

STUDIES ON
THE BACTERIAL FLORA OF RESPIRATORY TRACT
OF POULTRY WITH SPECIAL REFERENCE TO
PLEUROPNEUMONIA LIKE ORGANISMS

A THESIS SUBMITTED
TO
MAGADHI UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
M. Sc. (Vet.)
IN
BACTERIOLOGY

By
K. Palanichandran Pillay
B. V. Sc.

PRINTED BY THE
PATER
MAGADHI, 1955

**STUDIES ON
THE BACTERIAL FLORA OF RESPIRATORY TRACT
OF POULTRY WITH SPECIAL REFERENCE TO
PLEUROPNEUMONIA LIKE ORGANISMS**

**A THESIS SUBMITTED
TO
MAGADH UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
M. Sc. (Vet.)
IN
BACTERIOLOGY**

B
247

By

K. Padmanabha Reddy

BIHAR VETERINARY COLLEGE	
PATNA	
Acc. No.	4174
Date	15.12.66


**BIHAR VETERINARY COLLEGE
PATNA
DECEMBER, 1966.**

Shri P. B. Kuppu Swamy. B.A., G.M.V.C.,
B. V.Sc., P.G. Bact. (N.Z.), M.S.Path.
(Missouri),
Professor, Head of the Department of
Pathology and Bacteriology and Principal,
Bihar Veterinary College,
P A T N A.

PATNA

Dated

Certified that the work described in this thesis entitled
" STUDIES ON BACTERIAL FLORA OF RESPIRATORY TRACT OF
POULTRY WITH SPECIAL REFERENCE TO PNEUMONIA LIKE
ORGANISMS" is the bonafide work of KADITAM PADMANABHA
REDDY, carried out under my guidance and supervision.


(P. B. Kuppuswamy) . 11/12/61

ACKNOWLEDGEMENTS

I express my deep sense of gratitude to Shri P. B. Kuppaswamy, Professor, Head of the Department of Pathology and Bacteriology and Principal, Bihar Veterinary College, Patna, for his valuable guidance and constant encouragement. I am highly grateful to Dr. B. S. Mallik, Professor of Bacteriology U.P. College of Veterinary Science and Animal Husbandry, Mathura, for providing PPD diagnostic antigen without which the serological work on PPD could not have been possible. I am very much thankful to Shri S.A. Ahmad, former Principal, Bihar Veterinary College, Patna, for providing necessary facilities. I am thankful to Shri S.A. Ansari, Assistant Professor and Shri S. C. Shrivastava, Lecturer, Department of pathology and Bacteriology for their help in my work. I am also thankful to Shri J. S. Ahmad, Assistant Director and Shri Sacchidananda, Manager, Government Central Poultry Farm, Patna for permitting me to collect materials for my study.

(K. Padmanabha Reddy).

C O N T E N T S.

	<u>Page No.</u>
I. INTRODUCTION.	1-3
II. REVIEW OF LITERATURE.	4-49
A. Actiology	
Pleuropneumonia like organisms (PPLO).	4-10.
Bacterial flora other than PPLO.	10-17
B. Cultivation and growth requirements.	18-24
C. Morphology.	24-27
D. Biochemical reactions.	28-31
E. Serology.	32-40
F. Pathogenicity trials.	41-49
III. MATERIALS AND METHODS .	50-71
Materials.	50
Methods.	
Bacterial flora other than PPLO.	51-52
PPLO	
Culture media and culturing.	52-56
Isolation and identification.	57-63
Comparative study of growth rate and colony morphology.	64-65
Pathogenicity trials.	65-71
IV. RESULTS	72-87
PPLO? .	72-85
Bacterial flora other than PPLO.	86-87
V. DISCUSSION.	88-99
PPLO.	88-97
Bacterial flora other than PPLO.	97-99
VI. SUMMARY.	100-101
VII. REFERENCES.	1-x1
Photomicrographs and gross photographs (19)	

"STUDIES OF THE BACTERIAL FLORA OF RESPIRATORY
TRACT OF POULTRY WITH SPECIAL REFERENCE TO PNEU-
MONIA LIKE ORGANISMS"

I. I N T R O D U C T I O N

Poultry keeping in India dates back to very early times. India and neighbouring countries are the original homes of the "Jungle fowl" from which the domestic fowls have descended, (Naidu, 1964). Though India has got huge population of poultry, little attention was paid hither to ~~xxx~~ on scientific breeding, and so the birds became uneconomical for meat and egg production. The Royal Commission on Agriculture in 1927 recommended the establishment of an institute for poultry research in India. With the effect from 1938 poultry research at I.V.R.I. Izatnagar was established, In India the poultry population was 35 lakhs in 1951. The egg laying capacity per annum was 1908 millions and the value of the eggs and table poultry was 30 crores. In 1956 poultry and duck population was about 97 millions or one tenth of the world population. In 1961 the Poultry census showed 1,6,914129 (Naidu 1964). The annual contribution of Poultry to Indian economy (1961) was 100 million rupees (Krishna Mahan Rao, 1965).

Once poultry farming was the cinderella of agriculture in India, now it occupies an important position in live stock industry. The great drawback of intensive system of poultry keeping in which a large number of poultry are housed in a single unit, increases the risk of respiratory infections in

India - Ranikhet, (Newcastle disease). Fowlpox, Tickfever, coryza and coccidiosis of chickens. Just like Newcastle disease chronic respiratory disease is also a great obstacle for the poultry industry and respiratory diseases are becoming increasingly serious menace.

Since Nalson's report in 1935 regarding the pleuropneumonia like organisms in the respiratory diseases of poultry for the first time, the havoc caused by these organisms were viewed with great concern from that time. At the third world poultry congress in 1937, the coryza problem was clarified for the first time. The scientific research concerning coryza started from that time and in many aspects research work was undertaken. Due to heavy losses mainly in broiler industry in America a conference was called, in 1952 at Washington by the Bureau of Animal Industry U.S. Department of Agriculture, to ascertain the cause or causes of this condition and to formulate methods for control, (Wasserman et al. 1954). Though scientists all over the world have put up untiring efforts to control this danger to poultry, no satisfactory results have been achieved so far. The menace caused by these organisms lead to the organisation of world wide conference by the New York Academy of sciences in Jan. 1960 which discussed " Biology of Pleuropneumonia like organisms". The conference also discussed the morphology, isolation, propagation, physiology, serology pathology and chemotherapy of these organisms.

In India though it was reported long back (I.V.R.I. annual report 1954) it was not established till Pathak and Singh (1959) first reported and established it. Later on many workers worked on these organisms in various aspects. The out come of this condition so far is that the chronic respiratory disease

is mainly caused by M. gallisepticum-a pathogenic strain of PPLO and the other organisms of secondary importance are nonpathogenic PPLO (M. gallinarum) E. Coli, H. gallinarum and some filterable viruses. The co-incidence of other diseases such as Newcastle disease, Infectious bronchitis, Infectious laryngo-tracheitis Fowl cholera, Parasitic infestations and various stress factors enhanced the disease proper.

Economic importance :-

The above diseases of respiratory tract caused heavy economic losses in poultry farming by heavy mortality or depletion of egg production or lowering the quality of meat, continuous reduction in growth rate and hatchability of eggs. It was estimated that more than 50% of birds were lost annually due to diseases and other causes. Infectious diseases alone caused 40% losses in India (Naidu, 1964). The disease is prevalent in almost all parts of India and the All India Research Workers conference (1962) confirmed the same.

Scope for study :-

The disease was recorded in the Bihar State only a few years back (Bihar Animal Husbandry Bulletin ^{and I.R.S Annual report 1962-1963} 1958, 1959, 1965). There was an outbreak also in the poultry farming Patna in 1966 in which there was heavy mortality in chicks. So advantage was taken to survey the bacterial flora of respiratory tract of poultry with special reference to pleuropneumonia like organisms".

II. REVIEW OF LITERATURE.

INTRODUCTION

A review into the literature and the present work indicated that a variety of organisms such as (1) PPLD; (2) E. Coli (3) Staphylococci, (4) Micrococci, (5) Streptococci, (6) Klebsiella, (7) Proteus, (8) Pseudomonas, (9) Bacillus, and (10) other gram-negative bacteria (unclassified) have been isolated from the respiratory tract of poultry and associated with respiratory affections. But of all these organisms the PPLD which is associated with CRD is found to cause greatest havoc and as such a full account on the literature in respect of this organism is given. And a brief account of each of the other organisms accompany the same.

1. Pleuropneumonia like organisms : A. Aetiology.

They are also called coccobacilliform-bodies of Nelson's type II. They belong to an apparently more or less homogenous group of those fastidious micro-organisms which are filterable, soft, fragile, and highly pleomorphic, Pathak and Singh (1961). The disease produced by them is chronic respiratory disease which is other-wise called as Nelson's type II coryza, chronic coryza; and Airsac disease, in chickens and turkeys characterized by nasal discharge, respiratory rales, slow rate of spread, persistent symptoms, moderate decline in egg production and loss in body weight (Delaplane and Stuart 1943). Nelson (1935, 1936) first observed minute gram negative Coccobacilliform-bodies in the nasal exudates of birds suffering from chronic infectious coryza of fowls. He differentiated it from H. gallinarum the causal agent of

rapid onset. Dalaplam and Stuart (1943) studied a respiratory disease of chickens and named it as chronic respiratory disease. They thought it to be caused by a virus. ~~Wasserman~~ Groupé et al (1948) isolated a similar agent from cases of turkey sinusitis and reproduced the condition with it. Van-Heric and Eaton (1945) during their passages of the primary atypical pneumonia virus in chickens isolated an organism which was pleuropneumonia like in its characteristics. Growth of these organisms occurred in 10% horse beef heart infusion broth and on horse serum agar plates. Markham and wong (1952) isolated microorganisms similar to PPLO group on artificial medium from embryos inoculated with CRD agent. They termed them as pleuropneumonia like organisms. Jungherr and Luginbuhl (1952) grouped CRD and similar affections of sinuses and air-sacs under the term " Airsac infections". They considered CRD agent and ornithosis like agent were mainly responsible for this infection in poultry. Van-Roekel and Olesiuk (1953) supported the findings of Markham and Wong (1952). Lecce and Sperling (1954) considered PPLO as opportunists and play pathogenic role when the secondary bacteria invade the host. Their isolation was best made from trachea of CRD fowls. Johnson (1954), Osteen (1954) and Jungherr et al (1954) studied that PPLO were always present in CRD complex of chickens and produce the disease.

(Wasserman et al (1954) stated that PPLO infection was commonly associated with E. Coli and Crawley and Fahey (1955) and Fahey and Crawley (1956) indicated that the aetiology of CRD was complex and that PPLO was not the sole

cause of CRD. They have also stated that PPLO was responsible for chronicity of the disease and PPLO did not necessarily represent any past or future infection of the ~~xxx~~ flock with CRD. A PPLO-virus complex was the most probable aetiology. Tozzini (1955) isolated PPLO from the nasal exudate of chickens suffering from CRD like disease. Junghur (1955) recognised that PPLO are primary aetiological agents of a number of respiratory conditions in poultry and as possible cause of arthritis and sinuvitis Johnson and Domermuth (1956) avoided this confusion by proving that PPLO was an important agent in the aetiology of C.R.D. In confirmation of Johnson and Domermuth's opinion, Jordon (1956), Bissa (1956) and Tellogarust and Motracge (1956) isolated PPLO from CRD fowls. Ven Rackel et al. (1958) by adopting methods such as flock histories, conditions of outbreak, pathology, the results of bird and embryo inoculation studies, and isolation of PPLO, diagnosed CRD in fowls. Chu (1958) divided chronic respiratory diseases into 3 types:-

(1) Infections coryza-caused by coccobacilliform-bodies of Nelson (1936, a, b, c. and d) (2) C. R. D. of chickens according to the description of Delaplane and Stuart (1943) and (3) Infectious sinusitis of turkeys by coccobacilliform-bodies of Nelson's type III. He concluded that all these forms were due to PPLO and manifestations of the same disease. Fabricant et al. (1959) stated that although viral and bacterial agents play an important role in CRD under field conditions, PPLO were the essential factors in this disease. Stoenescu et al. (1960) isolated the CRD agent from a batch of imported cockrels and diagnosed the disease as CRD in them.

Loizelier Adres Blanco (1960) isolated M. Gallinarum from out breaks of CRD birds and stated that this agent was similar to pathogenic type S6. Brion (1960) suggested the name of mycoplasmosis for infection caused by M. Gallisepticum- a pathogenic ~~straining~~ strain but not to M. Gallinarum-a non pathogenic strain. This partially over ruled the statement of Loizelier-Adres-Banco (1960). Gentry (1960) recovered PPL0 by six cultural methods and divided them into pathogenic forms of my coplasma and non pathogenic forms which revert to bacteria. Brion (1961) and Bankowski (1961) considered that PPL0 was an integral part of the complex aetiology of the avian respiratory disease. They have also noted that PPL0 alone caused rather mild infections and did not alone serve to define the aetiology of the entire CRD syndroms. Skaika (1951) isolated PPL0 from the respiratory tract of infected fowls, but not from healthy birds. Kaybill et al. (1961) isolated PPL0 from patients with bronchopneumonia, bronchiactasis, and acuted respiratory disease. (Flir et al. (1962) isolated PPL0 in 235 out of 272 infected with CRD fowls and 229 out of 306 healthy fowls. Rao (1962) reported the isolation of a HA-ve strain of PPL0 which developed the typical disease involving the the lower respiratory tract as well, in 100 percent of the artificially infected cases. Kumar et al. (1963) isolated PPL0 from day ola poultts affected with high percentage of air sacculitis. Shiskov and Enchev (1962) and Prokofira et al. (1966) also isolated PPL0 from fowls. Fomia et al. (1964) found mycoplasmosis in one flock when the birds reached sexual maturity, and in the other at the peak of egg laying.

They have also found it in 2% eggs. Imported birds suffered heavily than local birds and in pure breed birds less mixed infection than in local breeds were found. The hatchability of eggs reduced by 8.5%, body weight at 90 days of age by 14%, and egg yield by 30%, and also mortality under unfavourable conditions 16%.

Kozusnik et al. (1966) observed the age incidence of mycoplasmosis in birds. The disease commenced in about 2 weeks of age, and became most common at 20-70 days of age. In young birds the course of the disease was found to be acute form where as in adults it was found to be chronic form.

Various stress factors lay a part either by enhancing the susceptibility of birds to CRD infection or intensify the infection.

Wasserman et al. (1954) stated that PPLO act as primary agent in CRD and the other organisms complicate air-sac infections in broilers.

Many workers like Fahey and Crawley (1955) Edward and Fraundt ~~gixax~~ (1956), Biddle and Cover (1957) Gross (1954, 1958) Simburt et al. (1958) noted that E. Coli is the most complicating factor in CRD. Levine (1962) and others confirmed the findings of Gross (1957, 1958). Barnes et al. (1960) noted that E. Coli and Staphylococci were frequent associating organisms of PPLO in the production of CRD. Environmental factors like chill, humidity, excessive heat, rain, bad hygiene and poor ventilation, also act as stress factors in CRD. Jungherr (1958) noted that poor ventilation increased the spread and

severity of the disease. Tournut et al. (1963) claimed that poor hygiene and management was responsible for sporadic CRD outbreaks. They observed an immediate improvement followed by an increase in Ventilation, or the reduction in the density of birds.

Brion (1960) reported that chemical caponisation has got the same effect. Adler and Shifrine (1960) observed that M. gallisepticum and E. Coli together incited severe cases of aerosacculitis, fibrinous perihpatitis and pericarditis. Not only viral and bacterial agents act as inciting agents of mycoplasmal infections, chemicals also have been shown to increase the disease severity. Shifrine et al. (1960) showed that histamine feeding to chicks reduced the disease resistance in chickens towards mycoplasmosis. Brion (1961) gave the list of stress factors under which the resistance of the birds were lowered for CRD as follows (1) virus infection (2) Parasitism (3) Transportation (4) Faulty management (5) Bad hygiene (6) and Changes in feeding.

Tournut et al. (1963) noted the predisposing factors such as poor hygiene in 76, newcastle disease in 25, parasitism in 4, and infectious bronchitis in 2 out of 107 respiratory diseases observed by him.

Adler (1955), Blake (1962) Van-Roukel^{etal (1957)}, Calnek and Levine⁽¹⁹⁵⁷⁾ and Gross (1961) reported the prevalence of Newcastle disease virus, and Infectious bronchitis virus in association with PPLD, complicated the disease -mycoplasmosis. Stubbse et al. (1955) observed that vaccination serve as stress factor in the development of CRD. Fomia et al. (1954)

found that the incidence and mortality due to mycoplasmosis was greatly increased in cold weather. He also reported mixed infection. (Mycoplasma, fowl pox, or laryngo tracheitis in poultry. Fabricant et al. (1959) stated that viral agents such as Newcastle disease, Infectious bronchitis and bacterial agents such as coliforms play an important role in CRD.

BACTERIAL FLORA OTHER THAN PPLO.

2. Escherichia species:

Gibbs (1931) isolated Escherichia species from 10% birds in his bacteriological survey from respiratory tract of healthy and diseased birds. Marcato (1936) isolated large number of coliform bacterial from nasal exudate of fowls infected with fowl coryza. He concluded that an infectious disease of fowls a generalized infection caused by an organism of coliaerogenus group with coryza as the main symptom. Kahler (1951) isolated E. Coli from lungs of 2583 fowls though the occurrence was not as common as in liver and intestine. Osteen (1954) found in most of the air sac infections only E. Coli and it was thought to be the main causative agent. Cover and Waller (1954) during their study on CRD in pipped eggs, closely observed the severe infection of the parent flock. The lungs and air sac of the birds showed coliaero-genus organisms in combination of streptococci. Luginbuhl et al. (1954) cultured blood from avian respiratory cases and isolated 26 E. Coli from 75 birds. The lung cultures on sabaureauds agar gave 22 coliforms along with other organisms.

Wasserman et al. (1954) examined 22 cases of air sac infection in poultry out of which 20 E. Coli and

coli-aerogenus forms from rest of the two cases were isolated. Fahey (1953) examined air sac infection associated with an exudative process occurring in the abdominal and thoracic air sacs of birds with CRD. In his bacteriological survey of exudates of air sacs, liver and pericardium of 187 chicks with CRD revealed 131 E. coli. He concluded that its presence in air sac infections and debilitation of particular birds by CRD alone or CRD with accompanying disease conditions acting in partnership with CRD, predispose the air sac infection. Fahey and Crawley (1955) recorded the species of *Escherichia* in autopsied birds of CRD. and concluded that they act as secondary invader. Geurden et al. (1956) isolated 78 E. coli strains from diseased foals, calves, pigs, dogs, cats, and fowls and in particular 086, and 026 B6 strains of E. coli from poultry only. Taylor and Fabricant (1957) in a study on the bacterial flora of 120 tracheas of poultry found 30 E. coli as the predominating species in 68 specimens along with other gram negative bacteria. Biddle and Cover (1957) reported the same findings of Taylor and Fabricant (1954) that 57% of E. coli species in 51 CRD cases were present. This supported the concept of close association of E. coli with CRD and their large part in the aetiology of the disease. Gross (1957) also studied the natural infection of E. coli in chickens and turkeys almost always in combination with CRD.

Price et al. (1957) studied the microflora of respiratory tract of healthy and CRD infections in chickens. He isolated only one E. coli species from pleura of healthy chicks. He observed an increase of 75% of bacterial forms in infected birds than in normal birds. Then he concluded that the increase

was mainly from gram positive to gram negative bacteria that too E. coli. Van-Roekel et al. (1957) found the same results in the respiratory system of poultry. Gross (1958) noted E. coli. as the most complicating factor of CRD. In 1960 Simburt et al. isolated E.coli and PPLO more frequently among the negative organisms from aerosacculitis in birds. Among 74 strains of E. coli isolated by Renault et al. ~~ixixixix~~ (1960) from respiratory diseases of fowls, 46% from the heart, 32% from the liver, 19% from the air-sacs and one strain from lungs and spleen were found. In 1960 Prasad isolated 2 Escherichia species from nostrils of healthy chicks and 8 Escherichia species from upper respiratory tract of chickens suffering from infectious coryza.

Brion (1961) in support of Nelson's view (1959) isolated predominantly E.coli and H. gallingaram among the secondary bacterial flora of CRD. He concluded that E. coli as the secondary invader capable of modifying the clinical aspects and special lesions. Adlakha (1961) isolated 34 E.coli from tracheas of 100 live birds, and .28 E. coli from tracheas, lungs and air sacs of 50 dead birds. Glantz et al. (1962) isolated 50 E. coli strains from CRD fowls and 72 E.Coli strains from salpingitis fowls. Shiskov (1964) examined bacterial flora of air-sacs of 157 birds with CRD and found various strains of E.Coli in 47 birds along with other gram positive and gram negative bacteria. Hensley and Holway (1965) found E. coli infection in 51,500 birds in out breaks associated with respiratory diseases. Fomia et al. (1965) isolated 41 E. coli strains from nasal sinuses, trachea, air-sacs, heart, brain and other organs of 22 fowls that have died from respiratory mycoplasmosis. Coisek (1965)

isolated 110 E. coli strains from diseased and 396 strains of E. coli from healthy fowls. Bereznai et al. (1965) isolated 422 E. coli from 171 carcasses of CRD and 30 from 11 healthy birds.

