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**COMPARATIVE STUDIES OF STRAINS OF PLEURO-PNEUMONIA-LIKE
ORGANISMS ISOLATED FROM CASES OF CHRONIC
RESPIRATORY DISEASE IN POULTRY**

BY

ORVILLE M. BRAND

**A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Department of
Bacteriology, South Dakota State
College of Agriculture
and Mechanic Arts**

December, 1960

COMPARATIVE STUDIES OF STRAINS OF PLEUROPNEUMONIA-LIKE
ORGANISMS ISOLATED FROM CASES OF CHRONIC
RESPIRATORY DISEASE IN POULTRY

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor

Head of the Major Department

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ONE

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INTRODUCTION

Chronic respiratory disease in chickens and infectious sinusitis in turkeys were first recognized as clinical entities in the United States in 1942. They are now considered as diseases of major economic importance throughout the poultry industry. The loss from chronic respiratory disease and infectious sinusitis to the United States poultry industry has been estimated at 100 million dollars per year. Losses from mortality are less than those occurring in other respiratory diseases. The greatest loss comes through decreased growth rate and loss of egg production. The slower growth requires more time to prepare birds for market and there is a greater chance of loss from secondary infections.

Work has been done on a pleuropneumonia-like organism vaccine in broilers, but without success. Serological testing has proved to be an effective means of checking for possible infection. It has been suggested that flock weeding similar to the pullorum program could be used for chronic respiratory disease and infectious sinusitis. This plan has not yet been put into effect. It fails to yield complete elimination of the disease or the agents when used on large flocks over a large area. Luginbuhl (37) stated that poultry management has played a major role in keeping poultry flocks free of the pleuropneumonia-like organisms.

At the present time it has been accepted that chronic respiratory disease and infectious sinusitis are caused by one or possibly

two infecting agents. The first is one of the group of pleuropneumonia-like organisms, and the second is a bacterium or virus, which is thought to be required in addition to the pleuropneumonia-like organisms to produce the severe form of chronic respiratory disease and infectious sinusitis. Luginbuhl (37) states "....CRD has been seen to be mild when infecting chickens by itself and severe when aided by secondary invaders, as bacteria or viruses". It is also generally accepted at the present time that the agent causing chronic respiratory disease of chickens can also cause infectious sinusitis of turkeys and that this agent is transmitted through the egg to the progeny from infected parents.

LITERATURE REVIEW

Nelson (41, 42) was one of the first investigators to study the pleuropneumonia-like organisms. He worked with strains from fowl coryza of both acute and chronic types of infection. Nelson (41, 42) found Haemophilus gallinarum to be the organism which causes an acute coryza, and organisms designated as "cocco-bacilliform" bodies in birds as causing chronic coryza. By mixing the two types of organisms, Adler and Yamamoto (3) were able to repeat Nelson's experiments with essentially the same results.

Van Herick and Eaton (53) isolated the pleuropneumonia-like organisms as a contaminant during virus passage in chick embryos. They found that these cultures agglutinated the erythrocytes, and that antibodies of a high level could be demonstrated by use of hemagglutination inhibition tests. Markham and Wong (38) demonstrated the role of the pleuropneumonia-like organisms in the etiology of turkey sinusitis and chronic respiratory disease. They were able to produce the chronic respiratory disease symptoms by means of agents serially cultured in embryonated eggs. Lecce and Sperling (35) were able to cultivate pleuropneumonia-like organisms from chickens that had recovered from chronic respiratory disease and had been around infected birds. They were not able to isolate the pleuropneumonia-like organisms from normal birds. Lecce and Sperling (35) found these organisms to be more common in the trachea than in the lungs.

Fahy and Crowley (22) examined avian strains of the pleuropneumonia-like organisms that differed from Mycoplasma gallinarum listed in

Bergey's manual (9) by being able to ferment glucose and other sugars.

Adler et al. (6, 7, 8) were able to differentiate two serological and pathogenic types of the pleuropneumonia-like organisms in chickens and turkeys. They were also able to demonstrate a difference in pathogenicity of two strains of the pleuropneumonia-like organisms. The nonpathogenic strain was readily propagated on artificial media, while the pathogenic strain did not readily grow on serum enriched agar. Yamamoto and Adler (58) were able to separate seven avian strains of the pleuropneumonia-like organisms into five groups on the basis of morphological, physiological and antigenic characteristics.

Biddle and Cover (10) studied the bacterial flora of the respiratory tract of 51 chickens and were able to isolate the following organisms: Escherichia, Staphylococcus, Pseudomonas, Proteus, Aerobacter, Salmonella, Streptococcus, gram positive, bacilli and yeasts. Only seven were harboring organisms resembling the pleuropneumonia-like organisms. Thallium acetate in a dilution of 1:2000 failed to inhibit growth of coliform organisms in exudates of disease chickens. Fahey (21) found similar results in the microflora of chickens affected with chronic respiratory disease.

The pleuropneumonia-like organisms have been placed in the class Schizomycetes order I of the Mycoplasmatales. They are described as non-motile, highly pleomorphic, gram-negative organisms, having filterable stages and very delicate, elastic structures. They are aerobic to facultatively anaerobic, grow poorly on agar and have exacting

nutritional requirements. These organisms are classified into 15 different species according to the hosts in which they are found. The avian strain isolated from the upper respiratory tract of the fowl has been named Mycoplasma gallinarum. This organism produces good colonies on rabbit or horse serum agar and does not ferment glucose in broth cultures. Methylene blue is reduced rather rapidly. The pleuropneumonia-like organisms are characterized by their "button-like" colonies that grow down into the agar. These colonies often possess opaque, granular, brown or yellowish central areas. This central area is surrounded by a translucent, flat zone of variable size (9).

Van Isteron and Ruys (54) studied the fine structure of three strains of the pleuropneumonia-like organisms with the electron microscope. The colonies grew in the agar surface and were covered with a membrane continuous with the surface of the medium. Vesicles of simple structure were found to be the basic element of the colonies; however, coccoid elements having a bacterial nature were found. They seemed to be more in favor of considering these organisms as L forms of minute cocci. Freundt (23) is of the opinion that the pleuropneumonia-like organisms from bovine sources are of a filamentous or mycelial nature. The mycelial filaments fragment to form elementary bodies.

Taylor and Fabricant (52) and Taylor (51) made isolations from inoculated embryonated eggs into a modified Gramble's liquid medium containing phenol red broth base, carbohydrate, serum fraction and inhibitors. They concluded that the isolation of these organisms from

tracheal exudates could be successfully accomplished from a modified carbohydrate liquid medium. This medium has an advantage of being less costly and time-consuming than the use of hen eggs produced from disease free birds. Taylor and Fabricant (52) were disturbed by the variation and inconsistency of the fermentation of sugars by different strains of the pleuropneumonia-like organisms. It was reasoned, on the basis of their findings, that there are strains of these organisms which do not grow in the artificial medium employed or will grow but not ferment the carbohydrates consistently.

Adler et al. (1, 5) was able to enhance growth by adding yeast hydrolysate to a revised Difco medium (17); also, he was able to make isolations of the pleuropneumonia-like organisms using only 10 per cent horse serum in broth cultures. Adler, et al. (1) compared the efficiency of his "R.M." medium with other media for the isolation of these organisms. This medium consisted of a 10 per cent horse blood agar slant overlaid with 20 per cent horse serum in a broth base. This medium proved to be the most satisfactory for isolation of the pleuropneumonia-like organisms.

Hofstad and Joder (31) obtained good results using an avian meat infusion medium composed of chicken and turkey broth made from minced chicken or turkey meat and blood, and enriched with inactivated serum from the birds whose meat was used.

Smith et al. (48, 49) studied the nutritional requirements of the pleuropneumonia-like organisms and were able to isolate a phospholipid which proved to be the major growth factor. Cholesteryl laurate,

lecithin and methanol were required for optimal growth in synthetic media. Smith (50) was able to demonstrate the cholesterol esterase activity toward cholesteryl esters of various fatty acids in the pleuropneumonia-like organisms. Those strains possessing a greater esterase activity required a lipo-protein growth factor. Hydrolysis of fatty esters resulted in equimolar quantities of free cholesterol and fatty acid. Coenzyme A is essential in both the synthesis and hydrolysis of the esters. These reactions are thought to be specific for 3-beta-hydroxy-delta-5 sterols.

Fertile eggs have become a common and useful medium for the cultivation and study of the pleuropneumonia-like organisms. Nelson (42), in his search for a culture medium, was one of the first to demonstrate the use of fertile eggs as a means of growing the pleuropneumonia-like organisms. Fertile eggs readily supported the growth of "cocccobacilliform" bodies from "chronic-coryza" of chickens. The best growth results were obtained with four day old eggs. Ten day old embryos are not affected by inoculation. During this period, he noted the growth of "cocccobacilliform" bodies was not dependent on the presence of living cells. Since this time, many workers have used fertile eggs for cultivation and experimental examination of the pleuropneumonia-like organisms. When it was found that these organisms grew so profusely in fertile eggs, workers began using them to gather information on different strains of the organisms and to check their pathological effects.

