CHARACTERIZATION OF AVIAN MYCOPLASMA

by

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INTRODUCTION

The present study was initiated in 1957, when it was just becoming apparent that there was more than one type of avian Mycoplasma. Dr. M. S. Hofstad[1] had isolated and maintained numerous cultures of Mycoplasma from turkeys, chickens and one pigeon during the previous 10 years, and had noted some differences among them in preliminary cultural, biochemical, morphological and pathogenic characterization studies. A need for more complete characterization studies was apparent.

The main purpose of this study was to characterize numerous isolates of Mycoplasma from chickens, turkeys and other birds in an effort to determine their possible significance in avian diseases.

Aspects to be studied were as follows:

1. Develop media and techniques for the isolation and cultivation of Mycoplasma from numerous types of avian specimens.
2. Investigate the colonial and cellular morphology of representative isolates.
3. Determine the significance of various biological and biochemical characteristics which might aid in the differentiation of

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Mycoplasma types encountered.

A. Hemagglutination

B. Hemolysis

C. Reduction of tetrazolium

D. Carbohydrate fermentation

4. Investigate serological procedures which might aid in establishing various serological groups or serotypes.

A. Rapid serum plate agglutination test

B. Tube agglutination test

C. Hemagglutination-inhibition test

5. Determine the pathogenicity of representative isolates by inoculating experimental hosts.

A. Embryonated chicken eggs

B. Chickens

C. Turkeys

6. Compare representative isolates with those from other laboratories to determine differences and similarities.

REVIEW OF LITERATURE

In 1898 Nocard *et al.* (135) cultivated the infectious agent of bovine pleuropneumonia in a cell-free medium composed of peptone bouillon with 5% bovine serum. The medium was placed in a collodion sac which was inserted into the peritoneal cavity of a rabbit for incubation. Their success in cultivating a filtrable agent in a nonliving medium has become a classic example in microbiology. In 1910 Bordet (22) and Borrel *et al.* (23) described the peculiar morphology of the pleuropneumonia organism. They noted tiny rods of various lengths, almost filamentous at times, rings and pseudo-vibrio forms in Giemsa stained preparations.

Similar organisms were eventually isolated from a variety of animals and man, and became known collectively as the pleuropneumonia group of organisms. The term pleuropneumonia-like organism was adopted to distinguish the various members from the original bovine pleuropneumonia organism. The lengthy designation of pleuropneumonia-like organism was early shortened to the simple PPLO abbreviation.

In the seventh edition of Bergey's Manual of Determinative Bacteriology Freundt (72) classified 14 species from animals and man and one saprophytic species under a single genus, Mycoplasma, which was the genus designation given by Nowak (137) in 1929. The earlier genus designation of Asterococcus, suggested by Borrel *et al.* (23) in 1910, was

invalid because it had previously been used as a generic name for algae. Freundt (72) designated the type species as Mycoplasma mycoides, retaining the specie designation of Borrel et al. (23). He designated Mycoplasmataceae as the single family within the order Mycoplasmatales. Thus, the pleuropneumonia group is now known as Mycoplasma.

Although this thesis is concerned with the characterization of Mycoplasma from avian sources, it is of interest to note that various Mycoplasma have been isolated from humans, cattle, sheep, goats, swine, dogs, cats, rats, mice and poultry as reviewed by Switzer (161).

The numerous observations and investigations which led to the isolation and identification of Mycoplasma associated with avian species have been conducted along several different paths during the past half century. In 1907 Graham-Smith (77) described "swollen head" in turkeys in England. He demonstrated transmission of the disease by direct contact and by inoculation of sinus exudate which caused sinusitis. Tyzzer (166) in 1926 apparently was the first to describe sinusitis in turkeys in the United States. He studied the use of Argyrol in the treatment of sinusitis.

During 1933 to 1939 Nelson (127, 128, 129, 130, 131, 132, 133, 134) reported on a series of studies concerning various forms of coryza in chickens. He observed that typical coryza in chickens was an acute upper respiratory disease which could

readily be transmitted by nasal exudate or by a small Gram negative bacillus, Hemophilus gallinarum, which could be cultivated on artificial medium with some difficulty. Later he noted that in some instances a typical coryza of rapid onset was followed by a coryza of unusually long duration, especially when crude nasal exudate was used as the experimental inoculum. Continued studies resulted in the selection of nasal exudates which caused coryza of rapid onset and short duration, coryza of rapid onset and long duration, or coryza of slow onset and long duration. He demonstrated tiny coccobacilliform bodies in nasal exudate from the lingering disease. He cultivated the coccobacilliform bodies in embryonated eggs and in chicken embryo tissue culture, but noted that the bodies would multiply in tissue culture fluids containing no viable tissue cells and possibly in broth at the base of veal infusion blood agar slants. Nelson mentioned the apparent similarity of the coccobacilliform bodies to pleuropneumonia organisms, but retained his term of coccobacilliform bodies.

In 1936 Beach and Schalm (19) reported that they inoculated 3 turkeys intranasally with nasal exudate from chickens with coryza and produced sinusitis in one turkey by the 5th day and in the other two after 10 days. The sinusitis persisted at least 50 days. In retrospect it appears that Nelson, and Beach and Schalm, actually encountered pathogenic

Mycoplasma of avian origin.

Dickinson and Hinshaw (44) named the disease "infectious sinusitis" of turkeys in 1938. Harr (91) described sinusitis of turkeys in California in 1943, and Hart (93) in 1940 reported on the disease in turkeys in Australia. They tended to believe that it was due to a virus.

In 1943 Delaplane and Stuart (43) reported on the isolation of a virus in embryonated chicken eggs which had been inoculated with exudate from a flock of laying chickens with a chronic respiratory disease. The upper respiratory form of the disease has since been designated as chronic respiratory disease, or more frequently as CRD. They were able to reproduce the typical prolonged respiratory disease in young and mature chickens.

Van Herick and Eaton (168) isolated an unidentified pleuropneumonia-like organism in chicken embryos during studies with atypical pneumonia of humans in 1945. They cultivated the organism in a beef heart infusion broth containing 10% horse serum following inoculation with the amniotic membranes from infected chicken embryos. They noted that the organisms caused hemagglutination, and devised a hemagglutination-inhibition test which they used to demonstrate specific PPLO antibodies in sera from the original supply flock of chickens. The significance of their findings was not immediately realized.

Smith et al. (156) reported on studies concerned with pleuropneumonia-like organisms of human derivation in 1948. They also studied a strain of Nelson's coccobacilliform bodies, which Nelson had adapted to in vitro cultivation. They cultivated his strain in liquid and agar media containing horse serum, and described its tiny colonies and coccoid bodies. They concluded that it was a strain of pleuropneumonia-like organisms.

In 1948 and 1949, apparently still unaware of the possible significance of the continued studies on Nelson's coccobacilliform bodies, Delaplane (42, 41) reported on the lesions produced by the virus of chronic respiratory disease in chicken embryos. In 1948 Groupé et al. (83) described the isolation of an agent resembling the Chlamydozoaceae group which they propagated in chicken embryos from turkey sinus exudate, and Groupé and Winn (82) described the agent in considerable detail in 1949. It had the characteristics of pathogenic PPLO as currently described.

Infectious sinusitis of turkeys was experimentally produced by inoculation of a chicken embryo propagated agent which was considered to be a virus by Jerstad and Hamilton (103) in 1948, and a rickettsia by Hitchner (96) in 1949. Hoyt et al. (102) merely called it the "agent" of infectious sinusitis.

In 1952 Van Roekel and Olesiuk (170) reported on the

successful cultivation of the agent of chronic respiratory disease and infectious sinusitis in Edward's medium, having been informed of studies in progress by Markham and Wong. Markham and Wong (118) in 1952 clearly demonstrated that similar pleuropneumonia-like organisms were the etiological agents of chronic respiratory disease of chickens and infectious sinusitis of turkeys. They cultivated the organisms from chickens and turkeys for several passages in artificial medium, then infected chicken embryos and produced sinusitis in turkeys by inoculating infective yolk into their sinuses. They reisolated the organisms in media and embryos. In 1952 Van Roekel et al. (172) also reported that embryo passaged chronic respiratory disease isolates produced sinusitis in inoculated turkeys.

After the causative agents of chronic respiratory disease in chickens and infectious sinusitis of turkeys were identified as pleuropneumonia-like organisms some of their cultural and biochemical characteristics were investigated. In 1953 Grumbles et al. (87) demonstrated that broth cultures of PPLO from chickens and turkeys fermented dextrose, maltose and sucrose with the production of acid, but not gas. Lactose and mannitol were not fermented. That same year Van Roekel and Olesiuk (171) studied 11 isolates of PPLO from chickens and reported that dextrose and maltose were consistently fermented, trehalose and sucrose were fermented by some

isolates, and lactose, dulcitol and mannitol were not fermented.

These preliminary findings were in general agreement with those reported for various other members of the pleuropneumonia group as summarized by Edward (49), although he reported that sucrose and trehalose were not fermented by the group.

Within the following 10 years a very large number of reports were published concerning numerous aspects of chronic respiratory disease (CRD) and infectious sinusitis. The problem became more complex as a somewhat different type of chronic respiratory disease became apparent, especially in broiler chickens, which came to be known as "air sac infection." Jungherr et al. (107) reviewed the concept of air sac infection in 1953, and reported Escherichia coli as the most common secondary bacterial invader. Aspects of Escherichia coli, Newcastle disease virus and infectious bronchitis virus in complicated chronic respiratory disease (air sac infection) have been investigated by numerous workers, especially Gross (79, 80, 81), Wasserman et al. (173), Smibert et al. (152), Sullivan et al. (160), Fahey (64), Fahey et al. (70), Van Roekel et al. (169), Biddle and Cover (20), Adler et al. (6), Blake (21), and Glantz et al. (76).

Gross (78) in 1961, and Fabricant and Levine (60) in 1962 studied the effects of PPLO, E. coli and infectious bronchitis

virus as single and various combination infections. They experimentally reproduced a severe air sac infection syndrome when all 3 agents were combined, and noted that E. coli could not readily infect the air sacs unless they were previously invaded by PPLO or PPLO and infectious bronchitis virus.

Most of the reports concerning chronic respiratory disease of chickens and infectious sinusitis of turkeys have been made by American investigators. However, the occurrence of these disease syndromes has been described in numerous other countries. Reports establishing, beyond reasonable doubt, respiratory infections associated with Mycoplasma in chickens have been made by Fahey et al. (70) and Fahey and Crawley (67) in Canada, by Garust and Nóbrega (73) in Brazil, by Quizon (146) in the Philippines, by Pathak and Singh (144) in India, by Cottew (34) and Hart (92) in Australia, by Tajima et al. (162) in Japan, by Chu (28, 29) in England, by de Blieck (40) in the Netherlands, by Hartwigk (94, 95) in Germany, by Stricker and Fisera (158) in Czechoslovakia, by Keller (109) in Switzerland, by Eissa (55) in Egypt, and by Philip (145) in Rhodesia.

Similar reports concerning infection in turkeys have been made by Graham-Smith (77) in England, by Hart (93) in Australia, and by Valcarenghi (167) in Italy. Infections in chickens and turkeys have been reported by Brion et al. (24) in France, and by Coles and Cumming (32) in South Africa.

The pathology of Mycoplasma infections in chicken embryos has been described in several reports since the very early observations of Nelson (132, 133), Van Herick and Eaton (168) and Delaplane and Stuart (43) concerning the propagation of their agents in embryonated chicken eggs.

Delaplane (41, 42), Hitchner (96), Van Roekel et al. (169), Van Roekel et al. (172), and Chute and Cole (31) described hemorrhage, edema and stunting of affected embryos with necrosis of the liver, varying degrees of pneumonia and airsaculitis, occasionally pericarditis, plaques on the chorioallantoic membrane and abscessation of various joints. Chute (30) described the "joint abscesses" as being primarily subcutaneous pariarticular granulomas with necrotic centers and a border of epitheloid cells, some of which had coalesced to form giant cells.

It is interesting to note that "joint abscesses" have been observed in chicken embryos infected with a variety of Mycoplasma isolates from chicken sources by Delaplane (41, 42) Van Roekel et al. (172), Van Roekel and Olesiuk (171), Van Roekel et al. (169), Chute (30), Chute and Cole (31), Thompson (164), Moulton and Adler (126), Yamamoto (179), Calnek and Levine (25), and Hofstad (98).

The pathology of infectious sinusitis in turkeys was described in 1949 by Hitchner (96), Cover and Prier (36) and Jungherr (106). The pathology of chronic respiratory disease

in chickens was described by Jungherr et al. (107) in 1953. In 1957 Van Roekel et al. (169) described the pathology of chronic respiratory disease in chickens and infectious sinusitis in turkeys. There was general agreement that the pathology was very similar in chickens and turkeys. Sinusitis was more extensive in turkeys than in chickens. The gross lesions were primarily a catarrhal inflammation of the nasal passages, trachea, bronchi and air sacs. The microscopic pathology was characterized mainly as a catarrhal inflammation with obvious focal areas of lymphoid hyperplasia which was designated as a lymphofollicular reaction.

In 1954 Johnson (105) considered the lymphofollicular lesions to have a definite value in establishing a diagnosis of CRD in chickens and turkeys. However, in 1962 Barber (16) observed similar lesions in apparently normal turkeys, and suggested that the presence of lymphofollicular lesions may be of limited diagnostic value.

Although most of the reports concerning avian Mycoplasma have been confined to isolates from chickens and turkeys, some isolates have been obtained from other avian species.

In 1953 Winterfield (177) isolated the "turkey sinusitis agent" from pigeons via chicken embryo inoculation. Embryo passage material produced sinusitis in turkeys, chronic respiratory disease in chickens, and conjunctivitis and tracheitis in young pigeons. Gianforte et al. (75) reported

that an isolate was cultivated in chicken embryos from pigeon air sacs and subsequently found it to be antigenically related to known turkey and chicken pathogenic PPLO. Mathey et al. (120) isolated a non-glucose fermenting, nonpathogenic PPLO from pigeons. Hartwigk (94) described 2 glucose fermenting PPLO isolates from pigeons in Germany.

Pathogenic Mycoplasma have been isolated from sinus exudate of pheasants by Osborn and Pomeroy (141). Keymer (111) isolated pathogenic and nonpathogenic PPLO from pheasants in England.

The isolation of pathogenic Mycoplasma from sinus exudate of partridges has been reported by Wichman (175) and in England by Keymer (111). Gianforte et al. (75) did not determine the pathogenicity of an isolate from partridge sinus exudate, but did report that it was antigenically related to known pathogenic chicken and turkey isolates. Alter et al. (5) reported on the isolation of nonpathogenic Mycoplasma from 2 partridges.

Wills (176) reported on the isolation of pathogenic Mycoplasma from the tracheal exudate of a peacock. Hartwigk (95) isolated PPLO from the sinus of a peacock in Germany, although he did not determine its pathogenicity.

Adler (1) isolated a PPLO from the air sac of a parakeet. The culture was determined to be nonpathogenic during 9 passages in chicken embryos, and was not antigenically

related to known pathogenic isolates from chickens and turkeys.

In 1955 Fahey (61) isolated nonpathogenic PPLO from ducks with a chronic respiratory disease in Canada. He isolated a virus which was considered to be the cause of the respiratory disease in the ducks. The properties of the virus were essentially the same as those described in 1954 by Fahey and Crawley (66) for a "CRD virus" of chickens. The agent is commonly called the Fahey-Crawley virus. Fahey (65) reported on the isolation of a similar virus from two flocks of turkeys in 1956. One flock was experiencing acute sinusitis, and pathogenic PPLO were also isolated. The second flock of turkeys was suffering from a mild transient respiratory disease with no apparent sinusitis, and PPLO were not isolated.

In 1960 Subramanyam and Pomeroy (159) studied the effects of experimental inoculation of chickens and turkeys with the Fahey-Crawley virus. They noted only slight effects and concluded that the possible role of the virus in the pathogenesis of chronic respiratory disease in chickens and infectious sinusitis of turkeys is not understood.

Failure to infect small laboratory mammals with avian Mycoplasma has been noted by those reporting such attempts. Mice were reported to be refractory by Van Roekel et al. (172) Jerstad and Hamilton (103), Chute and Cole (31), Groupé and

Winn (82), and Yamamoto (179). Van Herick and Eaton (168) reported negative results in mice, hamsters, rats and guinea pigs. They found that cotton rats developed extensive red edematous consolidation of the lungs following intranasal inoculation of broth cultures of PPLO and infected chick embryo suspensions. However, they determined that the lung reaction was due to a toxic effect of the inoculum rather than an infection by the PPLO, since Seitz filtrates and suspensions heated for 30 minutes at 90° C. also produced the reaction. Van Roekel and Olesiuk (171) found cotton rats to be refractory to isolates from chickens, although they readily infected guinea fowl and pheasants.

It is of interest to note that in 1956 Adler and Yamamoto (10) essentially reproduced Nelson's coryza of rapid onset and long duration in studies employing Hemophilus gallinarum and Mycoplasma gallisepticum. This is further evidence that Nelson's coccobacilliform bodies were actually PPLO.

In 1959 Smibert et al. (153) clearly demonstrated the significance of Mycoplasma gallisepticum in infectious sinusitis of turkeys when they were able to reproduce the typical disease in germ-free poults by the inoculation of either bacteria-free cultures or sinus exudates containing the PPLO. They also inoculated poults with a nonpathogenic serotype Mycoplasma and noted no lesions or agglutinins, although they readily isolated the PPLO from inoculated poults.

Evidence that the turkey sinusitis agent was egg transmitted was obtained in 1949 when Jerstad et al. (104) isolated the agent from pips and young poults. They also noted that sinusitis developed in some of the poults which hatched from experimentally inoculated embryonating eggs. Van Roekel et al. (172) obtained evidence that the chronic respiratory disease agent was egg transmitted when they isolated the agent from embryos and young chicks originating from naturally infected chicken flocks. Further evidence indicating egg transmission in chickens has been reported by Cover and Waller (37), Fahey and Crawley (67), and Olesiuk and Van Roekel (139). Egg transmission actually was suggested in 1945 when Van Herick and Eaton (168) isolated PPLO from chicken embryos and demonstrated homologous antibodies in the supply flock. Additional proof of egg transmission in turkeys was obtained by Mataney et al. (119) and Hofstad (99).

Adler et al. (5) considered the N serotype of avian Mycoplasma to be egg transmitted in turkeys, and Yoder and Hofstad (192) presented evidence suggesting that the Iowa 695 serotype could be egg transmitted in both chickens and turkeys.

The immunological response of chickens and turkeys following natural infection and artificial infection or vaccination with Mycoplasma gallisepticum was reviewed in 1962 by Domermuth (47). Although serum antibodies have been

demonstrable in most instances, resistance to subsequent infection by various routes of exposure has been quite variable. Olesiuk and Van Roekel (138) noted that mature chickens were at least partially refractory to infection after they appeared to have overcome a natural infection. McMartin and Adler (123) noted relatively good resistance to the production of air sac lesions in young chickens previously exposed intranasally with virulent culture. They inoculated graded doses of organisms directly into the air sacs at various intervals following initial exposure. Domermuth (47) injected virulent organisms intramuscularly in chicks 2-4 weeks old followed by challenge via the air sacs 2-4 weeks later. The number of chicks capable of resisting challenge, as indicated by failure to develop air sac lesions, was only moderately reduced following vaccination. Serum agglutinins were not demonstrable at the time of challenge.

It is apparent that results may vary depending upon the route of exposure following various routes of inoculation or vaccination. Similarly, the method of determining immunity may alter the conclusions.

Serological tests have been developed for the detection of Mycoplasma antibodies in avian hosts. Some of the procedures have been adapted for use in studies concerning the characterization of avian Mycoplasma serotypes.

The hemagglutination-inhibition (HI) test devised by

Van Herick and Eaton (168) has been found to be very useful in serological studies concerning natural and artificial infections by Jungherr et al. (107), Fahey and Crawley (68), Fahey (62), Hofstad (100), and Hall et al. (89). The HI test has been used in serological characterization studies by White et al. (174), Gianforte et al. (75), Kleckner (113), and Yoder and Hofstad (182).

The tube agglutination test has been used in studies by Jungherr et al. (108) and Hofstad (100). It has been used in serological characterization studies by Gianforte et al. (75), Kleckner (113), Yoder and Hofstad (182), and Noel et al. (136).

A rapid serum plate agglutination test was described by Adler (3), and has been used in studies by Hall et al. (89) and Adler (2). Yamamoto and Adler (180), and Moore et al. (124) used the plate test in the serological characterization of avian Mycoplasma.

Comparative studies employing the rapid serum plate, tube agglutination and HI tests have been reported by Hofstad (100), Barber (15) and Jungherr et al. (108). A whole blood plate test was described by Jungherr et al. (108) and Aftosmis et al. (13). Hall (88) reported on the production of antigens for the rapid serum plate, tube agglutination, and HI tests for use in large control program studies.

Fabricant (58) reported on the use of the colony inhibition test for the serotyping of avian Mycoplasma. Colonial

growth was inhibited on agar medium containing high levels of homologous antibody. Yamamoto (179) explored the use of homologous antibody in agar and broth media for the inhibition of Mycoplasma growth. Variable results were sometimes noted, especially with certain serotypes.

In 1960 Cover et al. (35) reported that heat inactivation of chicken serum somehow destroyed its ability to react in the tube agglutination test for Mycoplasma, but did not alter its reactivity in the rapid serum plate test. The destructive effect was noted after as few as 10 minutes at 56 ° C. Jungherr et al. (108) reported that heat inactivation was detrimental to agglutinating antibodies, but slightly enhanced the readability of the HI test.

The morphology of pleuropneumonia-like organisms has been investigated by numerous workers since the initial reports on the pleomorphic nature of the original bovine pleuropneumonia organism by Bordet (22) in 1910. Extensive reviews concerning the morphology of PPLO other than from avian sources have been made by Sabin (149), Dienes (45), Edward (50), Klineberger-Nobel (114) and Freundt (71). They all have described a tendency for most PPLO from human and animal sources to possess a peculiar developmental cycle starting with coccoid elementary bodies which swell to form large bodies which internally produce further elementary particles. Filamentous protrusions from the surface of the swollen cells have been

noted to become beaded and subsequently fragment into a series of small elementary particles. Freundt (71) noted that the length of the filaments, and degree of branching, varied considerably with the various strains.

Initial reports on the morphology of avian Mycoplasma from broth cultures by Markham and Wong (118) and Grumbles et al. (87) agreed with the earlier findings of Nelson (131), Hitchner (96), and Groupé and Winn (82) based on Giemsa stained exudate and yolk preparations. They all described the organisms to be coccoid to coccobacilliform bodies with a size range of approximately 0.2-0.5 micron in diameter. Their findings were in good agreement with the electron micrograph studies conducted with chicken and turkey PPLO by Reagan et al. (147), White et al. (174), Morton et al. (125), and Hofstad (98) although some of the electron micrographs showed slender filamentous strands adjacent to the elementary particles. In 1962 Shifrine et al. (151) studied the edge of growing colonies of Mycoplasma gallisepticum. Their electron photomicrographs demonstrated rather hexagonal elementary cells and larger cells which sometimes appeared to contain small particles. Tiny beaded filamentous protrusions were noted at the periphery of some of the larger cells. They postulated that a growth cycle was present, with elementary particles originating from within larger cells or by fragmentation of the peripheral filaments. The elementary

cells varied from 0.1-0.5 micron in diameter.

Ring forms have been described in Giemsa stained culture preparations by Fahay and Crawley (67), and Hofstad and Doerr (101). Yamamoto and Adler (181), Kleckner (113) and Chu (28) only found ring forms in cultures which proved to be non-pathogenic for chickens and turkeys.

Hofstad (98) determined that avian Mycoplasma were not completely retained by Selas 02, Berkfeld V and N, and Mandler 6 and 7 filters when dilute suspensions of chorio-allantoic membranes were employed. Selas 03, Berkfeld W, and Seitz sterilizing pads (EK) retained the organisms. Hitchner (96) was unable to produce sinusitis in turkeys with Seitz, and Berkfeld V and N filtrates of infected embryo membranes. Van Herick and Eaton (168) reported that the organisms passed through Berkfeld N, but not Seitz, filters. Nelson (131) noted that nasal exudate containing coccobacilliform bodies usually was noninfectious after being filtered through Berkfeld V filters. The type of material, volume filtered, and method of detection of organisms may have influenced the varied results reported.

The possible relationship of avian Mycoplasma to so-called L forms of bacteria and their reversion to bacteria was postulated by McKay and Taylor (121) in 1954. Gentry (74) reviewed the subject and described the results of continued studies in 1960. He isolated various bacteria, predominantly

Gram positive cocci, from 152 of 316 cultures of PPLO from avian sources which were propagated in media containing no penicillin or thallium acetate. Gentry (74, p. 408) concluded, "The pathogenic strains are classified as true PPLO or Mycoplasma. The nonpathogenic strains revert to bacteria and are classified as L forms." McKay and Truscott (122) also reviewed the subject, including reports concerning reversions of Mycoplasma from other than avian sources and presented their results from further studies in 1961. The subject is so controversial that the validity of various conclusions is difficult to evaluate. It seems apparent that some PPLO represent rather stable forms, and various bacteria may be demonstrated to revert to their L forms which in turn may revert to their typical bacterial forms. However, it has not been definitely established that bacteria may revert to reasonably stable typical PPLO forms. The maintenance of L forms is frequently difficult and in all studies the possibility of extraneous bacterial contamination must be considered.

The colony morphology of avian PPLO was found to resemble that of other members of the large group of pleuropneumonia-like organisms in the early studies by Smith et al. (156) in 1948, by Edward (50) in 1954, and by Hofstad and Doerr (101) in 1956. The colonies were very tiny to 0.2-0.3 mm in diameter with a rather flattened border surrounding a more dense, elevated center. By 1958 it was apparent that

different isolates of avian PPLO varied considerably as to the size of their usual colonies as reported by Chu (28), Adler et al. (5), and Hartwigk (95). Chu (28) and Hartwigk (95) also noted the absence of a definite central elevation in some colonies. Yamamoto and Adler (181) and Kleckner (113) demonstrated that certain serotypes of avian Mycoplasma produced colonies which were much larger than those of other serotypes.

The viability of avian Mycoplasma after prolonged storage has not been extensively mentioned. Hofstad (98) found that infective chicken turbinate material suspended in tryptose phosphate broth was still infective after 5 years at approximately -30° C. Conrad (33) studied the viability of lyophilized broth cultures of avian Mycoplasma which had been stored at 4° C. Several samples were viable for at least 14 months storage, and one was viable after 27 months.

The infectivity of rather high passage broth cultures has been recorded. Hofstad and Doerr (101) found that a chicken isolate produced turkey sinusitis after 33 serial broth passages, a turkey isolate was similarly infectious after 50 broth passages, and two other isolates were not infective after 75 and 95 broth passages. Yoder and Hofstad (182) reported that the Iowa 695 isolate produced embryo deaths and lesions after 190 broth passages. Yamamoto (179) demonstrated that a 44th broth passage of the S6 isolate produced air sac

lesions in young chickens. Fahey and Crawley (69) found that a 62nd broth passage culture was infective for turkeys. Grumbles et al. (86) found that the 43rd, but not the 100th, broth passage of a culture was infective for turkeys.

Studies concerning the relative sensitivity of avian Mycoplasma to various chemotherapeutic agents have aided in their overall characterization. However, only a selected few reports will be mentioned in an effort to simply denote the significant findings. It is extremely difficult to evaluate the large number of reports on field studies because varied combinations of agents have frequently been encountered under a wide variety of environmental conditions.

The resistance of Mycoplasma, as a group, to penicillin and thallous acetate is of considerable significance in that these agents have been widely used in various PPLO media to control contamination due to many other bacteria and some fungi as discussed by Edward (51), and Taylor and Fabricant (163).

Tyzzer (166), and Dickenson and Hinshaw (44) noted that certain silver preparations were reasonably effective in the treatment of infectious sinusitis in turkeys. Hitchner (97) was one of the first to report that streptomycin inhibited embryo passage of the sinusitis agent and was an effective antibiotic for the treatment of the disease in turkeys. Grumbles and Boney (84, 85) found that streptomycin,

chlortetracycline, chloramphenicol and oxytetracycline were of value for the treatment of turkey sinusitis. Van Roekel *et al.* (172) reported that streptomycin, chlortetracycline, oxytetracycline, and chloramphenicol were somewhat effective against the chronic respiratory disease agent. Crawley and Fahey (39) proposed a control program based on injecting chicken flocks with streptomycin prior to saving hatching eggs. Wong and James (178) reported that magnamycin was effective in their *in ovo* studies, as did Hamdy *et al.* (90) who also mentioned the value of erythromycin.

Domermuth and Johnson (48) included furazolidone and several sulfa drugs in their *in vitro* studies. They found furazolidone to be quite effective, but sulfadiazine, sulfamerazine, sulfamethazine and sulfaquinoxaline were not effective. Padgett and Schoenhard (142, 143) found that deoxycorticosterone was inhibitory *in vitro*, *in ovo* and *in vivo* for PPLO. Spiramycin was reported to be of some value in the treatment of infectious sinusitis in turkeys by Sanger and Gale (150). Barnes *et al.* (17) reported tylosin to be very effective in experimental PPLO infections in chickens and turkeys.

Not all of the chemotherapeutic agents mentioned are of equal value for all avian Mycoplasma under different conditions. In recent years reports have increased on the finding of antibiotic resistant strains. Fahey (63), Osborn *et al.*

(140), Domermuth (46), Kiser et al. (112), and Yoder et al. (183) have encountered avian PPLO which were very resistant to streptomycin. Resistance to erythromycin by various strains has been reported by Domermuth (46), Osborn et al. (140), and Barnes et al. (18). Kiser et al. (112) demonstrated the development of resistance to Spiramycin within 5 embryo passages when the antibiotic was present.

It is of interest to note that erythromycin and tylosin have been employed in egg dipping experiments by Levine and Fabricant (117). Experimentally PPLO infected eggs at 37° C. were dipped in solutions of erythromycin or tylosin at 4° C. for 5-30 minutes. The temperature differential caused diffusion of the cold dip solution into the egg, and enough of the antibiotics entered the egg contents to afford relatively good inhibition of the PPLO in the infected eggs.

Media and techniques for the isolation of avian Mycoplasma have been investigated by numerous workers since the initial isolation of the agents in embryonating chicken eggs was reported in 1943 by Delaplane and Stuart (43) and in 1948 by Jerstad and Hamilton (103). Markham and Wong (118) and Van Roekel and Olesiuk (170) used Edward's medium, as described by Edward (51) in 1947. His medium was essentially beef heart infusion broth with 20% horse serum and 10% yeast extract. Penicillin and thallous acetate were added as inhibitors for other bacteria. Grumbles et al. (87) used

Difco phenol red broth base supplemented with 1-1.5% Difco PPLO serum fraction and 1% maltose. In 1954 Adler et al. (11) evaluated several types of media for the isolation of PPLO from chicken and turkey exudates. They concluded that horse blood agar slopes overlaid with 20% horse serum broth afforded the highest rate of isolations. In 1956 Adler and Yamamoto (9) noted the beneficial effects of added yeast autolysate.

In 1956 Hofstad and Doerr (101) reported good results with broth media prepared from avian meat infusion supplemented with 20% chicken or turkey serum. In 1958 Fabricant (57) evaluated the use of Adler's agar slope overlays, modified Grumble's broth, and agar plates with 10% horse serum as compared to chicken embryos for the isolation of Mycoplasma from avian exudates. He concluded that none of the media tested was as good as embryo inoculation for the isolation of pathogenic PPLO. The nonpathogens were readily isolated in the media employed. This had been demonstrated by Taylor and Fabricant (163) in a previous study. In 1959 Fabricant (59) noted that swine serum was superior to horse serum in media used for the isolation of pathogenic avian Mycoplasma, and that added carbon dioxide (candle jar) was somewhat beneficial for growth on agar. In a later study Fabricant et al. (56) demonstrated that pathogenic avian Mycoplasma grew in various atmospheres with reduced oxygen tension, but that growth was not dependent upon added carbon dioxide. Excess moisture in

the air definitely stimulated growth on agar media.

Adler and Berg (4) evaluated various media containing chicken or horse serum in combination with yeast preparations, hemoglobin or yolk in meat infusion and commercial broths. They concluded that at least two types of media, one with chicken serum and another with horse serum, should be employed for best isolation results.

