間接酵素抗体法による鶏血清中の抗Mycoplasma gallisepticumおよび抗M. synoviae抗体価の測定

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Indirect Immunoperoxidase Technique for the Assay of Antibodies against *Mycoplasma gallisepticum* and *M. synoviae* in Chicken Serum

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The indirect immunoperoxidase technique (IIP) was applied to the assay of antibodies against *Mycoplasma gallisepticum* and *M. synoviae* in chicken serum by using colonies grown on the agar plate as antigen. The sensitivity and the specificity of IIP were evaluated by the use of sera from experimentally infected, field, and SPF chickens. As compared with tube agglutination and hemagglutination-inhibition tests, IIP was highly effective and specific for detecting antibodies against *M. gallisepticum* and *M. synoviae*.

For the detection of antibodies against *Mycoplasma gallisepticum* and *M. synoviae*, plate agglutination test(PA), tube agglutination test (TA), and hemagglutination-inhibition test (HI) have been used widely. It was apparent, however, that they sometimes showed a nonspecific reaction and that their sensitivity was not so high.[10,13]

Previously, the direct immunoperoxidase technique was successfully applied to the identification of *M. gallisepticum* and *M. synoviae*.[6] This study was carried out to evaluate the application of the indirect immunoperoxidase technique (IIP) to the detection of antibodies against *M. gallisepticum* and *M. synoviae* in sera from experimentally infected chickens and specific pathogen free (SPF) chickens.

MATERIALS AND METHODS

Antigen: Broth cultures of the 1RF strain of *M. gallisepticum*[11] and the 1-3SN strain of *M. synoviae*[12] stored at −70°C in small portions were used. They were diluted with Frey's broth medium[3] to a concentration of 2×10^5 colony forming units (CFU) per milliliter, and 0.05 ml of the dilution was spread evenly with a glass stick over Frey's agar plate 45 mm in diameter. Colonies grown on the agar plate after 2 weeks' incubation at 37°C in an atmosphere containing 5% carbon dioxide were used as antigen without fixation.

Sera from experimentally infected chickens: SPF day-old PDL-1[4] chicks were experimentally infected with the 1RF strain of *M. gallisepticum* and the 1-3SN strain of *M. synoviae* by aerosol or by foot-pad inoculation. Aerosol infection was carried out by using a cabinet, where 21 birds were kept in the air containing 10^4 CFU of organisms per liter of air for 5 minutes. Of them, six were injected with 0.2 ml of broth culture containing 10^8 CFU of organisms in the left foot pad. As controls, 18 birds were treated with broth medium.

Sera were collected over a period from 3 to 15 weeks after infection at 3-week intervals and stored at 4°C with 0.01% thimerosal. Three, 9, and 15 weeks after infection, 5

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birds each of the aerosol infected group were killed. Cotton swab samples were obtained from nasal cavity, sinus, trachea and air sac and submitted to culture.

Sera from field chickens: Sera were collected from the same 20 birds of 4 field flocks, 5 birds per flock, at 4, 10, 18, 24, and 30 weeks of age, respectively, and stored at −20°C until use.

Sera from SPF chickens: Sera were collected from the same 20 birds of the SPF PDL-1 flock at 42, 74, and 107 weeks of age and stored at −20°C until use. The SPF chickens were kept in an filtered air under positive pressure type house. All of them were serologically monitored by PA at 8-week intervals from 18 to 107 weeks of age for antibodies against *M. gallisepticum* and *M. synoviae*. When eight of them showed a positive reaction at 107 weeks of age (two with *M. gallisepticum* antigen and six with *M. synoviae* antigen out of 145 birds), they were subjected to autopsy and confirmed by culture to be free from *M. gallisepticum* and *M. synoviae* infection.

Conjugate: Anti-chicken immunoglobulin serum was prepared in a rabbit by injection with chicken immunoglobulin isolated from chicken serum by precipitation with one-half saturation of ammonium sulfate and mixed with Freund's incomplete adjuvant. The gamma-globulin fraction isolated from anti-chicken immunoglobulin serum by precipitation twice with one-third saturation of ammonium sulfate was labelled with peroxidase (type VI, Sigma) by the method of Nakane & Kawaoi.[8]

Purification of conjugate by gel filtration was omitted. The conjugate was stored in small portions at −70°C with 1% bovine serum albumin.

