Avidin-biotin immunohistochemical detection of *Mycoplasma gallisepticum* antigens in turkey respiratory tissues with rabbit polyclonal primary antibodies

by

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INTRODUCTION

Mycoplasma gallisepticum (MG) causes infectious sinusitis in turkeys (86). This pathogen was previously referred to as S-6 or pleuropneumonia-like organism (PPLO) (94). Like all mycoplasmas, MG is a specialized bacteria that lacks a cell wall and it survives only 1-3 days in feces at 68° F. MG attaches to the tracheal epithelium of poultry (122) and once a turkey is infected, that bird is considered to be infected for life. MG is transmitted from breeder hens to turkey poults through the egg (1, 65). The National Poultry Improvement Plan requires that turkey breeder flocks be tested for MG and must be negative before eggs can be hatched and sold to producers (97). Infected flocks of breeding birds must be depopulated. Consequently, all infected breeder flocks must be sent to slaughter.

Pathogenicity varies widely between isolates and turkeys are more susceptible to MG infection than chickens (70, 132). Infectious sinusitis has an incubation period that lasts from 6 to 10 days and the duration of clinical signs may range from weeks to months (132). MG infection typically causes an increase in mucus production at all levels of the turkey respiratory tract. Accumulation of mucus production is most obvious in the infraorbital sinuses which become markedly swollen. Mortality is less than 2% in uncomplicated cases but may be significantly elevated with concurrent bacterial or viral infections which contribute to elevated mortality, increased condemnations at processing, and reduced growth rate (72). Unfortunately, concurrent infections are the rule since each gram of poultry dust contains up to 800,000 *E. coli* and 10 to 15 percent of intestinal *E. coli* are pathogenic (60).

Mycoplasma gallisepticum (MG) continues to be a problem for the commercial turkey

industry in the United States. Commercial turkey producers lose money due to cost of medication, elevated mortality, reduced growth rate in affected flocks, and the necessity of sending all infected breeder flocks to slaughter. Condemnation of turkeys at the processing plant is a major cause of economic loss. Mucoid lesions in the air sacs can not be removed by suction devices at the processing plant resulting in condemnation of the entire carcass. In 1992, approximately 30-40% of carcasses from MG-infected commercial turkey flocks in Michigan were condemned (personal communication, BilMar Turkey Company).

Confirming a diagnosis of MG infection may be difficult and time-consuming in laboratories that do not routinely work with avian mycoplasmas. Faster identification of positive cases would reduce the opportunity for the organism to spread to other farms. A definitive diagnosis is based on the isolation and identification of the causative organism but isolation requires special media and may take weeks. Serology is useful but is not without problems. Serological tests include the serum plate agglutination test which is capable of detecting IgM antibody early in the course of infection and the hemagglutination inhibition (HI) test which detects IgG antibody (58). The National Poultry Improvement Plan (97) has ruled that HI titers of $\leq$1:40 are suspicious and flocks must be monitored whereas HI titers of 1$\geq$:80 are positive and the flock is presumed to be infected. Unfortunately, titers are often equivocal. In recent years, an ELISA test has become available but false positive results have been encountered in flocks that have previously been vaccinated with oil-adjuvanted vaccines. The serum plate agglutination test (SPA) and the enzyme linked immunosorbent assay (ELISA) test are sensitive but may yield false positive results (11). The polymerase

chain reaction (PCR) has the advantage of speed, sensitivity, and specificity. However, this technique is available only in few specialized laboratories, is expensive, and there is a danger of false positives due to contamination by environmental laboratory strains (84).

The avidin-biotin immunohistochemical technique has the potential to provide a rapid confirmation of *Mycoplasma gallisepticum* in turkeys with swollen infraorbital sinuses. With this procedure, primary antibodies directed against MG antigens are produced in laboratory animals, such as rabbits. An antigen-containing specimen is embedded in paraffin, sectioned, and displayed on a microscope slide. Primary antibodies are applied to the slide and, after rinsing the slide, are followed by biotinylated secondary antibodies produced in a different species of laboratory animal, such as a goat. If primary antibody attaches to the MG antigens, biotinylated secondary antibodies will attach to the primary antibodies. Incubation with chromogen results in a chemical reaction with peroxidase attached to the secondary antibodies and deposition of a red deposit. Avidin-biotin immunohistochemical tests are inexpensive, can be completed in hours, and are readily performed in most diagnostic laboratories. The immunoperoxidase technique can be run on tissues routinely fixed in 10% neutral buffered formalin . An immunoperoxidase procedure has been developed in rodents which allows visualization of individual mycoplasma organisms (62).

The purpose of this research was to develop an avidin-biotin histochemical test to detect *M. gallisepticum* infections in turkeys. This procedure should make it possible to confirm or deny the presence of MG in turkeys with swollen heads within 48 hours after specimens are submitted to a diagnostic laboratory. The avidin-biotin test must be

sufficiently sensitive to detect MG antigens in turkey respiratory tissues and it must be specific. The test must not cross-react with respiratory tissues from mycoplasma-free turkeys, turkeys infected with *Mycoplasma meleagridis*, or turkeys infected with *Mycoplasma synoviae*. The procedure should be cost-effective and amenable for routine use in a diagnostic laboratory.

REVIEW OF LITERATURE

This literature review includes three parts: characteristics of *Mycoplasma gallisepticum* organisms, immunohistochemistry techniques used in this research, and a review of the disease in turkeys caused by *M. gallisepticum* infection.

Mycoplasma gallisepticum. The earliest reports of infectious sinusitis in turkeys came from England. In 1905, Dodd described a disease that he called "epizootic pneumoenteritis of the turkey" and Gram-Smith reported "swollen head" in turkeys two years later (41, 81). Markham and Wong in 1952 discovered that infectious sinusitis of turkeys and chronic respiratory disease of chickens were caused by the same pleuropneumonia-like organisms (PPLO) (94). In 1960, Edward and Kanarek proposed the name *Mycoplasma gallisepticum* (MG) for these PPLO of poultry (45). Different strains of MG exist and the most strains used in research include S6, R, and F. The S6 strain of MG is the prototype strain which was originally isolated from the brain of turkeys with infectious sinusitis (135). F strain is a low virulence vaccine strain that originated from studies by van der Heide employing the Connecticut F strain (132). The pathogenic R strain was isolated in 1963 from a chicken with airsacculitis by Dale Richy at the University of Georgia Poultry Disease Research Center and is frequently used as a challenge strain in research (132).

Ultrastructural Morphology and Replication. In dense aggregates, *M. gallisepticum* have a cocco-bacilliform shape and are approximately 0.25-0.5 μm in diameter (4, 132). When separated, MG have an irregular, elongated shape (3). *M. gallisepticum* is a specialized bacteria with a lipoprotein cell membrane measuring about 80-100 Å thick but

does not possess a cell wall (101) . The lipoprotein cell membrane enables mycoplasma to adapt their shape according to the host cell surface contours (102). MG are capable of slow gliding movement which is facilitated by a tiplike terminal bleb which points in the direction of movement and adheres to solid surfaces (3, 27, 88). An infrableb region is composed of granular material (88). A system of submembrane tubules represented by smoothly curved loops is present in *M. gallisepticum* which may serve as a cytoskeleton. It has been postulated that submembrane tubules may reversibly anchor to surface proteins which enables a 'caterpillar' type of motility (78). Tubules have a diameter of 40 nm, a central core of 10 nm, and both ends of each tubule is anchored in the infrableb region (78). Replication of MG occurs by binary fission that begins with formation of a second bleb opposite the original bleb and by budding (44, 89).

Mycoplasma gallisepticum attaches to tracheal epithelium of poultry by a combination of hydrophobic and ionic bonds (22, 102). Host cell membrane receptors for attachment of MG's terminal bleb consist of the sialic acid moieties of sialoglycoproteins or sialoglycolipids (103). Terminal sialic acid residues have been identified on the apical surface of epithelial cells and endothelial cells in the trachea and lung of healthy turkeys (5). Glycophorin, the erythrocyte membrane glycoprotein carrying almost all sialic acid moieties of erythrocytes, is the major receptor for *M. gallisepticum* (15). Binding of MG to erythrocyte membranes results in hemagglutination (118). Banai has shown that adherence of MG to erythrocytes is not dependent upon ionic gradients across the mycoplasma cell membrane (16).

