SEROLOGICAL STUDIES WITH *MYCOPLASMA SYNOVIAE*

by

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INTRODUCTION

The mycoplasmas are bacteria-like organisms that differ from bacteria in several important respects: (1) They are smaller than most bacteria, the smallest reproductive units being less than 150 mm. (2) They lack a rigid cell wall. (3) They form a characteristic dense centered colony on agar which has a "fried egg" appearance. They also differ from most bacteria in that they are inhibited by specific antibody whether or not complement is also present. Additionally, most species require a complex growth medium which has been supplemented with serum or serum components.

Mycoplasma synoviae is a species of mycoplasma that is pathogenic for chickens and turkeys. At the time the present investigations were begun, commercial antigens were not available for this organism.

A grant made by a commercial biologics producer to the Veterinary Medical Research Institute, Iowa State University, was largely responsible for the initiation of this project. The purpose of the project was to study some of the problems that the firm had encountered in the development of a suitable serum plate agglutination (SPA) test antigen for use by laboratories involved with research into or eradication of Mycoplasma synoviae infection in poultry.

The first objective was to determine whether media were available which would support sufficient growth of the organism to make commercial antigen production feasible and, if not, to determine what changes could be made to improve the antigen yield. A second objective was to determine the conditions necessary for specific and sensitive reactivity during a

reasonably long storage period. A third objective was to develop other serological tests which could be used for evaluating the sensitivity of experimental SPA antigens and also as a verifying test for SPA tests run in the field.

LITERATURE REVIEW

Edward (1947) described a medium of ox heart infusion broth with 1 percent peptone, 20 percent horse serum, 10 percent yeast extract, penicillin and thallium acetate for growing bovine mycoplasmas. The same medium was used by Markham and Wong (1952) and Van Roekel and Olesiuk (1952) to cultivate the agent of turkey sinusitis and chronic respiratory disease of chickens, Mycoplasma gallisepticum.

Edward and Fitzgerald (1951) found that cholesterol, in combination with starch, bovine albumin and acetone-insoluble lipid of egg yolk, would enhance the growth of mycoplasmas. They postulated that cholesterol might act by neutralizing the toxic effects of fatty acids.

Grumbles et al. (1953) used Difco phenol red broth base supplemented with 1 to 1.5 percent Difco PPLO serum fraction and 1 percent maltose. Adler et al. (1954) found that the best medium for the isolation of mycoplasmas from CRD in chickens was broth containing medium supplemented with 10, 20 or 30 percent horse serum or Difco PPLO serum fraction. This was used as a broth overlay for blood agar slants. Fabricant (1958) compared these two media with yolk sac growth of two types of mycoplasmas and found need for improvement.

Hofstad and Doerr (1956) developed a new medium for the growth of M. gallinarum - a chicken meat infusion broth enriched with avian sera.

Adler and Yamamoto (1956) found that, of media enriched with yeast hydrolysate, yeast autolysate or yeast extract, medium with the autolysate was the best for growing Mycoplasma gallisepticum antigen.

Swine serum was used by Fabricant (1959) instead of horse serum with improved growth of avian mycoplasmas. Carbon dioxide also seemed to improve growth and yeast hydrolysate improved the growth of some strains, but not others.

Studying avian mycoplasmas, Adler and Berg (1960) found no one medium was satisfactory for growing the four strains under study. They compared Hofstad's medium, trypticase soy broth, tryptose phosphate broth and Difco PPLO broth with such nutrients as chicken hemoglobin, horse hemoglobin, chicken and horse sera, yolk, serum fractions and yeast hydrolysate.

None of the media which had been developed would successfully support the growth of M. synoviae. However, Lecce (1960) was able to grow the organism on heart infusion agar with the aid of a micrococcus nurse colony. Then Chalquest and Fabricant (1960) described a medium that would support the growth of the organism without the nurse colony. It consisted of Difco PPLO broth with 0.1 percent diphosphopyridine nucleotide (DPN) and 0.1 percent cysteine, 10 percent heat inactivated swine serum plus thallium acetate and penicillin. Chalquest (1962) determined the optimum concentration of DPN and cysteine but was unable to find a substitute for DPN. Cysteine hydrochloride would substitute for cysteine but sodium thioglycolate and glutathione would not.

A modification of Chalquest's medium was used by Olson et al. (1963) to grow antigen of M. synoviae. The medium was Difco PPLO broth, swine serum, DPN, cysteine, penicillin, thallium acetate and phenol red. They used this broth to overlay slants of the same medium supplemented with trypticase and soluble starch.

Fabricant et al. (1962) found that excess moisture and various atmospheric conditions of reduced oxygen tension aided the growth of mycoplasmas on agar. In another study by Fabricant et al. (1964), it was determined that rabbit, horse, turkey and swine serum supported optimum growth, whereas human serum was less satisfactory. PPLO serum fraction or bovine serum were poor and dog serum failed to support growth. They also analyzed some of the components of medium necessary for the growth of M. gallisepticum.

Tauraso (1967) found that the addition of DEAE dextran (10 mg/100 ml) to agar medium enhanced the growth of M. gallisepticum and M. pulmonis strains. He felt the effect was probably due to the ability of the dextran to bind sulfated polysaccharides in agar.

Frey et al. (1968) prepared five similar experimental media and compared them to three commercial and two fresh meat medium bases. The primary objective was to find a medium for the isolation and propagation of M. meleagridis as well as M. gallisepticum and M. synoviae. The experimental media fulfilled the primary objective and also were found to be suitable for propagation of all other avian strains. A commercial dehydrated medium base was made available as a result of this research.

Much work has been done on developing a good serological test for the detection of mycoplasma infections in several species. In avian serology, serum plate agglutination (SPA), tube agglutination (TA) and hemagglutination-inhibition (HI) tests are often used. However, other tests also have been used.

Domermuth and Johnson (1956) developed a freeze agglutination technique for detecting M. gallisepticum antibodies in chickens, and it gave good results when compared to HI and TA tests. Aftosmis et al. (1960) used a whole blood agglutination test to detect M. gallisepticum antibodies. The technique of growth inhibition by specific antisera has been used primarily to classify avian mycoplasmas, but it also has been used to identify new isolates. Fabricant (1960) used this technique for a preliminary classification. Chalquest and Halfhill (1962) worked with M. synoviae antigen and antisera using this procedure. Stanbridge and Hayflick (1967) used antiserum impregnated discs in growth inhibition tests for the identification of mycoplasma species.

Adler and DaMassa (1964) and Adler et al. (1964) developed an antiglobulin technique for the detection of mycoplasma antibodies in avian sera. In comparison to SPA and TA tests, the titers with the antiglobulin procedure were markedly higher and the test was more sensitive with no false negatives. Tests were done with M. gallisepticum, M. synoviae and M. meleagridis with little cross reaction, and freezing and thawing did not alter the endpoints.

