Response of swine to *Mycoplasma hyopneumoniae* vaccination

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INTRODUCTION

Since the recognition of chronic pneumonia of swine as a disease entity distinct from Swine Influenza, its etiology has been the subject of much conflict and confusion. The disease has been variously called "Infectious Pneumonia of Pigs", "Swine Enzootic Pneumonia", and "Virus Pneumonia of Pigs". Recent research by independent groups of investigators has established that the most common cause of chronic pneumonia in swine is a mycoplasmal infection. The causative mycoplasma has been characterized and named Mycoplasma hyopneumoniae. In recognition of the importance of etiology in the description of the disease, the name "Mycoplasmal Pneumonia of Swine" will be used in the present study.

Mycoplasmal pneumonia of swine is characterized clinically by non-productive cough, high herd morbidity, low mortality, loss of vigor, retarded growth, and decreased feed efficiency. Gross pneumonic lesions occur most frequently in the apical, cardiac, and intermediate lobes of the lungs, and are marked by grey to reddish colored areas clearly demarcated from normal lung tissues. The lesion is characterized by peribronchiolar and perivascular lymphoid hyperplasia, alveolar interstitial thickening, septal cell proliferation and neutrophil infiltration.

Mycoplasmal pneumonia of swine has been reported in almost all major swine producing countries of the world. The disease has been described as the world's most prevalent and most important respiratory disease of swine. Economic loss which the disease causes to swine industry has been estimated in hundreds of millions of dollars annually.

Efforts at control of the disease have been centered on measures which

break the cycle of infection. However, such measures as depopulation of infected herd and repopulation with surgically derived or specific pathogen-free (SPF) swine have not proved very successful. Chemotherapy and chemoprophylaxis have proved ineffective in the elimination of the causative mycoplasma from the lungs and in prevention of spread of the organism to in-contact pigs. There is, therefore, a need for research on the development of autogenous vaccines and on the application of immunization as an alternative or complementary control measure.

The present study was designed to evaluate the ability of M. hyopneumoniae vaccines to provide protective immunity in susceptible pigs, and to cure swine already infected with the organism.

REVIEW OF THE LITERATURE

Literature reviews on enzootic or mycoplasmal pneumonia of swine have been presented by Betts (1953), L'Ecuyer (1962), Maré and Switzer (1965), Maré (1969), Huhn (1970), Lam (1970), and Slavik (1971). The present literature review is not intended to cover all aspects of this disease but will concentrate on three relevant areas: (1) Major developments in the identification of the etiologic agent and the definition and nomenclature of the disease entity; (2) Serologic Tests for Mycoplasma hyopneumoniae; and (3) Immunization against Mycoplasma Infections in Humans and in Animal Species.

Mycoplasmal Pneumonia of Swine

Shope (1931) first described Swine Influenza in swine herds in eastern Iowa. The respiratory disease he studied was transmissible with a short incubation period. It was characterized histologically by atelectasis, peribronchial round cell infiltration and desquamation of bronchiolar epithelium. He demonstrated that swine influenza was caused by the synergistic action of the swine influenza virus and Hemophilus influenzae suis. In addition to swine influenza, he noted a second respiratory disease characterized by low morbidity, absence of prostration, and greater chronicity. He cautioned that swine influenza was not the only pneumonia present in swine.

In the decade following Shope's classic work, many investigators described any pneumonic condition in pigs as some form of swine influenza. Köbe (1932) applied the name "Ferkelgrippe" (piglet influenza) to a chronic respiratory disease of young pigs in Germany. He believed the

causative agent was a low-virulence variant of the swine influenza virus. Glasser (1939) observed that the disease reported by Köbe occurred in both young and old pigs; he therefore used the more general term "Schweingrippe" (swine influenza) to describe it.

Pullar (1948) reported the occurrence of a chronic pneumonia which he considered the most important disease of pigs in Australia. He differentiated it from swine influenza, noting that its etiologic agent was unknown. This respiratory condition appeared to be transmissible so he referred to it simply as "Infectious Pneumonia".

While attempting to isolate swine influenza virus in England, Gulrajani and Beveridge (1951) recognized a transmissible pneumonia which differed clinically and serologically from swine influenza. They were unable to isolate the virus of swine influenza from the naturally and experimentally transmitted cases. They isolated another filtrable agent which reproduced the chronic low-grade pneumonia following intranasal inoculation of pigs. The gross lesions of the pneumonia were apparent 12 to 16 days post-inoculation. Hemagglutination-inhibition tests for antibody against swine influenza virus were repeatedly negative. Gulrajani and Beveridge called the disease "Infectious Pneumonia of Pigs" because they considered the disease similar to that described by Pullar (1948) and because the name was noncommittal with regard to etiology.

In the following year, Betts (1952) reported clinical and epidemiological studies which supported the finding that infectious pneumonia was distinct from swine influenza. He described the macroscopic lesion as plum or greyish colored and clearly demarcated from the normal lung tissues. Pneumonic lesions were frequently confined to the cardiac

and apical lobes, with the diaphragmatic lobes less frequently affected. His attempts to detect mycoplasma-like organisms in smears of lung tissues and by culture were unsuccessful; the procedure and medium he employed were not indicated. The infective agent passed through bacteria-retaining filters and was re-isolated by intratracheal injection into susceptible pigs. Betts observed that the etiologic agent was susceptible to the antibiotic, Aureomycin. This was probably the first indication that the causative agent was nonviral. However, Betts considered the agent to be a large virus and applied the name "Virus Pneumonia of Pigs" (VPP) to the disease. During the next 14 years, while the concept of a viral etiology prevailed, this name became widely accepted.

Goodwin and Whittlestone (1963) introduced the designation "Enzootic Pneumonia" in their description of the chronic disease. They applied this name because the transmissible pneumonia was very common or "enzootic" in swine populations. They also preferred that name to "Virus Pneumonia of Pigs" because they believed the etiology had not been established satisfactorily.

Maré (1965) and Maré and Switzer (1966) have reviewed the attempts made by many investigators to propagate the agent in embryonated chicken eggs and in cell cultures. L'Ecuyer (1962) and L'Ecuyer and Switzer (1963) successfully propagated the etiologic agent in primary cultures of swine kidney and in human cervical carcinoma (HeLa) cell cultures. Respiratory disease-free pigs inoculated intranasally and intratracheally with the cell culture fluids developed typical gross lesions. These lesions were characterized by perivascular and peribronchiolar lymphoid hyperplasia and alveolar interstitial thickening. The fourth serial

passage in the cell cultures was the highest passage that reproduced the pneumonia. No cytopathic effects were detected in the inoculated cell cultures. L'Ecuyer suggested that the cells or the culture fluid allowed the agent to propagate in an inapparent manner.

Betts and Whittlestone (1963) and Goodwin and Whittlestone (1963) detected cytopathic effects in plasma-clot cultures prepared from the pneumonic lung of a pig experimentally infected with a bacteria-free Cambridge "J" strain of the infective agent. They also produced similar effects in pig-lung monolayer cultures inoculated with tissue culture fluids from the plasma-clot cultures. Early passage material inoculated into a pig produced typical lesions. They were able to detect pleomorphic organisms in the cell cultures and in the pneumonic lesions. They mentioned the likelihood that the organism might fall in the pleuropneumonia-like organism (PPLO) group.

Goodwin and Whittlestone (1964) propagated the "J" strain in boiled pig lung cell cultures and in cell-free medium enriched with infusion broth and yeast extract. They consistently detected the pleomorphic organisms in the cultures and in touch preparations of lesions experimentally induced with high dilutions of the culture fluids. Their work showed that the etiologic agent could not be a virus, and although they suggested that the J agent probably was a PPLO, they did not substantiate or prove it.

Maré (1965) and Maré and Switzer (1965) propagated strain 11 of the swine pneumonia agent they worked with in cell-free medium composed of Dulbecco Phosphate Buffer (DPB) and Beef Heart Infusion (BHI) with yeast extract enrichment. Seventh, eighth, and twenty-fifth serial passages of the fluid culture inoculated intranasally into respiratory disease-free

pigs produced the characteristic gross and histologic lesions of enzootic pneumonia. The agent was successfully reisolated in cell-free medium from the experimentally produced lesions. Microscopic examination of Giemsa-stained touch preparations of the pneumonic lungs revealed coccoid to cocco-bacillary organisms. After further characterization the organism was found to be between 110 and 220nm in diameter, ether- and chlortetracycline-sensitive, and penicillin-resistant. It formed very minute colonies on solid medium. On the basis of these findings, Maré and Switzer named the organism Mycoplasma hyopneumoniae (Mycoplasma of swine pneumonia). This constituted the first published evidence establishing that the etiologic agent of the disease was in fact a Mycoplasma.

Some months later, Goodwin, Pomeroy, and Whittlestone (1965) reported the propagation of their "J" strain agent on solid medium and observation of pleomorphic mycoplasma-type elements in stained touch preparations from the colonies. The organism was serially passaged on solid medium. Such cultures were used to induce typical lesions in experimental pigs. The morphology, growth requirements, and antibiotic sensitivity of the organism were generally similar to those described by Maré and Switzer (1965). They assured themselves that the agent was a Mycoplasma species and proposed the name Mycoplasma suipneumoniae (Mycoplasma of swine pneumonia) for it.

Estola and Schulman (1966) reported the isolation of mycoplasma strains from cases of swine enzootic pneumonia in Finland. They used embryonated hen's eggs, PPLO agar, and tissue cultures for the isolation of these organisms.

Goodwin, Pomeroy, and Whittlestone (1967) reported additional work on

their "J" agent which confirmed that it was a mycoplasma species. By the growth-inhibition and metabolic-inhibition tests they found that M. suipneumoniae and M. hyopneumoniae were antigenically indistinguishable. Both were, however, shown to be serologically distinct from known mycoplasmas isolated from the pig and other animal species.

L'Ecuyer (1969) recovered three strains of a mycoplasma species from the pneumonic lung of pigs in Canada. He was able to reproduce the disease by intratracheal inoculation of respiratory disease-free pigs with the mycoplasma propagated in an enriched cell-free medium or in pig testicle cell cultures. The mycoplasma was reisolated from the pneumonic lung of the experimentally infected pig. He did not attempt to assign any specific name to his mycoplasma isolate.

Hodges, Betts, and Jennings (1969) observed that earlier investigators in England and the United States had reproduced enzootic pneumonia in first and subsequent generation specific pathogen-free (SPF) or respiratory disease-free pigs but not in "germ-free" pigs. They pointed out the possibility that apparently nonpathogenic micro-organisms normally present in the respiratory tract of conventionally-reared SPF swine may have been involved in the production of the pneumonic lesions. They used as inoculum a mycoplasma which they isolated from the lungs of a pig experimentally infected with the "J" strain of enzootic pneumonia. A pure culture of the organism produced by terminal dilutions was found to be serologically indistinguishable from M. hyopneumoniae supplied by Switzer. They produced pneumonia in both gnotobiotic and SPF pigs by a single inoculation with the pure culture. From the SPF pigs they collected nasal washings which contained several bacterial species but no mycoplasma; the nasal

washings failed to elicit the pneumonia. Their work confirmed that M. hyopneumoniae is a primary etiology of swine enzootic pneumonia.

In Denmark, Friis (1969) reported the isolation from the pneumonic lung of pigs of an agent which he found to be culturally and serologically identical to M. hyopneumoniae.

In the same year Takatori (1969) demonstrated a serologic relationship between M. hyopneumoniae and the "M" strain of SEP in Japan which suggested that the "M" strain is M. hyopneumoniae.

Mycoplasma hyopneumoniae is the only mycoplasma which has been demonstrated, beyond any reasonable doubt, to cause enzootic pneumonia of swine. There are, however, conflicting reports regarding the role of a few other species of mycoplasma in this disease. One of these, Mycoplasma hyorhinis, a polyserositis--producing mycoplasma, was first isolated by Switzer (1955) from both normal and pneumonic lungs of pigs. Since then, several investigators have concluded that M. hyorhinis was a secondary invader in cases of mycoplasmal pneumonia (L'Ecuyer et al., 1961; L'Ecuyer and Switzer, 1963; Switzer, 1967; Goodwin et al., 1967, 1968; Goodwin and Hurrell, 1970). These investigators noted that M. hyorhinis readily overgrows M. hyopneumoniae and that this fact often creates difficulties in the isolation of the etiologic agent of swine mycoplasmal pneumonia. In order to overcome this problem of mixed cultures in the primary isolation, Goodwin et al. (1968) have suggested the use of the pig as a selective medium. Goodwin et al. (1970) and Friis (1971a) have developed an isolation technique that involves serial dilution of the pneumonic lung and a prolonged incubation period.

Some recent reports have incriminated M. hyorhinis in the primary

etiology of swine mycoplasmal pneumonia. In Czechoslovakia, Gois et al. (1968) and Gois et al. (1970) believed they produced enzootic pneumonia with "cloned" cultures of TR32 strain of M. hyorhinis inoculated into both conventional and gnotobiotic pigs. They classified the isolate as M. hyorhinis on the basis of colonial growth and metabolic-inhibition tests. There were no uninoculated control pigs in the experiment.

Poland et al. (1971) in England reported an extension of the work of Gois and his colleagues. Three of 9 gnotobiotic piglets exposed to an aerosol of the TR32 strain of M. hyorhinis developed pneumonic lesions. However, 2 out of the 3 also developed pleuritis; one pig had a broncho-pneumonia which histologically resembled typical enzootic pneumonia. Mycoplasma hyorhinis was reisolated from the lungs of the inoculated pigs, irrespective of the presence or absence of lesions, but was isolated from the serosa and joints only when lesions were present. All the inoculated pigs developed some type of serositis. This indicates that the primary lesion produced by M. hyorhinis infection is polyserositis.

