type A2. A rotating drum chamber was employed with controlled temperature, humidity, and aerosol concentration. Aerosols were generated from undilute allantoic fluid. Viral infectivity of aerosols was assayed in embryonated eggs.

Aerosol studies were conducted at 70 F and at 4 relative humidities (25%, 40%, 55%, and 80%). Highest viral infectivity was noted at the lowest tested relative humidity of 25% and lowest viral infectivity was noted at the highest tested relative humidity of 80%. The results indicated that under the test conditions, the effect of relative humidity on the prototype strain of Asian influenza virus closely parallel that of influenza virus, type A.

INTRODUCTION

The Asian strain of influenza virus (A2) has caused disease in one or more geographic areas of the world each year since 1957. The viruses responsible for epidemics of type A influenza since 1957 have been of the A2 sub-type, clearly related to viruses isolated in 1957 but showing antigenic shift (28).

The natural history, mechanism of spread, and epidemiology of Asian influenza are well documented (16,20,28,34). These reports clearly indicate the importance of Asian influenza as one of the primary causes of respiratory infections.

Experimentally, influenza virus has been shown to be transmitted by the aerosol route. Alford et al. (1) exposed human volunteers to small particle aerosols of the Bethesda 10/63 (type A2) strain of influenza virus. Infection and typical influenza resulted even when low doses of virus were administered. Low levels of serum neutralizing antibody were not completely effective in preventing infection and illness. Hood (14) demonstrated the infectivity of aerosols of influenza virus in mice by using the PR8 (type A) and Singapore (type A2) strain. Saslaw and Carlisle (29) infected rhesus and cynomolgus monkeys with the PR8 strain of influenza virus.

Viral agents other than influenza have also been shown to be infectious via the respiratory route (3,4,8,19,23). These experiments

further indicate the potential importance of the aerosolized state of viral agents in causing infections in man and animals.

For an airborne respiratory infection such as influenza, passage of the etiologic agent through the atmosphere is an essential part of the infectivity chain; therefore, the agent will be exposed to the effects of many environmental conditions. The observation that relative humidity has a significant influence on the survival of airborne influenza A virus was first made by Loosli and associates in 1943 (22). They observed that the infectivity of influenza virus decreases as the relative humidity increases.

Since the work of Loosli, many experiments involving the effects of relative humidity on viral and bacterial agents have been conducted. Lester (21) observed that the infectivity of influenza A virus decreased as the relative humidity was raised from 30% to 50% and then gradually increased as the relative humidity was elevated to 80% or lowered to 20%. Dunklin and Puck (5) observed this same phenomenon in the death rate of pnemococci, hemolytic streptococci and staphylococci atomized into the air of an experimental chamber. The concept that intermediate relative humidities are most lethal is supported by other investigators (6,31). Harper (9), working with vaccinia, influenza, Venezuelan equine encephalitis and poliomyelitis viruses found that with the exception of poliomyelitis virus, all survived best at the lowest relative humidities tested which ranged from 17% to 25%. Poliomyelitis virus survived best at the highest relative humidity tested, which was 81%. Hemmes, Winkler, and Kool (12) reported that relative

humidity was more important than temperature and absolute humidity in the survival of influenza and poliomyelitis virus aerosols. Also, the effects of relative humidity were not dependent on the composition of the medium surrounding the virus particle. They reported that influenza virus survives best at low relative humidity and poliomyelitis virus at high relative humidity.

In a more recent study, Songer (32) observed that virus loss on aerosol generation was lower at 90% relative humidity than at 10% or 35% relative humidity. With reference to aerosol stability, he observed that Newcastle disease virus and vesicular stomatitis virus survived best at 10% relative humidity, whereas infectious bovine rhinotracheitis virus and Escherichia coli B T3 bacteriophage survived best at 90% relative humidity. With aerosols of parainfluenza 3 and adenovirus type 4 and type 7, Miller and Artenstein (24) found that parainfluenza virus was more stable at lower relative humidity and the adenoviruses were more stable at higher relative humidity. Finally, Kelley (17) observed that the infectivity of aerosolized PR8 influenza virus was higher at 85% than at 50% relative humidity.