3. Staphylococci :

Gibbs (1931) isolated staphylococci in addition to other organisms from the respiratory tract of birds suffering from laryngotracheitis, pullorum disease, chronic laryngitis, and avianparalysis, and also from healthy birds. Cover and Waller (1954) isolated Staphylococci from lungs and air-sacs of birds suffering from CRD. Luginbuhl et al. (1954) from blood cultures of avian respiratory cases isolated 7 staphylococci from lungs on sabourauds agar. He concluded that high percentage of bacterial and fungi in respiratory diseases. Wasserman et al. (1954) isolated staphylococci from air-sac infection of poultry. He suggested that " Chronic respiratory disease" as " Airsac infection" is erroneous since the latter was caused by bacterial infection. Fahey (1953) isolated 43 staphylococci from air sacs, liver, and pericardium of 187 chicks. Suffering from CRD. Price et al. (1954) isolated staphylococci from nose of healthy and CRD chickens. Van-Roekel et al. (1957) also got the same results of price et al. (1957). Prasad (1960) isolated 4 Staphylococci from upper respiratory tract of 5 healthy and 13 staphylococci from 15 infectious coryza chickens. Adlakha (1961) isolated 39 staphylococci from tracheas of 100 healthy and 9 staphylococci from tracheas, lungs, and air sacs of 50 dead birds.

Inalis

Shiskov (1964) isolated 6 staphylococci from air sacs of 157 birds suffering from CRD.

4. Micrococci :

Gibbs (1931) isolated micrococci from the respiratory tract of healthy and diseased birds. He also isolated 40% species of Micrococci from 10 control birds and other micrococci from 20% of the birds. Fahey (1955) in bacteriological examination of exudates of air sacs, liver and pericardium isolated 73 micrococci along with other gram-positive and gram-negative bacteria. Fahey and Crawley (1955) recorded species of micrococci in autopsied cases of CRD, and concluded that they act as secondary invaders. Biddle and Cover (1957) in their study on bacterial flora of respiratory tract of 120 chickens found species of genus micrococci as second predominating organisms to E.coli. Van Roekel et al. (1957) also got the same findings of Biddle and Cover (1957). Simburt et al. (1960) isolated micrococci from nose, sinuses, trachea, lungs, and air-sacs of 6010 fowls with natural cases of aerobacteraemia. Prasad (1960) was able to isolate 1 micrococcus from nose of 5 healthy, and 2 micrococci from 15 infections caryza chicks.

5. Streptococci:

Buxton (1962) studied the disease caused by streptococcus zooepidemicus in poultry with diarrhoea, nasal catarrh congestion of liver, lungs and tracheitis, and infection of air sacs as constant feature. He concluded that the organisms entered by respiratory tract and the carrier birds acted as

a source of primary contamination. Cover and Waller (1954) have have isolated streptococci from lungs and air sacs of the birds with CRD. Taylor and Fabricant (1957) isolated 11 streptococci species from 120 Tracheas of CRD poultry. Price et al. (1957) in his studies on microflora of respiratory tract of healthy and CRD chickens, isolated streptococci from trachea and pleura along with other bacteria.

Simburt et al. (1958) isolated streptococci most frequently from respiratory tract of 20 chicks. Streptococci was absent from the nose and trachea but in 1960 the same ^{Jadhav} authors isolated it from the same sites. Prasad (1960) found 3 streptococci from the upper respiratory tract of 15 chicks suffering from infectious coryza. Adlakha (1961) could isolate 1 streptococcus from tracheas out of 100 live birds and 4 streptococci from the trachea, lungs and air sacs of dead birds. Neuton et al. (1962) isolated streptococcus species from congested lungs of fowls. It caused fibrinous bronchopneumonia and congestion of lungs.

6. Klebsiella :

In 1961 Adlakha in his bacteriological survey of respiratory tract of poultry found 20 klebsiella from tracheas of 100 live birds and 5 klebsiella from tracheas, lungs and air sacs of 50 dead birds.

7. Proteus :

Fahey (1955) has isolated 85 proteus from exudates of air sacs, liver, and pericardium of 187 chicks with CRD. Fahey and Crawley (1955) recorded proteus sps.

from autopsy CRD birds. Taylor and Fabricant (1957) isolated 9 proteus sps. from 120 tracheas of poultry suffering from CRD. Van Roekal et al. (1957) found proteus sps. in the respiratory system of poultry. In 1966 Prasad was able to isolate 1 proteus sps. from the respiratory tract of 15 chicks suffering from respiratory infection. Adlakha (1961) isolated 6 proteus sps. from the respiratory tract of live birds and 3 proteus sps. from dead birds.

8. Pseudomonas :

Gibbs (1931) isolated pseudomonas species from the respiratory tract of 56 adult fowls, 15 chicks of 6 weeks age and 10 control chicks. Fahey (1955) got 50 pseudomonas species from the respiratory tract of 187 chicks, suffering from chronic respiratory disease. Taylor and Fabricant (1957) in a study on the bacterial flora of 120 tracheas of CRD birds has isolated 3 pseudomonas organisms. Price et al. (1957) in their bacteriological survey of respiratory system of healthy and diseased birds, isolated only one species of pseudomonas from nose, and 2 from pleural cavity. In 1960 Prasad isolated 3 pseudomonas species from the nostrils of 15 chicks suffering from respiratory infection. Adlakha (1961) isolated 1 pseudomonas sps. from tracheas of 100 birds and 5 pseudomonas sps from 50 dead birds. Shiskov (1954) has isolated pseudomonas sps from air-sacs of only one bird out of 187 birds infected with CRD.

9. Bacillus :

Price et al. (1957) isolated Bacillus from the respiratory system of CRD chicks especially from nose, trachea

and pleural cavity. Simburt et al. (1958) isolated Bacillus from 20 chicks.

Indir Prasad in the year 1960 isolated 2 bacillus sps from nose of chicks infected with infectious coryza. Adlakha (1961) isolated 4 bacillus species from tracheas of 100 live birds and 4 bacillus species from tracheas, lungs, and air sacs of 50 dead birds.

10 Other gram negative bacteria(unclassified):

Gibbs (1931) isolated most of the other gram negative organisms other than previously described from the respiratory tract of healthy and diseased birds. Fahey (1955) isolated 21 other bacteria from respiratory system of 187 chicks suffering from CRD. Taylor and Fabricant (1957) also isolated 15 other gram negative bacteria from 120 tracheas of CRD poultry. Adlakha (1961) found 11 other gram-negative organisms from tracheas of 100 birds, and 6 other gram negative bacteria from tracheas, lungs and air-sacs of 50 dead birds. Shiskov (1964) in his examination of 157 air sacs of birds suffering from CRD isolated various gram-negative bacteria from 43 birds.

B. CULTIVATION AND GROWTH REQUIREMENTS-PPLD.

A brief account on cultivation and growth requirements of PPLD have been reviewed in the following literature.

Nelson (1936) cultivated coccobacilli-from bodies by tissue membrane method of " Wood-ruff and Good pasture" and by the tissue membrane method of " Li and Rivers". Swift(1941) grew PPLD on chorio-allantoic membrane of 11 - 12 days old chicken embryos. He emphasized the need of killing embryos by freezing before inoculation for better growth. Van Heric and Eaton (1945) isolated PPLD and grew it on 10% horse serum beef heart infusion broth and agar plates. Edward (1947) used the same media of Van Heric and Eaton (1945) and inhibitors. Fresh yeast extract was also used and claimed that it is necessary for good growth. Stubbs (1947) used formalinized horse serum in the basal medium for PPLD growth and succeeded. Smith et al. (1950) cultivated PPLD in the basal medium with the admixture of inhibitors viz Crystal violet and Potassium tellurite.

Edward and Fitzrald^{sc} (1951) stated that cholesterol or related compounds are necessary for growth of PPLD. White (1952) used Crystal violet, sulphamezathine and thallium acetate as bacterial inhibitors in the PPLD medium. Van Roekel and Olasink (1952) used 20% serum in the basal medium and also used embryonated eggs for PPLD growth. Markham and Wong (1952) grew PPLD in Edward's medium with 20% horse serum. Edward and Fitzrald^{sc} (1951) and Edward (1953) replaced serum with egg yolk and then with ether extract of egg yolk. The extract in turn was replaced by cholesterol in the presence of bovine albumen or starch. Grumbles et al. (1953) used

used phenolred broth with 1-1.5% serum fraction (Difco) for cultivation of C.R.D. agent and Turkey Infectious sinusitis agent (I.S.T.). He used sugars in the medium for fermentation reactions of PPLD. Lecce and Sperling (1964) used 1:2000 ~~usedxxx~~ thallium acetate in the basal medium for primary cultivation. Then they compared Bacto PPLD agar heart infusion agar, and Bacto heart infusion agar and found no discernible difference for PPLD isolation. Chu (1954) cultivated coccobacilli-form bodies in Tyrod's solution containing chick embryonic tissue. Fahey and Crawley (1954, b) studied PPLD on cell free media containing exudate of trachea and lung treated with 100 units of penicillin and 0.5 mgs of aureomycin per ml. of exudate.

Leibermiester (1954) showed that certain lots of agar were unsatisfactory for some strains of mycoplasma, but the inhibitory component was not identified. Edward, (1954) showed that certain strains of PPLD are dependent on the presence of DNA which cannot be replaced by Deoxyriboside.

Adler et al. (1954) employed a medium composed of 1% blood agar slope overlaid with either 20% horse serum broth or 2% serum fraction with penicillin 100 units per ml. and thallium acetate 1:2000 - 1:4000. This media was compared with other media and proved its superiority over all for PPLD isolation. Gianforte et al. (1955) used Mortin's (1952) fluid medium and modified it according to Edward's (1947) medium for PPLD growth. Smith (1955) described a synthetic medium for PPLD in which amino acid appeared to be the source of energy. Adler and Yamamoto (1956 C) supported the Nelson's (1939)

and Lancet et al. view (1953) that chicken embryo provided substances capable of supporting PPLO growth. Hofstad and Doerr (1956) used chicken infusion broth enriched with 20% inactivated chicken serum for isolation. Smith (1956) determined the quantitative growth rate of PPLO by measuring the turbidity of concentrated cultures, colony diameter, and the number of colonies on a plate. Shipkowitz and Clarke (1956) used thallium acetate as 5% solution in 0.15 ml. - 1 ml. broth suspension of tracheal serapings for isolation.

Taylor and Fabricant (1954) modified Grumbles medium (1953) by adding penicillin and thallium acetate for primary isolation. Taylor et al. (1957) used 4 in vitro methods for PPLO primary isolation and compared their efficiency and concluded that modified Grumble's medium was the most successful one for isolation, control of bacterial and mycotic contaminants, speed, simplicity and economy. Van Roekel et al. (1957) used bird and embryo inoculation methods for isolation and found that the former was more effective. Adler et al. (1958) modified their previous medium by adding 1% yeast hydrolysate Yamamoto and Adler (1958, a,b) used 10% horse serum in the media as modification for studying PPLO.

Smith and Lynn (1958) agreed with the view of Edward and Fitzgerald (1951). Freundt (1958) stated that sera from different animals vary in their ability to support growth of various strains. Adler and Shifrine (1960) studied the nutrition, metabolism and pathogenicity of mycoplasmas of different species. They used ascitic fluid or serum to the medium (Difco) to reach final concentration of 5 - 30% to

support growth. They used 1.5% agar in the solid medium. Growth was further improved when cholesterol was supplemented with and acetone insoluble lipid (A.I.L.) fraction of yolk to the basal medium. The balance of cholesterol and AIL for optimum growth differed with various strains used. High concentrations of either component tended to be inhibitory. Chu (1959) used streptomycin in addition to common inhibitors in the media and also used serum or ascitic fluid as necessary means for the growth of most strains of PPLO. Gentry (1959, 1960) used horse serum in different concentrations, 500 units /ml. penicillin, and 1:4000 thallium acetate in the broth and stated that the serum below 10% hampered the growth of some PPLO strains but helped to detect the contaminants. Domermuth and Gross (1959) studied the growth characteristics of PPLO on semi-solid agar medium as reported by Beveridge (1943) and Edward (1954) for the cultivation of PPLO. They used 0.42% agar in the basal medium. Fabricant (1959) used different combinations of media in different atmospheric conditions to study the efficiency of each for PPLO growth and measured the growth of colonies in micrometer units. He concluded that moisture was necessary for growth. Yeast hydrolysate enhanced the growth of non pathogenic where as CO_2 stimulated pathogenic strains mainly.

Chalquest and Fabricant (1960) grew PPLO strains in broth and on agar containing 0.1% Beta-dihyphosphopyridine, nucleotide, 10% heat inactivated pig serum thallium acetate, and penicillin. Adler and Berg (1960) suggested that trypticase-soy broth was better than tryptose agar or broth. Lecithin

(100-500 mgs per litre), gastric mucin 2% and 0.3 mgs cholesterol per ml. in the basal medium were inhibitory to PPLD growth. They compared all media and claimed that two media are superior for primary isolation of PPLD viz one containing chicken serum and the other containing horse serum.

Brion (1961) stated that the cultivation of PPLD was difficult as their nutritional requirements are still uncertain. Most of them require blood serum, cholesterol and deoxyribonucleic acid. Skalka (1961) used a broth containing 25% inactivated horse serum, 0.5% glucose, 0.5% maltose, 0.0025% phenol red, 5000 I.U. per ml. of penicillin and 1:25000 of thallium acetate for PPLD isolation.

Gill and James (1962) maintained the anaerobic growth of pathogenic PPLD by adding pyruvate as hydrogen recipient. They used 100 units penicillin per ml in stock cultures. They proved that peptone was not necessary for growth in the basal medium, but its inclusion increased the growth rate and slight turbidity. The addition of beef heart dialized and dializate gave good growth rate. Addition of glucose increased the growth rate and turbidity, but resulted in accumulation of acid (0.2%). Colusi (1962) stated that the growth of pathogenic strains of PPLD was as good on a basal medium enriched with 25% egg yolk as on the same medium enriched with 20% serum. Fabricant et al. (1962) experimentally proved that the growth of PPLD did not depend on increased CO₂ tension, but excessive moisture in the atmosphere stimulated the growth. Fabricant et al. (loc cit) showed that increased moisture in

atmosphere increased the growth of *M. gallisepticum* and similar growth in 100% nitrogen due to reduced oxygen tension rather than CO_2 stimulatory effect when used. The strains were not adversely affected by increased CO_2 tension as the growth did not depend on CO_2 tension.

Kelton (1962) described a pure culture technique for isolation of PPL0 in pure form by single colony method and stated that five such single colony transfers were sufficient to purify culture.

Shifrine and Mahe (1962) used glucose 0.05% in the PPL0 broth (PH. 7.6) and 2% agar for isolation. Rodwell (1962) accepted the view of Edward and Fitzgerald^{3c} (1951) that some species of mycoplasma require steroids for their growth. With some mycoplasma strains of human and avian origin, it was shown by Smith and Lynn (1958) that Beta sitosterol and cholesterol promoted better growth than did cholesterol, while ergosterol and stigmasterol were less effective.

Kumars et al. (1963) stated that the meat liver medium was superior to Difco PPL0 medium for primary isolation of PPL0. Schimmel (1963) studied the growth rate of mycoplasma by incorporating vitamin B1, and B12 into liquid and solid PPL0 media in place of yeast extract. Cattereu et al (1963) reported excellent growth of pathogenic PPL0 in enriched Difco's PPL0 broth. He incorporated cholesterol and peroxidase for more rapid and abundant growth and glucose for acceleration of the growth rate only. Salana Alonso (1964) used B.V.F. medium (Containing beef, ox liver, and pig stomach) enriched with

horse serum or rabbit serum for cultivation and preparation of mycoplasma antigens.

Fabricant et al. (1964) supplimented PPLO tissue culture medium with Swine serum which supported optimum growth. Growth was also stimulated by the addition of 5 monophosphate nucleotides (adenylic, cytidylic, guanylic, thymidylic acids). In some strains addition of four ribonucleotides and deoxyribonucleotides in place of peptone improved the growth, whereas the four ribonucleotides alone supported poor growth when substituted for peptone. The concentrations of nucleosides and nucleotides had a significant effect on growth. Although the nutritional factors of the pig serum was not defined, the effects of different sera on growth was investigated. Rabbit, horse, turkey, and pig, serum supported optimum growth, human serum supported less, whereas PPLO serum fraction (Difco) or bovine serum supported poor growth. Dog's serum did not support growth. Swaine (1965) described a medium which could support the growth of most of the PPLO strains.

Swaine's (1965) medium is supposed to be the good medium for cultivation of PPLO than all the media mentioned above.

C. Morphology of PPLO

Nelson (1936) described the organisms as coccobacilliiform bodies isolated from the upper respiratory tract infection of fowls. The description was so made according to the morphology of organisms. Sabin (1941) put forward criteria before admitting any organism into pleuropneumonia group based mainly

on their morphology and growth characters, of minute colonies on suitable solid media as described below.

C r i t e r i a :-

Their morphology on cell free media varying from minute filterable elementary bodies to pleomorphic structures including large protoplasmic masses with "Chromatin bodies"; rings, globules, filaments, of 125-250 μ in size. The development of these minimal reproductive units into tiny colonies as small as 10-20 μ and not larger than 500 μ with a central dark, nipple like structure or vacuolar mesh work on a suitable solid media. Their pleomorphism is the main characteristic feature mainly due to lack of cell wall. (Klieneberger Nobel, 1954).

Morton et al. (1954) examined 6 human, and 2 avian isolates of PPLO in liquid media and found that they were spheroidal to ellipsoidal in shape. On solid media coccoid forms, bizzare forms, varying from large circular masses to long filamentous forms with no rigid cell wall were seen. Adler et al. (1956 b) observed some rough colonies suggestive of PPLO in the culture exudate of mixed infection. Lecce and Sperling (1954 b) gave detailed technique of isolation, identification, and subculture of PPLO. They used 1:2000 thallium acetate as bacterial inhibitor for primary isolation of PPLO. They considered trachea to be the best site than lungs or air sacs for PPLO isolation and indicated that tracheal swabs may be taken during life. They evaluated Bacto PPLO agar, heart infusion agar, and Bacto heart infusion agar and found little or no difference among them for isolation.

Hagan and Bruner (1957) described the morphology and cultural characteristics of M. gallinarum. They were coccobacilli-form bodies with 53-60 m/u in diameter (average). They could be stained by Giemsa's stain. The colonies were found small and delicate just on the border line of visibility. With low power lens button like elevations in the centre of the colonies were seen. Domermuth (1958) developed the technique of staining PPLD by modifying Giemsa's staining procedure.

Chu (1958 a,b.) differentiated pathogenic from non pathogenic PPLD. The pathogenic types grew more on Edward's media and their cultures contained mainly of coccobacilli-form bodies whereas the saprophytes grew more rapidly and their cultures contained mainly of rings, globules and bipolar bodies.

Chu (1959) stated that the nipple like centre differ among different strains under different conditions and some did not produce definite centre. Coles and cuming (1959) observed the same findings of Sabin (1941) in their findings on M. gallinarum. Yamamoto and Adler (1959 b) studied the development cycle of 6 strains of PPLD as determined by periodic staining of smears from broth cultures.

Adler (1960) described cultural criteria of pathogenic S6 strain of PPLD. The organisms were mostly coccobacilli-forms bodies (125-500 m/u) with occasional rings. Colonies not exceeding 0.5 m.m. diameter grew into the agar and contained fine coccoid forms in a lacy network at the periphery, with central dense papillae. The typical colonies were unchanged by many subcultures in non inhibitory media. Merchant and Packer (1961) described the colonies of

pathogenic PPL0 M. gallisepticum a prototype of S6 isolate of Adler (1955) consists of an elevated central portion, a smooth surface and an entire periphery. Clarke et al (1961) described hot water fixation technique for studying PPL0 colonies microscopically. He also stated that fixation could be successfully carried out by hot aqueous solution of formalin 1%, acetic acid 1%, and trichlor acetic acid 5%. Rusy and Van Itersonw (1961) studies the characteristics of pathogenic PPL0 of avian origin on solid media. It grew into two types of colonies viz small ones typical of PPL0 and large ones apparently same as Nelson's coccobacilliform bodies (1936).

The detailed study ^{of PPL0} /on electron microscope by Reagan et al. (1953), White et al. (1954), Morton et al. (1954) Dumermuth et al. (1964) Dutta et al. (1965) and Maniloff et al. (1965) revealed many things. The studies of Maniloff et al (loc cit) showed that the nuclear material of M. gallisepticum contains DNA, RNA and protein etc. These findings furnish an information about the growth requirements of PPL0.

D. Biochemical reactions - of PPLO.

The biochemical reactions exhibited by some of these organisms were taken as a guide in identification and classification.

Van Roekel and Olesiuk (1953) used dextrose, maltose sucrose, lactose and mannite in the case of CRD and IST agents. They fermented dextrose, maltose and sucrose but not lactose and mannite. Grumbles et al. (1953) and Fahey and Grawley (1954 b) followed the same method and confirmed their findings. In the latter two author's work the fermentation reactions were maltose in 3-6 days, Dextrose in 5-7 days and sucrose in 8-12 days. Edward (1954) tried sugar tests on one strain only and it did not ferment glucose. Gianforte et al. (1955) analysed PPLO from cases of air sac infection in poultry into 7 strains by biochemical tests. The fermentation reactions were studied 4 days after incubation. All strains produced acid from dextrose, maltose, mannose, saccharose and trehalose, and slight acid in levulose broth. No acid was produced in adonital, galactose, inositol, innulin, lactose, mannitol raffinose, rahmnose, salicin, sorbitol and xylose, Gas formation was not demonstrated. Taylor ~~et~~ and Fabricant (1957) recorded the formentation rate and the number of carbohydrates attacked by PPLO strains in the liquid medium of the 68 isolates, 55 fermented 3 sugars (glucose, maltose and sucrose), one isolate fermented only glucose on the original inoculation.