Fahey and Crawley (1954) and Fahey (1954) made early reports on the use of the HI test for M. gallisepticum in chickens and turkeys. In chickens, it was found that titers appeared around 2 to 3 weeks post infection and peaked at 6 to 8 weeks. With the turkeys, many sera were tested and the results were reproducible. Later Crawley and Fahey (1957) suggested using HI tests for control and eradication of mycoplasma infections in poultry. In other tests with M. gallisepticum in chickens,

Crawley (1959) found HI titers starting from 9 to 29 days post infection and detected antibody levels for at least one year. However, Roberts (1964) found that in turkeys 44 percent still had lesions, but 23 percent had lost hemagglutination-inhibition titer 9 to 10 weeks after infection with M. gallisepticum. These birds were also refractory to reinfection. Newnham (1964) experimentally infected chickens and found HI results correlated with clinical signs. Titers were low until about 3 weeks post infection and titers persisted 19 to 34 weeks longer. Baharsefat and Adler (1965a) used a killed antigen in this test, but they found antigens killed with formalin or merthiolate did not give as good titers as those in glycerol. Neimark (1968) developed an indirect hemagglutination using gluteraldehyde fixed cells which were stable for six months. Manchee and Taylor-Robinson (1968) investigated hemagglutination and hemadsorption by mycoplasmas and found strain variations and little correlation between the two tests. They had best results with HI tests performed in microtiter plates. Working with M. synoviae antigen, Vardaman and Yoder (1969) obtained very good results using an HI test. They found the test to be nearly specific and not plagued with the cross reactions between M. gallisepticum and M. synoviae that are often seen with the serum plate agglutination.

The serum plate agglutination has been a popular test although several problems have been encountered. In a preliminary report, Adler (1954) successfully detected antibodies in sera of turkeys and chickens infected with M. gallisepticum. At 9 days, 6 of 19 birds were positive and all were positive at 13 days. Later Adler (1958) found that TA tests were

more sensitive than the SPA test in detecting antibodies in turkeys that had been infected with M. gallisepticum for almost one year. Hammar et al. (1958) had problems with false negative tests and found that sera which had been refrigerated or frozen and thawed contained interfering precipitates that had to be removed by centrifugation. Boyer et al. (1960) also encountered troublesome nonspecific agglutinations using mycoplasma antigens and turkey sera with antibodies to either mycoplasmas or erysipelas bacterin. Barber (1962) evaluated antigens for the detection of mycoplasma agglutinins in turkey sera and found great variability among these antigens. Olson et al. (1965), using the SPA and antiglobulin tests, reported cross reaction between M. synoviae and M. gallisepticum.

Olson et al. (1964) found the route of infection influenced the development of agglutinins to M. synoviae and infectious bronchitis. Footpad inoculation gave good results, but infection by the intranasal route resulted in only low titers at 5 weeks post inoculation. Sadler and Corstvet (1965) observed similar results with M. synoviae in chickens. Adler and Sadler (1965) used the SPA test in their studies on the response of turkeys to M. gallisepticum infection. They found younger birds were more susceptible and had greater SPA response. Antibodies detected were at all stages of experimental infection and in contact controls. In another experiment, Olson et al. (1968) found that many, if not all reactors to M. synoviae, were negative on retesting. Nagy (1968) reported good results with M. gallisepticum antigen except for doubtful positives. He also reported that good results were obtained with antigen containing up to but not in excess of 5 percent sodium chloride. Adler and DaMassa

(1968) found SPA antigens for M. gallisepticum grown in medium with dextrose to be less sensitive than those grown in medium without dextrose, although the dextrose definitely increased the yield. However, no buffering system was used in the medium and they felt that the drop in pH may have been responsible for decreased sensitivity. They also found that increasing the concentration of the antigen or resuspending it in buffered saline did not improve the sensitivity of the antigen.

Hromatka and Adler (1969) found pH, physical factors and preservatives had varying effects on the sensitivity of M. gallisepticum serum plate agglutination antigens. Of three buffers used, Sorenson saline buffer, citrate-phosphate buffer and tris buffer, the citrate-phosphate gave the best sensitivity. Optimum pH and molarity, related to sodium chloride, varied with the buffer used. Formalin and phenol were not satisfactory because they reduced sensitivity.

Lawson and Hertler (1969) gave a good report on their results using M. gallisepticum plate antigen. Using standardized antigens, they obtained a 90 to 100 percent agreement between SPA tests and HI tests on positive and negative sera. Although the SPA test lagged behind the HI test during the first 2 weeks post infection, the test was completely positive by the seventh week and the two tests gave the same results between the eighth and thirty-fifth weeks.

Many workers have compared the serum plate agglutination, the hemagglutination-inhibition and the tube agglutination tests for their ability to detect various mycoplasma infections. Also these three tests are often used to characterize the immune response to mycoplasma infections.

Hofstad (1957) conducted a serological study of infectious sinusitis in turkeys and experienced poor results with the SPA. However, the HI and TA tests were more successful. He found the TA test corresponded to the clinical signs but the HI test did not decline as rapidly. Hall (1962) worked with production of antigens of M. gallisepticum for the HI, TA and SPA tests. Baharsefat and Adler (1965b) compared the TA, HI and antiglobulin titers of chickens and turkeys infected with M. gallisepticum. There was close agreement between the TA test and HI test, although the TA was positive before the HI. However, they were able to detect antibodies earlier with the antiglobulin technique and with greater sensitivity than with the other two tests. Adler and DaMassa (1965) compared six strains of M. gallisepticum using SPA, TA and antiglobulin agglutination. The antigenicity of the six varied most in the SPA test and least in the antiglobulin test. Variation was not due to media or enrichments. In their serological studies of M. synoviae, Roberts and Olesiuk (1967) found positive serum plate agglutinations before positive tube agglutination. There was, however, a cross reaction between M. synoviae antiserum and M. gallisepticum antigen with SPA that did not occur in the HI and TA tests. There were also no cross reactions in capillary tube agglutinations and gel double diffusion. The cross reactions in the SPA test were attributed to a rheumatoid factor in the serum. Mohamed and Bohl (1968) conducted serological studies on M. meleagridis and found the TA test to be more sensitive than the SPA test at low titers, but there was 100 percent correlation between the two in higher titers. Route of inoculation also affected the results on experimentally infected turkeys. Thornton (1969)

found that the SPA test was positive before the HI test in studies with M. gallisepticum. He also found freezing and thawing of serum caused false positives and increased titers. However, this could be avoided by heating the sera to 56°C for 30 minutes before or after freezing and by using fresh serum or storing it at 4°C. He also found that by using 8 instead of 4 HA units nonspecific hemagglutination could be avoided. The effects of freezing and thawing were also noted by Cover et al. (1960). In addition, they reported that heating sera to 56°C for 10 minutes destroyed tube agglutination activity.

