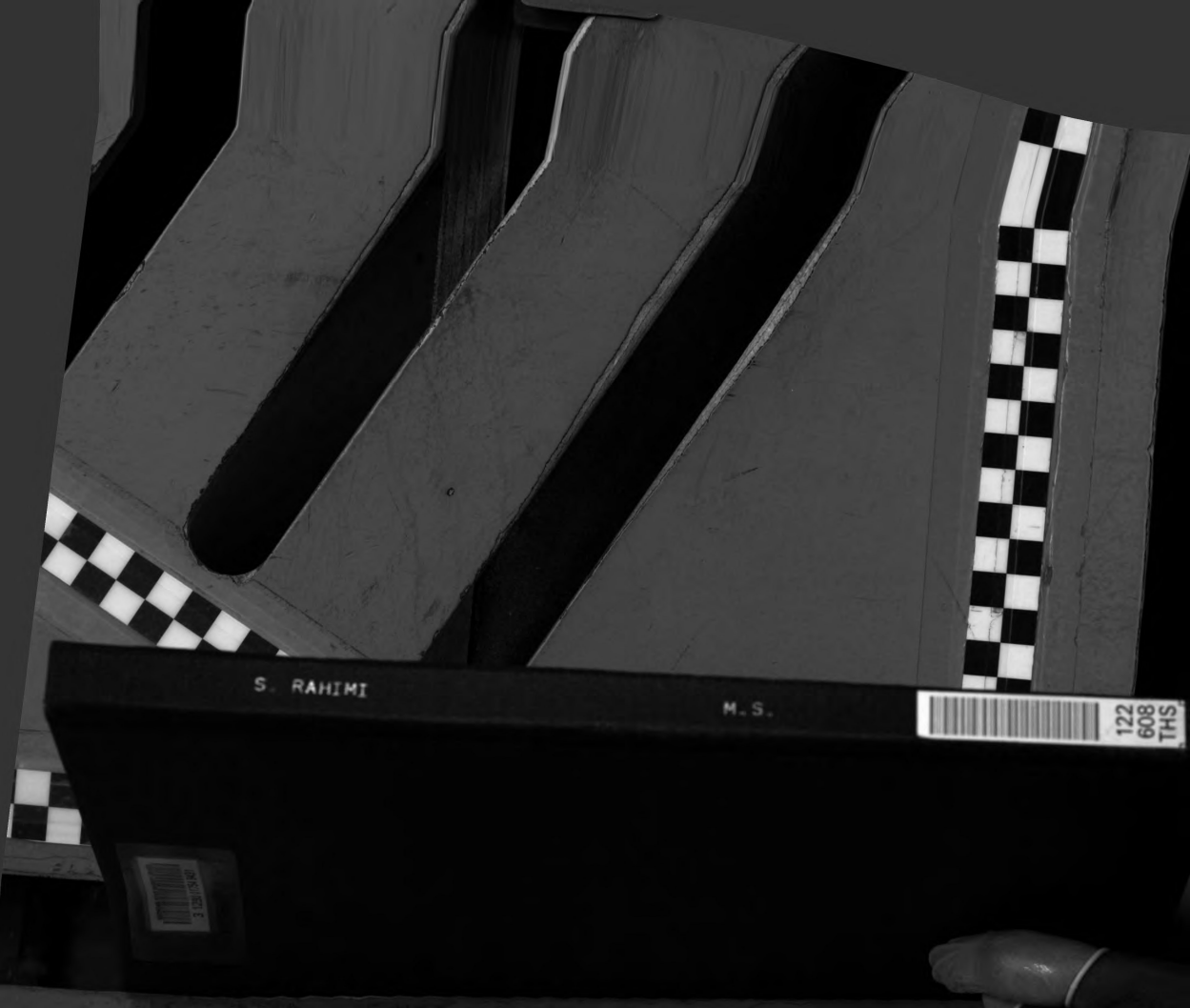
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This is to certify that the

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Shaban Rahimi

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PRODUCTION AND STANDARDIZATION OF MYCOPLASMA GALLISEPTICUM ANTIGEN

by

Shaban Rahimi

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ABSTRACT

PRODUCTION OF MYCOPLASMA GALLISEPTICUM

ANTIGEN

By

Shaban Rahimi

The economical importance of Mycoplasma gallisepticum (MG) infection in poultry makes control and eradication of this disease imperative. Because of world wide distribution of the disease, producing a low cost MG antigen, as well as good efficacy of the antigen for doing serological tests is important.

A medium was developed for producing MG antigen. This medium contains: PPLO-broth, brain heart infusion, trypticase, yeast extract, PPLO Serum Fraction and thallous acetate. The medium was used for MG antigen production for: serum plate test (SPT), tube test and hemagglutination-inhibation (HI) test.

A comparative study between the "test antigen" and a "control antigen" was performed. According to the results, the test antigen is superior to the control antigen.

Also, in comparing prices of the "test medium" with brain-heart infusion medium, PPLO medium, tryptose-phosphate medium, frey medium and control medium, the "test medium" cost the least to produce.

TO THE MARTYRS OF ISLAM

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INTRODUCTION

Mycoplasmosis is a term applied to diseases caused by Mycoplasma organisms. Mycoplasmas are the smallest free living bacteria, 200 to 800 nm in size, lacking cell walls and having complex nutritional requirements for multiplication. Approximately 20 serotypes of mycoplasma have been isolated in poultry but those most commonly associated with the diseases of poultry are Mycoplasma gallisepticum, Mycoplasma synoviae and Mycoplasma meleagridis. In poultry, the most important Mycoplasma problems involve the respiratory system or joints. It seems to be world-wide in distribution. Economic losses due to downgrading broiler products, reduction in egg production, inefficient use of feed and medication costs make mycoplasmosis one of the costliest diseases in the poultry industry. For example: In 1967 Mycoplasma airsaculitis cost 45 million dollars in the U.S. (USDA 1969). Also outbreak of MG infection from October 1979 through 1980 in market turkeys and broiler-breeder farms in north Carolina cost 2,000,000 dollars (Johnson, et al. 1981). Mason and Maiers (1984) reported that occurence of M. gallisepticum in turkey farms of the Monroe, North Carolina, between January and June of 1983 involved 304,000 turkeys and cost 550,000 dollars.

Isolation and identification of the Mycoplasma organism in infected birds is not always successful.

Serological tests are used routinely for detecting the diseases in a particular flock. The tests used most commonly to identify Mycoplasma gallisepticum (MG) are: the rapid serum plate agglutination test (RPT) and the tube agglutination test (TAT), which are followed by the hemagglutination-inhibition (HI) test for confirmation. Other methods also have been used to detect MG infection, for example: microtitration complement fixation (CF), fluorescent antibody technique (FA) and, recently, enzyme-linked immunosorbent assay (ELISA).

Because of the economical importance of MG infection in poultry, control and eradication of this disease is of great importance from an economic standpoint. The objective of this study was to develop, or choose, an MG antigen culture medium economical to produce as well as convenient to prepare to be used for a massive production of MG antigen, which in turn can be used for the purpose of eradication of Avian Mycoplasmosis. This would be beneficial to the poultry industries of both the developed and developing nations.

