Differentiation of Porcine *Mycoplasma* Strains

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SUMMARY

Porcine *Mycoplasma* strains, previously called 'swine enzootic pneumonia strains' are antigenically closely related to *Mycoplasma hyorhinis*. Other porcine *Mycoplasma* strains which are culturally and antigenically distinct from *M. hyorhinis* are more heterogeneous and fall into four serotypes. *M. granularum*, however, is antigenically unrelated to these strains.

INTRODUCTION

Different types of *Mycoplasma* have been isolated from pigs with enzootic pneumonia or atrophic rhinitis in Sweden (Bakos & Dinter, 1963). The majority of isolates were shown to be culturally closely related to a cytopathogenic agent cultivated by Wesslén & Lannek (1954) from pigs with a disease called swine enzootic pneumonia (SEP). These isolates were hence labelled SEP strains, although no evidence was presented that such strains cause a transmissible pneumonia in pigs (Goodwin & Whittlestone, 1964). A minor group of isolates, designated B strains, were culturally distinct from the SEP strains.

In this study the SEP and B strains have been compared with each other and with reference strains of *Mycoplasma hyorhinis* (Switzer, 1955) and *M. granularum* (Switzer, 1964)—the two hitherto described species of *Mycoplasma* occurring in pigs. The results show that porcine *Mycoplasma* strains are a heterogeneous group.

METHODS

Mycoplasma strains. Two strains of *Mycoplasma hyorhinis*, kindly supplied by Professor A. Freundt, University of Aarhus, Denmark, and Dr W. P. Switzer, Iowa State University of Science and Technology, Ames, Iowa, U.S.A., and one strain of *M. granularum*, kindly supplied by the latter, were investigated. The *M. hyorhinis* strains were labelled F and s7, respectively. Other strains used in this study belonged to our collection of isolates from pigs. The SEP strains labelled SEP/200, SEP/sk and SEP/G as well as six B strains labelled B 1 to B 6 were used. The strains SEP/G and B 6 were isolated from pig material sent by Dr H.-J. Neumann, Kiel, Germany. Stock cultures of the strains were prepared in PPLO broth, distributed in tubes in amounts of 5 ml. per tube and stored at -60° before use.

PPLO media. PPLO broth medium consisted of beef heart infusion with addition of 0.5% (w/v) yeast extract (Difco), 0.1% (w/v) glucose, 20% (v/v) unheated horse serum, penicillin (100 i.u./ml.) and 1% (v/v) of a 5% (w/v) solution of thallium acetate. PPLO agar medium was composed of the same ingredients with addition

of 1.2% (w/v) Special Agar-Noble (Difco). Penicillin and thallium acetate were omitted when satellite growth was required.

Viable cell count. Serial tenfold dilutions of a Mycoplasma suspension were made in PPLO broth and inoculated in amounts of 0.1 ml. on plates with PPLO agar. The plates were incubated at 37° and the number of colonies counted on day 6 after inoculation.

Satellite growth. The surface of PPLO agar was first inoculated with Mycoplasma and then, along the diameter of the plates, with staphylococci according to the method of Morton, Smith & Leberman (1949). The staphylococcus strain rc3II, kindly supplied by Dr J. I. Terpstra, Rotterdam, Holland, was used for this purpose.

Egg inoculation. Suspensions of Mycoplasma were inoculated into the yolk sac of chick embryos aged 5–7 days as previously described (Dinter, Wesslén & Lannek, 1957). Pools of amniotic and allantoic fluids were used for further passages in eggs.

Cell cultures. Primary cultures of calf cells were prepared from trypsinized kidneys of newborn calves and grown at 37° in tubes. Growth medium was Hanks's salt solution with 0.5% (w/v) lactalbumin hydrolysate (H + Lah) enriched with 10% (v/v) newborn calf serum. The maintenance medium consisted of H + Lah + 5% (v/v) unheated horse serum. Penicillin (100 i.u./ml.) was added to both media.