METHODS AND MATERIALS

Media Studies

Basal media

The formula for basal medium A was as follows:

Peptone "CS"[1]	10.0 gm
Yeast autolysate[1]	5.0 gm
NaCl	5.0 gm
KCl	0.4 gm
$MgSO_4$	0.2 gm
$Na_2HPO_4 \cdot 7\ H_2O$	1.6 gm
$NaH_2PO_4 \cdot H_2O$	0.1 gm
Dextrose	5.0 gm
Phenol red	0.02 gm
Penicillin	500,000 units
Thallium acetate	0.02 gm
Triple distilled H_2O	1000.0 ml

Basal medium B was identical in formula to A, but a commercially prepared base[1] consisting of the peptone, yeast autolysate and inorganic salts was used in place of the individual components.

The media were made double strength and the pH was adjusted to 8.0 with 10 percent sodium hydroxide. It was sterilized by filtration through Hormann D-5 and D-8 Seitz-type filter pads in a Hormann model 7B double

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[1]Pfizer Diagnostics (Albimi Laboratories), Flushing, New York.

pass filter.[1] First, 1 liter of triple distilled water per pad was filtered in order to rid the pads of contaminating ions. Before use, the stock medium was diluted 1/1 with sterile triple distilled water and was supplemented with 15 percent serum, 0.01 percent diphosphopyridine nucleotide (DPN) and 0.01 percent cysteine hydrochloride.

The additions to and/or changes made in the basal medium formula are summarized in Table 1. Each test medium was given a different number.

Serum supplements

Swine serum used in the media experiments was obtained from the normal herd at the Veterinary Medical Research Institute (VMRI) or from surplus stocks obtained from the Iowa State University Antigenic Laboratory. Pooled rabbit serum (type II) of slaughter house origin was obtained commercially.[2] Turkey serum was obtained from the VMRI mycoplasma-free turkey flock.

Some serum lots were acid treated by adding sufficient 1N hydrochloric acid to lower the pH to 4.2. The pH was held at that point for 2 to 18 hours, at which time 1N sodium hydroxide was added to restore the pH to the original value. The precipitate that formed was removed by centrifugation at 2600 X g for 15 minutes. To further clarify the serum, it was filtered through several thicknesses of gauze and then a Millipore prefilter[3] fitted in a Buchner funnel. In further preparation for

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[1]F. R. Hormann and Company, Inc., Milldale, Connecticut.

[2]Pel-Freeze Biologics, Rogers, Arkansas.

[3]Millipore Corporation, Bedford, Massachusetts.

Table 1. Additives and/or treatments of media in tests to improve the growth of M. synoviae[a]

Medium No.	Additives and/or treatments
M30	Unheated medium
M31	Heated medium
M32	Normal control medium
M33	Tween 80[b]: 0.015 ml/100 ml
M34[c]	Charcoal: 10 gm/300 ml
M35[c]	Tween 80 and charcoal as above
M36	Basal medium A substituting 0.815 gm/l NaH_2PO_4 and 0.050 gm/l KH_2PO_4 for buffer
M37	Basal medium A substituting 1.1 gm/l of tris[d] for buffer
M38	Turkey serum: Heat inactivated for 30 min. at 56°C
M39	Turkey serum: Heat inactivated for 15 min. at 60°C
M40	Turkey serum: Heat inactivated for 30 min. at 60°C
M41	Turkey serum: Not heat inactivated
M42	Rabbit serum: Heat inactivated for 30 min. at 56°C + DPN and cysteine-HCl at 0.01%, 0.002%, 0.001%, 0.0005%. These percents were used through M49.
M43	Rabbit serum: Heat inactivated for 15 min. at 60°C
M44	Rabbit serum: Heat inactivated for 30 min. at 60°C
M45	Rabbit serum: Not heat inactivated
M46	Swine serum: Heat inactivated for 30 min. at 56°C
M47	Swine serum: Heat inactivated for 15 min. at 60°C
M48	Swine serum: Heat inactivated for 30 min. at 60°C
M49	Swine serum: Not heat inactivated

[a]Basal medium B supplemented with swine serum and 0.01% DPN and cysteine-HCl was used unless otherwise specified.

[b]Difco Laboratories, Inc., Detroit, Michigan.

[c]pH readjusted and phenol red added again because removed by charcoal.

[d]Trishydroxymethylaminomethane (TRIS), General Biochemicals, Chagrin Falls, Ohio.

Table 1 (Continued)

Medium No.	Additives and/or treatments
M50	0.005% cholesterol + 0.025% Tween 80
M51	0.005% cholesterol + 0.005% Tween 80
M52	0.005% cholesterol + 0.001% Tween 80
M53	0.001% cholesterol + 0.025% Tween 80
M54	0.001% cholesterol + 0.005% Tween 80
M55	0.001% cholesterol + 0.001% Tween 80
M56	0.0002% cholesterol + 0.025% Tween 80
M57	0.0002% cholesterol + 0.005% Tween 80
M58	0.0002% cholesterol + 0.001% Tween 80
M59-M67	Same as M50 through M58 only used rabbit serum
M68	Filtered basal medium A + rabbit serum
M69	Autoclaved basal medium A + rabbit serum
M70	Autoclaved basal medium B + rabbit serum
M71	Filtered basal medium B + rabbit serum
M72	Swine serum: acid treated
M73	Swine serum: untreated
M74	0.05% DMSO[e]
M75	0.1% DMSO
M76	0.25% DMSO
M77	Basal medium A without dextrose and phenol red
M78	Basal medium A with dextrose and phenol red
M79	Basal medium A substituting 0.35 gm/l tris[f] base and 0.35 gm/l tris HCl[f] for buffer
M80	Basal medium A + acid treated swine serum
M81	Basal medium A + 1 ml of MEM vitamins[g]/50 ml media

[e]Dimethyl sulfoxide, Sigma Chemical Company, St. Louis, Missouri.

[f]Tris(hydroxymethyl)aminomethane base and Tris(hydroxymethyl)aminomethane HCl, Sigma Chemical Company, St. Louis, Missouri.

[g]Eagle's Minimum Essential Vitamins, 100X, Grand Island Biological Company, Grand Island, New York.

Table 1 (Continued)

Medium No.	Additives and/or treatments
M82	Basal medium A substituting HEPES[h] for buffer
M83	Basal medium A substituting TES[i] for buffer
M84	Basal medium A substituting 0.5 gm peptone "CS"/100 ml and 0.5 gm peptone B[j] for 1 gm peptone "CS"
M85	Basal medium A substituting 1/2 the amount of yeast autolysate

[h]N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid, Nutritional Biochemicals Corp., Cleveland, Ohio.

[i]N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid, Nutritional Biochemicals, Corp., Cleveland, Ohio.