The most accepted route of inoculation seems to be the yolk

sac. Hoyt et al. (32) thought the yolk sac route gave a more uniform death pattern; also, that the agent appeared to survive longer in the yolk than in the allantoic fluid. He found organisms in the yolk material were viable, when stored as long as 180 days at -40°C . All membranes, chorioallantoic fluid and yolk material contained appreciable concentrations of the pleuropneumonia-like organisms. Sheriff and Pierrey (47) found that the yolk sac route to be the most lethal; although, the organisms administered by chorioallantoic route appeared to be quite virulent for chick embryos. Keller and Morton (34) worked with six strains of the pleuropneumonia-like organisms from the human genitourinary tract. These strains were inoculated intra-allantoically into developed chick embryos. One strain failed to grow, and the other strains grew poorly. Strains that grew well in chick embryos were found to grow well in vitro. Yamamoto et al. (56) found a loss of all chick embryos in 72 hours, when inoculated via the chorioallantoic and yolk sac routes. They concluded that the death pattern depended on the virulence of the strain used, the route of inoculation and age of the embryo at the time of inoculation.

Delaplane (15) and Chute and Cole (13) agreed that the death pattern produced in chick embryos from the pleuropneumonia-like organisms was irregular for the first 13 passages. Hoyt et al. (32) obtained a fairly uniform death pattern after 5-7 days with most strains. It has been shown that passage of these organisms in the yolk sac increases the virulence of the organisms. This may suggest that saprophytic strains can become parasitic or pathogenic through

continued passage in hosts where the organisms grow best (33, 15, 13). Nasemann and Rockl (40) considered this question while working with saprophytic human strains of the pleuropneumonia-like organisms. They stated "...A definite answer can not be given as yet as to the questions of whether there are any PPLO strains primarily pathogenic for humans or under what conditions saprophytic PPLO may acquire secondary pathogenicity".

Heyt et al. (32) obtained 10^9 L.D.₅₀ for chick embryos per milliliter of yolk from infected embryos after 72 hours of growth.¹ Luginbuhl and Jungherr (36) were not able to detect measurable growth of the agents until the third day after inoculation. Growth then rose one logarithmic unit per day until it reached 10^6 L.D.₅₀ per milliliter on the fifth day. The embryos started dying on the seventh day, and developed a titer of 10^5 and 10^3 L.D.₅₀ per milliliter for all deaths after that period. They concluded that the yolk material harvested from living embryos just prior to the beginning or during the first day of embryonic death will give the greatest concentration of the infectious agent and the most uniform results in titrating.

Meller and Morton (34) working with six human strains of the pleuropneumonia-like organisms, found a generation time of 3.27 hours. These organisms seemed to follow the human bacterial growth cycle, when enumerated by the turbidometric and plate count methods.

¹L.D.₅₀ = The dose required to kill 50 per cent of the experimental animals.

Nearly all workers are in agreement as to the pathological effects produced by the pleuropneumonia-like organisms in chick embryos. The differences in these effects are thought to be the result of the virulence of the particular strains being used or the virulence built up during embryo passage. The degree of virulence appears to affect the death pattern and the course of the infection (33, 15, 13, 14, 56, 39). The main lesions in pathological infection seem to be cutaneous hemorrhages around the head, wings and feet. Embryos dying between eight and 14 days show extreme hemorrhage and edema. A transudate may ooze from all sides of the embryo. Metastatic hemorrhages are found in the skin, head, neck and lower thoracic region. Embryos dying in 14 to 21 days show similar changes, but they are more dwarfed. Some embryos show a body largeness and shortness of legs. The leg and wing joints may be arthritic, puffy and white and may contain exudate. The liver, heart, brain, kidneys and chorioallantoic membrane all show pathological conditions and edema. It appears that chronic lesions are more frequent in embryos inoculated with adapted strains of the pleuropneumonia-like organisms (33).

Chute and Cole (13) and Chute (14) noted that differences were produced by different cultures of the organisms and could be demonstrated in pathological appearances. The Giemsa stain was used to demonstrate the pleuropneumonia-like organisms in stained sections. Jungherr (33) stated that pathological response in the chick embryo could not be used for recognition of possibly pathogenic pleuropneumonia-like organisms. He described the pathogenic strains as hemagglutinating;

the nonpathogenic lacked this ability. He ascribed this to the fact that the heterophil is the only leucocytic type available for inflammatory mobilization throughout most of the embryonic life.

Nasemann and Rockl (40) used a process of "symbiosis-like" action to increase the number of the pleuropneumonia-like organisms per egg. Egg cultures which were infected simultaneously with the pleuropneumonia-like organisms and virus of ectromelia, produced a much higher percentage of growth of the pleuropneumonia-like organisms. Other viruses have been used with similar results. No mention was made of using this method as a stressing vector, nor of the pathological effects produced. They stated "...increase in the multiplication rate of the PPLO by a symbiosis-like process offers little confirmation for a stronger, primarily existing virulence of the organisms".

Gentry (24) studies the toxicity of certain antibiotics and furasolidone for chick embryos. Using ten day old embryos with ten different antibiotics, the E.L.D.₅₀ was calculated for each antibiotic.¹ Results indicated that the effect varied with the drug and route of inoculation. The most sensitive route was the allantoic cavity. Toxicity for fertile eggs with different drugs ranged from 0.96 milligrams to 152.5 milligrams.

Yamamoto and Adler (56) and Handy et al. (28, 29) were able to use embryos as a means of evaluating the effectiveness of antibiotics

¹E.L.D.₅₀ = The egg lethal dose. The dose required to kill 50 per cent of the embryos.

in ovo. They both found erythromycin to be quite effective. Handy (29) made a comparison of in vitro, in ovo, and in vivo results. Erythromycin and oxytetracycline in that order were found to be the most effective against the three strains used. Handy's group (29) was able to demonstrate a difference in the effectiveness between the antibiotics; also, they were able to demonstrate a difference in effectiveness for each antibiotic against the different strains used.

Hacemann and Rockl (40) compared their in vitro and in ovo results with antibiotics against the pleuropneumonia-like organisms. Their results were found to be quite comparable. They found no effect from the various sulfonamides or penicillin. Aureomycin and other tetracyclines exhibited some effectiveness. In ovo, at 5.0 micrograms per gram of egg or higher, Aureomycin produced complete inhibition of the micro-organisms.

Hitchner (30) inoculated infected turkeys with 150 milligrams of streptomycin in water at 0.6 milliliters per sinus. This amount would clear up sinus swelling, but internal lesions were found in 12 out of 26 turkeys so treated. One hundred milligrams per sinus showed regression of infectious sinusitis in five days, but relapses of the infection were noted after seven days.

Fahy (21) was able to produce F4 and F5 progeny free of chronic respiratory disease from infected F3 flocks treated with injections of 200 milligrams of dihydrostreptomycin, 120,000 units of procaine penicillin G and 40,000 units of potassium penicillin G. Symptoms of the disease were gone two weeks after treatment. Terrany-

cin and Aureomycin were found ineffective at 200 grams per ton of feed. This antibiotic mixture proved to be more effectively administered by injection than by feeding.

Adler and Yamamoto (2) were able to show that the agent of infectious sinusitis was passed through eggs from parent to progeny and that the stress of the infectious bronchitis virus enhanced the spread and severity of the disease; also, that egg-transmission of pleuropneumonia-like organisms was prevented in two flocks by medication with 25 milligrams per kilogram body weight of streptomycin-dihydrostreptomycin injections.

Of interest is the use of dihydrostreptomycin-sulfate-propylene glycol spray (50 grams dihydrostreptomycin sulfate, 300 milliliters of propylene glycol, 1.0 grams of metholatum and 1000 milliliters of water). Perk and Perk (45) used this mixture to spray flocks of different ages at designated time intervals. This spray was of value in preventing chronic respiratory disease for the first eight weeks of life, and the growth rate was found to be more uniform in the treated than in the untreated groups.

DeVelt and Gabuten (16) used Panmycin, Aureomycin, and Terramycin in commercial turkey starter mash for control of infectious sinusitis two days after exposure to the disease. It was concluded that the severity of the disease determined how much antibiotic to use. Tetracycline and Terramycin were similar in action; the chemoprophylactic action being roughly proportional to the concentration of the drug employed.

Duerre (20) fed Aureomycin to one month old chicks inoculated with the pleuropneumonia-like organisms. He found the uninoculated controls gained 70 grams more per bird over a four week period. The only indication of possible chronic respiratory disease was the slow weight gain in the infected birds. No isolation of the pleuropneumonia-like organisms could be made from autopsied birds.

Grumbles and Boney (27) found that Terramycin fed in concentrations of 0.5 per cent to 0.25 per cent for eight to 12 days was not as effective as Chloromycetin in treating infectious sinusitis. Chloromycetin in 0.1 per cent portions was not effective. Symptoms of infectious sinusitis recurred in two of 30 pullets.

Peterson (46), Gross (25), and Carson and Eaton (11) used Terramycin in oil as a therapeutic agent. Peterson used 11,000 pullets in two cases studied. Treatment was subcutaneous administration of Terramycin in mineral oil. Control groups showed evidences of the disease and loss of egg production, while the treated groups showed no infection and improvement in egg production. Gross (25) made a comparison of streptomycin in water, Terramycin in water and Terramycin in oil on experimentally induced infectious sinusitis. All birds receiving 3.125 milligrams per sinus or more of Terramycin in mineral oil recovered and showed no relapses. There were relapses of infections following treatment with antibiotics administered in water. Carson and Eaton (11) treated two lots of pullets infected with chronic respiratory disease with two milliliters of a suspension containing 25 milligrams of Terramycin per milliliter of mineral oil

injected subcutaneously into the upper neck region. Disease symptoms disappeared and egg production improved in seven days. Controls still continued to show chronic respiratory disease and low egg production.

