An avidin-biotin enhanced dot-immunobinding assay for the detection of serum antibodies to avian mycoplasmas

by

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GENERAL INTRODUCTION

The poultry industry in Europe and North America has steadily grown in accordance with consumer demand. In 1986, the world production of poultry products totaled 33 million metric tons of meat and 31 million tons of eggs. Europe and North America accounted for approximately 65% of that production. In that same year, per capita consumption of turkey and broiler meat in the United States exceeded that of beef for the first time (Shane, 1988). In order to meet this expanding market demand, the industry has responded by intensifying its production. Because of production practices which are commonly used in the poultry industry, large numbers of birds may be placed in high risk situations if the introduction of infectious agents such as mycoplasmas were to occur. Monitoring for the presence or past occurrence of disease agents is of paramount importance for effective and efficient disease control. For these reasons, the development of more sensitive and specific diagnostic methods would greatly benefit the poultry industry.

The purpose of this study was to develop an alternative serologic assay for detecting antibodies to avian mycoplasmas and to evaluate the proposed assay when used under commercial conditions.

Explanation of Thesis Format

As the findings of this work are to be submitted for publication, the alternate thesis format is being used. Following a literature review, two manuscripts are presented as separate sections. The first manuscript describes the development of an avidin-biotin enhanced dot-immunobinding (DAB) assay for the detection of serum antibodies to Mycoplasma gallisepticum and M. synoviae. The second manuscript documents the utilization of the DAB assay to monitor flocks of commercial turkeys for four species of avian Mycoplasma. A general summary of the entire thesis follows this section.

LITERATURE REVIEW

In 1898, Nocard and Roux (1898) isolated and characterized an organism from cattle with contagious bovine pleuropneumonia; the organism later was recognized as the first mycoplasma cultivated and described. Organisms with similar properties became known as PPLOs, pleuropneumonia-like organisms (Couch, 1985; Freeman, 1985; Gillespie and Timoney, 1981). These organisms were isolated from several species of animals and became recognized as the causes of a variety of diseases including mastitis, synovitis, respiratory disease, contagious agalactia, and conjunctivitis (Gillespie and Timoney, 1981). Because of their small size, the organisms were initially assumed to be viruses (Couch, 1985). Later, investigators discovered that eubacteria could give rise to "L-forms", bacteria which can be induced to survive without synthesizing a normal cell wall, and therefore have a structure and character similar to the PPLOs (Stanier et al., 1963). PPLOs later came to be called mycoplasmas, an informal and collective term for species in the class Mollicutes (Freeman, 1985; Razin, 1983). Minimum standards for the classification of organisms as Mollicutes were established by the Subcommittee on Taxonomy of Mollicutes in 1979 (International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of

Mollicutes, 1979). To date, a genetic relationship has not been established between L-forms and mycoplasmas (Jawetz et al., 1984).

Mycoplasmas are the smallest of the procaryotes (Razin and Freundt, 1984), are distinct from other bacteria in their inability to synthesize a cell wall and differ from viruses in their ability to grow on cell-free media (Couch, 1985; Razin and Freundt, 1984). The trilaminar membrane morphology of the mycoplasmas permits diverse pleomorphism (Freeman, 1985; Razin, 1983; Razin and Freundt, 1984). Mycoplasmal morphology varies with the culture techniques used and methods of examination (Razin and Freundt, 1984). Morphologic forms described include spheres, filaments, rings, branching or helical shapes, globules, and spirals (Freeman, 1985). On agar media, mycoplasma colonies tend to grow beneath the surface of the medium, resulting in a "fried egg" appearance (Freeman, 1985; Jawetz et al., 1984, Razin and Freundt, 1984). The lack of a cell wall contributes to the organism's sensitivity to lysis, its resistance to some antibiotics (e.g., penicillin), and its ability to pass through a 0.45um membrane filter (Freeman, 1985). The cells are small and vary in size from 50 to 300nm (Jawetz et al., 1984). They contain a genome of 0.5-1.0 x 10^9 daltons (Razin and Freundt, 1984), which appears

to be the minimum required for growth and reproduction (Razin, 1983). Replication processes that have been reported are binary fission (Razin, 1983), fragmentation (Couch, 1985), and budding (Couch, 1985; Razin, 1983). Motility has been reported for some species but most are nonmotile without resting stages. Mycoplasmas are facultatively anaerobic except for the obligate anaerobes which belong to the genus Anaeroplasmas.

Mollicutes

Three families have been established in the Class Mollicutes (Stanier et al., 1986). They are parasitic, commensal, or saprophytic and many of the species in these families are pathogenic for man, animals, plants, and insects. All require sterols for growth except for the genus Acholeplasma and some Anaeroplasma spp.

Family I Mycoplasmataceae has the smallest genome of 5 x 10^8 daltons, has the nicotinamide adenine dinucleotide (NADH) oxidase located in the cytoplasm, and contains two genera: Mycoplasma and Ureaplasma. The Mycoplasma may utilize carbohydrates and/or arginine as growth substrates and is urease negative whereas Ureaplasma is distinguished by its ability to utilize urea. Natural hosts include humans, animals, insects, and plants.

Family II Acholeplasmataceae, which does not require sterols for growth, has a genome of 1 x 10^9 daltons, with NADH oxidase localized in the membrane, and contains only one genus: Acholeplasma. The natural hosts include humans and animals.

Family III Spiroplasmataceae also supports a genome of 1.0 x 10^9 daltons. The NADH oxidase is found in the cytoplasm. The distinguishing properties of this family and its single genus Spiroplasma, are that these organisms are helical at some stage of development and may exhibit a swimming motility. Natural hosts include arthropods and plants.

The genus Anaeroplasma has not been assigned to a family. It may or may not require sterols for growth, utilizes carbohydrates, and exhibits a coccoid morphology. They are strict anaerobes and have been isolated from the rumens of cattle and sheep (Razin and Freundt, 1984; Stanier et al., 1986).

Species characterization usually depends upon biochemical and serological assays (Jawetz et al., 1984; Razin, 1983). Biochemical assays include the ability to ferment glucose and other carbohydrates, to hydrolyze arginine and urea, and to produce pigments (Razin and Freundt, 1984). Serological assays include growth

inhibition and immunofluorescence. The following techniques have been used to further characterize mycoplasma: DNA homology, electrophoresis of cell proteins, demonstration of phosphatase and proteolytic activity, detection of film and spot production, hemolysis and hemadsorption, metabolic inhibition tests, complement fixation test, and agglutination tests. Guanine plus cytosine content of the DNA has been determined for most species and is used for proper species description (International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of Mollicutes, 1979; Razin, 1983).

Mycoplasma

Seventy-eight different named species of the genus Mycoplasma have been reported (Bradbury and Forrest, 1984; Bradbury et al., 1983; Forrest and Bradbury, 1984; Freeman, 1985; Razin and Freundt, 1984). These species are distinguished by morphology, motility, nutritional requirements, and biochemical and other physiological properties (Freeman, 1985). Final classification is dependent on serological techniques; the most common are growth inhibition, immunofluorescence and metabolic inhibition assays.

Some species names of mycoplasmas bear the geographical locale (e.g., M. californicum and M. iowae) from which they

were first isolated. Other species names are descriptive of a particular disease condition or reaction such as M. agalactiae. Many mycoplasma species are host specific such as M. moatsi for the grivet monkey or M. molare for the dog. Some species, such as M. arginini, are found in a variety of hosts (sheep, goat, cattle). Several species, including M. genitalium, or M. gallisepticum, have specialized structures which may be involved in attaching to host cells (Razin and Freundt, 1984).

Mycoplasmas are widely distributed throughout the animal world. They have been isolated as commensals, saprophytes and parasites. Several are pathogenic for humans and animals. Mycoplasmas have been frequently found on animal mucous membranes, especially the respiratory and genital tracts and the synovial membranes of joint capsules (Stanier et al., 1986). A preference for the mesothelial cells of these membranes has been reported, e.g., pleura, peritoneum and synovial cells (Jawetz et al., 1984). Jawetz et al. (1984) observed that the parasitic mycoplasmas were host specific.

Those mycoplasmas that have been isolated from avian species are listed in Table 1 (Bradbury and Forrest, 1984; Bradbury et al., 1983; Forrest and Bradbury, 1984; Razin and Freundt, 1984; Yoder, 1984a).

Table 1. Mycoplasmas isolated from avian sources

SERO-TYPE	SPECIES	PATHOGENICITY	CHARACTERIZATION Substrate[a] GLU	ARG	Other	USUAL SOURCE
A	M. gallisepticum	airsacculitis, sinusitis	+	-	adsorbs RBCs "bleb" structure	chicken, turkey
B,M	M. gallinarum	mild airsacculitis	-	+	common parasite, film and spot	chicken
C,O	M. pullorum	mild airsacculitis	+	-		chicken
D,P	M. gallinaceum	abscess in chick embryos	+	-		chicken
E,G	M. iners	abscess in chick embryos	-	+	film and spot	chicken
F	M. gallopavonis	not known	+	-	agglutinates RBCs to high titers	chicken, turkey
H	M. meleagridis	airsacculitis	-	+		turkey
I,J,N Q,R	M. iowae	lethal to embryos	+	+		chicken, turkey

[a]Ability to hydrolyze glucose and/or arginine.

Table 1. (continued)

SERO-TYPE	SPECIES	PATHOGENICITY	CHARACTERIZATION Substrate GLU	ARG	Other	USUAL SOURCE
K	Unnamed	not known	+	-	adsorbs RBCs	
L	M. columbinasale	not known	-	+	film and spot	pigeon
S	M. synoviae	airsacculitis, synovitis	+	-	NAD required, film and spot, adsorbs RBCs	chicken turkey
-	M. columbinum	not known	-	+	film and spot	pigeon
-	M. columborale	not known	+	-	oral parasite	pigeon
-	M. anatis	not known	+	-	film and spot	duck
-	M. cloacale	not known	-	+		wildlife
-	M. lipofaciens	not known	+	+	film and spot	chicken, turkey
-	M. glycophilum	not known	+	-	adsorbs turkey RBCs	

Avian Mycoplasmas

Over twenty serotypes of mycoplasma have been characterized from poultry, including isolates from chickens, turkeys, pigeons, and ducks. Early designations represented by alphabetical letters consisted of serotypes A through S. Based on further studies, these serotypes were combined into 10 serogroups. Most of these serogroups have been given names (Yoder, 1984a). Later, new Mycoplasma species were reported (Bradbury and Forrest, 1984; Bradbury et al., 1983; Forrest and Bradbury, 1984).