[j]Pfizer Diagnostics (Albimi Laboratories), Flushing, New York.

sterilization, the serum was filtered through several prerinsed Hormann Seitz-type clarifying filters. The serum was filter sterilized using the same procedure as for the medium.

Culture methods

Various amounts of inocula were used throughout the experiments, although generally, an amount from a rapidly growing culture was used to give a final concentration of 1 percent. In experiments in which several media were being compared, the inoculations were made from pooled aliquots of the previous passage in each medium.

For antigen production, stationary flasks of inoculated medium containing 500 ml to 2000 ml were incubated at 37°C for various periods of time. When medium containing dextrose was used, an attempt was made to

harvest the antigen before the pH of the medium went below 7.0. This usually did not exceed an incubation period of 36 hours. However, when medium without dextrose was used, the incubation period was extended to 72 to 84 hours.

Measurement of growth

Change of pH was used as the criterion for measuring growth rate in early studies with media containing dextrose and phenol red. The culture tubes were observed at regular intervals beginning at about 18 hours incubation. Insofar as possible, the order of change from red to orange and from orange to yellow was recorded for all media. The results were recorded as color changes from eight to one - most growth to least growth.

Nephelometric determinations were made of the growth in most of the medium experiments. These were made by comparing medium from inoculated tubes with uninoculated medium incubated for the same period of time. Measurements were recorded in Beckman nephelos units.

Light refraction due to emulsified cholesterol decreased as growth progressed with media 59 through 67. This presumably was due to free cholesterol being incorporated into the cell membranes. However, the light refraction did not decrease in the uninoculated blanks, and this led to erroneously low nephelometric readings. The error was overcome by using a buffered saline blank instead of uninoculated medium for comparisons. Therefore, the organisms in each medium had to be centrifuged at 8500 X g for 15 minutes and resuspended in buffered saline.

In one experiment, growth curves were used to determine the rate of growth in medium with and without dextrose. A 1 percent inoculum was used

and 0.01 ml aliquots of 10^{-1} through 10^{-5} dilutions of each culture were plated onto agar at 8 hour intervals.

Antigen Studies

Harvest and standardization of antigen

Before harvesting the antigen, the broth growth was heat inactivated at 56°C for 30 minutes. The antigen was harvested by centrifugation of the culture medium at 2600 X g for 20 to 30 minutes. The pellet was drained well or resuspended in buffered saline and centrifuged again. Final resuspension was made in one of the following buffers:

1. Tris buffered saline

Tris(hydroxymethyl)aminomethane base[1,2]	0.8 gm
Tris(hydroxymethyl)aminomethane HCl[2]	6.86 gm
NaCl	7.93 gm
Na_2HPO_4	0.1 gm
Dextrose	1.0 gm
KCl	0.97 gm
Triple distilled H_2O	1000.0 ml

pH 7.2 - 7.4 (adjusted with HCl when tris base was used alone)

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[1]General Biochemical Company, Chagrin Falls, Ohio.

[2]Sigma Chemical Company, St. Louis, Missouri.

2. Phosphate buffer

	pH		
	6.5	7.0	7.2
a.	68.0 ml	37.0 ml	27.0 ml
b.	32.0 ml	63.0 ml	73.0 ml

a. KH_2PO_4 - 0.9078 gm/l

b. $Na_2HPO_4 \cdot 2\ H_2O$ - 0.178 gm/l

3. Phenolized Cox buffer

$NaH_2PO_4 \cdot H_2O$	4.6 gm
$Na_2HPO_4 \cdot 7\ H_2O$	17.3 gm
Phenol	2.5 gm
Triple distilled H_2O	1000.0 ml

pH 7.0

The first antigens were standardized to read 50 on the Klett-Summerson photoelectric colorimeter with a No. 54 filter. In some cases, antigens were standardized by packed cell volume (PCV) determinations made using a standard Hopkin's tube. The density was adjusted so that the PCV was 0.05 ml when 5.0 ml of the antigen was centrifuged in a Hopkin's tube in an International No. I centrifuge at 1000 X g for 1 1/2 hours. A listing of the antigens and their various treatments is given in Table 2.

Each of these antigens was tested at various time intervals for agglutination of a known positive serum and a known negative serum, precipitate formation and general appearance, i.e. granularity, contamination, etc.

Table 2. Additives and/or treatments of M. synoviae serum plate agglutination antigens in shelf life and sensitivity tests[a]

Diluent	pH	Additives and/or treatments
Tris buffered saline	6.5	0.1% phenol
Tris buffered saline	7.2	0.1% phenol
Tris buffered saline	7.0	Merthiolate, tightly capped
Tris buffered saline	7.0	Merthiolate, no cap
Tris buffered saline	7.0	Merthiolate, glutathione, tightly capped
Tris buffered saline	7.0	Merthiolate, glutathione, no cap
Phosphate buffered saline	7.0	Merthiolate, tightly capped
Phosphate buffered saline	7.0	Merthiolate, no cap
Phosphate buffered saline	7.0	Merthiolate, glutathione, capped
Phosphate buffered saline	7.0	Glutathione, no cap
Tris buffered saline	7.0	Merthiolate
Phosphate buffered saline	7.0	Merthiolate
Tris buffered saline	7.0	0.25% phenol
Phosphate buffered saline	7.0	0.25% phenol
Phosphate buffered saline	7.0	0.25% phenol, 1% rose bengal dye
Tris buffered saline	7.0	0.25% phenol, 1% rose bengal dye
Phenolized Cox buffer	7.0	No NaCl
Phosphate buffer	6.2	No NaCl
Phosphate buffer	7.0	No NaCl

[a]Antigens in first 4 groups were standardized with Klett-Summerson photoelectric colorimeter. Antigens in the last group were standardized in Hopkin's tube.

Physico-chemical variables

A study was made of the effect of several variables on antigen sensitivity and shelf life. These included buffers, pH, redox potentials, aging and concentration. The various buffers or treatments used are listed in Table 2.

Strain of Mycoplasma synoviae

The WVU 1853 strain of M. synoviae was used throughout the course of the study. It was originally isolated by Dr. N. O. Olson, West Virginia University, Morgantown, West Virginia. The immediate source of the strain was Dr. E. L. McCune, University of Missouri, Columbia, Missouri.

Serological Studies

Experimental infections

For the serological studies, three infection experiments were conducted. All birds for these experiments were obtained from the VMRI mycoplasma-free flocks at Iowa State University.

Experiment 1 Twelve roosters were bled and then half of the birds were injected in the footpad with 0.1 ml of 10^{-2} dilution of M. synoviae in yolk sac suspension, while the others were inoculated with 0.2 ml. All birds were bled at 5, 8, 12 and 22 days post inoculation.

Experiment 2 See Table 3 for inoculation schedule of adult chickens. These birds were bled before inoculation and then bled at 7, 14 and 21 days post inoculation.

