ASPECTS OF AVIAN MYCOPLASMOSIS

by

David Hugh Roberts

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Section 1

General Introduction

The work described in this theses relates to studies on diseases of poultry known to be caused by Mycoplasma (PPLO) or in which pathogenic Mycoplasma have been implicated. These diseases include:-

Chronic respiratory disease (CRD) of poultry

Infectious sinusitis of turkeys

Sinusitis of ducks

Infectious synovitis of chickens and turkeys

(A) Historical Review

Chronic respiratory disease in fowls is characterised by nasal discharge, respiratory rales, slow rate of spread, persistency of the clinical signs, moderate decline in egg production, and loss in body weight. Chronic respiratory disease is widespread in broilers, it interfers with growth and prevents the prompt marketing of birds, so that the birds have to be fed for longer periods than usual. Many broilers die of the disease and the carcases of the survivors are often of inferior standard.

Infectious sinusitis in turkeys has been recognised as a distinct clinical entity for a considerable time. The disease is characterized by shaking of the head, nasal discharge, frothy lacrimation, distension of the infraorbital sinuses with mucogelatinous exudate, dyspnea, coughing, pneumonia and inflammation of the air sacs.

Frequently CRD is complicated with other diseases that aggravate the severity of the infection and increase the mortality. These include:

Newcastle disease, infectious bronchitis and Escherichia coli infection (Stubbs et al 1954; Sullivan et al 1957; Gross 1958; Gross 1961; Adler et al 1962; Blake 1962; Fabricant and Levine 1962).

Early work suggested that chronic respiratory disease and turkey sinusitis were caused by a virus (Delaplane and Stuart 1943; Reagan et al 1951; Fahey and Crawley 1954a; Fahey 1956).

Nelson (1935, 1936a and b) reported the isolation of coccobacilliform bodies from flocks with fowl coryza. These coccobacilliform bodies were less than 0.5 μ in diameter and Gram negative. Nelson distinguished between infectious coryza of rapid and slow onset. Haemophilus gallinarum was invariably present with coryza of rapid onset with an incubation period of 1-2 days but with coryza of slow onset with an incubation period of 12 days or more, these coccobacilliform bodies were invariably present. Nelson (1936c) successfully cultivated the coccobacilliform bodies in fertile eggs and tissue culture. Nelson (1939) suggested that the coccobacilliform bodies were similar in size to the agent of bovine pleuro-pneumonia.

Smith et al (1948) studied Nelson's agent and found that it produced abundant growth when inoculated onto ascitic peptic digest plates and infusion broth enriched with 30% horse serum. Giemsa stained smears showed tiny round coccoid and minute rod shaped bodies. The appearance of the colonies, their habit of burrowing down into the agar, the size and morphology of the elements composing them and the reproduction by simple

fission and multipolar germination of large bodies led the authors to conclude that the strain of coccobacilliform bodies could properly be included in the group of pleuropneumonia-like-organisms (PPLO).

Delaphane (1949a and b) showed that the agents causing chronic respiratory disease in fowls and turkey sinusitis were identical.

Markham and Wong (1952) isolated micro-organisms essentially similar to those of the pleuropneumonia group from yolk sac suspensions of chick embryos inoculated with egg passage strains of the agents of chronic respiratory disease and turkey sinusitis. They used a beef heart infusion broth enriched with 20% horse serum. After 13 consecutive passages in artificial media, the agents induced typical swelling and exudates when injected into the infrorbital sinuses of turkeys and PPLO were isolated from the exudate from such birds. With Giemsa's stain, PPLO were demonstrated in sinus and tracheal exudates of field cases of turkey sinusitis and chronic respiratory disease.

Merck & Co., Inc. (1956) in a review summarized the position of CRD and turkey sinusitis thus: "the elucidation of its aetiology has been and still is in a state of flux, although it is generally agreed that the PPLO is the most likely cause. It is equally accepted that the causative agent is probably identical for both diseases."

Confusion arose because more than one species of the PPLO group were present in the respiratory tract of birds, some isolates being pathogenic and others not. In many investigations PPLO cultures produced a mild disease or none at all.

Chu (1954) isolated PPLO from fowls affected with a variety of diseases conditions as well as from apparently healthy fowls. He suggested that there were a number of different types of PPLO, of which some were sufficiently pathogenic to cause disease by themselves while others were not.

Gianforte et al (1955) examined seven different strains of PPLO from air sac infection in poultry. All strains possessed haemagglutinating properties, and tube agglutination tests indicated that the strains were identical. Van Herick and Eaton (1945) isolated from chick embryos an organism pleuropneumonialike in its characteristics with haemagglutinating properties during passages of the primary atypical pneumonia virus.

Adler et al (1957) found that two different serological and pathological types of PPLO were present in chicken and turkey tissues. The pathogenic strains were serologically related and could be distinguished from the non-pathogenic by agglutination, haemagglutination and ammonium sulphate flocculation procedures.

Yamamoto and Adler (1958) divided avian PPLO into 5 distinct antigenic types. All the pathogenic strains were antigenically related and were placed in one group.

It is now generally accepted that the disease entities, chronic respiratory disease in fowls and infectious sinusitis, are caused by a common agent, designated as one of the pleuropneumonia-like-organisms (Adler 1960; Edward and Kanarek 1960; Olesiuk and Van Roekel 1960b). Edward and Freundt (1956) suggested that the pleuropneumonia group should be called Mycoplasmatales. Edward and Kanarek (1960) proposed the name Mycoplasma gallisepticum for the agent responsible for CRD in fowls and infectious sinusitis in turkeys.

Fig. 1. Infectious sinusitis in turkeys, showing marked distension of the sinuses.

Transmission of CRD and infectious sinusitis is by contact and by airborne dust or droplets. Outbreaks of the disease are often started by carriers. Egg transmission of the agent has been demonstrated in turkeys (Mateney et al 1955; Jerstad 1956; Hofstad 1957; Richey et al 1958; Abbot et al 1960) and in fowls (Cover and Waller 1954; Fahey and Crawley 1954b; Van Roekel et al 1958; Fabricant et al 1959; Olesiuk and Van Roekel 1960a; Olson et al 1962). Olesiuk and Van Roekel (1960a) found that egg transmission may be of a higher rate in flocks that have had a recent outbreak of CRD than in flocks where CRD has subsided. Fabricant et al (1959) presented evidence that egg transmission is not due to infection of the reproductive tract as in pullorum disease. They suggest that egg infection is due to contamination of the ovules at the time they leave the follicle or in the infundibular portion of the oviduct. This contamination is made likely by the contiguity of the abdominal air sacs to the ovarian follicles and to the thin walled infundibular region of the oviduct.

The pathological changes in CRD and infectious sinusitis have been studied by Olesiuk and Van Roekel (1960b). The gross lesions consist primarily of catarrhal exudate in the nasal passages, trachea, bronchi and air sacs.

The infraorbital sinus mucosa is often oedematous and congested. The sinus cavity may contain clear or turbid mucous which often causes a marked distension of the wall in turkeys (Fig. 1). Complicating bacterial infections in the sinus may cause a purulent exudate. The mucosal thickening is due to infiltration with mononuclear cells and hyperplasia of the mucous glands. Focal areas of lymphoid hyperplasia are commonly

found. The sinus exudate contains mucocellular elements and epithelial fragments.

The gross pathology of the trachea, larynx, bronchi and nasal passages is similar to that observed in the sinuses. In the early stages of the disease the tracheal mucosa may reveal small raised follicles that present a roughened "beaded" appearance. The epithelial changes are characterised by the loss of cilia and elongation of the mucous glands.

In the lungs the gross findings are usually slight, but severe involvement usually is observed when the disease is complicated with other infections.

The air sac membranes in the early stage may reveal a slightly turbid and oedematous appearance that may be followed by increased vascularity. This is followed by a whitish yellow exudate covering the membranes. The microscopic pathology of the air sacs consists of focal proliferation of lymphocytes and an exudation consisting of fibrin, mononuclear cells, lymphocytes and heterophils.

Chronic respiratory disease in ducks was first described by Fahey (1955). The disease was characterised by depression, sore eyes, sneezing and head shaking. The gross pathological picture consisted of extensive pericardial and perihepatic exudation extending in some cases to the abdominal air sacs. A Mycoplasma antigenically distinct from M. gallisepticum and a virus was isolated. Dougherty and Fabricant (1953) have shown that the agent of CRD of fowls and turkey sinusitis will not cause chronic respiratory disease in ducks. Koppel et al (1956) described a mass infection in ducks characterised

by severe sinusitis where an influenza A virus was isolated. Influenza A viruses have been isolated from duck sinusitis by Simmins and Asplin (1956) and Tsimokh (1962).

Infectious synovitis is primarily a disease of broiler chickens. It was first reported in the United States by Wills (1954) and by Olson et al (1954) working independently. Later the disease was identified in turkeys (Snoeyenbos and Olesiuk 1955). The disease is observed most frequently in growing birds 4-10 weeks of age with low mortality but often a high morbidity rate. The clinical signs observed are those of lameness, retarded growth, breast bilsters and swelling of the joints. The hocks and feet are most frequently involved and in some birds the wing joints are affected. The birds become listless, dehydrated and emaciated. Post mortem examination revealed a grey to creamy yellow viscous exudate in the synovial membranes of the joints, tendon sheaths and bursal membranes of the keel. As the disease progresses the exudate becomes caseous. The infectious synovitis agent grows in chicken embryos and was thought to be a large virus or rickettsiae (Lecce et al 1955). Lecce (1960) reported the isolation of a Mycoplasma from allantoic fluid of infected embryos; this was followed by the work of Chalquest and Fabricant (1960) who identified the agent as a Mycoplasma

(B) Preliminary Investigations

Mycoplasma gallisepticum is the cause of chronic respiratory disease of fowls and infectious sinusitis of turkeys. The uncomplicated disease follows a definite clinical course, produces a typical pathological picture, and induces an antibody response which is detectable by serological means.

All the features can be reproduced by inoculating susceptible birds with M. gallisepticum.

Various procedures are used in the diagnosis of these diseases:

a) Culture

b) Serology

c) Egg propagation

d) Experimental transmission

Breed et al (1957) describes the Order Mycoplasmatales and Family Mycoplasmataceae as highly pleomorphic organisms which possess a peculiar mode of reproduction characterized according to some observers by the breaking up of filaments with more or less pronounced tendency to true branching into coccoid, filterable elementary bodies. The cell bodies are soft and fragile; without special precautions they are often distorted or entirely destroyed in microscopical preparation. They are non motile and Gram negative. Typical endospores are never produced. Growth occurs on media, although most of the species have exacting nutritional requirements.

The criteria for the identification of avian Mycoplasma are similar to those described for the Genus Mycoplasma (National Research Council 1959). These are:

1) Avian Mycoplasma will grow only in a medium containing enrichments such as serum or serum-like proteins.

2) Growth is usually fine, uniform and turbid in broth with no granular sediment after 3-6 days incubation at 37°C.

3) Numerous coccobacillary, coccoid bodies (125-500 mμ) and occasional rings, bipolar bodies visible in Giemsa stained preparations are found in broth culture.

4) Small characteristic (not exceeding 0.5 mm.) circular colonies are produced. The minute colonies have an opaque, granular, brown or yellowish central area growing down into the agar; the central area is surrounded by a raised translucent, smooth zone.

5) The same small typical colonies are maintained unchanged through many subcultures.

The choice of medium in the cultivation of avian Mycoplasma was difficult; none appeared to be completely satisfactory. Mycoplasma strains were obtained from Dr. H.P. Chu, School of Veterinary Medicine, Cambridge and from Dr. D.G.ff. Edward, Wellcome Research Laboratories, Beckenham. The strains obtained included M. gallisepticum and the nonpathogenic species M. iners and M. gallinarum (Edward and Kanarek 1960).

The media used and found reasonably satisfactory were:-

a) Difco PPLO agar or broth, plus

Horse serum 10%

Yeast autolysate (Albimi) 1%

Dextrose 0.1%

pH 7.8

Difco PPLO agar or broth has the following constituents per litre:-

Beef Heart, Infusion from 50 g.

Bacto-Peptone 10 g.

Sodium chloride 5 g.

b) Brucella agar or broth (Albimi Laboratories Inc.) enriched with 10% Horse serum with the pH adjusted to 7.0.

Brucella agar or broth has the following constituents per litre:-

Peptone "M"	20 g.
Dextrose C.P.	1 g.
Yeast autolysate	2 g.
Sodium chloride	5 g.
Sodium bisulphate	0.1 g.

To check bacterial contamination on primary isolation of the Mycoplasma, penicillin (1000 U/ml.) and thallium acetate (1:5000) were included in the media. The laboratory-adapted Mycoplasma strains all grew well on the above media.

The following procedures were used in the primary isolation of avian Mycoplasma from infected material. Exudate was taken from the sinuses, trachea and air sacs, and

1) Inoculated directly onto PPLO agar
2) Inoculated directly into PPLO broth
3) Diluted 1:3 in sterile broth containing 1000/ml. of penicillin

Six embryos 7 days old were each inoculated via the yolk sac route with 0.2 ml. of the exudate suspension using the method of Beveridge and Burnet (1946).

The PPLO agar plates were incubated aerobically in a moist atmosphere which was obtained by placing them in a closed vessel containing an exposed piece of moist cotton wool. Both the PPLO agar and PPLO broth were incubated at 37°C.

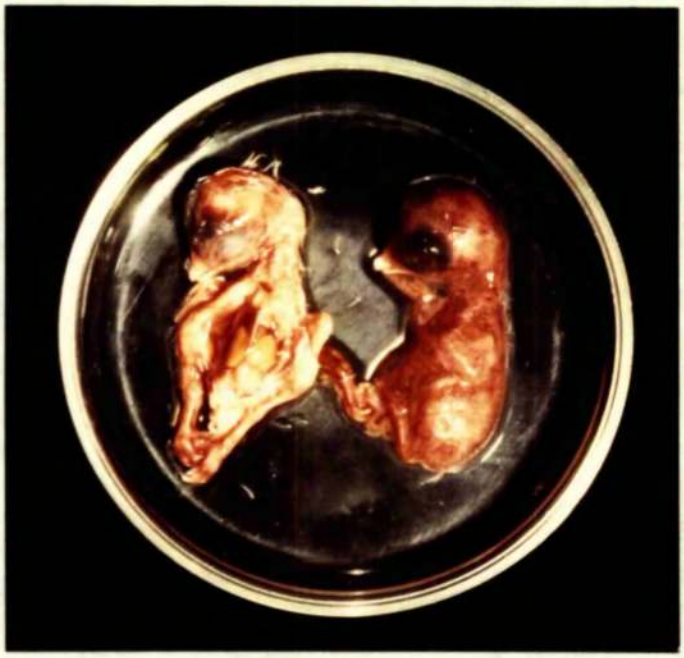


Fig. 2. Mycoplasma gallisepticum infected embryos, 11 days old.

The PPLO agar plates were examined daily for 5 days using a dissecting microscope (X 63 magnification). If mycoplasma colonies were not seen in 5 days, the PPLO broth was streaked onto PPLO agar and subcultured into a second PPLO broth. This blind passage procedure was repeated three times before a sample was determined negative and discarded.

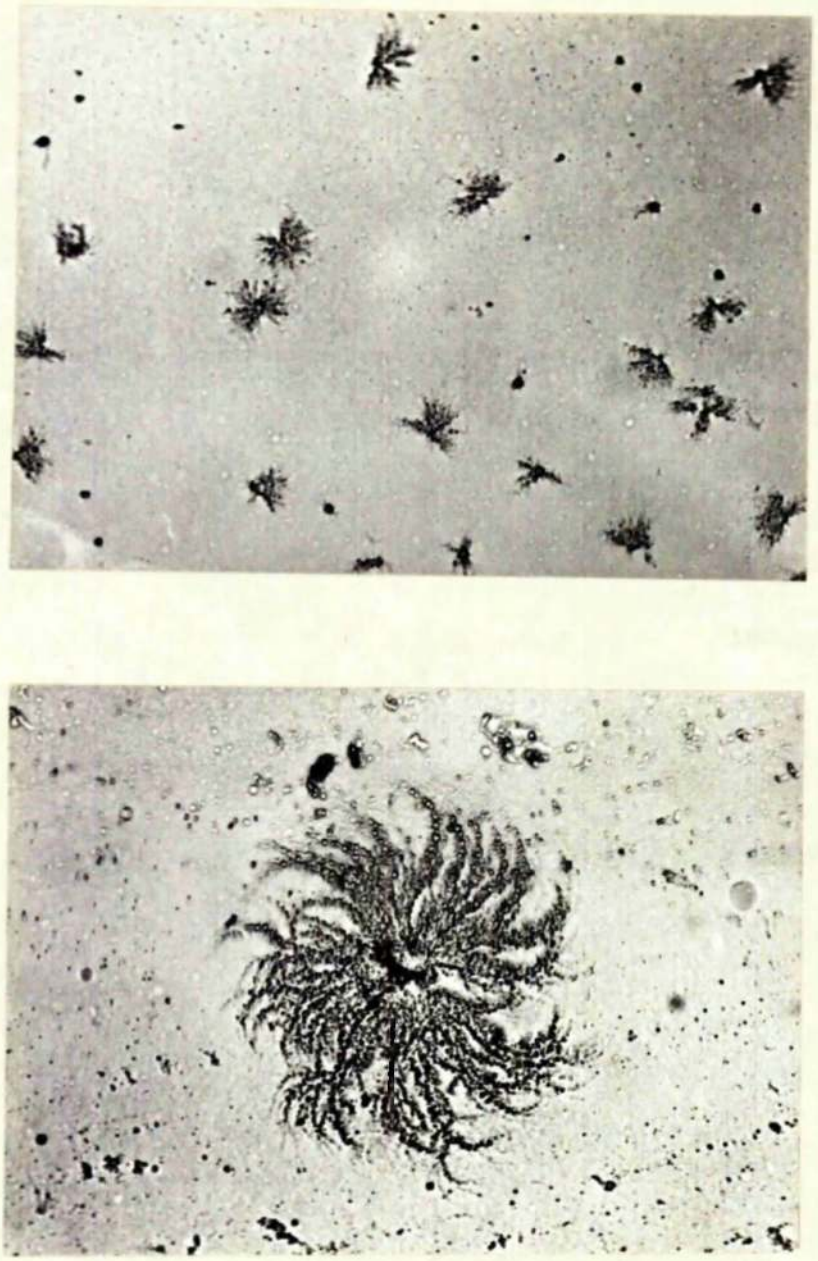
The chicken embryonated eggs used were obtained from a Mycoplasma gallisepticum free flock. All avian Mycoplasma strains grow rapidly in embryonated chicken eggs. The pathological response in chicken embryos to the pathogenic (M. gallisepticum) and nonpathogenic (non haemagglutinating) avian Mycoplasma was similar (Jungherr 1960). Mortality from avian Mycoplasma was at its highest about the 5th day after inoculation and continued erratically for the next few days of incubation. The gross pathology (Chute 1960) of the affected embryos consisted of stunting, oedema, congestion and haemorrhage (Fig. 2). Ecchymotic haemorrhages were noted frequently in the skin of the neck, head and ventral thoracic region. Congestion and necrosis with enlargement of kidneys and livers were frequently seen. Sparse feathering and thickened chorioallantoic membranes were common. If no embryonic deaths occurred by the 10th day following inoculation, yolk was harvested and inoculated into PPLO broth and onto PPLO agar.

When colonial growth was obtained and the colonies appeared to have the general characteristics of Mycoplasma, these colonies were examined under oil immersion using the stained agar technique of Dienes (1939). The method used was that described by Adler et al (1958).

The staining solution was made by dissolving 2.5 g. methylene blue, 1.25 g. azur II, 10 g. maltose, 0.25 g. Na_2CO_3 and 0.2 g. benzoic acid in 100 ml. distilled water. By means of a cotton swab, a thin film of staining solution was applied to coverslips and allowed to dry. Agar blocks containing the colonies were cut from the plate and placed colony side up on a slide and a stained cover slip was placed stain side down on the colonies. Staining was complete within a few minutes. In such preparations, colonies of Mycoplasma stained bright blue, with the centre of the colony staining more deeply than the periphery. Other bacterial colonies with the exception of Haemophilus gallinarum usually decolourized the stain (Adler et al 1958).

Subcultures were made into PPLO broth from single colonies by means of a Pasteur pipette drawn to a fine capillary point, or an ordinary loop. Growth in broth was usually fine, uniform, turbid with no deposit. Broth cultures were examined using the dark ground illumination method and the morphology of avian Mycoplasma was studied and stained by a modification of the method described by Goodburn and Marmion (1962):

1) Smears were prepared and allowed to dry in the air
2) The slide was flooded with methyl alcohol, 2 minutes were allowed for fixing.
3) The slide was washed with distilled water.
4) The slide was flooded with freshly prepared 1% solution of potassium permanganate for 2 minutes.
5) The slide was washed with distilled water.
6) The slide was placed face downwards overnight in a solution of 1:25 Giemsa's Stain (Improved Giemsa Stain, R66, George T. Gurr Ltd. London) in phosphate buffer pH 6.4.



a

b

Fig. 3. Pseudocolonies simulating those of Mycoplasma. x 155.

a) After 9 days incubation.

b) After 15 days incubation.

