The passive haemagglutination test for the detection of *Mycoplasma suipneumoniae* and the possible diagnosis of enzootic pneumonia of pigs

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SUMMARY

Fourteen cases of enzootic pneumonia, nearly all of which had presented diagnostic difficulties using the metabolic-inhibition test, were re-examined using specific pig antisera in the passive haemagglutination test (PHA). All proved positive for *Mycoplasma suipneumoniae*, indicating that the test, used in this manner, might be particularly valuable for routine diagnosis.

The PHA test was also used to demonstrate antibody to *M. suipneumoniae* in pneumonic tissue and the associated bronchial lymph nodes.

To allay our concern that cross-reactions might interfere with this and other serological tests—the complement-fixation test (CF) and precipitation in agar-gel—the specificity of our reagents and the antigenic relationships of *Mycoplasma hyorhinis*, *Mycoplasma granularum*, mycoplasma B 3, *Mycoplasma hyopneumoniae* and three strains of *M. suipneumoniae* (including cloned and uncloned isolates of the J strain) were studied in various ways. Antibodies to medium constituents occurred in rabbit antiserum but did not present a problem with pig antisera. These antibodies were successfully absorbed from the rabbit antisera but it was not possible to remove medium constituents from the antigens used to produce antisera in rabbits by repeated washing.

By all these tests, the main species of mycoplasmas studied seemed to be antigenically distinct. No major antigenic differences between the three strains of *M. suipneumoniae* were revealed by the PHA test and the CF test; a slight difference in the precipitation lines of one of these strains (MG) in agar-gel might have indicated an antigenic variation or been a measure of some other factor.

INTRODUCTION

Goodwin, Pomeroy & Whittlestone (1965, 1967) induced enzootic pneumonia of pigs with a mycoplasma, which they named *Mycoplasma suipneumoniae*. In the field, other infective agents may frequently be isolated from lungs affected with enzootic pneumonia but, as respiratory diseases often become complicated in this way in many species, this does not invalidate the proposition that *M. suipneumoniae* is the primary cause of enzootic pneumonia of pigs. The most direct way of diagnosing the disease, therefore, is to isolate and identify the causal agent.

Goodwin, Pomeroy & Whittlestone (1968), using liquid medium and the metabolic-inhibition test, were able to isolate *M. suipneumoniae* from 91 % of

a series of experimental cases; with the same techniques, however, they recovered *M. suipneumoniae* from only 13 % of field cases, and from only 18 % of field outbreaks when two specially selected cases were examined from each outbreak. Subsequently, Goodwin & Hurrell (1970) improved these figures to 45 and 75 %, respectively, and developed selective media that could well allow even higher isolation rates when tested over a comparable series of field cases. Nevertheless, several technical difficulties remained: there were problems in producing good antisera in both rabbits and pigs, and there were considerable, unpredictable variations in the ability of different batches of medium to support the relative growths of *M. suipneumoniae* and *Mycoplasma hyorhinis*. It was still uncertain, therefore, whether a stage had been reached where most cases of enzootic pneumonia might be diagnosed routinely by cultural examination in liquid medium with the aid of the metabolic-inhibition test. An alternative approach to the problem using the passive haemagglutination test was therefore investigated, and the results of this study are now described.

MATERIALS AND METHODS
Preparation of antigens and antisera

Antigens were grown in liquid medium and concentrated as described earlier (Goodwin, Hodgson, Whittlestone & Woodhams, 1969*b*); they were finally resuspended in one-hundredth of their original volume, thiomersal was added (1/10,000), they were ultrasonically disintegrated for 1 min. and then stored at −20° C. Whether they were cloned or uncloned is indicated, where appropriate, in the Tables.

The history of the J strain of *M. suipneumoniae* has been summarized elsewhere (Goodwin & Whittlestone, 1963). The CZ strain of enzootic pneumonia (Goodwin *et al.* 1968) derived from a sudden field outbreak in a previously unaffected herd. The MG strain of enzootic pneumonia (R. F. W. Goodwin & P. Whittlestone, unpublished) was obtained in similar circumstances, except that the outbreak was clinically atypical, in that all the adult stock remained unaffected. This adult stock, when taken to another farm, was used to establish a herd which has never shown any signs of enzootic pneumonia over a period of several years (R. F. W. Goodwin, unpublished). A problem remains with the nomenclature of *Mycoplasma hyopneumoniae* (Goodwin *et al.* 1968): the culture given that name in this publication derived from pneumonia-inducing fluids and not from the colonies on solid medium that were so named by Maré & Switzer (1965). The 603 strain of *M. hyorhinis* (Goodwin *et al.* 1967) was used throughout. The sources of *Mycoplasma granularum* and mycoplasma B3, and the rabbit antisera R2 and R7 were as before (Goodwin *et al.* 1967). The other rabbit sera were prepared in the same general way.