Serologic procedures, e.g., agglutination, hemagglutination-inhibition, and complement fixation, are the most common techniques used to identify avian mycoplasma isolates. Other procedures include growth inhibition, electrophoresis, agar gel immunodiffusion and immunofluorescent tests (Yoder, 1984a).

The avian mycoplasmas require media containing 10-15% animal serum and a yeast extract or yeast autolysate for growth. In addition, M. synoviae requires reduced nicotinamide adenine dinucleotide. The optimum growth temperature is 37-39^{o} C (Yoder, 1984a).

Several factors may determine the susceptibility of the avian host to infection. Those reported include tissue tropism, infecting dose, route of infection, concurrent

infection, environmental conditions, and the species, age, genetic makeup, and immune status of the host (Jordan, 1981).

The serotypes considered to be pathogenic for certain avian species and to be of significant economic importance include M. gallisepticum (MG), M. meleagridis (MM), and M. synoviae (MS) (Yoder, 1984a). M. gallisepticum has been isolated from several avian species but appears to be pathogenic only for chickens and turkeys. M. meleagridis infects turkeys, and MS infections have been more common in chickens than in turkeys. Young birds have been more susceptible than mature birds; embryos have become infected while older poults have been less susceptible (Jordan, 1981). Recently M. iowae has been recognized as a pathogen in turkeys (Jordan, 1985). Concurrent infections with organisms such as Newcastle disease virus, Escherichia coli, and infectious bronchitis virus may enhance or diminish the virulence of the invading mycoplasma. Diagnosis of avian mycoplasma disease has been dependent on isolation or demonstration of specific antibodies in the sera of infected birds (Jordan, 1981).

Mycoplasma gallisepticum

M. gallisepticum is the cause of chronic respiratory disease in chickens and infectious sinusitis in turkeys

(Yoder, 1984a). Different strains of MG vary in the degree and type of clinical signs and pathological lesions manifested by the host. For example, strain S6 can cause nervous signs and lesions in chickens and turkeys; strain A574 can cause unilateral enlargement of the eye (Jordan, 1981). Clinical signs associated with respiratory infections have included rales, coughing, sneezing, nasal discharge, and swelling of sinuses (Jordan, 1981). Clinical manifestation of disease may be slow to develop. Severe airsacculitis is usually the result of MG infection in conjunction with other infectious agents (Yoder, 1984b).

The organism has been isolated world wide. Transmission is often accomplished by contaminated oviducts and semen; MG can also be spread in aerosols of dust or water vapor. Direct contact with infected chickens or turkeys causes outbreaks in susceptible birds. The incubation period is from 6 to 21 days. Infection and antibody production can occur without affected birds exhibiting clinical signs. Outbreaks in broiler flocks at 4 to 8 weeks of age are usually associated with concurrent infections. Once introduced, MG infects almost all chickens or turkeys in a flock. Birds that recover from clinical disease can become carriers and transmit the disease by contact or egg transmission. Economic losses attributed to

MG infection have included the downgrading of carcasses; decrease in egg production, hatchability, and viability of chicks; and increased medication costs (Yoder, 1984b). Trauma, adverse environmental conditions (ammonia, dust, and cold) and social stress have been associated with increased severity of disease (Jordan, 1981).

Strict sanitation, isolation and serological monitoring of flocks are management procedures used to control and eliminate MG in poultry. Reactor turkey flocks are eliminated for breeding purposes; chicken breeding stock are immunized and medicated. Immunization has limited value and medication produces variable results. Antibiotics or clinical treatment may not be cost effective. Although egg dipping does reduce transmission, it is detrimental to hatching and may promote bacterial infection. However, this procedure has made possible the rearing of chicken and turkey flocks free of MG (Yoder, 1984b).

Mycoplasma iowae

Certain strains of M. iowae have caused a decrease in the hatchability of chicken and turkey eggs (Jordan, 1981). The organism produces lethal infections in turkey embryos that have been inoculated via the yolk sac. The air sac lesions are similar to those produced in turkeys infected with MG, MM, and MS (Rhoades, 1980). Bradbury and McCarthy

(1983) found variation among MI strains in their ability to reduce hatchability of chicken eggs. The majority of embryonic deaths occurred on or after 19 days of incubation. Congestion and stunting of the embryos were the most prominent abnormalities; mustard colored livers were frequently observed (Bradbury and McCarthy, 1983). Experimental studies have suggested that M. iowae infections can cause a temporary immunosuppression in poults (Bradbury, 1984).

The protein electrophoretic patterns of 21 strains, representing 6 serovars of MI, were compared. The patterns of the strains were similar except for strain 1805 (serovar K). These results suggested that this serovar may not be related to the other strains of MI. Agglutination tests using the same MI strains demonstrated antigenic variation within the species (Rhoades, 1984). Other investigators have found variation in electrophoresis, growth inhibition, and immunofluorescence results when attempting to identify organisms thought to be MI (Zhao et al., 1987).

Routes of infection were compared. One-day-old poults, which had been inoculated in ovo, were compared to poults which had been inoculated orally, via the thoracic air sac, or via the foot pad. The MI organism was most widely disseminated in the tissues of the birds which were

inoculated in ovo and the least disseminated in tissues of those birds inoculated orally. The in ovo route of inoculation produced a severe generalized disease with high mortality, whereas those birds inoculated via the air sac and foot pad developed poor feathering and chondrodystrophy. The organism could not be recovered from the birds at 12 weeks of age. Agglutinating serum antibodies to M. iowae were not detected (Bradbury et al., 1988).

Although MI has been reported to be pathogenic in both chickens and turkeys, MI infections have not been of commercial importance in chickens. There have been only a few clinical signs of disease observed in experimentally infected 1-day old chicks; exudative tenosynovitis was the most common finding (Bradbury and McCarthy, 1984).

Mycoplasma meleagridis

M. meleagridis has been isolated worldwide in turkeys. It has caused airsacculitis in progeny (Yamamoto, 1984), but infected birds rarely exhibit respiratory signs (Jordan, 1981). Skeletal abnormalities have occurred but have not been a consistent feature in adult birds. Infected poults have frequently exhibited stunting, perosis, and crooked necks. Primary wing feathers have developed at right angles to the body.

The organism has been isolated from the cloaca and bursa of Fabricius in young birds, but is found in the oviduct or phallus of mature birds (Jordan, 1981). High infectivity and low mortality have been reported.

M. meleagridis is primarily a vertically transmitted disease. Artificial insemination with infected semen plays a major role in sustaining egg transmitted rates of infection. Horizontal (airborne) transmission may result in up to 100% infection. Problems develop during the first 12 weeks of age; the greatest incidence occurs during the interval between the first and sixth weeks. Maternal antibodies can be detected in poults from infected hens. Continued exposure to MM can result in immune tolerance. The active immune response in infected birds will eliminate the organism when the source of infection is eliminated (Yamamoto, 1984).

Economic losses have been attributed to reduced hatchability and poor growth performance (Yamamoto, 1984). Exposure to ammonia, dust, and cold stress have been associated with increased severity of MM disease (Jordan, 1981). Secondary infections and the strain of MM involved have also been contributing factors. MM has been found to act synergistically with MI and MS, increasing the severity of disease (Rhoades, 1981).

Dipping of eggs in antibiotic solutions and the injection of antibiotics into eggs have been successful methods of eliminating MM in poults, however, strict sanitary precautions must be taken during collection of semen, insemination of hens, and the sexing of poults.

Mycoplasma synoviae

M. synoviae has been isolated worldwide and has been involved in upper respiratory infections and infectious synovitis of poultry. The natural hosts are chickens, turkeys, and guinea fowl (Olson, 1984).

An acute generalized disease usually occurs in chickens at 4-16 weeks and turkeys at 10-20 weeks of age. The acute disease occurs occasionally in adult birds. Chronic infection, affecting the joints or respiratory tract, may follow the acute disease and may persist five years or more (Jordan, 1981; Olson, 1984). Clinical signs in the chicken include pale comb, lameness, retarded growth, ruffled feathers, and/or shrinking combs which may have become bluish red. Chickens also exhibit swollen joints, sternal bursitis and may be listless, dehydrated and emaciated. Turkeys predominately exhibit lameness, weight loss, and poor growth. Respiratory symptoms are usually absent in infected turkeys. Morbidity and mortality in chickens may be low. Morbidity in turkeys is low, however, significant

mortality may result because of birds trampling each other and from cannibalism (Olson, 1984). Suboptimal environmental temperatures have been associated with acute infection (Jordan, 1981).

Vertical transmission is the primary route of spreading the disease. Transmission by direct contact usually results in 100% infection but with fewer joint lesions (Olson, 1984).

Isolation and identification may be easily accomplished from acute cases, but is extremely difficult from chronically ill birds. Diagnosis of MS is dependent on isolation or serology. Two to 4 weeks are required for the development of agglutinating antibodies following exposure to MS. Turkeys with respiratory infections produce low levels of antibodies (Olson, 1984).

Prevention, using MS free flocks, is the most effective method of control. Antibiotic treatment of breeders is not effective. Since serologic tests for turkeys are not always reliable, tracheae are also cultured as part of surveillance programs (Olson, 1984).

Serologic Nonspecificity

Nonspecific or false positive reactions have long been problems in avian mycoplasma serology. This causes

considerable concern to those individuals evaluating the status of flocks in regards to mycoplasma infection.