Adler et al. (1957) studied biochemical properties of PPLO. All strains produced acid in trehalose, maltose ,

starch, Sucrose, lactose, dulcitol, and mannose at 8 hours intervals. The non-pathogenic strains fermented sucrose within 48 hours. Yamamoto and Adler (1958 b) divided PPL0 isolates in to 8 strains on the basis of sugar fermentation reactions. All 7 strains attacked dextrose, levulose, galactose, sucrose, maltose, dextrin, and starch. Except one strain, mannose was also fermented by all strains. The 8th strain "N" did not ferment lactose, mannitol, dulcitol, and salicin. The non-pathogenic group IV and the mildly pathogenic group V organisms did not ferment any carbohydrate. Some strains classified in group II were also nonfermentors. Pathak and Singh (1959) conducted sugar fermentation tests of 104 PPL0 cultures isolated by them, 11 fermented glucose, and maltose, 57 glucose, maltose, and sucrose. None of them fermented lactose or mannitol on their studies the fermentation reactions of glucose, maltose, and sucrose appeared to be quite variable. Moore et al. (1960) performed sugar fermentation tests in 10 strains of avian PPL0. All fermented dextrose, maltose and sucrose in 1 - 7 days, but none of them fermented lactose or mannitol. Edward and Kanarek (1960), studied biochemical and serological properties of 40 strains isolated from the respiratory tract of fowls and turkeys. On the basis of biological properties, these strains were classified into 3 groups. Only organisms belonging to one of the groups represented by M. gallinarum and M. imers nor sp., did not ferment even glucose. According to Kleckner's (1960) observations, 5 serotypes of avian PPL0 were not able to ferment carbohydrates. The most

significant feature was that none of the pathogenic strains came in that group and this character has not diagnostic value in the biochemical analysis. Adlakha (1961) conducted sugar fermentation tests of 465 PPLD isolates. 55.9% of them fermented glucose, maltose, and sucrose, 33.5% fermented glucose, and matose, and 10.5% glucose only. Singh (1962) characterized 191 cultures out of 793 on the basis of biochemical reactions and other criteria. Swain (1965) studied the biochemical properties of PPLD of various species. He noted that M. gallinarum did not attack carbohydrates. He cautioned that horse serum is not suitable as an enrichment for the sugar media because it contains maltases and diastases which may engender false positive reactions. He also suggested that either Human or rabbit serum can be used for this study.

4. Tetrazolium reduction test-PPLD.

This test was first performed by Yamamoto and Adler (1958 b) as a means to differentiate the pathogenic and the non-pathogenic types of PPLD by the reduction of dye in correlation to time. Later on many workers used this on the same lines. Adlakha (1961) and Singh, (1962) have taken this as one of the criteria for differentiating pathogenic and nonpathogenic ~~and~~ PPLD.

Yamamoto and Adler (lac cit) used 0.75% semi solid agar medium with 0.0025 tetrazolium blue as indicator for this test. A stock solution (1%) sterilized by 121c for 15 minutes and stored in the refrigerator in a dark container to avoid non enzymic reaction. (Somerson and Morton 1953). Their results

indicated that group I organisms could be easily differentiated from group II organisms, the former reduced the dye in 24 hours, the latter in 72 hours and group III and IV organisms showed intermediate reactions in reducing the dye in about 36 and 48 hours respectively. The "N" strains group V did not reduce the dye.

E. SEROLOGY (PPLD)

Introduction :

The serology in avian PPLD has become a beacon light for detecting CRD in poultry and analysis of various strains. Though many tests were evolved, the most practicable and widely used tests are plate agglutination and tube agglutination tests, and so the literature has been reviewed accordingly.

Van Heric and Eaton (1945 b) were the first to demonstrate haemagglutination of PPLD from chicken embryos. Later on other workers tried Haemagglutination inhibition and agglutination tests with CRD and IST strains. Slide agglutination test was introduced by Priestly (1951).

Jungherr et al. (1953) were the first to devise a serologic test for CRD that demonstrated the PPLD to be antigenic and agglutinable by its specific immune serum. Adler (1954) described the slide agglutination test which appeared specifically to detect the presence of antibodies against PPLD in sera of birds exposed naturally or experimentally to the CRD agent. The lesions and symptoms of CRD correlated with the agglutination test. Bacteriological cultures of the affected birds yielded PPLD in almost all the cases.

Fahey (1954), and Fahey and Crawley (1954 b), reported the uses of H.I. test to study CRD and IST by using an antigen prepared from PPLD. The H.I. antibodies developed between second and third week after infection and reached their peak 6-8 weeks afterwards. Jacobs et al. (1954) examined CRD cases by haemagglutination, haemagglutination inhibition, tube agglutination

and rapid plate tests. They concluded that haemagglutination inhibition (H.I.) test was considered to be the most sensitive and the rapid ~~plate test~~ a relatively accurate method for the detection of chronic respiratory disease in fowls. Gianforte et al. (1955) conducted serological analysis of seven strains of PPLO from air sac infection by adopting tube agglutination, haemagglutination (H.A.), haemagglutination inhibition (H.I.), slide agglutination, cross absorption, precipitation and complement fixation tests. They gave a method of production of formalinized antigen for tube and slide agglutination and cross absorption tests. They also conducted precipitation, complement fixation, H. A., H.I., tests to detect PPLO infection in poultry. All strains had haemagglutinable properties in almost the same titre. When strains tested for homologous and heterologous sera, similar HI titres were obtained. For routine diagnosis complement fixation test was not possible due to technical difficulties. Because of low titres obtained in precipitation tests, it was also not the method of choice. Taking into consideration of the results obtained, organisms used and obtained from different geographical areas were shown to be identical. The results of their studies suggested that the slide agglutination and tube agglutination tests would be the earliest and most practicable ones to use in the diagnosis of CRD, since antigen for these tests could be made in ample quantity and stored for longer periods of time at 4C.

Newing (1956) developed whole blood plate agglutination test using stained antigen. Adler and Yamamoto (1956) developed a new cultural method for production of satisfactory antigen to detect PPLO. In CRD and IST they

demonstrated the usefulness of S-6 strain of Zander (1954) as an antigen for the detection of CRD and IST by the slide agglutination test. Jungherr et al. (1955.) tried to assess the significance of serological tests on the basis of cultural and isolation studies of PPLD. But the correlation was low due to technical difficulties found in the isolation of PPLD. They adopted H.I. test of Van Heric and Eaton (1955) and developed serum plate (S.P.), whole blood (W.B.) and tube agglutination (T.A.) tests. The sensitivity of tests ranged in the order of S.A., W.B., H.I., and T.A. They tested 15,266 chicks and turkeys and found some flocks were negative while others showed up to 95% infection. Hofstad (1957) performed plate agglutination test with equal volumes of sera and antigen and read the results after 3-4 minutes. He also used double the amount of antigen with equal parts of 20% sodium chloride solution in an attempt to hasten the reaction. Adler et al. (1957) studied strain differences of PPLD Avian origin. He found that atleast two different serological and pathological types of pleuropneumonia like organisms were present in chicken and turkey sinuses. These two groups were primarily differentiated by agglutination and ammonium sulphate flocculation procedures. Keller (1958) conducted agglutination test in 1581 fowls out of which sera of 82% were positive for infection. Yamamoto and Adler (1958 a,b.) carried out antigenic analysis of 7 strains of PPLD of avian origin. A comparison of serological data with the pathogenicity for chickens and turkeys indicated with an exception that there was close correlation of the lesions seen at necropsy, the agglutinations detected, and the reisolation of the organisms. Hammer et al. (1958) used spot plate aggluti-

-nation test for diagnosis of avian PPLD. He used 30,000 serum samples in 3 years period and confirmed that the test was simple, rapid, sensitive and specific. Olesiuk and Van Roekel (1960) in their pathogenicity trials on chicks of 6 weeks age, immunological observations of avian PPLD. The sera of 17 out of 18 chicks definitely agglutinated PPLD antigen after 14 days exposure to the agent. They also showed the possibility of contact transmission of CRD from naturally infected to susceptible chicks by agglutination test and determined the status of breeding flocks by rapid serum plate agglutination test. Moore et al. (1960) indicated that some PPLD did not haemagglutinate and good haemagglutinating antigens could not be made from others and therefore haemagglutination test could not be practiced in a serologic study of all organisms. They utilized agglutination reaction and found among their isolates that there were at least 4 serologic types of PPLD on the basis of serum plate test. Their results indicated that most, if not all, of the pathogenic strains of PPLD had common antigenic factor and could be detected with the available commercial antigens. The separation of pathogenic antigenic strains from the several nonpathogenic antigenic strains appeared to be accomplished best by serologic techniques because the pathogenic strains seems to possess a common antigenic factor or not sharing the nonpathogens.

Klecker (1960) studied the use of agglutinating antigens for the detection of CRD and infections sinusitis in chickens and turkey flocks and the antigenic relationship of avian PPLD with other biological characteristics. Aftosmis et al. (1960) adopted the whole blood test as described by

Jacobs et al. (1954) which provided a rapid method for detecting antibodies against the agent. He stated that the temperature has a marked effect on field whole blood test. If the temperature decreased, the rate of reaction decreased as shown by an increase in time required for agglutination and the small size of the agglutinating particles. The optimum temperature for the test was between 70-85F°. An artificial source of heat might be supplied when the heat goes below 50F°. He concluded that the comparative tests with standard plate antigen and with culture isolation showed agreement. Cover et al. (1960) stated that frozen serum used in PPL0 tube or plate agglutination test increased the ability of a positive serum to agglutinate the antigen. The duration of storage in the frozen state had no effect on the reaction and storing at 4C° for 32 days did not loose their agglutinating ability.

Brion (1961) stated that the negative result of M. gallisepticum was not conclusive proof since M. gallinarum and secondary bacteria over grew rapidly on the whole surface of media, hence the serological methods offered a valuable aid in the diagnosis of CRD due to M. gallisepticum.

Bankowski (1961) in his experimental studies on mycoplasma in chicks, the organisms produced neither symptoms nor mortality but resulted 100% serological response that persisted for at least 3 months. Coming (1961) in his survey of the incidence of avian PPL0 using plate agglutination test found 16 to 17 forms had incidence of antibodies to avian PPL0 in their flocks. Dunlop and Styout (1956.) gave M. gallisepticum by aerosol to birds of 4-22 weeks age and tested serologically.

All except one was positive to the test at 10 days post inoculation. Hall (1962) adopted procedures for the production of serum plate agglutinating antigens in Difco tryptose phosphate broth. He also described^a procedure by which either of 2 - M. gallisepticum strains (Iowa 801 or california 56) could be propagated in a simple production medium to produce satisfactory H.I. serum plate and serum tube agglutination tests. Ceccarelli (1962) used antigen prepared from PPLD of turkey origin for H.I. and rapid agglutination tests on fowls. In his studies in 12 flocks by using both tests, the results of each gave good agreement. Gullion et al. (1963) conducted HI tests with serum samples from fowls in four different age groups and found no significant results. He concluded that serological reactions did not constitute a severe indication of the presence or absence of mycoplasma. Colusi and Monchaca (1963) in their epidemiological survey of CRD used rapid slide agglutination test out of 179 serum samples from 56 regions tested, 114 were positive, 58 negative and 7 doubtful. Cattereau et al. (1963) used rapid slide agglutination test with stained M. gallisepticum antigen having the same antigenic properties as Adler's strain 56 for avian mycoplasmosis. They tested over 2500 serum samples from infected, suspected or healthy birds and got satisfactory results.

Sornicle and Zaibet (1963) in their serological studies, used extract of the blood clot from the heart or large blood vessels from the dead fowls as it was not possible to obtain liquid blood for the slide agglutination test. They claimed that the results were in close agreement with those of serum agglutination. Prokofera et al. (1963) prepared

antigen on the lines of Yamamoto and Adler (1956) and used it for diagnosis of avian mycoplasmosis by serum agglutination test in some thousands of serum samples and indicated that the method was specific. Shiskov (1964) infected birds with M. gallisept-
-ticum and tested ~~xxx~~ them by rapid agglutination test. They were positive to the test 40 days afterwards. Adler and Da Massa (1964) used comb's antiglobulin technique (using antiglobulin) for enhancement of mycoplasma agglutinating titres and to detect antibodies of M. gallisepticum, M. synoviae, and strain 'N' of
avian origin. The antibodies were found at high titres and the test was specific. Lucas and Luthgen (1964) studied the distribution of mycoplasmosis in poultry farms. They examined 3630 random serum samples from about 10% of the birds in each of 61 flocks. 1,510 (41.6%) birds contained mycoplasma agglutinins, and about 70% of the flocks were infected. In cross breed flocks reactors amounted to about 32% and in pedigree stock nearly 51%. Rislakki and Vasenius (1965) studied the mycoplasma infections in poultry by rapid slide agglutination test. Storr's mycoplasma antigen stained by crystal violet was used in the test. Of 1,512 blood samples used, 783 (51.8%) reacted positively. The reaction was higher in flocks where respiratory disease had occurred. Richey and Dirdjosebroto (1965) in their serological test against CRD in chicks, 40% of 54 flocks were positive to the PPLO slide agglutination test, and all were positive to H.I. test.

Serotypes :

The serotypes of avian PPLO were evolved by various

authors by adopting various serological tests.

White et al. (1954) and Gianforte et al. (1955) classified PPL0 into one serotype that was associated with respiratory infections of turkeys and chickens. Chu (1954) broadly classified the PPL0 into two serotype viz (1) pathogenic serotypes that produce disease by themselves and (2) non-pathogenic serotype which act as secondary invader. Later on Adler and Yamamoto (1957), Chu (1958, a, b) and Chu and Newham (1959) have confirmed the findings of Chu (1954). Yamamoto and Adler (1958) separated 5 serotypes as Sb, SA, C, O, and N. Kleckner (1960) antigenically differentiated 15 strains of avian PPL0, and grouped them into 8 serotypes as A, B, C, D, E, F, G, and H and his new serotypes as B.D. and E. Chal quest and Fabricant (1960) identified another new serotype which was associated with sinusitis of chickens and turkeys. The strain S6 was originally isolated by Zinder (1954) from the brain of a turkey torticollis. Yader and Hofstad (1962) reported an isolate from air sac lesions in turkey embryos and designated it as Iowa 695. Noel et al. (1962) analysed 8 strains of mycoplasma gallinarum into 4 serological groups. Group A - consisted of pathogenic strains-MD-2, M D-3, M D-8, and S-6., Group B-contained MD-6 which was although pathogenic strain of group C and contained specific antigenic components not present in other strains. Group C contained non pathogenic, and antigenically distinct strains of MD-4, and MD-5. Group D contained strains of T4 which was saprophytic and antigenically not related to any of the others.

Roberts (1963) isolated a previously unreported

avian mycoplasma serotype and designated it as "WRI". Yamamoto and Bigland (1964) isolated 5 mycoplasma strains from air sac lesions of poultry, sinus exudates of adult turkey and a Japanese quail, and classified them into 2 serological types N and 8. Yorder and Hofstad (1963) isolated 98 mycoplasma strains from chickens, turkeys, pigeons and partridges and classified 88 strains into 12 serotypes from A to L alphabetically. Among the isolates, A, B, C, D, E, G, I and K from chickens and A.F, H.I. and J from turkeys were found.

The genus and species designations were made in a few cases. *Mycoplasma gallinarum* as a non pathogenic type was suggested by Edward and Freundt (1956) and this was found out by Kleckner (1960) as B type. Yamamoto and Adler (1958) represented S6 strain to *M. gallisepticum* as pathogenic and C-strain to *M. gallinarum* as non pathogenic. Edward and Konarek (1960) classified 40 serotypes of mycoplasma into 11 strains of *M. gallinarum*, 8 strains as a new species *M. iners* and 15 strains regarded as *M. gallisepticum*. Adler and Yamamoto (1957) Adler, Yamamoto and Berg (1958), Chu (1958), Chu and Newham (1959) and Edward (1960) described 3 species of PPLO as given by Edward and Konarek (1960). Merchant and Packer (1961) described 3 species only with their prototypes as *M. gallinarum*-54-537 isolate of Kleckner (1960), *M. gallisepticum* S6 isolate of Adler (1955) and *M. iners*. '0' isolate of Yamamoto and Adler (1960). The most popular avian pathogenic S6 strain antigen was used in the diagnosis of CRD by many workers.

F. PATHOGENICITY TRIALS -PPL0.

Introduction :

The transmission of PPL0 is broadly classified into three routes. viz by direct contact, Air borne, and via eggs.

A. Natural Transmission : I. By direct contact and air.

Grumbles et al. (1952) reported that air borne spread of CRD and IST agents from layers with infectious sinusitis to chicks, resulted in a mild form of CRD. Van Roekel and Olesiuk (1952) observed that natural transmission occurs by direct contract or by exposure to contaminated equipment, droppings, water, dust, and fomities capable of carrying the CRD agent. Again the same authors in 1954 observed that turkeys placed in direct contact with infected fowls developed "chronic respiratory disease". The lungs and exudates from the infra-orbital sinuses, nasal cavities, trachea and air sacs of infected fowls were infective to other fowls and chick embryos. Crawley (1955) described that air borne dust or droplets or contact transmission of infection to susceptible chickens played an important role in the dissemination of CRD when PPL0 alone were transmitted then in combination of CRD virus by air borne route, the time of response to infection was prolonged and that the out breaks are often started by carriers. Berger et al. (1958) stated that the disease was considered to be transmitted by air contamination of feed, water, litter may be additional sources of infection.

Coles and Cuming (1959) observed that PPL0 spread by air currents since the nasal and oral secretions are often infected and also easily distributed by feed and water.

Lancaster et al. (1960) stated that PPIO spread rapidly by direct contact and by air over a short distance. Cuming (1961) pointed out that young chicks contract only when the pullets came in contact with adults in the farm. He was of the opinion that PPIO were not being transmitted widely through egg but mainly by contact with recovered " carrier" birds and so the lower incidence of PPIO in young birds. Merchant and Packer (1961) described that M. gallisepticum was transmitted by direct contact with infected poultry or contaminated equipment.

Mc Martin (1962) reported contact transmission of M. gallisepticum when infected and susceptible fowls were mixed. Yamamoto and Bigland (1964) reported natural infection of M. gallisepticum strain in chicks and turkeys. It reproduced significant increase in air sac inflammation. Heishman et al. (1966) reported the incidence of infection by M. gallisepticum by natural means.

2. Egg transmission :

Egg transmission of PPIO though not the only route of dissemination of CRD, plays a major role in addition to transmission by contact and air.

Van heric and Eaton (1945) were the first to discover PPIO in fertile hens' eggs accidentally while working with the primary a typical pneumonia virus of humans in chicken embryos. Olesiuk (1952) revealed that 10% of the cull chicks and 46% of pipped but unpatched embryos produced by an infected supply flock had lesions of the respiratory tract characteristic of CRD. Fahey and Crawley (1954, b, c.) studied CRD agent

from eggs and chicks and cautioned that egg transmission was not the only method of spread of PPLO infection although it was the major source of spread of infection. They followed the breeding stock of one hatchery through four generations, each of which developed symptoms of CRD and yielded ~~HEA~~ PPLO on culture. They claimed that the major means of spread of PPLO was through eggs. Cover and Waller (1954) examined pipped and dead embryos from a hatchery and found 72 % were infected with CRD agent. Calnek and Levine (1957) reported that egg transmission of CRD has been incriminated by many workers including Jungherr et al. (1955) Hofstad (1957), Fabricant et al. (1958, 1959), Beckman et al. (1959), Van Roekel et al. (1958) and Jarstaad et al. (1959) as one of the more important means of dissemination of CRD agent. Hofstad (1957) confirmed the findings of Van Roekel et al. (1952). Abbot et al. (1959) studied the egg transmission of mycoplasma. In one group there was high incidence of air-sac lesions in embryos or cull poult. In the other, the abnormal patterns of seasonal egg production and hatchability and they varied increasingly with the incidence of egg transmitted disease. There was an increase in death rate of pipped but unhatched eggs and increase in the early embryonic mortality. When there was severe disease prevailing, the egg production was affected due to egg transmission of CRD agent. Olesiuk and Van Roekel (1960) reported a high incidence of egg transmission of CRD agent in flocks in which the disease had established.

Merchant and Packer (1961) reported limited significant transmission of M. gallisepticum occurred from

hen to off-spring by means of egg. Brion (1960) noted that only pathogenic PPLO transmitted through egg but not nonpathogenic PPLO. Oleson et al. (1962) confirmed the findings of Merchant and Packer (1961). Fomia et al. (1964 isolated mycoplasma from 2% eggs. They have also observed full course of symptoms of the disease in the hatched chicks.

B. EXPERIMENTAL INOCULATION

Introduction :

Animal inoculation and reproduction of the disease has got great importance. Various workers taken this as the best method of classifying the strains into pathogenic and non-pathogenic and to determine their specificity. The course and intensity of disease varied according to the route, dosage and type of infection given.

(1) In Chicks :

Nelson (1935) was the first person to produce experimentally Coryza of slow on set and long duration by inoculating Coccobacilli-form bodies into fowls. They developed nasal discharge in 1-4 weeks after infection. Chu (1954) was unable to reproduce the disease by injection with cultures, though he was successful by intranasal instillation of the nasal exudate containing coccobacilli-form bodies. Alder (1955) reproduced sinusitis in turkeys with PPLO broth culture and did not succeed in chickens with the same culture. Fehey and Grawley (1956) produced mild disease in turkeys and chickens indistinguishable from that of Nelson's coryza type II.

Adler and Yamamoto (1956) were able to produce chronic coryza by infecting chickens using virulent exudate obtained after 20 passages of broth culture in chicks embryos and two passages in turkeys.