When determined by checkerboard titration, the optimal concentration of the conjugate was 1:16 or 1:32. The dilution 1:16 was used.

Indirect immunoperoxidase technique: A small square piece of filter paper about 5×5 mm in size was soaked in chicken serum which had been diluted by the twofold serial system in phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBST). It was placed on colonies grown on the agar plate. After incubation at 37°C for 2 hours the paper and excess serum were washed off with PBST. After washing for 30 minutes the agar surface was dried slightly at 37°C for 30 minutes.

Then, a small square piece of filter paper which had been soaked in the conjugate dilution in PBST was placed on the same spot of the agar plate as that soaked in chicken serum had been. After incubation at 37°C for 3 hours the paper and excess conjugate were washed off with PBST in the same manner as mentioned above. Then a substrate solution was freshly prepared by mixing 0.2 ml of 2% benzidine in ethanol, 2 ml of 4.5% magnesium sulfate in 0.01 M PBS, pH 7.2, and 0.01% of hydrogen peroxide. It was placed on the agar plate. This plate was incubated at room temperature for 15 minutes and then washed with PBST. Colonies were stained and observed by the stereoscopic microscope. The antibody titer was expressed with the reciprocal of the end dilution that showed positively stained colonies.

Hemagglutination-inhibition test: The HI was performed by the procedure mentioned by Kuniyasu et al.[7] and Sato et al.[12] Sera were titrated by the twofold serial system beginning with a 1:5 dilution.

Serum plate agglutination test and tube agglutination test: PA and TA were performed by the method of Ando et al.[1] Sera were titrated by the twofold serial system beginning with a 1:5 dilution.

RESULTS

Examination of optimal antigen conditions: Colonies grown after incubation for 2 to 3 weeks showed about two times as high an IIP titer as colonies grown after incubation for 1 week. When fixed in methanol, *M. gallisepticum* colonies decreased in staining specificity and were stained with sera from *M. synoviae* infected birds and SPF birds, as shown in Table1. The staining specificity

Table 1. *Decrease in staining specificity of M. gallisepticum colonies by fixation in methanol*

Colony	Fixation	Chicken serum	Density of colonies stainable with each serum dilution					
			10^1	10^2	10^3	10^4	10^5	10^6
MG	−	Anti-MG	###	###	###	+	−	−
		Anti-MS	∓	∓	∓	−	−	−
		Normal	−	−	−	−	−	−
	+	Anti-MG	###	###	###	##	+	##
		Anti-MS	##	##	##	+	##	##
		Normal	##	##	##	##	##	+
MS	−	Anti-MG	−	−	−	−	−	−
		Anti-MS	##	##	###	+	−	−
		Normal	−	−	−	−	−	−
	+	Anti-MG	−	−	−	−	−	−
		Anti-MS	###	###	###	+	−	−
		Normal	−	−	−	−	−	−

MG: *Mycoplasma gallisepticum*
MS: *M. synoviae*

Table 2. *Determination of optimal conjugate dilution by checkerboard titration with M. gallisepticum antigen*

Reciprocal of conjugate dilution	Anti-MG serum dilution														Anti-MS serum dilution		No anti-serum
	12.5	25	50	100	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400	10	100	
4	##*1	###	###	###	###	###	###	###	###	##	+	±	±	−	##	##	−
8	##	###	###	###	###	###	###	###	##	##	+	±	±	−	+	±	−
16	##	###	###	###	###	###	###	###	##	##	+	±	−	−	±	−	−
32	##	###	###	###	###	###	###	###	##	##	+	±	−	−	±	−	−
64	##	###	###	###	###	###	###	###	##	##	+	±	−	−	−	−	−
128	+	##	##	##	##	##	##	##	##	##	+	±	−	−	−	−	−
256	+	+	##	##	##	##	##	##	##	+	+	±	−	−	−	−	−
512	±	±	±	±	±	±	+	+	+	+	±	±	−	−	−	−	−

*1 ###～−: Grades of staining density of colonies of MG.
MG, MS: See the footnote of Table 1.

