Enzootic Pneumonia of Pigs: Complement-Fixation Tests for the Detection of Mycoplasma Antibodies in the Serum of Immunized Rabbits and Infected Swine

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SUMMARY

The direct, the modified direct and the indirect complement-fixation tests were investigated as methods for the detection of antibodies for the enzootic pneumonia mycoplasma and for Mycoplasma hyorhinis in the serum of infected pigs and of immunized rabbits.

Only the modified direct complement-fixation test in which the guinea-pig complement is supplemented with fresh, normal unheated calf serum was suitable for the detection of mycoplasma antibodies in sera of infected swine. Based on the close correlation between the production of typical lung lesions in experimentally infected pigs and the appearance of significant serum antibody titres, the modified direct complement-fixation test provides for the first time a sensitive, specific in vitro method for the detection of enzootic pneumonia in the live pig. This test also permitted the in vitro differentiation of the mycoplasma causing enzootic pneumonia from M. hyorhinis which causes polyserositis.

Antibodies in the sera of rabbits were demonstrable by the ordinary direct complement-fixation test. However, in contast to the observation made with swine sera, only a slight quantitative antigenic difference between the enzootic pneumonia mycoplasma and M. hyorhinis was seen when the tests were performed with rabbit serum antibodiies.

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RÉSUMÉ

Trois procédés de fixation du complément, l'épreuve directe, l'épreuve directe modifiée et l'épreuve indirecte furent essayés pour la mise en évidence des anticorps dans le sérum de porcs et de lapins respectivement infectés et immunisés avec la mycoplasma qui cause la pneumonie enzootique et avec M. hyorhinis.

L'épreuve directe modifiée de la fixation du complément dans laquelle le complément de cobaye est additionné de sérum frais, non-inactivé de veaux normaux, fut nécessaire pour démontrer les anticorps pour les mycoplasma dans le sérum de porcs infectés. La corrélation étroite entre les lésions d'hépatisation pulmonaire produites par l'infection expérimentale avec des cultures de mycoplasma et la montée des anticorps dans le sérum des mêmes porcs, nous permet de conclure que l'épreuve directe modifié de la fixation du complément nous fournit pour la première fois une méthode spécifique et sensible, pour déceler la pneumonie enzootique chez le porc vivant. Cette épreuve appliquée aux anticorps dans le sérum de porc, a permis de différencier les mycoplasma causant la pneumonie enzootique de M. hyorhinis l'agent de la polysérosite du porcelet.

Il fut possible de démontrer les anticorps dans les sérums de lapins par l'épreuve ordinaire de la fixation du complément. Toutefois avec les sérums de lapin, contrairement aux observations faites avec les sérums de porcs, seulement une légère différence quantitative à été démontrée entre les mycoplasma de la pneumonie enzootique et M. hyorhinis.

INTRODUCTION

Serological studies of enzootic pneumonia (EP) have been hampered by the inability to isolate the etiologic agent in culture and consequently to produce even partly purified antigens. Recent successes in propagating a new species of mycoplasma (9, 13, 14) from cases of EP and in demonstrating its primary etiologic significance have made possible the investigation of *in vitro* diagnostic tests and of methods for differentiating EP infection from that produced by other species of porcine mycoplasma. The broth medium described by L'Ecuyer (13) was found suitable for the production of mycoplasma organisms in relatively large quantities and in a sufficiently pure form to allow serological procedures to be developed.

