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Studies of pleuropneumonia-like organisms

John A. Duerre
STUDIES OF PLEUROPNEUMONIA-LIKE ORGANISMS

By

John A. Duerre

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Thesis Advisor

Head of the Major Department
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INTRODUCTION

The occurrence of a great number of poultry diseases which affect the respiratory systems of both chickens and turkeys has brought about extensive studies on control measures to curb losses due to these infections. The losses due to these infections are tremendous throughout the poultry producing areas of the world.

Infectious laryngotracheitis, Newcastle disease, fowl pox, infectious bronchitis, chronic respiratory disease, infectious sinusitis, fowl cholera and aspergillosis cause similar symptoms and gross lesions (19). Diagnosis and preventive treatment are of extreme importance. Fowl cholera and aspergillosis can be detected by cultural techniques. The other six diseases show differential diagnosis with histological examination of lungs, trachea and air sac. The respiratory diseases; chronic respiratory disease in chickens (CRD) and infectious turkey sinusitis (ITS) are of prime importance. There is a report of 90-100 per cent mortality in one flock of 500 birds (15), with two more examples of 100 per cent mortality in flocks of 500 birds each (15). The greatest losses have been reported in weight loss, loss of egg production, and of fertility in breeder stock (14,16,26). J.B. Prior (31) found in overall studies of ITS a morbidity of 25 per cent and with low mortality. McDowell (30) estimated for the 1961 Delmarva production of 167,000,000 broilers, an average loss of six per cent above normal or 10,000,000 birds.

The purpose of these experiments were to check the effects of Aureomycin in the diet of chickens as a possible control measure against CRD, and to study the methods of isolation and culturing of the agent
or agents involved.
REVIEW OF LITERATURE

In 1935 coco-bacilliform bodies were found by Nelson (20) to be associated with infectious fowl coryza. This organism was a Gram negative coco-bacilliform body found in the exudate and measuring from 0.1 to 0.5 micron in diameter. This agent caused a disease of low onset while Hemophilus gallinarum, a small Gram negative rod associated with fowl coryza, produced a disease of rapid onset. Lee, in 1942, (25) considered H. gallinarum as the most serious cause of sinusitis of turkeys. However, this disease is believed by many investigators (15) to be identical with CED in chickens.

The characteristics of the agent or agents involved have been studied by many investigators since that time. Laidlow and Elford in 1938 (23) demonstrated this group of organisms to be filterable and from 125 to 175 millimicrons in diameter.

Delaplane in 1944 (2) recovered a pleomorphic Gram negative rod from infectious sinusitis of turkeys. Small coccoid rods and filaments were observed in blood culture while those on agar showed long filaments. His studies indicated an organism probably belonging to the genus Pasteurella.

S.B. Hitchner in 1949 (15) found no evidence of bacterial cause of the disease. He classified the agent as possibly a rickettsial organism.

Justad and Hamilton in 1948 (21) thought that the causative agent was a virus. They found the virus could be cultivated on chick embryos, and that it also occurs naturally in turkey embryos from breeders infected with the respiratory form of the disease. They found that
mice and pheasants in all instances, and chickens in most instances, remained normal following inoculation with the virus.

Delaplane in 1949 (3) suggested that the virus of CRD of chickens and the virus of ITS were identical. He noted a non-bacterial agent which could not be differentiated from the virus of infectious sinusitus of turkeys, which was isolated from three groups of chickens exposed to turkeys affected with ITS. Jungherr in 1949 (18) and Johnson in 1951 (17) also could not differentiate an agent involved in air sac colds of broilers from the virus of turkey sinusitus.

Fahrey in 1955 (8) described chronic respiratory disease of ducks with the gross pathological picture the same as for CRD in chickens. However, the agent would not induce ITS or CRD. Morphologically the organisms appeared the same as other avian pleuropneumonia-like organisms (APPLO) but would not ferment sucrose or maltose, and dextrose was weakly fermented. There was a possibility that it played no role in the disease, for a virus could also be demonstrated to be present.

Boney et al in 1952 (1) found in turkeys a non-bacterial agent differing from agents recovered from CRD of chickens and capable of causing involvement of the air sacs. This viral agent isolated from turkeys easily falls into the classification of the lower form of turkey sinusitus. The lower respiratory form of CRD or ITS involves lungs and air sacs; while the upper respiratory form involves sinuses and trachea.

Grumbles et al in 1952 (12) found that the clinical symptoms of CRD were manifested concurrently with an outbreak of Newcastle disease. Respiratory symptoms were still present eight weeks after the onset of infection, instead of subsiding two weeks after the onset like typical Newcastle disease. Von Roekel et al in 1952 (35) also found
that bronchitis- and Newcastle refractory birds, both chickens and turkeys, when treated with a suspension of the agent recovered from ITS or CRD developed symptoms of the natural disease.

