

## Bacteriological and Serological Survey of Avian Mycoplasmosis in Peninsula Malaysia

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### Abstract

Some mycoplasmas were isolated from most of the oropharyngeal swabs taken from 7-month-old layer chickens in Ipon but not from 1-month-old broiler chickens in Sitiawan in Malaysia. It was noted that 16 of the 18 strains isolated from one layer chicken flock were arginini catabolizing mycoplasmas and identified as *Mycoplasma gallinarum* through metabolism inhibition test. On the other hand, 26 of the 31 strains from another layer flock were a glucose fermenter, one of which was identified as *M. synoviae*. This is the first isolation of this species in Malaysia. An improvement was made on the ELISA technique in order to precisely detect antibodies against *M. gallisepticum* and *M. synoviae*. The improved technique employed antigens made from young cultures, blocked the antigen coated plates, and diluted test serum with skim milk containing buffer. A serological survey of chicken flocks in Malaysia using the improved ELISA technique together with a serum plate agglutination and hemagglutination-inhibition test that both *M. gallisepticum* and *M. synoviae* infections prevailed in layer chickens at high incidences but not in broiler flocks in the middle part of West Malaysia.

**Discipline:** Animal health

**Additional key words:** ELISA, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*

### Introduction

Respiratory mycoplasmosis in chickens due to *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS) is one of the most serious problems in the poultry industry. It causes economic losses which result from the decreased egg production in layers and from the reduced weight gain and the lessened feed efficiency in broilers. Clinical signs are usually mild or inapparent, but the organisms attach to mucosal surfaces and remain in the upper respiratory tract for a long period of time. The disease is worldwide in distribution, wherever chickens are raised in large commercial flocks.

The present paper attempts to review results of the bacteriological and serological survey which was made in Malaysia to identify the morbidity of respiratory mycoplasmosis in chickens, since limited information of this disease is presently available in the country.

### Materials and methods

#### (1) Mycoplasma strains

*M. gallisepticum* PG-31 and S6, *M. gallinarum* PG-16, *M. gallinaceum* DD, *M. iners* PG-30, *M. meleagridis* 17529, *M. synoviae* WVU1853 were used for comparative study. The strains PG-31, PG-16, PG-30, 17529 were supplied by Dr. M. F. Barile,

National Institute of Health, Maryland and strains S6, DD and WVU1853 were originally obtained from Dr. J. Fabricant, Department of Avian Disease, Cornell University, New York.

## (2) Media

Modified K-broth or agar was used for isolation of mycoplasmas and preparation of antigens. PPLO broth w/o CV (Difco; 21 g) and yeast extract (Difco; 5 g) were dissolved in 800 ml of distilled water and sterilized at 121°C for 15 min, and then the following supplements were added: 10 ml of 2.5% sodium glutamate, 10 ml of 0.2% DNA, 10 ml of 1% cysteine, 10 ml of 1% nicotine adenine dinucleotide, 150 ml of horse serum, 10 ml of 2.5% thallos acetate, 1 million units of penicillin G. To prepare agar medium, Agar Noble (Difco; 12 g) was added before autoclaving. Urea-broth containing 0.1% urea and 0.004% phenol red for pH indicator in addition to K-broth with adjusted pH of 6.0 was also used for the isolation of ureaplasmas.

## (3) Samples

Oropharyngeal swabs to isolate mycoplasmas and ureaplasmas and serum samples for serological examinations were taken from chickens in three broiler farms in Sitiawan and two layer farms in Ipoh. Both cities are located in the middle part of West Malaysia. Broiler chickens in Sitiawan were 1 month of age and layer chickens in Ipoh were 7 months old. Twenty chickens were randomly selected for collecting samples in each farm.

## (4) Antigen preparation

*M. gallisepticum* S6, *M. gallinarum* PG-16, *M. gallinaceum* DD, *M. iners* PG-30, *M. meleagridis* 17529 and *M. synoviae* WVU1853 were subject to antigen preparation. One ml of logarithmic phase of the culture was inoculated into 1 l of modified K-broth and incubated at 37°C for 2 to 4 days. After centrifugation at 12,000 g for 30 min, the pellets were washed twice with phosphate buffered saline (PBS) and finally suspended in 10 ml of PBS and stored at -20°C until use.