In the studies reported above involving the effect of relative humidity on influenza virus, the PR8 strain of influenza virus, type A, was used. The results of these experiments yielded little information as to whether or not Asian strains of influenza virus, type A2, are affected in a similar manner. Asian influenza virus resembles earlier type A strains, but there are certain distinct strain differences. According to Robinson (27), the more important differences are: (a)

there are significant immunologic differences when compared with earlier prototype strains; (b) the hemagglutination-inhibition (HI) test, which is strain specific, reveals no antigenic relationship when compared against several isolations made during the period 1933-1957; (c) the Asian strains agglutinate erythrocytes to a considerably lower titer than the earlier type A strains, and they will agglutinate a broader spectrum of erythrocyte species; and (d) the Asian strains are not particularly virulent for mice.

The present study was undertaken to determine the effect of various relative humidities at 70 F (21 C) on the infectivity of the prototype strain of Asian influenza virus, Japan 305/57.

MATERIALS AND METHODS

Microorganisms

Spores of Bacillus subtilis were used as a physical tracer for determining physical decay rates and recovery ratios of aerosols. A stock spore suspension, prepared by Kelley (17), with a concentration of 6.0×10^9 spores/ml was used. From this stock suspension, a working stock containing 5.3×10^7 spores/ml was prepared in double distilled water. One-tenth ml of this working stock was added to the spray suspension.

Japan 305/57 strain of Asian (A2) influenza virus was used. The virus was obtained from the Research Reference Reagents Branch of the National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, Maryland. It was passed one additional time in embryonated eggs in our laboratory before the working stock to be used in these experiments was prepared. A virus working stock was prepared by inoculation of 0.1 ml of a 10^{-4} dilution of stock virus in Difco brain heart infusion broth into the allantoic cavity of 11-day-old embryonated eggs. After 48 hr incubation at 38 C, the embryos were candled and all viable embryos were refrigerated overnight. The allantoic fluid was collected aseptically, pooled, and stored at -65 C in 5-ml volumes. This working stock of Japan 305/57 influenza virus had an egg infective dose 50 (EID_{50}) titer of 10^7 infectious units/0.1 ml as determined by the method of Reed and Muench (26).

Aerosol Generation

A rotating drum chamber (7) housed in an environmental-controlled safety cabinet was used for the atomization, storage, and collection of aerosols. Characteristics and operating procedures for this apparatus have been reported in detail by Kelley (17).

Aerosols were generated from a sterile Devilbiss #40 nebulizer using a compressed air supply of 25 psi. The spray suspension was prepared by mixing 0.1 ml of working spore suspension with 4.9 ml of undilute allantoic fluid containing the working stock virus. The nebulizer was loaded with 4 ml of the spray suspension prior to atomization and then connected to the compressed air line. The aerosol was generated for 4 min at a total air flow of 250 liters/min. Secondary air was obtained from the environmental-controlled safety cabinet through a filtered valve. Air balance in the cabinet was maintained with the compressed air inlet valve. After atomization, the compressed air and vacuum lines were closed simultaneously. The secondary air valve was then closed, thus sealing the storage drum.

Aerosol Sampling

Aerosol samples were obtained with sterile all-glass impingers (AGI-30) (2). The impinger fluid consisted of 20 ml of sterile brain heart infusion broth containing 2 drops of antifoam. The impingers had a flow rate of 12.5 liters/min and each sampling period was 1 min. Aerosols were generated at 70 F at relative humidities of approximately 25%, 40%, 55%, and 80%. Initial samples were taken at 15 min after aerosolization. Subsequent samples were taken at intervals of 10 min,

30 min, or 60 min, depending on the relative humidity being tested. Spray and impinger samples were dispensed into tubes and stored at -65 C until assay could be conducted.

Physical Decay

Physical decay of the aerosols was determined by using the spores of B. subtilis as a tracer. The stability of this organism for aerosol studies is well established (11,25). Spore assays were made promptly on the spray suspensions prior to and after aerosolization, and on the impinger fluid of each sample. Ten-fold dilutions were made in distilled water. Assays of spray samples were made by spreading 0.1 ml of each dilution on each of 3 plates of Bacto nutrient agar. Impinger samples were assayed by spreading 0.2 ml of undilute impinger fluid on each of 3 Bacto nutrient agar plates. Dilutions of spore suspension yielding less than 300 colonies were counted.