Experiment 3 Fifteen 3 1/2 week old turkeys were bled and then injected in the footpad with 0.1 ml of 10^{-2} dilution of M. synoviae in yolk sac suspension. Each turkey was bled at 7, 14, 21 and 28 days post inoculation.

The sera from the above experiments were tested for antibodies by the serum plate agglutination, tube agglutination and hemagglutination-inhibition tests. The procedures for each of these tests were adapted from two U. S. Department of Agriculture pamphlets (ca. 1960a,b).

Table 3. Route of inoculation and dose of M. synoviae for chickens in experiment 2

Chicken No.	Route of inoculation and dose of infection
3991	0.1 ml of 10^{-2} dilution of yolk sac suspension[a], footpad
3992	0.1 ml of 10^{-2} dilution of yolk sac suspension, footpad
3993	0.1 ml of 10^{-1} dilution of yolk sac suspension, footpad
3994	0.1 ml of 10^{-1} dilution of yolk sac suspension, footpad
3995	0.1 ml of 1/5 dilution of yolk sac suspension, footpad
4197	0.2 ml of 10^{-2} dilution of yolk sac suspension, i.v.
3997	0.2 ml of 10^{-2} dilution of yolk sac suspension, i.v.
3998	0.2 ml of 10^{-2} dilution of yolk sac suspension, footpad and i.v.
3999	0.2 ml of 10^{-2} dilution of yolk sac suspension, footpad and i.v.

[a]10% suspension of M. synoviae in tris buffered saline.

Hemagglutination and hemagglutination-inhibition procedures

Growth from 500 ml of broth was harvested by centrifugation for 20 minutes at 2600 X g in a PR-1 International centrifuge. The antigen was washed in phosphate buffered saline and recentrifuged. The antigen was then resuspended in 10 ml of phosphate buffered saline and 10 ml of glycerol was added. The antigen was stored at -70°C in a Revco freezer.

The buffer used in the tests was made as follows:

NaOH	0.075 gm
NaCl	4.75 gm
KH_2PO_4	0.34 gm
Triple distilled H_2O	500.0 ml

pH 7.1 - 7.2

Before using the buffer 1 percent rabbit serum was added.

Chicken or turkey red blood cells were collected in an equal volume of Alsever's solution and washed three times in buffered saline before use. The final concentration used in the tests was 0.25 percent.

All HA and HI tests were done in microtiter plates. For the HA test, two dilutions of antigens were made - 1/10 and 1/15. In 0.05 ml volumes, doubling dilutions of antigen, 1/20 through 1/640 and 1/30 through 1/960, were made in duplicate. The plates were incubated for 60 to 75 minutes at 4°C after 0.05 ml of the 0.25 percent red blood cell suspension had been added. From these titrations, 8 HA units and 4 HA units were determined.

For the HI tests, doubling dilutions of serum, 1/2 through 1/4096 or 1/5 through 1/10,240, were made in 0.05 ml volumes containing 4 HA units of antigen. To each well, 0.05 ml of the 0.25 percent red blood cell suspension was added and the tests were incubated as before. Antigen, cell and antiserum controls were included in each series of tests. Also, the HA titer was determined with each new batch of red blood cells.

Tube agglutination procedures

The antigen was harvested as above except the final resuspension was made in 20 ml of phenolized Cox buffer. The suspension of antigen was

ground in a TenBroeck tissue grinder. The concentration of antigen was adjusted so that a 1/20 dilution of the antigen read 50 on a Klett-Summerson photoelectric colorimeter with a No. 54 filter. This concentrated antigen was stored at 4°C. It was not necessary to age this antigen before it was used.

Preliminary tube agglutinations were conducted on each serum sample. One ml of the diluted antigen was added to 0.08 ml, 0.04 ml and 0.02 ml of serum, giving a 1/12.5, 1/25 and 1/50 dilution, respectively. The tests were incubated at 37°C for 15 to 18 hours. If the serum sample was positive through a 1/50 dilution, another titration was made using doubling dilutions of 1/5 through 1/320 in 0.5 ml volume to which 0.5 ml of diluted antigen was added. Thus, the final dilutions were 1/10 through 1/640. These tests were incubated as above.

Serum plate agglutination procedures

Antigen preparations were previously described. To 0.02 ml of each serum on a glass plate, 0.03 ml of antigen was added. The two were mixed with a wooden applicator stick. Then the plate was rotated occasionally during 2 to 3 minutes after which time observations for agglutination were made. Often with turkey serum, the length of time had to be extended to 5 minutes. The sera and antigens were at room temperature and the glass plate was slightly warmer.

RESULTS

Media Studies

The media in earlier experiments were evaluated on the basis of rapidity of color change due to fermentation of dextrose and the simultaneous pH change.

In twenty separate trials, unheated medium (M30) and medium that had been autoclaved at 121°C for 15 minutes (M31) were compared. Little difference was observed in time required for pH changes; however, M30 appeared to be slightly better than M31.

The growth of M. synoviae was compared in eleven trials using control medium (M32), medium containing Tween 80 alone (M33), medium containing charcoal alone (M34), and medium containing Tween 80 and charcoal (M35). In every trial, M34 and M35 supported growth that was slightly more rapid than that in M32, while growth in M33 was consistently slower than in the control medium.

In seven trials, basal medium A with phosphate buffers (M36) showed definitely faster color change than basal medium A with tris buffer (M37).

No comparisons could be made using media containing turkey serum (M38 through M41) because M. synoviae failed to grow even after lengthy attempts to adapt it to this serum.

The results obtained with media supplemented with rabbit serum (M42 through M45) and with swine serum (M46 through M49), which had different serum heat inactivation treatments and various dilutions of DPN, are shown in Table 4. Comparisons among the media were made on the basis of color

Table 4. Comparison by color change and nephelometry of M. synoviae growth in medium 42 through 49

Medium No.	Serum Treatment	DPN 0.01%	DPN 0.002%	DPN 0.001%	DPN 0.0005%
M42	30 min. at 56°C	8[a]	8	8	8
M43	15 min. at 60°C	7	5	6	6
M44	30 min. at 60°C	6	7	5	4
M45	Not heat inactivated	6	4	4	4
M46	30 min. at 56°C	8	8	6	6
M47	15 min. at 60°C	8	7	7	5
M48	30 min. at 60°C	8	7	6	6
M49	Not heat inactivated	7	6	8	7
M46	30 min. at 56°C	64[b]	57	50	42
M47	15 min. at 60°C	45	40	36	30
M48	30 min. at 60°C	48	44	39	32
M49	Not heat inactivated	33	35	34	28

[a]Arbitrary units: 8 = most rapid growth; 1 = least rapid growth.