Markham and Wong in 1952 (27) suggested from experimental evidence that the agent of ITS or CRD of chickens belongs to the pleuropneumonia-like group of organisms. The sensitivity of the organisms to antibiotics, the chronic nature of the lesions, and the joint lesions in chick embryos, were consistent with pathology caused by certain strains of the PPLO group. The possibility of their being an "L" form of bacteria was excluded with finality (6).

The bovine pleuropneumonia-like group of organisms demonstrated by Sabin (33) can be cultivated on cell free media. They produce rings, globules, filaments and minute filterable bodies 125-250 millimicrons in diameter. The colonies produced on PPLO agar are in range of 10 to 600 microns in diameter. A possible life cycle proposed by Sabin suggested that the filterable viable elements develop into filamentous and ramifying bodies. These filamentous and ramifying elements when stained with Giemsa preparation appear as deeply stained chromatic areas, from which consolidation nodes appear, with further maniliform growth. Further pullulation of these elements by unipolar or multipolar pseudopodial budding furnishes the mass of polymorphic units, rings, spheres, filaments.

Several strains of PPLO have been isolated; Nocard in 1896, as described by Dienes (5) first isolated PPLO from cattle. He could not demonstrate individual cells. Bordet in 1910, as described by Dienes (5) succeeded in demonstrating individual cells in Giemsa preparations. Besides granules and round forms he saw branching filaments. To this
organism he gave the name Asterococcus. The second organism identified with this group was isolated by Kleineberger from agalactia in goats in 1923 (22). Since that time PPLO have been identified in various other lower animals and in man.

PPLO of rats, mice and man, in young cultures, are small bacilli with bipolar staining (5). The bacillary forms swell into large spheres. These either produce vacuoles and degenerate into empty blebs, or reproduce small bacillary forms inside a limiting membrane. Morton et al. in 1954 (29) showed in electronmicrographs of human PPLO, round bodies 0.5 - 3 microns in diameter associated with rod forms and filaments up to 5 microns in length.

PPLO appear to be associated with air sac infection in poultry (9). They are readily stained with Gram, Macchiavello or Giemsa stain. Morphologically they are extremely pleomorphic, manifesting blue stained, curved rod like structures and coccoid bodies.

Electronmicrographs by Reagan et al. (32) of four strains of the causative agents recovered from CRD showed spherical particles 200 to 250 millimicron in diameter, and virus-like particles 50 to 60 millimicron in width. Two strains possessed no virus like structures. This indicated the possibility that two agents are required to produce symptoms of disease. White et al. in 1964 (37) also demonstrated single cells from 0.1 to 0.5 micron in diameter by electronmicrographe; with an average diameter of 0.3 micron; large filaments about the diameter of single cells and smaller filaments were also observed. They could demonstrate no differences in three CRD strains and two ITS straine by electronmicrographs and hemagglutination studies. Hemagglutination
occurred with all strains and similar antibodies could be demonstrated by hemagglutination inhibition tests with either C9D or ITS strains. Gianforte et al in 1956 (9) also noted no difference in hemagglutination tests with seven different strains of PFLO; three strains from chickens, two from turkeys, one from a partridge and one from a pigeon. All strains showed approximately the same titer in hemagglutination inhibition tests. Physiological tests appeared similar, giving acid from dextrose, maltose, mannose, sucrose and trehalose, and slight acid from levulose. No gas was produced in any of the media. They refuted the possibility of virus origin because their organisms grew on artificial media. They classified the agent as belonging to the PFLO group of organisms; however, their classification does not completely satisfy the description of PFLO set down by Kleineberger (1934) (22) and Dienes (1939) (5). But it appears that at the present time the etiologic agent exists as one species and belongs to the PFLO group.

The methods of treatment and control of the disease have been studied by many investigators. Dickenson and Hinshaw (4) first treated infectious sinusitis of turkeys with argyrol and silver nitrate in 1938. Silver nitrate showed the best results, but it was very caustic.

Since the advent of antibiotics many investigators have undertaken studies of the effectiveness of various antibiotics in the treatment of the disease. Hitchner in 1949 (15) found that 20,000 units of penicillin per milliliter of infected allantoic fluid did not prove inhibitory, but as little as 2500 units of streptomycin per ml. of
infected allantoic fluid prevented infection in turkeys when directly inoculated into the infraorbital sinuses. Turkeys which developed swollen sinuses completely recovered in from seven to ten days following injection of 50 milligrams of streptomycin into the infraorbital sinuses. Birds infected with the lower form of the disease recovered in from eight to fifteen days when 150 milligrams of streptomycin was injected into the dewlap.

McArthur in 1950 (28) showed 90 per cent recovery out of 300 turkeys infected with ITS when injected with 100 to 150 milligrams of streptomycin into their infraorbital sinuses. The majority of the remaining ten per cent recovered upon retreatment, with only three birds dying.

