THE EFFECT OF DIFFERENT ROUTES OF INOCULATION ON THE ADAPTATION OF INFECTIOUS BRONCHITIS VIRUS TO EMBRYONATING CHICKEN EGGS

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THE EFFECT OF DIFFERENT ROUTES OF INOCULATION ON THE ADAPTATION OF INFECTIOUS BRONCHITIS VIRUS TO EMBRYONATING CHICKEN EGGS

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REVIEW OF LITERATURE

A. Infectious Bronchitis

1. Characteristics of the Disease

Infectious bronchitis is a respiratory disease of chickens, which has, thus far, not been reported as infectious for other fowl.

Originally, the disease was considered to be confined to chicks,[39] but has since been shown to infect chickens of all ages.[16] The morbidity is high, the disease spreading with marked rapidity and sometimes infecting the greater part of a flock over a short period of time.[3] In chicks, the mortality may be as high as 80 or 90 per cent, but in birds over six weeks of age the mortality is negligible.[2,3,4,16,17,28,39] Infectious bronchitis presents a serious economic problem in view of the frequency with which it has been noted in chicks and the marked decrease in egg production which usually results when a laying flock becomes infected.[18]

The incubation period may vary from 1 day to 6[42] or 7 days.[5] The duration of the disease is relatively short,

recovery occurring usually within 10 days to 21 days.[5,18,42] In some cases, the disease may follow a protracted course. Van Roekel et al.[42] observed that age has no influence on the course of the disease, as no difference was noted between chicks and sexually mature birds.

2. Geographical Distribution

Infectious bronchitis was first reported and described in 1931 by Schalk and Hawn,[39] who had encountered it in North Dakota in 1930. By 1933 the disease had been reported in Kansas by Bushnell and Brandly,[8] in Massachusetts by Gibbs,[24] and in California by Beach.[3] It was soon found that the disease was nationwide in its distribution. Recently, infectious bronchitis has been recognized in England,[1] in the Netherlands[40] and in Canada.[6]

3. Etiological Agent

The causative agent of the disease is a virus which has been shown to be capable of passing through all grades of Berkefeld, preliminary Mandler and Seitz filters.[2,4,8,16,17,28,39]

The virus has been shown to be air-borne for a distance of five feet.[34]

a. Morphology.

By means of electron microscopy the virus particles were shown to have round heads with filamentous projections.[37,38] In the initial study the heads were observed to have a mean diameter of 90 millimicrons,[37] but improved technique has demonstrated a mean diameter of 70 millimicrons.[38]

b. Distribution in the body.

The virus has been found most abundantly in tissues and exudates of the respiratory tract, and has also been isolated from the liver, kidney tissues and blood.[8] According to Fabricant,[22] the virus can usually be isolated from chickens throughout the respiratory phase of the disease.

c. Effect of physical agents.

Beach and Schalm[2] demonstrated that the virus in tracheal exudate, when frozen, dried, and stored in the refrigerator for

180 days, could incite the disease. Virus stored in 50 per cent glycerin remained viable for 80 days.

Delaplane and Stuart[17] reported that egg-propagated virus was viable after storage in the freezing compartment of a refrigerator for 4-1/2 months but not for 5-1/2 months, as indicated by egg tests. It survived at room temperature for 5 to 7 days but not for 14 days, and at 50° C. for 15 minutes.

Cunningham and Stuart[10] found that infected allantoic fluid, stored at -25° C. and at -70° C., produced a higher virus titer than infected allantoic fluid stored at -10° C. Allantoic fluid when dried from the frozen state, in vacuo, and stored at 4° C. for 7 days showed a hundred-fold decrease in virus activity when restored to volume. Freezing and thawing produced no effect on virus activity.

Levine and Hofstad[34] showed that air-borne virus could be destroyed by ultraviolet irradiation but concluded that under ordinary field conditions such treatment would be of no practical value in controlling the disease.

d. Effect of chemical agents.

Cunningham and Stuart[9] studied the effects of certain chemical agents on an egg-adapted strain of the virus. Infected allantoic fluid was subjected to the action of each agent for a period of 3 minutes, then injected via the allantoic cavity into embryonating chicken eggs. Inactivation of the virus was indicated by survival of inoculated embryos.

The following reagents were found to inactivate the virus: phenol, 3 per cent and 1 per cent; liquor cresolis saponatus, 3 per cent and 1 per cent; tincture of metaphen, undiluted and 1 per cent; potassium permanganate, 1:1000 and 1:10,000; ethyl alcohol, 95, 70, 40 and 25 per cent; tincture of zephiran, 1:1000; Lugol's solution, 1 per cent; sodium hydroxide, 1:20; neoprontosil, 5 per cent, and formalin, 1 per cent.

Tincture of iodine, 0.01 per cent, and boric acid, 4 per cent, were without effect on the virus.

e. pH stability.

The pH stability of the virus was studied by Cunningham and Stuart.[11] For the first 60 days the virus was more stable in an acid medium, but from the 60th day to the 170th day it

showed greater stability in an alkaline medium. The virus remained active for 170 days in a phosphate buffer (.05 M) at pH 7.79, and in allantoic fluid at pH 7.80 for 100 days.

4. Symptoms

Sneezing, coughing, gasping and respiratory rales are the symptoms most characteristic of the disease. Nasal discharge and swollen sinuses may be present in chicks. Severely-affected birds may show depression, weakness and decrease in feed consumption.[2,3,16,18,39]

A marked decline in egg production is evident when laying flocks contract the disease.[2,16,18,28] Losses as high as one dollar per bird may result from decreased egg production and abnormal egg quality, both of which frequently persist for a considerable length of time. Egg production may drop to as low as 2 to 3 per cent, and, although it increases on recovery, preinfection levels of production are seldom regained. Eggs are often misshapen, rough and thin-shelled with watery albumen.[41]

5. Lesions

Gross lesions are found mostly in the lungs, the bronchi and the lower trachea. These lesions consist of congestion and edema of the lungs, and serous or mucous exudates in the trachea and bronchi, the latter sometimes containing caseous plugs. In some instances, the air sacs may appear cloudy and contain accumulations of caseous, yellow exudate. In chicks, pericarditis, and coating of the nasal mucosa with a heavy, sometimes purulent, mucoid exudate may be found. Splenic changes are not well defined.[2,4,7,16,26,39]

The histopathologic lesions, as reported by Hofstad,[28] include thickening of the tracheal mucous membrane and submucosa, due primarily to edema and diffuse, leucocytic infiltration, with little evidence of tracheal and bronchial desquamation and complete absence of gross hemorrhage. No inclusion bodies were noted and no significant changes were observed in sections of liver, spleen and kidney. Beaudette[6] stated that infectious bronchitis produces no changes in nervous tissue.

