EGG TRANSMISSION OF AVIAN TUBERCULOSIS WITH OBSERVATIONS ON THE HEMAGGLUTINATION TEST

By

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AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Bacteriology and Public Health

Year 1953

Approved W. H. Mack

ACKNOWLEDGMENTS

The writer wishes to express his appreciation to Dr. W. N. Mack for his advice and encouragement offered during the course of this study.

He is also greatly indebted to Dr. H. J. Stafseth for his kind guidance and valuable help in checking the dissertation.

Grateful acknowledgment is also due Mr. Cleveland Allen, Department of Poultry Husbandry for his help in the insemination of the chickens.

The writer deeply appreciates the financial support of the Graduate Assistantship from the Department of Bacteriology and Public Health and the scholarship provided by Michigan State College.

Two methods were selected to determine whether a tuberculous bird could be hatched from an artificially infected hen. They were 1) inoculation of embryonated eggs to determine the ability of a tuberculous embryo to hatch and develop active tuberculous; 2) inoculation of the ovary of normal hens, collecting and hatching their eggs, and finally reisolating and identifying the tubercle bacilli from the hatched chick.

The hemagglutination activity of several sera was studied according to the Middlebrook-Dubos reaction.

Inoculation of 242 normal embryonated eggs with tubercle bacilli resulted in the death of 115 or 47.5 percent.

Only 85 of the 127 embryos which hatched were alive after eight months. Sixty or 70.5 percent of the birds gave a positive tuberculin reaction at eight months of age. Twenty-seven of these sixty tuberculin positive birds showed lesions characteristic of the disease whereas 25 (29.4 percent) of the 85 birds showed no reaction to the tuberculin test and were devoid of lesions.

Inoculation of 16 normal hens with tubercle bacilli, directly into the ovary resulted in the development of progressive fatal tuberculosis in six birds. Ten of the 16 hens were artificially inseminated once each week and eggs from each hen were collected. A total of 241 eggs were laid by these ten hens. Sixty-three were non-fertile, 18 fertile eggs failed to hatch

because of death of the embryo, and 160 eggs hatched. All chickens hatched from these 160 eggs were examined microscopically, culturally, and by embryo inoculation of normal embryos for tubercle bacilli.

Of the 81 eggs laid by these ten hens which failed to hatch, nine contained acid-fast bacilli. Three of these nine eggs contained virulent avian tubercle bacilli.

One hundred and sixty eggs from the injected hens hatched. Twenty-three of the 160 chicks died within 11 days after hatching. One chick, which died on the fifth day after hatching contained acid-fast bacilli in its yolk sac. Three of 139 birds were tuberculin positive six months after hatching. These three birds were hatched from eggs of hens which also laid non-fertile eggs containing tubercle bacilli.

A mixture of rooster semen and tubercle bacilli was introduced into the oviduct of two birds in an attempt to hatch tuberculous birds.

A hemagglutination reaction is described in which sera from tuberculous chickens and tuberculin sensitized sheep red blood cells are used. The sera of eight tuberculin negative chickens, previously inoculated in the embryonic stage, showed hemagglutinin in dilutions ranging from 1:4 through 1:16.

There was no significant increase in hemagglutinins in the serum of six apparently healthy chickens "stimulated " with tuberculin.

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INTRODUCTION

Tuberculosis in poultry, as observed by the poultry pathologist, comprises only a small percent of the specimens brought to the diagnostic laboratory. However, according to Smith (1947) tuberculin tests indicated that fifty percent of the flocks in 12 North Central States were affected; one state showed 65 percent infection with one or more birds diseased out of 542 flocks. The extent of infection may vary in different flocks; Smith (1947) reported seven percent infected in 88,000 older birds whereas he found only 0.4 percent infection in 25,000 pullets. Ballantyne and Bigland (1951) reported that in Canada 11.8 percent of 170 chickens were infected with tuberculosis.

Hinshaw, Niemann and Busic (1932) showed that there was a higher incidence of tuberculosis in the reporductive tract of turkeys than in the reproductive tract of chickens. Ducks may also exhibit tuberculous lesions caused by the avian tubercle bacillus. Rewell (1948) reported a tuberculoma of the brain in a Mandarin duck in captivity from which the tubercle bacilli had spread to other organs of the body including the ovary.

Wild birds, pigeons, gulls and sparrows have been shown to be naturally infected with avian tubercle bacilli whereas some migratory birds revealed no evidence of such an infection (Plum, 1942).

Tuberculosis of swine is most often contracted either directly or indirectly from tuberculous chickens (Dickey, 1945). The incidence of swine infection varies from less than one percent to 12 percent in the United States (Smith, 1943) with about 21 percent infection of swine in Canada (McIntosh, 1942). The highest incidence of swine tuberculosis is in Denmark. Forty to 97 percent of the hogs are infected in that country and the great majority by Mycobacterium avium (Bankier, 1946), and (Hoeden, 1941).

Although Mycobacterium avium can stimulate antibody formation in cattle with consequent sensitization to avian and possibly mammalian tuberculin, widespread infection is not often encountered. Sporadic cases of tuberculosis in cattle caused by avian tubercle bacilli have been reported by Timothy (1939), Stuart and Marshall (1952), and Ottosen (1944).

Several recent cases of tuberculosis in humans, proved by laboratory and pathogenicity experiments to be caused by avian tubercle bacilli are described by Finlayson (1948), Dragsted (1949), and Brandbury (1946). Rich (1950) sums up the question of avian tubercle bacilli in humans with the following paragraph: "In view of the various considerations presented in this discussion, one can only conclude that if progressive tuberculosis is ever produced in the human being by the avian tubercle bacillus it must be only very rarely, and it is highly desirable that more well-documented studies be brought to bear upon the question".

It is generally assumed that the avian tubercle bacilli are transmitted directly from bird to bird by ingestion of infected droppings. The incidence of intestinal lesions and the presence of tubercle bacilli in the intestinal contents of infected fowl indicate that many new cases occur by such a method (Lichtenstein, 1933), (Schalk et al. 1935), and (Feldman, 1938).

Just what role ovarian transmission plays in the dissemination of avian tuberculosis is not completely known. A review of the literature indicates that there is need for a study of this method of dissemination. Such an opportunity existed principally because of housing facilities in a new building. In addition, test animals were available for the study of antibody reaction in avian tuberculosis.

REVIEW OF LITERATURE

Ovarian transmission of tuberculosis in the fowl has not been sufficiently established to say that this method is of major importance in the spread of avian tuberculosis. The possibility that the avian tubercle bacillus might be transmitted through the egg from tuberculous hens has been suggested for over sixty years.

Several factors are inherent in the study of natural or artificial transmission of avian tubercle bacilli through eggs. They include: 1) adequate isolation of infected birds, 2) isolation and identification of tubercle bacilli and 3) establishing the pathogenicity of the bacilli for animals and other fowl.

A review of the literature was made for information concerning previous study of this problem but little information was found of an exacting nature that could not be challenged in relation to the factors listed above. Definite conclusions were, therefore, difficult to formulate. In part, this confusion of results was due to various techniques utilized, difficulty in obtaining homogenous suspensions of the organism, strict isolation of test birds, and especially lack of information concerning the virulence of the isolated organisms.

Avian tuberculosis was first described by Crisp (1868) who reported the occurrence of tubercles on and in the spleen, liver and peritoneal surface of diseased fowl. He was successful, after two years experimentation, in transmitting the disease from infected to healthy birds. This work was done several years before Koch discovered and cultured the mammalian tubercle bacillus in 1882. Simulatneously and independently Ribbert (1883), and Sutton (1884), in two widely separated laboratories, established the bacterial nature of avian tuberculosis.

One of the earliest publications concerning injection of embryonating chicken eggs with avian tubercle bacilli is that of Maffucci (1889), who inoculated 18 eggs. Eight chicks were hatched. Tubercle bacilli were demonstrated microscopically on necropsy in six of the eight hatched chicks.

Baumgarten (1891) reported that only two eggs out of 12 inoculated with a culture of avian tubercle bacilli hatched. One of the chicks died after four and one-half months and was found to have generalized tuberculosis. The other chick died a short time later but was not infected.

Maffucci (1892), and Milchner (1904) came to the same general conclusion that chicks hatched from eggs injected with avian tubercle bacilli usually became tuberculous and died from the disease. Proof of the pathogenicity of the tubercle bacilli, the presence of which was determined by microscopic examination, was not discussed.

Inoculation of embryonating chicken eggs with avian, human and bovine tubercle bacilli was studied by Koch and Rabinowitsch (1907). Of 14, 11, and seven eggs respectively, which were inoculated with the above types of bacilli only eight chicks hatched. One chick died at two months of age with generalized tuberculosis; three chicks which were injected with human bacilli developed no disease during four and one-half months; while of the four chicks injected with bovine bacilli, one died in one month with tuberculosis of the ceca and another in four and one-half months with tuberculous lesions in the liver. The two remaining chicks were sacrificed and no lesions noted. These authors did not discuss the infectivity of the different types of tubercle bacilli for the fowl. It is interesting to note that two of the four embryos inoculated with bovine bacilli developed tuberculosis one and four and one-half months, respectively, after hatching. Information today concerning the infectivity of bovine bacilli for the fowl leads one to believe that the bovine tubercle bacillus, in small numbers, is non-pathogenic for the chicken.

An experiment similar to that of Koch and Rabinowitsch (1907), was carried out by Rautmann (1931). Three groups of 25 eggs each were inoculated with avian, bovine and human tubercle bacilli. Hatchability was forty, twenty, and fifty percent, respectively. Most of the chicks hatched from avian bacilli inoculated eggs died of extensive tuberculosis. Chicks hatched from the bovine and human bacilli inoculated eggs did

not die of tuberculosis and generally no tissue changes were noted. It was concluded that fowls are resistant to the human and bovine types of bacilli.

Silva (1932) noted that inoculation of ten day-old embryonating chicken eggs resulted in a lower mortality as compared to that among those inoculated at an earlier date; that is, the sixth to eighth day of incubation. This procedure enabled the bacilli to invade tissues of the embryo resulting in a tuberculous bird. There is no doubt that inoculation of embryonating chicken eggs with avian tubercle bacilli results in a lower hatchability and higher mortality of the embryo when compared to the mortality rate of controls (Thorp and Graham, 1930) and (Fitch and Lubbehausen, 1928).

