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Interaction of porcine mycoplasmas with fresh animal serum

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SUMMARY

When fresh animal serum was dropped onto seeded mycoplasma agar plates, inhibition of growth frequently occurred. This effect was dependent on the mycoplasma serotype and on the animal species from which the fresh serum came. This activity of fresh animal serum was heat-labile and would not diffuse through the agar medium. Growth of all the porcine mycoplasma serotypes was inhibited by fresh sheep serum. *M. hyorhinis*, *M. hyopneumoniae*, B 3 and the P 45 strains were insensitive to fresh horse serum. The addition of fresh horse serum to specific *M. hyorhinis* rabbit antiserum-impregnated disks appeared to have a synergistic effect and the combination of *M. hyorhinis* antiserum-impregnated disk and fresh horse serum always inhibited the growth of *M. hyorhinis* strains.

INTRODUCTION

The disk growth-inhibition test (Clyde, 1964) has proved to be unsatisfactory for the identification of *Mycoplasma hyorhinis* (Dinter, Danielsson & Bakos, 1965; Dinter & Taylor-Robinson, 1969). *M. hyorhinis* strains when grown on solid medium have been subdivided into antiserum-resistant and antiserum-sensitive strains, and it has been suggested that the difference between sensitive and resistant strains is due to changes in the composition of the mycoplasma membranes (Dinter & Taylor-Robinson, 1969).

The effect of fresh animal serum on the disk growth-inhibition test was investigated with particular reference to *M. hyorhinis*. As normal serum is an essential component in the growth medium for many mycoplasma species, it has been difficult to evaluate the role of normal serum factors in the growth inhibition phenomenon.

The blood of animals which have recovered from contagious bovine pleuropneumonia is highly bactericidal for *M. mycoides*. The bactericidal action is dependent upon the presence of antibody and complement (Priestley, 1952). A heat-labile factor in rabbit serum is essential to neutralize *M. pneumoniae* infectivity for cell cultures (Eaton, Farnham, Levinthal & Scala, 1962). In the metabolic inhibition test, antiserum does not inhibit the growth of *M. pneumoniae* in broth if the horse serum in the growth medium has been heated; unheated guinea-pig serum is also essential for demonstrating the growth-inhibiting effect of specific antiserum against the Negroni strain of *M. pulmonis* (Taylor-Robinson, Purcell, Wong & Chanock, 1966). Complement-like substances play an important role in immune inactivation of *M. gallisepticum* (Barker & Patt, 1967).

Mycoplasma species of porcine origin have been classified into seven groups or serotypes (Dinter *et al.* 1965; Goodwin, Pomeroy & Whittlestone, 1967; Taylor-Robinson & Dinter, 1968). These groups include glucose fermenters and arginine-metabolizing strains of mycoplasma. *M. hyopneumoniae* (*suipneumoniae*), *M. hyorhinis*, *M. granularum*, *M. laidlawii* (B 4) and the B 3 strain are glucose fermenters while *M. gallinarum* (B 2) and *M. iners* (B 6) are arginine-metabolizing strains. Roberts & Gois (1970) reported the isolation from the porcine respiratory tract of mycoplasmas which were arginine-metabolizing and antigenically distinct from the other seven serotypes; these mycoplasmas are represented in this study by the P 45 strain. Using the disk growth-inhibition test, the P 45 strain has been found antigenically similar to *M. hyosynoviae* (Ross & Karmon, 1970) and *M. suidaniae* (Friis, 1970).

The experiments reported here were designed to study the effect of normal serum factors on the porcine mycoplasma serotypes.