The isolation and identification of Mycoplasma from infectious synovitis (tendovaginitis) of chickens required still further modification of media and techniques. Lecce et al. (116) isolated an agent in chicken embryos and studied it extensively. They described typical coccobacilliform bodies characteristic of PPLO, but considered the agent to be a rickettsia since they could not cultivate it in any of several media which supported growth of Mycoplasma from avian respiratory tracts. Their agent was most sensitive to the tetracycline antibiotics and resistant to penicillin; further characteristics suggesting that the agent was probably a PPLO. In 1960 Lecce (115) was attempting to isolate PPLO from fluids obtained from chicken embryos which had been killed by an agent isolated from swine with polyserositis. Very tiny colonies were observed on heart infusion agar supplemented with 10% swine serum following incubation in a candle jar. A definite satellite phenomenon by the PPLO colonies was observed around a contaminant Micrococcus colony. Mycoplasma

colonies were readily obtained from chicken embryo passaged avian synovitis material when the same technique was employed.

Chalquest and Fabricant (27) confirmed the results of Lecce (115) and demonstrated that cultures obtained from synovitis in chickens and turkeys were capable of reproducing the disease in chickens and turkeys. They found that diphosphopyridine nucleotide (DPN) could be substituted for the Micrococcus nurse colony, and also permitted the cultivation of the fastidious Mycoplasma in a liquid medium. Chalquest (26) reported on further modifications of media, but failed to find a substitute for DPN.

In general, the nutritional and environmental requirements of the Mycoplasma are rather complex with variations noted for certain isolates. All, except the saprophytic isolates, require a protein enriched medium. Smith and Morton (155) demonstrated that the essential growth factor was a low molecular weight protein which could be separated from serum and ascitic fluid. They also found that the growth factor was present in certain yeast preparations. Smith and Lynn (154) determined that the group possessed a lipid requirement which was satisfactorily supplyed by cholesterol.

Tourtellotte and Jacobs (165) included 6 isolates of avian Mycoplasma in their physiological studies. The avian isolates did not vary considerably from those of human and animal sources in their requirements for serum, and in the

production of primarily lactic, pyruvic and acetic acids by carbohydrate utilizing strains. Although there was general uniformity in the spectrum of carbohydrates which were fermented, variations by a single strain were noted. Only the saprophytic isolate would grow at room temperature and in the absence of serum.

Somerson and Morton (157) studied the use of various tetrazolium salts in media for the detection of electron transfer due to activity of Mycoplasma from human sources. Tetrazolium salts accept electrons and are converted from soluble colorless salts to a colored insoluble formazan in the reduced state. Some tetrazolium salts become blue in the reduced state, others become red. They concluded that 2, 3, 5 triphenyl tetrazolium chloride was satisfactory at a final concentration of 0.005% in media. It produced a red formazan upon being reduced.

Yamamoto and Adler (181) reported that most of their isolates of avian Mycoplasma reduced "tetrazolium-blue" when incorporated into broth medium at a final concentration of 0.0025%.

Hemolysis of horse erythrocytes incorporated into agar media has been described for certain isolates of Mycoplasma by Edward (50) and Adler et al. (7). Hemolysis of turkey and chicken erythrocytes in liquid medium was described by Yamamoto and Adler (181), although there was more of a

tendency for brown discoloration than for hemolysis.

The need for detailed characterization studies concerning Mycoplasma from various avian sources became apparent by 1957 when Adler and Yamamoto (8) reported on the isolation of pathogenic and nonpathogenic PPLO from a turkey with sinusitis. The two isolates were antigenically different. White et al. (174) had reported in 1954 that the agents of chronic respiratory disease in chickens and infectious sinusitis of turkeys were antigenically similar. This was in agreement with the earlier finding of Markham and Wong (118) that cultures from chicken and turkey sources appeared to be essentially identical. Further proof that Mycoplasma from avian sources were similar was demonstrated by Gianforte et al. (75) in 1955 when they found that PPLO from 3 chickens, 2 turkeys, 1 partridge and 1 pigeon exhibited the same biochemical and serological characteristics.

In 1957 Moulton and Adler (126) described the pathogenesis of arthritis in chicken embryos which was caused by a culture of PPLO obtained from the pericardial sac of a chicken. Their isolate did not ferment carbohydrates, and was not antigenically related to typical pathogenic avian PPLO. That same year Adler et al. (12) reported that at least 2 serological types of PPLO were isolated from chickens and turkeys, and that nonpathogenic isolates were obtained from the respiratory and genital tracts of chickens and

turkeys.

The first extensive study concerning the detailed characterization of various avian Mycoplasma was done in 1957 by Yamamoto (179). Yamamoto and Adler (180, 181) published the major aspects from his thesis. Yamamoto investigated the cultural, biochemical, morphological, serological and pathogenic characteristics of 8 isolates of Mycoplasma from chicken and turkey sources. He characterized 5 distinct serological groups (serotypes) among them, designated as I, II, III, IV, and V with typical isolates designated as S6, C, SA, 0 and N respectively. Only members of group I were obviously pathogenic for chickens and turkeys. The others were essentially nonpathogenic, although the N isolate had been obtained from poult air sac lesions and was capable of producing mild air sac lesions, but not sinusitis, in experimentally inoculated poults. Differences between the groups were also noted in their biochemical and morphological characteristics.

The origin of the S6 isolate was described by Zander (184) in 1961. He isolated it from the brain of a turkey in 1954. The S6 designation has been used extensively to denote the typical pathogenic serotype of avian Mycoplasma.

It is of interest to note that Chu (28, 29) reported on the isolation of pathogenic and nonpathogenic Mycoplasma from chickens in England in 1954 and in more detail in 1958. Adler *et al.* (5) reported in 1958 on the isolation of nonpathogenic

Mycoplasma from 8 chickens and 2 partridges. They also isolated N serotype PPLO from poult air sac lesions obtained from 8 of 10 supply flocks examined.

In 1960 Kleckner (113) reported on the characterization of 8 serotypes of avian Mycoplasma, including the 5 serotypes previously described by Yamamoto and Adler (181). Kleckner simply designated the serotypes as A, B, C, D, E, F, G, and H which were represented by typical isolates designated as S6, 54-537, C, NY, HPR-15, SA, O and N respectively. There were differences between the groups in their biochemical, colony morphological and pathogenic characteristics, but they could not be completely differentiated except serologically.

Also in 1960, Fabricant (58) employed the colony inhibition technique of Edward and Fitzgerald (52) to confirm the distinctness of serotypes conveniently designated as A, B, C, D, E and F of Kleckner (113) with typical isolate designations of 293, K18B, C, R39A, SLO and SA respectively. Isolate DPR-2 from Kleckner was found to belong in the E serotype.

That same year Moore et al. (124) reported the cultural, biochemical, serological and pathogenic characteristics of Mycoplasma from 7 chickens (Texas C1, C2, C3, C4, C5, C6 and C7) and 3 turkeys (Texas T1, T2 and T3). They demonstrated that at least 4 serological groups were represented, but they did not compare them with previously designated serotypes

other than typical pathogenic isolates. However, Fabricant (58) included several of the Texas isolates in his study and identified Texas T1, C1 and C6 as A serotype isolates. Texas C7 and C8 were placed in the D serotype.

In 1962 Yoder and Hofstad (182) characterized still another serotype of avian Mycoplasma, designated as Iowa 695, which was unrelated to the serotypes described by Yamamoto and Adler (181) and Kleckner (113). That same year Noel et al. (136) studied isolates of Mycoplasma from 4 chickens, 2 turkeys and known cultures of A and C serotypes. They found that 3 of their isolates (MD-2, MD-3 and MD-8) were of the A serotype, none were of the C serotype, and 3 were divided among 2 serotypes which were not further identified.

It has been mentioned that Mycoplasma have been isolated from humans, numerous animals and birds. Very little is known about the possible inter-relationships of various Mycoplasma and various hosts. In 1960 Tourtellotte and Jacobs (165) reported on the physiological and serological characteristics of 15 Mycoplasma from various sources; 2 human, 3 cattle, 1 goat, 1 sheep, 1 pig, 1 sewage, 1 turkey, 1 pigeon and 4 chicken isolates. The 15 isolates were divided among 10 serotypes. The isolates from sheep and goats belonged to a single serotype. The single turkey isolate (S6) was in the same serotype as 2 chicken isolates (A5969 and F), thus, representing the A serotype. Their chicken isolate C belonged

to the C serotype. The pigeon isolate was a non-carbohydrate fermenter which was serologically distinct from the others. None of the other serotypes contained isolates from more than 1 animal species.

The classification of pleuropneumonia-like organisms from avian sources has been attempted to a limited extent. Edward and Freundt (53) suggested a revised classification for the entire pleuropneumonia group in 1956. Freundt (72) published the major aspects of it in the 7th edition of Bergey's Manual of Determinative Bacteriology in 1957. A single genus, Mycoplasma, was placed in the Mycoplasmataceae family under the Mycoplasmatales order. The only avian member listed was Mycoplasma gallinarum for an isolate from the trachea of a chicken. That isolate, designated as Fowl, was described by Edward (50) in 1954 and originally named in 1956 by Edward and Freundt (53). Unfortunately, that organism was not representative of the typical pathogenic avian Mycoplasma (serotype A). Mycoplasma gallinarum is a nonfermenter and a nonpathogen which Fabricant (58) demonstrated to belong to the B serotype of Kleckner (113).

In 1960 Edward and Kanarek (54) named 2 more species from avian sources. They designated Mycoplasma gallisepticum for a typical pathogenic isolate, X95, and designated Mycoplasma iners for a nonpathogenic isolate, M. Fabricant (58) demonstrated that M. gallisepticum represented serotype A, and M.

iners represented serotype G of Kleckner (113).

In 1961 Adler et al. (7) reported on the isolation of a saprophytic Mycoplasma from the sinus of a chicken with coryza. They suggested that it be designated as Mycoplasma inocuum. Shifrine[1] indicated that further study suggested that it was very similar to Mycoplasma laidlawii B, the saprophytic member of the genus. Further information is needed before the exact identity of M. inocuum is established. However, it is important to recognize that saprophytic Mycoplasma may be isolated from avian sources. The designation, saprophyte, is used in the sense that such organisms can be cultivated in media containing no added serum for enrichment, and tend to grow reasonably well at room temperature.

A summary of the reported serotypes and their typical isolate designations is presented in Table 1 in an effort to simplify comparisons.

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[1]Shifrine, Moshe, Davis, California. Further studies on Mycoplasma inocuum. Personal communication. 1962.

Table 1. Summary of reported serotype, isolate, and species designations of avian Mycoplasma

Serotype designations[a]			Typical isolate designations	Genus and species
Kleckner (113)	Yamamoto and Adler (181)	Fabricant (58)		
A	S6	S6	S6, F, SV, W, VR, A5969, 293, T1, C1, C6, X95	*Mycoplasma gallisepticum*
B		K18B	54-537, HPR-5, Fowl	*M. gallinarum*
C	C	C	C, Tu, DIVA, CRDA	
D		R39A	NY, R39A, C7, C8	
E		SLO	HPR-15, DPR-2, SLO	
F	SA	SA	SA	
G	O		O, M	*M. iners*
H	N		N	

[a]Additional serotypes reported, but not compared in the above studies: Chalquest and Fabricant (27), ISD; Yoder and Hofstad (182), Iowa 695; Adler *et al.* (7), *M. inocuum* (*M. laidlawii* B).

MATERIALS AND METHODS

Sources of Mycoplasma Cultures

Cultures of Mycoplasma representing previously identified serotypes were obtained from several sources as shown in Table 2. Such cultures were obtained for comparative studies. An effort was made to obtain them from the person originally isolating and characterizing them. However, some cultures were obtained indirectly from one or more other persons, especially when original cultures were not available or were not viable as received and subsequently cultured.

The cultures of Mycoplasma selected from those which had been isolated and maintained by Dr. M. S. Hofstad are identified in Table 3.

All other cultures of Mycoplasma used during the course of this study were isolated from naturally infected birds, and are identified in Table 4. The 32 isolates from turkeys originated from 25 different flocks. In contrast, all but 5 of the 32 isolates from chickens were obtained from a single farm flock. This flock of 80 white leghorn type hybrid pullets and 8 roosters was obtained for detailed study because it was known to have a lingering respiratory disease, and subsequent serological and cultural studies demonstrated the presence of Mycoplasma gallisepticum infection. The flock was maintained in isolation at the Veterinary Medical Research Institute, College of Veterinary Medicine, Iowa State University of

Table 2. Source of avian Mycoplasma received from other laboratories

Isolate	Designated serotype: Kleckner (113)	Designated serotype: Yamamoto and Adler (181)	Origin: Bird	Origin: Site	Supplied by	Original source
S6	A	S6	turkey	brain	Adler[a]	Zander[b]
A5969	A	S6	chicken	trachea	Jungherr[c]	Van Roekel[d]
VR	A	S6	chicken	trachea	Kleckner[e]	Van Roekel
F	A	S6	chicken	trachea	Fabricant[f]	Adler
293	A	S6	chicken	trachea	Fabricant	Taylor[g]
54-537	B		chicken	trachea	Kleckner	Kleckner
K18B	B		chicken	trachea	Fabricant	Fabricant
DIVA	C	C	chicken	trachea	Kleckner	Kleckner
C	C	C	chicken	trachea	Fabricant	Adler
Tu	C	C	chicken	turbinate	Fabricant	Adler

[a]Adler, H. E. University of California, Davis, California.

[b]Zander, D. V. University of California, Davis, California.

[c]Jungherr, E. L. University of Connecticut, Storrs, Connecticut.

[d]Van Roekel, H. University of Massachusetts, Amherst, Massachusetts.

[e]Kleckner, A. L. University of Georgia, Athens, Georgia.

[f]Fabricant, J. Cornell University, Ithaca, New York.

[g]Taylor, J. Cornell University, Ithaca, New York.

Table 2. (Continued)

Isolate	Designated serotype: Kleckner (113)	Designated serotype: Yamamoto and Adler (181)	Origin: Bird	Origin: Site	Supplied by	Original source
NY	D		chicken	trachea	Kleckner	Markham[h]
DPR-2	E		chicken	trachea	Kleckner	Kleckner
C26	E		chicken	trachea	Fabricant	Fabricant
SA-1	F	SA	turkey	trachea	Adler	Adler
SA-2	F	SA	turkey	trachea	Kleckner	Adler
SA-3	F	SA	turkey	trachea	Fabricant	Adler
SA-4	F	SA	turkey	trachea	Adler	Adler
O	G	O	chicken	pericardium	Fabricant	Adler
N	H	N	turkey	air sac	Kleckner	Adler

[h]Markham, F. S. Lederle Laboratories, Pearl River, New York.

Table 3. Source of avian Mycoplasma supplied by Dr. M. S. Hofstad

Isolate	Origin			Storage		
	Bird	Site	Year	Material	Method	Year
187	chicken	turbinate	1947	turbinates	lyo[a]	1948
197	chicken	turbinate	1948	turbinates	lyo	1948
594	chicken	trachea	1952	CAM[b]	lyo	1952
595	turkey	sinus	1952	CAM	lyo	1952
640	chicken	trachea	1953	CAM	lyo	1957[c]
693	turkey	joint[d]	1955	culture	lyo	1957
694	pigeon	turbinate	1955	culture	lyo	1957
695	turkey	air sac[e]	1955	culture	lyo	1956
699	chicken	trachea	1956	culture	lyo	1956
801	turkey	air sac[e]	1955	culture	frozen	1956
1344	turkey	air sac[e]	1954	culture	lyo	1954

[a]Lyophilized ampoule stored at 4° C.

[b]Chorioallantoic membrane.

[c]CAM stored at -25° C. previous to 1957.

[d]Hock joint exudate, 1 week old poult.

[e]Pipped turkey egg.

Science and Technology, Ames, Iowa. During the following 26 months an attempt was made to isolate Mycoplasma from numerous sites, including the trachea, air sacs, ovary, oviduct, semen, and embryonating eggs from trapnested hens. Only 27

Table 4. Origin of Mycoplasma isolated from naturally infected birds during the course of this study

Isolate	Origin		Remarks
	Bird	Site	
24R	chicken	semen	rooster 24
26R	chicken	semen	rooster 26
27R	chicken	semen	rooster 27
108TA	chicken	trachea	hen 108, swab A
114C	chicken	CAM[a]	hen 114, embryo CAM
114TB	chicken	trachea	hen 114, swab B
116C	chicken	CAM	hen 116, embryo CAM
128VA	chicken	ovary	hen 128, infected 26 months
132VI	chicken	oviduct	hen 132, oviduct swab
158VI	chicken	oviduct	hen 158, oviduct swab
172C	chicken	CAM	hen 172, embryo CAM
734	turkey	sinus	exudate
755	chicken	CAM	hen E25, embryo CAM
756	chicken	CAM	hen W5, embryo CAM
796	turkey	yolk	dead embryo yolk
799-1	turkey	air sac	flock 1, pipped egg
799-4	turkey	air sac	flock 4, pipped egg
806C	turkey	air sac	flock C, pipped egg
807-5	turkey	air sac	flock 5, pipped egg

[a]Chorioallantoic membrane.

Table 4. (Continued)

Isolate	Origin		Remarks
	Bird	Site	
807-18	turkey	air sac	flock 1, pipped egg
814-5	turkey	air sac	flock 5, pipped egg
815-4	turkey	air sac	flock 4, pipped egg
822-4	turkey	air sac	flock 427, pipped egg
823C	turkey	air sac	flock C, pipped egg
833R	turkey	air sac	flock R, pipped egg
844-2	turkey	air sac	flock 212, pipped egg
849P	turkey	air sac	flock P, pipped egg
850-2	turkey	air sac	flock 2, pipped egg
851R	turkey	air sac	flock R, pipped egg
853M	turkey	air sac	flock M, pipped egg
854-2	turkey	air sac	flock 2, pipped egg
857-1	turkey	sinus	exudate
858-76	pigeon	turbinate	"normal" pigeon
878-50	pigeon	turbinate	"normal" pigeon
882	turkey	sinus	exudate
890A	pigeon	turbinate	"normal" pigeon
893	partridge	sinus	exudate
894	chicken	joint	hock exudate
933	turkey	air sac	2 weeks old
1010	chicken	trachea	hen 102, swab A, small colony A

Table 4. (Continued)

Isolate	Origin		Remarks
	Bird	Site	
1018	chicken	trachea	hen 102, swab A, small colony B
1021	chicken	trachea	hen 102, swab A, large colony
1022-2	turkey	air sac	1 day old
1042	turkey	yolk	flock W, dead embryo
1043	turkey	semen	flock W, pool
1075	turkey	semen	flock E, pool
1079-1	turkey	yolk	flock 18, pipped egg
1079-3	turkey	yolk	flock 32, pipped egg
1087-1	turkey	air sac	flock 14, pipped egg
1102-7	turkey	lung	flock 7, pipped egg
1103-5	turkey	yolk	flock 51, pipped egg
1104	turkey	sinus	exudate, 4 weeks old
1109	turkey	yolk	flock 25, 2 days old
1111	chicken	trachea	hen 111, swab A, large colony
1112	chicken	trachea	hen 111, swab A, small colony
1207	chicken	trachea	hen 111, swab B, large colony
1304	chicken	trachea	hen 116, swab A, large colony
1403	chicken	trachea	hen 116, swab B, small colony

Table 4. (Continued)

Isolate	Origin		Remarks
	Bird	Site	
1504	chicken	trachea	hen 176, swab, large colony
1605	chicken	oviduct	hen 176, oviduct suspension
1703	chicken	yolk	hen 108, embryo yolk
1805	chicken	oviduct	hen 108, oviduct swab
1900	chicken	CAM	hen 111, embryo CAM
2004	chicken	oviduct	hen 111, oviduct suspension
2600	chicken	trachea	hen 5, swab, "normal" 2 year old
2705	chicken	oviduct	hen 116, oviduct suspension
2805	chicken	trachea	hen 119, swab
5858	chicken	trachea	flock C, swab

representative isolates of Mycoplasma from various sites were selected for detailed study.

Isolate 2600 was obtained from the trachea of an apparently normal 2 year old hen from the Veterinary Medical Research Institute flock of white leghorn chickens which have been maintained as a closed isolated flock for approximately 10 years. Serological and cultural studies have consistently failed to demonstrate Mycoplasma gallisepticum infection in the flock.

Isolates designated as 858-76, 878-50 and 890A were obtained from the nasal turbinates of apparently normal pigeons from local barn lofts.

Solutions

Various solutions which were used in laboratory procedures were prepared as follows:

Saline solution (0.85%)

8.5 grams sodium chloride

1000 ml distilled water

Autoclave sterilized, 20 minutes at 120° C.

Cox phosphate buffer solution (0.1 molar, pH 7.0)

Cox *et al.* (38)

4.7 grams monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$)

17.3 grams dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$)

1000 ml distilled water

Selas[1] 03 filter sterilized

Phosphate buffered saline solution

950 ml 0.85% saline solution

50 ml Cox phosphate buffer solution

10 ml 1:1000 colorless merthiolate solution added as a preservative

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[1]Selas Corporation of America, Dresher, Pennsylvania.

Phenolized (0.25%) Cox phosphate buffer solution

1000 ml Cox phosphate buffer solution

2.5 grams phenol crystals

Alsever's solution Alsever and Ainslie (14)

2.05 grams dextrose

0.80 grams sodium citrate

0.42 grams sodium chloride

100 ml distilled water

Selas 03 filter sterilized

Citrate solution

2.0 grams sodium citrate

100 ml distilled water

Selas 03 filter sterilized

Tryptose phosphate broth

29.5 grams Difco[1] tryptose phosphate broth

1000 ml distilled water

Autoclave sterilized, 20 minutes at 120° C.

All of the solutions were stored in glass containers at 4° C. except the phenolized phosphate buffer solution which was stored at room temperature since relatively large amounts were used and it was prepared frequently.

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[1]Difco Laboratories Incorporated, Detroit, Michigan.

Erythrocyte Suspensions

A 10 ml glass syringe, fitted with a 20 gauge needle 1 1/4 inches long, containing 2 ml of sterile 2% solution of sodium citrate was used to aseptically withdraw 8 ml of chicken or turkey blood from a wing vein. Then 5 ml of the citrated blood was delivered into each of 2 sterile 40 ml graduated glass centrifuge tubes with screw caps. Sterile saline was added until the contents of each tube was 40 ml. The centrifuge tubes were gently inverted several times before being centrifuged for 10 minutes at 2000 RPM using a #269 swinging bucket head in a model 2-V International[1] centrifuge at room temperature.

The supernatant fluid was carefully poured off and the sedimented erythrocytes were resuspended by gently inverting the tubes after adding sufficient saline to make the total tube contents 40 ml. The tubes were again centrifuged for 10 minutes as described above and the supernatant fluid was then poured off.

The third washing was done in sterile Alsever's solution rather than saline. After resuspending the 40 ml contents of each tube they were centrifuged at 1000 RPM for 8 minutes. The volume of the packed erythrocytes in each tube was recorded before most of the rinsing fluid was carefully poured off. Then sterile Alsever's solution was added to prepare a 10%

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[1]International Equipment Company, Boston, Massachusetts.

suspension of triple washed erythrocytes. The suspension from each tube was poured into a separate sterile 100 ml Erlenmeyer flask and stored at 4° C. for future use. The stock 10% erythrocyte suspension in one flask was depleted before using the second flask in an effort to reduce deterioration due to bacterial contamination introduced during numerous withdrawals of small amounts.

Erythrocyte suspensions for hemagglutination and hemagglutination-inhibition studies were prepared by pipetting 1.0 ml of the 10% stock suspension into 39 ml of Cox buffered saline in a small flask. The resulting 0.25% erythrocyte suspensions were prepared fresh daily as needed.

Culture Media

The culture media employed were mainly various modifications of the avian meat infusion medium described by Hofstad and Doerr (101).

Media ingredients

Meat for infusion was obtained by removing the muscles from the breast, thighs, and legs of growing or mature turkeys. Excess fat and tissue debris were removed as the meat was cut in strips prior to being ground in an electric meat grinder. The meat was then stored frozen as 500 gram portions wrapped in aluminum foil for future use.

Turkey serum for media was harvested from blood which

was obtained from apparently normal turkeys. The blood was collected in 12 quart pails as the birds were being bled out on the processing line of a nearby turkey dressing plant. The clot was cut and the serum was allowed to separate for 3-4 hours at room temperature in large glass funnels lined with gauze. The serum was then clarified by the use of a standard type electric cream separator. Two passages at a slow flow rate afforded very clear serum with little hemolysis. The serum was then tested by the hemagglutination-inhibition procedure to establish that it was free from detectable antibodies for Mycoplasma gallisepticum before it was processed further. Then the serum was heat inactivated at 56° C. for 30 minutes in a magnetic agitated water bath with constant circulation of the serum by an electric motor driven glass stirring rod. The serum was stored frozen in one quart capacity plastic bags for future use. Horse serum was processed in a similar manner.

Serum was thawed just prior to medium preparation. It was partially clarified before being incorporated into the medium since it contained precipitates due to heating, freezing and thawing. The serum was filtered through 6 cm diameter filter pads in a Seitz filter assembly using a vacuum system. Clariflow[1] filter pads of D-0, D-2 and D-4 porosities were used consecutively to obtain well clarified serum.

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[1]F. R. Hormann and Company, Newark, New Jersey.

Turkey blood clot for use in media preparation was collected from the gauze lined funnels after the serum had separated and drained off. The blood clot was then processed in a food blendor and stored frozen in one half pint paper food containers for future use.

Yeast autolysate was prepared by adding 200 ml of distilled water to 4 ounces of dehydrated bakers' yeast[1] in a 6 liter Erlenmeyer flask which was incubated for 48 hours at 56° C. in a water bath. The supernatant fluid was harvested after centrifugation in 100 ml glass tubes in a #840 angle head at 4000 RPM for 30 minutes at 4° C. in a model PR-1 International refrigerated centrifuge. The yeast autolysate was stored frozen in one pint capacity plastic bags for future use.

Horse erythrocytes used in the preparation of media were obtained from defibrinated blood. Normal horse blood was aseptically collected in a flask containing glass beads with constant agitation to defibrinate the blood. The defibrinated blood was then dispensed at 25 ml per 30 ml capacity screw cap tubes which were then stored in a refrigerator at 4° C. When horse erythrocytes were needed for the preparation of agar medium for hemolysis studies they were obtained from the sedimented portion of such tubes.

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[1]Red Star Yeast and Products Company, Milwaukee, Wisconsin.

Preparation of broth media

Turkey meat infusion broth was prepared by the addition of 1 liter of distilled water to 500 grams of ground turkey meat in a 2 liter Erlenmeyer flask which was then stored in a refrigerator at 4° C. for approximately 20 hours.

The meat infusion was then cooked with frequent agitation in a boiling water bath for 30 minutes after the bath came to a rolling boil. The infusion was then filtered through #1 Whatman paper in an 18.5 cm diameter Buchner funnel using a vacuum system. Then 50 ml of blood clot, 5 grams of sodium chloride, and 10 ml of a 10% solution of sodium hydroxide were added to the infusion followed by cooking, with frequent agitations, in a boiling water bath for 25-30 minutes, until the red color of the blood clot was no longer visible. Clear amber colored infusion broth was obtained following another Buchner funnel filtration.

The infusion broth was cooled to room temperature before various ingredients were added depending upon the type of medium to be prepared. The various media were prepared as follows:

Plain turkey serum medium

1000 ml turkey meat infusion broth

200 ml turkey serum

adjusted to pH 8.0

Yeast autolysate medium

1000 ml turkey meat infusion broth

200 ml turkey serum

60 ml yeast autolysate

20 ml 1% solution of thallous acetate

200,000 units crystalline penicillin G potassium

adjusted to pH 8.0

Tetrazolium medium

1000 ml turkey meat infusion broth

200 ml turkey serum

60 ml yeast autolysate

4.5 ml 10% solution of thallous acetate

200,000 units crystalline penicillin G potassium

55 mg 2, 3, 5 triphenyl-2H-tetrazolium chloride[1]

adjusted to pH 8.0

Infusion medium with carbohydrate

300 ml plain turkey serum medium

3 grams desired carbohydrate substance

6 mg phenol red indicator

5 ml 1% solution of thallous acetate

50,000 units crystalline penicillin G potassium

adjusted to pH 8.0

Phenol red broth medium with carbohydrate

300 ml distilled water

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[1]Eastman Organic Chemicals, Rochester, New York.

4.8 grams Difco[1] phenol red broth base

30 ml turkey serum

3.3 grams desired carbohydrate substance

5 ml 1% solution of thallous acetate

50,000 units crystalline penicillin G potassium

adjusted to pH 7.8

Horse serum medium with mucin

1000 ml turkey meat infusion broth

10 grams swine gastric mucin powder[2]

150 ml horse serum

50 ml yeast autolysate

20 ml 1% solution of thallous acetate

200,000 units of crystalline penicillin G potassium

adjusted to pH 8.0

All of the broth media, with the exception of horse serum medium with mucin, were prepared by adding all of the listed ingredients to the base broth in a flask followed by adjustment of the pH with 10% sodium hydroxide solution. A Beckman[3] model G pH meter was used.

The incorporation of swine gastric mucin into the medium required a special procedure as devised by Ross (148). The specified portion of gastric mucin powder was mixed with a

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[1]Difco Laboratories Incorporated, Detroit, Michigan.

[2]Cudahy Packing Company, Omaha, Nebraska

[3]National Technical Laboratories, South Pasadena, Cal.

small portion of the still hot meat infusion broth, immediately following the second cooking and clarifying, to obtain a paste-like preparation which was then mixed into the remainder of the broth. The flask was placed in a boiling water bath for 15 minutes, and then approximately 20 grams of Celite[1] filter aid substance was mixed into the broth. Cooking, with frequent agitations, was continued for 10 more minutes. Clarification was accomplished with little difficulty by filtering the broth-mucin-Celite mixture in a 12.5 cm diameter Buchner funnel fitted with a Whatman GF/A glass filter disc. The mucin broth was then cooled to room temperature before the addition of the remainder of the ingredients and adjustment to pH 8.0.

Sterilization of completed broth media was accomplished by filtration through a graded series of Selas filter candles starting with porosity 10 followed by the 01, 015 and 02 porosities. Final sterilization was attained by the use of a Selas 03 filter candle adapted to a sterile dispensing flask. A Gast[2] vacuum pump was used in the filtration procedures. Several lengths and diameters of Selas filter candles were employed depending upon the volume of medium to be filtered. Batches of 1 liter or more required the use of candles approximately 20 cm long and 2.5 cm in diameter.

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[1]Johns-Manville, New York, New York.

[2]Standard Scientific Supply Corporation, New York.

Broth medium not containing added carbohydrate was aseptically dispensed at the rate of 8 ml per 16 X 125 mm screw cap Pyrex[1] culture tube. Medium to be used in the preparation of large batches of antigen was dispensed at approximately 200 ml per 500 ml Erlenmeyer flasks with cotton plugs. Medium for carbohydrate fermentation studies was dispensed at 5 ml per 13 X 100 mm screw cap culture tubes.

Carbohydrate medium was incubated at 37° C. for 2 days to detect bacterial contamination. Other broth media were routinely incubated at 37° C. for 7 days to detect bacterial and possible Mycoplasma contamination. Sterile media were stored in a refrigerator at 4° C. for future use. An effort was made to prepare media rather frequently to avoid storage for longer than 1-2 months.

Preparation of agar media

Several types of media containing 1.5% agar were prepared with turkey meat infusion broth. The desired volume of infusion broth was adjusted to pH 8.3 by the addition of 10% sodium hydroxide solution. Then 175 ml portions of the broth were poured into 200 ml capacity glass centrifuge bottles followed by the addition of 2.6 grams of agar to each bottle. The bottles were capped with heavy aluminum foil, placed in large centrifuge buckets, balanced in pairs by adding water

[1]Corning Glass Works, Corning, New York.

to the buckets, and then were autoclave sterilized at 120° C. for 35 minutes.

Immediately after autoclaving each centrifuge bottle was rotated to mix the contents with a swirling motion. They were centrifuged for 3-4 minutes at 1000 RPM in a #239 swinging bucket head in a model 2-V International centrifuge to sediment precipitates. The supernatant fluid from pairs of bottles was aseptically poured into 500 ml Erlenmeyer flasks. The flasks of agar broth were then placed in a 45° C. water bath. The above steps were done as quickly as possible to avoid solidification of the agar.

