DISTRIBUTION OF THE VIRUS OF INFECTIOUS BRONCHITIS OF CHICKENS IN EMBRYONATED CHICKEN EGGS

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DISTRIBUTION OF THE VIRUS OF INFECTIOUS BRONCHITIS OF CHICKENS IN EMBRYONATED CHICKEN EGGS

By

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INTRODUCTION

This study was undertaken in an endeavor to ascertain the activity and distribution of an egg adapted strain of the virus of infectious bronchitis in the extra-embryonic fluids and certain tissues of embryonated chicken eggs. Since the chicken embryo serves a useful means for viral diagnostic purposes in the laboratory, the results submitted may direct attention to suitable procedures for the production of high potency virus.

REVIEW OF LITERATURE

Infectious Bronchitis

Schalk and Hawn first described the disease in 1931 in North Dakota. By 1933 it had been recognised in several of the midwestern states and in California and since that time it has been reported in other states. The disease is infectious only to chickens.

Etiology:

Infectious bronchitis is caused by a virus which is found most abundantly in the exudate and tissues of the affected respiratory organs and it has been also demonstrated in the liver, spleen, kidney and blood.

Symptoms and lesions:

The most characteristic symptom is gasping although convulsive coughing may also be seen. Depression and weakness are seen in advanced stages of the disease. Nasal discharge and swollen sinuses are frequently observed. Chicks less severely affected exhibit such symptoms irregularly. Infected laying birds show symptoms similar to those observed in chicks.

At autopsy, varying amounts of clear or turbid liquid or viscid exudate are found in the lower trachea and bronchi. It is rarely of the thick yellowish and bloody character which is found in laryngotracheitis.

Lungs may show variable congestion and air sacs frequently are opaque in appearance.

The disease spreads rapidly and usually occurs most frequently in chicks under 3 or 4 weeks of age although it has been reported in chicks as young as 2 days old. Beach and Schalm (1936) observed that birds from 10 to 112 days old were equally susceptible to artificial infection.

Beaudette and Hudson (1937) reported the spread of the disease from chicks to adult birds on the same premises. Delaplane and Stuart (1939) observed the disease in semi-mature and older chickens as well as chicks.

In young chicks the mortality has been reported to be as high as from 70 to 80 per cent, whereas in semi-mature and adult birds the mortality is nominal in most outbreaks. Retardation of growth in semi-mature birds and decreased egg production which persists for several weeks in laying flocks are a considerable economic loss.

Diagnosis:

History of the case, clinical symptoms and autopsy findings are usually sufficient for recognition of infectious bronchitis. Transmission, cross immunity, or serum neutralization tests are often required for definite identification of the disease present.

Transmission and immunization tests:

The disease may be transmitted by exposure of the respiratory system to infected tracheal exudate and lung material, with a usual incubation period from 18 to 48 hours. Symptoms are frequently noted in from 24 to 48 hours. Subcutaneous and air sac inoculation will incite the disease.

Delaplane and Stuart (1939) observed that birds which had recovered from infectious bronchitis developed an immunity which completely protected them against subsequent contact or artificial exposure under field conditions. One bird was normal in every respect except for an almost unnoticeable nasal discharge which had persisted for 2 months from the time of infection. During this time the nasal exudate from the bird incited regularly the disease in susceptible chickens. The washing of the nasal passages from this bird after cessation of the exudate was no longer infective.

Neutralization tests:

It has been shown by Delaplane and Stuart (1939) that when serum from birds which were immune to infectious bronchitis was mixed with the virus and incubated for 1 hour at 37 degrees C, the mixture did not incite the disease when injected into air sacs of susceptible birds. This indicated neutralization of the virus by the serum. Birds that were injected subcutaneously with 0.25 cc of serum followed after 1 day by artificial inoculation of virus intratracheally developed the disease indicating no protection from the quantity of serum employed although this serum neutralized the virus in vitro.