Table 3. *Determination of optimal conjugate dilution by checkerboard titration with M. synoviae antigen*

Reciprocal of conjugate dilution	Anti-MS serum dilution														Anti-MG serum dilution		No anti-serum
	12.5	25	50	100	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400	10	100	
4	##*1	###	###	###	###	###	###	###	##	##	+	±	±	−	##	##	−
8	##	###	###	###	###	###	###	###	##	##	+	±	±	−	−	−	−
16	##	###	###	###	###	###	###	###	##	##	+	±	±	−	−	−	−
32	##	###	###	###	###	###	###	###	##	##	+	±	−	−	−	−	−
64	##	###	###	###	###	###	###	###	##	##	+	±	−	−	−	−	−
128	##	###	###	###	###	###	###	###	##	##	+	±	−	−	−	−	−
256	+	+	+	##	##	##	##	##	##	##	+	±	−	−	−	−	−
512	−	±	+	−	±	±	±	±	±	±	±	±	−	−	−	−	−

*1 ###～−: Grades of staining density of colonies of MS.
MG, MS: See the footnote of Table 1.

of *M. synoviae* colonies, however, was not affected by fixation. This indicates that unfixed colonies grown after incubation for 2 to 3 weeks are suitable for IIP antigen.

Determination of optimal conjugate dilution: Optimal conjugate dilution for the titration of antibodies against *M. gallisepticum* and *M. synoviae* was examined by checkerboard titration. The results obtained are shown in Tables 2 and 3. Each dilution from 1: 4 to 1: 256 gave the same IIP titer, although the higher the dilution, the paler the colonies stained. When the dilution was 1: 4, colonies were stained nonspecifically with a heterologous combination of antisera diluted 1: 100. Taking these results into account, 1: 16 and 1: 32 were determined as optimal dilutions. They made it possible to stain colonies specifically and read IIP titer with ease.

Sensitivity of IIP in experimentally infected chickens: Antibodies in the sera from experimentally infected chickens were titrated by IIP, HI and TA. By IIP, the sera were titrated by the twofold dilution steps beginning with 1: 100 dilution. The results obtained from the aerosol infected groups are shown in Figs. 1 and 2. The geometric mean of IIP titers reached a maximum 6 weeks after infection. The maximum mean titers were 1: 35,200 in the *M. gallisepticum* aerosol infected group and 1: 20,000 in the *M. synoviae* aerosol infected group. Throughout the experiment, the geometric mean of IIP titers was approximately 100 times as high as that of HI or TA titers. All the sera from the aerosol infected groups stained only homologous combination of colonies.

The serological response of the *M. gallisepticum* foot-pad infected group was almost the same as that of the *M. gallisepticum* aerosol infected group. All the sera from these groups stained only *M. gallisepticum* colonies.

The group of chickens infected with *M. synoviae* by way of the foot-pad showed the highest IIP titer of all the experimentally infected groups. The serum of 1 bird, however, reacted with not only *M. synoviae* colonies but also *M. gallisepticum* colonies 6 and 9 weeks after infection. The end dilution that could stain *M. gallisepticum* colonies was 1: 12,800 6 weeks and 1: 1,600 9 weeks after infection. The IIP titer of these sera was 1: 409,600 and 1: 204,800, respectively. It

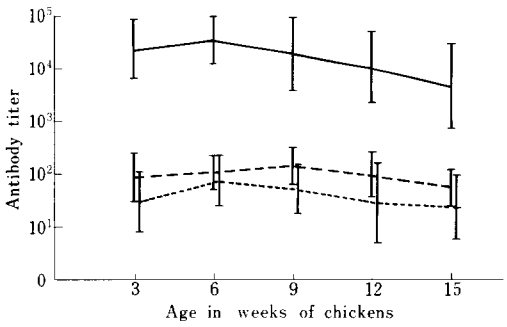

Fig. 1. Antibody titers determined by indirect immunoperoxidase technique (IIP), tube agglutination test (TA), and hemagglutination-inhibition test (HI) in chickens infected with *M. gallisepticum* by aerosol

—I—, —+— and ----I----: Mean titer ±95% confidence limit by IIP, TA and HI, respectively. Recovery rates of organisms from respiratory organs were 5/5, 4/5, and 4/7 at 3, 9, and 15 weeks of age, respectively.

