Influence of Incubation Temperatures on the Demonstrable Amount of PR8, Lee-B and FM-1 Strains of Influenza Virus in the Chorio-Allantoic Fluid of Chick Embryos

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The chick-embryo in the past few years has become increasingly important as an experimental host for influenza virus as well as being used for the commercial production of influenza virus vaccine. Many investigations have been conducted to ascertain the specific roles of a number of variables which are known to be involved in the relationship between the infective agent and the host. Some of the variables which have been studied are (a) the concentration of the virus in the inoculum, (b) the length of time of incubation of the infected embryos, (c) the age of the embryos at time of inoculation, (d) the temperature of incubation of the embryos before inoculation, and (e) the temperature of incubation of the infected embryos. Relative to the studies conducted concerning the temperature of incubation of the infected embryos the investigators have used on the most part small numbers of eggs in conducting their studies. It has been our experience, from a production view point in which as many as 100,000 eggs are inoculated per week, that one is not always able to obtain the same results when using a large number of eggs as an investigator who has published data in which only a few eggs have been used, though one endeavors to conduct ones production methods in an identical manner to the experimental methods. Miller (1) has conducted some studies upon the production of A virus (PR8 strain) at various temperature levels and found maximum virus production to occur when the embryos were incubated at 35°C. Beard (2) and his group of co-workers at Duke University suggest 39°C. while Henle (3) suggests 36-37°C. for this same strain. In studies upon the production of B virus (Lee strain) at various temperature levels Beard (4) found maximal infectivity as well as greater hemagglutinative activity when the embryos were incubated at 35°C. No reference in the literature could be found concerning the optimal temperature level for the production of the A virus (strain FM-1). This particular strain was isolated in 1947 and the National Institute of Health had all producers of influenza virus vaccine to substitute this strain for the Weiss strain which had formerly been included in their mixed vaccine product. The results obtained with all three strains of the influenzal virus are described in the present paper.

Materials and Methods

Stock Inoculum.—Infectious allantoic fluid obtained by the regular passage of the particular strain through eggs for seed purposes was used. The PR8 strain contained around 250 units of CCA activity per cc. (5) The Lee strain 100 and the FM-1 strain 100.

Inoculations.—Eggs for the experiments were obtained from a commercial hatchery which also supplies us with our eggs used for production purposes and had been incubated 11 days at 37.5°C. The eggs were inoculated, into the chorio-allantoic sac, at room temperature with 0.2 ml of a suitable dilution in saline of the stock virus by means of a mechanical egg inoculating machine developed in our own laboratories and capable of inoculating 6000 eggs per hour. The openings in the shells were not sealed since we have found it is unnecessary to do so. The eggs were then incubated for 48 hours at the desired temperature range, and the humidity kept at a high level. At the end of the 48 hour incubation period the eggs were candled and all dead embryos discarded. The eggs having living embryos were then chilled for approximately 24 hours at the end of which time the allantoic fluid was aspirated and tested for CCA activity.

Measurements of Virus Activity.—Assuming that virus infectivity and chicken red blood cell agglutination (CCA) activity are the properties of the same molecule (6), the relative amounts of virus were measured indirectly by means of determinations of CCA titers. The CCA titrations were carried out according to the method of Hirst and Pickels (7), as modified by Miller and Stanley (5).

Experimental Results

Table I shows the results of three separate trials using PR8-"A" virus at incubation temperatures of 33°-35°C., 35°-37°C., and 37°-39°C. In trial 1 it will be noted that the CCA titer of the eggs incubated at 33°-35°C. and 35°-37°C. is indentical while that of the eggs incubated at 37°-39°C., is less than one-third this amount. Trial 2 shows practically the same relation existing between the various temperature levels as did trial 1. In trial 3 the eggs incubated at 35°-37°C. had a slightly higher CCA value than those incubated at 33°-35°C. It will be noted in all three trials that the eggs incubated at 37°-39°C. had a much higher death rate than those incubated at the two lower levels. It was because of the much higher death rate at 37°-39°C. that the number of eggs inoculated in trials 2 and 3 for incubation at this level was reduced in comparison to the number inoculated for incubation at 33°-35°C. and 35°-37°C. A total of 1008 eggs was used in these three trials.

TABLE I

The red blood-cell hemagglutinative and CCA activity of chorioallantoic fluid from chick embryos incubated at various temperature levels after inoculation with PR8 influenzal virus.

Incub. Temp.	Trial	Length of Incub. Hrs.	No. Eggs Inoc.	Death Rate %	CCA Value
33°-35°C	1	48	118	11.85	250
	2	48	137	22.6	125
	3	48	150	10.0	225
35°-37°C	1	48	119	15.8	250
	2	48	137	30.7	125
	3	48	150	20.0	250
37°-39°C	1	48	119	63.8	70
	2	48	50	78.0	50
	3	48	28	100.0	..