In 1960, Hall demonstrated that a rapid serum plate (RSP) test was a reliable screening assay. This report was based on the results of a two-year effort to eradicate infectious sinusitis from a turkey breeder-hatchery in Texas. Occasional false positives occurred in the serum samples obtained from the 150,000 birds tested (Hall et al., 1960). Newnham (1964) reported that a detailed history of a flock was needed to interpret the significance of low hemagglutination-inhibition (HI) titers detected in sera from any given flock. Using a HI test with less than four hemagglutination (HA) units resulted in nonspecific reactions. Newnham (1964) recommended an 8-unit HA test be used for chickens and a 4-unit HA test be used for turkeys. Thornton (1969) compared the RSP and HI tests for MG and concluded that the RSP was the more sensitive test. He also concluded that an 8-unit HA antigen test was more reliable than a 4-unit HA antigen test and found that turkey serum contained less nonspecific activity than chicken serum. A higher percentage of MS positive sera which cross reacted with MG on the RSP occurred more often in sera obtained from mature birds than in sera obtained from younger birds (Thornton, 1969). A higher frequency of MG-MS cross

reactions was observed in 1975 than in 1957. Positive HI titers were also lower in younger birds than in older birds (Weinack and Snoeyenbos, 1975).

The culture that is used to produce antigens can be a factor in serologic variance. Serologic responses in chickens infected with three different strains of MG indicated that the RSP was more mycoplasma species specific and the HI was more strain specific (Roberts, 1969). MG cultures grown in media containing swine serum were found to lose their HA titers after continued passage (Bradbury and Jordan, 1972). HI titers varied, depending on the MS strain used for the HA antigen (Vardaman and Drott, 1980; Weinack and Snoeyenbos, 1975). MS was reported to exist as a mixed population of HA and non-HA cells. A shift in the relative population may account for changes in the HA activity of a culture (Rhoades, 1985). Glisson et al. (1983) reported the specificity and sensitivity to MG and MS differed depending on the antigen used for testing (two commercial antigens and an antigen the authors prepared were compared).

The process utilized for antigen production can have a significant influence on specificity. Antigenic components of the medium used to cultivate MG contributed to nonspecific reactions when the organisms were used as an inoculum for antiserum production or as an antigen in

serological tests (Jordan and Kulasegaram, 1968). Swine serum proteins in the growth medium adsorbed to viable and nonviable cells at low pH values and were not removed after several washings. In addition, serum protein had a blocking effect on the HA activity (Bradbury and Jordan, 1972).

In a plate antigen study, two variables affected the sensitivity: antigen concentration and dextrose in the cultivating medium. Antigen concentrated to USDA specifications produced more false positive reactions than a less concentrated antigen. Dextrose in the medium increased the growth rate but produced more false positive reactions on the RSP test when testing turkey sera than a medium without dextrose (Thorpe, 1970). An antigen that is too dilute or too concentrated gave misleading results (Crawley, 1960).

Other disease agents also affect the specificity of the mycoplasma tests. Low transient HI antibody titers were reported in adult flocks which were infected with Newcastle disease virus or infectious bronchitis virus (Crawley and Fahey, 1957). Serum from birds infected with M. synoviae, Erysipelothrix rhusiopathiae, and avian mycoplasma serotype P reacted with the MG RSP antigen. The nonspecific activity appeared to be associated with a rheumatoid-like factor which was adsorbed on the organism during cultivation. This

rheumatoid-like factor was induced in the chickens by infection with MS, E. rhusiopathiae, or mycoplasma serotype P (Roberts and Olesiuk, 1967). Transient reactions in the RSP may occur in serum from chickens vaccinated with commercial, inactivated products for avian encephalomyelitis, Newcastle disease and infectious bronchitis (Vardaman and Yoder, 1971; Roberts, 1970). These nonspecific reactions were associated with anti-gamma-globulin that could be detected in the serum (Roberts, 1970). Staphylococcus or Streptococcus infection stimulated the production of rheumatoid-like antiglobulins which react with MG antigen (Thornton, 1973). It was also found that vaccines produced with medium containing serum components were more involved in producing nonspecific reactions than those produced with serum-free medium (Glisson et al., 1983). Sera from birds inoculated with media containing swine serum produced nonspecific reactions (Wright and Meneely, 1972).

Improper storage of test serum can result in false RSP reactions. Freezing of serum (Thornton, 1969) or prolonged storage (Sahu and Olson, 1975; Wright and Meneely, 1972) were reported to increase nonspecificity.

Methods were devised to remove nonspecific factors. The nonspecific activity in sera previously frozen was

thermolabile at 56 degrees C (Thornton, 1969). Nonspecific reactions in the RSP test were removed by diluting the sera with an equal volume of treated guinea pig serum (Wright and Meneely, 1972). A preparation of MG antigen containing fifteen percent horse serum inhibited the nonspecific agglutination of chicken sera containing antibodies directed against Staphylococcus aureus and the following proteins: myxilin ET, horse protein, pig globulin, and pig albumen (Thornton, 1973). Nonspecific reactivity was removed by diluting sera used in the RSP test or by inactivating sera used in the HI test (Sahu and Olson, 1975). Trypsin was used to remove or neutralize components of antigen associated with nonspecific reactions that occurred in the MG RSP test (Newman and Simonson, 1984).

The antigenic variation between different strains of M. iowae presents an additional problem in serodiagnosing infection (Rhoades, 1984).

Other testing procedures have been suggested. Sahu and Olson (1975) suggested the use of agar gel precipitation and HI as well as RSP to evaluate MS suspected flocks. A neotetrazolium stained microagglutination test antigen was sensitive and specific for MG and MS antibodies. The advantage of this test was that a greater number of samples per milliliter of antigen could be tested as compared with

the RSP test (Yoder, 1982). More sensitive and specific diagnostic procedures are needed to control mycoplasmas in poultry (Jordan, 1985).

SECTION I. AN AVIDIN-BIOTIN ENHANCED DOT-IMMUNOBINDING ASSAY FOR THE DETECTION OF *MYCOPLASMA GALLISEPTICUM* AND *M. SYNOVIAE* SERUM ANTIBODIES IN CHICKENS

An avidin-biotin enhanced dot-immunobinding assay for the detection of *Mycoplasma gallisepticum* and *M. synoviae* serum antibodies in chickens

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SUMMARY

A dot-immunobinding assay was enhanced by the incorporation of avidin and biotin reagents into the test system (DAB assay). This assay was used to detect serum antibodies to M. gallisepticum (MG) and M. synoviae (MS) from chickens. Serum samples were tested by rapid serum plate, hemagglutination-inhibition, and DAB assay methods. These results were compared. The DAB assay was at least 20 times more sensitive in detecting antibodies for MS and at least 75 times more sensitive in detecting antibodies for MG than the hemagglutination-inhibition test. The DAB assay was as specific as the HI test. The DAB assay was also more sensitive and specific than the rapid serum plate test. Some cross reactions occurred when low dilutions of high titer sera were used in the DAB assay. Parameters for determining negative, suspicious and positive samples were established. The DAB assay for MG and MS may have several applications, including use as a screening test and/or a confirmatory test.

INTRODUCTION

Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS) are etiologic agents for chronic respiratory disease and infectious synovitis of poultry (Olson, 1984; Yoder, 1984). These diseases cause severe economic losses to the poultry industry (Yoder, 1984). In an effort to control these diseases, the National Poultry Improvement Plan (NPIP) has approved a rapid serum plate (RSP) test, a hemagglutination-inhibition (HI) test, and an enzyme linked immunosorbent assay (ELISA) for the detection of mycoplasmal serum antibodies in poultry (National Poultry Improvement Plan and Auxiliary Provisions, 1986). However, nonspecific serologic reactions with the ELISA (Avakian and Kleven, 1987) and false positive reactions with the HI and RSP tests (Yoder, 1987) have become a concern to those involved in the serologic testing of poultry flocks for MG and MS.

The dot-immunobinding (DOT) assay is a modified ELISA assay which employs a nitrocellulose membrane disk as a matrix for antigen or antibody. A positive reaction is indicated by a colored dot against a white background. The dot assay has been successfully used to screen hybridoma supernatants, detect serum antibodies (Hawkes et al., 1982), identify mycoplasmas from cell cultures, broth cultures, and clinical specimens (Kotani and McGarrity, 1985a), identify

mycoplasma colonies on agar from pure and mixed cultures (Kotani and McGarrity, 1985b) and characterize mycoplasma antigens (Kenny and Cartwright, 1984). Incorporation of avidin/biotin (Guesdon et al., 1979) reagents into the test procedure enhances the sensitivity of the ELISA (Kendall et al., 1983) and the DOT assay (Reynolds and Hughes, 1985). The DOT assay amplified with avidin/biotin (DAB assay) has been successfully used to detect and speciate mycoplasmas (Gabridge et al., 1986). An advantage of the DAB assay is that several agents may be adsorbed onto different locations of the same nitrocellulose disk, thereby, allowing a single serum sample to be tested against several different antigens simultaneously.

The objective of this study was to develop the DAB assay for the detection of MG and MS antibodies in chicken sera and to determine the specificity and sensitivity of the DAB assay.

MATERIALS AND METHODS

Mycoplasma Antigens

The MG antigen used in the DAB assay was strain A5969. It was cultivated in a production broth medium (Subcommittee on Avian Diseases, 1971) enriched with 10% horse serum. Cells were removed from an 18-hour broth culture of approximately 1400 ml by centrifugation at 6000 x g for 25 minutes. The pellet of cells was washed twice in phosphate buffered saline (PBS), pH 7.1, suspended in 100 ml 0.05 M carbonate-bicarbonate buffer, pH 10, and incubated for 45 minutes at 37°C. The suspension of cellular material was further diluted to 400 ml in PBS and centrifuged. The pellet was washed twice in PBS, suspended in 25 ml PBS, and frozen in small (1-5 ml) aliquots at -70°C. The test antigen was adjusted to an optical density of 0.10-0.12 at 560 nm (Varian^{R} Spectrometer 643) prior to use.

The MS antigen used in the DAB assay was strain WVU 1853. It was cultivated in a Frey formulated broth (Vardaman and Yoder, 1969) enriched with 12% swine serum. The MS antigen was prepared by following the procedure as described above for MG antigen.

The RSP and HI antigens for MG and MS were those prepared by the National Veterinary Services Laboratories (NVSL), Ames, Iowa, for use in diagnostic tests.

