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MYCOPLASMA SUIPNEUMONIAE AND MYCOPLASMA FLOCCULARE IN THE GROWTH PRECIPITATION TEST

By

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FRIIS, N. F.: *Mycoplasma suipneumoniae and Mycoplasma flocculare in the growth precipitation test.* Acta vet. scand. 1977, *18*, 168—175. — A number of laboratory and field strains of Mycoplasma suipneumoniae and Mycoplasma flocculare were subjected to a comparative examination by the growth precipitation test. It was found that all the strains could readily be identified by that test. Slight evidence of cross-reaction was noted for a few of the laboratory strains, but not until late in the observation period. Only some of the field strains would form precipitates when primary cultures (from tissue suspensions) were used, but all strains could be identified already in the second and third passages. The test therefore seems well suited for distinguishing the two species from each other.

mycoplasma suipneumoniae; mycoplasma flocculare; growth precipitation test.

For rapid serologic identification of mycoplasmas the disc growth inhibition test (DGI) as described by *Stanbridge & Hayflick* (1967) is extensively used. This method is based upon an inhibition of mycoplasma colonies on solid medium around a paper disc impregnated with homologous antibody. In the cases of Mycoplasma suipneumoniae (M. suip.) and Mycoplasma flocculare (M. floc.) DGI has the great disadvantage that a proper colony growth on solid medium may be difficult to obtain, unless the strains under test are well adapted to artificial medium.

An alternative to DGI is the growth precipitation test (GP) evolved by *Krogsgaard-Jensen* (1972). This test is carried out in agar gel, a growing broth culture of mycoplasma being deposited in a central well surrounded by antibody-impregnated discs. Pre-

cipitation lines will develop between the control well and discs with antibody for the mycoplasma concerned.

The purpose of the present work was to study the value of GP for identification of M. suip. and M. floc., and to examine the specificity of the test so far as the two species are concerned.

MATERIAL AND METHODS

Well-adapted, pure-cultured strains of M. suip. and M. floc. were tested against antisera for both species (Exp. I). Two types of antisera were used, viz., antisera produced in rabbits with antigen harvested from cultures in ordinary broth (ob antiserum) and antisera produced in rabbits with antigen harvested from cultures in rabbit broth (rb antiserum). The tests were repeated after two—four weeks. Later on, freshly isolated field strains of each species were tested (Exp. II) though only against rb antiserum.

Media

The medium for cultivation of strains and for use in the test has been described earlier (*Friis* 1975). For enrichment, a mixture of horse and swine serum was used at a final concentration of 20 %. Solid medium was prepared with Agar-Agar (Oxoid) 0.8 %, and DEAE-Dextran, 10 mg/100 ml.

Rabbit broth was prepared as follows: Muscle and heart of a rabbit were pooled and minced. One l of distilled water was added to 125 g meat under further blending. This suspension was heated to 90—100 C for 20 min. and thereafter centrifuged; the supernatant was enriched with Bacto peptone (Difco) 10 g, and NaCl, 5 g, was added. To 300 ml of this solution 200 ml of Hanks' balanced salt solution was added; further, after autoclaving, yeast extract, 25 ml, and 10 % glucose, 10 ml, were added. Finally, rabbit serum to 20 %, bacteriostatics, and phenol red were added. The pH was 7.6. For details, see *Friis*.

Strains of mycoplasma

Strains "J" (NCTC 10110) and Ms42 (NCTC 10143), i.e., the type strains of M. suip. and M. floc., resp., were tested in Exp. I together with nine Danish isolates of each of the two species. These were well adapted to artificial medium, and had all been cloned twice on solid medium before being identified by ordinary

DGI. In Exp. II, five crude strains of M. suip. and five of M. floc. were examined as well. These strains were isolated for this particular purpose from tissue suspensions stored for a few months at —25 C and previously shown by DGI to contain the mycoplasmas in question.

The GP test

A central well 8 mm in diameter was made in a plate of solid medium. The well was filled with a broth culture in which early growth was evidenced by a beginning colorshift of the phenol-red indicator. Discs which had previously been wetted with antiserum and allowed to dry were placed on the agar at a distance (rim to rim) of 10 mm from the well. Two discs were placed opposite each other on each plate; one with antibody for M. suip. and one with antibody for M. floc. The plates were incubated at 37 C in a high-humidity box with 5—10 % CO_2 in the atmosphere. Readings were made after four, six, and eight days in a black-background viewer giving indirect light. The intensity of the precipitation lines were scored according to the scale: 0 = no reaction, ? = dubious r., 1 = very weak r. (though definite), 2 = weak r., 3 = distinct r., 4 = very distinct r.