Table II shows the results of three separate trials using Lee "B" virus at incubation temperatures of 33°-35°C., 35°-37°C., and 37°-39°C. In trial 1 it will be noted that the CCA titer of the eggs incubated at 33°-35°C. and 35°-37°C. is identical while that of the eggs incubated at 37°-39°C. is practically nil. The results obtained from the 37°-39°C. incubation temperature substantiated previous tests not included in this paper and consequently no further tests were repeated at this temperature level. Trials 2 and 3 both indicate very little difference in virus activity between the two temperature levels. Comparing the death rate of the embryos at the various temperature levels there was 2.2% less deaths in the group incubated at 33°-35°C. than there was in the group incubated at 35°-37°C. The 37°-39°C. group again had a much higher death rate in comparison to the other two groups. Beard (8) suggests that the small quantity of Lee "B" virus demonstrable in the chorio-allantoic fluid of embryos incubated at 39°C. may be due to the inactivation or breaking down of the virus that is formed by exposure to adverse pH of the chorio-allantoic fluid, as the fluid descends into the acid region at 39°C. Miller (9) has found that the optimum pH range for the stability of influenza virus B is at or above pH 7.9 while that for optimum stability (9) of PR8-"A" is 6.5 to 7.0. A total of 1234 eggs was used in the study of the Lee B virus.

TABLE II

The red blood-cell hemagglutinative and CCA activity of chorio-allantoic fluid from chick embryos incubated at various temperature levels after inoculation with Lee "B" influenzal virus.

Incub. Temp.	Trial	Length of Incub. Hrs.	No. Eggs Inoc.	Death Rate %	CCA Value
33°-35°C	1	48	132	3.8	100
	2	48	204	11.2	150
	3	48	145	7.6	100
35°-37°C	1	48	273	6.0	100
	2	48	203	12.3	125
	3	48	145	11.0	100
37°-39°C	1	48	132	18.2	10
	2	Not Repeated			
	3	Not Repeated			

Table III shows the results of the trials made using the FM-1 virus. Trial 1 indicates about twice as much CCA activity in the chorio-allantoic fluid from the embryos incubated at 35°-37°C. than those incubated at 33°-35°C. and 10 times more than from the embryos incubated at 37°-39°C. Trials 2 and 3 indicate a slightly greater amount of CCA activity

TABLE III

The red blood-cell hemagglutinative and CCA activity of chorio-allantoic fluid from chick embryos incubated at various temperature levels after inoculation with FM-1-"A" influenzal virus.

Incub. Temp.	Trial	Length of Incub. Hrs.	No. Eggs Inoc.	Death Rate %	CCA Value
33°-35°C	1	48	104	24	50-75
	2	48	150	20	60
	3	48	100	16	125
35°-37°C	1	48	104	12.5	125
	2	48	150	26	80
	3	48	100	7	150
37°-39°C	1	48	104	15.3	10
	2	48	150	16	5
	3	48	100	12	50

in the chorio-allantoic fluid from the embryos incubated at 35°-37°C. than from those incubated at 33°-35°C. Only small amounts of CCA activity is indicated by trials 2 and 3 as being present in the chorio-allantoic fluid of the embryos incubated at 37°-39°C. Relative to the death rate of the embryos during the 48 hour incubation period there was approximately 5% less deaths in the 35°-37°C. range as compared to the 33°-35°C. group. There was a total of 1062 eggs used in this study of the FM-1strain.

The authors of this paper feel that mention should be made of the fact that some investigators, i. e. Salk (10), feel that conditions of cultivation of the various strains within the ddifferent laboratories may also have an effect upon many strain and line differences that are noted. If this be true, then the differences in optimal incubation temperature for the production of PR8 virus, that is mentioned by the different investigators in the beginning of this paper, can be explained. This same fact might also explain the findings of the present authors relative to the optimal temperature level of incubation for the production of PR8 and Lee virus being the same, as the seed for each strain has been carried in an identical manner in regard to the temperature of incubation since we received the original strains in 1944. Incubation of the seed has always been done in the same incubator in which our production eggs are incubated, and because of the size of this incubator room we have a temperature variation of at least ±1° at 36°C. Our present findings would seem to indicate that we would have more FM-1 virus produced if we incubated this strain at 35°-37°C. while 33°-35°C. would optimum for the production of the PR8 and Lee virus using the particular strains which we have in our laboratory.

Summary

1. Very little, if any, difference in demonstratable PR8 virus as measured by the CCA test and the hemagglutination test was noted between the chorio-allantoic fluid of eggs incubated for 48 hours at 33°-35°C. and 35°-37°C.

2. Very little difference in demonstratable Lee virus as measured by the CCA test and the hemagglutination test was noted between the chorio-allantoic fluid of eggs incubated for 48 hours at 33°-35°C. and 35°-37°C.

3. From an economic view point, because of a lower embryo death rate, the optimum temperature level of incubation would be 33°-35°C. for the particular strains of PR8 and Lee virus which we have in our laboratory.

4. The optimum temperature level of incubation for the maximum production of FM-1 virus in the present experiments was 35°-37°C.

5. The present experiments indicate that the 37°-39°C. temperature level of incubation was unsuitable for the cultivation of any one of the three strains studied.

References

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