Von Roekel et al in 1952 (25) showed that streptomycin, Aureomycin chloromycetin and Terramycin exerted an inhibitory effect on infection in chickens inoculated intrasinusoidally with a suspension of the agent recovered from CRD of chickens. Wong and Jones in 1953 (39) showed that only the antibiotics which are effective against the rickettsial group produce definite effects against this agent. Magnamycin and Terramycin being the most effective.

Grumbles and Boney in 1961 (11) showed that chloromycetin in concentrations of 0.5 per cent in mash resulted in 100 per cent recovery in ten days in turkeys with induced infections, and in 0.25 per cent concentration resulted in 100 per cent recovery in seven days, indicating 100 per cent recovery in all trials when 0.25 per cent and 0.5 per cent chloromycetin was used. Chloromycetin in 0.1 per cent concentration resulted in only ten per cent recovery in fourteen days; while, 0.5 per cent Terramycin in mash resulted in only 20 per cent
recovery in seven days.

Lecce and Sperling in 1955 (34) showed that if chickens were artificially infected with the agent of CRD and treated with Terramycin or Aureomycin in water or subcutaneous injection, PPLO could not be isolated from the tracheae of the treated birds. However, nine weeks after they discontinued treatment it was possible to isolate PPLO from these birds.

White-Stevens et al in 1954 (33), under commercial field conditions, indicated by feeding trials that broilers with sub-clinical infections of CRD maintained a normal growth level on a diet including 50 to 100 grams of Aureomycin per ton of feed. However, this amount was not sufficient to maintain or restore growth when a severe clinical outbreak occurred. Concentrations of approximately 400 grams per ton of feed or 360 milligrams per gallon of drinking water for a period of fourteen days followed by 50 to 100 grams per ton of feed would sustain growth until marketing.
EXPERIMENTAL DATA AND DISCUSSION

Several cultures of the causative agents of both chronic respiratory disease (CRD) in chickens and infectious turkey sinusitis (ITS) were received through the courtesy of Dr. G.S. Appelton of the American Scientific Laboratories, Madison, Wisconsin and from Dr. J. Markham of the Lederle Laboratories, Pearl River, New York. However, these strains were not sustainable on artificial media as recommended by Sabin et al. (33, 24) for the growth and recovery of pleuropneumonia-like organisms (PPLO).

Strain C-15 and C-17 of CRD agent received from Dr. Appelton and strain X-245 of ITS agent received from Dr. Markham produced typical mortality patterns in ten day old chick embryos (27). However, colony growth was never recognizable on Bacto PFLO Agar (7).

There were several cases of apparent turkey sinusitis present among breeder turkeys at the Poultry Farm, South Dakota State College. These birds manifested a typical clinical upper respiratory infection similar to that produced by PPLO (15). The gross symptoms readily recognizable were shaking the head, nasal discharge, coughing, and distension of one or both infraorbital sinuses by an almost colorless gelatinous exudate.

The sinuses of seven of these birds were aspirated using syringes with 16 gauge needles, recovering from four to six ml. of clear exudate from the sinuses of each bird. Of this exudate one ml. amounts from each bird were inoculated into two sets of tubes. The first set contained ten ml. of Bacto PFLO Broth Base with one per cent Bacto PFLO Serum Fraction (7); 1000 units of penicillin per ml. (15) and 0.01
gram of crystal violet per liter (7) were used as bacteriostatic agents. The second set of tubes contained 10 ml. of fresh beef heart infusion broth (35) prepared as follows: five hundred grams of fresh beef heart was ground in a Waring Blender and infused in 1000 ml. of double distilled water over night at 4°C, then heated to 60°C for one hour and boiled for five minutes. This broth was then filtered. The filtrate volume was reconstituted to 1000 ml. with distilled water; ten grams of Bacto Peptone and five grams of sodium chloride were then added. This mixture was heated to boiling and refiltered to produce a clear yellowish brown broth base. Phenol red was added as an indicator and the pH adjusted to approximately 7.8 with ten per cent NaOH. One gram of Bacto Glucose was added and the medium was autoclaved at 15 pounde pressure for fifteen minutes and cooled to room temperature. One per cent by volume of Bacto PPLO Serum Fraction was added. As bacteriostatic agents a Seitz filtered five per cent aqueous thallium acetate solution was added at the rate of 0.15 ml. per ml. (34) of the medium, and 1000 units penicillin per ml. This amount of thallium acetate brought about slight precipitation in broth and also proved in later studies to be inhibitory. The basal medium used in these studies proved highly satisfactory for growth and was used throughout the entire study as a means of isolation and cultivation of the agent under investigation.

These cultures were incubated at 38.5°C for a period of seven days. Then 0.2 ml. were pipetted onto a plate of Bacto PPLO Agar containing one per cent Bacto PPLO Serum Fraction, and spread with a bent glass rod. These plates were sealed with rubber bands to prevent excessive drying and allowed to incubate at 38.5°C. From the fourth
day on, daily microscopic examination of the plates, with the low power objective (100x) was undertaken to detect the presence of typical PPLO colonies (5). On the seventh day after inoculation typical colonies were detected on one set of plates which had been inoculated from one of the beef heart infusion cultures. This strain was designated as T-2.