One of the earliest reports of apparent ovarian transmission of tubercle bacilli in chickens is that of Sibley (1893), who observed tuberculosis in chickens hatched from eggs laid by naturally infected hens. He attempted to explain the origin of the disease by stating that "the disease appears to be a clear case of heredity".

Gartner (1893) was one of the first to scientifically carry out experiments to determine if tubercle bacilli could be transferred through the egg. In these experiments he inoculated one male and 12 female canaries intraperitoneally with virulent avian tubercle bacilli. He observed, microscopically, the presence of acid-fast bacilli in the peritoneal cavity of two guinea pigs inoculated with portions of each egg. In a

second experiment, two of 24 eggs contained acid-fast bacilli. Artault (1895), and Mohler and Washburn (1906) also observed by microscopic examination, acid-fast bacilli in rabbits and guinea pigs following inoculation of portions of one egg laid by a known tuberculous hen. However, these and the two preceeding experiments failed to mention ovary or oviduct infection. Higgen's (1912) report is similar to those of Gartner (1893), and Artault (1895) but he mentioned seeing tubercles macroscopically in two of 11 guinea pigs inoculated with material from three apparently infected eggs.

The apparent transmission of avian tubercle bacilli through the egg was summarized by Van Es and Schalk (1914): "It seems thus that transmission by means of the eggs must be given consideration, although the data are not sufficiently numerous to enable us to correctly estimate the extent of the danger".

Little work was done in this field during the next decade until the publication of Fitch et al. (1924). This group published results of a systematic experiment to determine whether avian tuberculosis is transmitted through the eggs of tuberculous fowls. They examined 876 eggs, 367 by cultural methods and 509 by inoculation of poultry. They found that the composite of nine eggs obtained from two of 43 tuberculous hens contained tubercle bacilli. It is interesting to note in this report one of the hens on being necropsied gave no evidence of tuberculosis but was tuberculin positive. Fitch and Lubbehausen (1928) completed experiments on the

same group of chickens and hatched 697 birds from two thousand eggs laid by 88 tuberculin positive hens. Approximately two-thirds of the chicks died. A bacteriological examination was performed on each bird and no evidence of tubercle bacilli was noted microscopically or culturally. The other one-third of the chicks matured normally and were later tuberculin tested. On necropsy, each bird was carefully examined as above. Tubercle bacilli were not found in any birds of this last group. The investigators concluded that transmission of tuberculosis through the egg, rarely, if ever occurs.

Raebiger (1938) reported the largest number of eggs revealing tubercle bacilli microscopically or by cultural methods. The hens that laid these eggs were experimentally infected. Approximately 36 percent of the eggs contained tubercle bacilli. The virulence of the acid-fast organisms isolated was not mentioned. Organisms resembling tubercle bacilli have been reported present in eggs laid by hens. The number of organisms reported by various investigators varied from zero to 13.9 percent (Harshfield, 1937; Eber, 1932; Klimmer, 1930; Liverani, 1934; Bigger, 1932; and Raebiger, 1929). The bacilli isolated were not proved to be virulent avian tubercle bacilli.

Bunyea (1929) hatched 276 chicks from eggs obtained from a flock of 1,075 hens. These hens were either tuberculin positive or revealed lesions of the disease when necropsied.

The experiment also included three generations of newly hatched chicks from the tuberculous parent stock. Of 361 chicks reared, none gave a positive tuberculin reaction and no tuberculous lesions were found.

Beller and Henninger (1930) conducted an ingenious experiment whereby they fed large numbers of virulent tubercle bacilli to 18 hens over a period of two years. Six hens were given avian, six bovine, and six human tubercle bacilli. Twelve contact controls were added to the group and all thirty birds placed in the same area. At necropsy, lesions of tuberculosis were found in only three birds and these had received avian tubercle bacilli. Only two of the 620 eggs laid by the thirty hens, contained acid-fast bacilli, and these organisms were identified as avian species by microscopic and cultural exaimination.

The possibility of rearing tuberculous chickens from eggs of artificially infected hens was studied by Stafseth et al. (1934). They reported no tuberculous birds after the hatched chicks were seven months of age.

Complete identification of tubercle bacilli is lacking in much of the literature reviewed. Infectivity studies of the isolated bacilli are essential for positive identification. Electron microscopic studies of pathogenic mycobacteria indicate that human tubercle bacilli are generally longer and more slender than bovine bacilli, and usually, that avian bacilli are more pleomorphic than mammalian bacilli, (Soltys,

Hill and Ansell, 1952). These investigators mentioned the variable microscopic characters of the three types of bacilli and stated that it is impossible to decide on morphological grounds alone whether the bacilli are of human, bovine, or avian origin. It is apparent that morphological characteristics of tubercle bacilli depend on the medium on which they are growing, the age of the culture, and on the variation of strains.

Middlebrook and Dubos (1948) described a hemagglutination reaction with the sera from experimental animals or from tuberculous patients. The reaction involved tuberculin treated sheep red blood cells in the presence of tuberculous antiserum. The authors did not elaborate on this reaction as a diagnostic procedure but presented results which showed that the sera from six patients with active pulmonary tuberculosis had titers of 1:8 or higher; whereas sera from eleven non-tuberculous patients showed no titer higher than 1:4.

Rothbard, Doonief and Hite (1950) tested sera from 216 normal adults and patients with syphilis or other non-tuberculous chest diseases in an attempt to demonstrate the presence of tuberculosis antibodies by the hemagglutination reaction. Their results showed that 203 of 216 sera gave no hemagglutination reaction, 12 showed reactions in serum dilutions of 1:2, and one showed a reaction in a 1:4 dilution. Ninety-two percent of the sera from 168 patients with active tuberculosis gave hemagglutination reactions. These reactions occurred in

dilutions ranging from 1:8 to 1:512. Thirty-one sera from 33 supposedly cured tuberculous individuals revealed no reaction. The other two sera showed slight reactions in a 1:2 serum dilution. They concluded that the hemagglutination reaction is apparently a relatively specific test for active tuberculosis.

Smith and Scott (1950) reported that the hemagglutinatinn test was usually negative in the far advanced or arrested types of tuberculosis. They theorized that the antibodies of the first type were neutralized by adsorption of products produced by the growing tubercle bacilli while agglutinins were absent in the second type because there was not sufficient antigenic stimulation from healed tuberculous lesions. They also found that of 58 students with positive tuberculin tests, 12 (22 percent) had hemagglutinins before "stimulation" with tuberculin. Of the remaining 46, 15 (32 percent), had no detectable hemagglutinins before stimulation with tuberculin, whereas, after stimulation the hemagglutinins were detectable. They also reported eighty percent positive hemagglutination reactions with 104 sera from active tuberculous patients.

Gernez-Rieux and Tacquet (1950), Sohier et al. (1950), Scott and Smith (1950), and Chang et al. (1952) presented similar results with sera from actively tuberculous patients.

Kirby, Bunnell, and O'Leary (1951) found that the hemagglutination reaction was positive in 123 of 251 sera from patients

exhibiting acute non-tuberculous infections. In a second group of 54 patients, all with active tuberculosis, 31 (57 percent) of the sera gave hemagglutination reactions in a 1:8 dilution or higher. Their conclusion was that the hemagglutination titer is of no diagnostic value.

MATERIALS AND METHODS

Cultures: Two strains of Mycobacterium avium, Van Es and St. Elizabeth, used in this study were obtained from the bureau of Animal Industry of the United States Department of Agriculture, Washington, D. C. The Van Es strain was sent to them by Dr. L. Van Es, University of Nebraska, Lincoln, Nebraska. The St. Elizabeth strain was obtained from the liver of a hen belonging to the St. Elizabeth Hospital flock, Washington, D. C.

Culture Media: Both strains of tubercle bacilli were cultured on Petragnani's medium prepared by the Laboratories of the Michigan Department of Health, Lansing, Michigan. Excellent growth was secured from small inocula after ten to 15 days incubation at 37° C. The same medium was used for primary isolation of tubercle bacilli from infected eggs, tissues, and body fluids. Stock cultures were preserved at refrigeration temperature on Petragnani's medium.

Before inoculation of embryonated chicken eggs or chickens, the tubercle bacilli were transferred from Petragnani's medium to sterile screw cap culture tubes containing eight ml. Difco Tb broth base plus ten percent Difco Tb medium albumin. This culture medium is a liquid medium developed especially for the dispersion of tubercle bacilli. These organisms grow in a suspension after three to five days incubation at 37° C.

Chicken embryos as cultures: Acid-fast organisms observed microscopically in eggs, embryos, or hatched chicks originating from injected hens were tested for virulence by yolk sac inoculation of embryonated eggs. All acid-fast organisms cultured on Petragnani's medium from the above tissues were tested for virulence by this method. Specimens in which acid-fast granules were observed were also injected into embryonated eggs. This procedure provided a rapid method of demonstrating the presence of the tubercle bacilli, if present in the specimen, and, although the original organisms were virulent, this procedure confirmed their pathogenicity. According to Brueck and Buddingh (1952) this method of demonstrating tubercle bacilli in materials from tuberculous patients was superior to the microscopic, cultural, and Petragnani inoculation methods. These authors reported tuberculous infection of the yolk, and rapid proliferation of the acid-fast organisms from 39 specimens later proved positive for tubercle bacilli by guinea pig inoculation. They also reported 11 specimens negative by both the yolk infection and guinea pig inoculation.

Approximately 0.2 ml. of digested and concentrated yolk material or other tissues suspected of containing the tubercle bacilli was inoculated into each of two embryonated eggs. Any embryo dead within 72 hours was considered to have died from trauma. Embryos dead after 72 hours were removed from the shell and concentrated by the method described by Brueck and Buddingh (1952). Two ml. of the yolk was mixed with twenty ml.

of 15 percent phenol solution in a large test tube. The tube was allowed to stand for two hours while the fatty layer floated to the surface. A Ziehl-Neelsen stain of a portion of the fatty material revealed many acid-fast bacilli in yolk material of infected embryos.

