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Investigation into rabbit infusion media for the growth of *Mycoplasma gallisepticum* antigens for inoculation into rabbits

By JANET M. BRADBURY and F. T. W. JORDAN
Poultry Research Section, University of Liverpool Veterinary Field Station 'Leahurst', Neston, Wirral, Cheshire

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SUMMARY

Serum cholesterol in rabbits was elevated, by intravenous inoculation of 20% Tween 80, to amounts thought to have been sufficient to support growth of *Mycoplasma gallisepticum* when 20% of such serum was added to rabbit infusion broth.

However, better growth of the organism was obtained with a supplement of 5% rabbit serum and commercial cholesterol. Investigation showed that normal rabbit serum may be inhibitory, which would explain these findings. The commercial cholesterol was not antigenic.

INTRODUCTION

Antigenic purity is an essential feature of mycoplasma antigens intended for animal inoculation for the production of specific antibodies. It has been shown that protein in the mycoplasma culture medium foreign to the animal to be inoculated can give rise to non-specific antibody (Smith, Dunlop & Strout, 1966; Kulasegaram, 1967; Jordan & Kulasegaram, 1968). To overcome this problem, the last-named workers used a medium that contained protein derived entirely from the species to be inoculated (chickens, turkeys and rabbits). In this laboratory *M. gallisepticum* has been grown satisfactorily in chicken and turkey infusion medium supplemented with 20% (v/v) chicken and turkey serum respectively, but difficulty has been experienced with rabbit meat infusion broth supplemented with 20% rabbit serum (Nutor, 1969).

Edward & Fitzgerald (1951*a*, *b*) and Edward (1954) attributed the poor growth of some mycoplasmas in medium supplemented with rabbit serum to a low cholesterol content in the serum. Taylor-Robinson, Somerson, Turner & Chanock (1963) and Eng (1967) successfully grew *M. pneumoniae* by supplementing their medium of rabbit meat infusion and rabbit serum with a suspension of commercially prepared cholesterol.

However, the incorporation of a suspension of cholesterol may not be the ideal way of increasing the supply of sterol because of the possible difficulty in preparing a stable suspension (Edward & Fitzgerald, 1951*b*) and because there may be a tendency for the suspension to sediment with the organism at harvest. In addition, although cholesterol itself is not antigenic, commercial cholesterol, derived from

bovine brain and spinal cord, may contain bovine protein and this might contaminate the prepared mycoplasma antigen (D. H. Roberts, personal communication).

This paper describes an attempt to prepare antigen free from the disadvantages described above in order to produce antisera in rabbits specific to *M. gallisepticum*. The medium for the growth of mycoplasma for this purpose was rabbit meat infusion containing rabbit serum in which the cholesterol concentration had been elevated *in vivo*. Its ability to support growth was compared with an orthodox medium, a rabbit infusion medium containing normal rabbit serum and infusion medium containing commercial cholesterol.

The paper also records the growth inhibitory effect observed in the sera of some normal rabbits.

MATERIALS AND METHODS

Mycoplasma gallisepticum *strains*

The following three strains were used: A 514, originally obtained from Dr H. Chu, University of Cambridge, which has now undergone numerous passages in artificial medium in this laboratory; S 6M, obtained from Dr D. H. Roberts, Central Veterinary Laboratory, Weybridge; and X 95, obtained from Dr R. H. Leach, Wellcome Research Laboratories, Beckenham.

Rabbit sera

After collection these were stored at 0–4° C.

Determination of total cholesterol content in normal rabbit, horse and swine serum

In order to confirm that the serum cholesterol concentrations of our normal rabbits were relatively low, the serum total cholesterol of 20 normal rabbits was determined. For comparative purposes, the concentrations were also estimated in three batches of horse serum and three of swine serum as used in routine mycoplasma culture media.

Determinations were made by the ferrous sulphate* method (Searcy & Bergquist, 1960) and optical densities were measured at 490 nm.

Elevation of serum cholesterol in rabbits

Six rabbits were inoculated intravenously with 20 % Tween 80 as described by Kellner, Correll & Ladd (1951). Two were given a single inoculation and blood was collected after 16 hr. The other four received two inoculations with a 16 hr. interval and blood was collected 16 hr. after the first inoculation and 8 hr. after the second.

Media

Broth medium (BM)

The medium used for routine maintenance of cultures was that described by Taylor-Robinson *et al.* (1963) except that it contained 1 % nicotinamide adenine

* Hyland Baxter, Thetford, Norfolk.

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dinucleotide (NAD), 1 % glucose and 0·002 % phenol red, while amphotericin was omitted.