Test Sera

Pooled reference antisera for the HI test were produced by inoculating specific-pathogen-free (SPF) chickens intramuscularly (IM) and/or intravenously (IV) with either MG (A5969) or MS (WVU 1853) cells washed and suspended in PBS. Birds used for the production of antisera which were used in this study, received inoculations in increasing doses. The doses were in amounts of 0.5 to 1.5 mls; optical densities were at 0.1 to 0.3 at 560 nm. Sera having the same HI titers were pooled. Thimerosal 1:5000 was added as a preservative. The sera were stored at 4°C. Fifteen positive control sera for MG, 14 positive control sera for MS and 17 negative control sera were tested.

Additional MG serum samples were produced by inoculating twenty 17-week-old SPF chickens subcutaneously (SC) at days 1 and 13 with MG in Freund's incomplete adjuvant. These chickens were also inoculated IM on day 26 and IV on day 41 with MG in PBS. They were bled on day 49 or 54. A total of 26 serum samples from days 49 and 54 were tested.

Additional MS serum samples were obtained from two groups of chickens. (1) Twenty 12-week-old SPF chickens were inoculated SC with MS in Freund's incomplete adjuvant on days 1 and 13. They were next inoculated IM on day 27

and IV on day 41 with MS in PBS. Sixteen preinoculation serum samples, 8 samples each from days 36 and 50, and 16 samples from day 55 were tested. (2) Forty 12-week-old SPF chickens were inoculated SC with MS in PBS on days 1 and 15. These were then inoculated IM on days 31 and 44. Twenty birds were exsanguinated on day 44. The remaining 20 were inoculated IM on day 44 and exsanguinated on day 52. Eight serum samples were collected from this group of chickens on days 0, 29, 44, and 52. The chickens were not individually identified and individual records were not kept.

Serum samples from 85 SPF chickens were obtained from the Biologics Virology Laboratory at NVSL and tested by using the DAB assay. These chickens were vaccinated IM at 51 days of age with various killed virus oil emulsion vaccines as indicated in Table 1. They were bled 31 days post inoculation.

Disk Preparation

Nitrocellulose membranes, pore size 0.2um (Schleicher & Schuell^{R}, Catalog #40-19790), were washed in distilled water. The membranes were handled with forceps or latex gloves throughout the preparation. While a membrane was still wet, disks were cut and removed by using a conventional 1/8-inch office paper punch. A disk was placed onto the bottom of each of the wells of a 96-well, flat-

Table 1. Viral agents used to produce chicken antisera for the comparison of the rapid serum plate, hemagglutination-inhibition and DAB assay methods

Viral agents[a]	Strain	No. of Samples
NDV/IBDV	B1, LaSota/Lukert	6
NDV/IBDV	B1 Clone/D-78	7
NDV/AEV	LaSota/ A3720	7
IBDV	Baxendale	7
IBDV	Lukert	14
TSV	R-1	8
NDV/IBV/IBDV	B1, LaSota/Mass H52/Lukert	15
NDV/IBV/IBDV/TSV	B1, Lasota/Mass M41/D-78/1733	6
NDV	Kimber	15
Total		85

[a]Abbreviations: NDV=Newcastle Disease Virus; IBDV=Infectious Bursal Disease Virus; AEV=Avian Encephalomyelitis Virus; TSV=Tenosynovitis Virus (Reo virus); IBV=Infectious Bronchitis Virus.

bottom polystyrene tissue culture plate (Costar^{R}, Catalog #3596). The disks were then allowed to dry thoroughly. An electronic multidispensing pipette (Rainin^{R}) was used to deliver 1 microliter of MG antigen (producing a dot) on the upper half of each disk. The disks were allowed to dry thoroughly. In the same manner, the MS antigen was applied

on the lower half of each disk. The plates were stored at 4°C or at room temperature.

Reagents

Tris buffered saline (TBS) was prepared by adding 4.84 gm Trizma-base and 58.48 gm NaCl to two liters of distilled water. The pH was adjusted to 7.5 with 0.5 N HCl. The wash solution consisted of 0.5% Tween 20 in TBS; the blocking solution consisted of 3.0% bovine serum albumin (BSA) in TBS; and the diluent consisted of 0.3% BSA and 0.5% Tween 20 in TBS. The diluent was used to dilute the test sera, antispecies antibody, and the conjugated enzyme. Biotinylated anti-chicken IgG (heavy and light chain), goat origin (Vector Laboratories, Catalog #BA-9010) was used at a dilution of 1:500. Avidin-D-Horseradish Peroxidase (HRP) (Vector Laboratories, Catalog #A2004) was used at a dilution of 1:2000. The chromogen was prepared as a stock solution of 300 mg of 4-chloro-1-naphthol in 100 ml of methanol and stored in a light-proof container at -70°C. A developing solution was prepared immediately before use by adding 6 ul of 30% H_2O_2 to 10 ml TBS and adding 2 ml of the chromogen stock solution.

Testing Procedures

The RSP and HI tests were performed by using standard testing methods (National Poultry Improvement Plan and Auxiliary Provisions, 1986). An HI titer of 1:20 or less was negative; 1:40 was suspect; and 1:80 or above was considered positive.

The DAB testing procedure was a modification of procedures previously described (Hawkes et al., 1982; Kendall et al., 1983). This assay was conducted at room temperature. Plates containing prepared disks were placed on a platform shaker (American Rotator VR) at 85 rpm for the duration of each incubation step as indicated in Figure 1. Wells containing disks with antigen were blocked by adding 100 ul/well of blocking solution and incubated for 60 minutes. The blocking solution was aspirated and the diluent solution was added at 100 ul/well in wells 2 through 12 of each row. One hundred uls of diluted (1:100) test serum were added to wells 1 and 2, resulting in dilutions of 1:100 and 1:200, respectively. The samples in well 2 of each row were serially diluted twofold through the 12th well by using a multichannel micropipette (Titertek^{R}) and the plates were placed on a platform shaker for 60 minutes. Following the primary antibody step, the plates were rinsed three times in succession with 100 ul/well of wash solution.

STEP	INCUBATION TIME (minutes)
BSA block	60
↓	
1^{o} Antibody (Test Sera)	60
↓	
3 rinses	
↓	
wash	15
↓	
BSA block	60
↓	
Biotinylated 2^{o} Antibody (Antispecies Antibody)	60
↓	
3 rinses	
↓	
wash	15
↓	
BSA block	60
↓	
Avidin-D-Enzyme	30
↓	
3 rinses	
↓	
wash	15
↓	
develop	10
↓	
rinse	
↓	
dry	variable

Figure 1. Flow diagram of the DAB assay

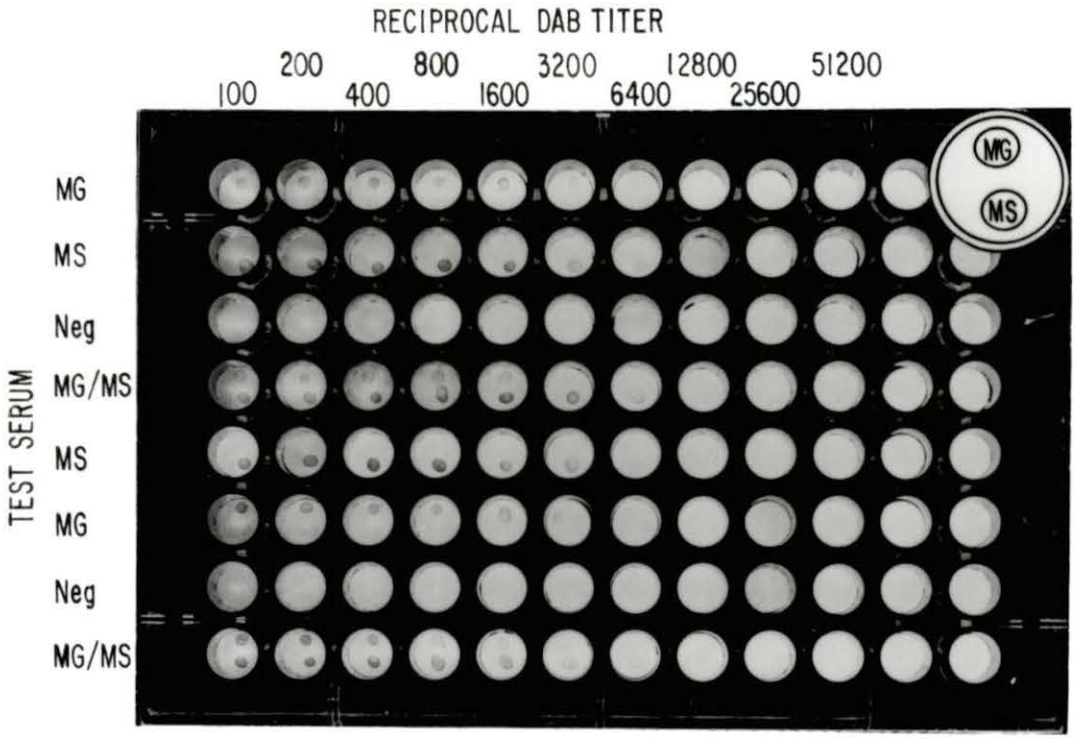
Wash solution was again added, the plates were placed on a shaker for 15 minutes and removed, and the fluid was aspirated. The plates were blocked as before. Diluted biotinylated antiserum was added at 100 ul/well and the plates were placed on the shaker for 60 minutes. Following this step, the plates were again rinsed, washed and blocked as before. Diluted avidin-D-HRP was added at 100 ul/well, the plates placed on the platform shaker for 20 minutes, and then rinsed and washed as before. Following washing, 100 ul/well of chromogen was added and the plates were placed in the dark for 10 minutes. To terminate development, distilled water was added at 200 ul/well. The fluid in the wells was aspirated, and the disks were allowed to dry prior to reading.

A control plate was set up each time the test was conducted and included the following titrations: in row A, the test was performed without the addition of test serum; in rows B, C, and D positive MG and MS sera were mixed and serially diluted; the wells in row B received diluent in place of biotinylated antibody; the wells in row C received diluent in place of Avidin-D-HRP. No substitutions were made in row D. Rows E, F, G, and H were tested with known HI positive and negative sera for MG and MS. A positive reaction (Figure 2) was indicated by a dark blue dot on a

white disk when the plate was observed at arm's length under fluorescent lighting.