Various ingredients were added to the 45° C. agar base depending upon the type of agar medium to be prepared. The various media were prepared as follows:

Turkey serum agar with yeast

700 ml turkey meat infusion agar

140 ml sterile turkey serum (Selas 03)

35 ml sterile yeast antolysate (Selas 03)

20 ml 1% solution of thallous acetate

200,000 units crystalline penicillin G potassium

Turkey serum agar with horse erythrocytes

200 ml turkey serum agar with yeast

4 ml sedimented horse erythrocytes

Horse serum agar with mucin

700 ml turkey meat infusion agar

140 ml sterile horse serum (Selas 03)

35 ml sterile yeast autolysate

20 ml 1% solution of thallous acetate

200,000 units crystalline penicillin G potassium

Serum and yeast autolysate for preparing agar media were Selas 03 filter sterilized and dispensed into 16 X 125 mm screw cap tubes at the rate of 14 ml for serum and 10 ml for yeast autolysate. The tubes were stored frozen at -30° C. for future use. When agar medium was to be prepared the desired number of tubes of sterile serum and yeast autolysate were thawed and kept in a 45° C. water bath until they were aseptically poured into the flask containing the turkey meat infusion agar. The crystalline penicillin was dissolved in the thallous acetate solution which was then pipetted into the agar medium just prior to dispensing.

Sterile horse erythrocytes were aseptically removed from the sedimented portion of stored tubes of defibrinated horse blood by a 5 ml pipette and were aseptically added to agar medium being prepared for hemolysis studies.

The agar media prepared by the methods described were then either poured at the rate of 15-20 ml into 100 mm diameter glass Petri dishes, or dispensed at approximately 4 ml per 16 X 125 mm screw cap tubes which were cooled in a slanted position to provide relatively long agar slopes. When preparing agar slants the agar was kept warm by wrapping

an electric heat tape around the sterile dispensing flask.

All agar media were stored in a refrigerator at 4° C. for future use. An effort was made to prepare such medium rather frequently so that it rarely was stored longer than 3-4 weeks. Groups of 4-6 agar plates were wrapped in aluminum foil to retard contamination and dehydration during storage.

Experimental Animals

Embryonated chicken eggs, young chickens, various age turkeys, and a small number of pigeons and quail were employed as experimental hosts. Young male chickens and young rabbits were used in the preparation of antisera.

The chickens and embryonated chicken eggs originated from normal flocks of white leghorns and Rhode Island reds maintained in isolation at the Veterinary Medical Research Institute at Iowa State University. Day old bronze turkeys were obtained from a nearby hatchery producing poults only from parent flocks found to be serologically negative when tested with Mycoplasma gallisepticum agglutination antigen. The purchased poults were brooded and reared in isolation units prior to being used as experimental birds.

No serological or cultural evidence was obtained which would suggest the presence of Mycoplasma in the chickens or embryonated chicken embryos employed. Serological and cultural studies failed to detect Mycoplasma gallisepticum

in the turkeys employed. However, the results of recent cultural procedures would suggest that a small percentage of the turkeys employed probably were not entirely free from all serotypes of Mycoplasma.

Young pigeons were obtained from several local barn lofts. Very little was known about their disease status when obtained, although they appeared to be reasonably normal. They were consistently free from detectable antibodies for Mycoplasma gallisepticum and no isolation of that serotype was obtained. However, isolations of other Mycoplasma were rather frequent.

The characteristics of Mycoplasma associated with experimental pigeons, and possibly associated with some of the experimental turkeys, will be discussed later in the thesis.

The few quail employed were obtained at one day of age from a nearby game farm. They appeared to be normal throughout their rearing period in an isolation unit, and were found to be free from detectable antibodies for Mycoplasma gallisepticum.

Young rabbits, mainly New Zealand whites, were obtained from a small local source. They were consistently free from apparent disease, including conjunctivitis. No serological evidence was found which would suggest the presence of Mycoplasma in the employed rabbits.

Specimen Procurement

Specimens were obtained and prepared in such a manner as to have sufficient volume of the material to inoculate culture media, and sometimes embryonated eggs, experimental birds, and a portion to store at -30° C.

Sinus exudate was aseptically removed from infraorbital sinuses of birds, mainly turkeys, by a glass syringe fitted with an 18 or 20 gauge needle. If less than 0.5-1.0 ml of fluid exudate was obtained by aspiration 2-3 ml of sterile tryptose phosphate broth was injected into the sinus and then it was withdrawn as a rinse.

In a few cases nasal turbinates were excised with reasonably aseptic technic and a suspension was prepared in a mortar with sterile alundum and 4-5 ml of tryptose phosphate broth.

The nasal cavity of pigeons was sometimes rinsed with 2-3 ml of tetrazolium medium injected through the palatine cleft with a blunt needle and collected into a tube of tetrazolium medium as it flowed from the nostrils of the pigeons held with their head down.

Tracheal exudate was obtained by inserting a cotton tipped applicator swab into the trachea of living birds. The swabs were then rinsed in 2-3 ml of broth or were rinsed directly in tubes of tetrazolium medium.

Air sac, lung, pericardium, ovary, oviduct, testicle, and other tissue specimens were aseptically excised and tissue

suspensions prepared in a mortar with alundum and 4-5 ml of tryptose phosphate broth.

Cotton swabs were inserted into the everted oviduct of chickens in some studies. The swabs were then rinsed in 2-3 ml of broth or were rinsed directly into tubes of tetrazolium medium.

Another means of obtaining material from oviducts was to aseptically remove the oviduct from chickens as soon as they were sacrificed. Then the lower end of an elevated portion of the oviduct was held closed while a 5 ml pipette was used to puncture the oviduct and rinse its lumen with 3-5 ml of tetrazolium medium which was withdrawn and pipetted directly into a tube of tetrazolium medium.

Rooster and turkey semen samples were collected in sterile tubes at the time of artificial manual ejaculation.

Exudate was sometimes obtained from the tendon sheath areas of the hock and foot pad of turkeys and chickens. The involved areas were cleansed with cotton pledgets wet with 70% ethanol, seared by a gas flame, and incised with a scalpel. A sterile cotton swab was inserted repeatedly to obtain exudate which was rinsed off in 2-3 ml of tryptose phosphate broth. Sometimes masses of caseated exudate were removed with sterile forceps and ground in a mortar with alundum and then suspended in 4-5 ml of broth.

Fluid yolk from embryonated eggs was sometimes obtained

by aseptic removal by a 5 ml pipette. Smaller portions of fluid yolk were sometimes obtained with a cotton swab or an inoculating wire with 3 loops at the end. The smaller portions of yolk were usually inoculated directly into culture medium.

Yolk from embryonated eggs was frequently too thick to be pipetted with ease. Then sterile thumb forceps were used to aseptically remove a portion of the yolk sac membrane with abundant adjacent yolk materkal. This was ground in a mortar with alundum and sufficient tryptose phosphate broth to provide a 25-50% suspension of yolk.

Cultural Procedures

Almost all of the specimens obtained, other than serum samples, were inoculated into tubes of tetrazolium medium for the isolation of Mycoplasma. Some of them were also inoculated onto turkey serum agar slants.

The tubes of tetrazolium medium were inoculated with approximately 0.5-1.0 ml of fluid specimen or prepared tissue suspension. Some specimens which were obtained by cotton swabs or inoculation loops were placed directly into tetrazolium medium.

The inoculated tubes were incubated at 37° C. for 5-7 days before they were examined for possible Mycoplasma growth as evidenced by a change in color of the medium from light amber to shades of red due to reduction of the tetrazolium

indicator. Then 0.5-1.0 ml was transferred to a fresh tube of tetrazolium medium with subsequent transfers at 7 day intervals.

All tubes were held at least 10 days before they were discarded as negative in the absence of a color change. Cultures which continued to reduce tetrazolium were examined further after 2-5 transfers. They were streaked onto agar plates which were incubated at 37° C. for 2-3 days in a moist chamber before the plates were examined for the presence of typical Mycoplasma colonies. Cultures which failed to give evidence of Mycoplasma colony growth were streaked onto ordinary horse blood agar plates for the detection of bacterial contaminants. If contamination was not apparent the cultures were again plated on agar medium for the detection of Mycoplasma colonies. Giemsa stained culture sediment was sometimes studied to aid in identifying Mycoplasma.

Cultures which were considered to be Mycoplasma were then maintained by weekly transfers in yeast autolysate medium for further studies.

Slants of turkey serum agar with yeast received approximately 0.5 ml of inoculum, 2.0 ml of yeast autolysate medium, and an additional 0.1 ml of a 1% solution of thallous acetate containing 200,000 units of crystalline penicillin G potassium per ml.

The agar slant tubes were incubated upright in racks at

37° C. for 5-7 days. Then a 1.0 ml pipette was used to rinse the surface of the slant with broth from the base of the slant and 0.5-1.0 ml was transferred to a fresh agar slant. After at least 2, but usually 3-4, agar slant passages a drop from the tip of the transfer pipette was distributed over the surface of a portion of the plate of agar medium for subsequent detection of Mycoplasma growth. This procedure was repeated 1 or 2 more times before agar slant cultures were discarded as negative due to the absence of Mycoplasma colony growth on the agar plate medium.

All isolates of Mycoplasma which were obtained from agar slants were maintained on agar slants by weekly transfers. In addition, they were transferred into tetrazolium medium to determine if they could be propagated in that broth medium with or without reduction of tetrazolium. If they did not grow in the tetrazolium medium they had to be maintained on agar slants. However, attempts were then made to adapt them to broth horse serum medium with mucin.

An effort was made to store at least one 4-5 ml portion of actively growing broth cultures in a mechanical freezer at -30° C. to maintain early passages of cultures for later pathogenicity studies. Numerous selected culture sediment suspensions were lyophilized for more permanent storage.

Isolates of Mycoplasma which were found to grow only on agar slant medium were maintained on slants by weekly

transfers, but storage of some slant cultures was attempted by adding 2-3 ml of yeast autolysate medium to the slant tubes which were then placed in a mechanical freezer at -30° C. in a relatively flat position so that the broth medium would freeze while it was covering the agar surface.

Broth cultures of Mycoplasma obtained from other investigators were inoculated into plain turkey serum medium or yeast autolysate medium. In addition, slants of turkey serum agar with yeast were sometimes inoculated. Isolates received in the form of lyophilized samples were similarly cultured after being reconstituted with a few drops of sterile distilled water. The isolates were then maintained in broth medium, unless they required medium in the form of agar slants.

Lyophilization Procedure

Tissue and exudate suspensions in tryptose phosphate broth, whole broth culture, and culture sediment from 2-3 tubes of broth medium resuspended in 3-4 ml of plain turkey serum medium were lyophilized at various times during this study. The procedure was the same as employed by Dr. M. S. Hofstad for the lyophilization of the isolates of avian Mycoplasma which he supplied.

Approximately 0.25-0.50 ml of fluid suspension was delivered into lyophilizing ampoules prepared from 7 mm outside diameter Pyrex glass tubing with a blown bulb diameter of 0.5-1.0 inch. Up to 2.0 ml of suspension was delivered

into ampoules with bulb dimensions of approximately 0.75 X 2.0 inches when larger volumes were desired. The ampoules were then rotated in an absolute alcohol-dry ice bath to freeze the suspension in a thin film. The ampoules were then attached to the mantle of a custom made high vacuum apparatus with a condenser submerged in an absolute alcohol-dry ice bath.

The 8-12 ampoules representing a typical batch were maintained on the apparatus for 6-7 hours before they were sealed off under vacuum, at only 2-5 microns of mercury, with an oxygen-gas flame. The lyophilized samples were then stored at 4° C.

Antigen Production

Antigen for preparing antiserum

Antigens were prepared fresh for each inoculation of roosters or rabbits when preparing specific antiserum. Three 8 ml tubes of yeast autolysate medium, or 4 slants each with 4 ml yeast autolysate medium for isolates adapted only to that medium, were inoculated with 1.0 ml of actively growing culture, and were incubated at 37° C. for 48-72 hours to attain adequate growth. Sediment from the broth was resuspended in 3 ml of sterile saline following centrifugation at 2500 RPM for 15 minutes at room temperature in a #269 swinging bucket head in a model 2-V International centrifuge.

The resulting antigen preparations varied in their

relative density. No attempt was made to standardize them as to density. However, larger volumes of the less dense preparations were employed.

Agglutination antigen

Antigens for the rapid serum plate agglutination and tube agglutination tests were prepared by the same procedure up to the step of turbidity standardization. Tubes of turkey meat infusion broth medium with turkey serum, and usually yeast autolysate, were inoculated with 0.5-1.0 ml of actively growing broth cultures of Mycoplasma and were incubated for 48-72 hours at 37° C. The contents of 2 tubes were then aseptically poured into each 200 ml portion of medium in flasks followed by incubation at 37° C. for 36-48 hours before being harvested.

The few agar slant isolates which were adapted to grow in horse serum medium with mucin often required longer incubation. However, adequate growth was obtained in 2-3 days using the above inoculation procedures followed by agitation of the flasks during incubation in a water bath as described by Ross (148). A model BB Burrell[1] wrist action shaker was used to gently agitate the flasks without excessive foaming of the horse serum medium with mucin.

Antigen was then harvested by pouring the broth cultures

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[1]Burrell Corporation, Pittsburgh, Pennsylvania.

into 100 ml plastic bottles which were centrifuged for 60 minutes at 4000 RPM in a #850 angle head in a model PR-1 International[1] refrigerated centrifuge at 4° C.

The supernatant fluid was poured off and the culture sediment was resuspended in 0.25% phenolized Cox phosphate buffer solution. A glass Tenbroek tissue grinder was used to obtain a uniform antigen suspension. The turbidity of the antigen was standardized so that a 1:20 dilution of stock antigen would afford a reading of 50 on a Klett-Summerson[2] photoelectric colorimeter in the presence of a #54 light filter. The stock antigens were then stored in a refrigerator at 4° C.

Stock antigens were employed without further dilution as plate agglutination antigens, or were diluted 1:20 in 0.25% phenolized Cox phosphate buffer solution for tube agglutination antigen in preliminary studies.

Agglutination antigens which were employed with extended serial two-fold dilutions of serum were adjusted, just prior to being used, to give a 60 reading on a Klett-Summerson photoelectric colorimeter with a #54 light filter.

Hemagglutination antigen

The hemagglutinating ability of various Mycoplasma was

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[1]International Equipment Company, Boston, Massachusetts.

[2]Klett Manufacturing Company, New York, New York.

studied by titration of broth culture sediment obtained during the procedures used in preparing antigens for either agglutination tests or for production of specific immune serum. Such portions were resuspended in Cox phosphate buffer without phenol. An effort was made to prepare rather dense suspensions, but they were not standardized as to their turbidity.

In the case of the A serotype, where larger volumes of hemagglutinating antigen were needed, antigen was harvested from several 500 ml Erlenmeyer flasks containing 200 ml of plain turkey serum medium or yeast autolysate medium as described for the preparation of agglutination antigens. The centrifuged culture sediment was resuspended in 3-4 ml of Cox phosphate buffer solution per flask of culture employed. A glass Tenbroek tissue grinder was used to prepare uniform antigen suspensions which were so concentrated that they were almost milky white. An equal volume of U.S.P. glycerin was added to most batches in an effort to further preserve the hemagglutinating potency in addition to being stored at -30°C.

Preparation of Specific Immune Serum

Antigens were inoculated into at least 3 roosters and/or 2 rabbits for the preparation of specific immune serum. Roosters received 3-4 injections of 0.5-1.0 ml into the leg muscles at 5-7 day intervals, followed by being bled out 5-7 days after the last injection. Rabbits were given 3-4 intravenous injections of 0.5-1.0 ml at similar intervals. More

recently prepared rabbit sera were not harvested until 8-14 intravenous inoculations were made. Roosters were maintained in cages kept in isolation rooms or in isolated outdoor areas. Rabbits were maintained in cages in isolation rooms.

The roosters and rabbits were bled by cardiac puncture with a 50 ml glass syringe fitted with an 18 gauge needle 2 inches long. The blood from each group of roosters or rabbits was pooled in a 250 ml glass beaker and allowed to clot at room temperature. The clot was cut and the serum allowed to separate for 2-3 hours followed by centrifugation to obtain the clear serum. Each lot of serum was dispensed into small screw cap vials.

Then rabbit sera were routinely heat inactivated at 56° C. for 30 minutes in a water bath prior to storage. Some of the rooster sera were also heat inactivated, but recently prepared ones were not heated. Vials of antisera were stored in a mechanical freezer at -30° C.

Morphological Studies

Colony morphology

A binocular dissecting microscope with 9X eye pieces and a 6X objective was used to examine inoculated agar plates for the presence of Mycoplasma colonies, and to study the colonies observed. Proper adjustment of the reflected indirect substage lighting afforded visualization of the colonies with a

shadowing effect to enhance differences of elevation and light density.

A speed graphic type camera was used to obtain photographs of Mycoplasma colonies. The front lenses of the camera were removed so that the camera could be adapted directly to one eye piece lens system of the microscope. The intensity of the light source was then increased and colonies could be clearly observed on the view screen of the camera prior to making each exposure.

Cell morphology

The morphology of Mycoplasma organisms was studied by microscopic examination of Giemsa stained smears of whole broth culture or culture sediment. Culture sediment was obtained following centrifugation of broth culture tubes at 2500 RPM for 15 minutes in a #269 swinging bucket head in a model 2-V International centrifuge at room temperature. The supernatant broth was poured off, and the sediment was resuspended in what little broth remained in the tube.

A small drop of broth culture or culture sediment was placed on a clean glass slide by a 1.0 ml pipette. The smear was air dried for several minutes before being flooded with 100% methyl alcohol for 5 minutes for fixation. The smear was then flooded for 1 hour with a 4% solution of Giemsa stain prepared in 20 ml of distilled water with 5 ml of Cox phosphate buffer solution. The smear was water rinsed,

blotted dry, and examined under the oil immersion lens of a binocular microscope at 950X magnification.

Biochemical Studies

Carbohydrate fermentation

Separate batches of media for carbohydrate fermentation studies contained 1% dextrose, maltose, sucrose, trehalose, galactose, mannitol and lactose. Inverted glass vials were included in tubes of all the media for a few initial studies for the detection of gas production.

Cultures maintained in medium containing yeast autolysate were passaged at least 10 times in medium free from yeast autolysate prior to initiating final carbohydrate fermentation studies to avoid possible effects of enzymes of yeast origin. However, numerous cultures growing in yeast autolysate medium were inoculated directly into carbohydrate media for initial screening type studies.

A 5 ml pipette was used to inoculate each series of tubes with 0.5 ml of actively growing broth culture of the various Mycoplasma. The tubes were incubated at 37° C. and observed daily for evidence of any decrease of the pH as noted by a change of the phenol red indicator from red to shades of yellow. Final readings were recorded after 5-7 days of incubation. Some series of tubes were held for 14-21 days.

A Beckman model G pH meter was employed to determine the

pH of various tubes in some series, especially for tubes with only partial color changes, and to establish definite acidity or alkalinity of other tubes.

Tetrazolium reduction

Most initial isolations of Mycoplasma were obtained by inoculating tetrazolium medium. The red color change due to reduction of the tetrazolium served as an indicator of Mycoplasma growth. Care was taken to determine that the reduction was due to the presence of Mycoplasma, and not due to the effects of tissue debris or contaminant bacteria.

The isolates of avian Mycoplasma which had been received as broth passages and those obtained from lyophilized samples were eventually studied to determine their ability to reduce tetrazolium. Reduction of tetrazolium by isolates which were originally obtained on agar slant medium was studied after those isolates had been adapted to grow in horse serum medium with mucin, to which tetrazolium was then added.

Biological Studies

Hemagglutination

Culture sediment preparations which were prepared as antigens for hemagglutination studies were titrated by exactly the same procedure as described for titration of hemagglutination antigen for use in hemagglutination-inhibition studies. The hemagglutinating titer of such antigens

was recorded as the reciprocal of the highest serial two-fold dilution of antigen which afforded complete agglutination of turkey erythrocytes in the 0.5 ml of 0.25% suspension used.

Hemolysis

Actively growing cultures of Mycoplasma were streaked onto plates of turkey serum agar with horse erythrocytes by a platinum inoculating loop. The plates were kept moist by placing them inverted in an instrument pan, containing a layer of gauze saturated with distilled water, which was then sealed shut with aluminum foil. The plates were incubated at 37° C. for 48-72 hours before final readings were recorded.

Hemolysis could best be detected by holding the plates toward a light source and observing them.

Cultural Studies

Media enrichment

Turkey serum enrichment Turkey meat infusion broth without turkey serum and yeast autolysate was prepared to determine if isolates of avian Mycoplasma actually required serum enrichment of that base medium for growth. Tubes, which contained 8 ml of the medium, were inoculated with 0.5 ml of actively growing broth cultures and were then incubated at 37° C. for 7 days with subsequent transfers of 0.5 ml portions at 7 day intervals. The presence of growth, as indicated by increased turbidity of the medium, was recorded

at each passage. However, final determination of growth was based on observation of colony growth on agar media which were inoculated with the broth cultures. One drop of inoculum was placed on the agar medium with a pipette, and then the drop was streaked over the surface of the agar by a platinum inoculating loop. Turkey meat infusion agar and Difco tryptose blood agar base were employed as agar plate media in addition to turkey meat infusion agar with 20% turkey serum and 5% yeast autolysate.

Yeast autolysate enrichment Broth media were prepared from turkey meat infusion broth with 20% turkey serum with and without yeast autolysate for studies to determine the relative growth promoting effect of yeast autolysate for avian Mycoplasma. Media with .5%, 2%, 5% and 10% yeast autolysate were compared in one preliminary study. However, most trials were conducted in media containing 5% yeast autolysate as compared to media without yeast autolysate. The media were inoculated in the usual manner and incubated at 37° C. for 7 days, with serial passages made at 7 day intervals. The relative amount of growth was determined by observation of increased turbidity of the media. Isolates of the H serotype required special procedures, which are described separately in the following section.

Enrichments for cultivation of H serotype isolates Isolates of the H serotype were studied separately since they

required special procedures. Initial isolation attempts employed broth tetrazolium medium and turkey serum agar slants, both containing 5% yeast autolysate. Additional studies were conducted to investigate the use of 15% horse serum and 1% swine gastric mucin in broth and agar media. Evidence of growth was based primarily on subsequent observation of colony growth on agar plate media inoculated with material from the various media employed.

Pathogenicity Studies

Pathogenicity studies were conducted with embryonated chicken eggs, young chickens, various age turkeys, and a small number of young pigeons and quail.

Embryonated chicken eggs were routinely inoculated via the yolk sac route at 7 days of incubation at 37° C. A 1.0 ml glass syringe fitted with a 24 gauge needle 1 inch long was used to inject 0.1 ml of serial ten-fold dilutions of broth culture, resuspended culture sediment, tissue suspension or fluid exudate into the yolk sac. Entry was made through a hole which was drilled in the egg shell approximately 1/2 inch below the air cell boundary. After inoculation the entry hole was sealed with Duco[1] cement and the eggs were placed in trays in an incubator at 37° C. until the 19th day of incubation, or until removed as dead when candled daily. All dead and

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[1]E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware.

surviving embryos were examined for gross lesions, and all materials harvested were cultured for the presence of bacterial contamination.

Chickens and turkeys were inoculated with broth culture, culture sediment suspension, tissue suspension or fluid exudate introduced into one or more of several sites. Initial studies involved the inoculation of 0.5-1.0 ml of inoculum into one thoracic air sac and several drops intratracheally. Turkeys also received 0.5-1.0 ml of the inoculum injected into one infraorbital sinus. A few turkeys also received intravenous inoculation.

Later studies included the injection of inoculum into the tendon sheath areas of the hock and foot pad regions of young chickens and turkeys. A standard inoculation procedure was eventually devised which consisted of inoculation of the right sinus (of turkeys only), left thoracic air sac, right hock region and left foot pad of chickens and turkeys.

All bird inoculations were made with a 1.0 ml glass syringe fitted with a 24 gauge needle 1 inch long. Air sacs were inoculated by inserting the needle in a dorso-anterior direction after penetrating the body wall just posterior to the last rib. The tendo-vaginal cavity of the hock region, not the joint cavity directly, was inoculated by inserting the needle from the medial surface of the hock so as to penetrate the relatively large cavity just beneath the tendon

over the posterior surface of the flexed hock. When 0.5 ml of inoculum was so injected it distended the tendon sheath both dorsal and ventral to the hock joint.

The few pigeons and quail employed as experimental hosts received several drops of inoculum intranasally, intratracheally and approximately 0.5 ml injected into the left thoracic air sac.

Most of the experimentally inoculated birds were housed in cages in separate isolation rooms, or in stainless steel box-type isolation units in an isolation room. Some groups of large turkeys were housed in small wire fence pens on various isolated pasture sites to provide isolation for several groups at the same time.

Serological Procedures

Plate agglutination test

Rapid serum plate agglutination tests were conducted by mixing one drop of standardized stock antigen from a 0.2 ml pipette with one drop of serum on a clear glass plate. Agglutination results were recorded after 2-3 minutes with occasional rotation of the plate. Positive agglutination was evidenced by obvious clumping of the unstained antigen employing indirect light from beneath the glass plate. Known positive and negative sera were included with each series of sera being tested.

Tube agglutination test

In preliminary tube agglutination studies a 0.2 ml pipette was used to deliver 0.08, 0.04, and 0.02 ml portions of test serum into 10 x 75 mm tubes followed by the addition of 1.0 ml of a 1:20 dilution of stock antigen in 0.25% phenolized Cox phosphate buffer solution to each tube. The resulting serum dilutions were 1:12.5, 1:25 and 1:50 respectively. Agglutination results were recorded after 15 to 18 hours incubation at 37° C. Positive agglutination was evidenced by obvious clearing of the upper portion of the fluid with clumpy sediment in the bottom of the tube when observed toward an indirect light source with a black background.

Final tube agglutination studies were conducted with extended dilutions of serum. A series of 10 x 75 mm tubes were prepared to contain 0.5 ml amounts of serial two-fold dilutions of serum from 1:5 through 1:320. Then 0.5 ml of stock antigen diluted to give a 60 reading on a Klett-Summerson photoelectric colorimeter was pipetted into each tube. This procedure provided final serial two-fold dilutions of 1:10 through 1:640. The tubes were incubated at 37° C. for 15 to 18 hours before agglutination results were observed and recorded.

Hemagglutination-inhibition test

The hemagglutination-inhibition test is a method to

detect serum antibodies which are capable of inhibiting the hemagglutinating effect of certain Mycoplasma antigen preparations. Serial two-fold dilutions of serum gave a reasonably quantitative titration procedure as described by Hofstad (98).

Since serial two-fold dilutions of serum were titrated against a constant amount of hemagglutinating antigen it was necessary to determine the hemagglutinating potency of the antigen just prior to its use. Serial two-fold dilutions of antigen were prepared in a series of 13 x 100 mm glass tubes.

The diluent used was Cox buffered saline containing normal rooster or turkey serum at the rate of 1:1000 to aid in a more uniform settling out of the erythrocytes. Tube 1 received 0.9 ml of diluent, and tubes 2 through 10 received 0.5 ml of diluent. Then 0.1 ml of antigen was pipetted into tube 1. A clean pipette was used to mix the contents of tube 1 and to transfer 0.5 ml to tube 2. A separate pipette was used to mix and then transfer 0.5 ml portions serially through tube 9 with a final 0.5 ml being discarded from tube 9. Thus, 0.5 ml portions of serial two-fold dilutions from 1:10 through 1:1280 were prepared. Tube 10 contained no antigen because it was a diluent and erythrocyte control.

An automatic pipetting syringe was used to deliver 0.5 ml of a 0.25% suspension of chicken or turkey erythrocytes into each of the 10 tubes. The contents of the tubes were mixed by shaking and were incubated at room temperature for 1-2 hours

before the results were recorded.

Complete hemagglutination was evidenced by a uniform pink sheet or film of agglutinated erythrocytes over the entire bottom of the tube. Negative hemagglutination was noted by a dense red button of nonagglutinated erythrocites at the center of the bottom of the tube. Partial hemagglutination was noted by a red doughnut-like ring on the bottom of the tube with a pink background indicating some hemagglutination.

The titer of the antigen was recorded as the reciprocal of the highest dilution which afforded complete hemagglutination. One hemagglutinating unit was considered to be contained in 0.5 ml of the antigen at that end point dilution. Thus, if a given antigen was found to have a titer of 640 then 1 hemagglutinating unit would be represented by 0.5 ml of a 1:640 dilution of that antigen. Two hemagglutinating units would be contained in 0.5 ml of a 1:320 dilution and 4 units in a 1:160 dilution.

Hemagglutination-inhibition tests were conducted by titrating serial two-fold dilutions of serum against 2 hemagglutinating units of antigen in a series of 13 x 100 mm glass tubes. Tube 1 received 0.8 ml of Cox buffered saline. Tube 2 received 0.5 ml of antigen with a potency of 4 hemagglutinating units, and tubes 3 through 9 received 0.5 ml of antigen with a potency of 2 hemagglutinating units.

When chicken serum samples were being tested it was

found to be more satisfactory to employ 8 hemagglutinating units in tube 2, and 4 units in tubes 3 through 9.

Then a 1.0 ml pipette was used to deliver and mix 0.2 ml of serum in tube 1, and to transfer 0.5 ml to tube 2. The mixing and transferring of 0.5 ml portions through tube 9 was done with the same pipette and a final 0.5 ml portion was discarded from tube 9. Thus, serial two-fold dilutions of serum from 1:10 through 1:1280 were prepared in 2 hemagglutinating units. Tube 1 served as a 1:5 dilution of serum in diluent with no antigen. Tube 10 was prepared to contain 0.5 ml of diluent only to serve as a diluent and erythrocyte control.

Then 0.5 ml of a 0.25% suspension of chicken or turkey erythrocytes was pipetted into each of the 10 tubes in the series. The contents of the tubes were mixed by shaking and were incubated at room temperature for 1-2 hours before the results were recorded as positive or negative hemagglutination. Negative hemagglutination indicated hemagglutination-inhibition due to specific antibodies in the test serum. The hemagglutination-inhibition titer of a given serum was recorded as the reciprocal of the highest dilution of serum affording complete inhibition of hemagglutination.

Known positive and negative serum samples were always included with each group of samples to be tested. In addition a separate titration of the calculated 2 hemagglutinating

units of antigen was prepared at the time it was being employed in a test series. This was done so that if any major deviation from 2 hemagglutinating units was encountered it would indicate some error in preparing the 2 units and would suggest in which direction to alter interpretation of inhibition results.

Chicken erythrocyte suspensions were used when testing chicken serum samples. Turkey erythrocytes were used when testing serum samples from turkeys, rabbits, pheasants, partridges, pigeons and quail. Low dilutions of rabbit serum usually caused agglutination of turkey erythrocytes. This reaction could be removed by absorbing 5 ml of serum with 2 ml of packed turkey erythrocytes for 15-30 minutes at room temperature followed by moderate centrifugation to remove the erythrocytes. Two absorptions were usually adequate. The procedure for absorbing smaller amounts of serum was to add 0.5 ml of serum to 2.0 ml of a 10% suspension of erythrocytes followed by similar incubation and centrifugation procedures. The resulting supernatant then served as a 1:5 dilution of the absorbed serum.

Hemagglutinin absorption study

Only one hemagglutinin absorption study was conducted employing 2 sets of antigens and their homologous antisera of rooster origin.

Hemagglutinating antigens were prepared by harvesting

the culture sediment from 3 flasks of medium for each antigen strain as described in the section on the preparation of hemagglutinating antigens. Glycerin was not added to the antigens in this procedure.

Three successive absorptions of each serum with the homologous and heterologous antigens were conducted on separate portions of the sera. Each absorption was done by adding 6.0 ml of serum to the centrifuged culture sediment obtained from 1.5 ml of very dense, but not standardized, hemagglutinating antigen. The culture sediment was resuspended in the added serum with the aid of a 1.0 ml pipette. The 16 x 125 mm tube containing the serum-antigen suspension was then placed in a model BB Burrell wrist action shaker and incubated for 1 hour at 37° C. with moderate agitation.

After each of the 3 absorptions the tube of serum-antigen suspension was centrifuged in a #269 swinging bucket head at 2500 RPM for 15 minutes in a model 2-V International centrifuge at room temperature. The supernatant fluid was then poured off into a clean 16 x 125 mm screw cap tube containing another portion of sedimented antigen. The supernatant fluid from the 3rd absorption was filtered through a Selas 03 filter candle to remove the excess antigen after a preliminary clarification by the described centrifugation procedure.

