Diagnosis of mycoplasmal pneumonia of swine and control of the disease by farrowing seronegative sows

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INTRODUCTION

Mycoplasmal Pneumonia of Swine (MPS) or Enzootic Pneumonia of Pigs (EPP) is caused by Mycoplasma hyopneumoniae. The disease is characterised by a low grade cough, high morbidity, growth retardation and decreased efficiency of food utilization. Mycoplasmal pneumonia of swine has a world wide distribution, more especially in areas of intensive swine raising. It is believed that this respiratory disease is the single most economically important swine disease and 40 to 50% of all swine in the world are estimated to be affected (Switzer 1969). In the United States, economic loss due to this disease was estimated in 1976 to be over 180 million dollars annually (Slavik 1976a). The economic losses encountered are the result of general unthriftness, poor feed efficiency and stunted growth and not because of a high death rate.

The clinical appearance of the disease is similar in many respects to other respiratory diseases of pigs making differential diagnosis of respiratory infections of etiologies different from M. hyopneumoniae, difficult. Furthermore, primary M. hyopneumoniae pneumonia is complicated by secondary involvement with bacteria, notably Haemophilus parasuis, Bordetella bronchiseptica and Pasteurella multocida. Although Mycoplasma hyorhinis has been isolated frequently from cases of MPS, its role as a primary pulmonary pathogen is a debatable issue. The secondary bacterial involvement often exaggerates the clinical signs and mortality patterns. In addition,

bacterial infection has the tendency of superimposing different gross and histopathological changes on primary MPS, thus making diagnosis by cultural techniques, gross and histopathological evaluations very difficult and sometimes misleading.

M. hyopneumoniae pneumonia is exclusively a disease of swine. No other natural or experimental host has been demonstrated, and all breeds and ages of swine are affected. Transmission of the disease is by the inhalation of infective droplets by a susceptible pig. There is also a generation to generation transmission from an infected sow to its progeny. Attempts at controlling this disease have been centered on breaking the cycle of transmission. The occasional outbreaks of MPS in Specific Pathogen Free herds (SPF) point to the inadequacy of this approach. Chemotherapeutic and chemoprophylactic measures, although useful in suppressing the development of lesions, do not effectively clear the mycoplasmal organisms from the lungs, and therefore these measures do not break the cycle of transmission.

This study was essentially done in two parts. In the first part, current diagnostic techniques, macroscopic and histologic evaluation of pneumonic lung and serologic evaluation for antibodies to M. hyopneumoniae, were compared with a recently developed direct immunofluorescence procedure and culture of the organism for diagnosis of the disease. The second part involved the evaluation of a control program based on farrowing of sows which were negative for complement-fixing antibodies to M. hyopneumoniae, and monitoring their progeny clinically, serologically, and by gross and microscopic evaluation of lungs for evidence of disease.

LITERATURE REVIEW

History and Nomenclature

The unravelling of the complex nature of porcine respiratory disease problems began when Lewis and Shope (1931) demonstrated that Haemophilus influenza - suis and a virus were the etiologic agents of swine influenza. Köbe (1933) described "Ferkelgrippe" or piglet influenza in Germany. Lamont (1938) discussed piglet influenza in Ireland. The piglet influenza described by Köbe and Lamont had clinical, epidemiological and pathological features similar to enzootic pneumonia of pigs. Pullar (1948) described a widespread pneumonia of pigs in Australia of unknown etiology, but distinct from swine influenza. In 1951, Gulrajani and Beveridge reported a chronic pneumonia of swine in the United Kingdom (U.K.) which also differed markedly from swine influenza. Although they could not isolate the "virus" they were able to transfer the disease to susceptible pigs. They observed macroscopic and histologic lesions 12 days post inoculation. Their report constituted the first experimental reproduction of the disease. The similarity between the disease and that described by Pullar was recognized by these two workers, consequently they decided to call the disease "Infectious Pneumonia of Pigs". Betts (1952) suggested the name virus pneumonia of pigs or VPP for the disease described by Gulrajani and Beveridge as infectious pneumonia of pigs. His criteria for suggesting such a description of the disease was based on the fact that filtrates of lung suspensions which passed through bacteria retaining filters were infective for pigs. Secondly, the infectivity was unaffected by penicillin

or sulfonamides. Hjarre et al. (1952) recognized that pneumonia in Sweden was caused by an agent differing from swine influenza virus, and consequently suggested the name "Enzootic Virus Pneumonia of Pigs" for the disease. Plowright (1953) succeeded in transmitting the disease by intranasal and intratracheal inoculation of pneumonic lung suspensions sterilized by Seitz filtration.

L'Ecuyer (1962) and L'Ecuyer and Switzer (1963) elicited typical gross and microscopic lesions in respiratory disease-free pigs inoculated intranasally and intratracheally with culture fluids harvested after 3 serial passages in HeLa cells.

Goodwin and Whittlestone (1963), Betts and Whittlestone (1963) reported the successful propagation of the J strain of the agent of VPP in cell cultures of swine lung. Goodwin and Whittlestone (1964) produced pneumonia in pigs with microorganisms grown in cell-free medium. The broth cultures included an agent of VPP which was transmissible to other pigs. The dilution of their original pneumonic lung suspension was so high that it precluded the mechanical carry-over of virus from the original material. They proposed that the term virus pneumonia of pigs be dropped and suggested, based on the epidemiology of the disease, the term "Enzootic Pneumonia of Pigs" (EPP).

Up until this time, two lines of evidence suggested that the causal agent of "VPP" might be a nonviral agent. Betts and Campbell (1956) demonstrated that the establishment of VPP lesions could be

prevented by the prior administration of tetracylcines. Whittlestone (1957) showed that the size of the smallest infective particle, as judged by testing the infectivity for pigs of filtrates of pneumonic lung, was between 0.2 µm and 0.45 µm. He also observed the presence of a delicate pleomorhic organism with individual particles in the size range of 0.2 µm to 0.7 µm in Geimsa stained preparations of M. hyorhinis-free lungs.

Mare and Switzer (1965) isolated a small coccobacillary organism in the cell-free medium from lesions of experimentally transmitted "Virus Pneumonia of Pigs". After the 7th and 8th serial passages, the organism was capable of eliciting the characteristic gross and microscopic lesions and the agent was successfully reisolated in cell-free medium. They found colonies of this organism on agar to possess the characteristic morphology associated with Mycoplasma spp. The name Mycoplasma hyopneumoniae was proposed for this organism. Two names were suggested for the disease by these two workers namely, Pulmonary Mycoplasmosis of Swine and Mycoplasmal Pneumonia of Swine (MPS). Huhn (1970) indicated that while the term Mycoplasmal Pneumonia of Swine is etiologically suggestive, it did not differentiate between the primary and secondary nature of the incriminated agents. He preferred the use of the term Enzootic Pneumonia of Pigs (EPP) which he thought was etiologically noncommittal. However, the term MPS is preferred in the United States.

Goodwin et al. (1965) reported that their J strain agent grew on solid medium and further observed that pleomorphic mycoplasma-type

elements could be seen in Giemsa-stained preparations from these colonies. Cultures were used to induce typical lesions in experimental pigs. They proposed that the organism was a mycoplasma species and consequently suggested the name Mycoplasma suipneumoniae for it.

Goodwin, Pomeroy and Whittlestone again reported (1967) additional work on the characterization of their 'J' strain. They found that their M. suipneumoniae and the M. hyopneumoniae of Mare and Switzer (1965) were indistinguishable by the growth and metabolic inhibition tests. By using the growth inhibition test, they demonstrated that M. suipneumoniae was distinct and unrelated to all of a wide range of other mycoplasmas - 42 strains examined.

The Subcommittee on the Taxonomy of the Mycoplasmatales (1974) recommended that strain J of M. suipneumoniae be accepted as the neotype strain of M. hyopneumoniae, because authentic cultures of strain 11, the type strain of M. hyopneumoniae, were no longer in existence. Persuant to this recommendation, Rose and associates (1979), exhaustively compared the biological and serological characteristics of M. hyopneumoniae, M. suipneumoniae and M. flocculare. They found M. hyopneumoniae and M. suipneumoniae to be indistinguishable. Subsequently they proposed that strain J, the type strain of M. suipneumoniae be the neotype strain of M. hyopneumoniae.

After the initial breakthrough of isolation and confirmation of M. hyopneumoniae as the causative agent of MPS various reports of the successful isolation of the organism appeared in the literature. Estola and Schulman (1966) reported the isolation of mycoplasmas from cases of swine enzootic pneumonia in Finland. L'Ecuyer (1969) recovered three

strains of a mycoplasma species from pneumonic lungs of pigs in Canada, and was able to reproduce the disease by intratracheal inoculation of respiratory disease-free pigs with mycoplasmas grown in cell-free medium. Friis (1969) in Denmark, reported the isolation of an agent which he found to be serologically and culturally identical to M. hyopneumoniae. Takatori (1969) also demonstrated a serologic relationship between his isolate "M" in Japan and M. hyopneumoniae.

Other mycoplasma agents isolated from pneumonic lungs of swine are M. hyorhinis, M. hyosynoviae, M. flocculare, and A. laidlawii.

The first isolation of mycoplasmas from the porcine respiratory tract was made by Carter and Mckay (1953). They isolated a PPLO from cases of atrophic rhinitis. Switzer (1955) isolated this organism from cases of atrophic rhinitis and from normal pig turbinates. He described and characterized his isolate and proposed the name Mycoplasma hyorhinis. Ross and Switzer (1963) proved that original isolates of mycoplasmas thought to be M. hyorhinis were not a single mycoplasma species, by using the indirect hemagglutination test. Mycoplasma hyorhinis has been isolated from chronic porcine pneumonia with a frequency which prompted early workers to suggest that it is a primary pulmonary pathogen. L'Ecuyer et al. (1961) found M. hyorhinis in approximately 50% of all cases of pneumonia.

Conflicting reports exist in the literature with respect to the role of M. hyorhinis in the initiation or exacerbation of MPS lesions. Workers in U.S. believe that M. hyorhinis is merely playing a secondary role in the production of porcine pneumonia, (Switzer 1967 and L'Ecuyer

1969). Their assertion was based on the fact that polyserositis and arthritis have been consistently produced experimentally in 3 to 6 week old swine by intraperitoneal or intranasal inoculation, but that primary pneumonia due to this agent has not been a constant feature.

Work in European countries has incriminated M. hyorhinis as a primary pulmonary pathogen in gnotobiotic pigs. Gois et al. (1968, 1971) produced pneumonia with cloned cultures of M. hyorhinis strain TR32, in both conventional and gnotobiotic pigs. Poland et al. (1971) produced pneumonia in 3 of 9 gnotobiotic piglets, infected by exposure to aerosols of M. hyorhinis. However, all pigs had serositis. Friis (1971b) inoculated 12 colostrum-deprived, hysterectomy-derived piglets. The organism was recovered from the brain and nasal cavities of inoculated pigs. Meyling, (1971) examined naturally occurring piglet pneumonia by the direct fluorescent antibody test. He observed that lungs which stained specifically for M. hyorhinis, were negative for M. hyopneumoniae and in these cases, M. hyorhinis was located on the surface of the bronchiolar epithelium, the same localization as observed in a number of pneumonias caused by other mycoplasmas.

Mycoplasma hyosynoviae is occasionally isolated from pneumonic lesions. Its role in the pathogenesis of pneumonia is not yet known. Friis (1970) examined 71 bacon pigs and found that 11% yielded A-M strains of mycoplasma (arginine metabolizing strains). Ross and Karmon (1970) characterized some (A-M) strains as M. hyosynoviae, and showed them to be biochemically, serologically and electrophoretically different

from M. granularum which was later classified as Acholeplasma granularum. Meyling (1971) failed to find specific fluorescence in 5 lungs positive on culture for M. hyosynoviae.

An acid producing mycoplasma was isolated from pneumonic lungs that was biologically similar to M. hyopneumoniae (Meyling and Friis, 1972). It was serologically different from M. hyopneumoniae. This organism grows at 30°C. The name M. flocculare was proposed. Rose et al. (1979) observed that although M. flocculare shared a number of biological features with strains of M. hyopneumoniae, it was serologically distinct not only from this mycoplasma, but also from all of the other currently recognized species of mycoplasmas. Friis (1974b) exposed piglets to aerosols of M. flocculare. At necropsy 3 of 15 piglets had small pneumonic lesions in which there were slight mononuclear and polymorphonuclear cell accumulations in bronchial tree and alveoli. The role of M. flocculare in pneumonia of pigs is uncertain. Infection of SPF pigs in Denmark is not associated with clinical outbreaks of pneumonia (Friis 1976). From the multiplicity of studies, it can be seen that so far, only Mycoplasma hyopneumoniae has been incriminated as a primary pulmonary pathogen, beyond any reasonable doubt.

Morphology and Staining Characteristics

Mare and Switzer (1965) described the morphologic features of their M. hyopneumoniae strain 11 isolate. They described it as having coccoid to short forms and filamentous and occasionally ring forms ranging in size from 0.5 µm in diamter. A similar morphologic

description was given by Goodwin et al. (1965) for their U.K. isolate of M. suipneumoniae. On solid agar, this organism appeared as distinct very minute colonies devoid of a central core, which is characteristic of the typical "fried egg" appearance among the mycoplasmas. At 7 to 10 days, their diameter may reach about 0.5 mm on a good medium. It does not produce a film on swine serum agar, and does not hemolyse swine, horse, cattle or chicken red blood cells in agar. Colonial morphology is of limited value in distinguishing one mycoplasma from another because it is influenced by many factors including the composition and texture of growth medium, the concentration of seeding, the humidity and gaseous conditions of incubation (Whittlestone 1973).

Primary Isolation of M. hyopneumoniae

The organism is extremely fastidious in its growth requirements. Growth occurs in Hank's balanced salt solution enriched with 20% heat inactivated pig serum from pneumonia-free swine, 0.5% lactalbumin hydrolysate and 0.01% Difco yeast extract. The type strain organisms are microaerophilic. The organism can be isolated in fluid medium containing bacterial inhibitors such as 200 IU of penicillin. M. hyopneumoniae resists thallium acetate at 1:4,000. Yamamoto et al. (1971), obtained a high isolation rate of M. hyopneumoniae by incorporating benzyl penicillin and thallium acetate in the growth medium. Wilson (1976) also reported the successful isolation of M. hyopneumoniae by incorporating ampicillin alone. He could not recover mycoplasma when benzyl penicillin and thallium acetate were incorporated into

the medium. Friis (1975) recommended the combined use of bacitracin and meticillin as bacteriostatic agents after demonstrating previously (1971a, 1974a) that penicillin G and other benzyl derivatives of penicillin had an inhibitory effect on the replication of M. hyopneumoniae and M. flocculare. The medium recommended by Friis (1975), has found wide usage in laboratories engaged in the isolation of M. hyopneumoniae. Primary isolation is performed in broth. A 10% suspension of ground tissue is made in broth and ten fold dilutions made by serial transfer of 0.2 ml amount to 10 ml plastic tubes containing 1.8 ml of broth. Growth is evidenced by an acid shift from red (pH 7.4) to yellow (pH 6.8). Occurrence of color shift may indicate time for subcultivation on agar or further passage in broth. The MH medium of Etheridge et al. (1979b) appears to aid in the recovery of Australian strains of M. hyopneumoniae from porcine pneumonia. The A26 medium described by Goodwin et al. (1969a) is also used for the successful isolation of M. hyopneumoniae.

Disease Symptoms and Pathology

The clinical signs of MPS were described by Betts (1952). He characterized the disease clinically as being a chronic pneumonia with a high herd morbidity and low mortality. Pigs evidence first signs of the disease between 3 and 10 weeks of age. The incubation period is between 10 and 16 days following exposure. A transient diarrhea has been noted to occur 2 or 3 days postexposure, which is followed by a dry nonproductive cough. Some pigs are never observed to cough.

Coughing is pronounced when the animals are aroused from rest. Respiratory movements are usually normal and affected pigs generally have good appetites but do not grow properly. Relapses do occur probably caused by stress or secondary bacterial infection. Huhn (1970) stated that the rate of weight gain was decreased in direct proportion to the severity of the lesions and that the average expected reduction in rate of gain was 0.05 kg/day or 7% per pig.

Betts (1952) fully described the gross pathologic characteristics of MPS. He observed that the lesions consisted of purple to gray areas of consolidation in the lungs that were well-demarcated from normal areas. He reported the lesion distribution to be predominantly anterio-ventral in location, affecting mostly the right cardiac and apical lobes and to a lesser degree the intermediate lobe. Many workers seem to agree with his description of the disease.

The microscopic pathological changes in MPS were characterized by Pattison (1956), Urman et al. (1958) and Hodges et al. (1969). Pattison (1956) described the histopathology as involving a gradation of changes beginning as an increase in cellularity of the interalveolar tissue with slight edema and a few large mononuclear cells in the alveolar spaces. Later, there is an increase in cellular exudate, edema and lymphoid hyperplasia related to bronchi and bronchioles. Urman et al. (1958) reported on the microscopic differentiation of swine influenza from MPS. They observed that the tissue response to introduction of swine influenza virus was much more rapid. Areas of edema and hyperemia were seen throughout the lungs within 24 hours after inoculation.

Alveoli and bronchi contained neutrophils and much cellular debris 3 days after inoculation. Six days after inoculation, the mononuclear perivascular reaction was more pronounced than was the peribronchiolar reaction. Later, peribronchiolar cuffing became the more prominent lesion. This contrasted with MPS, in which histologic lesions did not consistently appear until 13 days after inoculation.

Hodges et al. (1969) described the microscopic lesions found in gnotobiotic pigs inoculated with M. hyopneumoniae. Lesions consisted of infiltration with lymphocytes and plasma cells and an increase in reticulin content of peribronchial tissue and presence of neutrophils and mononuclear cells in lumina of bronchioles. Livingston et al. (1972) reported the first sequential histologic study of M. hyopneumoniae disease. They induced the disease by intranasal instillation of broth cultures of the organism. They described changes consisting essentially of small lymphoid nodules in the submucosa of bronchial epithelium as early as 6 days PI. By ten days the bronchial lesions appeared to be spreading into alveolar areas. Fourteen days PI bronchioles and blood vessels were surrounded by lymphocytic cuffs and there was exudate in bronchi and alveoli. By 22 days PI, there was extensive perivascular and peribronchiolar lymphoid hyperplasia and involvement of alveoli. The literature on interpretation of histological changes of porcine enzootic pneumonia was reviewed by Jericho (1977). He suggested that outbreaks of MPS should be recognized solely on the basis of epizootiologic studies and stressed that histopathologic characteristics common to MPS are not pathognomonic. He further cautioned that misinterpretation of these histopathological changes

may lead to an undue simplification or complication in the study of porcine respiratory disease.

In sequential studies on the pathogenesis of mycoplasmal pneumonia in swine, Livingston et al. (1972) observed that electron microscopy revealed mycoplasmas adjacent to cilia and plasma membrane of epithelial cells lining the bronchioles and bronchi. Mycoplasmas were confined to surface structures and were not within epithelial cells. Baskerville and Wright (1973) studied the sequential ultrastructural changes in experimental enzootic pneumonia. They used pneumonic lung inoculum containing Mycoplasma hyorhinis derived from the lungs of animals with natural enzootic pneumonia. They also found mycoplasmas consistently on mucosa of airways. Large masses of cytoplasma projected from the apex of many ciliated bronchiolar cells. However they could not determine whether the mycoplasmas on the mucosal surface of airways were M. hyopneumoniae or M. hyorhinis.

Serological Procedures used in Diagnosis of Mycoplasmal Pneumonia of Swine

Earlier attempts to develop a serological test for demonstrating antibodies in sera of pigs infected with M. hyopneumoniae were discouraging. These failures were due in part to the inability to obtain sufficient growth of antigen for the tests.

Lannek and Börnfors (1957) demonstrated that pigs which have recovered from experimental enzootic pneumonia were resistant to a second challenge with the same infectious agent. Goodwin et al. (1969a)

demonstrated that a strong immunity in pigs followed recovery from the disease.

Complement fixation test

Using the standard complement fixation test, Roberts (1968) detected antibodies to M. hyopneumoniae in pig sera. Boulanger and L'Ecuyer (1968) also demonstrated antibodies to the organism at 2 to 3 weeks after experimental infection by using the direct modified complement fixation test. They showed that peak titers were reached at about 35 days PI and persisted for 119 days.