The sensitivity of the DAB assay was determined by comparing the DAB assay, RSP test, and HI test results of serum from chickens with known exposure to the homologous mycoplasma. The specificity of the DAB assay was determined by comparing the test results of serum from chickens with exposure to heterologous antigens or uninoculated SPF chickens.

Figure 2. Demonstration of the DAB assay, illustrating the reactions of known positive and negative serum samples for Mycoplasma gallisepticum (MG) and M. synoviae (MS). Plate was photographed against a black background


RECIPROCAL DAB TITER
100
200
400
800
1600
3200
6400
12800
25600
51200
TEST SERUM
MG
MS
Neg
MG/MS
MS
MG
Neg
MG/MS
MG
MS

RESULTS

All of the 41 serum samples obtained from chickens inoculated with MG were positive when tested with the RSP test for MG and reacted at 1:40 or greater with the HI test for MG. They reacted with the DAB assay at titers ranging from 1:1,600 to 1:102,400. These DAB titers are compared to the MG titers obtained by the HI test (Table 2). The

Table 2. Comparison of the DAB assay titers to the corresponding hemagglutination-inhibition (HI) titers from 41 sera obtained from chickens inoculated with Mycoplasma gallisepticum (MG)

	Number of Observations					
DAB Titer[a]	MG HI Titer[a] 40	80	160	320	640	HI GMT[b]
1600	2	1				50
3200						
6400	1	1	4			110
12800	2	5	2	4		120
25600		1	7	3		180
51200			3	3	1	260
102400			1			160
DAB GMT[b]	4850	9900	21000	24000	51200	

[a]Reciprocal titer.

[b]Reciprocal geometric mean titer.

corresponding geometric mean titers for both assays are included. Nine of these samples (22%) were also positive by the DAB assay for MS at titers ranging from 1:100 to 1:400 (data not shown). All samples were negative for MS by the RSP and HI tests.

The 70 serum samples obtained from the chickens inoculated with MS reacted in the MS DAB assay at titers ranging from 1:400 to 1:512,000 (Table 3). These titers were compared to the MS titers obtained by the HI assay. The GMTs for both assays are included. Nine of the 70 samples were positive in the DAB assay for MG at titers ranging from 1:100 to 1:400 (data not shown).

Results obtained with samples which were periodically collected from a group of SPF chickens inoculated with MS over a period of 52 days are shown in Figure 3. Seven of eight samples collected at day 29 PI reacted at 1:800 or above in the DAB assay. One of these samples was positive at 1:80, two were suspect at 1:40, and the remaining five samples were negative at 1:20 or less by the HI test. Four of these eight samples were positive by the RSP test. All samples which were collected on 44 days PI were positive by the DAB assay at titers of 1:1600 or above, yet three of these eight samples were in the suspect range (1:40) by the HI assay. All samples were positive on the RSP test.

Table 3. Comparison of the DAB assay titers to their corresponding hemagglutination-inhibition (HI) titers obtained with 70 sera from chickens inoculated with Mycoplasma synoviae (MS)

	Number of Observations								
DAB Titer[a]	MS HI Titer[a] <20	20	40	80	160	320	640	1280	HI GMT[b]
400	1								<20
800	1	2	1						<20
1600	1		2	1					<40
3200			6	5	1	2			80
6400			1	6	5	2			120
12800				2	7	5	8	1	310
25600						2	4	2	640
51200							1	1	910
DAB GMT[b]	800	800	2600	5000	8900	10000	17700	25600	

[a]Reciprocal titer.

[b]Geometric mean titer.

At day 52 PI, all samples tested were positive in the DAB, HI, and RSP tests for MS; three of eight exhibited a cross reaction in the MG RSP test. All 24 samples tested were negative by the HI test and DAB assay for MG.

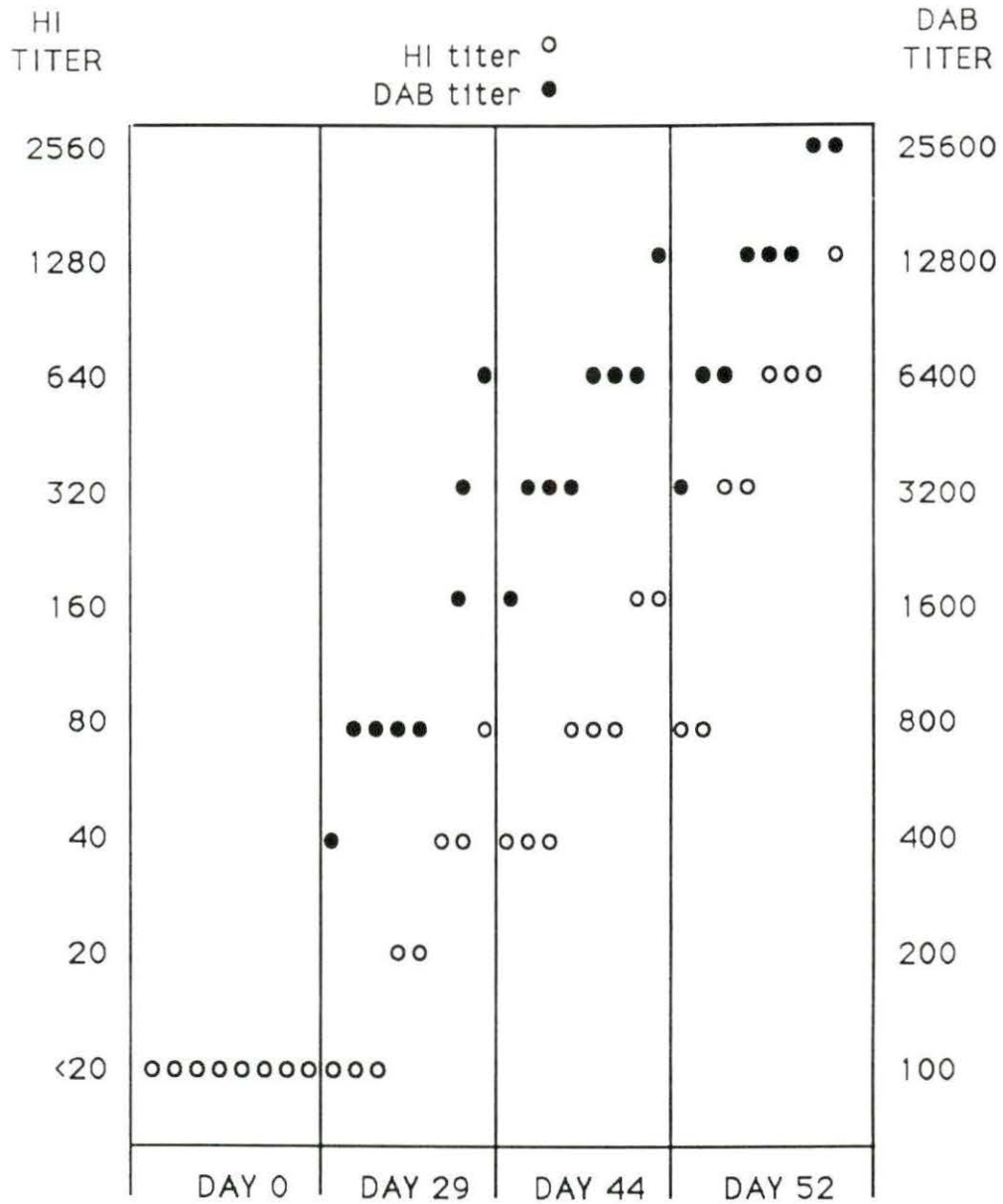


Figure 3. Comparison of the hemagglutination-inhibition (HI) and DAB reciprocal titers for M. synoviae antibodies in sera collected from chickens on days 0, 29, 44, and 52 post-inoculation (no data for DAB titer on day 0)

The test results obtained with the 85 serum samples from chickens inoculated with various viral vaccines and the 41 negative reference/control serum samples are shown in Table 4. The RSP assay yielded the highest number (26) of false positive reactions. The HI assay had the fewest false reactions: 1 suspicious reaction. The serum samples which were evaluated by the DAB assay resulted in 18 false positive reactions with both MG and MS. However, these false positive reactions occurred at serum dilutions of 1:200 or less. All samples which were positive for MS by the DAB assay were also positive for MG by the DAB assay. The DAB assay was positive for MG but not MS in two instances.

Sensitivity data for the three assay methods are shown in Table 5. Samples collected on day 29 of the MS group did not all react on the RSP and HI tests, whereas all samples reacted on the DAB assay. Samples collected on days 36 and 44 of the MS group and day 49 of the MG group were positive on the RSP test and DAB assay; the samples were suspicious or positive on the HI test. Samples collected after day 49 were positive by all three assays.

Specificity data for the MG and MS DAB assays are shown in Table 6. Cross reactions at titers greater than 1:200

were evident only in sera from chickens inoculated with mycoplasmas.

Table 4. Comparison of the results obtained with the rapid serum plate (RSP), hemagglutination inhibition (HI), and DAB assays with 85 serum samples obtained from chickens vaccinated with various viral agents and from 41 negative/control serum samples

Antisera[a]	Samples tested	RSP[b]		HI[b]		DAB ASSAY[c]	
		MG	MS	MG	MS	MG	MS
NDV-IBDV	13	0	2	0	0	1(100)	1(200)
NDV-AEV	7	0	3	0	0	1(100)	1(100)
IBDV	21	2	9	0	0	1(200)	1(200)
TSV	8	0	2	0	0	3(100)	2(200)
NDV-IBV-IBDV	15	2	4	0	0	2(100)	2(200)
NDV-IBV-IBDV-TSV	6	1	1	1(40)	0	0	0
NDV	15	0	0	0	0	2(100)	1(200)
Negative/ Control	41	0	0	0	0	0	0

[a]Viral agents used to produce antisera: NDV=Newcastle Disease Virus; IBDV=Infectious Bursal Disease Virus; AEV=Avian Encephalomyelitis Virus; TSV=Tenosynovitis Virus (Reo virus); IBV=Infectious Bronchitis Virus.

[b]Number of samples reacting.