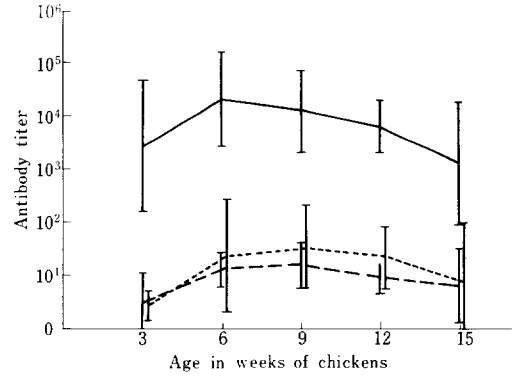

Fig. 2. Antibody titers determined by indirect immunoperoxidase technique (IIP), tube agglutination test (TA), and hemagglutination-inhibition test (HI) in chickens infected with *M. synoviae* by aerosol

—I—, —+— and ----I----: Mean titer ±95% confidence limit by IIP, TA and HI, respectively. Recovery rates of organisms from respiratory organs were 5/5, 4/4, and 4/4 at 3, 9, and 15 weeks of age, respectively.

Table 4. *Titration of sera collected from field chickens by indirect immunoperoxidase technique (IIP) and hemagglutination-inhibition test (HI)*

Flock	Chicken number	Test	Antibody titer with MG antigen					Antibody titer with MS antigen				
			4	10	18	24	30	4	10	18	24	30
			weeks of age					weeks of age				
A	1	HI	—	—	20	40	80	—	—	20	20	80
	2		—	—	10	40	80	—	—	5	20	80
	3		—	—	10	80	40	—	—	5	80	20
	4		—	—	20	20	20	—	—	5	5	40
	5		—	—	20	10	10	—	—	5	5	20
	1	IIP	—	—	12,800	3,200	3,200	—	—	3,200	3,200	6,400
	2		—	—	3,200	1,600	12,800	—	—	6,400	3,200	6,400
	3		—	—	3,200	6,400	3,200	—	—	1,600	6,400	1,600
	4		—	—	12,800	1,600	6,400	—	—	1,600	1,600	3,200
	5		—	—	—	800	3,200	—	—	3,200	1,600	1,600
B	1	HI	—	—	10	10	10	—	—	—	—	—
	2		—	—	10	40	20	—	—	—	—	—
	3		—	—	5	—	20	—	—	—	—	—
	4		—	—	5	10	10	—	—	—	—	—
	5		—	—	—	—	10	—	—	—	—	—
	1	IIP	—	—	1,600	1,600	1,600	—	—	—	—	—
	2		—	—	1,600	3,200	3,200	—	—	—	—	—
	3		—	—	1,600	3,200	3,200	—	—	—	—	—
	4		—	—	3,200	1,600	800	—	—	—	—	—
	5		—	—	—	1,600	6,400	—	—	—	—	—
C	1	HI	—	—	—	—	5	—	—	10	—	5
	2		—	—	—	—	5	—	—	—	5	5
	3		—	—	—	—	—	—	—	—	5	5
	4		—	—	—	—	—	—	—	—	5	5
	5		—	—	—	—	—	—	—	—	5	5
	1	IIP	—	—	—	—	—	—	—	—	6,400	400
	2		—	—	—	—	—	—	—	—	1,600	800
	3		—	—	—	—	—	—	—	—	1,600	3,200
	4		—	—	—	—	—	—	—	—	3,200	1,600
	5		—	—	—	—	—	—	—	—	800	1,600
D	1	HI	—	—	—	5	20	—	—	—	—	—
	2		—	—	—	—	—	—	—	—	—	—
	3		—	—	—	—	—	—	—	—	—	—
	4		—	—	—	—	—	—	—	—	—	—
	5		—	—	—	—	—	—	—	—	—	—
	1	IIP	—	—	—	—	—	—	—	—	—	—
	2		—	—	—	—	—	—	—	—	—	—
	3		—	—	—	—	—	—	—	—	—	—
	4		—	—	—	—	—	—	—	—	—	—
	5		—	—	—	—	—	—	—	—	—	—

MG, MS: See the footnote of Table 1.