Price et al. (43, 44) checked the influence of Terramycin and penicillin in the rations on the mortality, lesions and predominating microflora in experimental chronic respiratory disease. Penicillin was shown to have no effect on the course of the disease. Terramycin demonstrated a significant reduction in mortality rate and lesion severity and improvement in weight gain over control groups using 100 and 500 grams per ton of feed. Recovery of the organisms producing chronic respiratory disease was not influenced by any of the treatments although, a lag in development of antibodies against this agent was noted at the 100 and 500 grams per ton levels of feeding. Only higher levels were able to suppress secondary invasion of coliforms in the respiratory tract.

Chalquest and Fabricant (12) checked the survival of pleuropneumonia-like organisms injected into eggs previously dipped in antibiotic solution. The object of the study was to prevent egg transmission of the organisms by destroying those organisms within the hatching egg. This was done by immersing warm eggs into cold antibiotic solution. The antibiotic would be absorbed through the shell to destroy the organisms. Erythromycin, Terramycin and streptomycin were used against strains 29305 and 86. Analysis of the eggs for antibiotic showed 0.15 micrograms per gram in the yolk and 0.4 to 7.5 micrograms per gram in the albumin, which indicated that anti-

biotics could enter the egg by this method. In 75 trials with 6,500 chicken eggs and seven trials with 700 turkey eggs, there appeared to be no difference in hatchability between the dipped and undipped eggs. There were significantly fewer dipped eggs than untreated ones from which the micro-organisms could be isolated. One trial with Terramycin gave a similar result. This suggests that the pathogen may not survive this treatment.

Wong and James (55) claimed that Magnamycin and Terramycin were the most effective of 16 antibiotics tested in ovo; also it appeared that only the anti-rickettsial group of antibiotics exerted an unmistakable inhibitory effect on these pleuropneumonia-like organisms. These included Magnamycin, Terramycin, Aureomycin, chloramphenicol and streptomycin.

Gross and Johnson (26) stated that antibiotics influenced the killing properties of the organisms causing chronic respiratory disease and infectious sinusitis in ovo and in the following order of sensitivity: Terramycin, streptomycin, Aureomycin, neomycin and Chloromycetin. The drugs seemed to lengthen survival time of the embryo without completely destroying the micro-organism.

Yamamoto and Adler (57) tested the effects of antibiotics on pleuropneumonia-like organisms of avian origin. In vitro studies on several avian strains showed tetracycline and oxytetracycline to be most active; erythromycin, chortetraycline and streptomycin were all comparable, but less active. Dihydrostreptomycin was least active of all. In ovo testing showed erythromycin and viridogrisein were most

active and streptomycin - dihydrostreptomycin least active against chick embryos. Differences in antibiotic sensitivity were observed with avian pleuropneumonia-like organisms by both in vitro and in vivo methods.

Domermuth and Johnson (18) tested twelve drugs in vitro for their ability to inhibit avian strains of pleuropneumonia-like organisms. The drugs were found to be active in the following diminishing order: Magnamycin, Terramycin, streptomycin, furasolidone, Aureomycin, Chloromycetin, neomycin and penicillin. Magnamycin, Terramycin, streptomycin and furasolidone were tested and found to kill the organisms. The sulfa drugs were found to be inactive. Domermuth (19) found that streptomycin and furasolidone in vitro exerted similar effects after an initial lag phase. Subculturing of a strain growing in a streptomycin culture after 57 days suggested that this strain may develop a one-step resistance to streptomycin.

Reports by Lecce and Sperling (20) and Fahey and Crowley (22) and from unpublished work of Price et al., as stated by Price, (43) indicate that there is a decrease in the ease of isolation of pleuropneumonia-like organisms noted early in the course of antibiotic treatment, which suggests that the organisms were not eliminated by oral administration of the antibiotics. Fahey and Crowley (22) stated that the possible bacterial invaders in chronic respiratory disease are coliforms, Proteus, Micrococcus and the fungus Aspergillus.

METHODS AND MATERIALS

Cultures

Cultures of the pleuropneumonia-like organisms used for these experimental studies were obtained from several different sources. The S6 strain was received from Dr. E. B. Adler of the University of California, Davis, California. Strain 29365 was received from Dr. J. Frabricant of Cornell University, Ithaca, New York. Strain 780 was received from Dr. W. S. Hofstad of Iowa State University, Ames, Iowa. The VT strain was isolated in this laboratory from a chicken that had died of chronic respiratory disease. A strain designated as the M¹ strain was isolated from the yolk material of two eggs used as uninoculated controls in the course of this work. All of these strains appeared to produce good growth in broth and on agar. As a result of the large amount of work that has been done with strains S6, 29365, and 780, strain VT was selected for experimental study.

Culture Medium

Tubes and plates of Grumble's (52) modified phenol red medium were made up as follows: 860 milliliters of de-ionized water and 20 milliliters of yeast hydrolysate were added to 16 grams of Difco (17) phenol red broth base. The pH was adjusted to 7.8 - 8.0 with 10 per cent NaOH, and the medium was autoclaved at 121°C. for 15 minutes. One gram of a sugar and 0.05 grams of the inhibitor, thallos acetate, were dissolved in 100 milliliters of de-ionized water and sterilized by

filtration through a Seitz filter. The second inhibitor, crystalline potassium penicillin G, was added to give a final concentration of 1000 units per milliliter of medium. When the phenol red broth had cooled to 40-50°C., the sterile serum fraction, sugar and inhibitors were added aseptically. The sugars used most often for fermentation were glucose and maltose. The most common serums used were Difco (17) PFL0 serum fraction and horse serum. The phenol red broth was tubed in 16 x 150 millimeter screw cap tubes in 4.5 and 9 milliliter amounts with an aseptic dispenser.

For the preparation of agar plates, 1.5 per cent Difco agar was added to the phenol red broth base just before it was autoclaved, and the heat-labile components were added when the medium had cooled below 54°C. The phenol red agar was poured into petri dishes in layers about one centimeter thick. All agar plates and broth tubes were stored in the refrigerator at 10°C. until they were used.

Different serums were tried as sources of growth factors in phenol red broth and agar. The cultures were set up in dilutions of 2, 10, 16, and 20 per cent serum. If poor growth was obtained in these amounts of serum, a series of the serum dilutions were set up in broth cultures ranging from 0.0012 to 20 per cent serum. The controls contained no serum. This method demonstrated at what concentration the best growth could be obtained with the particular serum used. The growth factors tried were heat-inactivated, filter-sterilized bovine, swine and chicken sera.

Isolation and Cultivation

The following criteria were set up as a guide in the isolation, identification and recognition of pleuropneumonia-like organisms for the purpose of this study. (1) The organisms should produce acid in phenol red maltose broth containing the inhibitors thallic acetate and potassium penicillin G. (2) Growth in broth should be apparent as minute refractile granules suspended in a medium which is entirely free of turbidity. (3) The organisms should produce acid in phenol red maltose broth without inhibitors and the medium should show the refractile granules but no trace of turbidity. (4) The organisms should produce characteristic "button-type" colonies on phenol red maltose agar containing no inhibitors. Some strains of the pleuropneumonia-like organisms may not produce the characteristic pH change in broth, but they will produce the characteristic colony types on agar; therefore, the "button-type" colony on agar was used as the main identifying characteristic.

The procedure of isolation was essentially the same as that used by Adler (4) in his studies on the pleuropneumonia-like organisms. The specimens of trachea, lungs and exudate were each ground separately in a sterile mortar and pestle with sterile ground glass and 10 milliliters of phenol red maltose broth enriched with 20 per cent horse serum. From this ground material, 0.1 milliliters of the mixture were inoculated on 20 per cent serum-enriched phenol red maltose agar plates; one milliliter was added to 20 per cent serum-enriched phenol red maltose broth containing thallic acetate and potassium penicillin G.

The plates were sealed with masking tape to retain the moisture, and all cultures were incubated in a Thelco incubator at 38°C. Blind passages were made in broth and on agar plates at three day intervals until a pH change was noted in the broth and colonies were found on the agar plates. The cultures were discarded if no pH change or colonies were found after four blind passages at three day intervals (Figure 1.).

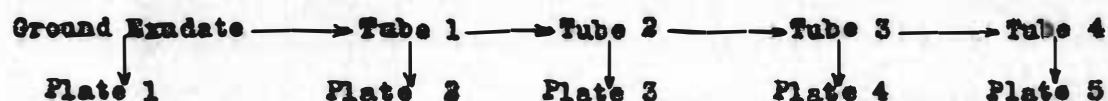


Figure 1. Isolation of Pleuropneumonia-like Organisms from Chickens having Chronic Respiratory Disease

Growth of the pleuropneumonia-like organisms was determined by observing a change in color of the phenol red maltose broth from red to yellow with a clear, granular appearance throughout the medium. The agar plates were checked for "button-like" colonies and the development of acidity. One milliliter of the phenol red maltose broth showing the positive characteristics of the pleuropneumonia-like organisms was transferred to a fresh tube of phenol red broth which contained no inhibitors. After several transfers in the inhibitor free-medium, the broth cultures proved to contain a pure culture of the pleuropneumonia-like organism and could be stored at -65°C.