Swine serum contains procomplementary substances which greatly increase the hemolytic activity of guinea pig complement. Cowan (1961) attributed the procomplementary activity of pig serum to high levels of the third component of complement. Roberts (1968) suggested that the procomplementary activity of swine serum could be reduced by heating at 60^{o}C for 30 min and by filtration through filter pads. Other problems inherent with using the CF test with swine serum are the presence of natural hemolysins and poor complement binding activity of swine antibody. Slavik and Switzer (1972) circumvented the procomplementary activity of swine serum by reconstituting lyophilised guinea pig complement with swine serum.

Takatori (1970) found that there was a good correlation between development of mycoplasmal pneumonia lesions and occurrence of detectable CF antibody levels. Nonspecific reactions and cross-reactivity with M. hyorhinis have been reported. Nonspecific reaction in the CF test

were in part explained by the work of Roberts and Little (1970b). They found that antitissue antibodies could be produced in the lungs following mycoplasma infection and suggested that the nonspecific CF reaction may be a measure of antilung antibodies. They also suggested that cross-reactivity between M. hyopneumoniae and M. hyorhinis in hyperimmune rabbit sera resulted from medium components being absorbed by the mycoplasmas.

Indirect hemagglutination test

Ross and Switzer (1963) adapted the indirect hemagglutination test (IHA) for the detection of antibodies to M. hyorhinis. They found serological variations between isolates of what were identified as M. hyorhinis.

Goodwin et al. (1969b) demonstrated the antibody response against M. hyopneumoniae by the use of the indirect hemagglutination test. They used tanned sheep erythrocytes sensitized with mycoplasma antigen, and found high IHA titers in sera from pigs that had been infected 16 or more weeks previously but not in sera collected 12 to 22 days PI. In a comparative study with the CF test, these workers found that IHA titers appeared later and lasted longer than the CF titers.

Lam and Switzer (1971a) observed that pigs experimentally exposed to M. hyopneumoniae developed antibody titers detectable by the IHA test 2 to 3 weeks PI. Titers reached a maximum level by the 8th to 11th week and remained there until the 28th week. Titers started decreasing but remained detectable for 47 weeks. They used tanned

swine erythrocytes, and treated their mycoplasma antigen with sodium dodecyl sulfate followed by ammonium sulfate precipitation.

Holmgren (1974a) used the IHA test for swine serum using formalinized, tanned swine erythrocytes. He detected antibodies in 89% of sera from slaughter pigs with enzootic pneumonia, and in 3% of pigs believed to be free from the disease. Holmgren (1974b) found indirect hemagglutinating antibodies in tracheobronchial secretions at 2 and 4 weeks after infection. This persisted through 13 weeks PI in one pig. The antibody activity was associated mostly with IgA.

Tube agglutination test

Fujikura et al. (1970) demonstrated antibody against M. hyopneumoniae by use of the tube agglutination test. They found a good correlation between presence of MPS lesions and detectable antibody levels, as well as no cross reaction between M. hyopneumoniae and M. hyorhinis. Their methodology involved incubating a mixture of antigen and antibody with a total reaction volume of 0.5 ml, in a water bath at 37°C for 2 hr for optimum sensitization. This was then placed in a cold room at 4 to 5°C for 18 hr. Tubes were observed macroscopically for agglutination. Roberts and Little (1970a) reported their failure to detect agglutinating antibodies to M. hyopneumoniae and in animals infected with Acholeplasma laidlawii by both the slide and tube agglutination techniques. The anti M. hyopneumoniae titers that they obtained were higher than by metabolic inhibition or IHA tests.

Pijoan and Boughton (1974) developed a tube agglutination test using an antigen suspension of M. hyopneumoniae in saline and sera from hyperimmune and naturally infected pigs. They found that temperature was critical for agglutination and obtained best results at 42°C, 52°C or 56°C rather than at 37°C. They stated that the test was effective only in the presence of macroglobulins and did not detect antibodies of smaller size. In sera of pigs with experimental infection, a gradual increase in CF titer was not paralleled by the appearance of agglutinating titers. They therefore concluded that the tube agglutination test was useful for the titration of hyperimmune sera from pigs.

Slavik (1976b) found latex agglutinating (LA) antibodies against M. hyopneumoniae in experimentally inoculated pigs at 2 to 3 weeks PI. He found LA antibodies to persist up to 48 weeks PI and in a study of field cases of MPS found a correlation between LA titers, CF titers and gross and microscopic pneumonic lung lesions.

Immunofluorescence test

The basic principle of the immunofluorescence technique is that antibody molecules can be conjugated with a fluorescent dye without affecting the biologic activity or specificity for a homologous antigen. When this dye is subjected to ultraviolet light at the appropriate wavelength, it emits visible green light thus identifying the location of any substance to which the antibody has attached.

The application of immunofluorescent technique to the study of mycoplasmas was reported in 1957 by Liu. He demonstrated by using this technique that the etiologic agent of atypical interstitial pneumonia of man was localized on bronchial epithelial surfaces of chick embryos experimentally infected with this agent.

Direct immunofluorescent staining of pneumonic lungs of pigs to identify M. hyopneumoniae antigen was reported by L'Ecuyer and Boulanger in 1970. Specific fluorescence was detected 25 days PI and lasted up to 49 days PI. Fluorescent staining was almost always localized on the surface of the bronchial and bronchiolar epithelium and in bronchial exudate. Meyling (1971) confirmed the findings of L'Ecuyer and Boulanger. By direct immunofluorescent staining of cryostat sections of pneumonic lungs of young pigs, Meyling observed that M. hyorhinis could often be demonstrated with distribution and localization similar but not identical to that of M. hyopneumoniae. In slaughter house material, he isolated M. hyopneumoniae, from 38 cases, and observed specific fluorescence in 35 of these. Holmgren (1974b) compared the direct immunofluorescence technique with the development of IHA titers in conventional pigs naturally exposed to infection. He found that M. hyopneumoniae antigen could be demonstrated by the IF technique in pneumonic tissues of pigs with high IHA titers in serum.

The IF staining of mycoplasma colonies directly on agar medium was reported by Del Giudice et al. (1967). The success and practicality of this technique depended on 2 factors; the preparation of large quantities of potent and specific fluorescein conjugated antisera to mycoplasmas, and the adaptation of incident illumination techniques to

ultraviolet microscopy. The specificity of this test has lent itself as a standard procedure in the identification of mycoplasmas.

L'Ecuyer and Boulanger (1970) used the immunofluorescence technique to stain smears from broth cultures of M. hyopneumoniae. They observed that intensity of fluorescence was good whether the smears were prepared from washed antigens or from unwashed broth culture sediments. Comparative studies made with acetone-fixed and unfixed smears indicated that no advantage was gained by fixation. Schuller et al. (1976b) reported the staining of M. hyopneumoniae colonies. They overlayed glass microscope slides with solid medium, and air-dried colonies grown on this medium for 5 days at 37°C in a moist atmosphere with 5% CO_2. They reported that air-dried colonies were resistant to removal by washing procedures, and that the drying of the colonies did not destroy their antigenicity.

Armstrong (1976) described the use of inoculated cellulose acetate filter membranes in immunofluorescent identification of M. hyopneumoniae cells. The inoculated membranes were placed in broth and incubated until there was a pH change to acid at which time the membrane was stained and examined by incident UV light.

Growth inhibition test

The term growth inhibition (GI) applies to those techniques which measure a decrease in the number of mycoplasma colonies formed on an agar medium, or a decrease in turbidity of mycoplasma broth culture medium. The inhibition of the growth of mycoplasma in a liquid medium

by immune serum was first demonstrated by Priestly (1952). He used M. mycoides var mycoides and fresh convalescent serum from cattle infected with the organism. Clyde (1964) used the principle of growth inhibition of mycoplasmas by homologous antibody to extensively compare and identify human mycoplasma isolates. Sterile discs were impregnated with hyperimmune serum and placed on agar plates inoculated with mycoplasma culture. A zone of inhibition similar to that seen in antibiotic sensitivity tests is seen if the antibody is homologous. To date, the GI test is one of the most common laboratory procedures used for the identification of mycoplasmas.

Metabolic inhibition test

The term metabolic inhibition applies specifically to those techniques in which inhibition of mycoplasma growth is detected indirectly via inhibition of metabolic activity. Such procedures have been based on the observation that actively growing mycoplasmas metabolize certain substrates. Specific antiserum inhibited metabolism of organisms and thereby prevented a color change of the medium from occurring. Jensen (1964) was the first to publish the application of metabolic inhibition to the study of human mycoplasmas. He utilized 2, 3, 5, triphenyl tetrazolium chloride which was colorless in the oxidized state and red in the reduced state. Taylor-Robinson et al. (1966) developed an MI test based on the fermentative action of some mycoplasmas. Purcell et al. (1966) used the MI test for mycoplasmas that utilized arginine with the liberation of ammonia which increased

the pH of media.

Goodwin et al. (1969b) observed that metabolic inhibiting antibody titers did not correlate with the immune status of pigs. Animals already shown by challenge to be immune had low titers whilst titers of 1:16 were obtained with over one third of samples from uninfected controls, or from pigs not yet exposed to infection. He concluded that the metabolic inhibition test might be of little value because nonspecific inhibitory substances were present in the sera of some pigs both before and after experimental infection. This nonspecific inhibition was sometimes reduced by heating the sera at 56^{o}C for 30 minutes.

Enzyme linked immunosorbent assay (ELISA) and immunoperoxidase tests

Engvall and Perlmann (1972) reported a sensitive method for the quantitative determination of antibody. Tubes coated with antigen were incubated with antiserum followed by the addition of an enzyme-labelled preparation of anti immunoglobulin. The enzyme remaining in tubes after washing provided a measure of the amount of specific antibodies present. Bruggmann et al. (1977) adapted this procedure for the detection of antibodies to M. hyopneumoniae as a sensitive test for the serological investigation of epidemiological cases, particularly clinical infections with M. hyopneumoniae. They utilized horseradish

peroxidase conjugated rabbit anti-swine IgG. They also showed that antibody to M. hyopneumoniae in experimentally infected pigs was detected several weeks before the clinical manifestation of MPS and sera were positive up to 50 weeks after inoculation. No cross reaction between M. hyorhinis, M. flocculare and M. hyopneumoniae was observed. The ELISA test was found to be highly sensitive and specific for the detection of antibodies to M. hyopneumoniae. The low concentration of antigen optimal for coating the polystyrene tubes (2.5 µg to 5 µg) indicated a high sensitivity in relation to the number of antibodies that could be detected. Bruggmann (1978) characterized the chemical nature of the antigenic determinants of M. hyopneumoniae. He observed that membrane antigen solubilized with sodium dodecyl sulfate (SDS) was superior to whole antigen in displacing antibodies from the absorbed antigen. This indicated, too, that the active antigenic determinant(s) is localized in the membrane. He hypothesized that the antigens are lipoproteins, the lipid portion being the determinant hapten and protein, the carrier.

Bruggmann et al. (1976) used the indirect immunoperoxidase test to detect antibodies in sera of pigs. Using this serological test, they found that there was positive correlation of 95.7% between presence of antibody and evidence of the disease in pigs. They concluded that the indirect immunoperoxidase test was more sensitive for detecting chronically infected pigs than the CF test.

Epizootiology of Mycoplasmal Pneumonia of Swine

Mycoplasmal pneumonia of swine (MPS) is exclusively a disease of swine. No other natural or experimental hosts have been demonstrated. Fomite or transplacental transmission of the disease has not been demonstrated and transmission of this infection is considered to occur exclusively after birth. Close contact between infected and susceptible swine is necessary for the transmission of the organism. Within chronically infected pig herds, the incidence of clinical pneumonia falls progressively with increasing age of the animals. Whittlestone (1973) observed that the incidence of pneumonia in sows was less than in bacon pigs and thus concluded that older sows in infected herds seem less likely to transmit the infection to their own litters. Introduction of the disease into a herd not previously infected can be traced to the purchase of coughing feeder pigs or asymptomatic adult carriers which are added as new breeding stock.

Healthy herds become infected without any recorded proximity of pigs affected with MPS and in these cases, the indirect transfer of M. hyopneumoniae in sputum of birds, animals or people is suspected (Whittlestone 1976). Since infection usually spreads by the aerosol route, climatic conditions could influence the severity of MPS by its effect on the survival rate of M. hyopneumoniae in infective droplets. Switzer (1967) observed that there is no apparent seasonal incidence of mycoplasmal pneumonia. However, clinical illness produced by secondary bacterial invaders such as P. multocida may be weather-associated. Goodwin (1972) put pigs into pens which had housed infected

pigs and had been vacated 5 minutes previously. None of the pigs came down with the disease nor was the organism recovered from any of them. Transmission of MPS usually occurs over short distances which suggests that relatively large particles which sediment rapidly are responsible for transferring the agent from one pig to the other. Unexplained infections do occur at greater distances and Whittlestone (1976) speculated that smaller particles which remain suspended for longer period may sometimes be responsible for spread of infection. Gordon (1963) noted that the incidence of pneumonia was lower in pigs from houses with a high relative humidity and high temperature.

Various agents within the respiratory system itself play a role in the severity of MPS. Mackenzie (1963) observed that lesions of MPS were more extensive in pigs with lungworm infection. Preston and Switzer (1976) studied two herds with lungworm infection. Lungworm larvae-infected earthworms were collected and fed to swine free of MPS. This procedure resulted in lungworm infection in recipient pigs, but failed to produce pneumonic lesions suggestive of MPS. Complement fixing antibody titers against MPS were not detected. Kasza et al. (1969) inoculated pigs with a pathogenic swine adenovirus and M. hyopneumoniae. The two agents combined produced a pneumonia of greater severity than either agent alone. L'Ecuyer (1962) found that the principal microorganism associated with MPS was Pasteurella multocida.

Incidence and Distribution

The disease is world wide and has been reported from all five continents. Switzer (1967) found the incidence of MPS in Iowa swine to be between 35 and 60%. Betts (1952) observed the incidence of MPS in England to be between 61% and 72%. Whittlestone (1967) confirmed Bett's observation. Mugera (1967) reported that 56% of pneumonia in swine observed in Kenya, East Africa resembled MPS. Norton (1976) reported a prevalence of 68% in 516 porkers in Northern Queensland, Australia.

Control of MPS

The control and attempts at eradicating MPS have been centered around chemotherapy and the specific pathogen free (SPF) swine program. Betts and Beveridge (1952) recognized the fact that the causative agent of MPS was sensitive to chlortetracycline, but not to penicillin or sulfonamides. Wesslen and Lannek (1954) demonstrated the in vitro susceptibility of M. hyopneumoniae to chlortetracycline and oxytetracycline. Bornfors and Lannek (1958), observed that tetracycline at a dosage of 20, 15 and 10 mg per kg body weight provided a prophylactic effect. Pigs that were given tetracycline in these dosages did not develop lesions of pneumonia when placed in contact with infected animals. Goodwin and Whittlestone (1960) believed that tetracycline antibiotics were the only means of preventing the development of MPS. This susceptibility to tetracycline was a factor in the realization

that the etiologic agent of MPS was indeed not a virus. Mare (1965) observed that tylosin tartrate had no prophylatic or therapeutic action on MPS. He also found furaltadone to be ineffective against the disease agent. Mare and Switzer (1966) showed that chlortetracycline was effective in preventing the formation of lesions of MPS at a dosage of 400 gm per ton in feed. Huhn (1971) observed that chlortetracycline at a dosage of 50 gm to 200 gm per ton of feed may be effective in preventing development of MPS lesions if given prior to exposure to M. hyopneumoniae. Etheridge et al. (1979a) reported that a strain of M. hyopneumoniae designated the Beaufort strain was resistant to chlortetracycline.

Durickovic et al. (1964) demonstrated that when nitrofurazone was given intramuscularly, the incidence and extent of pneumonia was reduced. In vitro studies by Ogata et al. (1971) correlated with the findings of Durickovic et al. Ogata and coworkers found that nitrofurans at low concentrations were inhibitory to M. hyopneumoniae. More recently, increasing information on the efficacy of Tiamulin - 14-deoxy(2-diethylaminoethyl) mercapto acetoxy hydrogen fumarate, has been reported. Schuller et al. (1976a) reported the in vitro efficacy of tiamulin in pigs infected with M. hyopneumoniae. They observed that animals infected with M. hyopneumoniae and medicated with 200 ppm of tiamulin showed a considerable resolution of inflammatory lung reaction. Johnson (1978) evaluated the use of this drug in naturally infected pigs. He concluded that tiamulin given in drinking water at 0.006% or 0.008% for 5 days was effective in controlling MPS.

Goodwin (1979) observed, in in vitro studies, that tiamulin inhibited mycoplasmal growth at a lower concentration than either oxytetracycline or tylosin. Treatment of infected pigs with tiamulin resulted in remarkable improvement characterized by healed lesions 49 days PI.

The fastidious nature of Mycoplasma hyopneumoniae has made the growth of enough antigen for vaccination studies difficult. Thus, initial experimentation on vaccination was done using vaccine prepared from pneumonic lung suspensions. Lannek and Bornfors (1957) showed that pigs which have recovered from experimental MPS developed a strong immunity to subsequent challenge. Goodwin et al. (1969a) infected pigs by intranasal inoculations of pneumonic lung suspensions. Sixteen weeks after infection, pigs were challenged with the same inoculum. No lung lesions were seen when the animals were killed 3 weeks after challenge. Again Goodwin et al. (1969b) used formalinized M. hyopneumoniae cells to induce immunity in pigs. Pigs were vaccinated two times intramuscularly or intradermally with Freund complete adjuvant. The third injection was given without adjuvant. The pigs were challenged intranasally with pneumonic lung suspension. The vaccinated pigs showed no evidence of resistance to challenge. However the possibility that the challenge inoculum might have been overwhelming was noted by the authors.

Lam (1970) and Lam and Switzer (1971b) reported the efficacy of five different vaccines. Vaccine A was prepared by ether extraction of mycoplasma cells in conjunction with Freund incomplete adjuvant. Vaccine F was prepared by alternately freezing and thawing mycoplasma

cells using solid carbon dioxide-alcohol bath. The preparation was administered in an incomplete Freund adjuvant. Vaccine G was prepared by lysis of cells with a 2% solution of sodium lauryl sulfate, and dialyzed against ammonium sulfate and distilled water. Vaccine H was formalin-inactivated broth culture. Vaccine H was administered intranasally on 3 consecutive days whilst the remaining 4 vaccines were given intramuscularly. Protection was observed in animals vaccinated with vaccines A,B,F and G. No protection was seen in the intranasally vaccinated pigs. Farrington (1976) demonstrated that a peanut oil base adjuvant coupled with heat-inactivated M. hyopneumoniae cells enhanced protection against lung lesion formation. Slavik et al. (1979) evaluated the effectiveness of several possible vaccines in preventing M. hyopneumoniae infection. Pigs vaccinated simultaneously with heat-inactivated M. hyopneumoniae by intravenous, footpad and subcutaneous routes were partially protected against subsequent M. hyopneumoniae challenge as indicated by a reduction in gross lesions of pneumonia.

Specific Pathogen Free Herds

In view of the fact that the etiologic agent of MPS is infectious, one of the methods that had been used to control the disease is the production of specific pathogen free pigs. Pigs are obtained by hysterectomy or Caesarian section without exposure to their dam's environment, and raised in laboratory isolation for 4 weeks. They are then transferred to and grown on farms from which all other swine have

been removed. These new "disease free" pigs form the nuclei of clean herds. Offspring in these clean herds can then be used as clean breeding stock. Certification schemes for maintaining MPS free herds are in operation in the U.S., Britain, Canada, and continental Europe.

In the U.S., the Nebraska certification scheme involved essentially stock derived directly by surgical removal, or progeny of these animals. The health requirement in the Nebraska scheme included freedom from MPS as ascertained by lung examinations at slaughter. Young and Underdahl (1960) recommended the examination of a sample of 10 lungs from each herd to determine whether a herd should be certified as free from MPS or not.

Keller (1969) reported that the Swiss certification scheme included 8,500 sows in 350 primary and secondary herds. Registered SPF herds were also required to remain free from certain other diseases as well. The British scheme was started in 1958 and the basis of operation was freedom from MPS. The central feature of this scheme therefore was routine post mortem examination of the lungs of pigs at slaughter (Goodwin and Whittlestone 1960).

Occasional breakdowns have occurred in SPF herds. Most outbreaks appeared to have occurred without any known possibility of contact with infected pigs. The possibility of indirect transfer of mycoplasmal agents cannot be ruled out.