Rabbit infusion broths (RB 1–3)

The growth-promoting properties of an infusion medium containing rabbit serum with cholesterol elevated *in vivo* was compared with one containing normal rabbit serum and one to which commercial cholesterol and normal rabbit serum had been added.

The basic infusion for all three media was similar to that described by Jordan & Kulasegaram (1968) but using rabbit meat in place of chicken meat, and the infusion was autoclaved at 10 lb./in.^{2}, 114° C., for 30 min. The supplements were as described except that 1 % glucose was used and the pH indicator was phenol red.

To prepare the experimental media the following additions were made to the above infusion:

Rabbit infusion broth 1 (RB 1). 20 % (v/v) rabbit serum with elevated cholesterol (98 mg./100 ml.).

Rabbit infusion broth 2 (RB 2). 20 % (v/v) normal rabbit serum with relatively low total cholesterol (30 mg./100 ml.).

Rabbit infusion broth 3 (RB 3). 5 % (v/v) of the same pool of normal rabbit serum and also 2 % (v/v) of the cholesterol* preparation as described by Taylor-Robinson *et al.* (1963).

For all three media the rabbit serum was heated at 56° C. for 30 min. immediately before incorporation.

Evaluation of the media for the growth of Mycoplasma gallisepticum

This was studied for three strains (A 514, S 6 M and X 95).

Inocula were prepared from a 24 hr. culture of the organisms in BM. The culture was harvested, washed once in 0·5 M sucrose containing 0·01 M phosphate buffer pH 7·0 as described by Rodwell & Abbot (1961), and resuspended to its original volume in phosphate-buffered sucrose.

The inoculum (10 %, v/v) was added to each medium and in addition a sample of the inoculum was immediately titrated for viable organisms by the method described below. The inoculated media were incubated at 37° C., and viable counts were conducted on all samples at 8, 24 and 48 hr.

Viable counts

The 'Most Probable Number' method (Taylor, 1962; Meynell & Meynell, 1965) was employed using colour change in the medium associated with fall in pH by at least half a pH unit as an indication of growth.

A series of tenfold dilutions to 10^{-10} was prepared from the culture in a diluent consisting of distilled water adjusted to pH 7·6–7·8 with 0·01 M-K_2HPO_4 and 1 % (v/v) BM (Butler & Knight, 1960). From every dilution 0·1 ml. was transferred to

* Koch Light, Colnbrook, Bucks.

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each of five vials containing 0·9 ml. of BM. All dilutions were then incubated at 37° C. for 10 days.

The value of the Most Probable Number of viable organisms was derived from tables and the validity of the method was confirmed for each strain of *M. gallisepticum* by application of Moran's test to the dilution counts (Moran 1954*a*, *b*, 1958).

Examination of the cholesterol preparation for bovine protein

The commercial cholesterol preparation was examined for bovine protein contaminants by attempting to produce an anaphylactic response in guinea-pigs, a method that will detect very small amounts of protein (Kabat & Mayer, 1961).

Four guinea-pigs were inoculated intraperitoneally with 5 ml. cholesterol suspension as used in medium RB 3 and four with 2 mg. bovine serum protein. After 14 days, two of the four guinea-pigs given cholesterol and two given bovine serum protein were challenged by intracardiac inoculation of 2·5 ml. cholesterol preparation. The other two of each group were similarly inoculated with 2 mg. bovine serum protein. The two guinea-pigs that were sensitized and also challenged with bovine serum protein were a positive control for the anaphylactic system.

Two normal guinea-pigs were given an intracardiac inoculation of 2·5 ml. of the cholesterol suspension to ensure that large particles or the ethanol content had no adverse effects.

All guinea-pigs were closely observed for signs of anaphylactic shock for 2 hr.

The effect of normal rabbit serum on the growth of Mycoplasma gallisepticum

The possible growth inhibitory effect of normal rabbit serum was examined because better growth was obtained with 5 % rabbit serum (RB 3) than with 20 % (RB 1) although both should have contained adequate cholesterol.

Sera from eight rabbits were tested both unheated and after heating at 56° C. for 30 min. The method employed was a modification of the metabolic-inhibition test in 'microtitre' plates (Taylor-Robinson, Purcell, Wong & Chanock, 1966) using BM, and rabbit serum replacing antiserum. The serum was incorporated in the medium in the following final concentrations: 20 %, 10 %, 5 %, 2·5 % and 1·25 %. Each was tested with two dilutions (10^{-3} and 10^{-4}) of each of the three test organisms. These dilutions were prepared from a 24 hr. culture of the strain in BM and each test-well received 20 % (v/v) of the dilution. Control wells contained the same medium and inoculum without rabbit serum.