LITERATURE REVIEW

Infectious sinusitis of the turkey was first recorded by Dodd (1905) in England. Then Tyzzer (1926) described the same infection in turkeys in the USA. Nelson (1935) reported the relationship of coccobacilliform organisms with a case of infectious coryza in chickens. Later, he isolated this organism from embryonated eggs, tissue culture, and cell-free medium. This organism was identified as the PPLO organism by Markham and Wong (1952). A combination of Haemophilus gallinarum and avian PPLO was described by Adler and Yamamoto in 1956b. Adler et al. (1958) isolated two species of PPLO from chickens and turkeys. The PPLO organism which caused disease only in the turkey was named M. meleagridis by Yamamoto et al. in 1965. Another PPLO organism that caused swelling of joints (arthritis) was named M. synoviae by Olson et al. in 1964.

A. SEROTYPING

Many experiments have been conducted to classify various isolates of avian Mycoplasmas. Yamamoto and Adler (1958 a, b.) characterized 5 serotypes of Mycoplasma spp. Kleckner in 1960 described 8 serotypes designated A-H including the previous 5 serotypes. Yoder and Hofstad (1964) characterized 12 serotypes (A-L) and Dierk et al. (1967) described 19 serotypes, each designated by a letter A through S. Serotypes I, J, K, N, Q, and R which are closely related have been named Mycoplasma iowa. The typical

pathogenic serotypes A, H, and S remained separate and distinct (representing Mycoplasma gallisepticum, Mycoplasma meleagridis, and Mycoplasma synoviae respectively).

B. CLASSIFICATION

In nature Mycoplasma spp. have been isolated from both animals and plants. A number of species of avian Mycoplasma have been isolated from poultry, but as mentioned before, only three species (M. gallisepticum, M. meleagridis, and M. synoviae) are associated with disease in poultry. They belong to the class Mollicutes, order Mycoplasmatales, family Mycoplasmaceae. M. gallisepticum was suggested by Edward and Kanarek (1960) to be a typical pathogenic species which causes chronic respiratory disease in chickens and turkeys and also infectious sinusitis of turkeys. Olson et al. (1964) reported that the cause of infectious synovitis is M. synoviae. In 1965, Yamamoto et al. believed that M. meleagridis belonged to the H serotype. Distinguishing characteristics of three mycoplasma species are presented in Table 1 (Appendix).

C. CHARACTERIZATION

Characterization of avian Mycoplasma depends on biochemical reactions and serotyping of the organisms. Biochemical characterization of mycoplasma spp. from avian sources was studied by many authors (Fabricant, 1969; Freundt, 1974; and Yoder, 1975, 1984).

Avian mycoplasmas are very fastidious organisms requiring highly enriched media. Serum (10-15%) or serum substances are necessary for their growth. A temperature of 37 degrees centigrade to 38 degrees centigrade is favorable for growing of these organisms.

They are resistant to penicillin and thallium acetate, therefore these materials are frequently added to growth media to inhibit bacterial and fungal contaminants. Typical Mycoplasma colonies may be observed as early as 24 hours postinoculation on the plate agar medium, but Mycoplasma colonies are not visible for 3 to 5 days. Agar plates should be examined microscopically (35-50x) under low light for typical colonies. Very small characteristic colonies that grow into the agar and that have a raised center are seen on the solid medium.

Yoder (1984) described the characterization of avian Mycoplasma for example: Some avian Mycoplasma species reduce the tetrazolium chloride which acts as a growth indicator. They agglutinate chicken or turkey red blood cells, and lyse horse erthrocytes. M. synoviae requires the addition of reduced nicotinamyse adenine dinucleotide to the growth medium.

Serological tests are necessary to definitely identify avian Mycoplasma. And as mentioned before agglutination tests are the most common procedures for the primary detection of mycoplasmosis in a flock. But some

other procedures may be required to confirm the agglutination results. More details about serologic tests will be explained in the section on diagnosis of diseases.

D. L-PHASE ORGANISMS

Mycoplasmas are the smallest free-living organisms without cell walls and have a potential reversibility to their original form (Gordon and Jordan, 1982). McKay and Taylor (1954), and Kelton et al. (1960) reported that PPLO organisms isolated from chicken were L. form bacteria. (L-Phase bacteria do not have a rigid cell wall and have a potential reversibility to the original form.) (Klieneberger, 1935).

Mycoplasma gallisepticum Infection

Mycoplasma gallisepticum is recognized as the causative agent of chronic respiratory disease (CRD) in chickens and infectious sinusitis in turkeys. It is also considered the primary agent of "airsac disease" in chickens, a disease complex of multiple etiology.

M. gallisepticum infection is characterized by respiratory rales, coughing, nasal discharge, facial swelling (especially in turkeys) which may cause closing of one or both eyes. M. gallisepticum infection by itself in chickens is usually a relatively mild respiratory infection. It can be complicated by secondary bacterial infection like: E. coli, fowl cholera, and infectious coryza. Also by the presence of "stress factors" such as: newcastle disease,

infectious bronchitis, vaccination, weather changes, poor management, and nutritional deficiency. These factors enhance the severity of Mycoplasma gallisepticum infection (Gross, 1961a).

"Airsac disease" which is particularly a problem in broilers is characterized by massive air sac inflammation, perihepatitis and pericarditis. In this case the liver and heart are covered by fibrinous or fibrino purulent materials. Infection is followed by reduced feed conversion, decreased egg production, loss of weight, downgrading of carcasses, increased medication costs, severe mortality that cause the high economic loses. It appears to be worldwide in distribution.

M. gallisepticum has no human public health significance (USDA, 1969). There is no evidence of involvement of Mycoplasma of avian origin in diseases of man or other animals.

a. Chemical Composition of M. gallisepticum

M. gallisepticum is a bacteria-like agent composed of a three-layered membrane, without bacterial walls. Plasma membrane covers the cell and its components. It consists of 50 to 60% protein, 30 to 40% lipid and a very little amount of carbohydrate (Razin, 1963). The membrane can be separated from the cell by osmotic lysis (Rottem et al. 1968) or by mechanical pressure using sonic or ultrasonic oscillators (Pollack, et al., 1965).

Approximately 80% of the whole cell component is water, and 20% dry weight containing protein, lipid, carbohydrate, RNA and DNA (Morowitz et al., 1962). Using electron microscopy by Morowitz et al. (1962) showed the absence of nuclear membrane in Mycoplasma gallisepticum. Also they found that the genome of Mycoplasma was much smaller than the genome of the common bacteria.

Maniloff et al. (1965), and Domermuth et al. (1964) reported that the ribosomes of MG were arranged in cylindrical or helical corncob structures of over 50 ribosomes each.

Protein synthesis in Mycoplasma can be inhibited by chloramphenical and erytheromycin (Turtellotte et al., 1967).

b. Growth Requirements

Growth of M. gallisepticum requires some enrichment materials. One of the major requirements is 10-15% heat-inactivated horse, avian or swine serum or serum factors. If avian serum is used it should be free of MG antiserum. The organism grows very well in suitable media at pH 7.8 and a temperature of 37 to 38 degrees centigrade. Nutritional requirements such as lipids (especially cholesterol), proteins (amino acids), and carbohydrates (dextrose) are usually added to the growth medium. Tween 80 and diacetyl tartaric acid ester of tallow monoglycorides (TEM-4T) as a source of fatty acids are found useful to the growth of

avian Mycoplasma (Lund and Shorb, 1966). Some kinds of vitamins such as nicotinic acid, thiamine, riboflavin, and nicotinamide adenine dinucleotide are required for the growth of mycoplasma (Tourtellotte et al., 1964). Yeast is beneficial to the growth of some mycoplasma. Formulations of some typical mycoplasma media which are very commonly used are presented in Table 2 (Appendix).