PART I. DIAGNOSIS OF MYCOPLASMA PNEUMONIA OF SWINE BY DIRECT IMMUNOFLUORESCENCE TECHNIQUE

MATERIALS AND METHODS

Experimental Animals

Thirty-five conventional purebred Yorkshire pigs that were farrowed in isolation units at the Veterinary Medical Research Institute (VMRI) were used for the study. The pigs were 6 to 8 weeks of age and were divided into five groups on the basis of results of bacteriologic culture of nasal secretions, sex and litter. The various groups were kept in individual isolation units and fed 16% protein grower ration with no added antibiotics. Each pig was ear-notched for identification.

Experimental Design

Three groups of pigs were to have been used in this study. However, bacteriologic examination of nasal secretions revealed that pigs in 2 of the 4 litters were infected with Bordetella bronchiseptica. Therefore, the pigs were alloted to 5 groups. Group 1 consisting of 12 pigs from litters culture negative for B. bronchiseptica was inoculated endotracheally with pneumonic lung suspension containing M. hyopneumoniae strain 11. Group 2 consisting of 6 pigs which were culture negative for B. bronchiseptica were inoculated with pneumonic lung suspension containing M. hyopneumoniae strain 11 and M. hyorhinis strain 7. Group 3 consisting of 6 pigs from the B. bronchiseptica-infected litters was inoculated endotracheally with pneumonic lung suspension containing M. hyopneumoniae strain 11. Group 4 consisting of 6 pigs

from B. bronchiseptica-infected litters was inoculated endotracheally with pneumonic lung suspension containing M. hyopneumoniae strain 11 and M. hyorhinis strain 7. Group 5 was the control group and consisted of 5 pigs derived from litters culture negative for B. bronchiseptica. Pigs in this group were inoculated endotracheally with sterile mycoplasma broth (Friis 1975).

Blood samples were collected from each pig prior to inoculation and at weekly intervals post inoculation (PI), until each pig was euthanized. Two pigs were necropsied before the start of the experiment to establish that no pulmonary lesions were present. Sequential post mortem examination of pigs was performed as presented in table 1.

Table 1. Post mortem examination of pigs and time interval of euthanasia

Group No.	No. pigs necropsied at 2 week intervals PI						Total No. of pigs necropsied
	2	4	6	8	10	12	
1	2	2	2	2	2	2	12
2	1	1	1	1	1	1	6
3	1	1	1	1	1	1	6
4	1	1	1	1	1	1	6
5	1	1	1	1	-	1	5

Pneumonic Lung Inoculum

The pneumonic lung inoculum (PLI) used in this study was derived by serial passage of M. hyopneumoniae strain 11 (Mare and Switzer 1965),

in pigs. The pneumonic lung was stored frozen in 50 ml vials at -20°C. On the day of inoculation, vials were removed and pneumonic lung was thawed. A ten percent suspension was made by grinding the approximate weight of lung tissue in a sterile Ten Broeck[1] glass tissue grinder using Friis broth medium without bacterial inhibitors as diluent. A 24 hour 15th passage culture of M. hyopneumoniae strain 11 grown in mycoplasma broth was added to the ground lung suspension in the ratio of 1:4. This mixture was centrifuged at 4°C at 800 rpm to remove large pieces of lung tissue. The supernate was kept in an ice bath until inoculation. For preparation of M. hyopneumoniae - M. hyorhinis inoculum, M. hyorhinis strain 7 grown in beef heart infusion-turkey serum (BHI-TS) broth (Ross and Switzer 1963, Ross and Karmon 1970) was used. This broth culture was added to a portion of M. hyopneumoniae strain 11 inoculum in a ratio of 1:4 and held in an ice bath until inoculation. Lung suspensions were inoculated on 5% horse blood agar with a nurse colony to determine that no bacteria were present. Color changing units (CCU) were determined for M. hyopneumoniae PLI. The CCU was defined as the highest dilution of PLI which changed the color of mycoplasma medium within 14 days incubation. The PLI used for the inoculation of pigs was found to contain 10^6 CCU of M. hyopneumoniae. The M. hyorhinis colony forming units (CFU) were determined by making 10 fold tube dilutions in BHI-TS broth. The CFU was calculated by counting the colonies formed on BHI-TS agar after 4 to 5 days incubation. The M. hyorhinis inoculum contained 10^5 CFU per ml.

[1]Fisher Scientific Company, Chicago, Illinois.

Inoculation of Experimental Pigs

Pigs were inoculated endotracheally by means of a rubber veterinary catheter. An assistant held the pig in a vertical position. A porcine mouth speculum was inserted between the pig's upper and lower jaw by another assistant. The tube was then passed quickly into the trachea while the pig was squealing. The glottis is unguarded during squealing. The presence of the tube in the trachea was indicated by forceful breathing sounds, some coughing and passage of air through the other end of the tube. Five ml of inoculum and 15 ml of air were drawn into a 20 ml syringe. The syringe was attached to a Luer-Lock adapter on the endotracheal tube, and the material was quickly inoculated into the lungs. The tube was held against the roof of the upper jaw during inoculation to prevent it from slipping out of position. Blowback was prevented by pinching the tube whilst the syringe was being removed. Control pigs were inoculated with an equal volume of sterile Friis broth medium. Pigs were observed daily for feed consumption, lethargy, coughing and dyspnea.

Bacteriology

Nasal secretions were collected prior to inoculation of pigs and examined for bacteria and mycoplasmas by culture technique. Calcium alginate swabs with flexible aluminum shafts[1] were used when pigs were between the age of 2 to 6 weeks. Thereafter, sterile cotton-tipped

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[1] Calgiswab, Inolex, Glenwood, Illinois.

applicators[1] with wood or plastic shafts were used. Nasal secretions were streaked on 5% horse blood agar, MacConkey agar and inoculated into 5 ml of BHI-TS broth containing 2,000 IU penicillin/ml and 1:4,000 thallium acetate. Staphylococcus epidermidis culture was streaked diametrically on each blood agar plate as a nurse colony. Plates were incubated aerobically at 37°C and examined after 24 and 48 hr incubation for colonies suggestive of Pasteurella multocida, Bordetella bronchiseptica and Haemophilus parasuis. Mucoid large colonies on blood agar with morphology and odor suggestive of P. multocida were picked with a straight inoculating needle, inoculated into tryptose phosphate broth[2] and incubated for 24 hr. Colonies with a bluish cast which were non-dextrose fermenters on MacConkey agar were picked after 48 hr incubation, inoculated into tryptose phosphate broth and incubated for 24 hr. Carbohydrate utilization was determined in phenol red broth base containing 0.5% of the following carbohydrates; dextrose[2], lactose[2] and mannitol[2]. Other biochemical activities were determined in urea agar[2], Simmons citrate agar[2] and sulfide-indole-motility (SIM) agar[2]. Small mucoid and smokey gray colonies satelliting the S. epidermidis nurse streak were picked with a bacteriologic loop and streaked on blood agar plate with a nurse colony for the confirmation of H. parasuis.

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[1] S/P Scientific Products, McGraw Hill, Illinois.

[2] Difco Laboratories, Detroit, Michigan.

BHI-TS broth was examined daily for opalescence, tetrazolium reduction as indicated by a color change to pink, pellicle formation and a smooth deposit that swirled when the tubes were agitated. A drop of culture medium showing any of the above changes was placed on BHI-TS agar plates, streaked for isolation and incubated at 37°C under 5% CO_2. The plates were examined daily for mycoplasma colonies. Direct plate epi-immunofluorescence was used to identify mycoplasma colonies (Del Giudice et al. 1967).

Blood Sample Collection

Blood samples were collected from the anterior vena cava at intervals already described. The pigs were restrained by means of a nose snare. Blood was allowed to clot at room temperature and serum was collected and centrifuged at 1700 rpm for 10 min to remove blood cells. Two ml aliquots of serum collected from each pig were stored at -20°C. Blood samples collected at necropsy were treated in the same manner.

Necropsy Technique

At the appropriate time interval, pigs were selected at random according to the protocol already described. They were electrocuted and exsanguinated by severing the right brachial blood vessels. The thoracic cavity was exposed aseptically by reflecting the right thoracic wall dorsally. Samples of pneumonic lung were collected aseptically for bacteriologic and mycoplasmal examination. In cases

where no lesions were visible grossly, approximately 2 gm of lung tissue was collected aseptically from the ventral portions of right and left cardiac lobes. Tracheal secretions were collected aseptically by incising the mid-trachea and inserting a sterile cotton-tipped swab. The lungs were then removed from the thoracic cavity and evaluated for gross lesions of pneumonia. The severity of lesions in each lobe was scored on a scale of 1 to 4 as follows: 1 = no gross lesions; 2 = up to 25% of lobe affected; 3 = 25 - 75% of lobe affected; or 4 = entire lobe affected. The total lesion score for each pig was calculated. Presence or absence of exudate in bronchi and bronchioles was recorded. Bronchial lymph nodes were evaluated for extent of enlargement.

Nasal secretions were collected by cutting the snout transversely at the level of the first premolar tooth with a band saw. The head was then cut into halves along the midline. The septum nasi was removed aseptically to expose the turbinates and cribriform plate. Nasal secretions were collected from the turbinates and anterior aspect of the cribriform plate by using sterile cotton-tipped swabs. The transverse section of the snout was evaluated for evidence of turbinate atrophy and septal deviation. Atrophy was scored on a scale of 0 to 5; 1 = mild atrophy of ventral scroll; 2 = moderate to severe atrophy of ventral scroll; 3 = mild atrophy of dorsal scroll; 4 = moderate to severe atrophy of dorsal scroll; 5 = severe atrophy of dorsal and ventral scrolls; and 0 = no atrophy of dorsal and ventral scrolls of turbinates.

Preparation of Tissues for Histologic Examination

Portions of pneumonic lungs and or grossly normal lungs, bronchial lymph node and trachea were collected. The samples were allowed to remain in 10% buffered formalin for 48 hr and processed by standard paraffin techniques. Sections 5 μm thick were cut and stained with haematoxylin and eosin as described in "Histologic and Special Staining Techniques" (Armed Forces Institute of Pathology, 1968).

The sections were examined with a light microscope for the following lesions; peribronchiolar lymphoreticular hyperplasia, alveolar thickening, presence of inflammatory cells in lumina of bronchi and bronchioles and infiltration of the lamina propria of trachea by inflammatory cells.

Preparation of Tissues for Direct Immunofluorescence Test

The procedure followed was that developed by Dr. C. N. Weng (Unpublished, 1978) at the VMRI. Portions of pneumonic and grossly normal lungs were collected and trimmed to a size of approximately 1 x 1 x 0.5 cm. The pleural surface was not trimmed. Samples were usually taken at the junction of grossly normal tissue and diseased tissue to include bronchus and several small bronchioles (Fig 1). One to two tracheal rings were taken about 3 cm proximal to the bifurcation of the trachea. Tissues were then embedded in OCT[1] fluid

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[1] Lab-Tek Products, Naperville, Illinois.

contained in small, flat-bottom plastic wells[1], 1.6 x 1.3 cm in dimension. These were frozen on dry ice. Frozen tissues were wrapped in aluminum foil and stored at -70°C. The tissues were cut 4 µm thick with a cryo-cut microtome[2] with a knife edge temperature of -20°C. Duplicate slides were made for each lung specimen. Specimens were fixed for 10 min in absolute methanol precooled to 4°C. The slides were air-dried and stored at 4°C.

Preparation of Fluorescein Isothiocyanate (FITC) Conjugated M. hyopneumoniae antibody

Hyperimmune serum against M. hyopneumoniae produced in a pig (#42) by Dr. D. O. Farrington, formerly of VMRI, was used. This antiserum had been stored at -20°C.

The antiserum was thawed and 12 ml of hyperimmune serum was centrifuged in a refrigerated Sorvall superspeed RC2-B[3] centrifuge at 10,000 rpm for 30 min to remove fat droplets. A saturated solution of ammonium sulfate was prepared and the pH was adjusted to 7.2 at 4°C with 1% ammonium hydroxide solution. Ten ml of saturated ammonium sulfate was added dropwise to 10 ml of clear serum to obtain a 50% saturation. This was allowed to stir for 1 hr at 4°C, and centrifuged for 20 min at 8,550 rpm. Globulins were precipitated twice more with ammonium sulfate. After the last precipitation, the supernate was discarded and the sediment was redissolved in 0.05M Tris buffer to

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[1] Linbro Scientific Inc., Hamden, Connecticut.
[2] American Optical Corp., Scientific Inst., Div., Buffalo, New York.
[3] Ivan Sorvall Inc., Newton, Connecticut.

Figure 1. Schematic drawing of pig lung showing sample collection sites for immunofluorescent examination.

RA = Right apical lobe

RC = Right cardiac lobe

RD = Right diaphragmatic lobe

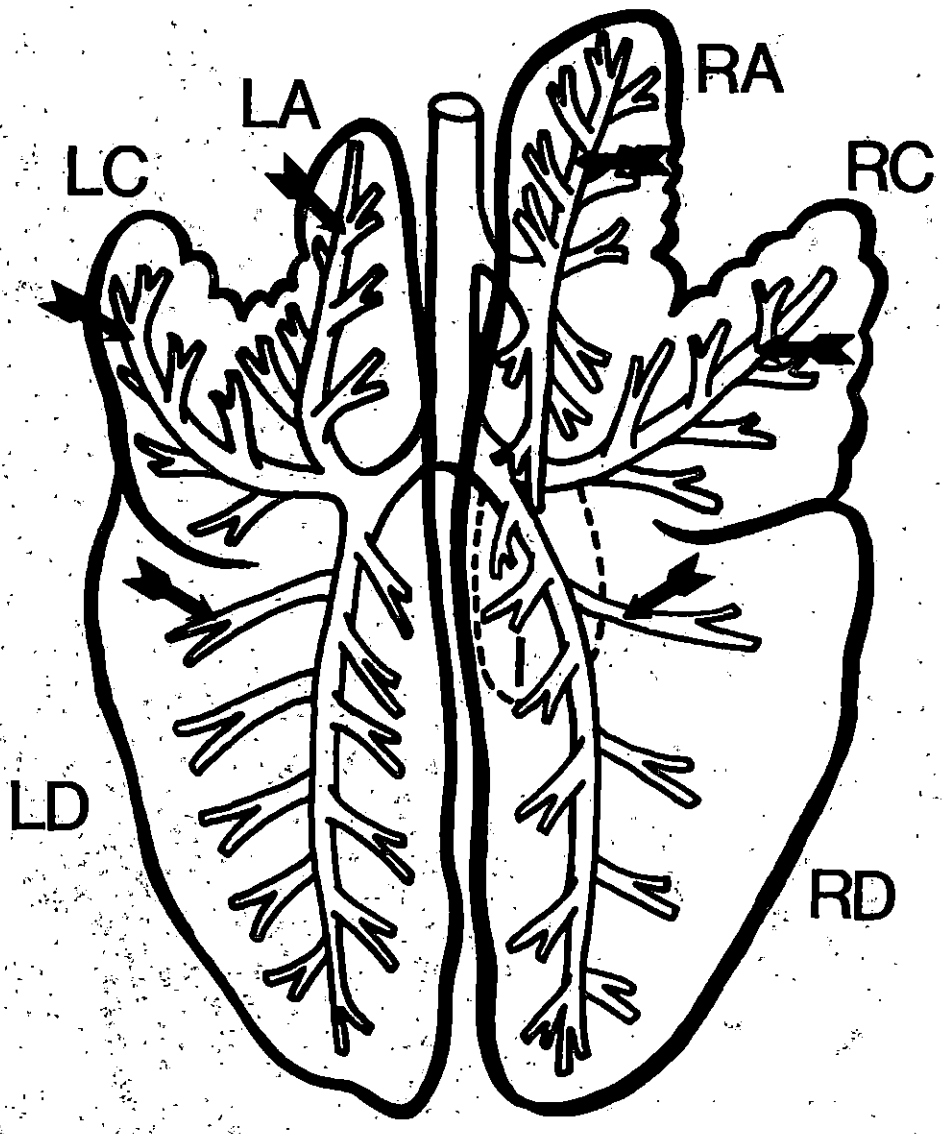
LA = Left apical lobe

LC = Left cardiac lobe

LD = Left diaphragmatic lobe

I = Intermediate lobe

Arrows indicate bronchi serving lobes and sites for collection of samples for immunofluorescent examination.



Schematic drawing of pig lung showing sample collection sites for immunofluorescent examination.

Adapted from Neurand, K., Wissdorf, H. and Messow, C. 1970 Beitrag zur Bronchialverzweigung beim Schwein. Berl. Munch. Tierartzl. Wach. 23:467-470.

half the original volume of serum. The Tris buffer contained 0.05M 2 Amino-2-(hydroxy-methyl)-1, 3 - propanediol (Tris), 0.1M NaCl and 0.1% sodium azide. It was adjusted to pH 8.0 with 1N HCl. The redissolved sediment was dialyzed against 0.05M Tris buffer at 4°C for 24 hr with 4 changes.

Sephadex G200[1] was swollen by adding 7 gm to 400 ml Tris buffer slowly with stirring. The mixture was allowed to settle for approximately 15 min and the supernate was removed by suction. This was repeated 5 times. Coarse Sephadex G25[1] was prepared by adding 5 gm to 50 ml Tris buffer. Sephadex G25 was allowed to swell for 5 hr at room temperature. A 1.6 x 100 cm column[1] was one quarter filled with Tris buffer. Seven ml of Sephadex G25 buffer mixture was added and allowed to settle. The Sephadex G200 buffer mixture was slowly added along the side of the glass to eliminate air bubbles in the column. After the Sephadex had settled, Tris buffer pH 8.0 was pumped through the column for approximately 15 hr.

The dialyzed protein sample was loaded on the Sephadex G200 column and eluted with Tris buffer. A polystaltic pump[2] was set to deliver 7 to 8 ml of eluate per hr. Fractions were recorded by an ultraviolet analyser UA-2[3], set at a wavelength of 280 nm. Tubes of the second peak fraction of the eluate were pooled and dialyzed against PBS, pH 7.2, for 24 hr at 40 times the original volume of serum

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[1]Pharmacia Fine Chemicals Inc., Piscataway, New Jersey.
[2]Buchler Instruments, Fort Lee, New Jersey.
[3]Instrumentation Specialties Co., Lincoln, Nebraska.

with 4 changes of buffer. A Beckman DB-G spectrophotometer[1] was used to determine the protein concentration of the dialysate. Ultraviolet light transmission at 260 and 280 nm wavelength was determined for 1:50 and 1:100 dilutions of IgG dialysate and the protein concentration was determined from standard reference tables. Fluorescein isothiocyanate (FITC), BBL isomer 1[2], was used for conjugation. The FITC was dissolved in carbonate-bicarbonate buffer at pH 8.8 in the proportions of 0.128 mg FITC/0.1 ml carbonate-bicarbonate buffer. The fluorescein to protein ratio was 1:90. The calculated amount of fluorescein carbonate-bicarbonate buffer solution was added slowly to the IgG fraction and the mixture was stirred slowly at 4° C overnight. Fifteen gm of Sephadex G25 (coarse) was swollen in 15 ml of deionized water for 2.5 hr. This was packed in a 2.5 x 40 cm column[3] and 0.01 M PBS, pH 7.4, with 0.1% sodium azide was run through the column for 6 hr. The conjugated IgG fraction was loaded on this column to remove free fluorescein. The conjugate was collected manually in 5 ml aliquots by visual tracing. The aliquots were pooled and concentrated to 12 ml by ultrafiltration using an Amicon ultrafiltration apparatus[4] fitted with filter No. 30[4].

The concentrated conjugate was absorbed with a desoxycholate (DOC) extract of M. hyorhinis strain 7. The DOC extract was prepared by the method of Ross and Karmon (1970). This was allowed to stir

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[1] Beckman Instrument Inc., Fullerton, California.
[2] BBL, Cockeysville, Maryland.
[3] Pharmacia Fine Chemicals, Piscataway, New Jersey.
[4] Amicon Corporation, Lexington, Massachusetts.

overnight at 4°C. The absorbed conjugate was centrifuged at 15,000 rpm for 1 hr. The supernate was stored frozen at -70°C in 1 ml aliquots.

Preparation of Chelated Azo-dye Counterstain

The counterstain was prepared by dissolving 15.6 mg of Eriochrome Black T[1] in 20 ml of N, N dimethyl formamide (Potgieter 1970). A chelating agent was prepared by mixing 50 ml of N, N dimethyl formamide, 20 ml distilled water, 10 ml of 0.1M aluminium chloride and 10 ml 0.1M acetic acid. The pH of the chelating agent was adjusted to 5.2 with 1N sodium hydroxide solution. Deionized water was added to the chelating agent to make a total volume of 100 ml. The 100 ml of chelating agent was added slowly with stirring to the Eriochrome Black T dissolved in N, N dimethyl formamide. The chelated azo-dye was stored at 4°C.