[c]Number of samples reacting (reciprocal titer).

Table 5. Comparison of results obtained with the rapid serum plate (RSP) test, hemagglutination-inhibition (HI) test, and DAB assay for *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS)

Samples			Assays[a]				
Source	Day PI[d]	Number Tested	RSP	HI[b]		DAB[c]	
				sus	pos	sus	pos
MG							
Reference[e]	N/A[f]	15	15	3	12	0	15
Group 1	49	16	16	2	14	0	16
	54	10	10	0	10	0	10
Total		41	41	5	36	0	41
MS							
Reference	N/A	14	14	2	12	0	14
Group 1	36	8	8	3	5	0	8
	50	8	8	0	8	0	8
	55	16	16	0	16	0	16
Group 2	29	8	4	2	1	5	3
	44	8	8	3	5	0	8
	52	8	8	0	8	0	8
Total		70	66	10	55	5	65

[a]Number positive.

[b]Suspect 1:40; positive >1:40.

[c]Suspect 1:400-1:800.

[d]Post inoculation day sample collected.

[e]Pooled control sera

[f]Information not available.

Table 6. Distribution of the DAB assay titers for *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS)

Sera	MG DAB Titer[a]					MS DAB Titer[a]					Total Tested
	100	200	400	800	>800	100	200	400	800	>800	
MG	0	0	0	0	41	6	2	1	0	0	41
MS	4	4	2	3	0	0	0	1	4	65	70
Other[b]	9	1	0	0	0	8	4	0	0	0	126

[a]Reciprocal titer.

[b]Serum samples obtained from uninoculated SPF chickens and from chickens inoculated with antigens other than mycoplasmas.

The DAB assay GMTs obtained from the MG and MS sera were compared with their corresponding HI titers (Tables 2 and 3). Using these titers, the sensitivity of the DAB assay as compared to the HI test was determined by a DAB:HI ratio (Table 7). The DAB:HI ratios ranged from 75 to 131 for MG and 20 to 65 for MS, indicating that the DAB assay was more sensitive than the HI test.

Table 7. Sensitivity of the DAB assay as compared to the HI test for M. gallisepticum (MG) and M. synoviae (MS) as determined by dividing the reciprocal geometric mean titer (GMT) of the DAB assay by the corresponding reciprocal HI titer

	MG			MS		
HI Titer	No. of Samples	DAB GMT	Ratio DAB:HI	No. of Samples	DAB GMT	Ratio DAB:HI
20				2	800	40
40	5	4850	121	10	2600	65
80	8	9900	124	14	5000	63
160	17	21000	131	13	8900	56
320	10	24000	75	11	10000	31
640	1	51200	80	13	17700	28
1280				5	25600	20

DISCUSSION

The DAB assay was at least 75 times more sensitive than the HI test when used for detecting antibodies to MG and at least 20 times more sensitive than the HI test when detecting antibodies to MS. The DAB assay, as compared to the HI test, was able to detect antibodies earlier in birds exposed to MS. Increases in DAB assay titers corresponded to increases in HI titers.

Cross reactions occurred with the DAB assay when low dilutions of individual serum samples were examined but not when pooled reference sera were tested. Cross reactions were observed with the MS DAB assay when high DAB titer MG serum was tested (Table 5). It is hypothesized that the positive reactions that occurred at dilutions of 1:200 or less were caused by nonspecific serum factors and should not be considered positive. A DAB assay reaction at serum dilutions greater than 1:800 was considered positive. Reactions occurring in the dilution range of 1:400-1:800 were considered suspicious. As the tests for MG and MS can be conducted simultaneously, a positive reaction for both mycoplasmas may indicate that previous infections of MG and MS have occurred. If the sample is positive at a high titer for one mycoplasma species and positive at a low titer for another mycoplasma species, then one must consider the

possibility that a false positive reaction has occurred due to nonspecific factors.

The DAB assay has a number of advantages. The antigen is secured to the nitrocellulose by high affinity binding, precluding the need for a capture antibody. Plates of disks can be prepared in advance and stored for long periods of time. Plates that had been stored at 4°C and at room temperature for several weeks were compared with plates stored for 2 days by using the same test sera. Comparable results were obtained using both types of plates. Several antigens can be dotted onto the same disk so that a single serum sample can be tested simultaneously against several different antigens. The test can be read without special equipment and the test plates stored for future reference.

Disadvantages of the DAB assay include the time consumed in disk preparation and the length of time needed to perform the assay (several hours). Although changes in the testing procedure were not attempted, it may be possible to combine reagents (e.g., the antispecies antibody/biotin complex and the avidin-D-HRP) or shorten (e.g., incubation times) steps of this assay.

The DAB assay may have limitations as a screening test for MG and MS because of the cost, time, and labor involved.

Because of its specificity and high sensitivity, it could be used as a confirmatory test for detecting the presence of antibodies to MG and MS in chicken sera.

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SECTION II. USE OF AN AVIDIN-BIOTIN ENHANCED DOT-IMMUNOBINDING ASSAY TO DETECT ANTIBODIES FOR AVIAN *MYCOPLASMA* IN SERA FROM IOWA MARKET TURKEYS

Use of an avidin-biotin enhanced dot-immunobinding assay to dedect antibodies for avian *Mycoplasma* in sera from Iowa market turkeys

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SUMMARY

A dot immunobinding assay, amplified with avidin and biotin (DAB assay), was used to test serum samples from 122 commercial market turkey flocks obtained from four Iowa processing plants. The samples were tested for the presence of antibodies to four species of Mycoplasma considered to be important pathogens for turkeys: M. gallisepticum (MG), M. iowae (MI), M. meleagridis (MM), and M. synoviae (MS). The prevalence of antibodies against these mycoplasmas, as determined by the DAB assay, were 5.7% for MG, 18.0% for MI, 77.9% for MM, and 9.8% for MS.

INTRODUCTION

Four species of Mycoplasma that are of concern to the turkey industry are M. gallisepticum (MG), M. iowae (MI), M. meleagridis (MM), and M. synoviae (MS) (Jordan, 1985; Yoder, 1984). Serodiagnostic tests currently used for MG, MM, and MS include a rapid serum plate (RSP) test, a hemagglutination-inhibition (HI) test and an enzyme linked immunosorbent assay (National Poultry Improvement Plan and Auxiliary Provisions, 1986b).

Mycoplasma iowae includes strains which formerly represented six different serotypes, designated as serovars I, J, K, N, Q, and R. Cultures representing five of these serovars were selected for this study. They were D2497 (R), PHN-D13 (N), L3-10 (Q), 693 (J), and 695 (I). Strain 1805 (K) differed from the other strains in biochemical reactions, plate and tube agglutination tests, and protein electrophoretic patterns (Rhoades, 1984). Therefore, strain 1805 was not included in this study. The antigenic variation of MI strains (Rhoades, 1984) and the inability to detect serum antibodies for MI in experimentally infected poultry (Bradbury et al., 1988) has made the detection of MI infection difficult.

A dot immunobinding assay amplified with avidin and biotin (DAB assay) was found to be a sensitive and specific

test for detecting antibodies to MG and MS in chickens (Cummins and Reynolds, 1989). Furthermore, the results of a preliminary study indicated that antibodies for MG, MM, and MS could be detected in sera from inoculated turkeys using the DAB assay (unpublished observation).

The objectives of this study were as follows: 1) to determine if serum antibodies to MI could be detected with the DAB assay; 2) to determine if the DAB assay could be used as a practical assay for testing large numbers of serum samples for the presence of antibodies to MG, MI, MM, and MS; and 3) to determine the prevalence of these four species of Mycoplasma in flocks of commercial market turkeys.

MATERIALS AND METHODS

Strains

Strains M. gallisepticum A5969, M. meleagridis 8M92, and M. synoviae WV 1853 have been used by the principal author at National Veterinary Services Laboratories (NVSL), Ames, Iowa for the production of diagnostic and reference reagents. M. iowae strains D2497, PHN-D13, L3-10, 693, and 695 were obtained from Dr. K. R. Rhoades, National Animal Disease Center (NADC), Ames, IA.

Antigens

The plate antigens for MG, MM, and MS were those produced and used by the NVSL. The MI plate antigens were produced and standardized by using the same criteria as were used for MG and MS (National Poultry Improvement Plan and Auxiliary Provisions, 1986a). Antigens for the DAB assay were produced as previously described (Cummins and Reynolds, 1989).

Antimycoplasma Sera

Antisera for MG, MS, and MM were those produced and used as reference sera at the NVSL.

The antisera for MI were prepared by using specific-pathogen-free turkeys obtained from a flock housed and maintained at the NADC. Antigens of each MI strain (washed

whole cells suspended in phosphate buffered saline) were prepared and standardized to an optical density of 0.1 at 560 nm. Antigen was inoculated intravenously into two or three turkeys at weekly intervals for three weeks at doses of 1 ml, 2 ml, and 1 ml. Samples were collected from the turkeys prior to inoculation and at 7, 14, and 21 days after the last injection.

Test Sera

Serum samples were obtained during a six-week period in late summer from market turkeys at four Iowa turkey processing plants. Ten to 20 individual bird samples (a total of 2390 serum samples) were obtained from each of 122 flocks. The samples were pooled and diluted, resulting in a final working dilution of 1:100 for each test serum. Four pools were prepared from each flock. A total of 488 pools were tested.

Test Procedures

The RSP test was conducted according to standard methods (National Poultry Improvement Plan and Auxiliary Provisions, 1986b). The DAB assay was conducted as previously described (Cummins and Reynolds, 1989). A test sample was considered positive by the DAB assay if the sample reacted at a dilution of greater than 1:800; a sample

reacting at a titer of 1:400 or 1:800 was considered suspicious; and a sample reacting at a titer of less than 1:400 was considered negative.

MI Specificity

The DAB assay and RSP were conducted on the sera obtained from the turkeys inoculated with the five strains of MI. Each MI strain was tested for homologous and heterologous reactivity.