[b]Beckman nephelos units.

changes which were rated eight through one, most rapid or complete color change to the least color change. The results shown in the table are representative of many determinations. Nephelometric determinations were made on both groups of media; however, only the results of M46 through M49 are shown. The results obtained with M42 through M45 are not shown as the incomplete color change in some tubes led to inaccurate readings. From these results, it was concluded that less than 0.01% DPN would support optimum growth in either rabbit or swine serum.

Many trials were made using media supplemented with swine serum and various amounts of cholesterol and Tween 80 (M50 through M58). In other experiments, media supplemented with rabbit serum and various amounts of cholesterol and Tween 80 (M59 through M67) were compared. Some of the earlier trials were excluded from the averaged results because dilutions had to be made in order to obtain a reading on the nephelometer.

Some of the uninoculated media containing cholesterol, particularly those also containing rabbit serum, had a high refractive index which interfered with the accuracy of nephelometric readings. For the final tests, media containing cholesterol and either rabbit or swine serum were centrifuged and the nephelometric determinations made of the sediments resuspended in 20 ml of phosphate buffered saline. In this way, the treatment of the two separate groups was identical. Therefore, the results of only three trials are shown in Table 5.

The results obtained by nephelometry on all of the remaining media used are given in Table 6. Basal medium A, which had been filter- or autoclave-sterilized (M68 and M69), was compared to filtered or autoclaved basal medium B (M70 and M71). Media supplemented with acid treated swine serum (M72) and untreated serum (M73) were tested. Various amounts of dimethyl sulfoxide were added to M74 through M76. Basal medium A without dextrose and phenol red (M77) was compared with basal medium A containing dextrose and phenol red (M78). Medium containing tris buffer from a different commercial source (M79), basal medium A plus acid treated swine serum (M80) and the same medium with additional vitamins (M81) were compared. HEPES buffer (M82) and TES buffer (M83) were compared with the

Table 5. Nephelometric determinations (expressed in Beckman nephelos units) of the growth of M. synoviae in medium 50 through 67

Medium No.	Trial 1	Trial 2	Trial 3	Average of 2 best trials	Treatment of basal medium B
M50	109	115	123	119	0.005% cholesterol + 0.025% Tween 80
M51	130	130	130	130	0.005% cholesterol + 0.005% Tween 80
M52	130	130	130	130	0.005% cholesterol + 0.001% Tween 80
M53	88	56	71	79	0.001% cholesterol + 0.025% Tween 80
M54	108	116	92	112	0.001% cholesterol + 0.005% Tween 80
M55	105	119	100	112	0.001% cholesterol + 0.001% Tween 80
M56	80	50	70	75	0.0002% cholesterol + 0.025% Tween 80
M57	100	86	89	94	0.0002% cholesterol + 0.005% Tween 80
M58	94	73	83	88	0.0002% cholesterol + 0.001% Tween 80
M32	92	69	69	80	Control medium
M59	68	45	70	69	0.005% cholesterol + 0.025% Tween 80
M60	92	91	81	91	0.005% cholesterol + 0.005% Tween 80
M61	95	86	73	90	0.005% cholesterol + 0.001% Tween 80
M62	75	67	81	78	0.001% cholesterol + 0.025% Tween 80
M63	92	60	80	86	0.001% cholesterol + 0.005% Tween 80
M64	90	70	74	82	0.001% cholesterol + 0.001% Tween 80
M65	94	78	85	89	0.0002% cholesterol + 0.025% Tween 80
M66	106	65	89	97	0.0002% cholesterol + 0.005% Tween 80
M67	91	74	82	86	0.0002% cholesterol + 0.001% Tween 80
ADRa	91	65	92	91	Control medium

Table 6. Nephelometric determinations (expressed in Beckman nephelos units) of the growth of M. synoviae in medium 68 through 85

Medium No.	Trial 1	Trial 2	Trial 3	Average of 2 best trials	Treatment of media
M68	62	64	100	82	Filtered A + rabbit serum
M69	30	36	46	41	Autoclaved A + rabbit serum
M70	33	35	43	39	Autoclaved B + rabbit serum
M71	48	44	58	53	Filtered B + rabbit serum
M72	130	130	130	130	B + acid treated swine serum
M73	5	11	10	10	B + untreated swine serum
M74	122	125	113	123	B + 0.05% DMSO
M75	125	129	120	127	B + 0.1% DMSO
M76	116	118	127	122	B + 0.25% DMSO
M77	84	45	71	77	A without dextrose and phenol red
M77a	130	81	130	130	Incubated 72 to 84 hours
M78	130	128	127	129	A with dextrose and phenol red
M32	122	117	112	119	Control medium
M79	56	77	59	68	A substituting tris buffers
M80	43	56	55	55	A + acid treated swine serum
M81	57	85	82	83	A + vitamins
M32	72	64	70	71	Control medium
M82	83	74	77	80	A substituting HEPES buffer
M83	90	85	81	87	A substituting TES for buffer
M32	90	70	81	85	Control medium
M84	118	71	126	122	A substituting peptone B and "CS"
M85	112	123	121	122	A substituting 1/2 yeast autolysate
M32	63	106	111	108	Control medium

usual phosphate buffered medium (M32). Medium with both peptone "CS" and peptone B (M84) was compared to medium in which the amount of yeast autolysate was halved (M85). The media were divided into five groups, within which the inocula and incubation conditions were identical. Strict comparisons were considered to be valid only within groups.

The results of the growth curves are given in Table 7. It was concluded that the organisms grew better in medium with dextrose in a 36 hour period. The length of time could not be extended further when using medium with dextrose due to the drastic change of pH. However, it was noted that yields from medium without dextrose were very good if the incubation time was extended to 72 or 84 hours (see Table 6, M77 and M77a).

Table 7. Growth curves of M. synoviae in media with and without dextrose expressed as colony-forming units per ml

Hours	Trial 1		Trial 2	
	Dextrose	No dextrose	Dextrose	No dextrose
8	2.1×10^8	ND[a]	3.9×10^7	ND
16	8.0×10^8	ND	6.6×10^8	3.8×10^4
24	ND	2.0×10^6	1.5×10^9	4.2×10^5
36	6.5×10^8	4.8×10^6	1.3×10^9	2.5×10^6

[a]Not determined.

From the above data it was concluded that only minor improvements in yield could be made over basal medium B. Acid treated swine serum was the best serum of those tested. The DPN concentration used was 0.002 percent with 0.002 percent cysteine-HCl. Dextrose and phenol red were used in most cases because of the decreased incubation time. MEM vitamins (20 ml/l of medium) were used; however, it was later found that half of this amount did not decrease the yield of antigen. Although cholesterol appeared to increase the yield considerably, it was not used in the final medium for reasons which are discussed later.