Titration of Conjugate on Frozen Lung Sections

Sections of lung from pigs experimentally infected with M. hyopneumoniae and cut at 4 µm were used. Two fold dilutions of conjugate were made in 0.01M PBS pH 7.4. A few drops of the appropriate dilution of conjugate was added to each specimen and spread evenly. The slides were placed in a rectangular dish containing moist filter paper and sealed with a microtiter plate sealer[2]. The conjugate-

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[1] Hartman Leddon Company, Philadelphia, Pennsylvania.

[2] Cook Engineering, Alexandria, Virginia.

coated slides were incubated for 30 min at 37°C. Excess uncombined conjugate was removed by rinsing the slides in precooled PBS pH 7.4 for 5 min on a stirrer. This was done twice. The sections were then counterstained with the chelated azo-dye for 30 sec and washed twice in precooled distilled water for 5 min. They were allowed to air dry at room temperature, mounted in phosphate buffered glycerin pH 7.4, and examined with incident ultraviolet light. The degree of fluorescence and background autofluorescence was noted. The intensity and extent of fluorescence in bronchi and bronchioles were scored on a scale of 1+ to 4+:1+ = granular fluorescent particles lining the bronchi with no fluorescence in bronchioles; 2+ = thin coating of fluorescent particles lining the bronchi, with no bronchiolar fluorescence; 3+ = intense fluorescent coating of bronchi and presence of scattered fluorescent granules in lumina of bronchioles; and 4+ = intense fluorescent coating of bronchi and bronchioles. No fluorescence was scored as 0.

Conjugated porcine antibody against M. hyopneumoniae was used to detect M. hyopneumoniae antigen and conjugated rabbit M. hyorhinis antibody was used to detect M. hyorhinis antigen in sections. The end-point was taken as that point where minimum nonspecific fluorescence and maximum bronchial and bronchiolar specific fluorescence were seen. Conjugate diluted 1:8 was chosen as the optimal dilution for direct immunofluorescent staining of cryostat sections of frozen lung tissues.

Fluorescence Microscopy

Fluorescence microscopy was achieved with a binocular Leitz Ortholux microscope[1] equipped with a dark field condenser and an Osram HBO 200 mercury vapor lamp. A blue excitatory filter (BG 12) and a yellow barrier filter (K530) were used. A 10X objective lens was used to examine stained sections.

Titration of Conjugate Using M. hyopneumoniae Colonies Grown on Solid Agar Medium

M. hyopneumoniae strain 11 was used. Forty-eight hr broth cultures of M. hyopneumoniae were inoculated on Friis agar medium and incubated at 37°C in an atmosphere of 5% CO_2.

The procedure used in this test was that reported by Del Giudice et al. (1967). Mycoplasma agar plates were examined every other day by using a 10X magnification binocular light microscope. After 8 days of incubation single large colonies were selected. About 3 ml of 0.01M PBS pH 7.4 was poured gently on the agar surface and allowed to stand for 20 min at room temperature. The buffer was poured off and teflon rings were placed on colonies that had been circled. A few drops of different dilutions of conjugate were put in each ring. The plates were incubated at room temperature for 30 min and washed 3 times with PBS pH 8.0. The colonies were examined under vertical U.V. illumination[2] from a 12 volt tungsten filament lamp and a blue

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[1] Ernst Leitz, Wetzlar, West Germany.

[2] American Optical Company, Scientific Instruments Div., Buffalo, New York.

excitatory filter. The highest dilution of conjugate that gave good colony fluorescence with a minimum of background autofluorescence was selected for subsequent epi-immunoflourescence examination. Conjugate diluted 1:4 was chosen as the optimal working dilution for the epi-immunofluorescence test.

Colonies of M. hyopneumoniae had the tendency to wash off the agar surface. A modification of the standard procedure was devised to prevent the loss of M. hyopneumoniae colonies during the washing process. Plates of solid agar medium with large colonies were kept in a refrigerator at $4^{o}C$ for 48 hr. The colonies were then fixed for 60 min with absolute methanol precooled to $4^{o}C$. The standard procedure was followed after fixation.

Specificity of Anti M. hyopneumoniae Conjugate

Various mycoplasmas and acholeplasmas of swine origin (table 2) were grown on either Friis or BHI-TS agar. The time required for maximum growth of colony size differed considerably among the various acholeplasma and mycoplasma species. Therefore, plates were examined daily for maximum colonial size. The isolates were then stained when appropriate and examined by the direct plate epi-immunofluorescence as described previously.

Table 2. Mycoplasma and Acholeplasmas used for the determination of conjugate specificity

Mycoplasma or Acholeplasma species	Strain
M. arginini	G 200
M. hyorhinis	7
M. hyosynoviae	S 16
M. flocculare	Ms 42
M. hyopneumoniae	11
M. hyopneumoniae	11 (Lung inoculum isolate)
M. hyopneumoniae	J
A. laidlawii	M 192
A. granularum	19168

Isolation of M. hyopneumoniae and M. hyorhinis from Lungs

M. hyopneumoniae and M. hyorhinis were isolated from lungs according to the procedure described by Friis (1975). Approximately 1 gm of lung tissue including a bronchus and several small bronchioles was collected aseptically. This was homogenized in a Ten-Broeck tissue grinder[1] using 5 ml of Friis broth medium without antibiotics as diluent. Serial ten-fold dilutions of homogenate were made by transferring 0.2 ml to each 1.8 ml Friis broth with antibiotics contained in rubber stoppered tubes. A final serial dilution of 10^{-7}

[1]Fisher Scientific Company, Chicago, Illinois.

was made. To facilitate the isolation of M. hyopneumoniae, four tubes containing 1.8 ml of Friis broth were inoculated with 0.2 ml of lung homogenate. To each of the 4 tubes, 0.2 ml of filter sterilized hyperimmune rabbit anti M. hyorhinis serum was added. Cycloserine was added to each tube at a concentration 0.15 mg/ml. This was done to suppress the growth of M. hyorhinis if present in the lung homogenate. All tubes were incubated at 37°C on a rotating drum. Initial passages from primary dilutions were made when there was a shift in pH as indicated by a color change of broth from pink to orange (Friis broth) and yellow to reddish color (BHI-TS broth). Tubes were discarded if no color change or turbidity occurred within 21 days of incubation. After 3 to 6 passages, a drop of Friis broth was inoculated on Friis solid agar medium and BHI-TS agar and streaked with a loop. Lack of growth on BHI-TS agar was a negative control check for M. hyopneumoniae, if growth occurred on Friis solid agar medium. A drop of BHI-TS broth was placed on BHI-TS agar and streaked for the isolation of M. hyorhinis. All plates were incubated in a moist chamber at 37°C with a 5% CO_2 atmosphere. The plates were examined every other day for mycoplasmal growth. Colonies were identified by the direct plate immunofluorescence technique.

Lung homogenate was also cultured for bacteria on 5% horse blood agar with a S. epidermidis nurse colony.

Complement Fixation Test

Complement fixing antibodies to M. hyopneumoniae and M. hyorhinis were detected according to a direct microtitration system (Laboratory Branch of the Communicable Disease Center), using the modification of Slavik and Switzer (1972). Lyophilized guinea pig complement[1] was reconstituted with normal unheated swine serum from a 6 to 8 week old respiratory disease-free pig to circumvent the procomplementary activity of swine serum. Five 50% hemolytic units of complement were used and the optimal concentration of antigen was determined by block titration. The endpoints were read as the highest dilution of serum with approximately 70% or more hemolysis. The antibody titers were expressed as reciprocals of the serum dilutions after addition of all test reagents.

Enzyme-Linked Immunosorbent Assay (ELISA) Test

The procedure used to detect antibodies to M. hyopneumoniae was that described by Bruggmann et al. (1977). This procedure was adapted to the use of microtiter plates by Theresa Young at the VMRI.

Microtiter wells were coated with M. hyopneumoniae strain J antigen solubilized with sodium dodecyl sulfate (SDS). Plates were washed with buffer and various dilutions of test serum were added to the microtiter wells. The plates were washed again with buffer and M. hyopneumoniae specific antibodies in test serum selectively bound by the

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[1]Grand Island Biological Company, Grand Island, New York.

antigen were detected by a rabbit anti swine immunoglobulin-peroxidase conjugate. The change in color of the fluid phase after addition of the enzyme substrate, 5-aminosalicylic acid and hydrogen peroxide, was taken as the equivalent of the amount of fixed conjugate. The amount of fixed conjugate is also equivalent to the amount of antibody bound by antigen. The degree of color change was read by the unaided eye on a microtiter plate mirror reader[1]. The endpoint of the test was taken as the color intensity corresponding to the intensity of negative control serum obtained from a respiratory disease-free pig.

Comparison of Sequential Development of Complement-Fixation and ELISA Antibodies

The correlation of the CF test results with results obtained by the ELISA test was determined in a different group of pigs. Fourteen SPF purebred Hampshire pigs farrowed in isolation units at the VMRI were used. The pigs aged between 10 to 12 weeks, were randomised on the basis of sex and litter into two groups. The two groups were kept in isolation and fed 16% protein grower ration with no added antibiotics.

Group 1, consisting of 9 pigs were inoculated endotracheally with pneumonic lung inoculum containing M. hyopneumoniae strain 11 as described previously. Group 2 consisting of 5 control pigs were inculated with sterile Friis broth medium. A preinoculation blood sample was taken. Thereafter, blood samples were taken at 4, 5 and 6 weeks PI.

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[1] Cook Engineering Co., Alexandria, Virginia.

Antibodies to M. hyopneumoniae were determined by the ELISA technique and the modified complement fixation test as described previously.

RESULTS

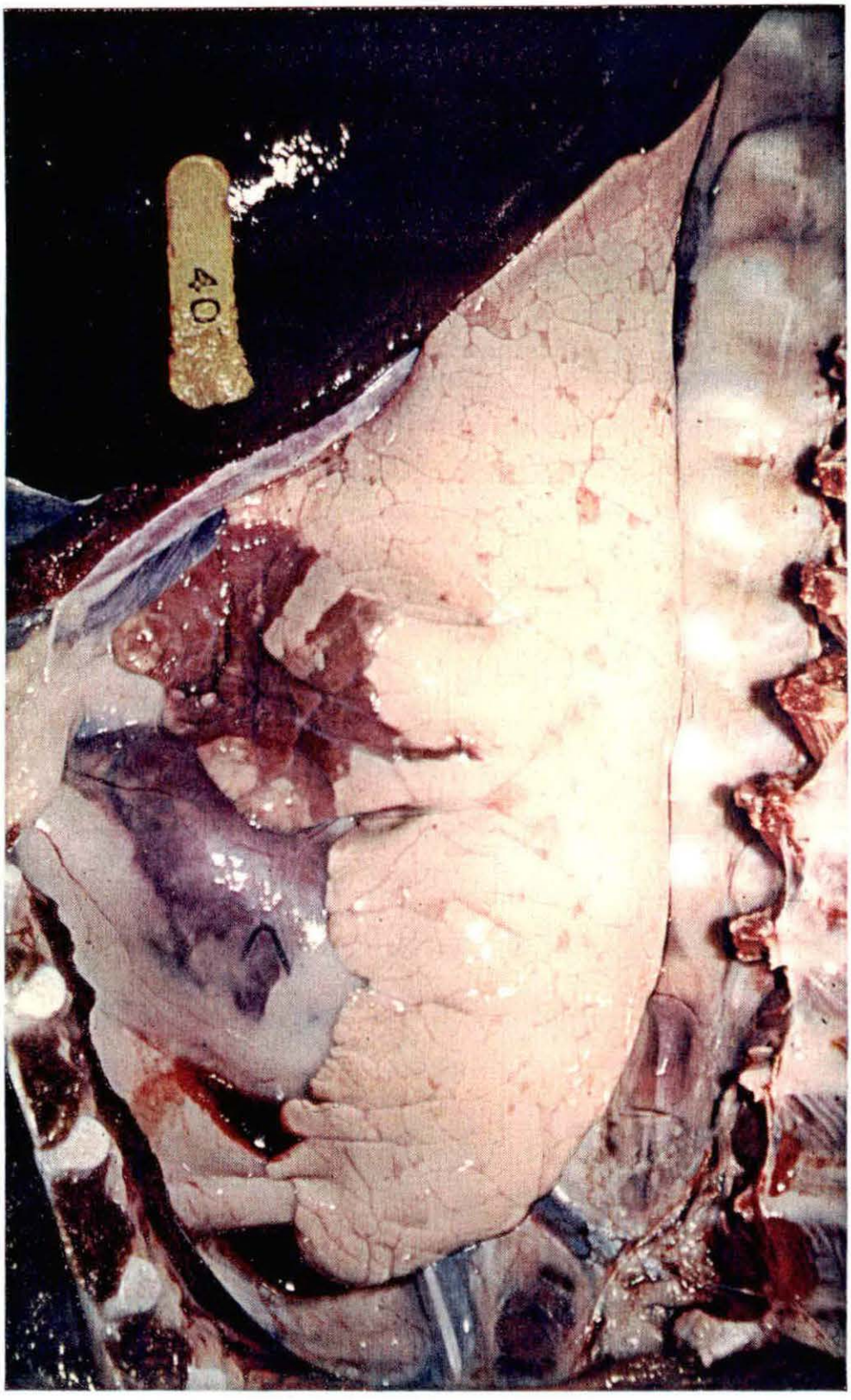
Experimental Inoculation of Pigs

Pigs were observed to be coughing 10 to 18 days post inoculation. Coughing was more pronounced when the pigs were aroused from rest. Appetite and general outlook of pigs remained unaffected throughout the course of the experiment. Dyspnea was not detected in any of the inoculated pigs. However, transient diarrhea of 5 days duration was observed in pig 407G. No treatment was given and none of the remaining pigs in the same pen developed diarrhea.

Necropsy Findings

Gross lesion scores are summarized in Table 3. In general, lesions were confined to the apical, cardiac and intermediate lobes of lungs although a few were seen in the diaphragmatic lobes as well. Lesions consisted of purple areas of consolidation in the ventral portions of the affected lobes (Figure 2). Exudate could be expressed from the cut surface of some pneumonic lesions between 2 and 6 weeks PI. Deep purple lesions were observed 6 to 8 weeks PI and these were clearly demarcated from the adjoining normal appearing lung. Pale tiny gray nodules were observed on the cut surface of affected lung 8 to 12 weeks PI. During this period, pneumonic areas of lung were depressed in comparison to adjoining normal appearing lung. Bands of gray fibrous tissue were present between the lobules. Exudate was minimal or absent when cut surfaces were expressed at 8 to 12 weeks PI. Bronchial lymph nodes

Figure 2. Gross lesions of MPS in lung of a pig inoculated with pneumonic lung suspension containing M. hyopneumoniae. Pig was killed 4 weeks PI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of all but 3 pneumonic lungs were enlarged.

Histopathology

For descriptive purposes, the microscopic pathology was divided into 3 stages; early acute (2-6 weeks PI), acute to subacute (6-10 weeks PI) and chronic (10-12 weeks PI). The histopathologic changes observed in the early acute phase of infection consisted of perivascular and peribronchiolar cuffs of lymphocytes. In some cases there was extension of peribronchiolar lymphoreticular hyperplasia into adjacent interstitial tissue leading to thickening of interstitium. Polymorphonuclear leukocytes (PMNs) were the predominant inflammatory cells in the lumina of bronchioles (Figure 3B). Alveolar spaces occasionally contained proteinaceous edema fluid and alveolar macrophages. Free erythrocytes were sometimes present in alveolar spaces. This change probably resulted from electrocution. Histopathologic changes in the trachea consisted of loss of cilia. In one pig (303B) there was clumping of cilia and a leukocytic infiltration in the lamina propria with PMNs and lymphocytes. No lymphoreticular hyperplasia or PMNs were seen in lung sections from control pigs (Figure 3A). However, atelectasis characterized by a folded appearance of bronchioles and collapse of alveoli was observed in some pigs. Lymph nodes of control pigs were histologically normal.

In the subacute stage, the lymphoreticular hyperplasia around blood vessels, bronchi and bronchioles was prominent and well-organized. In a few pigs, the surrounding lymphocytic reaction compressed the lumina of bronchioles. Septal cells and a few plasma cells were present

Table 3. Necropsy findings in pigs euthanized at various intervals

Pig No.	Group No.	Total lung[a] scores	Enlargement of lymph node	Bronchial Exudate	Turbinate atrophy score
		Two weeks PI			
401G	1	14	2[b]	+	2/1
405G	1	9	2	-	1/1
409G	2	9	2	+	1/1
627G	3	9	2	-	2/2
303B	4	11	3	-	0/1
620B	5	7	1	-	0/1
		Four weeks PI			
402B	1	17	2	-	0/1
611B	1	16	3	+	2/1
406B	2	9	2	-	1/2
305B	3	14	2	+	2/1
632G	4	7	2	-	1/1
413G	5	7	1	-	1/1
		Six weeks PI			
191B	1	11	1	-	2/2
407G	1	7	1	-	0/1
404G	2	12	2	-	2/3
629G	3	8	2	-	0/1
615B	4	7	1	-	2/2
415G	5	7	1	-	1/1

		Eight weeks PI			
280G	1	7	1	-	2/2
293G	1	7	3	-	3/3
302G	4	9	2	-	1/1
304B	3	8	2	-	1/1
612B	2	12	2	-	2/2
410B	5	7	1	-	1/1
		Ten weeks PI			
608G	1	10	2	+	1/1 remodeling
178B	1	14	2	-	1/1
176B	2	8	1	-	1/1
306B	3	8	2	-	1/1
619B	4	9	3	-	1/1
		Twelve weeks PI			
177B	1	10	2	-	2/1
408B	1	9	1	-	0/0
192B	2	7	2	-	0/0
628G	3	9	3	-	0/0
308G	4	14	3	+	0/1
282G	5	7	1	-	0/0

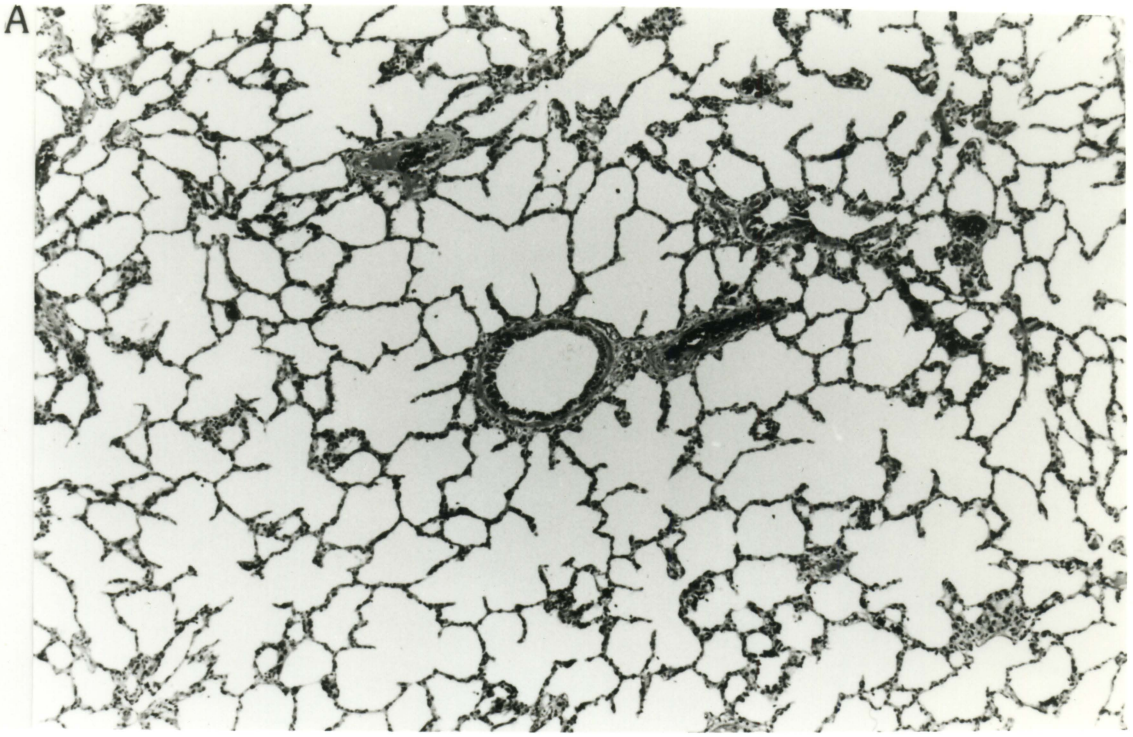
[a] A total score of 7 indicates no gross lesions were observed.
A total score >7 indicated lesions were observed.

