

From the Institute of Virology, Royal Veterinary College, and  
the National Veterinary Institute, Stockholm, Sweden.

EVALUATION OF SOME SEROLOGICAL  
TECHNIQUES FOR THE IDENTIFICATION  
OF MYCOPLASMA HYORHINIS  
AND MYCOPLASMA SUIPNEUMONIAE

By

*Rolf Jansson*

JANSSON, ROLF: *Evaluation of some serological techniques for the identification of Mycoplasma hyorhinis and Mycoplasma suis pneumoniae.* Acta vet. scand. 1974, 15, 274—282. — Eighteen strains of Mycoplasma hyorhinis and a strain of Mycoplasma suis pneumoniae were tested in 4 serological tests, i. e., disc growth inhibition, metabolic inhibition, indirect haemagglutination and indirect epi-immunofluorescence. Only with immunofluorescence could all tested strains of M. hyorhinis be shown; no cross-reactions between M. hyorhinis and M. suis pneumoniae could be detected. The other tests failed in many cases to identify strains of the same species, and they gave cross-reactions between M. hyorhinis and M. suis pneumoniae.

porcine mycoplasmas; serological tests.

Several different serological techniques have been employed for the identification of M. hyorhinis and M. suis pneumoniae, but they have not been evaluated in a comparative way, considering both these porcine species. In the present study the disc growth inhibition, metabolic inhibition, indirect haemagglutination, and epi-immunofluorescence have been compared using various strains of M. hyorhinis and a strain of M. suis pneumoniae. Although these tests are all based on reactions between antibodies and mycoplasmal surface antigens, the results of the present study demonstrate the superiority of epi-immunofluorescence over the other techniques employed.

## MATERIALS AND METHODS

*Mycoplasma strains.* The strains of *Mycoplasma hyorhinis* and the *M. suis* pneumoniae strain were kindly supplied by Z. Dinter of this Institute (F, FD, GDL, S, 200, K, S7, SK, G, W, and J), K. Bakos, National Veterinary Institute, Stockholm (KR and 378), N. F. Friis, State Veterinary Serum Laboratory, Copenhagen (201, 203, 872, and 859), T. Estola, State Veterinary Medical Institute, Helsinki (959), and J. G. Tully, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland (BTS-7). Dinter's strains were all "old" strains, passaged many times on culture media. The strains of Bakos, Friis, and Estola were all isolates with a low number of passages before testing. Stock cultures of all strains in liquid medium were stored at  $-20^{\circ}\text{C}$ .

*Mycoplasma media.* The liquid medium consisted of beef-heart infusion, 40 % (v/v), Hanks' balanced salt solution with 0.2 % (w/v) lactalbumin hydrolysate and 0.002 % (w/v) phenol red, 20 % (v/v) unheated horse serum, 2.5 % (v/v) extract of baker's yeast, benzyl penicillin (100 i. u./ml), and 0.05 % (w/v) thallium acetate; the pH was adjusted to 7.8 with N-NaOH. The agar medium was composed in the same way except that phenol red was omitted and 1 % (w/v) of Noble agar (Difco) was added.

*Preparation of antisera.* Rabbits were inoculated with mycoplasmas grown in liquid medium. The cultures were incubated for 3 to 4 days at  $37^{\circ}\text{C}$ , and the organisms were harvested by centrifugation for 1 hr. at  $31,000 \times g$  and  $4^{\circ}\text{C}$ . The sediment was then washed 5 times in isotonic saline. The washed sediment was resuspended in isotonic saline 1:200 of the volume of liquid medium, and the suspension was frozen and thawed from  $-20^{\circ}\text{C}$  to  $20^{\circ}\text{C}$  8 times. The suspension was then emulgated with an equal amount of Freund's complete adjuvant (Difco). Each rabbit was given 1 ml i. m. of the mycoplasma-adjuvant mixture and 1 ml i. p. for 2 subsequent days and 1.5 ml i. m. for 3 further days. After 6 weeks the rabbit was given 1.5 ml i. m. and serum was collected 1 week later. The sera were stored at  $-20^{\circ}\text{C}$ . Reference antiserum to the BTS-7 strain of *M. hyorhinis*, produced in the donkey, was kindly supplied by J. G. Tully, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