The early, essentially negative, results of serological studies on EP and like conditions has been reviewed (12). Immunity of infection was demonstrated by Lannek and Bornfors (11) who could not by the intratracheal (IT) route reinfect pigs that had recovered from the disease while control pigs of the same age readily developed pneumonia. It has been shown that swine exposed by the subcutaneous (SC) and intramuscular (IM) routes were not protected when subsequently exposed IT (2). Bakos and Dinter (1) used either pneumonic lung suspensions or chicken embryo yolk-sac suspensions of a strain of the Swedish swine enzootic pneumonia (SEP) agent to hyperimunize rabbits and obtained low-titre antisera that neutralized the SEP agent in calf kidney cell cultures. Using the indirect method they could demonstrate specific fluorescence in infected calf kidney cell cultures. Precipitation was also obtained with these sera and suspensions of the SEP-agent grown on solid medium. However, these authors questioned the primary etiologic significance of the SEP-agent in swine pneumonia. Subsequently Dinter *et al.*, (7) using antisera prepared in rabbits, demonstrated by gel diffusion an antigenic relationship between their SEP isolates and the polyserositis producing species *Mycoplasma hyorhinis*. They could not confirm these results using the same reagents in growth-inhibition tests carried out on agar medium. Estola and Schulman (8) have shown by growth inhibition or serum neutralization in chicken embryos and cell cultures that a number of mycoplasma isolates from Finland were identical to the SEP-agent.

Goodwin *et. al.*, (10) have shown an antigenic relationship between their EP isolate *Mycoplasma suipneumoniae* and the one described by Maré and Switzer (14) *Mycoplasma hyopneumoniae*, using both growth inhibition on agar and metabolic inhibition in broth medium. Similar tests were used to show that *M. suipneumoniae* was not antigenically related: (1) to the other swine species (*M. hyorhinis* and *M. granularum*); or (2) to a variety of other strains of mammalian and avian origin.

Swine antiserum was not, until recently, considered useful in complement-fixation (CF) tests because of its recognized procomplementary properties. In 1963, Robertson *et. al.*, (16) reported that non-complement-fixing porcine serum antibodies could be detected by the indirect CF test, thus indicating that the procomplementary properties of pig serum were not the only cause of poor fixation. Boulanger *et. al.*, (5, 6) have used a modified-direct CF test, supplemented with normal, unheated bovine serum, to detect hog cholera and African swine fever serum antibodies.

The direct, indirect and modified-direct CF tests were studied to determine their usefulness in detecting serum antibodies in hyperimmunized rabbits and in swine experimentally infected with our EP strains of mycoplasma. Pigs infected with *M. hyorhinis* were included in this study for comparative purposes.

MATERIALS AND METHODS

MYCOPLASMA STRAINS

Enzootic pneumonia strains, 28, 29 and 33, and *M. hyorhinis* strain 31 as described by L'Ecuyer (13) were used in the course of this study. Strains 28, 29 and 33 were maintained either in the frozen state at -70°C or were passaged at 48-72 hour intervals in the liquid medium (Broth A) described by L'Ecuyer (13). *M. hyorhinis* strain 31 was also stored frozen or was propagated in a modification of the beef heart infusion (BHI) broth described by Ross & Switzer (17).

ANTIGEN PRODUCTION

The appropriate liquid medium was pre-

TABLE I Results of the Titration of EP Mycoplasma and M. hyorhinis Antigens by the Complement-Fixation Test Using an Excess of Rabbit Homologous and Heterologous Antisera.

Antigens[1]	Antisera		Antigen CF titres[2]
	Strain	Rabbit	
EP29-32-	EP29	297	640
" "	EP28	294	640
"29-27-	EP29	297	1600
" " "	EP28	294	1600
EP28-3-	EP28	294	320
" " "	EP29	297	640
EP33-33-	EP33	291	640
" " "	" "	292	640
" " "	" "	293	320
" " "	EP28	294	640
" 33-34-	EP33	292	640
" " "	EP28	294	640
" " "	" "	296	640
" " "	M.h. 31	300	640
M. hyorhinis	M.h. 31	300	5120
31-4-	EP28	294	2560
" "	" "	296	2560
" "	EP29	297	1600
" "	EP33	292	2560

1. Number of broth passages since initial isolation indicated between dashes.
2. Reciprocal of the final dilution of packed cells which fixed 1 unit of complement in the presence of an excess of immune rabbit serum.

pared as described (13) and dispensed in 500 ml. quantities. Each flask was inoculated with a 1:20 to 1:30 dilution of a 48 hour stationary culture of the desired mycoplasma strain. Shaker cultures were used (13) and were incubated for 72-96 hours. The mycoplasma cells were removed from the broth medium by centrifugation at 5400 G for 30 minutes in an angle-head centrifuge. The sediment was washed once by diluting at least 1:40 in normal saline and recentrifuged. The sediment was then resuspended in normal saline and centrifuged on a swing head, in Kolmer tubes at 2200 G for one hour or in ten ml. tubes in an angle head, as above. The yield of packed cells was then measured and the sediment resuspended 1:10 in normal saline. The diluted antigen was stored frozen at -20°C until used.