These colonies were microscopic in size varying from 10 to 175 microns in diameter. They appeared to be amorphous in structure with a dense zone in the center and clearer periphery. Denser growth developed in the center of the colonies after seven to eight days from their first appearance. These particles appear as dense chromatic structures arising in the center or occasionally at the outer edge of the colony. The following photomicrographs were taken of organisms growing on PPLO agar. (Plates I and II pp. 13 and 14)

Several transfers were made after seventeen days of incubation by cutting out small agar blocks containing colonies and placing them in ten ml. of beef heart infusion broth containing no inhibitors. These cultures were then incubated for three days, and a pH change was noted in the broth. The broth was inoculated into chick embryos, into one month old chickens, and sub-cultured in beef heart infusion broth. Two tenths ml. of enrichment broth containing the agent was inoculated into six embryos, three via allantoic inoculation, and three by the yolk sac method (10).

When death of the embryos was noted, 0.3 ml. of allantoic fluid was inoculated into beef heart infusion broth, containing 1000 units of penicillin per ml. as a bacteriostatic agent. These cultures were incubated for one week and then spread on PPLO agar with a bent glass rod.
Plate I. PPLO (strain T-2) photographed on PPLO agar plate. 100X.
Plate II. PFLO (strain T-2) photographed on PFLO agar plate. 430X.
TABLE I. Pathology and Results of Culturing from Chick Embryos Infected with Strain 2-2 (PPLO)

<table>
<thead>
<tr>
<th>Embryo No.</th>
<th>Method of inoculation</th>
<th>Pathology</th>
<th>Results of culturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Allantoic</td>
<td>Died, six days; lesions in respiratory tract.</td>
<td>Typical colonies in four days</td>
</tr>
<tr>
<td>2</td>
<td>Allantoic</td>
<td>Died, six days; lesions in respiratory tract.</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>Allantoic</td>
<td>Died, eight days; lesions noted</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>Yolk sac</td>
<td>Died, eight days</td>
<td>Typical colonies</td>
</tr>
<tr>
<td>5</td>
<td>Yolk sac</td>
<td>Hatched and appeared normal on autopsy</td>
<td>No attempt was made to culture</td>
</tr>
<tr>
<td>6</td>
<td>Yolk sac</td>
<td>Hatched and appeared normal on autopsy</td>
<td>No attempt was made to culture</td>
</tr>
</tbody>
</table>

The following experiment was set up to clarify the above embryo study: two tenths ml. of in vitro culture of strain 2-2, after 14 in vitro transfers in beef heart infusion broth, was injected into ten day old embryos by yolk sac and allantoic methods. Twenty three eggs were inoculated, twelve via allantoic membrane and twelve eggs by yolk sac inoculation. These eggs were designated A-1 through A-12 and Y-1 through Y-11, respectively.

The results of these embryo studies indicate a typical mortality pattern in chick embryos for strain 2-2, with the causative agent readily recoverable in beef heart infusion broth plus one per cent Bacto PPLO Serum Fraction. Inoculation into the allantoic sac was the method of preference because of the ease of recovery and subculture of the allantoic fluid. The pH change in beef heart infusion broth plus 0.5 per cent dextrose used to culture yolk material and allantoic fluid indicated that a drop of pH could be used to indicate presence of growth in this medium.
<table>
<thead>
<tr>
<th>No.</th>
<th>Death no. days after inoculation</th>
<th>Pathology</th>
<th>Mode of recovery</th>
<th>pH change in enrichment broth days after inoculation</th>
<th>Results of plating</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>10</td>
<td>Respiratory involvement</td>
<td>Allantoic fluid</td>
<td>2nd.</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>Respiratory lesions</td>
<td>Allantoic fluid</td>
<td>2nd.</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>Slight respiratory involvement</td>
<td>Allantoic fluid</td>
<td>2nd.</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Hatched</td>
<td>Normal</td>
<td>Yolk sac in body cavity</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Hatched</td>
<td>Normal</td>
<td>Yolk sac in body cavity</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Hatched</td>
<td>Normal</td>
<td>Yolk sac in body cavity</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>Picked shell and died, normal</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>Normal</td>
<td>Allantoic fluid</td>
<td>1st.</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>Air sac involved</td>
<td>Allantoic fluid</td>
<td>2nd.</td>
<td>+</td>
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<tr>
<td>10</td>
<td>6</td>
<td>Normal</td>
<td>Allantoic and yolk</td>
<td>1st.</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>Hatched</td>
<td>Yolk material</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Hatched</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-1</td>
<td>11</td>
<td>Respiratory involvement</td>
<td>Allantoic fluid</td>
<td>2nd.</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>Normal</td>
<td>Allantoic and yolk</td>
<td>2nd.</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>Normal</td>
<td>Allantoic and yolk</td>
<td>2nd.</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>Picked shell and died, normal</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>Slight respiratory involvement</td>
<td>Allantoic and yolk</td>
<td>2nd.</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>Respiratory lesions, yolk</td>
<td>2nd.</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>Picked shell and died, apparently normal</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>Respiratory lesions</td>
<td>Yolk and allantoic</td>
<td>2nd.</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>11</td>
<td>Respiratory lesions</td>
<td>Allantoic</td>
<td>2nd.</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>Picked shell and died, apparently normal</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>Respiratory lesions</td>
<td>Yolk</td>
<td>2nd.</td>
<td>+</td>
</tr>
</tbody>
</table>
Sixty chickens were divided into five groups of twelve birds each. Each bird of group number one was inoculated with 0.2 ml. of a broth culture of strain T-2, six birds being inoculated intratracheally and six birds intrasinusoidally. Each bird of group two was inoculated with 0.2 ml. of a broth culture of strain X-245, six birds being inoculated intratracheally and six birds intrasinusoidally. Each bird of group three was inoculated with 0.2 ml. of a broth culture of strain 0-15, six birds being inoculated intratracheally and six birds intrasinusoidally. Each bird of group four was inoculated with 0.2 ml. of strain C-17, six birds being inoculated intratracheally and six birds intrasinusoidally. Group five served as control for groups one through four. All birds received regular mash diet.