[b] 1 = No gross enlargement of lymph node
2 = Slight enlargement of lymph node.
3 = Moderate enlargement of lymph node.
4 = Enlargement approximately equal to twice normal size of lymph node.

Figure 3. Histologic appearance of lung of a control pig inoculated with sterile mycoplasmal medium (A) and pig inoculated with M. hyopneumoniae (B).

A. Note absence of inflammatory cells and edema. H & E stain (100X magnification)

B. Early lymphocytic cuffs around bronchioles, presence of cellular infiltrate and edema in alveoli, and PMNs in lumina of bronchioles. Pig was euthanized 4 weeks PI. H & E stain (100X magnification).


A

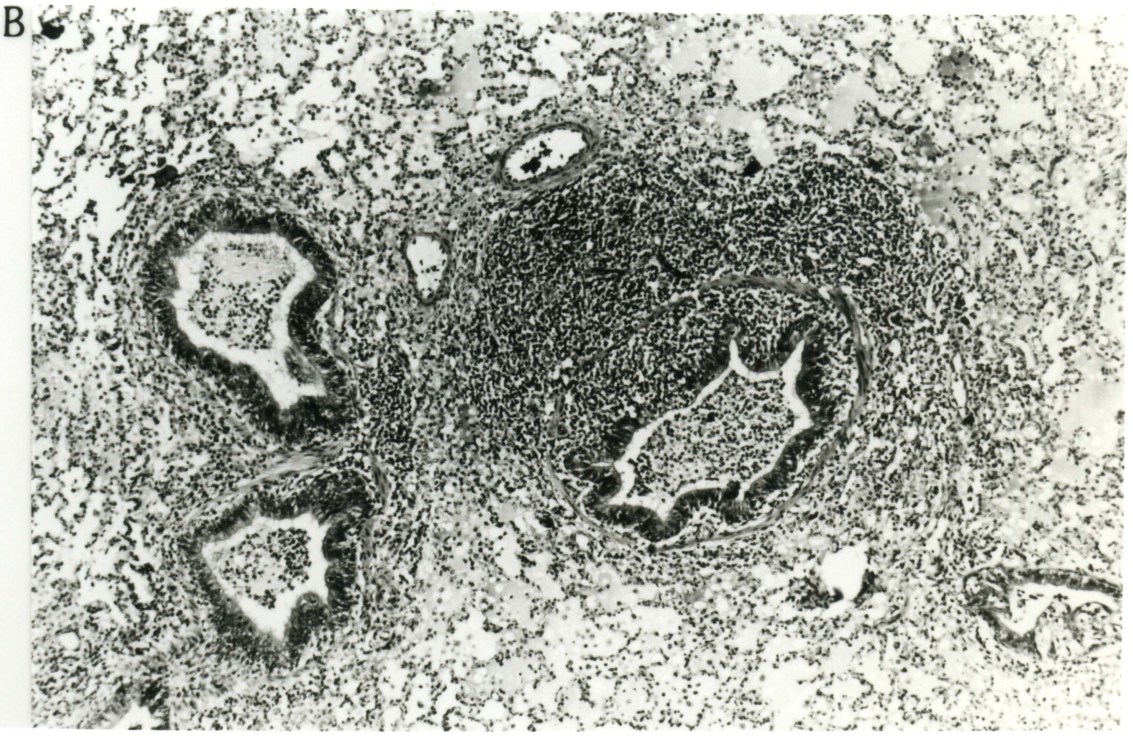
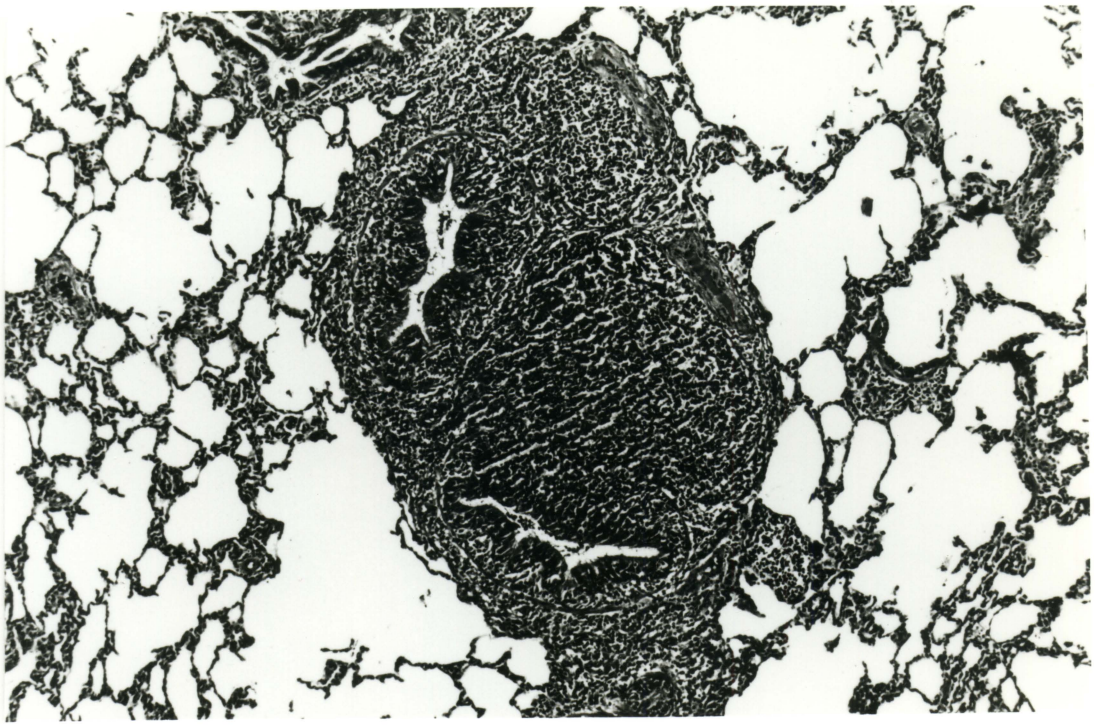

B

Figure 4. Microscopic section of lungs of a pig killed 10 weeks PI.

A. Hyperplastic lymphoid nodule surrounding bronchiole and absence of inflammatory cells and edema in alveoli. H & E stain (100X mangification).

B. Hyperplastic lymphoid nodule compressing lumen of bronchiole. H & E stain (250X magnification).

A



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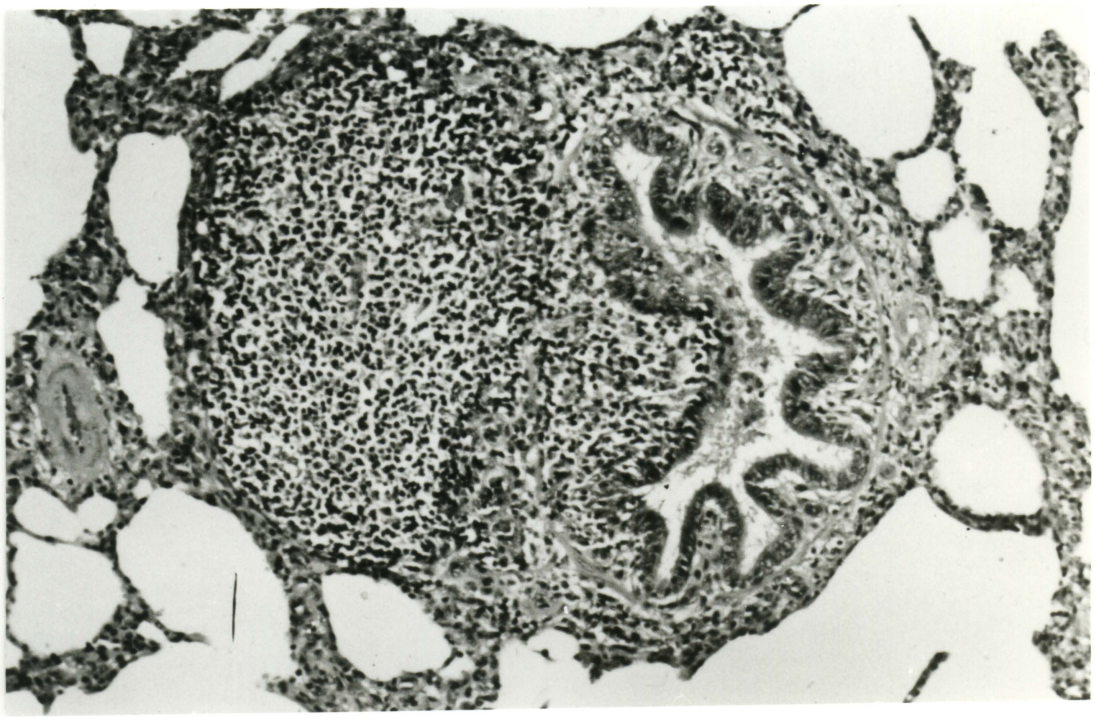


in alveolar spaces. PMNs could be found in the lumina of airways. Although no inflammatory cells were observed in lamina propria of tracheas, clumping and loss of cilia were prominent. Two pigs (632G, 407G) had no visible gross lesions. Microscopically, focal areas of lymphoreticular hyperplasia characteristic of MPS were found in these pigs. Lymph nodes were slightly hyperplastic in cortical areas.

In the chronic stage, the perivascular and peribronchiolar lymphoid nodules were well-organized often compressing the lumina of bronchioles (Figure 4). Exudation into alveolar spaces was minimal in most pigs. However, in pig (308G) there was a diffuse infiltration of PMNs in the interstitium. Lumina of bronchioles and bronchi were filled with PMNs. Moderate depletion of lymphocytes in the paracortical areas of lymph nodes was observed. The sharp line of demarcation between normal and pneumonic areas seen grossly was also observed histologically. Alveolar thickening was the only change found in the control pigs.

Isolation of Bacteria from Lungs, Trachea and Nasal Cavities of Pigs

Bacteria isolated from the lung, trachea and nasal cavities are summarized in Table 4. Pasteurella multocida was the most frequent bacterial isolate from the lungs. It was recovered from lungs and trachea of 8 pigs and nasal cavities of 11 pigs. Although B. bronchiseptica was isolated from nasal cavities of 5 pigs, the organism was not isolated from the lungs or tracheas of any of the pigs infected with this organism. H. parasuis was the principal bacterium isolated from nasal secretions of pigs.

Isolation of M. hyopneumoniae and M. hyorhinis from Lungs

Isolation of M. hyopneumoniae was confirmed by lack of growth on BHI-TS plates and by direct plate epi-immunofluorescence of growth on Friis agar. M. hyorhinis grew luxuriantly on BHI-TS agar and sometimes on Friis agar, if the original broth culture contained both M. hyopneumoniae and M. hyorhinis. M. hyopneumoniae was recovered from 17 of the 18 pigs inoculated with this organism alone. Isolation of M. hyopneumoniae was made in 8 of the 12 pigs inoculated with pneumonic lung inoculum containing M. hyopneumoniae and M. hyorhinis. M. hyorhinis was isolated from the lungs of 8 of the 12 pigs inoculated and from the nasal cavities of 4 of these 12 pigs. A summary of mycoplasma isolation results is presented in Table 5. No mycoplasmas were isolated from control pigs.

Specificity of Conjugate as Determined by Plate Immunofluorescence

Anti M. hyopneumoniae conjugate was found to be highly specific and fluorescence was seen with the lung inoculum isolate of M. hyopneumoniae, strain J and strain 11. No specific fluorescence was observed with colonies of any of the other mycoplasmas or acholeplasmas examined.

Table 4. Bacteria isolated from pigs at necropsy

Weeks PI	Nasal Cavities		
	B. bron.	*H. parasuis*	*P. multocida*
2	2/6[a]	3/6	2/6
4	1/6	3/6	3/6
6	1/6	3/6	1/6
8	0/6	1/6	2/6
10	1/5	0/5	2/5
12	0/6	1/6	1/6

[a]No. pigs positive over numbered cultured.

Trachea			Lungs		
B. bron.	H. parasuis	P. multocida	B. bron.	H. parasuis	P. multocida
0/6	0/6	2/6	0/6	1/6	2/6
0/6	0/6	2/6	0/6	0/6	2/6
0/6	1/6	1/6	0/6	1/6	1/6
0/6	1/6	0/6	0/6	0/6	0/6
0/5	0/5	2/5	0/6	0/6	3/6
0/6	2/6	1/6	0/6	0/6	0/6

Table 5. Mycoplasmas isolated from pigs at necropsy

Weeks PI	Lungs		Nasal Cavities
	M. hyopneumoniae	M. hyorhinis	M. hyorhinis
2	5/6[a]	2/6	2/6
4	4/6	1/6	0/6
6	5/6	1/6	0/6
8	5/6	1/6	1/6
10	5/5	2/5	1/5
12	5/6	1/6	0/6

[a]No. positive over no. cultured.

Direct Immunofluorescent Examination of Cryostat Sections of Lungs

Mycoplasma antigen was detected as bright fluorescent granules in lumina of bronchi and bronchioles or as a heavy fluorescent coating on the epithelial surface of the above structures. Table 6 summarizes the results of direct immunofluorescence with cryostat sections of lung. In general, specific fluorescence was seen in bronchioles and bronchi of lungs that had gross lesions indicative of MPS. The increase and subsequent decrease in fluorescence intensity during the course of MPS disease is demonstrated by Figure 5. Fluorescence was first detected 2 weeks PI and maximum fluorescence was observed 4 to 6 weeks (Figure 6). From 8 to 12 weeks PI, there was a gradual decrease in intensity of specific fluorescence on bronchiolar and bronchial epithelial surfaces (figure 7).

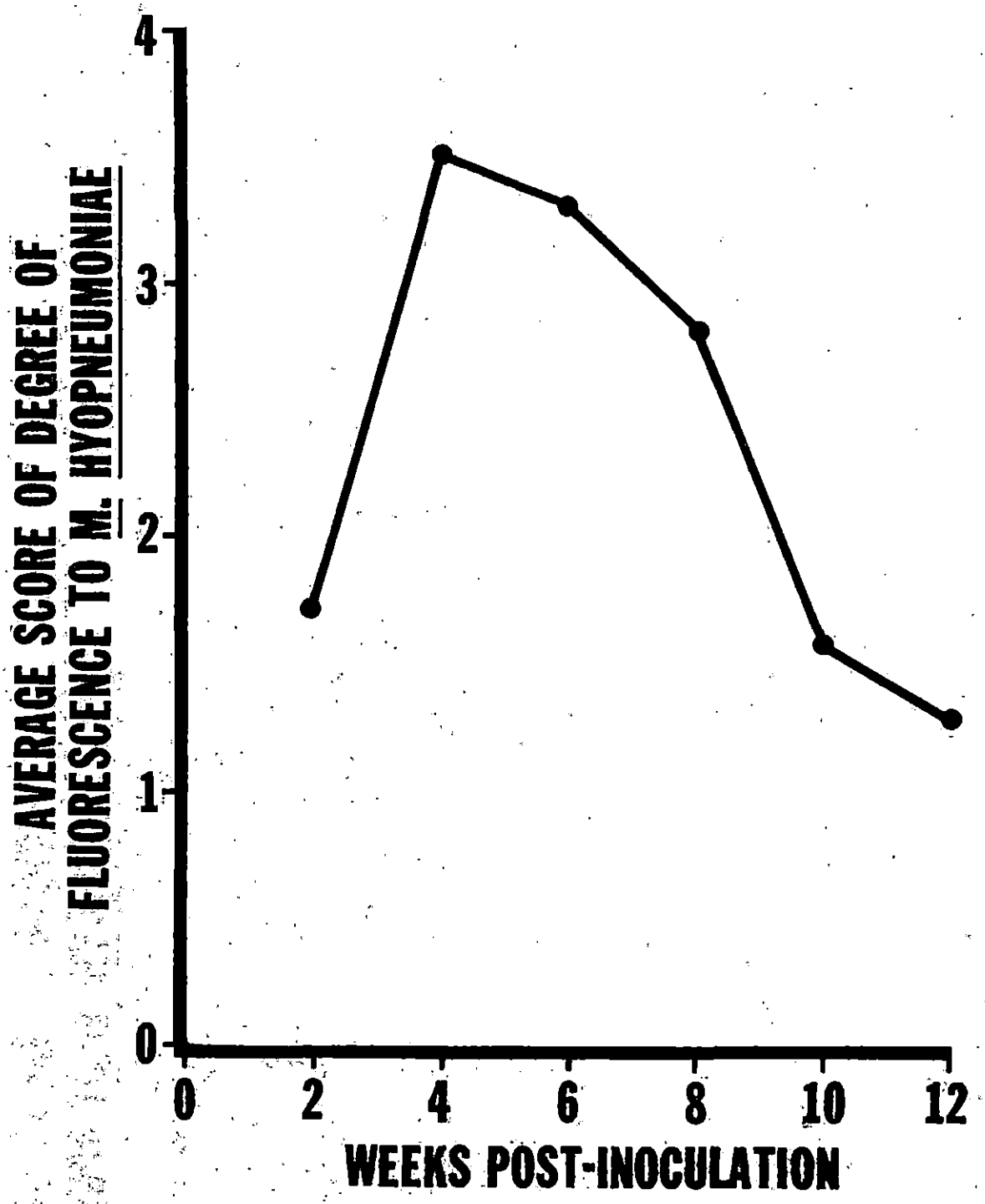
The counterstain produced a red background which contrasted sharply with the yellow-green fluorescence of bronchial and bronchiolar surfaces (Figure 7). Sections stained with conjugated rabbit antibody to M. hyorhinis had a diffuse, green nonspecific background. The counterstain reduced this nonspecific fluorescence but did not completely eliminate it.

Cryostat sections of trachea stained with conjugated M. hyopneumoniae antiserum, revealed specific fluorescence in the trachea in only 2 pigs (402B and 406B) at 4 weeks PI. Fluorescence was limited to the surface of ciliated epithelium of tracheas and no specific fluorescence was observed in lumina of tracheas. No fluorescence was observed in the lumen of any of the tracheal sections stained with rabbit anti M. hyorhinis conjugate.

Table 6. Summary of immunofluorescent examination of cryostat sections of pig lungs.

Weeks PI	No. pigs positive		Average score of degree of fluorescence	
	M. hyopneumoniae	*M. hyorhinis*	*M. hyopneumoniae*	*M. hyorhinis*
2	5/6	0/6	1.8+	0
4	4/6	0/6	3.5+	0
6	4/6	0/6	3.3+	0
8	4/6	1/6	2.8+	1+
10	5/5	1/6	1.6+	1+
12	4/6	0/6	1.3+	0

Figure 5. Relationship between degree of fluorescence and time interval post-inoculation.



Relationship between degree of fluorescence and time interval post-inoculation.