6. Transmission and Immunity Studies

The disease is spread through direct exposure of susceptible chickens to infected chickens or to "carrier" birds. Contaminated equipment or brooder houses may serve as indirect foci of infection. It is considered that infection is introduced through purchase of infected chicks or carrier birds. Delaplane[18] has suggested that other birds or animals may serve as carriers of the infection.

The disease has been artificially induced by inoculation of respiratory exudates intranasally, intratracheally and intraperitoneally.[4,8,16,39] Symptoms usually develop within 48 hours in birds inoculated by the above routes. Intratracheal inoculation has produced the disease as early as 18 hours. The disease has also been produced, although not so quickly, by inoculation via the larynx, air sacs, air spaces of bones and the thymus.[16] Subcutaneous and intramuscular inoculations were either non-infective or produced the disease after long incubation.

Birds recovered from the disease are generally considered to be refractory to subsequent infection[1,2,4,16,18,29,31] although Van Roekel et al.[42] reported a few instances in which

birds that were supposedly immune contracted the disease. Some recovered birds may continue as carriers of the disease.[16,18,31] Although Hofstad[31] was unable to demonstrate the persistence of the carrier state for as long as 80 days, Delaplane and Stuart[16] reported one instance in which a bird remained a carrier for as long as 8 weeks.

Serum from recovered birds is capable of neutralizing the virus.[1,2,4,16] Jungherr and Terrell[33] found that neutralizing antibodies were present in eggs laid by hens recovered from the disease, in the yolk through the 11th day of embryonation and in the tissues and serum after the 16th day. Chicks hatched from such eggs maintained high antibody levels for 2 weeks after hatching and lost them by the end of the 4th week. In contrast to this, Hofstad and Kenzy[32] were able to produce the disease in 4, 6, 7 and 10 weeks-old chicks hatched from eggs laid by immune hens. These observations would indicate that natural passive immunity is not sufficient to protect the chicks against a challenge dose of the virus.

No cross immunity exists with laryngotracheitis, Newcastle disease or fowl pox.[1,2,4,16]

7. Control Measures and Immunization Studies

No medicinal agents have been found that are of any value in preventing or controlling the disease.[18] Thorough cleansing and disinfection of houses and equipment after an outbreak, prevention of contact between recovered and susceptible birds, and purchase of stock only from "clean" sources are precautionary measures suggested by Beach.[3]

Formalized virus has failed to incite immunity. Inoculation of the mucous membrane of the cloaca and the bursa of Fabricius produced immunity but not sufficiently soon to prevent infection via the respiratory tract.[16] Beach[3] states that applying virus to the cloaca can produce infection of the respiratory organs and therefore is not applicable as a means of immunization.

In New England, a program of immunization has been adopted whereby infectious bronchitis is artificially induced in young birds at an age when the disease produces the least objectionable results, i.e., during the growing stage, beyond the period of high mortality but before the beginning of egg production. Two to 5 per cent of a flock of healthy birds not under 6 to 8 weeks and not over 16 weeks of age are inoculated

intratracheally with a chicken-propagated strain of the virus. The disease is spread by the inoculated birds to the remainder of the flock. The best results have been obtained with chicks from 10 to 14 weeks old. Little or no retardation in growth has been observed. In an occasional flock, insufficient immunity has been produced to prevent a subsequent natural outbreak.[41,42] In spite of the relative success of this method in providing protection against the disease during the laying season, Delaplane[19] recommended the exposure of growing pullets to infectious bronchitis only where poultry farms are in close proximity as in New England.

Egg-propagated virus has been used to produce the disease in chicks 6 to 8 weeks of age. As with chicken-propagated virus, this procedure has afforded protection to pullets during the laying season.[5,18] Beaudette[7] suggested that the duration of immunity produced by embryo-modified virus would not be so lasting as that produced by a fully virulent strain.

8. Diagnosis

The virus of infectious bronchitis does not cause agglutination of chicken red blood cells, and it does not inhibit

hemagglutination by Newcastle disease virus.[1,30] These phenomena have served to differentiate the two viruses.

The clinical history, symptoms and lesions found at autopsy are sometimes sufficient for diagnosis of infectious bronchitis.[2,16,18,28]

Serum neutralization tests in embryonating chicken eggs are of value in diagnosis of the disease if used not earlier than 3 weeks following exposure to the virus.[23,36] According to Cunningham[14] the serum neutralization titers of normal birds not previously exposed to infectious bronchitis would not be expected to exceed 36 neutralizing doses. Fabricant[23] concluded that a titer of 100 neutralizing doses or greater is diagnostic for the disease. Fabricant[21] and Van Roekel et al.[42] have used bird inoculation tests in combination with serum neutralization tests to substantiate diagnosis by the latter method. Susceptible birds inoculated intratracheally with tracheal exudates from suspected birds will show symptoms of the disease within 18 to 36 hours if the virus is present.[21]

Diagnosis is frequently based on characteristic lesions produced in embryonating chicken eggs when respiratory tissue suspensions or exudates from suspected birds are inoculated via

the allantoic cavity.[20,21] Hitchner, Reising and Van Roekel[26] suggested that diagnosis of infectious bronchitis by this method should be substantiated by the absence of hemagglutination by the allantoic fluid.

B. Cultivation of the Virus in Embryonating Chicken Eggs

Beaudette and Hudson[4] were the first to study the cultivation of the virus in embryonating chicken eggs. Inoculation of the virus on the chorioallantoic membrane proved to be relatively nonlethal to embryos in the first few passages. In subsequent passages the virus produced death in the majority of embryos. Gross lesions included dwarfing of the embryos to about one-half normal weight, and congestion of the liver. The yolk fluid was quite solidified, blood vessels were injected, residual albumen was watery and the chorioallantoic membrane appeared thinner than normal and was adherent to the inner shell membrane. No pock or plaque lesions were observed, but occasionally the membrane showed a few turbid areas.