These birds were kept for one month, showing no apparent gross involvement of upper respiratory tract. Upon autopsy there was no apparent involvement of trachea, lung or air sacs. Tracheal scrapings were taken from each chicken and inoculated into Bacto PPLO Broth containing crystal violet and 1000 units penicillin per ml. as bacteriostatic agents. These cultures were incubated at 38.5°C, after seven days 0.2 ml. of each culture was plated on Bacto PPLO Agar. These cultures were checked periodically for 21 days using the low power objective. No significant colonies were recognizable during this entire period.

Several methods of cultivation were undertaken to produce optimum conditions for isolation and growth of this organism. The following experiments were carried out because preliminary studies indicated that PH change in culture media could be used as an indication of optimum growth. Fresh beef heart infusion broth prepared as before was divided
into three lots. To lot one no dextrose was added, to lot two, 0.5 per
cent dextrose was added, and to lot three one per cent dextrose was
added. This medium was autoclaved at fifteen pounds pressure for ten
minutes and cooled, and one per cent Bacto PPLO Serum Fraction was added;
it was then distributed aseptically in tubes in 10 ml. amounts; 0.2 ml.
of actively growing broth culture being inoculated into each tube. (See
table III.)

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>PPLO enrichment broth containing phenol red</th>
<th>pH change</th>
<th>pH</th>
<th>Results</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No dextrose</td>
<td>Slight change six days</td>
<td>6.8</td>
<td>Large no. colonies</td>
<td>Large no. colonies appear in 24 hr. after plating</td>
</tr>
<tr>
<td>2</td>
<td>0.5% dextrose</td>
<td>Slight change six days</td>
<td>5.9</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1% dextrose</td>
<td>Slight change four days</td>
<td>6.0</td>
<td>Large no.</td>
<td>No. of colonies appearing on plate 1 greater than on plate 3</td>
</tr>
</tbody>
</table>

The pH determination was made using a Beckman type G pH meter. From
this experiment there was an indication that more rapid growth had
occurred with 0.5 per cent dextrose. Growth was not noted on plate two,
indicating that the death of the organisms had occurred before nine days.

The following experiment was set up to determine the growth rate
under the same conditions. (See table IV).
TABLE II. Effect of Dextrose on Daily Counts

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Media</th>
<th>pH change</th>
<th>Average no. of colonies per field each day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No dextrose</td>
<td>4</td>
<td>2  17  17  17  17  17  12</td>
</tr>
<tr>
<td>2</td>
<td>0.5% dextrose</td>
<td>2</td>
<td>7  13  0</td>
</tr>
<tr>
<td>3</td>
<td>1% dextrose</td>
<td>5</td>
<td>11  13  17  14  10  0</td>
</tr>
</tbody>
</table>

Determination of the number of colonies was made by pipetting 0.1 ml. of culture on to Bacto PPLO Agar and spreading it over an area of 15 square centimeters with a platinum loop and allowing these plates to incubate for four days, then counting the number of colonies per low power field. Ten fields were counted and the average determined.

The results of these studies indicate that the dextrose concentration of 0.5 per cent developes acid more rapidly with failure to subculture the organism 24 hours after complete acid change. However, to sustain growth in subcultures, no dextrose should be added.