Colony Morphology and Growth Requirements. *M. gallisepticum* colonies are usually no more than 0.2-0.3 mm in diameter and appear as tiny, smooth, circular, translucent masses with a dense, raised central area (132). Adjacent colonies frequently coalesce. *Mycoplasma gallisepticum* requires an enriched medium with 10-15% heat-inactivated swine, avian, or horse serum for optimum growth at pH 7.8 and 37-38° C. Colonies on agar media require a moist atmosphere and incubation for 2-5 days (132). Frey's media will support the growth of *M. gallisepticum*, *M. synoviae*, *M. meleagridis*, and many other avian mycoplasmas (53).

Biochemical properties. *Mycoplasma gallisepticum* has neuraminidase-like enzyme activity that is absent in other avian mycoplasmas (104, 112). MG ferments glucose and maltose resulting in production of acid but not gas. It does not ferment lactose, dulcitol or salicin. It is phosphatase negative and will agglutinate turkey and chicken erythrocytes (132). MG requires an exogenous source of cholesterol for its growth because it lacks the ability to synthesize cholesterol. During the growth period, cholesterol is first incorporated into the outer half of the MG lipid bilayer and subsequently moves from the outer surface of the membrane to the inner surface (108). Metabolic activity is probably required for motility and formation of the terminal bleb attachment structure (42).

Genetics. *M. gallisepticum* has a DNA genome. Studies with ribosomal RNA gene probes indicate that there is genotypic heterogeneity between different MG strains (133). Recently, Tigges reported that the total genome size of the R strain of MG is 998kb (121). A family of genes encoding for the adhesin of *M. gallisepticum*, pMGA, have been identified.

Variability in pMGA structure observed in MG may represent a mechanism for evasion of the immune response and probably results from expression of one variant pMGA gene from the gene family (93).

Antigens. The plasma membrane of *M. gallisepticum* contains at least 35 acylated polypeptides which represent the major antigens and immunogens (67). Palmitic acid is the major ester-bound fatty acid and linoleic acid is the major amide bound-linked fatty acid (67). Immunogenic integral membrane proteins specific to *M. gallisepticum* which have been intensively studied include proteins p64 (64 kDa) and p56 (56 kDa) (12, 115). Protein p64 was found in 19 of 19 strains of MG and p56 was detected in 18 of 19 MG strains. Markham has described a 67 kDa membrane protein (pMGA) which is responsible for hemagglutination. Avakian's p64 and Markham's pMGA may be identical lipoproteins specific to *M. gallisepticum* which function both as an adhesin and as a hemagglutinin (52, 67, 92). Lipoprotein p64 is located in the cell membrane near the base of the terminal bleb (54). In addition, a different surface protein of 155 kDa has been identified in the cell membrane at the tip of the terminal bleb organelle which may facilitate attachment (42).

Surface epitopes of *M. gallisepticum* may vary among colonies of the same passage, between passages of the same strain, and among different strains (55). Furthermore, monoclonal antibodies may recognize proteins of different sizes in different isolates (55). However, one membrane protein containing a conserved epitope present in most *M. gallisepticum* strains has been identified near the attachment organelle (47). This protein has a molecular weight of 98 kDa (p98) and has been found only in *M. gallisepticum* (47).

Immunogenicity. Kleven reported that an antibody response to MG can be detected by the serum plate agglutination test within a few days after infection (75) and by the hemagglutination-inhibition (HI) test two weeks post infection. The first antibody generated in response to MG infection is IgM which persists for up to 77 days. The IgM response is followed within a week by an IgG response which persists in the serum for months (6). The bursa of Fabricius has a vital role in development of the peripheral humoral immune response (6). Cellular immunity of the T-cell type against MG has been reported in the nasal cavity and the lungs of chicken after intramuscular injection (6). MG strains differ in their ability to induce an antibody response measured by hemagglutination-inhibition (HI) or enzyme-linked immunosorbent assay (ELISA) tests (40).

Pathogenicity. Pathogenicity varies widely between isolates (82, 83). Jordan reported that the severity of MG diseases is influenced by the age of the host, route of entry of mycoplasma, the number of infecting organisms, and the virulence of the infecting mycoplasma (70). Concurrent bacterial or viral infections associated with MG contribute to their pathogenicity (126).

Mycoplasma preferably colonize respiratory and genital mucosa (56, 123). Adsorption or adherence of mycoplasma to a host cell membrane is the initial step in infection. The ability to adhere tightly to target cells is essential for colonization and loss of adherence in MG has been associated with loss of virulence. *M. gallisepticum* possess integral membrane proteins called adhesins which facilitate attachment to host cell receptors (103). Receptors on host cell membranes consist of sialic acid moieties, such as *N*-acetyl

neuraminic acid, which are conjugated to glycoproteins or glycolipids (103, 126).

Prolonged mycoplasma adherence to host cell membranes is required to cause host membrane damage (102). However, the mechanism by which *Mycoplasma gallisepticum* injures the host cell is not clearly understood. Some strains of MG (S6) in turkeys have been found to possess non-exotoxin neurotoxins that are responsible for encephalitis and polyarteritis nodosa in the brain of turkey poults (119, 126). Hydrogen peroxide (126) and polysaccharides (56) are also produced by mycoplasma and may contribute to pathogenicity by causing membrane and tissue damage. The hemolysin of MG has been shown to be hydrogen peroxide which may be a virulence factor (31).

Multiplication of *M. gallisepticum* takes place on the cell surface and both the integrity of the host cell membrane and the host cell function may be altered (56). MG will inhibit ciliary activity in tracheal organ cultures. However, this cilia-stopping effect (CSE) is not affected by either neuraminidase which disrupts cell adherence nor by catalase which inactivates hydrogen peroxide (31). No change in the activity of dehydrogenase enzymes associated with the tricarboxylic acid cycle in respiratory epithelial cells was found in sinuses of turkeys experimentally infected with MG (22).

Immunoperoxidase Histochemistry. Four basic immunoperoxidase cytochemical techniques for tissue antigen demonstration have been described (23, 46). These procedures are described below.

1. Direct Method: Horseradish peroxidase is chemically linked to the primary antibody. Peroxidase-labeled antibody binds to a specific antigen in tissue and a colored end product

precipitates at the antigen site after a chromogen is applied. This technique has the advantage of being quick and producing few nonspecific reactions. The primary disadvantage is that a separate specific conjugated antibody must be produced to identify each individual, different antigen in tissue sections. Due to a lack of sensitivity, frozen sections must be used for consistent results.

2. Indirect Method: Primary unconjugated antibody is applied to tissue on a microscope slide and binds to a specific antigen. Subsequently, a peroxidase enzyme-labeled secondary antibody attaches to the primary antibody and a substrate is added to localize the reaction. Primary antibodies produced in one animal species, such as a rabbit, are excellent antigens when injected into a different animal species, such as a goat. This technique is more versatile than the direct method because the same conjugated secondary antibody produced in one animal species can be used to detect primary antibodies directed against a variety of antigens which are produced in a different species of laboratory animal. The indirect method takes twice as long as the direct method, has an increased chance of nonspecific reactions, and the procedure works best to detect antigen in frozen sections.

3. Peroxidase-Antiperoxidase Method. This three-layer technique employs primary antibodies specific for the antigen, secondary "link" antibodies which bind to the primary antibody and to a peroxidase-antiperoxidase (PAP) antibody complex. Primary antibody and antiperoxidase antibodies are produced in the same animal species. One Fab site of the link antibody binds to the primary antibody and the other Fab site of the link antibody binds to the PAP complex. Peroxidase enzyme is seen via a substrate-chromogen reaction. The PAP

method is more sensitive that the direct or indirect methods and can be used in formalin-fixed sections which makes retrospective studies possible.

4. Avidin-Biotin Method: This procedure utilizes the phenomenon that avidin of egg-white origin binds to 4 molecules of biotin in a nonimmunologic manner. Three reagents are used in this procedure: primary antibody directed against a specific antigen, secondary antibody conjugated to biotin, and a complex of peroxidase conjugated-biotin and avidin. Free sites on the avidin molecule bind to biotin conjugated to the secondary antibody and a red precipitate forms following addition of chromogen. This method is relatively sensitive and can be used on formalin-fixed specimens.

Endogenous peroxidase activity. The substrate-chromogen reaction is nonspecific in that it can not distinguish between peroxidase enzyme localizing the cellular antigen and pre-existing tissue peroxidase activity located primarily in erythrocytes and leucocytes. Erythrocytes contain pseudoperoxidase and myeloperoxidase is present in granulocytes (46). Removal of endogenous enzymatic activity at the beginning of the staining procedure is necessary to prevent nonspecific reactions (23). Endogenous peroxidase can be irreversibly denatured by applying 3% hydrogen peroxide directly to the tissue sample. This solution does not denature horseradish peroxidase. Other solutions that can be used to prevent this endogenous activity include 0.01% periodic acid followed by sodium borohydride and 0.2% acetic acid with 1% sodium nitroferricyanide.