Delaplane and Stuart[17] confirmed the observations of Beaudette and Hudson[4] with regard to the dwarfing of embryos and the thinness of the chorioallantoic membrane after several

passages of the virus via the chorioallantoic membrane. The virus increased in virulence for embryos with each succeeding passage until all embryos were killed at the 70th passage. Correspondingly the virus decreased in virulence for chicks so that saline washings of the chorioallantoic membranes from the 89th passage failed to incite the disease. Gross lesions consisted of whitish foci on the liver, congestion and swelling of the kidneys and occasional hemorrhages on the embryo's skin. Whitish, opaque circular lesions were present on the chorioallantoic membrane at the point of inoculation. Beaudette[5] suggested that Delaplane and Stuart must have used parentally-immune chicks since they had been able to produce the disease in older chickens, i.e., birds old enough to have lost any parental immunity which they might have had at hatching.

Delaplane[20] reported that the allantoic cavity route of inoculation was superior to the chorioallantoic membrane route for isolation of field strains of the virus, inasmuch as inoculation by the former route produced dwarfing on the first passage. He suggested the use of streptomycin to eliminate bacterial contamination of respiratory exudates, thus permitting their use for isolation of the virus in embryonating chicken eggs.

Asplin,[1] studying the English strain of the virus, noted few deaths in early passages. After the 14th passage the majority of embryos died within 2 to 7 days. Dwarfing was the only change observed in the embryos. He confirmed previous observations in the United States[4,17] that the virus produced a milder effect in chicks with successive passages in eggs.

Fabricant[21] confirmed the observations of Delaplane[20] that lesions were produced more rapidly when the virus was inoculated via the allantoic cavity than by the chorioallantoic membrane, and the findings of Beaudette and Hudson[4] that the yolk was of a thicker consistency than normal. Fabricant observed that, as in previous studies,[4,17] embryos were dwarfed and the chorioallantoic membrane appeared thinner than normal and adherent to the inner shell membrane. In addition, he noted a tendency for the embryos to be tightly curled in a ball-like form due to a bending of the longitudinal axis of the embryo around its ventral surface. Curling usually preceded dwarfing and sometimes occurred in infected embryos of normal size. Curling of the embryo was associated with a decrease in size of the amnion and a decrease in the amount of amniotic fluid. Occasionally the amnion was thickened, cloudy and tightly

adherent to the embryo. Curling was considered to be the significant lesion produced by the virus and was sometimes the only change evident in the first three passages. On this basis, virus isolation was successful in 92 per cent of cases with a typical clinical history of infectious bronchitis. Of 116 virus isolations, 46 per cent were diagnosed on the first passage, 33 per cent on the second passage, 20 per cent on the third passage and 1 per cent on the fourth passage.

Loomis, Cunningham, Gray and Thorp[35] studied the pathology of chicken embryos inoculated by the allantoic cavity route with a chicken-propagated strain of the virus. Their observations confirmed those of Fabricant[21] with regard to gross lesions produced by the virus in embryos. In addition, they noted that living embryos were sluggish in their movements, curled embryos had wry necks with lateral curvature, and deformed feet curled over the head. Feathers were drier than normal and immature in development. Jaundice was observed in about one-third of the embryos and dermal petechiae were occasionally present. In about 25 per cent of the embryos the cloaca was distended with white, fat-like droplets. Bones were retarded in development and softer than normal. Leg bones

and tarsal joints were deformed. Incomplete closure of the abdomen, due to retardation of development of the body walls, was observed. Proliferation of islands of tissue in the chorio-allantoic membrane, described by Delaplane and Stuart,[17] was not observed. Livers were abnormally dark and showed hemorrhagic and necrotic areas. Kidneys were swollen and edematous with some necrotic foci. Hearts and lungs were markedly smaller than normal, the latter being abnormally pale and soft in consistency. Spleens were twice normal size. Microscopic examination revealed edema of the chorioallantoic and amniotic membranes. Proliferation of mesodermal and ectodermal cells was observed. Evidence of pneumonia, with serious exudation and granulocytic and lymphocytic infiltration was found in the lungs. Perivascular "cuffing," hepatic hemorrhage, necrosis and abscess formation were observed in the liver. The kidneys showed interstitial nephritis and necrosis.

Hitchner, Reising and Van Roekel[27] observed that a strain of infectious bronchitis virus, which has apparently lost its identify as such and is now designated as the B1 strain of Newcastle disease virus,[26] caused curling, dwarfing and dryness of embryos, associated with tight adherence of the amnion to

the embryo, a marked decrease in amniotic fluid, an increase in the amount of allantoic fluid and thickening of the yolk. This evidence seems to refute Fabricant's observation[21] that curling of embryos is pathognomonic for infectious bronchitis. These workers suggested that curling and dwarfing may not be due primarily to growth of virus in the tissue, but to a secondary change due to dehydration as a result of a flow of fluids from the amniotic sac, embryo and yolk sac into the allantoic cavity. A similar explanation was expressed by Beaudette[7] who suggested that curling of the embryo may be caused by a shrinking of the amniotic membrane due to loss of fluid.

The properties of egg-adapted virus have been studied extensively. A strain of the virus which has been completely egg-adapted will kill all embryos within 48 hours.[10] Dwarfing of the embryos and congestion and swelling of the kidneys are usually the only lesions observed.[17] Groupe[25] reported the presence of a thermostable interfering material in allantoic fluid from infected dead embryos which had been stored at 36° C. for 24 hours after death. This material produced a markedly slower death rate in embryos.

When an egg-adapted strain was inoculated into embryonating chicken eggs via the allantoic cavity, the highest concentration of the virus was found in the chorioallantoic membrane, followed in decreasing order by allantoic fluid, amniotic fluid, and liver. Yolk was innocuous.[13]

Egg-adapted virus will not incite the disease in chicks but can be neutralized by immune serum.[33]

MATERIALS AND METHODS

This study was undertaken with the purpose of determining the effect of different routes of inoculation on the adaptation of infectious bronchitis virus to embryonating chicken eggs in the hope that it might contribute to general knowledge concerning the virus.

A. Inoculum Used for the First Passage of the Virus

The strain of infectious bronchitis virus used, designated as Lot 290, was supplied by Dr. Henry Van Roekel, Department of Veterinary Science, University of Massachusetts. The virus was received as a saline suspension of tracheal washings from infected chickens. This was a sample of a chicken-propagated strain which had not been cultivated in embryonating chicken eggs. In order to render the suspension bacteria free, penicillin and streptomycin were added in amounts of 10,000 units each, per milliliter of suspension. The suspension was centrifuged in a clinical centrifuge (International Equipment Co.) for ten minutes and the supernatant fluid was used for inoculum. For eggs inoculated via the allantoic cavity, chorioallantoic

membrane and yolk sac routes, 0.1 cc. of the undiluted virus suspension was injected per egg. The suspension was diluted with saline, one in five, for inoculation via the amniotic cavity, and 0.1 cc. was injected per egg. The dilution of the virus suspension was necessitated by a limited amount of the original material. Also, dilution was considered a means of avoiding the possibility of injecting an overwhelming dose of the virus into the amniotic cavity.