Bacterial Cell Counts: The approximate number of tubercle bacilli inoculated into the embryonated chicken eggs and test birds was determined by the Cenco-Sheard-Sanford Photelometer Type B2 and the Petroff-Hausser counting chamber. The method is as follows: sterile Tb broth base medium was placed in a standard photelometer cell and the cell inserted into the absorption compartment. With the green (5600Å) and blue (4500Å) glass filters in position the meter scale was adjusted to give a hundred percent light transmittancy. Using aseptic technique and with thorough mixing, a six-day-old suspension of tubercle bacilli was added to the sterile medium in the photelometer cell until the meter scale gave a ninety percent light transmittancy. Bacterial counts were made on this ninety percent light transmittancy suspension until results could be really duplicated. The procedure for counting the actual number of bacilli per ml. in the suspension was as follows: a staining solution of 0.5 ml. saturated alcoholic gentian violet and 50 ml. of five percent aqueous phenol was prepared. These reagents were mixed and filtered through No. 1 Whatman filter paper until free from small particles. Dilutions of the standard

suspension prepared above were made in one percent formalized saline solution by adding one ml. of the bacterial suspension to nine ml. of formalized saline solution. This gave a 1:10 bacterial suspension. One ml. of this dilution was added to ten ml. of saline solution. To this tube was added one ml. of the filtered gentian violet solution, giving a final 1:120 dilution of the standard ninety percent light transmittancy suspension. This final suspension containing the mixture of organisms and stain, was heated until the liquid boiled. The suspension was allowed to stand for three minutes before proceeding with the bacterial count.

The Petroff-Hausser counting apparatus was cleaned and a cover glass placed over the chamber. A capillary pipette was filled with the well-mixed and stained suspension, and the chamber filled by capillary attraction. The small 1/20 mm. squares in the chamber were observed with the high, dry objective. By raising and lowering the objective slightly the bacterial cells were counted in each small square until 20 squares had been counted. The results were calculated by dividing the total number of bacterial cells counted by 20 to obtain the average number for each square. The average number of cells per square was multiplied by 20,000 (which is the cubic dimension of each square in millimeters), by 120 (the dilution of tubercle suspension), by 1,000 (the number of cubic millimeters in a cubic centimeter). This result was

found to be the bacterial count per ml. of the ninety percent light transmittancy tubercle culture.

Inoculation of embryonated chicken eggs: Fertile white leghorn eggs, from disease free chickens were placed in the hatching incubator. The eggs were candled every day to observe development of the embryo. All eggs with dead or abnormal embryos were discarded.

When the embryonated chicken eggs were seven days old they were candled with the long axis in the horizontal plane and the yolk sac located. A mark was made on the shell over the yolk sac about half-way from the small end of the egg to the apex of the curvature of the shell. A small opening was drilled through the shell leaving the membrane intact. Tincture of metaphen (1 to 200) was applied to the opening and with the long axis of the egg in the horizontal plane, the inoculum was introduced slowly into the yolk sac. Injection was made with a 27 gauge, 1/2 inch needle on a 1/4 ml. syringe, the needle being inserted the full length. Approximately 50 tubercle bacilli were introduced into the yolk sac of each embryo. The opening was sealed with melted paraffin and the inoculated eggs returned to the incubator. All inoculated eggs were candled each day until hatching occurred. Dead embryos were subjected to bacterial, cultural, and microscopic examination. Chicks hatched were isolated in a heated brooder which had been thoroughly disinfected. Birds dying before eight months of age were examined for tuberculous lesions and other gross pathological symptoms.

Unabsorbed yolk material, yolk stalk tissue and its contents were subjected to microscopic and cultural examination for tubercle bacilli. Digestion of a portion of the above material with four percent sodium hydroxide for thirty minutes at 37° C. was routine practice. The sediment was neutralized with dilute hydrochloric acid. A portion of the sediment was stained by the Ziehl-Neelsen method and the rest distributed over the surface of four tubes of Petragnani's medium. The tubes were incubated for ten weeks at 37° C. until acid-fast bacilli were observed. Tubes exhibiting growth of organisms other than acid-fast bacilli were discarded. Yolk stalk, portions of the digestive tract, liver, and spleen were examined in the older birds. All birds alive after eight months of age were bled for collection of serum and then necropsied. Any suspicious lesions were subjected to cultural examination.

Inoculation of adult hens: Eighteen normal New Hampshire red hens, nine months of age, were purchased locally, wing-banded and isolated in individual cages. The cages were placed in a room that had never been used for animal quarters. Each bird was given an intradermal injection of approximately 0.25 avian tuberculin. Injection was made with a small syringe and 27 gauge needle into the skin of one wattle. A small bleb was produced at the site of injection. The injected wattle of each bird was examined at 24 and 48 hours using the other wattle for comparison. None of the 18 birds was sensitive to

the tuberculin. A sensitivity reaction produces a soft, edematous swelling, marked by discoloration, ranging from dirty pale yellow to dirty greenish yellow.

All 18 birds were quarantined under excellent housing conditions and did not experience any disease other than the one to which they were exposed.

The ovary of 16 of the 18 hens was inoculated with approximately 100 tubercle bacilli. The hens were deprived of food and water 12 to 18 hours prior to the ovarian inoculation. This permitted the intestinal contents to be reduced to a minimum resulting in a clear field of operation in the abdominal cavity and less danger of puncturing the intestine when the intercostal incision was made.

The hen was placed on the operating table on its right side. The wings were securely held together above the body with the legs fully extended. Assistance in restraining the bird during the operation eliminates the need of an anesthetic.

Some of the feathers were plucked from the region of the hip and ribs. The area was washed with 1:200 tincture of metaphen. The narrow region between the sixth and seventh ribs (last two) was located with the fingers of the left hand. The skin was drawn back over the hip and with it the thigh muscle. An incision was made about an inch long through the skin and intercostal structures which reached almost to the dorsal rib attachments or vertebral column.

Severing of the cutaneous vein which runs diagonally across the body from the thigh to the wing was avoided. A spreader was placed in the incision and the peritoneal membrane which forms the abdominal air sac punctured. The abdominal organs were visible after the membrane was punctured.

The single ovary that develops to maturity is situated in the sublumbar region of the abdominal cavity. It lies at the anterior end of the left kidney and slightly to the left of the midline of the body. The heavy stalk, or base, of the ovary is attached to the dorsal wall of the abdominal cavity by a fold of peritoneum, called the mesovarium. This fold keeps the ovary in position, suspended in the cavity. The inactive ovary is composed of many ova, gray-white in color, which becomes more intensely colored, soft to bright yellow, as they increase in size and age. This change in coloration indicates that yolk material is forming. One-tenth ml. of a suspension of the organisms (100) was injected into the yolk stalk of each hen. After inoculation, the spreaders were removed from the incision and the bird released from the table. Suture of the incision was not found necessary unless the tissues were badly torn. The skin and thigh muscle slipped back in place over the incision as the limbs regained their normal positions. Hard crusts or scabs were formed of exudate from the incised

tissue and healing took place rapidly. After inoculation the birds were transferred to isolated cages. Some of the birds developed wind puffs which were released by puncturing the skin with a 22 gauge, two inch needle.

Two of the 18 hens, not included in the group, were artificially inseminated with 0.2 ml. of semen from the roosters mixed with one hundred tubercle bacilli. This procedure was performed on the two hens each time the entire group was inseminated.

Artificial insemination of the hens: Four normal, well-developed male chickens were kept segregated in quarantine and used solely for the collection of semen. The semen was artificially introduced into the oviduct of the 18 hens. This method of insemination was desirable because the cages were too small for a rooster and a hen. Also there was less chance of cross infection.

The technique of collection of the semen and insemination of the hens was as follows: the male chicken was held under the right arm while resting on the right knee. The left hand held the legs securely. Stimulation of the bird was accomplished by placing the right hand on the bird's back and sliding the thumb and fingers backward toward the base of the tail. Stroking the male in this manner caused the protrusion of the copulatory organ. Extending the thumb and forefinger around the base of the tail brought about ejection

of the semen. The average volume of semen collected from each male was 0.5 ml. to 1.0 ml.

Insemination of the hen was performed by extruding the oviduct through the vent and injecting 0.2 ml. semen directly into the vagina with a one ml. syringe without needle. In order to extrude the oviduct, the hen was held under the left shoulder with the arm extending under the keel. Its head was placed in a downward position to the rear of the operator. The side of the hen was placed against the operator's body, the left thumb and fingers were used to exert pressure around the vent. The sudden and simultaneous pressure exerted by both hands caused the oviduct to protrude. Each hen was inseminated once a week as long as she was in production.

Examination of eggs laid by inoculated hens: The hens band number and date of laying were recorded on each egg collected. The eggs were then placed in the incubator and candled each day until non-fertility was established. For the bacteriological examination, the shells of the non-fertile eggs were washed with 70 percent alcohol by immersion for 15 minutes. The egg shell was broken and the contents released into a sterile Petri dish. A five ml. portion of each yolk was digested with an equal volume of four percent sodium hydroxide. After digestion of the specimen, the tube and its contents were centrifuged at 2500 revolutions for thirty minutes. The supernatant fluid (not the fatty material at

the surface) was discarded, and the sediment and fat material neutralized with 2.5 percent hydrochloric acid with brom thymol blue as the indicator. A portion of the sediment was streaked upon three tubes of Petragnani's medium and upon a clean glass slide. The inoculated culture tubes were incubated for eleven weeks at 37° C. A microscopic examination was made of the slide after staining by the Ziehl-Neelsen acid-fast method. Two normal embryonated eggs were inoculated with 0.2 ml. of the sediment as described under Chicken embryos as cultures. In order to determine the efficiency of these methods of isolating acid-fast bacilli from yolk material, control eggs were inoculated with approximately fifty tubercle bacilli. The yolks of two inoculated control eggs were processed, as described above, for every ten test eggs.

All fertile eggs laid by the inoculated hens were kept in the incubator until the embryos died or the chicks hatched. Each dead embryo was immediately removed from the incubator and necropsied. The unabsorbed yolk and any tissue suspected of containing tubercle bacilli were examined microscopically and cultured as described above. Hatched chicks were isolated in a brooder and quarantined from all sources of avian tubercle bacilli. As soon as any bird showed symptoms of the disease it was bled and the serum stored in the frozen state. A thorough bacteriological examination was made of suspicious lesions observed in each bird. Gross pathology was recorded.