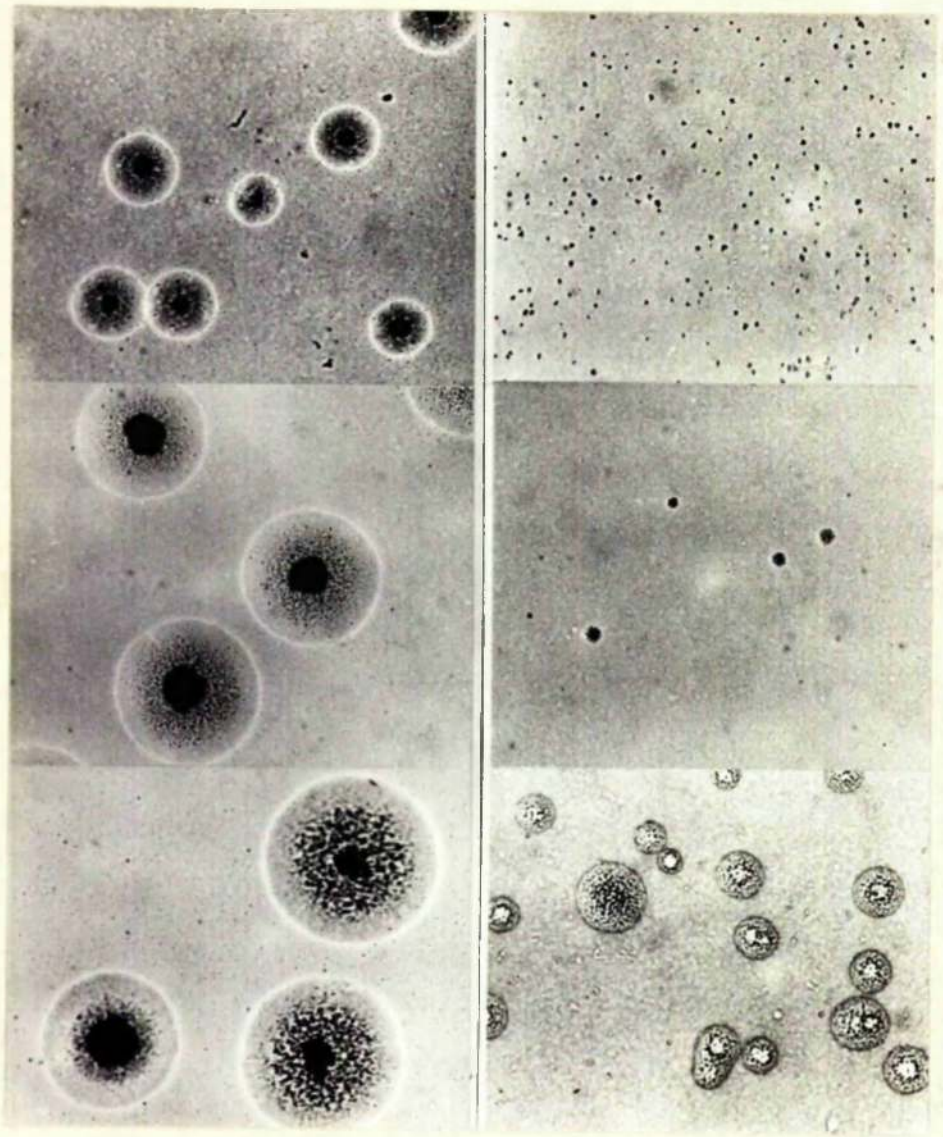
7) 0.5% acetic acid was used to differentiate, approximate time 20 seconds.

8) The slide was washed well in distilled water and allowed to dry in the air.

All avian Mycoplasma cultures isolated were passaged four times in non-inhibitory broth to test the possibility that the organisms were L-phase bacterial forms. McKay and Taylor (1954) and McKay and Truscott (1960 reported the reversion of cultures designated as avian Mycoplasma to an organism resembling Haemophilus gallinarum.

While attempts were being made to cultivate Mycoplasma from cases of CRD and turkey sinusitis pseudocolonies were encountered. These pseudo-colonies (Fig. 3) were similar to and could be confused with growth of filterable micro-organisms. Dr. E. Klieneberger-Nobel of the Lister Institute of Preventive Medicine, London was consulted and she drew attention to the work of Brown et al (1940) who studied similar colonies on horse serum agar while attempting to cultivate Mycoplasma from patients with rheumatic fever. These pseudocolonies began as globules on the surface of the PPLO agar and after a few days there developed around each globule granular filaments which radiated from the globule. Eventually a Catherine-Wheel-like appearance developed. Under cultural conditions, these pseudocolonies developed much more slowly than did those of Mycoplasma, a fully developed pseudocolony did not attain its maximum size for 10-15 days. Their macroscopic appearance on the surface of the media was that of a film like growth. When cultural techniques used in propagating Mycoplasma were applied, for instance, by

Fig. 4. Growth rate of Avian Mycoplasma. x 95.



cutting agar blocks containing pseudocolonies and smearing gently over a new plate, pseudocolonies multiplied to a remarkable degree (Fig. 3). These colonies have appeared on sterile plates after a sterile block of agar had been drawn over the surface of the medium. Brown et al (1940) showed that these pseudocolonies grew on horse serum agar containing formalin and merthiolate and were probably of a fatty nature as they stain typically with fat stains.

The isolation and identification of the Mycoplasma was followed by its characterization. It was essential to differentiate M. gallisepticum from the nonpathogenic strains. The following criteria were used:

	M.gallisepticum	Nonpathogenic Mycoplasma
Morphology (Stained preparation)	Coccoid and Coccobacillary	Very pleomorphic, with ring, bipolar, coccoid and coccobacillary forms
Rate of growth on PPLO agar (Fig.4)	Slow, colonies often less than 0.1 mm. after 72 hours incubation	Rapid, colonies often 0.5 mm. after 24 hours growth
Agglutination of chicken red blood cells	Positive	Negative
Pathogenicity	Pathogenic for chickens and turkeys	Fail to produce respiratory symptoms in chickens and turkeys
Serology	M.gallisepticum is antigenically distinct from the nonpathogenic types as revealed by: (a) Macroscopic serum plate agglutination test (b) Tube agglutination test (c) Haemagglutination-inhibition test	Nonpathogenic types are antigenically distinct from M.gallisepticum as revealed by:

(a)

(b)

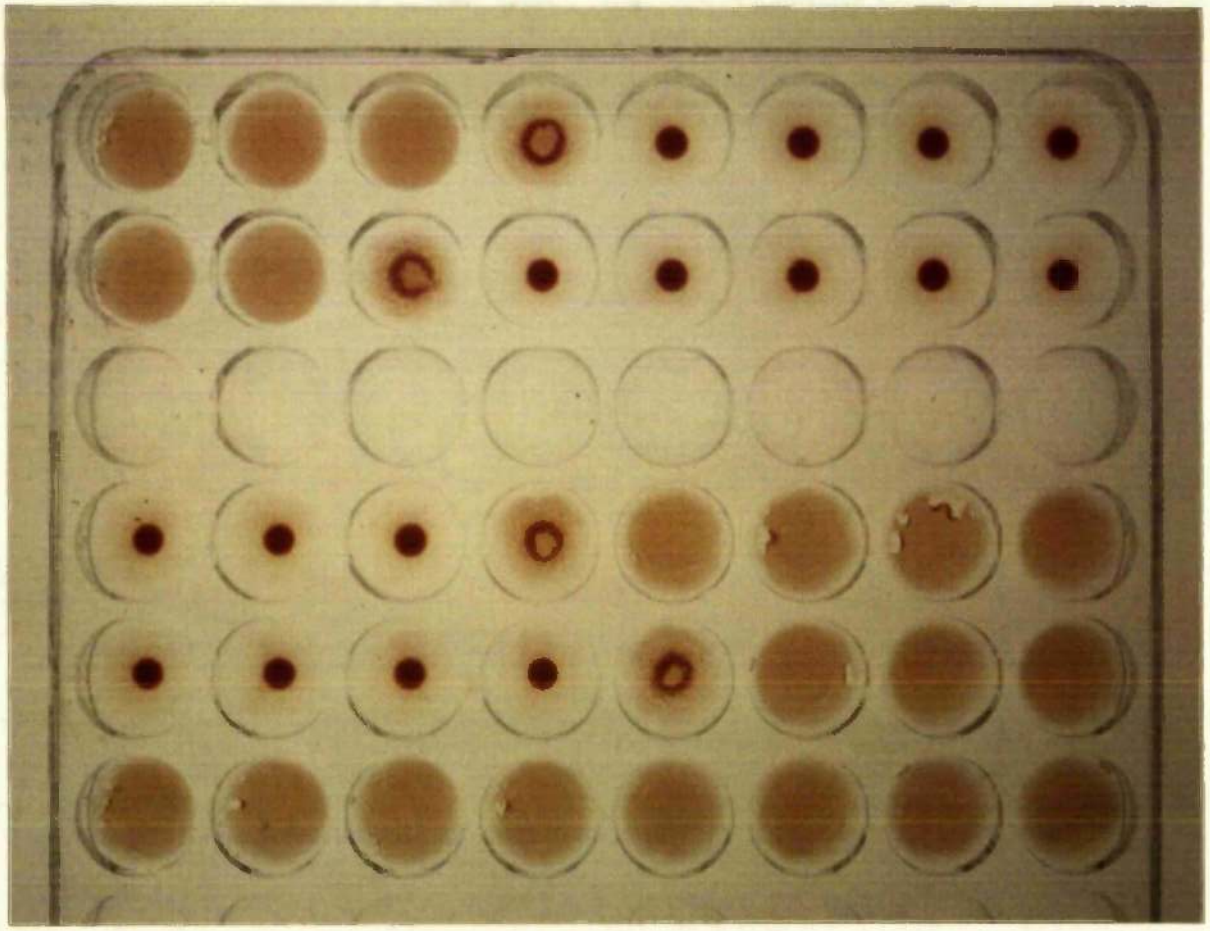


Fig. 5. Haemagglutination-inhibition Test.

a) Titration of antigen:

a.1. Titre of broth is 8 partial HA units

a.2. Titre of broth is 4 partial HA units

b) H.I. test: Serum dilutions begining 1/5 and using 4 partial HA units.

b.1. Positive serum, titre ++ 1/20, + 1/40

b.2. Positive serum, titre ++ 1/40, + 1/80

b.3. Negative serum sample.

Experimental transmission was one of the first procedures used in the diagnosis of the disease. Either yolk material harvested from embryos inoculated with a particular strain or broth cultures, was inoculated via the abdominal air sacs and infraorbital sinuses into 6 chicks or turkey poults. These birds which were 5-7 weeks old had been obtained from a M. gallisepticum free flock. Blood samples were taken at the time of inoculation. The birds were observed for 3 weeks and the presence or absence of clinical signs noted. Blood samples were taken again and the birds necropsied. The bloods were examined by either the haemagglutination-inhibition or agglutination test and an antibody response to M. gallisepticum was demonstrated.

Serological tests are the most rapid indicators of M. gallisepticum infection. These include:

1) Tube agglutination test (Jungherr et al 1953).
2) Haemagglutination-inhibition test (Fahey and Crawley 1954a) - Fig. 5
3) Serum slide agglutination test (Adler and Yamamoto 1956) - Fig. 6
4) Whole blood agglutination test (Aftosmis et al 1960).

The serological tests were used as a diagnostic aid and had to be supported by other procedures for accurate diagnosis of CRD and turkey sinusitis.

The most reliable method of isolating M. gallisepticum is by yolk sac inoculation of chick embryos (Fabricant 1958). All M. gallisepticum strains grow in embryonated chicken eggs (Adler 1962) but these strains are difficult to adapt to culture media. Mixtures of more than one type of Mycoplasma

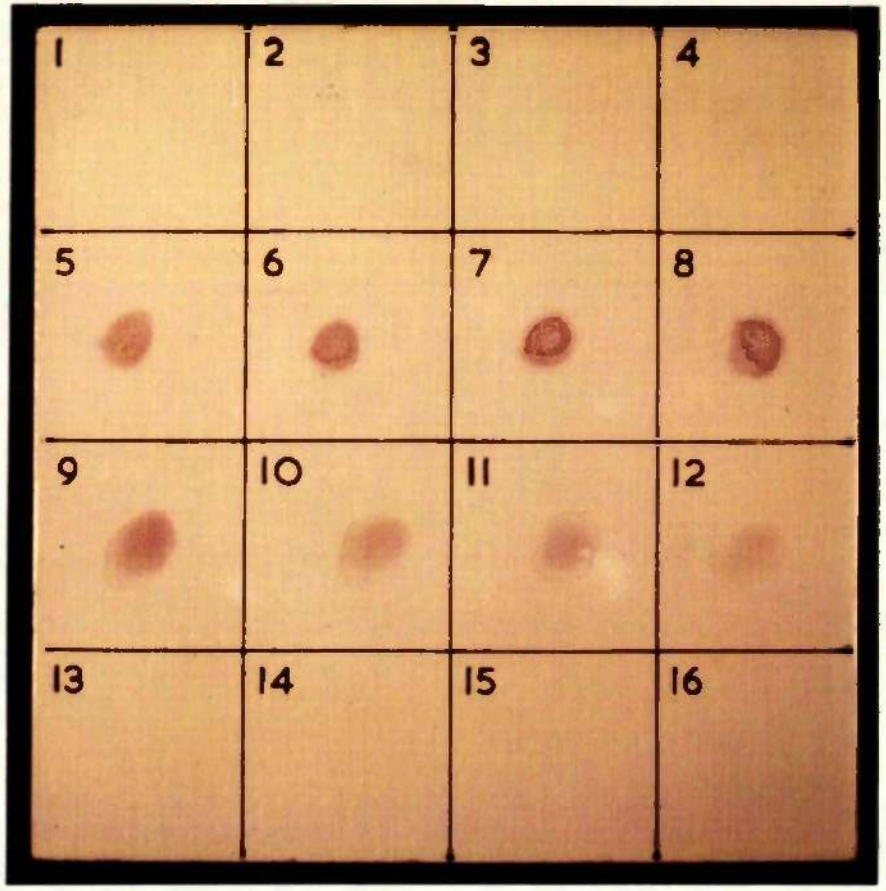


Fig. 6. Serum slide Agglutination Test. Drop of serum is mixed with a drop of crystal violet stained antigen. Plate is rotated and read within 2 minutes.
Top Row: Positive serum samples.
Bottom Row: Negative serum samples.

are often found in cases of CRD and turkey sinusitis, and the present cultural techniques favour the isolation of the nonpathogenic types. The nonpathogenic types grow rapidly and may completely cover the PPLO agar plate so that M. gallisepticum may not be detected.

In the absence of an isolation of M. gallisepticum a diagnosis of CRD or turkey sinusitis was based on a combination of history, symptoms, pathology and serological tests.

C. The Aims of the Investigations

Section 2 describes an outbreak of respiratory disease in turkeys where M. gallisepticum was not involved and from which a new Mycoplasma serotype was isolated. A survey was carried out to assess the incidence of Mycoplasma serotypes in poultry.

Section 3 describes an outbreak of sinusitis in ducks. An outbreak of sinusitis had occurred on the same farm in 1956 and an influenza A virus had been isolated, but the disease could not be reproduced in ducks with the virus. In 1962 another outbreak of sinusitis occurred on the same farm. The clinical picture presented was very similar to turkey sinusitis, with distension of the infra-orbital sinuses with the exudate at first gelatinous and later becoming caseous. The cause of the disease in turkeys is M. gallisepticum and in view of this attempts were made to isolate a Mycoplasma along with an influenza A virus.

Section 4 describes investigations into infectious synovitis. Recent reports in the U.S.A. indicate that the aetiological agent is a Mycoplasma. Four field outbreaks of the disease in chickens and one in turkeys were investigated in an attempt to isolate the agent.

The survey in section 2 suggested the high incidence of haemagglutinating inhibition antibodies in turkey sera to the WRI strain of Mycoplasma. The possibility that thes inhibitors are associated with a non specific reaction was investigated in Section 5.

Beta propio-lactone is used in this country in the preparation of Newcastle disease vaccines from infected embryonated chicken eggs. Egg transmission occurs with M. gallisepticum. Section 6 deals with the inactivation of Mycoplasma using beta propio-lactone because of the danger that might exist in the use of Newcastle disease vaccines prepared from eggs obtained from a M. gallisepticum infected flock.

Section 7 deals with the experimental infection of chickens with M. gallisepticum and subsequent re-isolation from the body tissues. The antibody response as determined by the haemagglutination-inhibition test, the clinical signs and lesions produced are described. Immunity to M. gallisepticum is discussed.

Section 8 describes the serological analysis of Mycoplasma strains isolated in this laboratory during investigations into outbreaks of respiratory disease in poultry.

SECTION (2)

The Isolation of a Previously Unreported Avian Mycoplasma Serotype and Some Observations on the Incidence of Mycoplasma in Poultry

Summary

A previously unreported avian Mycoplasma serotype designated as WRI is described. Results are given of a survey of avian blood samples to determine the incidence of WRI, S6 and Iowa 695 serotypes in certain areas of England and Wales as indicated by the Haemagglutination-inhibition test.

Introduction

Avian Mycoplasma have been subdivided into pathogenic and nonpathogenic types (Yamamoto and Adler, 1958; Chu and Newnham 1959). The typical avian pathogenic organisms designated as Mycoplasma gallisepticum by Edward and Kanarek (1960) and represented by the S6 serotype (Zander 1961), are characterized by their ability to agglutinate fowl red blood cells. An apparent exception to this generalization is the Iowa 695 strain (Yoder and Hofstad 1962), which agglutinates fowl red blood cells but is of doubtful pathogenicity.

The purpose of the first part of this paper is to report the isolation of a strain of Mycoplasma which agglutinates fowl red blood cells and is serologically distinct from both the S6 and Iowa 695 strains, and secondly to determine the incidence of WRI, S6 and Iowa 695 serotypes in certain areas of England and Wales as indicated by the H.I. test.

History

The original isolate of WRI was obtained from air sac lesions of adult Broad Breasted White turkeys which had been sent in for post mortem

examination. The turkeys were part of a breeding flock consisting of 8,000 birds in which there was considerable overcrowding in many of the units.

Widespread clinical air sac disease was evident throughout all sections of the flock. The birds were showing respiratory distress. Other features of the outbreak were the production of large numbers of soft shelled eggs and the presentation of nervous symptoms by a few of the birds e.g. torticollis and leg weakness. Egg production remained satisfactory, but there was a drop of 15 per cent in fertility and hatchability.

Post mortem examination revealed that the lesions were confined to the lower respiratory passages without involvement of the sinuses. The air sac membranes varied from slight cloudiness and oedema to pronounced thickening with a heavy coating of yellowish exudate.

Fifty blood samples were taken from the flock and all were negative to the haemagglutination-inhibition reaction with the S6 type antigen.

Materials and Methods

The culture media used were P.P.L.O. agar/or broth (Difco), enriched with 1% yeast autolysate (Albimi), 10% horse serum and 0.1% dextrose with the pH adjusted to 7.8. Fermentation reactions in the broth were determined by replacing the dextrose by 1% of other carbohydrates and adding phenol red as indicator.

Pathogenicity tests were carried out in 4-5 weeks old Rhode Island Red chickens and Broad Breasted White turkeys inoculated into the infra-orbital sinuses and abdominal air sacs with 0.5 ml. of a 24-hour broth culture. The birds were killed three weeks later and examined for lesions.

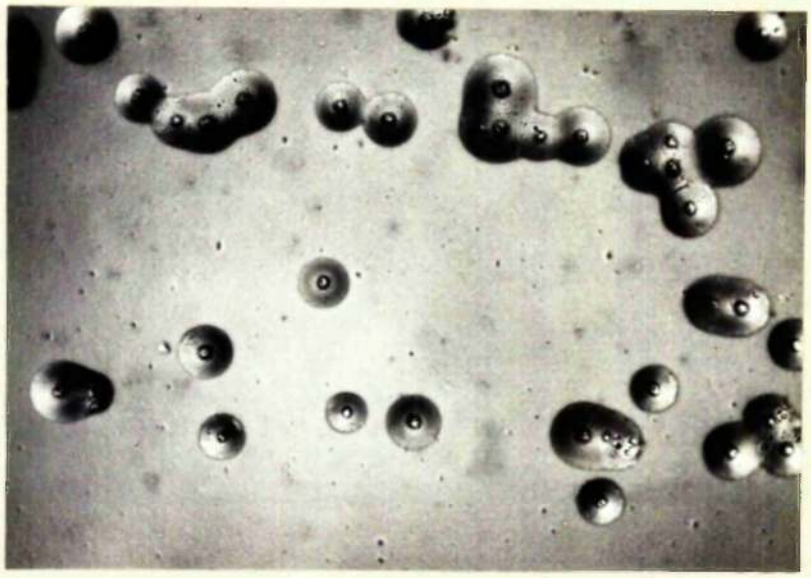


Fig. 1. Colonial appearance of WR1 after 24 hours incubation. X 63.