DAB Assay for Screening Market Turkeys

Nitrocellulose discs were prepared with antigens from four mycoplasma species, MG, MI, MM, and MS. One microliter of antigen from each of these species was placed on a different quadrant of each disc. The antigens for MG, MM, and MS were prepared as described under "antigens". Four MI antigen strains (693, D2497, PHN-D13, and L3-10) were pooled (optical density of 0.16 at 560nm) and one microliter was placed onto each disc (Figure 1).

Each test plate was divided into four sections (Figure 1). In each section, each of the four serum pools from a flock was serially titrated from a dilution of 1:200 to 1:6400. Pools from four flocks were tested on a plate. A control plate, containing known positive and negative sera, was included each time the assay was performed.

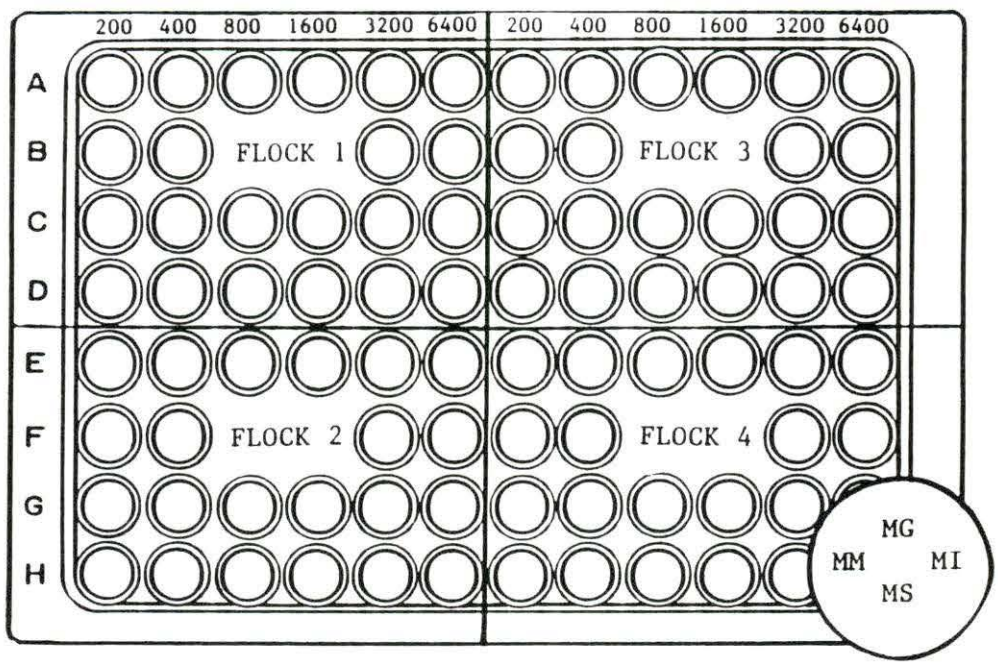

Figure 1. Illustration of test plate divided into 4 sections in preparation for testing the pooled samples from 4 market turkey flocks. Numbers at the top represent serial dilutions. Letters at the left represent pools. Insert illustrates placement of antigens on disk

RESULTS

Detection of MI Antibodies

The results of the DAB assay and RSP test with sera collected from turkeys inoculated with MI are presented in Table 1. The homologous titers for strains 693 and PHN-D13 were notably higher than for the other MI strains. Antisera against strain L3-10 reacted with most of the MI antigens that were tested. Homologous titers for all MI strains were equal to or greater than 1:1600. Positive reactions from sera that were homologous with the antigen were more intense in color than sera that were tested against heterologous antigens. Heterologous titers decreased more rapidly than homologous titers during the three weeks of sampling following the last exposure to antigen. The antigen prepared from strain 695 reacted with many of the sera tested from birds exposed to any of the other MI strains. The preinoculation serum samples were negative by the RSP test and DAB assay for all MI strains.

The RSP antigen prepared with strain 695 reacted with nearly all sera obtained from turkeys that had been exposed to the five MI strains. The RSP antigens prepared from strains PHN-D13 and 693 reacted only with their homologous antisera. The RSP antigens prepared from strains D2497 and L3-10 reacted with antisera against D2497 and L3-10. Some

Table 1. Results of the rapid serum plate (RSP) test and DAB assays for the detection of serum antibodies from turkeys inoculated with M. iowae (MI) strains D2497, L3-10, PHN-D13, 693, and 695

Turkey ID (MI strain)	Bleeding Day PI[a]	DAB ASSAY					RSP TEST				
		D2497	L3-10	PHN-D13	693	695	D2497	L3-10	PHN-D13	693	695
5161 (D2497)	Pre-inoc	-[b]	-	-	-	-	-	-	-	-	-
	7	6400[c]	12800	-	800	3200	+	+	-	-	+
	14	1600	1600	-	800	400	+	+	-	-	+
	21	1600	1600	-	800	400	-	-	-	-	+
5173 (D2497)	Pre-inoc	-	-	-	-	-	-	-	-	-	-
	7	NT[d]	NT	NT	800	1600	+	+	-	-	+
	14	NT	NT	NT	NT	NT	NT	NT	NT	NT	N
	21	1600	3200	400	800	800	+	+	-	-	+
5179 (D2497)	Pre-inoc	-	-	-	-	-	-	-	-	-	-
	7	12800	6400	12800	-	-	+	+	-	-	+
	14	NT	NT	NT	NT	NT	+	+	-	-	+
	21	NT	NT	NT	NT	NT	+	+	-	-	+

[a]Number of days after last injection of antigen.

[b]No reaction in the DAB assay; negative in the RSP test.

[c]Reciprocal titer.

[d]Not tested.

Table 1. (continued)

Turkey ID (MI strain)	Bleeding Day PI	DAB ASSAY					RSP TEST				
		D2497	L3-10	PHN-D13	693	695	D2497	L3-10	PHN-D13	693	695
5154	Pre-inoc	-	-	-	-	-	-	-	-	-	-
(L3-10)	7	3200	3200	3200	400	3200	+	+	-	-	+
	14	3200	3200	3200	1600	1600	+	+	-	-	+
	21	1600	1600	800	200	800	-	-	-	-	+
5155	Pre-inoc	-	-	-	-	-	-	-	-	-	-
(L3-10)	7	12800	12800	800	800	3200	+	+	-	-	+
	14	6400	12800	400	800	1600	+	+	-	-	+
	21	3200	3200	200	400	1600	-	-	-	-	+
5156	Pre-inoc	NT	NT	NT	NT	NT	-	-	-	-	-
(L3-10)	7	6400	6400	1600	3200	6400	+	+	-	-	+
	14	6400	6400	400	800	3200	+	+	-	-	+
	21	3200	3200	200	6400	6400	+	+	-	-	+
5147	Pre-inoc	NT	NT	NT	NT	NT	-	-	-	-	-
(PHN-D13)	7	400	400	6400	-	-	-	-	+	-	+
	14	NT	NT	NT	200	-	-	-	+	-	+
	21	-	NT	NT	-	-	-	-	+	-	-
5148	Pre-inoc	-	-	-	-	-	-	-	-	-	-
(PHN-D13)	7	800	800	51200	200	200	-	-	+	-	+
	14	200	200	51200	-	-	-	-	+	-	+
	21	-	-	12800	-	-	-	-	+	-	-

Table 1. (continued)

Turkey ID (MI strain)	Bleeding Day PI	DAB ASSAY					RSP TEST				
		D2497	L3-10	PHN-D13	693	695	D2497	L3-10	PHN-D13	693	695
5181	Pre-inoc	-	-	-	-	-	-	-	-	-	-
(693)	7	800	800	-	102400	3200	-	-	-	+	+
	14	800	800	-	25600	1600	-	-	-	+	+
	21	200	200	400	25600	800	-	-	-	-	+
5188	Pre-inoc	-	-	-	-	-	-	-	-	-	-
(693)	7	1600	1600	400	12800	6400	-	-	-	+	+
	14	NT	NT	NT	NT	NT	-	-	-	+	+
	21	NT	NT	NT	NT	NT	-	-	-	+	+
5198	Pre-inoc	-	-	-	-	-	-	-	-	-	-
(695)	7	3200	3200	800	6400	25600	+	+	-	-	+
	14	1600	1600	-	800	6400	+	+	-	-	+
	21	1600	1600	800	1600	3200	+	+	-	-	+
5200	Pre-inoc	-	-	-	-	-	-	-	-	-	-
(695)	7	3200	6400	1600	12800	25600	+	+	-	-	+
	14	1600	800	400	NT	NT	+	+	-	-	+
	21	1600	1600	400	12800	6400	+	+	-	-	+

of the turkeys with positive RSP tests at 7 and 14 days were negative at 21 days after the last exposure to an MI antigen.

Results obtained when antisera prepared against MG, MM, and MS were tested with MI antigens in the DAB assay are indicated in Table 2. One chicken serum sample, positive for MG, reacted at 1:1600 against MI antigens 693 and 695; the remaining serum samples in this table which cross reacted had titers of 1:800 or less. Negative control chicken sera showed some cross reactivity; negative control turkey sera did not react.

Twenty-six serum samples that were positive for MI on the RSP test and DAB assay were tested by the DAB assays for MG, MM, and MS. One of these samples reacted with the MG antigen at a titer of 1:800; five samples reacted with the MM antigen at titers of 1:400 or 1:800. There were no positive reactions with the MS antigen (data not shown).

Market Turkey Survey

Four hundred and eighty-eight pools, prepared from 2390 serum samples representing 122 flocks, were tested with the DAB assay (Table 3).

In several instances, all four pools from the same flock failed to react to the same antigen. The numbers of flocks with one or more pooled samples reacting in the DAB

Table 2. Results of testing reference antisera for M. gallisepticum (MG), M. meleagridis (MM), and M. synoviae (MS) and negative control sera in the DAB assay using five different strains of M. iowae as antigens

Reference[a] Serum	Sera Source	MI strains D2497	L3-10	PHN-D13	693	695
MG	Turkey	0/4[b]	0/4	0/4	0/4	0/4
MG	Chicken	3/7	3/7	2/7	2/7	2/7
MM	Turkey	2/12	2/12	0/12	4/12	4/12
MS	Chicken	3/12	3/12	1/12	1/12	1/12
Negative	Turkey	0/13	0/13	0/13	0/13	0/13
Negative	Chicken	0/12	1/12	0/12	0/12	1/12

[a]Pooled reference sera positive for the designated mycoplasma as determined by the RSP and HI tests.