Friis (1971b) inoculated 12 hysteroctomy-produced, colostrum-deprived pigs with high dilutions of M. hyorhinis isolated from various cases of swine pneumonia in Denmark. Two of the inoculated pigs developed pneumonic lesions. Mycoplasma, identified as M. hyorhinis by the growth-inhibition test, were recovered from the lesions as well as from normal areas of the lungs. The same mycoplasma were recovered from the brain and nasal cavity of inoculated pigs. The pneumonic lung lesions were negative for M. hyopneumoniae when examined by means of the fluorescent antibody test.

Dzu et al. (1971) reported the production of lesions typical of

enzootic pneumonia and atrophic rhinitis in specific pathogen-free (SPF) swine inoculated with both Mycoplasma laidlawii and Pasteurella multocida. Pigs inoculated with M. laidlawii strain J252 first and then Pasteurella strain B1297 developed more extensive lesions than those that received only mycoplasma, or pasteurella first and then mycoplasma. Most of the inoculated pigs also developed serositis. Antibodies against the mycoplasma strain were detected by the slide-agglutination, growth-inhibition, metabolic-inhibition, and the indirect hemagglutination tests. Reisolation of the inoculated strain of mycoplasma and pasteurella was reported successful in all test animals.

These independent groups of investigators have provided evidence that the etiologic agent of a widespread, chronic pneumonia of swine is mycoplasmal in nature. Therefore the use of the name "Virus Pneumonia of Pigs" (VPP) for this condition is inappropriate and misleading, and should be abandoned. Maré (1969) has stated that the term "Swine Enzootic Pneumonia" (SEP) is based solely on epidemiological characteristics of a field disease and evades the issue which has been the major concern of many investigators; that is, the etiology of disease. He favored the designation "Mycoplasmal Pneumonia of Swine" because it is more accurate and specific and makes allowance for additional species of Mycoplasma which may induce similar disease in pigs. Huhn (1970) stated that while the term "Mycoplasmal Pneumonia of Swine" is etiologically suggestive, it does not differentiate between the primary and secondary nature of the incriminated agents. He preferred the use of "Enzootic Pneumonia of Pigs" which he considered to be an etiologically neutral term. However, several workers, particularly in the United States, have used the designation

"Mycoplasmal Pneumonia of Swine" in their publications (Maré, 1969; Slavik, 1971; Lam and Switzer, 1972; Switzer, 1972). In 1971, the Committee on Nomenclature of the Colloquium of the American Veterinary Medical Association recommended the adoption of the term "Porcine Mycoplasmal Pneumonia". This indicates a recognition by the members of this committee, of the significance of the etiology in the definition and nomenclature of the disease entity.

Serologic Tests for *Mycoplasma hyopneumoniae*

Successful propagation and identification of the etiologic agent of mycoplasmal pneumonia of swine has made possible the production of specific antigen and the development and application of different serodiagnostic tests for the disease.

Metabolic-inhibition tests

Goodwin *et al.* (1967) indicated that immune sera produced inhibition of acid production by the swine pneumonia agent. They compared *M. hyopneumoniae* and *M. suipneumoniae* by the metabolic-inhibition test and found the two isolates to be serologically indistinquishable. Hodges *et al.* (1969) also used the metabolic-inhibition test to show there is a close antigenic relationship between *M. suipneumoniae* and *M. hyopneumoniae*.

Takatori *et al.* (1968) were unable to detect any metabolic-inhibition activity in the serums of pigs experimentally infected with *M. hyopneumoniae*. Goodwin *et al.* (1969a) also reported that they could not demonstrate specific metabolic-inhibition antibody against *M. suipneumoniae* in serums from infected pigs. Instead, they detected nonspecific inhibitory substances in some normal and infected pigs.

Takatori (1970) has reported that while metabolic-inhibition is an encouraging test for detection of mycoplasmal antibodies in other animals, it seems of little value for detection of Mycoplasma hyopneumoniae antibody in infected pigs.

Agglutination tests

Moore et al. (1965) described a direct plate agglutination technique for M. hyopneumoniae and four other mycoplasma species in swine. Antigens preserved in 0.24% phenol and antiserums serially diluted two-fold were used in the test. The test was performed in wells of a glass plate and consisted of 0.05ml of the antigen mixed with 0.1ml of the serum dilution. After overnight incubation at 4°C the test was read. These workers noted that inactivation of the antiserum prevented the agglutination reaction. They indicated that complement was probably necessary in the reaction.

Roberts and Little (1970a) employed a stained antigen plate agglutination test to determine the serologic response of pigs to two strains of M. hyopneumoniae. A mixture of one drop of the serum from an infected pig and an equal volume of the stained antigen was made on an enamel plate at room temperature. The plate was rotated and the test read after 3 minutes. No slide agglutination reaction was demonstrated.

Fujikura et al. (1970) reported a workable tube-agglutination test which was interpreted on the basis of clearing and settling of the antigen. Using this test, they demonstrated antibody against M. hyopneumoniae in infected pigs. The test serum was inactivated at 56°C for 30 minutes. They performed the test in glass tubes and utilized a total reaction volume of 0.5ml consisting of equal volumes of the antigen and two-fold

dilution of the inactivated antiserum. The mixture was first incubated in a water bath at 37°C for 2 hours and then in a cold room at 4 to 5°C for another 18 hours. There was a relatively good correlation between the presence of mycoplasmal pneumonia lesions and detectable agglutinating antibody titers. The test appeared to be specific since no cross-reaction was demonstrated between M. hyopneumoniae and M. hyorhinis antigens with the heterologous immune serums. In pigs less than 3 months of age, the agglutinating antibody was demonstrated at a higher frequency than was the complement-fixing antibody.

Indirect-hemagglutination tests

Moore et al. (1965) described an indirect-hemagglutination (IHA) method which they claimed could allow the grouping of M. hyopneumoniae and other swine mycoplasmas into species on a more precise basis. The respective antigens were standardized to a MacFarland Nephalometer reading of 10. An unspecified amount of the saline-suspended organisms was boiled in a water bath for 10 minutes, then cooled to room temperature and an equal volume of 5% washed sheep red cells was added. The mixture was incubated at 37°C for 2 hours. The cells were then washed three times in 0.85% saline and resuspended to a 5% concentration. The test was performed by mixing 0.05ml of the sensitized cells and 0.1ml of two-fold serial dilution of the test serum. After a 20-minute incubation period, the test was read by observing agglutination of the red blood cells. No evaluation of the test was given by the authors.

Goodwin et al. (1969a,b) used a tube indirect-hemagglutination test to demonstrate immune antibody against M. hyopneumoniae. They utilized

sheep red blood cells treated with tannic acid and sensitized with the mycoplasma antigen. Tubes containing the test serum dilutions and the sensitized red blood cells were incubated at 37°C for 2 hours. After initial reading, the tubes were incubated overnight at room temperature before the final reading was made. The highest dilution of serum giving a clearly positive agglutination was taken as the end-point. These investigators reported that the indirect-hemagglutination antibody appeared later and lasted longer than the complement-fixing antibody. Recovered pigs had antibody titers greater than 1:40,000, as measured by the IHA test.

Goodwin and Hodgson (1970) reported a passive hemagglutination test (PHA) for the detection of M. hyopneumoniae. The test was a modification of the IHA test described by Goodwin et al. (1969). These workers found that tannic acid in the final concentration of 1:100,000 yielded a superior final suspension of cells and that sensitization of the tanned red blood cells for 30 minutes at 37°C gave a better antigen. They used the PHA test to diagnose 14 cases of mycoplasmal pneumonia which had earlier presented diagnostic difficulties with the metabolic-inhibition test.

Lam and Switzer (1971) developed an indirect-hemagglutination test for the detection of antibodies to M. hyopneumoniae in experimentally and naturally infected pigs. The antigen was prepared by adding 2% sodium lauryl sulfate (SLS) to the concentrated mycoplasma suspension to obtain a final concentration of 0.2%. The suspension was dialyzed first against 5M ammonium sulfate overnight and then against phosphate buffered saline for 72 hours. Thimerosal was added to produce a final concentration of 1:10,000. In order to avoid the naturally occurring hemolysin in test swine serum to sheep red blood cells, these investigators used swine red

blood cells as antigen-carrier in place of the conventional sheep erythrocytes. A mixture of the antigen, buffer, and 10% tannic acid-treated erythrocytes was incubated in a water bath at 37°C for 20 minutes. The antigen-sensitized cells were then suspended to obtain a final concentration of 1%. The final test was conducted in trays, each well containing 0.05ml of the serum dilution and 0.025ml of the antigen-coated cells. The mixture was incubated at 37°C for 1 hour before reading the test. A titer of 1:12 or higher was considered positive for M. hyopneumoniae. The test detected antibodies in 92% of experimentally inoculated pigs that had pneumonic lesions and 78% of naturally infected pigs. There was a good correlation between pneumonic lesions and antibody titers. No serologic cross-reaction was demonstrated when the M. hyopneumoniae antigen was tested against antiserums to M. hyorhinis or M. granularum.

In further experiments, Lam and Switzer (1972) demonstrated that pigs experimentally exposed to M. hyopneumoniae developed antibody titers detectable by the IHA test 2 to 3 weeks after exposure. They found that these titers reached a maximum on the 8th to 11th week and remained at the maximal level until the 18th week. The antibody was still detectable 47 weeks post-inoculation.

Switzer (1972) has pointed out that while the IHA test detects extremely high titers of antibodies that appear early in mycoplasmal pneumonia of swine, the test requires considerable standardization of reagents and conditions and is rather difficult to depend on for consistent serologic investigations.

Immunofluorescence

L'Ecuyer and Boulanger (1970) developed a useful direct immunoflourescent technique for the demonstration of M. hyopneumoniae in smears of broth cultures and in pneumonic lesions. The pneumonic lung sections were fixed with cold acetone and the specific mycoplasmal immune serum conjugated with fluorescein isothiocyanate was applied for staining. When viewed in the fluorescent microscope the mycoplasma antigen was detected on the surface of the bronchial and bronchiolar epithelium and in the contained exudate. No fluorescence was demonstrated in known positive preparations stained with nonimmune pig globulin conjugates or in normal lungs stained with immune globulin conjugates. In experimentally infected pigs fluorescence was not detected until 25 days post-infection. The immune swine serum conjugates were found to be species specific; they reacted only with their homologous mycoplasma species--M. hyopneumoniae or M. hyorhinis. The authors noted that by specifically allowing the visualization of the etiologic agent, immunofluorescent staining could confirm at necropsy the diagnosis established by other serologic methods. The technique was, therefore, considered a practical alternative to isolation for identifying M. hyopneumoniae. However, the fluorescent antibody method appeared to be sensitive for the detection of the etiologic agent only in established cases of mycoplasmal pneumonia and not reliable during the early stages of the disease. They attributed this absence of fluorescence early in the disease to the difficulty in obtaining the appropriate sections of the lungs when the areas of consolidation were very limited. It could also be due to an insufficient amount of the organism (antigen) being present to permit visualization.

Meyling (1971) reported the demonstration of M. hyopneumoniae and M. hyorhinis in pneumonic swine lungs by the fluorescent antibody technique. Cryostat sections were prepared from pneumonic lungs of pigs collected at a slaughter-house, and from the lungs of dead piglets. Sections were also prepared from pneumonic lungs of SPF pigs experimentally infected with M. hyopneumoniae and M. hyorhinis. The lung sections were stained with the homologous immune serum conjugates. Mycoplasma hyopneumoniae was isolated by culture in 38 of the 50 slaughter-house cases, and in 35 of these the mycoplasma was demonstrated by the immunofluorescent method. In 18 cases the positive staining results for M. hyopneumoniae were not confirmed by isolation of the organism; however, in 15 of these M. hyorhinis was demonstrated. Meyling demonstrated with the FA test that M. hyorhinis had the same localization or distribution in the pneumonic lung as that typical of M. hyopneumoniae, and that M. hyorhinis was in most cases found in tissue sections which were negative for M. suipneumoniae. These findings suggested that M. hyorhinis may under certain conditions act as a primary etiologic agent. He stated that the sensitivity of the direct immunofluorescent staining depends both on the number of organisms present in the sections and on the accessibility of these organisms to the labeled antibody.

Complement-fixation test

Until recently, the complement-fixation (CF) test was not considered satisfactory for the detection of antibodies in swine serums. This was partly because of the "procomplementary" properties of swine serums which significantly enhanced the hemolytic activity of the guinea-pig complement

as well as the occurrence of heat labile sheep red blood cell hemolysins in swine serums (Bankowski et al., 1953).

Roberts (1968) first demonstrated the presence of immune antibodies against M. hyopneumoniae in unheated swine serum by using the direct tube complement-fixation test. Four units of a heat treated antigen were used. An antigen unit was the highest dilution of the antigen which gave complete fixation with the highest dilution of serum in two dimensional titrations. The antigen, the serum dilutions, and guinea-pig complement were allowed to react for 3 hours at 20°C. The hemolytic system, consisting of 3% suspension of sheep red blood cells sensitized with hemolysin was then added. The mixture was incubated for an additional 30 minutes at 37°C and allowed to cool to room temperature before the test was read. There was a direct correlation between the level of complement-fixing antibodies and the degree of lung lesions. However, possible cross-reactions of the test serums with other swine mycoplasma species were not determined. A CF reaction of 1:40 serum dilution was regarded as positive because a "hemolytic prozone" or procomplementary effect was evident in the unheated serums at dilutions of 1:10 and 1:20.