Embryonated chicken eggs have been used for propagation of M. gallisepticum. Yolk materials also have been used for storage of MG frozen at -20 to -80 degrees centigrade. Penicillin and thallium acetate frequently are used in Mycoplasma media to avoid growing other contaminant bacteria or fungi.

c. Colonial Morphology

After the incubation of inoculated agar plates for 3.5 days at 37 degrees centigrade and moist atmosphere, colonies appear as tiny, smooth, circular, translucent masses with an elevated, more dense center. The size of the colony is between 0.1 to 1 mm in diameter. The best way to observe the colonies is by using the indirect light microscope with approximately 20 to 50x magnification.

Colonies can be transferred very easily with a wire loop from the inoculated agar plate into broth media or inverted and spread over the surface of a new plate of agar medium. For transferring the colonies one may pick up a single colony or pick up an excised agar block.

d. Morphology and Staining

M. gallisepticum is a pleomorphic (coccoid, spherical or elongated) organism with a diameter of 0.15 to 0.5 um (Morowitz et al., 1962). In staining with Giemsa stain the organism becomes weakly gram-negative (Yoder, 1984).

d. Biochemical and Biological Properties

Hemagglutination of chicken and turkey red blood cells, and complete lysing of horse red blood cells was reported by Yamamoto and Adler (1958b). M. gallisepticum ferments glucose and maltose with production of acid but not gas. It does not ferment lactose, dulcitol, or aslicin. Sucrose is rarely fermented, but results with galactose, fructose, trehalose and manitol are variable. It is arginine negative (Yoder, 1984). Tetrazolium (2,3,5-triphenyl-2H-tetrazolium chloride) and phenol red are commonly added into the medium as a growth indicator (Yoder and Hofstad, 1964).

e. Resistance to Chemical and Physical Agents

M. gallisepticum is resistant to penicillin and thallous acetate (1:4000), thus to prevent bacterial contamination, penicillin and thallous acetate can be used as preservatives to MG culture media. Beta propiolactone can inactivate Mycoplasma spp. (Roberts, 1964). MG also is susceptible to some antibiotics such as: streptomycin and tylosin. Sensitivity of the organism to oxytetracycline and

chlotetracyclin has been reported by many researchers (Fahey, 1957; Domermuth, 1958; Kiser et al., 1961; and Yoder et al., 1961). Therefore, medication of infected breeders with specific antibiotics, and antibiotic treatment of hatching eggs from infected flocks, have been used to reduce the level of egg transmission. Also MG can be inactivated in infected hatching eggs by heating at 45.6 degrees centigrade for 12 to 14 hours (Yoder, 1970). Mycoplasma gallisepticum survives in frozen yolk materials at -20 to-80 degrees centigrade for several years (Chandiramini et al., 1966). Survival of the organism in liophilized milk, yolk, or serum broth at 4 degrees centigrade for several years was reported by Yoder and Hofstad (1964).

f. Strain Classification

There are various strains of M. gallisepticum such as California S6, Massachusetts A5969, Iowa 801, Fowls and 54-537. Only three strains (A5969, Iowa and S6) are used for antigen production.

g. Pathogenecity

Yoder in 1984 reported that pathogenecity of M. gallisepticum varies in different hosts based on the nature of isolate, method of propagation, route of infection, and number of organisms. Complications of disease with secondary infection, nutritional deficiency or stress factors increase the pathogenecity of the organism. Pathogenecity of MG in turkeys is more severe than in

chickens. Inoculation of the microbe into the yolk of chicken embryo eggs causes death of the embryo within 5-7 days post infection. Dwarfing, generalized edema, liver necrosis and enlarged spleen are typical lesions in dead embryos (Yoder and Hofstad, 1964).

h. Pathogenesis and epizootiology

(1) Natural and experimental hosts

Birds of any age may be affected but the severity of disease in younger birds is more than in older ones. Infection usually results in an immune response which provides some degree of protection. M. gallisepticum infection occurs naturally in chickens, turkeys and other species of poultry in the whole world.

(2) Transmission

M. gallisepticum can be transferred from infected parents (chickens and turkeys) through the egg to the embryos (Lin and Kleven, 1981). The organism spreads very rapidly by direct contact from bird to bird within a flock but transmission of disease between flocks or houses tends to be slow or may not occur. Spread of the microbe by dust or droplets was reported by Fahey and Crawley (1955a). The agent may be transmitted mechanically on shoes, feed sacks, and crates, but it can not survive more than several hours outside the host. Control of the disease on multiple-age intensive sites is difficult.

The spread of M. gallisepticum is influenced by other respiratory disease agents and by stress factors.

(3) Incubation period

In natural conditions, because of the influence of many other factors in the onset and extent of the infection, determining the exact data of exposure is difficult. Incubation periods of between 4-21 days in experimental transmission have been reported by some investigators.

i. Signs

M. gallisepticum mainly affects the respiratory system and causes difficulties in physiological function of this system. Clinical signs include coughing, sneezing, moist rales, breathing through the open beak, and swelling of one or both infraorbital sinuses, especially in turkeys, which may result in closing of the eyes. Also birds may be observed shaking their heads and attempting to clear their nostrils on the wing feathers. Watery eyes and conjunctivitis may also be present. The signs just described are usually seen in young or growing chicks or poults. In broilers 3-8 weeks old, signs are more severe; feed consumption and growth rate are reduced. In adults signs are usually mild and consist of slight eye or nasal discharge and occasional coughing and head shaking together with some loss of feed consumption and egg production. Egg production continues at a lower level.

If only the airsacs are affected and infected by MG there are no specific clinical signs. But presence of secondary infections (such as E. coli, fowl cholera, coryza) or stress factors (poor husbandry, malnutrition, etc.) with mycoplasmosis results in a complicated form of the disease, which is more severe than mycoplasma infection alone.

j. Morbidity and Mortality

The severity of MG infection in young chickens is more pronounced than in adults (Yoder, 1984). In broilers (3-8 weeks of age) mortality is variable but usually is higher than in laying hens (Whiteman and Bickford, 1983). In adult layers mortality is low, but there may be many unvigorous birds. Mortality in complicated infections is greater than in uncomplicated forms.

k. Gross Lesions

Catarrhal inflammation of the nasal passage, sinuses, trachea, bronchi and air sacs is presented. Sinusitis is most commonly seen in turkeys. Airsacculitis, perihepatitis and pericarditis are usually seen in this disease. The lesions are more severe in the complicated form of CRD (Gordon and Jordan, 1982).

l. Histopathology

Thickness of the mucous membrane of affected tissues from infiltration with mononuclear cells and hyperplasia of the mucous glands was reported by Van Roekel et al. (1957). Airsacs may be as much as 8 to 10 fold thicker than normal.

Multiple foci of epithelial cell hypertrophy, degeneration, and necrosis probably represents sites of attachment and colonization by mycoplasma organisms. Nodular lymploid cell foci and fibrous connective tissue are more commonly seen in older lesions (Kleven and Fletcher, 1983).

m. Diagnosis

The criteria for diagnosis are signs, lesions (if present), serological tests, isolation and identification of M. gallisepticum.