MATERIALS AND METHODS

Mycoplasma strains

M. hyorhinis, strain 7, *M. granularum* strain 39 and *M. hyopneumoniae* strain 11 were obtained from Dr W. P. Switzer, Iowa State University, U.S.A. The GDL strain of *M. hyorhinis* was obtained from Dr D. Taylor-Robinson, Salisbury. The B 2, B 3, B 4, B 6 strains and the F strain of *M. hyorhinis* were obtained from Professor Z. Dinter, Uppsala, Sweden. The M 244 strain of *M. hyopneumoniae* was obtained from the Mycoplasma Reference Laboratory, Colindale. The EP 33 strain of *M. hyopneumoniae* was obtained from Dr C. L'Ecuyer, Hull, Quebec. Other strains used in the study were isolated at the Central Veterinary Laboratory, Weybridge; these included the P 19, P 22, S 145 and S 150 strains of *M. hyorhinis*. These strains were typed using the gel diffusion method (Dinter *et al.* 1965) and the metabolic inhibition test (Taylor-Robinson *et al.* 1966). The P 45 strain has been described previously (Roberts & Gois, 1970).

Mycoplasma media

The following three media were used.

(1) Difco PPLO broth plus 20% unheated horse serum, dextrose 0·1% and yeast extract 10% prepared by the method of Marshall & Kelsey (1960). Penicillin G (500 i.u./ml) was added and the pH adjusted to 7·6. Agar plates were prepared using 1% ionagar (Oxoid). This medium was primarily used for growing *M. hyorhinis* and P 45 strains.

(2) Difco PPLO broth plus inactivated horse serum 10%, yeast autolysate (Albimi) 1%, dextrose 0·1%, penicillin G(1000 i.u./ml) and the pH adjusted to 7·8. Agar plates were prepared using 1% ionagar (Oxoid). This medium was used for growing *M. granularum*, B 2, B 3, B 4 and B 6 strains.

(3) Acellular medium described by Goodwin & Whittlestone (1966). Agar plates were prepared using 1% agarose (BDH). This medium was used for growing *M. hyopneumoniae* strains only.

Antiserum production

Specific antisera were prepared in rabbits against the mycoplasma strains by the method described by Roberts (1968). Antiserum to *M. hyorhinis* strain 7 was also prepared in pigs. All sera were Seitz filtered and stored at −20° C.

Cultivation

All cultures were incubated at 37° C. *M. hyopneumoniae* agar cultures were placed in sealed plastic bags and other mycoplasma agar cultures were placed in candle jar containers.

Fresh animal serum and disk growth inhibition

The disk growth inhibition technique was that of Hayflick & Stanbridge (1967). The dried antiserum-impregnated disks were stored at −10° C.

Fresh animal serum was obtained from a rabbit, horse, sheep and guinea-pig. Each serum sample was filtered through a 0·8 μ Millipore filter and stored at −20° C. until ready for use. Preserved guinea-pig serum (Wellcome) was included in the investigation.

Agar plates were prepared, and some were seeded with mycoplasma cultures. On these agar plates, both seeded and unseeded, were placed filter-paper disks (6 mm diameter), each soaked with 0·025 ml. of one of the fresh animal sera or of preserved guinea-pig serum. On similar agar plates dry filter-paper disks, and dried antiserum-impregnated disks for the disk growth-inhibition test, were placed, and 0·025 ml. of fresh animal serum or preserved guinea-pig serum was dropped on each of these disks. When the serum was dropped in this way it spread over the surface of the medium in the area surrounding the disks. A similar series of plates was set up in exactly the same way except that the fresh animal sera and the preserved guinea-pig serum had been heated at 60° C. for 30 min.

RESULTS

Inactivation of porcine mycoplasma by fresh animal serum

When fresh animal serum was added to the filter-paper disks and placed on mycoplasma-seeded agar plates inhibition of growth in the region of the disks was not observed. When, however, the filter-paper disks were placed on the seeded agar plates, and fresh animal serum was dropped onto the disk, so that the serum spread over the surface of the agar in the region of the disk, growth of mycoplasma in the area of the agar covered by fresh animal serum was often not observed. This inhibition of growth did not occur when the fresh serum was heated at 60° C. for 30 min. The results of adding fresh animal serum to the different porcine mycoplasma serotypes are recorded in Table 1. The absence of growth is demonstrated in Pl. 1, Fig. 1 with *M. granularum* and *M. laidlawii* (B 4). The effect of fresh animal serum on *M. hyopneumoniae* was difficult to assess. Owing to its slow rate of growth, the plates were incubated for 2 weeks and then examined, but even after this length of time, growth on agar is relatively sparse and it was not possible

by gross examination to assess any increase or decrease in growth. Even on microscopical examination it was difficult to assess, as colonies were found in the region of the disk. There were, however, differences between the various animal sera; sheep and guinea-pig serum appeared to inhibit growth to a greater extent than rabbit and horse serum.