Diluents

Serum from swine, cattle or chickens was separated from blood clots and filtered through a Whatman number one filter paper. The serum was then centrifuged in an International refrigerated centrifuge at 1000 times gravity for one hour. The clear serum was pooled and heated in a water bath at 56°C. for 40 minutes.

Yeast hydrolysate was made from dry baker's yeast. Three packages of the dry yeast cells were added to 500 milliliters of de-ionized water. The solution was incubated in a water bath at 56°C. for three days. The hydrolysate was clarified and sterilized by means of a Seitz filter and stored in the refrigerator at 10°C.

Physiological saline and 10 per cent NaOH were made up in 100 milliliter amounts, sterilized by autoclaving and stored in the refrigerator. The saline solution was used as a general diluent for egg and culture inoculations. The NaOH was used for pH adjustment of the phenol red medium.

Harvesting and Storing of Materials

Infected yolk material was obtained from dead embryos by using a 20 gauge needle and 10 milliliter syringe and placed in screw cap tubes. Specimens of materials to be cultured were placed in Petri dishes. Viable cultures in phenol red broth were stored in five and 10 milliliter amounts in 16 x 150 millimeter screw cap tubes. All the above materials were stored at -65°C. Broth cultures could be used and refrozen several times with little

or no loss in viability.

Smears and Staining

Six cultures were centrifuged at 2400 revolutions per minute or 1500 times gravity for one hour at 0°C. in an International refrigerated centrifuge. The supernatant was poured off and the sediment smeared on slides for examination with Gram's and Giemsa stains. The giemsa stain was most effective when left on the slide for 25 to 30 minutes. Phenol red maltose agar plates were also used to gather cells for staining. The plate was inoculated with a large volume of inoculum to produce profuse growth over the surface of the agar. Using an inoculating needle with a large loop, the agar surface was scraped from all sides into a central pile. The material was in a viscous state and could be picked up by the loop to smear on the slide.

Staining of colonies was accomplished by two methods. In the first method, agar plates containing the colonies were flooded with Giemsa stain. After an hour in the incubator at 38°C., the plates were examined with the low power lens of the microscope. The agar plate containing the stained colonies was cut out of the agar and mounted on a glass slide with the colonies uppermost. In the plate flooding method, care was used in placing the stain on the surface of the agar. Too much stain washing over the agar surface would wash out the delicate colonies and leave empty craters. Once the stain has been absorbed, the colonies were gently covered with a cover slip

and examined with the oil immersion lens of the microscope. In the second method used, agar blocks containing the colonies were cut out of the agar plates and placed on a glass slide with the colony side up. Giemsa stain was poured around the agar block using care not to flood the surface of the agar. The slide was placed in a Petri dish in the incubator at 38°C. for two to three hours. This period allowed the stain to diffuse through the agar to be absorbed by the colonies. After the colonies had absorbed the stain, a cover slip was gently laid over the agar block, and the stained colonies were examined with the oil immersion lens of the microscope. The agar block method showed better results but required more time for the stain to diffuse through the agar blocks. It proved more desirable to use a well filtered, diffusible stain and small agar blocks.

Experimental Animals

The chickens and feed used in these studies were obtained from the Poultry Department of South Dakota State College. These birds were all Single Comb White Leghorn chickens and were housed in wire cages of the Bacteriology animal room at South Dakota State College. The feed consisted of ground corn and soybean meal mixed with vitamins, minerals and two milligrams of penicillin per pound of feed. The birds were all tagged and numbered and blood was taken from each bird for testing of antibodies against pleuropneumonia-like organisms by the spot plate method using a commercially prepared antigen. All birds showing a positive reaction to the antigen test

were eliminated from the experimental flock. Blood was also taken at the end of these experiments at the time of autopsy to check for the presence of antibodies produced from experimental inoculation.

In the first experimental group, nine five month old birds were inoculated drop-wise nasally, intratracheally and with one milliliter in each of the posterior air sacs using one each of the three strains. The object was to induce chronic respiratory disease with the different strains of the organisms. This group contained six roosters and three hens. Three roosters were inoculated with strains 29395, three roosters with the 86 strain and three hens with strain 780. The birds were observed over a 30 day period for signs of chronic respiratory disease. Four birds were autopsied and the lungs and trachea were cultured in phenol red maltose broth enriched with 20 per cent horse serum. Blood was taken from each bird for testing for antibodies against the different strains with a commercially prepared antigen.

In a second set of experimental animals, seven hens and four roosters were selected for inoculation, and three hens and three roosters were used as controls. The birds were separated into four groups --- male and female controls and inoculated males and females. The cages of the controls were located in the next layer above the inoculated group. The birds, all 24 weeks old, appeared to be in good health. They were inoculated with a 35 hour broth culture of the 86 strain. The control birds received no inoculations. The experimental birds received injections of six milliliters of the viable

36 culture in the posterior thoracic air sacs, 0.5 milliliters intratracheally and dropwise by nasal inhalation. It was thought that chronic respiratory disease could be produced by different stresses on the host. In this trial, the birds were stressed in three ways. First, they were given nothing but water for 48 hours before inoculation. Second, an hour before inoculation, the birds were taken from their cages in the warm animal room and placed in cages in the snow at a temperature of -3°C . Third, a larger than normal amount of inoculum was given to each bird.

After the birds were inoculated by the above routes, they were returned to their cages in the warm animal room; however, the stress of chilling was continued at periods of 24, 48, 72, and 84 hours after inoculation. The chilling time and temperature were 4.0 hours at -3°C ., 3.5 hours at 0°C ., 11.5 hours at 0°C . and 4.0 hours at -3°C . They were again chilled at 8, 10, 14 and 16 days after inoculation for two four hour periods and two 10 hour periods at temperatures of -3° , -4° , -4° and -2°C . The birds were observed, and their reactions and symptoms were recorded over a 32 day period. The birds were all autopsied. The trachea, lungs and exudate from breast blisters and swelling eyes were taken for cultivation in broth and on agar enriched with 20 per cent serum. Blood was taken for testing for antibodies as previously mentioned.

In Vitro Antibiotic Testing

Oleandomycin phosphate, erythromycin thiocyanate, and oxy-tetracycline solutions were made up fresh just before titration in

screw cap tubes. Phenol red maltose broth with two per cent serum and without inhibitors was made in 500 milliliter amounts. A 40 milliliter amount of the broth was used for the preparation of each antibiotic. The method used for the in vitro testing of antibiotics was essentially the same as that used by Zolli et al. (59). The phenol red maltose broth was aseptically dispensed into screw cap tubes in one milliliter volumes, and the tubes were divided into groups of 14. The antibiotic was made up in 40 milliliters of the phenol red maltose broth at a concentration of 100 micrograms per milliliter.

Two-fold dilutions were made as follows: from the 100 microgram stock solution of antibiotic, one milliliter was pipetted into tube one, and into tube two containing one milliliter of the phenol red maltose broth, and these tubes were marked 100 micrograms and 50 micrograms. After mixing tube two, one milliliter of this 50 microgram dilution was transferred to tube three marked 25 micrograms. This two-fold dilution series was continued by the above procedure through tube 12, containing 0.05 micrograms of antibiotic. From this tube, one milliliter of the final dilution was discarded. Tube 13 and 14 were used as controls and contained no inhibitors.

The culture inoculum was prepared in the following manner. Each stock culture was diluted with nine parts of phenol red maltose broth, and incubated at 38°C. for 15 minutes. One milliliter of the freshly diluted culture was then inoculated into each of the screw cap tubes containing the different dilutions of antibiotic and into each of the controls. This gave a final volume of two

milliliters per tube. The culture tubes were incubated at 38°C. until a pH change was noted in the control tubes. The minimum inhibitory concentration was then determined by comparison with the control tubes. In making the medium and adding the antibiotic, care must be used in adjusting the pH. The antibiotic concentration may have some effect on the pH at these different dilutions and gave variable results.

Four cultures were selected at random from each of the three sets of 14 cultures made with each antibiotic. These cultures were subcultured on phenol red maltose agar without inhibitors. This method was used to check for the presence of any viable organisms after cultivation in the selected antibiotic for 72 hours. The presence of typical colonies on agar plates was regarded as an indication that the antibiotic was not able to kill the organisms. If colonies were produced in subcultures from antibiotic cultures that showed no pH change after 72 hours, the organisms were considered to be inhibited by the antibiotic but not killed.

Chick Embryos

Fertile eggs were obtained from the Poultry Department at South Dakota State College. The eggs were from Single Comb White Leghorn Chickens. All eggs were incubated at 38°C. in a Brower, model 55, Humidair incubator with the humidity maintained at 60 to 64 per cent saturation. After six days of incubation, the fertile eggs were candled and the air sac, embryo and the point of inoculation were out-

lined on the shell. On the seventh day, the air sac was swabbed with an iodine solution and a small hole for inoculation was placed near the edge of the air sac. The eggs were divided into groups according to the antibiotic dilution being used and inoculated in the yolk sac with 0.2 milliliters of broth culture or infected yolk material.