Titration of infectivity in cell cultures. Serial tenfold dilutions of a Mycoplasma suspension were made in maintenance medium and inoculated in 0.1 ml. amounts in each of 4 cell culture tubes. Four additional, non-inoculated cultures served as controls of the specificity of cell degeneration induced by Mycoplasma. All cultures were incubated at 37° in upright position and in a volume of maintenance medium sufficient to cover the entire cell sheet. The results were read on day 6 or 7 after inoculation. The titres were calculated according to Kärber and are expressed as log₁₀ units of TCD 50/0.1 ml.

Preparation of antigens for gel diffusion. The strains were transferred into PPLO broth by inoculating 200 ml. of this medium with 10 ml. of stock culture. A dense outgrowth was obtained after incubation for 2 or 3 days at 37°. The organisms were concentrated by sedimentation for 30 min. at 35,000g and 4° and then washed 3 times in isotonic saline. The washed sediments were resuspended in 1 ml. of distilled water per 100 ml. of the original volume and the suspensions frozen and thawed 5 times before their use as antigens. A similar technique has previously been applied by Taylor-Robinson, Somerson, Turner & Chanock (1963) for the preparation of antigens from human Mycoplasma strains. The antigen stocks were kept at –20°.

Preparation of antisera. Antisera were prepared in rabbits with PPLO broth cultures as antigens. The Mycoplasma strains were sedimented and washed as described above. The washed sediments were resuspended in 5 ml. of isotonic saline per 100 ml. of the original volume. The suspensions were then mixed thoroughly with an equal amount of Freund's complete adjuvant (Difco). Each rabbit was given 2 ml. of the antigen–adjuvant mixture intramuscularly into each leg. After 5 or 6 weeks the rabbits were injected intravenously with 1 ml. of a suspension without adjuvant prepared as described above. Serum samples were taken 1 week after the second injection of antigen and were stored at –20°. They were used unheated.

Absorption of antisera. Antisera used for gel diffusion were absorbed with the PPLO broth medium in order to remove antibodies to the ingredients of this medium. PPLO broth was concentrated 10 times by pressure dialysis in a collodion bag (Membranfilter AG, Göttingen, Germany). Equal amounts of concentrate and antiserum were mixed and the mixture kept for 16 hr at 4°. The mixture was then centrifuged for 30 min. at 4500g and 4° and the supernatant concentrated to the original volume by pressure dialysis as mentioned above.

Gel diffusion. The micro modification of the Ouchterlony method (Wadsworth, 1957) was used. Tests were performed on glass plates with a 0.4 mm. thick layer of agar gel consisting of 1% (w/v) Special Agar-Noble (Difco) in phosphate-buffered saline (PBS), pH 7.2. The wells were 3 mm. in diameter and made in 3 mm. thick plexiglass templates. The apertures connecting the wells with the gel were 2 mm. wide. Templates with 3, 5 or 7 wells at a distance of 5 or 6 mm. between the edges were used. When the agar had congealed the wells were filled once with reactants. The plates were kept in a humidified atmosphere at room temperature for 2 days. The templates were then removed and the precipitation patterns recorded. The plates were washed for a few days in PBS and then dried and stained with amido black as recommended by Wadsworth (1957).

Growth inhibition. The technique was that of Clyde (1964) as applied by Taylor-Robinson, Canchola, Fox & Chanock (1964) for the differentiation of human *Mycoplasma* strains. The strains were cultivated in PPLO broth for 2 or 3 days at 37° and the cultures then spread on PPLO agar in amounts of 0.1 ml. containing 10^6 to 10^8 colony-forming organisms. The PPLO agar was dried for 1 hr at 37° prior to inoculation. Filter-paper discs were soaked with 0.025 ml. of undiluted antiserum by means of wire loops invented by Takátsy (1955) and manufactured by Cooke Engineering Co., Alexandria, Virginia, U.S.A. The discs were then placed at appropriate distances from one another on the surface of PPLO agar. The results were read after incubation for 3 days at 37°. Zones of growth inhibition were measured as the distance between the margin of the disc and the margin of inhibition.