Table 5. *Comparison of specificity between indirect immunoperoxidase technique (IIP) and hemagglutination-inhibition test (HI) by use of sera from SPF chickens*

Age in weeks of chickens	Number of chickens	Number of chickens positive for HI		Number of chickens positive for IIP	
		MG antigen	MS antigen	MG antigen	MS antigen
42	20	1 (5)*1	0	0	0
74	20	3 (5, 5, 5)	1 (5)	0	0
107	20	5 (5, 40, 40, 40, 40)	1 (5)	0	0

*1 In parentheses are shown the HI titers of positive birds.
MG, MS: See the footnote of Table 1.

was the highest titer of all the groups of experimentally infected chickens.

The staining of *M. gallisepticum* colonies cross-stained with these sera was obviously different from that of those stained with a homologous combination of antisera. With these sera only about one tenth of colonies was stained partially. With a homologous combination of antisera, almost all the colonies were stained evenly.

Comparison of IIP with HI for detection of antibodies in field chickens: One hundred serum samples were obtained from the same 20 birds in the field and titrated by IIP and HI. As shown in Table 4, the antibody titers obtained by both methods correlated with each other in almost all the serum samples and IIP titers were always higher than HI titers. Four serum samples from 3 birds belonging to two different flocks, however, showed a positive reaction with *M. gallisepticum* antigen only by HI.

Specificity of IIP: Sixty serum samples were collected from the same 20 birds of the SPF flock and examined by IIP and HI. Table 5 shows the results obtained. All the serum samples showed a negative reaction to IIP at serum dilutions of 1: 25, 1: 50, and 1: 100. On the other hand, 9 serum samples showed positive reaction, five showing 1: 5 and four 1: 40, to HI with *M. gallisepticum* antigen, and 2 serum samples 1: 5 with *M. synoviae* antigen. It seemed that the number of HI-positive sera and the HI titer might have increased with the advance in chicken age.

DISCUSSION

In the present studies, it was shown that IIP was a very sensitive procedure to assay antibodies against *M. gallisepticum* and *M. synoviae*. In all the experimentally infected chickens IIP titers were always above 1: 100 and about 100 times as high as HI titers or TA titers.

In spite of the high sensitivity, IIP presented only one case of cross reaction among the chickens infected with *M. synoviae* by way of the foot-pad. This cross reaction apparently differed from the specific one because of the odd staining of colonies.

The sensitivity of IIP was also shown in field flocks, in which the IIP reaction correlated with HI reaction and presented IIP titers about 100 times as high as HI titers, as seen in the experimentally infected birds.

Furthermore, the IIP was found to exhibit higher specificity than the HI test when performed on a flock of SPF chickens. By HI, some positive reactions were seen. The number of HI-positive sera and the titer by HI increased with the advance in age. By IIP there was no positive reaction. From this result, it was considered that the positive reaction of the field flocks only to the HI test might be nonspecific.

An other advantage of IIP is that the procedure is very simple, especially for the preparation of antigen. Only colonies grown on the agar plate after 2-week incubation are necessary as antigen. No other com-

plicated procedures are required, contrary to enzyme-linked immunosorbent assay (ELISA) in which solubilized antigens have to be used. Although IIP seems to be unsuitable for assay of a large number of samples, it is very useful for assay on a laboratory scale. For the field application, ELISA had better be developed.

Studies were previously made on the ELISA applied to a small number of serum samples. However, whole-cell antigens of *M. gallisepticum* and *M. synoviae* soluble in sodium dodecyl sulfate,[2,9] Nonidet P40, and pH 10[5] showed nonspecific reactions. Whole-cell soluble antigens showed a nonspecific reaction to ELISA, and fixed antigen of *M. gallisepticum* to IIP. Taking these result into account, it was considered that the type-specific antigens of *M. gallisepticum* and *M. synoviae* were located on the cell surface.

In conclusion, IIP was shown to be a very sensitive, specific and simple technique for measuring antibodies against *M. gallisepticum* and *M. synoviae* in chicken serum. It seemed to be useful when antibody titers were very low and when there was a fear of nonspecific reaction induced by any other method.

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