## (5) Antiserum preparation

Equal amounts of antigen and Freund's complete adjuvant were mixed using a homogenizer. A rabbit weighing approximately 1.5 kg was injected with 4 ml of the mixture intramuscularly and subcutaneously (2 ml each) followed by the same procedure 1 week later. After 2 weeks of the second injection, 1 ml of the booster injection of antigen alone was

given intravenously in 3 to 4 successive days. Serum antibody titer was checked by metabolism inhibition (MI) test according to the method described by Purcell et al.<sup>5) 5 to 7 days after the last injection. When MI titer showed 1:1,240 or higher, the rabbit was exsanguinated. In case where sufficient titer was not obtained, booster injection was repeated.</sup>

## (6) Isolation procedures

Swab samples were taken from oropharynx of the randomly selected chickens in each farm and put into 0.5 ml of PPLO broth (Difco) supplemented with horse serum (15%) and ampicillin (1 mg/ml) and transported to the laboratory kept in ice box on the day of sampling. The 0.2 ml of the materials were inoculated into modified K-broth and Urea-broth. Serial ten-fold dilutions were made in each broth and incubated at 37°C. Urea-broth was examined daily for its color change to alkaline shift up to 7 days. K-broth was examined every 3 or 4 days for their turbidity up to 2 weeks and subcultured onto K-agar at 7 and 14 days of incubation. Agar plates were incubated at 37°C in the presence of 5-10% CO<sub>2</sub> in air and examined every 2 to 3 days up to 2 weeks before they were regarded as negative. Positive cultures were cloned 3 times on modified K-agar and then subjected to serological identification by the MI test using rabbit antiserum against *M. gallisepticum*, *M. synoviae*, *M. gallinaceum*, *M. iners* and *M. gallinarum*.

## (7) ELISA procedure

Flat-bottomed, 96 well microplates (Flow Laboratories) were coated overnight at 4°C with appropriate concentration of sonicated antigen (20 kc, 3 min) in carbonate buffer, pH 9.6. After coating, the plates were washed 3 times with Tris buffered saline solution containing 0.05% Tween 20 (Tw-TBS). The plates were then treated with 100 µl/well of blocking solution at 37°C for 1 hr. The blocking solution is a mixture of 1 volume of Tw-TBS with 5% skim milk (SM-Tw-TBS) and 19 volumes of Tw-TBS with 1% bovine serum albumin (BSA-Tw-TBS). After discarding the blocking solution, the plates were stored at -20°C until use. The test serums were diluted 1:20 by adding 10 µl to 190 µl of SM-Tw-TBS and 5 µl of 1:20 dilution was then dispensed into the antigen coated plate containing 100 µl/well of BSA-Tw-TBS. After 60 min incubation at 37°C, the plates were washed 3 times with Tw-TBS, and 100 µl of the appropriate dilution of horse radish

peroxidase conjugated anti-chicken IgG (ICN Immuno Biologicals) was placed in all wells, incubated at 37°C for 60 min and washed 3 times. Then 100 µl of freshly prepared substrate solution (30% H<sub>2</sub>O<sub>2</sub>; 100 µl, 0-phenylenediamine dihydrochloride; 100 mg, citrate buffer pH 5.0; 100 ml) was added. Substrate degradation was stopped by the addition of 50 µl of 3M H<sub>2</sub>SO<sub>4</sub> after incubation at room temperature for 15 min. Absorbance was measured at 492 nm with a Titertek Multiscan (Flow Laboratories). Positive and negative controls described below were included in each plate.

#### (8) Other serological tests

The serum plate agglutination (SPA) and hemagglutination inhibition (HI) tests were carried out after the method by Sahu and Olson (1975)<sup>6)</sup>. Antigens for SPA test were kindly supplied by Dr. T. Yagihashi, Nippon Institute for Biological Science. Hemagglutination antigens were prepared at the National Institute of Animal Health. Negative control serums were obtained from specific pathogen free (SPF) chickens aged 5 to 10 weeks. Positive control serums were obtained from SPF chickens which had been immunized with MG or MS antigens with Freund's complete adjuvant.