Plates were sealed with clear tape, incubated at 34° C. and results were read when the pH of the control wells had fallen by approximately half a pH unit. The end-point for inhibition by a serum was taken as the lowest concentration that inhibited a colour change.

Three batches of horse serum and three of swine were similarly examined and, to ensure that any inhibition of colour change by the different sera was not due to different buffering capacity, this was measured by titration against 0·1 M-HCl.

RESULTS

Determination of total cholesterol content in normal rabbit, horse and swine serum

The distribution of the serum total cholesterol values of 20 normal rabbits is shown in Fig. 1. The mean of the results was 41·0 mg. ± 16·0 mg. (S.D.)/100 ml. and this was considerably lower than the average total cholesterol of the horse and swine serum, 75 and 160 mg./100 ml. respectively.

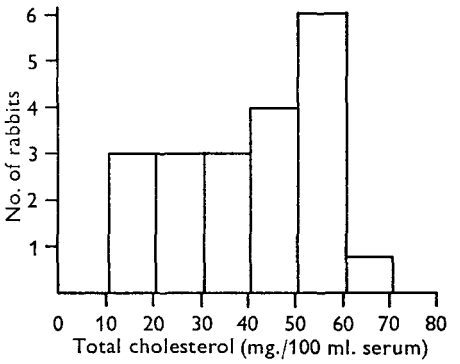

Fig. 1. Serum total cholesterol of 20 normal rabbits.

Table 1. *Serum total cholesterol (mg./100 ml.) of six rabbits inoculated with* 20% *Tween* 80

Rabbit no.	No. of inoculations	Time after initial inoculation (hr.) 0	16	24
1	1	37	54	.
2	1	61	75	.
3	2	39	65	155
4	2	69	87	104
5	2	34	73	181
6	2	18	ND	98

ND = not determined.

Elevation of serum cholesterol in rabbits

Cholesterol concentrations were considerably raised after a single intravenous inoculation of 20% Tween 80 and were further elevated after a second inoculation (Table 1).

Evaluation of the media

Table 2 shows the viable counts of A514, S6M and X95 in the four different media, the actual growth curves being shown in Figs. 2–4. Table 3 is a summary of the growth-promoting properties of the media for the three strains.

BM supported growth of all three strains to a higher titre than the other media. RB3 also promoted growth of A514 and S6M, while the titre of X95 was main-

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tained for 24 hr. RB1 supported growth of A514 but only appeared to maintain the titre of S6M after 24 hr. and did not support growth of X95. RB2 supported only A514.

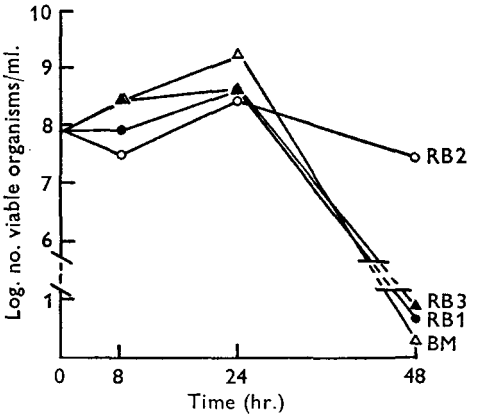

Fig. 2

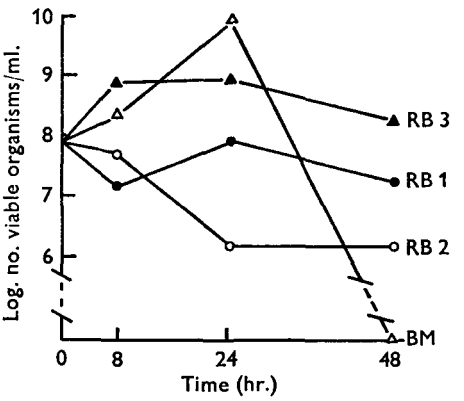

Fig. 3

Fig. 2. Growth of *Mycoplasma gallisepticum* A514. BM = Broth medium; RB1 = rabbit infusion broth 1; RB2 = rabbit infusion broth 2; RB3 = rabbit infusion broth 3.

Fig. 3. Growth of *Mycoplasma gallisepticum* S6M. BM = Broth medium; RB1 = rabbit infusion broth 1; RB2 = rabbit infusion broth 2; RB3 = rabbit infusion broth 3.