Boulanger and L'Ecuyer (1968) investigated both the direct and a modified-direct complement-fixation tests to determine their usefulness in detecting immune antibodies in pigs experimentally infected with strains of M. hyopneumoniae. In the direct CF test normal and immune test serums were heat-inactivated at 60°C for 30 minutes. At least two units of the titered antigen were added to two-fold serum dilutions in the presence of four and a half, 50% hemolytic units of guinea-pig complement. After an initial incubation of 18 hours at 9°C, 0.2ml of a 2.5% suspension of

sensitized sheep red blood cells was added, and then incubated another 30 minutes at 37°C. The same technique was used in the modified direct CF test, with the exception that the complement was supplemented with 1% pre-tested fresh normal, unheated calf serum. These workers found that only the modified direct test was suitable for the detection of mycoplasmal antibodies in the heat-inactivated serums of infected pigs. The procomplementary activity and poor complement binding of swine serum were masked by the increased sensitivity of the modified test. There was a close correlation between the development of pneumonic lesions in experimentally infected pigs and the appearance of detectable antibody titers. The specificity of the test was indicated by the absence of cross-reactions between the anti-M. hyopneumoniae serum and M. hyorhinis or M. granularum antigen; antibodies were demonstrated only with the homologous antigen.

Takatori et al. (1968) used another modified direct complement-fixation test to demonstrate immune antibodies in the serums of pigs experimentally or naturally infected with M. hyopneumoniae. The test was modified by the supplementation of the guinea-pig complement with 1% normal calf serum; two units of the modified complement in 0.1ml volume was used in the test. The CF antigen, consisting of a suspension of washed M. hyopneumoniae organism, was found to be highly anti-complementary; attempts were made to eliminate or reduce this effect by ether extraction, heating at 56°C, boiling, or trypsinization. Complement-fixing antibody was first detected in experimentally infected pigs 2 to 3 weeks after inoculation and tended to increase for at least 9 weeks post-inoculation. No cross-reactions were demonstrated between M. hyopneumoniae, M. hyorhinis, or M. granularum and the heterologous antiserum. Procomplementary activity

was observed only in serum dilutions below 1:8; the procomplementary property was unpredictably neutralized by the anti-complementary effect of the antigen.

Hodges and Betts (1969) demonstrated heat-labile antibodies against the "J" strain of M. hyopneumoniae by using a microtiter complement-fixation test. They were able to detect antibodies less than 2 weeks after infection and the antibodies persisted for at least 15 weeks. The procomplementary effect of pig serum prevented the detection of antibodies in titers below 1:20. Heat-inactivation of the test serums at 56°C resulted in very significant decreases in titer. The authors claimed a relatively high degree of specificity for the test.

Goodwin et al. (1969) used the CF test to study the immunity in experimentally induced mycoplasmal pneumonia of swine. They reported that there was little correlation between the CF titer and the extent of pneumonic lesions or immune status.

Wallis and Thompson (1969) carried out a modified direct complement-fixation test for the detection of antibodies against M. hyopneumoniae in unheated serum samples collected from field cases of the disease. They claimed that heat-treatment of the immune serums at 56°C for 30 minutes caused the total loss of complement-fixing capacity. They concluded that the complement-fixing capacity was heat-labile. Attempts to restore the complement-binding activity by adding normal, fresh pig serum or calf serum proved surprisingly unsuccessful. Procomplementary activity persisted in serum dilutions below 1:10, and efforts to reduce the procomplementary property by formaldehyde treatment were also unsuccessful.

Boulanger and L'Ecuyer (1970) disputed the conclusion of Wallis and

Thompson (1969) that their failure to obtain fixation with heat-inactivated swine serum meant the complement-fixing capacity was "irreversibly heat-labile". They noted that the noncomplement-fixing antibodies in heated swine serums have been shown to react with M. hyopneumoniae antigen in other modified CF tests (Boulanger and L'Ecuyer, 1968; Takatori et al., 1968). This fact indicated that the antibodies themselves are not denatured by heating at 56°C for 30 minutes; however, certain essential supplementing substances are destroyed. They discouraged the use of normal pig serum as a supplementing factor because of its procomplementary and hemolytic properties. They preferred fresh normal serum from calves between the ages of 3 and 12 months. Serums from calves below or above this age range, they said, often caused nonspecific complement-fixation in the presence of M. hyopneumoniae antigen.

Roberts and Little (1970a) described a modified CF test for M. hyopneumoniae that utilized unheated swine serum. Heat-inactivation substantially decreased the CF titers, and the addition of normal serum from 1-day old pigs failed to fully restore the titers. Attempts to remove the procomplementary activity with formalin resulted in partial and often total elimination of the CF titer of both heated and unheated serum samples. These authors also reported that M. hyorhinis occasionally induced pneumonic lesions; when these occurred, CF cross-reaction between the anti-M. hyorhinis serums and the M. hyopneumoniae antigen was detected. They reasoned that the cross-reactions and the nonspecificity of the test indicated a measure of anti-lung tissue response.

In another report, Roberts and Little (1970b) investigated the possibility of auto-immune response associated with mycoplasmal pneumonia

of swine. They used a heated extract of normal pig lung as a CF antigen. They titered 400 serum samples against both the lung extract and the M. hyopneumoniae antigen. Complement-fixing antibodies against the mycoplasma antigen and the lung extract were demonstrated. Serum samples which reacted positively against M. hyopneumoniae also gave similar positive reactions with the lung extract. These preliminary findings suggested to the authors that an auto-immune phenomenon might be involved.

Slavik (1971) and Slavik and Switzer (1972) have developed a workable modified direct microtitration complement-fixation test for the diagnosis of M. hyopneumoniae. Test serums were heat-inactivated at 54°C to 56°C for 30 minutes in order to destroy the natural hemolysins in pig serum. The lyophilized guinea-pig complement was reconstituted with fresh, normal serum from young, pneumonia-free pigs. This modification had the double effect of compensating for the procomplementary activity of swine serum on the guinea-pig complement and of restoring or supplementing the complement-binding capacity of the inactivated serum. The diagnostic CF antigen was prepared by diluting a 10% suspension of the harvested organism to a 1% concentration, and then heating at 52°C for 30 minutes. This antigen was titered in the CF test against serum from a pig known to be infected with M. hyopneumoniae. The CF test was performed in microtiter plates, according to the procedure outlined by the Laboratory Branch of Communicable Disease Center (1965), except for the modifications indicated. The sensitivity of the test was markedly increased, as antibodies were detectable at dilutions of the test serums as low as 1:8. The modified CF test was used to diagnose 90% or more of M. hyopneumoniae infections in pigs that had typical macroscopic lesions at necropsy. The sensitivity

of the test was evaluated by testing M. hyopneumoniae, M. hyorhinis, and M. hyosynoviae antigens against the heterologous and homologous antiserums. No cross-reactions were demonstrated.

In a recent discussion, Switzer (1972) has outlined the essential elements and the advantages of this modified complement-fixation test.

Immunization against Mycoplasma Infections in Humans and in Animal Species

Contagious bovine pleuropneumonia (CBPP)

Before the introduction of the modern program of vaccination against contagious bovine pleuropneumonia, native cattle owners in Africa were using a modification of Willem's method of rubbing infected pleural lymph into an incision made over the nasal bones (Lindley, 1965). Current field immunization against CBPP is carried out almost exclusively with attenuated live vaccines; the data on the value of inactivated vaccines are fragmentary and conflicting.

Walker (1921, 1922) recorded that the virulence of the etiologic agent, Mycoplasma mycoides var. mycoides, diminished as a result of serial passage in artificial medium. He demonstrated that by the 27th passage the agent became attenuated; he subsequently developed a "culture-virus" technique for protective inoculation. The attenuated vaccine was inoculated into the tail. Animals that showed no reaction to the vaccination were then injected in the shoulder, and if this still failed to elicit a reaction, a third inoculation was given. During this time the belief was that immunity would result only when a visible or palpable local reaction occurred, and it was necessary to continue the inoculation until such a

reaction was induced.

Benneth (1932) showed that immunity could be induced against CBPP without the production of the visible or palpable reaction. He advocated a single inoculation method for the nomadic Sudan herds.

Purchase (1939) tested the antigenicity of attenuated live vaccine derived from the 11th to 45th passage of the "culture-virus" on two local breeds of cattle in Kenya. Groups of cattle were given one, two, or three doses of the vaccine. The first inoculation was made in the tip of the tail and subsequent inoculations were given subcutaneously in the shoulder. Vaccinated cattle were challenged by contact-exposure to known infected herds or by subcutaneous injection of fresh, nonattenuated culture. Local reactions to the vaccination were mild. A strong immunity was induced in one breed of cattle but only 30 to 60% of the second breed were protected. The immunity was induced sooner when the number of doses of vaccine was increased. The author recommended, therefore, that for field vaccination, two doses of the vaccine be given at 21 days interval.

Priestley (1955a,b) reported that freeze-dried pleuropneumonia organisms reconstituted in saline or broth failed to elicit immunity on injection into cattle. When the reconstituted suspension was mixed with an equal volume of 1% agar in saline and then injected, solid immunity was readily and rapidly produced. The vaccine was prepared from a suitably attenuated strain obtained after the 12th serial passage and inoculation was made in the tail. Protective immunity was tested by intramuscular challenge 28 days after vaccination. When agar was used as the adjuvant, the number of organisms required to immunize was very much smaller. For field immunization, the author recommended a dosage of 0.2ml containing

10,000 viable organisms. He also investigated the adjuvant action of hens egg. Whole egg, and especially yolk, was found to be a good adjuvant, but not as good as agar.

Dafaalla (1956) investigated the adjuvant effect of 16 substances with the hope of finding some simple substitute for agar. Four classes of substances were tested with the dried CBPP vaccine of Priestley (1955): mineral salts, polysaccharides, peptone and proteins, and oils. Groups of serologically negative cattle were inoculated at the tail-tip with 0.2ml doses of the adjuvant-containing vaccines, and were challenged by intramuscular injection of the virulent culture. Six mineral salts, polysaccharides (starch and gum acacia), bovine gamma globulin, and sesame oil were ineffective as adjuvants, as specific immunity could not be detected. Bovine albumin, liquid paraffin, and mucin gave encouraging results; two of each group of 3 animals inoculated with vaccines containing these substances developed antibodies and resistance to challenge. Of 24 animals vaccinated with organisms in Shell ondina oil, 16 developed antibody and 17 resisted challenge.

Hyslop (1956) studied the duration of immunity in cattle vaccinated with egg-adapted (avianized) CBPP vaccine. At one month after vaccination none of the vaccinated animals reacted severely to subcutaneous challenge. In the group challenged at 14 months post-vaccination, only one of 7 reacted severely. Twenty-two months after vaccination, 2 of 10 vaccinates reacted severely. By the 43rd month post-vaccination, the number of severe reactors in the vaccinated group had increased to 6 out of 12. The increasing proportion of severe local reactors showed a decline in resistance during three and one half years. None of the vaccinated cattle

died, while 9 of 19 control animals died.

In view of the field evidence that highly attenuated avianized CBPP vaccines sometimes failed to stimulate protective immunity, Piercy and Knight (1958) carried out trials with vaccines prepared from organisms at differing levels of egg passage. Three of the 6 batches of vaccines were prepared from the 44th egg-passage of T_1 strain culture; the other 3 batches were prepared from the 137th, 162nd, and 185th egg-passage levels of the same strain. All the vaccines conferred a significant degree of protection, although not complete immunity. The increased passage through eggs did not lessen immunizing ability, since results at passage 185 were apparently better than at passage 44.

Hudson and Turner (1963) compared the efficacy of two types of Australian vaccine: the standard laboratory culture vaccine and the avianized vaccine. The culture vaccine was prepared from the 21st passage of M. mycoides strain V5; the avianized vaccine was prepared from lyophilized material made from the 6th egg-passage of the same strain. Serologically negative cattle were vaccinated in the tail-tip, and were then challenged by contact exposure to other cattle infected by intra-bronchial intubation with a virulent strain of M. mycoides. Groups of vaccinated cattle were challenged at 1 week, 1 month, 3 months, and 12 months post-vaccination. The number and severity of local reactions to vaccination was greater in the egg-vaccine group than in the broth vaccine group. Egg-vaccine produced almost complete protection against challenge exposure as early as one week after vaccination. Although the response to broth vaccine was slower, there was little difference between the efficacy of the two vaccines when challenge was made 1 or 12 months after vaccination.

Lindley (1965) showed that an avirulent or attenuated strain KH_3J of M. mycoides was a good immunizing agent when administered as a broth culture vaccine into Nigerian Zebu cattle. At varying periods after subcutaneous vaccination with fresh culture vaccine the animals were challenged by subcutaneous injections of lyophilized virulent strain. Thirty-one of 37 vaccinated cattle were resistant to challenge. In another trial, the author reported that 1% agar had an insignificant adjuvant effect when added to the lyophilized vaccine. This was in contrast to the results reported by Priestley (1955a,b). This investigator reasoned that the conflict was due to differences in the strain; the more virulent the strain, the more pronounced the effect of adjuvant.

Hudson (1965) evaluated the immunizing value, in Australian cattle, of the attenuated KH_3J strain obtained from Nigeria. He compared two groups of cattle vaccinated either in the tail-tip or subcutaneously behind the shoulder with the same dose of the same strain. There was an indication that tail-tip vaccination was more effective; there was no detectable local reaction and no evidence of lung lesions. The trial also demonstrated that the attenuated KH_3J vaccine produced a level of immunity about equal to that induced by the strain V5 broth vaccine.

Lloyd (1967) investigated the role of humoral antibodies in resistance to M. mycoides infection by passive transfer of immune serum. Serums, obtained from immune cattle that had resisted the local infection, were administered to susceptible cattle. These passively inoculated animals, together with the immune serum donors and nonimmunized controls, were challenged by subcutaneous inoculation with virulent strain of the organism. None of the immune-serum donors showed a local reaction to the

challenge injection. In contrast, all the cattle that received the immune serum as well as the controls developed characteristic Willem's reaction (severe local inflammatory swelling, edema, and necrosis). These results indicated that while active immunization produced resistance to re-infection, passively transferred immune serum was unable to provide protective immunity. The authors suggested that resistance of immune cattle to contagious bovine pleuropneumonia may depend very little, or not at all, on humoral antibodies.

