J. Hyg., Camb. (1980), **84**, 29
With 1 *plate*
Printed in Great Britain

Studies on the effect of growth medium composition on the antigenicity of *Mycoplasma bovis*

By C. J. THORNS and E. BOUGHTON
Ministry of Agriculture, Fisheries and Food,
Central Veterinary Laboratory, New Haw, Weybridge, Surrey

(*Received* 3 *July* 1978)

SUMMARY

Serum proteins adsorbed from the culture medium were detected in *Mycoplasma bovis* antigens, the number and type of proteins depending on the serum used in the medium. The α-globulins cross-reacted with α-globulins from different types of sera but the γ-globulins did not. The removal of non-specific medium antibodies by absorption showed that they affected the gel diffusion and growth precipitation tests, producing cross-reactions between *M. bovis* and *M. bovigenitalium*, but that the complement fixation, tube agglutination, and growth inhibition tests were not similarly affected. The presence of serum proteins in the antigens changed their specific reactivity in all the tests. The production of antibodies to serum proteins was increased by the use of an adjuvant, but it appeared that production of specific antibodies to the mycoplasmas was not.

INTRODUCTION

Adsorption of serum protein components from the growth medium to mycoplasma membranes has been recognized by many workers (Smith, Dunlop & Strout, 1966; Jordan & Kulasegaram, 1968; Bradbury & Jordan, 1972). Medium components have also been shown to bind to ureaplasmas (Masover, Mischak & Hayflick, 1975). Most workers agree that this adsorption is irreversible and cannot be eliminated by extensive washing, so that antimedium antibodies are produced if animals are inoculated with the washed antigens. Adequate methods are available to minimize antimedium antibody production (Freundt *et al.* 1973) although they may not be practicable with certain mycoplasma species. Adsorbed proteins may also mask antigenic determinants and so reduce the specific reactivity of the antigen. Bradbury & Jordan (1972) have shown that certain pig serum proteins adsorbed to *Mycoplasma gallisepticum* influence haemagglutination reactions, and Masover *et al.* (1975) found that growth and metabolism inhibiting reactions of ureaplasmas were altered by the adsorbed proteins. A number of different sera are used in mycoplasma media (Frey, Thomas & Hale, 1973). This report describes the effect of different types of serum in the media on the antigenicity of *M. bovis* and of adjuvant on the production of antimedium antibodies, and the importance of these antibodies in some serological tests.

0022-1724/80/0084-1978 $01.00

MATERIALS AND METHODS

Organisms

M. bovis strain 5M193, isolated at Weybridge from the lung of a calf.

M. bovigenitalium strain PG-11 from the FAO/WHO Collaborating Centre for Animal Mycoplasmas (Institute of Medical Microbiology, University of Aarhus, Denmark).

Media

Modified Eaton media as previously described (Thorns, 1978), with 20% of unheated horse, pig, ox, rabbit or chicken serum, were used in the study.

Antisera

Antisera were prepared in rabbits. Antisera to *M. bovis* were raised with or without Freund's complete adjuvant but those for *M. bovigenitalium* only with adjuvant. All sera were stored in 2 ml quantities at −70 °C.

Antigens

Antigens for inoculation of rabbits and for use in serological tests were produced in the same way. Strains were grown in broth, usually in 1 litre volumes, incubated in air at 37 °C for 48–72 h. The cells were precipitated by centrifugation at 18000*g* for 30 min, washed three times, and finally resuspended in phosphate-buffered saline of pH 7·2 and stored at 4 °C.

Growth inhibition (GI) and complement fixation (CF) tests

These were performed using the methods described by Thorns & Boughton (1978).*

Growth precipitation (GP) and gel diffusion (GD) tests

These were performed and recorded as described by Ernø & Jurmanova (1973).*

Tube agglutination (TA) tests

Antigens were standardized to match Brown's opacity tube no. 4 with isotonic saline solution. Doubling dilutions of sera were made in saline and equal volumes of antigen added. The tests were incubated in a water bath at 37 °C for 2 h, then read immediately and after 4 h at 4 °C.*

Immunoelectrophoresis

Immunoelectrophoresis, used to identify the serum proteins that attach to *M. bovis*, was performed as described by Morris & Hussaini (1974) on 82 mm square slides in 1·5% Ionagar in veronal buffer at pH 8·8 (I = 0·05) for 100 min at a constant current of 15 mA.*

* The number of repetitions of tests varied, but in no case do observations depend on a single set of results.