[b]Number of samples with titers ≥1:400/number of samples tested.

assay for MG, MI, MM, and/or MS are shown in Table 4. Seventy-two of the 95 flocks that were positive for MM in the DAB assay exhibited strong reactions at the highest dilution tested, 1:6400.

The distribution of the suspect and positive flocks, as determined by the DAB assay is shown in Table 5.

Table 3. Distribution of DAB assay titers for M. gallisepticum (MG), M. iowae (MI), M. meleagridis (MM), and M. synoviae (MS) that were obtained with the 488 pools of turkey sera

Antigen	Number of Pools Reacting Reciprocal DAB Titer						Percent
	(200)	400	800	1600	3200	6400	≥400[a]
MG	(13)	7	2	1	0	0	2.0
MI	(24)	28	12	1	0	0	8.4
MM	(0)	0	0	1	7	366	76.6
MS	(14)	6	7	3	7	8	6.4

[a]Percentage of the total number of pools reacting at titers equal to or greater than 1:400.

Table 4. Results from 122 market turkey flocks reacting on the DAB assay survey for M. gallisepticum (MG), M. iowae (MI), M. meleagridis (MM), and M. synoviae (MS)

Antigen	Suspect[a]	Positive[b]	Total Reacting
MG	6 (4.9%)	1 (0.8%)	7 (5.7%)
MI	21 (17.2%)	1 (0.8%)	22 (18.0%)
MM	0	95 (77.9%)	95 (77.9%)
MS	6 (4.9%)	6 (4.9%)	12 (9.8%)

[a]One or more pools from a flock with a DAB titer of 1:400 or 1:800.

[b]Positive: one or more pools from a flock with a DAB titer of 1:1600 or greater.

Table 5. Percentage of flocks positive and suspect for M. gallisepticum (MG), M. iowae (MI), M. meleagridis (MM), and M. synoviae (MS) as determined with the DAB assay using sera from four processing plants located in Iowa

Processor	No. of flocks	No. of pools	MG		MI		MM		MS	
			sus[a]	pos[b]	sus	pos	sus	pos	sus	pos
A	30	120	10%	0%	17%	0%	0%	80%	10%	7%
B	38	152	3	0	11	0	0	97	3	0
C	15	60	7	0	13	0	0	33	0	0
D	39	156	0	3	23	3	0	74	5	10

[a]Suspect: one or more pools from a flock with a DAB titer of 1:400 or 1:800.

[b]Positive: one or more pools from a flock with a DAB titer of 1:1600 or greater.

DISCUSSION

The DAB assay was capable of detecting serum antibodies to MI. There was a difference in the extent of reactivity between the MI strains when tested against each other. Therefore, it was decided to use four strains as a composite antigen. Strains D2497, L3-10N, PHN-D13, and 693 were selected on the basis of their reactivity to homologous and heterologous antisera as determined by the RSP test and DAB assay.

The DAB assay was a practical assay for detecting serum antibodies for avian mycoplasmas in turkey flocks. Four antigens (MG, MI, MM, and MS) were employed in the same assay procedure, allowing simultaneous testing for antibodies against each of the four agents. This permitted comparison of results, was efficient from a time and labor standpoint, and facilitated interpretation of the results. For example, if a serum had a high titer against one antigen and exhibited a low titer for another antigen, the possibility of cross reactivity should be considered. A few cross reactions occurred when MS and MM reference antisera were used in the MI DAB assay. Therefore, cross reactivity may have contributed to the high percentage of MI suspicious reactions. However, the processing facility with the highest number of flocks reacting with the MM DAB assay had

the lowest number reacting on the MI DAB assay. These results were unexpected considering that 75% of these pools reacted at 1:6400 or higher for MM.

The significance of the number of samples reacting at low titers on the MI DAB assay is unclear. The parameters that were used in defining a negative, suspect, and positive sample for MG, MM, and MS in the DAB assay may need to be redefined for the MI DAB assay. For example, a titer of 1:400 might be considered a negative rather than a suspect.

The slightly higher concentration of the composite antigen for the MI DAB antigen could also account for the large number of low titers occurring in the market survey. It is also possible that the MI antigen is reacting to other MI strains or other Mycoplasma spp. M. gallinarum, M. pullorum, M. gallinaceum, M. iners, M. gallopavonis, MM, and MI share antigens as demonstrated by double immunodiffusion in agar tests, but are serologically distinct by growth inhibition, metabolism inhibition, and immunofluorescence tests (Razin and Freundt, 1984). Jordan et al. (1987) reported that 6% of the sera tested resulted in false positive reactions when an indirect ELISA for MI strain 695 was used. We found 17.2% of the pools reacted in the suspect range and 0.8% of the pools were positive on the MI DAB assay.

The starting dilution of the serum used in this study was 1:200; however, if the starting dilution was 1:400 and the samples were diluted to 1:6400, 24 pooled samples representing 6 flocks could be tested on each test plate. Thus, the high sensitivity of the DAB assay could permit screening several flocks per plate with a minimum number of test samples. A pool containing more than 6 samples was not attempted; however, there was no indication that this would not be feasible.

In studies by Bencina et al. (1987a), the incidence of avian mycoplasma infections in 696 chickens and chicken embryos was 19% for MG, less than 2% for MI, and 22% for MS. Jordan and Amin (1980) examined respiratory tract, cloaca and brain tissue samples obtained from turkeys with respiratory and/or locomotive disease manifestations and from rejected carcasses. Out of 343 samples, 43 (12.5%) contained MG, 14 (4.1%) MS, 12 (3.5%) MI, and 102 (29.7%) MM. In the present study, the percentage of reactors (suspicious plus positive) in the 122 flocks was low for MG (5.7%) and MS (9.8%). It appears that efforts to control MG and MS infections in the Iowa turkey populations have been fairly successful. The 18.0% for MI is much higher than that reported by other workers (Bencina, 1987a; Jordan and

Amin, 1980). The 77.9% for MM indicates this organism is widespread in Iowa.

Over 70% of flocks from three of the four processing plants were positive by the MM DAB assay; however, only 33% of the flocks from the fourth plant were positive. Although fewer samples were obtained from the fourth plant, the weekly percentages of positive samples obtained from this facility remained relatively constant. A possible contributing factor for this might be the geographical location from whence the birds were reared or the source of the poults. We have no history of the status of the flocks tested. Different management systems in chicken flocks can significantly affect isolation yields and incidence of mycoplasma species (Bencina et al., 1987b).

Plates used in the DAB assay were easily preserved so they could be reexamined later for reference or comparison. Exposure to light for several days did not cause fading of the reactions on the discs.

We conclude that the DAB assay is an effective method to survey large populations of birds for several mycoplasma antigens simultaneously. The DAB assay may have similar applications for other avian pathogens.

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GENERAL SUMMARY

Several species of Mycoplasma are pathogenic for chickens and turkeys and have become of significant economic importance in a rapidly growing industry. Early detection and diagnosis requires sensitive and specific assays in order that steps may be taken to minimize losses.

The most commonly used diagnostic tools in avian mycoplasma serology are rapid serum plate (RSP) and hemagglutination-inhibition (HI) tests. Several problems have been associated with these tests. An alternative serologic assay, a dot-immunobinding assay amplified with avidin and biotin reagents (DAB assay) was developed and used to detect antibodies to MG and MS in sera from chickens and turkeys.

From the results of these studies, it was concluded that the DAB assay was a sensitive serologic test for detecting serum antibodies to avian mycoplasmas. The DAB assay was found to be more sensitive than either the RSP and the HI tests without loss of specificity. The DAB assay was capable of detecting serum antibodies in chickens experimentally exposed to MS earlier than the HI test. Experiments involving the capability of the DAB assay to detect early serum antibodies following exposure to other avian mycoplasmas were not conducted. Weekly sampling and

testing of birds inoculated with Mycoplasma species would better demonstrate how sensitive and how soon after exposure the DAB assay could detect antibodies.

Chickens can produce several classes of antibody in response to invading antigens. Immunoglobulin (Ig) A, IgG, IgM, and a homologue of IgD can be formed. IgA is present in secretions; IgG and IgM are found in highest concentrations in the blood; and IgD is found as an antigen receptor on lymphocytes. IgG levels in chicken serum are found at levels of 300-700 mg/100 ml serum. Chicken IgM is found at levels of 120-250 mg/100 ml serum (Tizard, 1982). IgM, formed primarily during the primary immune response in chickens, may be found in detectable levels sooner than IgG. The DAB assay employed a biotinylated antichicken-IgG in these studies. Improved detection of an early serum antibody response after exposure to a mycoplasma may be facilitated by substituting an anti-IgM reagent in place of the anti-IgG.

The parameters for establishing a positive, suspect, and negative titer for the MG and MS DAB assays were established in the first study. The same parameters were used for both the MM and MI DAB assays, however, these parameters were not extensively evaluated. Further studies may also be warranted to evaluate the antigens used in this

study by using heterologous sera from other avian mycoplasma.

The DAB assay was used to determine the prevalence of several mycoplasmas in a large population of turkeys. The increased sensitivity of the assay permitted samples to be pooled for testing. This method proved to be efficient, sensitive and specific.

The DAB assay has several advantages, including the ease in which plates can be stored before and after use, the ability to simultaneously test for several agents, and to perform and read the test without specialized equipment. Disadvantages include the time spent preparing the disks and the time required to perform the assay. Commercial products may become available which might reduce the time and facilitate preparation of the disks. Other aspects of the assay which could be investigated further include combining steps, e.g., mixing the avidin and biotin reagents before addition to the test plates; shortening the time of some of the assay steps, e.g., reducing the blocking time; and analyzing the effect of antigen concentration on the specificity.

The ability to reuse the plates (discs) may also be possible. Discs which did not exhibit a positive reaction when used on the DAB assay might be used again.

Additionally, it may be possible to chemically manipulate the discs which exhibit a reaction and allow the release of the antigen-antibody complex thus the rendering discs reuseable. The DAB assay may have a number of other uses as a diagnostic and research tool.

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