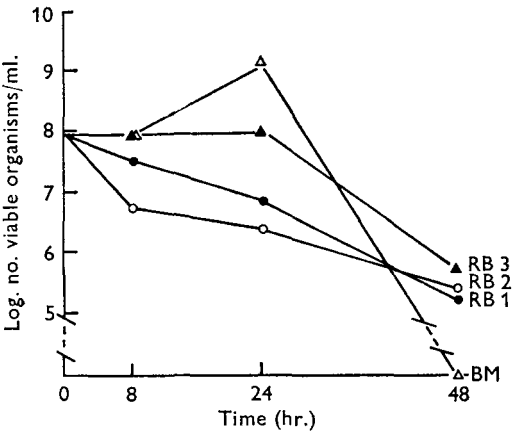

Fig. 4. Growth of *Mycoplasma gallisepticum* X95. BM = Broth medium; RB1 = rabbit infusion broth 1; RB2 = rabbit infusion broth 2; RB3 = rabbit infusion broth 3.

Examination of the cholesterol preparation for bovine protein

Of the ten guinea-pigs, only the two that were sensitized and challenged with bovine serum protein showed signs of anaphylactic shock. These showed classical anaphylactic symptoms and died in less than 5 min.

The guinea-pigs that received intracardiac inoculations of the cholesterol sus-

Table 2. *The number of viable organisms/ml. of* Mycoplasma gallisepticum *A* 514, *S* 6 *M and X* 95 *in media BM, RB_1, RB_2 and RB_3*

M. gallisepticum strain	Incubation time (hr.)	BM	RB_1	RB_2	RB_3
A 514	0	$8{\cdot}3 \times 10^7$	$8{\cdot}3 \times 10^7$	$8{\cdot}3 \times 10^7$	$8{\cdot}3 \times 10^7$
	8	$2{\cdot}9 \times 10^8$	$8{\cdot}3 \times 10^7$	$2{\cdot}9 \times 10^7$	$2{\cdot}9 \times 10^8$
	24	$2{\cdot}3 \times 10^9$	$4{\cdot}9 \times 10^8$	$2{\cdot}9 \times 10^8$	$4{\cdot}9 \times 10^8$
	48	2·0	4·5	$2{\cdot}9 \times 10^7$	7·8
S 6 M	0	$6{\cdot}4 \times 10^7$	$6{\cdot}5 \times 10^7$	$6{\cdot}4 \times 10^7$	$6{\cdot}4 \times 10^7$
	8	$2{\cdot}4 \times 10^8$	$1{\cdot}5 \times 10^7$	$5{\cdot}2 \times 10^7$	$9{\cdot}2 \times 10^8$
	24	$9{\cdot}2 \times 10^9$	$8{\cdot}6 \times 10^7$	$1{\cdot}5 \times 10^6$	$9{\cdot}2 \times 10^8$
	48	0	$1{\cdot}7 \times 10^7$	$1{\cdot}5 \times 10^6$	$1{\cdot}6 \times 10^8$
X 95	0	$9{\cdot}2 \times 10^7$	$9{\cdot}2 \times 10^7$	$9{\cdot}2 \times 10^7$	$9{\cdot}2 \times 10^7$
	8	$9{\cdot}2 \times 10^7$	$3{\cdot}5 \times 10^7$	$5{\cdot}2 \times 10^6$	$9{\cdot}2 \times 10^7$
	24	$1{\cdot}5 \times 10^9$	$6{\cdot}4 \times 10^6$	$2{\cdot}4 \times 10^6$	$1{\cdot}0 \times 10^8$
	48	0	$1{\cdot}6 \times 10^5$	$2{\cdot}4 \times 10^5$	$5{\cdot}2 \times 10^5$

BM = Broth medium; RB_1 = rabbit infusion broth 1; RB_2 = rabbit infusion broth 2; RB_3 = rabbit infusion broth 3.

Table 3. *Media RM, RB* 1, *RB* 2, *RB* 3 *and the growth of* Mycoplasma gallisepticum, *A* 514, *S* 6 *M and X* 95

	BM	RB 1	RB 2	RB 3
A 514	+	+	+	+
S 6 M	+	±	−	+
X 95	+	−	−	±

+ = growth; − = no growth; ± = viability maintained after 24 hr. incubation.

Table 4. *Growth inhibition of* Mycoplasma gallisepticum *by normal rabbit serum*

	Lowest % of rabbit sera A–H which inhibits growth of strain		
Serum	A 514	S 6 M	X 95
A	20	10	20
A_h	20	20	20
B	20	10	N
B_h	20	20	N
C	20	10	N
C_h	20	10	N
D	N	20	N
D_h	N	20	N
E	10*	10*	N
E_h	20	10*	N
F	20	20	N
F_h	20	20	N
G	20	5*	20
G_h	20	5*	20
H	20	20	N
H_h	20	N	N

h = serum heated at 56° C. for 30 min.; N = serum not inhibitory even at 20 %.
* Bactericidal (no growth after 7 days incubation).