The antibiotic was made up in saline solution in the desired concentrations and administered 12.0 hours after the eggs had been inoculated with the VT strain. The antibiotic was injected into the chorioallantoic fluid in 0.2 milliliter amounts and in concentrations ranging from 1.0 to 0.0062 milligrams per egg.

The eggs were turned at least four times a day, and twice a day they were removed from the incubator for a cooling period of 30 to 45 minutes. The eggs were candled twice a day for the first 72 hours after inoculation and once a day for the remainder of the incubation period. Eggs that died during the first 48 hours after inoculation were discarded. These eggs were considered as loss resulting from inoculation injuries. Materials from all eggs that died after 48 hours were cultured on phenol red maltose agar containing two per cent serum and no inhibitors. The eggs to be cultured were cleaned with 70 per cent alcohol and the shell covering the air sac was removed. The chorioallantoic membrane was removed with sterile forceps, and the yolk material extracted with a sterile 20 gauge needle and 10 milliliter syringe. A drop of yolk material was inoculated on phenol red maltose agar plates and the remainder placed in sterile screw cap tubes in the refrigerator. If the refrigerated yolk material

produced typical colonies on agar and showed no signs of contamination, it was then stored at -65°C . When the embryo yolk material was not saved, a loop of the discarded yolk was streaked on agar plates and inoculated into broth tubes. Cultures were also made in broth and on agar from those embryos that pipped their shells and died, or that had died just after hatching. Hatched chicks were kept for five to six days before they were autopsied and cultured for the pleuropneumonia-like organisms. During this period, the hatched chicks were observed for any clinical signs of chronic respiratory disease, swollen or infected joints and other abnormalities.

RESULTS

Culture Medium

Strains of the pleuropneumonia-like organisms studied grew well on Grumble's modified phenol red medium. Unless inhibitors were used, this medium would readily support the growth of contaminating bacteria or molds. Studies were made with Grumble's medium using different sera as sources of growth factors. Good growth was obtained with Difco (17) serum fraction or Difco (17) horse serum. The heated bovine and swine sera also provided equally good growth of the S6 and VT strains. Heated avian serum did not support good growth.

Cultures of the S6 and VT strains were tested for growth in phenol red broth with different dilutions of serum. The S6 strain produced good growth after 10 hours in duplicate tubes fortified with horse serum. Only one of five S6 cultures fortified with avian serum grew. Growth of the S6 strain on phenol red agar plates gave results similar to those with the liquid media using horse and avian sera.

The VT strain produced growth in 46 hours in all but two dilutions of avian serum. Cultures were set up in a series of serum dilutions to continue the test with the VT strain in avian serum. Culture five, containing 2.0 per cent avian serum, was the first to show growth and produced a pH change in 12 hours. Cultures containing from 0.005 to 8.0 per cent serum showed a pH change in 46 hours.

The control cultures and the cultures containing 18 and 20 per cent serum showed little or no pH change over a seven day period. Twenty-six per cent avian serum was required for abundant growth of the VT strain on agar plates. Plates with 22 per cent avian serum contained a few colonies of the VT strain. Plates with 12 and 18 per cent avian serum did not show any recognizable colonies.

Three sets of four culture tubes contained dilutions of 2, 10, 16 and 20 per cent swine serum in nine milliliters of broth. These dilutions were inoculated with one milliliter of the VT strain. All three groups gave similar results. Cultures containing two and ten per cent serum showed pH changes in 13 hours, while cultures containing 16 and 20 per cent swine serum did not show a pH change until the sixteenth hour. All cultures had developed exactly the same pH at 21 and 24 hours. Heated swine serum was used to fortify large amounts of medium and good growth of the VT and S6 strains was maintained in all trials and passages made.

Isolation and Cultivation

Specimens of lungs, trachea, and gray, cheesy exudate from the air sacs were taken from a chicken diagnosed as having chronic respiratory disease by the Veterinary Department at South Dakota State College. Cultivation of these specimens resulted in two types of colonies. One was quite granular, and appeared to resemble the S6 strain. The second designated as the VT strain, was larger and faster growing. The colonies of the VT strain were granular only in the center and maintained a smooth, entire margin. With successive transfers of the two cultures,

the granular, erose colonies were lost. These granular colonies were considered to be possible bacterial L forms that had been eliminated by passage in broth cultures. All the specimens taken from the lungs, trachea, heart and liver produced a good growth of the VT strain, which was readily reproducible on Grumble's modified medium.

Although good growth results were obtained with Grumble's medium, it was found that one must adhere to the criteria outlining the difference between the pleuropneumonia-like organisms and bacterial L forms. At different times contaminants were able to grow in the medium in spite of the inhibitors present. They were found to be more common during the time of isolation or after a culture had been stored in the freezer.

Strain Characteristics

One object of this study was to demonstrate the particular characteristics of the different strains of the pleuropneumonia-like organisms on Grumble's medium. The different strains were compared on the basis of the length of time required to produce a pH change in phenol red maltose broth, the time required to produce recognizable colonies on phenol red maltose agar, and differences in colony morphology and in the fermentation of sucrose, glucose, maltose and mannitol.

All four strains were able to ferment glucose and maltose quite readily, but they were not able to ferment mannitol. Sucrose fermentation was variable and much slower than the other sugars. The cultures that were not capable of fermenting mannitol were satisfactorily grown on phenol red maltose agar.

When fresh cultures were made up from cultures taken from the freezer or experimental animal, they required more time to produce a pH change; however, each strain demonstrated a characteristic time pattern for fermentation. Two or three passages through phenol red broth were required to adapt the strain to the medium sufficient to establish a fermentation pattern.

The S6 and VT strains were able to produce a pH change in the medium in 24 hours. The final pH of the medium was 5.0 to 5.5. In six passages of strain 780, the average time required for a pH change was 4.5 days. Strain 29305 was even slower, requiring 10 days to produce a definite pH change. The pH of cultures of strains 780 and 29305 was never found to be below 5.8 and was generally six.

The most characteristic difference between these strains was noted in colony morphology. Although the S6 and VT strains produced similar fermentations, their colony structure was not the same. They both started as small pock-marks in the agar surface; however, the S6 strain became completely granular throughout its entire surface, while the VT strain produced a smooth surface colony with a small granular center. The S6 colonies were irregular with an erose margin. The margins of colonies of the VT strain were circular and entire. Both strains formed recognizable typical colonies in 24 hours. Strain 29305 produced colonies very similar to S6, but it required a much longer growing period before the colonies were recognizable. Colonies of the 780 strain were granular and very irregular with an undulate or lobate margin. This strain also required a long growth period to pro-

duce recognisable colonies. Staining with a Giemsa stain was sometimes necessary to demonstrate the colonies of the 780 strain.

Smears and Staining

Stained smears of material centrifuged from broth cultures did not show any morphological forms that could be recognisable. The best material for staining came from agar plates. Smears from agar plates did not contain the extraneous debris that was always found in smears of centrifuged cultures. These organisms cannot be stained using the Gram technique.

The giemsa stain gave a more distinct picture of morphology. The organisms were stained light blue and many were minute, coccoidal forms. Extraneous material did not stain the same color and could be distinguished from the organisms. Staining of the colonies resulted in a color similar to that found on the giemsa stained slides. Best results were obtained by cutting agar blocks out of the plate and placing the stain around them. The Giemsa stain appeared to be specific for the colonies of the pleuropneumonia-like organisms, which made it relatively easy to distinguish them from the agar (Figures 2, 3, 4).

Experimental Animals

During a 30 day period, the first group of experimental birds displayed little or no reaction to injections of the three strains. Autopsy of the birds showed them all to be very fat and with no signs of internal lesions. Cultivation of material from the trachea and lungs in phenol red maltose broth and on agar did not yield recognis-

able colonies. Only the sera from the birds inoculated with the 29305 strain reacted with the commercial antigen.

The second set of experimental birds were stressed by different methods to see if symptoms of chronic respiratory disease could be produced. For the first 48 hours, the inoculated birds were all very inactive and ate very little. There was a difference between the sexes in the demonstration of symptoms of chronic respiratory disease. After inoculation the hens were continually shaking their heads and were less active than the roosters. All the hens lost these symptoms after four days. When they were placed out in the cold for a period of time, and then returned to the warm animal room, the head shaking and inactivity would again start. After nine days, the head shaking symptoms stopped and could not be induced again by stressing the hens with cold or starvation.

The effects of stress from cold affected the roosters in a similar manner; although, they did not show any of the clinical signs such as head shaking or coughing and sneezing during the first eight day period after inoculation. Signs of head shaking and inactivity became quite pronounced in the roosters 10 days after inoculation. Stress with cold and starvation appeared to have a marked effect on the activity of the male birds. These symptoms of chronic respiratory disease lasted about 24 days; after that period, stress of cold or starvation did not appear to induce any of the signs of chronic respiratory disease.