The following growth study was carried out to determine the optimum growth of T-2 in media containing various inhibitory agents. In all cases except media containing crystal violet, phenol red was used as an indicator with fresh beef heart infusion broth containing one per cent Bacto PPLO Serum Fraction, and 0.5 per cent dextrose as basal media. (Figures 1 and 2 p. 20)

Counts were determined by pipetting 0.05 ml. of culture on to Bacto PPLO Agar and spreading over an area of 8 square centimeters with a platinum loop. These plates were sealed with rubber bands and allowed to incubate at 38.5°C for five days, then counting the number of colonies using the low power objective. Ten fields were counted and the average determined. In the cases of high numbers of colonies per field, extreme crowding of colonies made accurate counting difficult.
Figure 1. Estimated growth of PPLO cultured in different concentrations of thallium acetate.

Figure 2. Estimated growth of PPLO cultured with different bacteriostatic agents.
However, the results of duplicate experiments indicate that crystal violet (0.01 gram/liter); potassium tellurite (1:10,000) and thallium acetate (1:2000) inhibit growth of strain T-2. Growth was not sufficient to bring about pH change in media containing potassium tellurite or thallium acetate (1:2000). The concentration of thallium acetate (1:3000 and 1:4000) showed a definite lag period over the same media containing no thallium acetate. Penicillin showed no inhibitory effect.

Studies were carried out on various cultures to determine growth characteristics of strain T-2 turkey sinusitis agent. These colonies grow out readily on Bacto PPLO Agar Base containing one per cent Bacto PPLO Serum Fraction. Upon first appearance they appear as small round granular colonies, varying in size from 10 to 175 microns in diameter. The size of the colonies appears to be related to the number of colonies on the agar, crowding producing the smaller colonies. Within from five to ten days these colonies develop larger granular particles which seem to develop into darker chromatic structures. These denser particles or chromatic structures appear to cover the entire colony in many cases. However, after several transfers of the organism in vitro, these denser bodies appear to develop much more slowly.

Several methods of staining these agents were attempted. The most rapid for gross morphology was the "fixation method of Kleineburger". A section of agar containing colonies was cut out and placed on a glass slide. A cover slip having been stained with an alcoholic solution of azure and methylene blue and allowed to air dry, was placed over the agar block. These sections were then sealed with paraffin and examined with a microscope. The following photo микронographs were taken.
from sections so prepared. (Plates 3, 4 and 5 pp. 23, 24, 25). Material from broth cultures from various experiments was centrifuged at 2000 revolutions per minute. The sediment was smeared on the slides and stained using Gram, Giemsa, or methylene blue stains. Of these, Giemsa showed most distinguishing characteristics; but no definite structural morphology could be noted, the material appeared of amorphous granular nature. Small filamentous structures were noted; however these were very irregular and perhaps artifacts.

The results of antibiotics studied were of considerable importance as a possible means of controlling ORD and ITS in poultry flocks (38). The following experiments were undertaken to determine the effect of Aureomycin in the form of Aurofac-10* mixed with the diet on the weight gain of chickens. Sixty eight one month old Plymouth Rock chicks were weighed and separated into four groups of seventeen birds each.

Group number one was fed a regular mash diet with the addition of one-half pound of Aurofac-10 per 100 pounds of feed. This gave a final concentration of 50 milligrams of Aureomycin per pound of feed. These birds were inoculated intratracheally with 0.2 ml. of actively growing broth cultures of strain T-2 which had been passed through twelve consecutive transfers in beef heart infusion broth containing no bacteriostatic agent since the original isolation. These birds were reinoculated intratracheally with 0.2 ml. of an actively growing broth culture of strain T-2 one week after the first inoculation.

The second group served as a control group for group one.

*Aurofac-10 Lederle Laboratories brand of antibiotic feed supplement containing 10 grams of chlortetracycline per pound.
Plate III. PPLO colonies (strain T-2) "Fixation method by Kleineburger". Showing more deeply stained chromatic granules which stain deep blue, while remaining colony stains light pink. 235X.
Group three was fed regular diet, but received no antibiotic. The birds were inoculated and reinoculated the same as group one. Group four served as a control for group three and as a control for Aureosycin fed birds of group two. Nine birds from each group were killed and autopsied two weeks after inoculation. No apparent infection of trachea, air sacs or lungs was noted in either group two or group four, the control groups. There was one bird in group one and one bird in group three which showed slight cloudiness of air sacs. These air sacs were removed and ground in sterile sand using 2 to 3 ml. of beef heart infusion broth containing 1000 units of penicillin per ml. and 0.01 gram crystal violet per liter as bacteriostatic agents. Tracheal scrapings were also inoculated from each chick in the same type of medium. These cultures were incubated at 38.5°C. After one week of incubation 0.2 ml. of each culture was pipetted on to Bacto PPL0 Agar and spread with a bent glass rod. These plates were periodically checked, after four days incubation, for a period of three weeks. No visible colonies were noted over this entire period.

Due to the apparent inability to recover PPL0 from these cultures with crystal violet as a bacteriostatic agent, potassium tellurite was used in place of crystal violet, with 1000 units penicillin per ml. as bacterial inhibitors in culturing from the remaining chickens. Ten ml. of Seitz filtered one per cent solution of potassium tellurite was added per liter of beef heart infusion broth, giving a final concentration of 1:10,000 potassium tellurite.