Johnson and Domermuth (1956) inoculated PPL0 broth culture into sinuses of turkeys and air sacs of chickens and produced very mild disease in which only microscopic lesions were seen. Tylor and Fabricant (1957) inoculated into sinuses and tracheas of chickens and young turkeys with cultures propagated in modified grumble's medium and embryos. They observed that PPL0 propagated in yolk-sacs was superior to the other in pathogenicity and chicks were less susceptible than turkeys on inoculation. Gross (1958) studied the role of E.Coli alone and in combination with yolk adopted CRD agent into right lesser abdominal air sacs of chicks and turkeys. In all the cases both the agents and in one case E.coli along as reported by Edwards and Ewing (1954) produced pericarditis and air sac infection. He concluded that E. coli along with CRD agent greatly increased the pathogenicity. Olesiuk et al. (1957) employed embryo adopted, turkey passaged and broth subcultured PPL0 of the same strains in chickens and turkeys by intravenous or via combined sinus and tracheal routes. The results varied clinically and pathologically in extent and severity of the disease, and 14th broth passaged PPL0 did not manifest any symptoms or lesions of CRD when given intravenously. Fabricant et al. (1959) were not able to reproduce as serious CRD in chickens as seen in out-breaks with known pathogenic strain (S6) of PPL0.

Gross (1958) studied the role of E.coli in CRD and

supported the view of Edwards and Ewing (1954). Simburt et al. (1959) used germ free birds to avoid the influence of other organisms. Four weeks old germ free and conventional turkeys were inoculated either with pathogenic or non-pathogenic strains or ^{of PPLo} bacteria free sinus exudate ^{of PPLo} intra-tracheally. Inflammation of air sacs was caused by pathogenic or sinus exudate, and no symptoms or lessions were produced by non-pathogenic strain. Gienforte et al. (1960) experimentally attempted to enhance CRD syndrome with PPLo alone and with PPLo and some stress factors. Ose et al. (1960) reported trials involving 1300 chicks to establish a reproducible system of mycoplasma infection based on the measurement of serology, weight gains, incidence of typical gross and histopathological lessions and recovery of the organisms of the various combinations - I/V, I/V air sac routes appeared most agreeable in producing a high incidence of air sac lesions and the greatest amount of weight suppression in chickens by pathogenic PPLo cultures in Grumbles medium. Bankowski (1961) studied experimentally the nature and extent of CRD produced by PPLo alone, PPLo and E.coli and E.coli/alone. He ~~He~~ concluded that E.coli acts as a self limiting opportunist and that serious disease resulting in mortalities can be expected from a combination of E.coli and other relatively mild agents such as mycoplasma. Adlakha (1961) and Khare (1961) in their pathogenicity trials in birds inoculated pooled PPLo cultures, egg passaged PPLo, E. Coli, and PPLo combined. The results suggested that uncomplicated PPLo infection remained inapparent but was accompanied by significant positive serological response. However the pathogenic

potentialities of PPLO could not be determined.

Domermuth and Charles (1962) produced experimentally distinct type of " breast blisters" in birds by 36 mycoplasma. Olerson et al. (1962) studied experimentally the most predilection seat of pleuropneumonia organisms and their survival. After 2 weeks highest percentage of organisms were isolated from tracheas. A sharp decline occurred when one day old chicks reached 8 weeks and this persisted during the 35-38 weeks experimental period. Rashaw and Popov^α (1963) reproduced CRD in chickens by intratracheal inoculation of cultures or material from affected turkeys in 16 - 24 days. Shiskov (1964) studied the contact transmission of PPLO in birds. Air-sac lesions were seen in most of them. Various workers like Rhodes et al. (1964), Naumets (1964) and Roberts (1964) also produced the disease in experimental animals.

2. AIR-BORNE INFECTION

The natural spread of infection (CRD) has been observed by various workers. Very few workers attempted to transmit the disease by air borne route.

Fahey and Crawley (1954 a,b) showed experimentally that CRD virus and PPLO associated with CRD could be transmitted by air. Gordan (1956) studied experimentally the air borne infection in healthy cockrels. The healthy birds kept at 4 feet height in pens above and the infected kept on the floor just below the healthy birds. After periodical H.I. test revealed that 3 out of 4 healthy birds were positive. The author concluded that PPLO can spread through the air over

a short distance. Mc Martin (1963) conducted experiments on air-borne transmission of mycoplasmosis. 14 birds infected by air borne route were grossly normal, and in 2 groups of 10 normal chicks infected by air borne route. But minute localised lessions in the abdominal air-sacs of one bird was exception in 14 birds on microscopical examination, all infected revealed typical lesions. Cultures from the respiratory tract of every bird produced 2 isolates of M. gallisepticum from the massal mucosa but none from the tracheas or air-sacs. Though there are number of experimental animals used by many authors, typical disease syndrome was produced only in chickens and turkeys.

3., EGG INOCULATION

Several workers have studied experimentally the pathogenicity of CRD agent in chicken embryos.

Fahey and Crawley (1954 b) reproduced CRD experimentally in 7 day old embryonated eggs and they died in 5th day. Chute and Gole (1954) studied the experimental transmission of CRD agent in 7 day old chicken embryos. They inoculated the diluted culture into the embryos by air-sac route. All embryos died in 24 hours. Cover and Waller (1954) in thier experimental studies inoculated 0.2 ml. of inoculum in each of the 7 day old chicks embryos. All embryos which died in first 8 hours were discarded as they were found to be due to traumatic cause. The positive results showed irregular morality and typical lesions characteristic of CRD. Adler and Yamamoto

(1956) indicated that the growth of PPLO in experimental chicken embryos reached a peak in 24 hours (10^8 organisms per ml. of yolk) and maintained this level through a 120 hour period.

Tellogarust and Nobraega (1956) passaged PPLO 3 - 4 times through 6 day old chick embryos. There was mortality of 70 - 100% and the organisms were recovered by staining. Fahy and Crawley (1956) experimentally showed that chick embryo inoculation with cultures of PPLO resulted in increased embryonic mortality and death of chicks at the time of hatching. Mortality was greater in first 72 hours in chicks hatched from infected eggs.

Calnek and Levine (1957) found that six chicks that hatched from inoculated fresh eggs and four out of four of chicks from embryos inoculated at 5 days were infected with PPLO. They also found that PPLO died at 37°C in unfertile eggs within 4 - 8 days and in fertile eggs it survived for 24 days at $58 - 60^{\circ}\text{F}$ and 7 days at 80°F . Fabricant et al. (1958) reported that in their experimental egg transmission uniform and reproducible results were not secured.

Yamamoto and Adler (1958) propagated PPLO strains in 5 - 7 day old chicken embryos by yolk sac route and isolated PPLO from the yolk of dead and live embryos after 4 - 7 days. Rashaw and Popov (1963) reproduced typical lesions in chicken embryos with M. gallisepticum. The embryos died after 3 - 4 days of infection. Yader and Hafstad (1964) studied the pathogenicity of their isolates in chicken embryos and A, D, E, I and J were found pathogenic for chicken embryos.

III. MATERIALS AND METHODS

Materials :

The materials were collected from Government Central Poultry Farm, Patna in this present investigation. In the present studies materials from tracheas of apparently healthy birds, ^{and} tracheas, lungs, and air-sacs of chicks died due to some respiratory disease, and yolk of pipped but unhatched chicks were collected. The method adopted for the diagnosis of a respiratory disease in poultry was essentially on the lines described by Chu (1958).

Table No.1

Showing details of materials used for cultural and serological examination from apparantly healthy birds, dead chicks and pipped but unhatched chicks.

Source	Kind of material	Cultural Examination			Serological examination.		
		Appera ntly healthy birds.	Dead chicks 6-10 weeks age.	Pipped but unhatched chicks. with part- ially observed yolk sac.	Live birds.	Dead chicks 6-10 weeks age.	Pipped but unhatched chicks with parti- ally obser- bed yolk sac.
Government	Tracheal	80	20				
Central	swabs.						
Poultry	Lung swabs	-	20				
farm,	Air sac						
Patna.	swabs	-	20				
	Yolk	-	-	50			
	Whole blood	-	-	-	80-250		
	Exudate of clotted blood.					20	50

Dis

1.

2.

I

1. Cultured in modified grumbles PPLO Dis PP
1. Cultures centrifused stained with Giemsa's stain and grams stain and examined. 1.
2. Serially passaged in PPLO broth. 2
3. Inoculated on PPLO agar. 3
4. Inoculated in chicken embryos. 4
5. Inoculated in chicks.
6. Materials collected from dead diseased and healthy experimental chicks and examined on the same line as described above.
7. Tested for sugar fermentation reactions.
8. Tested for tetrazolium reduction test.

plates,
urther

Methods :

The method of study for the isolation and propagation of PPLD were essentially the same as described by Taylor and Fabricant (1957) except for some minor changes in the preparation of the liquid medium and solid medium (vide infra).

Inoculum :

Sterile cotton swabs on sterile applicator sticks were used to remove the exudate, if any, from tracheas of apparently healthy birds, ^{and} tracheas, lungs, and air sacs of dead chicks and yolk of pipped but unhatched chicks. In the case of apparently healthy birds, this was achieved by opening the beak of the bird by holding comb and wattle and introducing the sterile cotton swab into the trachea and gently rotating it to remove the adhering exudate from trachea. In the case of dead chicks and pipped but unhatched chicks the swabs were collected after conducting postmortem from respective regions as detailed in the table No. 1 some times pieces of tracheas, lungs and air-sac membranes were also collected under sterile precautions from dead chicks, triturated by grinding in nutrient broth and then placed in 5 ml. nutrient broth (pH. 6.8) and thoroughly shaken.

Bacterial flora other than PPLD.

The primary inoculum was streaked on blood agar plates, incubated for 24 hrs and plates showing no growth were further

incubated for 24 hours and before being discarded as negative. Representative colonies were picked up and maintained for further study. The above said colonies stained by Gram's stain, and differentiated into cocci, gram positive and gram negative organisms. The gram negative organisms were streaked on Mac Conkey's agar. The lactose fermenters further streaked on E.M.B. agar and examined for metallic sheen a characteristic feature of E.coli species.

Pure cultures of most of the strains were identified and genus established on the basis of morphology, cultural characteristics, and biochemical activity. The strains were identified and classified according to Bergey's Manual of Determinative bacteriology. (Breed et al. 1957) and Edward's and Eving (1954).

Pleuropneumonia like organisms :

1. Culture media :

The method used in this studies for the isolation and identification of PPLO were the same as those described by Grumbles et al. (1953) and modified by Taylor and Fabricant (1957), and later on followed by many workers with little modifications.

Method of various inoculums used for inoculation in PPLO broth, PPLO agar, chicken embryos and chicks.

1. Modified Grumble's medium:

- (a) Tracheal exudate along with mucous membrane, pieces of lungs and air-sac, materials and membranes.
- (b) Yolk from infected embryos.

- (c) PPLO cultures in modified grumbles PPLO broth (1-2 serial passages).

2. PPLO agar :

- (a) PPLO culture in modified grumbles PPLO broth.
- (b) Direct~~o~~ cultures from apparently healthy birds and dead chicks.
- (c) Yolk from infected embryos.

3. Chicken embryos :

PPLO culture in modified grumbles PPLO broth.

4. Chickens :

- (a) PPLO culture in modified grumbles PPLO broth.
- (b) Yolk from infected embryos.

Method of various tests conducted in PPLO broth
PPLO agar, chicken embryos and chicks.

1. Modified grumble's PPLO broth.

- (a) Initial isolation of PPLO.
- (b) Fermentation reactions of different strains of PPLO.
- (c) As a medium for studying Pathogenicity trials in chicken embryos and chicks.

2. PPLO agar :

- (a) Initial isolation of PPLO.
- (b) Study of colony morphology of various strains of PPLO.
- (c) Study of growth rate and colony size of PPLO.

3. Chicken embryos :

- (a) Pathogenicity of various strains of PPLO in chicken embryos.
- (b) Study the transovarian passage of PPLO.

4. Chicks.

Study of pathogenicity tests of various strains of PPLO.

PPLD broth : (modified Grumble's medium)

Composition :-

Bovine heart infusion broth (PH 7.8-8)	800 ml.
Yeast extract (Difco)	5 gms.
Thallium acetate	1 G.
Phenol-red (as an indicator)	18 mg.
Sugars Glucose - Maltose (6-6 G.)	12 gms.
Sterile horse serum or healthy chicken serum	200 ml.
Penicillin G. sodium crystalline (1000 units per ml.)	10,00,000 units.
Deoxyribonucleic acid (Sodium salt of nucleic acid from thymus gland, (BDH) 20/U per ml. (DNA)	20 mg..

Preparation :

According to swain's (1965) description of PPLD medium, DN A was incorporated in the medium as a growth requirement.

Phenol red, thallium acetate and yeast extract were added to the infusion broth (P.H. 7.8-8) and autoclaved at 15 lbs pressure for 15-20 minutes. Thallium-acetate produced profuse turbidity, but it was brought into the solution again by little warming. Glucose and Maltose were added to the medium and autoclaved at 5 lbs pressure for 30 mts. This basal medium can be stored for any length of time. A stock solution of DN A (1%) was prepared by dissolving 200 mgs. in 20 ml. of distilled water and seitz filtered and stored in the refrigerator at 5°C. As it is denatured at 80°C, it was

added (2 ml. in 1000 ml.) in the medium along with serum at 56°C (approx). Penicillin was added only after cooling the medium sufficiently. The medium was finally distributed in 5 ml. amounts in sterile test tubes and stored in the refrigerator for a week.

PPL0 Serum agar : Composition.

Bovine heart infusion broth.	80 ml.
Yeast extract.	0.5 G.
Agar Powder(Italian) or	1.8 G.
Agar shreads (Japanese)	2.0 G.
Horse serum or healthy chicken serum.	20 ml.
Thallium-acetate.	0.1 G.
Deoxyribonucleic acid (DNA)	2 mg.
Penicillin G. sodium.	100000 units.

Preparation :

All ingredients except serum, penicillin, and D.N.A., were added and the broth was clarified by adding 0.5 gms. of egg. albumen powder (Preferably fresh egg albumen). The broth was warmed and filtered while hot and then autoclaved at 15 lbs. pressure for 15-20 mts. At approximately 56°C (as the serum will be denatured and clotted above 56°C) serum, and DNA were added and poured into petri-dishes. Penicillin was added at 45°C approximately (as agar solidifies at 42°C) and mixed by shaking the plate. Penicillin was added in cases where heavy contamination was suspected. In this study penicillin as an inhibitor of gram positive organisms, and thallium acetate as an inhibitor of gram negative organisms except PPL0, were used, mostly for primary

isolation of PPLO.

2. Culturing :

0.5 ml. of primary inoculum (from previously prepared stock) was transferred into 5 ml. modified Grumbles' medium. (PPLO broth). After inoculation the tubes were incubated for 3-8 days, and observed daily for signs of fermentation in tubes and growth on plates. The fermentation of sugars by as indicated by a change in the colour of indicator from red to canary yellow ~~mt~~ and slight turbidity and sedimentation by careful examination and gentle shaking of the tubes indicated the growth of PPLO. 2 or 3 blind passages were made in liquied medium at 24-48 hours intervals before growth determinations were made, as these were found necessary to eliminate bacterial contaminants because occasionally they would be too numerous to permit the isolation of PPLO. Gram's and Giemsa's (modified by Klieneberger's method) staining techniques were used for staining smears from fermented tubes to determine the presence of contaminating bacteria, the PPLO, or both under oil immersion of 1000 magnification. As soon as any of these changes appeared, the cultures from each tube was streaked uniformly on PPLO serum agar. The plates were kept in a closed container along with a moist cotton plug (to produce air saturated with moisture) incubated, and examined daily for one week for initial growth period to maximum growth rate. The plates were examined for gross bacterial contamination at 24 and 48 hours of incubation and if positive were discarded.

PPLD-Isolation and identification

The identification of PPLD was mainly studied on the basis of morphology, cultural characters, biochemical reactions, and sugar fermentation tests.

Microscopic examination of smears:

Smears were prepared from

1. The concentrated sediment of PPLD broth culture obtained by centrifugation at 3500 RPM for 30 mts.
2. Impression smears from yolk membrane and yolk material.
3. Picked up colonies from PPLD agar. Single colonies were picked up in a pure form along with portion of agar (as the growth penetrate into the agar) and rubbed on a glass slide.

Staining technique :

The procedure for staining by gram's stain was the same described by Wilkinson (Medical Microbiology 1965) and by Kilieneberger's method (Adler et al. 1958) the procedure was as follows.

1. Smear of the deposit was air dried and gently heat fixed.
2. Fixed again in bovins fluid for half an hour (Bovins' fluid was successfully replaced by methyl alcohol and the smear fixation time reduced to 5 minutes).
3. Washed thoroughly in running tap water.
4. Stained for half an hour with freshly prepared Giemsa's stain of 1:20 dilution in distilled water buffered at P.H. 7.0. The remaining procedure was the same as for

giemsa's stain described by Wilkinson (Medical Microbiology, 1965). Stained smears were examined under oil immersion objective.

Microscopic examination of colonies and growth rate.

The growth rate was studied by examining the plates daily for visible colonies. If the colonies were visible on first or second day, then the growth was considered to be rapid but when such growth appeared after two days, it was considered slow. The growth was further confirmed by sub-culturing on nutrient agar and found that PPL0 did not grow.

The colonies were studied at 50/^Xmagnification of the microscope. The colony size was comparatively studied according to growth rate for pathogenic and non pathogenic ~~exam~~ colonies. The pathogenic colonies appeared quite smaller without nipple like centre and their rate of growth was very slow where as the non pathogenic colonies appeared quite bigger and with faster growth rate. The colonies were also studied by Dienes staining technique described by Dienes and later modified by himself (Morton et al. 1953). It was also given by Adler et al. (1958) with certain modifications in the technique as follows.

Dienes stain : Composition.

Methylene blue	2.5 gms.
Azure II	1.25 gms.
Maltose	10 gms.
Sodium carbonate	0.25 gms.
Benzoic acid	0.2 gms.
Distilled water	100 ml.

Procedure :

Using thermometer metal case a portion of agar was cut where there was better growth of PPLO or with the help of a sharp safety razor blade very thin slice of agar containing growth was sectioned. When cut with metal thermometer the cut portion was lifted up by passing sharp teasing needle over very fine injection needle fitted to tuberculin syringe from one corner of cut edge downwards at 45° and thin slice of agar containing colonies was prepared. The slice was placed on a glass slide facing the colonies upwards and freshly prepared Dienes stain diluted 1:5 to 1:10 with distilled water was put with the help of finely drawn pastuer pipette over the slice just to cover the surface of the colonies. Clean grease free cover slip was placed gently on the agar block and sealed with molten paraffin. The staining was complete within 10-15 minutes and then examined at 50 X magnification. The colony morphology and organisms were studied according to the criteria set up by Sabin (1941) and Iecce and Sperling (1954).

Criteria :

1. Growth occurred only in the presence of enrichments such as serum or serum fraction.
2. The change in the colour of phenolred indicator from red to yellow and almost clear medium was an indication of PPLO growth.
3. The colonies grew not only on the surface of agar but also burrow into the medium, so that complete removal of

colonies by passing platinum loop, was not possible.

4. The characteristic colony size was ranging from 0.1 to 1 m. min diameter with morphology of smooth, circular and raised with or without central elevation.

5. The colonies were delicate, very easily distorted and loose their normal structure in the stained preparations. In stained preparations the organisms were cocobacilli-form bodies, and polymorphous in nature such as micrococci, filaments, rings, globules and very fine granules of indistinct morphology.

6. The ~~non~~ nonpathogenic colonies were having fried egg appearance with nipple like centre and the pathogenic colonies devoid of nipple like centre.

7. Their inability to revert to bacterial forms, isolation in pure culture, and ability to be passaged and maintained with characteristic colony morphology on sub-culturing.

8. The stained colonies (By Dienes technique) of PPLO took deep blue stain where as agar appeared light blue. The centrally elevated portion of non-pathogenic colonies stained more than at the periphery and the pathogenic colonies took uniform staining. Bacterial colonies, cellular debris and pollen grains took pink stain of Azure II.

4. Isolation of PPLO :

Pure culture technique :

Single colony method was the best for isolation of PPLO and maintained them in pure form than serial passage of PPLO cultures in the liquid media. Though it was a

difficult procedure, gave good results. Extra-ordinary care and great patience was required to fishout single colonies of PPLO from serum agar plates. Very fine injection needle with little bent at the point fixed to tuberculin syringe used for this purpose. The colonies were picked up under 50X magnification alongwith portion of agar where the growth was scarce and wide apart and inoculated into modified Grumble's medium (devoid of inhibitors) In cases when there was profuse growth on serum agar and the colonies were not wide apart, the culture was diluted by adding four or five drops in ^{5ml} modified Grumble's medium and plated. This gave good results. When using post mortem samples, bacterial colonies were observed intermingled with PPLO colonies and they either obscured PPLO or did not allow the transfer and hence the isolation of PPLO affected. In such circumstances, Lecce and Sprrling's (1954) method was followed. Agar containing such growth was cut and rubbed colony side down on the surface of serum agar plates and then few drops of penicillin G. Sodium (50,000 units per ml.) was added. The inhibitor was spread by gentle shaking or rotating the plate. PPLO colonies were obtained in pure form.

5. Maintenance of culture:

As PPLO are ~~xxx~~ very fragile even at 4°C, great difficulty was experienced in maintaining their viability. So subcultures were made at every 15 days in liquied media and the culture tubes were stored in the freezing chamber of the refrigerator. The method of sub-culturing was followed according to Gentry (1960). Broth tubes were tapped gently to suspend any of the organism that had

settled to the bottom of the tube and a sterile pipette was inserted until the tip touches the bottom of the tube and 0.3 ml. of the culture withdrawn.

6. Carbohydrate fermentation :

For the study of sugar fermentation reactions of PPLO, liquid medium without inhibitors was used. Only five sugars viz Glucose, Maltose, Sucrose, Mannitol and Mannose were used separately in final concentration of one percent. To 5 ml. of sugar medium 0.1 ml. of 36-48 hours broth culture of individual strain of PPLO was added, and the fermentation reactions were studied twice daily for a week.

7. Tetrazolium reduction :

2 - 3 - 5 triphenyl tetrazolium chloride was used in this test. The method adopted by Yamamoto and Adler (1958) was followed with some modifications.