Nonspecific background staining. Nonspecific background staining is not due to an antigen-antibody reaction (23). Nonspecific specimen staining is caused by attachment or

adsorption of protein antibodies to highly charged collagen and connective tissue elements in tissue. Secondary antibody with conjugated peroxidase nonspecifically attaches to charged sites resulting in a positive color reaction. Background staining can be prevented by the addition of a protein solution to the tissue specimen prior to applying the primary antibody. Nonimmune serum from the same animal species that produced the secondary antibodies will fill most of the charged sites and thereby prevent primary antibodies from binding to collagen (28). Nonimmune serum is used at a 1:5 to 1:20 dilution and allowed to remain for 10 to 20 minutes on the specimen. Excess nonimmune serum is tapped off leaving a thin layer coating the tissue. Other causes of nonspecific staining include: inappropriate antibody dilutions, incomplete removal of paraffin, improper rinsing of slides, incorrect substrate incubation, and hemolysis of erythrocytes by nonimmune serum.

Fixation. Tissue sections contain only a finite quantity of antigen and preservation of antigen is the primary concern in fixation. Antigen in a tissue section must be fixed to avoid diffusion or being washed out of the specimen. Adjacent cells must be fixed so that tissue morphology is preserved and a correct interpretation is possible. Overfixation may denature the antigen and may produce aldehyde cross-links that prevent primary antibody from reaching the antigen. Chemical fixation denaturates and stabilizes protein by coagulation or by forming additive compounds (29). Formaldehyde binds to amino groups on side chains of amino acids and cross-links proteins which may mask antigenic sites (29). Formaldehyde penetrates quickly but fixes slowly because significant time is required to cross-link tissue proteins (29). Methyl pentanedial glycol (MPG) forms fewer crosslinks than 10% neutral

buffered formalin so that cytoplasmic and membrane antigens are better preserved. In addition, MPG is non-toxic, non-corrosive, and non-inflammable (American Histology). Exposure of fixed tissues to proteolytic enzymes results in digestion of aldehyde linkages and exposes protein antigens.

Optimal fixation consists of the minimum time and minimum fixative concentration that will preserve tissue morphology. The following conditions should be considered when preparing specimens for immunohistochemistry (23, 113):

1. Tissue specimen size should not exceed an area of 2 cm square or 3-4 mm in thickness.
2. At least 15-20 volumes of fixative should be used for every volume of tissue.
3. Tissue samples should be placed directly into the fixative after being cut so that the specimens do not dry out.
4. Most fixation is done at room temperature (25° C).
5. Fixation time should be as short as possible to preserve tissue morphology. For formalin, 6 to 12 hours is usually adequate but the time required may vary depending upon the type and thickness of the tissue.
6. Different types of fixatives have been found to be useful for immunohistochemistry. These include 10% buffered neutral formalin, Bouin's fixative, Zenker's fixative, and methyl pentanedial glycol.

***Mycoplasma gallisepticum* Infection of Turkeys** (Infectious Sinusitis). Turkeys are more susceptible to *M. gallisepticum* infection than chickens and MG causes a disease of turkeys called infectious sinusitis (39, 70, 86, 132). The disease was originally called "epizootic pneumoenteritis" by Dodd in 1905 but other names include swell head, airsacculitis and PPLO infection. All ages of turkeys are susceptible and poults may show signs of infection as early as 3-4 weeks old (63). MG has been re-isolated from the lung, trachea, air sacs, brain, kidney, and spleen in turkeys experimentally inoculated via the trachea (100). In turkeys, the incubation period before appearance of clinical signs is 6 to 10 days and infection may persist for weeks or months in untreated flocks (132). Age of the host, route of infection, virulence of organisms, and predisposing stress factors, such as chilling, overcrowding, or high ammonia levels, can affect the severity of infectious sinusitis (70, 116). Mortality is less than 2% in uncomplicated cases but may be significantly elevated with concurrent bacterial or viral infections (72). Concurrent infections frequently occur since each gram of poultry house dust may contain up to 800,000 *E. coli* (60). Economic losses result from elevated mortality, reduced feed efficiency, medication costs, and the necessity to send all infected breeder flocks to slaughter (96, 132).

Natural and Experimental Hosts. *Mycoplasma gallisepticum* occurs primarily in domestic chickens and turkeys but other host species include pheasants, guineas, peafowl, chukker partridge, bobwhite quail, pigeons, Japanese quail, and house finches (132). Geese and ducks may become infected with MG (19, 56, 98, 129). In addition, MG has been isolated from the respiratory tract of wild turkeys showing clinical signs and lesions typical

of infectious sinusitis (38, 68).

Transmission. Transmission may occur by vertical or horizontal mechanisms. Because *M. gallisepticum* is transmitted from infected female birds to their offspring through the egg (1, 19, 65, 116), the National Poultry Improvement Plan requires that all participating turkey breeder flocks be free of this disease (97). Horizontal transmission can occur by direct contact with infected carrier turkeys or chickens or via air-borne dust or droplets (132). Contaminated litter, equipment, or people and inadequate rodent control have been implicated in outbreaks (69). *Mycoplasma gallisepticum* numbers peak in the upper respiratory tract of chickens 2 weeks after infection, and it is likely that transmission occurs most readily during this acute phase of the disease (116). *Mycoplasma gallisepticum* can survive 4 days on feathers and feed, 3 days on human hair, 2 days on cotton and rubber or in water, and 24 hours in the human nose (33).

Clinical Signs. Clinical signs in naturally infected turkeys include shaking of the head, coughing, sneezing, snicks, ocular discharge, labored breathing, and moist tracheal rales. Mild keratoconjunctivitis with frothy exudate in the eye may be the only sign during early stages of the disease (72). Swelling of one or both infraorbital sinuses by a gelatinous exudate which becomes caseous after few weeks of infection is a classical clinical sign of infectious sinusitis and bulging of severely distended sinuses may interfere with vision (53, 63, 70, 87). Swollen sinuses is associated with attachment of MG organisms to respiratory epithelial cells lining the sinuses by the terminal bleb (21, 109). Mucoid nasal discharge is believed to represent an overflow of exudate from affected infraorbital sinuses (59). Some

strains of *M. gallisepticum* produce a fatal encephalopathy in turkey that is associated with torticollis and opisthotonos (32, 36, 37, 126). Intravenous inoculation of turkeys with MG strain S6 usually results in nervous signs and localization within cerebral arteries (35). Commercial turkeys with infectious sinusitis experience a reduced rate of growth (87). Domesticated and wild turkey breeding hens infected with MG may experience a significant drop in fertility, egg production and hatchability (1, 107, 132)

Macroscopic Pathology. Macroscopic lesions in the respiratory system result from mucus accumulation at all levels of the respiratory tract including the nasal passages, sinuses, trachea, bronchi, lungs, and air sacs (132). At later stages of infection, inflammatory exudate turns purulent, fibrinous, or caseous and caseous exudate may persist indefinitely in infraorbital sinuses (59, 87). Salpingitis characterized by caseous plugs has been reported in turkey poults experimentally infected with *M. gallisepticum* via the yolk sac (43). Fibrinous perihepatitis and adhesive pericarditis, probably due to concurrent secondary infections, has been reported in turkeys experiencing infectious sinusitis (127).

Microscopic Pathology. Infected hosts react to mycoplasma infections with serofibrinous inflammation and activation of cell-mediated defense systems. Transformation of T-cell lymphoblasts may be initiated by a mutagenic substance resulting in extensive proliferation of immature lymphocytes (56). Consequently, lymphoid hyperplasia in respiratory tract subepithelial connective tissue is a characteristic lesion associated with mycoplasma infections. Histopathology has been observed in the respiratory tract, brain, joints, and oviduct of turkeys infected with *Mycoplasma gallisepticum.*

Sinuses and nasal passages of turkeys infected with MG are characterized by mucus accumulation, dilation of intraepithelial glands by mucus, hyperplasia and hypertrophy of goblet cells, and infiltration of the lamina propria by lymphocytes, reticulocytes, and plasma cells during the early stages of disease. Later, hyperplastic lymphofollicular structures with germinal centers appear in the submucosa of the sinuses, nasal passages, lungs and air sacs which are highly suggestive of infection by *M. gallisepticum* (17, 56, 70, 73). In the lung, lymphocyte infiltration in air capillary walls may result in thickened air capillary walls and atelectasis of air capillary lumina with consolidation and emphysema in adjacent lobules.

