Enhancement of Mycoplasma Agglutination Titers by Use of Anti-Globulin.* (29318)

H. E. Adler and A. J. DaMassa (Introduced by C. Stormont)
Department of Avian Medicine, School of Veterinary Medicine, University of California, Davis

Although the anti-globulin technique(1), as reintroduced by Coombs *et al*(2), has proved its efficacy in enhancement of the titer of agglutinins against red blood cells and bacteria and in detecting the presence of incomplete antibodies (cryptoagglutinins), the procedure has not previously been used in studies of Mycoplasma.

The present communication describes the use of anti-globulin in agglutination tests with antisera directed against 3 different Mycoplasma of avian origin.

Materials and methods. The 3 Mycoplasma used in the study were *M. gallisepticum, M. synoviae,* and a strain known simply as the N strain, which was originally isolated from the air sacs of a turkey(3). All 3 strains are pathogenic for either chickens or turkeys.

Antibodies to *M. gallisepticum* were produced by infecting chickens into the abdominal air sacs with 4.5×10^6 organisms. This inoculum incited severe air sac lesions. Serum samples were taken at 8-hour intervals for 3 days following the injection, then daily for the next 5 days, and on days 10, 21, and 31. *M. synoviae* antisera was obtained from Dr. N. O. Olson, West Virginia University, Morgantown. The N strain antiserum was obtained from Dr. R. Yamamoto of this School.

M. gallisepticum and N antigen were prepared as described previously(4) while *M. synoviae* antigen was produced by the method of Olson *et al*(5). For comparative purposes we also used antigen and antisera against *Haemophilus gallinarum.* These were provided by Dr. R. Yamamoto.

Anti-chicken globulin serum of rabbit origin was obtained from a local commercial source (Antibodies Inc., Davis, Calif.).

Regular saline agglutination or tube agglutination tests were performed using antigens which had been standardized to the #10 density on the McFarland scale(4), a density which has proven to be satisfactory for such tests. One-half ml of antigen was added to each of a number of 10 ml plastic tubes containing 2.5 ml of doubling antiserum dilutions made in 0.85% NaCl. The tests were incubated for 2 hours at 37°C and then were held at 5°C overnight. The tube agglutination titer was then determined. Agglutination was recorded in degrees ranging from 4+ (complete agglutination) to 1+ (a barely visible reaction). In the text or tables which follow titers are expressed as reciprocals of the dilutions and represent a reaction intensity of 2+ or greater.

For the anti-globulin tests the sediment (Mycoplasma antigen with attached antibody) in each of the tubes used in the saline agglutination tests was washed 3 times to eliminate free, unattached Mycoplasma antibodies. After the last washing this sediment was resuspended in 0.5 ml of saline and once again examined for agglutination. A drop (approx. 0.05 ml) of the resuspended sediment was placed on a plate and to it was added a loopful (2.0 mm inside diam.) of diluted anti-globulin serum. The mixture was gently rotated for 3 minutes, at which time the degree of agglutination was assessed.

Results. An antiserum against *M. gallisepticum* having a saline agglutination titer of 1:320 was used to determine the optimum dilution of anti-globulin for enhancement of agglutination. That dilution was 1:10, as indicated by the data in Table I. Using larger amounts of anti-globulin than that contained in one loopful of anti-globulin diluted 1:10 did not further augment the agglutination end-point. Accordingly, one loopful of a 1:10 dilution of anti-globulin was used in all subsequent tests.

To reassess the optimum amount of Mycoplasma antigen needed for anti-globulin end points 3 different concentrations were evaluated (0.5, 1.0, and 2.0 × McFarland #10). Since greater or lesser concentrations than

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TABLE I. Agglutination of *M. gallisepticum* Non-agglutinating Antibody with 4 Dilutions of Anti-globulin.

Dilution of anti-globulin	Dilution of anti-serum*			
	1280†	2560	5120	10,240
10†	++++	++++	+++	—
20	++++	+++	++	—
40	++	++	—	—
80	+	+	—	—

* Anti-serum end point without anti-globulin (saline titer) = 1:320.
† Reciprocals of the dilution.

1 × McFarland #10 did not enhance the agglutination end point when a 1:10 dilution of anti-globulin was employed, this concentration was used in all future tests.

In Table II are shown the saline and anti-globulin titers from 5 chickens at various intervals following their inoculation with *M. gallisepticum*. As may be noted, maximum titers were usually obtained with antiserum taken between the 7th and 21st day post-inoculation, the only exception being bird #209 in which the antiserum reached its highest titer at day 31. Generally, the titers were markedly augmented by the use of anti-globulin.

Similarity between the 3 Mycoplasma was evaluated on the basis of cross-agglutination tests (Table III). No similarity is observed when saline titers are compared whereas some antigenic resemblance occurs when anti-globulin titers are evaluated.

Antigenic relationships between *M. gallisepticum* and *H. gallinarum* were also assessed. The anti-globulin titer of *H. gallinarum* antiserum with *M. gallisepticum* antigen was only 1:40 as contrasted with a titer of 1:320 with the homologous antigen.

