APPLIED MICROBIOLOGY, Apr. 1968, p. 558-562

Vol. 16, No. 4
Printed in U.S.A.

Effect of Dextrose in Medium for the Preparation of *Mycoplasma gallisepticum* Plate Antigens

H. E. ADLER AND A. J. DAMASSA

Department of Epidemiology and Preventive Medicine, University of California, Davis, California 95616

Received for publication 18 December 1967

Antigens for *Mycoplasma gallisepticum* were prepared from organisms propagated in media with and without dextrose supplementation. The antigens made from organisms produced in medium enriched with dextrose were less sensitive than the others in slide agglutination tests.

In early studies of *Mycoplasma gallisepticum* antigen production, researchers differed considerably in their opinions of the sensitivity of the rapid serum-plate test (RSP). Until recently, most investigators preferred either tube agglutination or hemagglutination-inhibition tests for the detection of *M. gallisepticum* antibody, particularly with turkey serums.

We have observed that the addition of dextrose to the culture medium used for antigen production, although increasing the total yield of organisms, reduces the sensitivity of the RSP test. A brief review of the literature reveals that Jungherr et al. (5) used 0.5% glucose with aeration in preparing *M. gallisepticum* antigen. This antigen, produced commercially, was satisfactory for testing chicken sera for *M. gallisepticum* antibodies but was less reliable in detecting agglutinins in turkey sera. At that time, Adler and Yamamoto (3) developed an antigen, prepared from organisms propagated in a medium without a glucose substrate, that was sensitive for the testing of *M. gallisepticum* antibodies of turkey origin. Hall (4) used 0.25% maltose without aeration in his protocol, whereas Vardaman (6) recommended the addition of 0.3% dextrose to his basal medium. An antigen production procedure in which glucose can be added as an optional component is described by the U.S. Department of Agriculture Agricultural Research Service (Suggested protocol for production of *M. gallisepticum* antigens, Pamphlet 1-5).

We are not familiar with any detailed investigation in which the only variable is an added carbon source. Such an investigation is reported here, with comparisons made of the sensitivity of the RSP test of antigens prepared from organisms propagated in a basal medium with and without added dextrose.

MATERIALS AND METHODS

The basal medium used for the growth of *M. Gallisepticum* was Difco PPLO Broth (without crystal violet) supplemented with 1% Yeast Autolysate (Albimi Laboratories, Flushing, N.Y.). After reconstitution of the medium in distilled water, the *p*H was adjusted to 7.2 with 1 N NaOH, and the medium was sterilized for 30 min at 121 C. To ensure that the basal medium was identical for all flasks, a large quantity was made in one container, and 4-liter samples were placed in 6-liter flat-bottom boiling flasks. For each trial (Tables 1, 2), 50% of the flasks were supplemented with 1% D-glucose before sterilization. This was the only variable. At inoculation, each flask received 10% heat-inactivated (56 C for 30 min) equine serum from a single container and either 10% (Table 1) or 1% (Table 2) seed, each from a common flask. The seed (S_6 strain of *M. gallisepticum*), an actively growing 48-hr culture grown in basal medium without sugars, had an approximate titer of 10^9 viable organisms per ml. Penicillin (500 units/ml) was added to each flask. Incubation at 37 C lasted for 168 (Table 1) or 192 hr (Table 2). In trial 1 (Table 1), the flasks were agitated on a shaking apparatus oscillating approximately 120 times/min in a horizontal plane for one continuous 4-hr cycle each day. For trial 2 (Table 2), the flasks were agitated in the same way, except that the instrument operated for 12 hr daily (2 hr on, 2 hr off, etc.). After incubation, the flasks were checked for purity by staining with Giemsa solution and plating on Difco PPLO Agar containing 15% heat-inactivated equine serum. Harvesting and standardization of the antigen were done as described previously (2).

The *p*H, approximate number of viable organisms per milliliter, and plate counts (primarily to ensure purity) were determined at selected intervals post-inoculation. The hydrogen ion concentration was measured in an expanded-scale *p*H meter (model 76; Beckman Instruments, Inc., Fullerton, Calif.). Purity and approximate plate counts were ascertained by streaking a loopful (26-gauge wire, 5-mm inner

diameter) on a serum agar PPLO plate prepared as described previously (no bacterial inhibitors). Growth on plates was recorded as 4+ (too many colonies to count), 3+ (over 100), 2+ (over 10), and 1+ (1 to 10 colonies). Concentrations of viable organisms were determined by making $\log_{10}$ dilutions in basal media (PPLO Broth plus 1% Yeast Autolysate) containing 1% D-glucose and 2 ml of a 1% aqueous suspension of phenol red per liter. The *p*H was adjusted to 7.5 before sterilization at 121 C for 15 min. The last tube showing acid production, indicated by a deep yellow color upon 10 days of incubation, was taken as the approximate number of viable organisms per milliliter.