Davies (1969) investigated the persistence of M. mycoides in the host following vaccination with live T_1 broth vaccine. Cattle were vaccinated in the tail-tip and were slaughtered at intervals to determine the spread of the immunizing organism. The organism was recovered only from the site of vaccination and the regional lymph nodes draining the site. Isolations were successful for only 14 days post-vaccination.

Gilbert et al. (1970) tested the effectiveness of the T_1 strain broth vaccine by carrying out "in contact" challenge 6 and 12 months after a single primary vaccination. No CBPP lesions were seen and no isolations of M. mycoides were made from any of the cattle challenged 6 months after vaccination; all controls developed lesions from which the organism was isolated. In the cattle challenged 12 months post-vaccination, isolations were made from the respiratory tract of 3 of the vaccinates, although none of them developed CBPP lesions. This finding indicated that a single injection of the vaccine produced a high level of immunity for 6 months, but the protection was decreasing by 12 months. The authors recommended annual vaccination as a practical immunoprophylactic procedure.

In Vom, Nigeria, Karst and Mitchell (1972) investigated the effect of

intranasal vaccination of cattle with an attenuated Gladysdale strain of M. mycoides. The attenuation was carried out by passaging the strain 20 times in bovine kidney cell culture. When inoculated subcutaneously in more than 1000 cattle, the attenuated strain, G/20, did not produce any adverse effect. The strain was then passaged 10 times in cattle by intranasal instillation and it did not revert to virulence. The G/20 strain was used for the production of CBPP vaccine which was inoculated intranasally in 17 cattle. The vaccinates were challenged by contact exposure to cattle affected with CBPP. The vaccine had an efficacy of 77% when it was inoculated 24 hours before challenge, and of 83% when inoculated 6 months before challenge.

Avian Mycoplasma infections

Nelson was probably the first to demonstrate immunity to Chronic Respiratory Disease, an infection of chickens and turkeys caused by Mycoplasma gallisepticum (McMartin and Adler, 1961). He observed that chickens which recovered from the chronic disease were resistant to a second exposure.

Adler et al. (1960) also observed that recovery from the natural infection conferred measurable immunity in turkeys and chickens. They were unable to induce immunity with a vaccine prepared by sonic disruption of the organism. They also failed to immunize turkeys with a formalin-inactivated vaccine. Aluminium hydroxide adjuvant did not influence the response. An attenuated live culture administered intramuscularly or intravenously conferred protection against the air-sac route of challenge inoculation.

McMartin and Adler (1961) reported that birds vaccinated intranasally with live M. gallisepticum were refractory to abdominal air-sac challenge inoculation.

Domermuth (1962) reported that 38% of immature chicks vaccinated subcutaneously with a live culture of M. gallisepticum were immune to subsequent challenge by the air-sac route. Multiple vaccination of chickens with M. gallisepticum had no significant effect on either egg production or hatchability; the causative agent could not be cultured from the eggs of the vaccinated birds, and no evidence of immunity was detected in their progeny.

Warren et al. (1968) noted that intranasal or subcutaneous inoculation of formalin-inactivated culture vaccine of M. gallisepticum increased the resistance of 1-day old chicks to air-sac inoculation of the homologous strain. The increase in resistance was not accompanied by significant increase in IHA antibody level. These investigators also noted that the earlier failure of Adler et al. (1960) to obtain protection with an inactivated vaccine might be due to three factors: the destruction of the antigen by prolonged sonication (30 minutes), the higher concentration of formaldehyde used (0.5%), or the use of older chickens (6 to 7 weeks) in their trials.

Adler and Lamas Da Silva (1970) compared the immunogenicity of inactivated and live M. gallisepticum vaccines; they also studied the effect of dosage and route of inoculation on the immune response. Chickens inoculated intravenously with killed organisms were partially protected against challenge. However, intranasal instillation of the thimerosal-inactivated vaccine did not provide protection against challenge.

Intranasal inoculation of live vaccine induced a high degree of immunity to both air-sac and intravenous challenge. The resistance appeared to be immunizing-dose dependent; lesser numbers of organisms apparently provided little protection against severe challenge. There was slight correlation between serologic (HA) titer and immune response, after intranasal vaccination.

Few extensive studies have been reported on immunity to Mycoplasma synoviae, the etiologic agent of Infectious Synovitis. Wichmann et al. (1960) inoculated chickens with attenuated culture of M. synoviae, strain C-1. The birds were protected against footpad challenge with virulent cultures. Immunity varied from complete resistance to resistance against systemic infection and death; synovial lesions were less extensive in partially protected birds.

Carnaghan (1962) demonstrated that birds recovered from experimental infection with M. synoviae developed strong immunity against challenge. Chickens that were infected by the footpad with strains maintained by serial egg-passage, developed typical lesions. The surviving birds were challenged by intravenous inoculation, 39 days after infection. No systemic reaction or fresh lesions developed in the recovered birds. Passive immunity was also demonstrated by intramuscular inoculation of susceptible chickens with serum from convalescent fowls collected 12 and 16 weeks after infection.

Olson et al. (1964) infected chickens with M. synoviae administered by the intranasal route. Four weeks later, some of the birds were killed and histologic lesions were demonstrated. The surviving birds were then challenged in the footpad and were found to be refractory to challenge.

Vlaovic and Bigland (1971) reported an attempted immunization of turkey hens against Mycoplasma meleagridis, the etiologic agent of Airsacculitis. Female turkeys were given five weekly injections of the live organism, starting 10 days prior to collection of eggs. The inoculations failed to induce any immunity or reduce the rate of egg transmission of the infective agent. The attempted immunization proved detrimental, as the progeny of the vaccinated turkey hens showed a higher incidence of more severe air-sac lesions than were observed in the controls.

Human primary atypical pneumonia

The capacity of M. pneumoniae to produce characteristic disease in experimental animal models has led to some imaginative investigations directed toward the development of attenuated live vaccines. However, attempts to develop inactivated vaccines against M. pneumoniae infection in man have been more successful.

Jensen et al. (1965) prepared a formalin-inactivated vaccine from a M. pneumoniae strain adapted in a chemically defined medium. Hamsters intramuscularly inoculated with 0.5ml of the inactivated vaccine developed resistance to intranasal challenge. Human volunteers who received intramuscular injection of the vaccine developed no adverse local reactions. No challenge inoculation or evaluation of resistance in the vaccinated human subjects was done.

Smith et al. (1967) evaluated the protective value of an inactivated M. pneumoniae vaccine in volunteers. The vaccine was prepared from M. pneumoniae grown in chloroform egg-yolk extract medium and was inactivated with formalin. Volunteers were given 0.5ml of the vaccine

intramuscularly with a booster injection 5 weeks later. They were then challenged with a live suspension of the organism. Only one of 10 volunteers with vaccine-induced antibody became ill, whereas 7 of 9 who failed to respond serologically developed definite respiratory tract disease. Vaccinated subjects who did not produce detectable serum antibody developed more severe disease than nonvaccinated subjects. These findings suggested that the inactivated vaccine had a protective effect in those volunteers with vaccine-induced antibody, while the serologically nonresponding individuals might have been sensitized. The investigators could not determine whether the protection was a function of the serum antibody.

Mogabgab (1968) described a formalin-inactivated vaccine in which M. pneumoniae was grown in a "serum free" medium. When administered intramuscularly with alum adjuvant, the vaccine produced a 45% reduction of mycoplasmal pneumonia in a population of approximately 21,000 military personnel.

Fernald (1969) compared the antibody production stimulated by intranasal infection of hamsters with that which followed parenteral immunization with killed M. pneumoniae vaccine. Both intranasally infected and actively immunized hamsters developed serum antibodies, but the parenterally immunized group developed higher circulating antibody titers. Among the animals that were previously infected but recovered and then were challenged, no pneumonia developed. In the group that was actively immunized, all were infected on challenge and developed pneumonia in spite of the high serum antibody titers. This finding demonstrated a lack of correlation between serum antibody and resistance. The studies also

suggested that local antibody, either humoral or cellular, or both may be involved in resistance to M. pneumoniae respiratory infections.

Lipman et al. (1969) derived 3 different virulent and avirulent M. pneumoniae strains by serial passage of parent strains in artificial medium or in hamsters. The 3 strains were characterized in an effort to determine the factors associated with virulence. The avirulent strain derived after 169 passages had lost both the ability to produce pneumonia in hamsters and the capacity to adsorb to erythrocytes (cytoadsorption). The loss of hemoadsorption accompanied with total loss of virulence suggested that cytoadsorption is a requisite for virulence. This finding indicated the possible use of the avirulent strain in the production of live vaccines.

Fernald and Clyde (1970) investigated the influence of route of vaccination and of the virulence of mycoplasma strains on the induction of immunity against intranasal challenge with M. pneumoniae. Intranasal immunization with virulent strains of M. pneumoniae yielded a 71% reduction in pneumonia in the population. Despite high level of serum antibody, intraperitoneal and subcutaneous immunizations with the same virulent strains produced only 61% and 56% reductions, respectively. When the attenuated strain was used, resistance to pneumonia resulted after intranasal immunization but not after parenteral vaccination. This finding demonstrated that serum antibody levels did not correlate with protection and that the route of vaccination was an important factor in immune response to M. pneumoniae. The investigators demonstrated growth-inhibiting activity in bronchial washings of challenged hamsters. This suggested the induction of a local antibody response.

Steinberg et al. (1971) employed genetic selection to develop

temperature sensitive mutants of M. pneumoniae strains which could be used as live vaccines. They produced 14 temperature-sensitive (ts) mutants by exposing the organisms to N-methyl-N-nitro-N-nitrosoguanidine (NTG). The ts mutants were able to grow at 32°C, the temperature of the human upper respiratory tract. Their in vitro replication was impaired at 37°C, the normal temperature of the human lower respiratory tract. Hamsters infected intranasally with the ts mutants did not develop pneumonia, whereas 64% of animals inoculated with the parent strain developed lung lesions. This was an indication that the ts mutants were unable to invade the lungs. Infection with the ts mutants induced definite resistance to challenge. The authors noted that the application, in humans, of these ts mutants to stimulate resistance will depend on the ability of such mutants to grow and stimulate effective immunity in the upper respiratory but not in the lower respiratory tract of man.

Fernald (1972) investigated the role of cell-mediated immunity in human mycoplasmal pneumonia by use of the technique of stimulation of peripheral lymphocytes in volunteers with natural M. pneumoniae infection. Lymphocytes were collected from the peripheral blood of donors and were stimulated by treatment with phytohemagglutinin (PHA-P) and specific M. pneumoniae antigen. Lymphocyte stimulation was determined by the incorporation of tritiated-thymine in cultures derived from the peripheral cells. Cellular transformation occurred in nearly all individuals with confirmed M. pneumoniae infection who had specific antibodies. No such cellular reactivity was demonstrated in serologically negative subjects. The specific reactivity of peripheral lymphocytes persisted for years. This observation suggested that a population of antigen-sensitive

lymphocytes remained in the circulation following naturally acquired infection. The study also suggested that immunity to M. pneumoniae infection is mediated by circulating small lymphocytes.

Swine mycoplasmal pneumonia

Betts, Whittlestone and Beveridge (1955) indicated there was no field evidence that a natural infection of enzootic pneumonia induced any significant degree of immunity.

Lannek and Börnfors (1957) showed, however, that pigs which had recovered from experimentally produced enzootic pneumonia developed a strong immunity to subsequent challenge. The pigs were infected intranasally with a pneumonic lung suspension and the presence or absence of pneumonia was judged by X-ray examinations. By 118 days post-infection the pigs had recovered (as determined by radiological examination). They were then challenged by a second inoculation or by contact-exposure to pneumonic pen-mates. All the challenged pigs were free from lesions of the disease at slaughter; in contrast, all control animals with no previous exposure developed pneumonia.

In another experiment, Börnfors and Lannek (1958) tested the ability of the same infectious strain to induce immunity (1) by intranasal inoculation of pigs which were receiving prophylactic doses of tetracyclines, and (2) by repeated intramuscular injections of pneumonic lung. Pigs that were inoculated intranasally at the time they were on feeds containing tetracycline and that did not develop pneumonia during the 3-week period of antibiotic treatment were challenged with the infectious material after removal from the antibiotic treatment. Almost all the

animals which had been effectively protected by tetracycline treatment developed pneumonia following the challenge inoculation. The result demonstrated that while tetracycline prevented the production of pneumonic lesions, a primary infection of mycoplasmal pneumonia was essential for the development of immunity. Pigs that were given repeated intramuscular injection of the pneumonic lung suspension and subsequently challenged intranasally, developed pneumonia. This suggested that intramuscular inoculations of the pneumonic agent did not induce immunity.

In Sweden, Pfizer (1968) marketed a tissue-culture-derived vaccine against mycoplasmal pneumonia of swine. The commercial vaccine was formalin-inactivated and contained aluminium hydroxide as an adjuvant. The manufacturers claimed the vaccine was capable of freeing pigs in infected herds from the disease within a few months after vaccination. The vaccine had been prepared from a culture that was later identified as M. hyorhinis. A number of reports (L'Ecuyer, 1962; Goodwin, 1967; Slavik, 1971) have shown the absence of serologic cross-reaction between M. M. hyorhinis and M. hyopneumoniae, the cause of swine mycoplasmal pneumonia. This makes it rather difficult to understand the strong protective immunity claimed for this commercial vaccine.