RESULTS

Cultural characteristics of SEP strains and Mycoplasma hyorhinitis

The occurrence of different *Mycoplasma* strains in pigs was evident at the time of isolation (Bakos & Dinter, 1963). The SEP strains were easier to isolate in tissue cultures (Wesslén & Lannek, 1954) or in the yolk sac of chick embryos (Dinter *et al.* 1957) than on PPLO growth media. Similar results were obtained when isolating *Mycoplasma hyorhinitis* (L'Ecuyer, Switzer & Roberts, 1961).

A property common to SEP strains was that their growth on PPLO agar was promoted by the simultaneous cultivation of staphylococci. This phenomenon was most pronounced with SEP strains not adapted to cell-free media—the number and size of the colonies formed by SEP strains increased closer to the streak of staphylococci (Bakos & Dinter, 1963). The strains, however, were easily adapted to cell-free media after a few passages in PPLO broth.

The adapted strains showed an extensive growth on PPLO agar. The colonies were embedded in the agar and had a granular appearance with the larger colonies showing dark centres. Smears from colonies stained with Giemsa stain (Kebo AB,

Stockholm) showed particles with an approximate size of 0.2μ . A similar colonial structure was observed after cultivation of reference strains of *Mycoplasma hyorhinae*. When the method of satellite growth was applied, the colonies were enlarged adjacent to the staphylococci.

Three-day-old cultures in PPLO broth were titrated in cultures of calf kidney cells. The SEP strains and *Mycoplasma hyorhinae* produced cytopathic changes of the same type as described for the strains of the cytopathogenic agent isolated by Wesslén & Lannek (1954). The titres of infectivity varied between $10^{6.25}$ and $10^{7.75}$ TCD₅₀/0.1 ml. The SEP strains and *M. hyorhinae* proved to be lethal for chick embryos but the mortality rates were irregular as described by Dinter *et al.* (1957) for the cytopathogenic agent of Wesslén & Lannek (1954).

Table 1. *Differentiation of porcine Mycoplasma strains by some cultural properties*

Organism and strain	Promoted by satellite growth	Cytopathogenic for calf kidney cells	Lethal for chick embryos
<i>M. hyorhinae</i> F	+	+*	(+) [†]
<i>M. hyorhinae</i> s7	+	+	(+)
SEP/200	+	+	(+)
SEP/sk	+	+	(+)
SEP/G	+	+	(+)
<i>M. granularum</i>	—	(+) [‡]	—
B1	—	(+)	—
B2	—	(+)	—
B3	—	+	+§
B4	—	(+)	—
B5	—	(+)	—
B6	—	(+)	—

* Degeneration and cell death.

[†] Irregular rates of mortality.

[‡] Various degrees of cytoplasmic granulation.

§ Regular rates of mortality.

Cultural characteristics of B strains and Mycoplasma granularum. Five out of six B strains were isolated directly on PPLO agar. One strain, B6, had to be enriched in PPLO broth prior to cultivation on PPLO agar. The growth of B strains and *Mycoplasma granularum* was not stimulated by satellite cultivation but was dependent upon the presence of serum. The viable cell count gave 2 to 7×10^7 colony-forming units per 0.1 ml. of 2-day-old PPLO broth cultures. The colonies were embedded in the agar. Most colonies were of the 'fried egg' type, although the colonies of one strain, B6, differed from those of the other strains by having a structure similar to that described for *M. pneumoniae* (Channock, Hayflick & Barile, 1962). The size of the organisms in the colonies of B strains was approximately 0.2μ .

One of the B strains, B3, was shown to be strongly cytopathogenic for cultures of calf kidney cells and regularly lethal for chick embryos. Acid production was observed in cell cultures inoculated with this strain. The other B strains and *Mycoplasma granularum* have been passaged 3 times in eggs but proved to be non-lethal for chick embryos. Cell cultures inoculated with these strains showed various degrees of a cytoplasmic granulation.