In Exp. II each strain was tested in dilutions 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} of the first passage (a), except for four strains of M. floc. which did not grow beyond 10^{-2}. The strains were further examined in the second and third passages (b) but only in dilution 10^{-2}, which dilution was likewise used as seeding material for the subsequent passage.

RESULTS

The results of the experiment with culture-adapted strains (Exp. I) are given in Table 1. It appears that most strains of either species reacted better with rb antiserum than with ob antiserum. All the M. suip. strains were easily identified after four days of incubation with either ob or rb antiserum, whereas in the case of M. floc. many of the strains had to be incubated for six to eight days before a precipitation would occur, and satisfactory results were obtained only with rb antiserum. Cross-reaction was observed in a few cases towards the end of the observation period, and for strain "J" of M. suip. and strain Ms31 of M. floc. it was

Table 1. Results of growth precipitation test with adapted strains of M. suipneumoniae and M. flocculare (Exp. I).

Antigen	Antiserum											
	after 4 days				after 6 days				after 8 days			
	ob		rb		ob		rb		ob		rb	
	s	f	s	f	s	f	s	f	s	f	s	f
Strains of M. suip.												
strain "J"*	2—3	0—0	4—3	0—0	3—2	0—0	4—4	?—0	4—3	0—0	4—4	1—0
Ms5	3—1	0—0	4—2	0—0	3—1	0—0	4—3	0—0	3—2	0—0	4—4	0—0
Ms6	2—1	0—0	2—4	0—0	2—2	0—0	4—4	0—0	2—2	0—0	3—4	0—0
Ms7	2—3	0—0	4—3	0—0	3—2	0—0	4—3	0—0	1—3	0—0	4—3	0—0
Ms8	2—2	0—0	4—3	0—0	3—1	0—0	4—4	0—0	2—2	0—0	4—4	0—0
Ms9	3—3	0—0	2—3	0—0	3—2	0—0	4—4	?—0	2—3	0—0	3—3	?—0
Ms10	2—3	0—0	4—3	0—0	1—2	0—0	4—3	0—0	1—3	0—0	4—4	0—?
Ms11	3—3	0—0	4—3	0—0	2—2	0—0	4—4	0—0	2—4	0—0	4—4	0—0
Ms12	3—4	0—0	4—4	0—0	3—4	0—0	4—4	0—0	3—4	0—0	4—4	0—0
Ms13	3—3	0—0	4—2	0—0	3—3	0—0	4—2	0—0	3—3	0—0	4—2	0—0
Strains of M. floc.												
Ms42*	0—0	?—?	0—0	?—2	0—0	1—?	0—0	2—2	0—0	2—?	0—?	3—2
Ms26	0—0	?—?	0—0	2—2	0—0	?—?	0—0	2—2	0—0	?—?	0—0	3—1
Ms30	0—0	0—1	0—0	2—2	0—0	?—1	?—0	4—2	0—0	1—2	?—0	4—2
Ms31	0—0	?—?	0—0	2—2	0—0	0—1	0—?	2—3	0—0	0—1	0—1	3—4
Ms45	0—0	0—0	0—0	0—0	0—0	0—0	0—0	0—0	0—0	0—0	0—0	?—2
Ms46	0—0	0—0	0—0	0—0	0—0	0—?	0—0	0—?	0—0	0—1	0—0	1—2
Ms47	0—0	1—0	0—0	0—1	0—0	1—?	0—0	2—2	0—0	1—1	?—0	4—2
Ms48	0—0	0—0	0—0	0—0	0—0	0—?	0—0	0—?	0—0	0—?	0—0	1—1
Ms49	0—0	0—0	0—0	0—0	0—0	0—?	0—0	0—1	0—0	0—1	0—0	2—1
Ms50	0—0	1—1	0—0	2—2	0—0	?—2	0—0	2—2	0—0	?—2	0—0	2—2

* type strain of species cornerned.
ob, rb = antiserum from rabbits immunized with antigen grown in ordinary broth and in rabbit broth, resp.
s, f = M. suip. and M. floc., resp.
Figures indicate degree of precipitation, as read at the 1st and 2nd performance of the test at intervals of about three weeks.

Table 2. Results of growth precipitation tests with M. suipneumoniae and M. flocculare in primary cultures from tissue suspensions (Exp. IIa).