Goodwin et al. (1969a) infected pigs by intranasal inoculations of pneumonic lung suspensions. Sixteen weeks after the primary infection, when it was presumed the pigs had recovered, they challenged the survivors with the same strain. No lung lesions were observed when the animals were killed 3 weeks after challenge. These findings suggested that pigs acquired strong immunity after recovery from infection with mycoplasmal pneumonia. In the same year Goodwin et al. (1969b) tested the ability of

formalinized M. hyopneumoniae vaccine to induce immunity in pigs. The pigs were vaccinated twice with antigen prepared from the "J" strain. The first injections were given intramuscularly or intradermally with Freund's complete adjuvant, and the second injection was given without adjuvant. The immune status of the pigs was tested by intranasal challenge with doses of pneumonia lung suspensions. The vaccinated pigs showed no evidence of resistance to challenge inoculation. The authors noted a possibility that any immunity induced had been overwhelmed by the large dose of challenge inoculum (5ml of 1:10 suspension of the pneumonic lung). When a lower challenge dose was used, some protection occurred in 3 of the vaccinated pigs. In another trial, they injected a pregnant sow twice with nonformalinized antigen without adjuvant. The sow's litter was subsequently exposed to infection at 7 days of age, after they had suckled naturally from birth. The piglets were not protected against challenge inoculation. There was no correlation between the vaccine-induced serum antibody level and protective immunity.

Lam (1970) and Lam and Switzer (1971b) evaluated the ability of five vaccinal preparations of M. hyopneumoniae to prevent the infection in swine. Vaccine A was prepared by ether extraction of a concentrated suspension of the organism and contained Freund's incomplete adjuvant. Vaccine B was also prepared from ether extract but was formalin-inactivated. Vaccine F was prepared by alternate freezing and thawing in a solidified carbon-dioxide-alcohol bath; it too contained incomplete adjuvant. Vaccine G was prepared by lysis with 2% sodium lauryl sulfate (SLS) followed by dialysis against ammonium sulfate and distilled water. The fifth vaccine, Vaccine H, was a formalin-inactivated broth culture. Four

of the vaccine preparations were administered intramuscularly to groups of pigs; the pigs were vaccinated 3 times at one-week intervals. Vaccine H was administered intranasally to a fifth group of pigs on 3 consecutive days. All the vaccinated pigs and controls were challenged intranasally, two weeks after vaccination. None of the 4 pigs that received Vaccine A (ether extract with incomplete adjuvant) developed gross pneumonic lesions whereas all 4 nonvaccinated controls developed pneumonia; 5 of 8 pigs given the formalinized ether extract (Vaccine B) were protected whereas 7 of 8 controls had pneumonic lesions; Vaccine F (freeze-thawed) protected 19 of 26 pigs while 19 of 23 controls had lesions; and the SLS-lysed preparation (Vaccine G) protected 15 of 18 pigs while 15 of 19 control pigs developed pneumonia. None of the intranasally vaccinated pigs was protected. The results indicated that intramuscular injection of the vaccine preparations provided some protective immunity. These findings are in contrast with the result reported by Goodwin et al. (1969b); it is possible that the ether extraction, freezing-thawing disruption, and SLS lysis of the M. hyopneumoniae antigen enhanced its immunogenicity.

MATERIALS AND METHODS

Source of Mycoplasma hyopneumoniae

Mycoplasma hyopneumoniae strain #11 was used throughout this study. L'Ecuyer (1962) originally obtained transmission of pneumonia in pigs he inoculated with pneumonic lung material from a pig in a large swine herd with chronic pneumonia. He obtained the infectious material from the eleventh herd he studied and therefore identified it as number eleven. When inoculated intranasally into respiratory disease-free pigs, the pneumonic lung suspension consistently induced clearly demarcated, reddish-grey pneumonic lesions, frequently confined to the apical, cardiac, and intermediate lobes of the lungs. The lesion was characterized by perivascular and peribronchiolar lymphoid hyperplasia, alveolar interstitial thickening, and alveolar exudation of neutrophils, lymphocytes and septal cells. The organism was serially passaged several times in the pig (L'Ecuyer, 1962) and was grown in cell-free medium (Maré, 1965). Maré and Switzer (1965) characterized the infectious agent and named it Mycoplasma hyopneumoniae. Huhn (1969) has given a detailed diagrammatic passage history of the swine pneumonia organism.

Broth passaged cultures of M. hyopneumoniae strain derived from a lyophilized culture of the 20th passage were employed in the vaccine production and in most experimental inoculations in the present study.

Growth Medium for M. hyopneumoniae

Liquid medium

The cell-free medium for the propagation of M. hyopneumoniae consisted of Eagle's Minimum Essential medium, Hepes-Buffer

(N-2-hydroxyethyl piperazine-N-2 ethane sulfonic acid), yeast extract, lactalbumin hydrolysate, and swine serum (Switzer, 1972). The medium contained the following ingredients:

A. Eagle's Minimum Essential Medium[1]

B. Hepes buffer (N-2-hydroxyethyl piperazine-N-2 ethane sulfonic acid)[2]

C. Yeast Extract

   a. Two-hundred and fifty grams Fleischman's type 20-40[3] dry yeast were added to 1 liter distilled water.

   b. The mixture was heated to boiling, with frequent stirring.

   c. The mixture was then filtered through two sheets of Whatman #1 filter paper.

   d. The pH was adjusted to 8.0, with 1N NaOH.

   e. The yeast extract was dispensed in 5 to 8ml aliquots, autoclaved at 251°F (15 pounds pressure) for 15 minutes.

   f. It was stored at -20°C until used. Only the clear supernatant was used in the medium.

D. Lactalbumin hydrolysate (enzymatic)[4]

E. Swine serum

   a. Swine serum was adjusted to a pH of between 4.3 and 4.5 with 1N HCl and was allowed to stand at 4°C for 2 to 18 hours.

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

[2]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3]Standard Brands Incorporated, New York, New York.

[4]Nutritional Biochemicals Corporation, Cleveland, Ohio.

b. This acid-precipitated swine serum was then centrifuged at 2000 rpm (590XG) for 15 minutes. The sediment was discarded and the supernatant was clarified through a Whatman GF/a fiberglass filter pad and Selas #10, #01, #015, and #02 porcelain filters.[1]

c. The pH was adjusted to 7.0 with 1N NaOH, and the swine serum was stored at -20°C until used.

The complete medium was prepared by mixing the following components in the proportions indicated:

1.	Eagle's minimum essential medium	9.8gm
2.	Ion exchange column water	1000ml
3.	Hepes buffer	5.96gm
4.	Lactalbumin hydrolysate	10.0gm
5.	Yeast extract	10.0ml
6.	Swine serum	200.0ml

The pH of the medium was adjusted to 7.6 with 1N NaOH. The medium was clarified by passing through Selas #10 and #01 filter candle, and sterilized through a Selas #02 porcelain filter. The medium was dispensed in screw-capped glass tubes (16 x 125mm) or screw top flasks and incubated at 37°C for 24 hours as a sterility check and stored at 4°C until used.

Liquid medium used for the primary isolation of M. hyopneumoniae from lung lesions contained thallium acetate and penicillin at a final concentration of 1:4000 and 1,000 units per ml respectively.

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[1]Selas Corporation of America. Fluid processing division. Limekiln Pike and Dreshertown Rd., Dresher, Pennsylvania.

Solid medium

A. Preparation of the agar

1.	Ion Agar No. 2[1]	3.6gm
2.	Ion exchange column water	200ml

The agar was added to the water in a 1000ml Erlenmeyer flask. The mixture was boiled in a water bath to dissolve the agar. The dissolved agar was sterilized at 121°C for 15 minutes and then allowed to cool to 45°C.

B. Eagle's base (2x) medium: A 2x concentration of the complete Eagle's liquid medium was formulated according to the following procedure:

1.	Eagle's minimum essential medium	9.8gm
2.	Ion exchange water	500ml
3.	Hepes buffer	5.96gm
4.	Lactalbumin hydrolysate	10.0gm
5.	Yeast extract	10.0ml
6.	Acid adjusted swine serum	70.0ml

The pH of the medium was adjusted to 7.6 with 1N NaOH. Penicillin and thallium acetate were added to give a final concentration of 1:1000 and 1:4000, respectively. The liquid medium was then clarified through Selas #10, #01, and #015 filters and sterilized through a Selas #02 filter.

C. The medium was brought to the same temperature as the dissolved

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[1]Colab Laboratories, Inc., Box 66, Chicago Heights, Illinois.

agar (45°C) and the two were aseptically combined in equal parts. After mixing thoroughly and carefully by gentle swirling to avoid bubble formation, the agar medium was poured into plastic petri dishes[1] and allowed to solidify. The plates were incubated at 37°C for 24 hours for sterility check and stored in plastic bags at 4°C.

Maintenance of M. hyopneumoniae in Liquid Medium

Screw-capped glass tubes containing approximately 6ml of complete Eagle's base medium were inoculated with 0.5ml of M. hyopneumoniae fluid culture. Inoculated tubes were incubated at 37°C and serial transfers were made at intervals of 2 to 3 days. Selected passage levels were lyophilized in 0.2ml amounts and stored at -20°C.

Growth of M. hyopneumoniae on Solid Medium

Five-tenth ml of a 3-day old culture was inoculated onto the surface of agar plates. The plates were carefully rotated so the culture fluid evenly covered the agar surface. Inoculated plates were incubated in a 5% CO_2 incubator at 37°C, in an inverted position. The plates were examined daily for 5 to 6 days for M. hyopneumoniae colonies with the aid of 100X magnification utilizing a compound light microscope.

Experimental Swine

All the experimental pigs were obtained from the respiratory disease-free herd maintained at the Veterinary Medical Research Institute,

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[1]Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, California.

Iowa State University. The original stock was established from surgically derived pigs and has been maintained in quarantine for 20 years. The pigs were housed in isolation units during the experiments. All the pigs were either Yorkshires or Hampshires and were 4 to 24 weeks of age at the beginning of the experiments. They were fed a contract bid formulated pig grower ration of 19% protein that did not contain any added antibiotics.

Infection of Experimental Pigs

Pigs were exposed to M. hyopneumoniae by intranasal instillation of 3-day old fluid culture, or of pneumonic lung suspension, depending on the experiment. Each pig was given 5ml of the inoculum. The snout of the pig was held in an upright position and the nozzle of the syringe (without needle) was inserted into the nare. The culture was then instilled slowly; instillation of a small amount of inoculum was synchronized with the inspiration of the pig. The pigs could be observed to swallow much of the inoculum. The inoculum was administered daily for three consecutive days. The pigs were kept under observation for a varying number of weeks, as determined by the particular experiment.

Necropsy Examination

At the termination of each experiment, pigs were killed by electrocution and exsanguination, and necropsy was performed. The lungs were examined for gross mycoplasmal pneumonia lesions and lung specimens were taken for histological study. When desired, portions of pneumonic lungs were collected aseptically for isolation of M. hyopneumoniae.

Preparation of Lung Tissues for Histological Examination

Portions of the lungs were fixed in 10% formalin immediately after death. After 3 days fixation, the specimens were trimmed and embedded in Paraplast[1] tissue-embedding medium. They were sectioned at six micrometers and mounted on glass slides with an albumin fixative. Sections were then stained with Harris' hematoxylin and counterstained with eosin Y (U.S. Armed Forces Institute of Pathology, 1960).[2]

Isolation of M. hyopneumoniae from Pneumonic Lesions

A portion of the aseptically collected pneumonic lung was ground in a TenBroeck grinder, in the presence of about 10ml sterile complete Eagle's medium containing penicillin (1000 1 U/ml) and thallium acetate (1:4000 final concentration). Five-fold serial dilutions of the lung homogenate were made in screw-capped tubes containing Eagle's base medium with inhibitors. Inoculated tubes were incubated at 37°C. Initial passages from the primary dilutions were made at 4 to 14 days; subsequent passages were made at 3 to 5 day intervals. An inoculated tube was considered negative for M. hyopneumoniae if growth could not be demonstrated after 14 days incubation. A presumptive positive isolation was indicated by the following factors: an acid pH change and an increased opalescence or turbidity to the medium; presence of a slight sediment which formed a delicate spiral when the tube was gently swirled; demonstration of typical

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[1]Sherwood Medical Industries, Inc., St. Louis, Missouri.

[2]Sectioning, mounting, and staining done by Pathology Laboratory, VMRI.

mycoplasmal elements in a Giemsa stained film prepared from the sediment obtained after centrifugation of the fluid culture; the absence of growth on 5% horse blood agar or in NIH thioglycolate broth;[1] lack of growth in beef-heart-infusion-turkey serum[1] plates. Confirmation of isolation was made by inoculation of Eagle's base agar plates with the fluid subcultures and by comparison of the colonial morphology of the isolate with colonies formed by the laboratory-maintained M. hyopneumoniae.

Blood Collections

Throughout the course of the experiments, blood samples were collected from the anterior vena cava of the pigs. Collected blood was allowed to clot for 1 hour at room temperature or was left overnight at 4°C. The blood sample was then centrifuged at 1700 rpm (600 x G) for 10 minutes. The resulting serum was aspirated and stored at -80°C until used in the serologic test.

Production of M. hyopneumoniae Vaccine and Complement-Fixation Test Antigen

The following procedures were followed in the production of immunizing vaccines and complement-fixation (CF) antigen.

1. Five mililiter of a 48-hour high passage (greater than 200) fluid culture of M. hyopneumoniae was used to inoculate 250ml of sterile complete Eagle's base medium in a 500ml screw-capped flask. The inoculated flask

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[1]Difco Laboratories, Detroit, Michigan.

was incubated at 37°C for 3 days and the culture was then mixed with equal parts of a sterile 24% sucrose solution and dispensed aseptically in 5ml. amounts into sterile screw-cap tubes. The tubes were stored at -80°C.

2. Appropriate numbers of the frozen culture tubes were thawed and each 5ml of culture was inoculated into a screw-capped 1-liter flask containing approximately 500ml of sterile Eagle's base medium. The flasks were incubated for 3 days at 37°C in a shaking waterbath; the shaker was set at 10 to 15 oscillations per minute. This gentle shaking was found to be an important factor in production of a high titer antigen. An accelerated rate of agitation of the flasks caused precipitation of serum proteins from the culture medium, while stationary incubation resulted in low antigen yields.