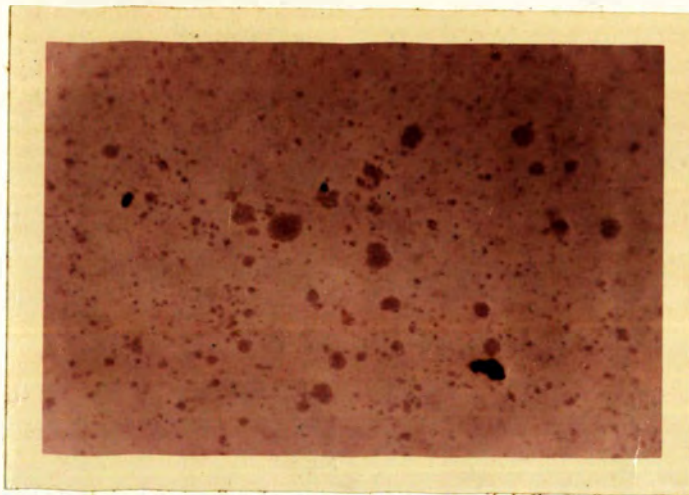


Figure 2. Colonies of the S6 Strain Stained with Giemsa Stain. x 100

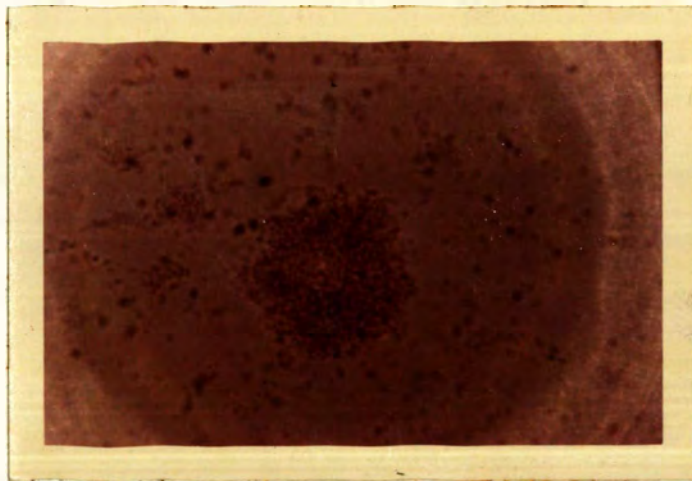


Figure 3. A Colony of the S6 Strain Stained with Giemsa Stain. x 970

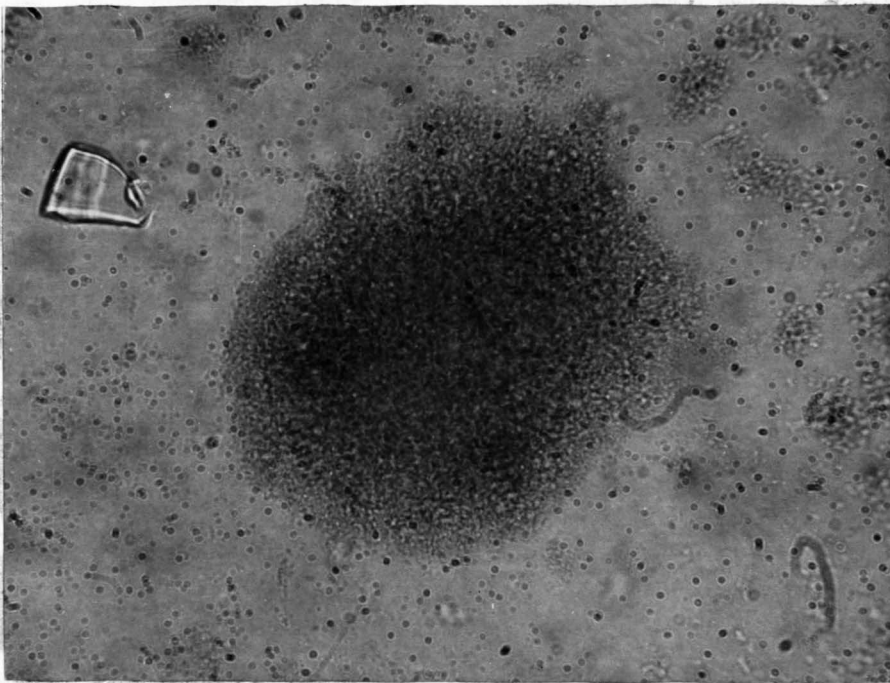


Figure 4. A Colony of the S6 Strain Stained
with Giemsa Stain. x 970

The birds were autopsied after 32 days. The hens were extremely fat and showed no lesions. Three of the roosters had breast blisters and large swellings over their eyes. Two roosters had cheesy exudate around their lungs and air sacs, and their livers were quite discolored. Exudate aspirated from the eyes and breast blisters contained gram positive cocci. None of the pleuropneumonia-like organisms were isolated from any of these experimentally infected birds.

There was a marked difference in the appearance of the fat found on the inoculated and control groups. The fat on the control birds appeared to be much healthier. This difference was also noted between the inoculated roosters and hens. The inoculated roosters carried very little fat and did not appear to be in good health. Tracheal rales were heard in two of the roosters, but they did not maintain these symptoms longer than 48 hours. A small amount of ocular discharge was noted from the swollen eyes of three roosters. This ocular exudate was cultured in phenol red maltose broth with inhibitors and yields only gram positive cocci, that grew in the presence of the two inhibitors.

Symptoms of chronic respiratory disease such as nasal discharge, swelling of the infraorbital sinuses, excessive mucus in the trachea and arthritic joints were not found in any of the birds examined.

Blood serum tests for antibodies were positive for two roosters and one hen of the inoculated group. No reactors were found in the control group. During this 32 day observation period, the controls did not display any of the symptoms of chronic respiratory

disease and appeared to be quite healthy.

In Vitro Antibiotic Testing

The control cultures containing the VT strain showed a pH change in 16 hours. The minimum inhibitory concentration of oleandomycin for the VT strain was 25 micrograms per milliliter. However, at the end of 32 hours, all the dilutions of oleandomycin allowed growth in spite of the early inhibitory effect of the antibiotic (Table I). Subcultures made from selected cultures of this group resulted in abundant growth of the VT strain (Table III).

Erythromycin was inhibitory for the VT strain down to a concentration of 25 micrograms per milliliter. At the end of 72 hours, all but the cultures containing 100 and 50 micrograms showed growth (Table I). Subcultivation of selected cultures of the erythromycin group gave negative results for the 100 microgram amounts, but subcultures were positive for 50 micrograms and below (Table III).

The VT strain in cultures with Terramycin produced the first pH change in 29 hours. The minimum inhibitory concentration was below 0.05 micrograms per milliliter. After 72 hours, the cultures in tubes 10, 11 and 12 were positive (Table I). Subcultivation of the 100, 12.5 and 0.8 and 0.1 microgram cultures on phenol red maltose agar showed the 100 microgram cultures to be negative. The 12.5, 0.8 and 0.1 microgram subcultures were positive after four days incubation (Table III).

Controls of the S6 strain showed a pH change in 14 hours. This strain was not able to grow in cultures containing more than 6.25 micrograms of oleandomycin (Table II). The cultures were difficult to read because of the variation in pH throughout the group. The minimum inhibitory concentration was estimated to be 12.5 micrograms per milliliter. There was no change in pH above 6.25 micrograms after 72 hours (Table II). Subcultivation did not produce any S6 colonies from cultures that contained more than 6.25 micrograms of oleandomycin (Table IV).

Cultures of the S6 strain containing dilutions of erythromycin showed a minimum inhibitory concentration of 25 micrograms per milliliter at 14 hours. Inhibition was still complete for the 100 and 50 microgram cultures at the end of 72 hours (Table II). Subcultivation of the 100, 50, 25, and 12.5 microgram cultures revealed no colonies in the microgram subcultures, but colonies were found on agar plates from the 50, 25, and 12.5 microgram cultures, showing that these organisms were inhibited but not killed (Table IV).

The S6 strain in cultures with Terramycin did not display a pH change until 20 hours after inoculation. The inhibitory concentration for this group was below 0.05 micrograms per milliliter. At the end of 72 hours, cultures in tubes 10, 11, and 12 showed growth (Table II). Subcultivation of 100, 12.5, 0.8 and 0.2 microgram cultures showed negative results for the 100 microgram cultures, but colonies were found for 12.5, 0.8 and 0.2 microgram subcultures from Terramycin (Table IV).

TABLE I. THE AVERAGE IN VITRO EFFECTS OF ANTIBIOTICS
ON THE GROWTH OR INHIBITION OF THE VT STRAIN

Antibiotics	Time of reading in hours	Tube numbers												Control	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		Concentration of Antibiotics in ugr./ml.													
		100	50	25	12.5	6.2	3.1	1.6	0.8	0.4	0.2	0.1	0.05	0.0	0.0
Cleandomycin	16	-	-	-	+	+	+	+	+	+	+	+	+	+	+
	32	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Erythromycin	16	-	-	-	+	+	+	+	+	+	+	+	+	+	+
	72	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Terramycin	16	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	29	-	-	-	-	-	-	-	-	-	-	-	+	+	+
	72	-	-	-	-	-	-	-	-	-	+	+	+	+	+

Legend + = Growth at time of reading.
- = No growth at time of reading.