*Adsorption of antisera.* All antisera were adsorbed by concentrated mycoplasma medium in order to remove antibodies to this medium. The mycoplasma medium was concentrated 10 times by dialysis with polyethylene-glykol in a dialysis tube. Equal amounts of concentrated mycoplasma medium and antiserum were mixed and kept at  $4^{\circ}\text{C}$  for 18 hrs. The sediment was removed by centrifugation for 30 min. at  $4500 \times g$  and  $4^{\circ}\text{C}$ . The clear supernatant was then concentrated to the original volume by dialysis.

*Check for specificity of antisera.* The adsorbed antisera were tested by gel diffusion with concentrated mycoplasma medium as antigen; the antisera did not react with this antigen. The test was carried out as previously described by Dinter *et al.* (1965).

*Disc growth inhibition.* The technique used in this test has previously been described by *Clyde* (1964), but included the details described by *Dinter & Taylor-Robinson* (1969).

*Metabolic inhibition.* This test was carried out with sterile microtitre plates as described by *Taylor-Robinson et al.* (1966).

*Indirect haemagglutination.* The technique used was that of *Krogsgaard-Jensen* (1971) with some modifications. The mycoplasmas were grown in the mycoplasma medium as described above, and the antisera were the same as those used in the other tests. The supernatant of the sonicated mycoplasmas was used as antigen, and the tests were carried out with fresh, tanned sheep erythrocytes. The buffer used was phosphate-buffered saline.

*Indirect epi-immunofluorescence.* This test was carried out as described by *Rosendal & Black* (1972) on unfixed agar colonies by indirect immunofluorescence. Eight agar blocks were incubated with antiserum in dilutions of 1:80 to 1:2560; a non-immune rabbit serum and phosphate-buffered saline were used as controls. A commercial horse anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate (Central Laboratorium van de Bloed transfusedienst van het Nederlandsche Roode Kruis) diluted 1:10 was used. The microscope was a Leitz Ortholux with lamp housing 250 equipped with a Philips lamp type CS 15014 and a Ploem illuminator system.

## RESULTS

### *Sensitivity of mycoplasma strains to antisera in disc growth inhibition test*

All mycoplasma strains were tested against antisera to 11 strains of *M. hyorhinis* (BTS-7, F, FD, GDL, S, 200, K, S7, SK, G, and W) and the J strain of *M. suis*pneumoniae. In the disc growth inhibition test the inhibition pattern was very divergent (Table 1). Antiserum against the *M. hyorhinis* strain BTS-7 inhibited the growth of all strains except the W strain. This strain was insensitive and was only inhibited by its own antiserum. Another extremely insensitive strain was the G strain which was only inhibited by the BTS-7 antiserum. The most sensitive strain was the KR strain which was the only one inhibited by all 11 antisera against *M. hyorhinis* strains. Antisera to the *M. hyorhinis* strains S, 200, SK, and G inhibited the smallest number of strains.

Between *M. suis*pneumoniae strain J and *M. hyorhinis* there were cross-reactions in as much as the J strain was inhibited by antisera against the *M. hyorhinis* strains F, K, 200, S7, SK, and W. On the other hand, no *M. hyorhinis* strain was inhibited by antiserum to the J strain.

Table 1. Sensitivity of *Mycoplasma hyorhinis* and *Mycoplasma suis* pneumoniae to inhibition by antiserum discs.