(I) Bacteriological diagnosis

Trachea, air sacs, sinuses, nasal passages, and respiratory exudates can be sampled with sterile swabs and directly cultured in suitable broth or agar overlay medium. Serial passages to a new broth tube every two to three days enhance the multiplication of the organisms. Culture on agar medium should be incubated at 37 degrees centigrade in a high humidity for 3-5 days. The colonies appear small and smooth, with dense elevated centers (fried egg colonies) under low power of stromicroscope.

An individual colony can be picked with an inoculating needle and transferred to a broth medium. The broth is incubated for 3 days at 37 degrees centigrade and plated again. This procedure is repeated two or three times to obtain the homogeneous pure culture.

Another method to obtain a pure culture is to cut aseptically on agar block that contains only a single colony of Mycoplasma, then place the colony upside down on the surface of another agar plate and use "quadrant streak method" to isolate the colonies. Biochemical examinations are useful to identify the organism. Fermentation of dextrose and maltose is positive but for lactose and sucrose is negative.

Cultures can be further identified by preparing Giemsa-Stained smear that should reveal small cocoid organisms often in clumps when examined through the oil immersion lens of a microscope.

The inoculation of 7-day old embryonated chicken eggs via the yolk sac with original exudates may be used for further means of isolating M. gallisepticum. The inoculated embryos will die within 5-8 days.

(II) Serological diagnosis

Serological tests, including the serum plate test (SPT), tube agglutination test, and the hemagglutination inhibition test (HIT) are commonly used for diagnosing of M. gallisepticum infection in poultry.

(1) Serum plate test

In this method stained antigen of M. gallisepticum (0.03 ml) and serum (0.02 ml) from a chicken or turkey are mixed on a glass or porcelain plate. Positive serum causes clumping of the antigen within 2 minutes. Flocculation

occurring after 2 minutes should be considered as a doubtful reaction.

A variety of factors, such as: human errors, improper temperature of serum and antigen, inadequate amount of serum and antigen, and improper light for reading results, can influence the accuracy of the test.

The serum plate test is very useful in the preliminary determining of M. gallisepticum infection in the flock rather than individual bird infection.

(2) Tube agglutination test (TAT)

The unstained serum plate antigen can be used for tube agglutination test. The stock antigen should be diluted (1:20) with Phenolized (0.25 percent) phosphate-buffered saline for this test. The MG tube test is conducted by mixing 0.02-0.08 ml of test serum with 1.0 ml of diluted (1:20) antigen in a clean glass tube (12 x 75 mm) and incubating the tube for 18-24 hours at 37 degrees centigrade. The test is read against a dark background under indirect fluorescent light. Positive reaction appears as a clearing of the supernatant fluid with visible sediment in the bottom of the tube. Incomplete reaction is considered as suspect. Positive results should be confirmed by the hemagglutination inhibition (HI) test. For control of the tube test, positive and negative control serum must be included.

(3) Hemagglutination inhibition (HI) test

The HI test is used almost routinely to confirm various serum plate agglutination tests and tube agglutination tests. In this test system, positive serum should inhibit the hemagglutination (HA) activity of the antigen. Negative serum should have no effect. Inhibition will be evidenced by the formation of a free-flowing button of cells in the bottom of the tube. The titer of the serum can be calculated as the reciprocal of the highest dilution of serum that produces complete hemagglutination inhibition. In this test with 4HA units of antigen, the HI titers of 80 or greater are definitely considered as positive, while titers of 40 or less are suspicious; however, titers of 10 or 20 are considered negative. If serologic results from agglutination tests complemented by the HI test are inconclusive, for exact judgement repeating the test after 2 or 3 weeks is suggested (Yoder, 1975).

Positive and negative serum controls should be incorporated with this test. Cross reaction between M. synoviae and M. gallisepticum antisera and antigens has been reported by some investigators by doing serum plate test. Such reaction is not observed in the hemagglutination inhibition test.

Some other serology tests which have been used to determine M. gallisepticum infection in poultry are:

(a) Complement Fixation Test (CFT)

The complement fixation test to detect the antibody of MG in chickens and turkey's sera, has been described by Frey and Hanson (1969), Marguardt and Newman (1971). They found that sensitivity and specificity of the CFT was as good as the HI test.

(b) Metabolic inhibition test (MIT)

This method was used by Barber and Fabricant 1971 for classification of MG. They used hyperimmune rabbit antisera for this test. Sensitivity of this test was reported.

(c) Fluorescent antibody technique (FA) (Immune-Fluorescent technique = IF).

The fluorescent antibody test is one of the most important diagnostic techniques in modern medicine. It can be used to diagnose many pathogenic agents such as: viral, bacterial, parasitic, and mycotic infection as well as different strains of mycoplasmas. For diagnosing of the M. gallisepticum by FA technique, many different materials can be used such as: tissue sections, tissue cultures, smears from various organs, exudates or frozen tissues (Goldman, 1968).

Two methods are very common for performing the FA test: the direct method and the indirect method. In the first method, fluorescein-labeled antibody is applied to a preparation containing the corresponding antigen. The indirect method consists of a two-step procedure: the first step allows the reaction of the antigen with a specific antibody. The second step entails the addition of antiglobulin labeled with fluorescent dye.

The immunofluorescent technique has been used successfully to identify MG infection (Corstvet and Sadler, 1964 and Noel et al., 1964).

(d) Agar gel Precipitin test (AGP)

Aycardi et al. (1971) used the gel-diffusion and growth inhibition tests to classify avian mycoplasmas. Using this test they classified 19 serotypes of mycoplasma into 9 groups. The agar-gel precipitin test was used to evaluate chicken serum for MG infection studied by Sahu and Olson (1976). They found it to be very useful in confirming reactor flocks and detected antibodies to isolate WVU 907, which did not produce meaningful HI titers to standard MG antigens.

(e) Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA has recently been adapted for the diagnosis of several viral and bacterial infections in human medicine and veterinary medicine as well as infection in poultry.

The ELISA technique was used for the first time in 1980 by Ansari to detect Mycoplasma infection in chickens sera (also see Ansari et al., 1982). They found this test more sensitive than the agglutination tests, but cross-reaction of MG and MS was reported.

(f) Immunoperoxidase test

The immunoperoxidase test system was reported as a simple and reliable method for identification of MG and MS cultures (Imada et al., 1979). And in comparing with TAT, and HI, the immunoperoxidase test was highly effective and specific for detecting antibodies against MG and MS.

n. Differential Diagnosis

In chickens MG infection must be differentiated from other respiratory diseases such as: infectious bronchitis, Newcastle disease, coryza, M. synoviae and fowl cholera. Certain serological tests and cultural methods are valuable to identify the main pathogenic agent (Yoder, 1984). In turkeys M. meleagridis, M. synoviae, fowl cholera, ornithosis, avian influenza and vitamin A deficiency may cause respiratory system problems and sinusitis as well as M. gallisepticum (Yoder, 1984).

o. Treatment

Treatment of M. gallisepticum-infected flocks can be helpful to prevent respiratory signs, airsacculitis and drops in egg production. Different antibiotics, such as: tylosin, tetracyclines, erythromycin, a combination of

spectinomycin and lincomycin, gentamycin and some other broad-spectrum antibiotics, have been used for the treatment of MG infection (Womermuth, 1958 and 1960; Domermuth and Johnson, 1955; Hamdy et al. 1957; Fahey, 1957; Kister et al., 1961, and Yoder et al., 1961). Tylosin is one of the major antibiotics which is used for the treatment of M. gallisepticum infection.

p. Prevention and Control

Because of the economic significance of the MG infection, control and prevention of this disease in the poultry industry has become very important.