By the RSP test, each lot of antigen obtained (30, 33, 36, Table 3) was tested against turkey and chicken antisera from artificially infected birds. One drop (approximately 0.025 ml) of each serum dilution was placed on a glass plate by use of a Pasteur pipette. An equivalent drop of antigen was added to each drop of antiserum dilution, and the end point of agglutination was recorded at the conclusion of a rotation period of 2 min with chicken antiserum and 3 min with turkey or goat serum. Agglutination was recorded in degrees ranging from 4+ (complete agglutination) to 1+ (a barely visible reaction). The highest antiserum dilution representing a reaction intensity of 2+ or greater was taken as the agglutination end point. Fresh and lyophilized antisera were used. Lyophilization, in our estimation, is perhaps the best method of preserving MG antiserum and stock cultures of viable MG.

Table 4 outlines RSP titers with antigens used at various concentrations. Standard antigens were sedimented for 10 min at 27,000 × *g* and were reconstituted to the original volume (standard, washed once), or to 50% (2×) or 33% (3×) of the original volume. This was done to determine whether "insensitive" antigens could be improved by concentration.

RSP titers were also conducted on chicken and turkey sera with antigens grown with or without dextrose and prepared for as long as 32 months previously (Table 5).

RESULTS

With a basal medium containing dextrose, MG growth caused a drop in *p*H to approximately 5.3 to 5.6 and was accompanied by a rapid loss of

TABLE 1. *Effect of dextrose on growth of Mycoplasma gallisepticum (S₆ 208 g27 P10), trial 1*[a]

Flask	Preincubation values		Values at selected time postinoculation					
	Media only after autoclaving	Media after addition of serum, seed, and penicillin	24 hr	96 hr	120 hr	144 hr	168 hr	192 hr
Flask 1[b]: (w dextrose)								
*p*H	6.95	7.0	7.0	6.9	6.5	5.8	5.6	5.6
Titration	N[c]	10^{8}[d]	10^{8}	10^{8}	10^{8}	10^{8}	10^{4}	N
Plate growth	N	4 +	4 +	4 +	4 +	4 +	1 +	N
Flask 2[b]: (w dextrose)								
*p*H	6.95	7.1	7.05	6.6	6.3	6.0	5.8	5.8
Titration	N	10^{7}	10^{9}	10^{8}	10^{7}	10^{7}	10^{4}	10^{4}
Plate growth	N	4 +	4 +	4 +	4 +	4 +	4 +	4 +
Flask 3[e]: (w/o dextrose)								
*p*H	7.6	7.6	7.4	7.2	7.1	7.15	7.2	7.2
Titration	N	10^{7}	10^{8}	10^{9}	10^{9}	10^{8}	10^{8}	10^{7}
Plate growth	N	4 +	4 +	4 +	4 +	4 +	4 +	4 +
Flask 4[e]: (w/o dextrose)								
*p*H	7.6	7.65	7.35	7.2	7.1	7.15	7.2	7.2
Titration	N	10^{7}	10^{9}	10^{9}	10^{8}	10^{8}	10^{8}	10^{7}
Plate growth	N	4 +	4 +	4 +	4+	4 +	4 +	4 +

[a] Each flask contained 4 liters of Difco PPLO Broth (w/o crystal violet) + 1% Yeast Autolysate + 10% equine serum. The basic medium was made in a single batch, and 4-liter samples were removed for each flask. Two flasks were supplemented with 1% D-glucose (flasks 1, 2). All flasks received equine serum (10%) and seed (10%) from a common lot. All flasks were agitated for 4 hr each day on a shaking apparatus oscillating approximately 120 times/min in a horizontal plane (one continuous 4-hr cycle each day).

[b] Growth harvested into one antigen pool (30W). Total antigen yield was 2.8%.

[c] Negative.

[d] Approximate numbers of organisms per milliliter.

[e] Growth harvested into one antigen pool (300Wo). Total antigen yield was 2.6%.