Source of animals

Adult healthy rabbits were used for immunization. Pigs were from a caesarean-derived minimal-disease swine herd maintained under isolation and shown by repeated clinical and pathological examinations to be free of respiratory diseases. The pigs were farrowed and reared normally.

Inoculation and bleeding procedures

The method used to infect pigs by the IT route with the various strains has been described (13). Rabbits were hyperimmunized by a series of ten daily intravenous (IV) inoculations of 1.2 ml. of mycoplasma antigen diluted 1:50 in saline. Where necessary, further IV inoculations were made.

Cardiac puncture was used to bleed all rabbits and small pigs. Large pigs which could not be held in a bleeding trough were bled either from the anterior vena cava or from an ear vein.

Several experiments were set up in which groups of pigs were experimentally infected and were subsequently bled at intervals, as indicated in Tables IV and V. All pigs were killed by exsanguination while under deep barbiturate narcosis.

Complement-fixation tests

The direct, the modified direct and the indirect CF tests were investigated. In these methods, the general procedure described by Wadsworth (18) for the titration of complement and amboceptor was followed closely. Unless otherwise indicated, each reagent employed in the test was added in 0.1 ml. amounts, giving in every case a final volume of 0.5 ml. The evaluation of the reaction in all methods was based on the 50% haemolytic unit, using 2.5% maximally sensitized sheep red blood cells. The density of the non-sensitized cell suspension was adjusted to a five per cent concentration by means of a photoelectric colorimeter. The reading of the degree of haemolysis was made with the aid of a color standard prepared with the day's reagents. All dilutions were made in veronal buffered saline containing Mg and Ca ions.

Direct CF test. Even though this method was investigated in the testing of swine sera, it was used mainly in the testing of rabbit sera. The normal and immune test sera, were heat inactivated for 30 minutes

TABLE II. Complement-Fixation Reactivity with Homologous and Heterologous Antigens of Sera from Rabbits Hyperimmunized with EP Mycoplasma and M. hyorhinis

Antiserum					
Strain	Rabbit	Bleeding days[1]	Booster[1]	Antigen[2]	Antiserum[3] CF titres
EP 28	294	25	—	EP29-32-	640
" "	295	25	—	EP29-32-	640
" "	296	47	39	EP29-32-	640
" "	296	47	39	EP28-3	640
RP-29	297	25	—	EP29-32-	640
" "	298	47	39	EP29-32-	1280
" "	298	47	39	EP28-3	1280
" "	299	47	39	EP29-32-	640
" "	299	47	39	EP28-3-	640
EP33	291	45	29	EP-33-34-	320
" "	291	45	29	M.h.31-4-	320
" "	292	45	29	EP33-34-	320
" "	292	45	29	M.h.31-4.	320
" "	293	45	29	EP33-34-	160
" "	293	45	29	M.h.31-4-	160
M. hyorhinis	300	32	16	M.h.31-4-	640
EP31	300	32	16	EP33-34-	80
"	301	32	16	M.h.31-4-	320
"	301	32	16	EP33-34-	40
"	699	32	16	M.h.31-4-	640
"	699	32	16	EP33-34	80

1. Number of days after the first antigen dose.
2. Number of broth passages indicated between dashes.
3. Reciprocal of the dilution of antiserum which fixed 1 unit of complement in the presence of at least two units of antigen.

at 60°C, on the day of the test. In the preliminary titration of the reagents, doubling dilutions of the antigen were tested in the presence of complement including an excess of normal or immune rabbit sera respectively. After the potency of an antigen was determined as above, at least two units of the antigen were used to titrate doubling dilutions of the sera in presence of complement. Four and a half, 50% haemolytic units of complement were used. The period of primary incubation for fixation was 18 hours at 9°C; that of secondary incubation after the addition of 0.2 ml. of a 2.5% suspension of maximally sensitized sheep red blood cells, was 30 minutes at 37°C.