Specific immune serum was prepared in rabbits against S6, Iowa 695 and the WRI strains of avian Mycoplasma. Two hundred ml. of PPLO broth were inoculated with 10 ml. of stock culture and incubated at 37°C for 72 hours. The culture was then centrifuged at 3,500 r.p.m. for 30 minutes; the supernate was decanted, and the broth sediment was resuspended in physiological saline. The cells were washed 3 times and finally resuspended in 12 ml. physiological saline and stored at -10°C in 2 ml. amounts. Rabbits were given 6 intravenous injections of 0.5-1.0 ml. of the antigen over a period of 14 days and were bled out 7 days after the last injection.

Serum titres and cross agglutination titres were determined by tube agglutination with the strains mentioned above, using as antigen a 24 hour broth culture. All antigens were standardized for turbidity with the nephelometer using sterile broth as diluent. The rabbit sera were diluted with physiological saline by twofold dilutions starting 1/4; 0.3 ml. of antigen was added to 0.3 ml. amounts of serum dilutions. The tubes were shaken and incubated in a water bath at 56°C for 24 hours. Controls were run with each test.

Results

Cultural and Morphological Characteristics Isolates of WRI produce within 24 hours colonies showing the typical fried egg appearance characteristic of the so-called non-pathogenic strain of avian Mycoplasma on PPLO agar (Fig. 1).

Smears made from cultures and stained by Giemsa's method show pleomorphic organisms in the form of rings, bipolar bodies, coccoid and coccobacillary forms. Colonies stain readily (Fig. 2) using a modified Dienes method (Adler et al 1958).

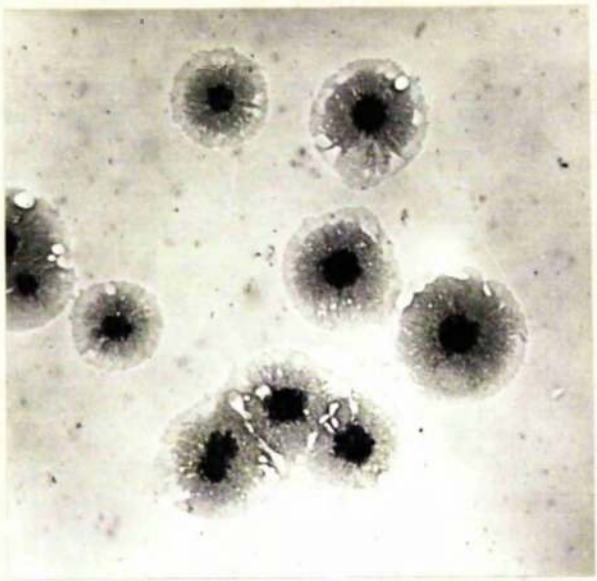


Fig. 2. WR1 colonies stained by modified Dienes method(Adler et al 1958). X 63.

Other features of WRI are:

a) Growth occurs on sheep blood agar but neither on nutrient agar nor on Difco PPLO agar minus horse serum and Yeast autolysate.

b) Broth cultures agglutinate fowl red blood cells.

c) Haemolysis of both sheep and fowl red blood cells occurs when these cells are incorporated into PPLO agar medium.

d) Smooth growth is produced in PPLO broth.

e) Growth is not inhibited by 2000 units of Penicillin per ml.

f) Growth occurs at 30°C and 37°C but not at 25°C and 44°C.

Biochemical reactions

The strain ferments glucose, maltose, dextrin, starch and sucrose to produce acid only. No acid was produced when lactose, levulose, mannose and galactose were used in the media.

Pathogenicity Studies

Sinusitis was not observed in any of the turkeys inoculated. Four of the 20 turkey poults inoculated showed moderate lesions of air sacculitis and the WRI strain was isolated from the air sacs of these four birds. No gross lesions were seen in any of the chickens inoculated with the culture.

The results of the tube agglutination tests using specific immune sera are summarized in table 1.

Table 1 **Tube Agglutination Studies**

	Antisera		
Antigens	S6	Iowa 695	WRI
S6	1/256	<1/4	<1/4
Iowa 695	<1/4	1/256	<1/4
WRI	<1/4	<1/4	1/9,600

Incidence of antibodies in Poultry sera to S6, WRI, and Iowa 695 serotypes as determined by the H. I. test

Blood samples are received at the Central Veterinary Laboratory from poultry flocks in various parts of England and Wales for testing for Newcastle disease antibodies. Sera from these bloods were also tested for Mycoplasma antibodies, by means of the Haemagglutination-inhibition test. Initially two antigens were used, S6 and WRI, but at a later stage when Iowa 695 became available, bloods from a small number of flocks were tested against this antigen also.

The Haemagglutination-inhibition test was carried out by the following method:

a) The antigen used was a 24 hour broth culture of Mycoplasma. Serial two-fold dilutions were made in saline, using 0.25 ml. volumes to which 0.25 ml. of a 1% suspension of washed fowl red blood cells were added, followed by 0.25 ml. of saline.

b) Readings were made after the plates had been left at room temperature for 50 minutes. Fifty per cent haemagglutination was regarded as the end point and at this titre one partial haemagglutinating dose of Mycoplasma was contained in 0.25 ml.

c) Stock mycoplasma suspensions were prepared by dilution of broth cultures in physiological saline. The antigens S6 and Iowa 695 contained 4 partial H.A. units in 0.25 ml., but the WRI antigen contained 8 partial H.A. units in 0.25 ml. to minimise the possibility of a non-specific reaction.

d) Serial twofold dilutions of serum commencing 1/10 were then made; to 0.25 ml. of each dilution was added 0.25 ml. of the stock suspension. The mixture was allowed to stand for a few minutes, after which 0.25 ml. of a 1% suspension of washed fowl red blood cells were added.

e) The plates were shaken and then left at room temperature and read after 50 minutes.

Any serum causing complete inhibition at 1/10 was recorded as positive (Crawley 1960).

The samples examined do not comprise a random sample as they were collected from flocks under investigation for Newcastle disease. Although they had been derived from flocks in 29 counties in England, and 4 in Wales, the majority had originated from an area in Eastern England where Newcastle disease was prevalent at the time. Some of the samples had come from premises adjoining poultry farms where Newcastle disease was suspected.

The number of samples examined varied from 1 to 50 with an average of 11 samples per flock. It was notable that when a positive reaction to the S6 antigen occurred in turkeys and chickens, at least 70% of the samples from that flock were positive. In turkey flocks in which a positive reaction to WRI antigen was obtained, the incidence was often as high as 90%. By contrast in chicken flocks showing a positive reaction to WRI, the percentage of bloods showing a reaction was only 27%.

The H. I. titre of the WRI positive serum samples and the S6 positive serum samples varied from 1/10 to 1/80. With both chicken and turkey sera, partial inhibition of haemagglutination was noted frequently when the WRI antigen was used. All flocks which showed a positive H. I. titre to Mycoplasma reacted either to the WRI antigen or to the S6 antigen; there was no case of a positive reaction to both antigens with the same sample.

The results of the tests with S6, WRI and Iowa 695 antigens are summarised in Tables 2 - 10.

Discussion

A new serotype of avian Mycoplasma designated WRI is characterised by properties similar to both the pathogenic and non pathogenic types. In limited experimental studies it does not appear to be particularly pathogenic but on present evidence it is more so for turkeys than fowls. Despite its relative lack of pathogenicity it does stimulate an antibody response in both species.

The survey indicates a high incidence of WRI antibodies in turkeys (37.3%) compared with 1.5% in chickens. The incidence of S6 antibodies in turkeys (4.9%) is much lower than might be expected in view of the large

number of cases of air sacculitis in young turkey poults that are sent to this laboratory for post mortem examination.

There was no significant difference in the incidence of S6 antibodies in chickens from Newcastle disease free flocks (23.8%) from those in Newcastle disease infected flocks (26.25%), nor was there any marked difference in the occurrence of WRI antibodies in Newcastle disease infected and non-infected poultry flocks. The small number of samples from Newcastle disease positive turkey flocks is a limiting factor in assessing the relevance of these results.

Blood samples from flocks of geese and ducks did not show any reaction to any of the antigens.

The incidence of H. I. antibodies to the Iowa 695 strain in turkeys and chickens was nil.

The experimental results indicate that WRI is pathogenic for turkeys but not for fowls. Nevertheless the results of the survey indicate that a small percentage of fowls possess antibody against this serotype but it is not known whether this resulted from clinical, subclinical disease, or if it was a non-specific reaction.

The properties of WRI particularly in relation to its cultural and morphological features are very similar to those ascribed to avian non pathogenic Mycoplasma. The N. strain (Adler et al, 1958) is another non pathogenic type that produces air sac lesions in turkeys, but it differs from WRI in that it neither agglutinates fowl red blood cells nor ferment sugars. It is suggested that the arbitary division of avian Mycoplasma into pathogenic and non-pathogenic types by virtue of their cultural, morphological and serological characteristics is not a reliable guide. Certain strains which

might be classified on the above criteria as non pathogens, may in fact be pathogenic for turkeys and possibly but to a lesser extent, for fowls.

Table 2

Incidence of H. I. antibodies to the S6 and WRI strains

Flock status as indicated by Newcastle disease H. I. test	No of Premises	No. of samples S6	No. of Premises with samples positive to S6	No. of premises with samples positive to WRI
FOWLS: Newcaslte disease-NEGATIVE	1038	9246	247(23.8%)	12(1.15%)
FOWLS: Newcastle disease-POSITIVE	259	3225	68(26.25%)	7(2.7%)
TURKEYS: Newcastle disease-NEGATIVE	62	896	3(4.8%)	22(35.5%)
TURKEYS: Newcastle disease-POSITIVE	21	783	1(4.8%)	9(42.8%)
DUCKS AND GEESE: Newcastle disease NEGATIVE	27	231	Nil	Nil

Table 3

Incidence of H. I. antibodies to the Iowa 695 strain

	No. of Premises	No. of Samples	Incidence of Iowa 695
Fowls	148	2121	Nil
Turkeys	18	306	Nil

Table 4

Geographical incidence of H.I. antibodies to S6 and WRI strains in NEWCASTLE DISEASE infected flocks : FOWLS

	Number of premises	Number of samples	Number of premises with samples positive to S6	Number of premises with samples positive to WRI	Flock incidence of S6	Flock incidence of WRI
Norfolk	126	1526	19	5	15.08%	3.97%
Suffolk	46	536	15	1	32.61%	2.17%
Lincolnshire	46	557	19	1	41.30%	2.17%
Warwickshire	14	238	6	-	43.0%	-
Essex	8	91	1	-		
Hants	6	98	2	-		
Sussex	4	69	1	-		
Oxfordshire	1	8	-	-		
Wiltshire	2	32	1	-		
Lancashire	2	20	2	-		
Dorset	1	12	-	-		
Leicestershire	1	12	1	-		
Kent	1	6	1	-		
Glamorgan	1	20	-	-		
	259	3225	68	7	26.25%	2.70%

Geographical incidence of H.I. antibodies to S6 and WRI strains in Newcastle Disease free flocks : FOWLS

	Number of premises	Number of samples	Number of premises with samples positive to S6	Number of premises with samples positive to WRI	Flock incidence of S6	Flock incidence of WRI
Norfolk	190	1798	30	5	15.8%	2.6%
Suffolk	169	1512	31	2	18.3%	1.18%
Lincolnshire	307	2230	86	2	28.0%	0.65%
Warwick	64	507	17	-	26.56%	
Kent	48	446	17	-	35.4%	
Hants.	38	470	9	2	23.7%	5.26%
Essex	46	297	7	-	15.2%	
Wiltshire	23	234	5	-	21.7%	
Leicestershire	18	155	8	1	44.4%	5.56%
Lancashire	14	117	4	-	29.0%	
Hunts	17	115	9	-	53.0%	
Dorset	8	131	-	-		
Sussex	10	113	4	-		
Northants	11	118	2	-		
Cambridge	19	101	4	-		
Berkshire	6	83	3	-		
Devon	4	87	-	-		
Staffordshire	4	56	2	-		
Gloucestershire	2	16	-	-		
Worcestershire	2	12	1	-		
Cornwall	1	10	-	-		
Cheshire	1	50	-	-		
Oxford	1	12	-	-		
Hereford	3	68	1	-		
Nottinghamshire	1	9	1	-		
Bedfordshire	2	16	-	-		
Derbyshire	3	35	-	-		
Hertfordshire	4	84	2	-		
Surrey	5	61	1	-		
Glamorgan	9	56	1	-		
Flintshire	1	7	-	-		
Carmarthen	1	8	1	-		
Montgomery	3	32	1	-		
Ireland	3	200	-	-		
	1038	9246	247	12	23.79%	1.15%

Table 6

Geographical incidence of H.I. antibodies to S6 and WRI strains in Newcastle Diseases free flocks : TURKEYS

	Number of premises	Number of samples	Number of premises with samples positive to S6	Number of premises with samples positive to WRI	Flock incidence of S6	Flock incidence of WRI
Kent	2	14	-	-		
Suffolk	14	201	2	3		
Northants	11	193	-	2		
Cheshire	2	22	-	-		
Essex	2	7	-	-		
Norfolk	21	311	1	12		
Lincs	2	23	-	1		
Hants	3	65	-	2		
Surrey	1	6	-	1		
Sussex	1	20	-	-		
Warwick	2	14	-	1		
Worcester	1	20	-	-		
	62	896	3	22	4.84%	35.5%

Table 7

Geographical incidence of H.I. antibodies to S6 and WRI strains in Newcastle Disease infected flocks : TURKEYS

	Number of premises	Number of samples	Number of premises with samples positive to S6	Number of premises with samples positive to WRI	Flock incidence of S6	Flock incidence of WRI
Suffolk	9	310	1	6		
Hants	2	50	-	1		
Norfolk	8	308	-	1		
Northants	1	95	-	1		
Gloucester	1	20	-	-		
	21	783	1	9	4.76%	42.85%

Table 8

Examination of sera from Ducks and Geese

	Number of premises	Number of samples	Number of premises with samples positive to S6	Number of premises with samples positive to WRI	Flock incidence of S6	Flock incidence of WRI
Suffolk	7	87	-	-		
Essex	2	6	-	-		
Northants	2	4	-	-		
Norfolk	5	84	-	-		
Warwick	3	7	-	-		
Gloucester	2	8	-	-		
Lincolnshire	4	11	-	-		
Staffordshire	1	20	-	-		
Devon	1	4	-	-		
	27	231	-	-	0	0

Table 9

Source of Fowl sera tested against Iowa 695 strain

	No. of Premises	No. of Samples	Flock status as indicated by Newcastle Diseases H.I. test		Flock incidence of Iowa 695
			H.I. results positive	H.I. results negative	
Essex	21	215	12	9	-
Sussex	2	22	-	2	-
Lincolnshire	6	96	1	5	-
Norfolk	55	768	28	27	-
Suffolk	13	145	3	10	-
Lancashire	20	297	10	10	-
Hampshire	1	50	-	1	-
Wiltshire	11	131	1	10	-
Warwickshire	2	10	1	1	-
Shropshire	3	36	1	2	-
Bedfordshire	1	4	1	-	-
Gloucestershire	1	12	-	1	-
Leicestershire	1	10	1	-	-
Cambridgeshire	2	40	2	-	-
Ireland	1	200	-	1	-
Cheshire	5	54	3	2	-
Derby	1	6	-	1	-
Staffordshire	1	5	-	1	-
Kent	1	20	-	1	-
	148	2121	64	84	Nil

Table 10

Source of Turkey sera tested against Iowa 695 strain

	No. of Premises	No. of Samples	Flock status as indicated by Newcastle Diseases H.I. test		Flock incidence of Iowa 695
			H.I. results positive	H. I. results negative	
Essex	3	52	2	1	-
Norfolk	7	145	2	5	-
Shropshire	2	24	-	2	-
Staffordshire	1	24	-	1	-
Surrey	1	10	1	-	-
Cheshire	1	20	-	1	-
Hampshire	1	10	-	1	-
Kent	1	10	-	1	-
Oxfordshire	1	11	-	1	-
	18	306	5	13	Nil

Section 3

The Isolation of an Influenza A Virus and a Mycoplasma associated with Duck Sinusitis

Summary

An outbreak of Duck Sinusitis in 1962 is described. An earlier outbreak had occurred on the same farm in 1956, where an influenza A virus was isolated. In the second outbreak in 1962, two agents were isolated, a Mycoplasma designated Mycoplasma anatis (Weybridge) and an influenza A virus serologically distinct from the virus isolated in 1956.

Introduction

Chronic respiratory disease in ducks was first described by Fahey (1955). His findings indicated the presence of two distinct entities, these being a virus and a Mycoplasma. The Mycoplasma was antigenically distinct from M. gallisepticum.

Koppel et al (1956) described a fatal mass infection in ducks aged 10-21 days, characterized by severe sinusitis with involvement of the lower respiratory passages in some; 1250 ducklings out of a total of 3,000 succumbed to the disease. A virus which belonged to the influenza A group was isolated. In 8 out of the 13 cases examined, Salmonella typhimurium was isolated from the parenchymatous organs.

Tsimokh (1962) describes three outbreaks of duck sinusitis in the Ukrain where three serologically related influenza A viruses were isolated.

History

Two outbreaks of duck sinusitis (chronic respiratory disease) have occurred on the same farm. The first outbreak occurred in 1956 and the

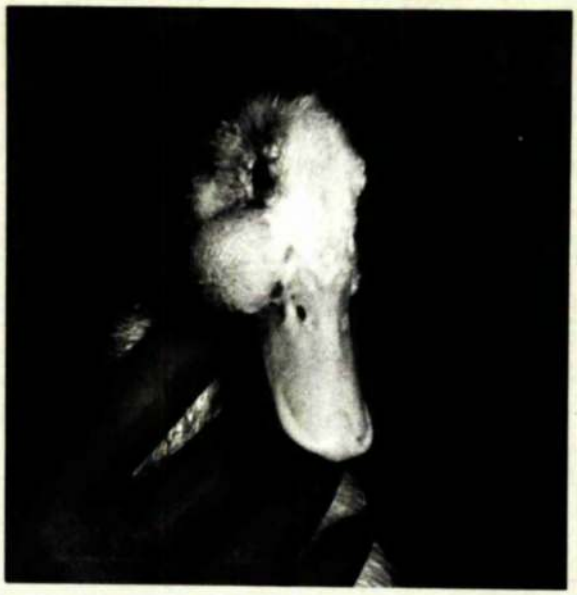


Fig. 1. Field cases of duck sinusitis showing distended sinuses.

second in 1962. In the 1956 outbreak Simmins and Asplin (1956) isolated a Myxovirus belonging to the influenza group A. This report deals with the second outbreak in 1962 where two agents were found to be involved; another influenza group A virus and a Mycoplasma. In 1956 no attempt had been made to isolate a Mycoplasma.

Widespread clinical disease was evident throughout all sections of the flock; the birds showed respiratory distress and their sinuses were distended (Fig. 1); some birds were shaking their heads.

Post mortem examination revealed that the sinuses were swollen with a mucoid-gelatinous exudate which had become rather caseous in some birds. The air sac membranes appeared cloudy and oedematous.

Despite the high morbidity rate, the mortality rate was very low and the affected birds recovered without any treatment.

Blood samples taken from the flock, were all negative in the haemagglutination inhibition reaction with the S6 type Mycoplasma antigen.

Materials and Methods used in the Isolation and Characterization of the Mycoplasma

The culture media used were PPLO agar or broth (Difco), enriched with 1% yeast autolysate (Albimi), 10% horse serum and 0.1% dextrose with the pH adjusted to 7.8. Fermentation reactions in the broth were determined by replacing the dextrose by 1% of other carbohydrates and adding phenol red as indicator.

Pathogenicity tests were carried out with Khaki Campbell ducklings.

Specific immune serum was prepared in rabbits (see Section 2) against M. anatis (Weybridge) and other haemagglutinating avian Mycoplasma, which

included the S6 strain of M. gallisepticum (Zander 1961), Iowa 695 strain (Yoder and Hofstad 1962) and WRI strain (Section 2). Serum titres and cross agglutination titres were determined with the strains mentioned above by the method described in Section 2.

Results

Material from the sinuses and air sac membranes was inoculated into PPLO broth which contained penicillin (1,000 U./ml.) and thallium acetate (1/5000).