Antigen	Antiserum (rb)											
	after 4 days				after 6 days				after 8 days			
	10^{-1}	10^{-2}	10^{-3}	10^{-4*}	10^{-1}	10^{-2}	10^{-3}	10^{-4*}	10^{-1}	10^{-2}	10^{-3}	10^{-4*}
	s f	s f	s f	s f	s f	s f	s f	s f	s f	s f	s f	s f
Strains of M. suip.												
Mp469	1 0	0 0	0 0	0 0	2 0	2 0	? 0	0 0	2 0	2 0	? 0	0 0
Mp503	nd	0 0	0 0	0 0	nd	1 0	2 0	0 0	nd	2 0	2 0	0 0
Mp513	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
Mp537	1 0	nr	0 0	1 0	3 0	2 0	0 0	2 0	3 0	2 0	1 0	3 0
Mp573	? 0	? 0	0 0	0 0	2 0	2 0	? 0	0 0	2 0	3 0	? 0	0 0
Strains of M. floc.												
Mp407	0 0	0 0			0 0	0 0			0 0	0 0		
Mp433	0 0	0 0			0 0	0 ?			0 0	0 1		
Mp480	0 0	0 ?			0 0	0 3			0 0	0 3		
Mp482	0 0	0 ?	0 0	0 0	0 ?	0 1	0 0	0 ?	0 ?	0 1	0 ?	0 ?
Mp559	0 ?	0 0			0 1	0 0			0 2	0 1		

* dilutions of antigen.
nd = not done. nr = not read. open space = no growth.
For further explanation, see legend to Table 1.

Table 3. Results of growth precipitation test with field strains of M. suipneumoniae and M. flocculare during the 1st, 2nd, and 3rd passage from tissue suspensions (Exp. II b).

Antigen	Antiserum (rb)											
	after 4 days						after 6 days					
	1st p.		2nd p.		3rd p.*		1st p.		2nd p.		3rd p.*	
	s	f	s	f	s	f	s	f	s	f	s	f
Strains of M. suip.												
Mp469	0	0	1	0	1	0	2	0	2	0	2	0
Mp503	0	0	1	0	?	0	1	0	2	0	2	0
Mp513	0	0	?	0	0	0	0	0	2	0	?	0
Mp537	nr		1	0	?	0	2	0	3	0	1	0
Mp573	?	0	3	0	2	0	2	0	3	0	3	0
Strains of M. floc.												
Mp407	0	0	0	0	0	0	0	0	0	0	0	1
Mp433	0	0	0	1	0	1	0	?	0	2	0	2
Mp480	0	?	0	?	0	?	0	3	0	2	0	2
Mp482	0	?	0	?	0	1	0	1	0	1	0	2
Mp559	0	0	0	0	0	0	0	0	0	?	0	?

Antigen	Antiserum (rb)					
	after 8 days					
	1st p.		2nd p.		3rd p.*	
	s	f	s	f	s	f
Strains of M. suip.						
Mp469	2	0	3	0	2	0
Mp503	2	0	4	0	2	0
Mp513	0	0	2	0	1	0
Mp537	2	0	3	0	2	0
Mp573	3	0	4	0	4	0
Strains of M. floc.						
Mp407	0	0	0	1	0	2
Mp433	0	1	0	2	0	3
Mp480	0	3	0	1	0	3
Mp482	0	1	0	2	0	2
Mp559	0	1	0	1	0	1

* passage of cultures used as antigen.
For explanation of symbols, see legends to Tables 1 and 2.
Each passage was tested in dilution 10^{-2}, which was also used as seeding material for the subsequent passage.

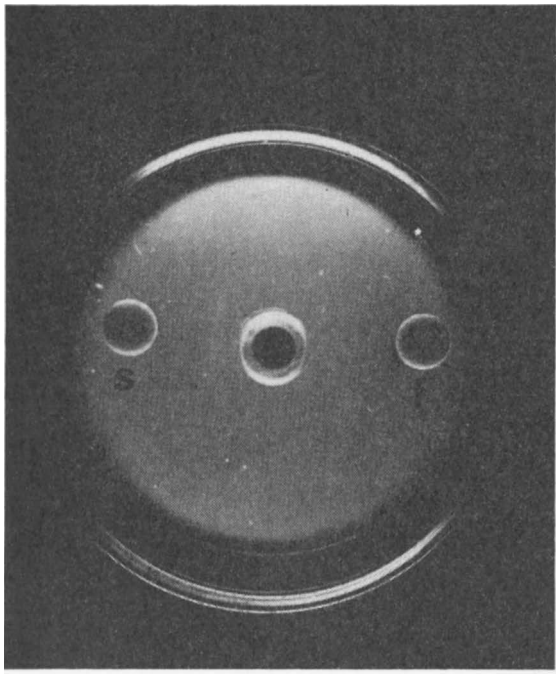

Figure 1. Growth precipitation test with M. suipneumoniae. "Score-3" reaction after five days of incubation.