Several methods have been used to control MG infection in chickens and turkeys. Most important of these methods are:

(1) Egg dipping

Antibiotic treatment of hatching eggs from infected flocks has been used to reduce the level of MG egg transmission. This may be done either by immersing pre-incubation eggs (heated to 37 to 38 degrees centigrade) into the solution at 2 to 4 degrees centigrade or by immersing the eggs in a solution at room temperature in a special container, reducing the pressure by evacuation of air and then permitting the pressure to return to atmospheric. By both methods the antibiotic solution is drawn through the shell into the egg (USDA, 1969).

(2) Egg inoculation

Embryo inoculation with antibiotics has been done experimentally by some scientists for eliminating egg transmission of M. gallisepticum. Smit and Hoekstra (1967) injected tylosin into the air cell of hatching eggs, but their preliminary results were inconclusive. Truscott and Ferguson (1975) reported that inoculating of hatching eggs with a combination of lincomycin-spectinomycin was effective in control of MG transmission through the eggs.

(3) Egg heating

Heat treatment of hatching eggs to break the transmission cycle of M. gallesepticum was reported by Yoder (1970). In this study the embryonated eggs were incubated in a forced-air incubator for 12 to 14 hours until the internal temperature of eggs reached 46.1 degrees centigrade. By doing this, M. gallisepticum and M. synoviae became inactivated, but hatchability was reduced 8 to 12 percent.

(4) Medication of Breeders

The medication of infected breeder flocks or their progeny with antibiotics reduces the rate of MG infection, but it does not make entirely infection-free flocks.

(5) Immunization

Vaccination of the replacement pullets during the rearing period with live F strain of MG vaccine has been used with some degree of success to eliminate MG infection.

Glisson and Kleven (1983) reported lower levels of egg transmission of MG and higher egg production in vaccinated chickens than in unvaccinated controls. Also Hildebrand et al. 1983 reported higher production, and larger grade of eggs and better feed conversion in chickens vaccinated with MG-Bacterin, than in chickens vaccinated with live-culture. They concluded that the oil-emulsified MG bacterin is safe and highly effective.

(6) Management and sanitation

- Eradication of M. gallisepticum in primary breeder stocks.
- Keep breeder flocks clean of MG infection.
- Acquire hatching eggs from flock free of MG.
- Maintain breeder flocks on farms separate from grower flocks.
- Minimize contact of breeder flocks with game and free-flying birds.
- Prevent transmission from outside sources by indirect contact through contaminated equipment, footwear, clothing, and vehicles.
- Keep the rodent population and other pests under control.
- Provide adequate isolation for breeder flocks to avoid airborne transmission from other flocks.
- Sanitation of buildings and premise.
- Cleaning, disinfecting, and fumigating of hatcheries.
- Fumigation of incubators.
- Disposal of hatching waste.

- Dispose of all dead birds properly and promptly.
- Keep hatchery and incubator room well isolated from battery room.

MATERIAL AND METHODS

Bacterial selection for antigen production: To produce a high quantity of Mycoplasma antigen the bacteria strain must possess the following properties:

1. High yeild in a good quality growth media.
2. Suitable for the production of plate agglutination, tube agglutination, and hemagglutination antigens.
3. Highly specific with minimal nonspecific reaction.
4. Good stability.

Three strains have been most commonly used for antigen production, California S6, Iowa 801, and Massachusetts A5969.

M. gallisepticum strain S6 was selected for the preparation of antigen in our experiment. This isolate was received from the USDA, Agricultural Research Service, Ames, Iowa, via the clinical laboratories, College of Veterinary Medicine, Michigan State University, East Lansing,Michigan.

Production Media:

The medium chosen for antigen production would depend on: (1) the adaptability of the MG strain to the medium, and (2) the availability of ingredients M. gallisepticum requires a rather complex medium with 10 to 15% heat-inactivated (30 minutes at 56 degrees centigrade) and filterized horse, swine or turkey (MG free) serum. Three commercial media were commonly used as a base for large-volume antigen production:

a) Brain heart infusion

b) Tryptose phosphate broth

c) PPLO-broth

Ingredients of these media are presented in Table 3 (Appendix).

The culture medium that was used in this experiment was formulated as follows:

PPLO broth	10.5 g
Brain heart infusion	18.5 g
Trypticase	5.0 g
Yeast extract	2.5 g
Thallium acetate	0.1 g
Distilled water	1000.0 ml

The pH was adjusted to 7.8 with 10 percent NaoH. The medium was dispensed in tubes (4 ml each) and Erlenmeyer flasks (500 ml, 2000 ml). They were sterilized for 15 minutes at 121 degrees centigrade with 15 pounds of steam pressure. The sterile media was held at 4 degrees centigrade until used.

Before the inoculant of Mycoplasma gallisepticum culture into the broth medium, 10 percent filtered sterilized and heat inactivated horse serum or 0.5 percent PPLO serum fraction was added to the medium.

The agar medium which was used in this study was PPLO-agar medium. For preparing this medium, 35 grams of PPLO agar was completely dissolved in 1000 ml of distilled

water, then sterilized by autoclaving at 15 pounds pressure and 121 degrees centigrade for 15 minutes. The sterile agar was cooled to 50 degrees centigrade in a water bath, then heat-inactivated horse serum was added (10%) and mixed by gentle swirling, and the enriched medium was poured into sterile petri dishes. Agar plates were left at 37 degrees centigrade overnight to ascertain their sterility and then stored at 4 degrees centigrade until used.

Storage of the organism

Some cultures of M. gallisepticum were injected into specific pathogen-free (SPF) embryonated chicken eggs (7 days) and stored in the frozen egg yolk at -40 degrees centigrade. The procedure for the propagation of the organism in yolk was as follows:

1) Twenty 7-day old SPF chicken eggs embryos were obtained at three different times from the USDA, Regional Poultry Research Laboratory, East Lansing, Michigan.

2) The large end of the eggs was sterilized with the alcohol or iodine.

3) A small hole on the top of the cleaned (sterilized) portion of the eggs was made.

4) Approximately 0.2 ml of M. gallisepticum suspension were inoculated into the yolk sac, using a 27 gauge, 1/2 inch needle.

5) The hole was sealed with liquid paraffin.

6) The inoculated, embryonated eggs were incubated at 37 to

38 degrees centigrade. Death of inoculated embryos occurred usually 4-5 days after inoculation.

7) After cleaning the large end of the dead embryonated eggs with alcohol or iodine, that portion of the shell was removed with scissors.

8) The dead embryo was picked out with a sterile forceps.

9) Yolk material was poured into a sterile petri dish. Approximately 0.2 ml of this yolk was injected again into the yolk sac of a 7 day chick embryo (5 embryonating eggs). Serial passages were made three times in chick embryos.

10) Yolk material was dispensed into small vials and held frozen at -40 degrees centigrade.

11) For controlling contamination, each time before the new passage, approximately 0.2 ml of cultured yolk was inoculated into the broth medium and incubated for 24 hours at 37 degrees centigrade. Then 0.1 ml of this medium was transferred onto the agar plate for cloning.

Production of MG antigen

The broth medium described previously was used for antigen production. Three different volumes were used in subsequent stages:

1) Tubes containing 4 ml of the broth medium were used for serial passages and stock cultures to obtain the organism in maximum growth. .2 ml to .3 ml of MG Seed was transferred into each one of these tubes. Usually tubes were inoculated and incubated at 37 degrees centigrade for 24-48 hours.