The remaining birds were killed, weighed and autopsied four weeks after the first inoculation. No apparent involvement of lungs, air
sac or trachea was noted in birds of groups two and four, the control
birds. One bird from group one and one bird from group three had slightly
cloudy air sacs. This material was ground in sterile sand with 2 to 3
ml. of broth. Five tenths ml. of this material was inoculated into the
above described medium. Tracheal scrapings from each bird were inoculated
into the same type of medium. A reduction of potassium tellurite
occurred indicating contamination of all cultures within from two to
three days after inoculation. No further attempt to recover the agent
was made from these cultures.

<table>
<thead>
<tr>
<th>Group no.</th>
<th>No. of birds</th>
<th>Wt. of birds in grams before inoculation</th>
<th>Wt. of birds in grams 28 days after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 inoc.</td>
<td>8</td>
<td>2785</td>
<td>6937</td>
</tr>
<tr>
<td>Aureomycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 uninoc.</td>
<td>7</td>
<td>2522</td>
<td>6675</td>
</tr>
<tr>
<td>Aureomycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 inoc. no</td>
<td>9</td>
<td>2933</td>
<td>6949</td>
</tr>
<tr>
<td>Aureomycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 uninoc. no</td>
<td>7</td>
<td>2662</td>
<td>6106</td>
</tr>
<tr>
<td>Aureomycin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The control birds fed antibiotic diet gained 75 grams more per
bird than inoculated birds on same diet. The control birds fed
regular diet containing no antibiotic gained 61 grams more per bird
than the inoculated birds fed the same diet. (See Table V).

The increase in weight gain of control birds (group 2 and 4) over
inoculated birds (group 1 and 3) indicates no significant effect of
the antibiotics on decrease in weight gain due to inoculation.
The control birds fed antibiotic diet gained 87 grams more per bird than the controls receiving no antibiotics.

The apparent reduction in weight gain of both regular and antibiotic fed birds inoculated with PPLO (groups 1 and 3) was further confirmed by the following experiment.

<table>
<thead>
<tr>
<th>Group no.</th>
<th>No. of birds</th>
<th>Wt. of birds in grams before inoculation</th>
<th>Wt. of birds in grams 14 days after inoculation</th>
<th>Wt. of birds in grams 28 days after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 inoc. no</td>
<td>10</td>
<td>3030</td>
<td>6060</td>
<td>8424</td>
</tr>
<tr>
<td>Auromycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 uninoc. no</td>
<td>10</td>
<td>3051</td>
<td>6061</td>
<td>8809</td>
</tr>
<tr>
<td>Auromycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 inoc. Auromycin</td>
<td>10</td>
<td>3127</td>
<td>6301</td>
<td>8862</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 uninoc. Auromycin</td>
<td>10</td>
<td>3192</td>
<td>6862</td>
<td>9728</td>
</tr>
</tbody>
</table>

There was no difference in weight gain in inoculated chickens fed regular diets over their controls two weeks after inoculation. At the end of four weeks the controls gained 37 grams more per bird than the inoculated birds. (See Table VI).

After two weeks the control birds fed antibiotic diet gained 40 grams more per bird than inoculated birds fed antibiotic. This weight gain difference was further increased in four weeks to 81 grams per bird.

The control birds fed antibiotic fed diet gained 78 grams more per bird than controls receiving no antibiotic. All the chickens from the above experiment were killed, autopsied, and examined at the end of the
28 day period. There was no visible involvement of air sacs, lungs or trachea.

Tracheal scrapings from each chicken were inoculated into beef heart infusion broth containing thallium acetate (1:2000) and 1000 units of penicillin per ml. as bacteriostatic agents. After ten days incubation 0.2 ml. of each culture was plated on Bacto PPLO Agar. There were no visible colonies noted in 21 days.

The following experiment was carried out to check the effect of inoculation with strain T-2 PPLO on one month old turkey poultets. Poultets were weighed and divided into three groups. Group one consisting of four poultets was inoculated with 0.2 ml. of actively growing broth cultures into sinuses and 0.2 ml. intratracheally. Culture T-2 was used after having been passed thru chick embryos for four consecutive passages. These poultets were fed a regular mash diet with no antibiotics.

Group two consisting of four poultets was inoculated with 0.2 ml. actively growing broth culture of T-2 in its 17th in vitro passage after isolation. These poultets received the same diet as group one.

Group three consisted of four poultets which served as control for the other two groups.

Three of the four birds in group one showed slight swelling of the infraorbital sinuses within six to eight days after inoculation (photograph A, plate VI p. 30). The swelling became more pronounced within nine to ten days after inoculation, with coughing and nasal discharge. (Photograph B plate VI p. 30). The fourth bird of this group showed no visible symptoms.
Plate VI. Photograph A. Six week old turkey one week after inoculation with pleuropneumonia-like organisms. Onset of disease is noted by slight distension of infraorbital sinuses.