Table 1. *Inhibition of growth of porcine mycoplasmas by animal sera*

Mycoplasma species	Animal sera: Rabbit	Sheep	Horse	Guinea-pig	Preserved guinea-pig
B 2	++	+++	++	−	+
B 3	++	++	−	−	−
B 4	−	+++	++	+++	++
B 6	++	++	++	−	±
M. granularum	++	+++	++	+++	+++
P 45	++	+++	−	+++	+++
M. hyopneumoniae					
Strain 11	−*	±*	−*	±*	±*
M. hyorhinis					
Strain GDL	+	+++	−	++	+++
Strain 7	+	+++	−	++	+++

+++ Inhibition zone round disk > 3 mm.
++ Inhibition zone round disk > 2 mm. and < 3 mm.
+ Inhibition zone round disk > 1 mm. and < 2 mm.
± Inhibition zone round disk < 1 mm.
− No inhibition.
* Microscopic assessment only

M. hyorhinis strains were insensitive to fresh horse serum. With rabbit antiserum-impregnated disks it is often difficult to type *M. hyorhinis* strains; the growth of mycoplasma in the region of the disk on solid medium is often not inhibited (see Pls. 1, 2, Figs. 2 and 3), although the gel diffusion and metabolic inhibition tests indicate that the strains are similar. The addition of fresh horse serum, dropped onto these rabbit antiserum-impregnated disks appeared to have a synergistic effect and the combination of *M. hyorhinis* antiserum-impregnated disk and fresh horse serum always inhibited *M. hyorhinis* strains. The results are shown in Table 2. Plate 1, fig. 2 and Pl. 2, fig. 3 show the synergistic effect of fresh horse serum and rabbit antiserum-impregnated disks on *M. hyorhinis* strains F and 7.

Precipitation rings were often observed in the agar medium around the disks containing rabbit antisera, similar to those described by Dinter & Taylor-Robinson, (1969). This occurred with both *M. hyorhinis* and *M. hyopneumoniae* strains. A precipitate or halo was often seen in the disk growth-inhibition test with *M. hyorhinis* strains and *M. hyopneumoniae* antiserum-impregnated disks onto which fresh horse serum had been dropped, as demonstrated in Figs. 2 and 3. Occasionally in the disk growth-inhibition test with *M. hyorhinis* strains and *M. hyorhinis* antiserum-impregnated disks, a zone of mycoplasma growth occurred between the disk and the zone of inhibition. This zone of mycoplasma growth was

Table 2. *Disk growth-inhibition tests with* Mycoplasma hyorhinis *strains: antiserum-impregnated disks with and without fresh horse serum*

	Antiserum-impregnated disks																	
	Without horse serum					With horse serum												
M. hyorhinis strain	F	7	S 150	P 22	P 19	F	7	S 150	P 22	P 19	B 2	B 3	B 4	B 6	P 45	MG	HP	C
F	+++ P	+	+ P	+ P	+ P	+++	+++	+++	+++	+++	−	−	−	−	−	−	−	−
7	−	+++	−	−	−	+++	+++	+++	+++	+++	−	−	−	−	−	−	−	−
GDL	+++ G	++ G	+ G	++ G	++ G	+++ G	+++	+++	+++	+++	−	−	−	−	−	−	−	−
S 150	+ P	−	++	−	−	+++	+++	+++	+++	+++	−	−	−	−	−	−	−	−
P 22	+++ P	+++	++ P	++ P	++ P	+++	+++	+++	+++	+++	−	−	−	−	−	−	−	−
P 19	−	−	−	−	++ P	+++	+++	+++	+++	+++	−	−	−	−	−	−	−	−