RESULTS

Influence of adjuvant on the production of antimedium antibodies

When antisera were produced using antigens without the addition of the adjuvant no antimedium antibodies were detected by GD. The antimedium precipitins detected in the antisera produced with *M. bovis* and adjuvant are shown in Table 1. Seven different antibodies to horse serum were detected by GD using rabbit antiserum to organisms grown with horse serum (RASH), and two of these

Table 1. *Number of precipitins produced from media containing different sera*

Serum as antigen	Rabbit antisera prepared* from *M. bovis* antigens grown with the following serum supplements				
	Horse	Pig	Ox	Rabbit	Chicken
Horse	7	2	0	0	0
Pig	2	2	0	0	0
Ox	1	0	0	0	0
Rabbit	0	0	0	0	0
Chicken	0	0	0	0	3

* Using adjuvant.

were the same as the two antibodies to pig serum detected using antiserum to organisms grown with pig serum. There was also cross-reaction between RASH and ox serum. Antibodies to chicken serum were also detected by GD, but no cross-reactions were shown. No precipitins were detected with any of the antisera when tested against medium without serum.

Identification of the proteins that produce antimedium antibodies

Immunoelectrophoresis showed that RASH contained antibodies to horse IgM, IgG, transferrin, albumin, glycoprotein and lipoprotein (Plate 1), and the antibodies to horse glyco- and lipoproteins cross-reacted with chicken and ox sera. Rabbit antiserum to organisms grown with chicken serum contained a similar range of antibodies except that anti-albumin was not detected; antibody to chicken lipoprotein cross-reacted with horse and ox sera. Antiserum to organisms grown with ox serum did not contain antibodies to γ-globulin but some antibodies to lipoprotein were produced; no cross-reactions with any other sera were shown. Rabbit antisera to organisms grown with rabbit serum contained antibodies to lipoprotein which cross-reacted with ox serum.

Influence of serum supplements on serological tests

Growth inhibition tests

The results are presented in Table 2. It can be seen that, when antigen was used with adjuvant, growth inhibition was in many cases less than when the adjuvant was not used. No cross-reactions occurred between *M. bovis* antisera and *M. bovigenitalium* antigens or vice versa.

Complement fixation tests

Table 3 shows the variation of complement fixation titres when different serum supplements were used for growing the antigen, either for use in the tests or for raising antiserum. Where horse or ox serum was used in preparing the antigen for antiserum production higher titres were raised with adjuvant than without, but those raised with adjuvant gave their highest titres in heterologous systems where-

Table 2. *Influence of serum supplements on the growth inhibition test* using* M. bovis *strain 5M*193

Antigen grown in medium containing	Rabbit antisera prepared from *M. bovis* antigens grown with the following serum supplements									
	Horse		Pig		Ox		Rabbit		Chicken	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Horse serum	11	—	10	6	10	—	11	—	7	8
Pig serum	8	—	8	—	10	—	10	—	11	8
Ox serum	7	8	10	7	8	10	12	10	8	10
Rabbit serum	6	12	7	10	8	12	6	12	8	10
Chicken serum	—	—	—	—	—	—	—	—	—	—

a, Antisera produced without adjuvant. b, Antisera produced with adjuvant.
—, No growth inhibition.
* Growth inhibition in millimetres.