Immunization studies:

Beaudette and Hudson (1937), and Delaplane and Stuart (1939) investigated the possibility of active immunization of chickens by inoculating the cloacal mucous membrane and the bursa of Fabricius with infectious tracheal exudate. Mild "takes" were noted within 24 hours to 48 hours which completely subsided after 72 hours following inoculation. The latter authors showed by transmission tests that the virus had grown in the cloacal mucous membrane. This did not develop immunity against the disease in the respiratory tract in 3 to 5 days after inoculation. The failure to do so was attributed to insufficient production of antibodies to prevent infection through the respiratory system.

Delaplane and Stuart (1939) were unsuccessful in producing immunity with a vaccine consisting of 20 per cent lung and tracheal exudate suspension in 0.85 NaCl and formalized to 0.5 per cent. This vaccine was inoculated in 2 cc amounts either subcutaneously or in the air sacs. The vaccinated birds were inoculated with the virus in the larynx and trachea 2 weeks later and developed the disease.

Cultivation of the Virus in Embryonated Chicken Eggs

During the first few egg passages of field strains of virus obtained from tracheal exudate from infected birds, there were no deaths or distinctive or characteristic lesions of the embryo. Delaplane and Stuart (1939) demonstrated growth of the virus in the embryo at this stage by the appearance of typical symptoms of the disease in chicks hatched from inoculated eggs. With each successive transfer the virus became more virulent to the embryo. From the fifth to the tenth transfers some embryos died 5 to 6 days following inoculation. As egg passages were continued embryos died at the fourth, then the third day, and finally at about the 60th passage the bulk of the embryos died between the second and third days. Apparently the ability of the virus to kill the embryo became fixed.

Delaplane and Stuart (1941) studied the modification of the Rhode Island strain and Beaudette's strain of virus in embryonated eggs, and did not note any marked difference in the effect on embryos. The Rhode Island strain at the 89th, 90th and 91st egg passages was avirulent for baby chicks exposed by spraying of the respiratory tract or the inoculation of bursa of Fabricius. Beaudette's strain of virus retained its virulence and incited typical symptoms of the disease in baby chicks at the 120th to 125th transfers during the winter months but only irregularly so during the summer months. This would indicate that seasonal variations as well as different strains of the virus have an influence on the virus activity.

Beaudette's embryo propagated strain (117th passage) incited symptoms in chickens. When re-isolated and passed in eggs, little change in embryo death rate was noted which would indicate that such a chicken passage failed to return the embryo-adapted virus to the field type.

Effect of Physical Agents

Beach and Shalm (1936) showed that tracheal exudate dried after freezing and stored in a refrigerator remained viable for 180 days. Under the same conditions, the virus when kept in 50 per cent glycerin, was viable for 80 days.

Delaplane and Stuart (1941) found that the embryo propagated virus survived storage in the fresh frozen state for 4½ months but not for 5 months. It survived room temperature for 5 to 7 days, and at 50 degrees C for f, 10 and 15 minutes as indicated by egg tests.

Cunningham and stuart (1947) reported that infected allantoic fluid which had been dehydrated from the frozen state at 0.15 mm mercury pressure for 8 hours, sealed in vacuo, and stored at 4 degrees C for 7 days showed a 100 fold decrease in activity when restored to volume. Freezing and thawing were without effect on the virus and loss of virus activity was probably due to dehydration.

MATERIALS AND METHODS

Virus

A strain of infectious bronchitis virus which had been adapted to cultivation in embryonated chicken eggs was used for inoculum. This virus was capable of killing the majority of the embryos by the end of the second day and the remainder by the end of the fourth post-inoculation day. The virus suspension consisted of pooled allantoic fluid and had a titer of $10^{-7.6}$ as indicated in Table I.