Plate VI. Photograph B. Seven week old turkey two weeks after inoculation with pleuropneumonia-like organisms. Swelling of infraorbital sinuses has become quite pronounced with nasal discharge.
Two of the four birds in group two showed visible symptoms with slight distention of infraorbital sinuses within eight to nine days after inoculation. These symptoms were in slower onset than group one, with swelling becoming pronounced within 15 to 16 days after inoculation, with nasal discharge. One bird of this group developed no visible symptoms and the fourth killed itself two days after inoculation, by strangling in the cage mesh.

None of the birds in the control group showed visible symptoms.

These birds were all weighed, killed and autopsied 21 days after inoculation. All the birds in group one showed respiratory involvement. There was evidence of lower respiratory infection in two birds of this group indicated by the presence of small yellow spots on the air sacs. One of these birds also had lesions on lung tissue and abnormal fluid in the lung cavity.

Two of the birds in group two showed involvement of upper and lower respiratory tract with visible spots on the air sacs. Group three showed no respiratory infection.

Tracheal scrapings and sinus exudate were taken from each bird and inoculated into fresh beef heart infusion broth, with one per cent Bacto PPLO Serum Fraction and 0.5 per cent dextrose. Thallium acetate (1:2000) and 1000 units of penicillin per ml. were used as bacteriostatic agents. These cultures were incubated at 38°C until a pH change was recognisable and then plated on PPLO Agar. Tubes which did not show pH change were plated at 10 and 16 days.
TABLE VII. Results of Plating Cultures from Turkeys- incubated with PPLO

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III (cont'd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>choana aspirate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tracheal scrapings</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table VII shows that groups one and two developed definite symptoms which can definitely be attributed to the inoculation with PPLO strain 7-2, with the organism readily recoverable from the infected birds. There was also an increase in weight gain of control birds over inoculated birds of group one and two.
SUMMARY AND CONCLUSIONS

All attempts to recover PPLO from cultures received through the courtesy of Dr. F. Markham and Dr. Geo. Appleton failed to produce visible colonies on Bacto PPLO Agar. An organism designated as strain T-2 was isolated from breeder turkeys with turkey sinusitis from the poultry farm, South Dakota State College. It produced typical PPLO colonies on Bacto PPLO Agar, produced typical mortality patterns in chick embryos with gross lesions, and produced symptoms of turkey sinusitis in one-month-old turkey poult's, when inoculated with an actively growing broth culture or infected allantoic fluid by infraorbital or intrasinusoidal route. The symptoms readily recognizable, were distension of one or both infraorbital sinuses, nasal discharge, and coughing. On autopsy, the air sacs of the inoculated birds were cloudy, the tracheas contained abnormal amounts of fluid and there were lesions present in the lungs.

The possibility of a virus being present in the inoculum can be eliminated because the organism infecting these birds in one group had been passed through broth cultures for 17 consecutive passages.

The organism produced good growth in freshly prepared beef heart infusion broth containing one per cent Bacto PPLO Serum Fraction, bringing about visible pH change with slight turbidity in five to nine days. Turbidity could not always be observed and therefore can not be used to indicate growth. The addition of 0.5 per cent dextrose to the medium brought about rapid production of acid, with death of the organism occurring within 24 hours. Five tenths per cent dextrose can be used in original isolation to stimulate growths, but should not be
used to maintain the organism in subcultures. Strain 2-3 is inhibited
giving only slight growth with the addition of crystal violet
(1:100,000), potassium tellurite (1:10,000) or thallium acetate in
concentrations higher than 1:3000.

The organism is extremely hard to grow and morphologically
difficult to study. The colonies vary from 10 to 175 microns in diameter
with dense areas in the center and clearing toward the periphery. For
growth morphological study the colonies stain readily with a mixture of
azure and methylene blue dyes. Individual cells are extremely difficult
to demonstrate; centrifugation of broth cultures occasionally produces
visible particles when stained with Giemsa, Macchiavelle or Gram
stains. Definite cell structures could never be distinguished.

The addition of 50 milligrams of Aureomycin to the diet of one
month old chicks inoculated with actively growing broth cultures of
strain 2-3 failed to accelerate their growth to a rate that might be
expected if the antibiotic exerted a curative effect on the PFLO
infection. The control birds in all cases had gained approximately 70
grams more per bird over inoculated birds at the end of four week period,
regardless of antibiotic.

In the inoculated birds the only indication of infection was the
clower weight gain over non-inoculated birds. On autopsy a few of the
inoculated birds showed slight coidiness of the air sacs, but this
could not definitely be attributed to the inoculum. In none of the
birds was the organism recoverable.

This work confirms the opinion of Markham and Wong that infectious
turkey sinusitis is caused by a PFLO, but fails to shed light on the
etiology of chronic respiratory disease of chickens.
LITERATURE CITED