Portions of unabsorbed sera and sera following each

absorption were saved for final hemagglutination-inhibition studies employing the absorbing antigens as the hemagglutinating antigens. A normal rooster serum sample was included with each series of inhibition studies for control purposes.

EXPERIMENTAL

Morphological Studies

Colony morphology

Colony morphology was essentially the first aspect studied because it became apparent very early that observation of colony growth was an accurate and simple procedure to identify growth of Mycoplasma after a few broth passages of original cultures. The use of Giemsa stained smears of early passage culture sediment will be described later in this thesis.

Results It was noted that a range of colony sizes was almost always present when cultures were streaked on agar medium. These size differences were assumed to be related to environmental conditions, since the larger colonies tended to be the most distant from others. Actual colony size within the densely colonized initial streak lines was difficult to determine because of crowding, and larger colonies often appeared to be the result of several small colonies which had coalesced. However, it was noted that colonies from some isolates were consistently larger than from other isolates. Most all of the colonies were similar in their general appearance in that they were usually smooth, entire and possessed an obvious center which was elevated above the periphery of the colony and was of greater optical density.

They tended to grow into the agar, as evidenced by the fact that they resisted being removed by an inoculating wire loop.

Colonies from certain isolates not only differed by their greater diameter, some also varied in the relative size and height of their centers in relation to their periphery. It soon became apparent that some original cultures contained colonies with a spectrum of features suggesting the presence of 2 colony types. Well isolated colonies were either large with relatively low and broad centers, or were small with relatively tall and less broad centers.

An attempt was made to pick typical large colonies and typical small colonies by cutting out a small block of agar which contained the desired colony. The block was pierced with the pointed scalpel blade used to cut the block, and was transferred to a tube of broth medium. When increased turbidity indicated reasonable growth in the broth, it was streaked on agar medium. The process of picking colonies, culturing in broth and streaking on agar was repeated 3 to 5 times to obtain pure large colony and pure small colony lines. It was more difficult to obtain pure large colony lines than pure small colony lines. Large and small colonies were often obtained from selected large colonies. This probably was due to the presence of small colonies adjacent to, or possibly under, the selected large colony. However, pure large colony lines were obtained after repeated selections.

The original broth culture obtained from tracheal swab A from hen 102 produced large and small colony types. A pure large colony line, isolate 1021, was obtained. A pure small colony line, isolate 1018, was also obtained by selection of colonies. Another small colony line, isolate 1010, was obtained from the sinus exudate of a turkey which developed sinusitis following inoculation of the original tracheal swab culture. Isolates 1010, 1018 and 1021 were determined to represent 3 separate serotypes as will be discussed later in this thesis.

The original broth culture obtained from tracheal swab A from hen 111 produced large and small colonies. A large colony line, isolate 1111, and a small colony line, isolate 1112, were obtained. The original culture from tracheal swab B, from the same hen at a later date, produced only large colonies, isolate 1207. These 3 isolates also represented 3 different serotypes.

Several other cultures from chickens in the same flock appeared to produce only large or only small colonies even after attempts to select for possible differences in colony sizes. Large colony isolates so studied are identified as 1304, 1504, 1805 and 2805. Isolates 1403, 1703, 2004 and 2705 originated from cultures which proved to consistently produce only small colonies.

Although isolate 594, as described in this thesis,

consistently produced only very large colonies it is interesting to note that early chicken embryo passage material yielded both large and small colonies. A small colony line was selected, and produced tendovaginitis in 1 of 3 chickens experimentally inoculated. All 3 chickens developed agglutinins for the A serotype. The small colony line was discarded without further study, since the large colony line was of major interest at the time.

As colonies from the various isolates were observed an attempt was made to record the diameter of typical well isolated colonies, excluding the few exceptionally large colonies. All of the recordings were made from colonies grown on turkey serum agar with yeast autolysate. The majority of the plates were incubated 48 hours, although some were incubated for 72 hours.

The results of these studies are presented in Table 5, and photomicrographs of colonies representing the various serotypes appear in Figure 1. In an effort to report the results in some organized manner, the isolates are arranged by serotypes, although these relationships were not established at the time many of these studies were being made.

The colony morphology of isolates within a given serotype was relatively similar, although not always characteristic enough to distinguish them from colonies of certain other serotypes. Isolates within serotypes A, H, I and J produced

Table 5. Diameter of typical well isolated colonies of avian Mycoplasma on turkey serum agar with yeast autolysate

Serotype	Isolate	Colony diameter in mm	Serotype	Isolate	Colony diameter in mm
A	A5969	.25	A	894	.20
	F	.20		1010	.30
	S6	.20		1079-1	.15
	VR	.20		1112	.20
	24R	.25		1344	.20
	26R	.20		1403	.15
	128VA	.30		1605	.20
	187	.20		1900	.15
	197	.20		2705	.25
	293	.20	B	K18B	.60
	595	.20		54-537	.75
	699	.20		114TB	.70
	734	.25		1207	.75
	755	.20		1304	.80
	756	.20		1504	.70
	796	.20		2600	.70
	801	.25		5858	.65
	857-1	.20	C	C	.75
	882	.20		DIVA	.70
	893A	.20		Tu	.80

Table 5. (Continued)

Serotype	Isolate	Colony diameter in mm	Serotype	Isolate	Colony diameter in mm
C	108TA	.70	I	799-1	.15
	1021	.80		799-4	.15
	1111	.80		806C	.15
	2805	.70		807-5	.15
D	NY	.70		807-18	.15
	594	.85		814-5	.20
E	C26	.70		815-4	.15
	DPR-2	.75		822-4	.15
	640	.65		823C	.15
F	SA-1	.90		833R	.15
	SA-2	.85		844-2	.15
	SA-3	.80		849P	.15
G	0	.70		850-2	.20
H	N	.20		851R	.15
	933	.15		853M	.15
	1022-2	.20		854-2	.15
	1104	.20		1018	.15
I	114C	.15		1703	.25
	116C	.20		2004	.15
	172C	.20	J	693	.15
	695	.25		1075	.15

Table 5. (Continued)

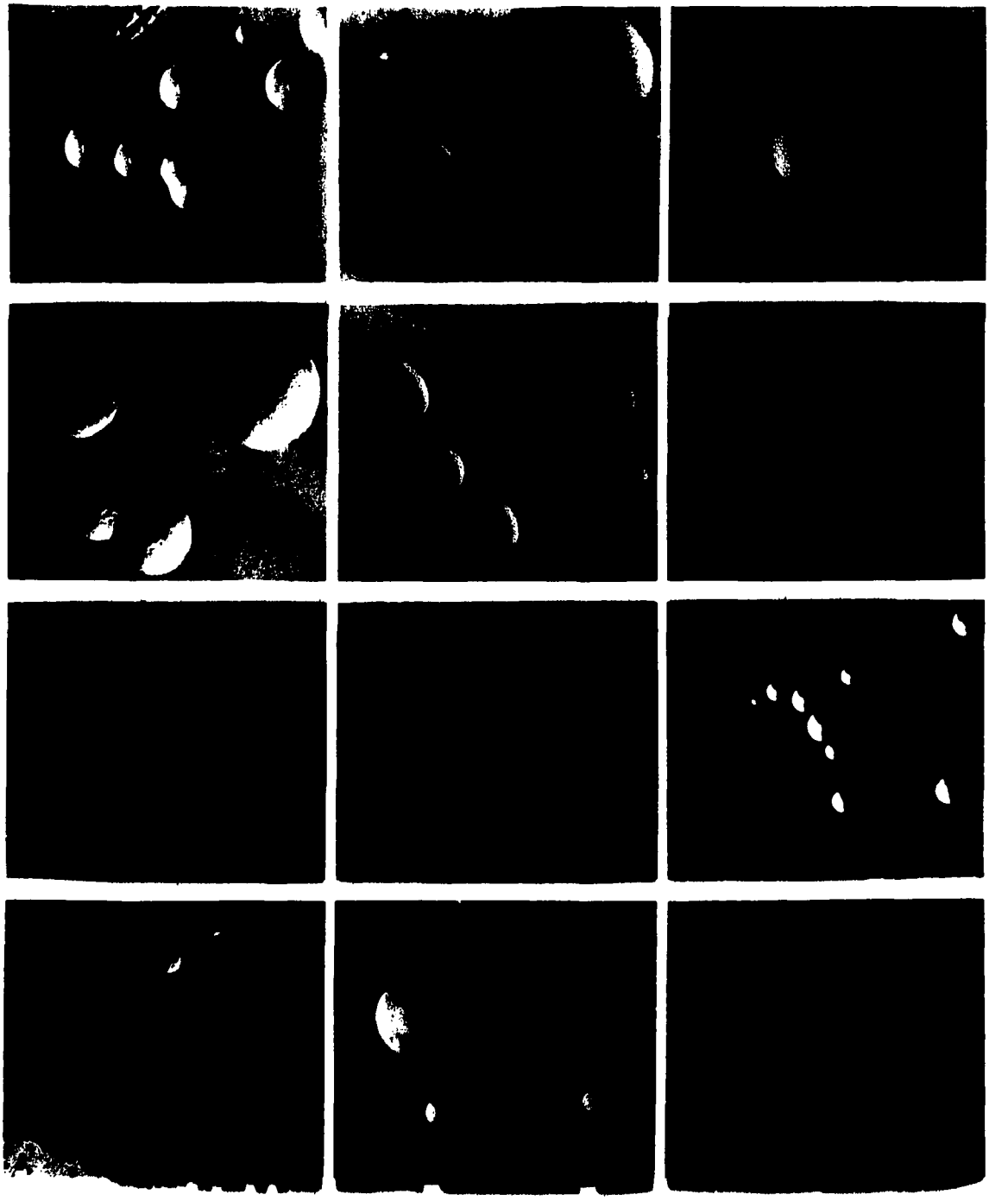
Serotype	Isolate	Colony diameter in mm	Serotype	Isolate	Colony diameter in mm
K	132VI	.60	Unclas-sified	890A	.40
	158VI	.60		1042	.15
	1805	.70		1043	.15
L	694	.45		1079-3	.15
	858-76	.40		1087-1	.15
	878-50	.40		1102-7	.15
Unclas-sified	SA-4	.60		1103-5	.15
	27R	.35		1109	.15

very small colonies, while those within serotypes B, C, D, E, F, G and K produced large colonies. Colonies of the isolates in the L serotype were intermediate in size. The relatively broad dense centers noted within the D serotype were characteristic but were not greatly different from those of the F serotype. The general topography of colonies in the H serotype tended to be distinct enough to differentiate them from other small colony serotypes which usually were not as flat.

In addition to differences in colony size and somewhat in general topography, their morphological stability was

Figure 1. Typical colonies of avian Mycoplasma isolates representing serotypes A through L as indicated. Approximately 48 hours incubation at 37° C. on turkey serum agar with 5% yeast autolysate. X 25.

Serotype	Isolate
A	128VA
B	54-537
C	DIVA
D	594
E	DPR-2
F	SA-3
G	O
H	N
I	1703
J	693
K	132VI
L	694



noted to vary following incubation beyond 48 to 72 hours. The isolates from pigeons (694, 858-76, 878-50, and 890A) produced colonies which tended to degenerate very rapidly. They frequently were so disorganized after only 48 hours incubation that a repeat plating followed by only 24 hours incubation was necessary to observe typical smooth colonies. Colonies from B serotype isolates were sometimes noted to begin degeneration earlier than the remaining serotypes. Figure 2 is a photomicrograph which shows typical early degeneration of colonies of pigeon isolate 878-50 at 48 hours incubation.

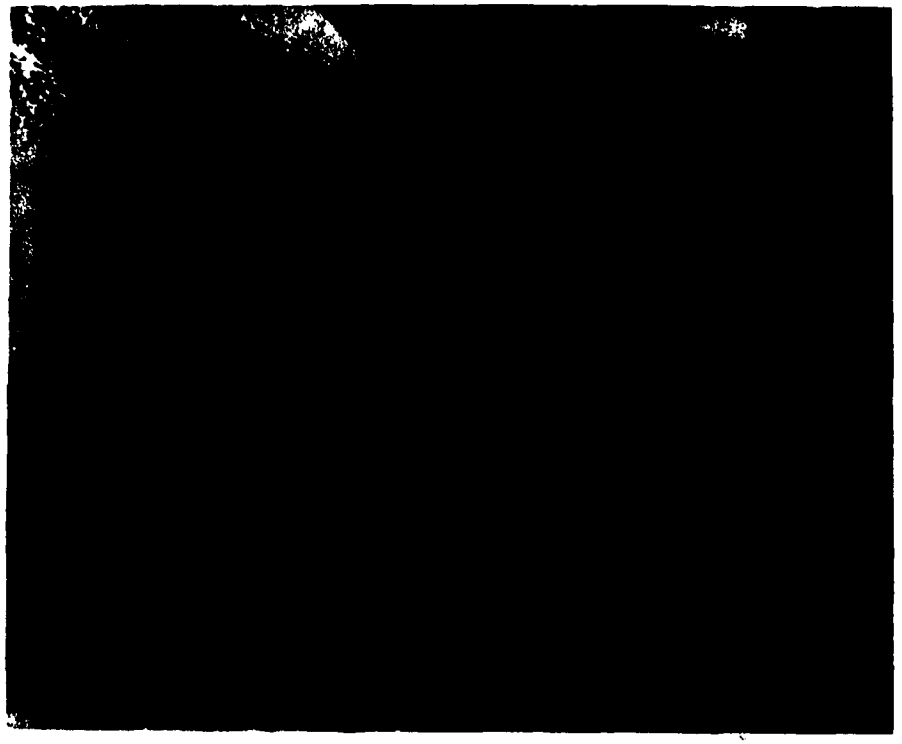
Discussion It is apparent that differences in colony morphology are significant between various serotypes, but are of limited value in differentiating isolates from 12 or more serotypes. At least large colony serotypes B, C, D, E, F, G and K can be differentiated from small colony serotypes A, H, I and J.

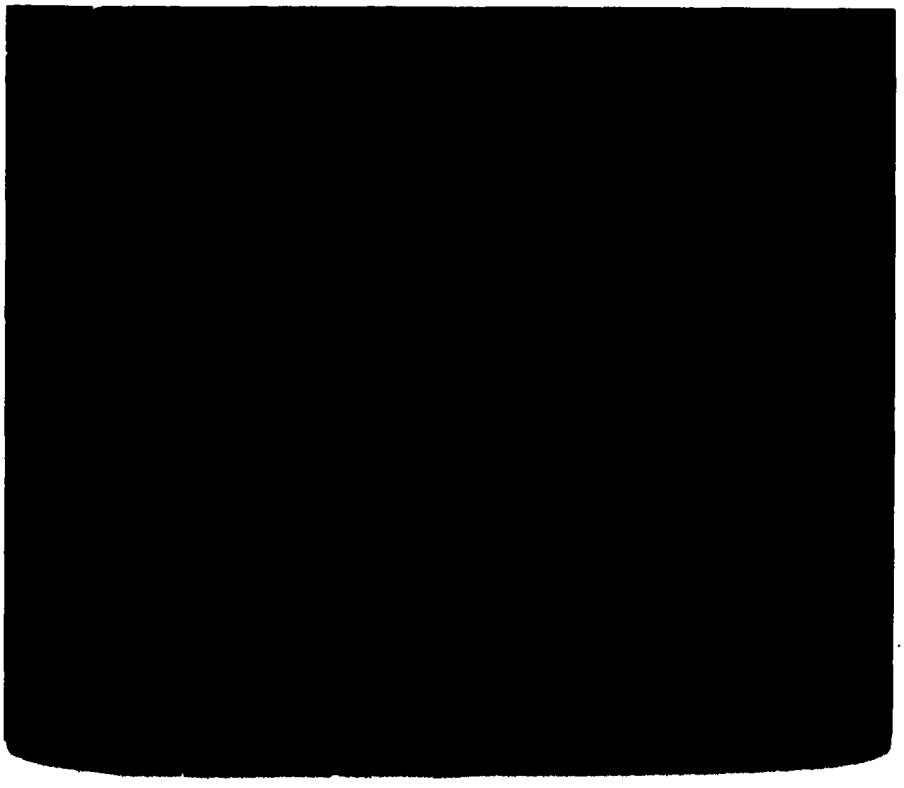
Yamamoto and Adler (181) reported that isolates representing A, G and H serotypes produced colonies 0.1 mm in diameter, those from the C serotype were 0.2 mm and those from the F serotype were 0.4 mm in diameter. Kleckner (113) reported that colonies from the A serotype were small, and those from serotypes B, C, D, E, F, G and H were large.

Differences in media could well account for differences in actual colony size, but it is not clear why Yamamoto and Adler (181) found very small colonies from the G serotype as

Figure 2. Typical early degeneration of colonies of serotype L isolate 878-50 on turkey serum agar with 5% yeast autolysate. Incubated 48 hours. X50.

Figure 3. Hemolysis of horse erythrocytes incorporated into turkey serum agar with 5% yeast autolysate. Isolate SA-3 of serotype F at 48 hours incubation. Dark colonies surrounded by clear zone of hemolysis. Approximately X2.





compared to the large ones described for the same isolate studied by Kleckner (113) and also in this study. Similarly, Kleckner (113) found large colonies to be typical for the H serotype while Yamamoto and Adler (181) considered them to be very small, as found in this study.

It should be mentioned that pure culture technique is not a simple procedure when dealing with Mycoplasma. The colonies are very small, the largest rarely attaining 1 mm, cannot easily be transferred with an inoculating wire, and are difficult to get well streaked out on agar medium. In addition, intermittent passage in chick embryos and birds may alter the actual spectrum of subsequent cultures reisolated, and further possible errors may occur when isolates are passed along via a series of laboratories some years after original isolation. Still another factor of practical significance is that different isolates, probably different serotypes, vary in their ability to grow in chicken embryos and various media as reported by Fabricant (57). All of these factors complicate the task of obtaining and maintaining pure cultures.

Cellular morphology

In the very early part of this study Giemsa stained smears from early passage culture sediments were frequently prepared and examined. It soon became evident that it was seldom possible to positively identify Mycoplasma organisms when they were few in number as compared to the debris in the

preparations. Coccoid, coccobacilliform, slender rod and ring forms were noted. However, further examination of stained smears for the purpose of identifying positive Mycoplasma cultures was abandoned in favor of observing colony growth on agar medium.

Very late in the study, once most all of the isolates of Mycoplasma had been serotyped, an effort was made to study their cellular morphology in greater detail on a comparative basis. Twenty-six isolates representing the various serotypes were selected for study. They were passaged twice at 2 day intervals to obtain actively growing cultures. Then 0.3 ml of each culture was inoculated into an 8 ml tube of yeast autolysate medium except for isolate N which was cultivated in horse serum medium with mucin. One drop of broth culture was removed at 12, 24, 48 and 72 hours of incubation to prepare smears which were Giemsa stained. At 96 hours centrifuged culture sediments were prepared for staining. Uninoculated medium was included for control purposes, and a repeat study was made with isolates of the K serotype.

Results No cellular forms were observed in the medium control smears, and the results of a repeated study with 3 isolates of the K serotype were in agreement with the first observations. An amazing spectrum of cellular morphological forms was observed in the 150 stained smears examined, although there was little difference noted between isolates

within the same serotype. It would require an extensive series of photomicrographs to present all of the details. The major forms observed from a typical isolate representing each serotype are presented in Table 6, and photomicrographs of several cellular forms are presented in Figure 4.

Minute cocci, coccobacilli and slender rods were the basic cellular forms noted. However, rings, short slender filaments, filaments in clusters, beaded filaments, sacculated or swollen rods and filaments, and cellular fragments were also observed.

Isolates of the A and H serotypes were characterized by their simplicity of coccoid cellular forms with little variation in size. The cells from isolates in the L serotype were essentially cocci, but variable coccobacilli were numerous. The serologically unclassified SA-4 isolate produced somewhat larger cocci which varied considerably in diameter. The I and J serotype isolates produced short rods of varying dimensions and some coccoid forms.

Serotype B, C and D isolates produced primarily slender rods and abundant rings. The F serotype isolates appeared to have essentially slender rods, but during rapid growth they were almost entirely ring forms. Isolates within the E and G serotypes also tended to produce slender rods, but relatively short slender filamentous forms were abundant. The ends of their rods and filaments often were swollen or sacculated,

Table 6. Summary of cellular morphological forms of avian Mycoplasma cultures

Serotype	Isolate	Forms observed at indicated hours of growth[a]				
		12 hours	24 hours	48 hours	72 hours	96 hours
A	1010	cocci .4-.5[b] coccobacilli	cocci coccobacilli	cocci coccobacilli	cocci in clumps	cocci .4-.5 in clumps
B	1304	rods .3x1.5 rings .7-1	rods .4x1.5 rings	rods rings .7-1	rods rings	rods .3x1 coccobacilli
C	Tu	rods .3x1.5 rings .7-1	rods .4x1.5 short chains	rods rings	rods .3x1.5 rings 1-1.5	rods .3x1 coccobacilli
D	594	rods .3x1.5 rings 1-1.5	rods .4x1.5 short chains	rods rings	rods rings 1-2	rods .3x1 in clumps
E	DPR-2	rods .3x1-2 filaments .1-.2x3-5	rods clusters[c] loops[d] .8-1	rods clusters loops	rods loops fragments	coccobacilli fragments

[a]Giemsa stained smears from broth culture at 12, 24, 48 and 72 hours, and from the centrifuged culture sediment at 96 hours of growth. Observed at 950X with oil immersion lens.

[b]Approximate dimensions in microns, via eye piece micrometer.

[c]Clusters of filaments which sometimes appear branched.

[d]Loops designate sac-like swellings at ends of rods or filaments.

Table 6. (Continued)

Sero-type	Isolate	Forms observed at indicated hours of growth[a] 12 hours	24 hours	48 hours	72 hours	96 hours
F	SA-3	rings 1-2 coccobacilli	rings 1-2	rings	rings fragments	coccobacilli fragments
G	0	rods .3x1-2 filaments .1-.2x3-8	rods .4x102 clusters loops .8-1	rods clusters loops	rods .3x1-2 loops .8-1 fragments	coccobacilli rods .3x1
H	N	cocci .4-.5 in clumps	cocci in clumps	cocci coccobacilli	cocci coccobacilli	cocci .3-.5 in clumps
I	695	rods .3x1.5 short chains	rods short chains	rods .4x1.5	rods coccobacilli	coccobacilli cocci .4-.6
J	693	rods .3x.8 short chains	rods cocci .4-.6	rods .3x1 cocci	rods in clumps	coccobacilli cocci .4-.6
K	1805	rods, rings beaded filaments .5x10-20	rods .3x1.5 beaded and swollen filaments	swollen filaments 1-2x30-50 loops	filaments fragments rods	coccobacilli fragments rods .3x1.5
L	694	cocci .4-.6 coccobacilli	coccobacilli cocci	coccobacilli cocci	cocci coccobacilli	cocci .5-.7 coccobacilli
Unclassified	SA-4	cocci .5-.7	cocci	cocci	cocci	cocci .6-.8

Figure 4. Cell morphology of isolates of avian Mycoplasma from broth cultures. Giemsa stained smears prepared at indicated hours of growth. Photographed with a green (#58 light filter. X1000.

1. Isolate 1010 of serotype A, 72 hours. Coccoi to coccobacilli 0.4-0.5 micron.

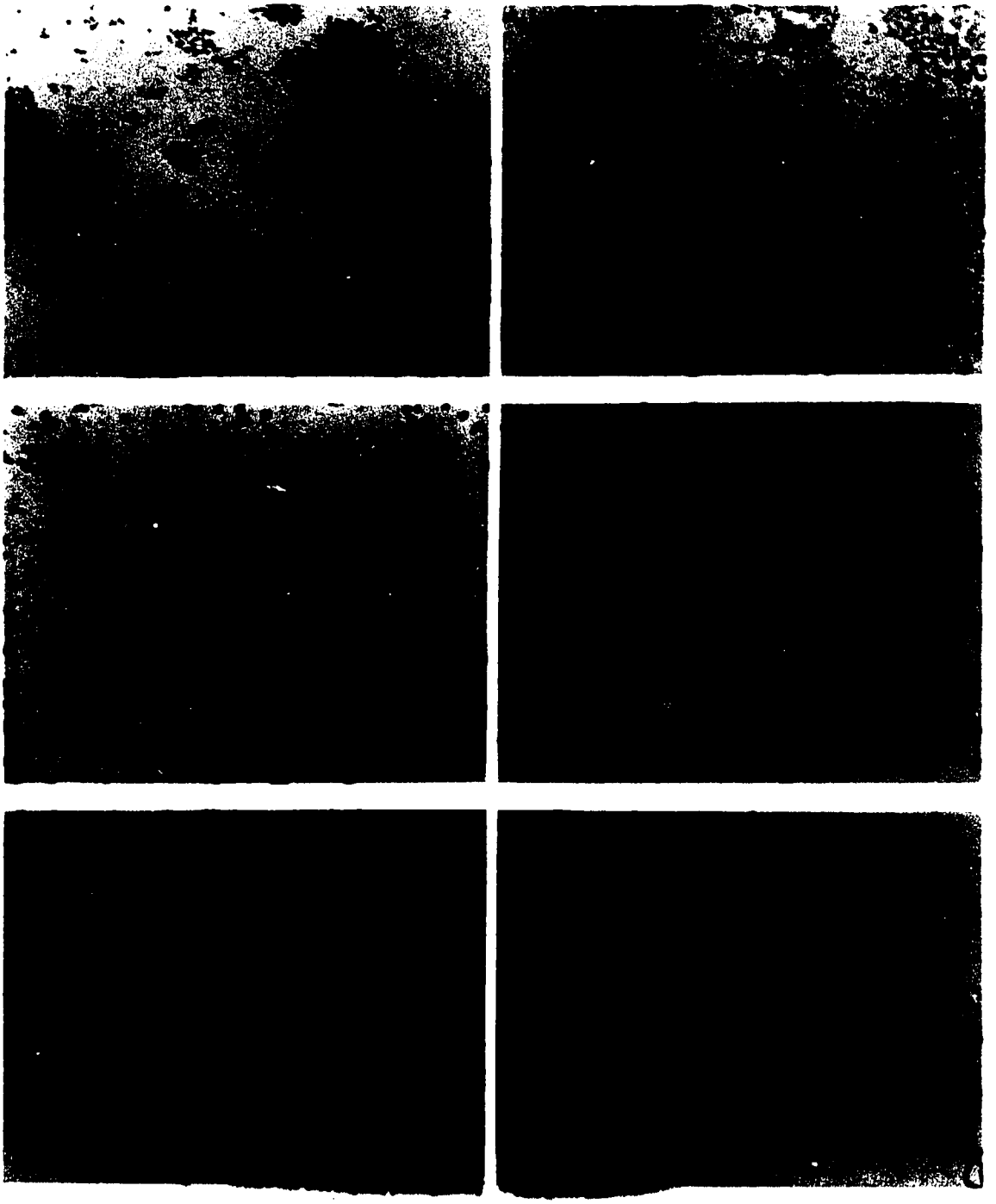
2. Isolate 695 of serotype I, 48 hours. Rods approximately 0.3 x 1.5 microns.

3. Isolate SA-3 of serotype F, 24 hours. Rings 1.0-2.0 microns in diameter.

4. Isolate 1805 of serotype K, 12 hours. Rods, rings and beaded filaments. Filaments to 20 microns in length.

5. Isolate 1805 of serotype K, 24 hours. Rods, loops in ends of rods or small filaments, long beaded and sacculated filaments.

6. Isolate 1805 of serotype K, 48 hours. Mass of swollen filaments with sacculated ends.



resembling an inoculating loop or a sphere with a tail on it. Regardless of the serotype the slender rods were usually slightly curved or bent.

Isolates representing the K serotype consistently produced slender rods, a few rings and numerous relatively large filaments which later were beaded and sacculated, and then apparently fragmented.

Discussion The cellular morphology of various avian Mycoplasma has not been studied extensively. Yamamoto and Adler (181) observed Giemsa stained culture preparations from isolates S6, SA, O, C and N. Their results were very similar to those in this study for the same isolates. Coccoid to coccobacilliform bodies have been associated with pathogenic avian Mycoplasma ever since the initial reports by Nelson (127), and Van Herick and Eaton (168).

The pecular ring forms, apparently representing swollen cells, have been described by Fahey and Crawley (67), Hofstad and Doerr (101), Chu (28), Yamamoto and Adler (181), and Kleckner (113). The rings were usually considered to be present in cultures of relatively nonpathogenic avian Mycoplasma, a concept further supported by the present study.

Extensive filamentous forms apparently have not been described from avian Mycoplasma in previous studies. Thus, the complex filamentous forms noted for the K serotype seem to be quite unique among Mycoplasma of avian origin studied

to date.

It is apparent that most all of the isolates possessed some degree of complexity during their growth period. However isolates of the K serotype exhibited more extensive forms strongly suggesting a complex growth cycle similar to that of Mycoplasma mycoides variety mycoides as described by Freundt (71). He studied several Mycoplasma from human and animal sources, and noted a wide variety of filamentous forms. Most of the filaments observed were quite slender and short for isolates other than M. mycoides variety mycoides.

The designation, pleomorphic organisms, seems almost too limited in scope to define the cellular forms of avian Mycoplasma which range from tiny cocci to complex mycelial-like structures and various intermediates.

Biochemical Studies

Carbohydrate fermentation

The determination of the ability of avian Mycoplasma isolates to ferment dextrose, maltose, sucrose, galactose, mannitol, trehalose and lactose required repeated studies. For the sake of clarity each study is described separately, and only the results for typical serotypes are mentioned in the preliminary studies.

Fermentation of carbohydrates in yeast autolysate medium

Isolates of serotypes A, B, C, I, K and L cultivated in either

yeast autolysate medium or in plain turkey serum medium were inoculated into tubes of yeast autolysate medium with carbohydrate and phenol red. Inverted glass vials were included in each tube for the detection of gas production. Evidence of carbohydrate fermentation was based on the color change of phenol red during an incubation period of 7 days.

Results Isolates of serotypes B and L did not ferment any of the carbohydrates. Isolates of serotypes A, C, I and K produced acid, without gas, only in dextrose, maltose and sucrose within 2-7 days as based on the presence of a deep yellow color in the medium. None of the isolates produced a color change in control medium with phenol red, but without added carbohydrate.

Fermentation of carbohydrates in Difco phenol red broth base medium Isolates of serotypes A, B, I, K and L cultivated in either yeast autolysate medium or in plain turkey serum medium were inoculated into tubes of Difco phenol red broth base with turkey serum and carbohydrate. Evidence of carbohydrate fermentation was based on the color change of phenol red during an incubation period of 11 days.

Results Isolates of serotypes B and L did not ferment any of the carbohydrates. Isolates of serotypes A, I and K fermented dextrose and maltose within 2-5 days and galactose within 4-7 days. Some of these isolates fermented sucrose within 2-5 days, but only if they had been cultivated

in yeast autolysate medium. The fermentation of dextrose, maltose and galactose did not appear to be influenced by the presence of yeast autolysate in the inoculum. None of the isolates produced a color change in control medium with phenol red, but without added carbohydrate.

Fermentation of carbohydrates in plain turkey serum medium Isolates of each serotype except H cultivated in either yeast autolysate medium or in plain turkey serum medium were inoculated into tubes of plain turkey serum medium containing carbohydrate and phenol red. Evidence of carbohydrate fermentation was based primarily on the color change of phenol red at the end of 10 days incubation, although the pH of selected tubes was measured at 10-14 days and many of the tubes were observed for 21 days.

Results Isolates of serotypes A, C, D, F, I, J and K fermented dextrose and maltose within 2-5 days when they had been cultivated in either yeast autolysate medium or in plain turkey serum medium. They also fermented sucrose within 2-7 days if they had been cultivated in yeast autolysate medium, and some isolates of serotypes C and D apparently fermented sucrose even though they had been cultivated in plain turkey serum medium for at least 8 passages. Many of the isolates were noted to produce at least a yellow-orange color in galactose as did a few in mannitol. None of the isolates fermented trehalose or lactose. Isolates of

serotypes B, E, G and L did not ferment any of the carbohydrates, even when they had been cultivated in yeast autolysate medium.

The pH of selected tubes was measured at 10-14 days incubation. The recorded pH value of deep yellow medium was approximately 5.5-6.4 and red medium was approximately 7.0-7.4. Uninoculated control medium was found to have a pH of 7.6-7.8 following incubation although it was approximately 8.0 when prepared. Medium which was yellow-orange had a pH of 6.6-6.9.