TABLE II. THE AVERAGE IN VITRO EFFECTS OF ANTIBIOTICS
ON THE GROWTH OR INHIBITION OF THE S6 STRAIN

Antibiotics	Time of reading in hours	Tube numbers											Control		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		Concentration of Antibiotics in ugr./Ml.											0.0	0.0	
		100	50	25	12.5	6.2	3.1	1.6	0.8	0.4	0.2	0.1	0.05	0.0	0.0
Oleandomycin	14	-	-	-	-	+	+	+	+	+	+	+	+	+	+
	72	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Erythromycin	14	-	-	-	+	+	+	+	+	+	+	+	+	+	+
	72	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Terramycin	14	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	20	-	-	-	-	-	-	-	-	-	-	-	+	+	+
	72	-	-	-	-	-	-	-	-	-	+	+	+	+	+

Legend + = Growth at time of reading.
- = No growth at time of reading.

TABLE III. RECOVERY OF STRAIN VT ON SUBCULTURING AFTER EXPOSURE TO ANTIBIOTICS

Antibiotics	Tube numbers														Control
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
	100	50	25	12.5	6.2	3.1	1.6	0.8	0.4	0.2	0.1	0.05	0.0	0.0	
	+	o	o	+	o	o	o	+	o	o	+	o	+	o	
Oleandomycin	+	o	o	+	o	o	o	+	o	o	+	o	+	o	
Erythromycin	-	+	+	o	o	o	+	o	o	o	o	o	+	o	
Terramycin	-	o	o	+	o	o	o	+	o	o	+	o	+	o	

Legend (o) = Not subcultured
 (-) = No growth on subculture
 (+) = Growth on subculture

TABLE IV. RECOVERY OF STRAIN S6 ON SUBCULTURING AFTER EXPOSURE TO ANTIBIOTICS

		Tube numbers											Control		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		Concentration of Antibiotics in ugr./Ml.													
Antibiotics	100	50	25	12.5	6.2	3.1	1.6	0.8	0.4	0.2	0.1	0.05	0.0	0.0	0.0
Oleandomycin	-	o	-	-	o	o	o	o	o	o	o	o	o	o	o
Erythromycin	-	+	+	+	o	o	o	o	o	o	o	o	o	o	o
Terramycin	-	o	o	+	o	o	o	+	o	o	o	o	o	o	o

Legend (o) = Not subcultured
 (-) = No growth on subculture
 (+) = Growth on subculture

In Ovo Antibiotic Testing

The experimental eggs were inoculated with cultures grown in two types of culture media. The eggs treated with oleandomycin were inoculated with cultures of the VT strain that had been through seven passages in phenol red maltose broth. The erythromycin treated group of eggs was inoculated with the VT strain in yolk material that had been transferred serially in fertile eggs four times. The VT cultures for Terramycin group had been transferred serially in fertile eggs five times. Egg yolk material was found to be an excellent culture medium for growth of the S6 and VT strains.

The death pattern was studied in eggs inoculated with 0.2 milliliters of a viable VT strain in phenol red broth and with yolk material. Eggs inoculated with the broth cultures survived for an average of 10.1 days. The ratio of survival was two per 19 eggs. Eggs inoculated with second passage yolk material survived for an average of 10.6 days and the ratio of survival was four per 32 eggs.

The yolk material taken from each of these embryos contained the pleuropneumonia-like organisms. The pleuropneumonia-like organisms were isolated from either the lungs, trachea, or both of the embryos that died just before hatching or after pipping their shells. Embryos dying two to five days after inoculation showed hemorrhage of the body, neck and head. Embryos dying five to 10 days after inoculation showed some hemorrhage of the head, legs and feet. The back of the head and neck commonly showed edema. Hatched chicks often displayed edema of

the feet and legs. In several cases in the Terramycin treated group, the chicks lost the use of their legs two to three days after hatching. Lesions appeared at the joints of the legs, and the legs and feet became edematous before death. Cultivation of material aspirated from the edematous legs of a four day old chick produced pleuropneumonia-like organisms and gram positive cocci.

Respiratory infection was found in chicks that had died two or three days after hatching. The lungs of three four day old chicks all contained cheesy exudate and congested air sacs, lungs and trachea. Several chicks had discolored livers, and one chick had an enlarged heart with some exudate around it. Pleuropneumonia-like organisms were isolated from this exudate. The yolk sac had not been properly enclosed into the ventral cavity in many of the chicks. These chicks had hatched and died or had pipped their shells and died. Cultivation of material from these external yolk sacs produced colonies resembling the VT strain. The pleuropneumonia-like organisms were isolated from the lungs and trachea of two of the eight chicks that appeared to be in good health eight days after hatching.

Oleandomycin in excess of 100 micrograms per egg appeared to be toxic to chick embryos. In both sets of embryos tested with oleandomycin, 0.05 milligrams per egg gave the best results. Concentrations of 0.1 milligrams gave signs of being toxic and a concentration of 0.0025 milligrams was not potent enough to be of any therapeutic value. Oleandomycin did not prove to be effective in preventing growth of the VT strain in chick embryos (Tables V and VI).

Erythromycin, like oleandomycin, appears to be toxic in large amounts. Mortality rates seemed to be the highest soon after injection of the antibiotic. The survival ratios of sets one and two were the same; however, the average time of survival was much longer in set two. The survival time appears to be lengthened when using erythromycin in smaller doses (Tables VII and VIII).

Terramycin has been tried by other workers and appears to have some therapeutic value against the agents of chronic respiratory disease. One milligram of Terramycin per egg did not appear to be toxic to chick embryos. The Terramycin treated eggs showed the highest survival ratio of the three antibiotics tried. It also prolonged the survival time of the embryos. Embryos that died before hatching or that hatched and died, contained pleuropneumonia-like organisms resembling the VT strain. These organisms were also isolated from one eight day old chick that appeared to be in good health (Table IX).

TABLE V. THE INHIBITORY EFFECT OF OLEANDOMYCIN
AGAINST THE VT STRAIN IN OVO SET I

Dosage in milligrams per egg	0.0	0.1	0.05	0.025	0.0125
No. of eggs per dosage of antibiotic	15.0	15.0	15.0	15.0	15.0
Per cent surviving	13.4	0.0	13.4	6.6	0.0
Per cent of embryos that died before or after hatching	40.0	0.0	13.4	6.6	0.0
Per cent mortality	86.6	100.0	86.6	93.4	100.0
Average survival time in days	10.9	6.0	6.3	5.5	4.9

TABLE VI. THE INHIBITORY EFFECT OF OLEANDOMYCIN
AGAINST THE VT STRAIN IN OYO SET II

Dosage in milligrams per egg	0.0	0.06	0.025	0.0126	0.0062
No. of eggs per dosage of antibiotic	10.0	10.0	10.0	10.0	10.0
Per cent surviving	0.0	0.0	30.0	0.0	10.0
Per cent of embryos that died before or after hatching	20.0	40.0	20.0	50.0	30.0
Per cent mortality	100.0	100.0	70.0	100.0	90.0
Average survival time in days	7.0	13.3	12.2	11.1	11.1

TABLE VII. THE INHIBITORY EFFECT OF ERYTHRONICIN
AGAINST THE VT STRAIN IN OVO SET I

Dosage in milligrams per egg	0.0	1.0	0.5	0.25
No. of eggs per dosage of antibiotic	5.0	12.0	12.0	12.0
Per cent surviving	40.0	8.4	16.6	25.0
Per cent of embryos that died before or after hatching	0.0	16.6	0.0	0.0
Per cent mortality	60.0	91.6	83.4	75.0
Average survival time in days	7.8	4.1	6.2	6.3

TABLE VIII. THE INHIBITORY EFFECT OF ERYTHROMYCIN
AGAINST THE VT STRAIN IN OVO SET II

Dosage in milligrams per egg	0.0	0.05	0.025	0.0125	0.0052
No. of eggs per dosage of antibiotic	9.0	13.0	12.0	12.0	13.0
Per cent surviving	0.0	15.4	8.3	16.6	0.0
Per cent of embryos that died before or after hatching	11.1	38.5	33.3	50.0	7.7
Per cent mortality	100.0	84.6	92.9	83.4	100.0
Average survival time in days	6.0	9.0	9.2	11.2	6.9

TABLE IX. THE INHIBITORY EFFECT OF TERRAMYCIN
AGAINST THE VT STRAIN IN QVQ SET I

Dosage in milligrams per egg	0.0	1.0	0.5	0.25
No. of eggs per dosage of antibiotic	18.0	18.0	17.0	18.0
Per cent surviving	11.1	16.6	29.3	16.6
Per cent of embryos that died before or after hatching	48.8	50.0	35.3	48.8
Per cent mortality	88.9	83.4	70.7	83.4
Average survival time in days	8.1	10.5	11.8	9.8

DISCUSSION AND CONCLUSIONS

Culture Medium

From the results obtained, it is evident that Grumble's modified phenol red medium was able to support growth of the different strains of pleuropneumonia-like organisms studied. The use of different sera as sources of growth factors has revealed a difference in the strains of pleuropneumonia-like organisms and suggests that growth requirements may differ between the strains. The difference in growth in the presence of these different sera could possibly be used in helping to classify these organisms. The fact that the VT strain was the only one able to grow in avian serum shows a difference between it and the other strains. It may also indicate that the VT strain was able to use or do without certain growth factors that may be required by the other strains.