Arteries in brains of commercial and experimental poults infected with *M. gallisepticum* showed endothelial cell swelling and hyperplasia, fibrinoid necrosis of the tunica media, and a transmural infiltration of lymphocytes, plasma cells, and macrophages (35, 119). Small focal areas of necrosis in the brain were presumed to be due to occlusion of blood vessels (32, 36).

M. gallisepticum has been demonstrated to occur in subsynovial and periarticular vessels by immunofluorescence prior to development of clinical signs of arthritis. By 30 days postinoculation, turkeys had clinical signs of acute arthritis and MG was cultured from synovial fluid. Periarticular arteries had sclerotic changes and lymphoplasmacytic cellular infiltrates. Also, hypertrophy of synovial membranes was observed. (34).

Inoculation of MG into yolk sacs of day-old poults resulted in infiltration of oviduct walls with numerous lymphocytes and a few heterophils (43).

Ultrastructural Pathology. Electron microscopy has been used to study the interaction

between *M. gallisepticum* and infected cells in the infraorbital sinuses, trachea, and blood vessels.

Colonization of turkey infraorbital sinus epithelial cells by *M. gallisepticum* results in loss of cilia and microvilli. Localization of mycoplasma in crypts and among cilia on the host cell surface may provide a protection mechanism against phagocytosis (102). In transmission electron micrographs, individual MG organisms are located between and within the cytoplasm of sinus epithelial cells and appear as large, round, dense bodies surrounded by a narrow zone of granular particles (21). Degeneration of sinus epithelial cells is limited to the distal part of the cell with nuclei and basal areas remaining unaffected (21). Scanning electron microscopy of MG-infected sinuses reveals swollen, dome-shaped epithelial cells and an absence of necrosis or ulceration (109).

Tracheal injury following infection by *M. gallisepticum* can be detected by scanning electron microscopy and initial changes consist of swelling of individual respiratory epithelial cells and edema of the mucosal surface. Edema is followed by accumulation of mucus and fibrin which may solidify and partially obstruct the trachea (64). In chicken tracheal explants, *Mycoplasma gallisepticum* organisms were observed in close proximity to cilia and microvilli of epithelial cells. Following sloughing of degenerating epithelium, MG invaded the basement membrane and attached to collagen fibers in the lamina propria (3).

A remarkable degree of endothelial swelling without endothelial cell proliferation was observed in brains of turkeys intravenously injected with the S6 strain of MG. Endothelial cell cytoplasm and nuclei were greatly enlarged resulting in nearly total occlusion of the

lumen of capillaries (90).

Diagnosis. A definitive diagnosis is based upon isolation and identification of the causative organism. Five to 10 tracheal cultures from turkeys or chickens are adequate in acute infections but 30 to 100 birds should be cultured in chronic cases. *Mycoplasma gallisepticum* can be grown in Frey's media which contains penicillin and thallium acetate to inhibit bacterial and mycotic contamination. Phenol red in Frey's media turns from red to orange to yellow as acidity increases. Four to 5 days are required for colonies to become visible on agar plates incubated at 37° C (76). In many laboratories, colonies of MG on agar plates are identified by fluorescein-labeled rabbit antibodies directed against MG (76).

Serological tests available to detect antibodies against *M. gallisepticum* include serum plate agglutination, hemagglutination inhibition, and an enzyme-linked immunosorbent assay. The serum plate agglutination (SPA) test can detect an antibody response within a few days after infection. The SPA test is more sensitive than the hemagglutination inhibition (HI) test and is capable of detecting IgM antibody early in the course of infection (70). Absorption of gamma globulins to *Mycoplasma gallisepticum* during incubation may cause nonspecific reactions to MG antigen used in the SPA test (24). Other causes of nonspecific agglutination include storing serum at 4° C, freezing and thawing serum, and vaccinating birds with oil emulsion vaccines (10, 77). The hemagglutination inhibition test does not become positive until two weeks post infection. The HI test is more specific than the SPA test and detects IgG antibody (58, 70). The National Poultry Improvement Plan has determined that HI titers less than or equal to 1:40 represent suspicious flocks which must be

monitored and HI titters of 1:80 or greater are positive and indicate an infected flock (97). In wild turkeys, antibodies against *M. gallisepticum* could be detected by SPA for one year after infection while HI titers declined after three months (106). In recent years, the enzyme-linked immunosorbent assay (ELISA) test has become available but false positive results have been encountered in flocks previously vaccinated with oil-adjuvanted vaccines (10, 11, 77). The sensitivity of ELISA, HI, and SPA are affected by the antigen strain used in the test (11).

Additional diagnostic tests for *M. gallisepticum* have been developed. DNA probes have been used to detect MG but the sensitivity of DNA probes is limited to detection a large number of organisms (10^6) which are found only during the acute phase of infection (96). Polymerase chain reaction (PCR) tests are much more sensitive and can detect 10^{-6} picograms of MG DNA which is less than the total chromosomal content of one MG organism (96). Although PCR is highly sensitive and specific, there is always a danger of false positive reaction from environmental contamination (84). A radioimmunoassay has been used experimentally in chickens to detect MG antibodies in serum, yolk fluids, and tracheal washings (74). Flow cytometry to identify MG has been described which requires specific antibodies labeled with fluorescein isothiocyanate (95).

Treatment. Treatment of infectious sinusitis will frequently alleviate signs but will not eliminate MG from infected turkeys. Antibacterials that have been successfully used to treat this disease include tylosin, chlortetracycline, oxytetracycline, erythromycin, spectinomycin, lincomycin, and streptomycin (72, 85, 132). Fluoroquinolones, including enrofloxacin and

danofloxacin, have been found to be effective against MG (71, 72). Antibiotics may be administered by water, feed, or subcutaneous injection (132). Some strains of MG have developed resistance to commonly used antibiotics.

Prevention. A series of rigid biosecurity measures are essential to prevent horizontal transmission. Flock caretakers and servicemen need to wear clean clothes and footwear when moving between flocks. Employees in contact with commercial turkeys should not own game birds or chickens. Contaminated litter should be hauled in covered trucks to avoid airborne transmission. Loading chutes and other equipment must be thoroughly cleaned before being moved from farm to farm. Livehaul trucks need to be cleaned and covered with netting to avoid dropping feathers and other debris from infected birds along the roads. Predators should not have access to dead carcasses. Rodent control is necessary to prevent rodents from moving from MG-infected houses onto neighboring premises (69).

Dipping turkey eggs in antibiotic solutions, injecting antibiotic directly into eggs, and heating turkey eggs have been used to reduce the incidence of vertical transmission. Egg treatments do not completely eliminate *M. gallisepticum* from turkey poults and are seldom used today. Dipping warm eggs in tylosin or erythromycin for 30 minutes proved to be of value. A temperature differential of 37° C between the eggs and a solution containing 800 ppm erythromycin yields the highest levels of antibiotic within the eggs (8). Inoculation of lincomycin-spectinomycin into the albumen ensures that a known volume of antibiotic enters the egg (72). Heating infected hatching eggs to 114° F within 12 hours will inactivate *M. gallisepticum* but hatchability will be reduced 8-12% (131).

Immunization. Mechanisms of acquired immunity against *M. gallisepticum* in poultry is not completely understood (124). Cell mediated immunity does not protect chickens from infection or development of air sac lesions following exposure to MG (79, 102). Barker reported that a complement-like substance may play a role in inactivation of MG by the immune system in laboratory animals (18). Resistance of chickens immunized against *M. gallisepticum* is mediated by bursal dependent lymphoid cells (80). Protection does not correlate with circulating antibody titers (117), but local immunoglobulin titers do correlate positively with immunity in chickens (128). Chickens infected with *M. gallisepticum* produce IgA, IgM, and IgG on mucosal surfaces of the respiratory tract (14, 128). Development of secretory antibodies in chicken tracheas is associated with a sharp decline in numbers of *M. gallisepticum* colonizing the trachea and a decline in tracheal lesions (128). Secretory immunoglobulins inhibit attachment of MG to chick tracheal rings in an organ culture system and protect against ciliostasis in a dose-dependent manner (14). Protection of chickens following immunization may be due to immunoglobulins on mucosal surfaces which prevent attachment of *M. gallisepticum* to epithelial cells on mucosal surfaces (80).

Vaccines consist of live *M. gallisepticum* with reduced virulence or killed organisms in bacterins (7). In most instances, the ability of *M. gallisepticum* to induce acquired immunity is directly proportional to its ability to cause disease (125). F strain is a naturally occurring isolate of MG that is commercially available for vaccinating chickens and displaces virulent field strains of MG in vaccinated layer flocks (57, 72). Unfortunately, F strain vaccine has been shown to reduce egg production by more than 5% in white leghorn chickens (26). F

strain has low transmissibility and transmission between birds diminishes with time (57). Also, F strain is fully virulent for turkeys and has been transported from vaccinated chicken flocks to turkeys resulting in severe disease (49, 57, 86).