The results recorded from observation of tubes which were held for 21 days incubation were essentially the same as those recorded at 10 days.

Effect of yeast autolysate on sucrose fermentation

In an attempt to definitely establish that yeast autolysate influenced the fermentation of sucrose yeast autolysate medium was serially diluted in tubes of plain turkey serum medium with phenol red and 1% sucrose following the procedure for routine culture transfers. Then 0.5 ml of an A serotype isolate culture in plain turkey serum medium was inoculated into each of the tubes containing approximately serial ten-fold dilutions of the yeast autolysate medium.

Results Enough yeast autolysate was present in the first 4 tubes to allow fermentation of sucrose. This suggested that cultures required at least 4 routine passages

in medium free from yeast autolysate before valid sucrose studies could be made.

Similar evidence that yeast autolysate influenced sucrose fermentation resulted when it was noted that a culture of Salmonella typhimurium failed to ferment sucrose, but readily did so when sterile yeast autolysate was added to the medium.

Fermentation of carbohydrates in horse serum medium The only broth medium in which isolates N, 933, 1022-2 and 1104 of the H serotype were adapted to grow in was horse serum medium with mucin and yeast autolysate. Similar medium was prepared with added carbohydrate and phenol red. Each isolate was inoculated into tubes of the carbohydrate medium and the pH of each tube was measured at the end of 10 days incubation.

Results The H serotype isolates did not ferment dextrose, maltose, sucrose, galactose, mannitol, trehalose or lactose as indicated by pH readings of 7.0-7.3

Carbohydrate fermentation without the influence of yeast autolysate This study was conducted to determine the carbohydrate fermenting spectrum of representative isolates of each serotype except H based on actual pH values in medium free from the influence of yeast autolysate. Isolates which had been passaged a minimum of 12 times in turkey serum medium free from yeast autolysate were inoculated into tubes of

plain turkey serum medium with carbohydrate and phenol red indicator. The pH of the carbohydrate medium in each tube was determined with a Beckman pH meter at the end of 10 days incubation at 37° C. Medium with a pH of 6.5 or less was considered as evidence of carbohydrate fermentation.

Results The results of this study are presented in Table 7. Isolates of serotypes B, E, G and L did not ferment any of the carbohydrates employed. Isolates of serotypes A, C, D, F, I, J and K consistently fermented dextrose and maltose, but not trehalose or lactose, and the results with sucrose, galactose and mannitol were variable.

Sucrose was definitely fermented by isolates DIVA and 1111, but not Tu, of serotype C and by isolates NY and 594 of serotype D. None of the isolates of other serotypes was considered to ferment sucrose, since the lowest pH recorded for any of them was 6.8 and readings of 6.9-7.2 were the most common.

Galactose was fermented by isolates 158VI and 1805, but not 132VI, of the K serotype. None of the isolates of other serotypes was considered to ferment galactose although a pH of 6.6 was recorded for isolates 1605 and 734 of serotype A, isolates DIVA, Tu and 1111 of serotype C, isolate 833R of serotype I, isolate 1075 of serotype J and unclassified isolate 1043. The pH values for most other isolates were 6.7-7.2.

Table 7. Carbohydrate fermentation of avian Mycoplasma without the influence of yeast autolysate

Serotype	Isolate	Dextrose	Maltose	Sucrose	Galactose	Mannitol	Trehalose	Lactose
A	A5969	+[a]	+	-[b]	-	-	-	-
	S6	+	+	-	-	-	-	-
	293	+	+	-	-	-	-	-
	734	+	+	-	-	-	-	-
	801	+	+	-	-	-	-	-
	1605	+	+	-	-	-	-	-
B	K18B	-	-	-	-	-	-	-
	5858	-	-	-	-	-	-	-
C	DIVA	+	+	+	-	-	-	-
	Tu	+	+	-	-	+	-	-
	1111	+	+	+	-	-	-	-
D	NY	+	+	+	-	-	-	-
	594	+	+	+	-	-	-	-
E	DPR-2	-	-	-	-	-	-	-
	640	-	-	-	-	-	-	-

[a]Positive indicates a pH of 6.5 or less in plain turkey serum medium with carbohydrate and phenol red at the end of 10 days incubation.

[b]Negative indicates a pH of 6.6 or higher. A Beckman pH meter was employed.

Table 7. (Continued)

Serotype	Isolate	Dextrose	Maltose	Sucrose	Galactose	Mannitol	Trehalose	Lactose
F	SA-1	+	+	-	-	-	-	-
	SA-2	+	+	-	-	-	-	-
	SA-3	+	+	-	-	-	-	-
G	O	-	-	-	-	-	-	-
I	695	+	+	-	-	-	-	-
	833R	+	+	-	-	-	-	-
	850-2	+	+	-	-	-	-	-
J	693	+	+	-	-	-	-	-
	1075	+	+	-	-	-	-	-
K	132VI	+	+	-	-	+	-	-
	158VI	+	+	-	+	-	-	-
	1805	+	+	-	+	-	-	-
L	694	-	-	-	-	-	-	-
	878-50	-	-	-	-	-	-	-
Unclas-sified	SA-4	+[c]	+	-	-	-	-	-
	890A	-	-	-	-	-	-	-
	1043	+	+	-	-	-	-	-
Control medium		-	-	-	-	-	-	-

[c]Isolate SA-4 completely decolorized phenol red in all of the tubes within 2 days.

Mannitol was fermented only by isolate Tu of serotype C and by isolate 132VI of serotype K. A pH of 6.6 was recorded for isolate 1605 of serotype A, isolate DIVA of serotype C, and isolates 695 and 850-2 of serotype I. The pH values for most other isolates were 6.7-7.2.

Isolate SA-4 of the unclassified group completely decolorized phenol red within 2 days. The fermentation of dextrose was indicated by a pH of 6.1 and fermentation of maltose was indicated by a pH of 6.2.

Discussion Yeast autolysate was considered to be a valuable enrichment in media for the isolation and maintenance of avian Mycoplasma, but it certainly was not appreciated during carbohydrate fermentation studies. However, it did not appear to alter the results obtained in studies with carbohydrates other than sucrose. It was concluded that yeast autolysate contained sucrase which hydrolyzed sucrose into its components, dextrose and fructose. Then dextrose, and possibly fructose, was available for fermentation.

Dextrose was readily fermented by all of the isolates which fermented any of the carbohydrates and lactose medium remained neutral to alkaline. Thus, it seems to be justifiable to extract at least the results from dextrose and lactose from all of the trials, regardless of the presence of yeast autolysate. Then it can be concluded that all isolates representing serotypes A, C, D, F, I, J and K

fermented dextrose and not lactose, and that all of the isolates of serotypes B, E, G, H and L did not ferment either dextrose or lactose.

Sucrose was definitely fermented by both isolates of serotype D and by at least 2 of the isolates of serotype C, but not by any other isolates when yeast autolysate was not present. The very rarely observed fermentation of mannitol possibly is not significant. The few definitely confirmed results indicating galactose fermentation are also of questionable significance.

Irregularities were frequently encountered; depending upon the isolate, the medium employed, the medium in which the isolate was cultivated, the specific carbohydrate employed and the method of determining acid production. However, the irregularities encountered in this study are matched by those reported in the literature. In 1957 Adler et al. (12) reported that dextrose, galactose and sucrose were fermented by pathogenic Mycoplasma, but not mannitol or lactose. In 1958 Adler et al. (5) reported that pathogenic Mycoplasma did not ferment galactose, but did ferment dextrose, trehalose and sucrose. Yamamoto and Adler (181) listed dextrose, maltose, sucrose and galactose as fermented. Klecker (113) reported that isolates of serotype A fermented dextrose, maltose, sucrose, galactose and trehalose. Gianforte et al. (75) reported that isolates representing serotype A fermented

dextrose, sucrose and trehalose, but not galactose. Tourtellotte and Jacobs (165) reported that avian Mycoplasma usually fermented dextrose, sucrose and galactose.

It is almost meaningless to attempt comparison of the present results with those previously reported. However, all of the reported results seem to be in agreement with the proposed concept that avian Mycoplasma can be divided into 2 main groups; those that ferment dextrose and those that do not. The simplicity of such a division is significant.

Then the results of the present study are in very good agreement with those reported for the serotypes studied by Yamamoto and Adler (181) and Kleckner (113); isolates representing serotypes A, C, D and F fermented dextrose and isolates representing serotypes B, E, G and H did not ferment dextrose.

Tetrazolium reduction

Tetrazolium was primarily employed as an indicator of Mycoplasma growth in original isolation procedures. However, it was later employed in studies to determine if isolates of all of the serotypes of avian Mycoplasma were capable of reducing it. Reduction of tetrazolium was indicated by the production of a bright red color in the medium. Isolates which failed to reduce tetrazolium were carried for at least 5 serial passages at 7 day intervals before they were recorded as negative.

Results The results of studies to determine the ability of isolates of avian Mycoplasma to reduce tetrazolium are presented in Table 8.

Isolates within serotypes A, B, C, D, I, J, K and L readily reduced tetrazolium, while isolates within serotypes E, G, and H did not. The F serotype isolates did not produce obvious reduction of tetrazolium although a pink color sometimes developed in the lower portion of the broth following 7 days of incubation. Only isolate SA-4 in the unclassified group failed to reduce tetrazolium.

Discussion Somerson and Morton (157) reported that isolates of Mycoplasma from humans reduced various tetrazolium salts with the production of colored insoluble formazans. They found that triphenyltetrazolium chloride was satisfactory when incorporated in medium at 0.005%, but was inhibitory at 0.1%.

Yamamoto and Adler (181) studied the reduction of "tetrazolium-blue" by avian Mycoplasma and reported that isolates representative of serotypes A, C, F and O, but not H, reduced it. Isolates within serotypes F and O essentially did not reduce tetrazolium in the present study. These differences may well be due to the fact that different tetrazolium compounds were employed in different types of media.

Table 8. Ability of avian Mycoplasma to reduce tetrazolium in broth medium

Serotype	Isolate designations	Reduction of tetrazolium
A	A5969, F, S6, VR, 24R, 26R, 128VA, 187, 197, 293, 595, 699, 734, 755, 756, 796, 801, 857-1, 882, 893A, 894, 1010, 1079-1, 1112, 1344, 1403, 1605, 1900, 2705	+[a]
B	K18B, 54-537, 114TB, 1207, 1304, 1504, 2600, 5858	+
C	C, DIVA, Tu, 108TA, 1021, 1111, 2805	+
D	NY, 594	+
E	C26, DPR-2, 640	-[b]
F	SA-1, SA-2, SA-3	0[c]
G	0	-
H	N, 933, 1022-2, 1104	-
I	114C, 116C, 172C, 695, 799-1, 799-4, 806C, 807-5, 807-18, 814-5, 815-4, 822-4, 823C, 833R, 844-2, 849P, 850-2, 851R, 853M, 854-2, 1018, 1703, 2004	+
J	693, 1075	+

[a]Positive reduction of tetrazolium in broth medium within 3-5 days, as indicated by a red color change, was caused by each of the isolates of the indicated serotype.

[b]Negative reduction, absence of a red color change, was recorded for all of the isolates of the indicated serotype after at least 5 serial passages in tetrazolium medium.

[c]Isolates of the F serotype essentially did not reduce tetrazolium, although a pink color sometimes developed in the lower portion of broth following 7 days of incubation.

Table 8. (Continued)

Serotype	Isolate designations	Reduction of tetrazolium
K	132VI, 158VI, 1805	+
L	694, 858-76, 878-50	+
Unclassified	27R, 890A, 1042, 1043, 1079-3, 1087-1, 1102-7, 1103-5, 1109	+
	SA-4	-

Biological Studies

Hemagglutination

The ability of concentrated culture sediment preparations to agglutinate erythrocytes was studied by preparing serial two-fold dilutions of antigen followed by the addition of the erythrocyte suspension. Refrigeration, freezing and glycerin were employed in attempts to enhance the stability of the hemagglutinating activity of antigens.

Results Preliminary studies showed that antigens prepared from isolates of the A serotype agglutinated chicken and turkey erythrocytes. Erythrocytes from several animal species were tested once with a hemagglutinating antigen prepared from isolate 801 of the A serotype. Erythrocytes from a day old chick, a chicken, turkey, guinea pig, raccoon

and a dog were agglutinated. The avian erythrocytes gave the highest titers. Erythrocytes from a horse, calf, sheep, pig, cat, rat and a mouse were not agglutinated to any extent. Human type O erythrocytes were only partially agglutinated by low dilutions of the antigen.

Chicken and turkey erythrocytes afforded consistently good hemagglutination, although turkey cells often yielded titers which were 1 two-fold dilution higher. For the sake of uniformity turkey erythrocytes were employed to determine the hemagglutinating ability of antigens prepared from isolates in this study. The results are presented in Table 9.

Almost all of the isolates of serotypes A, I, J and K caused agglutination of turkey erythrocytes with titers of 80 or higher, considering the titer as the reciprocal of the highest dilution causing complete hemagglutination. Repeated attempts failed to detect higher titers for the few isolates with titers below 80. Seven of the 10 isolates in the serologically unclassified group consistently caused hemagglutination. None of the isolates from serotypes B, C, D, E, F, G, H and L was ever noted to cause hemagglutination at or above the 1:10 starting dilution. Unclassified isolates SA-4, 27R and 890A also were nonhemagglutinating. Regardless of the serotype the hemagglutination reaction was very stable, with almost no elution of erythrocytes during 24-48 hours at room temperature.

Table 9. Hemagglutination of turkey erythrocytes by avian Mycoplasma antigens

Serotype	Isolate	Hemagglutination titer[a]	Serotype	Isolate	Hemagglutination titer[a]
A	A5969	40	A	801	1280
	F	160		857-1	80
	S6	160		882	40
	VR	160		893A	160
	24R	80		894	80
	26R	40		1010	160
	128VA	160		1079-1	40
	187	80		1112	40
	197	160		1344	160
	293	320		1403	320
	595	160		1605	320
	699	40		1900	40
	734	160		2705	80
	755	--[b]	B	K18B	--
	756	20		54-537	--
	796	320		114TB	--

[a]Titers are recorded as the reciprocal of the highest serial two-fold dilution of antigen which caused complete hemagglutination.

[b]Negative at the 1:10 starting dilution.

Table 9. (Continued)

Serotype	Isolate	Hemagglutination titer[a]	Serotype	Isolate	Hemagglutination titer[a]
B	1207	--	H	N	--
	1304	--		933	--
	1504	--		1022-2	--
	2600	--		1104	--
	5858	--	I	114C	320
C	C	--		116C	160
	DIVA	--		172C	160
	Tu	--		695	640
	108TA	--		799-1	--
	1021	--		799-4	--
	1111	--		806C	160
	2805	--		807-5	640
D	NY	--		807-18	160
	594	--		814-5	1280
E	C26	--		815-4	1280
	DPR-2	--		822-4	160
	640	--		823C	640
F	SA-1	--		833R	640
	SA-2	--		844-2	80
	SA-3	--		849P	40
G	O	--		850-2	1280

Table 9. (Continued)

Serotype	Isolate	Hemagglutination titer[a]	Serotype	Isolate	Hemagglutination titer[a]
I	851R	640	L	858-76	--
	853M	640		878-50	--
	854-2	40	Unclassified	SA-4	--
	1018	40		27R	--
	1703	320		890A	--
	2004	--		1042	640
J	693	320		1043	40
	1075	640		1079-3	320
K	132VI	160		1087-1	320
	158VI	160		1102-7	320
	1805	80		1103-5	160
L	694	--		1109	160

The stability of the hemagglutinating activity of antigens prepared from isolates of the A serotype was far superior to that from other serotypes. Attempts to employ antigens from serotypes other than A as hemagglutinating antigens in subsequent hemagglutination-inhibition tests failed with one exception, as will be described in a later section.

Methods to enhance the stability of the hemagglutinating activity were investigated to a limited extent with antigens from serotype A and I isolates. Typical results from studies employing refrigeration, freezing and glycerin are presented in Table 10.

The stability of the hemagglutinating activity of the A serotype isolate antigen was definitely enhanced by storage at -30° C., and by the addition of an equal folume of glycerin to the original antigen suspension as compared to storage at 4° C. The preparation with 50% glycerin stored at -30° C. was the most stable.

In contrast, the same treatments were detrimental to the stability of the hemagglutinating activity of the antigens from the I serotype isolates. The untreated original antigen preparations were also much less stable at 4° C. than the A serotype antigen.

Discussion In 1945 Van Herick and Eaton (168) prepared hemagglutinating antigens from a Mycoplasma encountered during studies employing embryonated chicken eggs. Jungherr et al. (107) reported that antigen prepared from isolate A5969 caused hemagglutination of chicken erythrocytes. These early reports and those of White et al. (174), Fahey and Crawley (68), Gianforte et al. (75) and Hofstad (100) described the hemagglutinating ability of avian Mycoplasma which apparently represented the A serotype.

Table 10. Stability of hemagglutinating activity of concentrated antigens of avian Mycoplasma

Antigen treatment	Hemagglutination titer[a]		
	Serotype A Isolate 801	Serotype I Isolate 850-2	Serotype I Isolate 823C
Original antigen	1280	640	640
Original stored 24 hours at 4° C.	1280	320	160
Original stored 24 hours at -30° C.	1280	10	--[b]
50% glycerin added, stored 24 hours at 4° C.	640	10	--
50% glycerin added, stored 24 hours at -30° C.	640	--	--
Original stored 10 days at 4° C.	640	--	--
Original stored 30 days at 4° C.	320		
Original stored 1 year at 4° C.	20		
50% glycerin added, stored 1 year at 4° C.	320		
50% glycerin added, stored 2 years at 4° C.	80		
50% glycerin added, stored 1 year at -30° C.	640		
50% glycerin added, stored 2 years at -30° C.	320		

[a]Titer recorded as the reciprocal of the highest silution which caused complete hemagglutination of turkey erythrocytes.

[b]Negative at the 1:10 starting dilution.

Yamamoto and Adler (181) reported that isolates representative of the A serotype were hemagglutinating while isolates representative of the C, F, G and H serotypes did not cause agglutination of avian erythrocytes. Kleckner (113) confirmed their findings, and also reported that isolates of serotypes B, D and E did not cause hemagglutination.

Thus, the results of the present study are in very good agreement with previous reports. The hemagglutinating ability of isolates within serotypes I, J and K has not been studied by other workers to date, although the hemagglutinating characteristic of the I serotype was reported in 1962 by Yoder and Hofstad (182).

The significance of the hemagglutinating ability of serotypes I, J and K apparently is minor from a practical standpoint since such preparations were not stable enough to permit their use in hemagglutination-inhibition studies. However, ability to produce hemagglutination has been a characteristic which was formerly thought to be associated only with the A serotype. From the standpoint of determining the characteristics of various avian Mycoplasma it is significant that isolates from serotypes I, J and K may also cause hemagglutination to some extent.

Hemolysis

Hemolysis of erythrocytes incorporated into turkey serum agar medium was investigated by streaking broth cultures of

Mycoplasma isolates onto plates of such medium. The plates were observed for evidence of hemolysis after incubation for 48 hours at 37° C. in a moist chamber.

Results A zone of hemolysis around colonies of some isolates of avian Mycoplasma grown on turkey serum agar with erythrocytes was noted in preliminary studies. Colonies which caused hemolysis tended to hemolyze horse, sheep, turkey and chicken erythrocytes to approximately the same degree. Horse erythrocytes were selected for all subsequent studies because their bright red color was found to be very stable during the 48 hour incubation period, even when the agar plates had been stored for 2-3 weeks at 4° C. before being inoculated.

The ability of each of the isolates of avian Mycoplasma to cause hemolysis of horse erythrocytes in agar medium was investigated. Isolates which failed to produce hemolysis were plated a minimum of 3 times. The results of this study are presented in Table 11.

All of the isolates of serotypes A, B, C, D, F, H, K and L were hemolytic. None of the isolates from serotypes E, G, I and J was hemolytic. Isolates SA-4 and 890A of the unclassified group were hemolytic, and others in that group were nonhemolytic.

Hemolysis was of the clear (colorless) type with only an occasional tendency toward green hemolysis at the periphery.

Table 11. Hemolysis of horse erythrocytes in agar medium by avian Mycoplasma

Serotype	Isolate designations	Clear hemolysis
A	A5969, F, S6, VR, 24R, 26R, 128VA, 187, 197, 293, 595, 699, 734, 755, 756, 796, 801, 857-1, 882, 893A, 894, 1010, 1079-1, 1112, 1344, 1403, 1605, 1900, 2705	+[a]
B	K18B, 54-537, 114TB, 1207, 1304, 1504, 2600, 5858	+
C	C, DIVA, Tu, 108TA, 1021, 1111, 2805	+
D	NY, 594	+
E	C26, DPR-2, 640	-[b]
F	SA-1, SA-2, SA-3	+
G	0	-
H	N, 933, 1022-2, 1104	+
I	114C, 116C, 172C, 695, 799-1, 799-4, 806C, 807-5, 807-18, 814-5, 815-4, 822-4, 823C, 833R, 844-2, 849P, 850-2, 851R, 853M, 854-2, 1018, 1703, 2004	-
J	693, 1075	-
K	132VI, 158VI, 1805	+
L	694, 858-76, 878-50	+
Unclassified	SA-4, 890A	+
	27R, 1042, 1043, 1079-3, 1087-1, 1102-7, 1103-5, 1109	-

[a]Positive indicates that colonies of all the isolates of a given serotype caused clear hemolysis.

[b]Negative indicates that all of the isolates of a given serotype failed to cause hemolysis.

The extent of hemolysis, as based on the diameter of the zone, seemed to be directly related to the size of the colonies. The sharpness of hemolysis was just as distinct for small colony isolates as it was for large colony isolates. Figure 3 on page 98 is a photograph which shows the hemolytic effect of isolate SA-3, a very large colony isolate which produced large zones of hemolysis.

Discussion Very little information has been published concerning hemolysis by isolates of avian Mycoplasma. In 1954 Edward (50) reported that isolate Fowl (later designated as Mycoplasma gallinarum, and representative of serotype B in this study) was hemolytic when grown on horse serum agar medium containing horse erythrocytes. Yamamoto and Adler (181) studied the ability of broth cultures to hemolyze horse erythrocytes which were added to the broth. They noted a brown discoloration, rather than true hemolysis, for isolates of serotypes A and C. Their G serotype, isolate O, caused hemolysis on occasion, depending upon the type of serum in the medium and the source of erythrocytes used.

The results of the present study are in agreement with the above results with exception of the variable results previously reported for the O isolate, which was consistently found to be nonhemolytic on agar medium in this study. It appears that determining the hemolytic ability of avian Mycoplasma on agar medium is more satisfactory than by the broth method.

Cultural Studies

Media enrichment

Turkey serum enrichment Twenty-six isolates of avian Mycoplasma, representing each serotype, were employed in a study to determine if they actually required serum enrichment of turkey meat infusion broth for growth. Gross evidence of growth in turkey meat infusion broth without serum was recorded at each passage and observation of colony growth on agar medium was recorded following the 6th and 8th broth passage levels.

Results The results of observation for evidence of growth in the first 6 passages in broth medium and for subsequent colony formation on agar medium are presented in Table 12. Only isolate SA-4 was able to grow sufficiently in the turkey meat infusion broth without serum to produce grossly visible turbidity beyond the 3rd passage. Many of the isolates failed to produce visible turbidity beyond the 1st broth passage. However, it was surprising that 9 of the isolates produced distinct colony formation on turkey meat infusion agar with 20% turkey serum and 5% yeast autolysate when broth from the 6th passage was plated.

The isolates which apparently were growing in the broth, as evidenced by subsequent colony formation, were transferred 2 more times before they were again plated on agar media. Three types of agar media were inoculated from the 8th

Table 12. Growth of avian Mycoplasma during 6 passages in plain turkey meat infusion broth without serum

Serotype	Isolate	Highest passage with visible turbidity in broth without serum[a]	Distinct colony formation on infusion agar with serum[b] from 6th passage in broth without serum
A	1010	1	-
	1605	1	-
B	1504	1	-
	54-537	1	-
C	DIVA	2	-
	Tu	2	-
D	NY	1	-
	594	1	-
E	C26	2	+
	DPR-2	2	+
	640	3	+
F	SA-1	1	-
	SA-3	1	-
G	O	1	-
H	N	-[c]	-
I	695	1	-
J	693	3	+
	1075	1	-

[a]Turkey meat infusion broth without serum and yeast autolysate.

[b]Turkey meat infusion agar with 20% turkey serum and 5% yeast autolysate.

[c]Visible turbidity not present in the 1st through 6th passage.

Table 12. (Continued)

Serotype	Isolate	Highest passage with visible turbidity in broth without serum[a]	Distinct colony formation on infusion agar with serum[b] from 6th passage in broth without serum
K	132VI	1	+
	1805	1	+
L	694	1	-
	878-50	1	+
Unclassified	890A	1	+
	1042	1	-
	1043	2	-
	SA-4	6	+

passage cultures; Difco tryptose blood agar base, turkey meat infusion agar, and turkey meat infusion agar with 20% turkey serum and 5% yeast autolysate. None of the isolates produced any evidence of growth on the plates of Difco tryptose blood agar base. The results of observations for evidence of colony growth on the plates of turkey meat infusion broth and turkey meat infusion broth with 20% turkey serum and 5% yeast autolysate are presented in Table 13. Only isolate SA-4 produced distinct colonies on turkey meat infusion agar without serum or yeast autolysate, although isolates C26, 640 and 890A produced small irregular masses which suggested that some degree of growth was present.

Table 13. Growth of avian Mycoplasma during 8 passages in plain turkey meat infusion broth without serum

Serotype	Isolate	Distinct colony formation on designated agar media from 8th passage in broth without serum[a]: Turkey meat infusion agar without serum or yeast autolysate	Distinct colony formation on designated agar media from 8th passage in broth without serum[a]: Turkey meat infusion agar with 20% turkey serum and 5% yeast autolysate
E	C26	0[b]	+
	DPR-2	-	-
	640	0	+
J	693	-	-
K	132VI	-	-
	1805	-	-
L	878-50	-	-
Unclassified	890A	0	+
	SA-4	+	+[c]

[a]Turkey meat infusion broth without serum and yeast autolysate.

[b]Distinct colony formation was not present, but small irregular masses were noted which suggested that some degree of growth was present.

[c]Colonies were very numerous, and were up to .45 mm in diameter.

Isolate SA-4 produced abundant relatively large colonies from the 8th passage in serum-free broth when plated on turkey meat infusion agar containing 20% turkey serum and 5% yeast autolysate. Isolates C26, 640 and 890A also produced

definite colonies on that medium. The other isolates plated from the 8th broth passage failed to produce evidence of growth on this relatively well enriched agar medium. Isolate SA-4 was growing so abundantly in the serum-free broth by the 6th passage that a subculture was initiated at room temperature (approximately 25° C.). That subculture line was also transferred at 7 day intervals and growth was abundant during the subsequent 3 passages recorded to date. In addition, it was noted that the 8th broth passage of isolate SA-4, maintained at 37° C., produced colony growth with moderate hemolysis when plated on standard horse blood agar maintained at room temperature for 4 days.

Discussion In 1940 Sabin (149) reviewed numerous aspects concerning almost all of the members of the pleuropneumonia group which were known at that time and concluded that only the saprophytic member of the group, later designated as Mycoplasma laidlawii, could be cultivated in media without serum protein and at 22° C. However, Smith and Morton (155) demonstrated that serum, ascitic fluid, yeast and to some extent hemoglobin contained the growth factor which was essential for the cultivation of most Mycoplasma. The growth factor was described as a low molecular weight protein or a large polypeptide derivative. Freundt (72) reviewed the characteristics of Mycoplasma in 1957 and considered Mycoplasma laidlawii to be saprophytic as compared to the other

species which he designated as parasitic, since only the saprophytic specie could be cultivated in non-protein enriched media and at 22° C.

Adler et al. (7) designated Mycoplasma inocuum for a saprophytic specie isolated from the infraorbital sinus of a chicken. They reported that it was readily cultivated in broth media without serum, and at 20° C. as well as at 37° C. It produced colonies on standard horse or bovine blood agar, and was hemolytic. Shifrine[1] indicated that further studies suggested that M. inocuum was very similar to M. laidlawii B.

Thus, at least 1, and possibly 2, species of Mycoplasma have been reported which could be cultivated at approximately 22° C. and 37° C. in non-protein enriched media.

In the present study isolate SA-4 grew abundantly in non-serum enriched broth and at approximately 25° C. as well as at 37° C. However, it seems apparent that isolates C26, 640 and 890A not only survived during the 8 passages, but must also have reproduced to some extent. Eight consecutive transfers of 0.5 ml into tubes containing approximately 8 ml of broth represents a dilution of approximately 1:98,000,000,000. It is difficult to assume that one drop streaked on agar medium from the 8th passage would have yielded relatively numerous colonies if some multiplication

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[1]Shifrine, Moshe, Davis, California. Further studies on Mycoplasma inocuum. Personal communication. 1962.

had not occurred.

It should be mentioned that turkey blood clot was included in the preparation of the turkey meat infusion broth employed in the present study. Although serum was not added, it is possible that such broth actually was not entirely free of the "essential growth factor".

The validity of conclusions which can be drawn from the present study, which has not yet been terminated, is somewhat limited. However, it does seem obvious that isolate SA-4 was less fastidious in regard to its growth requirements than the other isolates studied.

Yeast autolysate enrichment The relative growth promoting effect of yeast autolysate for avian Mycoplasma was studied to a limited extent.

Results A preliminary study in 1958 showed that isolates 172C and 1703 of the I serotype definitely grew more abundantly in media containing 5% and 10% yeast autolysate than in media containing 2%, .5% or no yeast autolysate. The amount of growth appeared to be similar with 5% and 10% yeast autolysate during 3 passages. Growth of isolate 801 of the A serotype was enhanced only slightly, if at all, by even 10% yeast autolysate.

It was also noted that 17 of 49 chorioallontoic membrane suspensions from embryonated chicken eggs, from a flock with chronic respiratory disease, reduced tetrazolium when

inoculated into medium containing 8% yeast autolysate. The same 49 suspensions failed to reduce tetrazolium when cultured in similar medium without yeast autolysate. Unfortunately, only 2 of the 17 possibly positive cultures were selected for confirmation and both yielded typical Mycoplasma colonies.

Following these preliminary studies 5% yeast autolysate was extensively employed in media for the isolation and maintenance of avian Mycoplasma. It appeared that yeast autolysate medium was superior for the isolation of most serotypes, but further investigation proved that it was not essential for growth. Representative isolates from each of the serotypes except H were transferred into turkey serum medium without yeast, and were maintained in such medium for numerous passages. Although the isolates studied did not specifically require yeast autolysate for reasonable growth, most of the isolates, especially those of serotypes D, E, F and G, definitely grew more abundantly when maintained in medium with it. Only isolates of the A serotype appeared to be little influenced by the presence of yeast autolysate. However, they required several passages to adjust to medium without it, as did all other isolates which had previously been cultivated in medium with yeast autolysate. The effects of yeast autolysate enrichment of media for isolates of the H serotype are described in the next section.

Discussion Edward (51) found that different

batches of horse serum varied in their growth promoting effects for Mycoplasma isolates from humans and mice. He further noted that 10% freshly prepared yeast extract tended to enhance growth when the less effective batches of horse serum were employed. Saprophytic Mycoplasma were not influenced by the presence of yeast extract, but some of the isolates from mice were actually inhibited when more than 1% yeast extract was employed.

Adler and Yamamoto (9), Yamamoto and Adler (181), Adler and Berg (4) and Kleckner (113) employed yeast preparations in media for the cultivation of avian Mycoplasma. In general, the yeast preparations tended to enhance growth. Yamamoto and Adler (181) determined the value of 1% yeast hydrolysate in medium with 10% horse serum for the growth of avian Mycoplasma isolates represented by S6, C, Tu, SA-1 and O of the present study. All of their isolates except S6 grew more abundantly when yeast hydrolysate was included in the medium. Thus, the results reported in this thesis are in very good agreement with their observations on the specific isolates mentioned.

Enrichments for cultivation of H serotype isolates

Several combinations of possible enrichments were employed in studies to devise satisfactory media for the isolation and maintenance of members of the H serotype. Turkey serum, yeast autolysate, and horse serum with swine gastric mucin were employed in broth and agar media.