(Hemagglutination Studies)

Preparation of chicken sera: Approximately five ml. of blood was drawn by cardiac puncture from each chicken and allowed to clot at room temperature. The serum was removed after centrifugation and stored in the frozen state. Stored in this manner the titer remains constant for several months.

Each serum was diluted with an equal volume of buffered saline solution and inactivated by heating to 65° C. for three minutes. This temperature was slightly higher than that often employed in inactivating sera. The chicken serum was not harmed by this inactivation procedure and time was saved by employing this method. The inactivated serum-saline solution was stored in the frozen state. Adsorption of natural hemagglutinins with normal sheep red blood cells was performed before proceeding with the test proper.

Buffered saline solution: The buffered saline solution was made as follows:

Nacl - - - - - - - - - - 170 grams

KH_2PO_4 - - - - - - - - 2.7 "

Na_2HPO_4 - - - - - - - - 11.3 "

Distilled water to 1000 ml.

Before use, it was diluted 1:20 with distilled water.

Preparation of Alsever's solution:

Dextrose	2.05 grams
Na citrate	0.8 "
Na chloride	0.42 "
Citric acid	0.005 "
Distilled water to	100 ml.

The Alsever's solution was sterilized by Seitz filtration.

Preparation of washed, packed sheep red blood cells: Approximately 100 ml. of sheep's blood was collected aseptically in a flask containing 100 - 200 ml. Alsever's solution. The sheep red blood cells can be used satisfactorily for several months if kept in this solution in the refrigerator at 4° C. When ready for use, the necessary amount was centrifuged at 2000 revolutions per minute for 15 minutes. The supernatant fluid was removed and the sheep cells were washed three times with about five volumes of buffered saline solution. After the fourth washing and centrifugation as much as possible of the supernatant fluid was removed, leaving the packed, washed sheep red blood cells. These sheep red blood cells were used within 48 hours.

Adsorption of natural hemagglutinins from the sera: Natural hemagglutinins were removed from the chicken sera by adding normal, washed and packed sheep red blood cells in 0.2 ml. amounts to each tube containing the serum-saline solution. The tube was shaken and allowed to stand at room

temperature for twenty minutes. The mixture was centrifuged at 2500 revolutions per minute for six minutes and the supernatant fluid transferred to another tube containing 0.2 ml. amounts of previously washed, unsensitized sheep red blood cells. Two adsorptions do not always remove all the natural hemagglutinins from the serum and more adsorptions may be necessary until no agglutination occurs. The adsorbed serum was used to test for anti-tuberculosis antibodies by the hemagglutination test. This serum can be stored indefinitely in the frozen state.

Preparation of sensitized sheep red blood cells:

Old Tuberculin (Lederle Laboratories)* 4X strength, in 0.8 ml. amounts, was added to 11.2 ml. buffered saline solution. After shaking this mixture, 0.2 ml. of washed, packed sheep red blood cells was added to the mixture and incubated in a 37° C. water bath for two hours. Settling of the sheep red blood cells can be avoided by shaking the mixture every 15 minutes during the incubation period. Adequate sensitization was accomplished during the two hour period. The mixture was centrifuged at low speed (about 1000 revolutions per minute) for six to eight minutes and supernatant fluid discarded. The sheep red blood cells were washed three times by resuspending in 12 ml. buffered saline solution and centrifuging as above. Low speed centrifuging prevented the formation of

*Courtesy of Dr. H. D. Piersma, Lederle Laboratories, Pearl River, N. Y.

tightly packed sheep red blood cells which were difficult to resuspend without producing hemolysis. After the last centrifuging the cells were suspended in 50 ml. of buffered saline solution and used as an antigen in the hemagglutination test. This final concentration of sensitized red blood cell suspension was approximately 0.4 percent, and may be used up to three days if stored at four degrees centigrade.

Hemagglutination test procedure: The protocol for the test procedure is presented in Table I.* Nine standard Wassermann tubes were placed in a rack. Tubes one through seven contained serial serum dilution tubes, tube nine contained unsensitized sheep red blood cells-serum control, and tube eight contained sensitized sheep red blood cells-saline control. Tubes one, two and eight received 0.5 ml. adsorbed test serum diluted 1:2. Serial dilutions were then made from tube two by transferring 0.5 ml. of that mixture to tube three, 0.5 ml. of the mixture in tube three to tube four and so through tube seven, the content of which was reduced to the standard volume by discarding 0.5 ml. All tubes received 0.5 ml. of the sensitized sheep red blood cells except tube eight which received 0.5 ml. of unsensitized sheep red blood cells. Final dilutions of the adsorbed serum were from 1:4 in tube one to 1:256 in tube seven.

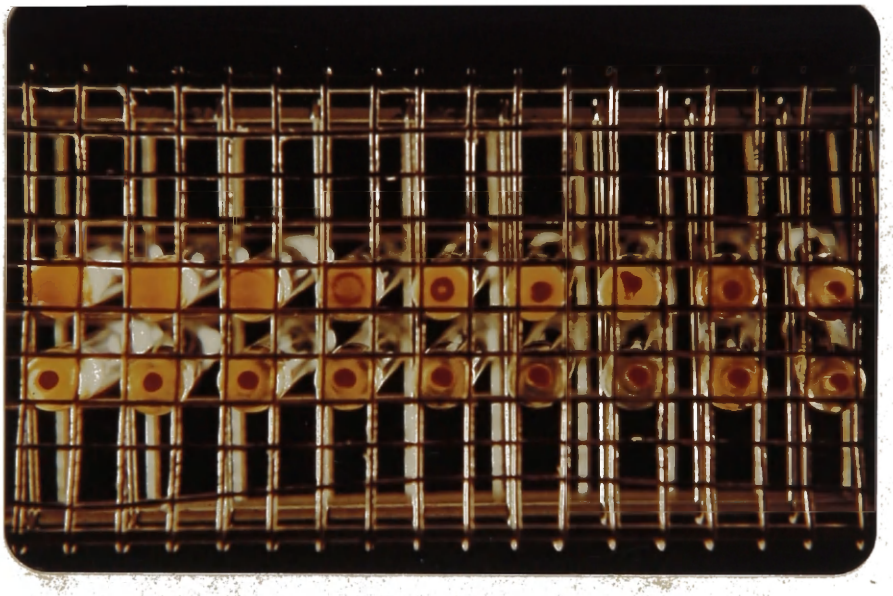
*With permission of the authors and publisher, Isabelle Gilbert Schaub and M. Kathleen Foley, chap. 15. Diagnostic Bacteriology, 4th ed. St. Mo., The C. V. Mosby Co. p. 290.

All tubes were shaken and then incubated for two hours in a 37° C. water bath. After partial settling of the sheep red blood cells the tubes were shaken vigorously and left undisturbed at room temperature overnight. Figure 1 shows the appearance of a positive and negative hemagglutination reaction.

Figure 2* shows diagrams of the hemagglutination text procedure. They are similar to those of the usual research and laboratory diagnostic hemagglutination reactions.

*Modified from chart by Armstrong, A. R. and J. Orlicki, 1951. The technique of the sensitized sheep cell hemagglutination test for tuberculosis. Can. Jour. Med. Tech. 2:67-75.

Figure 1



Kodochrome print from transparency showing
a positive and a negative hemagglutination reaction

TABLE I

PROTOCOL OF HEMAGGLUTINATION TEST

Tubes Nos.	1	2	3	4	5	6	7	8	9
Amt. phosphate buffered saline	--	0.5ml.	0.5ml.	0.5ml.	0.5ml.	0.5ml.	0.5ml.	--	0.5ml.
Amt. of adsorbed test serum (1:2)	0.5ml.	0.5ml.	0.5ml. of tube 2	0.5ml. of tube 3	0.5ml. of tube 4	0.5ml. of tube 5	0.5ml. of tube 6	0.5ml.	--
Amt. of sensitized sheep red blood cells	0.5ml.	0.5ml.	0.5ml.	0.5ml.	0.5ml.	0.5ml.	0.5ml.	--	0.5ml.
Amt. of unsensitized sheep red blood cells	--	--	--	--	--	--	--	0.5ml.	--
Final dilution of serum	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:4	--