Modified direct CF test. The technique (3, 4, 5, 6) was the same as for the direct method with the exception that the veronal buffer used in making the complement dilution contained one per cent pretested normal unheated bovine serum, as supplementing factor. It was impossible with the present antigen, to use a five per cent concentration of bovine serum for supplementing factor as previously employed with other antigens. Every bovine serum tested as source of supplementing factor reacted non-specifically with the antigen when used in a five per cent concentration but they satisfactorily supplemented the test in a one per cent concentration. The supplementing bovine serum should come preferably from a six month old calf (3), it should be stored frozen and be used within four weeks after collection.

Indirect CF test. The method employed was an adaptation of the one described by Rice (15). Dilutions of inactivated test sera were mixed in 0.1 ml. amounts with 0.05 ml. of the antigen and incubated at 9°C for 18 hours. The antigen was employed in the highest dilution of which 0.05 ml. had given complete fixation of four and a half, 50% haemolytic units of complement in the antigen titration. Following the first incubation, 0.1 ml. of a complement dilution containing 4.5 haemolytic units was added followed by 0.05 ml. of inactivated rabbit

TABLE III Behavior of Swine Serum Antibodies in Various Types of Complement-Fixation Test with Homologous Antigen.

Pig No. 2510	Complement-fixation tests		
	Direct	Modified	Indirect
Pre-infection	0	0	0
28 days post-inf.	0	80[1]	40
78 days post-inf.	Trace 20	320	40

1. Reciprocal of the dilution of antiserum which fixed 1 unit of complement in the presence of at least two units of antigen.

immune serum containing an excess of antibody. The tubes were shaken and the test incubated a further 18 hours at 9°C. Finally 0.2 ml. of a 2.5% suspension of maximally sensitized sheep red blood cells were added and the test was incubated at 37°C. in a water bath for 30 minutes. The serum titre was recorded as the highest dilution with which 50% haemolysis or more was obtained in the presence of antigen.

RESULTS

ANTIGEN PRODUCTION

The production of antigen for the serological tests was facilitated by the development of a satisfactory broth medium, described previously (13), for the growth of large quantities of organisms. The volume of packed, washed mycoplasma cells obtained varied between 1.4 ml. and three ml. per liter of medium. This variation seemed to depend at least in part, on the concentration and activity of the inoculum. An inoculum consisting of a 1:30 dilution of an active 48 hour stationary culture gave fairly consistent results. The incubation of the inoculated broth medium for 72 hours appeared to be optimum to provide a reasonable volume of active antigen.

Giemsa stained smears of satisfactory antigens contained small to medium-stained ring-formed organisms and a minimal amount of amorphous pink-stained precipitate. A few lots of antigen prepared with broth enriched with some pools of swine serum yielded growth containing an excessive amount of amorphous precipitate. These lots were as a rule of poor antigenic activity.

POTENCY OF ANTIGENS WITH IMMUNE RABBIT SERA

The potency of the various lots of antigen was determined by the direct CF test using an excess of pre- and post-immune rabbit sera. Anti-complemetary activity was found with some antigens particularly those of strain EP 28. A few lots of antigens were also non-reactive and invariably these were found to contain large amounts of amorphous precipitate derived from the culture medium. The potency of various antigens tested against rabbit immune sera is listed in Table I. Antigens prepared with strains EP 29 and EP 28 reacted to the same level with homologous and heterologous rabbit antisera indicating

TABLE IV Specificity of Swine Serum Antibodies for Enzootic Pneumonia or M. hyorhinis Antigens Using the Modified Direct Complement-Fixation Test