Semisolid medium : Composition.

Heart infusion broth (P.H. 7-8-8)	80 ml.
Yeast extract (Difco)	0.5 gms.
Agar powder (Italian)	0.5 gms.
Tetrazolium salt.	4 mgs.
Sterile horse serum	20 ml.

1% stock solution of the tetrazolium salt was sterilized by autoclaving at 15 lbs pressure for 15 mts. and stored in the refrigerator in a dark container to avoid non-enzymic reaction. Stock solution was added in appropriate concentration while adding horse serum to the semisolid medium and the medium was dispensed in 5 ml. amounts in

narrow test tubes. The medium was stabbed with a loopful of 18-24 hours culture and examined every 12 hours for a period of 3-4 days for the time and degree of reduction. The positive reaction mented when the medium turned pink.

8. Whole blood plate agglutination test.

This test was conducted to determine the incidence of active and latent carrier birds and the relation of this test to the symptoms and isolation of PPLO.

This test was performed by using PPLO diagnostic (coloured) antigen and samples of blood from the superficial ulnar vein of birds. 2 drops (0.1 ml) of antigen was placed on a clean, grease free dry glass plate measured off in one inch squares. One loopful (0.02 ml.) blood was mixed with wire loop, mixed gradually and read the results. A positive reaction was characterized by a purple granular precipitation usually starting from periphery within $\frac{1}{2}$ minute and completing in 2-3 minutes. The loop was rinsed in distilled water and dried on cloth every time. In the case of dead chicks the procedure adopted by Sornicle and Zaibet (1963) was followed. The extract of the blood clot from the heart or large blood vessels was used. The method of Random testing of about 10% of the birds was adopted in the Poultry farm.

(1) A comparative study of agglutination and possible cultural isolation of PPLO strains were made in 80 apparently healthy birds.

(2) and a comparative study of agglutination and P.M. lesions and possible cultural isolation of PPLO strains were studied in 20 dead chicks and 50 pipped but unhatched chicks.

IX A comparative study of growth rate and colony morphology of PPLO with different nutrients and in different atmospheric conditions.

This experiment was conducted according to the method described by Fabricant (1959). 3 pathogenic and 3 non pathogenic strains of PPLO were used and the method was as follows:-

Separate ^{Serum agar} plates for each strain of PPLO were used.

1. All plates incubated in air.
2. All plates incubated in moisture saturated air.
3. All plates incubated in moisture saturated air with 10% CO_2 tension.
4. All plates not having yeast extract incubated in moisture saturated air.
5. All plates containing DNA incubated in moisture saturated air.

Chicken serum was used in the beginning of the experiment and later on horse serum was used. There was no noticeable difference in the growth of PPLO and so it was not used in the experiment to compare with horse serum. The 10% CO_2 tension was produced according to Swain's (Medical Microbiology, 1965) description.

Examination of plates :

All plates were examined for growth rate and colony characters at intervals up to 6th day of incubation. A total of 100 colonies out of which 50 having maximum diameter and 50 having typical diameter were measured with ocular micrometer from each plate and read in stage micrometer. The average of the 3 pathogenic and the average of the 3 non-pathogenic PPLO strains were taken from each observation.

35 divisions of ocular micrometer coincided with 18 divisions of object micrometer and so the colonies measured with ocular were multiplied by the factor 19.44 to get the colony readings in microns (/U). The colony size and growth rate were compared on the basis of these readings and initial growth period respectively.

X. Pathogenicity Trials :

The object is to study the pathogenic potentialities of PPL0 strains isolated. These trials were studied in stages as follows.

1. Transovarian passⁱⁿage of PPL0 fertile eggs.
2. Pathogenicity of PPL0 in 7 day old chicken embryos.
3. Pathogenicity of PPL0 chicks.

1. Transovarian passage :-

It was pointed out by VanHerick and Eaton (1945), VanRoekel and Olesiuk (1952) that the major source of PPL0 transmission is through eggs causing CRD in birds. 24 fertile eggs from disease free flock were taken, washed thoroughly in tap water and sterilized with absolute alcohol. 4 strains of PPL0 which have fulfilled all the criteria described by Sabin (1941) and Lecce and Sperling (1954) were used. 20 eggs were inoculated with 0.1 ml. of PPL0 broth culture in each and 4 control eggs with PPL0 broth. The aperture was closed with molten paraffin. They were studied twice daily $37 \pm 0.5^{\circ}\text{C}$ in incubation for the death of germ plasm. The yolk was cultured and examined for the viability of PPL0 strains.

2. Pathogenicity of PPL0 in 7 day old chicken embryos:

Studied experimentally on the lines given by

Blake (1962). The number of strains used and sterilization adopted were the same as described in transovarian inoculation of PPLO. All 24 chicken embryos of 7 days old were candled before inoculation for livability of developing embryo and inoculated in 20 eggs with ~~2x2~~ 0.2 ml. of inoculum each by intra-yolk-sac route after noting the position of air-cell and head region. 4 control embryos were inoculated with PPLO broth. The aperture was closed with molten paraffin. The eggs were incubated at 37 ± 0.5 C and studied twice daily for pathogenic effects of PPLO strains. The dead embryos removed kept in the refrigerator for 2 hours and examined for lesions. The yolk and yolk sac membrane of each embryo were cultured and studied for the viability of PPLO strains.

3. Pathogenicity of PPLO in chickens:-

All the chicks taken from the same geographical condition but were of different age groups.

<u>Age group</u>	<u>Number</u>
Chicks of 5 weeks	10
" 6 "	10
" 7 "	8
" 8 "	8
	<hr/> 36

The principles adopted in this experiment were fundamentally the same as followed by Joseph-Blake (1962)

The chicks were tested / serologically for antibodies, culturally for the presence of PPLO, and found negative for PPLO. The inoculums made use of in this trials were from

1. PPLO broth culture of 24-36 hours.
2. Yolk from experimentally infected embryonated eggs diluted with N.S.S. 1:5.
3. and 18-24 hours broth culture of E.coli.
3. Strains of E.coli were grown in plain broth for

18-24 hours and tested for pathogenicity in 12 mice by inoculating

0.2 ml. in each. I/peritorially. In the first batch 6 mice were inoculated and the rest in the second batch. 4 control mice were maintained separately. The mice died in 24-48 hours. Heart Wood was directly cultured on Mac-Conkey's agar and in plain broth, and examined for growth characteristics, and the stained smears for E.coli respectively. In the second group broth culture was similarly given into 6 mice. The mice died in 24-48 hours and again similarly examined for E.coli organism. The most pathogenic strain among them

was selected for experimental inoculation in chickens.

Method of infecting chicks :

The inoculum was given intra-tracheally with hypodermic syringe with 2" - 20 gauge blunt pointed needles. The needle was introduced one inch deeper into the trachea. Regurgitation of culture by coughing was prevented by holding the tongue. Intra-thoracic inoculation was made by injecting into the left posterior thoracic air sacs by pushing the 3/4" long and 20 gauge needle about 1 mm. deep in between the last second and third ribs. 0.5 of E.coli strain was inoculated in 6 weeks age chicks as detailed in Table No. III.

The dosage of PPLO given in experimental chicks was as follows.

Age.of the chicks.	Amount of culture inoculated in mls.	
	I/Tracheal.	I/Thoracic.
5 weeks	0.2 ml.	0.1 ml.
6 weeks.	0.2 ml.	0.2 ml.
7.weeks.	0.3 ml.	0.2 ml.
8 weeks	0.3 ml.	0.3 ml.
<u>When trachea alone was inoculated</u>		
5 weeks	0.3 ml.	
6 weeks.	0.4 ml.	
7weeks.	0.5 ml.	
8 weeks.	0.6 ml.	

Table No. III.

Showing the details of Inoculation experimental chicks by various routes.

Group No.	Chick leg band No.	Age group	Inoculum		Strain No. of PPLO.	Route of infection.
			PPLO	E.coli with strain no.		
1.	2.	3.	4.	5.	6.	7.
1	901-904 (Control chicks)	5 weeks	PPLO broth.	-	-	I/TR
	915,938	5 weeks	Yolk	-	5 P.	IAS
	941,939	"	MGB	-	5P1	ITR
	942,930	"	"	-	5P1	ITR, IAS
2	905,906 (Control chicks)	6 weeks	PPLO broth.	-	-	ITR
	950,964	"	Yolk	811Tb	15 P, 7 NP	IAS
	924,931	"	MGB	"	5 P	ITR
	934,911	"	-	"	-	IAS
	937,952	"	MGB	"	7 P, 7 NP	ITR, IAS

1.	2.	3.	4.	5.	6.	7.
3	907, 908 (control chicks) 7 weeks		PPLO broth	-	-	ITR, IAS
	953, 921	"	Yolk	-	16 P, 7 NP	IAS
	919, 920	"	Yolk	-	5 P	ITR, IAS
	923, 947	"	MGB	-	16 P, 7 NP	ITR
4	909, 910 (control chicks) 8 weeks		PPLO broth	-	-	ITR, IAS
	1000, 914	"	Yolk	-	5 P, 7NP	ITR
	913, 912	"	MGB	-	4 NP, 7P	IAS
	918, 932	"	Yolk	-	16P, 4NP	ITR, IAS

Index

MGB.	Modified Grumbles PPLO broth.
ITR.	Intra tracheal.
IAS.	Intra Air Sac.
P.	Pathogenic PPLO.
NP.	Non Pathogenic PPLO.

All five groups were kept separately in isolated places. They were fed on standard balanced ration. They were daily exposed to Sunlight for one hour and studied for nasal and eye discharges, and respiratory symptoms. Cultural and serological examinations were done at the end of 2nd, 3rd and 4th weeks. The dead chicks were examined for lesions, and from tracheas lungs and air sacs were cultured in PPLO broth and on serum agar plates for PPLO isolation. Direct smears were made, stained by Giemsa, and examined for PPLO.

Preparation of E.coli antigen.

The most pathogenic strain of the used was grown on nutrient agar for 24 hours. The growth was seeded into normal saline and autoclaved for 2½ hours in running steam. Then it was stored in the refrigerator at 4°C and used for serum agglutination test in the experimental chick. The chicks were tested at the end of 2nd, 3rd and 4th weeks.

Clinical symptoms in the chicks were studied as follows:

1. Nil :- Chicks were healthy clinically.
2. Mild :- Mild serous or mucous nasal discharge.
3. Severe :- Profuse offensive, thick nasal discharge, dyspnoea cough, rales, and loss of body weight.

Lesions in autopsied chicks were studied as follows:

1. Nil :- No gross pathological lesions.
2. Mild :- Little congestion of tracheal mucosa with serous or mucous exudate, mild pneumonic changes.

3 Severe : Trachea highly congested, petechial
haemorrhages, and plogues of cheesy exudate.

Lungs Consolidation, Pneumonia

Air sacs - Thickening of membreme, plugs of
caseous exudate or beading.

IV. R E S U L T S

PPL0 and gramnegative and grampositive organisms as presented in the table no. 3 and 9 constituted the bulk of the bacterial flora isolated during the previous studies from the trachea, lungs and air sacs of birds and chicks from a flock which had a history of CRD.

T A B L E N O. 4

Showing the number of pathogenic and nonpathogenic PPL0 isolated from live birds and dead chicks.

No. of birds & chicks exa- mined.	Pathoge- nic PPL0.	Nonpathoge- nic PPL0.	Total number of PPL0 isola- ted.
80 live birds.	18	59	77
20 dead chicks (6-10 weeks age)	5	14	19
50 dead unhatched & chicks.	28	-	28

The table no. 4 shows the number of PPL0 isolated on serum agar and maintained in modified Grumbles medium (devoid of inhibiteres). The nonpathogenic PPL0 predominated in healthy and dead chicks (6-10 weeks age) only pathogenic PPL0 were isolated from dead unhatched chicks in a lesser percentage. In many occasions bacterial contamination was so high that it became rather very difficult to isolate PPL0 in pure culture. Even higher concentrations of thallium-acetate (1:500) and penicillin G-sodium (10,000 units per ml.) did not inhibit the contaminants

on repeated passages. Pure culture technique of PPIO by single colony method from serum agar and subculturing them in modified Grumble's medium gave good results. Even several transfers of contaminated cultures in liquid medium containing inhibitors did not yield pure cultures of PPIO. Smear examination of centrifused broth culture, yolk-sac membrane, Yolk, and isolated colonies from serum agar plates by Gram's and Klieneburger's method revealed minute cocci, coccobacilli from bodies rings and very fine granules of indistinct morphology at 1000X magnification. It was very difficult to describe complete morphology at 1000 X magnification as the organisms were too small to be appreciated at that magnification. Figure No.1 showing coccobacilli-form bodies stained by Klineberger's method. The size and growth rate of various strains of PPIO was quite variable. Many of the PPIO colonies were seen at 50 X magnification within 24-48 hours of incubation; whereas several others were only seen after 72-96 hours of incubation. In acute CRD the growth rate and colony size of PPIO were quite significantly increased. Figures 2 and 3 give the appearance of colonies with variable sizes.

The PPIO isolates were differentiated into strains on the following lines. The colonies of one type of strains were minute , circular, shining, raised, and smooth without central elevation. The colonies of other type of strains were large circular, and smooth with nipple like centre or heaped up granular centre and a lacey

periphery giving the colony characteristic of fried egg appearance or button like appearance or dome shaped appearance. The physiological characteristics of the former producing pathogenic type of colonies were in majority of them evinced a slower growth rate and rapid tetrazolium reduction. The latter producing nonpathogenic type of colonies with rapid growth rate and did not reduce the tetrazolium salt or reduce in a comparatively longer time. Figures nos. 7 and 8 showing pathogenic and nonpathogenic colonies.

The pathogenic type of colonies took uniform dense stain by Diene's method figure no. 9 showing stained (Dienes stain) colonies of pathogenic PPL0. The nonpathogenic colonies took more deeper stain at the centre and lighter stain at the periphery. The size of the colonies were very much variable. The discrete individual colonies were many times larger than the densely packed ones. Generally the change in colour of the phenol red indicator from red to yellow was appreciated after 48-72 hours incubation. There was no turbidity or sediment even after prolonged incubation in some cases. The plates were examined daily for 7 days at 37°C incubation. Most of the pathogenic strains grew after 48 hours and nonpathogenic strains grew in 24-48 hours.

Some observations of PPL0 colonies

1. Rough Colony :

They were the typical colonies of pathogenic PPL0 having rough surface irregular edges and raised. They were

comparatively bigger in size also. The colonies had borrowing growth on serum agar. They did not possess any nipple like centre in the colony. They were mostly found in acute cases of CRD. They were confirmed as ~~path~~ pathogenic type by the criteria fixed up for pathogenic PPL0 viz colony characters growth rate, sucrose and mannitol fermentation, and tetrazolium reduction. Figure No. 4 showing rough colonies.

2. Colonies having small central elevation :

They were the unique type of colonies found in association with pathogenic colonies. in chronic cases of CRD. They were smooth circular, translucent and raised. They possessed very small central elevation which is an exception in pathogenic colonies. The cultures of this strain fulfilled all the criteria of pathogenic strains (vide supra). They were presumed that they belong to pathogenic PPL0. Figure No. 5 showing the colonies of PPL0 as described above.

3. Colonies having rings in the centre:

These were observed in pipped but unhatched chicken embryos with partially absorbed yolk sac. The colonies were having two rings - one at the centre and the other at periphery. They were smooth, shining, circular and raised in their character. The size was slightly increased in discrete colonies and typical where the growth was densely packed. They also fulfilled all the criteria

of pathogenic colonies (vide supra). The broth culture examination revealed that they were possessing regular coccobacilli-form bodies. Figures No. 6 showing ring forms of PPLO.

Among 124 isolates of PPLO, 13 fermented all five sugars viz Glucose, Maltose, Sucrose, Mannitol and Mannose 49 - Glucose, Maltose and Sucrose, 8 - Glucose and Maltose, 8 Sucrose and Mannitol, 16 Mannitol, 8 Sucrose, 17 Mannose, 5 Glucose, and 3 Maltose. 16 strains did not ferment any of the five sugars. Only acid without gas was produced in all the cases.

Out of 400 ^{blood} samples tested in apparently healthy birds, dead chicks (6-10 weeks age) and pipped but unhatched embryos, 316 were positive (79%) and 84 were negative in which apparently healthy birds were more positive and the chicks were less positive and the pipped but unhatched chicks were least positive for whole blood plate agglutination test.

Table No. IV

Showing the comparative study of PPLO in different atmospheric conditions and growth requirements.

Treatment of the serum agar plates.	In microns (/U)			
	Ave.max. dia of Path PPLO colonies.	Ave.typ. dia. of path PPLO colonies.	Ave.max. dia of non-path. PPLO colonies.	Ave. typical dia. of non-path. PPLO colonies.
1.	2.	3.	4.	5.
All plates incubated in air.	75.816	40.824	219.672	118.584
All plates incubated in moisture saturated air.	161.352	73.972	314.948	200.232

	2.	3.	4.	5
All plates incubated in moisture saturated air with 10% Co ₂ tension.	161.358	73.978	314.840	200.110
All plates not having yeast extract incubated in moisture saturated air.	151.632	69.984	244.056	140.068
All plates containing DNA incubated in moisture saturated air.	167.184	75.816	318.891	202.174

In atmospheric air the growth of PPLO was meagre and the colonies were very much reduced in size. In moisture saturated air the growth was abundant and the colony size was nearly double to that of the colony grown in air. There was no significant change in colony size and growth rate when grown in moisture saturated air with 10% Co₂ tension. The colonies of non pathogenic strains were very much reduced in size than the colonies of pathogenic strains in the absence of yeast extract. The growth rate was not such effected. The additional of DN A to serum agar stimulated the growth rate of PPLO and the colony size also slightly increased. The colony size was indicated in microns as shown in the table No. I V. Figure No. 10 showing the colony size when incubated in moisture saturated air with 10% Co₂ kxtention. Figure No. 11 showing the colony size when incubated in moisture saturated air with DNA.

Table No. V.

Showing the comparative study of cultural, pathological and serological examination apparently healthy birds, dead chicks and pipped but unhatched chicks.

Cultural examination.	Pathological Examination.	Serologic- ical Exmn.	Results of examination						
			Culturally		Pathologically		Serologically		Total
			+ve	-ve	+ve	-ve	+ve	-ve	
80 Tracheal swabs of live birds.	-	Whole blood from 80 birds.	54	26	-	-	63	17	70
Tracheal, lungs Postmortem examination		Exudates of clotted blood of 20 dead chicks (1) for inflammation of tracheas lungs and air sacs (2) exudates in the tracheas and air sacs (3) and thickening of air sac membranes.	11	9	13	7	12	8	55
swabs of 20 dead chicks (6-10 weeks age).									60
Yolk sac swabs of pipped but unhatched chicks.	-do-	Exudates of clotted blood of 50 pipped but unhatched chicks for examination.	28	22	26	24	29	21	56
		Pathological examination.							52
		Serological examination.							58
									78.8

Index: C = Cultural examination.
P = Pathological examination.
S = Serological examination.

The table No. 5 presented the association of cultural test, pathogenic test and the plate test in assaying the incidence of PPLO infection in flock appeared to be statistically significant and also that if a bird was positive to PPLO infection where cultural isolation was present, the pathogenic and plate tests also gave similar reaction. The isolation of the organisms approximately coincided with the serological findings in the live birds. In dead chicks though the percentage of isolation, pathological lesions and serological reactions were comparatively less showed some agreement among the three. In pipped but unhatched chicks the cultural, pathological and serological results revealed a very low percentage of pathogenic PPLO or the low transmission of PPLO possible through eggs. The relationship of all gave a definite conclusive idea that the disease was prevailing in the farm and the comparative studies of these 3 aspects indicated that a fairly accurate diagnosis of CRD is possible.

Table No. VI

Showing the experimental transversion infection of PPL0.

[illegible]

Among the 5 pathogenics strains used, the yolk cultures of 16 P and 15 P caused death of germ plasma within a short period. The 8th and 9th day dead embryos' yolk sacs were congested. In rest of the cases only death was observed. PPLO was isolated from all cases except control chicks. The experiment showed the pathogenic potentialities of PPLO strains in fertile eggs. The egg yolk passaged strains showed increased pathogenicity than broth cultures of PPLO.

Table No. VII

Showing experimental infection of 7 days old chicken embryos.

Sl. No.	PPLO broth culture & strains	No. of embryos inoculated.	Death of embryos in days.	Lesions	PPLO isolation.
1.	2.	3.	4.	5.	6.
1.	5 P.	1	2 days	Hæmorrhages all over the embryos.	+ Ve
		2	3 days	Congestion of the yolk sac membrane.	
		3	2 days	Petechial hæmorrhages over yolk-sac membrane	
		4.	3 days	Oedema & congestion of the chick embryo with unabsorbed yolk.	
		5	3 days	Congestion and oedema.	
2.	15 P.	1 Embryo	4 days	Moderate congestion.	+ Ve
		2 "	6 "	Petechial hæmorrhages on head and neck.	
		3 "	7 "	Echymosis on head and neck.	

1.	2.	3.	4.	5.	6.
		4 Embryos	8 days	Transudate oozing out from all sides of the body. Oedema of the head and neck region	+ Ve
		5 "	8 "	Congestion of yolk-sac	
3	7 P.	1 Embryo	3 days	Mild congestion of yolk sac membrane.	
		2 "	4 "	Congestion of the yolk sac membrane	
		3 "	6 "	Severe congestion all over the surface of yolk sac membrane (moderate)	+ Ve.
		4 "	7 "	Congestion and oedema of neck and head (moderately)	
		5 "	9 "	Petechial haemorrhages all over the body. Stuning and unabsorbed yolk sac.	
4	16 P.	1 Embryo	2 days	Mild congestion of yolk sac membrane.	
		2 "	3 "	-do-	
		3 "	4 "	Moderate congestion of yolk sac membrane.	+ Ve.
		4 "	3 "	Mild congestion and slight oedema over the head region.	
		5 "	3 "	Petechial haemorrhages of yolk sac membrane.	
5	PPLO broth	4 control embryos	-	-	-Ve for PPLO.