Figure 2

Diagrams illustrating
the procedure of the hemagglutination reaction

HEMAGGLUTINATION PROCEDURE

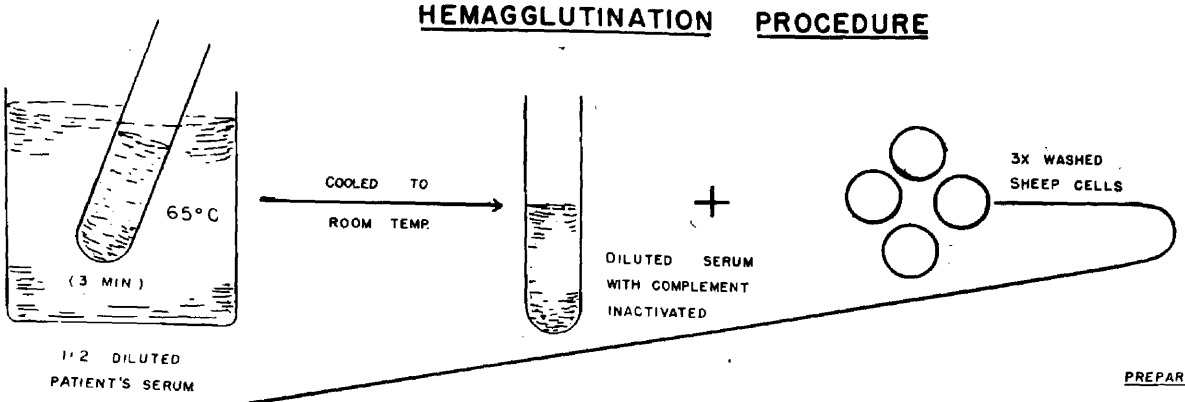


DIAGRAM 1

PREPARATION OF PATIENT'S SERUM

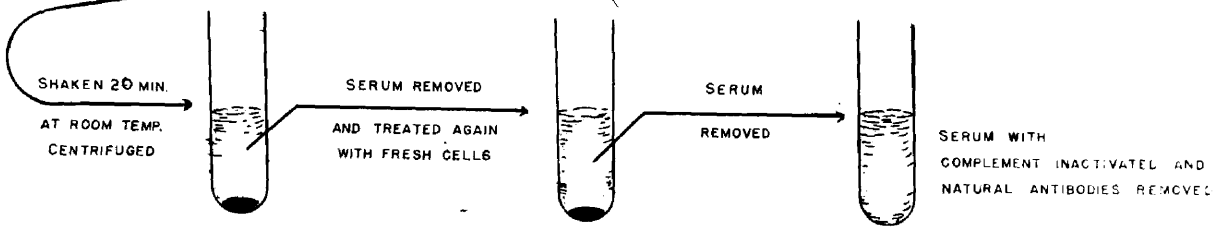
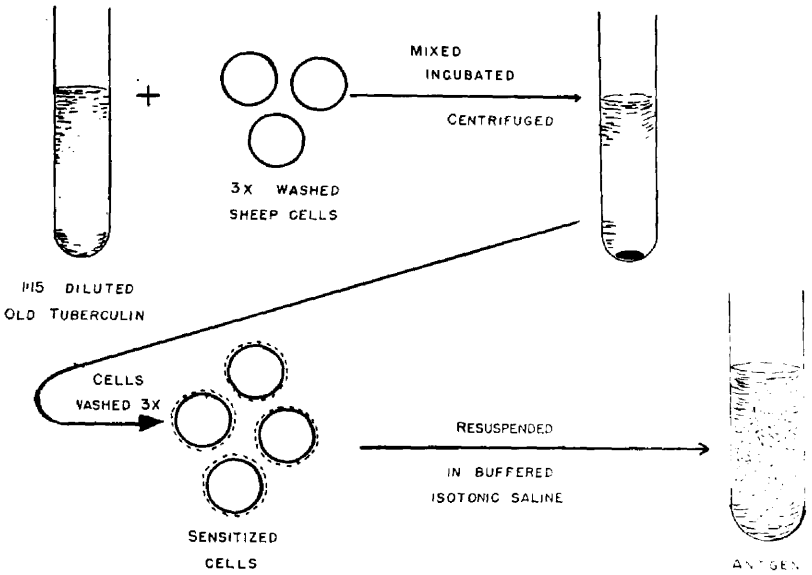


DIAGRAM 2

PREPARATION OF ANTIGEN



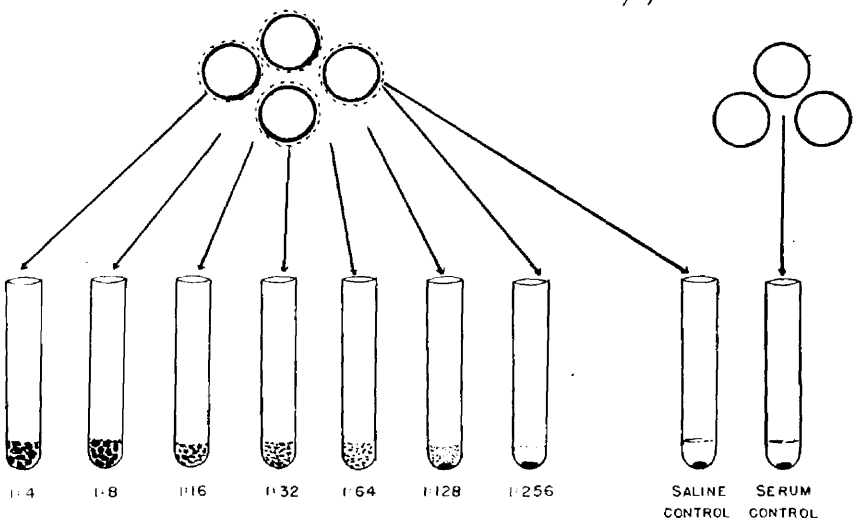


DIAGRAM 3

RESULTS

Inoculation of embryonated chicken eggs: A total of 242 seven-day-old embryonated chicken eggs were inoculated each with approximately fifty tubercle bacilli by the yolk sac method. The mortality of the infected embryos was high; 115 or 47.5 percent failed to hatch. In comparison, the mortality of the culture medium inoculated controls was two out of 16 or 12.5 percent. These results are given in Table II. There is no doubt that inoculation of embryonated chicken eggs with avian tubercle bacilli affects their hatchability, and that the action of the virulent tubercle bacilli was instrumental in their destruction. Figure 3 illustrates a six-day-old chick hatched from an inoculated embryo and a six-day-old chick hatched from a culture medium inoculated control.

Because of the action of the tubercle bacilli many of the 127 hatched chicks died during the first few weeks of life. They exhibited general weakness, inappetence, pasty vent and were often found dead in the cage. A composit summary of the development of tuberculosis in these birds is given in Table III. It will be noticed that the mortality of the hatched chicks showing infection was highest during the first week. Approximately fifty percent of the deaths occurred within the first week. Birds living to be six weeks of age usually survived.

TABLE II

MORTALITY RESULTS OF THE INOCULATION OF 242 EMBRYONATED EGGS

Embryo age (days)	Number dead (242 inoculated)	Controls dead (16 inoculated)
1-5	33	0
6-10	24	1
11-15	18	1
16-21	40	0
Mortality totals	115 (47.5%)	2 (12.5%)
Total no. hatching	127 (52.5%)	14 (87.5%)

Figure 3

A six-day-old chick hatched from a tubercle bacilli inoculated embryo (left)

A six-day-old chick hatched from a culture medium inoculated embryo (right)

TABLE III

DEVELOPMENT OF TUBERCULOSIS IN 127 CHICKS HATCHED FROM EMBRYONATED EGGS INJECTED WITH 50 TUBERCLE BACILLI

Age at death	Number of birds	Organ Diseased				
		Intestine	Yolk or yolk stalk	Liver	Spleen	Lung
1 week	19	2	19			
2 weeks	4		3			
3 weeks	6		6			
4 weeks	3		3			
5 weeks	3	1	2			
6 weeks	4		1	4		
5 mo.	3		2			
8 mo. (sacrificed)	85	24	9	5	2	2
Total	127					

It is of interest to note that the tuberculous lesions in the birds sacrificed at eight months of age were most prevalent in the intestinal tract. The lesions projected outward from the intestinal wall and were irregular in shape. Often the nodular lesions on the exterior of the intestine extended through the wall into the mucosa. The yolk stalk was also a common site of infection which was filled with tubercle bacilli. At necropsy it was often noticed that the yolk stalk was infected and apparently closed, producing a cryptic infection. The liver was infected in five instances, and the spleen and lungs in two birds.

Tests on the 85 birds alive at six months showed that sixty or 70.5 percent gave a positive tuberculin reaction. Twenty-five or 29.5 percent of these birds showed no reaction to the tuberculin test. Table IV shows these results. It is difficult to explain why these 25 "infected" birds reacted negatively to the test. It is known that tuberculin testing is not one hundred percent specific but the susceptibility of the individual bird must be recognized. The 25 tuberculin negative birds showed no tuberculous lesions at necropsy. Twenty-seven of the birds (29.5 percent) reacting to the tuberculin test at six months, showed tuberculous lesions. It would be interesting to know whether the birds became tuberculin positive and develop active tuberculosis if allowed to survive.

In summary, it can be noted that inoculation of embryonated eggs with fifty tubercle bacilli produced death in about fifty

percent of the embryos. Approximately fifty percent of the chicks that hatched died during the first week. Sixty of the birds were tuberculin positive at six months of age but 25 remained negative.

The lesions of the disease were found to predominate in the intestinal tract.

TABLE IV

TUBERCULIN REACTION OF 85 CHICKENS HATCHED FROM INFECTED EMBRYONATED EGGS

Total Number of Birds	Tuberculin Positive	% Tuberculin Positive	Number with Lesions	Tuberculin Negative	% Tuberculin Negative	Number with Lesions
85	60	70.5%	27	25	29.5%	0

Inoculation of normal adult hens: Sixteen of the 18 normal adult hens were inoculated with avian tubercle bacilli as described under Materials and Methods. These hens were inoculated in order to determine whether an ovarian infection would permit passage of the organisms through the egg to the embryo and produce a tuberculosis infected bird. For the same reason an attempt was made to infect the last two birds via the oviduct during artificial insemination.

Six of the 16 inoculated birds exhibited symptoms of tuberculosis within 14 to 21 days after injection of the ovary with approximately 100 tubercle bacilli. Because of the rapid development of the disease, artificial insemination was not practiced on these birds. Gradual loss of weight, wasting of the muscles, paleness of the comb, dullness, sleepiness and diarrhea with yellowish-green droppings were noted. All six of these chickens died of progressive tuberculosis within 12 weeks after inoculation; three dying in 19, 20, and 21 days, respectively, after inoculation, one six weeks, one eight and one-half weeks, and one 12 weeks after inoculation. Table V shows the results of this experiment. A necropsy was performed on all these birds and extensive ovarian, and some intestinal, liver, and spleen lesions of tuberculosis observed. Figure 4 shows a picture of bird number 1933 taken three days before death.