B. Routes of Inoculation

Single Comb White Leghorn embryos were used throughout the study. Eggs were inoculated via four routes: (1) allantoic cavity, (2) chorioallantoic membrane, (3) amniotic cavity and (4) yolk. With the exception of the first passage, for which a suspension of tracheal washings was used, inocula used for all subsequent passages corresponded to the route of inoculation, i.e., allantoic fluid was injected into the allantoic cavity, amniotic fluid into the amniotic cavity, etc. Seven serial passages of the virus were carried out by each route.

C. Techniques of Egg Inoculation

Asepsis was maintained throughout all procedures. Teasing needles were flamed to a red heat then cooled for about thirty seconds before use. Fine, curve-tipped forceps were kept in a Coplin jar of ethyl alcohol and flamed just before use. Syringes and needles were sterilized by autoclaving. Vials of inocula were kept in cracked ice in "Thermos" laboratory vessels during the process of inoculation in order to minimize thermal inactivation of the virus.

An inoculum of 0.1 cc. per egg was used for the first passage of the virus due to the limited amount of the original virus suspension. For all subsequent passages, an inoculum of 0.2 cc. per egg was used since that amount had been employed in previous studies of the virus.[11,35] A sterility test was conducted on a sample of each inoculum. Each sample was seeded on a nutrient agar slant (Difco), which was then incubated at 99° F. for at least 48 hours. Thirty eggs were usually injected by each route of inoculation. From those, ten eggs, selected at random after inoculation, were marked for subsequent harvesting. Where less than thirty eggs were used, as occurred in the first passage, a corresponding proportion of

"harvest" eggs was marked. Uninoculated embryos were used as controls, a range of 3 to 12 being used per route of inoculation per passage.

All incubation was at 99° F. (wet bulb 86-88° F.) in an electric, forced-draft incubator (Jamesway, Model 252), in which the eggs were turned automatically every two hours.

1. Allantoic Cavity Inoculation

Thirty ten-day embryos were inoculated for each passage. To determine the site for injection into the allantoic cavity, eggs were transilluminated for selection of an area of the chorioallantoic membrane, on the side opposite the embryo, about 3 mm. below the air space and free from large blood vessels. This point was marked with a pencil. A similar mark was made on the shell over the air cell. By means of a small drill (Handee Speed Drill, No. 10, Chicago Wheel and Mfg. Co.), attached to the chuck of an electric motor (Deluxe Handee Model, Chicago Wheel and Mfg. Co.), a hole, just large enought to allow passage of the inoculating needle, was drilled at each pencil-mark, without damaging the underlying shell membranes. With the eggs supported, air cell uppermost, in

cardboard flats, tincture of metaphen was applied to the holes and allowed to dry. With a teasing needle, the outer shell membrane over the air cell was pierced to serve as an air vent, permitting equalization of pressure produced by injection of inoculum, and preventing leakage of the inoculum and embryonic fluid from the site of injection. A 27-gauge, 1/2-inch needle, attached to a B-D Yale, 1-cc. capacity tuberculin syringe, was inserted, to a depth of about 1/4 inch, through the hole in the side of the egg, and the inoculum was injected. After inoculation, the holes in the shell were sealed with melted paraffin. The eggs were then returned to the incubator.

2. Chorioallantoic Membrane Inoculation

Thirty ten-day embryos were used for each passage of the virus. The "artificial air cell" method was used, to make sure that all the inoculum was deposited on the chorioallantoic membrane.

Eggs were transilluminated to determine the location of the embryo. A pencil-line was made on the shell directly over the embryo, parallel to the long axis of the egg and equidistant from the ends of the egg. A mark was made on the

shell over the air space. Using a small carborundum disc (Rubber Cutting Disc, No. 25, Chicago Wheel and Mfg. Co.) attached to the chuck of an electric motor, a groove, 1 cm. long and about 1 mm. wide, was made along the pencil-line, sufficiently deep to penetrate the shell without piercing the shell membranes. A small hole was drilled through the shell over the air space to provide an air vent. Tincture of metaphen was applied to the groove cut by the disc, and to the hole over the air space, and allowed to dry. The exposed outer shell membrane over the air cell was punctured with a teasing needle. An egg candler with its opening at one side was placed on the bench and the room was darkened. While held in the hand, with the long axis in the horizontal plane and with groove uppermost, the egg was transilluminated. The bend of an angularly-tipped teasing needle was placed in the groove. While slight downward pressure was applied on the elbow of the bend, the point of the needle was pressed gently downward sufficiently to rupture the shell membranes without piercing the chorioallantoic membrane. When the pressure thus applied was insufficient to cause the chorioallantoic membrane to drop from the shell membranes, as determined by transillumination, further

pressure on the elbow of the teasing needle usually sufficed to produce this desired result, thus creating an artificial air cell on the side of the egg. In a few instances, it was necessary to apply gentle suction, by means of a medicine dropper bulb, at the hole over the normal air cell. With the egg supported, groove uppermost, in an egg flat, a 27-gauge, 1/2-inch needle, fitted to a B-D Yale, 1-cc. capacity tuberculin syringe, was inserted diagonally, for a length of about 1/4 inch, through the intact shell membranes over the artificial air cell, and the inoculum was deposited on the chorioallantoic membrane. In this way, the rupture made previously in the shell membranes served as an air vent, preventing leakage of the inoculum at the site of injection. Melted paraffin was applied to the groove and to the hole over the normal air space. After inoculation, the eggs were returned to the incubator, supported in egg flats in the same horizontal position in which they were inoculated, to prevent any disturbance in the position of the artificial air cell. After not less than 24 hours in this position, the eggs were placed in the racks of the incubator.