The chicken embryos inoculated died within 2-9 days period after enoculation. The lesions were mostly congestion, haemorrhages and oedema of the dependent part in most of the cases. PPLO was isolated in all the cases except the control chicks. The experiment showed pathogenic potentialities of PPLO in chicken embryos and possible transovertian passage embryonated eggs. 5 P and 15 P were proved to be more pathogenic due to their pathogenic potentialities in chicken embryos. Figure No. 12 showing unabsorbed yolk sac petechial hemorrhages oedema of head region and stunted growth of chicken embryo.

Sl. No. Chick leg band no. Date of inoculation

1. 2. 3.

1 908 23.10. to all the cl

2 902 -

3 903 -

4 904 -

5. 915 -

6. 938 -

7. 941 -

8. 939 -

9. 942 -

10. 930 -

11. 905 -

12. 906 -

13. 950 -

14. 964 -

15. 924 -

16. 931 -

The pathogenic potentialities of PPL0 strains as shown in table no. VIII were recorded according to their ability to produce CRD in chickens. The chicks developed symptoms in 3rd week and also antibodies in blood. Cases numbering 931 and 913 did not develop antibodies by third week but were positive culturally and pathologically. Case No. 905 took infection by fourth week through air from the infected chicks kept at the close proximity. The disease caused mortality mostly in chicks between the ages of 5 to 6 weeks. The disease produced by PPL0 alone was very mild. The disease syndrome was more intensive in chicks when inoculated with PPL0 and E. coli and/or nonpathogenic PPL0. This was evidently seen by pathological lesions developed on P.M. examination at the symptoms exhibited. The intra air-sac route inoculation has proved to be more pathogenic than intra-tracheal inoculation as because the organism reached the more sensitive organs early and produced the disease. This was evidenced by symptoms and lesions found in chicks comparatively more early and prominent respectively. Number 934 and 911 cases showed that E.coli alone could not produce CRD, but could produce pericarditis.

Figure No. 13 showing chicks suffering from CRD.

- | | | | |
|----|----|----|--|
| ,, | 14 | ,, | caseous exudate in trachea. |
| ,, | 15 | ,, | petechial haemorrhages in trachea. |
| ,, | 16 | ,, | Cervical air sac filled with caseous exudate and thickening of the air sac membrane. |
| ,, | 17 | ,, | Thoracic air sac filled with caseous exudate in beaded form. |
| ,, | 18 | ,, | Abdominal air sac showing caseous exudate. |
| ,, | 19 | ,, | Chicken embryo with partially absorbed yolk sac died due to PPL0. |

The pathogenic potentialities of PPIO strains as shown in table no. VIII were recorded according to their ability to produce CRD in chickens. The chicks developed symptoms in 3rd week and also antibodies in blood. Cases numbering 931 and 913 did not develop antibodies by third week but were positive culturally and pathologically. Case No. 905 took infection by fourth week through air from the infected chicks kept at the close proximity. The disease caused mortality mostly in chicks between the ages of 5 to 6 weeks. The disease produced by PPIO alone was very mild. The disease syndrome was more intensive in chicks when inoculated with PPIO and E. coli and/or nonpathogenic PPIO. This was evidently seen by pathological lesions developed on P.M. examination at the symptoms exhibited. The intra air-sac route inoculation has proved to be more pathogenic than intra-tracheal inoculation as because the organism reached the more sensitive organs early and produced the disease. This was evidenced by symptoms and lesions found in chicks comparatively more early and prominent respectively. Number 934 and 911 cases showed that E.coli alone could not produce CRD, but could produce pericarditis.

Figure No. 13 showing chicks suffering from CRD.

- | | | | |
|----|----|----|--|
| ,, | 14 | ,, | caseous exudate in trachea. |
| ,, | 15 | ,, | petechial haemorrhages in trachea. |
| ,, | 16 | ,, | Cervical air sac filled with caseous exudate and thickening of the air sac membrane. |
| ,, | 17 | ,, | Thoracic air sac filled with caseous exudate in beaded form. |
| ,, | 18 | ,, | Abdominal air sac showing caseous exudate. |
| ,, | 19 | ,, | Chicken embryo with partially absorbed yolk sac died due to PPIO. |

Table No. IX

Showing the frequency of bacterial flora from trachea, lungs, and air sacs of apparently healthy birds, dead chicks and pipped but unhatched chicks.

Kinds of material examined.	Gram negative organisms					Gram positive organisms				Total no. of gr-ve organisms	Total no. of gr-ve organisms
	E. coli.	Klebsiella.	Prot. eus.	Pseudo- monas	Other gr-ve cocci	Staphylococci	Micrococci	Staphylococci	Bacillus		
80 Tracheal swabs of 80 live birds	28	9	13	3	8	33	3	3	5	61	44
20 Tracheal											
20 lung &											
20 air sac											
swabs of											
20 dead											
chicks											
(6-10 weeks											
age)	12	4	4	3	5	12	1	3	4	28	20
50 yolk											
swabs of											
50 pipped	-	-	-	-	-	-	-	-	-	-	-
but unhat-											
ched chicks.											

(only PPLO that too pathogenic type could be isolated)

The results of the bacterial flora isolated in the present work as shown in the Table No. IX revealed that various gram positive and gram negative bacteria in isolation with CRD agent existed. Among the various isolates of gram negative organisms E. coli predominated and among the gram positive organisms Staphylococci predominated ~~the~~ mostly in healthy birds. Among the CRD infected chicks there was a shift from predominately gram positive to gram negative organisms that too E. coli. The other gram negative and gram positive organisms such as Klebsiella Proteus, Pseudomonas and unclassified gram negative organisms, and Micrococci Streptococci and Bacillus were isolated in a lesser percentage due to their lower incidence in the environment in which the birds were raised. Among the isolates E. coli acted as complicating factor in CRD and other organisms as secondary invaders.

V. DISCUSSION

PPL0.

The isolation of normal flora in health and disease depends very much on the technique that is employed and the flora also varies with the place, environment, age of the birds etc. for example there are even records of seasonal variation (Topley and Wilson, 1957).

The isolation and cultivation of PPL0 modified Grum-ble's medium with certain modifications supported the contention of Taylor and Fabricant (1957) that the liquid medium inoculation is a sensitive method for the cultivation of PPL0. In the present studies Grumble's medium was employed using two sugars namely glucose and maltose. The sugars used in Grumbles medium were not fermented by all the strains of PPL0 and so many organisms were missing during isolation. Such findings were also made by Adler et al. (1958), Pathak (1959) and Singh (1962). The cause might be due to the fastidious nature in growth requirements of these organisms as stated by Brion (1961) and according to Chu (1959), Adler and Berg (1960) and Fabricant (1960) that there is no perfect medium yet developed which will support the growth of all PPL0 strains. Taylor et al. (1957) and Taylor and Fabricant (1957) considered that this medium was superior to those of Edward (1950) Lecce and Sperling (1954) and Adler et al. (1954). This media due to its simplicity, speed of isolation and economy is superior to other media. Further it eliminated the danger of simultaneous involvement of any viral agent, which may

vitiate the results as is the case in egg inoculation. Many of the organisms isolated in the present studies did not produce turbidity and sedimentation which was in agreement with the findings of Singh (1962) that most of his isolates also did not fulfilled this criteria. And so cultures from all tubes were tested on serum agar to confirm the presence of PPLO. DNA was also added to the serum agar according to Swaine (Medical Microbiology, 1965) on which PPLO was isolated successfully. So it is considered that this is the most satisfactory method that can be adopted for the isolation of PPLO. Adler and Yamamoto (1956 b, 1957) and Abbot et al. (1960) stated that certain strains grew only in broth and failed to colonize on serum agar. In the present study this was clarified by examining the broth cultures for PPLO that did not produce colonies by centrifugation and staining by Klieneberger's method as described under materials and methods. The isolation of 82.7% of PPLO in this medium is in accordance with the results of isolation of PPLO 90% by Pathak (1959), 78% by Singh (1962), and 98% by Taylor et al. (1957). It was found that adult birds yielded more percentage of PPLO isolation than chicks (6-10 weeks age) and pipped but unhatched chicks. The cause being that the older progeny have more chances of contracting infection on account of direct or indirect contact and the younger progeny have lesser chances of contracting the disease. PPLO was also isolated from a few serologically negative cases which ⁱⁿ support of the findings of Jungherr

11. (1958) who isolated 5% of 36 strains from a group of serologically negative flocks. A large number of nonpathogenic PPL0 were isolated from apparently healthy birds and dead chicks. The significance of presence of nonpathogenic PPL0 in birds with or without a clinical signs of disease has not yet been found out so far (Adler et al. 1958 and Chu 1958 b.). Only pathogenic PPL0 could be isolated in the present studies from the pipped but unhatched chicks which corresponds to the observations of Adler and Yamamoto (1957) and Merchant and Packer (1961).

124 PPL0 isolates were characterized on the basis of colony characters, rate of growth, fermentation of sugars glucose, maltose, sucrose and mannitol and mannose and tetrazolium reduction. All the isolates were divided into pathogenic and non pathogenic on the basis of colony morphology which was according to the reports of Chu (1958a,b) Adler et al. (1958), Yamamoto and Adler (1958 b), Kleckner (1960) and Brion (1960). Most of the nonpathogenic type strains grew within 24 hours and very few in 2 days. The pathogenic strains grew on serum agar in 2-3 days but some grew in 5-6 days which is in full agreement with the works of Chu (1958 a,b.) Adler et al. (1958) Yamamoto and Adler (1958 b), Kleckner (1960).

Only one isolate was having small central elevation in the colony which is quite similar to the description of Merchant and Packer (1961) that all the strains of PPL0 viz M. gallisepticum, M. gallinarum, and M. iners were having central elevation in their colonies. So the present

findings is perhaps the first record after Merchant and Packer (loc cit).

Rough colonies were isolated from an acute case which was suffering from CRD and Newcastle disease. Similar findings were reported by Adler and Yamamoto (1956) in CRD due to PPL0 and H. gallinarum and also recorded by Agarwal (1964) from acute CRD cases. They were rough in shape with irregular edges, did not possess nipple like centre or fried egg appearance. They were also characterised by delayed fermentation of sucrose and mannitol, slow growth rate on serum agar and tetrazolium reduction in 24 hours. So from the above criteria the colonies which were found rough are very likely of pathogenic type of PPL0. Of-course a biological test is therefore indicated for the confirmation of their pathogenicity.

Ring from colonies were isolated from pipped but unhatched chick. As pointed out by Adler and Yamamoto (1957) and Merchant and Packer (loc cit) only pathogenic strains were able to pass through eggs and Wasserman et al. (1957) noted that pathogenic PPL0 remained viable for 21 days incubation period and multiplied. In support of their findings it was presumed that only pathogenic PPL0 is able to pass through eggs and viable for 21 days. The multiplication of these organisms might not have reached mature stage and so they might have got ring shape in the colonies. Their pathogenicity was further confirmed by other 5 criteria (vide supra) and in addition to that they were tested on nutrient agar for growth characteristics. They did not grow on it. and so they were considered to be pathogenic type of colonies

is in agreement with the findings of Agarwal (1964) who also observed some colonies in CRD cases and classified them under pathogenic forms of PPL0.

The sugar fermentation reactions gave varied results and hence the strains that fermented all 5 sugars (vide supra) and those which fermented mainly Sucrose and Mannitol in 3 days and 7 days respectively were regarded as pathogenic after taking into consideration other 5 criteria (vide supra). This is similar to the method adopted by Singh (1962) for strain differentiation of PPL0. The delayed fermentation of sucrose is also in agreement with the findings of Adler et al.(1957, 1958) and Yamamoto and Adler (1956 b) and the delayed fermentation of mannitol by pathogenic strains is also similar to findings of Kleckner (1960). More et al.(1960) in contradiction reported that some non-pathogenic strains also ferment these sugars slowly. But Merchant and Pecker (1961) reaffirmed the findings of Adler et al. (loc cit) Yamamoto and Adler (loc cit) and Kleckner (loc cit).

Tetrazoline chloride reduction was taken as a guide to differentiate pathogenic and nonpathogenic PPL0. The strains which reduced the dye in 24-48 hours were taken as pathogenic and very few which fulfilled other criteria reduced the dye in 72 hours were also considered as pathogenic. This is in corroboration with the findings of Yamamoto and Adler(1958 b) that their first group pathogenic strains reduced the dye in 24 hrs. II group in 72 hours and the III and IV groups

gave intermediate reactions i.e. in 36 and 48 hours. respectively.

The comparative study of morphology and growth rate of 6 selected (3 pathogenic and 3 nonpathogenic) strains has thrown light for further studies. The atmospheric air is not all suitable for growth of PPL0 as the growth was insignificant and the colonies were more smaller in size as indicated by micrometry Table no. 4. And so these findings are in support of Fabricant's (1959) view. The moisture saturated air stimulated the growth of PPL0 and ~~the~~ the colonies were quite bigger i.e. approximately double the size of colonies grown in air which is suggestive of the need of moisture for their growth. This similar to the findings of Fabricant et al. (1962) and Fabricant (1959). There was no significant change in the colony morphology and growth rate ^{under 10% co2 tension} and so it was presumed that is not essential for the growth of PPL0. ^{This is} in full agreement with the findings of Fabricant (1962). Due to the absence of yeast extract in the medium the colony size of PPL0 greatly reduced. Though the colony was very much reduced but growth rate was not much affected. Regarding colony size in this present work the results have fully corroborated with the observations of Fabricant (1959). The inclusion of DNA as growth supplement in the medium alongwith peptone has stimulated the growth rate significantly and slightly the colony size, which is similar to the findings of Fabricant et al. (1964) and Swaine (Medical Microbiology 1965).

The serological examination of about 10% of the flock on random sampling basis was followed by many workers as recognized method for detecting PPIO infection with the lowest percentage of reactors. The serological findings by whole blood plate agglutination test revealed that out of 400 birds 318 were positive in which 79% apparently healthy birds, 60% 6-10 weeks dead chicks, and 58% pipped but unhatched chicks showed positive reaction. The results corroborate with the findings of Agarwal (1964), that 77.6% of birds and 60% of 12-20 weeks chicks were positive by plate test. This method was also followed by many workers specially in India by Adlakha and Singh (1961), Singh (1962), Agarwal (1964) and Gurumurti and Ghouse Mohiuddin (1964). Olesiuk and Van Roekal (1960), Crawley (1960) and Coles and Cuming (1959) also stated that the antibodies were widely spread in an infected flock and testings of a few birds would indicate whether infection is present. A very higher percentage of reactors in adult birds and lesser percentage in dead chicks and least in pipped but unhatched chicks is in accordance with the findings of Raymond et al. (1960) who showed that more chicks were serologically negative but became positive following exposure to carrier birds. This factor was also presumed by Pathak and Singh (1961) that PPIO infection which extended to the posterior portion of the abdominal cavity i.e. involving abdominal air sacs only carry infection from the infected visera to the egg. As such a low transmissibility of infection occurred in eggs that too pathogenic

PPIO, which was according to the description of Merchant and Packer (1961).

The yolk culture of PPIO killed the germ plasma early as they were highly virulent and more concentrated. These findings are in support of Adler and Yamamoto (1965). Their regulation was an evidence of transovarian infection which is analogous to the reports of Fahey and Crawley (1954b) and Mataney et al. (1955).

There was heavy mortality in 7 day old chicken embryos which was an indication of pathogenic potentialities of pathogenic PPIO strains in chicken embryos and also substantiate the transovarian passage of CRD. In almost all the dead embryos congestion haemorrhages and oedema in dependent parts occurred. The above findings correspond with the observations of Fahey and Crawley (1954 b 1956) Chute and Cole (1954) Cover and Waller (1954), Telloarust and Nobrega (1956) Galnek and Levine (1957) Yamamoto and Adler (1958) Rashaw and Popov (1963) and Yader and Hofstad (1964).

In chicks the pathogenicity trials revealed the pathogenic potentialities of the selected strains used. Yolk passaged cultures produced typical lesions in chicks due to exaltation of the virulence of the organisms and the higher concentration. This is similar to the observations of Adler and Yamamoto (1956). The disease could not be reproduced typical to the out breaks of CRD in the field due to small number of the organisms in the inoculum, which is in agreement with the observations of various workers such as Adler (1958), White et al. (1954) and Yamamoto and Adler (1954). Chicks

produced more pronounced lesions by air-sac route of PPLO inoculation which corroborates with the findings of Singh (1962). Pathogenic PPLO alone when given produced a mild form of mycoplasmosis in chicks. This is analogous to the reports of Simsburt et al. (1959) Gianforte et al. (1960), Adler and Yamamoto (1956), Brion (1961), and Bankowski (1961), In combination of E. coli and nonpathogenic PPLO, the pathogenic PPLO produced acute type of disease. The synergistic effect of the three, complicated the disease which is in accordance with the observations of Crawley and Fahey (1957), Gross (1956, 1957), Adler and Yamamoto (1956) and Bankowski (1961). Some chicks which did not exhibit any clinical symptoms were found positive on cultural, serological and postmortem examinations. The disease might be in subclinical stage and established in the body. This is similar to the findings of Ose et al. (1960) and Gianforte et al. (1960). Out of 10 control chicks only one was found positive after 4 weeks serologically, culturally and pathologically but was negative clinically. The chick might have got infection through air from the infected chicks kept in close proximity. This corroborates with the findings of several workers like Fahey and Crawley (1954, a, b. 1955), Berger et al. (1958), Grumbles et al. (1952) Lancaster et al. (1960) and McMartin (1963).

Various stress factors such as Newcastle disease, E. coli, vaccination against New-castle disease, and excessive heat lowered the resistance of the flock to infection and when PPLO assumed virulence by bird passage from subclinical

stage, the lowered resistance and the presence of all the factors mentioned above acted synergistically and produced an outbreak of CRD. This is in agreement with the reports made by Wasserman et al. (1954), Grass (1955, 1956), Stubbs et al. (1955), Fahey (1955), Biddle and Cover (1957) and Simburt et al. (1958).

Bacterial flora other than PPLO

E.coli :-

Among 61 gram negative organisms, 28(35%) of E.coli sps. were isolated from tracheas of live birds. This in agreement with findings of Adlakha (1961) who isolated 34 (34%) E.coli sps. from tracheas 100 live birds similarly Tylor and Fabricant (1957) isolated 30 (42.12%) E.coli sps. from tracheas of 68 birds.

An increase of 63.75% gram negative organisms from gram positive organisms that too E.coli sps. were found in tracheas, lungs and air sacs of 20 chicks died due to CRD which is analogous to the findings of Price et al. (1957) who found an increase of 75% gram negative organisms from gram positive organisms that too E.coli sps. in CRD cases. 12 (60%) isolates of E.coli species out of 28 gram negative organisms with an increase of 25% E.coli sps. as compared to the healthy (vide supra) were isolated which corroborates with the findings of Adlakha (1961) who isolated 23(56%) E.coli sps. with an increase of 22% E.coli sps. from tracheas, lungs and air sacs of chicks.

From the above findings it is apparent that E.coli sps. played an important role along with PPLO to produce CRD which is in agreement with reports of Fahey and Crowley (1955) Edward and Fraudt (1956) Biddle and cover (1957) Gross (1954, 1958) and Simburt et al.(1958).

Staphylococci :

33 Staphylococci out of 44 gram positive organisms from tracheas of 80 live birds and 12 staphylococci out of 20 gram positive organisms from tracheas lungs and air-sacs of 20 dead chicks were isolated. Similar findings viz 39 Staphylococci out of 44 gram positive organisms from 100 live birds and 48 staphylococci out of 61 gram positive organisms from 50 necropsied chicks were isolated by Adlakha (1961). The isolation of staphylococci along with PPLO from CRD cases and its role in CRD as secondary invader corroborates with the findings of Barnes et al.(1961), Cover and Waller (1954), Fahey (1953) and Adlakha(1961)

Micrococci :

3 micrococci from 80 live birds and 3 micrococci from 20 dead chicks were isolated. The part played by them in chicks as secondary invaders in association with PPLO CRD of chicks is in agreement with the findings of Fahey (1955) Biddle and Cover (1957) and Van Roekel (1954).

Streptococci :

In this present work 3 streptococci out of 44 gram positive organisms from live birds and 3 streptococci out

of 20 gram positive organisms from 20 chicks died due to CRD were isolated. The present findings shows that streptococci acted as secondary invader in CRD which agrees with the similar reports made by Cover and Waller (1954), Taylor and Fabricant (1957), and Adlakha (1961). The presence of *Klebsiella proteus*, *Pseudomonas*, *Bacillus* and other gram negative organisms (unclassified) dead chicks might be due to their secondary invasion of respiratory-tract. The small percentage of *Klebsiella proteus*, *pseudomonas*, *Bacillus* and other gram negative organisms (unclassified) in live birds probably due to their lower incidence in the environment in which the birds were raised as is also suggested by the work of Price et al. (1957). The association of gram negative organisms with PPIO appears to be significant observation which gives indirect indication of the presence of CRD in a flock. This is also according to the contention of Price et al. (1957).

The serological, pathological and cultural findings were correlated and they showed close agreement which was analogous to the discription of Adler (1954). The results on PPIO along with associated organisms indicated that often fairly accurate diagnosis of CRD is possible. As this work was mainly with reference to PPIO which consumed major part of the time, the work on *H. gallinarum* which was encountered during the studies could not be undertaken.