A smooth, fine uniform and turbid growth was produced in the broth. There was no granular sediment. The broth was subcultured on to PPLO agar, and within 24 hours colonies showing the typical fried egg appearance were produced, characteristic of the so called non-pathogenic strain of avian Mycoplasma, as described by Chu and Newnham (1959).

The colonies stained readily using a modified Dienes method (Adler et al 1958). Smears, made from cultures and stained by Giemsa's method, showed coccoid bodies and occasional ring forms. The coccoid bodies appeared larger than those of the S6 type cultures.

Other features of the Mycoplasma are:

(a) Growth occurred on sheep blood agar with a zone of haemolysis around the colonies. Growth did not occur on nutrient agar.

(b) Broth cultures agglutinated duck red blood cells but not fowl red blood cells.

(c) Growth was not inhibited by 2,000 units of penicillin per ml.,

(d) Growth occurred at 30°C and 37°C but not at 25°C and 44°C.

Biochemical Reactions

The Mycoplasma fermented maltose, levulose, dextrin and starch to produce acid only. Glucose and mannose were weakly fermented. No acid was produced when sucrose, lactose and galactose were used in the media.

Pathogenicity Tests

One, two and three week old ducklings were inoculated via the infra-orbital sinuses with 0.2 ml. of a 24 hour broth culture of the Mycoplasma. No gross lesions were seen in any of the birds killed 3-4 weeks later. Serum samples from the ducklings were negative when tested by the H.I. test. The method used in carrying out the H.I. test was similar to that described in Section 2. The antigen used was a 24 hour broth culture of the Mycoplasma. The HA titre of the broth was 4 units.

A 1% suspension of washed duck red blood cells was used.

The Mycoplasma was designated Mycoplasma anatis (Weybridge).

The results of the tube agglutination tests using specific immune sera are summarised in Table 1.

Table 1

Tube agglutination studies

	Antisera			
Antigens	M.anatis (Weybridge)	S6	Iowa 695	WRI
M.anatis (Weybridge)	1/128	<1/4	<1/4	<1/4
S6	<1/4	1/256	<1/4	<1/4
Iowa 695	<1/4	<1/4	1/256	<1/4
WRI	<1/4	<1/4	<1/4	1/9,600

Materials and Methods used in the Isolation of the Influenza A Viruses

Embryonated chicken eggs were used in the primary isolation. Pathogenicity tests were carried out with young Rhode Island Red chickens and Khaki Campbell ducklings. All eggs, ducks and chicks used came from CRD free flocks.

Specific immune serum to the duck influenza viruses was prepared in 12 week old Rhode Island Red chickens. The antigen, consisting of 0.5 ml. allantoic fluid containing live duck influenza virus, was inoculated into the sinuses of these birds and the birds were bled 2-3 weeks later.

The haemagglutination-inhibition test was carried out by the following method:-

1. Serial twofold dilutions of the virus were made in saline using 0.25 ml. volumes, 0.25 ml. of a 1% suspension of washed fowl red blood cells were then added, followed by 0.25 ml. saline.
2. Readings were made after the plates had been left at room temperature for 50 minutes. 50% haemagglutination was regarded as the end point and at this titre one partial haemagglutinating dose of virus was contained in 0.25 ml.
3. A stock virus suspension containing 8 partial haemagglutinating doses in 0.25 ml. was prepared.
4. Serial twofold dilutions of serum were then made: to 0.25 ml. of each dilution was added 0.25 ml. virus suspension containing 8 partial haemagglutinating doses. To this was added 0.25 ml. of a 1% suspension of washed fowl red blood cells.
5. The plates were left at room temperature and read after 50 minutes.

Results

Sinus material and air sac material were suspended in broth containing penicillin (1000 U./ml.). Each suspension was inoculated into 7 day old embryos, 0.2 ml. each, via the yolk sac. The embryos were incubated at 37°C.

The embryos inoculated with the sinus material died within 24 hours. Yolk sac fluid and allantoic fluid were harvested. The allantoic fluid was found to agglutinate a 1% suspension of fowl red blood cells to a titre of 1/512. When yolk sac fluid and allantoic fluid were inoculated into PPLO broth, a Mycoplasma was isolated from the broth. The Mycoplasma did not haemagglutinate fowl red blood cells.

The allantoic fluid containing the haemagglutinating virus and Mycoplasma was filtered through a Gradacol membrane, with a maximum pore size of 120 mμ. The filtrate was then inoculated into broth and into 9 day old embryos by the allantoic route, using 0.1 ml. amounts. Mycoplasma was not isolated from the filtrate. The embryos were candled daily and started dying two days post inoculation and all were dead by the 4th day. The allantoic fluid again agglutinated a 1% suspension of fowl red blood cells to a very high titre (1/512).

The embryos inoculated with the suspension of air sac material did not die. They were killed after 7 days incubation: their allantoic fluid did not agglutinate fowl red blood cells and Mycoplasma was neither isolated from yolk sac fluid nor from the allantoic fluid.

Blood samples taken from the infected ducks 2 weeks post inoculation and tested by the H.I. test using the influenza virus, were all positive with titres of from 1/5 to 1/20. None of the bloods reacted with the virus

isolated in 1956.

Both the viruses stimulated an antibody response in chickens, but no gross lesions were seen.

The results of the haemagglutination-inhibition test using specific immune sera prepared in chickens against the two viruses are summarised in table 2.

Table 2

Antisera	Duck influenza virus 1956 H.I. titre	Duck influenza virus 1962 H.I. titre
Duck influenza virus 1956	1/20	-
Duck influenza virus 1962	-	1/20

Both the viruses isolated were sent to Dr. H. G. Pereira at the National Institute of Medical Research, London. The complement fixation test indicated that both belong to the influenza A group. Dr. Pereira did haemagglutination-inhibition tests against a variety of influenza A antisera and these are summarised in table 3.

Duck/Czech./56 refers to the duck influenza virus isolated by Koppel et al (1956); F/Scot./60 refers to an influenza strain isolated by Wilson (1960) from fowls suffering from a fowl plague like condition in Scotland. Duck/England/56 and Duck/England/62 refers to the viruses in this paper. The remainder of the influenza strains are well known and need no clarification.

The results suggest that Duck/England/56 and Duck/England/62 are quite distinct from each other and quite distinct from any other strain so far isolated.

Pathogenicity tests

Three week old ducklings were inoculated via the infra-orbital sinuses with 0.5 ml. allantoic fluid containing live duck influenza virus (Duck/Eng/62). No gross lesions were seen in any of the birds killed 3 weeks later, but an antibody response as indicated by the H. I. test was stimulated. The titres varied from 1/10 to 1/40.

Attempts were made to reproduce clinical disease as seen in the field by inoculating 3 week old ducklings via the infra-orbital sinuses with:-

a) A mixture of influenza A virus and M. anatis

b) Influenza A virus followed 3 days later by inoculation with M.anatis.

c) M. anatis followed 14 days later by inoculation with influenza A virus.

The disease as seen in the field was not successfully reproduced. With method (c), however, the birds showed signs of respiratory distress 2-3 days after inoculation with influenza A virus. Sera from these birds gave a positive reaction 2-3 weeks later as indicated by the H.I. test to M.anatis. The titres varied from 1/10 to 1/40.

Discussion

Antigenic variation is highly characteristic of the influenza A group and the process is one of a continuing evolution of new serotypes. In this respect the duck influenza A viruses isolated resemble human influenza A viruses. Both the duck influenza viruses belong to group A but are

serologically distinct, as indicated by the haemagglutination-inhibition test.

Influenza virus infection in humans has a distinct influence in promoting bacterial invasion. In experimental animals, pre-existing bacterial infection is activated to produce more severe disease. Introduction of organisms of low virulence after establishment of virus infection enhances their virulence (Rivers and Horsfall 1959). This also appears to be the case with duck sinusitis. We were unable to stimulate an antibody response to M. anatis when the Mycoplasma is inoculated via the infra-orbital sinus by itself, but if influenza A virus was inoculated along with the Mycoplasma or even at a later stage an antibody response to the Mycoplasma was obtained. Hence influenza A viruses in ducks certainly promotes bacterial invasion.

Shope (1931) showed that swine influenza in its characteristic form was caused by the associated effects of an influenza type A virus and Haemophilus influenza-suis. The virus alone caused a relatively trivial infection and the bacillus alone caused no detectable illness. It appears that this might be the case with duck sinusitis; however, since we have failed to reproduce the disease with both agents e.g. M. anatis and influenza A virus, a third stress factor may be involved which is as yet unknown.

This third factor might be associated with the Mycoplasma. It has been assumed with human Mycoplasma that once it is grown in broth and subcultured a few times it loses its pathogenicity (Edward 1954). This might be the case with M.anatis

In the northern hemisphere outbreaks of human influenza due to type A tend to recur every2-3 years, those due to type B every 3-6 years. It will be very interesting to see whether or not there will be an outbreak of duck

sinusitis on the same farm in 5-6 years' time.

It must be borne in mind that influenza A viruses other than those of Newcastle disease and fowl plague can infect fowls without showing detectable symptoms, and that there is a danger of mistaking these for Newcastle disease and fowl plague unless full investigation procedures are followed.

Table 3

Results of the Haemagglutination-inhibition tests using specific immune sera against a variety of influenza A viruses

Antisera: H.I. titre

Strain	Duck/Eng/56	Duck/Eng/62	Duck/Czech/56	A-Swine	A-PR8	A1-FM1	A2/Sing/57	F/Scot/60	Fowl Plague	Newcastle Disease
Duck/Eng/56	80	<10	<10	<10	<10	<10	<10	<10	<10	<10
Duck/Eng/62	<10	60	<10	<10	<10	<10	<10	<10	<10	<10
Duck/Czech/56	<10	<10	<20	<10	<10	<10	<10	<10	<10	<10
A-Swine	<10	<10	<10	100	<10	<10	<10	<10	<10	<10
A-PR8	<10	<10	<10	<10	1920	<10	<10	<10	<10	<10
A1-FM1	<10	<10	<10	<10	<10	240	<10	<10	<10	<10
A2/Sing/1/57	<10	<10	<10	<10	<10	<10	400	<10	<10	<10
F/Scot/60	<10	<10	<10	<10	<10	<10	<10	1280	<10	<10
Fowl Plague	<10	<10	<10	<10	<10	<10	<10	<10	60	<10
Newcastle Disease	<10	<10	<10	<10	<10	<10	<10	<10	<10	640

Section 4

L-PHASE BACTERIAL FORMS ASSOCIATED WITH INFECTIOUS SYNOVITIS IN CHICKENS AND TURKEYS

Summary

Isolation of L-phase like bacterial forms of Bacillus licheniformis from four outbreaks of infectious synovitis in broilers and from one outbreak of infectious synovitis in turkeys is described, followed by an account of pathogenicity studies with these L-phase forms in association with other agents isolated. The importance of these L-phase forms is discussed.

Introduction

Infectious synovitis was first reported in this country by Carnaghan (1959). The infective agent was presumed to be a large virus or rickettsiae until Lecce (1960) and then Chalquest and Fabricant (1960), both in the U.S.A. reported the isolation of Mycoplasma. Lecce (1960) reported the isolation of a Mycoplasma from allantoic fluid of infected embryos; Chalquest and Fabricant (1960) isolated Mycoplasma from field cases and chick embryo cultures of the infective agent. In view of these findings, attempts were made to isolate Mycoplasma from four outbreaks of infectious synovitis in chickens and from one outbreak of infectious synovitis in turkeys. All these cases had been submitted for diagnosis to the Central Veterinary Laboratory. The outbreaks were considered typical of infectious synovitis as seen in this country, with swelling of the hocks and feet and sternal bursae, and in some birds the wing joints were affected. The swellings were found to contain exudate which varied from a grey viscous fluid to a caseous creamy yellow material in advanced cases.

ISOLATION OF INFECTIVE AGENTS

Materials and Methods

The culture media used were PPLO agar or broth (Difco), enriched with 5% yeast extract and 10% inactivated pig serum; included in the broth was 0.01% L-Cysteine. The pH was adjusted to 7.8.

The culture media differed from that used by Chalquest and Fabricant (1960), who used beta-diphosphopyridine nucleotide (DPN) powder dissolved in distilled water at a final concentration of 0.1%, but in this investigation

Fig. 1. Zone of haemolysis produced along the line of inoculation on sheep blood agar(7026).

DPN was supplied by a yeast extract prepared by the method of Marshall and Kelsey (1960). Fifty grammes of yeast were suspended in 100 ml. of 0.2 M KH_2PO_4, heated at 80°-85°C for 20 minutes, filtered or centrifuged, sterilised by Seitz filtration and stored in the cold, or preferably frozen.

Samples of synovial fluid were taken from the affected joints of birds from each outbreak and bulked. These bulked samples were inoculated into PPLO broth, onto PPLO agar and sheep blood agar. All plates were incubated in a candle jar at 37°C and all broth media were incubated aerobically at 37°C.

Results

Full details of the procedure of investigation and the results are given with outbreak A only; for the subsequent investigations the results only are given.

Outbreak A (317). Infectious Synovitis in broilers

Synovial fluid from the infected birds plated on sheep blood agar produced a zone of haemolysis along the line of inoculation (Fig. 1). This change has been mentioned by several observers (Cover, Galeta and Waller, 1956; Carnaghan, 1959). It was found that this haemolytic zone could be transferred (Fig. 2), and by the second or third passage bacterial growth had become evident. The method of subculture was similar to that used for Mycoplasma organisms, in which blood agar blocks containing the haemolytic zone are cut out of the plate and smeared gently over a new plate. The blood agar plates were subcultured every 2-3 days.

The bacterial growth consisted of four colonial types, which were:-

1. A haemolytic coagulase positive Staphylococcus with a phage pattern 6/7/47/53/54/75/83A/+ at Routine Test Dilution.

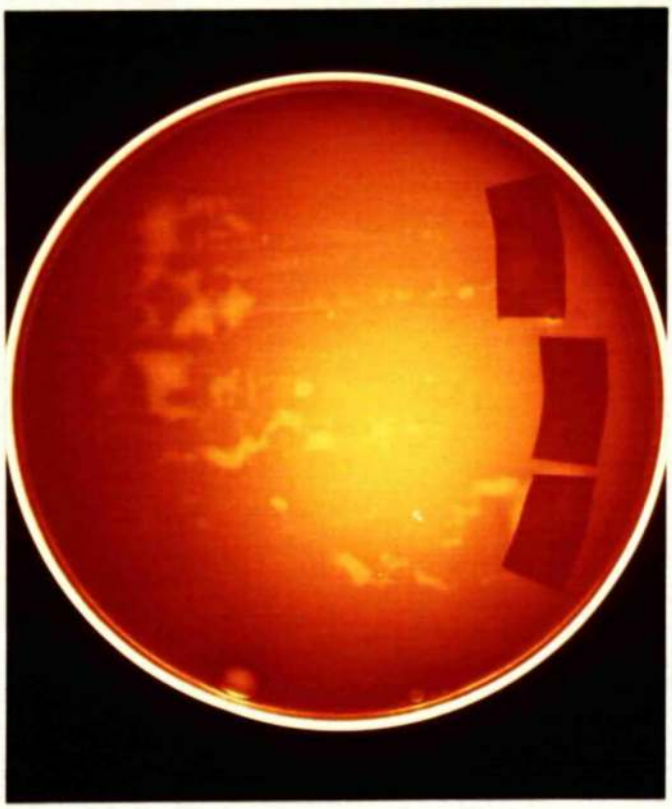


Fig. a. The transfer of the haemolytic zone(7026).

Fig. 3. B.licheniformis L-phase bacterial forms(1091).

Gram stained. X 1350.

2. A non-haemolytic coagulase negative Staphylococcus. Its biochemical reactions are shown in table 4.

3. An organism which produced small, circular, effuse smooth colonies with an entire margin, which after 24-28 hours growth had a diameter of approximately 0.2 mm. These colonies grew into the medium and were difficult to remove completely with a loop.

When colonies were stained using Gram's method, the majority of the organisms were Gram positive. The organisms appeared very pleomorphic, varying in size from very small granules of about 250 mμ to very long filaments. Some filaments appeared to branch; large round or oval bodies were seen with a diameter of about 10 mμ; there were many cocco-bacillary forms. There was distortion of the cocci and rods. The filaments were often Gram negative with Gram positive granules situated inside the filaments and there were large aggregates of Gram positive granules often apparently embedded in a weakly Gram negative material. Numerous other irregular forms were also demonstrated (Figure 3).

The appearance of these organisms suggested that they were L-phase or L-phase-like bacterial forms. The colonies were subcultured into PPLO broth and incubated at 37°C. Within 24 hours a pellicle developed on the broth and when this was sub-cultured on to blood agar and incubated, a colony was produced which proved to be a spore bearing organism of the bacillus group.

Similar organisms were seen on PPLO agar. The L-phase-like bacterial colonies appeared granular with a crenate margin. Isolations from Outbreak D (3495) gave similar colonial forms (Fig. 4).

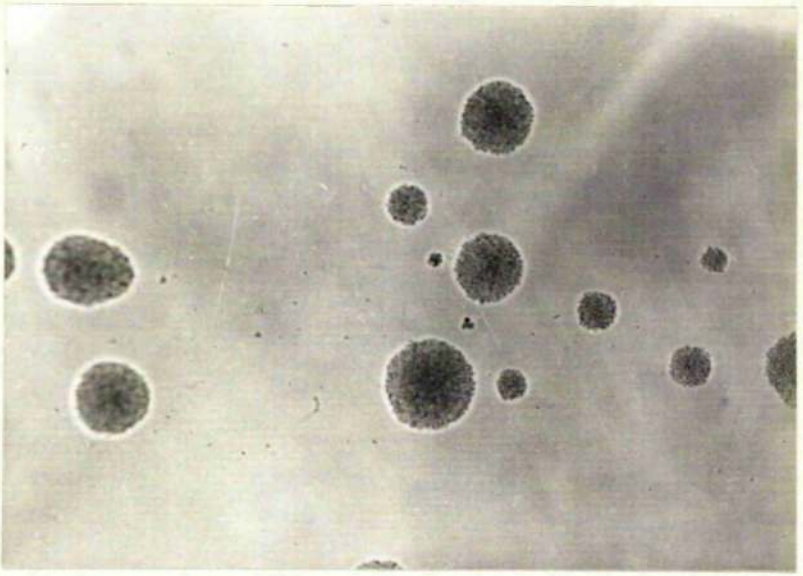


Fig. 4. B.licheniformis L-phase colonies(3495). X 145.

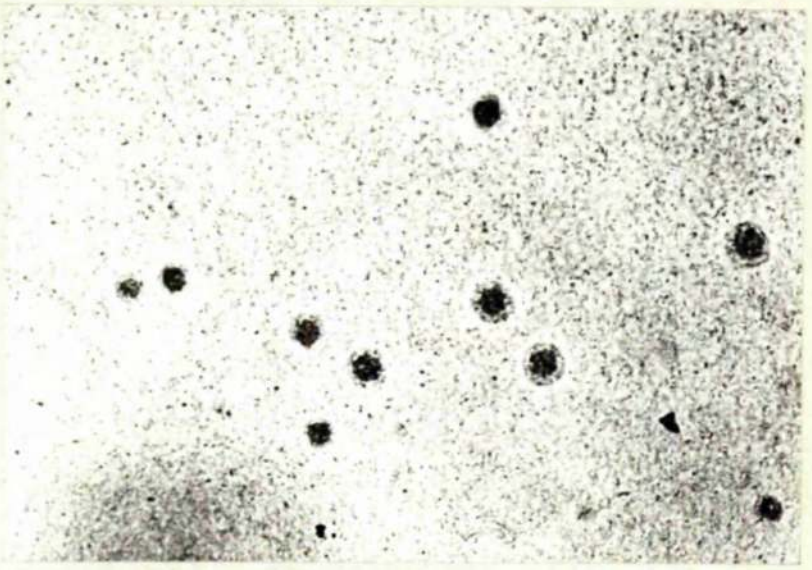


Fig. 5. Mycoplasma-like colonies isolated from cases of infectious synovitis(317). X 155.

The isolation of the L-phase-like organism was repeated on numerous occasions. Attempts to isolate the two staphylococcal strains from the same inoculum were not, however, consistently successful.

4. On two occasions a Mycoplasma-like colony was grown on the PPLO agar (Fig. 5). The colonies showed the typical fried egg appearance, and although on subculturing, the colonies grew, on the second passage the colonies appeared to die, and the PPLO agar plate became overgrown with the bacillus organism.

Several attempts were made to grow the Mycoplasma-like colony on the PPLO agar. Pure beta-diphosphopyridine nucleotide was substituted for yeast extract, as recommended by Chalquest and Fabricant (1960), in an attempt to adapt the Mycoplasma like colony to the medium, but without success.