Procedure

Ten-day-old embryonated chicken eggs were used throughout this experiment except in two instances when 9 and 11-day-old eggs were used. Inoculation was via the allantoic route. Eggs were transilluminated for selection of an area on the chorio-allantoic membrane free from large blood vessels, about 3 mm below the air sac, and a mark made for the site of injection. A small hole was drilled through the shell at this point without piercing the inner shell membrane. Another hole was made through the shell over the top of the air sac. Before inoculation, tincture of metaphen was painted over the holes and allowed to dry. The shell membrane in the hole at the top of the air cell was punctured. This allowed equalization of pressure within the egg when the inoculum was injected into the allantoic sac and prevented leaking out of the inoculum from the site of injection.

Collection of Material

Twenty-five 10-day-old embryonated eggs were inoculated with 0.1 ml of the virus suspension via the allantoic sac, and the eggs were returned

to the incubator for harvest of certain tissues and extra-embryonic fluids from living and dead embryos at 12 hour post-inoculation intervals. The eggs were inoculated at 8 p.m. for convenience of harvesting every 12 hours and with the expectation that the majority of the embryos would die between the 36th and 48th hours, 8 a.m. to 8 p.m., so that the eggs could be candled frequently during this time to select embryos as near to these hours as possible.

Eggs were painted with tincture of metaphen over the region of the air cell and the shell over the air cell was cracked and removed with forceps. Separate sterile instruments were used for each operation. The materials were collected in the following manner and order. Allantoic fluid was removed with a 5 ml syringe and needle. The shell membrane was removed with forceps to expose the embryo in the amnionic sac. Using another 5 ml syringe the amnionic fluid was removed. Yolk material was also collected in the same manner. The chorio-allantoic membrane was ruptured and the embryo and fluids poured into a sterile Petri dish. Then the chorio-allantoic membrane was removed from the shell and the albumen adhering to the membrane was washed in physiological saline and drained of excess fluid. The liver was collected from the embryo by dissection with forceps, and the embryo was examined for gross pathological lesions.

Harvested materials were placed in separate 10 cc screw cap vials which were labeled with pertinent information and frozen at -35 degrees C. All the materials were bacteriologically sterile as shown by inoculation of agar slants.

Titrations

Vials containing allantoic fluid, amnionic fluid and yolk were thawed at room temperature, centrifuged at 2500 r.p.m. for 5 minutes and the supernatant fluid used for titration. The chorio-allantoic membranes

and livers were thawed at room temperature, weighed and ground with sand in a mortar and pestle. Broth was added to the ground material in the proportions of 9 parts by volume of broth to 1 part by weight of the material. This was considered to be a 10^{-1} dilution of the material. In two instances the weight of the liver material was so little that a 10^{-1} dilution would have been insufficient for inoculation purposes. In these cases broth was added in the proportions of 99 parts of broth to 1 part of liver material to make a 10^{-2} dilution.

After thorough emulsification, the fluid was aspirated with a pipette and transferred to a vial. A few drops of the fluid were transferred to agar slants for bacteriological sterility tests and the vial returned to the freezer.

In all titrations 5 eggs per dilution were used and the eggs were incubated for 4 days. This was considered to be a significant number of eggs for detection of viral activity in 10-fold serial dilutions. Knight (1944) working with influenza virus reported that when using 5 eggs per dilution, one can scarcely expect to detect with any certainty differences less than 4 or 5 fold.

Procedure:

The procedure which has been described was used in all titrations. The titer was considered to be the highest dilution which killed more than 50 per cent of the embryos inoculated (Cunningham and Stuart 1943).

Inoculum consisted of supernatent fluid from samples which had been frozen at -35 degrees C, thawed at room temperature and then centrifuged. Difco nutrient broth was used as a diluent. Serial 10-fold dilutions of the fluid were made in the ratio of 1 part virus (0.5 ml): 9 parts diluent (4.5 ml) in screw cap vials (17 x 65 mm) from which

serial dilutions were made using a separate pipette (1.0 ml, graduation interval 0.01 ml, to deliver to the tip) for each dilution which was aspirated into and expelled from the pipette 10 times. After mixing the virus and diluent, the vial was vigorously shaken 100 times. Progressing from highest to the lowest dilution, using one syringe for the entire operation, one egg per dilution was injected with 0.1 ml of inoculum.