S U M M A R Y

1. The literature on the studies pertaining to the bacterial flora with reference to PPL0 - its nature and infections in chickens, and chicken embryos have been reviewed and discussed along with brief notes on the bacterial flora isolated.
2. The examination of 80 apparently healthy birds, 20 dead chicks and 50 pipped but unhatched chicks revealed 89 gram negative organisms, 64 gram positive organisms, and 51 pathogenic and 73 non-pathogenic PPL0 strains.
3. Modified Grumbles PPL0 broth and Swain's (Medical Microbiology, 1965) serum agar medium gave extremely satisfactory results for isolation of PPL0.
4. PPL0 appeared on smears as fine coccobacilli, singly or in groups with indistinct morphology. The colonies grew on the surface or into the agar by penetration into the substrate of serum agar and could not be removed in their entirety from the plates. They were soft and fragile, had fried egg appearance with nipple like centre in nonpathogenic and devoid of both in pathogenic colonies. Rough colonies, ring form colonies and colonies with small central elevation of pathogenic PPL0 were seen.
5. A comparative study of growth rate and colony size of PPL0 revealed the necessity of moisture, saturated air, yeast extract and DNA for increased PPL0 growth rate and colony size.
6. 124 PPL0 isolates were grouped into 51 pathogenic and 73 nonpathogenic strains on the basis of colony morphology growth rate, fermentation of sugars and tetrazolium reduction.

7. The serological studies on whole blood samples of 330 live birds and clotted blood samples of 20 dead chicks and 50 pipped but unhatched chicks revealed that 79% of live birds 60% of dead chicks and 58% of pipped but unhatched chicks were positive for CRD.
8. A high degree of positive correlation was found between cultural, pathological and serological tests.
9. The egg transmission of PPLO produced dead in germs and proved that indirect transmission of PPLO through eggs is possible to convey infection to the next progeny.
10. The chick embryo infection by PPLO is characterized by variable patterns of mortality and lesions. More pathogenic strains produced early embryonic mortality in 48-96 hours with congestion and oedema and mild pathogenic strains killed embryos on prolonged period 4-9 days with stunted growth, petechial haemorrhages unabsorbed yolk sac and oedema.
11. The chicks infected with PPLO by I/Tr and I/air-sac routes developed CRD in 14-18 days. The yolk passaged PPLO was found to cause disease earlier with more intensity of lesions. Air borne infection was found that it is one of the sure means of spreading infection.
12. E.coli appeared to have played as complicating factor in CRD complex and other stress factors such as New-castle disease, vaccination and excessive heat were also found to have aggravated the CRD syndrome in the farm.

REFERENCES.

1. Abbot, U.K.; D.A. Mac Martin; H.E. Adler and F. H. Kartzer (1959) Poult. Sci. 38:1185 (Abstract).
2. Adler, H.E.; R. Yamamoto, and Bankowski (1954). J. Vet. Res. 15:463-465.
3. Adler, H.E., (1955) Ph. D. Thesis University of California, cited by Merck (1955).
4. Adler, H.E. and R. Yamamoto (1956 b) Cornell. Vet. 46: 337-348.
* Adler, H.E. and Yamamoto (1956 c) Poult. Sci. 35: 1396-1397.
5. Adler, H.E. and R. Yamamoto (1956a). Amer. J. Vet. Res. 17:290-292.
6. Adler, H.E., R. Yamamoto (1957) Amer. J. Vet. Res. 18: 655-56.
7. Adler, H.E. R. Yamato and J. Berg (1957). Av. Dis. 1:19-26 (V. B. 28:394.)
8. Adler, H.E. (1958) Poult. Sci. 39:1116-1123.
9. Adler, H.E., J. Babricant, R. Yamamoto and J. Berg. (1958) Amer. J. Vet. Res. 19:440-447.
10. Adler, H.E. (1960) Ann. N. Y. Acad. Sci. 79:703-712.
11. Adler, H.E., and J. Berg (1960). Av. Dis. 4:3-12.
12. Adler, H.E., and ^{M.}Shifrine, (1960) Ann. Rev. Microbiol. 14:141.
13. Adler, H.E. ^{W.W.}A.J. Demasa, and ^{W.W.}Sadler, (1964) Av. Dis., 8:570-579. (V. B. 35:280).
14. Adlakha, S.C. (1961) M. V.Sc. thesis Agra University.
15. Aftosmis J. G., M.E. Faurtellotto and R.E. Jacobs (1960) A.V. Dis. 4:491 (V.B. 31:638).
16. Agarwal K.K. (1964). M.V.Sc. thesis Agra University.
17. Annual report I.V.R.I. Izatnagar (1954).
18. Annual report L.R.S. Patna (1962-63).
19. Bankowski, R.A. (1961) Brit. Vet. J. 117:306-315.
20. Barnes, L.E., E.E. Ose, and F.O. Gassett (1960) Poult. Sci. 39:1376.
21. Bereznai, T. and L. Stipkovits (1965) Magyar Allatorv. Lap 20:403-405 (V.B. 36:402).

22. Beckunow J.R. and W.R. Dunlop (1959) Poult. Sci.
38:807-816.
23. Berger E.H. , L.E. Card and B.S. Pomeroy (1958) Disease
and Parasitosis of Poultry Lea and Fabiger Philadelphia.
24. Biddle E.S. and M.S. Cover (1957). Amer. J. Vet. Res.
(8:405-408.
25. Bihar Animal Husbandry Bulletin (1958) 3:21.
26. -do- -do- (1959) 3:23-24.
27. -do- -do- (1965) 1:118-121
28. Blake J. T. (1962) Amer. J. Vet. Res. 23:847-853.
29. Brion J. , M. Fontaine and^{m.P.}Fontaine (1960) Rec. Med. Vet.
136:605 as cited by Brion (1961).
30. Brion J. (1960) Rec. Med. Vet. Alfort. 136:605-626
(Biol Abs. 39:587.)
31. Brion J. (1961) Brit. Vet. J. 117:296.
32. Buxton J. C. (1952) Vet. Rec. 64:221-223 (V.B.53:442).J.F.
Crowley & and J.E. Fahey (1955) Poult. Sci. 34:706-707.
33. Calnek B.W. and K.P.P. Leuins (1957) A.V. Dis. 1:208
34. Ceccarelli A. (1962) Zooprofilassi 17:499-507
(V.B.33:180).
35. Chalquest R.R. and J. Fabricant (1960) Av. Dis. 4:515.
36. Chu H. P. (1954) Proc. Tenth Poult. Congr. Edinburgh
26-251.
37. Chu. H.P. (1958) The Vety. Annual. Bristol. Johnwright
and Sons Ltd.
38. Chu H.P. (1958 b) Vet. Rec. 70:1064.
39. Chu H.P. (1959) Amer. J. Vet. Res. cited by
Jungherr (1960).
40. Chu, H.P. (1959) The Vety. Annual edited by W.A. Pool,
Rubb. John. Wright and Sons Ltd. Bristol P. 101.
41. Chu H.P. and Newnham A. (1959) Proc. XVI Ind. Vet. Congr.
Electron microscopy. London. P. 245 cited by Chu (1959).
42. Chute H.L. and C.R. Cole (1954) Amer. J. Vet. Med. Ass.
30:5-14.
43. Clark Harold W., G. Richard, Fowler and^{MCP}Thomas Bron J.(1961)
J. Bait 81:500-502.

44. Coisek, D. (1965) Medycynowet 21 : 74-77 (V.B. 35 : 609).
45. Coles, J.D., and W.A. Cumming R.B. (1959) J.S. Afr. Vet. Med. Ass 30: 5-14.
46. Colusi, A.D. (1962) Rev. Med. Vet. B. Aires 43 : 99-101. (V.B. 33 : 130).
47. Colusi A.D. and Menchaca E.B. (1963) Rev. Vet, Bares 44: 255-259 (V.B. 34 : 264).
48. Cottereau P, Valette, L.R; and Papagaorgior, C (1963). Rev. Med. Vet. 26 : 106-113.
49. Cover M.S. and Waller E.F. (1954). Amer. J. Vet. Res. 15 : 119-121.
50. Cover M.S; Benton, W.J; and Whelan S.R. (1960) Ann. N.Y. Acad. Sci. 79: 567.
51. Cumming R.B. (1961) Aust. Vet. J. 37: 221-224.
52. Delaplane J.P. and H.O. Stuart (1943). Amer. J. Vet. Res 4 : 325-332.
53. Domermuth Ch. (1958). Poult. Sci. 2 : 147-153.
54. Domermoth C.H. and Gross W.B. (1959) Av. Dis 3: 452-456. Cited by Gorss (1960).
55. Domermoth C.H. (1962) Av. Dis 6 : 135-140 (Biol. Abs. 3 39-1965).
56. Domermoth C.H., Nielsen M; Freundt E.A. and Birch Andersen. A. (1964) J. Bout 33 : 1423-1432.
- * Dunlop, W.R. and Strout, R.G (1956). Proc. 60th An. Meet, U.S. Livestock Sanit. Assoc. Chicago, Illinois, 197-202.
57. Dutta S.K. Dierks R.E. and Pomeroy B.S. (1965) Av. Dis 9: 241-251. (V.B; 35: 683).
58. Edward D.G. 66. (1947) J. Gew. Microbial 1 : 233.
59. Edward. D; G, ff; and Fitzgerald W.A. (1951) J. Gew. Microbiol 5: 576.
60. Edward D.G. ff (1953) G. Gew. Microbiol. 3: 256.
61. Edward. D.G. ff. (1954) J. Gew. Microbiol 10: 27-64.
62. Edward D.G. ff and Ewing W.H. (1954) Cornell Vet. 44 50-56 cited by gross. W.B. (1956).
63. Edward D.G. ff and Fraundt; E.A. (1956). J. gan. Microbiol. 14 : 197.
64. Edward D.G. ff (1960) Am. N.Y. Acad Sci. 79: 309.
65. Edward, D.G. ff, and Kanarek, A.D. (1960) Ann N.Y. Acad. Sci. 79: 696-702.

66. Bissa Y.M.(1956) Dull.off.Int.Epiz 46: 170-183.V.B.26: 615.
67. Fabricant J; Levine P.P.Calmek.B.W; and Adler H.E.(1958)
Poult Sci. 37: 1203.
68. Fabricant J.(1959) Av.Dis 3: 423-439.
69. Fabricant J, Levine P.P, Calmek B.W; Adler H.E. and Berg J.R.
(1959). Av.Dis 3 : 197-222.
70. Fabricant J, Levine P.P; Calmek B.W., H.E. Adler and Berg, J.R.
(1959) Av.Dis 3: 197-222.
71. Fabricant C.G, Vandemark P.J. and Fabricant J.(1962)
Av.Dis 6 : 323-332.
72. Fabricant C.G. Van Roekel P.J; and Fabricant J.(1962).
Av.Dis 6 : 6.
73. Fabricant C.G; Fabricant J. and Vandemark P.J.(1964) J.Gen.
Microbiol 35 (V.B. 34 : 646)...
74. Fabricant C.G, P.J. VanRoekel, and Fabricant J. (1962) Av.Dis 6:3.
75. Fahey J.E. and Crawley J.F.(1953) Caud.J.Camp.Med 19:53-56.
76. Fahey J.E. and Dunlop W.R.(1953) Caud.J.Camp.Med and Vet.
Sci. 17: 294.
77. Fahey J.E.(1954a) Proc.Soc.Exp.Biol N.Y. 86: 33-40(V.B.25:
35-68).
78. Fahey J.E. and Crawley J.F.(1954 b) J.Camp.Med.
13: 67-75.
79. Fahey J.E.(1955) Poult.Sci.34: 982-984.
80. Fahey J.E. and Crawley J.F.(1955) Canad .J.Comp.Med. 20:7-19.
81. Fahey J.E.and Crawley J.F.(1955) Caud J.Comp.
Med 19 : 53-56.
82. Fahey J.E. and Crawley, J.F.(1956) Canad J.Comp.Med.
20: 7-19.
83. Flier K, and V.Sprockhoff, H.(1962) Zbl.Bakt 18 : 349-366.
(V.B. 33: 297).
84. Fomia, A.Ya; Grosheva, G.A; and askoskov, V.S.(1964) Veterinariya
Moscow 41 No 11P.P. 37-40(V.B. 35: 422).
85. Fomia A.Ya; Grosheva G.A. and Shubin V.A. (1965). Veterinariya.
Mascow.42, Pol.PP 27-30(V.B. 35 : 609).
86. Fraundt.(1953). Aarhus stifts bogtry Kkerie Mungsgerard.
Cited Adler and Shifrine (1961).

87. Gentry, R.F. (1959). A.V. Dis 3: 175-181.
88. Gentry, R.F. (1960). Av. Dis. 4 : 436-443.
89. Gentry, R.F. (1960). Ann N.Y. Acad. Sci. 79: 403-409.
90. Geurden L.M.G; Devos A. and Van Den wyngaert, M; (1956) Vlaan dier Genesk T. Ydschr 25: 207-217. (V.B. 27: 273).
91. Gibbs, C.R. (1931). J. Bout 21 : 97-109.
92. Gianforte, E.M; E.L. Jungherr and E.E. Jacobs (1955). Poult. Sci. 34 : 662-669.
93. Gianforte E.M; Skamser L. Mand R.G. Brown (1960). Ann. N.Y. Acad. Sci. 79: 713-717.
94. Gill, James, W; (1962) B J. Bact. 33: 213-218.
95. Gross, W.B. (1955). Amer Z. Vet. Res. 19: 443.
96. Gross, W.B. (1956) Poult. Sci. 35: 765-771.
97. Gross, W.B. (1957). Amer J. Vet. Res. 18: 724.
98. Gross, W.B. (1958) Amer. J. Vet. Res. 19: 443-452.
99. Gross, W.B. (1958). Amer. J. Vet. Res. 19: 443-452.
100. Gross, W.B. (1961). Av. Dis. 5 : 431.
101. Groupe, V; Winn, J.D. and Jungherr, E. (1938). Proc. Soc. Exp. Biol. and Med. 67 : 397-398 Cited by Merchant & Packer (1961).
102. Grumbles, L.C; Boney W.A. and Delaplane J.P. (1952) Poult. Sci. 31 : 809.
103. Grumbles, L.C ; Phillips, E. Boney W.A, and I.P. Delapleme (1953) South Western Vet. 6 : 166 gluoted by Jaylor and Fabricant (1957).
104. Gullion, J.C; Renault; L; and Polisse, M; (1963). Bull Acad, Vet. Fr. 36: 57-60 (V.B. 33: 492).
105. Gurumurthy, V., and Ghouse Mahioddin (1964). I. Vet. J. 41: 174-180.
106. Hagen, W.A. and Bruner; D.W. (1957). The anfectious diseases of Domestic animals with special reference to New York, Constock Publishing associates 3rd edn.
107. Hall, C.F., (1962). Av. Dis. 6: 359-362 (Biol Abs. 41: 563).
108. Hammer, A.H; Rixx, Ginherr; P.E., Price, R.J. and Markham F.S. (1958) Av. Dis. 2 : 213-216.
109. Hamsley, L.A. and Harrey (1965) Vet. Rec. 77 : 103-107. (V.B: 35: 431).
110. Heishman, J.O.; abson, N.O; and Cunningham, C.J. (1966). Av. Dis. 10: 189-193.

111. Hofstad, M.S.; (1957). Av. Dis. 1 : 170-179.
112. Hofstad M.S. and Doerr (1956). Cornell Vet. 46 : 439-446.
113. Jacobs, R.E.; Luginbuhl; R.E. Burr, W.E. and
114. Jerslaad, A.C.; Hamilton C.M. and Smith V.E. (1949)
Vet. Med. 44 : 274.
115. Johnson, E.P. (1954) Cornell Vet. 44 : 230-239.
116. Jorden, R.F. ; (1956). Vet. Rec. 63: 547.
117. Johnson, E.P. and Domermuth, C.H. (1956) Cornell Vet. 46:
409-413.
118. Jungherr, E.L. and Luginbuhl (1952). Proc. 56th Ann. Meet.
V.S. Livestock San A. 275-283. Cited by Ven Ralkel and Obsiuk.
119. Jungherr, E.L.; Luginbuhl, R.E. and Jacobs, R.E. (1953)
Ann. Proc. Book. Amer. Vet. Med. Assoc. 303-311.
120. Jungherr, E.L.; (1954). Proc. 26th Ann. Meet. Conf. Lab. wkrs. Pull.
Dis. Contr. S. Raleigh, N.C. (V.B. 25: 56).
121. Jungherr, E.L.; Luginbuhl; R.E. and J.R. Avampato, J.E. (1954)
Proc. 26th Ann. Meet N.E. Conf. Lab. Wkrs. Pull. Dis. cont;
Raleigh, N.C. (V.B. 25 : 332).
122. Jungherr, E.L.; (1955) Arch. Exp. Vet. Med. 9: 212-217.
123. Jungherr, E.L.; Luginbuhl; R.E. Tavertellottee, M. and Burr
W.E. (1955). Proc. Book. Amer. Vet. Med. Assoc. 315-322.
124. Jungherr, E.L. (1958) Amer J. Vet. Res. 19: 464-467.
125. Keller, H. (1958) Schureiz Arch. Tierheik 100: 45-51.
126. Kelton, W.H.; (1962) Bact J. 3.3: 948-955.
127. Khare, J.P.; (1961) M.V.Sc. thesis Agra University.
128. Kleckner, A.L. (1960) Amer J. Vet. Res. 21 : 274-280.
129. Kohler, H. (1951). Expt. Med. 5.P.P. 75+94.
(V: B 53:400)
130. Kozusnik, Z; Cernovsky, J; and Burilora, E (1966).
Veterinariystvi 16: 18-22 (V.B 36: 365).
131. Kraybill; William H; York E; Crawford, John, C; Maisel and
Michael W. Rytel (1962) ————— Bact. Proc. 62: 157 (Abstract.)
(Biol. Abs. 41: 537.)
132. Krishna Mohan Rao, B. (1965) Outlines of Poultry Husbandry.
From the author's copy.
133. Kumar, S; Dierks, R.E; Newman J.A; Pfo W.C.J. and Pomery B.S.
(1953). Av. Dis. 7 : 376-385.

134. Lancoaster, J. E; Goswami, J. N. and Rienzi, A. A. (1960) J. Comp. Med. 24 : 10-16 (V.B. 30: 1375).
135. Lecce J. G. and Sperling , F. G. (1954a) Proc. 26th Ann. Meet. N.E. Conf. Lab. Wkrs. Publ. di. contv; Raleigh, N. C. (V.B. 25 : 55).
136. Lecce, J. G. and Sperling, F. G. (1954b) Cornell Vet. 44 : 441-449.
137. Leibermiester, K; (1954) Hyz. Z. Infektion. Krankh 140:132.
138. Levine PP and Fabricant J. (1961) 23rd N. E. Conf. Av. Dis. June 19-21.
139. Loezellier Adres Blanco (1960) Rev. Patron Biol Animal 6:5-10. (Biol. Abs 36 : 6090).
140. Lucas, H. and Luthegen, W. (1964) Dt. tieraztt. uschr 71:270-273.
141. Luginbuhl, R. E; Jungherr, E. L.; Jacobs, R. B; and Winn, J. D. (1955) Proc. 26th Ann. Meet. N. E. Conf. Lab. Wkrs. Pull. Dis. Contv; Raleigh N. C. (V.B. 25 : 331).
142. Maniloff, J; Morwitz, H. J; and Barnett, R. J; (1965) J. Bact. 90: 93-204 (V.B. 35: 764).
143. Marcoto, A; (1936) Nuova Vet. 14 : 235-243 and 272-275. (V.B. 9 : 453).
144. Markham, F. S. and Wong, S. C; (1952) Poult. Sci. 31 : 902-904.
145. Markham, F. S. and Wong, S. C.; (1952) Poult. Sci. 31: 921. 923
146. Mataney, C. F; Pomeroy, B. S; and Osborn, O. H. (1955) Proc. Ann. Vet. Med. Assoc. P. 310.
147. McMartin, D. A; (1962) Poult. Rev. 2: 43.
148. McMartin, D. A; (1963) Proc XVII World Vet. Congr. Hanover.
149. Merchant I. A. and Packer R. A. (1961) Veterinary Bacteriology and Virology. 6th edn. The Iowa State College Press, Ames, Iowa.
150. Moore, R. W; Grumbles L; and Beasely, J. N; (1960) Ann. N. Y. Acad. Sci. 79: 369-370.
151. Morton, H. E; Lecce, G. G. Askey J. J. and Coy , N. H. (1954) J. Bact. 69:7-717.
152. Naidu, P. M. N (1964) Poultry keeping in India 1st Edn. I. C. A. R. Publication.
153. Nelson, J. B; (1935) Science 82: 43.
154. Nelson, J. B. (1936a) J. Exp. Med. 64: 759-769.
155. Nelson, J. B. (1936b) Journ. Exp. Med. 63: 515.