Strain	Zones of inhibition (mm)* with antisera to											
	BTS-7**	F	FD	GDL	S	200	K	S7	SK	G	W	J
<i>M. hyorhinis</i>												
BTS-7	2—3	3	(3)	3	3	0	0	2—3	0	(2)	3	0
F	3.5	4	0	3	4	2—3	3	3	(4)	0	4	0
FD	(2)	0	3	0	(3)	0	3	(3)	0	0	(4)	0
GDL	4	0	0	1.5	(3)	(3)	2	0	(4)	0	3	0
S	(2)	2	3	3	3	0	0	3	0	0	4	0
200	3	4	2—3	2	0	2—3	0	3	0	0	2	0
K	2.5	3	(2)	0	0	(2.5)	2.5	0	0	0	2—3	0
S7	3	(3.5)	3	0	0	0	2—3	(3.5)	0	0	3	0
SK	3	0	0	3	0	0	0	0	2	3	3	0
G	1.5	0	0	0	0	0	0	0	0	0	0	0
W	0	0	0	0	0	0	0	0	0	0	(2)	0
KR	3	4	4—5	3	6	7	3	5—6	4	2	3—4	0
872	3	4	4	4	0	2	2—3	5	3—4	3	6	0
378	(2)	(2)	2	(2—3)	0	0	2	2—3	2—3	2	2	0
859	2	3	1—2	0	2	3	2—3	2	2	0	0	0
959	(2)	1—2	0	2	(2)	0	2—3	1—2	0	3	2—3	0
201	3—4	3	2	(2)	0	0	2—3	0	0	0	3	0
203	2—3	0	2	0	0	0	0	0	(2)	0	0	0
<i>M. suis</i> pneumoniae J												
	0	2	0	0	0	2	3	1—2	3	0	(2)	2

\* Zones less than 1 mm in diameter are indicated with 0.

\*\* Reference serum.

Figures in brackets indicate solitary colonies of mycoplasmas in the zone of inhibition.

*Metabolic inhibition test (MIT)*

All strains were tested against antisera to *M. hyorhinis* strains GDL, S, 200, K, G, and W, and *M. suis* pneumoniae strain J (Table 2). In disc growth inhibition test, antisera GDL, K, and W inhibited a large number of strains whereas antisera S, 200, and G inhibited a small number of strains. When the antisera were tested in MIT, 5 strains (SK, 872, 378, 859, and 203) were not inhibited by antiserum 200, whereas 1 strain (959) was inhibited, but not by all antisera and even then only at a low serum dilution. There was a difference between titres obtained with the "old" strains and the "fresh" isolates (Table 2). The "old" strains reacted with nearly all the antisera at high serum dilutions whereas the "fresh" isolates reacted mainly at low serum

Table 2. Sensitivity of *Mycoplasma hyorhinis* and *Mycoplasma suis-pneumoniae* to rabbit antisera in the metabolic inhibition test.

Strain*	Reciprocal titres of rabbit antisera to						
	GDL	S	200	K	G	W	J
<i>M. hyorhinis</i>							
F	1280	1280	2560	2560	640	1280	<10
FD	640	1280	160	1280	320	2560	<10
GDL	1280	1280	320	640	640	2560	<10
S	1280	1280	1280	1280	320	2560	<10
200	20	2560	320	2560	320	2560	<10
K	640	2560	1280	1280	160	2560	<10
S7	1280	2560	160	2560	640	1280	<10
SK	160	1280	<10	640	2560	320	<10
G	640	1280	320	640	640	2560	<10
W	2560	2560	1280	2560	1280	2560	<10
KR	20	320	80	160	20	640	<10
872	80	1280	<10	1280	40	80	<10
378	640	40	<10	10	320	160	<10
859	20	640	<10	640	20	80	<10
959	20	<10	10	80	10	80	<10
201	2560	80	40	640	80	1280	<10
203	40	1280	<10	640	20	320	<10
<i>M. suis-pneumoniae</i> J	<10	<10	<10	<10	<10	<10	20

\* *M. hyorhinis* strains F through W are all "old" strains, whereas all the other strains are "fresh" isolates (see Materials and Methods).

dilutions. *M. suis-pneumoniae* was not inhibited by any of the *M. hyorhinis* antisera, and the *M. suis-pneumoniae* antiserum did not inhibit any of the tested *M. hyorhinis* strains.