The 6/85 vaccine strain of *M. gallisepticum* induces significant protection against air sac lesions when administered to chickens via aerosol at 2 weeks of age (49). Chickens vaccinated with this product exhibit significantly lower egg production loss than nonvaccinated control birds (50). Fortunately, the 6/85 strain has minimal virulence for chickens and turkeys (49). The 6/85 strain is genetically stable after passage in chickens and turkeys as indicated by consistent DNA fragmentation caused by several endonucleases and consistent protein patterns following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (51).

A temperature sensitive vaccine strain has been created by exposing a culture of *M. gallisepticum* to a chemical mutagen, nitrosoguanidine, and selecting a clone capable of growing at cooler temperatures found in the trachea (125). This live clone, called ts-11, has retained the ability to colonize the trachea of chickens and can stimulate a protective immune response (125). Temperature sensitive mutants grow 100 times more efficiently at the permissive temperature (33° C) than at the restrictive temperature (39.5° C) (124). Ts-11 vaccine administered by eye drop protects chickens against loss of egg production and air sac lesions and is nonpathogenic for turkeys (124).

Bacterins are available to the commercial poultry industry which can be used to immunize chickens or turkeys. One or two doses of MG bacterin in white leghorn chickens

have been found to protect against drops in egg production and to reduce transmission of *M. gallisepticum* via eggs (111). Immunization of turkeys with commercially available MG bacterins (7, 61) is possible but two doses are required to develop an effective immunity.

Since *M. gallisepticum* does not possess a cell wall, membrane proteins interact with host immune cells during infection and probably represent the antigens responsible for inducing immunity in chickens. Antibodies against protein p64 inhibit growth and attachment of MG to chick tracheal rings (13, 54). Future vaccines for control of *M. gallisepticum* in chickens and turkeys may consist of protein subunits, such as p64 or p98, (13, 47). However, variable expression of MG surface epitopes must be taken into account during production of vaccines and diagnostic antigens (55).

MATERIALS AND METHODS

Experimental Turkeys. Forty eight (48) Nicholas Large White male turkeys were purchased from a commercial flock and randomly assigned to one of 4 experimental groups (Frey's media-inoculated controls, *Mycoplasma gallisepticum* (R strain)-inoculated, *Mycoplasma synoviae*-inoculated, and *Mycoplasma meleagridis*-inoculated). Prior to inoculation at 46 days of age, each turkey was examined and found to be negative for pre-existing mycoplasma infections by tracheal culture and serology. Serological testing included both the serum plate agglutination and hemagglutination inhibition tests for *Mycoplasma gallisepticum*, *Mycoplasma meleagridis* (MM), and *Mycoplsma synoviae* (MS) . Tracheal cultures and serological tests were performed at the National Veterinary Services Laboratories in Ames, Iowa using protocols established and approved by the National Poultry Improvement Plan. Feed and water were provided *ad libitum*. Turkeys were euthanized by intravenous injection of sodium pentobarbital immediately prior to tissue collection.

Experimental Design. Tissues were collected at 1, 2, 3, and 4 weeks postinoculation (PI) as indicated in Table 1.

Inocula. *Mycoplasma gallisepticum*, *M. synoviae*, and *M. meleagridis* were propagated in Frey's medium for 24 hours at 37 °C to achieve a broth culture containing approximately 1 X 10^8 color change units/ml. Each mycoplasma-inoculated turkey received an injection of 0.2 ml of infectious broth culture into each infraorbital sinus and 0.25 ml was injected into the trachea. Turkeys in the control group received an injection of 0.2 ml of Frey's media into each infraorbital sinus and 0.25 ml was injected into the trachea.

Table 1. Collection of respiratory tissues from turkeys at 1, 2, 3, and 4 weeks following inoculation with Frey's media (C), *Mycoplasma gallisepticum* (MG), *Mycoplasma meleagridis* (MM), or *Mycoplsma synoviae* (MS).

Tissue Collection Times at Weeks Postinoculation	Experimental Groups			
	Control	MG	MS	MM
1	3*	3	3	3
2	3	3	3	3
3	3	3	3	3
4	3	3	3	3
Total	12	12	12	12

* Number of turkeys

Mycoplasma Isolation and Serology. To ensure that experimental birds became infected with the appropriate mycoplasma species and remain free of other mycoplasma, a serum sample was taken from each of the 48 experimental turkeys immediately prior to euthanasia. Each serum sample was tested for antibodies against MG, MS, and MM by the serum plate agglutination (SPA) and hemagglutination inhibition (HI) tests (97). Also, tracheas were collected from each turkey at necropsy and cultured in Frey's media.

Tissue Collection, Fixation, and Scoring. Respiratory tissues collected from each of the 48 experimental turkeys include the outer wall of the left and right infraorbital sinus, left and right lung, and tissue from the upper third and lower third of the trachea. Two different fixatives, 10% neutral buffered formalin and methyl pentanedial glycol (Excell, American Histology Reagent Company, Lodi, California 95241), were used to fix each tissue. Each tissue from each turkey was subdivided into four portions. One portion of each tissue was

fixed for 6 hours in 10% buffered formalin, one portion was fixed for 6 hours in methyl pentanedial glycol, one portion was fixed for 24 hours in 10% buffered formalin, and the remaining portion was fixed for 24 hours in methyl pentanedial glycol. Following fixation, tissues were transferred into 70% ethyl alcohol, processed, and embedded in paraffin within 48 hours. The intensity of staining was semiquantitatively measured by assigning a numerical staining score. Staining scores ranged from 0 to 3; 0 = no staining, 1 = mild staining with less than 30% of the cell population stained, 2 = moderate staining with less than 60% of the cell population stained, and 3 = strong staining with the majority of the cells stained.

Avidin-Biotin Immunoperoxidase Procedure. Sections 3μm thick were mounted on 3-aminopropyl-triethoxy-silane glass slides, dehydrated and deparaffinized in 2 changes (5 minutes each) of xylene and rehydrated through graded alcohol baths to ultrapure water. Antigen retrieval was accomplished by microwaving tissues in citrate buffer at pH 6.0 for 2.5 minutes at 800 watts followed by 10 minutes at 180 watts. Slides were cooled for 20 minutes in a freezer at -20°C, washed in phosphate buffered saline, and placed on slide racks in a humidified chamber. Avidin blocking (Zymed Laboratories, South San Francisco, CA) for 15 minutes was followed by washing in buffer, 15 minutes of biotin blocking (Zymed Laboratories, South San Francisco, CA), and another wash in the buffer solution. Tissues on each slides were incubated in 300 μl of 20% normal goat serum (Sigma Chemical Co., St. Louis, MO) for 15 minutes at room temperature and then washed in buffer. Next, tissues were exposed to 300μl of pooled primary rabbit anti-MG antibody (National Veterinary

Services Laboratories, Ames, Iowa) diluted 1:5000 with reagent diluent (BioGenex, San Ramon, CA) for 16 hours at 4° C in a humidified chamber. Each slide was washed three times in phosphate buffered saline. To inactivate endogenous peroxidase, specimens were subjected to three sequential 15 minute treatments with 300 μl 3% hydrogen peroxide (Fisher Scientific, Itasca, Il) with a buffer wash between each treatment and 3 buffer washes after the final exposure to hydrogen peroxide. Three hundred microliters of biotinylated goat anti-rabbit immunoglobulins (Vector Laboratories, Burlington, CA) diluted 1:200 with reagent diluent was applied to each slide for 15 minutes at room temperature. Three buffer washes was followed by a 15 minute exposure at room temperature to horse radish peroxidase labeled steptavidin (Zymed Laboratories, South San Francisco, CA) diluted 1:200. After an additional 3 washes in buffer, sections were allowed to react with 300 μl of freshly made 3-amino-9-ethylcarbazole (The New Polyscience, Warrington, PA) for 15 minutes at room temperature. Each tissue was washed 3 times in buffer. Slides were reassembled on a rack, counterstained for 60 seconds with 1/4 strength instant hematoxylin (Shandon, Pittsburgh, PA), washed 3 times in ultrapure water, and placed for 30 seconds in Scott's tap water. Next, 300 μl of SuperMount (BioGenex, San Ramon, CA) was applied to each slide and slides were placed in an oven at 58 °C for 1 hour. Finally, 150 μl of Refrax Mounting Medium (BioGenex, San Ramon, CA) was placed on each slide and slides were coverslipped.