Table 3. *Influence of serum supplements on the complement fixation test* using* M. bovis *strain 5M*193

Antigen grown in medium containing	Rabbit antisera prepared from *M. bovis* antigens grown with the following serum supplements									
	Horse		Pig		Ox		Rabbit		Chicken	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Horse serum	640	640	320	ND	320	640	10240	160	—	10
Pig serum	320	1280	640	ND	640	2560	5120	80	—	—
Ox serum	320	640	640	ND	640	320	5120	160	10	10
Rabbit serum	320	10240	320	ND	320	2560	10240	160	—	—
Chicken serum	ND	40	80	ND	40	80	160	40	—	80

a, Antisera produced without adjuvant. b, Antisera produced with adjuvant.
ND, Not done.
—, No complement fixation at a dilution of 10 or more.
* Titres expressed as reciprocal of highest dilution showing fixation of complement.

as those raised without gave their highest titres in the homologous systems. Conversely, with rabbit serum titres were higher where adjuvant was not used, but the same relationship, if less clearly defined, obtained in respect of the highest titres. The most dramatic effect was the apparent inhibition of titre when chicken serum was used as the serum supplement. None of the *M. bovis* antisera cross-reacted with *M. bovigenitalium* antigens or vice versa. Removal of non-specific medium antibodies by absorption did not alter the titre in any system.

Tube agglutination

Table 4 shows the marked effect of antigen composition on the TA test. The variation in titres was more obvious than with the CF tests, and again chicken serum appeared to have an inhibitory effect. No cross-reactions between *M. bovis* and *M. bovigenitalium* were observed, and removal of non-specific antibodies did not affect the titres of the sera.

Table 4. *Influence of serum supplements on the tube agglutination test* using* M. bovis *strain* 5*M*193

Antigen grown in medium containing	Rabbit antisera prepared from *M. bovis* antigens grown with the following serum supplements									
	Horse		Pig		Ox		Rabbit		Chicken	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Horse serum	—	64	—	ND	—	—	16	8	—	—
Pig serum	128	128	1024	ND	1024	256	2048	—	—	—
Ox serum	—	64	—	ND	—	256	—	64	—	—
Rabbit serum	64	64	32	ND	16	128	128	128	—	—
Chicken serum	—	128	—	ND	—	128	—	32	—	—

a, Antisera produced without adjuvant. b, Antisera produced with adjuvant.
ND, Not done. —, No agglutination at a dilution of 10 or more.
* Titres expressed as reciprocal of highest dilution giving agglutination.

Table 5. *Influence of serum supplements on the growth precipitation test* using* M. bovis *strain* 5*M*193

Antigen grown in medium containing	Rabbit antisera prepared from *M. bovis* antigens with adjuvant grown with the following serum supplements				
	Horse	Pig	Ox	Rabbit	Chicken
Horse serum	c d	ND	c d	c d	c d
Pig serum	a b c d e	ND	c d e	c d e	c d e
Ox serum	a b c d e	ND	c d e	c d e	c d e
Rabbit serum	a c d e	ND	d e	d e	d
Chicken serum	a c d e	ND	—	—	d

ND, not done
* Recorded as single lines of precipitation represented by one lower case letter per common line.

Growth precipitation

Only antisera produced from antigens with adjuvant contained any growth precipitins (Table 5). RASH cross-reacted with *M. bovigenitalium* when pig, rabbit or chicken serum was used in the growth medium but not when horse serum (in effect absorbing anti-horse serum antibodies) was used. It can be seen that the number of precipitin lines is dependent upon the serum used in the growth medium.

Gel diffusion

The results of the GD tests are summarized in Table 6, and were similar to those for GP. Sera absorbed with media revealed that a number of lines were specifically attributable to antimedium antibodies, as has been shown by other workers. Similarly the type of serum used in the growth medium altered the number and type of precipitin lines. Cross-reactions similar to those in the GP test were observed.

Table 6. *Influence of serum supplements on the gel diffusion test* using* M. bovis *strain* 5*M*193

Antigen grown in medium containing	Rabbit antisera prepared from *M. bovis* antigens with adjuvant grown with the following serum supplements				
	Horse	Pig	Ox	Rabbit	Chicken
Horse serum	x y (z)	ND	y	y	—
Pig serum	x (z)	ND	(z)	(z)	—
Ox serum	x y	ND	x y	x	x
Rabbit serum	x y	ND	x y	x y	(z)
Chicken serum	x y	ND	y	y	x y (z)

ND = Not done.

( ) = Precipitin lines absent after absorption with growth media.