2) Production flask no. 1: 1000 ml Erlenmeyer flasks containing 500 ml of broth medium. 12 ml of 24-48 hour culture (from 1) was transferred to the 500 ml of medium. After mixing by gentle swirling, it was incubated for 1 or 2 days at 37 degrees centigrade. A sample of this medium was cultured on a PPLO agar plate to determine whether it is free from contaminants. The inoculated agar plate was incubated at 37 degrees centigrade and very moist conditions for 5-10 days. After 24 hours, plate was examined each day for growth of the organism by indirect light microscope at 20x-50x lens power. Typical colonies .1-1mm in diameter with a raised center were observed microscopically after 48 hours incubation.

3) Production flask no. 2: 3000 ml Erlenmeyer flask containing 2000 ml of broth medium. The contents of flask no. 1 were transferred into the flask no. 2 and mixed with gentle rotating, then incubated at 37 degrees centigrade for 40-48 hours. A culture on PPLO agar for control of contamination was carried out.

Harvesting

After sufficient incubation, contents of production flask no. 2 were centrifuged by using refrigerated sharples centrifuge for 30 minutes at 7000 rpm and 4 degrees centigrade. Phosphate buffer saline containing 0.01 percent merthiolate was used as a diluent and for washing media. Phosphate buffer saline was buffered to a pH of 6.0 for

plate antigen and 7.0 for tube antigen. The concentrated paste or suspension was kept at 4 degrees centigrade until final standardization. Buffered saline without merthiolate at pH 7.1 was used for harvesting the hemagglutination antigen. Equal quantities of this buffered saline, and glycerol were added to dilute the paste taken from the centrifuge. The HA antigen was kept frozen at -40 degrees centigrade until final standardization tests.

Before storing the harvested cells, they were homogenized by using an electrical shaker and magnetic bar for approximately 4 hours. Merthiolate was used as preservative for the antigen.

Standardization of MG antigen

The suspended antigen was diluted to a final concentration approximately equal to 2 times no. 10 McFarland nephelometer. Comparing the suspended antigen with the McFarland scale no. 10 was done visually toward a bright source (window).

Staining of antigen

After standardization of the antigen it was stained by 1% crystal violet as a final dye concentration of 1:10,000 for the serum plate test. Tube antigen and HA antigen are not required for staining.

Storage of the antigen

After adding 1% bovine albumin fraction v powder to the plate antigen and tube antigen, both of those antigens

were stored at 4 degrees centigrade. For the HA antigen equal quantities of buffered saline and glycerol were added and kept frozen at -40 degrees centigrade. No other preservative was added to the HA antigen.

Evaluation of the antigen for sensitivity, specificity, and stability

Before using the M. gallisepticum antigen for routine serology tests it should be standardized by doing certain tests. Those tests are run after 2-4 weeks of keeping the antigen at 4 degrees centigrade.

1. Sterility tests:

After storing the antigen for 2 weeks, sterility tests were conducted to determine the bacterial and fungal contamination and inactivation of MG. The following media were used for these tests: blood agar, PPLO agar, and Sabouraud dextrose medium (broth and agar).

2. pH determination:

Satisfactory pH for M. gallisepticum plate antigen is 6.0-6.3 and for MG tube antigen, 7.0 + or - 0.1. pH of antigens was determined by using the pH meter.

3. Homogeneity

The spot plate test was used to determine homogeneity of the plate antigen. With this method the plate antigen must be free of grossly visible particles.

4. Sensitivity and specificity tests

Sensitivity of the antigen should be checked by standard chicken and turkey antiserum of various titers. A known standard antigen was used for direct comparison of the results. The test antigen and the reference antigen should have the same agglutination reaction with most of the positive serums. And negative serum should have negative reaction in this test.

Specificity of MG antigen is determined by cross titration with antiserum of M. synoviae and M. meleagridis.

Procedures for serology tests were based on the methods previously described in the serological diagnosis of M. gallisepticum infection. MG-positive and MG-negative serum should be included in all the tests. Mycoplasma gallisepticum antiserum and MG reference standard antigens were obtained from The National Veterinary Service Laboratory (NVSL), Ames, Iowa.

Test Procedures:

The following tests were used to evaluate the sensitivity of the antigens:

1) Rapid serum plate agglutination test.

The tests were conducted by contacting and mixing 0.02 ml of each test serum with 0.03 ml of serum plate antigens placed on a glass plate at room temperature. The plate was rotated for a few seconds. At the end of the first minute, the plate was rotated again for 5 seconds. The results were read in two minutes. Degree of reactions

was recorded.

2) Tube agglutination test

The tests were conducted by mixing 0.08 ml of test serums with 1.0 ml of diluted (1:20) antigen in a glass tube (12 x 75 mm) and incubating the mixture overnight at 37 degrees centigrade. The results were read at the end of incubation time.

3) Hemagglutination inhibition test

The tests were conducted by the constant antigen decreasing-serum method. A 4 unit antigen system was used for this test.

Hemagglutination (HA) antigen titration performed as follows:

1. Racked a series of 11 clean glass tubes 12 x 75 mm and labelled the tubes from 1 to 11, left to right.
2. Put 0.8 ml of PBS in tube 1, put 0.5 ml of PBS in each of tubes 2-11.
3. Added 0.2 ml of HA antigen to tube 1 (dilution of antigen 1:5).
4. After mixing the contents of tube 1, transferred 0.5 ml to tube 2 and continued making serial twofold dilutions of antigen through tube 10. Pipette was discarded after each transfer. Antigen dilution ranging from 1:5 to 1:2560. 0.5 ml of contents of tube 10 was discarded.
5. Added 0.5 ml of 0.25 percent of chicken washed RBC's with Alsever's solution to tubes 1-11. Tube 11 was

saline/RBC control.

6. Mixed contents in the tube by shaking the racks and incubated at room temperature until the cells in tube 11 settled into a compact button at the bottom of the tube.

7. Steps 1 to 6 should be repeated with turkey RBC when testing turkey sera.

HA antigen titration is illustrated in Table 3. After titration of hemagglutination antigen, the hemagglutination inhibition (HI) test proceeded as follows:

1. Racked 11 clean glass tubes 12 x 75 mm. The tubes were labeled 1 to 11 from left to right.

2. Put 0.8 ml of PBS in tube 1, 0.5 ml of 8 unit antigen in tube 2, 0.5 ml of 4-unit antigen in tube 11.

3. Added 0.2 ml of serum to tube 1 (serum control). After mixing transferred 0.5, ml from tube 1 through tube 10. Discarded 0.5 ml from tube 10. Serial dilution of the serum was 1:5 - 1:2560. Pipette was changed at each transfer.

Table 3. Titration of Hemagglutination (HA) Antigen

Reagents (ml)	Tube 1	2	3	4	5	6	7	8	9	10	11(a)
Saline	0.8	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Antigen	0.2										
Transfer	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5(c)	
0.25% RBC (b)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ant. dilution	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	

(a) Tube 11, Saline/RBC Control

(b) RBC, red blood cells

(c) Discard 0.5 ml

4. Racked five 12 x 75 glass tubes and labeled them from 1-5, left to right to set up an antigen control.

5. Put 1.0 ml of 4 unit antigen in tube 1, and put 0.5 ml of PBS in tubes 2-5.

6. Transferred 0.5 ml from tube 1 to 2 and continued through 5. Discarded 0.5 ml from tube 5. Series units of antigen were 4, 2, 1, 1/2 and 1/4.