Total Decay

Total decay of virus was determined for each relative humidity tested. Aerosol storage periods were as follows: (a) 25% relative humidity, 360 min; (b) 40% relative humidity, 195 min; (c) 55% relative humidity, 65 min; and (d) 80% relative humidity, 55 min. Aerosol samples were taken at storage times from 15 min to end of storage period.

Biological Decay

Biological decay was determined by subtracting the $\log_{10}$ spray virus/spore ratio from the $\log_{10}$ impinger virus/spore ratio. To this

figure was added the log of 100, thus giving the log per cent of infectious virus present for a particular aerosol sample.

Erythrocyte Suspension

Blood taken aseptically by cardiac puncture from chickens was suspended in Alsever's solution (15) and stored at 4 C. Prior to use, a 0.5% (v/v) suspension was prepared in 0.85% NaCl by washing the cells 3 times in saline solution and centrifuging at 1000 rpm for 5 min.

Virus Titration

The titrations of the infective virus concentration of stock preparations and aerosol samples were carried out in 11-day-old embryonated eggs. Brain heart infusion broth was used for making serial dilutions. Eggs were inoculated by the allantoic route with 0.1 ml of the test material and incubated at 38 C for 44 to 48 hours. Live embryos were subsequently chilled at 4 C overnight. After chilling, approximately 0.5 ml of clear allantoic fluid was removed from the egg and checked for hemagglutinin. Fifty per cent end points were calculated as suggested by Hirst's (13) application of the Reed-Muench technique (26).

Preliminary titrations were carried out by using 10-fold dilutions and 6 eggs/dilution. This was necessary in order to establish approximate end points and aerosol storage times. Final titrations were based on 3-fold dilutions and 10 eggs/dilution.

Serology

Immune guinea pig serum which had previously been determined to contain strain specific hemagglutination-inhibiting antibody to Japan 305/57 influenza virus was used to confirm the identity of virus in stock and aerosol samples. A microtechnique as described by Sever (30) was used in making these determinations.

RESULTS

Preliminary Assay of Virus

Preliminary aerosol experiments with the Japan 305/57 strain of influenza virus were conducted in order to establish whether or not changes in relative humidity had any effect on viral infectivity. Relative humidities selected for these experiments were 25%, 40%, 55%, and 75%. Aerosol samples were taken at 15 min, 60 min, 120 min, 180 min, 240 min, and 300 min after aerosolization. The results of these experiments are summarized in Table 1. Preliminary viral assays revealed that at lower relative humidities the virus maintained its infectivity for embryonated eggs longer than it did at the higher relative humidities. These results provided the necessary information for continuation of the studies. It was apparent, because of the different decay rates, that the intervals between sampling had to be adjusted for each relative humidity. This was necessary in order to provide sufficient points for decay rate determinations. With this information it was decided that future samples would be taken at intervals of 60 min for 25% relative humidity, 30 min for 40% relative humidity, and 10 min for 55% and 80% relative humidity.

Final Viral Assays

Viral titrations were conducted on all spray suspensions and impinger samples. Assays of spray samples revealed the following $\log_{10}$

Table 1. Recovery of airborne Japan 305/57 influenza virus. Preliminary titrations of impinger samples.[a]

Relative Humidity (per cent)	Storage Period (min)					
	15	60	120	180	240	300
	EID_{50} Concentration[b]					
25	2.65	2.63	2.67	1.75	1.63	0.75
40	2.90	2.48	1.85	1.25	0.63	0.33
55	2.10	0.05	0.00	0.00	-[c]	-[c]
75	2.30	0.00	0.00	-[c]	-[c]	-[c]

a. Titrations in embryonated eggs, 10-fold dilution, 6 eggs/dilution.

b. Concentration expressed as EID_{50}/0.1 ml ($-\log_{10}$).

c. Not done.

titers: (a) 25% relative humidity, 6.546; (b) 40% relative humidity, 6.903; (c) 55% relative humidity, 6.588; and (d) 80% relative humidity, 6.736. At the 95% confidence limits there is no significant difference in titers. These results indicate that under the test conditions the influence of relative humidity had little or no effect on viral infectivity of the spray suspensions during aerosolization. The geometric mean titer (6.693) of spray suspensions described above was used in making future calculations.