On the basis of some cultural properties indicated in Table 1, the porcine *Mycoplasma* strains can be differentiated into two groups, one represented by *Mycoplasma hyorhinis* and the other by *M. granularum*.

Differentiation by gel diffusion. In preliminary experiments, unabsorbed antisera and antisera absorbed with the PPLO broth medium were tested with this medium as antigen. Some unabsorbed sera gave one precipitation line with PPLO broth but most unabsorbed sera as well as all absorbed sera did not react with this medium. The results of cross-testing eleven absorbed antisera against suspensions of 12 strains are shown in Table 2. According to these results a close relationship exists between *Mycoplasma hyorhinis* and the SEP strains. The precipitation lines showed reactions of identity. SEP strains are thus identified as strains of *M. hyorhinis*. No cross-reactions were found between the group of strains represented by *M. hyorhinis* and the B strains including *M. granularum*.

Table 2. *Differentiation of porcine Mycoplasma strains by gel diffusion*

Organism and strain	Antiserum against									
	<i>M. hyorhinis</i>					<i>M. granularum</i>				
	SEP		B			1		2		
	F	s7	200	sk	G	1	2	3	4	6
Number of precipitation lines										
<i>M. hyorhinis</i> F	3	4	3	2	5	0	0	0	0	0
<i>M. hyorhinis</i> s7	3	4	3	2	5	0	0	0	0	0
SEP/200	3	4	3	2	5	0	0	0	0	0
SEP/sk	3	4	3	2	5	0	0	0	0	0
SEP/G	3	4	3	2	5	0	0	0	0	0
<i>M. granularum</i>	0	0	0	0	0	4	0	0	0	1
B1	0	0	0	0	0	0	5	5	0	1
B2	0	0	0	0	0	0	5	5	0	1
B3	0	0	0	0	0	0	0	0	8	0
B4	0	0	0	0	0	1	0	0	0	4
B5	0	0	0	0	0	0	5	5	0	1
B6	0	0	0	0	0	0	1	1	0	4

Within the group comprising the B strains and *Mycoplasma granularum*, a reciprocal relationship was established between the strains B1 and B2. Since the antigen of the B5 strain was precipitated by antisera to strains B1 and B2, these three strains were considered to belong to the same serotype. Reciprocal cross-reactions of low order were noted between *M. granularum* and strain B4 as well as between strain B6 and the strains B1, B2 and B5. With heterologous antisera one precipitation line was formed by these strains whereas homologous antisera gave with them four or five lines (Table 2). No cross-reactions were observed between strain B3 and the other strains although B3 produced at least 8 lines when tested against homologous antiserum. According to these results the strains of the present study fall into six serotypes (Table 3).

Differentiation by growth inhibition. Antisera used in gel diffusion tests were also examined in growth inhibition tests. The results are summarized in Table 4. Antisera to SEP strains and strains of *Mycoplasma hyorhinis* inhibited the growth of the strain F of *M. hyorhinis* but had no inhibitory effect on the s7 strain of *M.*

hyorhinis. All SEP strains were inhibited by homologous antisera and two of them also by certain heterologous antisera. The reciprocal relationship between SEP strains and strains of *M. hyorhinis* as shown by gel diffusion could thus not be demonstrated by growth inhibition. Concerning the B strains and *M. granularum*, the specific reactions seen in gel diffusion tests were also observed in growth inhibition tests (Table 4). A close relationship was established between strains B1, B2, and B5. The other three B strains and *M. granularum* were inhibited only by homologous antisera. Cross-reactions of low order seen between some strains in gel diffusion tests were not observed in growth inhibition tests.