Figure 6. Cryostat section of lung of pig killed 4 weeks PI. Heavy fluorescent coating of M. hyopneumoniae is present on bronchial surface. Fluorescing M. hyopneumoniae antigen is present in lumen also. (150X magnification)

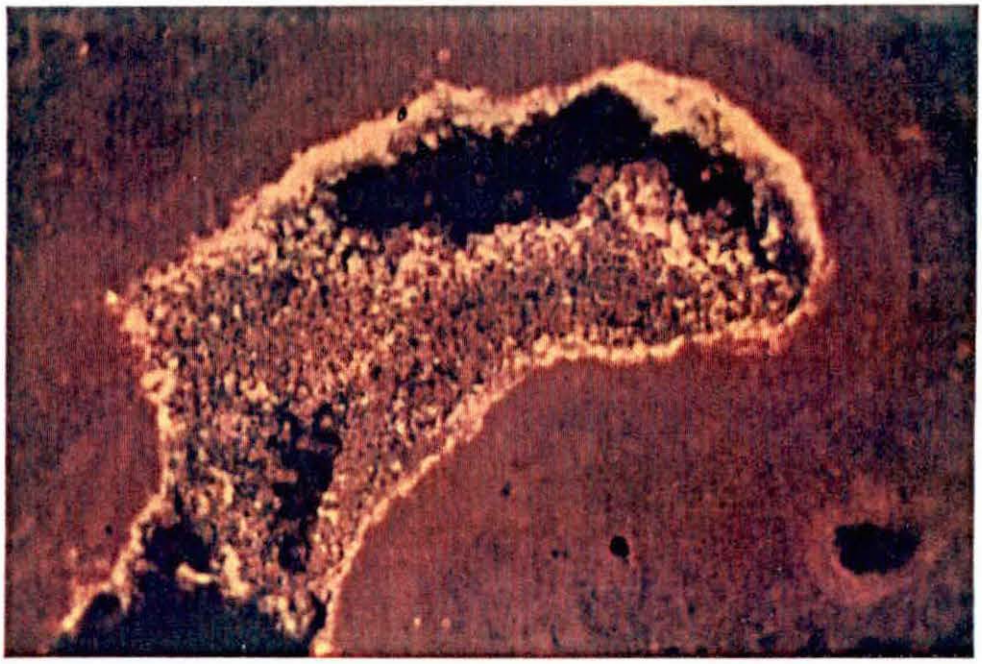
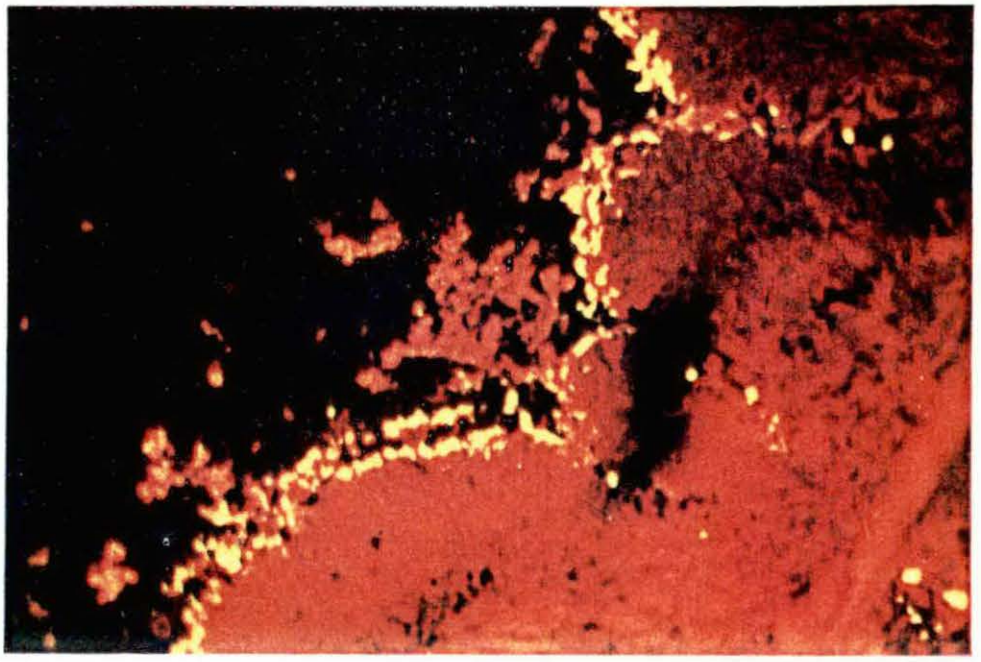


Figure 7. Cryostat section of lung of pig killed 10 weeks PI.

A. Note thin coating of M. hyopneumoniae antigen on bronchial epithelial surface. (150X magnification)

B. Same section not counterstained. Diffuse nonspecific staining is present. (150X magnification)

A



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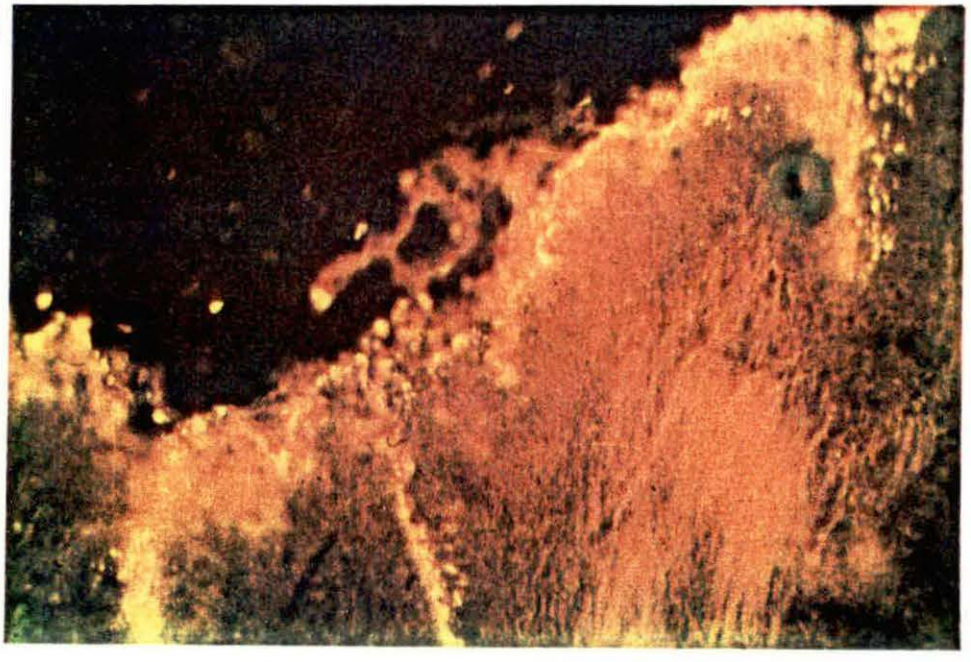


Complement Fixation Test

Complement fixing antibodies to M. hyopneumoniae were detected in sera from pigs collected 2 weeks PI. Control pigs remained serologically negative throughout the course of the experiment. CF antibodies to M. hyorhinis were not detected in any of the pigs inoculated with this organism. Antibody titers of pigs euthanized at various intervals are presented in table 7. At 2 weeks PI, 3 of the 5 pigs inoculated with M. hyopneumoniae had titers to the organism. Maximum seroconversion was demonstrated at 4 weeks PI when 4 of 5 pigs inoculated with M. hyopneumoniae had CF antibodies to the organism.

Comparison of Sequential Development of Complement Fixation and ELISA Antibodies

The results obtained from the CF and ELISA tests are presented in Tables 8 and 9. Preinoculation sera collected at the start of the experiment had no detectable CF or ELISA antibodies to M. hyopneumoniae. Seven of nine pigs inoculated with M. hyopneumoniae PLI had CF antibodies at 4 weeks PI. At 5 weeks PI, 8 of 9 pigs were CF positive and 6 of 9 were CF positive 6 weeks PI. ELISA tests done on the same aliquots of serum revealed that 8 of 9 pigs had antibodies at 4 weeks PI. Eight of 9 pigs were positive at 5 weeks and 7 of 9 pigs were positive at 6 weeks PI. The 5 control pigs were negative for antibodies to M. hyopneumoniae at all bleeding intervals.

Table 7. Complement-fixing antibody titers of pigs euthanized at various intervals

Pig No.	Time interval of Euthanasia	Complement fixing antibody titer	
		M. hyopneumoniae	*M. hyorhinis*
401	2 weeks PI	64[a]	<4
405		8	<4
303		<4	<4
409		32	<4
627		<4	<4
620		<4	<4
	4 weeks PI		
402		64	<4
611		16	<4
406		128	<4
305		128	<4
632		<4	<4
413		<4	<4
	6 weeks PI		
191		8	<4
407		<4	<4
629		8	<4
404		16	<4
615		<4	<4
415		<4	<4

8 weeks PI		
302	16	<4
304	<4	<4
293	128	<4
612	<4	<4
280	<4	<4
410	<4	<4
10 weeks PI		
608	<4	<4
176	<4	<4
178	128	<4
619	<4	<4
306	<4	<4
12 weeks PI		
177	<4	<4
192	<4	<4
308	8	<4
282	<4	<4
628	16	<4
408	<4	<4

[a]Reciprocal of final serum dilation.

Table 8. Complement-fixing antibody titers of pigs inoculated with *M. hyopneumoniae*

Pig No.	Treatment	Weeks PI 0	4	5	6
358	*M. hyopneumoniae* inoculum	<4[a]	32	128	128
364	"	<4	32	64	64
371	"	<4	4	8	<4
372	"	<4	4	16	16
373	"	<4	8	16	<4
375	"	<4	64	64	64
381	"	<4	<4	<4	<4
383	"	<4	<4	16	128
384	"	<4	<4	16	16
	Sterile Mycoplasma broth				
376		<4	<4	<4	<4
377	"	<4	<4	<4	<4
378	"	<4	<4	<4	<4
379	"	<4	<4	<4	<4
380	"	<4	<4	<4	<4

[a]Titers are expressed as reciprocal of highest dilution of serum in which 70% or more of complement was fixed.

Table 9. ELISA titers of pigs inoculated with *M. hyopneumoniae*

Pig No.	Treatment	Weeks PI 0	4	5	6
358	*M. hyopneumoniae* inoculum	<20[a]	1280	320	320
364	"	<20	320	320	320
371	"	<20	80	40	20
372	"	<20	20	160	80
373	"	<20	80	320	80
375	"	<20	320	320	320
381	"	<20	<20	<20	<20
383	"	<20	160	320	320
384	"	<20	40	160	40
376	Sterile Mycoplasma broth	<20	<20	<20	<20
377	"	<20	<20	<20	<20
378	"	<20	<20	<20	<20
379	"	<20	<20	<20	<20
380	"	<20	<20	<20	<20

[a]Reciprocal of final serum dilution.

DISCUSSION

Various procedures have been utilized in the diagnosis of MPS. Notable among these are gross and histologic morphological changes, serology, isolation of the organism and the fluorescent antibody test.

Isolation of the organism is the most ideal for a definitive diagnosis of MPS. However, this is hampered by some factors. The organism is extremely fastidious and exacting in its growth requirements. Secondly, other fast-growing organisms like P. multocida and M. hyorhinis, which are common secondary invaders in MPS, have the tendency to overgrow the slow-growing M. hyopneumoniae. The effect of this overgrowth is to deprive M. hyopneumoniae of nutrients and to cause a pH shift which is unsuitable for the growth of M. hyopneumoniae. Recent suggestions on isolation of the organism by Friis (1975) appear to aid recovery of the organism from a large number of field cases. However, it is tedious, time-consuming and an expensive venture.

Gross and histopathologic changes of MPS are characteristic but not pathognomonic for the disease. Concurrent bacterial infections may superimpose certain morphological changes on primary MPS lesions thus making diagnosis based solely on macroscopic and histologic changes confusing and misleading.

Serological procedures like the complement fixation, indirect hemagglutination and ELISA tests have been used to diagnose MPS. Although antibodies to M. hyopneumoniae have been detected by these procedures, some pigs seroconvert during the late recovery stages of

the disease or not at all. Their use, therefore, appears to be related to diagnosis of MPS on a herd basis.

The main thrust of this work was to evaluate the sequential detection of M. hyopneumoniae antigen in lungs of infected pigs by means of the direct immunofluorescence technique and to compare this with the serological and histological evidence of disease and isolation of the organism. The role of certain bacteria known to be pathogenic for the respiratory system was evaluated also.

M. hyopneumoniae antigen was found by means of the immunofluorescence test on the surface of bronchiolar and bronchial epithelial cells, a finding identical to that reported by L'Ecuyer and Boulanger (1970), Meyling (1971), Livingston (1972) and Giger et al. (1977). Consistent fluorescence was seen only in bronchi and bronchioles. It is therefore important that samples for immunofluorescence examination should always be taken to include these airways, else a false negative result could be obtained.

Maximum fluorescence was observed at 4 to 6 weeks PI and fluorescence persisted up to 12 weeks PI. Possibly the maximum fluorescence observed with M. hyopneumoniae at 4 to 6 weeks PI may be related directly to maximum number of organisms attained at this period. L'Ecuyer and Boulanger (1970) reported that specific fluorescence with M. hyopneumoniae antigen was observed first at 25 days PI and persisted up to 49 days PI. They also observed less intense fluorescence in chronic field cases of MPS collected at slaughter and in experimentally infected pigs in which the lesions were less extensive.

A decrease in the degree and areas of fluorescence with M. hyopneumoniae antigen was also observed in the chronic stages of infection in this study. This reduced fluorescence intensity could indicate a decrease in number of organisms as the disease progressed. The number of mycoplasma antigens detected could have been decreased due to combination with locally produced or transudated immunoglobins especially IgA and IgG. Necrotic epithelial cells were shed from the surface of bronchi and bronchioles and with them mycoplasma organisms. These could be found at times in the lumina of the bronchi and bronchioles by immunofluorescence. During coughing or normal breathing, some of these organisms are shed into the environment thereby helping to spread the infection whilst at the same time causing a decrease in number of these organisms in the lung.

Specific fluorescence was not observed in alveolar walls or spaces. This may be due to the specific requirement of mycoplasmas pathogenic for the respiratory tract to be closely associated with ciliated cells. There are no ciliated epithelial cells in the alveoli; thus, the mycoplasmas could not persist under those conditions. The presence of alveolar macrophages in alveoli may also play an important role in the rapid clearance of mycoplasma cells from the alveoli, thus preventing establishment of infection in these sites.

The chelated azo-dye (Eriochrome Black T) provided an excellent red background which contrasted sharply with the greenish-yellow fluorescent areas of lungs. This good color contrast offset reduced

intensity of fluorescence resulting from use of the counterstain and had the obvious advantage of permitting unambiguous histologic interpretation of cryostat sections of pneumonic lungs and trachea. A disadvantage with the counterstain is the possibility of fluorescence quenching when sections are allowed prolonged contact with the dye because the pH of the dye is lower (5.2) than that of the fluorescein (7.4). The dye did not significantly reduce the diffuse nonspecific staining observed with sections stained with rabbit anti-M. hyorhinis conjugate.

The main objective of adding M. hyorhinis to PLI containing M. hyopneumoniae was to determine whether that organism could be distinguished from M. hyopneumoniae in infected lung tissue by immunofluorescence. M. hyorhinis was detected by IF in only 2 of the 12 pigs inoculated with this organism although the organism was isolated from a higher number of pigs (8). This finding may relate to the ability of M. hyorhinis to colonize the tracheobronchial epithelial surface.

Nonspecific fluorescence is an inherent problem with immunofluorescence techniques. It can be reduced with counterstain or eliminated by treating conjugates with DEAE cellulose (Potgieter 1970), or gel filtration using Sephadex G25. Counterstains often utilized in immunofluorescence are chelated azo-dye and albumin conjugates of lissamine rhodamine RB200 (Malizia et al. 1961). Uncombined dye is a common cause of nonspecific staining (Nairn, 1964; Riggs et al. 1960). Dilution of conjugate is often used to reduce nonspecific fluorescence (Clark et al. 1963). This was found to be true especially in the case

of the plate epi-immunofluorescence test. An original working dilution of 1:4 was found to produce a strong nonspecific background fluorescence and a weak specific fluorescence. Dilution of conjugate to 1:16 produced no nonspecific fluorescence and a weak specific fluorescence for M. hyopneumoniae. A modification of the standard procedure was carried out. M. hyopneumoniae colonies were fixed in situ with cold absolute methanol. By this procedure the nonspecific background fluorescence was eliminated and a strong specific fluorescence was obtained.

The rapid freezing of lung tissue resulted in a remarkable preservation of normal tissue architecture. It also had the additional useful effect of immobilizing antigens which otherwise might diffuse into the tissues during slow freezing and cause confusion in the final interpretation of results. The precise anatomic location of M. hyopneumoniae is a useful guide in the FA diagnosis of lung tissues containing this organism thus histologic preservation of tissues by rapid freezing is necessary. Another possible advantage with freezing is that enzymatic alteration of mycoplasmal antigen may be reduced or totally inhibited thereby preserving the antigen for a considerable length of time.

A negative FA result with positive isolation of M. hyopneumoniae was observed in 3 pigs. This could be explained on the basis of presence of low numbers of M. hyopneumoniae antigen in bronchi and bronchioles of lung. Other possibilities could have been the selection of sample in an area low in M. hyopneumoniae cells, or that samples collected for the test were not identical microbiologically.

The serological indication of M. hyopneumoniae infection was observed in 50% of pigs inoculated with this organism whilst FA results indicated 87% positivity in pigs experimentally inoculated with the organism. Isolation of the organism was achieved in 97% of pigs inoculated. It appears, therefore, that there is a high degree of correlation between FA results and isolation of the organism.

Histopathologic changes observed in the lungs in this study were similar to those described by Pattison (1956), Urman et al. (1958), Hodges et al. (1969) and Whittlestone (1972). The peribronchial and perivascular infiltration and accumulations of mononuclear cells are considered a classical and nearly universal feature of mycoplasmal lung disease (Ross 1978). This lesion is assumed to be part of an immune response to M. hyopneumoniae. The work of Taylor et al. (1974) tends to support this assumption. Using the M. pneumoniae-infected hamster as a model, they demonstrated that treatment of infected hamsters with antithymocyte serum resulted in reduction of peribronchiolar lymphoid infiltration. Cole et al. (1978) demonstrated the mitogenic effect of mycoplasmas in in vitro studies on the interaction of mycoplasmas with lymphocytes of normal mouse, rat, guinea pig and human origin.

The acute stage of the disease was characterized by exudation of edema fluid in alveoli and the presence of PMNs in lumina of bronchioles and bronchi. This response could be partially attributed to the presence of bacteria such as P. multocida. In the chronic stage where PMNs and edema fluid were minimal or absent, P. multocida was not isolated. However, this organism was isolated in one pig at 12 weeks PI. Diffuse edema fluid in alveoli and PMNs in lumina of bronchioles and bronchi

were seen. Therefore, concurrent presence of other bacteria in MPS has the tendency to modify the classical histologic picture of MPS lesions. PMNs were also observed in the lumina of bronchi and bronchioles of lungs from which no bacteria were isolated. In such cases, the migration of leukocytes from peripheral blood to these sites could possibly be in response to the generation of chemotactic factors as a result of complement-mediated antigen-antibody reactions.

In this study, the degree of atrophic rhinitis was not found to relate to the degree of pneumonia. Pigs 280 and 293 had no visible gross lesions of pneumonia yet atrophic rhinitis scores were 2/2 and 3/3 respectively. It is possible that turbinate damage could enhance development of pneumonia if environmental contamination with pulmonary pathogens is high. This is expected to be so because in atrophic rhinitis one of the hosts' barriers against the entry of pathogens into the lungs is compromised, thereby allowing pathogens to enter the lungs more easily.

The pneumonic lung inoculum was found to induce pneumonia in pigs readily. Whittlestone (1979) also observed that the induction of MPS with cultures of M. hyopneumoniae was less certain than with pneumonic lung suspension. A possible explanation could be that the lung particles produced enough mucociliary damage to enhance the colonization between cilia of respiratory surfaces by M. hyopneumoniae.

The method of inoculation might have had an effect on the extent of lesion development. With the endotracheal inoculation, the exact amount of inoculum was delivered direct into the lungs with minimal

loss. This contrasts with intranasal inoculation where it is difficult to quantitate the actual size of inoculum entering the lungs because most of it is swallowed by the pig. The endotracheal procedure does not require use of general or local anesthetics. However, there are two drawbacks to this procedure. Three or 4 people are required to inoculate animals. Secondly, damage to tracheobronchial ciliated epithelium could occur due to the presence of the catheter in the trachea. Trauma to the oropharyngeal mucosa could occur during the process of probing for the larynx.

The strain of M. hyopneumoniae (11) used in this study required 3 to 6 passages in broth before good growth could be obtained on agar. This finding is in agreement with the observation of Etheridge et al. (1979b). They found that the Beaufort strain of M. hyopneumoniae required similar passages in broth medium before good growth could be obtained on agar. The established strains of M. hyopneumoniae are relatively easy to isolate. This contrasts sharply with the isolation of wild-type, unadapted M. hyopneumoniae strains. Successful isolation of wild-type M. hyopneumoniae strains requires an initial high titer of organisms in the lungs (Ross 1979, personal communication). Initial passage of broth cultures containing wild type M. hyopneumoniae might require a heavy inoculum during further passage in broth. This may be so, because for fast adaptation to artificial medium, it may be necessary to reintroduce enzymes produced by the organism into fresh broth medium. Different strains of M. hyopneumoniae may have different nutritional requirements. This phenomenon was observed by Etheridge and

coworkers (1979b). They found that primary isolation of the Beaufort strain of M. hyopneumoniae was not successful in liquid medium described by Friis (1975). However, they achieved good growth in their MH medium when DNA and β-NAD were added to the medium.

Complement-fixing antibody titers were detected in pigs as early as 2 weeks PI. This result concurs with the findings of Boulanger and L'Ecuyer (1968), Takatori et al. (1968), Hodges et al. (1969) and Slavik and Switzer (1972). The early detection of CF titers in these pigs might relate to the massive dose of inoculum given to the pigs. Eight pigs had no CF titers at the time of euthanasia yet all these pigs had lesions suggestive of MPS at necropsy. A possible reason for this finding may be the short duration of time between inoculation and necropsy. The immunological variability and competence of each pig in developing complement fixing titers to M. hyopneumoniae also has to be taken into account. Pig 628 had a persistent CF titer from 2 weeks PI to 12 weeks PI. Switzer (1973) found that the CF test was capable of demonstrating active infection in 83% of pigs infected with M. hyopneumoniae. It therefore appears that the greatest value of the CF test lies in the epizootiological detection of MPS in a particular herd.