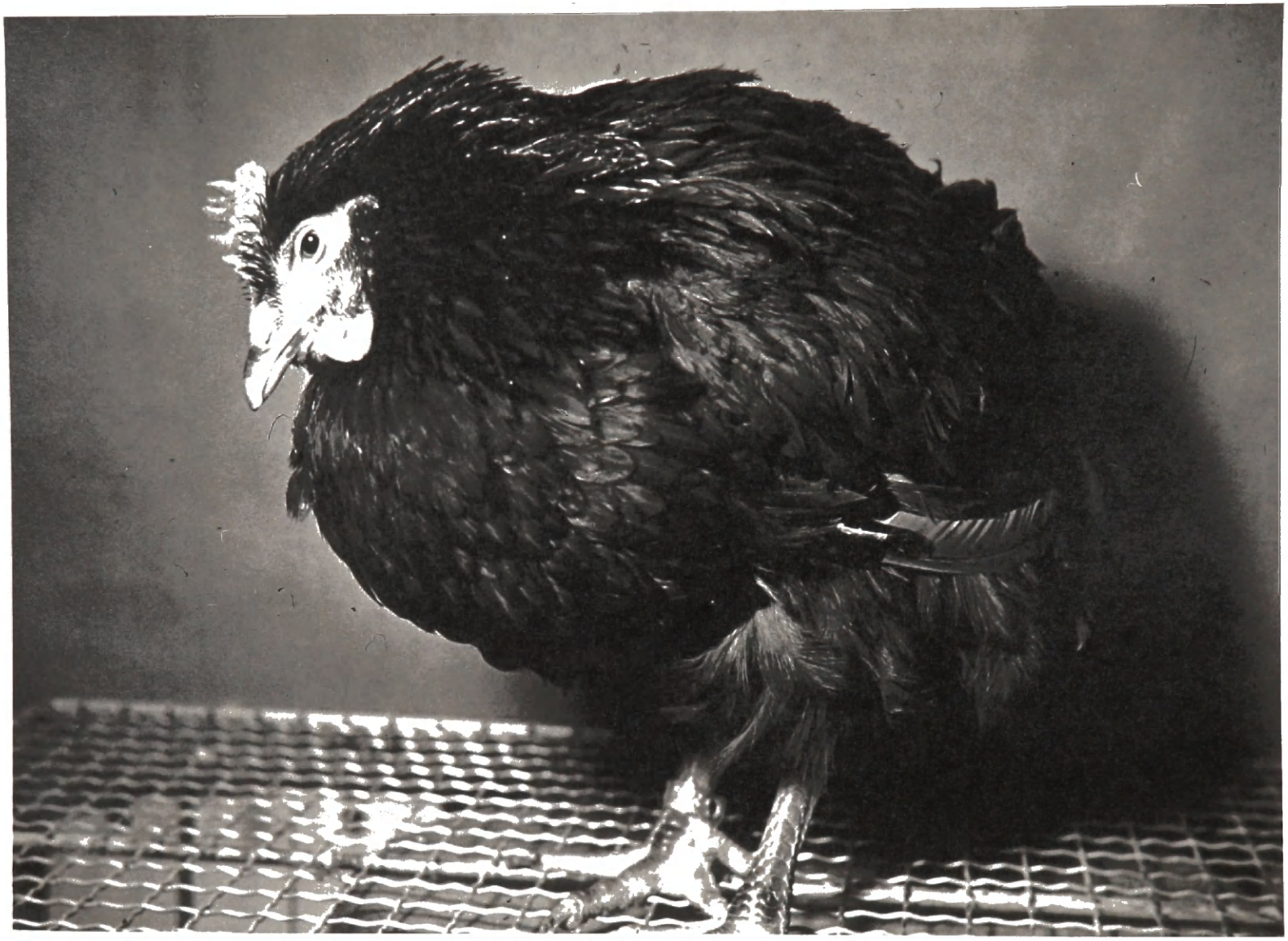
TABLE V

RESULTS OF THE EXAMINATION OF SIX CHICKENS DEVELOPING RAPIDLY FATAL TUBERCULOSIS

(No eggs collected from this group)

Bird No.	Time of death	Results
1917	19 days	Extensive ovarian and some digestive lesions. No nodules on liver of spleen. Ovaries non-functional at death. Acid-fast organisms observed in stained smears.
1995	20 "	
1906	21 "	
1989	6 weeks	
1935	8½ weeks	As above plus small miliary foci in liver and spleen. Many nodules on peritoneum and mesentery.
1933	12 "	

Figure 4



Terminally ill bird inoculated experimentally with avian tubercle bacilli

The other ten hens of this group were inoculated with approximately 100 tubercle bacilli directly into the ovary and isolated in individual cages. Each bird was inseminated artificially with 0.2 ml. of pooled semen obtained from three healthy roosters. Insemination was performed each week while the birds were in production.

A total of 241 eggs were collected during the eight months of observation. One egg was the smallest number collected from an individual hen. One bird produced 46 eggs in eight months. Four eggs were found broken giving a total of 237 eggs incubated for hatching. One hundred and sixty (67 percent) of these eggs hatched (Table VI).

Hens numbered 1909, 1942, and 1970 did not lay eggs at constant intervals of time as did the other seven hens. The egg laid by hen 1909 was obtained four weeks after inoculation. Three of the six eggs obtained from hen 1970 were collected four weeks after inoculation, two nine weeks, and one eleven weeks after inoculation.

Hen 1942 laid five of its six eggs 16 weeks after inoculation and the last egg 21 weeks after inoculation. Although each hen was inoculated with approximately the same number of organisms at the same time, and in the same organ, there was much variation as to the progress of the disease in the individual birds.

It can be observed from Table VI that 160 chicks were hatched from 241 eggs laid by ten hens. Of the 81 eggs failing

to hatch, 63 were found to be non-fertile. The remaining 18, which were fertile, failed to hatch either because of a dead embryo or death during hatching. A representative portion of the contents of each egg failing to hatch was examined microscopically and culturally for acid-fast bacilli resembling Mycobacterium avium.

The stage of development of the ovary at the time of inoculation of each hen seemed to have no correlation with its ability to lay eggs. Hens 1942 and 1970 each laid six eggs; the ovary of the former hen was not mature while that of hen 1970 was mature. It can be noticed from Table VI also that the two hens laying the largest number of eggs did not possess ovaries in the same stage of development.

Examination of 81 eggs, from injected hens, failing to hatch: Every egg which failed to hatch laid by these injected hens was examined microscopically, culturally, and by embryo inoculation for tubercle bacilli. It was approximately four weeks after injection of the ovary of the hens that acid-fast organisms were first observed in some of the eggs failing to hatch. Table VII presents the results of the examination of 81 eggs failing to hatch.

From this table it can be seen that three hens, 1924, 1977, and 126, laid eggs in which acid-fast bacilli were detected. Hen 1924 laid one non-fertile egg and one fertile egg (not hatching) which contained acid-fast bacilli.

TABLE VI

EGG PRODUCTION AND HATCHABILITY OF EGGS FROM 10 HENS

INOCULATED WITH 100 AVIAN TUBERCLE BACILLI

Bird	Inoculation age	Condition of reproductive organ	Number of eggs laid	Number of non-fertile eggs	Number of eggs with dead embryo	Number of chicks hatched
1909	7-8 mo.	productive	1	1	0	0
1942	"	non-productive	6	3	0	3
1970	"	productive	6*	2	1	3
1924	"	non-productive	22**	7	1	14
1966	"	non-productive	23	5	2	16
396	"	productive	27	5	2	20
344	"	productive	31	11	2	18
325	"	productive	34***	12	3	19
1977	"	non-productive	45	10	4	31
126	"	productive	46	7	3	36
10 birds			241	63	18	160
			* 1 broken ** 2 broken *** 1 broken			
10 birds			237 eggs placed in incubator	63	18 (67%)	160 hatched

TABLE VII

SUMMARY OF THE EXAMINATION OF 81 EGGS, FROM INJECTED HENS, FAILING TO HATCH

Hen	Unembryonated eggs	Embryonated eggs	Microscopic examination results *	Petragnani culture results *	Embryo inoculation results (from egg, yolk sac, or culture) *	Death in weeks **	Necropsy results
1909	1	0	Negative	Negative		K-20W	Tuberculosis of ovary, digestive tract, and mesentery
1942	3	0	Negative	Negative		K-20W	Tuberculosis of ovary and lung
1970	2	1	Negative	Negative		D-18W	Extensive tuberculosis of ovary, lungs and omentum
1924	6	2	1 egg had A-F organisms 1 embryo had A-F organisms	No growth from egg Growth of A-F organisms from yolk of 1 embryo at 9 weeks	No growth from egg Growth from culture killed embryo in 6 days. A-F bacilli seen	K-21W	Extensive tuberculosis of ovary, intestine, two small lesions in spleen
1966	6	1	Negative			D-16W	Ovary slightly tuberculous
396	6	1	Negative	Negative		K-14W	No lesions noted

* A-F --- Acid-Fast

** K -- Killed
D -- Died

TABLE VII (Cont.)

SUMMARY OF THE EXAMINATION OF 81 EGGS, FROM INJECTED HENS, FAILING TO HATCH

Hen	Unembryonated eggs	Embryonated eggs	Microscopic examination results *	Petragnani culture results *	Embryo inoculation results (from egg, yolk sac, or culture) *	Death in weeks **	Necropsy results
344	10	3	A-F granules seen in 2 eggs	Negative	Negative	K-14W	Few small nodules in intestine and mesentery
			No A-F organisms seen in embryos	Negative	Negative		
325	12	3	Negative	Negative		K-14W	No lesions noted
1977	9	5	Three eggs had A-F organisms	Scant growth of A-F bacilli from 1st. egg in 10 weeks	Growth from first egg killed both embryos in eight days. A-F bacilli seen	K-20W	Tuberculosis of ovary, intestine, and mesentery
				A-F granules from 2nd egg	Embryo not affected		
				No growth from 3rd egg	Embryo not affected		
126	8	2	Two eggs had A-F organisms	Growth of A-F bacilli from 1 egg in 7 weeks	Growth from culture killed embryo in 6 days. Many A-F bacilli seen	K-14W	Tuberculous lesions of intestine, and ovary
			No A-F organisms in embryos	No growth from embryos			
10 Hens	63	18					

* A-F --- Acid-Fast

**K -- Killed
D -- Died

Inoculation of a representative portion of the non-fertile egg on Petragnani's medium resulted in no growth after 11 weeks incubation at 37° C. Also the inoculation of two normal embryonated eggs with this material gave negative results. It was assumed that these acid-fast organisms either were not virulent or were dead tubercle bacilli. One dead embryo from this hen contained acid-fast organisms which were recovered on Petragnani's medium after nine weeks incubation. A portion of this growth was inoculated into the yolk of two normal embryonated eggs. One embryo died six days, the other seven days after inoculation; and many acid-fast organisms were observed microscopically in the yolk material. The infectivity of the ovarian transmitted bacilli was thus established.