#### *Indirect haemagglutination (IHA)*

Seventeen strains of *M. hyorhinis* and a strain of *M. suis-pneumoniae* were tested in IHA to 10 *M. hyorhinis* antisera and 1 *M. suis-pneumoniae* antiserum (Table 3). The antiserum to *M. hyorhinis* strain GDL did not give positive reactions with 4 other strains of the same species (F, FD, G, and W), nor the antiserum to the S7 strain with the FD and W strains. The haemagglutination by the K and 378 strains was observed only at low serum dilutions throughout. *M. suis-pneumoniae* gave either no haemagglutination or only reactions with *M. hyorhinis* antisera at very low dilutions.

Table 3. Cross-reactions in indirect haemagglutination between *Mycoplasma hyorhinis* strains and *Mycoplasma suis pneumoniae*.

Strain	Reciprocal titres of rabbit antisera to										
	F	FD	GDL	S	200	K	S7	SK	G	W	J
<i>M. hyorhinis</i>											
F	2048	1024	<2	32	16	32	64	128	64	128	<2
FD	2048	2048	<2	16	32	32	<2	256	64	128	<2
GDL	512	256	64	256	64	512	128	128	128	256	<2
S	512	128	128	256	128	512	256	64	128	512	2
200	512	1024	64	512	512	512	512	256	512	512	<2
K	128	64	64	64	64	64	64	64	32	128	<2
S7	512	256	32	256	16	128	128	32	128	1024	<2
SK	2048	1024	32	128	256	32	64	512	128	512	<2
G	512	256	<2	32	32	128	32	32	32	64	<2
W	4096	32	<2	32	16	64	<2	64	64	128	<2
KR	256	1024	128	256	1024	512	256	512	256	1024	8
872	256	256	128	512	256	512	512	256	256	2048	4
378	128	256	64	64	64	128	128	64	64	128	8
859	256	512	512	512	256	512	512	1024	256	1024	4
959	512	1024	128	512	1024	512	512	256	256	1024	4
201	256	512	128	256	512	512	256	256	256	1024	<2
203	128	256	256	256	256	128	128	128	128	512	2
<i>M. suis pneumoniae</i> J											
	<2	<2	<2	<2	<2	4	<2	2	<2	<2	64

*Indirect epi-immunofluorescence (IF)*

Eighteen strains of *M. hyorhinis* and a strain of *M. suis pneumoniae* were tested in IF with 10 *M. hyorhinis* antisera and the *M. suis pneumoniae* antiserum (Table 4). All the *M. hyorhinis* strains gave strong immunofluorescence to a homologous antiserum in a high dilution, but did not react in any case with the *M. suis pneumoniae* antiserum. *M. suis pneumoniae* gave only IF with antiserum to the homologous strain. The IF was the test that gave the most homogenous results. The difference in titres obtained is partly due to dissimilarities in size and structure of the mycoplasma colonies.

## DISCUSSION

In this study serological differentiation between *M. hyorhinis* and *M. suis pneumoniae* was carried out by disc growth inhibition, metabolic inhibition, indirect haemagglutination and in-

Table 4. Antigenic relationship of *Mycoplasma hyorhinis* and *Mycoplasma suipneumoniae* in the indirect epi-immunofluorescence technique.

Strain	Reciprocal titres of rabbit antisera to										
	F	FD	GDL	S	200	K	S7	SK	G	W	J
<i>M. hyorhinis</i>											
BTS-7	1280	2560	1280	1280	1280	640	2560	1280	640	1280	<80
F	1280	640	160	320	320	1280	2560	320	640	1280	<80
FD	640	640	160	640	640	640	640	640	320	640	<80
GDL	1280	1280	320	320	320	1280	320	640	640	640	<80
S	1280	1280	320	1280	320	1280	2560	320	1280	1280	<80
200	2560	2560	1280	1280	1280	1280	1280	1280	1280	1280	<80
K	320	320	320	160	160	640	2560	320	320	640	<80
S7	320	640	640	640	1280	1280	1280	320	640	640	<80
SK	1280	1280	1280	2560	1280	1280	2560	1280	1280	640	<80
G	1280	640	320	640	320	1280	640	1280	640	1280	<80
W	1280	2560	1280	2560	1280	1280	1280	1280	1280	1280	<80
KR	640	1280	1280	1280	1280	640	1280	1280	160	1280	<80
872	1280	1280	1280	1280	1280	1280	640	640	1280	1280	<80>
378	1280	1280	1280	1280	640	1280	2560	640	1280	2560	<80>
859	1280	1280	1280	1280	1280	1280	1280	1280	640	1280	<80>
959	640	640	320	640	1280	1280	1280	640	640	1280	<80>
201	640	1280	1280	1280	640	1280	1280	640	640	1280	<80>
203	1280	1280	320	1280	640	320	640	320	320	640	<80>
<i>M. sui-</i>											
<i>pneumoniae</i> J	<80	<80	<80	<80	<80	<80	<80	<80	<80	<80	320