Results Initial attempts to isolate members of the H serotype from young poults with air sac lesions were unsuccessful when tetrazolium medium (broth) was employed. However, when essentially the same type of medium was employed in the form of agar slants the results were excellent. During the entire course of this study a total of 53 isolations of what appeared to be isolates of the H serotype were obtained on such slants from 63 suspensions of affected air sacs from poults 1 day to 2 months of age. Not a single isolation was obtained from the same suspensions inoculated into tetrazolium medium, nor did representative isolates from the agar slants produce visible turbidity when subsequently inoculated into tubes of tetrazolium medium. It has already been mentioned that isolates of the H serotype did not reduce tetrazolium. It appeared that turkey meat infusion agar with 20% turkey serum and 5% yeast autolysate was satisfactory for the isolation, and maintenance of H serotype isolates. However, it soon became apparent that few studies were possible with such isolates which could not be cultivated in broth medium.

Turkey meat infusion broth with 15% horse serum, 5% yeast autolysate and 1% swine gastric mucin was prepared in the form of broth and agar slant media. Isolates N, 933, 1022-2 and 1104 of the H serotype were inoculated into tubes of the horse serum broth and agar, and were transferred at 7 day intervals for 5 passages. Colony growth was visible on

the agar slants, but growth was not visibly detectable in the broth cultures. However, subsequent observation of colony growth on agar plate medium suggested that the isolates were growing in the horse serum broth. Approximately 20 "blind" passages were made in the broth before visible turbidity was appreciable. Transfers were continued for 10-30 more passages before growth was sufficient to attempt antigen production. Then flasks of media were inoculated and were incubated with agitation for 4-5 days. Continued broth passages with subsequent inoculation of flasks of medium resulted in the production of very good antigens from all 4 of the H serotype isolates included in this study.

Only recently 0.005% tetrazolium was included in the horse serum broth with mucin. The 4 isolates did not reduce tetrazolium, but growth appeared to be slightly improved.

No attempt was made to determine the relative value of swine gastric mucin. A small study was conducted to determine the relative values of turkey serum, horse serum and yeast autolysate with isolates N, 1022-2 and 1104. Slants and plates were prepared from turkey meat infusion agar with 20% turkey serum and from turkey meat infusion agar with 15% horse serum. Slants and plates were also prepared from portions of these serum enriched media to which 5% yeast autolysate was added. After 3 and 5 slant passages the cultures were streaked on homologus agar medium in plates. Subsequent observation

of colony growth demonstrated that there was no obvious difference between turkey serum and horse serum in the agar slant preparations. However, colonies were rarely noted from slants which did not contain 5% yeast autolysate, suggesting that it was relatively important. The study was not continued long enough to prove beyond doubt that yeast autolysate was essential for growth. It was considered to be essential for maximum growth and was included in all subsequent media for the cultivation of H serotype isolates.

Discussion In 1954 Adler et al. (11) prepared agar slant medium containing 10% defibrinated horse blood. They added 2.0 ml of horse serum broth to the base of the slants and reported good results from attempts to isolate avian Mycoplasma. In 1958 Adler et al. (5) employed the same type of agar slants overlaid with broth and obtained the original N isolate, and other similar ones, from the air sacs of day old poults. They were not able to cultivate such isolates in broth media, although Yamamoto and Adler (181) subsequently cultivated the N isolate in broth medium containing 10% horse serum. They did not employ yeast hydrolysate in that medium. However, Adler and Berg (4) did employ 1% yeast hydrolysate in broth media containing horse serum and observed that growth of the N isolate was definitely enhanced. They further noted that the N isolate did not grow in broth medium containing 10-20% chicken serum with or

without 1% yeast hydrolysate.

It appears that horse serum is superior to chicken or turkey serum for the cultivation of isolates of the H serotype in broth medium, although turkey serum is quite satisfactory in agar medium. The reason for such differences is not apparent, nor is it obvious which substance or substances in yeast preparations cause enhancement of growth.

Growth characteristics

Observations concerning some of the characteristics of avian Mycoplasma growth in broth media were made during the course of this study.

Results The most obvious characteristic of avian Mycoplasma growth in broth cultures was the moderate degree of turbidity produced as compared to the turbidity in broth produced by most other types of bacteria. Following repeated rapid passages, at 1-2 day intervals, well adapted isolates produced considerably more turbidity than when maintained on a 7 day transfer schedule. Isolates of serotypes E, G and H tended to grow more slowly and produce less turbidity than isolates from other serotypes.

Obvious sediment was present at the bottom of tubes of broth cultures of all serotypes, although it was more abundant for isolates of serotypes B and L where it also appeared to be more chalk-like. The centrifuged culture sediment from such isolates was not as pasty as that from other serotype

isolates, and tended to slide off the bottom of inverted centrifuge tubes.

A very delicate pellicle was often noted on the surface of broth cultures of isolates of serotypes B, C, D, E, F, K and L in tubes which were incubated 2-3 weeks or longer. It was customary to keep at least the previous 3 passages of each isolate in racks during cultivation periods.

When a tube became broken or contaminated it was consistently possible to retrieve the culture from older tubes of culture in the rack. The actual duration of survival of cultures kept incubated for prolonged periods of time was not determined. The number of contaminated cultures encountered during the entire course of this study was very low. Gram positive cocci were the usual bacteria incriminated. Such cultures were discarded since it was possible to obtain the Mycoplasma from a previous passage.

Antigen preparations were often noted to contain black or gray amorphous granules which were especially obvious when the centrifuged culture sediment was still on the wall of the centrifuge tube. Similar black particles were often present at the bottom of broth cultures of isolates of the I serotype. It was almost possible to determine which cultures represented the I serotype by that characteristic when it was observed. However, it was not consistently present.

Discussion Yamamoto and Adler (181) considered

isolates of serotypes A and H to grow slowly as compared to the rapid growth of isolates of the other serotypes. Kleckner (113) reported similar results except that he designated isolate N of the H serotype as a rapid grower. The growth of isolates of serotypes E, G and H was considered to be slow in the present study. These differences may simply reflect differences in media employed.

Pellicle formation apparently has not been mentioned in previous reports. The black amorphous particles noted in culture sediment possibly were deposits of calcium and magnesium soaps as described by Edward (50) although he only mentioned such deposits on agar media.

No attempt was made to explore the controversial subject of whether bacterial contaminants were possibly reversions of Mycoplasma to vegetative bacterial forms. They were only rarely encountered, and furthermore, the most predominant bacterial contaminant noted in routine bacteriological procedures in the laboratory was also Gram positive cocci.

Storage Studies

Viability of Mycoplasma in frozen preparations

The viability of avian Mycoplasma in broth cultures which had been stored at -30° C. was determined by cultural procedures, and the viability of Mycoplasma in frozen chicken embryo materials was determined by observing the effects

produced in subsequently inoculated chicken embryos.

Results The storage of broth cultures at -30° C. was found to be so satisfactory and convenient that many isolates were kept frozen except during various studies. Results are presented in Table 14 for only a few typical isolates since nonviable cultures were rarely encountered. Existing records contain only the 2 nonviable subculturing attempts presented in the table, although a few others possibly were not recorded earlier in this study. Many cultures were found to be viable when subcultured following storage at -30° C. for 4 years and 3 months, the longest storage period examined to date. Others were viable following 2-4 years storage. Isolates 26R and 27R were viable at 3.5 but not at 4 years.

All chicken embryo passage materials, mainly yolk, were frozen between passages. Subsequent passages were usually made from the most recent material stored, and further passages were similarly made. Thus, materials were rarely stored as long as 1-2 years. Furthermore, the major part of the numerous results are limited to isolates of serotypes A, D, E, I and J which were studied extensively because they were pathogenic for chicken embryos. The repeated successful chicken embryo passages made with materials which were consistently stored at -30° C. is evidence that Mycoplasma of serotypes A, D, E, J and I were viable following the rather limited periods (to 1-2 years) of storage which have been

Table 14. Viability of avian Mycoplasma in broth cultures stored at -30° C.

Serotype	Isolate[a]	Approximate years stored	Viability[b]
A	26R	4	-
	26R	3.5	+
	801	4	+
B	1504	4	+
C	1111	4	+
D	NY	2.5	+
E	C26	3	+
F	SA-1	2.5	+
G	O	2	+
H	N	2.5	+
I	1703	4	+
J	693	3.5	+
K	1805	3.5	+
L	694	3	+
Unclassified	27R	4	-
	27R	3.5	+
	SA-4	2.5	+
	890A	3	+

[a]Results are presented for typical isolates of each serotype which were stored the longest time, except when a nonviable culture is recorded, then an earlier result is also recorded.

[b]Viability was determined in yeast autolysate medium except for isolate N of the H serotype which was frozen and subcultured on turkey serum agar with yeast autolysate.

been evaluated to date.

Discussion Studies on the viability of avian Mycoplasma in frozen preparations have rarely been reported, although freezing is a very common means of preserving biological materials. Hofstad (98) reported that infective chicken turbinate material suspended in tryptose phosphate broth was still infective after 5 years storage at -30° C.

The storage of embryo passaged Mycoplasma at -30° C. in this study was successful, and it was anticipated that it would be. However, the results obtained with frozen broth cultures of avian Mycoplasma were better than anticipated. The high serum content of the culture medium probably was beneficial.

Viability of Mycoplasma in lyophilized preparations

The viability of avian Mycoplasma in lyophilized preparations which had been stored at 4° C. was determined by cultural procedures. Lyophilized materials which had been prepared and stored by Dr. M. S. Hofstad were included in this study.

Results The viability of lyophilized preparations was determined primarily at times when the represented isolates of avian Mycoplasma were desired for other studies. A few lyophilized samples were subcultured specifically to determine viability. Since nonviable samples were not encountered only a few results are presented in Table 15 which represent

Table 15. Viability of avian Mycoplasma in lyophilized preparations stored at 4° C.

Serotype	Isolate[a]	Material lyophilized[b]	Approximate years of storage	Viability[c]
A	187	chicken turbinates	14	+
	197	chicken turbinates	13	+
	197	culture	7.5	+
B	1504	culture sediment	3.5	+
C	DIVA	culture sediment	3.5	+
D	594	embryo CAM	9	+
E	DPR-2	culture sediment	3	+
	640	embryo CAM	2	+
F	SA-3	culture sediment	1	+
G	O	culture sediment	3	+
H	N	culture sediment	2.5	+
I	695	culture	6	+
J	693	culture	4	+
K	1805	culture sediment	3.5	+
L	694	culture sediment	5	+

[a]Lyophilized preparations of isolates 187, 197, 594, 640, 695, 693 and 694 were supplied by Dr. M. S. Hofstad.

[b]Chicken turbinates and chicken embryo chorioallantoic membrane (CAM) suspensions in tryptose phosphate broth. Centrifuged culture sediment was resuspended in plain turkey serum medium for lyophilization.

[c]Viability was determined by subculturing in yeast autolysate medium except turkey serum agar with yeast autolysate was used for isolate N.

isolates from each serotype which were stored the longest time.

It is apparent that avian Mycoplasma representing each serotype were viable in the lyophilized preparations employed, although some of them had not been stored for more than 1-3 years. Several of the preparations had been stored for more than 5 years, and isolate 187 of the A serotype was viable when subcultured following 14 years of storage. No evidence was available to determine the final duration of survival nor to indicate if Mycoplasma survived longer in tissue suspensions than in culture preparations.

Discussion The viability of avian Mycoplasma in lyophilized broth cultures was reported by Conrad (33). He found that numerous lyophilized broth cultures were viable when examined during a 14 month period, and that one sample was viable after 27 months storage at 4° C. Only approximately 20% of the cells were viable on the day following lyophilization and at the end of 27 months storage.

The number of viable cells was not determined in the present study. However, the purpose of lyophilizing concentrated culture sediment was to partially compensate for the probable reduction of total viable cells during lyophilization and prolonged storage.

It is interesting to note that Dr. M. S. Hofstad had lyophilized preparations containing avian Mycoplasma of

serotypes A, D, E, I, J and L although their identity was not evident at the time. The chicken turbinate suspensions containing isolates 187 and 197 of serotype A were lyophilized several years before cultural procedures for avian Mycoplasma were employed. These are excellent examples of the practical application of lyophilization to maintain avian Mycoplasma.

Pathogenicity Studies

Pathogenicity of avian Mycoplasma for chicken embryos

Almost all of the isolates of each serotype were inoculated into the yolk sac of 7 day embryonated chicken eggs to determine the pathogenicity of avian Mycoplasma for chicken embryos. Evidence of pathogenicity was based on embryo mortality and grossly visible lesions. All surviving embryos were examined on the 19th day of incubation. Subsequent yolk passages were frequently made in an attempt to establish the relative pathogenicity of various isolates.

Results The results obtained from inoculating various preparations of 88 isolates representing each serotype and the serologically unclassified group are summarized in Table 16.

Nineteen of the 25 isolates of serotype A caused embryo deaths, usually within 5-10 days postinoculation. Stunting of some embryos was observed, especially if they survived until late in the embryo incubation period. Subcutaneous hemorrhage was rather frequently observed, as was a

Table 16. Pathogenicity of avian Mycoplasma for chicken embryos

Serotype	Isolate	Inoculum[a]	Embryo pathogenicity[b] Initial inoculum	Embryo pathogenicity[b] Yolk passages
A	F	23rd[c] culture	-	---[d]
	24R	10th culture	-	0/1[e]
	26R	18th culture	+	3/3
	128VA	9th culture	+	1/1
	187	10th culture	+	1/1
	197	12th lyo. CAM[f]	+	1/1
		33rd lyo. CAM	+	1/1
		42nd culture	+	---
	293	3rd culture	+	3/3
	595	4th lyo. CAM	+	---
	699	7th culture	+	---
	734	1st sinus ex.[g]	+	1/1
		34th culture	+	---

[a]Approximately 0.1 ml of undiluted and 1:10 dilution of inoculum injected into the yolk sac of 7 day embryonated chicken eggs. At least 5 eggs were inoculated per dilution.

[b]Pathogenicity indicated by embryo deaths and lesions; hepatitis, splenomegaly and frequently stunting of embryos.

[c]Does not include possible passages accumulated before the isolate was received from another laboratory.

[d]No yolk passages made.

[e]Number of times pathogenic/number of times passaged.

[f]Lyophilized chorioallantoic membrane suspension as inoculum.

[g]Turkey sinus exudate.

Table 16. (Continued)

Sero-type	Isolate	Inoculum[a]	Embryo pathogenicity[b] Initial inoculum	Embryo pathogenicity[b] Yolk passages
A	755	18th culture	+	1/1
	756	17th culture	-	0/1
	796	5th culture	+	---
	801	105th culture	-	---
	857-1	1st sinus ex.[g]	+	---
	882	1st sinus ex.	+	---
		9th culture	+	1/1
	893A	4th culture	+	2/2
	894	7th culture	+	1/1
		1st sinus ex.	+	3/3
		9th culture	+	1/1
	1010	25th culture	+	1/1
		1st sinus ex.	+	1/1
		3rd culture	+	4/4
		52nd culture	+	---
	1079-1	9th culture	+	---
	1112	39th culture	+	2/2
	1403	10th culture	+	1/1
	1605	29th culture	+	1/1
		1st sinus ex.	+	1/1
		3rd culture	+	4/4
	1900	11th culture	-	0/2
	2705	11th culture	-	---
B	54-537	5th[c] culture	-	0/2

Table 16. (Continued)

Sero-type	Isolate	Inoculum[a]	Embryo pathogenicity[b]	
			Initial inoculum	Yolk passages
B	114TB	13th culture	-	0/2
	1304	19th culture	-	---
	1504	30th culture	-	---
	2600	5th culture	-	0/1
	5858	10th culture	-	0/2
C	C	24th[c] culture	-	1/1
	DIVA	6th[c] culture	-	0/2
	Tu	26th[c] culture	-	---
	108TA	21st culture	-	---
	1021	9th culture	-	0/2
	1111	11th culture	-	0/2
D	NY	9th[c] culture	-	0/2
	594	1st yolk	JA[h]	2/2
E	C26	20th[c] culture	-	---
	DPR-2	15th[c] culture	-	0/1
	640	6th lyo. CAM[f]	JA[h]	2/2
F	SA-1	14th[c] culture	-	0/2
	SA-3	20th[c] culture	-	0/1
G	0	15th[c] culture	-	0/1

[h]Joint abscesses observed; see Table 17 for details.

Table 16. (Continued)

Serotype	Isolate	Inoculum[a]	Embryo pathogenicity[b] Initial inoculum	Yolk passages
H	933	8th culture	-	0/1
	1022-2	10th culture	-	0/2
I	114C	9th culture	+	1/1
		32nd culture	+	1/1
	116C	9th culture	-	---
	172C	30th culture	-	0/1
	695	83rd lyo. cult.[i]	+	5/5
		88th culture	+	4/4
		179th lyo. cult.	+	1/1
		190th culture	+	---
	799-1	6th culture	+	1/1
	799-4	10th culture	+	2/2
	806C	16th culture	+	1/1
	807-5	5th culture	+	1/1
	807-18	4th culture	+	2/2
	814-5	11th culture	+	2/2
	815-4	12th culture	+	2/2
	822-4	4th culture	+	---
	823C	4th culture	+	---
	833R	3rd culture	+	---

[i]Lyophilized culture employed as inoculum.

Table 16. (Continued)

Serotype	Isolate	Inoculum[a]	Embryo pathogenicity[b] Initial inoculum	Embryo pathogenicity[b] Yolk passages
I	844-2	4th culture	+	---
	849P	19th culture	+	---
	850-2	10th culture	+	2/2
	851R	3rd culture	+	---
	853M	4th culture	-	0/1
	854-2	4th culture	+	---
	1018	14th culture	-	0/1
	1703	22nd culture 26th culture	- -	0/2 0/1
	2004	9th culture	-	0/3
J	693	16th lyo. cult.[i] 34th culture	+ +	4/4 2/2
	1075	35th culture	+	2/2
K	132VI	12th culture	-	0/5
	158VI	9th culture	-	0/4
	1805	14th culture	-	0/3
L	694	11th culture	-	0/2
	858-76	7th culture	-	0/2
	878-50	7th culture	-	0/2
Unclassified	SA-4	11th[c] culture	-	0/1
	27R	19th culture	-	0/2

Table 16. (Continued)

Serotype	Isolate	Inoculum[a]	Embryo pathogenicity[b]	
			Initial inoculum	Yolk passages
Unclassified	890A	2nd yolk	JA[h]	---
	1042	6th culture	+	1/1
	1043	43rd culture	+	1/1
	1079-3	36th culture	+	1/1
	1087-1	33rd culture	+	---
	1102-7	30th culture	+	---
	1103-5	29th culture	+	---
	1109	26th culture	+	---

edema. The most obvious and constantly observed lesions were hepatitis and splenomegaly. The liver was usually enlarged, congested, and mottled or marginated with areas of necrosis. In less severe stages of hepatitis the liver appeared to be reasonably normal, although it frequently was some shade of green. The spleen was usually 2-3 times its normal size, and occasionally contained small white foci suggestive of necrosis. Pericarditis, pneumonia and airsacculitis were rarely extensive enough to be grossly visible.

Embryo deaths or lesions were not observed for 6 of the serotype A isolates. However, isolate 801, and probably F,

was inoculated at a relatively high culture passage level.

None of the isolates of serotypes B or C were found to be pathogenic for chicken embryos, even after 1 or 2 yolk passages.

Isolate 594 of serotype D and isolate 640 of serotype E caused embryo deaths, hepatitis, splenomegaly, sometimes pericarditis and frequently joint abscesses. Similar lesions were produced by isolate 890A of the serologically unclassified group. Further consideration will be given to joint abscesses later in this section.

The other isolates studied of serotypes D and E were not pathogenic for chicken embryos, nor were isolates of serotypes F, G and H.

Seventeen of the 23 isolates of serotype I were pathogenic for chicken embryos. The death patterns and lesions produced were indistinguishable from those produced by isolates of the A serotype. Isolate 695 was pathogenic at the 88th, 179th and 190th culture passage levels, the highest levels employed. It was also pathogenic when the 83rd culture passage was inoculated as reconstituted lyophilized material which had been stored at 4° C. for 2 years, and recently from another ampoule which had been stored for 6 years.

Isolates 693 and 1075 of serotype J and 7 isolates of the serologically unclassified group also produced embryo death patterns and lesions which were indistinguishable from those

produced by isolates of serotypes A and I.

The isolates representing serotypes K and L were nonpathogenic, as were isolates SA-4 and 27R of the serologically unclassified group.

Thus, serotypes A, D, E, I and J contained isolates which were pathogenic for chicken embryos and those of serotypes B, C, F, G, H, K and L were nonpathogenic. Eight of the 10 isolates of the serologically unclassified group were also pathogenic.

Information concerning the occurrence of joint abscesses in chicken embryos inoculated with isolates 594, 640 and 890A is presented in Table 17 and a photograph of affected embryos is presented in Figure 5. The joint abscesses were creamy white to yellow nodules which occurred in the subcutaneous areas adjacent to mandible, wing, hip, hock or toe joints. They were mainly periarticular, seldom involving the articulation to a visible extent.

Joint abscesses became more numerous following yolk or joint abscess passages and were more evident in embryos which survived longer than 6-8 days postinoculation. Abscesses were not produced by culture passage material for isolate 594, although embryo deaths and hepatitis were produced by the 2nd culture passage from the 1st yolk. Culture from joint abscesses did produce subsequent joint abscesses for isolate 640. The 3 cultures from isolate 594 embryo materials and

Table 17. Joint abscesses of chicken embryos produced by avian Mycoplasma

Sero-type	Isolate	Inoculum[a]	Embryo pathogenicity		
			Deaths	Hep.[b]	Joint abs.[c]
D	594	3rd CAM[d]	+	+	-
		1st yolk	+	+	+
		2nd culture	+	+	-
		2nd yolk	+	+	+
		1st joint abscess	+	+	+
		3rd culture	-	-	-
		2nd joint abscess	+	+	+
		5th culture	-	-	-
		3rd joint abscess	+	+	+
		4th joint abscess	+	+	+
E	640	6th CAM[d]	+	+	+
		1st yolk	+	+	+
		2nd yolk	+	+	+
		1st joint abscess	+	+	+
		4th culture	+	+	+
		2nd joint abscess	+	+	+

[a]Approximately 0.1 ml of undiluted and 1:10 dilution of inoculum injected into the yolk sac of 7 day embryonated chicken eggs. At least 5 eggs were inoculated per dilution.

[b]Hepatitis.

[c]Joint abscesses - periarticular granulomas in region of mandible, wing, hip, hock or toe joints. See Figure 5.

[d]Lyophilized chorioallantoic membrane suspension.

Table 17. (Continued)

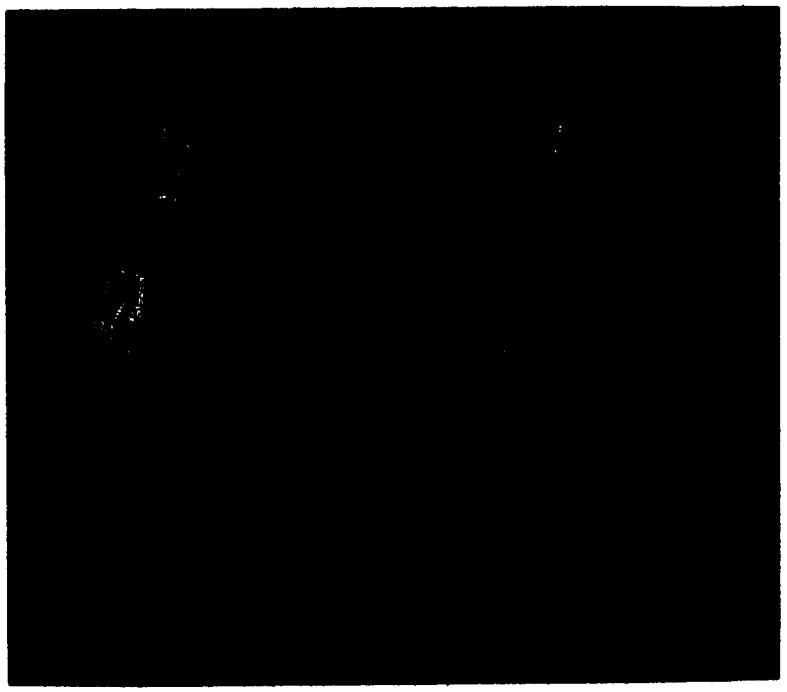
Serotype	Isolate	Inoculum[a]	Embryo pathogenicity		
			Deaths	Hep.[b]	Joint abs.[c]
E	640	3rd joint abscess	+	+	+
		3rd culture	-	+	+
Unclassified	890A	10th culture	+	+	-
		1st yolk	+	+	-
		2nd yolk	+	+	+
		1st joint abscess	+	+	+
		2nd joint abscess	+	+	+

the 2 cultures from isolate 640 embryo materials were eventually employed in agglutination procedures and were found to represent their respective serotypes.

The 10th culture passage of isolate 890A produced only embryo deaths and hepatitis. A single abscess was observed following inoculation of the 2nd yolk passage material, and numerous joint abscesses were present in embryos inoculated with the 1st, and especially the 2nd, joint abscess passage material.

Discussion The results obtained are in good agreement with those reported for isolates of serotypes A, C, F and H by Yamamoto (179). However, he did observe embryo

Figure 5. Joint abscesses of 19 day old chicken embryos produced by isolate 640 of serotype E. Embryo at left is normal. Note creamy yellow abscesses in region of joints of mandible, wing, hip, stifle, hock and toe.



deaths and joint abscesses for isolate O of serotype G. Moulton and Adler (126) had previously described the joint abscesses produced by that isolate. Isolate O was not pathogenic in the present study, although it probably was employed at a relatively high culture passage level.

Kleckner (113) reported that isolates of serotype A were pathogenic for chicken embryos and isolates of serotypes B, C, D, E, F, G and H were nonpathogenic. Thus, the present results differ only in that one isolate each of serotypes D and E were found to be pathogenic.

The pathogenicity of isolates of serotype I was reported in a previous publication by Yoder and Hofstad (182). They mentioned that joint abscesses had been observed in chicken embryos inoculated with up to the 88th culture passage of isolate 695. However, repeated passages of isolate 695 and the 22 other isolates of serotype I made during the course of the present study did not produce joint abscesses in embryos. The serotype of isolate 890A, which also produced joint abscesses, is not evident at this writing, although it does not appear to be related to serotypes D, E or G.

Chute and Cole (31) described the gross and microscopic pathology of Mycoplasma infections in chicken embryos. They determined that joint abscesses actually were subcutaneous periarticular granulomas, with necrotic centers, surrounded by epitheloid cells which had coalesced to form giant cells.

Joint abscesses have also been reported by Delaplane (42), Van Roekel et al. (169, 172), Thompson (164), Calnek and Levine (25) and Hofstad (98), all apparently employing isolates from the trachea of chickens with chronic respiratory disease.

Hofstad (98) observed joint abscesses produced by isolate 640 which was found to represent the E serotype in the present study. Calnek and Levine (25) observed joint abscesses when studying an isolate designated as 39. Fabricant[1] obtained his R39A isolate from embryo material from isolate 39. Then Fabricant (58) found that R39A was in the D serotype of Kleckner (113).

It was mentioned in the section on colony morphology that isolate 594 of serotype D actually was obtained from chicken embryo material which also contained Mycoplasma of the A serotype. The original inoculum was obtained from the trachea of a chicken. Another apparent example of serotypes A and D being represented in one preparation is presented in the following discussion, which is a bit cumbersome because of the literature searching and correspondence conducted in obtaining the information.

Kleckner (60) described the characteristics of isolate NY. It was a nonpathogen, and the only isolate of his D

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[1]Dr. Julius Fabricant, Cornell University, Ithaca, New York. Personal communication. "The source of isolate R39A". 1963.

serotype. The original yolk material had been pathogenic for embryos, but only the nonpathogen was isolated by cultural procedures. Kleckner[1] indicated that the original yolk material was designated as strain X-95 when received from Markham[2], who obtained the initial inoculum from the trachea of a chicken.

Edward and Kanarek (54) designated X-95 as the representative strain of Mycoplasma gallisepticum, which is further represented by typical pathogenic isolates of serotype A. Edward[3] indicated that strain X-95 material had been obtained from Dr. F. S. Markham. Thus, strain X-95 actually is considered to be the representative isolate of serotypes A and D, which is a bit unusual, but probably only reflects the presence of 2 serotypes of Mycoplasma in original material.

The above discussion makes it obvious that it is extremely difficult to evaluate many of the early reports based on studies with only chicken embryo propagated Mycoplasma. It is also apparent that some isolates of at least serotypes D, E and G may produce joint abscesses in chicken embryos. Joint

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[1]Dr. Albert L. Kleckner, University of Geortia, Athens, Georgia. Personal communication. "The source of isolate NY". 1963.

[2]Dr. Floyd S. Markham, Lederle Laboratories, Pearl River, New York. Personal communication. "The origin of strain X-95". 1963.

[3]Dr. D. G. ff. Edward, Wellcome Research Laboratories, Beckenham, Kent, England. Personal communication. "The source of strain X-95". 1963.

abscesses apparently have not been described for known pure isolates of serotype A.

Pathogenicity of avian Mycoplasma for young chickens

The pathogenicity of avian Mycoplasma for chickens 3-8 weeks of age was determined for various isolates representing most of the serotypes. Evidence of pathogenicity was based on the production of lesions within 2-3 weeks after inoculation of preparations into the left thoracic air sac, right hock region tendo-vaginal cavity and the left foot pad. The tube agglutination test was employed for the detection of antibodies for the homologous serotype and also for at least serotype A. Most of the reactions with serotype A antigen were confirmed by the hemagglutination-inhibition test.

Results In preliminary studies broth cultures of avian Mycoplasma isolates were inoculated only into the trachea and left thoracic air sac of chickens. Obvious tracheitis was rarely observed and moderate airsacculitis was only occasionally produced by the isolates employed. Chickens were not employed in further studies until it was observed that the hock region tendo-vaginal cavity and the foot pad tended to exhibit more obvious lesions than the air sacs of inoculated chickens. The results obtained during subsequent studies are presented in Table 18.

Moderate airsacculitis was produced only by isolates of

Table 18. Pathogenicity of avian Mycoplasma in young chickens

Serotype and isolate	Inoculum[a]	Lesions in chickens		Serology[c]	
		Airsac-ulitis	Tendo-vagin-itis[b]	Homol-ogous	Sero-type A
Serotype A					
128VA	11th culture	0/2[d]	0/2	2/2[e]	2/2
	14th culture		0/2	2/2	2/2
796	10th culture	1/2	2/2	2/2	2/2
882	3rd culture	1/2	2/2	2/2	2/2
894	7th culture	1/3	1/3	3/3	3/3
	1st yolk	2/3	3/3	3/3	3/3
1010	5th yolk	1/2	2/2	2/2	2/2
	6th culture	0/3	2/3	3/3	3/3
1605	5th yolk	1/3	2/3	3/3	3/3
Serotype B					
1504	9th culture	0/3	0/3	0/3	0/3
5858	14th culture	0/3	0/3	0/3	0/3

[a]Approximately 0.5 ml inoculum was injected into the left thoracic air sac, right hock tendo-vaginal cavity and left foot pad of chickens 3-8 weeks of age.

[b]Tendo-vaginitis in the region of the hock or foot pad.

[c]Tube agglutination serology, with most of the positive reactions for serotype A confirmed by the hemagglutination-inhibition procedure.

[d]Number with lesions/number inoculated.

[e]Number with positive serological reaction/number tested.

Table 18. (Continued)

Serotype and isolate	Inoculum[a]	Lesions in chickens		Serology[c]	
		Airsac-ulitis	Tendo-vagin-itis[b]	Homol-ogous	Sero-type A
Serotype C					
108TA	7th culture	0/3	0/3	0/3	0/3
1111	10th culture	0/3	0/3	1/3	0/3
Serotype D					
594	3rd joint abs.[f]	0/3	0/3	3/3	0/3
	5th culture	0/4	0/4	2/4	0/4
Serotype E					
640	3rd yolk	0/3	0/3	1/3	0/3
	3rd joint abs.	0/3	0/3	0/3	0/3
	5th culture	0/4	0/4	1/4	0/4
Serotype F					
SA-1	10th culture	0/3	0/3	2/3	0/3
SA-3	14th culture	0/3	0/3	1/3	0/3
Serotype I					
695	2nd yolk	0/3	3/3	2/3	0/3
799-1	12th culture	0/2	1/2	0/2	0/2
807-18	8th culture	0/2	2/2	0/2	0/2
814-5	10th culture	---[g]	2/2	1/2	0/2
822-4	8th culture	---	3/4	0/4	0/4

[f]Chicken embryo joint abscess suspension.