3. Antigen was harvested by centrifugation of the culture at 12,000 rpm (15,000 x G) at 4°C for 30 minutes in a Beckman Model L-2-65 (Head #19) preparative ultracentrifuge. The sediment was resuspended in veronal buffered diluent without gelatin (see subsequent section for composition) to 2% of the original volume of incubated medium. The suspension was washed two times by centrifugation at 2,500 rpm (1300 x G) for 45 minutes in 15ml centrifuge tubes. The packed mycoplasma was reconstituted to a 10% concentration with veronal buffered diluent. Usually, 0.5 to 0.75ml of packed mycoplasma was obtained from 1500ml of inoculated medium. The 10% suspension was then dispensed in vials in 1ml quantities and stored at -80°C until used as CF antigen or as vaccine.

4. Growth Initiating Titer was determined for each lot of M. hyopneumoniae antigen or vaccine produced. Before the culture was harvested, 5ml of the incubated flask culture was aseptically aspirated.

Five-tenths ml of this culture was inoculated into 4.5ml of sterile Eagle's medium in a screw-cap tube and the serial ten-fold dilution was continued until a final dilution of 10^{-10} of the original culture resulted. Inoculated tubes were incubated at 37°C for 7 to 10 days and were checked daily for growth of M. hyopneumoniae. The highest dilution in which growth occurred was recorded and the reciprocal of that dilution marked the growth initiating titer. A growth initiating titer of 10^{-6} or more in the flask culture was considered satisfactory.

5. The pH of the incubated flask culture was determined before the antigen was harvested. Fifteen to 20ml of the culture was transferred into a 50ml beaker and the pH was read with the aid of a pH meter.

6. Checks of the flask cultures for bacterial contamination were made by inoculating 0.25ml of the culture onto blood agar plates and into thioglycolate broth. Beef-heart-infusion-turkey serum plates were also inoculated with 0.5ml of the culture to check for other mycoplasmal contamination. The inoculated plates and broth were incubated at 37°C and examined daily for any indication of growth. The flask culture was regarded as negative for contaminants if no growth was observed on the plates or in the broth after 5 days of incubation.

Complement-Fixation Test

Serum samples were tested for the presence of specific antibodies against M. hyopneumoniae by the modified direct microtitration complement-fixation (CF) test.[1] The CF tests were conducted according to the modified

[1]The CF tests were performed by M. F. Slavik, Veterinary Medical Research Institute.

technique developed by Slavik (1971). The preparation of the reagents, the titration of complement, hemolysin, and antigen, and the procedure for the diagnostic test are briefly outlined.

Veronal buffered diluent (VBD) with gelatin

A 5X stock solution was prepared by combining the following components in a 2 liter volumetric flask in the order listed:

NaCl	83.00gm
Na-5, 5-diethyl barbiturate	10.19gm
Distilled water	500.00ml
1N HCl	30.00ml
Stock solution containing 1 molar $MgCl_2$ and 0.3 molar $CaCl_2$ (20.3gm $MgCl_2 \cdot 6H_2O$ and 4.4gm $CaCl_2 \cdot 2H_2O$ in 100ml distilled water)	5.00ml

The flask was filled to the mark with distilled water and the ingredients were mixed thoroughly. One hundred ml of 5X stock solution of VBD, 400ml of distilled water, and 16ml of 2.5% gelatin in distilled water were mixed thoroughly in a graduated cylinder. This solution made a 1X concentration of VBD-with-gelatin for daily use and was stored in 1 liter plastic bottle at $4^{o}C$.

Sheep red blood cells (RBC) suspension

Five ml of sheep blood, preserved in an equal volume of Alsever's solution (Karrer et al., 1950) was added to 10ml VBD with gelatin and the mixture was centrifuged at 1700 rpm (600 x G) for 5 minutes. After removal of the supernatant and white cell layer (buffy coat) more VBD was added

to give a total volume of 15ml. The RBC were re-suspended and were washed two more times by centrifugation. Following the final wash, the supernatant was removed by aspiration and the volume of the packed cells was recorded. Thirty-four and seven-tenths volumes of VBD were added for each volume of packed sheep red blood cells; this made a 2.8% RBC suspension which was stored at 4°C and was used on the day of preparation.

Hemolysin

Glycerinated anti-sheep hemolysin[1] was initially diluted to 1:100 by mixing 0.2ml of the 50% hemolysin antiserum and 9.8ml of 0.85% saline. Titration of the hemolysin was performed as follows: Six dilutions from 1:1000 to 1:8000 were made in VBD and 1ml of each hemolysin dilution was added to equal volume of 2.8% sheep RBC. The mixtures were incubated at 37°C for 15 minutes. Two-tenth ml of the sensitized RBC (from each hemolysin-dilution) was mixed with 0.4ml of cold VBD and 0.4ml of complement. The mixtures were again incubated at 37°C in waterbath for 1 hour and then centrifuged for 5 minutes. The percentage of hemolysis was found by comparison with freshly prepared hemoglobin color standards and the optimum dilution of hemolysin was determined from the plotted linear graph.

Sensitization of red blood cells

One volume of "optimal" hemolysin dilution was added to an equal volume of 2.8% sheep red blood cell suspension. The mixture was incubated

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[1]Difco Laboratories, Detroit, Michigan.

at 37°C in a water bath for 15 minutes. This procedure was carried out just before the use of the sensitized cells in the final CF test.

Guinea-pig complement

Commercial lyophilized guinea-pig complement[1] was used. The complement was modified by reconstituting it with fresh, normal serum from young, pneumonia-free pigs. The effects of this modification were a compensation for the procomplementary property of swine serum on the guinea-pig complement and the restoration of the complement-binding capacity of the inactivated test serum. Dilutions (1:400 and 1:500) of the reconstituted complement (C') were made in VBD plus gelatin. Each dilution of C' was titered by adding varying amounts of a particular dilution to appropriate volumes of chilled VBD in 4 serologic tubes. A constant volume of the sensitized RBC was added to each tube to give total reaction volume of 1ml. The mixtures were incubated in a 37°C waterbath for 30 minutes and then centrifuged. The percentage of hemolysis of each tube was found by comparison with a hemoglobin color standard. The $C'H_{50}$ (reciprocal of complement dilution producing 50% hemolysis) was determined by a logarithmic graphing method and the equivalent of five $C'H_{50}$ units (the working concentration in the CF test) was calculated algebraically.

Antigen

The diagnostic M. hyopneumoniae antigen was prepared by diluting the 10% suspension of the harvested organism (see above) to a 1% concentration,

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[1]Difco Laboratories, Detroit, Michigan.

and then heating at 52°C for 30 minutes. The antigen was then titered against a standard serum from a pig known to be infected with M. hyopneumoniae. Antigen titrations were conducted in plastic microtitration plates[1] containing 96 U-shaped wells. Dilutions were made with VBD plus gelatin. To each well containing the appropriate antigen and serum dilution, 0.05ml (5 $C'H_{50}$ units) of the complement reconstituted with normal swine serum was added. The plates were shaken and incubated overnight at 4°C. Sensitized sheep RBC (0.025ml) was then added to each well; the plate was re-incubated at 37°C for 30 minutes. After centrifugation, the percentage of hemolysis in each well was determined. The antigen dilution that gave the greatest fixation of complement (0 to 30% lysis) with the highest dilution of antiserum was taken as the working dilution for use in the diagnostic CF test. This working antigen dilution must, at the same time, show no anti-complementary effect.

Antiserums

Test serums were inactivated by heating at 54°C to 56°C for 30 minutes.

Procedure for complement fixation (CF) test

The CF test was performed in microtiter plates. Into each well of the first row was added 0.05ml of the different heat-inactivated test serums. Veronal buffered diluent plus gelatin (0.025ml) was dropped into all other wells. Two-fold dilutions of the serums were made and the plate

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[1]Linbro Chemical Company, Inc., New Haven, Connecticut.

was chilled at 4°C for 30 minutes. Twenty-five-thousandth ml of a working dilution of the antigen cooled to 4°C was added to each well; five units (0.05ml) of chilled complement was also added to each well. The plate was shaken and placed at 4°C for 15 to 18 hours. After this initial incubation, 0.025ml of the sensitized sheep RBC was added to all of the wells. The plate was re-incubated at 37°C for 30 minutes and then centrifuged at 2500 rpm (1300xG) for 5 minutes. The percentage of hemolysis in each well was determined with the aid of a test reading mirror[1] and recorded. Test serums were considered positive if they showed 70% or more intact RBC (30% or less hemolysis) at serum dilutions of 1:8 or greater. Titers were expressed as reciprocal of the highest dilution of the test serum, that exhibited the 70% or more intact RBC. Controls of the appropriate reagents were included in each CF test.

Experimental Design

Four separate immunization trials were conducted. The first trial was designed to evaluate the ability of a M. hyopneumoniae vaccine to eliminate lesions and promote recovery in pigs already infected with the organism. The other three experiments were carried out to determine the ability of M. hyopneumoniae vaccines to provide resistance to challenge infection.

Following the persistent failure of the vaccinated and especially the nonvaccinated control pigs in one of the immunization trials to develop a serologic response following challenge exposure, other

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[1]Cooke Engineering Company, Alexandria, Virginia.

experiments were designed. One experiment was performed to evaluate both the virulence of the test organism and the effect of age of the experimental swine on susceptibility to experimental mycoplasmal pneumonia. Two experiments were conducted to investigate the presence in the feed of a M. hyopneumoniae inhibiting substance.

Immunization Trials

Experiment I

Six- to 8-week old pigs which had been naturally infected with M. hyopneumoniae were tested serologically. Ten of those pigs that exhibited two consecutive positive CF antibody titers were chosen for the trial. By random selection the 10 pigs were assigned to two equal groups. One group served as infected pigs to be immunized and the other group served as infected but nonimmunized controls. The vaccinated and nonvaccinated groups were housed in separate isolation units.

On each day of immunization, the vaccine was prepared as follows: The frozen 10% concentration of M. hyopneumoniae packed cells was thawed. An equal volume of incomplete Freund's adjuvant was added to each vial and a water-in-oil emulsion was made by thoroughly mixing the contents with a syringe and 20 gauge needle.

Each of the vaccinated pigs was inoculated with 1ml of the adjuvant-live vaccine mixture. Injections were by the subcutaneous route in the flank region. Pigs were vaccinated three times at one week intervals. Blood was collected each week from all the pigs. Complement-fixation tests of the serums were performed and the serologic titers were recorded.

At no other time during the course of the trial were these pigs

reinfected or challenged by intranasal inoculation of M. hyopneumoniae culture. Seven weeks after the last vaccination, all pigs were killed and a necropsy was performed. The incidence and extent of gross pneumonic lesions were recorded.

Experiment II

Two types of M. hyopneumoniae vaccines were used in this trial. One of the vaccines was prepared from the 10% concentration of the harvested flask culture, as described in Experiment I. However, this vaccine was heat-inactivated at 52°C for 30 minutes. The vaccine contained equal parts of incomplete Freund's adjuvant and was designated as Flask-antigen. The growth initiating titer of the flask culture from which it was prepared was 10^{-7} prior to harvesting and concentration. The other vaccine was prepared from a 25% suspension of pneumonic lung obtained from pigs inoculated in previous research with M. hyopneumoniae strain 11. The frozen lung material was thawed and the suspension was reconstituted to a 10% concentration with sterile Eagle's based medium. The material was then thoroughly mixed with incomplete Freund's adjuvant. This vaccine was designated as Lung-antigen.

Sixteen pigs, 3 to 4 weeks old, were used in the trial. All the pigs were serologically negative to M. hyopneumoniae infection as determined by three complement-fixation tests performed at one week intervals. The animals were randomly assigned to 4 equal groups: pigs to be immunized with the flask-antigen (Flask-antigen vaccinates); pigs to be vaccinated with the pneumonic lung preparation (Lung-antigen vaccinates); pigs not immunized but later challenged (positive controls); and pigs not immunized

and not challenged (negative controls). The nonexposed negative control pigs remained in the supply herd until necropsy. The other 12 animals were housed in individual plexiglass cages in a large isolation unit. Two pigs were placed in each cage, thus making a duplicate of each experimental group. One ml of the respective antigen was administered subcutaneously at the flank region to the appropriate pigs. One week after the first vaccination, a booster inoculation was administered by the same route. A third inoculation was given two weeks after the second injection. Four weeks after the final inoculation, all the vaccinated pigs and the positive control pigs were challenged by intranasal instillation with a low passage (passage 21) M. hyopneumoniae fluid culture. The challenge inoculum had a growth initiating titer of 10^{-7}. Challenge inoculations were administered on three consecutive days. Blood was collected from the pigs each week and the serums were tested for antibodies against M. hyopneumoniae. Four weeks after the challenge all pigs were necropsied. Lung specimens were collected for histological examination.

Experiment III

This trial was designed to evaluate the cumulative effect of M. hyopneumoniae vaccines administered by various routes in inducing resistance of swine to challenge infection with the organism.

Twenty-four pneumonia-free (serologically negative) pigs consisting of equal numbers of vaccinates and nonvaccinates were used in the trial. The animals were housed in 3 isolation rooms, each room containing 4 vaccinates and 4 nonvaccinates, randomly selected. Each of the vaccinates was inoculated by the subcutaneous, footpad, intraperitoneal, and

intratracheal routes.

The vaccines consisted of a 10% suspension of M. hyopneumoniae antigen harvested from flask cultures. For the subcutaneous and footpad immunizations, live vaccine containing equal volume of incomplete Freund's adjuvant was used. Intraperitoneal inoculation was also made with a live vaccine but no adjuvant was added. The vaccine for intratracheal inoculation was heat-inactivated in a waterbath at 52°C for 75 minutes; this vaccine contained no adjuvant.