3. Amniotic Cavity Inoculation

Thirty eight-day embryos were inoculated for each passage of the virus, with the exception of the first passage for which twenty eggs were inoculated. Eggs were transilluminated and the location of the embryo was marked on each shell. A circle was drawn parallel to and about 5 mm. above the base of the air cell. By means of a small, carborundum disc, the shell was cut through at the circle, without piercing the outer shell membrane. Tincture of metaphen was not applied to the groove cut by the disc, as it was considered possible that diffusion of the disinfectant along the inner shell membrane may have contributed to the death of embryos in previous experiments. With the egg supported blunt end uppermost, the cap of shell over the air cell was removed with sterile forceps, care being taken so that fragments of shell did not fall on the inner shell membrane. A drop of sterile physiological saline was placed on the inner shell membrane directly over the amniotic cavity, the position of which had been determined by candling. The point of the 27-gauge needle, fitted to the syringe used to dispense the saline, was placed on the inner shell membrane, below the drop of saline, at an angle of 45^{o}. Gentle pressure was

applied downward and backwards so as to produce a small slit in the membrane without damaging the chorioallantoic membrane. The saline drop then passed through the slit, flowed between the inner shell membrane and the chorioallantoic membrane and caused their separation. With the point of the needle, a small portion of the inner shell membrane was retracted very carefully to give complete visibility of the route for amniotic cavity inoculation. A 27-gauge, 3/4-inch needle, fitted to a B-D Yale, 1-cc. capacity, tuberculin syringe, was inserted, by a short, sharp thrust, through the chorioallantoic membrane, in an area free of large blood vessels, and through the amnion without piercing the embryo. The presence of the needle within the amniotic cavity was confirmed by changes in the location of the embryo corresponding to sidewise movements of the needle. After injection of the inoculum, each egg was capped over the air cell with a sterile souffle cup from which the rim had been removed. The junction of the egg and the cap was sealed and the entire paper cup was covered with melted paraffin to prevent evaporation. This procedure provided an advantage over the use of scotch tape or adhesive tape since these materials

cannot be sterilized. The eggs were maintained with long axis vertical, in egg flats, throughout the period of incubation, as previous experiments showed that this treatment minimized mortality of embryos.

4. Yolk Sac Inoculation

Thirty six-day embryos were used for each passage of the virus, with the exception of the first passage, for which ten embryos were inoculated. Each egg was transilluminated with the long axis in a vertical plane, and the yolk sac was located. On the side of the egg opposite the embryo, a pencil-mark was made over the yolk sac about half way from the small end of the egg to the apex of the curvature of the shell. A similar mark was made on the shell over the air space. A small hole was drilled through the shell at each mark without piercing the shell membrane. Tincture of metaphen was applied over the holes and allowed to dry. The exposed outer shell membrane over the air cell was pierced with a teasing needle. With the long axis of the egg in a vertical position, a 20-gauge, 1-inch needle fitted to a B-D Yale, 1-cc. capacity tuberculin syringe, was inserted, horizontally, to a depth of

about 1/2 inch, through the hole in the side of the egg, and the inoculum was injected. The holes in the egg were sealed with melted paraffin and the eggs were returned to the incubator. For not less than 24 hours after inoculation, the eggs were incubated in flats in the same position in which they were inoculated, after which time they were placed in the racks of the incubator.

D. Harvest and Preparation of Material for Subsequent Egg Passage

On the third post-inoculation day, from eggs previously marked for harvesting, five living embryos, per route of inoculation, were selected for collection of infected material to be used as inocula for the next passage of the virus. Before harvesting, the eggs were chilled in the refrigerator for not less than four hours. Chilling causes contraction of the yolk and constriction of the blood vessels and thereby facilitates collection of the fluids. Separate sterile instruments were used for the collection of each material. Allantoic fluid, chorioallantoic membranes, amniotic fluid and yolk were harvested from eggs inoculated by these respective routes. Materials harvested from each route were pooled in sterile, 30-cc. capacity,

screw-cap vials, which were kept in "Thermos" laboratory vessels full of cracked ice, to minimize thermal inactivation on the virus.

A small sample was removed from each pool for a sterility test performed in a manner similar to sterility tests on inocula.

The materials were stored immediately after collection at -45° C. until ready to use as inocula for the next passage.

After collection of the desired fluid or membrane, the inner shell membrane was removed with forceps from the upper pole of the chorioallantoic membrane, which was then ruptured and the egg was inverted to deposit the contents in a Petri dish. The embryo and chorioallantoic membrane were examined for gross lesions typical of infection with infectious bronchitis virus.

1. Collection of Allantoic Fluid

Fluid was collected from the allantoic cavity from eggs inoculated via the allantoic cavity. With the eggs supported vertically, blunt end uppermost, tincture of metaphen was applied to the shell over the air cell, and allowed to dry. The shell

in this region was then cracked and removed with forceps to within 5 mm. of the base of the air cell. Care was exercised so that fragments of shell did not fall on the inner shell membrane. A 20-gauge, 1-inch needle, attached to a 5-cc. Luer syringe, was inserted through the inner-shell membrane and chorioallantoic membrane into the allantoic cavity, and 5 cc. of allantoic fluid was aspirated. Pooled allantoic fluid harvested from the five eggs per passage was stored at -45° C. until ready for the next passage. Just prior to inoculation, the fluid was thawed at room temperature, then centrifuged in the clinical centrifuge for five to ten minutes, in order to remove the precipitate formed by freezing and thawing.[10] The supernatant fluid was used for inoculation.

2. Collection of Chorioallantoic Membranes

Chorioallantoic membranes were harvested from eggs inoculated via the chorioallantoic membrane. The eggs were supported with the long axis in a vertical plane, air cell uppermost. The shell over the air cell was painted with tincture of metaphen, allowed to dry, and then was cracked and removed with forceps to within 5 mm. of the base of the air cell. The

inner shell membrane was removed from the upper pole of the chorioallantoic membrane, and the chorioallantois was ruptured. The egg was inverted and the embryo, yolk sac and extra-embryonic fluids were deposited in a Petri dish. In most instances, the chorioallantoic membrane adhered to the inner shell membrane and remained in the shell. The albumen was removed with forceps and the chorioallantoic membrane was separated from the inner shell membrane. The chorioallantoic membrane was "cleaned" of extraneous fluids by holding it with one pair of forceps and "stripping" it with another pair, before being pooled in a screw-cap vial with other harvested membranes. The membranes were prepared for inoculation by grinding them in a mortar with a pestle and sterile sand. The ground membranes and sand were suspended in Difco nutrient broth, 3 cc. of broth being added for each membrane. The suspension was centrifuged for five to ten minutes in the clinical centrifuge to remove sand and gross tissue particles from the suspension. A small sample of the supernatant was taken for a sterility test, before the suspension was stored at -45^{o} C. Just before the inoculation for the next passage, the material was thawed at room temperature.