The formula for the basal medium was as follows:

Peptone B	5.0 gm
Peptone "CS"	5.0 gm
Yeast autolysate	5.0 gm
NaCl	5.0 gm
KCl	0.4 gm
$MgSO_4$	0.2 gm
$Na_2HPO_4 \cdot 7\ H_2O$	1.6 gm
$NaH_2PO_4 \cdot H_2O$	0.1 gm
Thallium acetate	0.2 gm
Penicillin	500,000 units
Dextrose	5.0 gm
Phenol red	0.02 gm
Triple distilled H_2O	1000.0 ml

pH 7.8 - 8.0 with 10 percent NaOH

Antigen Studies

All of the antigens listed in Table 2 were checked periodically with known positive and negative chicken serum. Most of the antigens, whether standardized with the Klett-Summerson photoelectric colorimeter (dilute in appearance) or standardized in the Hopkin's tube (concentrated in appearance), gave good results over a one to two year testing period. Good results were considered to be definite, flocculant agglutination with the known positive serum and smooth, nonclumped appearance with the negative serum.

Although larger aggregates were obtained when the concentrated antigens were reacted with positive serum, the dilute antigens gave definite, unmistakable results, too. There was a greater problem with false positive reactions when the concentrated antigens were used. However, it appeared that part of the problem was due to the treatment of the serum. It was found that recently thawed sera gave false positive results even with some of the dilute antigens. Centrifuging the serum usually did away with the false positives although the concentrated antigens sometimes still agglutinated the negative serum. Storing the serum at refrigerator temperature for a few days after thawing and centrifuging also seemed to help prevent the false positive results. This problem was encountered also with the experimental sera and centrifuging after thawing and refrigerating became part of the routine procedure.

The addition of dye to the antigen might be considered an aid in reading the test, but those antigens without it were easily read and interpreted.

There was no appreciable difference in the antigens due to their various methods of treatment. All of the antigens were stable and no precipitate formation was seen. Only one tube of antigen was contaminated and that was by a mold. Also these antigens did not give positive results with sera containing M. gallisepticum antibodies.

Serological Studies

In the chicken experiments, positive tests appeared earliest with the tube agglutination procedure, but the results of the three tests correlated closely. Again in the turkey experiment, the tube agglutination tests were positive first, but as before all three tests correlated very closely. See Tables 8, 9 and 10 for the results of the serological studies.

Considerable difficulty was encountered when turkey sera were used in the serum plate agglutination. Use of an antigen grown in medium with dextrose resulted in very poor reproducibility and many false positive reactions. After many unsuccessful trials with the various antigens listed in Table 2, an antigen grown in medium without dextrose was produced. This was used primarily for testing turkey sera and not subjected to the repeated trials of the other antigens. This antigen was very grainy and had to be sonicated for 1 1/2 minutes. The results obtained with this antigen are those given in Table 10. Reproducibility was excellent and there was no problem of false positive results. Antigens at pH 6.5 and 7.2 were tested, but in these limited trials, it was impossible to determine if one was better than the other.

It was also found that tube agglutination antigens produced in filtered rather than autoclaved media were more sensitive. Several titrations with different lots of antigen were conducted and the sensitivity of the filtered media antigens was always higher than that of the autoclaved media antigens.

Table 8. Results of serum plate agglutination, tube agglutination and hemagglutination-inhibition tests performed on sera from M. synoviae infected chickens in experiment 1

Chicken No.	SPA				TA[a]				HI[a]			
	Days post inoculation				Days post inoculation				Days post inoculation			
	5	8	12	22	5	8	12	22	5	8	12	22
1680	-	-	-	+	-	-	-	25	-	-	-	40
1681	-	-	-	+	-	-	-	160	-	-	-	80
1682	-	-	+	n.s.[b]	-	80	160	n.s.	-	-	80	n.s.
1683	-	-	-	+	-	-	12.5	320+	-	-	-	320
1684	-	+	+	+	-	25	50	320+	-	40	320	320
1685	-	-	-	-	-	-	12.5	160	-	-	-	160
1686	-	-	-	-	-	20	12.5	25	-	-	-	20
1687	-	-	-	+	-	-	20	25	-	-	-	-
1688	-	-	-	-	-	-	12.5	20	-	-	-	-
1689	-	-	-	+	-	n.s.	10	-	-	n.s.	-	-
1690	-	-	-	-	-	-	25	25	-	-	-	80
1691	-	-	-	+	-	25	12.5	25	-	-	-	80

[a]Titer expressed as reciprocal of highest reacting dilution.

[b]No serum.

Table 9. Results of serum plate agglutination, tube agglutination and hemagglutination-inhibition tests performed on sera from M. synoviae infected chickens in experiment 2

Chicken No.	SPA Days post inoculation 7	14	21	TA[a] Days post inoculation 7	14	21	HI[a] Days post inoculation 7	14	21
3992	-	-	+	-	80	160	-	160	640
3993	-	+	+	80	320	320	-	320	2560
3994	-	+	+	80	320	320+	-	320	2560
3995	-	+	killed	40	160	killed	-	160	killed
3997	-	+	+	-	160	160	-	160	640
3998	-	+	+	160	320	160	-	320	1280
3999	-	+	killed	-	160	killed	-	160	killed

[a]Titer expressed as reciprocal of highest reacting dilution.

Table 10. Results of serum plate agglutination, tube agglutination and hemagglutination-inhibition tests performed on sera from M. synoviae infected turkeys in experiment 3

Turkey No.	SPA Days post inoculation 7	14	21	28	TA[a] Days post inoculation 7	14	21	28	HI[a] Days post inoculation 7	14	21	28
3586	-	-	-	-	-	-	25	25	-	-	64	256
3587	-	-	-	+	-	-	25	25	-	-	32	512
3588	-	-	-	-	-	-	-	-	-	-	-	-
3589	-	-	-	-	-	-	-	-	-	-	-	-
3590	-	-	+	+	-	-	12.5	50+	-	-	64	1024
3591	-	-	-	-	-	-	-	-	-	-	-	-
3592	-	-	+	+	-	-	160	320	-	-	128	512
3593	-	-	-	+	-	-	25	320	-	-	64	512
3595	-	-	-	-	-	-	-	-	-	-	-	-
3596	-	+	+	+	-	25	25	50+	-	16	256	4096
3597	-	-	-	-	-	-	-	-	-	-	-	-
3598	-	-	-	-	-	-	12.5	-	-	-	-	16
3599	-	-	+	+	-	-	80	80	-	-	128	128
3600	-		-	-	-	-	-	-	-	-	-	16

[a]Titer expressed as reciprocal of highest reacting dilution.

DISCUSSION

Although minor increases in yield were obtained with some of the modifications tested, fairly good yields of M. synoviae were obtained with basal medium A supplemented with only swine serum and DPN. Therefore, it seems that antigen production is feasible with this medium. Some of the changes that improved the growth of the organism were not incorporated into the final medium because the improvement in growth was not sufficient to warrant the expense or the time involved.