Outbreak B (1091). Infectious synovitis in broilers

Results: The following organisms were isolated:

1. A non-haemolytic coagulase negative Staphylococcus. Its biochemical reactions are given in table 4.
2. L-phase or L-phase-like bacterial forms which reverted in broth to the characters of the Bacillus species.

Outbreak C (7026). Infectious synovitis in broilers

Results: The following organisms were isolated:

1. Haemolytic, coagulase negative Staphylococcus. The biochemical reactions are given in table 4.
2. An organism with reactions similar to Aerococcus viridans as described by Cowan and Steel (1961). It produced alpha haemolysis on sheep blood

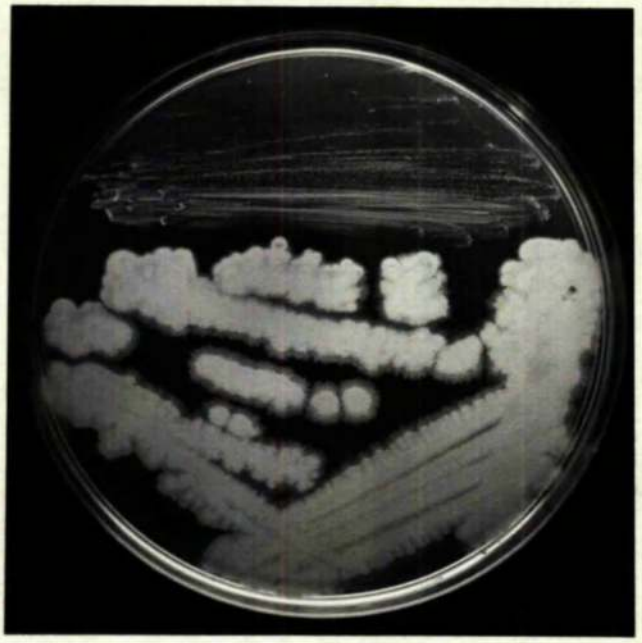


Fig. 5. Colonial appearance of B. licheniformis bacillary form and L-phase form(3495) on PPLO agar.

agar and could not be placed immunologically into any of Lancefield's groups. The organism grew on media containing bile salts, in the presence of 6.5% sodium chloride and at a pH of 9.6. Growth occurred at 45°C. and survived heating at 60°C for 30 minutes. It fermented trehalose and lactose; it did not ferment sorbital, mannite, raffinose, salicin and inulin. It reduced litmus milk with clot formation. It did not hydrolyse sodium hippurate, starch, arginine or aesculin. It was catalase negative.

3. L-phase or L-phase-like bacterial forms which reverted, as in Outbreak A.

Outbreak D (3495). Infectious synovitis in broilers

Results: The following organisms were isolated:

1. A coagulase positive haemolytic Staphylococcus with phage pattern 52A/79/+ at Routine Test Dilution.

2. L-phase or L-phase like bacterial forms which reverted as in Outbreak A, (Fig.6)

Outbreak E. Infectious synovitis in turkeys

Synovial fluid from infected turkeys plated on to sheep blood agar did not produce the zone of haemolysis along the line of inoculation, but on subculturing bacterial growth became evident on the first or second passage.

Only one type of colony was seen on both sheep blood agar and the PPLO agar. The culture was very similar to the L-phase or L-phase-like colonies isolated from chicken synovitis. Growth with pellicle formation occurred in broth which on subculturing onto blood agar produced an aerobic spore bearing colony of the Bacillus group.

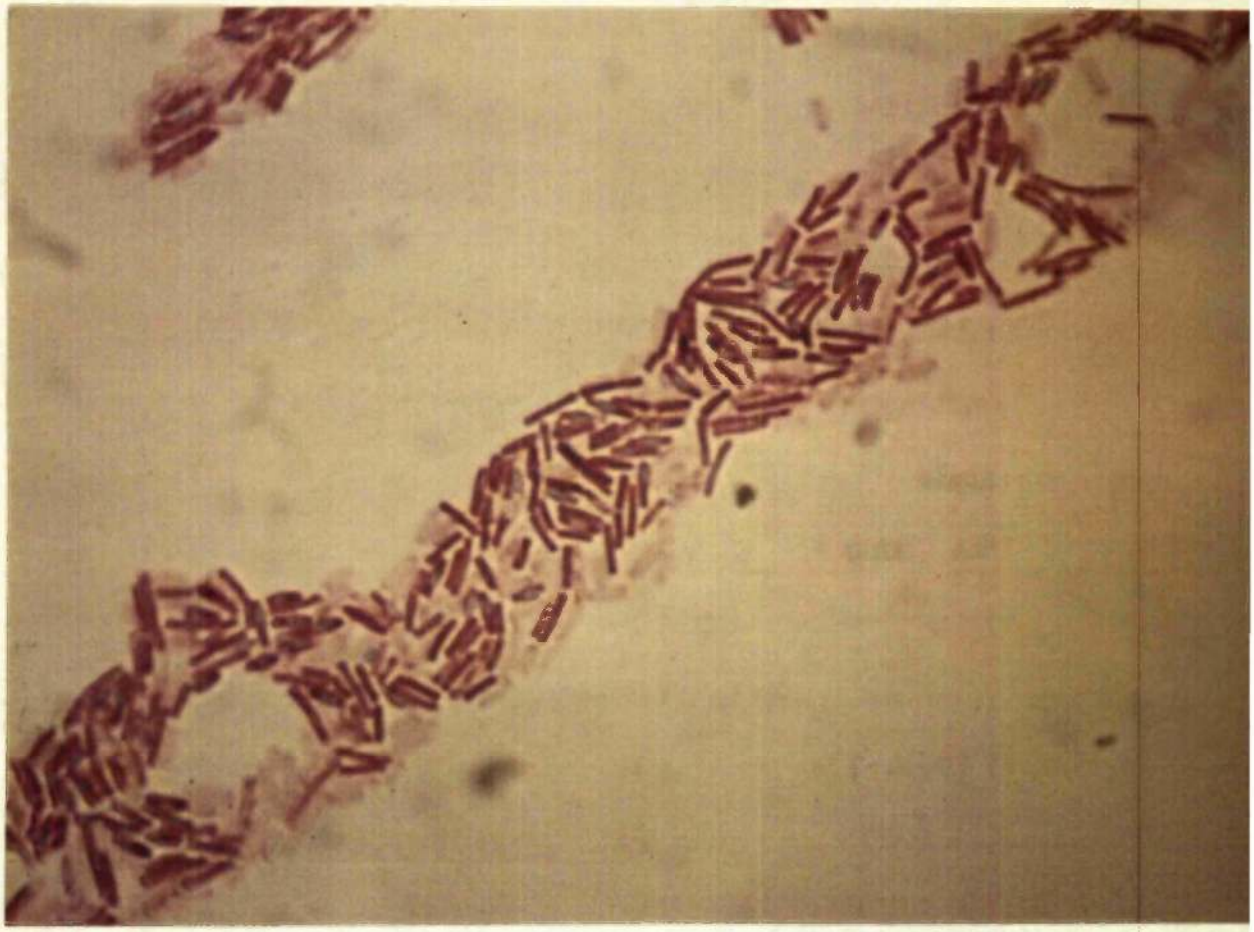


Fig.7. B.licheniformis bacillary forms(1091). Stained by Malachite green-safranin method. X 1575.

IDENTIFICATION OF THE BACILLUS SPECIES ISOLATED

L-phase or L-phase-like bacterial forms were isolated from all the specimens of infectious synovitis examined. All these L-phase-like forms when subcultured into broth reverted to aerobic spore bearing bacteria and tests were made to identify the five species isolated.

Materials and Methods

Smith, Gordon and Clark (1952) and Knight and Proom (1950) have described special tests which are of value in this group. Their techniques were used.

The genus is divided into three major groups on microscopic appearance of the organisms. Smears of young cultures grown on nutrient agar were Gram stained. The spores were stained using the malachite green-safranin method (Fig. 7).

Nutrient broth contained 5 gm. peptone, 3 gm. beef extract, 1000 ml. distilled water. The pH was adjusted to 7.0 Nutrient agar was prepared by adding 1.5% agar.

Potato plugs Cores, 10-15 mm. in diameter were cut from potatoes and autoclaved with a few ml. of distilled water in potato tubes.

Digestion of Starch Cultures were inoculated onto plates of nutrient agar containing 1% potato starch. After incubation the plates were flooded with 95% alcohol. If the starch was hydrolysed a translucent zone appeared around or underneath the growth but if it remained unchanged the medium became white and opaque.

Production of acetylmethylcarbonol The medium used contained 5 gm. glucose, 5 gm. NaCl. and 1000 ml. of distilled water. Five ml. portions in 18 mm. tubes were inoculated and incubated, at 30°C. and tested by O'Meara's method after 2-6 days.

Sugar fermentation The basal medium used was:

$(NH_4)\ HPO_4$	1.0 gm.
KCl	0.2 gm.
$MgSO_4$	0.2 gm.
yeast extract	0.2 gm.
Agar	15.0 gm.
distilled water	1000 ml.

Twenty ml. of a 0.04% solution of bromoresol purple was added as indicator. The medium was tubed and autoclaved and sterile carbohydrate was added to make a final concentration of 0.5%. Strains were examined for growth, acid and gas production from sucrose, glucose, arabinose and xylose.

Digestion of gelatin Plates of nutrient agar containing 0.4% gelatin were inoculated and after incubation were flooded with the following solution:

$HgCl_2$	15.0 gm.
Conc. HCl	20.0 ml.
Distilled water	100 ml.

Hydrolysis of gelatin was indicated by a clear zone around or underneath the growth in contrast to the white opaque precipitate of the unchanged gelatin.

Digestion of Casein Plates containing nutrient agar plus 50% skim milk were inoculated and observed for clearing.

Reduction of nitrate to nitrite The cultures were grown in nutrient broth containing 0.1% KNO_3 and incubated. The reduction was tested by the Greiss-

Ilosvay method.

Gibson and Abdel-Malek Test (1946) This test was used for the production of acid from gas under semi-anaerobic conditions. To litmus milk was added glucose to 5%; to 5 parts of this was added 1 part of melted nutrient agar and this was then inoculated. Nutrient agar was then added gently so as to form a layer 2-3 cms. deep.

Egg yolk reaction The yolk of one egg washed free of white was beaten in 250 ml. saline, spun and Seitz filtered. One part of this was added to nine parts of nutrient agar and plates prepared. The cultures were inoculated as a blob. Lecithinase production was indicated by a zone of opacity around or underneath the colony.

Growth on nutrient agar pH 6.0 was examined. The strains were tested for the production of catalase, motility, ability to grow anaerobically on nutrient agar. The urea medium of Christensen (1946) containing phenol red as indicator was used for the detection of urease.

The temperature of incubation for all the tests was 30°C.

Results

Smith, Gordon and Clark (1952) divide the genus Bacillus into three groups based on the size and shape of the mature spores and the shape of the sporangium.

In the five cultures isolated, the sporangia were only swollen slightly or not at all. The spore walls were thin; the spores were oval and found in a central position. The strains were all motile and Gram positive. The strains belonged to group 1.

The general appearance and size of the colonies of the five strains on sheep blood agar appeared to be the same.

On nutrient agar the colonies were rough and of whitish appearance with hairy outgrowths. On sheep blood agar there was a greenish zone of haemolysis surrounding the colonies.

The results of the biochemical reactions are summarised in table I; they indicate that all five organisms were Bacillus licheniformis, which is also known as B. subtilis Ford type. B. licheniformis differs from B. subtilis only in its ability to grow anaerobically, and from B. pumilus in its ability to hydrolyse starch and reduce nitrates. B. licheniformis differs from B. cereus in its inability to produce lecithinase, the diameter of the bacillus and the fermentation of arabinose and xylose.

FURTHER OBSERVATIONS ON THE L-PHASE-LIKE ORGANISM

Three cultures of the L-phase like bacterial forms, 1091, 317 and 7026 were grown on blood agar. The organisms were washed off and suspended in physiological saline and to each was added penicillin to make a final concentration of 1000 U. per ml. Each suspension (0.2 ml.) was inoculated via the yolk sac into 6-7 day old embryonated chicken eggs which had been obtained from a Mycoplasma gallisepticum free flock. The embryos started to die 3 days after inoculation and were all dead within 8 days of inoculation. L-phase like bacterial forms were isolated from the yolk sac material and allantoic fluid of all the dead embryos.

The results of egg inoculation are shown in table 2.

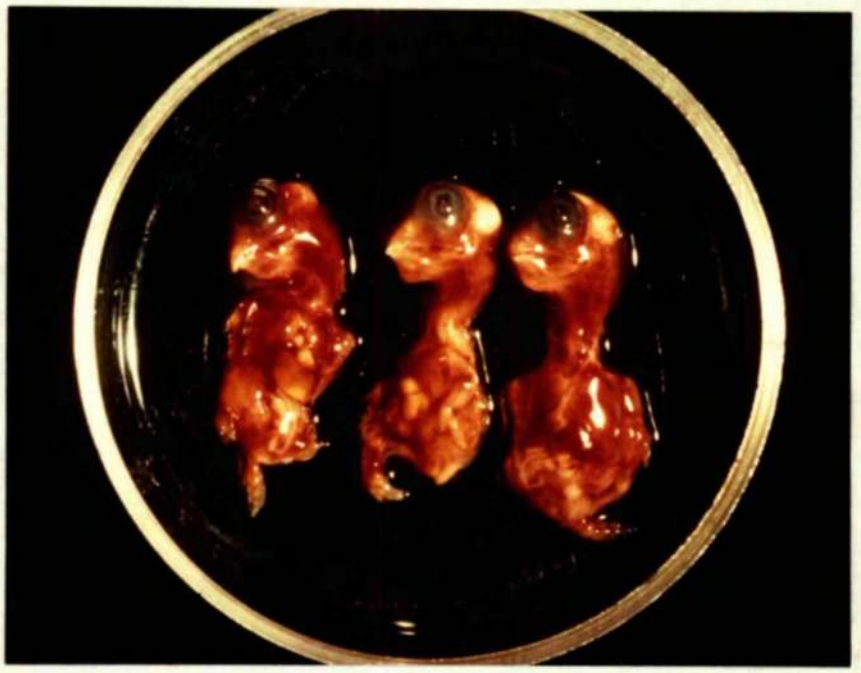


Fig. 8. B.licheniformis L-phase form(1091) infected embryos, 10 days old.

Table 2 Results of Yolk sac inoculation of chick embryos with L-phase-like bacterial forms

Strain	Embryo age (days)	No. of eggs	No. of dead embryos - days post inoculation							
			1	2	3	4	5	6	7	8
1091	7	6	0	0	1	3	1	1	0	0
317	6	6	0	0	0	3	1	1	1	0
7026	6	6	0	0	0	3	2	0	0	1

The gross lesions seen in the dead embryos were similar to those associated with Mycoplasma inoculation (Chute 1960) for they showed extensive haemorrhage and oedema and in some the livers were enlarged and necrotic (Fig. 8).

Penicillin sensitivity of the L-phase-like bacterial forms was determined. Nutrient agar plates were prepared containing 10, 50, 100 and 200 units of penicillin per ml. The surface of the agar was inoculated with three L-phase-like cultures, 1091, 317 and 3495. Growth was observed only on the nutrient agar plate containing 10 units per ml.

The fermentative properties of the L-phase-like forms differed from the bacillary forms as shown in table 1.

The stability of the L-phase-like forms increased with the number of subcultures. This was particularly so with strain 1091 which did not revert to its bacillary form on subculturing into broth from blood agar.

PATHOGENICITY TESTS

Pathogenicity tests were carried out in groups of six in 3-4 week old Rhode Island Red chickens obtained from a Mycoplasma gallisepticum free flock. Uninoculated control groups were included with each transmission.

Suspensions of the following organisms were prepared:

a) L-phase-like forms of B. licheniformis (1091)

b) Coagulase positive Staphylococcus (317)

c) B. licheniformis bacillary form (1091)

d) Mixture of (a) and (b)

e) Mixture of (b) and (c)

f) Non-haemolytic coagulase negative Staphylococcus (317)

g) Mixture of (c) and (f)

h) Haemolytic coagulase negative Staphylococcus (7026)

i) Mixture of (c) and (h)

The cultures were grown on blood agar, washed off and suspended in physiological saline; 0.25 ml. of each suspension was inoculated into the plantar pad of the left foot of each bird. The birds were examined daily for 10 days.

Results Suspension (a). L-phase B. licheniformis forms (1091). Swelling which appeared at the site of inoculation, subsided within a few days.

Suspension (b). Coagulase positive Staphylococcus (317). Swelling appeared at the site of inoculation; the birds became emaciated and dehydrated and a fatal septicaemia developed in 3 out of the 6 birds.

Suspension (c). Bacillus licheniformis (1091). Severe swelling appeared at the site of inoculation with a tendency to spread up the leg to the hock joint; breast blisters were seen in 2 out of the 6 birds.

Suspension (d). Mixture of L-phage B. licheniformis forms and coagulase positive Staphylococcus (317). Swellings were seen at the site of inoculation; birds became dehydrated and emaciated and 3 out of the 6 birds died.

Suspension (e). Mixture of coagulase positive Staphylococcus (317) and B. licheniformis (1091). Birds developed swellings at the site of inoculation and in 3 out of the 6 birds the hock joint immediately above also became swollen and filled with mucoid exudate. There were breast blisters in 3 of the birds.

Suspension (f). Non-haemolytic coagulase negative Staphylococcus (317). Slight swelling appeared in 2 of the 6 birds at the site of inoculation.

Suspension (g). Mixture of B. licheniformis (1091) and non-haemolytic coagulase negative Staphylococcus (317). Swellings developed at the site of inoculation. One of the 6 birds developed a swollen hock joint with a mucoid exudate.

Suspension (h). Haemolytic coagulase negative Staphylococcus (7026). No lesions seen.

Suspension (i). Mixture of B. licheniformis (1091) and haemolytic coagulase negative Staphylococcus (7036). Swellings developed at the site of inoculation and in 3 of the 6 birds breast blisters were seen.

Coagulase positive Staphylococci were isolated from the birds which died in groups (b), (d) and (e). The remaining birds were killed 10 days after inoculation and bacteriological cultures taken from the lesions. Isolations of B. licheniformis were obtained from each bird inoculated with B. licheniformis. No isolation of coagulase negative or coagulase positive Staphylococcus was obtained.

The results are summaraized in table 3.

The above tests were repeated with suspensions (a) to (e). The results were in general agreement with those described above.

Discussion

Four field outbreaks of infectious synovitis in broiler chickens and one in turkeys were investigated. Bacillus licheniformis in its L-phase-like form was isolated from each outbreak. From 2 out of 4 outbreaks of infectious synovitis in broilers, a coagulase positive Staphylococcus was isolated and in 3 out of the 4 a coagulase negative Staphylococcus was also isolated. The L-phase-like form of B. licheniformis was the only organism isolated from infectious synovitis in turkeys.

With most bacteria in which the L-phase variation is known, abnormal culture conditions are generally necessary to induce the development of L-type colonies on solid media. Dienes (1949) produced L-phase bacterial forms from a Gram positive spore bearing bacillus after growing the bacillus on horse serum containing penicillin. L. forms of Streptobacillus moniliformis have been cultivated directly from rats and human beings (Dienes 1960).

Streptobacillus moniliformis causes a spontaneous disease in mice characterised by multiple arthritis, often involving the joints of the feet and leading to swellings of the feet and legs. L form cultures of S. moniliformis are non-pathogenic when inoculated into mice, if reversion to the bacillary form in the inoculated animal is prevented (Breed, Murray and Smith, 1957).

The disease in mice caused by S. moniliformis is similar to infectious synovitis in chickens and turkeys. The diseases resemble each other in the clinical picture presented, and in the isolation of L forms from the pathological process.

The significance of these B. licheniformis L forms is difficult to assess. Infectious synovitis as seen in the field was not produced and the nearest resemblance to the disease was seen when the bacillary form of B. licheniformis and coagulase positive staphylococci were inoculated together. The bacillary forms of B. licheniformis cause a severe swelling at the site of inoculation with a tendency for the swelling to spread up the leg.

There is an abundance of evidence to support the theory that egg transmission occurs with CRD (Mycoplasmosis). The transmission of infectious synovitis via the egg has been reported by Carnaghan (1961); Thayer, Strout and Dunlop (1958); and Sneoyenbos and Basch (1958). There are similarities between Mycoplasma and L forms and some workers (Minck and Kirn 1960; Pease and Bisset 1962) believe that Mycoplasma are L forms of bacteria. Egg transmission may occur with L forms and, although the workers cited above did not identify the infectious synovitis agent, it is possible that they were dealing with transmission of L forms of bacteria.

The embryonic lesions and mortality pattern caused by L. phase forms and by Mycoplasma are very similar. L phase bacterial forms are very easily produced by the addition of antibiotics, in particular penicillin, and in the primary isolation of infective agents this factor must be borne in mind.