Hen 1977 laid three non-fertile eggs in which acid-fast organisms were seen microscopically. From one of these eggs tubercle bacilli were cultivated on Petragnani's medium. This culture produced death of two normal embryonated eggs eight days after inoculation. Many acid-fast organisms were observed microscopically in the yolk sac material. Typical mycobacterial growth was not observed on Petragnani's medium inoculated with material from the second egg. The granular, acid-fast material from this medium was inoculated into two normal embryonated eggs. The embryos were unaffected.

No growth was observed on Petragnani's medium within eight weeks after inoculation of a representative portion of the yolk from the third egg.

Hen 126 laid two eggs in which acid-fast organisms were seen microscopically. Growth of these organisms was evident on Petragnani's medium seven weeks after inoculation. Acid-fast organisms in large numbers were observed microscopically in the yolk sacs of the two normal embryonated eggs that died six days after inoculation.

Hen 344 laid three eggs (one fertile) which contained acid-fast granular material. Inoculation of this material on Petragnani's medium and into two normal embryonated eggs gave negative results.

Each inoculated hen was necropsied immediately after death and all lesions recorded. Eight of the ten hens developed lesions of tuberculosis within the eight month observation period. Avian tubercle bacilli were isolated, cultured, and identified from the eggs of three of these hens.

Results of the examination of 160 eggs, from injected hens, which hatched: The 160 eggs, from injected hens, which hatched were collected daily, dated, and source recorded. They were placed in the hatching incubator and candled each day to determine fertility and development of the embryo. When hatching occurred, the shell was removed from the incubator and the chicks left undisturbed for six to eight hours. Each chick was wing-banded and quarantined in a clean, electrically heated brooder for further observation. Detailed

TABLE VIII

SUMMARY OF THE RESULTS OF THE EXAMINATION OF 160 EGGS, FROM INJECTED HENS, WHICH HATCHED

Hen	Egg (s) Number	History after hatching*	Microscopic examination**	Petragnani's** medium	Embryo Inoculation	Tuberculin test 6 mo.	Necropsy
1942	1	A-7 mo.				-	Negative
	2	A-7 mo.				-	"
	3	D-4 days	Negative	No growth			"
1970	1	A-7 mo.				-	"
	2	A-7 mo.				-	"
	3	A-7 mo.				-	"
1924	1-6	A-7 mo.				all -	"
	7	A-7 mo.				+	"
	8-9	A-7 mo.				both -	"
	10	D-3 days	Negative	No growth			"
	11	D-5 days	Few A-F bacilli	Growth of A-F bacilli in 9 weeks	Killed embryo (6 days)		
1966	12-14	A-7 mo.				both -	"
	1-3	A-7 mo.				all -	"
	4	D-2 days	A-F granules	No growth	Embryo unaffected		
	5	D-11 days	Negative	No growth			"
	6-11	A-7 mo.				all -	"
	12-13	A-7 mo.				both -	"
	14	D-8 days	Negative	No growth			"
	15-16	A-7 mo.				both -	"
396	1-12	A-6 mo.				all -	"
	13	D-5 days	Negative	No growth			"
	14-16	A-6 mo.				all -	"
	17	D-4 days	Negative	Negative			"
	18-20	A-6 mo.				both -	"

* A - Alive
D - Dead

** A-F — Acid-Fast

TABLE VIII (Cont.)

SUMMARY OF THE RESULTS OF THE EXAMINATION OF 160 EGGS, FROM INJECTED HENS, WHICH HATCHED

Hen	Egg (s) Number	History after hatching*	Microscopic examination**	Petragnani's medium**	Embryo Inoculation	Tuberculin test 6 mo.	Necropsy
344	1-14	A-6 mo.				all -	Negative
	15	D-2 days	Negative	Negative			"
	16	D-1 day	Negative	Negative			"
	17-18	A-6 mo.				both -	"
325	1	D-8 days	Negative	Negative			"
	2-8	A-6 mo.				all -	"
	9	D-1 day	Negative	Negative			"
	10-17	A-6 mo.				all -	"
	18	D-3 days	Negative	Negative			"
	19	A-6 mo.				-	"
1977	1-4	A-7 mo.				all -	"
	5	D-3 days	A-F granules	No growth	Embryo unaffected		"
	6-19	A-7 mo.				all -	"
	20	D-4 days	Negative	Negative			"
	21	D-4 days	Negative	Negative			"
	22-25	A-7 mo.				all -	"
	26	A-7 mo.				+	"
	27-29	A-7 mo.				all -	"
	30	A-7 mo.				+	"
	31	D-3 days	Negative	Negative			"
126	1-4	A-6mo.				all -	"
	5	D-2 days	Negative	Negative			"
	6-18	A-6 mo.				all -	"
	19	D-4 days	Negative	Negative			"
	20	A-6 mo.				all -	"
	21	D-2 days	Negative	Negative			"
	22-31	A-6 mo.				all -	"
	32	D-5 days	A-F bacilli	No growth	Embryo unaffected	-	"
	33-36	A-6 mo.				all -	"
Total	160						

* A - Alive
D - Dead

** A-F — Acid-Fast

records were kept of the history of these birds. This information is presented in Table VIII.

It can be seen from this table that 21 chicks died within 11 days after hatching. The unabsorbed yolks were examined for acid-fast bacilli microscopically and culturally as described in the previous paragraphs.

Egg number 11 laid by hen 1924 was fertile and hatched. The chick died at five days of age. Ziehl-Neelsen staining of the yolk material revealed a few acid-fast bacilli. Growth of these organisms occurred after nine weeks incubation on Petragnani's medium. The pathogenicity of the organism was established by inoculation of two normal embryos which died on the sixth day. Many acid-fast bacilli were observed microscopically in the yolk sac material from this egg.

Acid-fast granules were observed in the yolk sac of two chicks which died within two and three days respectively after hatching. One egg was laid by hen 1966 (its fourth) and the other came from hen 1977 (its fifth). Inoculation of this suspicious material on Petragnani's medium resulted in no growth after 11 weeks incubation.

All birds hatched from tubercle bacilli injected dams and alive at six months were tuberculin tested. The results of these tests are shown in Table VIII, and reveal that 136 birds were tuberculin negative while three birds were tuberculin positive. These three tuberculin positive birds were hatched

from eggs laid by hens 1924 and 1977. From Table VII it may be seen that these tuberculin positive hens laid non-fertile eggs containing tubercle bacilli. In addition, hen 1924 laid one fertile egg which hatched an infected chick.

(Results of hemagglutination tests)

Results of the hemagglutination tests with sera from chickens injected with Mycobacterium avium: The results of the hemagglutination tests with sera of five chickens injected five months previously with avian tubercle bacilli show that a titer of 1:32 or above was obtained with each serum. Table IX show the titers obtained. The serum from chicken 1909 (Table I) gave a titer of 1:256 and, as can be seen, the end point was not reached. All these birds reacted to 0.25 ml. of avian tuberculin administered intradermally one month after injection with the organisms. There was correlation between a positive hemagglutination test and tuberculin sensitivity. All these birds died from the disease during the next five weeks.

Tuberculin and hemagglutination reactions of 41 chickens hatched from eggs experimentally injected with tubercle bacilli: Sera were collected from 41 seven-month old chickens hatched from eggs experimentally injected with tubercle bacilli and stored in the frozen state. Each chicken was tuberculin tested

TABLE IX

RESULTS OF HEMAGGLUTINATION TESTS WITH SERA FROM FIVE HENS INJECTED WITH MYCOBACTERIUM AVIUM

Serum Dilutions	Hens 126	1909	1942	1924	1977	Negative Serum Control
1:4	++++	++++	+++	+++	++++	-
1:8	+++	+++	++	+++	++++	-
1:16	+++	+++	+	+++	+++	-
1:32	+	+++	+	++	+++	-
1:64		+++		+	++	-
1:128		+++			++	-
1:256		+++			+	-
serum control (unsensitized sheep cells-serum)	-	-	-	-	-	-
saline control (sensitized sheep cells-saline)	-	-	-	-	-	-
Tuberculin reaction	+	+	+	+	+	

and sacrificed for necropsy at the end of the eight-month observation period. Seven chickens reacted to tuberculin and each showed lesions of the disease with tubercle bacilli present. The sera from these seven birds exhibited hemagglutination in a dilution of 1:16 or higher. The remaining 34 chickens varied in their reaction to the tuberculin test and none showed lesions of the disease. Whereas 23 or 68 percent of these 34 chickens reacted positively to the tuberculin test, 31 or 91 percent revealed hemagglutinins in a dilution of 1:4 or highers. Three sera contained no hemagglutinins and the chickens from which they were taken did not react to the tuberculin test. The results of this experiment are given in Table X.

Hemagglutination results with sera from six chickens before and after the administration of avian tuberculin: It has been observed by Smith and Scott (1950) that "stimulation" of humans with tuberculin often elicits a positive hemagglutination reaction. Therefore, an attempt was made to determine if avian tuberculin administered to normal adult chickens could produce hemagglutinins sufficient to give a positive hemagglutination reaction. Ten cubic centimeters of serum from each of six normal adult chickens was collected and stored in the frozen state. Each chicken was then given intradermally in one wattle 0.25 ml. avian tuberculin. All were tuberculin negative. Seven days later the sera from four

TABLE X

TUBERCULIN AND HEMAGGLUTINATION REACTIONS OF 41 CHICKENS HATCHED FROM EGGS EXPERIMENTALLY INJECTED WITH TUBERCLE BACILLI

Hemagglutination Titers	Number of chickens showing hemagglutination titer	Number of chickens showing lesions and tubercle bacilli	Tuberculin positive	Number of chickens not showing lesions	Tuberculin positive	Tuberculin negative
0	3			3		3
1:4	4			4	2	2
1:8	5			5	2	3
1:16	13	1	1	12	9	3
1:32	6	1	1	5	5	0
1:64	6	2	2	4	4	0
1:128	4	3	3	1	1	0
1:256	0			0		
Serum Control	-					
Saline Control	-					
Totals	41			31 (91%) 1:4 or above	23 (68%)	11 (32%)

of the birds were collected and stored in the frozen state, and three weeks later the sera from the remaining two birds were collected. Hemagglutination tests were performed with each serum. Every serum was negative except one which was positive 21 days after the administration of tuberculin.