Statistics. An analysis of variance (ANOVA) method, with the block effect of different weeks, provided an F test of the null hypothesis that the population means for different levels of each variable are identical. Variables tested consisted of treatment (Frey's media-

inoculated controls, *Mycoplasma gallisepticum* (R strain)-inoculated, *Mycoplasma synoviae*-inoculated, or *Mycoplasma meleagridis*-inoculated), fixative (10% neutral buffered formalin or methyl pentanedial glycol), fixation time (6 hours or 24 hours), and type of tissue (infraorbital sinus, trachea, or lung). The significance level used was 0.05 (110).

RESULTS

Clinical Signs and Macroscopic Lesions. By two weeks postinoculation, *M. gallisepticum*-inoculated turkeys began showing clinical signs of coughing and infraorbital sinus swelling. Gelatinous exudate was present both in the left and right infraorbital sinuses of the MG inoculated turkeys 2 weeks postinoculation and air sac had a light cloudiness. No clinical signs or macroscopic lesions were seen in the control, MM, or MS groups.

Mycoplasma Isolation. *M. gallisepticum* was re-isolated from tracheas of 100% of the turkeys inoculated with MG (Table 2). All turkeys inoculated with MS were culture-positive for *M. synoviae* except for one individual necropsied at 7 days postinoculation (Table 3). *Mycoplasma meleagridis* was not re-isolated from any turkeys inoculated with MM culture broth (Table 4).

Serology. All turkeys in the *M. gallisepticum* group were positive by the serum plate agglutination test (SPA). Antibody titers measured by the hemagglutination inhibition (HI) test ranged from 1:20 to 1:80 and were detected in all turkeys except for one bird necropsied at 7 days post inoculation (Table 2). In the *M. synoviae* group, no antibody response was detected at 1 week post inoculation. Thereafter, the humoral antibody response varied between turkeys, between weeks, and between serological tests. Hemagglutination-inhibition antibody titers were lower in MS turkeys than in turkeys inoculated with *M. gallisepticum* (Table 3). *Mycoplasma meleagridis*-inoculated turkeys showed inconsistent antibody responses with variability similar to that observed in the *M. synoviae* group (Table 4).

Avidin-biotin Immunoperoxidase Test. Results applying the avidin-biotin

immunoperoxidase test to tissues from turkeys infected with *M. gallisepticum* are shown in Table 5. The intensity of staining was semiquantitatively measured by assigning a numerical staining scores that ranged from 0 to 3 (Fig. 1, 2, 3, and 4). In most instances, staining of *M. gallisepticum* antigen was significantly more intense on infraorbital sinus epithelium than on respiratory epithelium from the trachea or lung ($P<0.05$; Table 5). Sinus mucosa from all six turkeys necropsied at the first week or fourth week postinoculation were positive by avidin-biotin immunoperoxidase staining. Tracheas showed variable results from negative (0) to strong (3) staining. One lung from one turkey at one week postinoculation and one lung from one turkey at three weeks postinoculation showed moderate staining on the epithelium adjacent to primary bronchi. Both of these lungs were fixed in 10% neutral buffered formalin.

When data from formalin and methyl pentanedial glycol fixation were analyzed by ANOVA, it was determined that the six hour fixation time offers better antigen preservation than 24 hours in a fixative ($P<0.05$). Statistical analysis indicated no difference in antigen staining between tissues fixed in methyl pentanedial glycol and tissues fixed in 10% neutral buffered formalin.

There was a difference in staining intensity between weeks in turkeys inoculated with *Mycoplasma gallisepticum*. The intensity of *M. gallisepticum* antigen staining on infraorbital sinus epithelium was greatest at the first and fourth weeks postinfection (Table 5). All turkeys tested at these times showed some degree of positive staining. In contrast, sinus tissues collected at the second and third week postinoculation showed variable results that

ranged from negative (0) to moderate (2) staining.

The specificity of the avidin-biotin immunoperoxidase test was not complete. The intensity of staining was significantly greater in tissues from turkeys inoculated with *M. gallisepticum* ($P<0.05$). None of the tissues from the *M. meleagridis* and control groups were stained. However, some staining was observed for *M. gallisepticum* antigen in the ciliated brush border of tracheal epithelial cells from turkeys inoculated with *M. synoviae*. No staining was detected on sinus epithelium from turkeys infected with *M. synoviae*.

Table 2. Serum plate agglutination reactions, hemagglutination inhibition titers, and reisolations at 1, 2, 3, and 4 weeks following inoculation of turkeys with *Mycoplasma gallisepticum*.

Weeks Postinoculation	Turkey Number	Serum Plate Agglutination	Hemagglutination Inhibition	Culture
1	1	+	-	+
	2	+	1:20	+
	3	+	1:20	+
2	4	+	1:80	+
	5	+	1:20	+
	6	+	1:40	+
3	7	+	1:80	+
	8	+	1:80	+
	9	+	1:80	+
4	10	+	1:80	+
	11	+	1:80	+
	12	+	1:40	+

Table 3. Serum plate agglutination reactions, hemagglutination inhibition titers, and reisolations at 1, 2, 3, and 4 weeks following inoculation of turkeys with *Mycoplasma synoviae.*

Weeks Postinoculation	Turkey Number	Serum Plate Agglutination	Hemagglutination Inhibition	Culture
1	1	-	-	-
	2	-	-	+
	3	-	-	+
2	4	+	1:20	+
	5	+	1:20	+
	6	+	-	+
3	7	+	1:20	+
	8	-	-	+
	9	-	-	+
4	10	+	-	+
	11	+	1:20	+
	12	+	1:40	+

Table 4. Serum plate agglutination reactions, hemagglutination inhibition titers, and reisolations at 1, 2, 3, and 4 weeks following inoculation of turkeys with *Mycoplasma meleagridis*.

Weeks Postinoculation	Turkey Number	Serum Plate Agglutination	Hemagglutination Inhibition	Culture
1	1	+	1:40	-
	2	+	1:40	-
	3	+	-	-
2	4	-	-	-
	5	-	-	-
	6	-	-	-
3	7	-	-	-
	8	-	-	-
	9	+	1:20	-
4	10	+	1:20	-
	11	+	1:20	-
	12	+	1:20	-

Table 5. Avidin-biotin immunoperoxidase staining for *mycoplasma gallisepticum* in turkey sinus, trachea, and lung at 1, 2, 3, & 4 weeks postinoculation following fixation in methyl pentanedial glycol (MPG) or formalin for 6 hours or 24 hours.

Weeks Postinoculation	Fixative-Time (hours)	Sinus	Trachea	Lung
1	MPG-6	2.8#*	2.2	0.0
	MPG-24	3.0*	2.2	0.0
	Formalin-6	2.0*	1.8	0.7
	Formalin-24	1.5*	2.2	0.3
2	MPG-6	0.8	1.0	0.0
	MPG-24	0.0	0.0	0.0
	Formalin-6	0.5	0.7	0.0
	Formalin-24	0.5*	0.7	0.0
3	MPG-6	1.7*	2.0	0.0
	MPG-24	0.0	0.7	0.0
	Formalin-6	0.8	1.0	0.3
	Formalin-24	0.5	0.7	0.0
4	MPG-6	2.8*	0.7	0.0
	MPG-24	1.5*	0.7	0.0
	Formalin-6	2.0*	0.3	0.0
	Formalin-24	1.5*	0.2	0.0

Mean staining scores from two observations on the sinus, lung, or trachea from each of 3 turkeys (n=6).

* Values within a row are significantly different ($P\langle 0.05$).