C = Fresh horse serum control.
HP = *M. hyopneumoniae* Str. 11.
MG = *M. granularum*.
P = Precipitation rings were noted.
G = Growth occurring in region of disk with inhibition of growth in peripheral area. See Table 1 for key.

very extensive with disks prepared from pig antiserum to *M. hyorhinis* strain 7, placed on *M. hyorhinis* seeded agar plates, followed by dropping fresh sheep serum onto the disk. The mycoplasma growth zone was then often 3 mm or more, at the periphery of which a zone of inhibition occurred. This phenomenon is demonstrated in Fig. 3.

A feature of the dropping of either fresh or heated animal serum onto filter-paper disks or antiserum-impregnated disks was the constant appearance of pseudocolonies (Hayflick, 1965; Brown, Swift & Watson, 1940) on solid medium 3. These agar plates were incubated for 2 weeks and the pseudocolonies appeared in the region of the disk where the animal serum had come in contact with the solid agar. These pseudocolonies developed on both seeded and unseeded agar plates. Pseudocolonies and mycoplasma colonies were not found together in the same region of the agar plate.

DISCUSSION

It was not established what effect fresh animal serum had on seeded mycoplasma agar plates, whether it was inactivation, inhibition of growth or lysis. Heat-labile accessory factors in fresh serum may either potentiate growth-inhibiting or metabolic inhibiting antibody titres or stabilize the titres so that they remain at their original level on continued incubation (Taylor-Robinson 1968). In the experiments reported here, fresh animal serum appears to have a far greater effect; this effect was dependent on the mycoplasma serotype and on the animal species from which the fresh serum came. This activity of fresh animal serum was heat-labile and would not diffuse through the agar medium; the factor involved could possibly be complement, for agar is known to be strongly anti-complementary (Jerne, Nordin & Henry, 1963).

The disk growth-inhibition test is not very satisfactory for the typing of *M. hyorhinis*. The addition of fresh horse serum to the rabbit antiserum-impregnated disks aided in the typing of *M. hyorhinis*. There was certainly a synergistic effect observed and, on the limited observations carried out, it seems possible that all *M. hyorhinis* cultures could be typed in this way. Gois, Cerny & Veznikova (1970) showed that *M. hyorhinis* produced colonies in liquid media in the presence of specific rabbit antiserum and of pig serum obtained from *M. hyorhinis*-infected herds. A similar phenomenon was seen in these investigations, but it occurred on solid medium, as demonstrated in Fig. 3. This phenomenon was clearly seen when fresh sheep serum was dropped onto pig antiserum-impregnated disks. Growth of mycoplasmas occurred immediately next to the disk and at the periphery of the growth zone an area of inhibition occurred. An explanation of this phenomenon is not apparent, but the ability of *M. hyorhinis* to grow in the presence of antibodies might explain why *M. hyorhinis* cannot be typed satisfactorily using the disk growth-inhibition test. The interaction of fresh animal serum and the various porcine mycoplasma serotypes could also be used as an aid in their classification. For example, *M. hyorhinis* and the P 45 serotype are sensitive to rabbit, sheep and guinea-pig, and insensitive to horse serum, of these two serotypes only *M. hyorhinis* ferments glucose. Differences between other serotypes can be seen in Table 1.

The growth-inhibition, lysis or inactivation effect of fresh animal serum on porcine mycoplasma in the absence of antiserum cannot be adequately explained. It is hardly likely that heat-labile natural antibodies are to be found in sheep to all the porcine mycoplasma serotypes. Non-specific attachment of serum proteins from the growth medium onto the mycoplasma organism may sensitize the organism to complement. Adsorption of medium components onto *M. hyorhinis* and *M. hyopneumoniae* cells have been shown to occur (Roberts & Little, 1970). This does not, however, explain why all porcine mycoplasmas are sensitive to fresh sheep serum whereas only four serotypes are sensitive to fresh horse serum. A bacteraemia rarely occurs in animal mycoplasmosis. When it does occur, it is associated with stress and sometimes it results in arthritis (Switzer, 1964; Roberts & McDaniel, 1967). Priestley (1952) demonstrated that the blood of cattle, which had recovered from contagious bovine pleuropneumonia, was highly bactericidal for *M. mycoides* and that the action was dependent upon the presence of antibody and complement. The blood of animals dying of contagious bovine pleuropneumonia was not bactericidal, and this was due to a deficiency of complement and not to any deficiency of antibody. It would be interesting to see if the blood of animals under stress is deficient in complement.