Bisset (1959) put forward the hypothesis that Gram positive bacteria, under the influence of a parasitic mode of life, evolve with a progressive loss of such structures as spores and flagella, and of Gram positivity, catalase and other enzyme systems, with the result that from a morphologically complex saprophyte is derived a Gram negative nutritionally exacting parasite.

The development of B. licheniformis might lend support to this hypothesis which could account for the appearance of mycoplasma-like colonies in Outbreak A (317). It appeared possible that the mycoplasma-like-colony was another variant of the Bacillus organism which was unstable and reverted to its bacillary form.

The transformation of bacteria into L forms of Mycoplasma takes place in a series of stages. This may be an example of the transformation of a saprophyte into a parasite by the use of antibiotics, for broiler food is widely supplemented with penicillin at 5-10 gram per ton.

Chalquest and Fabricant (1960) isolated Mycoplasma from cases of infectious synovitis which was serologically distinct from Mycoplasma gallisepticum. They could not reproduce the disease after the Mycoplasma had been passaged in artificial media and they suggested that the Mycoplasma had lost its pathogenicity, but they claimed that one of the cultures remained pathogenic for birds after several passages and produced typical

synovitis when inoculated into birds. From the same bird they isolated Staphylococcus epidermis. The broth medium they used in the isolation of Mycoplasma contained penicillin and thallium acetate, substances which do not prevent the growth of L forms. They did not isolate Mycoplasma from all the field cases examined and were unable to account for this lack of consistency.

Carnaghan(1959) failed to reproduce the disease with Seitz EK filtrate of synovial fluid from affected birds. As he failed to grow any bacteria or Mycoplasma on artificial media, he suggested that the agent was probably a virus. The evidence presented by Carnaghan(1959) could suggest that the aetiological agent of infectious synovitis is bacterial. The failure of so many workers to reproduce the clinical entity might be due to inhibition by antibiotics of the growth of the organism concerned.

The significance of B. licheniformis and its L forms has not been finally established as inoculated pure cultures did not produce typical infectious synovitis. The cause of infectious synovitis, however, may not be a single agent but a combination of agents, and inoculations of mixed cultures produced lesions similar to infectious synovitis.

Table 1.

Biochemical reactions of the Bacilli strains and three of the L-phase like bacterial forms

Strains		Bacillary forms					L-phase bacterial forms		
		7016	1091	317	3495	IS.Turkeys	1091	317	3495
Growth on	Nutrient agar pH 7	++	++	++	++	++	+	+	+
	Nutrient agar pH 6	+	+	+	+	+	+	+	+
	Potato	+	+	+	+	+	+	+	+
Production of acid-sugars	Sucrose	+	+	+	+	+	-	-	-
	Glucose	+	+	+	+	+	-	-	-
	arabinose	+	+	+	+	+	-	-	-
	Xylose	+	+	+	+	+	-	-	-
Vokes-Proskauer reaction		+	+	+	+	+	-	-	-
Hydrolysis of	Starch	+	+	+	+	+	-	-	-
	casein	+	+	+	+	+	-	-	-
	gelatin	+	+	+	+	+	-	-	-
Nitrate reduced to Nitrite		+	+	+	+	+	-	-	-
Urease formation		-	-	-	-	-	-	-	-
Anaerobic growth		+	+	+	+	+	+	+	+
Gibson Abdel Malek test (1946)		+	+	+	+	+	-	-	-
Egg yolk reaction		-	-	-	-	-	-	-	-
Catalase		+	+	+	+	+	+	+	+
Motility		+	+	+	+	+			

++ = abundant growth on nutrient agar pH 7

Table 3 Summary of the Pathogenicity tests

Suspension	Mortality over 10 day period	Lesions			
		At site of injection	No. affected	Due to spread from inoculation site	No. affected
a) L-phase like form(1091)	-	+	6	-	-
b) Staph.coagulase positive(317)	3/6	++	6	-	-
c) Bacillus licheniformis (1091)	-	+++	6	±	
d) Mixture of (a) plus (b)	3/6	++	6	-	-
e) Mixture of (b) plus (c)	1/6	+++	6	+	3/6
f) Non-haemolytic coagulase negative Staphs. (317)	-	+	2	-	-
g) Mixture of (c) plus (f)	-	+++	6	+	1/6
h) Haemolytic coagulase negative Staph. (7026)	-	-	-	-	-
i) Mixture of (c) plus (h)	-	+++	6	±	

Key to column 3 +++ = severe swelling
++ = moderate swelling
+ = slight swelling

In column 4 ± = inconclusive

Table 4 **Biochemical reactions of the coagulase negative Staphylococci isolated**

	Catalase	Coagulase	Nitrate reduction	Gelatin liquefaction	Urease	Voges-Proskauer reaction	Glucose	Red pigment
Case A (317)	+	-	+	-	+	-	A	-
Case B (1091)	+	-	+	-	-	-	A	-
Case C (7026)	+	-	+	-	-	-	A	-

Section 5

Non Specific Serum Inhibitors present in Turkey Sera to the WRI Strain of Mycoplasma

Summary

The presence in turkey sera of non specific serum inhibitors to the haemagglutinating activity of the WRI strain of Mycoplasma is described. The serum inhibitors are heat stable and were removed with the receptor-destroying enzyme (RDE) of Vibrio cholerae and other chemical agents. RDE treated fowl red blood cells were not agglutinated by the WRI strain of Mycoplasma.

Introduction

Using the haemagglutination-inhibition test a previous survey (see Section 2) revealed a high incidence of antibodies in turkey sera to the WRI strain of Mycoplasma.

It was decided to investigate the possibility that the inhibition of haemagglutinating activity of WRI antigen by turkey sera was a non specific reaction associated with the presence of non specific serum inhibitors.

Preliminary studies indicated that the receptor-destroying enzyme (RDE) from Vibrio cholerae removed the H.I. titres in a number of turkey sera examined. The inhibitory substance was not inactivated at a temperature of 65°C for 30 minutes.

Materials and Methods

Twelve 9 week old Broad Breasted White Turkey poults, were obtained from a PPLO free flock. Three of the birds were inoculated intravenously with 0.5 ml. of a 24 hour broth culture of Mycoplasma gallisepticum; three

birds were inoculated intravenously with 0.5 ml. of a 24 hour broth culture the WRI strain of Mycoplasma, and the remaining six were used as controls. The birds were bled before inoculation and subsequently at regular intervals.

The sera were treated with RDE and then assayed by the haemagglutination-inhibition technique using the antigens M. gallisepticum and WRI. At a later stage a small number of turkey sera were treated with kaolin, bentonite and trypsin; the results were compared with the RDE treatment in an attempt to assess the nature of these non-specific inhibitors.

In the H. I. test the M. gallisepticum antigen consisted of a 24 hour broth culture; the WRI antigen was prepared by inoculating 200 ml. of broth with 10 ml. of stock culture; 24 hours later the broth was centrifuged at 3,084 G. for 30 minutes. The deposit was re-suspended in 50% phosphate buffered glycerol (pH 7.2).

Serial 2-fold dilutions of both antigens were made in saline using 0.25 ml. volumes; 0.25 ml. of a 1% suspension of washed fowl red blood cells were then added. Readings were made after the plates had been left at room temperature for 50 minutes. Fifty per cent haemagglutination was regarded as the end point and at this titre one partial haemagglutinating dose of Mycoplasma was contained in 0.25 ml.

Stock Mycoplasma suspensions were prepared by diluting both antigens in physiological saline so that 4 partial H.A. doses were contained in 0.25 ml.,

Serial 2-fold dilutions in physiological saline of the treated and untreated sera ranging from 1/5 to 1/320 were made; to 0.25 ml. of each dilution was added 0.25 ml. of the stock Mycoplasma suspension. The mixture was allowed to stand for a few minutes, after which 0.25 ml. of a 1% suspension of fowl red blood cells were then added. The plates were shaken,

left at room temperature and read after 50 minutes.

The culture media used was PPLO broth (Difco) enriched with 1% yeast autolysate (Albimi), 10% horse serum and 0.1% dextrose; the pH was adjusted to 7.8.

Treatment of Sera

a) Receptor-destroying enzyme (RDE) of Vibrio cholera (Wellcome) RDE was reconstituted in saline containing 0.1% calcium chloride according to the manufacturers' directions. To one volume of serum was added four volumes of RDE and the mixture was incubated at 37°C for 18 hours. Excess RDE was inactivated by incubation at 56°C for one hour. Controls contained four volumes of saline containing 0.1% calcium chloride instead of RDE.

b) Kaolin The effect of kaolin was tested by the method of McFerran (1962). To 10 ml. phosphate buffered saline (pH 7.2) was slowly added 2.5 g. acid washed kaolin, stirring rapidly. Equal volumes of this suspension and the serum sample were mixed and allowed to stand at room temperature for 30 minutes and shaken occasionally. The mixture was centrifuged for 30 minutes at 1,400 G. and the supernatant withdrawn and tested for activity. Controls contained one volume of phosphate buffered saline instead of the suspension of kaolin.

c) Bentonite The effect of bentonite was tested by the method of Clarke and Cassals (1955). A 1% suspension of bentonite in phosphate buffered saline was made in the same way as the kaolin

suspension and used similarly for treatment of the serum.

d) Trypsin A stock solution of 4% trypsin (X 2 crystallised) in N/100 HCl was diluted 1 in 2 using phosphate buffered saline pH 8.0. To one volume of serum was added 4 volumes of typsin solution and the mixture was incubated at room temperature for 15 minutes. Excess trypsin was inactivated by heating at 56°C for 30 minutes.

The serum samples after treatment with RDE, kaolin, bentonite, trypsin, and their controls were all absorbed with one drop of washed packed fowl red blood cells. The samples were then centrifuged at 300 G. and the supernatant tested for activity against M. galliseptioum and WRI antigens using the H.I. technique. The H.I. titres in the tables are expressed as the initial dilution of serum present at the end point.

Treatment of fowl red blood cells with receptor-destroying enzyme:

RDE was reconstituted in saline containing 0.1% calcium chloride according to the manufacturers' directions. Serial two-fold dilutions of RDE were made with the same diluent, ranging from 1/2 and 1/512. To 0.5 ml. of RDE was added 0.5 ml. of a 1% suspension of washed fowl red blood cells and the tubes containing the mixture were incubated for 30 minutes at 37°C. The tubes were then centrifuged at 300 G. for 10 minutes, the supernatant fluid was removed from the cells and the cells were resuspended in 0.5 ml. of saline. To 0.25 ml. amounts of the resulting cell suspension was added (a) 0.25 ml. of WRI and (b) 0.25 ml. of M. galliseptioum antigens containing 8 partial haemagglutinating units. The test was performed in perspex plates, which were left at room temperature for 50 minutes and the presence

of absence of agglutination noted.

Results

The serological responses of the turkey poults to M. gallisepticum are shown in table 1. The tires of the RDE treated and untreated serum samples are given. For the first 8 weeks after inoculation the treatment of the serum samples with RDE had no observable effect on the inhibitory titre. After 8 weeks however the untreated serum samples had a slightly higher titre than the treated serum samples; this period was associated with a gradual decline in the H.I. titre. None of the control birds developed a titre of M. gallisepticum.

The effect of other chemical and enzyme treatments on the H.I. titre of specific antibodies against M. gallisepticum are given in table 3. None of the techniques employed had any observable effect on the inhibitory titre.

The serological responses of the turkey poults to WRI antigen are given in table 2. Untreated serum samples from all the birds before inoculation had titres to WRI; there was no titre when the serum samples were treated with RDE. The inhibitory activity was assayed against 4 partial haemagglutinating doses of WRI antigen, which resulted in a high titre of the untreated serum samples of both the inoculated and control birds. In the RDE treated samples a titre was found only in the inoculated birds and these were relatively low compared with the response to M. gallisepticum. The titres fell rapidly and were hardly detectable 5 weeks after inoculation. The titres of the untreated serum samples of the inoculated birds were higher than the controls, however, when 4 partial haemagglutinating doses of WRI antigen were used, the end point was difficult to assess in view of the

gradual transformation from inhibition to a definite agglutination pattern. A more definite end point is obtained with 8 partial haemagglutinating doses of WRI antigen, but the titres of the RDE treated serum samples of the inoculated birds may not have been detected.

The effect of chemical and enzyme treatments on the H.I. titre of the inhibitory substance and specific antibodies against WRI antigen are shown in table 4. The inhibitory substance was removed from all the sera from "normal" turkeys with RDE, kaolin, and bentonite. Trypsin treatment reduced the inhibitory activity of these sera to a very low level but did not remove them completely.

Testing the sera of the inoculated birds against these chemicals and enzymes revealed that RDE, bentonite and kaolin removed the non specific inhibiting substance but had no effect on the specific antibody. With one trypsin treated sample the titre was higher than the RDE, kaolin and bentonite treated samples.

The treatment of fowl red blood cells with RDE resulted in the red cell receptors to Mycoplasma being removed for the WRI antigen but not for M. gallisepticum. RDE treated fowl red blood cells were agglutinated by M. gallisepticum but not by the WRI strain of Mycoplasma. The results are given below.

Antigen	RDE dilutions added to fowl red blood cells								
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
WRI	-	-	-	-	-	-	±	+	+
M.gallisepticum	+	+	+	+	+	+	+	+	+

+ denotes agglutination - denotes no agglutination

Discussion

Non specific inhibitors in fowl serum to influenza A viruses have been reported on many occasions. Sampaio and Isaacs (1953) state that the inhibitor in fowl serum to influenza A viruses is similar to the Francis inhibitor (Francis 1947), which is heat stable and readily inactivated by the receptor-destroying enzyme (RDE).

Soluble inhibitors of viral activity obtained from tissues possess a variety of chemical compositions identified as proteins, mucoproteins, glycoproteins, mucopolysaccharides and lipoproteins (Ginsberg 1960). RDE removes mucoproteins (Burnet 1951) which inhibit the activity of influenza viruses. Kaolin and bentonite are used in the removal of non specific inhibitors to the arbor viruses; their action is based on the selective adsorption of protein and lipoprotein under standardised conditions, the gamma globulins being left unadsorbed (Clarke and Casals 1955, 1958). Kaolin has been used successfully in the removal of the non specific inhibitors in human and fowl sera to Asian influenza viruses (Spence 1960).

Crystalline trypsin has been found to be highly active in destroying Chu and Francis inhibitors (Sampaio and Isaacs 1953).

The results indicate the presence of a heat stable, non specific inhibitor in turkey sera to the WR1 antigen. It was removed by the receptor destroying enzyme, kaolin and bentonite. Trypsin did not remove the inhibitor completely, indicating that the trypsin like enzyme, which destroys the Chu and Francis inhibitors(Sampaio and Isaacs 1953) and is present in RDE, is not sufficient in removing completely the inhibitors to WR1.

The inhibitor appears to be very similar to the Francis inhibitor, in that both are heat stable, and both are removed by RDE. The Francis inhibitor is thought to be a mucopolysaccharide, mucoprotein or glycoprotein, and it has already been shown to exist in fowl serum (Sampaio and Isaacs 1953).

When fowl red blood cells are treated with RDE, virus receptors are removed and the cells are no longer agglutinated by any of the Myxoviruses. Kasel,Rowe and Nemes(1960) have also shown that the agglutinability of human O cells by adenoviruses is abolished by treatment of the cells with RDE. It was surprising to find that the RDE removed the cell receptors to the WR1 strain of Mycoplasma. The RDE treated cells were still agglutinated by M. gallisepticum and this emphasized the difference between the two strains of Mycoplasma.

Table 1. Serological response of the turkey poults to M. gallisepticum showing the H.I. titres of the KDE treated and untreated samples

Poult No.		H.I. titre at inoculation	Post inoculation period in weeks						
			3	6	8	9	11	14	16
M.G.1	Birds inoculated with M. gallisepticum	R -	R ++1/40	R ++1/40	R ++1/20	R ++1/10 +1/20	R ++1/5	R ++1/5	R +1/5
		U -	U ++1/40	U ++1/40	U ++1/20	U ++1/20	U ++1/10	U ++1/10	U ++1/5
M.G.2		R -	R ++1/80	R ++1/80	R ++1/20	R ++1/10	R -	R -	R -
			U ++1/80	U ++1/80	U ++1/20	U ++1/20	U ++1/5	U ++1/5	U -
M.G.3		R -	R ++1/80	R ++1/80	R ++1/20	R ++1/10	R ++1/20	R ++1/20	R ++1/5 +1/10
		U -	U ++1/80	U ++1/80	U ++1/40	U ++1/20	U ++1/40	U ++1/40	U ++1/10
C. 1	Uninoculated Controls	R -	R -	R -	R -	R -	R -	R -	R -
		U -	U -	U -	U -	U -	U -	U -	U -
C. 2		R -	R -	R -	R -	R -	R -	R -	R -
		U -	U -	U -	U -	U -	U -	U -	U -
C. 3		R -	R -	R -	R -	R -	R -	R -	R -
		U -	U -	U -	U -	U -	U -	U -	U -

++ denotes complete inhibition
+ denotes partial inhibition
R = treated serum samples
U = untreated serum samples

Table 2 Serological response of the turkey poults to WRI showing the H.I. titre of the RDE treated and untreated serum samples

Poult No.		H.I. titre at inoculation	Post inoculation period in weeks			
			2	3	4	5
W.1	Birds inoculated with WRI	R - U ++1/20	R ++1/10 U ++1/160	R ++1/10 U ++1/320	R +1/5 U ++1/320	R +1/5 U ++1/320*
W.2		R - U ++1/20	R ++1/20 U ++1/320	R ++1/20 U ++1/320	R ++1/5 +1/10 U ++1/320	R ++1/5 +1/10 U ++1/320*
W.3		R - U ++1/10	R ++1/20 U ++1/320	R ++1/10 R ++1/320	R +1/5 U ++1/320	R +1/5 U ++1/320*
C. 4	Uninoculated Controls	R - U ++1/20	R - U ++1/80	R - U ++1/80	R - U ++1/80	R - U ++1/80
C. 5		R - U ++1/20	R - U ++1/80	R - U ++1/80	R - U ++1/80	R - U ++1/80
C.6		R - U ++1/20	R - U ++1/40	R - U ++1/80	R - U ++1/80	R - U ++1/80

++ denotes complete inhibition
+ denotes partial inhibition
R = treated serum samples
U = untreated serum samples
* H.I. titre possibly higher

Table 3 Effect of Chemical and Enzyme treatments on the titre of specific antibodies against *M. gallisepticum*

Serum treatment	H. I. titre					
	Positive serum		Negative serum			
	1	2	1	2	3	4
No treatment	1/40	1/20	-	-	-	-
R.D.E.	1/40	1/20	-	-	-	-
Kaolin	1/40	1/20	-	-	-	-
Bentonite	1/40	1/20	-	-	-	-
Trypsin	1/40	1/20	-	-	-	-

Table 4 Chemical and Enzyme treatments on the titre of inhibitory substances and specific antibodies against WRI antigen

Serum treatment	H. I. titre								
	Negative serum						Positive serum		
	1	2	3	4	5	6	1	2	3
No treatment	1/40	1/80	1/20	1/20	1/20	1/320	1/320	1/320	1/320
R.D.E.	-	-	-	-	-	-	1/10	1/5	1/10
Kaolin	-	-	-	-	-	-	1/10	1/5	1/10
Bentonite	-	-	-	-	-	-	1/10	1/5	1/10
Trypsin	1/5	1/5	1/5	1/5	1/5	1/20	1/20	1/5	1/10

Section 6

The Inactivation of Mycoplasma using Beta-propiolactone

Summary The inactivation of five strains of Mycoplasma with beta-propiolactone is described. Inactivation of all strains occurred at a concentration of 0.1%.

Introduction

Beta-propiolactone (BPL) has been used successfully in the preparation of killed Newcastle disease vaccines produced from infected embryonated chicken eggs. The concentration of BPL used by the different workers has varied, Winmill and Weddell (1961) and Simmins and Baldwin (1963) used 0.25%; Haig et al (1962) used 0.033%; Sullivan et al (1958) and Gill et al (1959) used a concentration of 0.1%. The concentration of BPL in the commercial vaccines used in this country is 0.1%.

LoGrippo et al (1955) studied the concentration of BPL required to inactivate viruses, fungi, bacteria and desiccated spores. The concentration required to inactivate 8 viruses in undiluted allantoic fluid was 0.05%, that required to kill 5 pathogenic fungi in buffered phosphate solution ranged from 0.25% to 0.5%. that required to kill vegetative bacteria ranged from 0.1% to 0.2%, and to kill desiccated spores from 0.5% to 0.75%.

LoGrippo and Hartman (1954) found that virus inactivation using BPL occurred within a pH range of 5.0 to 9.0.

Chronic respiratory disease (CRD) is one of the important diseases in poultry and this is especially true in the broiler industry. It has been generally accepted that Mycoplasma gallisepticum is the cause of chronic

respiratory disease. Egg transmission has been demonstrated by a number of workers to be a common source of dissemination of the disease from one generation to the next (Cover and Waller 1954; Fahey and Crawley 1954; Van Roekel et al 1958; Fabricant et al 1959; Olesiuk and Van Roekel 1960; Olson et al 1962).