7. Added 0.25 percent washed homologous RBC to each tube. After mixing the contents by shaking the racks then were incubated for hemagglutination titration.

In the hemagglutination inhibition (HI) test the positive serum inhibited the HA activity of antigen by the formation of a free-flowing button of cells in the bottom of the tube. The titer of the serum was calculated as the reaction of the highest dilution of serum that produced complete HI. The hemagglutination-inhibition (HI) test is presented in Table 4. The antigen control test is illustrated in Table 5.

With this test system and four units of antigen, HI titers of 80 or above were considered positive, titers of 40 or less were suspicion, and titers of 20 or less were considered negative.

Table 4. Hemagglutination Inhibition (HI) Test

Reagents (ml)	Tube										
	1(a)	2	3	4	5	6	7	8	9	10	11(b)
Saline	0.8	0	0	0	0	0	0	0	0	0	0.5
8 unit antigen	0	0.5	0	0	0	0	0	0	0	0	0
4 unit antigen	0	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0
Test Serum	0.2	0	0	0	0	0	0	0.0	0	0	0
Transfer	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5(c)
0.25% RBC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Serum dilution	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	

(a) Tube 1, serum control

(b) Tube 11, Saline/RBC control

(c) Discard 0.5 ml

Table 5 Antigen Control

	Tube				
Reagents (ml)	1	2	3	4	5
4-unit antigen	1.0	0	0	0	0
Saline	0	0.5	0.5	0.5	0.5
Transfer	0.5	0.5	0.5	0.5	0.5(a)
0.25% RBC	0.5	0.5	0.5	0.5	0.5
unit ant/tube	4	2	1	1/2	1/4

(a) Discard 0.5 ml

References:

1. Anonymous (1972)
2. USDA (1966)
3. USDA (1966)
4. USDA (1985)
5. Yoder (1975)
6. Yoder (1984)

RESULTS

1. Growth of organism

The culture of Mycoplasma gallisepticum was received in unknown passage from Clinical laboratories, College of Veterinary Medicine, Michigan State University. After taking the organism from a frozen state it was passed 10 times in the production medium until maximum growth was obtained in 24 hours. A 2% innoculation of a 24 hour culture was introduced into the 500 ml production medium and incubated for 40-48 hours at 37 degrees centigrade. After showing enough turbidity it was transferred to 2000 ml of antigen production medium and allowed to grow for 40-48 hours at 37 degrees centigrade depending on the pH and turbidity of the medium. Incubation was stopped when the pH had dropped to the appropriate degree (6.6 - 6.9).

Production flasks were always checked to be free from contaminants by culturing broth cultures on PPLO agar plates.

2. Harvesting the organism

Harvested cells become homogenous by using a magnetic bar and electrical shaker for 4 hours.

3. Standardization of MG antigen

The standardization tests were performed for both "test antigen" and "control antigen".

a) Density requirements

MG antigen was diluted by PBS to a concentration approximately equal to 2 times no. 10 McFarland nephelometer. After determining the concentration of the antigen it was stained by crystal violet (1:10000) for preparing plate antigen. No stain was used for the tube antigen. The HA antigen was diluted by equal quantities of buffer saline and glycerin and held at -40 degrees centigrade.

b) Homogeneity

No autoagglutination or gross visible particles were observed in plate antigen ("test" or "control").

c) Purity and sterility

The results of these tests are presented in Table 6.

Table 6 Sterility Tests

	Culture Media		
Antigen	Blood agar	Sabouraud's Dextrose	PPLO agar
Test antigen			
Plate antigen	-	-	-
Tube antigen	-	-	-
HA antigen	-	-	MGx
Control antigen			
Plate antigen	contaminated	-	contaminated
Tube antigen	-	-	-
HA antigen	-	-	MGx

d) Hydrogen ion concentration

The hydrogen ion concentration was determined with a pH meter which had been standardized with a pH buffer just prior to use. The pH of the MG plate antigen was 6.0, and the pHs of the tube and HA antigens were approximately 7.1-7.2.

e) Sensitivity

1. Serum plate test

After mixing the test MG plate antigen with MG antiserum a positive reaction showed immediately by blue colored flocculation. (Table 7).

2. Tube test

Positive agglutination was observed by clearing of the fluid in the upper portion of the tube with aggregates of the cells in the bottom of the tube. (Table 8)

3. Hemagglutination (HA) test

The end point in the antigen titration was the highest dilution of antigen which gave complete agglutination of all the RBC as evidenced by a complete and uniformly thin deposit over the entire inner surface of the tube after incubation.

Table 9 shows the HA titration results. Hemagglutination occurred in the first 8 tubes (end-point 640) of test antigen. Therefore titer of the "test antigen" was 640 and each 0.5 ml of this dilution had one HA unit. And tube 6 (dilution 1:160) had 4 HA units and dilution 1:80 had 8 HA units. In control tubes hemagglutination occurred in first 5 tubes (end-point 80).

Table 7 Serum Plate Test

Antigen	Positive Serum	Negative Serum
Test antigen	Blue color clumps observed quickly after mixing antigen and antiserum ++++	No agglutination -
Control Antigen	Pink color clumps showed slowly within 2 minutes of mixing antigen and antiserum +++	No agglutination -

Table 8 Tube Agglutination Test

Antigen	Positive Serum	Negative Serum
Test Antigen	Large visible sediments occurred in the bottom of the tube	No agglutination
	++++	-
Control Antigen	Agglutinates were less than "Test antigen"	No agglutination
	+++	-

Table 9. Result of Hemagglutination (HA) titration of antigens

	Tube										
	1	2	3	4	5	6	7	8	9	10	11(a)
Ant. dilution	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Test Antigen	+	+	+	+	+	+	+	+	-	-	-
Control Antigen	+	+	+	+	-	-	-	-	-	-	-

+, HA; -, no HA

Tube 11, Saline Control

4. Hemagglutination inhibition (HI) test

Table 10 shows the results of HI titers of MG positive serum against Mycoplasma gallisepticum. Table 11 shows the results of HI test with MG negative serum.

The HI titer (1:80) was the highest serum dilution exhibiting complete hemagglutination as evidenced by a free flowing button of the cells on the bottom of the tube no. 5.

Beside the HI test an antigen control test was performed. Table 12 shows the results of the antigen control test.

All of the standardization tests were carried out simultaneously for the "test antigens" and known standard antigens as control. It was concluded that all test antigens results were in close agreement with the reference antigen.

Table 11. Result of Hemagglutination-inhibition (HI) test with Negative Serum

	Tube										
	1	2	3	4	5	6	7	8	9	10	11(b)
Serum dilution	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Test Antigen	-	-	-	+	+	+	+	+	+	+	+
Control Antigen	-	-	-	-	+	+	+	+	+	+	+

(a) Tube 1, Serum control

(b) Tube 11, Saline/RBC control

Table 12 Results of Antigen Control

	Tube				
	1	2	3	4	5
Unit ant./tube	4	2	1	1/2	1/4
Test Antigen	+	+	-	-	
Control Antigen	+	+	-	-	

+, HA; -, no HA

Economical Point

The price of ingredients for producing the experimental MG antigen was compared with some other media which are commonly used for MG antigen production. Table 13 shows the results of this comparison.