3. Collection of Amniotic Fluid

Amniotic fluid was collected from eggs inoculated via the amniotic cavity. The eggs were supported, air cell uppermost, in an egg flat, and the paper caps were removed just previous to harvesting. The shell around the air cell was painted with tincture of metaphen and allowed to dry. The allantoic fluid was collected, in the manner described above, and discarded. This was done to prevent mixture of the allantoic fluid with the amniotic fluid, while the latter was being collected. The inner shell membrane was removed from the upper pole of the chorioallantoic membrane with forceps, and the chorioallantoic membrane was ruptured and reflected. The amnion was grasped with forceps and a portion pulled upward to form a "tent." A 20-gauge, 1-inch needle, attached to a B-D Yale, 2-cc. capacity tuberculin syringe, was inserted through the amnion and the amniotic fluid was aspirated. A smaller capacity syringe than that used for collecting allantoic fluid was found to be more effective for harvesting amniotic fluid, as less suction was applied by the smaller syringe, thus preventing the needle from becoming clogged with feathers and from drawing the embryo against the lumen.

Before inoculation for the next passage of the virus, the amniotic fluid was removed from the freezer, thawed at room temperature, then centrifuged for five to ten minutes in the clinical centrifuge. The supernatant fluid was used as inoculum.

4. Collection of Yolk

With the egg supported air cell uppermost, tincture of metaphen was applied to the shell over the air cell. The shell in that region was then cracked and broken away, with forceps, to within 5 mm. of the base of the air cell. The exposed inner shell membrane was removed and the chorioallantoic membrane reflected. An 18-gauge, 1-inch needle attached to a 5-cc. capacity Luer syringe was inserted vertically into the yolk. About 5 cc. of yolk was drawn into the syringe and expelled into a large sterile test-tube. Test tubes proved more desirable for storing yolk, since they did not crack as did the flat-bottom vials when the yolk was frozen.

Yolk was thawed at room temperature before inoculation. No centrifugation was deemed necessary, inasmuch as the thawed yolk material appeared to be homogeneous.

E. Examination of Embryos and Chorioallantoic Membranes

Eggs were candled twice daily. Embryos dying within 24 hours after inoculation were discarded and were not included in the calculation of embryo mortality rates, as death was considered to be due to trauma or other non-specific causes. Thereafter, embryos which died were examined for gross pathologic alterations typical of infection with the virus. On the seventh post-inoculation day, all surviving and control embryos of the same age were examined and compared. The shell over the air cell was cracked and removed with forceps, the chorioallantois was ruptured, and the contents of the egg were deposited in a Petri dish. Inoculated embryos were examined for evidence of gross lesions considered typical of the action of infectious bronchitis virus. Curling and dwarfing of the embryo and distension of the cloaca with fat-like droplets were considered to be the significant lesions produced by the virus. The chorioallantoic membranes were examined for evidence of adherence to the shell membrane, abnormal thickness and the presence of whitish, opaque lesions.

RESULTS AND DISCUSSION

The effect of the virus on embryos inoculated by the allantoic cavity route is given in Table I. The mortality showed a tendency to increase gradually from 30 per cent in the first passage to a peak of 95 per cent in the sixth passage. A drop to a mortality of 85 per cent in the seventh passage was observed, but the difference between these last two passages is probably not significant inasmuch as one less death in the sixth and one more in the seventh passage would have given a mortality of 90 per cent in both passages. No explanation can be offered for the drop in mortality in the third and fourth passages. The majority of surviving embryos in all passages, when examined on the seventh postinoculation day, showed the gross lesions characteristic of infection with the virus. Marked curling and dwarfing associated with distension of the cloaca with fat-like droplets were the most significant lesions noted and these occurred as early as the third postinoculation day in all passages.

The results of serial passages of the virus by amniotic inoculation are shown in Table II. Inoculation of the virus by

TABLE I

EMBRYO MORTALITY RATES FOLLOWING INOCULATION VIA THE ALLANTOIC CAVITY

	Serial Passages						
	1	2	3	4	5	6	7
Number of embryos inoculated*	20	20	17	20	20	20	20
Number of surviving embryos	14	5	10	8	5	1	3
Mortality rate: No. of dead embryos / No. inoculated	6/20	15/20	7/17	12/20	15/20	19/20	17/20
Per cent mortality	30.0	75.0	41.2	60.0	75.0	95.0	85.0

* Number inoculated minus number dying due to trauma and other non-specific causes.

TABLE II

EMBRYO MORTALITY RATES FOLLOWING INOCULATION VIA THE AMNIOTIC CAVITY

	Serial Passages						
	1	2	3	4	5	6	7
Number of embryos inoculated*	12	17	19	19	16	13	18
Number of surviving embryos	4	1	3	3	3	2	4
Mortality rate: No. of dead embryos / No. inoculated	8/12	16/17	16/19	16/19	13/16	11/13	14/18
Per cent mortality	66.7	94.1	84.2	84.2	81.3	84.6	77.8

* Number inoculated minus number dying due to trauma and other non-specific causes.

this route produced a consistently higher mortality than by any of the other routes of inoculation compared in this study. In all passages, surviving embryos, examined on the seventh post-inoculation day, showed characteristic lesions. Dwarfing occurred to the same extent as in embryos inoculated via the allantoic cavity, but did not occur on the third postinoculation day until the third passage. The amniotic fluid in all harvested embryos was markedly decreased in amount compared with that found in uninoculated embryos.

Inoculation via the amniotic cavity exposes the inner epithelial lining of the amnion, the epithelium of the lungs and the epidermal epithelium of the chicken embryo to the virus, since these tissues are in direct contact with the amniotic fluid. Swallowing movements by the embryo, beginning on the ninth day, cause the amniotic fluid to enter the trachea and gastro-intestinal tract. These factors may account for the high mortality resulting from inoculation of the virus by this route.

Table III shows the embryo mortality produced by inoculation of the virus on the chorioallantoic membrane. The high mortality in the first passage is probably not significant in view

TABLE III

EMBRYO MORTALITY RATES FOLLOWING INOCULATION VIA THE CHORIOALLANTOIC MEMBRANE

	Serial Passages						
	1	2	3	4	5	6	7
Number of embryos inoculated*	6	18	17	20	17	19	20
Number of surviving embryos	3	9	7	7	9	10	9
Mortality rate: No. of dead embryos / No. inoculated	3/6	9/18	10/17	13/20	8/17	9/19	11/20
Per cent mortality	50.0	50.0	58.8	65.0	47.1	47.4	55.0

* Number inoculated minus number dying due to trauma and other non-specific causes.

of the small number of inoculated embryos on which the calculation was based, but it is nevertheless in keeping with the general trend observed in subsequent passages. The highest mortality, 65 per cent, occurred in the fourth passage. Although the per cent mortality showed fluctuations from passage to passage, there seems to have been a tendency for it to remain in the range of 50 to 55 per cent. Embryos showed characteristic lesions in all passages. Dwarfing was neither so pronounced nor so frequently observed as in embryos inoculated by the allantoic and amniotic cavity routes.