This report deals with the inactivation of four Mycoplasma species with BPL. These included two strains of Mycoplasma gallisepticum, S6 (Zander 1961) and X95; Mycoplasma gallinarum (Edward and Kanarek 1960); Mycoplasma iners (Edward and Kanarek (1960) and the WRI strain of Mycoplasma (See Section 2). Dr. D.G. ff. Edward kindly supplied X95 strain which was originally isolated by Markham.

Materials and Methods

The culture media used were PPLO agar or broth (Difco), enriched with 1% yeast autolysate (Albimi), 10% horse serum and 0.1% dextrose; the pH was adjusted to 7.8.

BPL in its concentrated form was kept at -40°C until ready for use. BPL solutions of 10% and 1% were made with distilled water at 4-5°C, the drug and diluent being kept at this temperature. This was done as quickly as possible as BPL hydrolyses rapidly in water.

BPL was added to 24 hour broth cultures of the Mycoplasma strains to obtain final concentrations of 0.01, 0.05, 0.1, 0.15 and 0.2% solutions. The number of organisms in these experiments exceeded 200 million per ml. as estimated by the method of Miles and Misra (1938). The broths were then incubated at 37°C and the rate of Mycoplasma inactivation assayed in broth

at 15, 30, 45, 120 and 180 minutes. At each interval 0.5 ml. of broth was subcultured into 3 x 10 ml. broth; the broths were incubated at 37°C and examined daily for 7 days. Mycoplasma inactivation was indicated by the lack of growth in the broth. Growth of Mycoplasma was established by subculturing the broths onto PPLO agar and examining for typical colonies. With the haemagglutinating strains, growth was also indicated by the haemagglutinating activity of the broth.

Results

The varying concentrations of BPL and times required to inactivate the five strains of Mycoplasma are recorded in tables 1 to 5.

The two strains of M. gallisepticum and M. gallinarum were inactivated by 0.1% concentration of BPL; the WRI strain and M. iners were inactivated with 0.05%.

Table 1 M. gallisepticum (X95)

BPL Concentration	Time in minutes				
	15	30	45	120	180
0.2%	3/3	0/3	0/3	0/3	0/3
0.15%	3/3	0/3	0/3	0/3	0/3
0.1%	3/3	0/3	0/3	0/3	0/3
0.05%	3/3	3/3	3/3	3/3	3/3
0.01%	3/3	3/3	3/3	3/3	3/3

Table 2 M. gallisepticum (S6)

BPL Concentration	Time in minutes				
	15	30	45	120	180
0.2%	3/3	0/3	0/3	0/3	0/3
0.15%	3/3	1/3	0/3	0/3	0/3
0.1%	3/3	3/3	2/3	0/3	0/3
0.05%	3/3	3/3	3/3	3/3	3/3
0.01%	3/3	3/3	3/3	3/3	3/3

Table 3 M. iners

BPL Concentration	Time in minutes				
	15	30	45	120	180
0.2%	3/3	0/3	0/3	0/3	0/3
0.15%	3/3	0/3	0/3	0/3	0/3
0.1%	3/3	2/3	0/3	0/3	0/3
0.05%	3/3	3/3	3/3	0/3	0/3
0.01%	3/3	3/3	3/3	3/3	3/3

Table 4 M. gallinarum

BPL Concentration	Time in minutes				
	15	30	45	120	180
0.2%	3/3	0/3	0/3	0/3	0/3
0.15%	3/3	0/3	0/3	0/3	0/3
0.1%	3/3	0/3	0/3	0/3	0/3
0.05%	3/3	3/3	3/3	3/3	3/3
0.01%	3/3	3/3	3/3	3/3	3/3

Table 5 WRI Strain of Mycoplasma

BPL Concentration	Time in minutes				
	15	30	45	120	180
0.2%	3/3	0/3	0/3	0/3	0/3
0.15%	3/3	0/3	0/3	0/3	0/3
0.1%	3/3	0/3	0/3	0/3	0/3
0.05%	3/3	2/3	2/3	0/3	0/3
0.01%	3/3	3/3	3/3	3/3	3/3

The failure to inactivate the Mycoplasma as indicated by the presence of growth is expressed as the numerator in the above tables. The denominator gives the number of broths inoculated.

Discussion

Inactivation of *Mycoplasma gallisepticum* occurred in serum broth cultures containing BPL at a concentration of 0.1%; inactivation did not occur at 0.05 and 0.01%. These results indicate that it would be unwise to use BPL at concentrations less than 0.1% in the preparation of Newcastle disease vaccines, unless the embryonated eggs were obtained from a *M. gallisepticum* free flock. It must be noted, however, that in these inactivation experiments serum broth cultures were used, and not Mycoplasma-infected embryonated eggs.

Experimental Infection of Chickens with Mycoplasma Gallisepticum and subsequent re-isolation of the Organism from the Body Tissues

Summary

Three groups of birds were experimentally infected with Mycoplasma gallisepticum via the sinus, trachea and abdominal air sacs. Up to 9 weeks after inoculation, the organism was re-isolated from the sinus, trachea and abdominal air sacs, regardless of the original route of inoculation. The antibody response as determined by the haemagglutination-inhibition test, is described. Immunity to M. gallisepticum is discussed.

Introduction

In fowls, McMartin and Adler (1961) produced results to show that the intranasal instillation of Mycoplasma gallisepticum produces resistance against homologous challenge in the abdominal air sacs. These authors called this resistance immunity, and they produced results to show the inability of M. gallisepticum to progress from the nasal cavity to the abdominal air sacs after instillation in the nasal cavity.

When Olson et al (1962) infected birds intranasally with M.gallisepticum, they were able to recover the organism from the trachea readily up to 6 weeks post-inoculation, but after this a rapid decline occurred in the percentage of recoveries.

The experiments reported here provide additional information on the experimental infection of fowls with M. gallisepticum and the recovery of the organism from various sites at varying post inoculation periods; the antibody

response as determined by the haemagglutination-inhibition test, and the clinical signs and lesions produced.

Materials and Methods

Four week old Rhode Island Red chicks were obtained from a respiratory disease free flock. Groups of 20 birds were inoculated with 0.25 ml. of a 24 hour broth culture of Mycoplasma gallisepticum via 1) intranasal sinus, 2) trachea, and 3) posterior abdominal air sac. Each group was kept in complete isolation.

Once a week for the first 9 weeks following inoculation and then at varying periods, the birds were examined and bled. Each blood sample was tested for haemagglutination-inhibition antibodies to Mycoplasma gallisepticum using 4 partial haemagglutinating units by the method described in Section (2). Each week also during the first 6 weeks, 2 birds were killed from each group, and at each later blood sampling, one bird was killed.

Attempts were made to re-isolate M. gallisepticum from the killed birds. Cultures were made into broth from,

1) abdominal air sacs,

2) infraorbital sinuses,

3) trachea

To ensure that the strain of Mycoplasma was the same as that inoculated, broth cultures were tested for their haemagglutinating activity and, if they were positive, the culture were tested serologically by means of the H.I. test using specific M. gallisepticum immune serum. If the broth cultures were so badly contaminated that the contamination could not be checked by the use of

penicillin and thallium acetate, the broth cultures were plated on to agar, and if any Mycoplasma colonies were present they were picked from the agar plates and propagated in broth.

The culture media used were: PPLO agar or broth (Difco), enriched with 1% yeast autolysate (Albimi), 10% horse serum and 0.1% dextrose with the pH adjusted to 7.8. For re-isolation of M. gallisepticum from the killed birds, penicillin (1000 Units per ml;) and thallium acetate (1:5000) was added to the media.

Results

The recovery of M. gallisepticum from the body tissues at varying periods after inoculation are summarized in table 1.

Group (1). Intra-sinus inoculation. It was possible to recover the organism up to 7 weeks after inoculation. During this 7 week period 12 birds were killed and the organism was isolated from (a) sinus: 9 times, (b) trachea: 9 times, (c) abdominal air sacs: 5 times.

Group (2). Intra-tracheal inoculation. The organism was recovered from the tissues up to 8 weeks after inoculation. Out of 12 birds killed, the organism was isolated from (a) sinus: 10 times, (b) trachea: 10 times, (c) abdominal air sacs: twice.

Group (3). Posterior abdominal air sac inoculation. It was possible to recover the organism from the tissues upto 8 weeks after inoculation. Out of 12 birds killed, the organism was isolated from (a) sinus: 5 times, (b) trachea: 8 times, (c) abdominal air sacs: 6 times.

The serological response to M. gallisepticum by the various routes of inoculation is given in table 2. Blood samples taken one week after inoculation showed that the number of positive reactors was greater after intra-tracheal inoculation with 70% of the birds reacting; with intra-sinus inoculation only 30% of the birds reacted, and with posterior abdominal air sac inoculation only 20% reacted. All birds gave an H.I. response 3-4 weeks after inoculation. The highest titre (1/160) was obtained 4 weeks after inoculation. This titre was obtained in 57% of those inoculated via the trachea, in 21% of those inoculated via the abdominal air sacs and in 7% of those inoculated via the infraorbital sinus. After 4 weeks, the titres in the three groups gradually declined. After 18 weeks when there were 4 birds left in each group, the titres varied from 1/5 to 1/40. The details are given below:

Birds inoculated via	H. I. titres			
	1	2	3	4
(1) Sinus	1/40	1/10	1/5	1/20
(2) Trachea	1/10	1/20	1/20	1/10
(3) Abdominal air sacs	1/20	1/20	1/5	1/40

The clinical signs and gross pathological lesions seen were similar to those ascribed to chronic respiratory disease in fowls (See Section 1.).

Discussion

Mycoplasma gallisepticum was recovered from the sinuses, trachea and abdominal air sacs of birds in the three groups and the route of inoculation did not appear to have much bearing on the frequency or site of recovery. A similar number of isolations of M. gallisepticum was obtained from the abdominal air sacs of birds inoculated intra-sinously and of those inoculated via the posterior abdominal air sacs. These results do not agree with those of McMartin and Adler (1961), who inoculated 8 susceptible birds intranasally with 10^8 organisms and found that in only one bird did air sac lesions occur and that the organism was subsequently recovered from only the air sacs of that bird. One explanation of this discrepancy could be the conditions under which the experiments took place. The presence of other diseases could have facilitated the spread of M. gallisepticum from the sinuses to the abdominal air sacs but examination of pooled serum samples taken from the 3 groups, did not reveal the presence of antibodies to Newcastle disease or infectious bronchitis. The explanation could be in differences in the technique and media used in the re-isolation of M. gallisepticum, but the media used in these investigations was essentially the same as that used by McMartin and Adler (1961). The only difference in the current experiments was in the use of fresh inactivated horse serum, instead of commercial horse serum as better growth was thereby obtained. A third explanation could be in the number of organisms inoculated. When a large inoculum is injected into the sinus, Mycoplasma will certainly be inhaled into the trachea and abdominal air sacs, whereas with a small inoculum the organism might be retained in the sinuses. An estimate of the number of organisms inoculated was not made in these investigations.

Eight weeks after inoculation M. gallisepticum was not recovered from the body tissues from any birds in the 3 groups. The antibody titre to M. gallisepticum persisted for 26 weeks. It was not possible in view of the numbers involved to investigate fully the recovery of the organism from each site (see Section 1). It should however be noted that Olesiuk and Van Roekel (1960) showed that birds which had contracted the disease 12 months previously, and were serologically positive to M. gallisepticum were capable of eliminating the organism through the egg and transmitting the infection to negative birds through cohabitation.

The decline in recovery appeared to be associated with a decline in respiratory signs. The failure of recovery might be associated with a change in habitat of the Mycoplasma, from extracellular to intracellular. It might also, be the result of a change in the organism itself; the organism having to re-adapt itself to the PPLO media, in a similar manner to field strains of the organism.

It has been demonstrated (Klieneberger-Nobel 1962) that Mycoplasma remain latent in the tissues of certain animals and when circumstances arise which lower the host's resistance they multiply and become actively pathogenic with the production of disease. Latency is known to occur in virus infections, for example herpes simplex. Here the virus is continually present in the tissue and so is circulating antibody to the virus. The disease manifests itself only under the stimulus of irritation, loss of sleep, infection with the virus of the common cold etc. The evidence suggests that M.gallisepticum occurs in the form of a latent infection, as the organism persists in the tissues for a considerable period of time and possibly throughout the life of

the fowl; the persistence of serum antibody adds further support to this theory. An equilibrium is set up between circulating antibody and the presence of M. gallisepticum in the tissues. This equilibrium is upset by the occurrence of other diseases such as Newcastle disease, infectious bronchitis and bacterial infections such as E. coli.

It is important to establish whether or not M. gallisepticum occurs in fowls as a latent infection, if it does the efficacy of vaccination would be in doubt. Fabricant and Levine (1963) have used live cultures of M. gallisepticum in the vaccination of young chickens in an effort to control egg transmission of the organism. When the birds inoculated during their growing period reach maturity, they are less likely to transmit the organism via the egg, in this way producing a Mycoplasma gallisepticum free flock from an infected parent flock. Fabricant and Levine (1963) showed that M. gallisepticum was not shed in the eggs after challenge with a virulent culture. These results might further the view that M. gallisepticum exists as a latent infection and that challenge did not upset the equilibrium between the latent organism and the circulating antibodies.

Various authors (Domermuth 1962; McMartin and Adler 1961; Adler, McMartin and Shifrine 1960) claim that Mycoplasma gallisepticum infection, both natural and experimental, produces demonstrable immunity both in chickens and turkeys against homologous challenge via the air sacs. If the organism, however, is already latent, homologous challenge would not have any effect unless the equilibrium between the organism and circulating antibodies was upset.

Olesiuk and Van Roekel (1960b) studied contact transmission between naturally infected and susceptible stock measuring the transmission by the agglutinin response in the susceptible flock. It took 3 months before all the birds gave a positive reaction. The authors suggest that the delayed appearance of agglutinin could mean that the widespread transmission in a flock may be related to the development of active cases of disease among the birds. This lends support to the theory that the disease can become latent, and when the equilibrium between circulating antibody and organism is upset, for example some stress factor, the organism is liberated into the respiratory tract and transmission by airborne infected dust or droplets takes place. In a flock where there are no clinical signs of CRD but positive serological reactors to M. gallisepticum, the organism is possibly latent in the majority of birds with the disease active in individual birds. This could account for the slow rate of spread.

The question of latency occurring with Mycoplasma gallisepticum has not been adequately investigated and further work is necessary to elucidate this point.

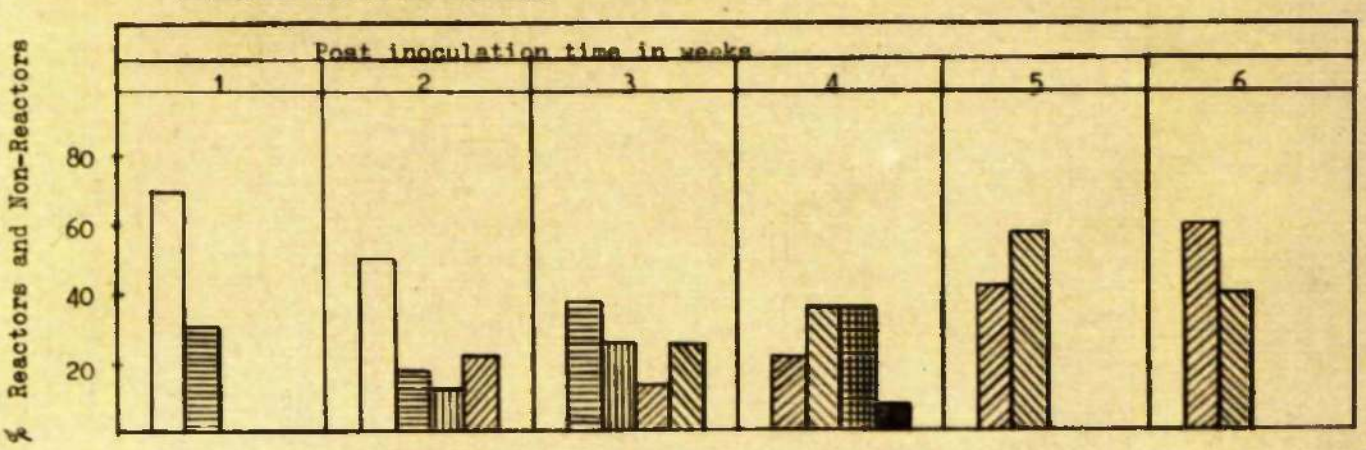
Table 1

Recovery of M. gallisepticum at varying post inoculation periods

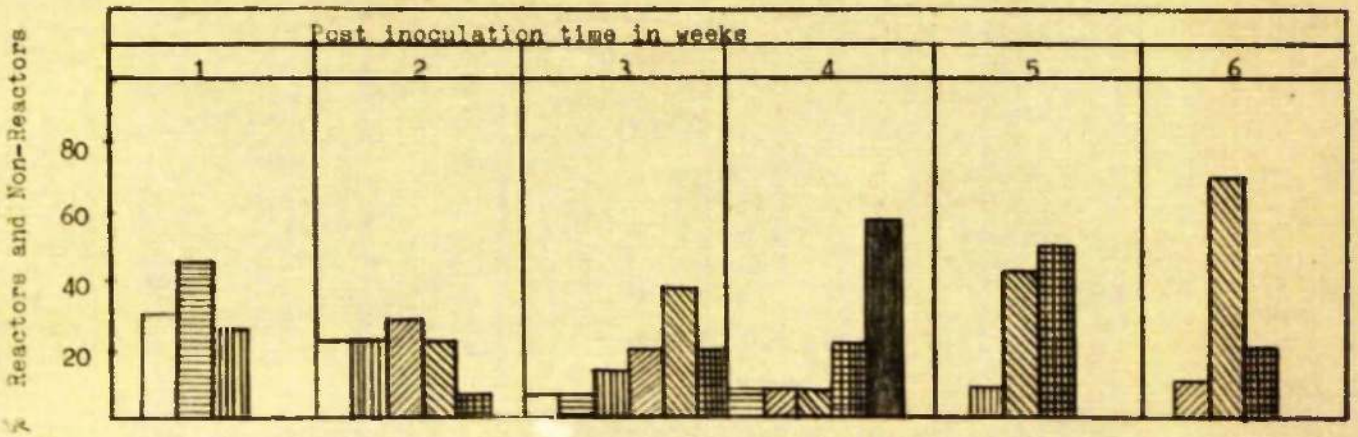
Route of injection	Post inoculation period in weeks												
	1	2	3	4	5	6	7	8	9	11	13	18	22	26
Intrasinus inoculation	1) S+ T- A- 2) S+ T+ A-	1) S- T- A- 2) S- T- A-	1) S- T+ A- 2) S+ T+ A-	1) S+ T+ A+ 2) S+ T+ A+	1) S+ T+ A+ 2) S+ T+ A-	S+ T+ A+	S+ T+ A+	S+ T- A-	S- T- A-	S- T- A-	S- T- A-	S- T- A-	S- T- A-	1) S- T- A- 2) S- T- A-
Tracheal inoculation	1) S+ T+ A- 2) S+ T- A-	1) S+ T- A- 2) S+ T- A+	1) S+ T+ A- 2) S+ T+ A-	1) S+ T+ A- 2) S+ T+ A-	1) S- T+ A- 2) S+ T+ A-	S- T+ A-	S+ T+ A+	S+ T+ A-	S- T- A-	S- T- A-	S- T- A-	S- T- A-	S- T- A-	1) S- T- A- 2) S- T- A-
Posterior Abdominal Air sac Inoculation	1) S- T+ A- 2) S+ T+ A-	1) S+ T+ A+ 2) S+ T+ A-	1) S- T+ A+ 2) S- T+ A+	1) S+ T+ A- 2) S- T- A+	1) S- T- A+ 2) S- T- A-	S- T- A-	S+ T- A+	S- T+ A-	S- T- A-	S- T- A-	S- T- A-	S- T- A-	S- T- A-	1) S- T- A- 2) S- T- A-

Table 2. Serological Response to M. gallisepticum.