Table 13 Comparing price of different media for producing MG agtigen

Media	Amount of Culture Media	Cost range based on different ingredients, amount of serum and different companies
1) Brain heart infusion medium	1000.0 ml	11.71-18.36 dollars
2) Tryptose phosphate medium	1000.0 ml	14.53-38.26 dollars
3) Suggest medium with USDA	1000.0 ml	8.89-20.32 dollars
4) PPLO medium	1000.0 ml	10.21-18.05 dollars
5) Mycoplasma broth medium (Frey)	1000.0 ml	12.11-18.79 dollars
6) Experimental medium	1000.0 ml	
a) with .5% PPLO serum fraction		4.21-4.55 dollars
b) with 1% PPLO serum fraction		6.34-6.68 dollars

DISCUSSION

A. Culture Medium

The broth medium which was used in this experiment for producing of M. gallisepticum antigen had the low amount of basic requirements to grow M. gallisepticum (S6).

Using 1% "bacto PPLO serum Fraction" substitute of horse serum did not show difference in quality of MG antigen. Therefore it is economical to use 0.5% PPLO serum fraction instead of using 10-20% some other serums in large amount of antigen production.

Thallium acetate 0.01% was added to the medium to inhibit bacterial contamination. Penicillin is very commonly used in mycoplasma media to control gram positive bacteria, I did not add penicillin to the culture medium and never had a contamination problem.

Incubation time of cultured medium was very important in antigen production. Stopping of the incubation depended upon the pH and turbidity of the medium. Usually it was between 24 to 48 hours.

B. Standardization

Merthiolate was used as a preservative for plate and tube antigens. Also 1% "bovine albumin fraction V" was added to these antigens. No other preservative was added to hemagglutination (HA) antigen except glycerol (equal quantity of buffered saline).

Using crystal violet for staining of plate antigen was useful to make more visible the flocculation of antigen-antibody. After staining the plate antigen if large particles are observed grossly in the homogeneity test, the antigen can be filterized.

Use of the electric shaker and magnetic bar was beneficial in making the homogenous antigen.

In comparing sensitivity tests of the experimentally made antigen with control antigen it was concluded that the MG "test antigen" had a stronger degree of agglutination in the serum plate test and tube test than did control antigen. Also hemagglutination (HA) titer of the "test antigen" (640) was higher than the HA titer of control antigen (80).

In using MG antigen for determining the status of flocks in respect to Mycoplasma gallisepticum infection, if the serum plate test or tube agglutination test is negative, the flock is considered MG free. If the tube agglutination or the serum plate test is positive, and hemagglutination-inhibition (HI) titer is 1:80 or greater, the flock is considered positive. An HI titer of 1:40 or less is suspicious, and final judgement should be based on further samplings and history and observation of the flock.

In comparison with membrane antigen, whole cell antigen was less costly and time-consuming to produce than membrane antigen (Villegas et al., 1976). Also in comparing the price of ingredients which are used in this experiment

with other media that are listed in appendix this medium costs less than others. Therefore, use of this medium for producing a large quantity of MG antigen is economical.

Major difference is due to using a low amount of PPLO serum fraction (0.5%) in this study rather than using a large quantity of other serum (10-20%).

APPENDIX

Table 1. Distinguishing characteristic of M. gallisepticum, M. synoviae, and M. melagridis.

	MG	MS	MM
Ferments sugar	+	+	-
Hemagglutinates	+	+	-
Hemadsorbs	+	?	-
Requires Diphosph-opyridine-nucleotid	-	+	-
Infects chickens	+	+	-
Infects turkeys	+	+	+
Grows on direct agar	+	-	-
Requires co2	-	+	-

Table 2 Media for M. gallisepticum

1. Brain heart infusion medium

Brain heart infusion broth	37.0	g
Yeast autolysate (Albimi)	5.0	g
Thiamin HCl	0.005	g
Dextrose	3.0	g
Tris buffer	3.0	g
Trypticase	0.5	g
Swine Serum	100.0	ml
Thallium acetate	0.1	g
Penicillin (crystalline g)	100,000.0	units
Distilled water	1,000.0	ml

Adjust pH to 8.0 with 10% NaOH

2. Tryptose phosphate medium

Tryptose Phosphate broth	29.5	g
Yeast autolysate (Albimi)	10.0	g
Maltose	2.5	g
Turkey Serum	200.0	ml
Thallium acetate	0.1	g
Penicillin	100,000.0	units
Distilled water gs	1,000.0	ml

Adjust pH to 8.0 with 10% NaoH

3. Suggested medium with USDA

Brain heart infusion broth	37.0	g
Yeast autolysate (Albimi labs)	5.0	g
Thiamine	0.005	g
Dextrose	3.0	g
Tris buffer (Tris Hydroxymethyl) aminomethane	3.0	g
Serum (inactivated)	100.0	ml
Thallium acetate	0.1	g
Penicillin (crystalline G)	100,000	u/l
Distilled water	1,000	ml

4. PPLO medium

PPLO(a) broth without crystal violet	21.0	g
Yeast autolysate	10.0	g
Dextrose	5.0	g
Horse Serum	100.0	ml
Thallium acetate	0.1	g
Penicillin	100,000.0	units
Distilled water	1,000.0	ml

Adjust pH to 7.9-8.0 with 10% NaOH

(a) PPLO, pleuropneumonia-like organism.

5. Mycoplasma Medium (Frey)

Mycoplasma broth base	22.5	g
Dextrose	10.0	g
Swine or horse serum	100-150	ml
Phenol red	25.0	mg
Penicillin G potassium	1000.0	u/ml
Thallium acetate (1:4000-1:2000)	2.5-5.0	ml 10% sol.
Distilled water	1000.0	

Adjust pH to 7.8

6. Avian Meat Infusion Medium (Hofstad and Doerr, 1956)

This is an excellent medium for isolation of mycoplasma specially M. gallisepticum. It is composed of: avian meat infusion broth with 20% heat-inactivated (56% for 30 minutes) avian, swine or horse serum plus yeast autolysate and 2,3,5-triphenyl-2H-tetrazolium chloride.

ANTICOAGULANT

Alsever's solution

Sodium citrate	12.0	g
Sodium chloride	4.2	g
Dextrose	20.5	g
Distilled water	1,000	ml

The sodium citrate and sodium chloride are dissolved in 800 ml distilled water and sterilized at 15 pounds of pressure for 15 minutes. Dissolve and sterilize separately the dextrose in 200 ml distilled water, and sterilize by seitz filtration. Add aseptically to the sterile saline-

citrate solution.

BUFFERS

1. Phosphate buffer saline (PBS), pH 7.1-7.2

Sodium hydroxide	0.15	g
Sodium chloride	8.5	g
Potassium dihydrogen phosphate (KH_2PO_4)	0.68	g
Distilled water	1,000	ml

The pH of the buffered saline will be 7.1-7.2 if all reagents are accurately measured.

2. Phosphate buffer saline with merthiolate

Monobasic sodium phosphate ($NaH_2PO_4H_2O$)	4.6	g
Dibasic sodium phosphate ($Na_2HPO_4 7H_2O$)	17.3	g
Distilled water	1,000	ml

50 ml Phosphate Buffer + 950 ml Saline = 1,000 ml PBS

1000 ml PBS + 0.1 gram merthiolate = 1000 ml PBS with merthiolate.

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