Viral Infectivity

The $\log_{10}$ ratio of infectious virus titer to spore tracer in the spray suspensions is shown in Table 2. The variations in $\log_{10}$ virus/spore (V/S) ratio are caused by the different concentrations of spore tracer used in the spray suspensions.

The log per cent infectious virus recovered from the impinger samples was determined by applying the formula:

$$\text{log per cent infectivity} = (2.0 + \log_{10} \text{impinger V/S ratio}) - \log_{10} \text{spray V/S ratio}$$

The amount of infectious virus recovered under various experimental conditions is presented in Tables 3, 4, 5, and 6.

The manner in which relative humidity affects the infectivity of airborne Japan 305/57 influenza virus is presented in Figure 1. The lowest tested relative humidity favors viral survival and the higher relative humidities are the most detrimental. As the relative humidity was increased from 25% to 55%, the capacity of this virus to survive

Table 2. Virus/spore ratios of spray suspensions at test relative humidities.

Relative Humidity (per cent)	Log_{10} Virus Spray Titer[a]	Log_{10} Spore Count	Log_{10} Virus/Spore Ratio
25	6.693	5.580	1.113
40	6.693	5.041	1.652
55	6.693	4.845	1.848
80	6.693	4.903	1.790

a. Geometric mean of all spray sample titers at 4 min.

Table 3. Infectivity of Japan 305/57 influenza virus aerosol recovered at 25% relative humidity from drum chamber.

Time in Min After Atomization	Log_{10} Impinger Titer[a]	Log_{10} Spore Count	Log_{10} Impinger V/S Ratio	Log Per Cent Infectivity[b]
15	3.073	1.813	1.260	2.147
60	3.307	1.799	1.508	2.395
120	3.020	1.756	1.264	2.151
180	2.744	1.699	1.045	1.932
240	2.524	1.681	0.843	1.730
300	2.442	1.716	0.726	1.613
360	2.309	1.643	0.666	1.553

a. Concentration expressed as EID_{50}/0.1 ml.

b. Based on a $\log_{10}$ spray V/S ratio of 1.113.

Table 4. Infectivity of Japan 305/57 influenza virus aerosol recovered at 40% relative humidity from drum chamber.

Time in Min After Atomization	Log_{10} Impinger Titer[a]	Log_{10} Spore Count	Log_{10} Impinger V/S Ratio	Log Per Cent Infectivity[b]
15	3.102	1.0	2.102	2.450
45	2.648	0.954	1.694	2.042
75	2.586	0.954	1.632	1.980
105	2.309	0.978	1.331	1.679
135	2.113	1.0	1.113	1.461
165	1.865	1.0	0.865	1.213
195	1.607	0.978	0.629	0.977

a. Concentration expressed as EID_{50}/0.1 ml.

b. Based on a $\log_{10}$ spray ratio of 1.652.

Table 5. Infectivity of Japan 305/57 influenza virus aerosol recovered at 55% relative humidity from drum chamber.

Time in Min After Atomization	Log_{10} Impinger Titer[a]	Log_{10} Spore Count	Log_{10} Impinger V/S Ratio	Log Per Cent Infectivity[b]
15	2.777	0.954	1.823	1.975
25	2.524	1.0	1.524	1.676
35	2.386	1.021	1.365	1.517
45	1.960	1.0	0.960	1.112
55	1.841	0.978	0.863	1.015
65	1.798	0.757	0.757	0.909

a. Concentration expressed as EID_{50}/0.1 ml.

b. Based on a $\log_{10}$ spray V/S ratio of 1.848.

Table 6. Infectivity of Japan 305/57 influenza virus aerosol recovered at 80% relative humidity from drum chamber.

Time in Min After Atomization	Log_{10} Impinger Titer[a]	Log_{10} Spore Count	Log_{10} Impinger V/S Ratio	Log Per Cent Infectivity[b]
15	2.270	0.699	1.571	1.781
25	2.061	0.740	1.321	1.531
35	1.860	0.778	1.082	1.292
45	1.021	0.740	0.281	0.491
55	0.997	0.740	0.257	0.467

a. Concentration expressed as EID_{50}/0.1 ml.

b. Based on a log_{10} spray V/S ratio of 1.790.