direct epi-immunofluorescence. Only in disc growth inhibition was the growth of *M. suipneumoniae* inhibited by some *M. hyorhinis* antisera (Table 1). However, it is impossible to say why such cross-reactions occurred.

Disc growth inhibition showed the most irregular pattern of all the tests. *Dinter & Taylor-Robinson* (1969) were able to divide 9 strains of *M. hyorhinis* into sensitive and non-sensitive groups based on the inhibition of growth by antisera. However, it became obvious that such a grouping is not consistently reproducible, i. e., when the very same strains of *M. hyorhinis* were tested on different occasions (cf. the present results with those of *Dinter et al.* 1965, and *Dinter & Taylor-Robinson*). The inhibition of growth results from the combination of antibody with specific reactive sites of the cell surface. Consequently, the inconsistent results of disc growth inhibition might be an expression of fluctuating changes in the composition of the myco-

plasmal membrane, changes probably induced by different growing conditions on non-synthetic media. It must be admitted, however, that also in our tests 2 strains, G and W, were remarkably resistant to inhibition while 2 other strains, KR and 872, were remarkably sensitive (Table 1).

In a study by *Roberts & Pijoan* (1971), it was found that disc growth inhibition could be used for testing both laboratory strains and recent isolates of *M. hyorhinis* by using fresh horse serum instead of commercial horse serum. By using their method no changes in the irregular pattern of growth inhibition could be obtained. It is therefore concluded that disc growth inhibition is an unsuitable test for typing *M. hyorhinis* and for differentiating that species from *M. suis pneumoniae*.

Both in the metabolic inhibition test (MIT) and the indirect haemagglutination test, some strains of *M. hyorhinis* failed to react with antisera to some other strains. This problem might be solved by using an antiserum which has given strong reactions with all the tested strains, but there is no evidence that such an antiserum would react with all *M. hyorhinis* strains on every occasion. It is worth mentioning that in MIT the "old" strains were much more sensitive to antisera than the "fresh" isolates, but the nature of this discrepancy cannot be explained. In short, the MIT and the indirect haemagglutination test are not suitable for the differentiation of *M. hyorhinis* from *M. suis pneumoniae*.

The indirect epi-immunofluorescence would appear to be the most suitable method for typing porcine mycoplasmas, because it gives distinct titres and no reactions which are difficult to interpret.

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#### SAMMANFATTNING

*Utvärdering av några serologiska metoder för identifiering av Mycoplasma hyorhinis och Mycoplasma suis pneumoniae.*

Aderton stammar av *Mycoplasma hyorhinis* och en stam av *Mycoplasma suis pneumoniae* har testats med följande serologiska metoder: växtinhibition på fast substrat, växtinhibition i flytande substrat, indirekt hemagglutination och indirekt epi-immunofluorescens. Endast med epi-immunofluorescens var det möjligt att klassificera alla undersökta stammar med avseende på art. De övriga metoderna visade sig vara mindre pålitliga eftersom reaktionen mellan testsera och vissa stammar var svag eller negativ. Dessutom förekom korsreaktioner mellan *M. suis pneumoniae* och vissa stammar av *M. hyorhinis*.

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Reprints may be requested from: Rolf Jansson, National Veterinary Institute, S-104 05 Stockholm 50, Sweden.