Currently, the CF test is not used as a diagnostic tool in the certification scheme for continued participation in the SPF programme. Freedom from MPS, as judged by slaughter checks of lungs of representative pigs from a particular herd, is inadequate. The inadequacy of this

approach is based on the fact that gross or histopathologic lesions of MPS, although typical, are not pathognomonic. Secondly, absence of lesions does not necessarily mean absence of M. hyopneumoniae. The absence of lesions in some cases may be due to concurrent use of antibiotics in the feed or drinking water which have the tendency to suppress development of lesions but not completely eliminate the organisms. When slaughter checks reveal gross lesions, histopathologic examination could be supplemented with the direct fluorescent antibody test for a more definitive diagnosis of MPS. The use of FA test as a rapid specific test in routine diagnostic work could further help to elucidate the role of M. hyopneumoniae in porcine respiratory disease.

PART II. CONTROL OF MYCOPLASMAL PNEUMONIA OF SWINE BY FARROWING SERONEGATIVE SOWS

INTRODUCTION

This study was based on a concept that older sows, which are seronegative for M. hyopneumoniae by the complement fixation test and presumably recovered from the disease, will not transmit the organism to their offspring. The development of this concept was based on work done by Switzer and Preston (1974). Surveys of swine of various ages indicated that sows 2 years of age or older had a very low incidence (10%) of CF antibodies to M. hyopneumoniae.

To test the concept, two trials were conducted wherein CF-negative older sows derived from a herd known to be infected with M. hyopneumoniae were farrowed in a specially designed 10 pen modified open front building. In the third trial, it was planned that five pens would be filled with CF-negative sows and 5 with CF-positive sows. The rationale behind this experimental design was to find out whether the experimental building would prevent the transmission of M. hyopneumoniae from infected pens to noninfected pens by aerosol transmission and also from infected dams to their offspring. Unfortunately, no CF-positive sows could be detected, therefore a replicate of the first and second trials was carried out. The fourth trial was designed to determine the relative ease with which M. hyopneumoniae could be transmitted when some pens in the housing unit contained pigs inoculated with M. hyopneumoniae and other pens contained pigs farrowed by seronegative sows.

MATERIALS AND METHODS

Experimental Animals

The herd maintained by the Animal Science Department at Iowa State University and known as the Swine Teaching Herd was the source of old sows. This herd, consisting of purebred and crossbred swine, is conventional in origin and is known to have a high prevalence of pneumonia caused by M. hyopneumoniae.

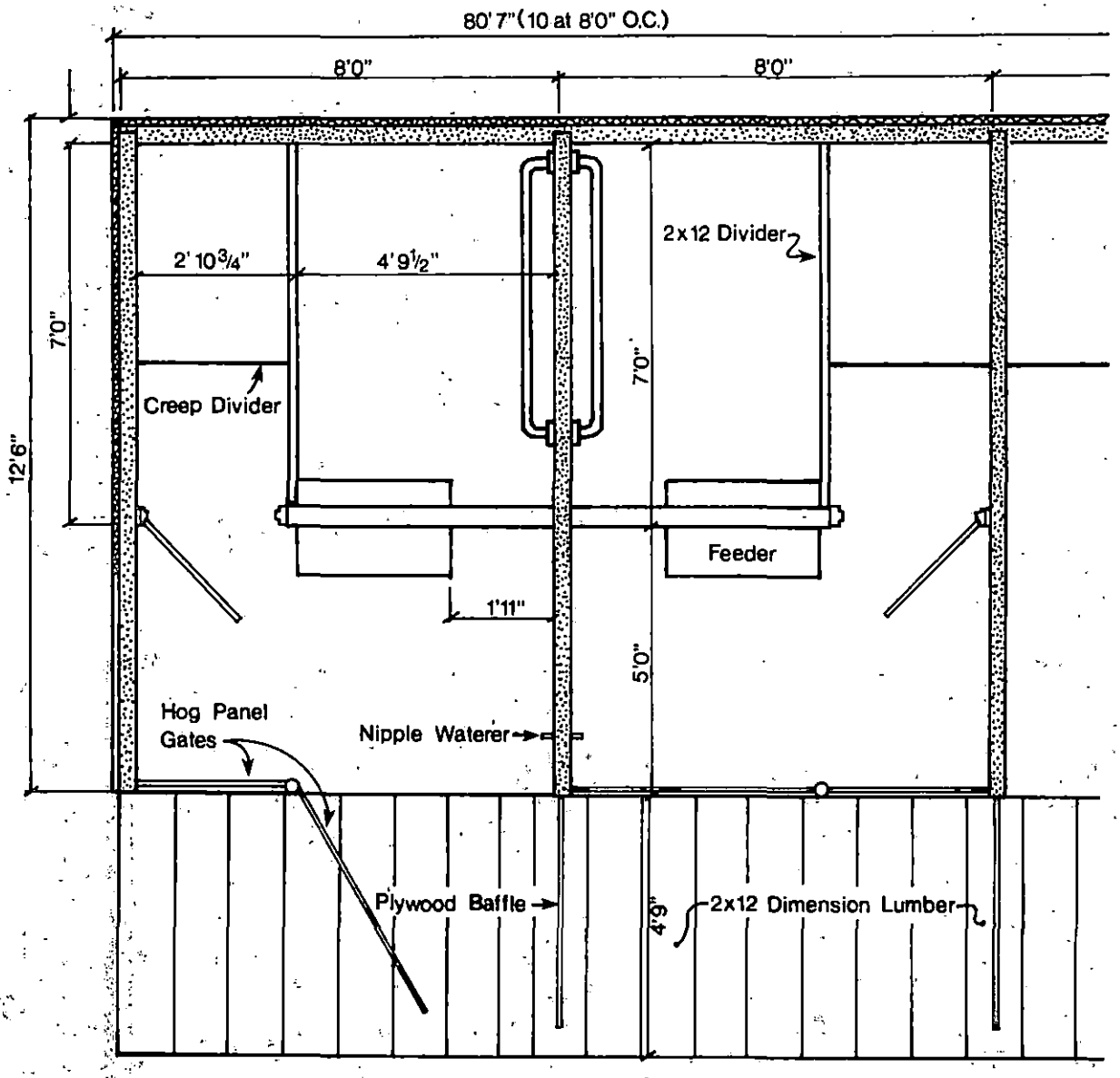
Housing

A building specially designed to minimize aerosol transmission between pens and to prevent direct contact between pigs in adjacent pens was used. The building consisted of ten open-front units separated by floor to ceiling concrete partitions. Each unit was designed to accommodate a sow during farrowing and lactation. Baffles extended 4 feet to the front of each partition. This feature was incorporated into the design to minimize the aerosol transmission between pens. Individual feeders were placed in each unit. Temperature of the floor in each pen was thermostatically controlled. Dimensions of the building are presented in Figure 1.

Experimental Design (Trials 1, 2 and 3)

Sows two years of age or older were selected from the resident herd during late gestation. Selection was based on two successive negative tests for CF antibodies to M. hyopneumoniae and two negative nasal swab culture examinations for B. bronchiseptica. In all the

Figure 1. Floor plan of modified open front unit used for experiment.


80' 7" (10 at 8'0" O.C.)
8'0"
8'0"
2x12 Divider
2' 10 3/4"
4' 9 1/2"
7'0"
7'0"
Creep Divider
12'6"
Feeder
1'11"
5'0"
Hog Panel Gates
Nipple Waterer
Plywood Baffle
4'9"
2x12 Dimension Lumber

three trials, blood samples were collected once before farrowing and once before weaning to ensure that the negative CF status had been maintained. Sera were collected from weaned pigs at 2 weeks of age, 4 to 10 weeks of age and at about 20 weeks of age. Following the 20 week collection of blood samples, some pigs were taken to slaughter and evaluated for lesions of pneumonia and atrophic rhinitis. The remaining swine were returned to the resident herd. Trial one was conducted in the Fall and Winter of 1976-77 by Dr. K. S. Preston. Trial two was conducted in the Spring and Summer of 1977 and trial 3 was conducted in the Fall and Winter of 1977-78. Trial 4 was conducted in the Spring and Summer of 1978.

Origin of Pigs and Experimental Design for Trial 4

Eighteen purebred Hampshire pigs aged between 3 and 4 weeks were obtained from a conventional herd in Fremont, Iowa. The pigs were kept in isolation at the VMRI initially and fed a 16% protein grower ration. When they were 11 to 12 weeks old, 12 of the 18 pigs were inoculated with a pneumonic lung suspension containing M. hyopneumoniae strain 11 as described previously. Six pigs were used as noninfected in-contact controls. Preinoculation blood samples and nasal secretions were collected. Groups of 4 infected pigs and 2 noninfected in-contact control pigs were put in pens 3, 6 and 9. Meanwhile, multiparous sows (2 years or over) were selected from the swine teaching herd as described previously. Pigs weaned from these sows were used for the study. Experimentally inoculated pigs were introduced into the building when

pigs weaned from seronegative sows were between 6 and 8 weeks of age and pigs inoculated with M. hyopneumoniae were in early acute phase of the disease.

Experimental Inoculation of Animals (Trial 4)

The pneumonic lung inoculum (PLI) was prepared as described previously. Pigs were anesthetized intravenously with Thiamylal Sodium (Surital)[1], placed in dorsal recumbency and the skin over the trachea was cleaned with cotton soaked in 70% ethanol. A 2½ inch 16 gauge needle was inserted into the trachea by immobilizing it between the thumb and index finger. A flexible 6 inch tubing attached to a 22 gauge needle was inserted into the trachea through the 16 gauge needle. Five ml of PLI was drawn into a 20 ml syringe to include 15 ml of air. The syringe was attached to the 22 gauge needle and the inoculum was injected quickly into the trachea, with the air helping to clear the tube of inoculum. Twelve pigs were inoculated according to this technique.

Three of the inoculated pigs were necropsied at 4 weeks PI to determine that active infection with M. hyopneumoniae was present. Direct fluorescent microscopy of frozen lung sections and isolation of M. hyopneumoniae from pneumonic lesions of the 3 pigs were performed as described previously.

All pigs in the trial were monitored daily for coughing. Nasal secretions and blood samples were collected at 0, 3, 4, 9, 15 weeks PI.

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[1] Park Davis and Company, Detroit, Michigan.

Slaughter Plant Procedures (All Trials)

Pigs to be slaughtered were weighed, tattooed and taken to Perry, Iowa the day before slaughter. At the slaughter plant the following day, lungs from each pig were collected in plastic bags. The snout of each pig was cut at the level of the first premolar tooth with a hacksaw. The lungs and snouts were quickly transported to the VMRI for evaluation. Lungs were evaluated and scored for gross lesions of pneumonia as described previously. Pieces of lung were collected and processed for histopathology and direct immunofluorescence examination. Nasal turbinates were evaluated for evidence of turbinate atrophy based on a scoring system described previously.

RESULTS

Trial 1 (Conducted by K. S. Preston)

Ten litters of pigs weaned from 10 aged sows grew to market weight without any clinical evidence of respiratory disease. Sera collected from 46 pigs at three intervals (weaning, midway between market weight, and market weight) were negative for CF antibodies to M. hyopneumoniae. A summary of results of CF tests is presented in Table 1. Lungs of 11 pigs representing the various litters were examined at slaughter (Table 2). Two pigs had small focal lesions of pneumonia. Histologic examination of the pneumonic areas of lung showed mild nodular lymphoid hyperplasia around bronchi and bronchioles (Table 3). Sera collected from the 10 sows at 2 weeks postpartum and 4 weeks postweaning indicated none had CF antibodies to M. hyopneumoniae.

Trial 2

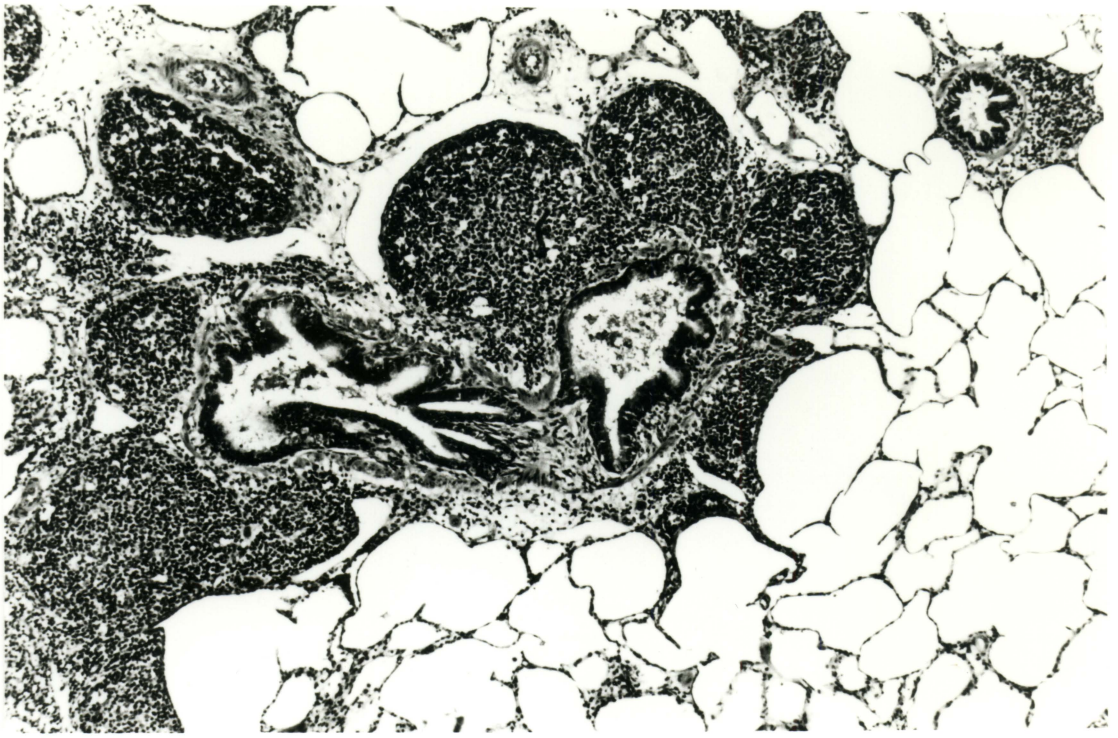
Fifty pigs were weaned from the 10 aged sows. All pigs were negative for CF antibodies to M. hyopneumoniae at each of four intervals from weaning to market weight (Table 1). Clinical evidence of respiratory disease was not observed. Examination of lungs from 27 pigs revealed 3 with focal lesions of pneumonia (Table 2). Histologic examination of the 3 pneumonic lungs revealed nodular lymphoid hyperplasia around bronchi, bronchioles and blood vessels (Figure 2A). Two of the 3 pneumonic lungs had multifocal areas of granulomatous pneumonia characterized by the presence of multinucleated giant cells (Figure 2B) and birefringent crystalline materials in macrophages in

Figure 2. Microscopic lung lesions in slaughter-weight pigs.

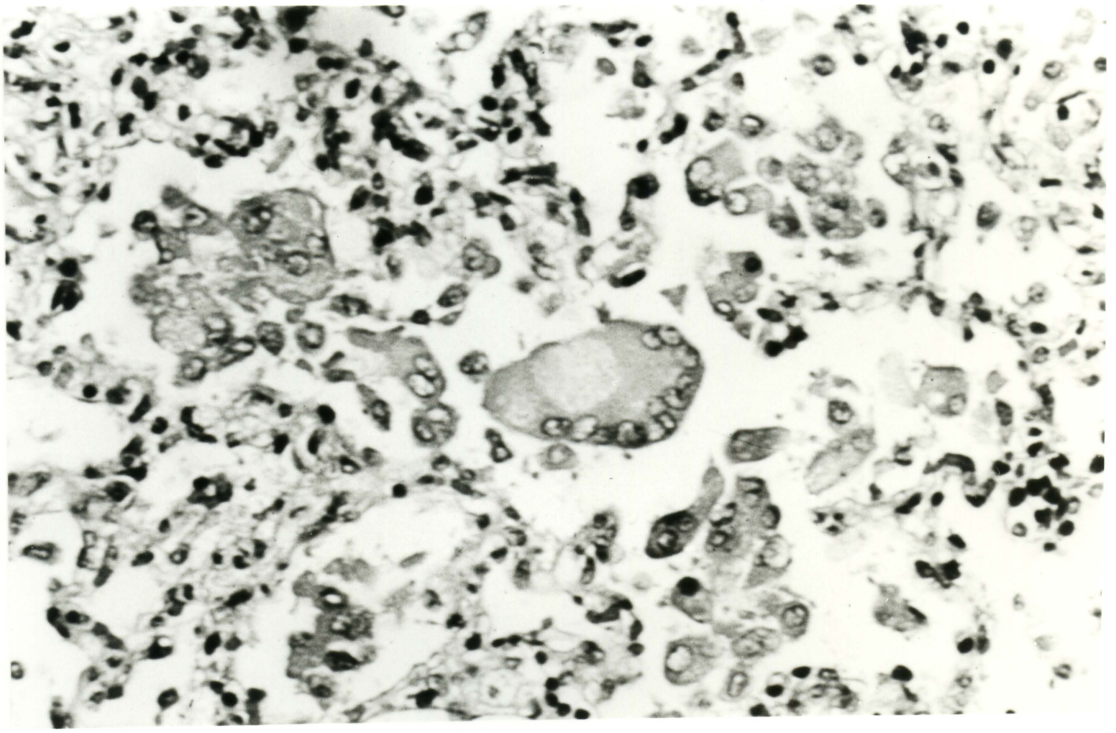
A. Hyperplastic lymphoid nodules surround bronchioles and blood vessels. H & E stain (100X magnification).

B. Diffuse granulomatous pneumonia. Note multinucleated giant cell in the center. H & E stain (400X magnification).

A



B



the lung. H. parasuis and B. bronchiseptica were isolated from the nasal cavities of 2 of the pigs with gross and microscopic lesions of pneumonia. H. parasuis was isolated from the nasal cavities of 10 of 50 pigs weaned from seronegative sows, at the preslaughter bacteriologic check of nasal secretions.

Trial 3

Eighty five pigs were weaned from 10 sows seronegative for CF antibodies to M. hyopneumoniae. Sera collected at 4 intervals from weaning to market weight were negative for CF antibodies to M. hyopneumoniae (Table 1). Clinical evidence of respiratory disease was indicated by mild coughing of about 50 days duration in 2 pens. Post mortem examination of lungs from 28 pigs at slaughter revealed 4 pigs with pneumonia (Table 2). Histopathologic examination of lungs revealed that all 4 of the grossly pneumonic lungs showed multiple areas of nodular lymphoreticular hyperplasia around bronchi and bronchioles. Two of the pneumonic lungs also had multifocal granulomatous pneumonia characterized by multinucleated giant cells, and crystalline materials in macrophages (Table 3). H. parasuis and B. bronchiseptica were isolated from nasal cavities of 2 of the pigs known to have gross and microscopic lesions of pneumonia. No bacteria were isolated from the nasal cavities of the remaining 2 pigs with lesions. H. parasuis was isolated from 12 pigs and B. bronchiseptica from 4 of the 85 pigs weaned from seronegative sows at the preslaughter bacteriologic check of nasal secretions.

Trial 4

A total of 54 pigs were weaned from 7 aged sows CF-negative for M. hyopneumoniae. Experimentally inoculated pigs were observed to begin coughing 10 to 18 days post inoculation. Coughing was more noticeable when pigs were aroused from rest. Very mild coughing was observed in 4 of the in-contact control pigs 5 weeks after contact. The pigs in pens 2, 4, 7 and 10 (seronegative sow origin) were observed to be coughing about 8 weeks after the start of the experiment. Mild coughing was detected 10 weeks postexposure in pigs in pens 1 and 8 (seronegative sow origin). H. parasuis was the predominant bacterium isolated from the nasal secretions of 15 pigs in trial 4. B. bronchiseptica was isolated from the nasal cavity of only 1 pig at the start of the experiment. A summary of bacteria isolated from pigs (seronegative sow origin) is presented in Table 4. H. parasuis was isolated from 4 of the experimentally inoculated pigs and 2 of the in-contact control pigs.