Tween 80 was added to the medium in an attempt to produce antigen that could be more readily suspended and also to determine if the growth of M. synoviae would be enhanced by its addition. Charcoal was added to counteract the toxic effect of the short chain fatty acids in Tween 80. This procedure was described by Hirsch (1954). Charcoal was also used in a medium without Tween 80 to determine if any effect seen was due to removal of toxic substances in the basal medium instead of in the Tween 80.

It was reported by Edward and Fitzgerald (1951) that cholesterol improved the growth of mycoplasmas. According to Hirsch (1954), added cholesterol may supplement the serum cholesterol or may act much the same as charcoal. Although the cholesterol markedly improved growth, it was not used due to problems encountered with nephelometric standardization and autoagglutination. More work needs to be done to determine whether these problems could be circumvented.

Treatment of swine serum with hydrochloric acid, which was described by Boulanger (1954), successfully removed procomplementary substances

which interfered with the complement-fixation test. Later Huhn (1969) used this procedure for swine serum which was incorporated into culture media and found that it improved the growth of mycoplasmas.

Basal medium A could be used equally well with or without dextrose. However, the incubation period had to be extended from 36 hours to 72 or 84 hours to provide acceptable antigen yields in media without dextrose. Also it was found that 0.002 percent DPN supported growth equal to that obtained in medium containing 0.01 percent DPN, as recommended by Chalquest (1962).

No one method for measuring the growth of mycoplasmas was satisfactory because variable results were obtained from one test to another. Some of the variations could be attributed to high incubator temperatures due to a malfunctioning thermostat. The number of viable organisms in the inocula was another factor causing variations from test to test. If the pH was too low, many of the organisms in the inoculum were not viable, and the readings of the subsequent passage were quite low at 24 hours. Since these variations led to erroneously low values, the averages in the tables were made from the two trials with the highest values.

In the SPA antigen studies, it was found that only two variables influenced the sensitivity of the antigens - concentration of the antigen and dextrose in the culture medium. Those antigens standardized in the Hopkin's tube were much more concentrated than those standardized by colorimetry. The concentrated antigens gave excellent agglutination with positive sera; however, they also agglutinated some negative sera. This problem was not seen with the less concentrated antigens, although the

agglutination with positive sera was not as easy to see. Nevertheless, the agglutination with the less concentrated antigens was very apparent and distinct with close observation. It is felt that more work needs to be done to determine whether or not the antigen concentration should be as high as present U. S. Department of Agriculture standards dictate.

When turkey sera were used in the SPA test with an antigen grown in medium containing dextrose, many nonspecific agglutinations occurred and reproducibility was very poor. Adler and DaMassa (1968) also found that SPA antigens grown in medium with dextrose were less sensitive. They did not use a buffering system in their medium. A buffering system was used in the medium for antigen production in these experiments, but there was still a marked drop in pH, and this was probably responsible for the decreased sensitivity.

Buffer solutions at two different pH's (6.2 and 7.4) were used to resuspend the antigens used in SPA tests with turkey sera and both gave satisfactory results. However, more work needs to be done to determine the optimum pH for maximum sensitivity.

It is felt that concentration of the antigen and dextrose in the medium may be responsible for some of the reports of high incidence of infection in the absence of other signs. In these experiments, no cross reactions were observed between sera containing M. gallisepticum antibodies and M. synoviae antigens.

In the SPA tests, many agglutinations were encountered which were thought to be nonspecific and apparently caused by freezing of the sera. Thornton (1969) reported that nonspecific agglutination due to freezing

and thawing sera could be avoided by heating the sera at 56°C for 30 minutes. Hammar et al. (1958) used centrifugation to remove false negative results. In the present experiments, only centrifugation removed the non-specific agglutinations, for all sera were routinely heated to 56°C for 30 minutes. Heating the sera in this manner did not destroy their tube agglutinating activity as reported by Cover et al. (1960) and Yoder and Hofstad (1964).

It was also found that TA antigens were less sensitive when grown in media that had been autoclaved than those which had been filter sterilized.

In the experiments with chicken sera, it was found that the TA tests gave positive results first. Positive results were obtained as early as 7 to 8 days post inoculation whereas the SPA and HI tests were not positive until 8 to 14 days post inoculation. However, by 21 days all results were positive.

The three tests correlated very closely in the experiments with turkey sera. Titers did not appear until 14 to 21 days post inoculation. All tests were positive by 28 days post inoculation.

These results agree with the findings of Fahey and Crawley (1954), Crawley (1959), Newnham (1964), Adler (1954)(1958), Baharsefat and Adler (1965a), Mohamed and Bohl (1968) and Lawson and Hertler (1969). However, Thornton (1969), working with M. gallisepticum, found the SPA test gave positive results before the HI test. Also Roberts and Olesiuk (1967), working with M. synoviae, found the SPA test to be positive before the TA test.

SUMMARY

A medium which had been used previously for isolation of Mycoplasma synoviae was tested and found to be satisfactory also for antigen production. Many additives and treatments were tried in attempts to improve the yield of antigen. Several changes were beneficial. It was found that filtered medium supported faster growth than autoclaved medium. Swine serum which had been treated with 1N hydrochloric acid was better than rabbit or turkey serum. Tris buffers were not as good as phosphate buffers. The concentration of DPN could be decreased from 0.01 percent to 0.002 percent and optimum growth was still obtained. Dextrose markedly increased the growth rate of M. synoviae and decreased the incubation time necessary for maximum growth. However, if the incubation time was extended from 36 hours to 72 or 84 hours, the yield in medium without dextrose was about equal to that in dextrose medium, and fewer false positive reactions were observed with the antigen produced in the medium without dextrose. Additional vitamins improved the growth of M. synoviae and were included in the final formula. Cholesterol also increased the growth of M. synoviae, but due to problems with standardization and autoagglutination, it was not incorporated into the final medium.

Studies were made of antigen shelf life and reactivity. It was found that antigens produced in these studies were stable and were acceptable from the standpoint of serological sensitivity. Buffers, preservatives, redox potentials, dyes and pH's were included in these studies. None of these variables affected the sensitivity of the antigens. However,

concentration of the antigen and dextrose in the medium were definite factors effecting the reactivity of the antigen. Antigens which were standardized according to USDA specifications tended to give more false positives than those antigens which were not as concentrated. Antigens grown in medium containing dextrose gave more nonspecific reactions than those grown in medium without dextrose, particularly when turkey sera were used.

The serological response of chickens and turkeys to M. synoviae infection were studied using serum plate agglutination, tube agglutination and hemagglutination-inhibition tests. It was found that all three tests correlated fairly well. The TA test was the most sensitive to early titers but all were positive by 3 to 4 weeks post infection. False positive SPA reactions were often encountered unless certain precautions were taken in handling of the test sera. Sera positive for M. gallisepticum antibodies were not positive with M. synoviae SPA antigens.

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