Table 3. Serotypes of porcine *Mycoplasma* strains as shown by gel diffusion

Serotype	Organism and strain
1	<i>M. hyorhinis</i> : F, s7; SEP: 200, sk, G
2	<i>M. granularum</i>
3	B3
4	B4
5	B1, B2, B5
6	B6

Table 4. Differentiation of porcine *Mycoplasma* strains by growth inhibition

Strain	Antiserum against									
	<i>M. hyorhinis</i>		SEP			<i>M. granularum</i>	B			
	F	s7	200	sk	G		1	2	3	4
	Zone of inhibition in millimeters									
<i>M. hyorhinis</i> F	5	3	4	3	4.5	—	—	—	—	—
<i>M. hyorhinis</i> s7	—	—	—	—	—	—	—	—	—	—
SEP/200	2.5	1.5	3	—	2.5	—	—	—	—	—
SEP/sk	—	—	—	2	—	—	—	—	—	—
SEP/G	2	—	2	—	2	—	—	—	—	—
<i>M. granularum</i>	—	—	—	—	—	5	—	—	—	—
B1	—	—	—	—	—	—	5	3	—	—
B2	—	—	—	—	—	—	5	3	—	—
B3	—	—	—	—	—	—	—	—	3.5	—
B4	—	—	—	—	—	—	—	—	—	4
B5	—	—	—	—	—	—	5	3	—	—
B6	—	—	—	—	—	—	—	—	—	5

— = no inhibition.

DISCUSSION

The porcine *Mycoplasma* strains isolated in this country could be divided at isolation into two groups. One group comprised isolates which originally grew better in cultures of animal cells and the yolk sac of chick embryos than on PPLO media unless satellite growth was employed. Isolates with such properties were encountered in more than 60 % of pigs suffering from enzootic pneumonia or atrophic rhinitis (Bakos & Dinter, 1963). These isolates were provisionally called 'SEP strains'.

The present study was carried out using SEP strains well-adapted to growth on

PPLO media but these strains retained the properties of being cytopathogenic, irregularly lethal for chick embryos and promoted by satellite growth. It is interesting that they were shown to be closely related to *Mycoplasma hyorhinis*, originally isolated from pigs with atrophic rhinitis (Switzer, 1955) but considered to be a secondary invader (Switzer, 1964).

The strains of the other group were provisionally called B strains. The growth of these strains was dependent upon the presence of serum in the medium but was not stimulated by animal or bacterial cells as shown for SEP strains and *Mycoplasma hyorhinis*. *M. granularum* showed similar properties to the B strains. The existence of two different species, *M. granularum* and *M. hyorhinis*, was first established when porcine Mycoplasma strains were compared by indirect haemagglutination (Ross & Switzer, 1963).

In the present study serological differentiation was carried out by gel diffusion and growth inhibition. These tests were found reliable for differentiation of human Mycoplasma strains (Taylor-Robinson *et al.* 1963, 1964). The gel diffusion tests showed that strains of *Mycoplasma hyorhinis* and SEP strains are antigenically identical. The growth inhibition test, on the other hand, proved to be unsatisfactory when applied for identification of *M. hyorhinis*. Antisera effective against one strain were less effective or ineffective against the other; one strain was not inhibited even by homologous antiserum. Klieneberger-Nobel (1962) and Huijsmans-Evers & Ruys (1956) made similar observations when working with other Mycoplasma strains but no explanation is available for this phenomenon.

Both tests, the gel diffusion and growth inhibition, demonstrated that the B strains, including *Mycoplasma granularum*, are distinct from strains of *M. hyorhinis* but show great heterogeneity within the group. Only three out of six B strains belong to a single serotype whereas the other three B strains and *M. granularum* each represent separate serotypes. Although some heterologous strains showed cross-reactions in gel diffusion tests, the homologous reactions were sufficiently complex to identify the strains as separate serotypes. Similar observations were made with other Mycoplasma strains by Lemcke (1965).

Finally, a classification of porcine Mycoplasma strains must consider many more isolates than the small number studied here. Of special interest are the results of investigations carried out by the Cambridge group in this field. Goodwin & Whittlestone (1964) cultivated a visible pleomorphic organism, the J agent, from pigs with enzootic pneumonia, which was shown to induce a transmissible pneumonia in pigs. This agent is probably a Mycoplasma.

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