156. Nelson, J.B. (1966) Journ. Exp. Med. 64: 749.
 * Nelson J.B. (1936d) Journ. Exp. Med. 64: 759-769.
157. Nulton, L.G; Connole, M.O. and Romby, P.D. (1962).
 Proc. XII World's Poult. Congr. Sydney Sept; papers pp.
 335-359 (V.B. 33 : 228).
158. Newnham, A.G. and Chue, H.P. (1965) J. Hyg. Comb 63 : 1-123
 (V.B. 493).
159. Newing (1956), Bull. Expiz. Dis. Afr. 4 : 197.
160. Noel, J.K; Devalt, H.M. and Faber, J.E. (1962).
 Poult. Sci. 41 : 580-587.
161. Olesiuk, O.M. and Van Ralkel, H (1952) Av. Dis. 4
 348-368.
162. Olesiuk, O.M. and Van Ralkel (1960) Av. Dis. 4 : 348-368.
163. Olesiuk, O.M. and Van Roekel, (1960) Ann. N.Y. Acad. Sci. 79: 727.
164. Oleson N.O; Harsh, T.R; Heishman, J.D. and Campbell, A (1962)
 Av. Dis. 6 : 191.
165. Oleson, N.O; Heishman J.O; and Shilton, D.C. (1962).
 Av. Dis. 6 : 171-177.
166. Ase, E.E; Barnes, R L.E. and Garsett, F.O. (1960) Ann. N.Y.
 Acad. Sci. 79: 713-726.
167. Osteen, O.L. (1954) Proc. Book. Ann. Vet. Med. Assoc.
 337-340.
168. Pathak, R.C. and Singh, C.M. (1959) Poult. Sci. 38: 956-959.
169. -do- (1961) Agra. Univ. J. Res. 10: 155-170.
170. Prasad, A.K; (1960) Dissertation, Madras University.
171. Price, K.E, Zalli, Z, Jr, WyB. Hardie, W.B ; and Callian, M.J. (1957)
 Poult. Sci. 36 : 219-225.
172. Priestly, F.W. (1951) Vet. Rec. 63: 427.
173. Prokofeva, M.T; Gurova, E.1 and Kiprichi, V.V. (1963) Veterinaria
 Moscow 40 : 60-63. (V.B. 34 : 325).
174. Prokofiva, M.T, Gurova, E.1; Kiprich, V.V. and German, V.V. (1964)
 Veterinariya, Moscow. 41 : 32-35. (V.B. 34 : 455).
- * Rao S B.V. (1962) Note for the first conference of Poultry Research workers, Jan. 1962. cited by Gurusamudram
 and G. Mohideen (1964)
175. Rashaw, H. and Popov, T. (1965) Izv. Vet. Instzarey Parazit
 Balesti, Sofia 8: 165-170. (V.B. 34 : 139).
176. Raymond, R.G; B. Zander, D.V. and Siddle P.J. (1960) Poult.
 Sci. 39: 1237.
177. Reagan, R.L; Day W.C. and Bruckner, A.L. (1953). Poult. Sci.
 32: 960-965.

178. Renault, L; Guilvon, J.C. and Palisse, M. (1960) Bull. Acad. Vet. Fr. 33: 495-499. (V.B. 31: 307).
179. Richey, D.J. and Dirdjosoebroto, S. (1966). Vet. Bogor. 9: 3-12. (V.B. 36: 285).
180. Rislakki, V. and Vasenius (1965) Nord. Vet. Med. 17: 213-219. (V.B. 35: 552).
181. Roberts, D.H. (1964) Vet. Rec. 76: 798-800 and 801.
182. Rhodes, K.R; Kelton, W.H. and Healeston (1964). Amer J. Vet. Res. 25: 766-767.
183. Rusy, A.C and Van Iterson (1961) J. Microbiol. 27: 129-133. (V.B. 32: 282).
184. Sabin, A.B. (1941), Bout. Rev. 5: 1-63.
185. Salona Alonso, A. (1964) Revla. Patromato. Biol. anim, Madria 8: No. 3. PP 33-91. (V.B. 35: 206).
186. Shelka (1961) Sborn Vys, Sk. Zemedeesk. Brno Ser B 9: 303-314. (V.B. 32: 742).
187. Shifrene, M; Adler, H.E., and Ousterhout, L.E. Aviondis 4: 12. (1960) Cited by Adler H.E. and Shifrine (1960).
188. Shifrine M. ^{Mohe; Jack-Pomgorn and Henry. and H.E. Adler} (1962) J. Boet. 83: 187-192. (Biol. Abs. 38 : 1134).
189. Shipkouritz, N.L. and Elarke, M.K. (1956). sci. 122: 969-970 (V.B. 26: 424).
190. Shiskov and Enchev, S (1962) Izv. Vet. Inst. Zaraz-Parait Zolesta, Sofia 3 : 103-119. (V.B. 3: 431.)
191. Shiskov, N. (1964). Vet. Med. Nauki, Sofia 9: 13-18 (V.B. 35: 609).
192. Simburt, R.M; Devolt, H.M; and Faber, J.E; (1958). Poult. Sci. 37: 157-166.
193. Simburt, R.M; Faber J.E; and Devolt, H.M. (1959) Poult. Sci. 1393-1404 & 676-684.
194. Singh, S.B. (1962). M.V.Sc. thesis Agra. University.
195. Smith, P.F; Morton, H.E; and Leberman, P.R. (1950). Proc. Soc. Exp. biol Med. 74: 552-555.
196. Smith P.F. (1955) J. Bout. 70: 552-556.
197. Smith, P.F. (1956). App. V.B. 26: 424).
198. Smith, P.F. and Lynn, R.J. (1958) J. Bout, 76: 264.
199. Somerson, N.L; and Morton, H.E; (1953) J. Bout. 65: 245-251.
200. Sornicle, J. and Zaibet, R (1963) Rec. Med. Vet. 139: 561-562 (V.B. 34 : 189).

201. Staub, A (1947) Ann. Inst. Pastur. 73: 430-432. (V.B. 18: 292).
202. Stoenescu, C. C; Surden, M; Staneschu; Sendulescu S and C. Vior (1960) Probl. Epiz. Vet, Bucerst. P: 56-68 (V.B. 30: 556)
203. Stublas, E. L; Sperling; F. G. and Lecue J. G. (1954).
Proc 10th World Poult. Congr. Edinburgh 244-246.
204. Swain, R. H. A. (1965) Medical Microbiology 11th edn. The
english language Book Society E. & S. Livin, Gtone Ltd.
205. Swift, H. F. (1941) J. Exp. Med. 74 : 557-568.
206. Tajima, M; Miyemoto T; and Agashi, H. N; (1958) N. J. B. S.
Bull Biol. Res. Tokyo . 3: 43-52 (V.B. 30: 1756).
207. Tazzini, F (1955) . Zooprofilarri 10: 531-5. W. (V.B. 26: 262).
208. Tallogarust, A; and Nobraeya, P; (1956) Arch. Inst. Bool. S. Poullo
23; 35-38. (V.B. 27: 230).
209. Tylor, J. R; Fabricant, J; (1957) Av. Des. 1: 101-104.
210. Tylor, J. R; Fabricant, j; (1957) Cornell Vet. 47: 112-126.
211. Taylor, J. R; Fabricant, J; and Levine, P. P. (1957).
Av. Dis. 1: 101-104.
212. Van Heric W. V; and Eaton, M. D. (1945). J. Bact. 50 : 47-55.
213. Ven Raekel, H . and Obsick, O. M. (1952) Proc. Book. Amer.
Vet. Med. Assoc. 271-275.
214. Van Raekel, H; and Obsiuk, O. M. (1953). Proc. Book. Ann. Vet. Med.
Assoc. 90th Annval. Meeting. Toronto 289-302.
215. Ven Raekel, H; and Obsick (1954) 26th Ann. Conf. Lab. Wkrs. Pub. Dis.
Cont. P. 2 Cited by Merck (1955).
216. Van Raekel, H; Gray, J. E; & Shipkowitz, N. L.; Clarke, M. K. and
Luchini, R (1957).
217. Van Raekel, H; Obsiuk, O. M; and Beninato, L. P. (1953) Amer J. Vet.
Res. 19: 453-463.
218. Wasserman, B; Yates. V. Z; and Fry, D. E. (1954) Poult. Sci. 33: 622-
623.
219. White, E. H. (1952) Brit. Vet. J. 108: 111-123.
220. White, E. H; Wallav, G. I; and Alberts, J. O. (1954). Poult.
Sci. 33 : 500-507.
221. Wilson, G. S. and Miles, A. A.; (1954) "Topley and Wilson's
principles of Bacteriology and Immunology" London, Edward
Arnold (Publishers) Ltd. 4th edn.

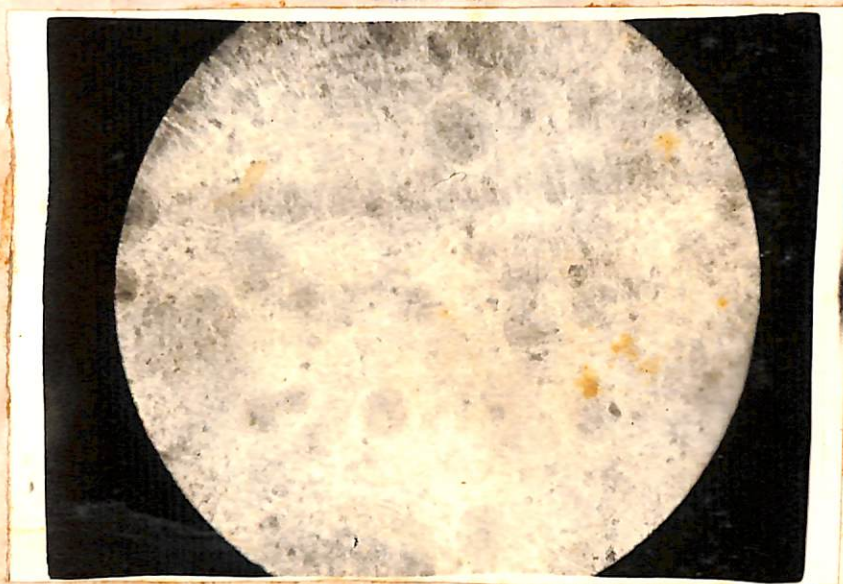
222. Wilkinson, J. F.; (1965) "Medical Microbiology" 11th Edn.
The English language. Book Society and E. and S. Livingstone, Ltd.
223. Yader, H. W. and Hofstad, M. S.; (1962). Av. Dis. 6: 147-160.
224. Yader, H. W. and Hofstad; (1964) Av. Dis. 481-512.
225. Yamamoto, R; and Adler, H. T.; (1953a) J. Inf. Dis. 102: 143-152.
226. Yamamoto, R; and Adler, H. E.; (1953b). J. Inf. Dis. 102: 243-250.
227. Yamamoto, R; and Bigland, C. H.; (1964). Av. Dis. 8: 523-531.

FIGURE -1



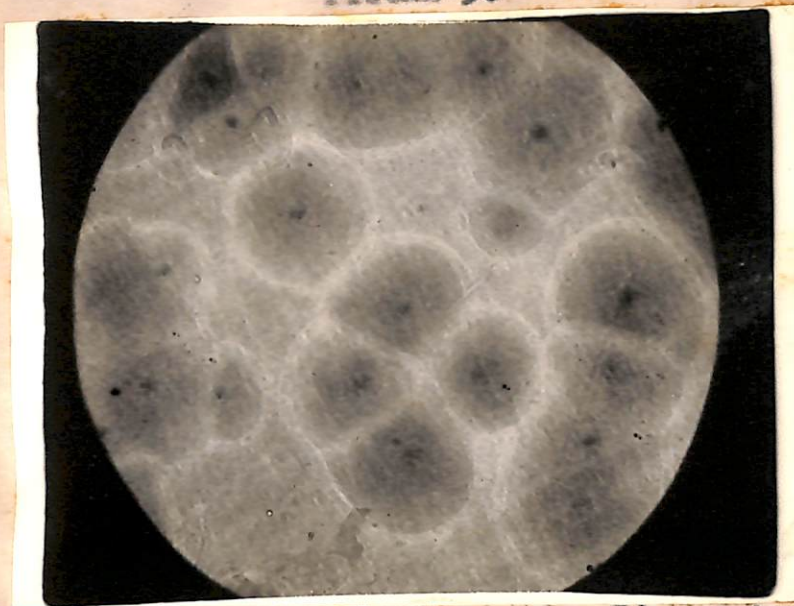
Giemsa stained smear of PPLO made from the sediment of centrifused
PPLO birth culture (1000 X)

FIGURE -2



Pathogenic PPLO colonies - Bigger type (60 X).

FIGURE-3.



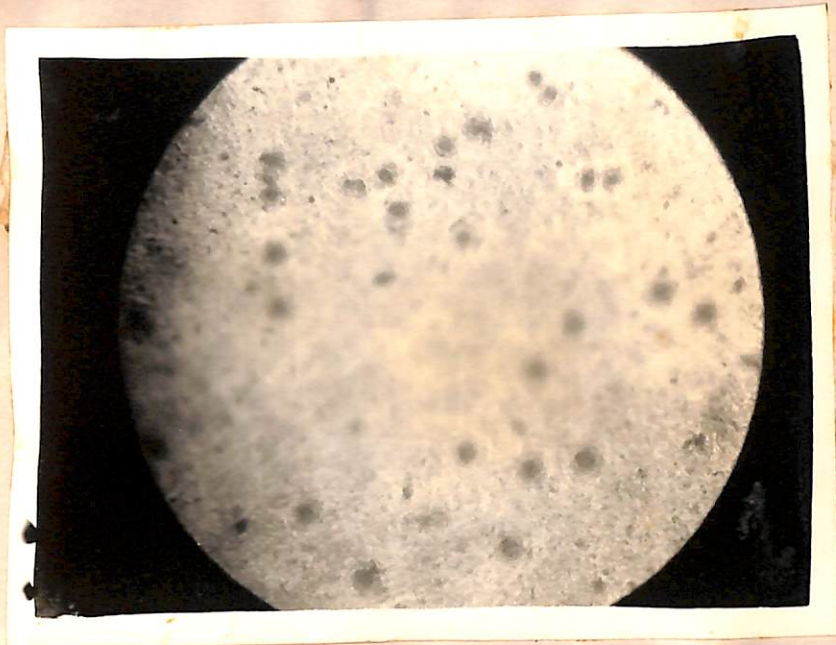
Non pathogenic PPLO colonies - Bigger type (50 X).

FIGURE - 4.



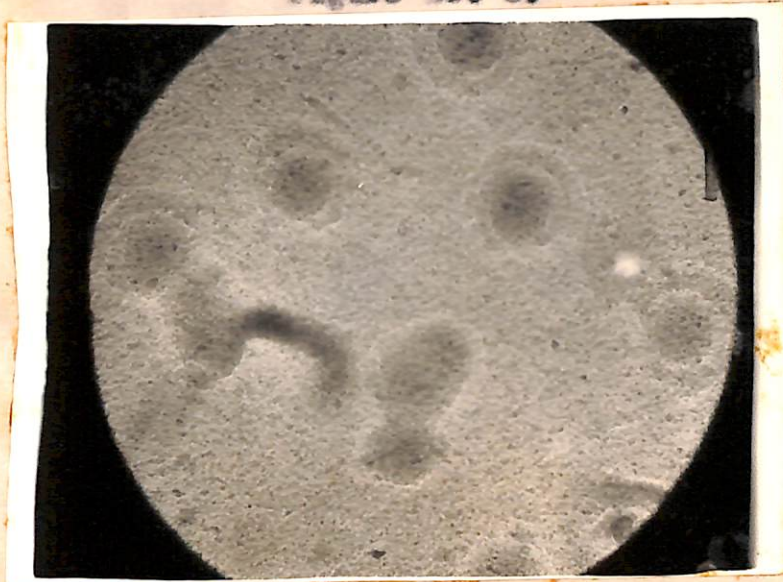
Rough colonies of pathogenic PPLO - (50 X).

Figure No. 5.



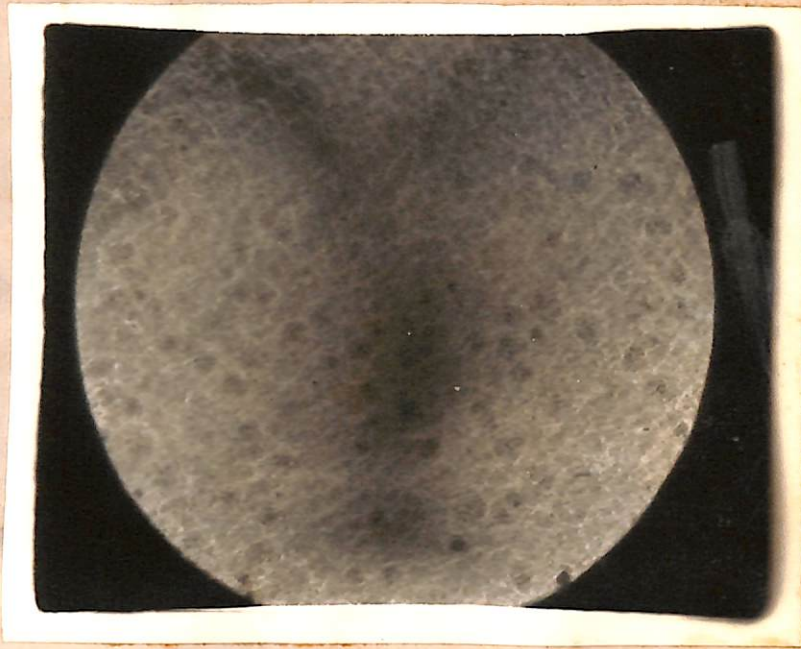
PTIO Colonies with small central elevation - Pathogenic type (50X)

Figure No. 6.



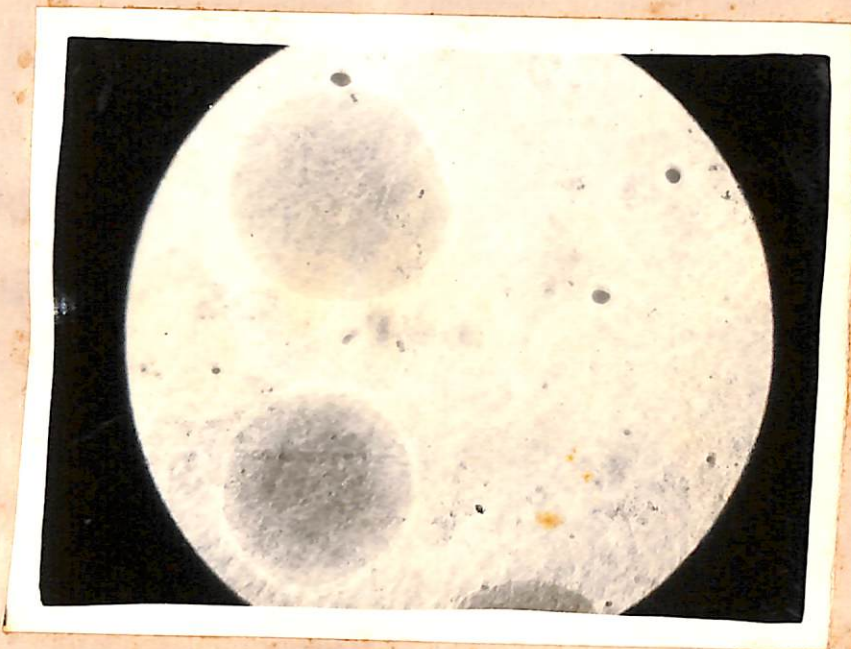
Irregular form colonies of pathogenic PTIO (50X)

Figure-7.



typical pathogenic PFLO colonies (50X)

Figure-8.



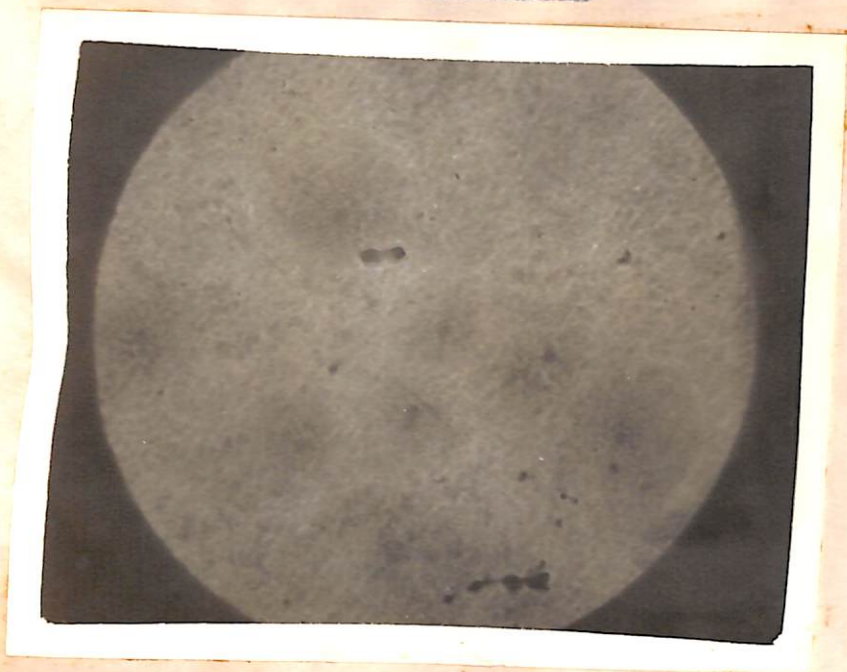
typical non pathogenic PFLO colonies (50X)
- with prominent nipple like center.

Figure-9.



Uniformly stained (Dienes stain) Pathogenic PPLO colonies (50x)

Figure-10.



Colony size of ^{nm} non-pathogenic PPLO after incubation at 10% CO₂ tension. (161.358 μ)

Figure-11.

Colony size of nonpathogenic PPL0 on serum agar when DNA added (167.187/u)

Figure-12.



Chicken embryo with unabsorbed yolk sac, petechial haemorrhages, oedema of head region and stunted growth (Experimental case).

Figure- 13.



Artificially affected with CRD chick showing emaciation and conjunctival discharge.

Figure-14.



Trachea with caseous exudate (Experimental case).

Figure-15.



Group - with pericardial hemorrhages (Experimental case)

Figure-16.



Cervical air sacs filled with caseous exudate and thickening of air sac membrane (Experimental case).

Figure-15.



Trachea - with petechial hemorrhages (Experimental case)

Figure-16.



Cervical air saces filled with caseous exudate and thickening of air sac membrane (Experimental case).

Figure-17.



Thoracic air sac filled with caseous exudate in beaded form
(Experimental case).

Figure-18.



Abdominal air sac with caseous exudate (Experimental case).

Figure-19.



Chicken embryo with partially absorbed yolk sac died due to natural PPLO infection.