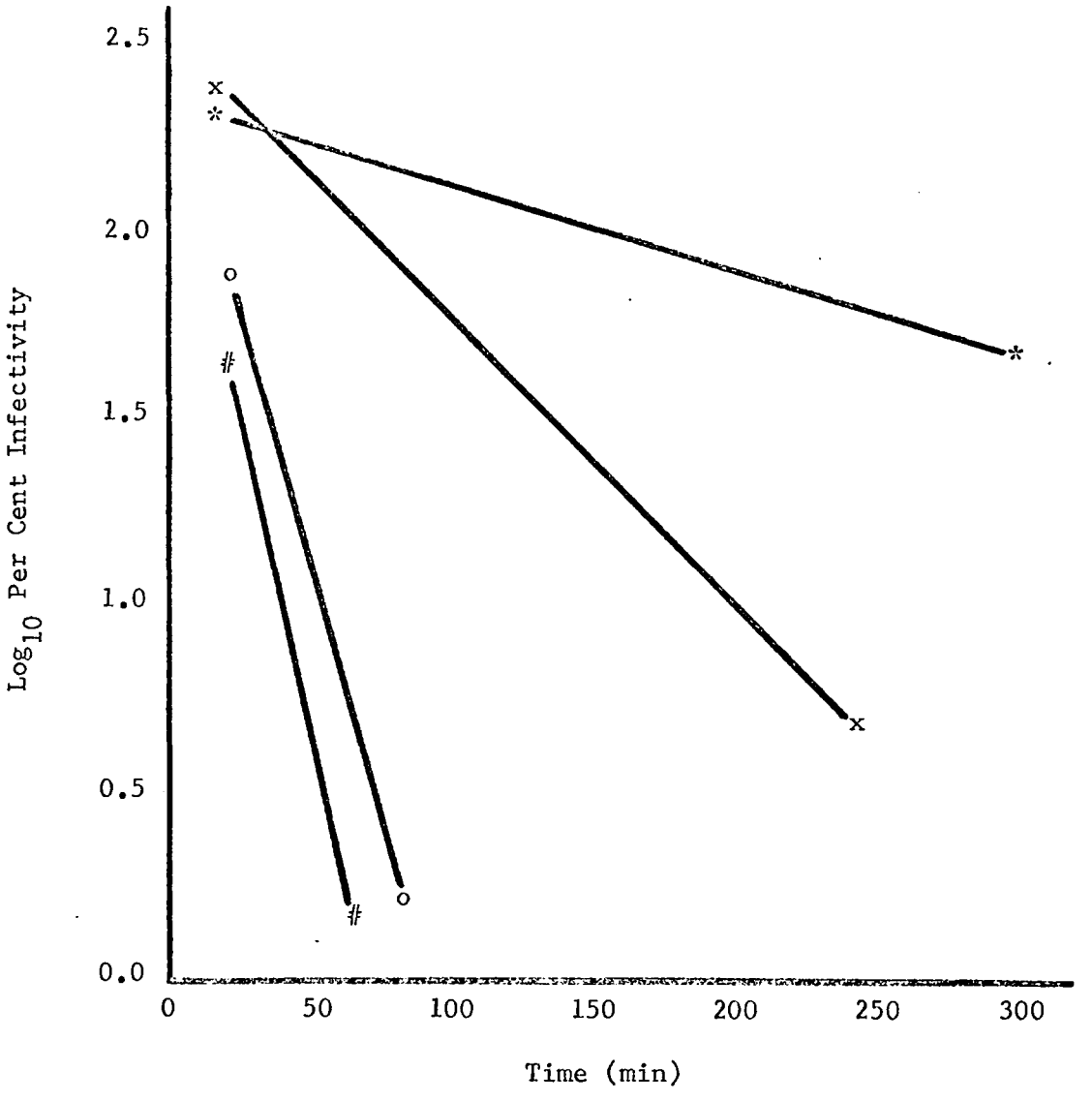

Figure 1. Loss of infectivity of aerosols of Japan 305/57 influenza virus, A2.