Antiserum			Antigen strain	Antiserum titre[1]
Infecting strain	Pig	Days PI		
EP29-24-	2503	0	EP29	0
,,	,,	7	,,	0
,,	,,	14	,,	10
,,	,,	21	,,	20
EP29-80-	2304	0	EP33	0
,,	,,	54	,,	20
,,	,,	130	,,	40
EP29-24-	2510	0	EP29	0
,,	,,	28	,,	80
,,	,,	78	,,	320
EP29-24-	2510	0	**M.h. 31**	0
,,	,,	28	,,	0
,,	,,	78	,,	0
M. hyorhinis	2505	0	**M.h. 31**	0
EP31-5-	,,	35	,,	160
,,	,,	68	,,	320
,,	2505	0	EP33	0
,,	,,	35	,,	0
,,	,,	68	,,	0
M. hyorhinis	2506	0	**M.h. 31**	0
EP31-5-	,,	35	,,	160
,,	,,	68	,,	640
,,	2506	0	EP33	0
,,	,,	35	,,	0
,,	,,	65	,,	0

1. Reciprocal of the dilution of antiserum which fixed 1 unit of complement in the presence of at last two units of antigen.

TABLE V. Pattern of Complement-Fixation Antibody Titres in the Sera of Pigs Experimentally Infected with Enzootic Pneumonia.

Pig No.	Strain	Route[1]	Days post-infection 0	7	14	21	28	35	42	49	56	78	119	142	175	203	232	267	Pneumonia[2]
1204	28	IT	0	0	10[3]	20	40	80	—[4]	160	80	—	—	—	—	—	—	—	++++
1205	"	"	0	0	0	10	20	80	—	80	—	—	—	—	—	—	—	—	+
1208	"	"	—	0	0	0	10	20	—	40	80	—	—	—	—	—	—	—	+
1201	"	CT	0	0	0	0	0	0	—	0	0	—	—	—	—	—	—	—	—
1202	"	"	0	0	10	20	20	40	—	80	80	—	—	—	—	—	—	—	+++
2401	29	IT	0	0	10	10	20	40	40	—	—	20	10	5	5	10	5	5	+++
2405	"	"	0	0	0	5	0	10	10	—	—	80	160	40	10	20	10	10	++++
2502	"	"	0	0	10	40	80	160	160	—	—	160	160	20	20	10	10	10	+
2508	"	"	0	0	0	10	10	40	80	—	—	160	80	20	40	20	20	20	+++
2509	"	"	0	0	5	40	80	160	160	—	—	160	80	40	40	40	40	20	+
2510	"	"	0	0	5	20	80	80	160	—	—	320	40	40	40	20	20	20	+

1. IT — intratracheal, CT — contact infection.
2. Severity of pneumonia graded from negative —, slight consolidation +, mild ++, moderate +++, severe ++++.
3. Reciprocal of the dilution of antiserum which fixed 1 unit of complement in the presence of at least two units of antigen.
4. — = not done

no antigenic difference. Likewise antigens prepared with strain EP 33 reacted to approximately the same degree with the rabbit immune sera prepared for strains EP 33, EP 28, and *M. hyorhinis* strain 31. Considering that *M. hyorhinis* strain 31 caused a different pathological picture in swine than that produced by the three enzootic pneumonia strains studied, it was not expected that cross reactivity would occur. However cross reactivity was also obtained when the *M. hyorhinis* strain 31 antigen was tested with the homologous rabbit serum and also with the three EP strains.

In general the potency of the various lots of antigen was such that the packed cell volume could be diluted between 320 and 5120 times and still fix complement in the presence of an excess of immune rabbit serum.

Cross reactivity of immune rabbit sera

As indicated in Table II, the antisera from rabbits immunized with EP strains 28, 29 and 33, gave equivalent titres when tested with a minimum of two units of antigens EP 28, 29 and 33 and *M. hyorhinis* strains 31 respectively. However the antisera of rabbits immunized with the *M. hyorhinis* strain had eightfold higher titres when tested with their homologous antigen than when tested with the heterologous EP 33 antigen. This suggested that the *M. hyorhinis* antigen contained one or more antigenic components not present in the three EP strains.