CF antibodies were not detected in pre-exposure sera of pigs weaned from seronegative sows or from inoculated and in-contact control pigs. CF antibodies were detected in sera from the inoculated pigs at 3 weeks PI and persisted up to 15 weeks PI. CF titers of inoculated pigs are presented in Table 5. Three of the 54 pigs weaned from seronegative sows had CF antibodies to M. hyopneumoniae between 3 to 9 weeks post-exposure. The number of pigs CF antibody positive are presented in Table 6. CF antibodies were detected in pigs in 4 pens, namely 1, 2, 4 and 7.

Table 1. Complement fixing antibodies to M. hyopneumoniae in pigs farrowed from sows negative for CF antibodies to M. hyopneumoniae

Trial No.	No. of Litters	No. of Pigs Weaned	No. of pigs CF-positive at various intervals[a]			
			1[a]	2	3	4
1	10	49	0/49[b]	1/46[c]	0/46	ND[d]
2	10	50	0/50	0/50	0/50	0/50
3	10	85	0/85	0/85	0/85	0/85

[a]Interval 1 = 2 weeks post farrowing
2 = 4 weeks post weaning
3 = 10 weeks post weaning
4 = pre-slaughter

[b]Number positive over number tested.

[c] One of 46 pigs was suspicious for CF antibodies to M. hyopneumoniae.

[d] Not done.

Table 2. Macroscopic evaluation of lungs of pigs farrowed from sows negative for CF antibodies to M. hyopneumoniae

Trial No.	No. of litters	No. of pigs weaned	No. of lungs examined at slaughter	Evidence of pneumonia
1	10	49	11/49	2/11
2	10	50	27/50	3/27
3	10	85	28/85	4/28

Table 3. Microscopic evaluation of lungs of pigs farrowed from sows negative for CF antibodies to M. hyopneumoniae

Trial No.	No. of litters	No. of pigs weaned	No. of lungs examined at slaughter	Microscopic lesions	
				Type A[a]	Type B[b]
1	10	49	11/49	2/11	0/11
2	10	50	27/50	3/27	2/27
3	10	85	28/82	4/28	2/28

[a]Nodular lymphoid hyperplasia around bronchi and bronchioles.

[b]Multifocal granulomatous pneumonia.

Three of the experimentally inoculated pigs (Nos. 630, 632, 639) were euthanized by electrocution at 4 weeks PI and necropsies were performed. Gross lesions typical of MPS were observed in all 3 pigs. Lesions consisted of consolidated areas of purple to gray coloration in the ventral portions of the affected lobes. Exudate could be expressed from the bronchi and bronchioles. H. parasuis was isolated from the nasal cavities and tracheas of pigs 630 and 632 whilst B. bronchiseptica was isolated from the nasal cavity of pig 639 and α hemolytic Streptococcus sp was isolated from the lungs of all 3 pigs.

Direct fluorescent antibody staining of cryostat sections of pneumonic lungs of pigs 630, 632 and 639 revealed a fluorescent coating of M. hyopneumoniae antigen in bronchi and bronchioles. Fluorescent particles were present in the lumina of the same structures. Scores of degree of fluorescence in lumina of bronchioles and bronchi are presented in Table 7. M. hyopneumoniae was isolated from all 3 pigs (Table 7). Histopathologic evaluation of H & E sections of the 3 pigs euthanized to verify active M. hyopneumoniae disease indicated lesions typical of MPS. Lymphoreticular hyperplasia around bronchi, bronchioles and blood vessels was seen. Proteinaceous edema fluid and septal cell proliferation were seen in the alveoli.

Gross and histologic evaluations of lungs of pigs weaned from seronegative sows are presented in Tables 8 and 9. Gross lesions of pneumonia were seen in 17 of 34 pig lungs examined at slaughter.

Histologic lesions characterized by lymphoreticular hyperplasia around bronchi and bronchioles were seen in 21 pigs. Healed lesions were seen in lungs of pigs inoculated with M. hyopneumoniae. Lesions consisting essentially of areas of scar tissue and deep fissures were seen in affected lobes. Bronchial lymph nodes were markedly enlarged. Histologically, nodular areas of hyperplasia were seen in 4 of the 6 in-contact controls and in 1 of the 9 experimentally inoculated pigs.

Direct fluorescent antibody examination of lungs of 34 pigs obtained at slaughter from pigs weaned from seronegative sows revealed no M. hyopneumoniae antigen on bronchial and bronchiolar epithelial surface. Lungs from 9 experimentally inoculated pigs and from 6 in-contact control pigs obtained at slaughter also revealed no M. hyopneumonia antigen on bronchial and bronchiolar epithelial surface when examined by the direct fluorescent antibody test.

Table 4. Bacteria isolated from nasal secretions collected from pigs farrowed by sows negative for CF antibodies to M. hyopneumoniae (Trial 4)

Pen No.	No. of Pigs	No. of pigs culture positive at various intervals[a]														
		B. bronchiseptica					H. parasuis					P. multocida				
		0	3	4	9	15	0	3	4	9	15	0	3	4	9	15
1	8	0/8	0/8	2/8	1/8	1/8	1/8	3/8	5/8	4/8	1/8	0/8	0/8	0/8	0/8	0/8
2	12	1/12	1/12	2/7	1/7	2/7	1/12	2/12	1/7	0/7	0/7	0/12	0/12	0/7	0/7	0/2
4	6	0/6	1/6	0/6	0/6	0/6	2/6	4/6	3/6	4/6	2/6	0/6	0/6	0/6	0/6	0/6
5	5	0/5	0/5	0/5	0/5	0/5	0/5	3/5	1/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5
7	8	0/8	1/8	3/8	1/8	0/8	5/8	6/8	2/8	4/8	0/8	0/8	0/8	0/8	0/8	0/8
8	8	0/8	1/8	1/8	3/8	0/8	3/8	2/8	2/8	1/8	0/8	0/8	0/8	0/8	0/8	0/8
10	7	0/7	0/7	2/7	2/7	0/8	3/7	2/7	4/7	1/7	0/7	0/7	0/7	0/7	0/7	0/7

[a]Interval of nasal culture in weeks.

Table 5. Complement fixing antibody titers[a] in swine inoculated with M. hyopneumoniae (Trial 4)

Pen No.	Pig No.	Weeks post inoculation				
		0	3	4	9	15
3	626G	<4	16	8	32[b]	16
	632G	<4	64	64	-	-
	634B	<4	64	64	16	<4
	635G	<4	128	128	<4	<4
	645B[c]	<4	<4	128	64	16
	646B[c]	<4	<4	<4	16	4
6	628G	<4	128	128	64	16
	633B	<4	64	64	16	<4
	639B	<4	128	128	-	-
	642G	<4	64	64	8	16
	641G[c]	<4	<4	8	16	8
	647B[c]	<4	<4	<4	16	<4
9	630B	<4	32	32	-	-
	637B	<4	32	32	8	<4
	638B	<4	64	>128	64	<4
	643B	<4	128	>128	64	32
	644B[c]	<4	<4	<4	32	32
	648G[c]	<4	<4	8	16	<4

[a]CF titer is expressed as reciprocal of highest serum dilution which fixed 70% or more complement.

[b]Pigs were killed. Not done.

[c]Noninoculated in-contact control pigs.

Table 6. Complement-fixing antibody response in pigs weaned from sows seronegative for M. hyopneumoniae (Trial 4).

Pen No.	No. of pigs used for study	No. of pigs CF positive at various weeks post exposure				
		0	3	4	9	15
1	8	0/8	2/8	1/8	4/8	1/8
2	12	0/12	1/12	1/7[a]	3/7	2/7
4	6	0/6	0/6	2/6	0/6	0/6
5	5	0/5	0/5	0/5	0/5	0/5
7	8	0/8	0/8	1/8	6/8	2/8
8	8	0/8	0/8	0/8	0/8	0/8
10	7	0/7	0/7	0/7	0/7	0/7

[a]Five pigs were removed to avoid overcrowding.

Table 7. Direct immunofluorescent test and isolation of M. hyopneumoniae from lungs of pigs inoculated (Trial 4) with the organism and euthanized 4 weeks PI

Pig # and Lobe of lung	Degree of Fluorescence		Isolation of	
	M. hyopneumoniae	M. hyorhinis	M. hyopneumoniae	M. hyorhinis
630LC[a]	1+	-	+[b]	-
630RC[c]	1+	-		-
632LC	2+	-	+	-
632RC	3+	-		+
639LC	4+	-	+	-
639RC	4+	-		+

[a]Left cardiac lobe.

[b]Isolation was made from pooled samples of left and right cardiac lobes.

[c]Right cardiac lobe.

Table 8. Macroscopic evaluation of lungs and turbinates of pigs farrowed from sows negative for CF antibodies to M. hyopneumoniae (Trial 4)

Pen #	No. of pigs used for study	No. of lungs examined at slaughter	Evidence of pneumonia	Evidence of turbinate atrophy
1	8	1/8	1/1	1/1
2	12	7/7	3/7	2/7
4	6	6/6	2/6	3/6
5	5	5/5	1/5	0/5
7	8	7/8	5/7	4/7
8	8	4/8	3/4	2/4
10	7	4/7	2/4	1/4

Table 9. Microscopic evaluation of lungs of pigs farrowed from sows negative for CF antibodies to M. hyopneumoniae (Trial 4)

Pen #	No. of lungs examined at slaughter	Microscopic lesions Type A[a]	Type B[b]
1	1/8	1/1	0/1
2	7/7	3/7	1/7
4	6/6	1/6	1/6
5	5/5	5/5	0/5
7	7/8	5/7	0/7
8	4/8	3/4	0/7
10	4/7	3/4	0/4

[a]Lymphoreticular hyperplasia around bronchi, bronchioles and blood vessels.

[b]Diffuse granulomatous pneumonia.

DISCUSSION

The fundamental hypothesis of this study was that older sows seronegative for M. hyopneumoniae by the CF test do not transmit the disease to their offspring. Results obtained from the first three trials tend to support this hypothesis. None of a total of 184 pigs weaned from 30 seronegative sows evinced antibody responses to M. hyopneumoniae. One pig had a suspicious titer during the 2nd bleeding interval but this was not observed again during the 3rd bleeding. The seronegative status maintained by pigs weaned from seronegative sows is in agreement with the observations of Preston (1976). Also, none of the sows seroconverted before or after farrowing. This indicated that transmission of MPS from the resident herd, which was only 100 yards away from the experimental building, did not occur and that the sows did not experience an exacerbation of old MPS infections. The aged multiparous sows CF negative to M. hyopneumoniae could have evolved this status possibly by being actively immune to most pathogenic organisms including M. hyopneumoniae that are enzootic in the herd. By the time these sows farrow for the 2nd or 3rd time, they may no longer be excreting many of the potential pathogens enzootic in the herd or are excreting them at a low rate.

Gross lesions of pneumonia were observed in 9 of 66 lungs examined at slaughter in trials 1, 2, and 3. Lesions of MPS are not pathognomonic and it is therefore dangerous to ascribe the gross lesions observed in this study to M. hyopneumoniae. Microscopic lesions characteristic

of MPS were observed in 9 lungs showing gross lesions of pneumonia. This is an indirect evidence of M. hyopneumoniae involvement but it is not conclusive. Multifocal granulomatous pneumonia was seen in 4 lungs in trials 2 and 3. The presence of birefringent crystalline material in macrophages might have been due to the inhalation of either fiber glass used for insulation or plant material inhaled during feeding. Since plant materials are biodegradable, it is possible to speculate that the birefringent crystals could have been inhaled fiber glass particles used in insulation. However, the exact nature of these crystals was not determined.

Gross pneumonic lesions were observed in 17 of 34 pigs weaned from seronegative sows in trial 4. Histologic lesions very characteristic of MPS were observed in 21 pigs. The number of pigs showing histologic lesions in this trial was more than those in trials 1 to 3 in which no M. hyopneumoniae infected pigs were put into adjacent pens. This might have suggested MPS was spread to adjacent pens, thereby, increasing the number of pigs affected with the disease. The nine pigs experimentally inoculated with M. hyopneumoniae had resolved lesions characterized grossly by deep fissures and fibrous bands in affected lobes. Histologic lesions were resolved in 8 of 9 pigs inoculated with M. hyopneumoniae. Resolution of MPS lesions begins about 6 weeks PI if primary infection is not complicated by other pulmonary pathogens. The absence of lesions in these pigs, killed 4½ months PI may also indicate successful clearance of M. hyopneumoniae from the lungs. Histologic lesions in pigs weaned from seronegative sows in trial 4

were found to be more active, with lymphoid nodules festooning the bronchi and bronchioles. Exudation in alveoli was minimal.

Antibodies to M. hyopneumoniae were detected in experimentally inoculated pigs at 3 weeks PI. CF titers in uninoculated, in-contact control pigs were detected first at 4 to 9 weeks PI. The development of CF antibodies at 4 weeks post contact exposure may be a useful guide in checking pigs for CF antibodies to M. hyopneumoniae. It can also be used as a time interval guide in the epizootic investigation of field cases of mycoplasmal pneumonia of swine. Complement-fixing antibodies to M. hyopneumoniae were detected in 3 pigs weaned from seronegative sows at 3 weeks post-exposure, in 5 pigs at 4 weeks post-exposure, 13 pigs at 9 weeks post-exposure and 5 pigs at 15 weeks post-exposure. The detection of CF antibody titers in pigs that were originally CF negative may indicate two things. Firstly that, MPS might have spread from pen to pen from the experimentally inoculated pigs. Secondly, some of the seronegative sows might have been harboring infection which was not detected serologically by the CF test. Preston (1976) utilized the microtitration CF test for a year to detect and remove CF positive breeding animals from 10 herds. This resulted in a definite reduction of the incidence of clinical signs in 9 herds. However, at the end of one year, CF positive animals were disclosed in 9 of the 10 herds. The reasons for these outbreaks were not known. Nonspecific reactions in the CF test have been observed. Whether this nonspecific reaction is due to shared antigenic determinants between other respiratory pathogens and M. hyopneumoniae is not known.

M. hyopneumoniae antigen was not detected by immunofluorescence in 34 lungs of pigs weaned from seronegative sows, 9 lungs of pigs inoculated with M. myopneumoniae and 6 in-contact noninoculated control pigs. This observation may be taken as an extrapolation of the findings in the first section of this thesis, where minimal fluorescence with M. hyopneumoniae was observed 8 to 12 weeks PI.

In the 4th trial, the principal bacterium isolated from the nasal cavities of pigs weaned from seronegative sows was H. parasuis. A greater number of pigs was found to harbor this organism in their nasal cavities at 3 weeks post-exposure. A decrease in number of pigs positive for the organism was observed through 4, 9, 15 weeks post-exposure. A possible explanation for this decrease could be that local secretory IgA production by immunocompetent cells in the nasal submucosa, reduced or eliminated the Haemophilus organisms from the nasal cavities of these pigs. B. bronchiseptica was isolated from a few pigs. In this case too, the number of positive pigs decreased as the pigs became older. Although B. bronchiseptica was not isolated from any of the sows used for this study, the presence of B. bronchiseptica in the pigs might indicate a very low level of infection in the sows, indeed too low to be detected by the nasal culture technique. These bacteria, commonly associated with respiratory disease syndromes in pigs, were evaluated to determine their contribution to pneumonia in pens where coughing was observed. Turbinate atrophy was highest in pigs in pen 7 of trial 4 from whom H. parasuis and B. bronchiseptica were

consistently isolated. Five out of 7 pigs from this pen examined at slaughter had gross lesions of pneumonia.

The eventual control of MPS necessitates the availability of a serologic diagnostic procedure that is capable of detecting active or latent infections in pigs, and the subsequent elimination of such positive carriers. Since the pig is the only known reservoir for M. hyopneumoniae, periodic testing of swine and removal of any serologically positive pigs might enable MPS free herds to be established.

Adegboye (1978) observed a skin hypersensitivity reaction in pigs characterized histologically by perivascular mononuclear cell accumulation in the dermis. This observation was made in tests performed when pigs were in the late recovering stage of infection with M. hyopneumoniae. The test should be further evaluated on the lines of the intradermal tuberculin test, to determine its efficacy and applicability in detecting swine chronically infected with the organism.

Slavik and Switzer (1979) observed that latex agglutination antibodies in pigs persisted longer than CF antibodies and suggested the use of the LAT and CF to detect both active and chronic MPS in a herd. Armstrong et al. (1980) compared the ELISA, indirect hemagglutination, indirect immunofluorescence and complement fixation tests as immunodiagnostic tools for MPS. They concluded that the CF and IHA tests were the most useful and practical serological tools for the diagnosis of MPS.

It is therefore necessary to use a combination of tests in diagnosing

MPS as a prelude to controlling the disease in a herd. This opinion is shared by McKean et al. (1976) who observed that individual animal and herd data revealed considerable variation in the presence of gross and histological lesions, CFT, and LAT titers and the isolation of M. hyopneumoniae.

Although the CF test suffers from some degree of nonspecificity, it is currently the diagnostic serological test of promise in the overall control of the disease. Its use in selecting aged CF-negative sows may, over the long run, contribute to the possible control of the disease. However, the practicality of this procedure may depend on other auxiliary measures. Older sows are less frequently infected with M. hyopneumoniae than gilts, thus piglets born to old sows are less likely to be infected with the organism than piglets nursing gilts. New breeding stock can be kept in isolation for about 4 weeks during which time the CF test and daily clinical observation of animals could be done. Replacement gilts where possible should be purchased from "MPS-free" herds or SPF herds.

SUMMARY

An improved direct fluorescent antibody test was evaluated for specificity and efficacy in diagnosing mycoplasmal pneumonia of swine. A sequential study was carried out in which pigs inoculated with a pneumonic lung suspension containing M. hyopneumoniae strain 11, were killed at 2, 4, 6, 8, 10 and 12 weeks post-inoculation (PI).

Fluorescent coating of M. hyopneumoniae antigen was detected primarily on bronchial and bronchiolar epithelial surfaces of lungs with gross lesions of pneumonia. Fluorescence was observed to be most intense at 4 to 6 weeks PI with a tendency to decrease in intensity from 8 to 12 weeks PI. This suggested a decrease in number of M. hyopneumoniae cells in the more advanced stages of the disease. The use of a counter-stain (chelated azo-dye) provided an excellent color contrast and permitted unambiguous interpretations of results.

Isolation of M. hyopneumoniae correlated well with positive immunofluorescence results. The FA technique seems ideal for MPS diagnosis in situations where isolation of the organism is impracticable, especially when tissues have been heavily contaminated with other bacteria notably P. multocida and M. hyorhinis.

Presently, macroscopic and microscopic evaluation of lungs of slaughter-weight pigs are used in certification for continued participation in the Specific Pathogen Free (SPF) herd program. Supplementation of histopathology with the direct fluorescent antibody test could provide a more definitive diagnosis and help in accurately

pinpointing herds with MPS problems.

The hypothesis that older sows seronegative for M. hyopneumoniae by the CF test do not transmit the disease to their offspring was evaluated. Seronegative multiparous sows (2 years or older) were selected from herds known to have a high prevalence of M. hyopneumoniae disease, and farrowed in modified open front units. None of 184 pigs weaned from 30 seronegative sows evidenced clinical mycoplasmal pneumonia disease or developed complement-fixing antibodies to M. hyopneumoniae. The aged sows did not transmit the disease to their offspring possibly because they were no longer shedding M. hyopneumoniae or were shedding the organism at a very low rate.

Between pen transmission of MPS was evaluated further in the modified open front building using pens alternately filled with pigs weaned from seronegative sows and pigs inoculated with M. hyopneumoniae. Complement-fixing antibodies to M. hyopneumoniae were detected in 3 pigs weaned from seronegative sows at 3 weeks post-exposure, in 5 pigs at 4 weeks post-exposure, in 13 pigs at 9 weeks post-exposure and in 5 pigs at 15 weeks post-exposure. Eighteen weeks after the M. hyopneumoniae inoculated pigs had been introduced into the modified open front building, 34 pigs (seronegative sow origin) were taken to slaughter. Gross lesions of pneumonia were observed in 17 of 34 pigs weaned from seronegative sows whilst histologic lesions characteristic of MPS were observed in 21 pigs (seronegative sow origin). Fluorescent antibody test evaluation of lungs from pigs weaned from seronegative sows as well as those inoculated 18 weeks previously with M. hyopneumoniae

were negative for fluorescing M. hyopneumoniae antigen in bronchi and bronchioles when examined after slaughter. This may suggest minimal concentration of antigen or clearance of mycoplasmal organism in chronic stages of the disease. The results of the 4th trial suggest that MPS was spread from pen to pen but that disease in seronegative pens was very mild.

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