TABLE 2. *Effect of dextrose on growth of Mycoplasma gallisepticum (S6 208 g27 P10), trial 2*[a]

Flask	Preincubation values		Values at selected time postinoculation			
	Media only after autoclaving	Media after addition of serum, seed, and penicllin	24 hr	48 hr	96 hr	168 hr
Flask 1[b]: (w dextrose)						
*p*H	7.2	7.25	7.25	5.6	5.35	5.3
Titration	N[c]	10^{6}[d]	10^{8}	10^{10}	N	N
Plate growth	N	4 +	4 +	4 +	N	N
Flask 2[b]: (w dextrose)						
*p*H	7.2	7.25	7.2	5.5	5.4	5.3
Titration	N	10^{7}	10^{8}	10^{9}	N	N
Plate growth	N	4 +	4 +	4 +	N	N
Flask 3[e]: (w/o dextrose)						
*p*H	7.45	7.5	7.5	7.45	7.45	7.4
Titration	N	10^{6}	10^{9}	10^{10}	10^{8}	10^{6}
Plate growth	N	4 +	4 +	4 +	3 +	4 +
Flask 4[e]: (w/o dextrose)						
*p*H	7.45	7.55	7.5	7.45	7.4	7.4
Titration	N	10^{7}	10^{9}	10^{9}	10^{8}	10^{7}
Plate growth	N	4 +	4 +	4 +	4 +	4 +

[a] Each flask contained 4 liters of Difco PPLO Broth (w/o crystal violet) + 1% Yeast Autolysate + 10% equine serum. The basic medium was made in a single batch, and 4-liter samples were removed for each flask. Flasks 1 and 2 were supplemented with 1% D-glucose. Each flask received 400 ml of equine serum (10%) and 40 ml of seed (1%) from common lots. Throughout the incubation period, the flasks were agitated on a shaking apparatus oscillating approximately 120 times/min in a horizontal plane. The shaking apparatus was operated by a timer so that the instrument functioned 50% of the time (2 hr on, 2 hr off, etc.).

[b] Growth harvested into one antigen pool (33W). Total antigen yield was 14.6%.

[c] Negative.

[d] Approximate numbers of organisms per milliliter.

[e] Growth harvested into one antigen pool (33Wo). Total antigen yield was 5.2%.

TABLE 3. *Effect of addition of dextrose to the growth medium on Mycoplasma gallisepticum antigens used for rapid serum-plate titers*[a]

Serum	Antigens used					
	30Wo	30W	33Wo	33W	36Wo	36W
17C (lyo)	40[b]	40	80	20	40	40
21C (fresh)	80	40	80	20	40	20
31C (fresh)	40	20	80	10	20	10
49C (lyo)	640	40	640	40	320	40
53C (lyo)	80	40	160	20	80	20
7T (lyo)	80	20	80	N	160	N
15T (lyo)	40	20	80	20	40	20
30T (fresh)	80	N	160	N	40	N
37T (lyo)	40	U	40	N	20	N
54T (lyo)	80	40	80	U	40	U
36G (lyo)	2,560	640	2,560	320	2,560	640

[a] Abbreviations: Wo = antigen prepared without dextrose; W = antigen prepared with 1% dextrose; C = chicken serum; T = turkey serum; G = goat serum; lyo = reconstituted, lyophilized serum; N = negative; U = undiluted;

[b] Rapid serum-plate titers expressed as reciprocal of serum dilution.

viability (Tables 1, 2; flasks 1, 2). Organisms propagated in the medium lacking dextrose (flasks 2, 4) remained viable, and the number of viable organisms did not decrease more than 2 $\log_{10}$.

Yields of antigen were markedly different in the two trials (Tables 1 and 2). Total yield values were 2.8% with dextrose and 2.6% without dextrose in trial 1 (Table 1), and 14.6 and 5.2%, respectively, in trial 2 (Table 2). The difference was attributed to the difference in agitation: one 4-hr continuous cycle each day for trial 1 and a total of 12 hr (2 hr on, 2 hr off) for trial 2. In an additional trial, all the details of Table 2 were duplicated, and antigen yields were approximately equal to those in Table 2 (12.4% yield in media with dextrose, 5.2% yield in the corresponding media without carbohydrate). These latter antigens are labeled 36Wo and 36W (Table 3).

The six antigens prepared from three separate trials in which the only variable added was dextrose (30Wo, 30W, 33Wo, 33W, and 36Wo, 36W in Table 3) were compared for sensitivity by use of 11 different sera of chicken, turkey, and goat origin. Dextrose substrate added to the basal

TABLE 4. *Effect of antigen concentration on rapid serum-plate titers*[a]

Serum	Antigen lot	Antigen concn			
		Standard	Standard, washed once	2× concentrated	3× concentrated
30T (fresh)	15[b]	320[c]	320	320	320
31C (fresh)		80	80	80	80
37T (lyo)		320	160	160	160
49C (lyo)		1,280	640	640	1,280
30T (fresh)	27[d]	40	20	80	80
31C (fresh)		20	20	40	40
37T (lyo)		10	20	40	40
49C (lyo)		80	160	160	160
30T (fresh)	28[d]	U	N	10	10
31C (fresh)		20	20	20	20
37T (lyo)		10	20	20	20
49C (lyo)		80	160	160	160
30T (fresh)	29[d]	10	10	10	20
31C (fresh)		20	20	20	20
37T (lyo)		10	20	20	10
49C (lyo)		80	160	160	160

[a] Abbreviations: T = turkey serum; C = chicken serum; lyo = reconstituted, lyophilized serum; U = undiluted; N = negative.