Sterility tests:

Every original specimen used, as well as every dilution, was checked for bacterial contamination. Vials containing the diluted material, as well as inoculated nutrient agar slants,were kept in the incubator and inspected every day until the fourth day on which inoculated eggs were discarded.

RESULTS

The distribution of the virus in the tissues and extra-embryonic fluids is shown in Table II.

In 12 hour living and 24 hour dead embryos the titer of the virus was $10^{-5.6}$; and in 24 hour living embryos it was $10^{-5.8}$. A maximum titer of $10^{-7.1}$ was attained in 36 hour living embryos. In 36 hour dead embryos the titer was $10^{-4.5}$.

Amnionic fluid:

The titer of amnionic fluid in 12 hour living embryos was $10^{-3.6}$, $10^{-5.8}$ in 24 hour living embryos after inoculation and $10^{-4.3}$ in 24 hour dead embryos. In 36 hour living embryos the titer was $10^{-4.5}$ and in 36 hour dead embryos it was $10^{-4.3}$.

Chorio-allantoic membrane:

In 12 hour living embryos the titer was $10^{-5.5}$. At 24 hours the titer was $10^{-6.1}$ in dead embryos, and $10^{-6.8}$ in living embryos. It reached a $10^{-7.6}$ titer at 36 hours in living embryos and declined to $10^{-5.6}$ at the time of death.

Livers:

The livers showed variable titers. After 12 hours, it was $10^{-3.1}$. In dead embryos it was $10^{-1.5}$ after 24 hours. In living embryos it was $10^{-3.7}$ after 24 hours. The titer attained $10^{-3.5}$ in living embryos at 36 hours and it reached $10^{-2.1}$ in dead ones in the same length of time.

Yolk:

Viral activity in yolks was insignificant.

Virus concentration was higher in the chorio-allantoic membrane and allantoic fluid than in the amnionic fluid and livers at corresponding periods. In the yolk virus was scarcely detectable. Maximum titers of the chorio-allantoic membrane and allantoic fluid were obtained in 36-hour living embryos. The low titer obtained from the 36-hour dead embryos may be attributed to the thermolability of the virus. These embryos were kept at the incubation temperature from the time of death to approximately 8 hours before the collection of material.

The tendency of the virus to propagate most freely in the chorio-allantoic membrane can probably be attributed to the natural tendency of the virus to invade the respiratory organs of chickens. To clarify the point of thermolability of the virus, the experiment was repeated.

The original inoculum was retitrated and 25 eggs were inoculated. Chorio-allantoic membranes and allantoic fluids were harvested at 24, 36, and 46 hours from dead and living embryos. In order to observe the death of embryos and harvest materials between the 24th and 36th hours, the critical period as previously described, eggs were inoculated at 8 a.m. instead of at 8 p.m. as was done in the first experiment. It was not possible to harvest embryos at the time of death between the 36th and 48th hour. This period of time happened to be between 8 p.m. and 8 a.m.

Table I shows the number of inoculated embryos and their death rate in the first and second experiments.

In the first experiment it was noticed that the death rate of the embryos was high between the 24th and 36th hours after inoculation. In the second experiment, the majority of embryos died after the first 24 hours following inoculation. Eggs were candled every 2 hours from the 24th to the 36th hour and those with dead embryos were refrigerated until the 36th hour when pooled material was harvested from the embryos dead

Table I

Number of Inoculated Embryos and their Death Rate
in the First and Second Experiments

Exp.	Orig. Inoc.	Titer	No. of Embryo Dead per Hour			No. of Chick Embryos Harvested							
						12		24		32		48	
			24	36	48	Living	Dead	Living	Dead	Living	Dead	Living	Dead
1	All. fluid	$10^{-7.6}$	8	10	-	3	-	3	3	1	3	-	-
2	All. fluid	$10^{-6.9}$	12	6	1	-	-	3	3	3	3	-	1

by this time. Only one embryo survived which died sometime between the 36th and 48th hours. This embryo showed weak movement when candled at the 36th hour. Table III shows the results of the second experiment which were practically the same as in the first experiment. Virus concentration was higher in the chorio-allantoic membrane than in the allantoic fluid, the maximum titer being in the 36-hour living embryo.