## Results

### 1) Isolation of mycoplasmas from oropharyngeal swabs

The mycoplasmas were isolated from oropharynx of sixty 1-month-old broiler chickens at 3 farms in Sitiawan and of forty 7-month-old layer chickens at 2 farms in Ipoh. Chickens did not show any detectable clinical signs of respiratory diseases. As shown in Table 1, mycoplasmas were isolated from 14 of 20 chickens in farm D and from 19 of 20 chickens in farm E, both of which were layer farms. On the other hand, only one of 60 broiler chickens harbored mycoplasmas in the oropharynx. Ureaplasmas were not isolated from any of the chickens examined. The strains isolated were grouped into the following 3 categories: glucose fermentation positive-arginine catabolization negative; glucose fermentation negative-arginine catabolization positive; and both positive. It was noted that most of the strains isolated from farm D were arginine catabolizers, while 26 of the 31 strains derived from farm E were glucose fermenters (Table 2). All the strains which

Table 1. Isolation of mycoplasmas from oropharyngeal swabs of apparently healthy broiler and layer chickens in Malaysia

Farm	Location	Type of chickens	Age (months)	Mycoplasma	Ureaplasma
A	Sitiawan	Broiler	1	0/20	0/20
B	Sitiawan	Broiler	1	1/20	0/20
C	Sitiawan	Broiler	1	0/20	0/20
D	Ipoh	Layer	7	14/20	0/20
E	Ipoh	Layer	7	19/20	0/20

Table 2. Biochemical characterization of mycoplasmas isolated from oropharyngeal swabs of apparently healthy chickens

Farm	Incidence of mycoplasma	No. of strains cloned	No. of glucose positive strains	No. of arginine positive strains	No. of both positive strains
B	1/20	1			1
D	14/20	18	2	16 <sup>a)</sup>	
E	19/20	31	26 <sup>b)</sup>	3 <sup>a)</sup>	2

a): All of these strains were identified as *M. gallinarum* by metabolism inhibition test.

b): One of these strains was identified as *M. synoviae*.

catabolized arginine were identified as *M. gallinarum* with MI test. Though one of the glucose fermenting strains isolated from farm E was identified as *M. synoviae*, other glucose fermenters were not identified in the present investigations.

### 2) Specificity of ELISA

Cross-reactivity of MG or MS antigen with heterologous antisera in ELISA was examined using positive control sera obtained by immunizing 20 SPF chickens with MG or MS antigens (10 birds each). The highest absorbance value of MS antisera with MG antigen did not exceed 0.1 (Fig. 1). On the other hand, cross-reactivity of MS antigen with MG antisera was slightly higher than that of MG antigen with MS antisera, but still lower than 0.2 (Fig. 2). The results obtained here suggest that absorbance values of 0.2 or higher for MG antigen and 0.3 or higher for MS antigen be regarded as a positive reaction.

### 3) Serological survey of MG and MS infections

Detection of anti-MG and MS antibodies was

carried out using SPA test, HI test and ELISA. In 1-month-old broiler chickens, antibodies to MG and MS were not detected by any of the methods employed. On the other hand, layer chickens were positive for MG and MS antibodies, though the positive rate differed among the methods employed. The positive rate for MG and MS antibodies by SPA

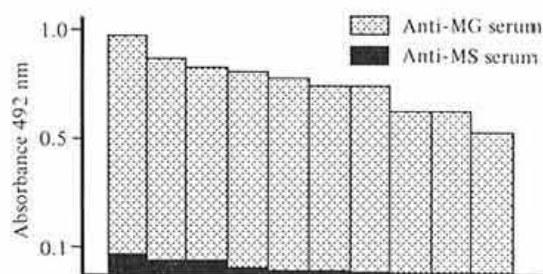


Fig. 1. Distribution of absorbance value of anti-*M. gallisepticum* and *M. synoviae* chicken serum with *M. gallisepticum* S6 antigen

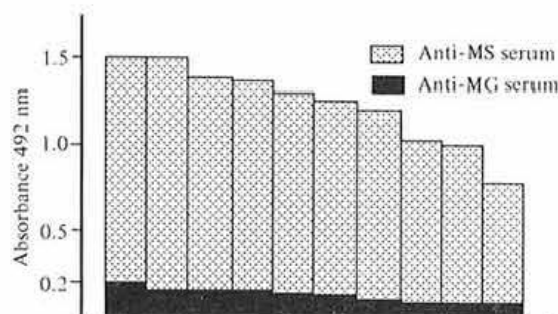


Fig. 2. Distribution of absorbance value of anti-*M. gallisepticum* and *M. synoviae* chicken serum with *M. synoviae* WVU1853 antigen

test was always lower than that of HI test or ELISA, especially in farm D. The results obtained by ELISA agreed to those by HI test except MG antibodies in farm D, in which ELISA showed a higher positive rate than HI test (Table 3).