[b] Organisms grown in medium without added sugar.

[c] Reciprocal of the serum dilution.

[d] Organisms grown in medium with added sugar.

TABLE 5. *Rapid serum-plate (RSP) titers with antigens prepared with and without added dextrose*[a]

Antigen lot	Dextrose added	Age of antigen since preparation (months)	RSP titer[b]	
			Turkey serum[c]	Chicken serum[c]
1	+	32	20	160
1A[d]	+	32	10	160
2	−	30	40	320
2 (stained)	−	30	40	320
3 (stained)	−	29	80	640
7	−	25	160	1,280
7 (stained)	−	25	80	640
8	−	25	160	1,280
8 (stained)	−	25	160	640
9	+	25	10	160
9 (stained)	+	25	20	160
12	−	22	320	1,280
13	−	22	160	1,280
14	−	20	80	1,280
15	−	20	320	2,560
16	−	20	80	1,280
20	−	18	160	1,280
22	−	11	160	1,280
25	−	6	160	2,560
26	−	6	80	640
27	+	4	40	80
28	+	3	40	80
29	+	1	40	80

[a] In evaluating antigens, a single turkey and a single chicken serum were used throughout.

[b] Reciprocal of the serum dilution.

[c] Reconstituted, lyophilized pooled serum.

[d] Antigen prepared with 10% inactivated bovine serum.

medium reduced the sensitivity of the antigens, particularly with turkey antisera.

Information from a number of laboratories has suggested that the concentration of the antigen increases sensitivity. Increasing the concentration up to three times (Table 4) had little, if any, effect on the RSP titer.

Table 5 presents additional information on the effect of dextrose on RSP titers with antigens prepared for as long as 32 months previously. Again, those prepared with dextrose show lower titers.

DISCUSSION

The addition of 1% dextrose to the basal medium (without added buffers) used to prepare *M. gallisepticum* antigen increased the total yield of organisms but decreased their sensitivity as RSP antigens. This reduction in titer was, in general, more evident with turkey antisera than with chicken antisera.

Many investigators studying the production of *M. gallisepticum* antigens used basal medium which contained a carbohydrate substrate. This may account for their failure to prepare satisfactory RSP antigens, particularly in detecting antibody in turkeys. Reduced sensitivity may result from the addition of carbohydrates, without proper control, in the preparation of other mycoplasma antigens.

Without question, the addition of dextrose to the basal media described here increased the total yield of organisms, particularly with aeration during incubation. Without proper safeguards, however, irreversible damage to the antigen seems to result from the low *p*H and other changes in the cell membrane, since sensitivity as an RSP antigen does not seem to be improved by increasing the concentration and resuspending in buffered saline diluent.

It should be emphasized that the results in this investigation are limited to the RSP test. With other procedures, sensitivity may not be affected. Previously, for example, it was indicated (1) that an "insensitive" MG antigen is satisfactory for the antiglobulin procedure with avian sera.

These findings do not preclude the use of carbohydrates in antigen production. An adequate buffering system and other substrates may provide a medium for optimal growth without affecting sensitivity.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-01726 from the National Institute of Allergy and Infectious Disease.

LITERATURE CITED

1. ADLER, H. E., AND A. J. DAMASSA. 1965. Antigenicity of six isolates of *Mycoplasma gallisepticum*. Avian Diseases **9**:205–211.
2. ADLER, H. E., AND A. J. DAMASSA. 1967. Use of formalinized *Mycoplasma gallisepticum* antigens and chicken erythrocytes in hemagglutination and hemagglutination-inhibition studies. Appl. Microbiol. **15**:245–248.
3. ADLER, H. E., AND R. YAMAMOTO. 1956. Preparation of a new pleuropneumonia-like organism antigen for the diagnosis of chronic respiratory disease by the agglutination test. Am. J. Vet. Res. **17**:290–293.
4. HALL, C. F. 1962. *Mycoplasma gallisepticum* antigen production. Avian Diseases **6**:359–362.
5. JUNGHERR, E. L., R. E. LUGINBUHL, M. TOURTELLOTTE, AND W. E. BURR. 1956. Significance of serological testing for chronic respiratory disease. Proc. Am. Vet. Med. Assoc., 92nd Ann. Meeting, 1955, p. 315–322.
6. VARDAMAN, T. H. 1967. A culture medium for the production of *Mycoplasma gallisepticum* antigen. Avian Diseases **11**:123–129.