The pig antisera P3018 and P3065 against *M. suipneumoniae* were from animals described by Goodwin *et al.* (1969*b*). The other two pig antisera (P3093, P3094) were obtained from animals inoculated with *M. hyorhinis* (Goodwin & Hurrell, 1970).

Serum samples were stored at about −20° C.

<center>*Serological tests*</center>

Passive haemagglutination (*PHA*)

This test was performed as previously described (Goodwin, Hodgson, Whittlestone & Woodhams, 1969a), except for the following two modifications. After trying different concentrations of tannic acid, it was found that a concentration of 1/50,000 (final concentration 1/100,000 when mixed with the suspension of sheep red cells) gave a slightly superior final suspension. Secondly, as a result of trying different times, it was found that a better antigen was obtained if the tanned red cells were sensitized for 30 min. at 37° C.

Pig antisera P 3065 and P 3094 were used to obtain all the results in Table 1.

Experiments on the specificity of this test have been described elsewhere (Goodwin *et al.* 1969a).

Complement fixation (*CF*)

This test was performed as previously described (Goodwin *et al.* 1969b), except that two units of complement were used and, with rabbit antisera, fixation was at 4° C. for 3 hr., instead of overnight. All rabbit antisera were treated overnight with an equal volume of normal pig serum (inactivated at 56° C. for 30 min.) and then centrifuged.

Precipitation in agar-gel

The double-diffusion method of Ouchterlony (1964) was used. The pig antisera used were as for the PHA test.

<center>*Isolation of mycoplasmas from pneumonic tissue and preparation of antigen*</center>

Mycoplasmas were isolated from pneumonic tissue in standard liquid medium (Goodwin *et al.* 1969a) as previously described (Goodwin & Hurrell, 1970). The field cases examined were from the series studied by Goodwin & Hurrell (1970). If growth was obtained, as judged by the production of acid, the culture was passaged into a larger volume (100–200 ml.) of the same medium, and the eventual antigen was harvested as previously described (Goodwin *et al.* 1969b). After being resuspended in phosphate-buffered saline (PBS) with 1/10,000 thiomersal, it was ultrasonically disintegrated for 30 sec. and stored at $-20°$ C. until required.

<center>*Extraction of antibody from pneumonic tissue and lymph node*</center>

About 25–30 g. of wet pneumonic tissue was cut up finely with scissors and freeze dried. It was then ground with a pestle and mortar, although this proved rather difficult, owing to its spongy nature, and extracted with PBS, pH 8·4. After centrifugation, the supernatant fluid was dialysed against 20,000 polyethylene glycol, dialysed against saline, and adjusted to an appropriate volume.

The lymph-node extract was prepared in the same way, but from much less tissue.

RESULTS

Detection of M. suipneumoniae *and* M. hyorhinis *in pneumonic tissue*

Goodwin & Hurrell (1970) examined 45 cases of pneumonia that were believed to be enzootic pneumonia by inoculating liquid medium with dilutions of pneumonic tissue and subjecting any acid-producing agents to the metabolic-inhibition test. *M. suipneumoniae* was recovered from 19 cases; there was an atypical result, suggesting the presence of both *M. suipneumoniae* and *M. hyorhinis* in five cases; *M. hyorhinis* alone was recovered from nine cases; a doubtful result was obtained

Table 1. *Comparison of the metabolic-inhibition test, the passive haemagglutination test and precipitation in agar-gel for detecting* M. suipneumoniae *in field cases of enzootic pneumonia*

		PHA test		Precipitation in agar-gel	
Pig	Metabolic-inhibition test*	*M. sui- pneumoniae*	*M. hyo- rhinis*	*M. sui- pneumoniae*	*M. hyo- rhinis*
991	⎰*M. suipneumoniae* ⎱*M. hyorhinis*	+	+	−	+
998	⎰*M. suipneumoniae* ⎱*M. hyorhinis*	+	−	+	−
1022	*M. hyorhinis*	+	+	−	−
1026	*M. hyorhinis*	+	+	−	−
1030	*M. hyorhinis*	+	+	ND	+
1031	*M. hyorhinis*	+	+	−	+
1048	*M. hyorhinis*	+	+	−	+
1052	DR	+	+	−	+
1057	⎰*M. suipneumoniae* ⎱*M. hyorhinis*	+	+	−	+
1064	*M. hyorhinis*	+	+	−	−
1065	⎰*M. suipneumoniae* ⎱*M. hyorhinis*	+	+	−	−
1070	DR	+	+	−	+
1084	*M. suipneumoniae*	+	+	+	−
1085	*M. hyorhinis*	+	+	−	ND

* The results in this column are taken from Goodwin & Hurrell (1970), Table 1.