Table II

Titer of Infectious Bronchitis Virus in Living and Dead Chick Embryo Tissues at Certain Intervals after Inoculation

Time after Inoculation	Material Tested	Titer: No. of Dead Embryos / No. of Inoculated Eggs								LD50*
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
	Undiluted Inoculum	5/5	5/5	5/5	5/5	5/5	5/5	4/5	2/5	7.6
12 hours, living	Allantoic fluid	5/5	5/5	5/5	5/5	5/5	1/5	0/5	0/5	5.6
	Amnionic fluid	5/5	5/5	5/5	1/5	0/5	0/5	-	-	3.6
	Chorio-allantoic memb.	-	-	5/5	5/5	4/5	2/5	2/5	0/5	6.0
	Liver	5/5	4/5	3/5	1/5	-	-	-	-	3.1
	Yolk	0/5	2/5	0/5	0/5	0/5	-	-	-	0.0
24 hours, dead	Allantoic fluid	5/5	5/5	5/5	5/5	5/5	1/5	0/5	0/5	5.6
	Amnionic fluid	-	-	5/5	5/5	4/5	0/5	-	-	5.3
	Chorio-allantoic memb.	-	-	-	5/5	5/5	3/5	0/5	0/5	6.1
	Liver	5/5	2/5	1/5	0/5	-	-	-	-	1.5
	Yolk	0/5	1/5	1/5	-	-	-	-	-	0.0
24 hours, living	Allantoic fluid	-	-	-	5/5	5/5	2/5	0/5	0/5	5.8
	Amnionic fluid	-	5/5	5/5	3/5	1/5	-	-	-	4.3
	Chorio-allantoic memb.	-	-	-	5/5	5/5	5/5	2/5	-	6.8
	Liver	-	5/5	4/5	2/5	0/5	0/5	-	-	3.7
	Yolk	1/5	1/5	-	-	-	-	-	-	0.0
36 hours, living	Allantoic fluid	-	-	-	5/5	5/5	4/5	3/5	1/5	7.1
	Amnionic fluid	5/5	5/5	5/5	5/5	0/5	0/5	0/5	-	4.5
	Chorio-allantoic memb.	-	-	-	5/5	5/5	5/5	5/5	1/5	7.6
	Liver	5/5	5/5	4/5	1/5	-	-	-	-	3.5
	Yolk	0/5	1/5	0/5	-	-	-	-	-	0.0
36 hours, dead	Allantoic fluid	5/5	5/5	5/5	3/5	2/5	0/5	0/5	-	4.5
	Amnionic fluid	5/5	5/5	3/5	1/5	0/5	0/5	0/5	-	3.2
	Chorio-allantoic memb.	-	-	-	5/5	5/5	1/5	0/5	0/5	5.6
	Liver	4/5	3/5	1/5	-	-	-	-	-	2.2
	Yolk	1/5	0/5	0/5	-	-	-	-	-	0.0

*** Negative Exponent of 10.**

Table III

Titer of Infectious Bronchitis in the Chorio-Allantoic Membrane and Allantoic Fluid of Living and Dead Embryos at Certain Intervals after Inoculation. Experiment 2.