* ———— * 25% relative humidity
x ———— x 40% relative humidity
o ———— o 55% relative humidity
———— # 80% relative humidity

was proportionally reduced, the most rapid decay occurring above 50% relative humidity. Under the test conditions, the biological half-life for Japan 305/57 influenza virus was found to be: (a) 25% relative humidity, 140 min; (b) 40% relative humidity, 60 min; (c) 55% relative humidity, 13 min; and (d) 80% relative humidity, 10 min.

Hemagglutination-inhibition Test and Other Controls

Hemagglutination-inhibition tests confirmed the identity of the stock virus preparation as the Japan 305/57 strain of influenza virus, type A2. This procedure also established that the hemagglutinins produced in embryonated eggs following inoculation of impinger samples were induced by the same virus. Stock spore preparation, when inoculated into embryonated eggs did not stimulate the production of hemagglutinins. Therefore, the hemagglutinins produced from impinger samples were established as being the result of infection of the embryonated eggs with Japan 305/57 influenza virus, type A2.

DISCUSSION

The results of preliminary trial assays indicated that the Japan 305/57 strain of influenza virus was affected by the various changes in relative humidity. The recovery of the stable spore tracer in aerosol samples eliminated doubt that the loss of viral activity was due to physical decay (settling out). The size and density of the virus particle when compared with that of the B. subtilis spore is much less. Therefore, it can be assumed that under the same experimental conditions physical decay of the virus would not exceed that of the spore, and any loss of viral activity is the result of biological decay ("death" of virus).

The decay rate for the Japan 305/57 influenza virus aerosols stored at the test relative humidities was comparable to that reported by Hemmes et al. (12), Harper (9), and Loosli et al. (22) with type A influenza strains. The decay rates, however, do not compare with the type A influenza viruses reported by Shechmeister (31) and Lester (21). In separate studies, they (31,21) observed highest viral recovery at high and low relative humidities, and most decay in the intermediate relative humidity range. Whether or not these differences were due to the nature of the suspending medium is not known. Previous studies (10,18,33,35) have shown that the composition of the spray suspension exert a great influence on the rate of biological inactivation.

The experimental results showed that relative humidity had little or no significant influence on the infectivity of the viral spray suspension during aerosolization. They did not, however, provide information necessary to determine which relative humidity was the most detrimental to the viral cloud immediately (within the first minute) following aerosolization. Songer (32) observed with several viral agents that loss of infectivity on aerosol generation was consistently lower at 90% than at 10% or 35% relative humidity. In that this point was not investigated with the Japan 305/57 strain of influenza virus, it is not known whether generation loss, as well as storage loss, must be considered when fully evaluating the effects of relative humidity on airborne Japan 305/57 influenza virus.

The findings observed are based on a spray suspending medium of undilute allantoic fluid and a collection medium of brain heart infusion broth. It would have been interesting to establish whether or not other suspending and collection menstrua would have given the same results. Such experiments would have indicated whether the stability of aerosolized Japan 305/57 influenza virus observed in this study represent a true property of the virus, or the added influence of the medium surrounding the viral particle.

The epidemiologic implications of these observations on Asian influenza virus, as well as those of Dunklin and Puck (5) on bacteria, may well be important. It seems likely that, in most environments, greater numbers of the infective agents dispersed from the respiratory tract are destroyed than survive, and many factors undoubtedly play a

role in this process. The results of this study, along with many others, strongly suggest that relative humidity is one of the more important environmental factors in determining the survival of respiratory viral agents issuing from an infected host.

SUMMARY

The effects of relative humidity on the infectivity of airborne Japan 305/57 influenza virus, type A2, were studied in an environmentally controlled rotating drum chamber. Aerosols were generated from allantoic fluid at relative humidities of 25%, 40%, 55%, and 80%.

The infectivity of Japan 305/57 influenza virus aerosols was determined by the ability of the virus particle to produce hemagglutinins in embryonated eggs. The infectivity of the tested viral agent is greatly influenced by relative humidity. Highest viral infectivity was noted at the lowest relative humidity and lowest viral infectivity at the highest relative humidity.

The results of these experiments compare with the findings of other workers who have studied influenza virus, type A. It is therefore concluded that relative humidity affects the prototype Asian strain of influenza virus much the same as it does type A influenza virus strains.

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