Note. The results bracketed together mean that both mycoplasmas seemed to be present in the metabolic-inhibition test, but a normal definite result was not obtained with either.

DR = doubtful result; ND = not done.

in five cases; and no result or no acid-producing agent was obtained with the remaining seven cases. One of the cases from which *M. suipneumoniae* had been identified, four of the five cases that had given a mixed result, seven of the cases that had yielded only *M. hyorhinis*, and two of the cases that had given a dubious result were examined in parallel by the PHA test and by precipitation in agar-gel. The results are shown in Table 1. The positive results in the PHA test had serum titres of 1/80 or above, whereas the corresponding titre in the single negative result was less than 1/10. In most cases, the serum was not fully titrated, the highest dilution usually being 1/320 or 1/640. However, as the

growth of the *M. hyorhinis* antigen appeared to be particularly good when the pneumonia from pig 1057 was being tested, the serum titration was taken to its limit in this case and the titre was about 1/82,000.

When the test was first performed on some of the cases listed, negative results were obtained for *M. suipneumoniae* with pigs 1022, 1030, 1048 and 1070. It was felt that this could be due to a particularly low yield of mycoplasma from the bulk culture, as growth occurred very slowly in the batch of liquid medium then being used to grow up the antigen from the primary isolation. A new batch of liquid medium was prepared, therefore, in which much better growth was obtained; the antigen prepared from this gave positive results in all four cases.

All the 14 cases in Table 1 gave a positive result for *M. suipneumoniae* in the PHA test. A positive result for *M. hyorhinis* was obtained by the same test in 13 out of the 14 cases.

As judged by precipitation in agar-gel, however, *M. suipneumoniae* was present in only 2 of the 13 cases so examined, and *M. hyorhinis* was present in only 7 of the 13 cases examined. It was concluded from this that the precipitation test was less sensitive than the PHA test, in that it probably required a greater yield of antigen for a positive result.

Detection of antibody to M. suipneumoniae *in lung tissue and bronchial lymph node*

These examinations are summarized in Table 2. The normal lung was obtained from an enzootic-pneumonia-free herd. Field case number 1 was of particular

Table 2. *Detection of antibodies to* M. suipneumoniae *and* M. hyorhinis
in tissue from the respiratory tract

Tissue examined		Antibody titre* against	
		M. suipneumoniae	*M. hyorhinis*
Normal lung		—	ND
Pneumonic tissue from field cases of enzootic pneumonia	1	640	< 4
	2	40,960	< 4
	3	> 640	< 4
	4	> 1280	< 4
	5	> 2560	< 4
Pneumonic tissue from experimental cases of enzootic pneumonia	A†	640	ND
	B‡	< 4	ND
Bronchial lymph nodes from Pig A		> 80	ND

* The figures shown are the reciprocals of the dilution.
ND = not done.
† Killed about 5 months after infection.
‡ Killed 20 days after infection.

interest: this active case of enzootic pneumonia had been repeatedly cultured by Goodwin & Hurrell (1970) in routine batches of liquid medium, and in three different types of selective medium, and had always yielded only *M. hyorhinis*

(case 1048 in Tables 1 and 6 of that publication and in Table 1 of this paper). By the PHA test, however, not only was *M. suipneumoniae* demonstrated, but also the antibody to this mycoplasma. Experimental case B was specially selected because it had been killed 20 days after infection, at a time when antibody to *M. suipneumoniae* could not be detected in its serum by the PHA test. It is of interest, therefore, that at this time antibody could not be detected in the lung either.