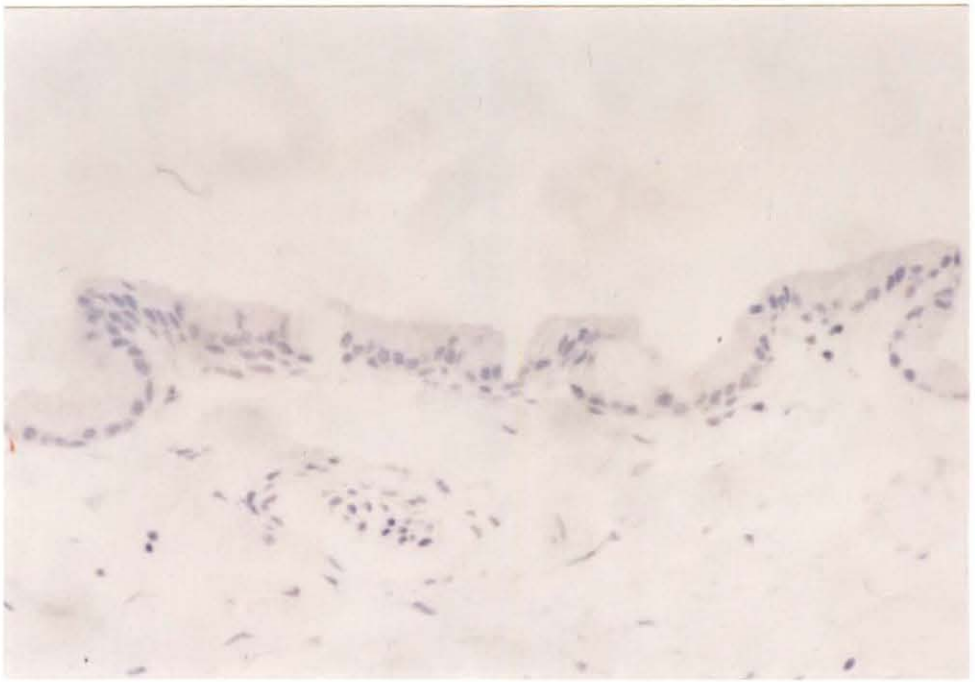
Table 6. Avidin-biotin immunoperoxidase staining for *mycoplasma gallisepticum* antigen on turkey sinus epithelium at 1, 2, 3, & 4 weeks postinoculation following 6 hours or 24 hours of fixation.

Weeks Postinoculation	Fixation Time	
	6 hours	24 hours
1	2.40*	2.33
2	0.67	0.00
3	1.25	0.27
4	2.36	1.50

* Mean staining scores of four observations from each of 3 turkeys on sinus epithelium fixed in formalin or methyl pentanedial glycol (n=12).

Fig. 1. Normal epithelium covering the mucosal surface of the infraorbital sinus from a turkey inoculated with Frey's media. No staining is present on the apical surface of respiratory epithelial cells which results in a staining score of 0. Magnification = 160X.

Fig. 2. Weak avidin-biotin immunoperoxidase staining of *M. gallisepticum* antigen in the ciliated brush border of infraorbital sinus surface epithelial cells is characteristic of tissues assigned a staining score of 1. Red-brown stain deposits are small and widely separated. Magnification = 160X.



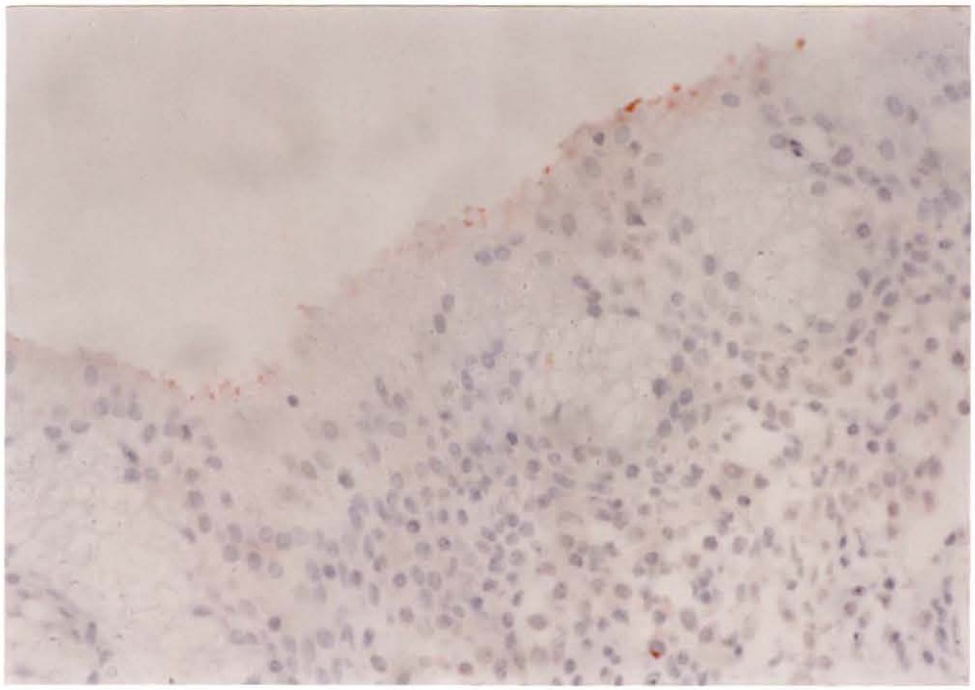
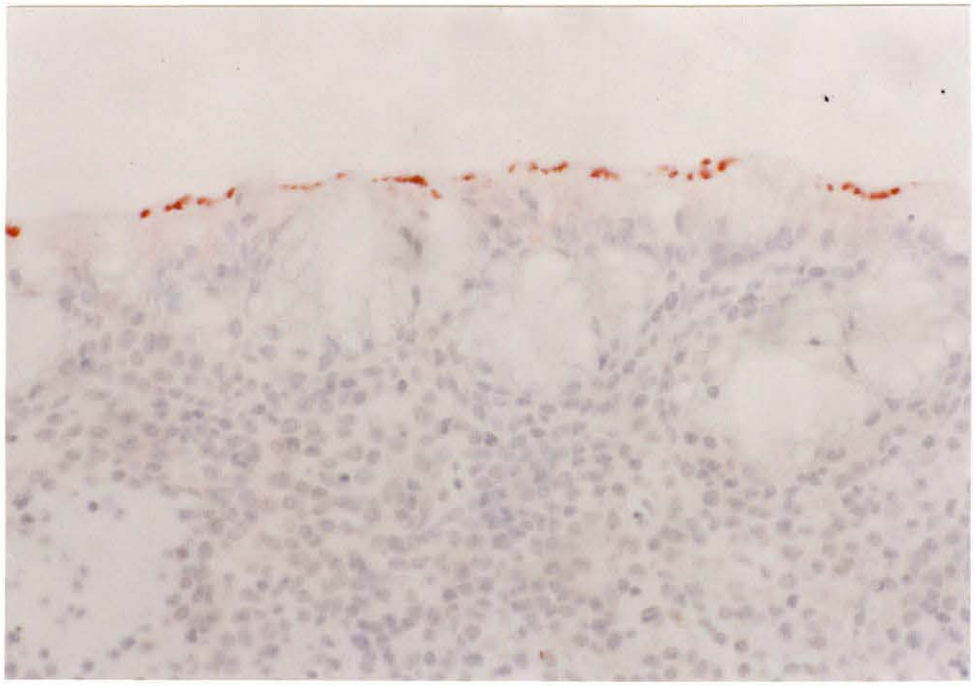
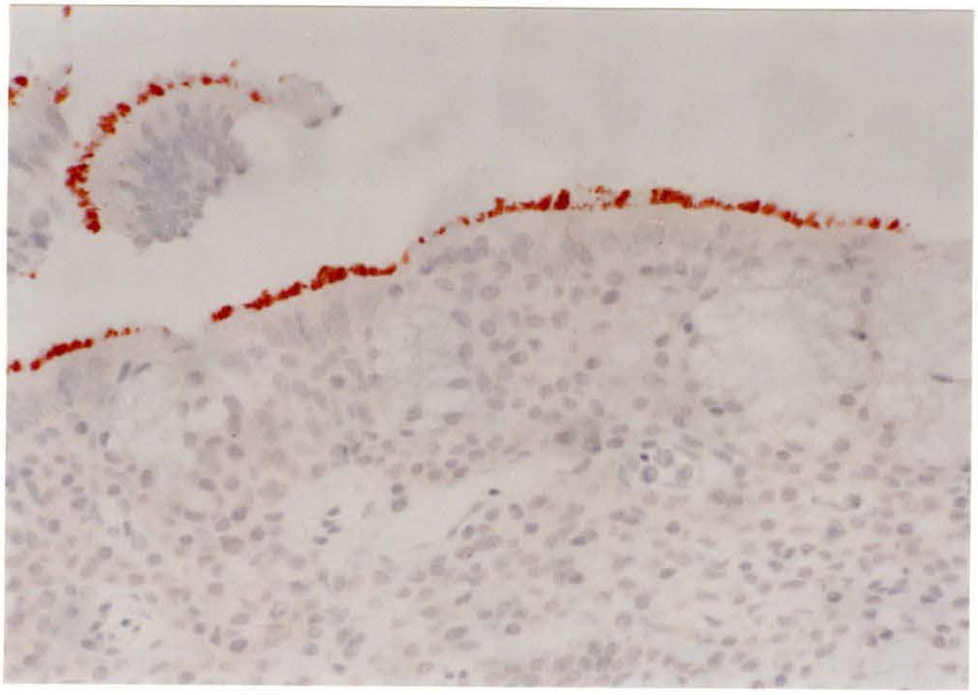


Fig. 3. Moderate avidin-biotin immunoperoxidase staining of *M. gallisepticum* antigen in the ciliated brush border of infraorbital sinus epithelial cells is representative of tissues assigned a staining score of 2. Red-brown stain deposits are of moderate size and nearly contiguous. Magnification = 160X.