[g]Air sac not inoculated and airsacculitis not present.

Table 18. (Continued)

Serotype and isolate	Inoculum[a]	Lesions in chickens		Serology[c]	
		Airsac-ulitis	Tendo-vagin-itis[b]	Homol-ogous	Sero-type A
850-2	5th culture	---	1/2	0/2	0/2
Serotype J					
693	2nd yolk	0/3	2/3	3/3	0/3
1075	20th culture	0/3	1/3	1/P[h]	0/P
Serotype K					
1805	16th culture	0/3	0/3	1/3	0/3
Serotype L					
694	8th culture	0/3	0/3	0/3	0/3
Unclassified					
890A	2nd yolk	0/3	0/3	0/3	0/3
1042	2nd yolk	0/3	2/3	1/3	0/3
1043	28th culture	---	2/3	---[i]	0/P
1079-3	20th culture	---	1/3	---	0/P
1087-1	18th culture	---	1/3	---	0/P
1102-7	14th culture	---	1/3	---	0/P
1103-5	13th culture	---	0/3	---	0/P
1109	11th culture	---	1/3	---	0/P

[h]Sera for all 3 chickens pooled.

[i]Sera not tested with homologous antigen.

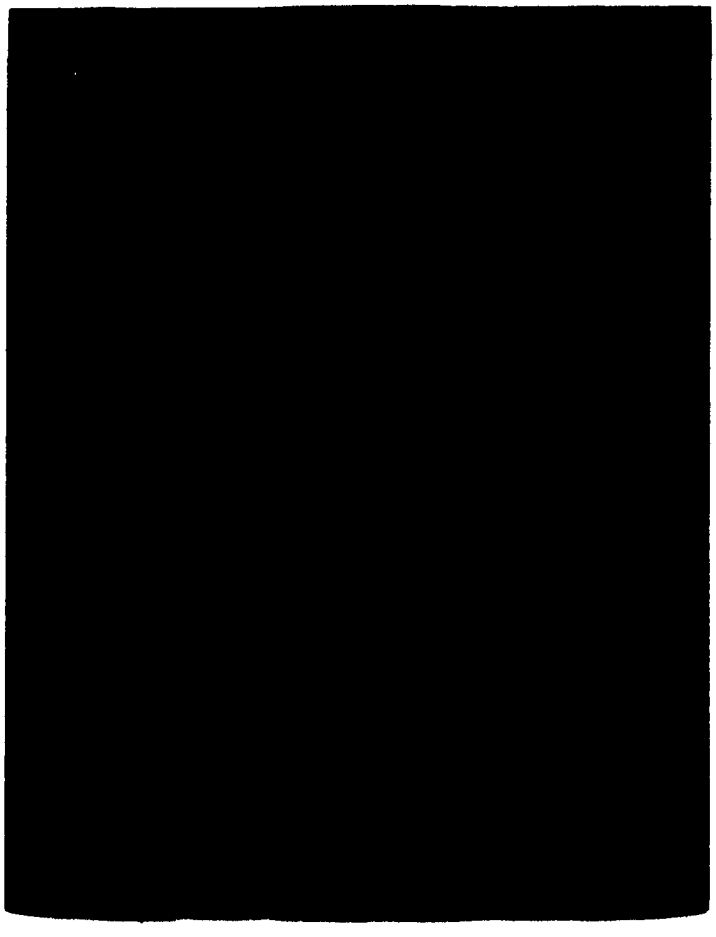
serotype A, and only in some of the chickens employed for such isolates. However, moderate to severe tendo-vaginitis was produced by most of the isolates of serotypes A, I and J as well as by 6 isolates of the serologically unclassified group. Tendo-vaginitis was more frequently observed in the region of the hock than in the foot. Figure 6 is a photograph of an involved hock region showing that the tendo-vaginitis was most obvious dorsal to the hock rather than in the vicinity of the articulation. Tendo-vaginitis was evident along the tendons above and below severely distended foot pads.

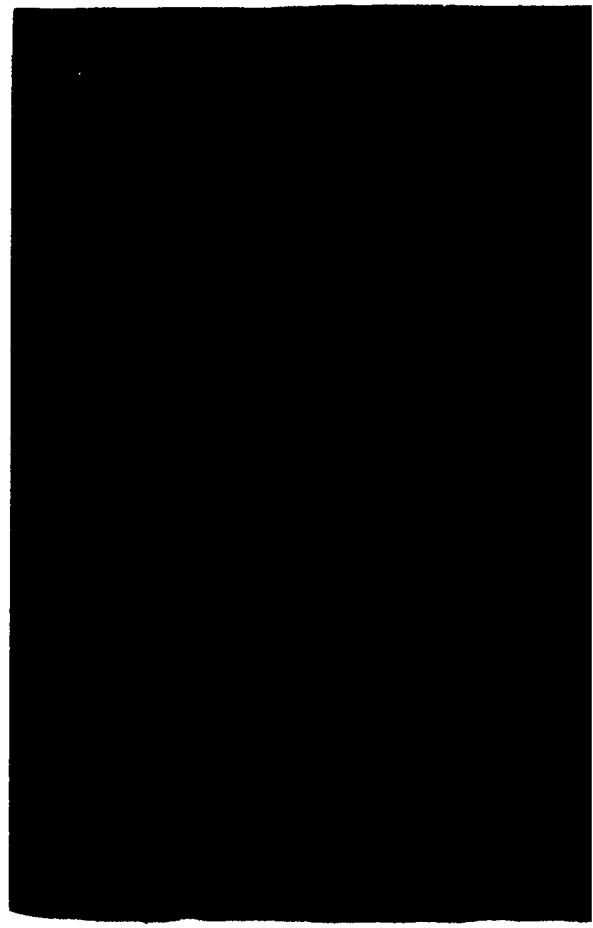
When the involved tendo-vaginal areas were incised a viscous fluid flowed from the incision. Such fluid was sometimes almost colorless although it usually was creamy white to yellow and contained flecks or masses of caseated exudate.

Homologous antibodies were considered to be present in the serum of inoculated chickens when a serum dilution of 1:12.5 or hither caused complete agglutination of antigens employed in tube agglutination studies. Only sera from chickens inoculated with serotype A isolates reacted with serotype A antigen. Such sera usually reacted in at least the first 2 dilutions of the 1:12.5, 1:25 and 1:50 serum dilutions employed. Isolate 128VA produced homologous antibodies although lesions were not visible.

Isolates of serotype I did not produce very significant antibody titers in early studies when sera were tested 2

Figure 6. Typical swelling of the hock region of a chicken indicating tendo-vaginitis. Produced by isolate 894 of serotype A.





weeks postinoculation. The 2nd yolk passage inoculum for isolate 695 of the I serotype did produce a reasonable antibody response when serum was harvested 3 weeks postinoculation.

Homologous antibodies were produced in some of the chickens inoculated with isolates of serotypes C, D, E, F and K although lesions were not visible.

Inoculated controls as such were not employed. However, it was considered that adequate controls were available throughout the study since airsacculitis was observed only in chickens which were inoculated with isolates of serotype A and tendo-vaginitis was observed in chickens inoculated with most of the isolates of serotypes A, I and J, but not with isolates of serotypes B, C, D, E, F, K and L.

Uninoculated controls were maintained. Chickens for experimental purposes were hatched and reared in groups of 80-150 in a separate isolation room. Pathogenicity studies were conducted with 6-24 chickens on a series of occasions during a period of approximately 5 years. Thus, the great majority of the chickens reared were confined as normal uninoculated controls, although some of them were employed for other purposes. Pools of sera from such chickens were employed as control sera for each series of serological tests which were conducted with sera from inoculated chickens and were consistently found to be negative. Tendo-vaginitis

was not grossly visible in any of the control chickens and airsacculitis was not observed in any of the more than 200 control chickens which were sacrificed and examined at various times.

Discussion The results of the present study are in good agreement with those reported for isolates representing serotypes A, C, F and G by Yamamoto and Adler (180), although they observed air sac lesions in chickens inoculated with the C isolate. However, they demonstrated serotype A antibodies in such chickens which were inoculated with yolk passage material. They also inoculated the foot pads of chickens and concluded that isolates of serotype A produced lesions and isolates of serotypes C, F and G were nonpathogenic. Kleckner (113) did not employ chickens in pathogenicity studies. Most other reports contain information concerning only isolates representing serotype A.

Gross (78) and Fabricant and Levine (60) reported that airsacculitis was difficult to produce in chickens inoculated with serotype A Mycoplasma although severe airsacculitis was produced when E. coli and infectious bronchitis virus were also included in the inoculum.

Thus, it appears that chickens are of limited value in pathogenicity studies employing inoculation of only avian Mycoplasma via the respiratory tract. In contrast, young chickens were found to be satisfactory experimental hosts

for pathogenicity studies when inoculation of the tendo-vaginal cavity of the hock region and foot pad was employed.

Antibody response was relatively good when serotype A isolates were employed although it was less consistent for isolates of serotype I. Antibodies were also detected in some of the chickens inoculated with isolates of serotype C, D, E, F and K in the absence of visible lesions. Such results may simply reflect that antibody production is more related to the relative antigenicity of isolates than it is to their relative pathogenicity.

Pathogenicity of avian Mycoplasma for turkeys

The pathogenicity of avian Mycoplasma for turkeys 4-16 weeks of age was determined for various isolates representing most of the serotypes. Evidence of pathogenicity was based on the production of lesions within 2-3 weeks after inoculation of preparations into the right infraorbital sinus, left thoracic air sac, right hock region tendo-vaginal cavity and the left foot pad. The tube agglutination test was employed for the detection of antibodies for homologous serotype and also for at least serotype A. Most of the reactions with serotype A antigen were confirmed by the hemagglutination-inhibition test.

Results Only the infraorbital sinus and thoracic air sac of turkeys was inoculated in preliminary studies with isolates of avian Mycoplasma. Sinusitis and moderate to

severe air sac lesions were produced only by a few isolates which also produced antibodies for the A serotype. In later studies the hock and foot pad regions were also inoculated. Then it became apparent, as in chickens, that some isolates produced little or no effect in the respiratory tract but did produce moderate to extensive tendo-vaginitis. The results of almost all of the studies conducted in turkeys are presented in Table 19.

Only isolates of serotype A produced sinusitis in turkeys, although isolates 24R, 26R and 128VA of that serotype did not produce sinusitis, but did produce homologous antibodies in some of the inoculated turkeys. Isolate 187 produced sinusitis in the single turkey inoculated with reconstituted lyophilized chicken turbinate passage material which had been stored at 4° C. for 9 years.

Moderate to severe airsacculitis was produced by most of the isolates of serotype A. Less extensive airsacculitis was observed following inoculation of some of the serotype I isolates. The 88th culture passage of isolate 695 produced moderate airsacculitis in 1 of 4 turkeys which were inoculated intravenously as well as by the usual sinus and air sac routes. The 3rd culture passage from that air sac lesion was injected intravenously into each of 4 turkeys which were also inoculated into the sinus and air sac with a suspension of the 1st air sac lesion. One turkey again was noted to have moderate

Table 19. Pathogenicity of avian Mycoplasma in turkeys

Serotype and isolate	Inoculum[a]	Site of lesions			Serology[c]	
		Sinus	Air sac	Tend.[b]	Homologous	Serotype A
Serotype A						
24R	17th culture	0/4[d]	1/4	---[e]	1/4[f]	1/4
26R	4th yolk	0/4	0/4	1/4	1/4	1/4
128VA	11th culture	0/2	0/2	---	2/2	2/2
187	lyo. turb.[g]	1/1	0/1	---	1/1	1/1
	1st sinus[h]	2/2	2/2	---	2/2	2/2
734	1st sinus	5/6	2/6	---	6/6	6/6
796	10th culture	1/2	2/2	---	2/2	2/2
882	2nd yolk	2/2	1/2	1/2	2/2	2/2

[a]Approximately 0.5-1.0 ml inoculum injected into the right sinus, left thoracic air sac, right hock tendo-vaginal cavity and left foot pad of turkeys 4-16 weeks of age.

[b]Tendo-vaginal area of hock or foot pad.

[c]Tube agglutination serology, with most of the positive reactions for serotype A confirmed by the hemagglutination-inhibition procedure.

[d]Number with lesions/number inoculated.

[e]Tendo-vaginal areas not inoculated and tendo-vaginitis not present.

[f]Number with positive serological reaction/number tested.

[g]Lyophilized chicken turbinate suspension stored at 4° C. for 9 years.

[h]Turkey sinus exudate.

Table 19. (Continued)

Serotype and isolate	Inoculum[a]	Site of lesions			Serology[c]	
		Sinus	Air sac	Tend.[b]	Homol-ogous	Sero-type A
Serotype A						
893A	3rd culture	2/2	1/2	---	2/2	2/2
894	1st yolk	2/2	2/2	2/2	2/2	2/2
1010	25th culture	3/3	2/3	---	3/3	3/3
	1st sinus	2/2	1/2	---	2/2	2/2
	5th yolk	2/2	2/2	2/2	2/2	2/2
1079-1	4th culture	2/3	1/3	2/3	3/3	3/3
1112	17th culture	1/2	1/2	---	2/2	2/2
1605	6th culture	2/2	2/2	---	2/2	2/2
	1st sinus	2/2	1/2	---	2/2	2/2
	5th yolk	2/2	2/2	2/2	2/2	2/2
Serotype B						
1504	9th culture	0/2	0/2	0/2	0/2	0/2
5858	14th culture	0/2	0/2	0/2	0/2	0/2
Serotype C						
108TA	7th culture	0/2	0/2	0/2	0/2	0/2
1111	10th culture	0/2	0/2	0/2	1/2	0/2
Serotype D						
594	3rd jt. abs.[i]	0/2	0/2	0/2	2/2	0/2
	5th culture	0/2	0/2	0/2	1/2	0/2

[i]Chicken embryo joint abscess suspension.

Table 19. (Continued)

Serotype and isolate	Inoculum[a]	Site of lesions: Sinus	Site of lesions: Air sac	Site of lesions: Tend.[b]	Serology[c]: Homologous	Serology[c]: Serotype A
Serotype E						
640	3rd yolk	0/2	0/2	0/2	1/2	0/2
	1st jt. abs.	0/4	0/4	0/4	0/4	0/4
Serotype F						
SA-1	10th culture	0/2	0/2	0/2	1/2	0/2
SA-2	14th culture	0/2	0/2	0/2	1/2	0/2
Serotype H						
1022-2	18th culture	0/2	2/2	---	2/2	0/2
Serotype I						
114C	9th culture	0/3	0/3	---	0/3	0/3
695	88th culture[j]	0/4	1/4	---	2/2P[k]	0/2P
	1st air sac[l]	0/4	1/4	---	4/4	0/4
	2nd yolk	0/6	0/6	3/6	6/6	0/6
799-1	1st yolk	0/3	1/3	---	0/3	0/3
806C	7th culture	0/2	1/2	---	0/2	0/2
807-5	4th culture	0/2	0/2	---	0/2	0/2

[j]Culture injected intravenously, in addition to sinus and air sac inoculation.

[k]Two pools of 2 sera each.

[l]Third culture from 1st air sac lesion injected intravenously, culture and 1st air sac lesion suspension used for sinus and air sac inoculation.

Table 19. (Continued)

Serotype and isolate	Inoculum[a]	Site of lesions			Serology[c]	
		Sinus	Air sac	Tend.[b]	Homol-ogous	Sero-type A
Serotype I						
807-18	9th culture	0/2	0/2	---	0/2	0/2
814-5	1st yolk	0/3	0/3	---	0/3	0/3
	2nd yolk	0/2	0/2	2/2	1/2	0/2
815-4	1st yolk	0/3	0/3	---	1/3	0/3
822-4	9th culture	0/2	0/2	---	0/2	0/2
850-2	1st yolk	0/2	0/2	---	1/2	0/2
	2nd yolk	0/2	0/2	2/2	2/2	0/2
851R	5th culture	0/3	0/3	---	0/3	0/3
1703	23rd culture	0/3	1/3	---	0/3	0/3
Serotype J						
693	2nd yolk	0/5	0/5	4/5	3/5	0/5
1075	5th culture	0/3	0/3	3/3	2/3	0/3
Serotype K						
1805	16th culture	0/2	0/2	0/2	0/2	0/2
Serotype L						
694	8th culture	0/2	0/2	0/2	1/2	0/2
Unclassified						
890A	3rd yolk	0/2	0/2	0/2	0/2	0/2
1042	1st yolk	0/2	0/2	2/2	1/2	0/2

Table 19. (Continued)

Serotype and isolate	Inoculum[a]	Site of lesions			Serology[c]	
		Sinus	Air sac	Tend.[b]	Homol-ogous	Sero-type A
Unclassified						
1043	12th culture	0/2	0/2	1/2	1/2	0/2
1079-3	5th culture	0/2	0/2	0/2	---[m]	0/2
1087-1	3rd culture	0/2	0/2	0/2	---	0/2

[m]Sera not tested with homologous antigen.

airsacculitis. Antibodies for serotype I antigen were detected in the sera from the employed turkeys.

Moderate airsacculitis was also observed in one turkey each following inoculation of isolates 799-1, 806C and 1703 of serotype I. None of the turkeys inoculated with isolates of serotypes B, C, D, E, F, J, K, L or of the serologically unclassified group exhibited grossly visible airsacculitis. Both turkeys inoculated with isolate 1022-2 of serotype H had moderate airsacculitis as did at least 1 control turkey. This will be mentioned in greater detail later in this section.

Isolates of serotypes A, I and J which were inoculated into the hock region tendo-vaginal cavity and into the foot pad produced tendo-vaginitis in 1 or more of the turkeys employed in each group. Tendo-vaginitis was not produced by

the employed isolates of serotypes B, C, D, E, F, K and L. Two of 6 isolates of the serologically unclassified group produced tendo-vaginitis.

Homologous antibodies were most consistently produced by isolates of serotype A, although homologous antibodies were also produced in some of the turkeys which were inoculated with isolates of serotypes C, D, E, F, H, I, J and L. Isolates 1042 and 1043 of the unclassified group produced antibodies which were detected by homologous antigen preparations.

It was mentioned in the section on materials and methods that turkeys were purchased at 1 day of age from a nearby hatchery, and then were raised in isolation until employed at 4-16 weeks of age. Several groups of 30-100 poults each were raised during the course of this study, and only a portion of each group was employed in pathogenicity studies. Most of the remaining turkeys in each group served as uninoculated controls.

Neither sinusitis nor tendo-vaginitis was observed in any of the control turkeys, and airsacculitis was not observed in any of the more than 100 controls which were examined at 16-24 weeks of age. However, airsacculitis was observed in 1 of 6 control poults which were examined at 6 weeks of age. Mycoplasma were not isolated from that air sac lesion which was inoculated into tetrazolium medium. Sera from those 6

control poults did not react with serotype A antigen, nor did other samples from older control turkeys. Antigen for serotype H was not available at that time, but the sera were stored frozen.

Subsequent studies demonstrated that airsacculitis was rather common in young poults obtained from farm flocks and in pips and cull poults obtained from hatcheries. Mycoplasma were frequently isolated from such lesions when turkey serum agar slants with yeast autolysate were inoculated. Isolates 933 and 1022-2 of serotype H were obtained during such studies.

Serotype H antigen was eventually produced after horse serum medium with yeast autolysate and mucin was employed. Then agglutination studies were conducted employing accumulated turkey serum samples. Both sera from poults which had been inoculated with isolate 1022-2 of serotype H gave positive reactions as did the serum sample from the control poult which had airsacculitis. The 5 sera from the other young control poults did not react with serotype H antigen, nor did 2 pools of sera from older control turkeys.

Further testing detected 12 more reactor sera from young poults which had airsacculitis. Such samples were obtained from poults which had been inoculated with air sac lesion isolates of Mycoplasma which were not actually included in the group selected for detailed study.

Discussion The results just mentioned concerning Mycoplasma of the H serotype associated with airsacculitis in young poults strengthen the concept that isolates of serotype H are significant pathogens. The same results suggest that airsacculitis may also have been present in some of the young turkeys employed in pathogenicity studies. However, it does appear to be significant that moderate to severe airsacculitis was observed in 1 or more turkeys in 15 of the 18 groups which had been inoculated with preparations of serotype A isolates and that airsacculitis was not observed in any of the turkeys which had been inoculated with 19 preparations of isolates of serotypes B, C, D, E, F, J, K, L and the serologically unclassified group.

Airsacculitis was inconsistently observed in turkeys which had been inoculated with preparations of serotype I isolates. Such results are difficult to evaluate, and may well be of questionable validity.

Results obtained from pathogenicity studies based on the production of sinusitis and/or tendo-vaginitis appear to be reasonable and valid.

Yamamoto and Adler (180) reported that only isolates of serotype A produced sinusitis in turkeys, isolates of serotypes A and H produced airsacculitis, and only isolates of serotype A produced tendo-vaginitis when inoculated into the foot pad. Isolates of serotypes F and G were nonpathogenic.

Homologous antibodies were detected in turkeys inoculated with isolates of serotypes A, H and F. They initially considered that isolate C of serotype C produced airsacculitis, but determined that the yolk inoculum also contained Mycoplasma of serotype A. Thus, only isolates of serotypes A and H were considered to be pathogenic.

Kleckner (113) reported that isolates of serotype A produced sinusitis and airsacculitis in experimentally inoculated turkeys. Isolates of serotypes B through H were nonpathogenic. However, his isolate N of serotype H probably was employed at a relatively high culture passage level.

The results of the present study are essentially in agreement with those just reviewed for serotypes A through H, although Kleckner (113) did not observe airsacculitis in turkeys inoculated with isolate N of serotype H. It is significant that only isolates of serotype A have been noted to produce sinusitis. Isolates of serotypes I, J and K have not been employed in comparative studies by other workers. However, pathogenicity studies with isolates of serotype I were mentioned in a previous report by Yoder and Hofstad (182).

Pathogenicity of serotype A Mycoplasma for pigeons

Only a limited number of attempts were made to determine the pathogenicity of isolates of serotype A for pigeons. The pigeons were sacrificed and examined for grossly visible

lesions 2-3 weeks after receiving several drops of inoculum intranasally, intratracheally and 0.5 ml injected into the left thoracic air sac.

Results Visible lesions were not present in the 2 pigeons from each group which were inoculated with the 4th culture passage of isolate 857-1, 3rd culture of isolate 882, 2nd culture of isolate 1010 and the 3rd yolk passage of isolate 1010. Preinoculation serum from each pigeon was found to be negative when tested by the hemagglutination-inhibition procedure with serotype A antigen. Postinoculation serum from only 1 of the 8 pigeons had a detectable hemagglutination-inhibition titer. That serum, which was obtained from 1 of the pigeons inoculated with isolate 1010 yolk material, only had a titer of 40.

Approximately 2 weeks before the pigeons were employed in pathogenicity studies an attempt was made to isolate Mycoplasma from their nasal cavity. Tetrazolium medium was employed to rinse the nasal cavity, and then the rinse fluid was cultured in tetrazolium medium. Three non-dextrose fermenting isolates of Mycoplasma were obtained from the 8 pigeons. One of the isolates was included in the group for detailed study and is identified as isolate 878-50 of serotype L. It should also be mentioned that isolate 858-76 of serotype L and isolate 890A of the serologically unclassified group were obtained from apparently normal pigeons employed

for other purposes.

Discussion The significance of the presence of Mycoplasma of at least serotype L in the nasal cavity of some of the employed pigeons is not known. However, Mycoplasma of serotype A were not isolated from any of the employed pigeons nor was there any evidence of serotype A antibodies in preinoculation sera.

It is apparent that the pigeons were resistant to infection with serotype A Mycoplasma and, with only 1 exception, did not produce detectable homologous antibodies. These results are not in agreement with other reports. Winterfield (177) isolated the "turkey sinusitis agent" from pigeons via chicken embryo inoculation. Embryo passage material produced sinusitis in turkeys and conjunctivitis and tracheitis in young pigeons. Gianforte et al. (75) reported that an isolate of Mycoplasma was obtained in chicken embryos inoculated with air sac lesions from pigeons. They found it to be antigenically related to known chicken and turkey pathogenic Mycoplasma (serotype A).

However, there apparently is no report in the literature on attempts to infect pigeons with known pure isolates of serotype A Mycoplasma, and apparently no isolations of serotype A Mycoplasma directly in culture from pigeons has been reported.

Mathey et al. (120) reported in 1956 that a non-dextrose

fermenting isolate of Mycoplasma was obtained from pigeons with a mild respiratory disease. Their isolate was nonpathogenic for chickens and turkeys and was unrelated antigenically to known chicken and turkey pathogenic Mycoplasma (serotype A). Isolate 694 of serotype L was obtained by Dr. M. S. Hofstad from the nasal turbinates of a pigeon with a mild respiratory disease. This isolate also is a non-dextrose fermenter and is nonpathogenic for chickens and turkeys.

Thus, the existing information does not seem to be sufficient to definitely establish the pathogenicity of serotype A Mycoplasma for pigeons.

Pathogenicity of serotype A Mycoplasma for quail

An attempt was made to determine the pathogenicity of isolate 894 of serotype A for quail. The quail were sacrificed and examined for evidence of infection 3 weeks after receiving several drops of inoculum intranasally, intratracheally and 0.3-0.5 ml injected into either the left sinus or the left thoracic air sac.

Results No evidence of infection was noted in the 12 quail which were inoculated with the 1st yolk passage of isolate 894. The 4 pools of sera from the 12 inoculated quail and the 1 pool of serum from 6 uninoculated control quail were negative for serotype A hemagglutination-inhibition antibodies.

Discussion The employed quail were resistant to

infection with serotype A mycoplasma, although the same inoculum had previously been found to be pathogenic for chickens and turkeys. Reports apparently have not been made concerning natural or experimental Mycoplasma infection in quail.

Serological Studies

Plate agglutination serology

The rapid serum plate agglutination procedure was employed to a limited extent in preliminary studies only.

Results Plate agglutination antigen prepared from isolate A5969 of serotype A was employed when the first few specific immune sera were being prepared in roosters. It appeared to be a reasonably satisfactory procedure for detecting sera of serotype A. However, when antigens were prepared from relatively early passage levels of isolates of other serotypes some degree of auto-agglutination and non-specific cross reactions became apparent. Attempts to titrate sera proved to be difficult because relative degrees of agglutination were present. Furthermore, some sera tended to react rather rapidly and others more slowly. Many sera were noted to clump various antigens when the test plate was rotated for more than 3-5 minutes. The rapid serum plate agglutination test was not employed in further studies for the serological characterization of isolates of avian

Mycoplasma.

Discussion It is obvious that the rapid serum plate agglutination procedure was abandoned without extensive use of serum dilutions, better quality antigens and investigations with specific rabbit antisera. However, the hemagglutination-inhibition and tube agglutination procedures appeared to be more satisfactory for quantitative serological procedures and were employed in all subsequent serological characterization studies.

Yamamoto and Adler (181) employed the rapid serum slide agglutination procedure in serological characterization studies and considered the results to be satisfactory. They classified 5 serotypes, designated as I, II, III, IV and V, which are represented respectively by serotypes A, C, F, G and H of the present study and that of Kleckner (113) who employed the tube agglutination procedure.

Tube agglutination serology

The tube agglutination procedure was very extensively employed for the serological characterization of avian Mycoplasma. Most of the preliminary screening type studies were conducted with serum dilutions of 1:2.5, 1:25 and 1:50 employing specific immune rooster sera. Serial two-fold dilutions of specific immune rabbit sera from 1:10 through 1:640 or higher were employed in final characterization studies.

Results Seventy-three pools of specific immune rooster sera were prepared for preliminary studies. Such rooster sera were satisfactory to at least indicate possible relationships of various isolates. Then 25 pools of specific immune rabbit sera were prepared and employed in final characterization studies. Thus, very few results from the use of rooster sera will be presented.

Rooster sera prepared for isolates of serotype A were especially valuable for use in hemagglutination-inhibition tests, as will be described in a later section. However, it became apparent that heat inactivation of initially prepared rooster sera was somewhat detrimental for sera employed in tube agglutination studies. The results from a limited attempt to investigate the effect of inactivation of rooster serum at 56° C. are presented in Table 20. The tube agglutination reaction was seriously affected following as few as 10 minutes inactivation although plate agglutination and hemagglutination-inhibition reactions were not affected. The effects of heating were not overcome by the addition of 10% normal unheated rooster serum to the antigen diluent in a repeat tube agglutination trial.

A definite reduction of the tube agglutination titer was noted when heated rooster sera prepared from isolates of other serotypes were tested, but the prozone effect was rarely noted. Subsequently prepared rooster sera were not

Table 20. Effect of heat inactivation of specific immune rooster serum prepared from isolate 894 of serotype A

Minutes serum heated at 56° C.	Reactions with serotype A antigens				
	Tube[a]			Plate[b]	HI[c]
	12.5	25	50		
0	+	+	+	+ + +	640
6	+	+	+	+ + +	640
10	-	-	±[d]	+ + +	640
20	-	-	±	+ + +	640
30	-	-	±	+ + +	640

[a]Serum dilutions of 1:12.5, 1:25 and 1:50 were employed for the tube agglutination test.

[b]Three plus indicates complete agglutination of 1 drop of antigen with 1 drop of serum on a glass plate.

[c]Hemagglutination-inhibition titers are recorded as the reciprocal of the highest serial two-fold dilution of serum which completely inhibited hemagglutination of chicken erythrocytes.

[d]Partial agglutination of antigen in the 1:50 dilution of serum, but not in the previous dilutions; indicating a prozone.

heat inactivated.

It is interesting to note that turkey sera prepared from isolates 894, 1010 and 1605 of serotype A exhibited a moderate prozone effect when heated for 30 minutes. However, the tube agglutination reaction was not completely destroyed following as long as 60 minutes at 56° C. Tube agglutination titers of

rabbit sera were not altered following 30 and 60 minutes at 56° C.

Only representative isolates of each serotype were selected for detailed tube agglutination studies with a complete spectrum of rabbit antisera for the various serotypes. Every isolate of each serotype was employed in tube agglutination tests with at least several possibly related antisera. Since the titers were reasonably high in homologous systems, and cross reactions were minor, only the homologous titers for each isolate are presented in Table 21 and the results of more complete studies are presented in Table 22. Figure 7 is a photograph showing typical tube agglutination reactions.

The results indicate that 88 isolates were classified in 12 serotypes designated as A through L with relatively minor cross reactions. Isolates SA-4 and 890A remain unclassified since their antigen preparations have consistently settled out in attempted agglutination studies. Antigens prepared from isolates 27R, 1042, 1043, 1079-3, 1087-1, 1102-7, 1103-5 and 1109 have been essentially unreactive when employed with known serotype antisera, however homologous rabbit antisera have not been prepared to determine if such antigens are reactive at all.

Discussion The detrimental effect of heat inactivation of chicken antisera for use in tube agglutination studies

Table 21. Tube agglutination titers of avian Mycoplasma antigens with immune rabbit serum of the homologous serotype

Antigens	Homologous[a] serotype antisera	Antigens	Homologous[a] serotype antisera
Serotype A	801 serum	Serotype A	801 serum
A5969	640[b]	755	160
F	320	756	320
S6	320	796	320
VR	320	801	640
24R	640	857-1	320
26R	320	882	320
128VA	640	893A	640
187	320	894	640
197	320	1010	320
293	320	1079-1	320
595	320	1112	160
699	320	1344	320
734	320	1403	320

[a]Only the results with sera of the homologous serotype are tabulated. Normal rabbit serum and serotype I antiserum were also employed with antigens of serotype A, and normal rabbit serum and serotype A antiserum were employed with antigens of serotypes B through L with no titers of 20 or higher noted.

[b]Titers are recorded as the reciprocal of the highest serial two-fold dilution of serum causing complete agglutination.

Table 21. (Continued)

Antigens	Homologous[a] serotype antisera	Antigens	Homologous[a] serotype antisera
Serotype A	801 serum	Serotype D	NY serum
1605	320	NY	320
1900	160	594	320
2705	320	Serotype E	640 serum
Serotype B	1504 serum	C26	80
K18B	160	DPR-2	320
54-537	160	640	320
114TB	160	Serotype F	SA-3 serum
1207	320	SA-1	640
1304	320	SA-2	640
1504	320	SA-3	1280
2600	160	Serotype G	O serum
5858	320	O	320
Serotype C	DIVA serum	Serotype H	1022-2 serum
C	160	N	640
DIVA	640	933	160
Tu	640	1022-2	320
108TA	320	1104	320
1021	160	Serotype I	695 serum
1111	160	114C	160
2805	160	116C	160

Figure 7. Tube agglutination reaction indicated by clearing of the fluid with clumpy sediment noted in center 2 tubes. Outer 2 tubes are controls. Tubes had just been shaken before being photographed. X2.