Possible antigenic relationships between various strains of porcine mycoplasmas

As a result of the work described above and previous studies in this laboratory (Goodwin *et al.* 1969*a*, *b*), we felt that we might be using serological methods, especially the PHA test, increasingly in the future in the diagnosis of enzootic pneumonia and for studying the epidemiology and immunology of this disease. If so, we were interested in the specificity of the test in relation to possible antigenic cross-reactions among porcine mycoplasmas. Taylor-Robinson, Somerson, Turner & Chanock (1963) had already pointed out the dangers of non-specific serological reactions when growth-medium constituents were common to the immunizing materials and the antigens used in serological tests. As it was difficult to remove contaminating growth constituents from suspensions of organisms used for immunization, these authors grew the different strains of mycoplasmas that were to be injected into rabbits or guinea-pigs in a rabbit or guinea-pig infusion medium, with corresponding rabbit or guinea-pig serum, respectively. We approached this problem another way, but likewise our main object was to satisfy ourselves that the rabbit and pig antisera used were specific.

Attempts to remove contaminating growth medium constituents by washing the antigen

A control antigen was prepared from uninoculated liquid medium and treated as described in the method for producing mycoplasma antigens. A rabbit (R 192) was injected with this control antigen, and the resulting antiserum—together with

Table 3. *Attempt to remove medium constituents by repeated washing of antigen*

Number of washings of antigen	Reciprocal of serum titre in PHA test	
	Serum R 192*	Serum P 3065†
3	10,240	5120
4	10,240	20,480
5	10,240	5120
6	10,240	10,240
7	5120	10,240
8	5120	20,480
9	2560	5120

* From a rabbit injected with medium constituents alone.
† From a pig injected with *M. suipneumoniae* antigen (J strain, uncloned) and challenged with the same strain.

Table 4. *Examination of rabbit and pig antisera using four strains of M. suipneumoniae and four other porcine mycoplasmas in the PHA test*

		Control*		M. suipneumoniae			M. hyo-pneumoniae (cloned)	M. hyorhinis (strain 603, cloned)
Serum	Organisms used to produce serum	Before absorption	After absorption	Strain J (uncloned)	Strain CZ (uncloned)	Strain MG (uncloned)		
R 2	M. suipneumoniae (strain J, uncloned)	81,920	< 20	20,480†	10,240	5120	20,480	< 20
R 7	M. suipneumoniae (strain J, cloned)	40,960	< 20	10,240	10,240	5120	20,480	< 20
R 360	M. suipneumoniae (strain J, uncloned)	ND	< 20	20,480	10,240	10,240	20,480	< 20
R 191	M. suipneumoniae (strain MG, uncloned)	10,240	< 20	2560	2560	5120	5120	< 20
R 211	M. hyopneumoniae (cloned)	20,480	< 40	5120	1280	1280	5120	20
R 11	M. hyorhinis (strain 603, cloned)	40,960	< 40	< 40	< 40	< 40	< 40	81,920
R 45	M. hyorhinis (strain 603, cloned)	20,480	< 20	< 20	< 20	< 20	< 20	20,480
R 140	M. granularum (cloned)	81,920	< 20	< 10	< 10	< 10	< 20	< 20
R 149	Mycoplasma B3 (cloned)	10,240	< 40	< 40	< 20	< 20	< 20	< 40
R 192	Control antigen*	40,960	< 20	2560	5120	2560	81,920	10,240
P 3018 }	M. suipneumoniae (strain J, uncloned)	< 20	—	20,480	10,240	10,240	10,240	< 20
P 3065 }		< 20	—	10,240	10,240	5120	10,240	< 20
P 3093 }	M. hyorhinis (strain 603, cloned)	ND	—	< 20	< 20	< 20	< 20	20,480
P 3094 }		< 20	—	< 10	< 10	< 10	< 20	20,480

Antigens used in PHA test

* Control antigen prepared from uninoculated liquid medium.

† Serum R 2 gave the same titre against the cloned, J-strain antigen.   ND = not done.

Note. All figures shown are the reciprocal of the serum titre.

pig serum P3065—was tested against an antigen made from a culture of *M. sui-pneumoniae* in the usual way with three washings. The antigen was then washed a further six times, and after each washing and resuspension a sample was taken. All these antigenic samples were then tested against the two sera in the PHA test, and the results are shown in Table 3. It can be seen that relatively little impression on the serum titres was made by repeated washing of the antigen.

During the course of this work, two useful observations were made. First, the control antigen, when resuspended, gave a considerable opacity reading with Brown's tubes. We therefore discarded such opacity measurements; for they seemed irrelevant to the amount of mycoplasma antigen obtained from inoculated cultures. Instead, as an arbitrary standard, all final antigenic suspensions were made up to one-hundredth of the volume of the original liquid-medium culture. Secondly, we observed that if the liquid medium were centrifuged before use, *M. suipneumoniae* grew just as well in the supernatant fluid, independently of the deposit. The deposit obtained from uninoculated medium could be a consequence of storing the medium frozen but we did not pursue this possibility experimentally at this stage.