Heating to 56°C for 30 minutes, freezing and thawing does not seem to alter the anti-globulin end-point. Titers on specific serum samples have been compared on several dates. Even with the utilization of different antigen lots, titers have not varied more than one tube when 2-fold dilutions are employed.

TABLE II. Comparison of Saline and Anti-globulin Agglutination Titers on Chicken Sera at Various Intervals Following *M. gallisepticum* Exposure.

	Saline and anti-globulin titers at various intervals																	
			Days post inoculation															
Bird	Pre-bleeding		4 days		5 days		6 days		7 days		8 days		10 days		21 days		31 days	
No.	S*	AG†	S	AG	S	AG	S	AG	S	AG	S	AG	S	AG	S	AG	S	AG
208	N‡	N	N	N	N	40	N	80	10	160	80	1280	80	10240	Nec	Nec	Nec	Nec
209	N	N	10	10	10	10	20	40	40	80	80	320	80	1280	160	1280	2560	5120
210	N	20§	10	20	10	40	20	640	40	320	40	640	80	2560	80	2560	80	1280
211	N	N	N	20	N	20	N	40	10	80	40	320	40	2560	80	2560	80	1280
212	N	N	N	N	N	N	N	N	N	10	10	40	10	320	40	1280	20	1280
213‖	10	10	N	N	N	N	N	N	N	N	N	N	N	N	N	N	20	20

* S = Saline titer.
† AG = anti-globulin titer.
‡ N = Negative.
§ Reciprocals of dilutions.
‖ = contact control.

Nec = Necropsied 14 days after exposure (saline titer 80, anti-globulin titer 1280).
The earlier blood samples did not have titers above 1:20 (post inoculation days 1-3).

TABLE III. Cross Agglutination Titers of 3 Mycoplasma by Saline and Anti-globulin Procedure.

	Antigens					
	M. gallisepticum		*M. synoviae*		*N. mycoplasma*	
Antisera	Saline*	AG†	Saline	AG	Saline	AG
M. gallisepticum	1:80	1:2560	<1:10	1:160	1:80	1:80
M. synoviae	<1:10	1:40	1:10	1:320	<1:10	1:20
N. mycoplasma	<1:10	1:20	1:10	1:40	1:10	1:320
Normal serum	<1:10	<1:10	<1:10	<1:10	<1:10	1:10

* Saline titer.
† AG = Anti-globulin agglutination titer.

Discussion. Serologic tests for Mycoplasma in many instances leave much to be desired. Although sensitive tests have been developed through the years for a number of Mycoplasma the majority require tedious laboratory techniques. At present the most sensitive test for detection of *M. pneumoniae* antibodies is the immunofluorescence test of Liu(6). This is an application of the anti-globulin procedure. It is a very sensitive and specific test but requires frozen sections of lungs of infected embryos, and the numbers of manipulations required in this system are usually large. The anti-globulin procedure described herein should be applicable for detection of *M. pneumoniae* antibodies. It may also be useful in determining antigenic similarity. In cross agglutination tests, common antigenic components of other species of Mycoplasma were detected.

Summary. Application was made of the anti-globulin procedure for detection of antibody to 3 Mycoplasma of avian origin. The test was specific, and antibodies were indicated at higher titers.

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Propagation of Encephalomyocarditis Virus in Swine Embryo Kidney Cells.* (29319)

H. ROUHANDEH, R. R. CHRONISTER AND M. L. BRINKMAN
(Introduced by H. A. Wenner)
Section of Virus Research, Department of Pediatrics and Department of Microbiology, School of Medicine, University of Kansas, Kansas City, Kansas

Encephalomyocarditis (EMC) virus has been recovered from tissues of naturally-infected pigs by Murane *et al*(1) and from pigs fed mouse-passaged EMC(2). In the course of our work we found that EMC virus produced cytopathic effect (CPE) on swine embryo kidney (SEK) cells. Reported herein are some quantitative data on the effects of EMC virus on SEK cells.

Materials and methods. Kidneys from fetal swine were removed aseptically and dispersed with trypsin by the method of Youngner(3) and suspended in medium LG(4) plus 10% calf serum. Test tubes (16 × 150 mm) received 1 ml each of a 0.20% cell suspension and were stoppered and incubated on a slant at 37°C. Petri plates (60 mm) received 8 ml each of 0.20% cell suspension and were kept in humidified atmosphere of an incubator continuously flushed with a mixture of 3% CO_2 and 97% air. Sheets developed in both tubes and plates in 5-8 days. Mouse L-cells were prepared as previously described by Rouhandeh(5). Tubes received 2 ml each of cell suspension.

EMC virus was supplied by American Type Culture Collection as a mouse-brain suspension. The virus was serially passaged 40 times in L-cells in our laboratory. It was plaque-purified by 3 serial isolations, and plaque-purified pools were prepared in L-cells. These stocks averaged 2-4 × 10^8 plaque-forming units (PFU) per ml in L-cells and in mice.

Tubes containing SEK cell sheets were washed twice, each time with 2 ml of phosphate-buffered saline (PBS)(6) and then inoculated with 0.1 ml each of L-cell passaged virus containing approximately 1,000 PFU

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