Fig 4. Strong avidin-biotin immunoperoxidase staining of *M. gallisepticum* antigen in the ciliated brush border of infraorbital sinus epithelial cells is typical of tissues assigned a staining score of 3. Red-brown stain deposits are abundant, large, and contiguous. Magnification = 160X.





DISCUSSION

Polyclonal antibodies directed against *M. gallisepticum* were not as specific as desirable in a test intended to differentiate between common mycoplasma pathogens of turkeys. The use of polyclonal primary antibodies for immunoperoxidase staining of *Mycoplasma gallisepticum* antigens in histology sections is inexpensive, relatively fast, and easy to conduct. In addition, polyclonal antibodies will recognize more epitopes than a monoclonal antibody preparation. However, in the current study, a low level staining reaction was initiated by interaction between MG primary antibodies and *M. synoviae* on epithelial surfaces of the trachea but not on epithelial cells of the infraorbital sinus. It is possible that *M. synoviae* colonization is heavier in the trachea than in the infraorbital sinus. Cross reactivity between *M. gallisepticum* antigen and *M. synoviae* antibodies has been reported with ELISA testing (99). Bradley showed that 53 kDa and 88 kDa MS polypeptides cross reacted with MG antisera and a 70 kDa MG polypeptide cross reacted with MS antisera (25). In addition, *M. gallisepticum* and *M. synoviae* share 44 kDa and 36 kDa epitopes which react to antisera against MG and antisera against MS (2). *M. gallisepticum* and *M. synoviae* possess common antigenic determinants which are encoded in the genome of both organisms (134).

Infraorbital sinuses were the preferred site for *M. gallisepticum* colonization within the turkey respiratory tract. Infraorbital sinuses from all MG-inoculated turkeys showed positive staining by the avidin-biotin procedure. Furthermore, intensity of staining was significantly greater in the infraorbital sinuses than in the trachea or lungs. This finding is consistent with

previous reports that lesions of *Mycoplasma gallisepticum* may be restricted to the swelling of the infraorbital sinuses (127). The micro-environment within the sinuses apparently provide an ideal environment colonization by *M. gallisepticum*. The apical surface of infraorbital sinus epithelium would appear to be the preferred site for utilization of diagnostic procedures designed to demonstrate MG antigens or genetic material in infected turkeys.

Fixation for 6 hours in either formalin or methyl pentanedial glycol resulted in more intense avidin-biotin staining for *M. gallisepticum* than fixation for 24 hours. Morphology of respiratory epithelial cells on mucosal surfaces was well preserved following 6 hours in either fixative. Optimal preservation of antigens in tissue sections requires that the duration of fixation be limited to the minimum time required to preserve tissue morphology. Time required for complete fixation depends on the rate of fixative penetration and the action of the fixative. Fixation activity by formalin progressively improves after a tissue has been completely penetrated (66). Approximately one half of formaldehyde uptake by tissue occurs in 8 hours but fixation may not be completed until 7 days (29). The rate of penetration and mechanism of action of methyl pentanedial glycol is not known.

Three modifications to the standard avidin-biotin immunoperoxidase procedure were necessary to successfully stain *M. gallisepticum* adhering to epithelial cells in the respiratory tract of turkeys. Non-specific staining associated with mucus glands was observed in both the tracheal and infraorbital sinuses. Use of avidin-biotin blockers successfully removed the non-specific staining from the mucosal surfaces of the respiratory tract. This finding is consistent with Cauli's observation of endogenous avidin binding activity in ducts of human

salivary glands (30). Endogenous peroxidase activity was suppressed by use of hydrogen peroxide at three different steps in the procedure. Erythrocytes contain pseudoperoxidase and myeloperoxidase is present in granulocytes (46). In avian species, eosinophils have peroxidase activity but heterophils do not (9, 91). Antigen retrieval by microwave heating of tissue sections increased the intensity of *M. gallisepticum* antigen staining in turkey respiratory tissues fixed in formalin or in methyl pentanedial glycol. Microwave heating is believed to disrupt cross-linking of proteins caused by tissue fixatives and, in some tissues, may eliminate the need for pre-digestion by protease enzymes (114).

Mycoplasma gallisepticum and *M. synoviae* were readily reisolated from the tracheas of inoculated turkeys. This was not the case with *Mycoplasma meleagridis* which was not reisolated from any of the inoculated turkeys. Many isolates of *M. meleagridis* grow poorly or not at all in broth (48, 130). However, antibodies against MM were detected by both the serum plate agglutination test and the hemagglutination-inhibition test at 7, 21, and 28 days postinoculation indicating that infection did occur.

CONCLUSION

An avidin-biotin immunoperoxidase diagnostic test was developed to facilitate rapid identification of *Mycoplasma gallisepticum* in respiratory tissues of turkeys. This procedure used polyclonal primary antibodies produced in rabbits. Forty eight turkeys were randomly assigned to one of four experimental groups of turkeys and inoculated into the infraorbital sinus and trachea with the R strain of *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma meleagridis,* or Frey's media. The outer wall of the infraorbital sinuses, lungs, and tracheas were collected and fixed in either 10% neutral formalin or pentanedial methyl glycol at 1, 2, 3, 4 weeks postinoculation. Tissues were subdivided and remained in each fixative for 6 or 24 hours.

The avidin-biotin immunoperoxidase diagnostic test was sufficiently sensitive to detect *M. gallisepticum* antigen at 1, 2, 3, 4 weeks postinoculation. Staining of *M. gallisepticum* was significantly more intense on infraorbital sinus epithelium than on respiratory epithelium from the trachea or lung. Statistical analysis indicates that the six hour fixation time offers better antigen preservation than 24 hours in a fixative. There was no difference in intensity of *M. gallisepticum* antigen staining in tissues fixed in methyl pentanedial glycol when compared to tissues fixed in 10% neutral buffered formalin. Significant differences in staining intensity were observed between weeks.

Specificity of the avidin-biotin immunoperoxidase test was not complete. None of the tissues from the *M. meleagridis* and control groups showed staining. No staining was observed in the ciliated brush border of infraorbital sinus epithelial cells from turkeys

infected with *Mycoplasma synoviae*. However, weak to moderate staining was observed in several tracheas of turkeys inoculated with *M. synoviae*. Improved specificity of an avidin-biotin immunoperoxidase diagnostic test to detect *M. gallisepticum* in respiratory tissues of turkeys probably will require the use of monoclonal antibodies directed against a specific epitope in the cell membrane of *Mycoplasma gallisepticum*.

APPENDIX A: FREY'S MEDIUM FOR AVIAN MYCOPLASMA ISOLATION

Mycoplasma broth base	22.5 g
Dextrose	3.00 g
Swine serum	120.0 ml
Cysteine hydrochloride	0.1 g
Nicotinamide adenine dinucleotide (NAD)	0.1 g
Phenol red (1%)	2.5 ml
Thallium acetate (10%)	5.0 ml
Penicillin G potassium	1,000,000 units
Distilled water	SQ 1000.0 ml

Adjust pH to 7.8 with 20% NaOH, and filter-sterilize

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APPENDIX B: IMMUNOHISTOCHEMISTRY SOLUTIONS

1. OptiMax Wash Buffer (20X) (500ml)

   500 ml 20X concentrated phosphate buffer saline at pH 7.4± 0.2 with surfactant

   10 L deionized water

   Label, date, initial, and refrigerate at 4 °C

2. Concentrated Reagent Diluent (reagent diluent)(100ml)

   Phosphate buffer saline at pH 7.6

   1% Bovine Serum Albumin

   0.1 % Tween-20

   0.09% Sodium azide

   Stable for 18 months at 2-8 °C

3. 3% Hydrogen peroxide

   10 ml 30% H_2O_2

   90 ml ultrapure water

4. Sodium Acetate Buffer (good for 6 months)

   210 ml 0.10N Acetic acid (5.75 ml GAA/1L H_2O)

   790 ml 0.10M Sodium acetate.$3H_2O$ (13.61 g/ 1 L H_2O)

5. Working AEC (3-amino-9-ethylcarbazole) (make fresh) (filter with plunger)

   2 ml Sodium acetate buffer

   3 drops AEC

   3 drops 1% hydrogen peroxide

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