On the first day, each of the vaccinates in isolation units #3 and #21 was given subcutaneous and footpad inoculations. One ml of the adjuvant-containing vaccine was administered subcutaneously and 0.2 to 0.3ml was administered into the footpad. Vaccinates in isolation unit #20 received only the subcutaneous inoculation. On the 5th day, 40 to 50ml of mineral oil was administered intraperitoneally into each vaccinate. The mineral oil was administered to cause an accumulation of immune-responsive peritoneal macrophages. Six days after the administration of the mineral oil, all the vaccinates were intraperitoneally vaccinated with 1ml of vaccine. One week after the intraperitoneal immunization, the second subcutaneous and footpad inoculations were made; this time, the vaccinates in unit #20 also received the footpad injections. After another 7 days, all vaccinates were given an intratracheal inoculation of 0.5ml of the heat-inactivated vaccine per pig. Two weeks after the intratracheal injection (six weeks after the first vaccination), all the vaccinates and nonvaccinates were challenged intranasally with low passages (passage 29 to 31) of M. hyopneumoniae fluid culture. Challenge inoculations were administered on 3 consecutive days. The challenge culture had

growth initiating titers of 10^{-5} to 10^{-6}. Blood for serologic tests was collected at regular intervals from the pigs. Since five weeks after the challenge exposure to M. hyopneumoniae, none of the control animals had reacted serologically to the challenge, all the pigs were challenged again. Both pneumonic lung suspension and the laboratory-maintained high passage (p. 324) fluid culture were used in the second challenge. The high passage culture had a growth initiating titer of 10^{-8}. All the pigs were killed three weeks after the second challenge and necropsy was performed.

Experiment IV

The vaccine used in this trial was a 10% suspension of concentrated M. hyopneumoniae harvested from the flask culture. Three batches of the vaccine were prepared from flask cultures which had growth initiating titers of 10^{-7}, 10^{-9}, and 10^{-9}. On the day of immunization, the vaccine was heat-inactivated at $52^{o}C$ for 30 minutes and incomplete Freund's adjuvant was added.

Twelve pneumonia-free pigs 12 to 14 weeks old were used. The pigs were randomly grouped into 7 vaccinates and 5 nonvaccinated controls. The animals were housed in three isolation rooms: unit #8 housed 4 vaccinates; unit #7 contained 3 vaccinates and 1 nonvaccinate, and unit #3 housed the other 4 nonvaccinates. All the pigs were fed a pig grower ration which was mixed and supplied by the Iowa State University Swine Nutrition Farm. This feed is known to contain no antibiotics. It was necessary to use this special feed because the regular stock of commercial swine feed at the Veterinary Medical Research Institute was suspected of containing some type of M. hyopneumoniae inhibiting substance (see

appropriate section below). Although all the pigs had been on the commercial VMRI feed before they were selected for the experiment, they were fed only the Swine Nutrition feed for the entire duration of the trial. The vaccinates were immunized three times at one-week intervals; 1ml of the vaccine was administered subcutaneously in the flank to each pig. In addition to the subcutaneous injections, footpad inoculations (0.2ml per pig) were given to the 3 vaccinates in isolation unit #7. The footpad booster injections were administered at the same time as the second and third subcutaneous injections.

Two weeks after the third immunization, the vaccinates and non-vaccinated controls were challenged by intranasal instillation of low passage (passages 39 and 40) of M. hyopneumoniae fluid culture. Challenge inoculations were administered on three consecutive days. Blood was collected weekly for serologic tests. Five weeks after challenge, all pigs were necropsied.

Studies on the Virulence of the Challenge Organism (M. hyopneumoniae) and the Effect of Age of the Pigs on Susceptibility to Experimental Infection

The unsuccessful attempts to infect any of the 12 nonvaccinated control pigs in Immunization Experiment III and the failure to infect 3 pre-trial pigs by intranasal inoculations with broth cultures of M. hyopneumonia (low and high passages) prompted the speculation that the virulence or pathogenicity of the test organism might have been lost or significantly reduced under the laboratory conditions. The fact that the pigs were 6 to 8 months old at the time of challenge inoculations and

that none of the pigs responded to challenge exposure with mycoplasmal pneumonic lung suspension raised the possibility that age resistance might be responsible for the failure of the pigs to develop pneumonia. The following experiment was then designed to determine the validity of either or both of these possibilities.

A total of 12 mycoplasmal pneumonia-free pigs, consisting of 6 young pigs (4 weeks old) and 6 old pigs (8 months and older) were used in the trial. The young pigs were housed in two isolation units, each unit containing 3 young pigs. The old pigs were similarly housed in two separate isolation units, with 3 pigs in each unit. The pigs were fed the regular VMRI pig grower throughout the course of the experiment. Three young pigs and 3 old pigs were inoculated intranasally with low Passage broth culture of M. hyopneumoniae; 3 young pigs and 3 old pigs were inoculated with high Passage broth culture.

The following is a brief history of the low passage culture used as inoculum:

> A lyophilized vial of 18th passage of M. hyopneumoniae which had been stored at -20°C since December, 1965 was reconstituted in May, 1971 and three more serial passages were made at 3-day intervals. Thirty-six-hour broth cultures of passage 21 were stored frozen at -20°C. After the first 24 hours in the freezer, one tube of the 21st passage had a growth initiating titer of 10^{-7}. In March, 1972 one tube of the frozen passage 21 was thawed and serial passages were made at 3 to 4-day intervals. The 24th, 25th, and 26th passages were used in the intranasal inoculation of the experimental pigs; these cultures

had growth initiating titers of 10^{-4}, 10^{-5}, and 10^{-6}, respectively.

The high Passage inoculum consisted of the 271st and 272nd passages of broth cultures of M. hyopneumoniae which were maintained in the laboratory and continually transferred at 2 to 3-day intervals. The cultures had growth initiating titers of 10^{-8} or greater.

The pigs were exposed intranasally with the respective cultures on three consecutive days. Blood was collected weekly from all the pigs. They were observed for 6 weeks and were then necropsied.

Effect of Swine Feed on Resistance to Infection

The continued lack of success in efforts to infect pigs with broth cultures of M. hyopneumoniae or with pneumonic lung suspension suggested the possibility that the new supply of feed at the Veterinary Medical Research Institute (VMRI) contained some kind of inhibiting substance. Two experiments were designed to test the validity of this possibility.

Experiment I

Preparation of feed filtrate Approximately one-half of one pound (225gm) of the pelleted VMRI swine feed was blended in the presence of an equal amount of distilled water. The resulting material was centrifuged at 1,700 rpm (600 x G) for 20 minutes. The supernatant was passed through Whatman #1 filter paper and sterilized through 0.45 Millipore filters. The filtrate was designated VMRI Feed Filtrate. A small amount of the filtrate was inoculated onto horse blood agar plates and the plates were incubated for 24 hours at 37°C as a sterility check.

Sensitivity tests Two types of sensitivity tests were performed on Eagle's base medium agar plates. About 0.5ml of a 2-day broth culture of M. hyopneumoniae (high passage) was inoculated onto the plates. The plates were carefully rotated to allow the culture to uniformly cover the surface. Sterile, blank filter paper discs[1] were soaked in the VMRI feed filtrate. After ridding the discs of excess moisture, they were placed on the inoculated plates. Two to 3 discs were placed on each plate. Sterile, blank discs that were not dipped in feed filtrate were placed on a number of inoculated plates as controls.

For the other type of sensitivity test, 2ml of broth culture of M. hyopneumoniae (high passage) was mixed with equal volume of the feed filtrate. About 0.5ml of the mixture was inoculated onto Eagle's base medium agar plates. Plates inoculated with the culture alone were also used as controls.

Both types of sensitivity tests were repeated, using the low passage culture of M. hyopneumoniae. All inoculated plates were incubated in a CO_2 incubator at 37°C. Plates were examined daily for 4 to 5 days with the aid of a light microscope.

Comparison of VMRI feed and feed from Swine Nutrition Farm[2] A filtrate of the Swine Nutrition feed was prepared in the same manner as the VMRI feed filtrate. The Swine Nutrition feed was known to contain no antibiotics. The inhibition activity of the different feed filtrates was

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[1]Difco Laboratories, Detroit, Michigan.

[2]This feed was mixed and supplied by the Iowa State University Swine Nutrition Farm by special arrangement.

compared by the disc sensitivity test, as described above.

Both filtrates were also compared by a tube-dilution technique. Two-fold serial dilution of each filtrate was made in glass tubes containing Eagle's base medium. The dilutions of each filtrate were made in duplicates. One row of the tube-dilutions of each filtrate was inoculated with 0.5ml of M. hyopneumoniae culture. The second row of serial dilutions of each filtrate served as uninoculated controls. All tubes were incubated at 37°C. Inoculated tubes were checked daily for 7 days for growth of M. hyopneumoniae, each inoculated dilution being compared with its equivalent uninoculated control.

Sensitivity tests of individual components of the swine feed

Attempts were made to determine if any one of the various components of the feed accounted for the inhibition activity. The individual ingredients used in compounding the VMRI commercial feed were not available. However, the Swine Nutrition feed contained similar components as the VMRI feed. Therefore, filtrates of each of the following components of the Swine Nutrition feed were prepared: ground corn, soybean meal, vitamin premix, dicalcium phosphate, calcium carbonate, iodized salt, and trace mineral premix. The disc sensitivity tests were performed with the filtrate prepared from each feed component.

Experiment II

This trial was carried out to verify the effect of source of feed on the response of pigs to infection with M. hyopneumoniae.

Eight pneumonia-free pigs, 4 weeks old, were housed in two isolation units, each unit containing 4 randomly selected animals. Pigs in one unit

were fed the VMRI feed throughout the course of the experiment. The pigs in the other unit were placed on the Swine Nutrition feed. Each of the pigs was inoculated intranasally with mycoplasmal pneumonic lung suspension. The pneumonic lung came from one of the infected nonvaccinated controls (pig #7092) in Experiment IV. Mycoplasma hyopneumoniae was recovered from this lung. No bacteria were detected. The pigs were exposed intranasally on three consecutive days. Five weeks after exposure all the pigs were necropsied.

In addition to the two experiments, samples of the pelleted VMRI feed were sent to the Animal Science Department, Iowa State University for antibiotics assay.[1] Feed samples were also sent to the American Cyanamid Company, and to the Eli Lilly Company for antibiotic assay.

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[1]Antibiotic assay was done by Dr. M. H. Jurgens, Assoc. Prof. Animal Science Department.

RESULTS

Growth of M. hyopneumoniae

Liquid medium

The growth of M. hyopneumoniae in the complete Eagle's base medium was evidenced after 2 or 3-day incubation by an acid pH change and by an increased turbidity to the medium. Slight sediment was produced which formed a delicate spiral when the incubated tube was gently swirled.

Solid medium

Colonies formed by M. hyopneumoniae on Eagle's base medium agar plates were not visible macroscopically but were visible at 100X magnification of the compound light microscope. The colonies had a tendency to grow into the agar, making it necessary to focus slightly lower than normal to see them. M. hyopneumoniae colonies lacked the "fried egg" or "film and spot" appearance characteristic of other swine mycoplasma. Typical colonies were translucent, coarsely granular, and 0.01 to 0.05mm (10 to 50μ) in size. Early colonies (2 to 3 days growth) were round and slightly raised with uniform borders; occasionally young colonies with irregular borders were observed. By the 4th to 8th day of incubation, the colonies became larger, less translucent, more granular, and irregular in shape.

Immunization Trials

Experiment I: Vaccination of pigs already infected with M. hyopneumoniae

The results of the serologic response of pigs naturally infected and then immunized and of the infected nonimmunized controls are summarized in Table 1. One of the vaccinates died about 20 minutes after receiving the

Table 1. Complement-fixing antibodies[a] against M. hyopneumoniae in pigs infected and then vaccinated

Pig nos.	Pre-vaccination	Weeks after first vaccination									Pneumonia score (%)
		1	2	3	4	5	6	7	8	9	
6100	8	32	128	256	512	2048	512	512	512	512	3
6102	16	16	128	256	512	1024	1024	1024	512	512	0[b]
6150	32	32	128	128	256	512	512	512	256	256	0
6162	64	128	128	128	256	256	128	128	64	64	0[b]
Average	30	52	128	192	384	960	544	544	336	336	
		Controls (infected but not vaccinated)									
6101B	8	8	16	16	32	64	64	32	16	32	0
6101G	8	8	16	32	64	32	32	16	32	32	3
6130	32	128	128	512	512	512	256	256	256	128	6[b]
6161	32	64	64	128	64	64	32	32	32	32	0
6154	128	64	64	64	32	64	32	64	64	64	3[b]
Average	52	54	58	150	141	147	84	80	80	58	

[a]CF titers are expressed as the reciprocal of the highest serum dilution in which 70% or more of the RBC remained intact.

[b]Scars in the lung.

second inoculation; the animal probably died of shock as no gross pathology was observed at necropsy. Both the infected vaccinates and infected nonvaccinated controls remained serologically positive throughout the duration of the trial. However, the vaccinates maintained higher levels of CF antibody than did the nonvaccinates. Although the vaccinates had slightly lower pre-vaccination average titers than the controls, the post-vaccination average titers of the vaccinates were higher than those of the controls (Figure 1). The vaccinates attained maximal antibody level, ranging from 256 to 2048, during the fifth week after primary immunization.

One of the 4 infected vaccinates (pig #6100) had gross pneumonic lesions (3%), whereas 3 of the 5 infected nonvaccinates had gross lung lesions, ranging in extent from 3 to 6% of the total lung. Two of the 3 vaccinates without gross lesions, and 2 of the controls with lesions had scars in the lungs which probably indicated healing. Although pig #6100 had a CF antibody titer as high as 2048 in the 5th week, it was the only vaccinate that had gross lesions at necropsy. Similarly, pig #6130, which maintained the highest titer among the controls, had the highest pneumonic score (6%).