Specificity of sera

Passive haemagglutination. Three of four pig antisera were screened in the PHA test against the above control antigen for antibodies to non-porcine growth-medium components. Negative results were obtained. The four sera were then tested against *M. hyorhinis* and four strains of *M. suipneumoniae* (Table 4).

The rabbit antisera were likewise tested against the control antigen, but they had substantial titres. The titre of the control antigen had previously been established by block titration against its homologous rabbit antiserum. Each rabbit antiserum was then absorbed by adding an equal volume of either normal pig serum or freeze-dried liquid medium, or both, and holding the mixtures at 4° C. for 24 hr. After centrifugation, the clear serum was removed and tested again. Reabsorption was carried out as found necessary. These antisera were then tested against the same mycoplasmas as the pig sera, and the results are also shown in Table 4.

Complement fixation. As a further check on possible cross-reactions between *M. suipneumoniae* and *M. hyorhinis*, some comparisons were next made using the CF test (Table 5).

Precipitation in agar-gel. Finally, the antigens prepared from the J, CZ and MG strains of *M. suipneumoniae*, together with the *M. hyopneumoniae* antigen, were diffused against a standard *M. suipneumoniae* antiserum. All the antigens, except for the MG strain, shared two or more precipitation bands with each other, but only one band with the strain-MG antigen. The antigen prepared from the MG strain of *M. suipneumoniae* consistently failed to produce a strong band of precipitate which formed close to the antigen well, but shared an inner band of precipitate with the other three strains.

Table 5. *Examination of rabbit and pig antisera using*
M. suipneumoniae *and* M. hyorhinis *in the CF test*

Serum	Organism used to produce serum	Antigens used in CF test		
		Control*	*M. suipneumoniae*	*M. hyorhinis*
R 363		ND	640†	320
R 363 (absorbed)	*M. suipneumoniae* (J strain, un-uncloned)	ND	640	< 20
R 360		1280	1280	640
R 360 (absorbed)		< 20	1280	< 20
R 100 (absorbed)	*M. hyorhinis* (strain 603, uncloned)	ND	< 20	5120
R 11‡ (absorbed)	*M. hyorhinis* (strain 603, cloned)	ND	80	2560
R 2‡ (absorbed)	*M. suipneumoniae* (J strain, uncloned)	ND	1280	80
P 3065		ND	640	< 20
P 3094	*M. hyorhinis* (strain 603, cloned)	ND	< 20	320

* Control antigen prepared from uninoculated liquid medium.
† All figures are the reciprocal of the serum titre.
‡ With each of these antisera, the titre of the serum control was 1/80.
ND = not done.

DISCUSSION

The difficulty hitherto in attempting to isolate *M. suipneumoniae* from pneumonic tissue has not primarily been an inability to culture acid-producing organisms in liquid medium. Goodwin *et al.* (1968) obtained such a pH change in 16 out of 23 cases examined (70 %), and Goodwin & Hurrell (1970) achieved a similar result with 40 out of 45 cases (89 %). The problem has arisen thereafter—in attempting to identify *M. suipneumoniae* by the metabolic-inhibition test, when more than one mycoplasma was probably present. One method of combating this difficulty is to suppress the interfering mycoplasma (usually *M. hyorhinis*), and this possibility was investigated by Goodwin & Hurrell (1970), but an alternative approach is to use a method that will recognize *M. suipneumoniae* antigen in the presence of *M. hyorhinis* antigen. The results presented here indicate that the PHA test was able to do this in a series of 14 field cases, nearly all of which had been specially selected for the difficulty they had already presented with the metabolic-inhibition test. A positive diagnosis was obtained in every case, and if these results can be confirmed on a larger number of unselected field cases it appears that cultural diagnosis could become a reliable routine procedure.

From our study of the specificity of this test and our observations on the purity of our diagnostic reagents, as judged by the CF test and precipitation in agar-gel

also, we concluded that the different mycoplasmas listed showed no major antigenic relationships, neither were any major antigenic differences noted between the different strains of *M. suipneumoniae*. A possible exception to this last statement arises from the fact that the MG strain of *M. suipneumoniae* behaved slightly differently in the precipitation test. At this stage, however, we have an open mind as to whether this represents a true antigenic difference or a variation associated with a possibly poorer yield of antigen.

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