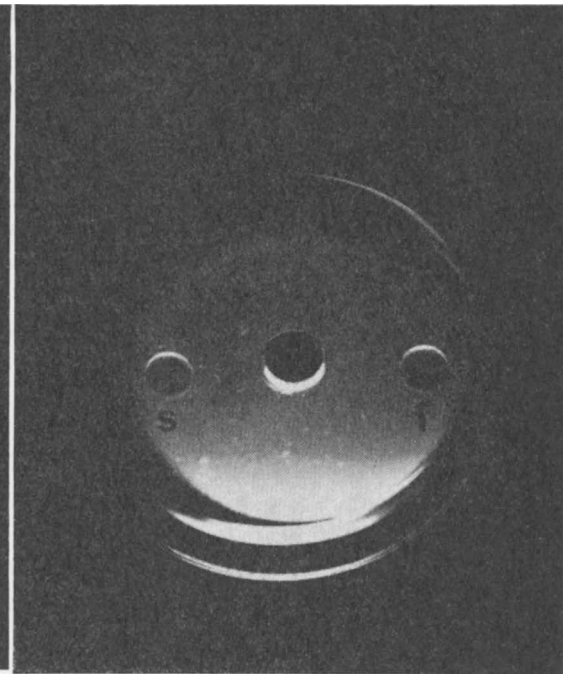

Figure 2. Growth precipitation test with M. flocculare. "Score-2" reaction after six days of incubation.

s, f = discs containing antiserum for M. suipneumoniae and M. flocculare, resp.

well defined, though weak. In both cases the homologous reaction was very distinct.

Most of the field strains isolated direct from tissue suspensions (Exp. IIa) were found to develop lines of precipitation already in their first passage (Table 2). It is noteworthy, however, that by far the best reactions were found with the 10^{-2} dilution, and, at least so far as M. suip. is concerned, the poorest ones with the 10^{-4} dilution. Already in the second and third passages (Exp. IIb, Table 3) all the strains formed precipitates without difficulty. None of the field strains showed cross-reaction.

In the great majority of cases just one line of precipitation occurred, which was usually situated 1 or 2 mm from the central well (Figs. 1 and 2). In the few cases where a second line was formed (usually score ? or 1) only the score for the main line is given in the tables.

DISCUSSION

The work with culture-adapted strains of M. suip. and M. floc. has shown (Table 1) that the growth precipitation test is well suited for identification purposes, and more especially for distinguishing between the two species mentioned. The same holds true for field strains already during the process of primary isolation. However, it appears recommendable (Table 3) to subcultivate primary cultures once before submitting them to the test, in that many strains have been found to give poor precipitation, if any, in their first passage (Table 2). This lack of reactivity seems most pronounced in aging cultures (dilutions 10^{-3} and 10^{-4}).

From Table 1 it appears that antisera produced with antigen grown in rabbit broth were superior to antisera produced with antigen grown in ordinary broth. This difference may be due to variations in the potency of the antisera or to unknown causes.

The serviceability of the test for identification of M. suip. has earlier been demonstrated by *Goiš & Kuksa* (1975) who identified 20 strains and found them all unrelated to Mycoplasma hyorhinis. The study did not include M. floc.

In the present study the incubation time necessary for precipitates to develop was found relatively long for many strains of M. floc. This phenomenon possibly reflects the extremely slow growth which is characteristic of M. floc., even as compared to M. suip.

Although some of the adapted strains showed a low degree of cross-reaction towards the end of the observation period, this does not necessarily indicate a relation at species level, since as discussed by *Freundt* (1974) such a phenomenon is well known to occur between other species of mycoplasma. On the other hand, the cross-reactions noted most likely do represent an antigenic relation, since in work with the double immunodiffusion test strains of M. suip. and M. floc. have been found to cross-react substantially (*Friis*, unpublished).

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SAMMENDRAG

Mycoplasma suipneumoniae og Mycoplasma flocculare sammenlignet ved vækstpræcipitationsmetoden.

Et antal laboratorieadapterede, såvel som nyisolerede stammer af Mycoplasma suipneumoniae og Mycoplasma flocculare er blevet underkastet en sammenlignende undersøgelse ved hjælp af vækstpræcipitationsmetoden. Samtlige stammer fandtes at kunne identificeres ved metoden, men for nogle laboratoriestammers vedkommende påvistes dog en lavgradig krydsreaktion sent i observationsperioden. Selvom nogle af de nyisolerede stammer var i stand til at danne præcipitater allerede efter isolation fra vævssuspensionen, fandtes det dog nødvendigt at foretage yderligere 1 eller 2 passager før regelret identifikation kunne iagttages for samtlige stammers vedkommende.

Metoden synes herefter anbefalelsesværdig til identifikationsformål, såvel hvad angår M. suipneumoniae som M. flocculare.

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