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pension, including the two unsensitized ones, showed varying degrees of incoordination for approximately 30 min., which was attributed to the small amount of ethanol in the inoculum.

There was thus no evidence for the presence of bovine protein in the cholesterol preparation.

Inhibitory properties of normal rabbit serum

The results of this investigation are shown in Table 4. Inhibition was found to be independent of the number of organisms in the initial inoculum. Many of the sera were inhibitory to the growth of A514 and S6M at 20% concentration, and some sera were inhibitory at lower concentrations, although none showed any inhibition at below 5%. X95 was inhibited by only two sera and never at less than 20% concentration.

In a few cases there was no mycoplasma growth, even after incubation for 1 week, and these sera were considered to be bactericidal for that organism, whilst others were merely bacteriostatic (Davies, 1969).

The growth-inhibitory effect was occasionally reduced by heating the serum at 56° C., but only once was the effect abolished.

There seemed to be little uniformity in the effect of the sera on the three strains; for example, serum E, which was bactericidal to A514 and S6M, and still inhibitory after heating, did not show any inhibition of X95. None of the horse or swine sera were inhibitory to any of the strains.

The buffering action of the rabbit sera was very similar in all samples, small differences did not correlate with the inhibitory properties and were therefore not considered to have influenced the results significantly.

DISCUSSION

These studies confirm that comparatively low concentrations of total cholesterol are present in normal rabbit sera, and also that they can be raised fairly rapidly by intravenous inoculation of 20% Tween 80. Two inoculations raised serum total cholesterol to values above those found in horse serum but below those in the swine serum. When 20% of such rabbit serum was added to rabbit infusion broth it supported growth of one of three *M. gallisepticum* strains and maintained viability of another. Medium that contained 20% normal rabbit serum supported growth of one of the strains while the other two strains lost viability. However, broth that was supplemented with 5% rabbit serum and a cholesterol suspension supported growth of two strains and maintained viability of the third. Its apparent superiority over that containing rabbit serum in which the cholesterol was elevated *in vivo* may be due to a number of factors. For instance, it is possible that the serum from the inoculated rabbits did not provide a favourable ratio of sterol and phospholipid or fatty acid for the organism (Smith, Lecce & Lynn, 1954; Edward & Freundt, 1956; Smith, 1960; Rodwell, 1963) since intravenous inoculation of Tween 80 elevates serum phospholipid and other lipids as well as total cholesterol.

Another influencing factor may have been the amount of free cholesterol in the

total. The parasitic mycoplasmas incorporate free cholesterol more readily than the esters (Rodwell, 1963; Argaman & Razin, 1965) and in this work the relative amounts of free and esterified cholesterol were not determined. Further, the presence in rabbit serum of natural inhibitors to the growth of *M. gallisepticum* might also explain why the organism was more likely to grow in the medium containing the lower percentage of rabbit serum. Such inhibition has been reported to exist against *M. pneumoniae* (Fernald, Clyde & Denny, 1967) and against *M. gallisepticum* (Friedrich, 1970), and has been confirmed in our studies. In these, growth inhibition of *M. gallisepticum* by rabbit sera varied with the strain of the organism. It is difficult to explain why S6M should be inhibited by all eight sera examined, A514 by seven of the eight while X95 was inhibited by only two of them. It is interesting also that heating the sera had little effect, which is in contrast with the observation of Friedrich (1970) that the inhibitors were heat-labile. The exact nature of the inhibition is not known, but Kenny & Grayston (1965) reported that the sera of some normal rabbits contain complement-fixing antibody to *M. pneumoniae*.

Since sera have been found to be inhibitory to the growth of *M. gallisepticum* when incorporated in the medium at 20%, 10% and even in one case at 5%, it is advisable to screen rabbit serum intended for media supplements.

The rabbit infusion medium that best supported growth of *M. gallisepticum* was that containing commercial cholesterol. It is therefore of value to note that the cholesterol preparation did not evoke an immune response. This means that if commercial cholesterol were used in the growth of *Mycoplasma* and excess was deposited at harvest, it would be unlikely to influence the antigenic properties of the organism.

A report by Sammons, Gardner & Dienst (1968) indicated that a satisfactory high cholesterol serum could be produced by feeding rabbits on a high cholesterol diet. Rabbit infusion broth with 5% of the resulting serum supported growth of several species of human *Mycoplasma*. No mention was made of inhibitory properties of serum. A comparison of the medium with one containing commercial cholesterol for the growth of *Mycoplasma* would be of value.

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