Table IV shows the effect of serial passages of the virus in the yolk. The high mortality observed in the first passage cannot be considered significant in view of the small number of embryos used. A mortality of 35 per cent in the fifth passage showed a considerable variation from results obtained in other passages. Within one hour after inoculation by the yolk route in the fifth passage, it was necessary to transfer the eggs to a bacteriological incubator room to permit cleaning of the egg incubator. The low humidity of the bacteriological incubator associated with handling of the eggs, which occurred in moving them, may have been a contributing factor to death of the embryos.

TABLE IV

EMBRYO MORTALITY RATES FOLLOWING INOCULATION VIA THE YOLK

	Serial Passages						
	1	2	3	4	5	6	7
Number of embryos inoculated*	6	20	20	20	20	20	20
Number of surviving embryos	3	18	19	18	13	19	20
Mortality rate: No. of dead embryos / No. inoculated	3/5	2/20	1/20	2/20	7/20	1/20	0/20
Per cent mortality	60.0	10.0	5.0	10.0	35.0	5.0	0.0

* Number inoculated minus number dying due to trauma and other non-specific causes.

Eggs inoculated via the chorioallantoic membrane were also moved but no marked increase in mortality resulted. The more advanced development of embryos inoculated by this route may have enabled them to withstand the adverse conditions more readily than the eggs in an earlier stage of embryonation used for yolk inoculation. It seems doubtful that the mortality which occurred in the majority of passages by the yolk route is significant. No specific changes were observed in embryos that died or in living embryos examined on the seventh postinoculation day of each passage. In a previous experiment, chicks were allowed to hatch from eggs obtained from the same source as those used for egg inoculation. Since intratracheal inoculation of the virus failed to produce bronchitis in these chicks, it seems likely that the eggs had been laid by immune hens, in which case, neutralizing antibodies would be present in the yolk through the eleventh day of embryonation.[33] Serum neutralization tests, performed on birds from the hatchery supplying the eggs, indicated that the flock had been previously infected with infectious bronchitis.[15] Neutralization of inoculated virus by antibodies in the yolk could account for the absence of significant mortality and of characteristic

lesions in the embryos in this study. This evidence seems to confirm the findings of Jungherr and Terrell,[33] i.e., that neutralizing antibodies are present in the yolk of eggs laid by hens recovered from the disease.

No whitish, opaque lesions were observed on the chorioallantoic membranes in eggs inoculated by any of the four routes, although in a number of instances, the membranes, upon removal from the shell, appeared edematous and thicker than normal.

Although amniotic cavity inoculation apparently produced earlier adaptation of the virus to eggs than did allantoic cavity inoculation, the former route would provide no advantage over the latter for the isolation of field strains of the virus. Inoculation via the allantoic cavity is less time-consuming and produced lesions, typical of infection with the virus, as early as the third postinoculation day of the first passage. This substantiates the observations of Delaplane[20] who considered allantoic cavity inoculation superior for isolation of field strains of the virus since dwarfing of embryos occurred on the first passage.

A comparative study of mortality rates as an indication of the effect of different routes of inoculation on egg-adaptation of a virus is subject to certain limitations. Embryos of the same age cannot be used effectively, because the optimal age for inoculation varies with different routes. Younger embryos might show a higher natural mortality and be less capable of surviving the effects of inoculation than embryos in a more advanced stage of embryonation. Also, lesions could not be expected to be so readily identified in younger embryos as in older ones, in view of the more complete development of the extra-embryonic membranes in the latter. The allantois does not attain full size until the ninth day.

The results obtained in this study indicate a need for further investigation, especially of the effects of amniotic and yolk inoculation on the adaptation of the virus to embryonating chicken eggs. Additional serial passages would possibly produce more conclusive results. Further studies of the effect of yolk inoculation would be clarified by the use of hens known to have been free from infection with infectious bronchitis.

SUMMARY

A study was made of the effect of different routes of inoculation on the adaptation of infectious bronchitis virus to embryonating chicken eggs. Embryo mortality rate was the criterion used as an indication of adaptation.

Four routes of inoculation were used: allantoic cavity, amniotic cavity, chorioallantoic membrane and yolk. Seven serial passages of the virus were carried out by each route.

The highest embryo mortality rate was produced by amniotic inoculation, followed in decreasing order by allantoic cavity and chorioallantoic membrane inoculation. Lesions considered characteristic of infection with the virus were observed following inoculation by these three routes.

Low mortality rates followed yolk inoculation, and no evidence of specific changes in the embryos was observed. It seems probable that the eggs used in the study were laid by hens immune to infectious bronchitis and the yolk therefore contained antibodies which neutralized the virus.

In spite of earlier adaptation by the amniotic route of inoculation, the allantoic cavity would seem to be the more

desirable route for isolation of field strains of the virus in view of the greater convenience of inoculation by the latter route, and the appearance of dwarfing as early as the third day following allantoic cavity inoculation.

REFERENCES

1. Asplin, F. D. Identification of infectious bronchitis in England. Vet. Rec., 60, (1948):485-486.

2. Beach, J. R. and O. W. Schalm. A filterable virus, distinct from that of laryngotracheitis, the cause of a respiratory disease of chicks. Poult. Sci., 15, (1936): 199-206.

3. Beach, J. R. Chapter 19. Infectious Bronchitis. Biester, H. E. and L. H. Schwarte, Editors. Diseases of Poultry. The Iowa State College Press, Ames, Iowa, 1948. 1154 pp.

4. Beaudette, F. R. and C. B. Hudson. Cultivation of the virus of infectious bronchitis. J.A.V.M.A., 90, (1937): 51-60.

5. Beaudette, F. R. Twenty years of progress in immunization against virus diseases of birds. J.A.V.M.A., 115, (1949):367-377.

6. Beaudette, F. R. Infectious bronchitis. (Differential diagnosis from Newcastle disease). Can. J. Comp. Med. and Vet. Sci., 14, (1950):24-27.