Time after Inoculation	Material Tested	Titer: No. of Dead Embryos / No. of Inoculated Eggs								LD50*
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
	Undiluted inoculum	-	-	-	5/5	5/5	5/5	0/5	-	6.5
24 hours, living	Allantoic fluid	-	-	-	5/5	5/5	4/5	0/5	-	6.3
	Chorio-allantoic memb.	-	-	-	5/5	5/5	4/5	3/5	-	7.0
24 hours, dead	Allantoic fluid	-	-	-	5/5	5/5	1/5	0/5	-	5.6
	Chorio-allantoic memb.	-	-	-	5/5	5/5	4/5	1/5	-	6.5
32 hours, living	Allantoic fluid	-	-	-	5/5	5/5	3/5	0/5	-	6.1
	Chorio-allantoic memb.	-	-	-	5/5	5/5	5/5	4/5	-	7.3
32 hours, dead	Allantoic fluid	-	-	-	5/5	4/5	1/5	0/5	-	5.5
	Chorio-allantoic memb.	-	-	-	5/5	5/5	3/5	0/5	-	6.1
48 hours, dead	Allantoic fluid	-	-	-	5/5	5/5	0/5	0/5	-	5.5
	Chorio-allantoic memb.	-	-	-	5/5	5/5	1/5	0/5	-	5.6

* Negative Exponent of 10.

EFFECT OF DILUTED INOCULUM ON TITER INFECTIVE FOR EGGS

Working with influenza virus Henle and Chambers reported that when embryos were inoculated with a low concentration of virus the resulting titer of harvested material was higher than that obtained with an inoculum of a high virus concentration. This was confirmed by Miller.

Parallel to experiment 2, an additional lot of 25 eggs were inoculated to study the effect of the concentration of inoculum on the resulting titers of the chorio-allantoic membrane and of the allantoic fluid at the same time intervals as in experiment 2. A 10^{-3} dilution of the original inoculum in experiment 2 was used. The experiment did not indicate any advantage in using this low concentration of virus as the results obtained were, for the most part, nearly the same as those obtained in experiment 2 with the undiluted inoculum. See Table IV.

Table IV

Titer of Infectious Bronchitis Virus in Living and Dead Chick Embryos at Certain Intervals after Inoculation with a 10^{-3} Dilution of the Stock Virus. Experiment 3.

Time after Inoculation	Material Tested	Titer: No. of Dead Embryos / No. of Inoculated Eggs								LD50*
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
	Undiluted Inoculum	-	-	-	5/5	5/5	5/5	0/5	-	6.5
24 hours, living	Allantoic fluid	-	-	-	5/5	5/5	0/5	0/5	-	5.5
	Chorio-allantoic memb.	-	-	-	5/5	5/5	4/5	1/5	-	6.5
24 hours, dead	Allantoic fluid	-	-	-	5/5	5/5	1/5	0/5	-	5.6
	Chorio-allantoic memb.	-	-	-	5/5	5/5	3/5	0/5	-	6.1
32 hours, living	Allantoic fluid	-	-	-	5/5	5/5	2/5	0/5	-	5.8
	Chorio-allantoic memb.	-	-	-	5/5	5/5	5/5	0/5	-	6.5
32 hours, dead	Allantoic fluid	-	-	-	5/5	5/5	0/5	0/5	-	5.5
	Chorio-allantoic memb.	-	-	-	5/5	5/5	1/5	0/5	-	5.6
48 hours, dead	Allantoic fluid	-	-	-	4/5	5/5	0/5	0/5	-	5.4
	Chorio-allantoic memb.	-	-	-	5/5	5/5	0/5	0/5	-	5.5

Material used for correction of material represented above is 10^{-3} of the undiluted material.

* Negative Exponent of 10.

SUMMARY

1. The maximum activity of the infectious bronchitis virus in the different tissues and fluids of the chick embryo using the allantoic sac inoculation route was as follows, from the highest titer to the lowest:

a. Chorio-allantoic membrane

b. Allantoic fluid

c. Amnionic fluid

d. Livers

e. Yolk

2. The virus reached its maximum titer by the 36th hour after inoculation.

3. Fluids and tissues had a higher titer in living embryos than in dead embryos harvested at the same time.

4. The low titer of fluids and membranes from dead embryos that were held for as long as 8 hours after death may be attributed to the thermolability of the virus (incubating temperature 99 degrees F).

5. Inoculum with low virus concentration yielded results comparable to those obtained with the inoculum possessing high virus concentration.

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