* Recorded as single lines of precipitation represented by one lower case letter per common line.

DISCUSSION

The findings of Jordan & Kulasegaram (1968) and Bradbury & Jordan (1972) have been confirmed. Serum proteins were the only detectable precipitinogens from the broth medium. Serum proteins from various animal species adsorbed to *M. bovis* organisms and were not removed by extensive washing, and specific antibodies to them were produced in rabbits. Bradbury & Jordan found that a low pH in the culture medium produced maximum serum protein adsorption. Asmar (1965) found that certain chicken serum proteins adsorbed to *M. gallisepticum* at pH 8·0. The results above show that *M. bovis* adsorbs a wide range of serum proteins at pH 6·8–7·2, supplying further evidence that adsorption is not confined to low pH conditions. Bradbury & Jordan suggested that certain pig or chicken serum proteins were adsorbed preferentially, in view of the absence of antibody to albumin, which is a powerful antigen. Immunoelectrophoretic analysis revealed that a variety of horse serum proteins, including IgG, IgM, transferrin and albumin elicited an antibody response, indicating non-selective adsorption. In contrast only a few chicken and ox serum proteins produced an antibody response. From these results it appears that the degree of adsorption of serum proteins to *M. bovis* depends on the type of serum used. Purely on the number of precipitins present it is clear that more antibodies are produced to horse serum than to the sera of other species tested. In immunoelectrophoresis the α-globulin proteins cross-reacted with α-globulins from other species of animal. Unexpectedly, antibody to rabbit albumin was produced in rabbits; possibly the conformation

of the albumin was altered by adsorption, or allotypic antigens may have been involved.

Not surprisingly, the results have shown that the use of adjuvant enhanced the production of antibodies to serum proteins; in fact they could not be demonstrated without its use. The use of adjuvants has long been advocated for the production of high-titred mycoplasma antisera (Morton & Roberts, 1967) but some species of mycoplasma consistently fail to produce certain types of anti-mycoplasma antibodies in rabbits (Carroll, Rollins & Jasper, 1976) even when an adjuvant is used. The results presented reveal that for *M. bovis* the use of an adjuvant is desirable for production of specific precipitins in the GP and GD tests, but it was also shown that a number of anti-medium precipitins were produced which cross-reacted with *M. bovigenitalium* antigen. The homologous system of antigen grown in rabbit serum does produce non-specific antibody to serum protein, probably anti-albumin, and furthermore cross-reactions between various serum proteins caused variation in the precipitins detected. It is clear, however, that the antibodies to γ-globulins did not cross-react, suggesting that they do not affect the result in the way that the other globulins do.

In the GD and GP tests the presence of antibody to serum constituents influences the test by producing false positives. In the CF, GI and TA tests their effect is more obscure. No cross-reactions were found between *M. bovis* and *M. bovigenitalium* antisera, but there were marked differences depending on the type of serum used to grow the antigen. Bradbury & Jordan found that the presence of pig serum in the medium reduced the haemagglutination titre of *M. gallisepticum*. Our results show that the medium formulation for the growth of *M. bovis* causes differences in specific reactivity of the antisera against *M. bovis*, possibly by masking specific antigenic determinants. When adjuvant is used for the production of antisera for the CF, GI or HA test non-specific antibody production is enhanced but the results indicate that the specific antibody production is not equally increased. Whilst precipitins against serum proteins were demonstrated they did not appear to have any effect on CF, GI or TA tests. This was supported by the observations that (i) antiserum to serum protein does not inhibit growth of *M. bovis*; (ii) antiserum absorbed with growth medium gives the same CF and TA results as unabsorbed serum; and (iii) no cross-reactions between *M. bovigenitalium* antigen grown with horse serum and *M. bovis* antisera were demonstrated.

The failure of CF and TA titres to develop when chicken serum was used to make antigen, for either production of antiserum or use in the tests, is difficult to explain. The simplest explanation would be that quantitatively serum protein adsorption is at its maximum, masking most of the specific antigenic determinants.