Other workers have found fractions in bovine serum that inhibit growth of the pleuropneumonia-like organisms. The lack of growth of the VT strain in high concentrations of chicken serum could possibly be the result of such inhibitors. No inhibitory action was noted for the large quantities of bovine or swine sera, and these sera were able to support good growth of each strain. If inhibitory substances are present, they are not in high enough concentration to be effective against the growth of these strains.

Isolation and Cultivation

The primary isolation of the pleuropneumonia-like organisms from exudate and autopsy specimens required 20 per cent serum in the culture medium. After the primary isolations were complete, growth of the organisms could always be maintained on medium with one per cent serum.

The results show that the criteria used for isolation were successful in delineating the pleuropneumonia-like organisms from bacterial L forms. However, bacterial L forms were a constant problem whenever primary isolations were made. The L forms were able to grow in the presence of the inhibitors and were not always completely eliminated by successive passage in inhibitor-free medium.

Gram positive cocci were the most common bacteria present during primary isolation of the VT strain. They were also found in cultures with the VT strain from chicks suffering from acute infections. The Gram positive cocci may act as secondary invaders, and this could account for the more severe infections found in some chicks. Since secondary invaders do appear to play an important role in the severity of chronic respiratory disease, cultures of these bacteria should also be obtained at the time of primary isolation. This would help to determine which bacteria are able to enhance the virulence of the pleuropneumonia-like organism and whether a symbiotic relationship exists between the two groups.

A second problem of concern deals with the isolation of all

strains of the pleuropneumonia-like organisms present in specimens during primary isolation. Workers have found several strains present in the same host and suggest that this is the cause of some acute cases of chronic respiratory disease. The VI strain was able to grow on serum-enriched media, while the more granular colonies were lost. These granular colonies may have been a strain of the pleuropneumonia-like organisms, that were not able to grow or grew poorly on the medium used, or they may have been a bacterial L form eliminated by passage. This again illustrates the need of a more selective medium ^{te by in vitro} for the isolation of these organisms.

Strain Characteristics

Differences were found in colonial morphology, pH change, nutritional requirements and inhibition by different antibiotics. By these methods, it was possible to differentiate between the strains of the organisms studied, and these methods are suggested as a possible means for classification of the different strains of pleuropneumonia-like organisms. The death pattern and pathogenicity of the S6 and VI strains of chick embryos were irregular and did not show a difference between the two strains. Because of this irregularity, it would be difficult to try to classify the strains studied on this basis.

Experimental Animals

The inoculation of in vitro cultures into chickens did not prove to be effective in producing chronic respiratory disease.

Stresses of cold and starvation may have some influence on the chronic form of the disease. Symptoms of the disease produced after stress of cold were more prominent in the males than in the females. No pleuro-pneumonia-like organisms could be isolated even though several of the birds' sera were positive to the antigen tests. This may be the result of allowing the disease to progress for too long a period before isolation attempts were made, which gave the host time to eliminate the organisms. This suggests that isolation attempts be made soon after the first signs of the disease have appeared. It is difficult to say that the stresses of cold and/or starvation aid in producing the chronic head shaking symptoms found in the experimental birds, but these stresses did appear to have some effect on the predominance of the symptoms. Certainly some consideration should be given to the effects that stresses of cold and starvation have upon the severity of the disease in poultry flocks not well cared for.

Smears and Staining

The gram stain was used mainly to check for the presence of contaminating organisms in broth cultures. Results show the pleuro-pneumonia-like organisms are not readily stained with the gram stain, but they are quite receptive to the giemsa stain. Staining of smears and colonies was used mainly as a diagnostic aid for identification of the different strains studied.

Pathological Effects of the VT Strain on Chick Embryos

Cultivation in the yolk sac of embryonating eggs proved to be the most effective method used for the S6 and VT strains. Inoculation of embryos by the yolk sac route was the most effective method in producing lethal infections. The results show that the death patterns produced by the two strains were quite irregular when inoculated with either the in vitro or embryo passed cultures.

The pathological effects produced in chick embryos were similar to those found by other workers. The fact that the VT strain was isolated from the edematous feet and legs of a four day old chick demonstrates the possible influence of this strain in producing the pathology found. The finding of gram positive cocci in the same cultures also suggests that the severity of the infection may have been increased by a secondary organism. However, the delayed pathological effects in the legs of these four day old chicks could also be the result of embryo passage of the VT strain, since no evidence of such lesions was found in hatched chicks inoculated with in vitro broth cultures. This would then indicate that such virulence was built up during passage of the VT strain through chick embryos and would have a marked effect on the death pattern and course of the infection. A third possible cause of the delayed pathological effects could be the inhibitory action of the antibiotics, which the VT strain was able to overcome after the chicks had hatched.

In Vitro and In Ovo Antibiotic Comparison

The in vitro and in ovo results using the selected antibiotics against the VT strain are to some extent comparable. Terramycin and erythromycin proved to be most effective against the VT strain in ovo but oleandomycin showed no inhibitory action. Similar results occurred in the in vitro cultures. Results in in vitro studies suggest that Terramycin and erythromycin are able to kill the S6 and VT strains when introduced in quantities of 100 micrograms per milliliter or more. Oleandomycin was not effective against the VT strain, but it produced inhibition of the S6 strain and was able to kill the organisms when used in larger amounts.

Erythromycin and oleandomycin in high concentration appeared to be toxic to chick embryos. Erythromycin thiocyanate did not prove to be as effective as other erythromycin derivatives tried, however, this again may depend on the strain of pleuropneumonia-like organisms studied and the culture medium used. Oleandomycin phosphate showed no inhibitory effect against the VT strain in vitro but its effectiveness against the S6 strain in vitro indicates it may have some value.

From these and the results of other workers, it appears that certain antibiotics may have some effect against the different strains of the pleuropneumonia-like organisms, and that these effects will vary with the amount and type of antibiotic used; also, these effects will vary with the strains of the pleuropneumonia-like organisms against which they are being used. This suggests that the use of antibiotics

as a means of control for the pleuropneumonia-like organisms will not be effective until a better understanding and classification of these organisms has been developed.

SUMMARY

Grumble's modified medium supported good growth of the strains studied and was easy to work with. Heated, filter-sterilised sera were comparable to Difco PPLO serum fraction and horse serum for growing the strains studied. Bovine and swine sera always gave good growth, but chicken sera were unpredictable for the different strains at varied concentrations.

The procedure and criteria used for isolation and identification of the pleuropneumonia-like organisms from chronic respiratory disease were successful. Contaminants were able to grow in the phenol red broth and agar in spite of the inhibitors present. The gram positive cocci were the most common bacterial contaminants found in primary isolations.

Differences were noted between strains of the pleuropneumonia-like organisms studied. These differences were observed in the time required to produce a pH change in phenol red maltose broth, growth in different sera, colony morphology and the variation of inhibition by different antibiotics.

Three of the in Vitro strains were not able to produce any noticeable symptoms of chronic respiratory disease in one group of inoculated chickens. Symptoms of chronic respiratory disease were produced in a second group of birds by stressing them with cold, starvation and large amounts of inoculum containing the S6 strain. No pleuropneumonia-like organisms could be isolated from these birds; however, four birds were positive to antigen tests.

The strains studied were not readily stained with the gram stain and no distinct morphological forms could be seen. Giemsa stain gave good results for slides or colonies, when the stain was allowed to be absorbed by the organisms. The giemsa stain demonstrated blue to purple stained organisms and had little effect on the agar material. Colonies were best stained by diffusion of the giemsa stain into agar blocks containing the colonies.

Oleandomycin showed little or no inhibitory effect against the VT strain, but it was able to inhibit the S6 strain of the pleuro-pneumonia-like organism. Erythromycin was not effective in amounts smaller than 50 micrograms per milliliter for either of the strains studied. The VT and S6 strains could not be subcultured from broth cultures containing more than 50 micrograms of erythromycin. Terramycin was found to be inhibitory against both strains of the pleuro-pneumonia-like organisms in concentrations below 0.05 micrograms per milliliter. These strains could not be subcultured from cultures containing 100 micrograms of Terramycin.

The yolk sac in fertile eggs proved to be an excellent growth medium for the strains of organisms studied. The yolk sac route was effective in causing death of embryos. No definite death pattern could be established for the S6 and VT strains. Cultures of yolk material of the egg passed VT strain produced a more severe infection in chick embryos than did in vitro cultures of the VT strain.

Oleandomycin appeared to be toxic for chick embryos in the larger amounts and was also ineffective against the VT strain.

Erythromycin was toxic for chick embryos in large amounts but it had some therapeutic value in lower concentrations; however, the VT strain could be isolated from birds of hatched or pipped eggs. Terramycin was not toxic to embryos in any of the amounts used. It produced the highest ratio of survival and prolonged the life of the embryos. Isolation of the VT strain in broth and agar cultures was accomplished from nearly all embryos that had been treated with the three antibiotics.

Pleuropneumonia-like organisms resembling the VT strain were isolated from the edematous feet and legs of the four day old chick. This chick had been inoculated with the VT strain by the yolk sac route and treated with Terramycin. Pleuropneumonia-like organisms were not commonly isolated from hatched chicks six to eight days old.

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