7. Beaudette, F. R. Infectious bronchitis and Newcastle disease. Can. J. Comp. Med. and Vet. Sci., 15, (1951): 65-71.

8. Bushnell, L. D. and C. A. Brandly. Laryngotracheitis in chicks. Poult. Sci., 12, (1933):55.

9. Cunningham, C. H. and H. D. Stuart. The effect of certain chemical elements on the virus of infectious bronchitis of chickens. Am. J. Vet. Res., 7, (1946):466-469.

10. Cunningham, C. H. and H. O. Stuart. Cultivation of the virus of infectious bronchitis of chickens in embryonated chicken eggs. Am. J. Vet. Res., 8, (1947):209-212.

11. Cunningham, C. H. and H. O. Stuart. The pH stability of the virus of infectious bronchitis of chickens. Cornell Vet., 37, (1947):99-103.

12. Cunningham, C. H. and A. H. El Dardiry. Distribution of the virus of infectious bronchitis of chickens in embryonated chicken eggs. Cornell Vet., 38, (1948):381-388.

13. Cunningham, C. H. A Laboratory Guide in Virology. Burgess Publishing Co., Minneapolis, Minn., 1948. 70 pp.

14. Cunningham, C. H. Newcastle disease and infectious bronchitis neutralizing antibody indexes of normal chicken serum. Am. J. Vet. Res., 12, (1951):120-133.

15. Cunningham, C. H. Personal communication. (1951).

16. Delaplane, J. P. and H. O. Stuart. Studies of infectious bronchitis. Rhode Island Agric. Exper. Sta., Bull. 273, 1939.

17. Delaplane, J. P. and H. O. Stuart. The modification of infectious bronchitis virus of chickens as the result of propagation in embryonated chicken eggs. Rhode Island Agric. Exper. Sta., Bull. 284, 1941.

18. Delaplane, J. P. The differentiation of the respiratory diseases of chickens. Rhode Island Agric. Exper. Sta., Bull. 288, 1943.

19. Delaplane, J. P. Panel discussion on poultry diseases. J.A.V.M.A., 106, (1945):91-103.

20. Delaplane, J. P. Technique for the isolation of infectious bronchitis or Newcastle virus including observations on the use of streptomycin in overcoming bacterial contaminants. Proceedings of the Nineteenth Annual Pullorum, Raleigh, N. C., 1947.

21. Fabricant, J. Studies on the diagnosis of Newcastle disease and infectious bronchitis of fowls. II. The diagnosis of infectious bronchitis by virus isolation in chick embryos. Cornell Vet., 39, (1949):414-431.

22. Fabricant, J. Studies on the diagnosis of Newcastle disease and infectious bronchitis of fowls. III. The differential diagnosis of Newcastle disease and infectious bronchitis. Cornell Vet., 40, (1950):39-48.

23. Fabricant, J. Studies on the diagnosis of Newcastle disease and infectious bronchitis. IV. The use of the serum neutralization test in the diagnosis of infectious bronchitis. Cornell Vet., 41, (1951):68-80.

24. Gibbs, C. S. Bronchitis of baby chicks. Poult. Sci., 12, (1933):46.

25. Groupe, V. Demonstration of an interference phenomenon associated with infectious bronchitis virus (IBV) of chickens. J. Bact., 58, (1949):23-32.

26. Hitchner, S. B. and E. P. Johnson. A virus of low virulence for immunizing fowls against Newcastle disease (avian pneumoencephalitis). Vet. Med., 43, (1948): 525-530.

27. Hitchner, S. B., G. Reising and H. Van Roekel. Characteristics of the B1 strain of Newcastle disease virus. Am. J. Vet. Res., 12, (1951):246-249.

28. Hofstad, M. S. A study of infectious bronchitis in chickens. I. The pathology of infectious bronchitis. Cornell Vet., 35, (1945):22-31.

29. Hofstad, M. S. A study of infectious bronchitis of chickens. II. Observations on the carrier status of chickens recovered from infectious bronchitis. Cornell Vet., 35, (1945):32-35.

30. Hofstad, M. S. A study of infectious bronchitis of chickens. III. Attempts to utilize the chicken red cell agglutination test as diagnostic aid in infectious bronchitis. Cornell Vet., 35, (1945):60-61.

31. Hofstad, M. S. A study of infectious bronchitis of chickens. IV. Further observations on the carrier status of chickens recovered from infectious bronchitis. Cornell Vet., 37, (1947):29-34.

32. Hofstad, M. S. and S. G. Kenzy. Susceptibility of chicks hatched from recovered hens to infectious bronchitis. Cornell Vet., 40, (1950):87-89.

33. Jungherr, E. L. and N. L. Terrell. Naturally acquired passive immunity to infectious bronchitis in chicks. Am. J. Vet. Res., 9, (1948):201-205.

34. Levine, P. P. and M. S. Hofstad. Attempts to control air-borne infectious bronchitis and Newcastle disease of fowls with sterilamps. Cornell Vet., 37, (1947): 204-211.

35. Loomis, L. N., C. H. Cunningham, M. L. Gray and F. Thorp. Pathology of the chicken embryo infected with infectious bronchitis virus. Am. J. Vet. Res., 11, (1950): 245-251.

36. Page, C. A. Antibody response of chickens exposed to infectious bronchitis virus. Unpublished M.S. thesis, Michigan State College, (1950). 39 pp.

37. Reagan, R. L., J. E. Hauser, M. G. Lillie and A. H. Craige, Jr. Electron micrograph of the virus of infectious bronchitis of chickens. Cornell Vet., 38, (1948): 190-191.

38. Reagan, R. L., A. L. Bruechner, and J. Delaplane. Morphological observations by electron microscopy of the viruses of infectious bronchitis of chickens and the chronic respiratory disease of turkeys. Cornell Vet., 40, (1950):384-386.

39. Schalk, A. F. and M. C. Hawn. An apparently new respiratory disease of baby chicks. J.A.V.M.A., 31, (1931):413-422.

40. Swierstra, D. Bronchitis infectiosa by Keppen in Nederland. Tijschr. Diergeneesk, 72, (1947):745-746. (Cited by Page[35]).

41. Van Roekel, H., K. L. Bullis, O. S. Flint and M. K. Clarke. Poultry Disease Control Service. Mass. Agric. Exper. Sta. Ann. Rep., Bull. 388, (1942):99-102.

42. Van Roekel, H., M. K. Clarke, K. L. Bullis, O. M. Olesiuk and F. G. Sperling. Infectious bronchitis. A.J.V.M.A., 12, (1951):140-146.