## Discussions

In the present investigations, *M. synoviae* was isolated from an oropharyngeal swab taken from one of the 20 chickens in farm E. This is the first isolation of this species in Malaysia. On the other hand, *M. gallisepticum* was not isolated from any of the samples examined, though it was confirmed serologically that the organism prevailed in this country. This may be caused by the fact that only oropharyngeal swabs were subjected to isolation. Most of the glucose fermenting isolates were not identified, though all of the arginine catabolizers were identified as *M. gallinarum*. In the present investigation, antisera against *M. gallisepticum*, *M. gallinaceum* and *M. synoviae* were used to identify glucose fermenters, but there were several other glucose fermenting mycoplasma species in chickens. Further serological examination is necessary to identify these organisms. Glucose and arginine positive strains may tentatively be identified as either *M. iowae* or *M. lipofaciens*. However, the confirmative identification will have to be made serologically in this respect.

In previous reports<sup>1-4</sup>), cross-reactions between MG and MS were pointed out to be a serious problem in ELISA to assay for MG or MS antibodies. In the present investigations, however, absorbance values of cross-reactivity were much lower than those

Table 3. Serological survey of *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS) infections in broiler and layer chickens in Malaysia

Farm	Type of chickens	Age	SPA <sup>a)</sup>		HI <sup>b)</sup>		ELISA <sup>c)</sup>	
			MG	MS	MG	MS	MG	MS
A	Broiler	1	0/20 <sup>d)</sup>	0/20	0/20	0/20	0/20	0/20
B	Broiler	1	0/20	0/20	0/20	0/20	0/20	0/20
C	Broiler	1	0/20	0/20	0/20	0/20	0/20	0/20
D	Layer	7	3/20	4/20	5/20	20/20	17/20	20/20
E	Layer	7	15/20	11/20	18/20	20/20	20/20	20/20

a): Serum plate agglutination test.

b): Hemagglutination-inhibition test.

c): Absorbance value of 0.2 or greater for MG antigen and 0.3 or greater for MS antigen was considered as positive reaction.

d): No. positive/no. examined.

described previously. This discrepancy may be attributed to the following two differences: one is the incubation time for preparing antigens, and the other is the modification of blocking buffer and test serum diluent. Antigens harvested after longer incubation time showed much higher cross-reactivity, especially for MS antigens. In fact, in the present investigation, antigens were harvested after 40 hr incubation. The use of skim milk containing buffer for blocking and making initial 1:20 test serum dilutions reduced the background value to less than 0.1.

A serological survey was made to identify the morbidity of respiratory mycoplasmosis in Malaysia. For this purpose, ELISA were compared with SPA and HI tests, since the latter two methods are the most commonly used assays for serological detection of avian mycoplasmosis. The result indicated that both MG and MS infections had prevailed in layer flocks in Malaysia at high percentages. Among the serological methods employed, SPA showed the lowest positive rate. This might have been caused by the fact that the stage of infection was too late for SPA test at the time of sampling, since the SPA test detected IgM antibodies which appeared in early stage of infection and disappeared rapidly. ELISA agreed to HI test except MG antibodies in farm D, where ELISA showed a higher positive rate than HI test. Sahu and Olson<sup>7)</sup> described the existence of MG strains which did not elicit HI antibodies. Furthermore, Talkington et al.<sup>8)</sup> described that HI test was strain-specific and would not always detect antigenic variants that differ from the test strain. As no MG strains could be isolated from any of the chickens examined in the present investigations, a reason of this discrepancy could not be explained.

The present study indicated that both MG and MS infections prevailed in at least some layer chicken flocks in Malaysia. Further studies are required to identify distribution and morbidity of respiratory mycoplasmosis in Malaysia.

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