Figure 8. Typical hemagglutination-inhibition reactions with antigen prepared from isolate 801 of serotype A. Photograph shows the bottoms of the tubes approximately X 3/4. Sharp central button indicates no hemagglutination of hemagglutination-inhibition. Even film of erythrocytes on bottom of tube indicates hemagglutination. Tubes in 4 horizontal rows. Tubes 1-9 from left to right.

Row 1. Normal turkey serum. First tube is 1:5 serum control. No inhibition.

Row 2. Strong positive turkey serum, titer of 320 with partial inhibition in tube 8.

Row 3. Weak positive turkey serum, titer of 40.

Row 4. Antigen titration starting with 1:10 dilution. Titer of 640. Tube 9 is cell control.

Figure 7. Tube agglutination reaction indicated by clearing of the fluid with clumpy sediment noted in center 2 tubes. Outer 2 tubes are controls. Tubes had just been shaken before being photographed. X2.

Figure 8. Typical hemagglutination-inhibition reactions with antigen prepared from isolate 801 of serotype A. Photograph shows the bottoms of the tubes approximately X 3/4. Sharp central button indicates no hemagglutination of hemagglutination-inhibition. Even film of erythrocytes on bottom of tube indicates hemagglutination. Tubes in 4 horizontal rows. Tubes 1-9 from left to right.

Row 1. Normal turkey serum. First tube is 1:5 serum control. No inhibition.

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Row 4. Antigen titration starting with 1:10 dilution. Titer of 640. Tube 9 is cell control.

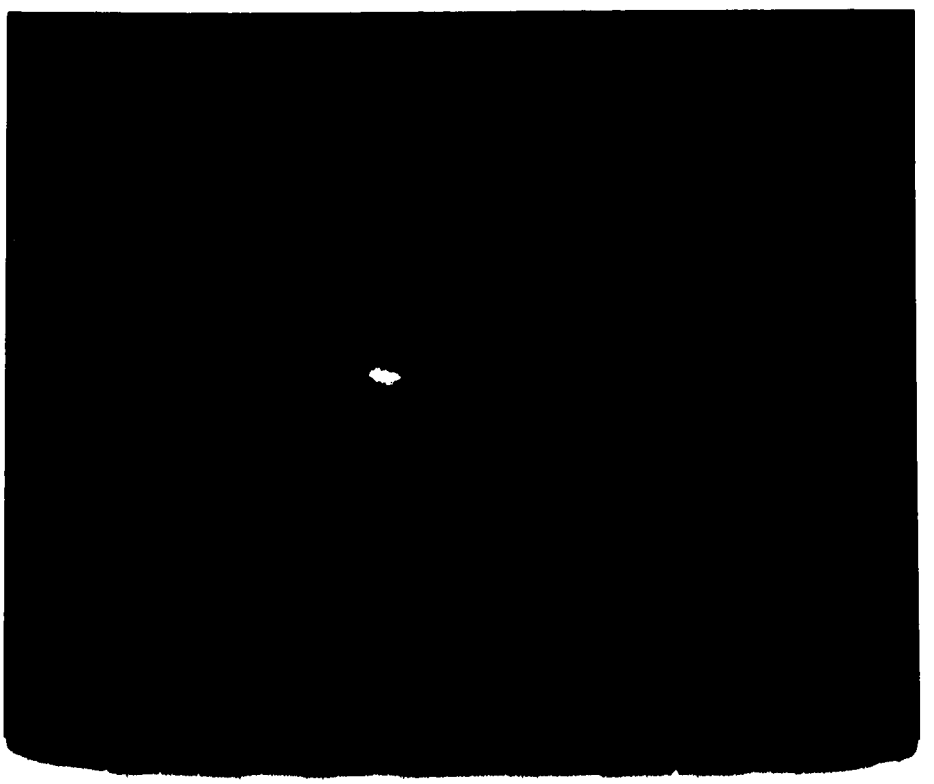

Table 22. Homologous and heterologous tube agglutination titers of avian Mycoplasma antigens with immune rabbit sera

Serotype	Antisera	Antigens A A5969	A S6	A 801	B 54-537	B 1304
Control	Normal	-[a]	-	-	-	-
A	801	640[b]	320	640	-	-
B	54-537	-	-	-	320	160
C	DIVA	-	-	-	10	-
D	NY	-	-	-	-	-
E	640	-	-	-	-	-
F	SA-3	-	-	-	-	-
G	0	-	-	-	-	-
H	1022-2	-	-	-	10	10
I	695	-	-	-	-	-
J	693	-	-	-	-	-
K	1805	-	-	-	-	-
L	694	-	-	-	10	10
Homologous		---[c]	---	640	320	320

[a]Negative indicates no agglutination in the 1:10 starting dilution.

[b]Titers are recorded as the reciprocal of the highest serial two-fold dilution of serum causing complete agglutination.

[c]Rabbit antiserum not prepared for this isolate.

Table 22. (Continued)

Serotype	Antisera	Antigens B 1504	C DIVA	C Tu	C 1111	D NY
Control	Normal	-	-	-	-	-
A	801	-	-	-	-	-
B	54-537	320	-	-	-	-
C	DIVA	20	640	640	160	-
D	NY	-	-	-	-	320
E	640	-	-	-	-	-
F	SA-3	-	-	-	-	-
G	0	-	-	-	-	-
H	1022-2	20	-	-	-	10
I	695	-	-	-	-	-
J	693	-	-	-	-	-
K	1805	-	-	-	-	-
L	694	10	-	-	-	-
Homologous		320	640	---[c]	320	320

Table 22. (Continued)

Serotype	Antisera	Antigens D 594	E DPR-2	E 640	F SA-1	F SA-3
Control	Normal	-	-	-	-	-
A	801	-	-	-	-	-
B	54-537	-	20	10	-	20
C	DIVA	20	-	-	-	-
D	NY	320	-	-	-	-
E	640	-	320	320	-	-
F	SA-3	-	-	-	640	1280
G	0	-	20	20	-	-
H	1022-2	-	20	10	10	20
I	695	-	-	-	-	-
J	693	-	-	-	-	10
K	1805	-	-	-	-	-
L	694	-	10	10	-	-
Homologous		---[c]	160	320	640	1280

Table 22. (Continued)

Serotype	Antisera	Antigens G 0	H N	H 1022-2	I 116C	I 695
Control	Normal	-	-	-	-	-
A	801	-	-	-	-	-
B	54-537	20	10	10	-	-
C	DIVA	-	-	-	-	-
D	NY	-	-	-	-	-
E	640	20	-	-	-	-
F	SA-3	-	-	-	-	-
G	0	320	-	-	-	-
H	1022-2	10	640	320	-	10
I	695	-	-	-	160	320
J	693	-	-	-	160	320
K	1805	-	-	-	-	-
L	694	10	20	10	-	-
Homologous		320	320	320	---[c]	320

Table 22. (Continued)

Serotype	Antisera	Antigens I 1703	J 693	J 1075	K 158VI	K 1805
Control	Normal	-	-	-	-	-
A	801	-	-	-	-	-
B	54-537	-	-	-	10	-
C	DIVA	-	-	-	-	-
D	NY	-	-	-	-	10
E	640	-	-	-	-	-
F	SA-3	-	-	-	-	-
G	0	-	-	-	-	-
H	1022-2	-	10	20	10	10
I	695	320	-	-	-	-
J	693	-	640	1280	-	-
K	1805	-	-	-	320	640
L	694	-	-	-	10	-
Homologous		320	640	---[c]	320	640

Table 22. (Continued

Serotype	Antisera	Antigens L 694	Antigens L 878-50
Control	Normal	-	-
A	801	-	-
B	54-537	10	20
C	DIVA	-	-
D	NY	-	-
E	640	-	-
F	SA-3	-	-
G	0	-	-
H	1022-2	10	20
I	695	-	-
J	693	-	-
K	1805	-	-
L	694	320	160
Homologous		320	---[c]

was noted by Jungherr et al. (108). They also noted that heated sera were still reactive when tested by the hemagglutination-inhibition procedure. Cover et al. (35) reported that the destructive effect of heat inactivation was not apparent when the serum was employed in the rapid serum plate test.

The present results are in agreement with these reports except that the noted prozone effect was not mentioned before. Heating seems to block the agglutination reaction until some inhibitory substance is diluted enough to allow the usual agglutination. Antibody does not seem to be destroyed. Plate agglutination may not be blocked due to a difference in the proportions of antigen, antibody and inhibitory substance. Hemagglutination-inhibition is not influenced, but it can not be stated for sure that the same antibody system is involved. A complete understanding of the phenomenon is lacking.

The results obtained from agglutination studies with rabbit antisera are in excellent agreement with those previously reported for serotypes A through H by Kleckner (113) and for serotypes A, C, F, G and H reported by Yamamoto and Adler (180, 181). The serological characteristics of isolates of serotype I were mentioned in a previous study by Yoder and Hofstad (182). Serotypes J, K, and L have not been studied by other workers.

Kelton and Van Roekel (110) reported that isolates representative of serotypes E and G were distinctly different as determined by the tube agglutination procedure. However, their results with the same isolates employed in colony inhibition tests indicated that they were homologous. Fabricant[1] designated his serotype SLO to include isolates representative of serotypes E and G which were also found to be homologous by colony inhibition studies. Thus, tube agglutination studies have consistently demonstrated that 2 distinct serotypes are represented although colony inhibition studies have not shown that difference. It is possible that agglutinins are not entirely the same as the substance which causes inhibition of colony growth.

Hemagglutination-inhibition serology

Specific immune rooster sera were employed in the hemagglutination-inhibition procedure in preliminary studies. The sera from experimental chickens, turkeys, pigeons and quail were also employed in subsequent studies as were specific immune rabbit sera. Hemagglutination antigen was usually prepared from isolate 801 of serotype A although antigens from a few other isolates were also employed.

Results Some of the results obtained with specific

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[a]Dr. Julius Fabricant, Cornell University, Ithaca, New York. Personal communication. Avian Mycoplasma representing serotype SLO. 1962.

immune rooster sera employed in hemagglutination-inhibition tests are presented in Table 23. The identity of many isolates of serotype A were readily determined, but the identity of isolates of other serotypes had to be determined by agglutination procedures.

Various antigens prepared from isolates of serotypes I, J and K were noted to have relatively good hemagglutinating titers, but when employed in hemagglutination-inhibition studies the hemagglutinating activity was rarely present. However, a single hemagglutinating antigen was prepared from isolate 695 of serotype I which proved to be stable enough to permit a series of studies. Glycerin was not added to the antigen and it was employed to absorb homologous rooster antiserum and rooster antiserum from isolate 801 of serotype A. Similarly, antigen prepared from isolate 801 was employed to absorb both sera. Then a series of hemagglutination-inhibition tests was conducted employing both antigens with the various sera. The results of this study are presented in Table 24. It is apparent that serotypes A and I are distinctly different. Repeated attempts to prepare another stable hemagglutinating antigen from isolate 695 consistently failed, so it was not possible to test numerous sera in that hemagglutination-inhibition system.

Numerous sera from chickens, turkeys, pigeons and quail employed in pathogenicity studies were tested by the

Table 23. Hemagglutination-inhibition titers of rooster antisera with isolate 801 antigen

Sero-type	Rooster antisera	HI titer	Sero-type	Rooster antisera	HI titer
Control	Normal	-[a]	B	54-537	-
A	A5969	320[b]		1304	-
	S6	640	C	DIVA	-
	VR	320		108TA	-
	24R	80	D	NY	-
	26R	160		594	-
	187	320	E	DPR-2	-
	197	320		640	-
	595	640	F	SA-1	-
	699	640	G	O	-
	734	160	H	N	-
	755	160	I	695	-
	756	320		815-4	-
	801	640	J	693	-
	894	640	K	132VI	-
	1344	320		1805	-
	1605	320	L	694	-

[a]Hemagglutination-inhibition titer of 20 or less.

[b]HI titers are recorded as the reciprocal of the highest dilution of rooster serum causing complete inhibition of agglutination of chicken erythrocytes.

Table 24. Hemagglutination-inhibition titers of homologous and heterologous absorbed rooster antisera[a]

Sera	Hemagglutination-inhibition titers	
	801 HA antigen	695 HA antigen
Normal	-[b]	-
801	320[c]	-
801 absorbed with 801	10	-
801 absorbed with 695	80	-
695	10	640
695 absorbed with 801	-	320
695 absorbed with 695	-	10

[a]Specific immune rooster sera for isolates 801 and 695 were absorbed 3 times with each antigen for 1 hour at 37° C. with constant agitation followed by centrifugation and the 3rd absorption sera were Selas 03 filtered to remove excess absorbing antigen.

[b]Negative indicates no inhibition of hemagglutination in the 1:10 starting dilution.

[c]Hemagglutination-inhibition titers are recorded as the reciprocal of the highest serial two-fold dilution causing complete inhibition of hemagglutination of chicken erythrocytes.

hemagglutination-inhibition procedure employing antigen prepared from isolate 801 of serotype A. The results were very good when chicken erythrocytes were employed with chicken sera and turkey erythrocytes were employed with

turkey, pigeon and quail sera without preliminary absorption of the sera with the erythrocytes. Normal chicken sera occasionally reacted in the 1:10 or 1:20 dilutions. Thus, titers were not considered significant unless they were 40 or higher. Detailed results are not tabulated since most of the information has already been employed in the section on pathogenicity studies. Figure 8 is a photograph showing a typical series of hemagglutination-inhibition tests.

One series of specific immune rabbit sera representing each serotype was tested by the hemagglutination-inhibition procedure employing antigen prepared from isolate 801 of serotype A. Two absorptions of each rabbit serum with turkey erythrocytes for 30 minutes at room temperature were necessary before the sera could be employed in the test with turkey erythrocytes. The hemagglutination-inhibition results are presented in Table 25.

Only rabbit sera representing isolates of serotype A reacted beyond the 1:10 starting dilution of serum.

Discussion The finding that only antisera prepared from isolates of serotype A reacted in the hemagglutination-inhibition test is in agreement with the results reported by Kleckner (113). Yamamoto and Adler (180) did not employ the hemagglutination-inhibition procedure in characterization studies.

Van Herick and Eaton (168) apparently were the first to

Table 25. Hemagglutination-inhibition titers of rabbit antisera with serotype A antigen prepared from isolate 801

Serotype	Antisera[a]	HI titer[b]	Serotype	Antisera[a]	HI titer
	Normal	-[c]	G	O	-
A	VR	160	H	N	-
	24R	640		1022-2	-
	801	1280			
			I	695	-
B	54-537	-		1703	-
C	DIVA	-	J	693	-
D	NY	-	K	158VI	-
				1805	-
E	DPR-2	-			
	640	-	L	694	-
F	SA-3	-			

[a]Specific immune rabbit antisera were absorbed twice with turkey erythrocytes at room temperature for 30 minutes.

[b]Hemagglutination-inhibition titers are recorded as the reciprocal of the highest dilution of serum causing complete inhibition of hemagglutination of turkey erythrocytes.

[c]No inhibition in the 1:10 starting dilution.

employ the hemagglutination-inhibition procedure in studies with avian Mycoplasma. They noted its ability to detect antibodies in chickens which were apparently infected with pathogenic Mycoplasma representing serotype A. The hemagglutination-inhibition procedure has become valuable in programs for the control of pathogenic avian Mycoplasma.

It is unfortunate that hemagglutinating antigens prepared from isolates of serotypes I, J and K could not readily be employed in the hemagglutination-inhibition procedure. However, it is significant that sera prepared from such hemagglutinating antigens did not cross react with serotype A in hemagglutination-inhibition tests.

DISCUSSION

In 1933 Nelson (129) described a lingering type of coryza in chickens which has since become of economic importance to the poultry industry of many countries in the world. Coccobacilliform bodies associated with the condition were first described by Nelson (127) in 1935 and have been the subject of major concern in numerous subsequent investigations, including the present one. Such bodies are now considered to be organisms of the genus Mycoplasma.

Relatively little information has been obtained concerning the intricate details of the structure and metabolism of the organisms as such, but a large volume of literature has accumulated concerning the more readily determined characteristics of avian Mycoplasma. The pathogenicity of some Mycoplasma for chickens and turkeys has obviously been a major factor in directing the course of most investigations.

The very early reports of Markham and Wong (118), White *et al.* (174) and Gianforte *et al.* (75) suggested that Mycoplasma from avian sources were essentially identical. However, the reports by Chu (28, 29), Adler and Yamamoto (8) and Adler *et al.* (5, 12) made it evident that nonpathogenic avian Mycoplasma also existed. Taylor and Fabricant (163), Adler and Yamamoto (8) and Fabricant (57) further noted that nonpathogens were more readily cultivated in artificial media than were pathogens.

Thus, it was apparent that detailed studies were necessary to characterize various Mycoplasma of avian origin. A rather extensive study was conducted by Yamamoto (179) in 1957. Yamamoto and Adler (180, 181) published the major results from his study of 8 isolates of varied origin presented in his doctoral thesis. They described the characteristics of 5 serotypes, represented by serotypes A, C, F, G and H of the present study. Only serotype A contained isolates which were obviously pathogenic for chickens and turkeys although isolate N of serotype H produced moderate air sac lesions in turkeys and isolate O of serotype G was only pathogenic for chicken embryos. They also noted differences in the colonial and cellular morphology of isolates of different serotypes and that only isolates of serotypes A, C and F were able to ferment carbohydrates.

Then in 1960 Kleckner (113) studied 15 isolates of avian Mycoplasma and characterized 8 serotypes, designated as serotypes A through H. Again only isolates of serotype A were found to be pathogenic for chickens and turkeys. Isolates of serotypes A, C, D and F fermented carbohydrates.

Similar studies were reported by Moore et al. (124) although their results are difficult to evaluate since they did not extensively employ previously described isolates or serotype designations. Tourtellotte and Jacobs (165) and Noel et al. (136) only included identifiable isolates of

serotypes A and C in their serological studies, with results in agreement with those of Yamamoto and Adler (181) and Kleckner (113).

Fabricant (58), Kelton and Van Roekel (110) and Fabricant[1] employed the colony inhibition technic to further investigate the serological characteristics of previously reported serotypes. Their results were in general agreement with previous findings, except that serotypes E and G were not separable by the colony inhibition technic.

The classification of avian Mycoplasma has been attempted to a limited extent. Edward and Freundt (53) designated Mycoplasma gallinarum for an isolate from the trachea of a chicken. Unfortunately, that organism was not representative of the typical pathogenic avian Mycoplasma (serotype A). Mycoplasma gallinarum is a nonfermenter and a nonpathogen which Fabricant (58) demonstrated to belong to the B serotype of Kleckner (113).

In 1960 Edward and Kanarek (54) named 2 more species from avian sources. They designated Mycoplasma gallisepticum for a typical pathogenic isolate, X95, and designated Mycoplasma iners for a nonpathogenic isolate, M. Fabricant (58) demonstrated that M. gallisepticum represented serotype A, and M. iners represented serotype G of Kleckner (113).

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[1]Dr. Julius Fabricant, Cornell University, Ithaca, New York. Personal communication. Avian Mycoplasma representing serotype SLO. 1962.

Thus, the present study is primarily a continuation and reevaluation of investigations which have been conducted since Nelson (129) first described a lingering coryza of chickens. The spectrum of avian Mycoplasma investigated is relatively amazing, and may account for some of the confusion apparent in studies conducted during at least the 1950's. It is no wonder that Chu (29) was reluctant to accept the concept of Markham and Wong (118) that Mycoplasma caused chronic respiratory disease in chickens and infectious sinusitis of turkeys when he rather consistently only isolated nonpathogenic Mycoplasma, even from normal chickens. Nor is it difficult to conceive how strain X-95 has come to be the type strain for both serotypes A and D. Similarly, the apparent egg transmission of avian Mycoplasma of serotype A first detected by Van Herick and Eaton (168) is now known to occur also with at least isolates of serotypes H and I, which may have erroneously been assumed to be isolates of Mycoplasma gallisepticum when encountered without further detailed identification.

The results of the present study are essentially so much in agreement with the results of studies with serotypes A through H by Kleckner (113) and with serotypes A, C, F, G and H by Yamamoto and Adler (180, 181) that little more needs to be said than has already been discussed in each section. However, it is worthy of mention that only isolates of

serotype A were pathogenic for chickens and turkeys, that isolates of serotypes A, I and J produced tendo-vaginitis in experimental chickens and turkeys, that isolates of serotype H produced airsacculitis in turkeys and that isolates of serotypes A, D, E, I and J were pathogenic for chicken embryos. Thus, the simple designations of pathogenic and nonpathogenic Mycoplasma are meaningless unless the site inoculated and the host employed are described.

Similarly, attempts to identify various serotypes by their colony morphology, biochemical and cultural characteristics is difficult when different media and technics are employed in various laboratories. Even with well standardized environmental conditions it is not possible to completely identify each serotype without the aid of serological procedures. It remains to be determined whether agglutination, colony inhibition, or some other procedure is superior for such serological studies.

The available evidence suggests that isolates 1042, 1043, 1079-3, 1087-1, 1102-7, 1103-5 and 1109 of the unclassified group are most closely related to serotypes I or J, but definite proof is lacking. Similarly, isolate SA-4 may well be a saprophyte, but such was not proved beyond doubt in the medium employed. Isolate 890A represents an exception to the finding that pigeon isolates were entirely nonpathogenic. However, its serotype is not evident, nor is that of

isolate 27R which was obtained from rooster semen.

One of the most intriguing studies included in the present work was that on cellular morphology. It is unfortunate that it was not more extensively explored. The numerous forms noted, especially with isolates of serotype K, are just as curious today as similar forms must have been for Bordet (22) and Borrel et al. (23) in 1910. The coccoid to coccobacilliform bodies of Mycoplasma gallisepticum apparently are the same type as those observed by Nelson (127) in 1935.

It is interesting to note the origin of isolates representing the various serotypes as presented in Table 27 in the Appendix. Isolates of serotype A were obtained from several sites from chickens, turkeys and a partridge while isolates of serotype B, D and E were only obtained from the trachea of chickens. Isolates of serotype C were obtained from the trachea of chickens with the exception of isolate Tu which was of nasal turbinate origin. The O isolate of serotype G was obtained from the pericardial sac of a chicken. In contrast, isolates of serotypes F and H were obtained only from turkeys. The 3 isolates representing serotype F actually were replicates of the original SA isolate which was obtained from the trachea of a turkey. Isolates N, 933 and 1022-2 of serotype H were obtained from poult air sacs while isolate 1104 was obtained from the

sinus exudate of a poult.

Isolates of serotype I were primarily obtained from poult air sacs although some were obtained from the chorioallontoic membrane and yolk of embryonated chicken eggs, and from the trachea and oviduct of chickens. Only isolates from turkeys were found to represent serotype J, all of the isolates of serotype K were obtained from the oviduct of chickens and only isolates from the nasal turbinates of pigeons represented serotype L.

Egg transmission of Mycoplasma of serotypes A and I in chickens is suggested by the isolation of such Mycoplasma from embryonated eggs. Isolates of serotype A were also obtained from chicken ovary, oviduct and semen, and 1 isolate of serotype I was obtained from the oviduct of a chicken. Similarly, egg transmission of serotypes A, H and I in turkeys is suggested since such Mycoplasma were obtained from embryonated eggs and very young poults. In addition, it may be of some significance that isolate 1075 of serotype J was obtained from turkey semen and isolate 693 of serotype J was obtained from the hock joint of a poult only 1 week old.

All 3 of the isolates of serotype K were obtained from chicken oviducts, but evidence of egg transmission is not apparent.

It was mentioned in the section on materials and methods that 27 isolates of avian Mycoplasma included in this study

originated from a single flock of chickens. These isolates were found to represent a relatively wide spectrum of serotypes. Isolates of serotypes A, C and I originated from a tracheal swab culture obtained from hen 102. Isolates of serotypes A, B, C and I originated from various cultures obtained from hen 111, and isolates of serotype K originated from oviduct swab cultures obtained from hens 108, 132 and 158. Thus, isolates of serotypes A, B, C, I and K were obtained from one flock of chickens.

It is difficult to comprehend how 1 flock could become involved with so many serotypes of avian Mycoplasma. The possibility of the occurrence of mutations of the Mycoplasma within the affected chickens can not be overlooked. However, the isolation of serotypes A, B, C and I from chickens and/or turkeys from other flocks tends to weaken the concept of possible mutations unless such mutants are relatively common. It may well be that initial infection with serotype A Mycoplasma somehow predisposes the chickens to infection by other serotypes, although the origin of the other serotypes is still not explained.

SUMMARY

The colony and cell morphology, biochemical, biological, cultural, serological and pathogenic characteristics of avian Mycoplasma obtained from chickens, turkeys, pigeons and a partridge were studied. Eighty-eight of the 98 isolates employed were classified by the tube agglutination procedure as representative of 12 serotypes designated A through L. The main characteristics of each serotype are summarized in Table 26.

Colonies on agar medium were essentially smooth, entire and possessed a more dense central elevation. The approximate average diameter in millimeters of well formed colonies was 0.2 for serotypes A, H, I and J, 0.4 for serotype L, 0.6 for serotype K, 0.7 for serotypes B, C, D, E and G and 0.8 for serotype F.

The cellular morphology in broth culture was extremely pleomorphic within the entire group. The cells of serotypes A and H were predominantly coccoid to coccobacilliform, approximately 0.5 microns in diameter. Cells of serotypes I and J were coccobacilliform to rods approximately 0.3 x 1.5 microns and those of serotype L were coccoid to smaller rods. Cells of serotypes B, C and D were slender rods and abundant rings, and those of serotype F were almost entirely rings 1.0-2.0 microns in diameter. Serotypes E and G produced slender rods and relatively short slender filaments which

sometimes had sacculated ends. Slender rods, a few rings and short beaded filaments to sacculated filaments approximately 1.0-2.0 x 30-50 microns in dimension were observed in cultures of serotype K.

Serotypes A, C, D, F, I, J and K fermented dextrose and maltose, but not trehalose or lactose. Fermentation of sucrose, galactose and mannitol was only infrequently noted. Serotypes B, E, G, H and L did not ferment dextrose, maltose, sucrose, galactose, mannitol, trehalose or lactose.

Serotypes A, I, J and K caused hemagglutination of turkey erythrocytes although only antigens prepared from serotype A were usually stable enough to employ in the hemagglutination-inhibition test.

Hemolysis of horse erythrocytes incorporated into agar medium was produced by serotypes A, B, C, D, F, H, K and L.

Triphenyl tetrazolium chloride was reduced by broth cultures of serotypes A, B, C, D, I, J, K and L.

Serotypes A, D, E, I and J were pathogenic for chicken embryos, causing deaths, hepatitis and splenomegally. Joint abscesses were produced in embryos by 1 isolate each of serotypes D and E.

Only serotype A produced airsacculitis in chickens although serotypes A and H produced airsacculitis in turkeys. Only serotype A produced sinusitis in turkeys and serotypes A, I and J produced tendo-vaginitis in chickens and turkeys.

Numerous broth cultures were found to be viable when subcultured after 3-4 years storage at -30° C. Several lyophilized preparations of avian Mycoplasma were found to be viable when subcultured after being stored at 4° C. for as long as 5-14 years.

One of the 10 serologically unclassified isolates appeared to be a saprophyte.

Isolates of serotypes A, B, C, D, E and I were obtained from the trachea of chickens, isolates of serotypes A, H and I were obtained from the air sacs of turkeys, and isolates of serotypes A and H were obtained from turkey sinuses.

Evidence was obtained which suggested egg transmission of serotypes A and I in chickens and serotypes A, H and I in turkeys.

Isolates of serotypes A, B, C, I and K were obtained from 1 flock of chickens, isolates of serotypes A, B, C and I were obtained from a single chicken, and isolates of serotypes A, C and I were obtained from a single tracheal swab culture from 1 chicken.

Table 26. Summary of characteristics of avian Mycoplasma serotypes

Characteristics	Serotypes A	B	C	D	E	F	G	H	I	J	K	L
Approx. colony size, mm.	.2	.7	.7	.7	.7	.8	.7	.2	.2	.2	.6	.4
Dextrose fermentation	+	-	+	+	-	+	-	-	+	+	+	-
Hemagglutination	+	-	-	-	-	-	-	-	+	+	+	-
Hemolysis	+	+	+	+	-	+	-	+	-	-	+	+
Tetrazolium reduction	+	+	+	+	-	-	-	-	+	+	+	+
Pathogenicity chicken embryo	+	-	-	J[a]	J	-	-	-	+	+	-	-
chicken air sac	+	-	-	-	-	-	-	-	-	-	-	-
hock and/or foot pad	+	-	-	-	-	-	-	-	+	+	-	-
turkey sinus	+	-	-	-	-	-	-	-	-	-	-	-
air sac	+	-	-	-	-	-	-	+	-	-	-	-
hock and/or foot pad	+	-	-	-	-	-	-	0[b]	+	+	-	-

[a]Joint abscesses were produced by 1 isolate each of serotypes D and E.

[b]Not determined.

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APPENDIX

Table 27. Summary of the origin of isolates of avian Mycoplasma of each serotype

Serotypes and isolates	Origin	Serotypes and isolates	Origin
Serotype A		Serotype A	
A5969	chicken trachea	801	turkey air sac
F	chicken trachea	857-1	turkey sinus
S6	turkey brain	882	turkey sinus
VR	chicken trachea	893A	partridge sinus
24R	chicken semen	894	chicken joint
26R	chicken semen	1010	chicken trachea
128VA	chicken ovary	1079-1	turkey yolk
187	chicken turb.[a]	1112	chicken trachea
197	chicken turb.	1344	turkey air sac
293	chicken trachea	1403	chicken trachea
595	turkey sinus	1605	chicken oviduct
699	chicken trachea	1900	chicken CAM
734	turkey sinus	2705	chicken oviduct
755	chicken CAM[b]	Serotype B	
756	chicken CAM	K18B	chicken trachea
796	turkey yolk	54-537	chicken trachea

[a]Nasal turbinates.

[b]Chorioallantoic membrane.

Table 27. (Continued)

Serotypes and isolates	Origin	Serotypes and isolates	Origin
Serotype B		Serotype E	
114TB	chicken trachea	DPR-2	chicken trachea
1207	chicken trachea	640	chicken trachea
1304	chicken trachea	Serotype F	
1504	chicken trachea	SA-1	turkey trachea
2600	chicken trachea	SA-2	turkey trachea
5858	chicken trachea	SA-3	turkey trachea
Serotype C		Serotype G	
C	chicken trachea	0	chicken per.[c]
DIVA	chicken trachea	Serotype H	
Tu	chicken turb.[a]	N	turkey air sac
108TA	chicken trachea	933	turkey air sac
1021	chicken trachea	1022-2	turkey air sac
1111	chicken trachea	1104	turkey sinus
2805	chicken trachea	Serotype I	
Serotype D		114C	chicken CAM[b]
NY	chicken trachea	116C	chicken CAM
594	chicken trachea	172C	chicken CAM
Serotype E		695	turkey air sac
C26	chicken trachea	799-1	turkey air sac

[c]Pericardium.

Table 27. (Continued)

Serotypes and isolates	Origin	Serotypes and isolates	Origin
Serotype I		Serotype J	
799-4	turkey air sac	1075	turkey semen
806C	turkey air sac	Serotype K	
807-5	turkey air sac	132VI	chicken oviduct
807-18	turkey air sac	158VI	chicken oviduct
814-5	turkey air sac	1805	chicken oviduct
815-4	turkey air sac	Serotype L	
822-4	turkey air sac	694	pigeon turb.[a]
823C	turkey air sac	858-76	pigeon turb.
833R	turkey air sac	878-50	pigeon turb.
844-2	turkey air sac	Unclassified	
849P	turkey air sac	SA-4	turkey trachea
850-2	turkey air sac	27R	chicken semen
851R	turkey air sac	890A	pigeon turb.
853M	turkey air sac	1042	turkey yolk
854-2	turkey air sac	1043	turkey semen
1018	chicken trachea	1079-3	turkey yolk
1703	chicken yolk	1087-1	turkey air sac
2004	chicken oviduct	1102-7	turkey lung
Serotype J		1103-5	turkey yolk
693	turkey joint	1109	turkey yolk