Behavior of swine serum antibodies in the CF test

No fixation or only trace reactions were demonstrable in the direct CF test of known immune swine sera with its homologous antigen, Table III. The same swine sera when tested by the modified direct CF test gave complete fixation in dilutions of 1:80 and 1:320 respectively. The swine serum antibodies were demonstrable also by the indirect CF test but the titres obtained were lower than in the modified direct test. Consequently the remaining tests with swine sera were performed with the modified direct CF test.

Specificity of Swine Serum Antibodies

The quantitative difference observed between the *M. hyorhinis* strain and strain EP 33 with rabbit antisera proved to be a complete antigenic difference when the tests were performed with swine antisera. In Table IV, sera from swine 2503, 2304 and 2510 infected with strain EP 29 contained antibodies detectable by antigens prepared with strains EP 29 and EP 33 but no reaction was detectable when the test was performed with antigen of *M. hyorhinis* strain 31. The absence of cross-reactivity between the *M. hyorhinis* strain and the EP 33 isolate was also complete with sera from pigs 2505-2506 infected with *M. hyorhinis* strain 31. As indicated in Table IV, the antibodies in their sera were demonstrable only with the homologous antigen.

Antibody Response of Experimentally Infected Pigs

As indicated in Table V at least 14 days elapsed between IT exposure and the appearance of detectable CF antibodies in the sera of swine. Titres of 1:160 were not reached until 35 days after exposure and in some instances titres of this magnitude were still present 119 days after exposure. Thereafter the serum antibody titres decreased gradually to reach a low but serologically significant level in five out of six pigs, 267 days after exposure.

At necropsy four of the five pigs killed at 56 days PI had active areas of consolidation graded from mild to severe and affecting the anteroventral portions of the lungs. The microscopic lesions observed were typical of those described for EP. The six pigs killed at 267 days PI had lung lesions characterized by moderate to severe scarring of the anteroventral portions with accompanying zones of atelectasis and some emphysema. Two of the six pigs had small areas of apparently active consolidation.

DISCUSSION

As was the case in previous studies (5, 6) with certain viral infections, the modified direct CF test which incorporates fresh, normal, unheated calf serum to supplement the guinea pig complement, was necessary to detect mycoplasma antibodies in the sera of infected swine. The procomplementary activity of swine sera, which once was thought entirely responsible for their poor fixability, is masked by the increased sensitivity of the modified test. The specificity of the modified direct CF test with swine sera permits the differentiation of the mycoplasma causing enzootic pneumonia from those causing polyserositis. In the sera of experimentally infected swine, the present method permits the detection of antibodies as early as 14 days after IT exposure and for at least 267 days after. This test provides us for the first time with a specific, and reliable diagnostic method in the live pig for the detection of infection or previous exposure to EP. On the basis of our results in experimentally infected pigs it is now possible to determine the status of an individual pig in relation to EP. In no case were antibodies detected in the sera of pigs prior to exposure or in pigs which failed to develop lesions following IT exposure and conversely antibodies were always detected in pigs showing lesions at necropsy. The test promises to be also a valuable indicator of the specificity and potency of immune swine sera to be used in immunofluorescence studies.

The modified direct CF test is not needed and should be avoided in the testing of rabbit serum antibodies. In the sera of this species the antibodies are detectable by the ordinary CF test. Even though a slight quantitative antigenic difference was observed between the enzootic pneumonia strains and *M. hyorhinis* when tested with rabbit serum antibodies, there is enough cross-reactivity to render this source of antibodies impractical for differentiating species. The results obtained in the CF test with rabbit serum confirm those of Dinter *et al* (7) who demonstrated by gel diffusion an antigenic relationship between their SEP isolates and the serositis producing agent *M. hyorhinis*. Like the growth-inhibition test performed by them the modified direct CF test with swine sera proved to be a realiable method to differentiate the two species of swine mycoplasma.

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