DISCUSSION

The transmission of avian tubercle bacilli through the eggs of tuberculous chickens has been suggested for several years. Many investigators have observed acid-fast organisms in eggs laid by naturally or artificially infected hens (Artault, 1895; Mohler and Wasburn, 1908; and Bigger, 1932).

It is also known that inoculation of embryonated eggs with avian tubercle bacilli has resulted in the hatching of infected chicks which usually develop active tuberculosis. (Feldman, 1938). However, the transmission of the tubercle bacillus through the egg of the diseased hen to infect the hatched chick has not been proved.

The main purpose of this study was to determine whether a tuberculous bird could be hatched from an artificially infected hen. Two methods were selected to attain this objective. They were 1) inoculation of embryonated eggs to determine the ability of a tuberculous embryo to hatch and develop active tuberculosis, 2) inoculation of the ovary of normal adult hens, collecting and hatching their eggs, and finally re-isolating and identifying the tubercle bacilli from the hatched chick. The objective was accomplished by both methods.

The number of tuberculous birds hatched from infected hens or embryonated eggs was not deemed overly important to this problem. Definite proof of the transmission of virulent avian tubercle bacilli through one egg to the offspring would establish the fact that tuberculosis may be transmitted to a flock in this manner.

Granular forms of acid-fast organisms have been known for many years. Koch concluded the granular segments were spores but their resistance to high temperature lead the majority of bacteriologists to doubt his theory. Much (1907) found that tubercle bacilli do break up into small segments or separate granules of various size, some acid-fast and other non-acid-fast. The significance of these granules is not completely understood. Yegian et al. (1947) presented evidence that a beaded or granular appearance may represent a staining artifact. Others believe them to be non-infective fragments of dead bacilli or an active stage of the life cycle of the bacillus. Kahn (1929) and Sweany (1928) conducted experiments which lead them and others to believe that granules can produce progressive tuberculosis in animals. It is generally concluded, however, that the evidence in favor of the pathogenicity of granules or filtrates of tuberculous cultures or lesions is insufficient to be regarded as a fact.

In the course of this study each embryonated egg and normal adult hen was inoculated with approximately the same number

of organisms. One striking observation was the marked variation in the infectivity of the organisms and disease processes in the individual egg and birds. An explanation of the factors that determine the extent of any tuberculous infection is not known. To explain these results it would be necessary to know 1) the virulence of the bacilli, 2) the number of bacilli that initiate infection, 3) the degree of native resistance, 4) the degree of acquired resistance, 5) the tissues in which infection occurs, and 6) possibly, the degree of hypersensitivity.

Inoculation of 242 normal embryonated eggs with 50 tubercle bacilli resulted in the death of 115 or 47.5 percent. Only 85 of the 127 (242-115) embryos which hatched were alive after eight months. This high mortality was due to the action of the tubercle bacilli as determined by a comparison with the mortality controls of which two out of 16 died. The specific action of the tubercle bacilli and their products in the tissue is not known. These organisms contain materials which incite the type of reaction characteristic of that caused by many foreign substances. The bacilli contain other materials which aid in setting up a state of hypersensitivity. This hypersensitivity permits the organisms to act as toxins for the altered tissues of the host.

Tuberculin tests were performed on the 85 chickens hatched from injected embryonated eggs when the birds were eight months old. Sixty or 70.5 percent of the birds gave

a positive tuberculin reaction. Twenty-seven of these 60 tuberculin positive birds showed lesions characteristic of the disease. It is of interest to note that 25 of the 85 birds (29.4 percent) showed no reaction to tuberculin and were devoid of lesions. In view of the fact that each of these 85 birds was infected in the embryonic stage, the cause of these negative tuberculin reactions is difficult to explain.

A positive tuberculin reaction means that the individual has been rendered hypersensitive through infection or vaccination with living or dead bacilli, and does not offer any evidence regarding the degree of infection. In human medicine, a persistent negative tuberculin test is, in part, evidence against the presence of active tuberculosis. However, one should not regard a negative test as evidence that the host was never infected by the tubercle bacillus.

It is very possible that some of these tuberculin negative birds might have developed active tuberculosis and become tuberculin positive if allowed to survive. This statement is made not only with a knowledge of the limitations of the tuberculin test but also because of the observation that cryptic infection developed in the yolk stalk of several birds. The tubercle bacilli were present without noticeable lesions in the yolk stalk and could be disseminated into other organs and tissues at some later time.

Inoculation of six normal adult hens with 100 tubercle bacilli, directly into the ovary resulted in the development of progressive tuberculosis in all. No eggs were laid by these birds and all died within 12 weeks after inoculation.

Ten hens inoculated in a similar manner were artificially inseminated each week. A total of 241 eggs were laid by these ten hens. Sixty-three eggs were non-fertile, 18 fertile eggs failed to hatch because of death of the embryo, and 160 eggs hatched. All chickens hatched from these 160 eggs were examined microscopically, culturally, and by inoculation of normal embryos for tubercle bacilli. By examining each egg, embryo, and hatched bird individually the transmission of tubercle bacilli through the egg was accurately ascertained. Infectivity studies of the organisms from each specimen was also performed.

Of the 81 eggs laid by these ten hens, which failed to hatch, nine contained acid-fast bacilli. Isolation and identification of tubercle bacilli was made by microscopical examination, cultural growth, and inoculation of normal embryos. Three of the nine eggs contained virulent avian tubercle bacilli.

One hundred and sixty eggs from the injected hens hatched. The chicks were quarantined in strict isolation in new quarters which had never housed animals. They were kept under observation for eight months. Twenty-one of these 160 chicks died within 11 days after hatching. Each was examined microscopically, culturally, and by inoculation of normal embryos for tubercle bacilli. One chick, which died on the fifth day after hatching

contained acid-fast bacilli in its yolk sac. These organisms were cultivated on Petragnani's medium. They produced characteristic mycobacterial growth on this medium and their infectivity was established by inoculation of normal embryos. Two chicks died on the second and third day, respectively, after hatching and acid-fast granules were observed microscopically in their yolk sacs. No growth occurred on Petragnani's medium after 11 weeks of incubation. The two embryos inoculated with a portion of the yolk sac material were unaffected. A third chick died on the fifth day after hatching. Acid-fast organisms were observed microscopically in its yolk sac but upon transfer to Petragnani's medium no growth occurred. Inoculation of normal embryos with this material did not show evidence of tubercle bacilli.

Avian tuberculin was administered intradermally into one wattle of each bird six months after hatching. Three of the 139 birds remaining (160-21) reacted to tuberculin. It is of interest to note that these three birds were hatched from eggs of hens which also laid non-fertile eggs containing tubercle bacilli.

It is known that most human beings with active tuberculosis possess antibodies in their serum which give hemagglutination reactions with tuberculin sensitized sheep red blood cells. This report indicates similar results with the serum of tuberculous chickens and sensitized sheep red blood cells. Strong

hemagglutination reactions were obtained with sera from five actively tuberculous chickens which correlated with their tuberculin reactions.

In another experiment serum was collected from 41 chickens (seven-months-old) which had been injected with avian tubercle bacilli as seven-day-old embryos. Tuberculin tests were then performed on all these birds which were later sacrificed for postmortem study. Seven birds showing lesions with tubercle bacilli were tuberculin positive and their sera revealed hemagglutinins in high dilution. Twenty-three of the remaining 34 chickens were also tuberculin positive. The sera of 31 possessed hemagglutinins in a dilution of 1:4 or above. It is of interest to note that the sera of eight of 11 tuberculin negative chickens revealed hemagglutinins in dilutions of 1:4 through 1:16. Of equal interest are the sera of nine tuberculin positive chickens which possessed high agglutination titers.

Intradermal inoculation of six birds with avian tuberculin revealed no increase in the hemagglutination content of the sera of five birds. The serum of one bird showed agglutinins up to 1:4 dilution 21 days after stimulation. Larger numbers of sera should be checked before definite statements are made concerning the value of the hemagglutination reaction in avian tuberculosis. Whereas the test does not yet have prognostic or diagnostic value even in human tuberculosis, it is of interest to know that chickens possess antibodies for the avian tubercle

tubercle bacillus. Information obtained from the hemagglutination reaction provides another useful tool in the study of tuberculosis.

SUMMARY

1. Inoculation of embryonated eggs with virulent avian tubercle bacilli resulted in the death of approximately fifty percent of the embryos. Injection with sterile culture medium resulted in the death of two out of 16 embryos.

2. Sixty of 85 (70.5 percent) chickens hatched from inoculated eggs gave a positive tuberculin reaction at six months of age. Twenty-five (29.5 percent) of the 85 birds were tuberculin negative at six months of age.

3. Rapidly fatal tuberculosis developed in six birds out of 16 inoculated (37 percent) with avian tubercle bacilli directly into the ovary. Ten of these 16 inoculated birds laid a total of 241 eggs. Eighty-one of these eggs failed to hatch.

4. Acid-fast organisms were seen microscopically in nine of the 81 eggs (11 percent) failing to hatch. Virulent avian tubercle bacilli were isolated and identified from three of the nine eggs.

5. One hundred and sixty eggs, laid by the ten hens, hatched. Twenty-one of these (16.1 percent) chicks died within 11

days after hatching. Avian tubercle bacilli were isolated and identified from one of the dead chicks.

6. Three of the ten inoculated hens produced 1) three eggs which failed to hatch and which contained avian tubercle bacilli; 2) one chick dying on the fifth day which contained avian tubercle bacilli; and 3) three eggs which hatched and became tuberculin positive at six months of age.

7. Tuberculin testing of 139 birds hatched from tuberculous dams showed that three (2.1 percent) were sensitive to avian tuberculin.

8. A hemagglutination reaction is described in which sera from tuberculous chickens and tuberculin sensitized sheep red blood cells are used.

9. The sera of five tuberculin positive chickens revealed a titer of 1:32 or above with each serum.

10. The sera of eight tuberculin negative chickens, previously inoculated in the embryonic stage, showed hemagglutinins in dilutions ranging from 1:4 through 1:16.

11. The sera of 23 tuberculin positive chickens exhibiting no lesions had agglutination titers from 1:4 through 1:108.

12. There was no significant increase in hemagglutinins in the serum of apparently healthy chickens "stimulated" with tuberculin.

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