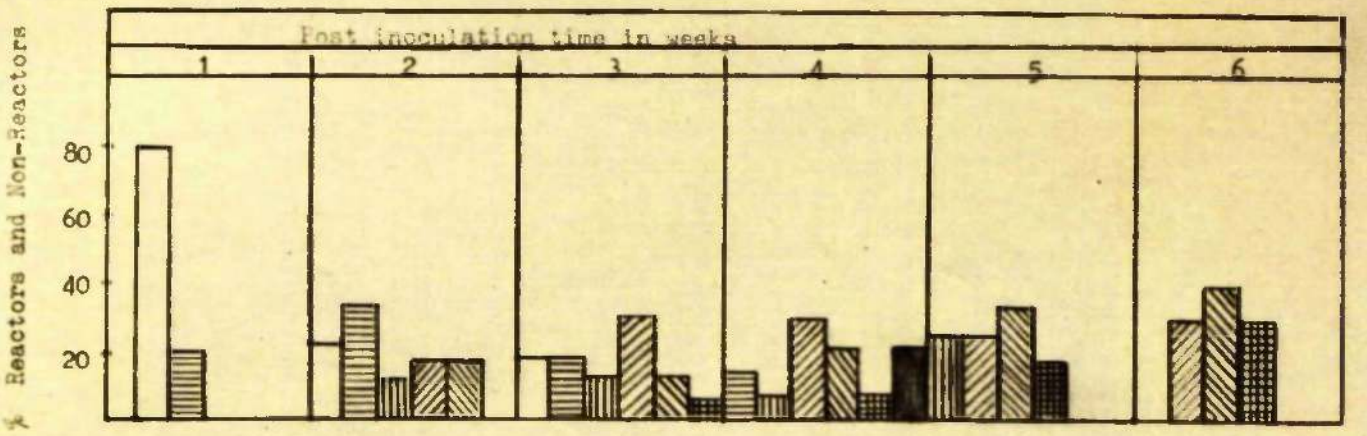
(a) Intra-sinus inoculation.



(b) Intra-tracheal inoculation.



(c) Posterior Abdominal Air Sac inoculation.



Section 8

Serotypes of Avian Mycoplasma

Summary

Thirty one strains of Mycoplasma of avian origin were classified serologically into 8 groups by the tube agglutination test. These antigenic groups were confirmed with the haemagglutination-inhibition test. The haemagglutinating activity of the Mycoplasma strains were investigated using erythrocytes of various mammals and birds. All avian Mycoplasma strains were found to agglutinate mouse red blood cells. Their ability to ferment carbohydrates and other cultural characteristics were also investigated and their results correlate with the serological classification.

Introduction

Recent studies have shown that various antigenic and pathogenic types of Mycoplasma are present in poultry tissues. Adler and Yamamoto(1957) isolated two distinct strains of Mycoplasma from a turkey with infectious sinusitis: a pathogenic strain which produced sinusitis in turkeys and respiratory lesions in chickens, and a non pathogenic strain.

Yamamoto and Adler(1958) characterized 5 distinct serotypes designated S6, SA, C, O, N. Kleckner(1960) confirmed these five and characterized three additional serotypes and simply designated the eight serotypes as A, B, C, D, E, F, G and H. His new types were B, D, and E.

Chalquest and Fabricant(1960) isolated a Mycoplasma associated with infectious synovitis in chickens and turkeys. Yoder and Hofstad(1962) isolated another serotype from air sac lesions in turkeys characterized and identified as Iowa 695.

This makes a total of ten serotypes of Avian Mycoplasma described by these workers, and those reported pathogenic for birds are the S6 serotype of Yamamoto and Adler(1958), the Iowa 695 strain of Yoder and Hofstad(1962) and the agent of infectious synovitis of Chalquest and Fabricant(1960). Generic names were assigned to three of these groups(Edward and Kanarek 1960): the name M. gallisepticum was given to the S6 serotype of Yamamoto and Adler(1958) and represented by Kleckner (1960) group A; the name M. gallinarum was given to the serotype represented in Kleckner(1960) group B; the name M. iners was given to the O serotype of Yamamoto and Adler(1958) which belonged to group C of Kleckner(1960). The work mentioned above has been done by American workers with strains isolated in the U.S.A.

This report describes the classification of avian Mycoplasma isolated in Great Britain. The strains were isolated from chickens and turkeys at the Central Veterinary Laboratory, Weybridge during investigati into respiratory diseases. Included in the investigations were M. gallisepticum, M. gallinarum, M. iners, M. anatis(see Section 3), WR1 strain of Mycoplasma(see Section 2). M. gallisepticum(S6 strain) was obtained from Dr.D.A.McMartin, Veterinary Laboratory,Lasswade;

M. gallinarum and M. iners were obtained from Dr. D. G. ff. Edward, Wellcome Research Laboratories, Beckenham.

The strains were grouped on the basis of the tube agglutination test. The Mycoplasma were tested for their ability to ferment carbohydrates and their ability to agglutinate red blood cells of various mammals and birds.

Materials and Methods

The culture media used were PPLO agar or broth(Difco), enriched with 1% yeast autolysate(Albimi), 10% horse serum and 0.1% dextrose with the pH adjusted to 7.8. Fermentation reactions in the broth were determined by replacing the dextrose by 1% of other carbohydrates, adding phenol red as indicator and adjusting the pH to 7.4. The isolates were tested for their ability to utilize the following carbohydrates: lactose, maltose, sucrose, glucose, galactose, trehalose, mannose, dextrin and starch. The tubes were inoculated with three drops of a 24 hour broth culture of the Mycoplasma strains and the tubes incubated at 37°C and examined daily for 10 days.

All Mycoplasma strains examined were submitted to a purification process to minimise the possibility of studying mixed cultures. The strains were cultured onto PPLO agar and passaged many times to ensure uniformity of such characteristics as colony type and growth rate. Finally single colonies were picked from the agar plates and propagated in broth.

Specific immune serum was prepared in rabbits against the Mycoplasma strains. Two hundred ml. of PPLO broth was inoculated with 10 ml. of stock culture and incubated at 37'C for 72 hours. The culture was then centrifuged at 3,500 r.p.m. for 30 minutes; the supernate was decanted, and the broth sediment was resuspended in physiological saline. The cells were washed four times and finally resuspended in 12 ml. physiological saline and stored at -10'C in 2 ml. amounts. Rabbits were given 6 intravenous injections of 0.5-1.0 ml. of the antigen over a period of 21 days and were bled out 7 days after the last injection.

Serum titres and cross agglutination titres were determined by tube agglutination with the Mycoplasma strains using as antigen:

a) a 24 hour broth culture; all antigens were standardized for turbidity with a nephelometer using PPLO broth as diluent.

b) A washed suspension of Mycoplasma cells, resuspended in physiological saline and standardized to 'Wellcome' opacity tube No. 4.

The rabbit sera were diluted with physiological saline by twofold dilutions starting 1/4; 0.3 ml. of antigen was added to 0.3 ml. amounts of serum dilutions. The tubes were shaken and incubated in a water bath at 56'C for 18-24 hours. Controls were run with each test.

The mammalian and avian red blood cells used in the haemagglutination studies were prepared by adding freshly drawn blood to a 4% solution of potassium oxalate, which was shaken gently to prevent coagulation. The cells were centrifuged and washed three times in

physiological saline and finally packed by centrifugation at 1,750 r.p.m.. A 1% concentration of red blood cells was then prepared from the packed cells for the respective tests. The Mycoplasma strains were tested for their ability to agglutinate red blood cells of the following species: fowl, pheasant, turkey, gull, pigeon, duck, rabbit, mouse, sheep, guinea-pig, horse and human.

The antigens used in the haemagglutination studies were prepared by growing the strains in PPLO broth. Twenty four or forty eight hours later depending on the rate of growth, the broth cultures were centrifuged at 3,500 r.p.m. for 30 minutes and the cells resuspended in 50% phosphate buffered glycerol(pH 7.2). Haemagglutination of the red blood cell suspension was studied by the titration of the Mycoplasma suspension using 0.25 ml. amounts with equal volumes of a 1% suspension of washed red blood cells. Readings were made after the plates had been left at room temperature for at least 60 minutes. Suspensions of the mammalian red blood cells often took much longer to settle.

Results

Serological Studies: Table 1 gives the results of the homologous and cross agglutination titres. The strains may be separated into 8 distinct antigenic types. In the agglutination tests it was found that antigen (a), the 24 hour broth culture, was much more stable and reliable than antigen (b), the washed suspension of Mycoplasma cells; frequently spontaneous agglutination occurred with antigen (b). The isolates other than M. gallisepticum produced the 'large flake' type of agglutination with antigen (a), with large easily visible clumps, whereas with M. gallisepticum isolates the agglutination was of the 'small flake' or finely granular type. These tests were often difficult to read. With the M. gallisepticum strains the results in table 1 are those using antigen (b) as the floccules obtained were more easily visible to the naked eye, whilst with all the other strains the results are those obtained with antigen (a).

A large number of strains isolated fell into group 8, represented by M. gallinarum which was included in group B of Kleckner(1960). Antiserum was prepared against M. gallinarum and 4 other strains placed in group 8. Thirteen strains were placed in group 8; all thirteen reacted with M. gallinarum antiserum, but not all the strains reacted with the 4 other antisera as shown in table 1. On this basis group 8 can be subdivided, but the exact relationship between the subgroups has not been ascertained.

No Mycoplasma isolated were found to react with Iowa 695 and M. iners. Isolate 186 was found to be antigenically distinct from the other isolates and was placed in group 6. Group 7 contained 4 isolates which appeared identical. Group 3 included the WR1 strain(see Section 2) and three other isolates which were found to be antigenically similar. Broth cultures of WR1 agglutinated 1% suspension of washed fowl red blood cells but broth cultures of the other three did not.

Carbohydrate Fermentation: The activity of the isolates in the fermentation of carbohydrates is summarized in table 2. The Mycoplasma could be conveniently subdivided into fermenters and non fermenters. The fermentation reactions of the isolates in each group were similar. None of the isolates in groups 5, 6 and 8 fermented any of the sugars, whereas the isolates in groups 1, 2, 3, 4 and 7 were fermenters.

In the fermenter groups, all strains were positive to maltose, glucose, dextrin and starch. In groups 1 and 2 only was mannose fermented, and in groups 3 and 7 only was sucrose fermented. Lactose, galactose and trehalose was not fermented in any of the groups.

The rate of growth and colony size obtained on PPLO agar are summarized in table 4. Two major types occurred: groups 1 and 2 produced small colonies with slow rate of growth, requiring 48-72 hours incubation; in groups 3-8 the rate of growth was rapid, often requiring only 24 hours incubation, and larger colonies were produced.

The results of the <u>haemagglutination tests</u> are shown in table 3. One strain from each group was tested except in group 3 where 2 isolates, WR1 and W120, were tested. All the strains examined agglutinated mouse red blood cells. The strains S6, WR1 and Iow 695 agglutinated red blood cells of all species examined except those of the horse and even with these cells S6 caused partial agglutination. M. anatis agglutinated the red blood cells of horse, human and all the avian species except fowl. M. inerc and isolate 186 agglutinated fowl, other avian and mammalian red blood cells. Pheasant red blood cells were the only avian cells agglutinated by M. gallinarum, but it did agglutinate human and guinea-pig red blood cells. Isolate W120 was unique in that mouse red blood cells were the only cells agglutinated. Isolate 658 agglutinated gull red blood cells and other mammalian cells.

It was possible to type the Mycoplasma strains into 5 groups using the H.I. technique as described in Section 2, using fowl red blood cells and specific rabbit immune serum absorbed with fowl red blood cells. These groups were represented by the strains S6(group 1), Iowa 695(group 2), WR1(group 3), M.inera(group 5), and isolate 186 (group 6). It was possible to type M. gallinarum(group 8) and M.anatis (group 4) with the H.I. test using respectively pheasant and duck red blood cells. In the H.I. tests specific immune serum to group 7 did not inhibit any of the above strains. The serological groupings obtained with the tube agglutination test were confirmed using the H.I. test.

Discussion

The thirty one Mycoplasma strains examined fall into 8 serological groups. The strains isolated at the Central Veterinary Laboratory fell into 6 groups; strains were not isolated which could be included in the two groups containing Iowa 695 and M. iners. These serological groupings were confirmed with the haemagglutination-inhibition test. Yamamoto and Adler(1958) indicated the presence of 5 distinct antigenic types. Kleckner(1960) confirmed these five and characterized three additional types. His results showed that cross agglutination occurred to some degree with most of the isolates and no group was antigenically distinct from any other, with many of the isolates possessing common antigenic components. When Kelton and Van Roekel(1963) re-examined the 8 serotypes of Kleckner(1960) using the tube agglutination test, they found that the serotypes E and G were related and placed them in the same serological group. Their results showed no or very little cross agglutination between the groups.

M. gallisepticum strains have been called 'avian pathogenic Mycoplasma' whilst all the other strains are called non pathogenic. The M. gallisepticum strains isolated(MG1-5) were all similar antigenically to the S6 strain in their rate of growth, cultural characteristics and biochemical reactions. The characteristics of M. gallisepticum were distinct and different from all other Mycoplasma strains isolated at the Central Veterinary Laboratory.

Kleckner(1960) reported that haemagglutinating activity would appear to be significant characteristic of the pathogenic avian Mycoplasma. One might assume that all strains which agglutinate the red blood cells of the various species are in fact pathogenic for those species. It was shown in Section 3, however, that M. anatis stimulates an antibody response in ducks only in the presence of some stress factor such as duck influenza virus. It seems likely that despite their haemagglutinating ability, Mycoplasma other than M. gallisepticum are pathogenic only under adverse conditions. In group 3, while WR1 agglutinates fowl and other avian red blood cells, the other three isolates do not, yet the four strains appear antigenically similar. It can be assumed that the WR1 strain is probably more pathogenic for these species than the other three isolates. The change from non-haemagglutinating to haemagglutinating activity might be associated with the change from a saprophytic to a parasitic mode of life.

To some extent the growth medium and the method of handling the culture govern the results of haemagglutination. The haemagglutinating activity is very sensitive to pH, so that the concentration of dextrose is important. When the fermentation of dextrose occurs, the drop in pH is related to the concentration of dextrose. If the concentration of dextrose is 0.1% or less, the pH of the medium seldom drops below 7.2 and this does not affect the haemagglutinating activity. The culture must be harvested at the right time, with a well adapted strain 24 hours

incubation of the broth is sufficient.

The carbohydrate fermentation reactions correlated with the serological groupings. The strains were either fermenters or non fermenters. Of the fermenters, the reactions of the isolates in each group were very similar. The M. gallisepticum strains, Iowa 695 and M. anatis did not ferment sucrose over a 10 day period while the rest of the fermenters did. Yamamoto and Adler(1958) showed that the S6 strain fermented sucrose at a remarkably slower rate than the non pathogenic strains and they suggested that the strains can be distinguished by this. The S6 strain did not ferment mannose but the British strains(MG1-5) did. The only other strain to ferment mannose was Iowa 695. These results indicate that the inability or slow fermentation of sucrose and the fermentaion of mannose might distinguish M. gallisepticum from the other avian fermenters.

Table 1. Serological Classification of Avian Mycoplasma

	S6	MG1	MG2	MG3	MG4	MG5	Iowa 695	WR1	W120	M. anatis	M. iners	186	658	1743	1744	W106	M. gallinarum	XP1	W57	271	K2
1. M.gallisepticum(S6)	1024	1024	1024	512	512	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MG1	512	1024	512	1024	1024	1024	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MG2	512	1024	1024	512	1024	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MG3	1024	512	512	512	1024	1024	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MG4	1024	1024	**512**	**512**	1024	1024	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MG5	512	512	1024	512	1024	1024	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2. Iowa 695	-	-	-	-	-	-	1024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3. WR1	-	-	-	-	-	-	-	1024	512	-	-	-	-	-	-	-	-	-	-	-	-
W120	-	-	-	-	-	-	-	1024	512	-	-	-	-	-	-	-	-	-	-	-	-
W124	-	-	-	-	-	-	-	1024	512	-	-	-	-	-	-	-	-	-	-	-	-
W128	-	-	-	-	-	-	-	1024	512	-	-	-	-	-	-	-	-	-	-	-	-
4. M. anatis	-	-	-	-	-	-	-	-	-	2048	-	-	-	-	-	-	-	-	-	-	-
5. M. iners	-	-	-	-	-	-	-	-	-	-	1024	-	-	-	-	-	-	-	-	-	-
6. 186	-	-	-	-	-	-	-	-	-	-	-	128	-	-	-	-	-	-	-	-	-
7. 658	-	-	-	-	-	-	-	-	-	-	-	-	1024	1024	2048	512	-	-	-	-	-
1743	-	-	-	-	-	-	-	-	-	-	-	-	1024	1024	2048	512	-	-	-	-	-
1744	-	-	-	-	-	-	-	-	-	-	-	-	1024	1024	2048	512	-	-	-	-	-
W106	-	-	-	-	-	-	-	-	-	-	-	-	1024	512	2048	512	-	-	-	-	-
8. M.gallinarum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2048	64	256	256	2048
XP1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32	256	-	1024	2048
645	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2048	-	128	128	32
W57	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	128	32	512	256	128
W115	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	256	32	64	128	128
W63	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	128	32	512	128	32
W95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	512	16	128	64	128
1036	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	512	-	265	128	256
K2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1024	-	128	128	128
815	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1024	-	256	128	128
W62	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	64	64	512	128	128
649	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2048	-	16	64	64
271	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	64	-	-	1024	256

Table 2. Carbohydrate Fermentation Reactions of Avian Mycoplasma

	Lactose	Maltose	Sucrose	Glucose	Galactose	Trehalose	Mannose	Dextrin	Starch	
M.gallisepticum	NC	A	NC	A	NC	NC	NC	A	A	
MG1	NC	A	NC	A	NC	NC	NC	A	A	Group 1.
MG2	NC	A	NC	A	NC	NC	A	A	A	
MG3	NC	A	NC	A	NC	NC	A	A	A	
MG4	NC	A	NC	A	NC	NC	A	A	A	
MG5	NC	A	NC	A	NC	NC	A	A	A	
Iowa 695	NC	A	NC	A	NC	NC	A	A	A	Group 2.
WR1	NC	A	A	A	NC	NC	NC	A	A	Group 3.
W120	NC	A	A	A	NC	NC	NC	A	A	
W124	NC	A	A	A	NC	NC	NC	A	A	
W128	NC	A	A	A	NC	NC	NC	A	A	
M. anatis	NC	A	NC	A	NC	NC	NC	A	A	Group 4.
M. inere	NC	NC	NC	NC	NC	NC	NC	NC	NC	Group 5.
186	NC	NC	NC	NC	NC	NC	NC	NC	NC	Group 6.
658	NC	A	A	A	NC	NC	NC	A	A	Group 7.
1743	NC	A	A	A	NC	NC	NC	A	A	
1744	NC	A	A	A	NC	NC	NC	A	A	
W106	NC	A	A	A	NC	NC	NC	A	A	
M.gallinarum	NC	NC	NC	NC	NC	NC	NC	NC	NC	Group 8.
XP1	NC	NC	NC	NC	NC	NC	NC	NC	NC	
645	NC	NC	NC	NC	NC	NC	NC	NC	NC	
W57	NC	NC	NC	NC	NC	NC	NC	NC	NC	
W115	NC	NC	NC	NC	NC	NC	NC	NC	NC	
W63	NC	NC	NC	NC	NC	NC	NC	NC	NC	
W95	NC	NC	NC	NC	NC	NC	NC	NC	NC	
1036	NC	NC	NC	NC	NC	NC	NC	NC	NC	
K2	NC	NC	NC	NC	NC	NC	NC	NC	NC	
816	NC	NC	NC	NC	NC	NC	NC	NC	NC	
W62	NC	NC	NC	NC	NC	NC	NC	NC	NC	
649	NC	NC	NC	NC	NC	NC	NC	NC	NC	
271	NC	NC	NC	NC	NC	NC	NC	NC	NC	

A=Acid only
NC=No Change

Table 3. Haemagglutinating Activity of Avian Mycoplasma

	Fowl	Duck	Pheasant	Turkey	Gull	Pigeon	Rabbit	Sheep	Guinea-pig	Mouse	Horse	Human	
M.gallisepticum	+	+	+	+	+	+	+	+	+	+	+*	+	Group 1
Iowa 695	+	+	+	+	+	+	+	+	+	+	−	+	Group 2
WR1	+	+	+	+	+	+	+	+	+	+	−	+	Group 3
W120	−	−	−	−	−	−	−	−	−	+	−	−	
M. anatis	−	+	+	+	+	+	−	−	+	+	+	+	Group 4
M. iners	+	+*	+	+	+	+*	−	+	−	+	−	+	Group 5
186	+	−	+	+	+*	−	−	−	−	+	−	−	Group 6
658	−	−	−	−	+	−	+	−	−	+	−	+	Group 7
M.gallinarum	−	−	+	−	−	−	−	−	+	+	−	+	Group 8

+ indicates agglutination

+* indicates partial agglutination

Table 4.

Group	Culture Characteristics		Carbohydrate fermentation	Haemagglutinating Activity: Fowl Erythrocytes
	Rate of Growth	Colony Size		
1	Slow	Small	+	+
2	Slow	Small	+	+
3	Rapid	Large	+	±
4	Rapid	Large	+	-
5	Rapid	Large	-	+
6	Rapid	Large	-	+
7	Rapid	Large	+	-
8	Rapid	Large	-	-

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