The addition of fresh and heated animal serum to medium 3 invariably produced pseudocolonies in the absence of mycoplasma colonies. These colonies form on agar with a high serum content and are composed of calcium and magnesium soaps which form crystalline structures on the agar surface (Hayflick, 1965; Brown *et al.* 1940).

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Inactivation of *M. laidlawii* (B 4) and *M. granularum* by fresh animal serum.

Fig. 2. Disk growth-inhibition tests with the F strain of *M. hyorhinis*, rabbit antiserum-impregnated disks with and without fresh horse serum. MHr.F = F strain of *M. hyorhinis*. MHr. 7 = 7 strain of *M. hyorhinis*. M.Hp. 11 = 11 strain of *M. hyopneumoniae*. MG = *M. granularum* and C = fresh horse serum control. Other symbols as designated in text.

PLATE 2

Fig. 3. Disk growth-inhibition tests with strain 7 of *M. hyorhinis*, rabbit antiserum-impregnated disks with and without fresh horse serum, and antiserum-impregnated disks with fresh sheep serum. Hr. = *M. hyorhinis*, Hr. 7 R and Hr. 7 P indicate rabbit and pig antiserum respectively; remaining disks are prepared from rabbit antiserum. Other symbols, see fig. 2 and text.

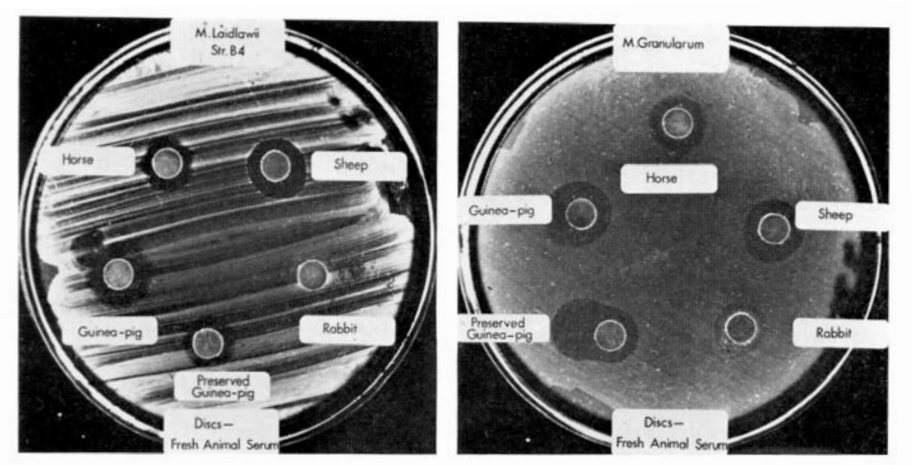

Fig. 1

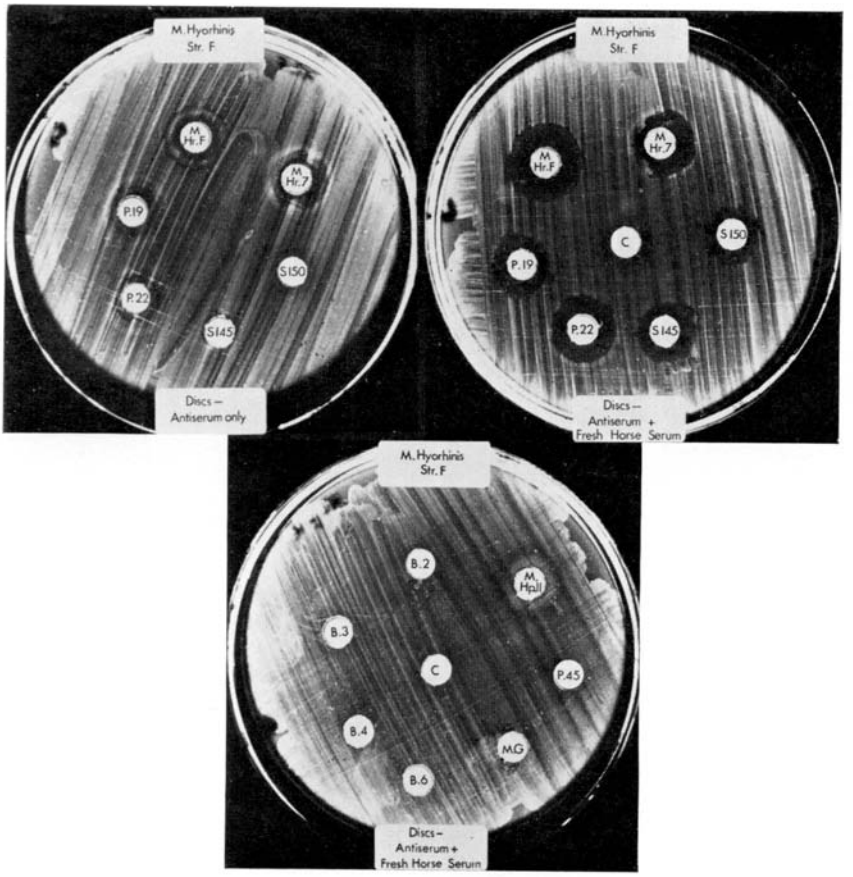

Fig. 2

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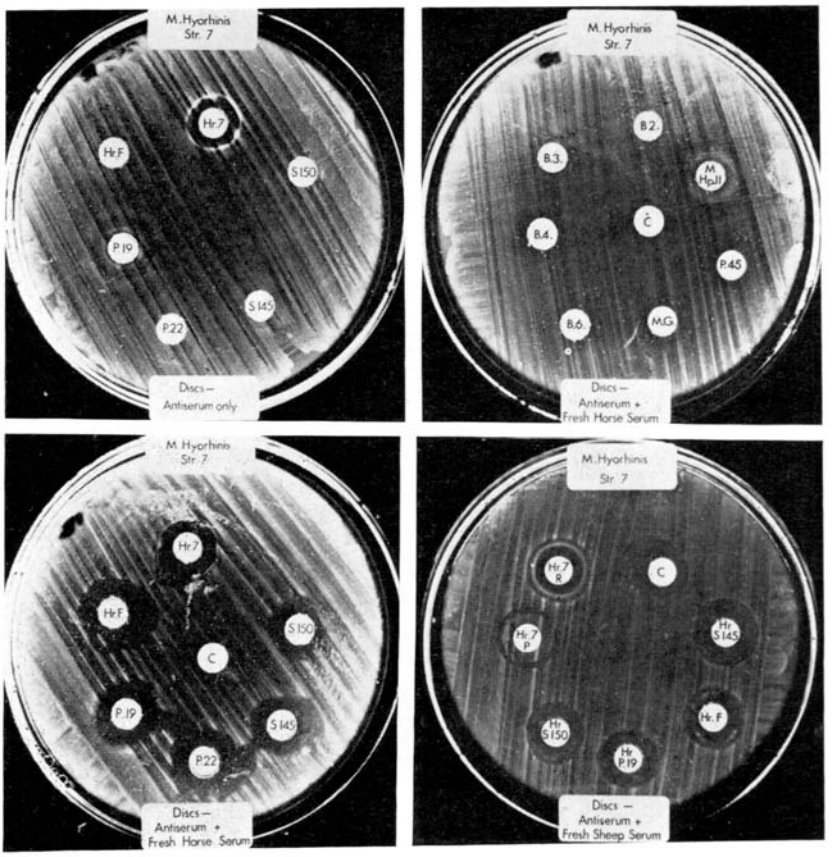

Fig. 3

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