It has been shown that non-specific antibodies to growth medium do not affect the results obtained with the CF, GI or TA systems used, whereas the presence of serum proteins on the antigen does affect the results. This problem can only be minimized by the use of supplements other than sera, or by standardization of the use of serum, for antigen production for serological tests. Serum protein adsorption to *M. bovis* is clearly a problem, and it would be interesting to determine whether other species of mycoplasma adsorb serum proteins to a comparable degree.

REFERENCES

ASMAR, J. A. (1965). Chromatographic and immunoelectrophoretic characterization of chicken and turkey agglutinating antibodies to *Mycoplasma gallisepticum*. Ph.D. thesis, University of California.

BRADBURY, J. M. & JORDAN, F. T. W. (1972). Studies on the adsorption of certain medium proteins to *Mycoplasma gallisepticum* and their influence on agglutination and haemagglutination reactions. *Journal of Hygiene* **70**, 267.

CARROLL, E. J., ROLLINS, M. & JASPER, D. E. (1976). The immune response of rabbits to 3 strains of *Mycoplasma agalactiae* var. *bovis* isolated from mastitic bovine udders. *Cornell Veterinarian* **66**, 143.

ERNØ, H. & JURMANOVA, K. (1973). Bovine mycoplasmas: serological studies by double immunodiffusion, growth precipitation and growth inhibition. *Acta veterinaria scandinavica* **14**, 524.

FREUNDT, E. A., ERNØ, H., BLACK, F. T., KROGSGAARD-JENSEN, A. & ROSENDAL, S. (1973). Evaluation of reference reagents for mycoplasmas. *Annals of the New York Academy of Sciences* **225**, 161.

FREY, M. L., THOMAS, G. B. & HALE, P. A. (1973). Recovery and identification of mycoplasmas from animals. *Annals of the New York Academy of Sciences* **225**, 334.

JORDAN, F. T. W. & KULASEGARAM, P. (1968). Non-specific antibodies in chickens inoculated intratracheally with *Mycoplasma gallisepticum*. *Journal of Comparative Pathology* **78**, 407.

MASOVER, G. K., MISCHAK, R. P. & HAYFLICK, L. (1975). Some effects of growth medium composition on the antigenicity of a T-strain mycoplasma. *Infection and Immunity* **11**, 530.

MORRIS, J. A. & HUSSAINI, S. N. (1974). Characterization of the antibodies detected by the microscopic agglutination test for bovine leptospirosis. *Journal of Hygiene* **73**, 425.

MORTON, H. E. & ROBERTS, R. E. (1967). Production of anti-mycoplasma (PPLO) antibodies in rabbits. *Proceedings of the Society for Experimental Biology and Medicine* **125**, 538.

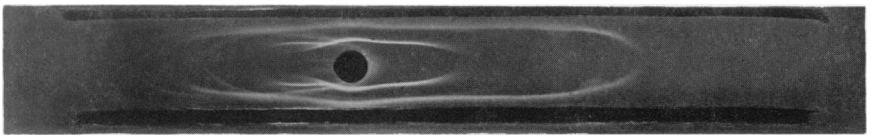
SMITH, S. C., DUNLOP, W. R. & STROUT, R. G. (1966). Effect of culture medium on antigenic structure of mycoplasma. *Avian Diseases* **10**, 173.

THORNS, C. J. (1978). Identification of the products of the 'Film and Spots' reaction of *Mycoplasma bovis* and *Mycoplasma bovigenitalium*. *Journal of Biological Standardization* **6**, 127.

THORNS, C. J. & BOUGHTON, E. (1978). Studies on film production and its specific inhibition with special reference to *Mycoplasma bovis* (*M. agalactiae* var *bovis*). *Zentralblatt für Veterinärmedizin*, B **25** 657.

EXPLANATION OF PLATE

Normal horse serum was placed in the large well and electrophoresis was performed at 15 mA for 100 min at room temperature. Rabbit antiserum against *Mycoplasma bovis* antigen grown with horse serum was placed in the troughs and left for 2 days at 4 °C. A number of lines can be seen, which probably include IgG, IgM, transferrin, glycoprotein, lipoprotein and albumin.



C. J. THORNS AND E. BOUGHTON

(*Facing p.* 36)