Experiment II

A summary of the CF antibody titers of pigs vaccinated with either the flask-antigen or the lung-antigen and the response of the pigs to challenge inoculation is presented in Table 2. All 4 negative controls (nonvaccinated and noninfected) remained serologically negative and were pneumonia-free at necropsy. Three of the 4 positive controls (nonvaccinated but infected) became serologically positive following the

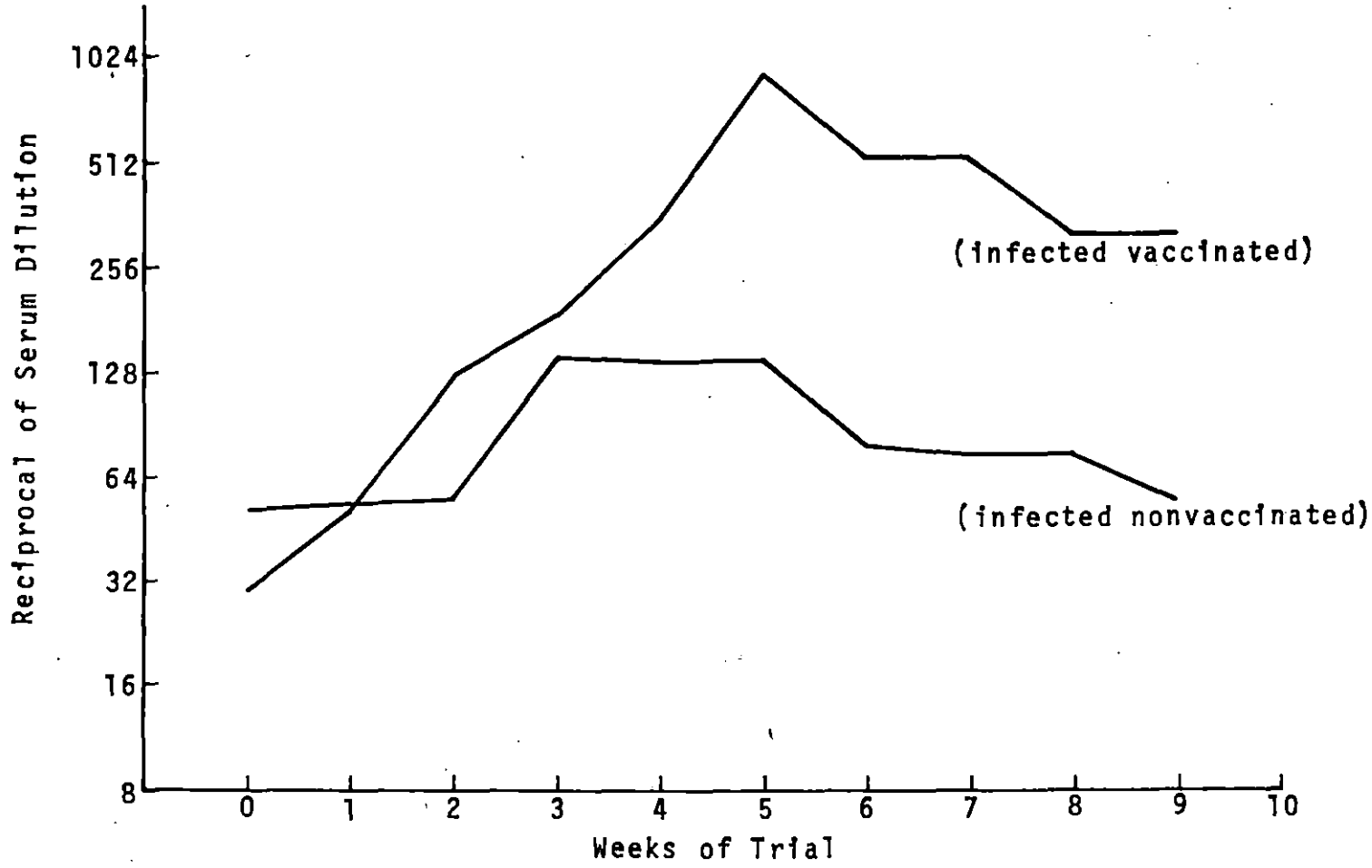


Figure 1. Average CF titers in pigs naturally infected and then vaccinated and in pigs infected but not vaccinated

Table 2. CF antibody titers[a] and response to challenge exposure in pigs vaccinated subcutaenously with flask-antigen or lung-antigen

Treatment	Pig nos.	Pre-vaccination	Weeks after first vaccination: 1	2	3	4	5	7 (challenge)	11 (necropsy)	Pneumonia score (%)	Microscopic lesions
Flask-antigen	6450B	<8	<8	64	256	512	2048	512	64	0	Negative
	6421B	<8	<8	128	512	512	1024	512	64	3	Positive
	6420B	<8	<8	64	256	256	512	512	32	0	Negative
	6422G	<8	<8	128	256	256	256	128	16	0	Negative
											Total = 1/4
Lung-antigen	6451B	<8	<8	<8	<8	<8	<8	<8	<8	0	Positive
	6450G	<8	<8	<8	<8	<8	<8	<8	<8	0	Positive
	6452B	<8	<8	<8	<8	<8	<8	<8	<8	0	Negative
	6454B	<8	<8	<8	<8	<8	<8	<8	<8	4	Positive
											Total = 3/4
Positive control	6460B	<8	<8	<8	<8	<8	<8	<8	<8	0	Negative
	6455B	<8	<8	<8	<8	<8	<8	<8	32	3	Positive
	6453G	<8	<8	<8	<8	<8	<8	<8	64	3	Positive
	6461B	<8	<8	<8	<8	<8	<8	<8	16	2	Positive
											Total = 3/4
Negative control[b]											

[a]CF titers are expressed as the reciprocal of the highest serum dilution in which 70% or more of RBC remained intact.

[b]All the 4 negative control pigs remained serologically negative and all were pneumonia-free at necropsy.

challenge inoculations and all 3 had gross pneumonic lesions at necropsy. The lesions were frequently confined to the apical, cardiac (Figure 2) and the intermediate lobes, and appeared as purple to grey colored areas clearly demarcated from the normal lung tissues. Microscopically, the lesions were characterized by peribronchiolar and perivascular lymphoid hyperplasia, alveolar interstitial thickening, and alveolar and bronchiolar exudation of septal cells, lymphocytes and neutrophils (Figures 4, 5, 6).

No complement-fixing antibodies against M. hyopneumoniae were detected in the serums of pigs vaccinated with the lung-antigen, and all 4 pigs remained serologically negative after the intranasal challenge inoculation. One of these 4 lung-antigen vaccinates had gross pneumonic lesions and two vaccinates without gross lesions had microscopic lesions of mycoplasmal pneumonia (Figure 7).

Antibodies were not detected in the flask-antigen vaccinates until two weeks after the primary vaccination. The CF antibodies of these vaccinates at the time of challenge inoculation ranged from 128 to 512. One of the 4 flask-antigen vaccinates had gross lesions of pneumonia.

Experiment III

The complement-fixing antibody titers produced in pigs immunized by the subcutaneous, footpad, intraperitoneal, and intratracheal routes are shown in Table 3. Three weeks after primary immunization, the antibody titers of vaccinates in isolation units #3 and #21 which received both the subcutaneous and footpad inoculations, ranged from 128 to 2048. These vaccinates maintained high CF titers at the time of the challenge inoculations (6th and 11th week). On the other hand, the vaccinates in unit #20

Figure 2. Gross pneumonic lesion from a pig infected intranasally with broth culture of Mycoplasma hyopneumoniae. Arrow points at clearly demarcated grey colored area in the cardiac lobe of the lung

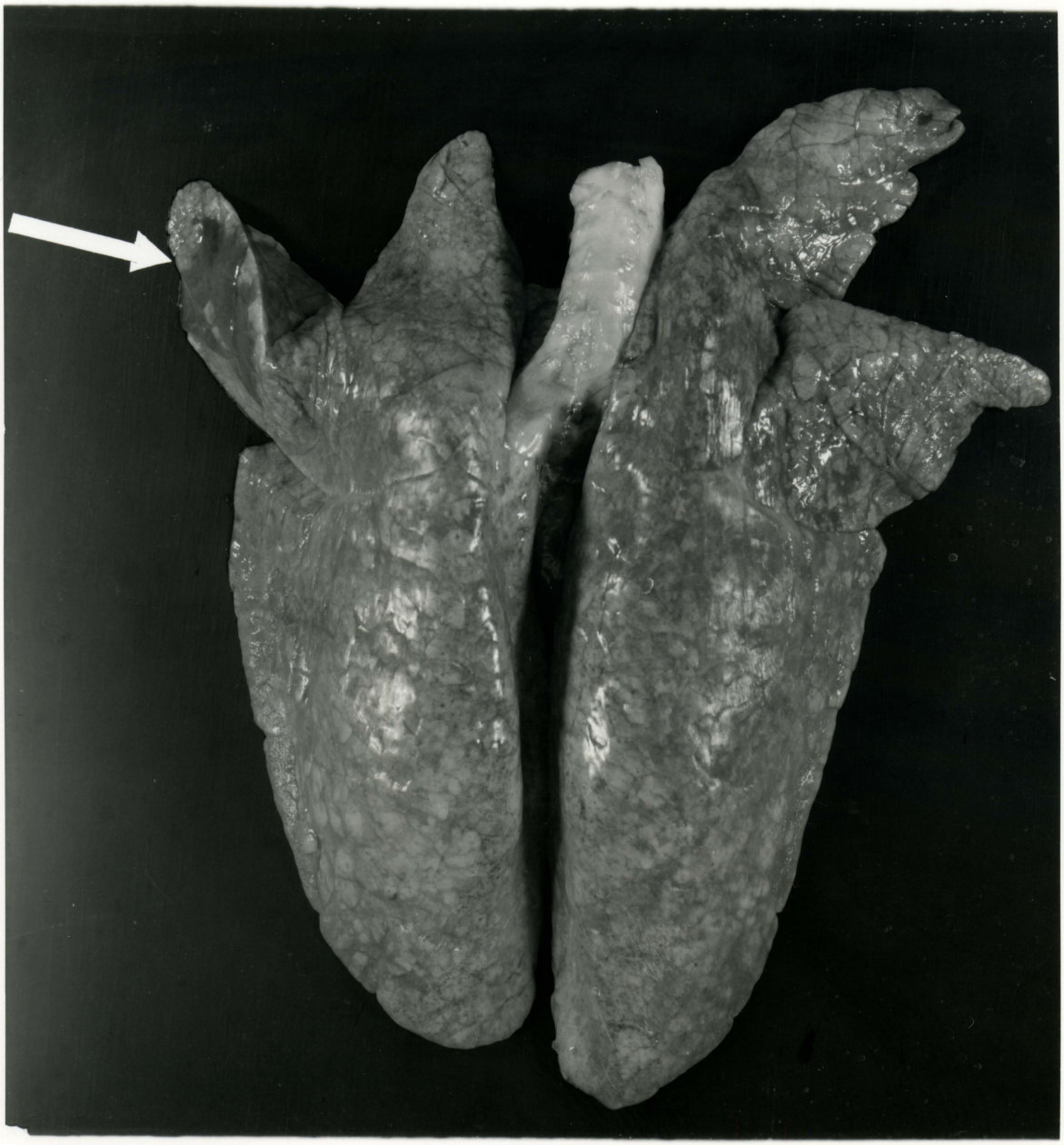
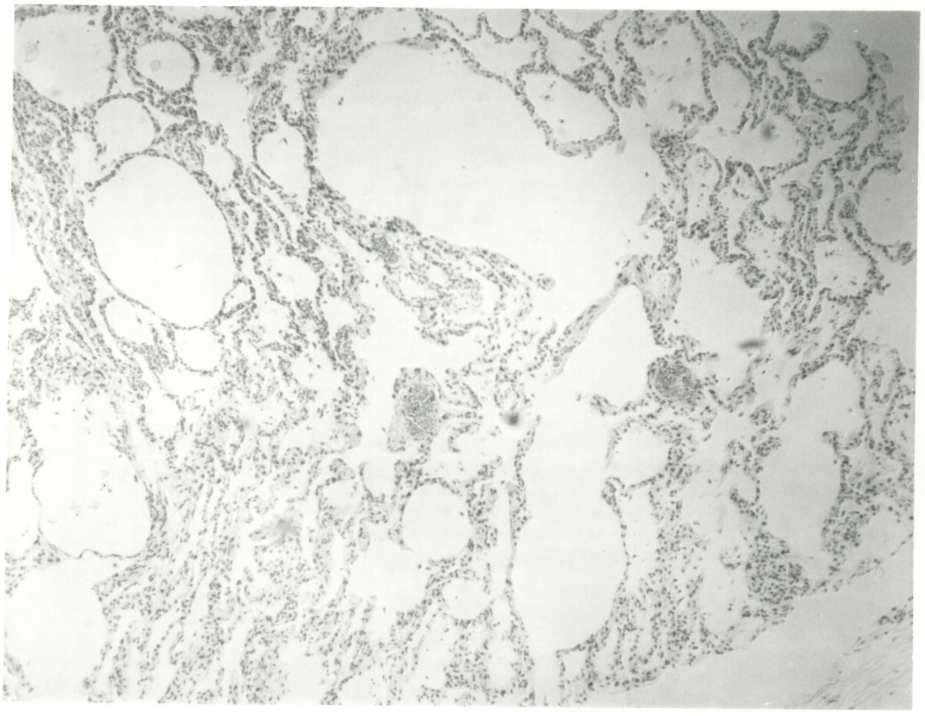


Figure 3. Lung from uninoculated control pig. Note absence of lesions. Hematoxylin and eosin stain. X100

Figure 4. Peribronchiolar and perivascular lymphoid hyperplasia, alveolar interstitial thickening, and septal cell proliferation. Lesions from control (nonvaccinated) pig inoculated intranasally with M. hyopneumoniae broth culture. Hematoxylin and eosin stain. X100



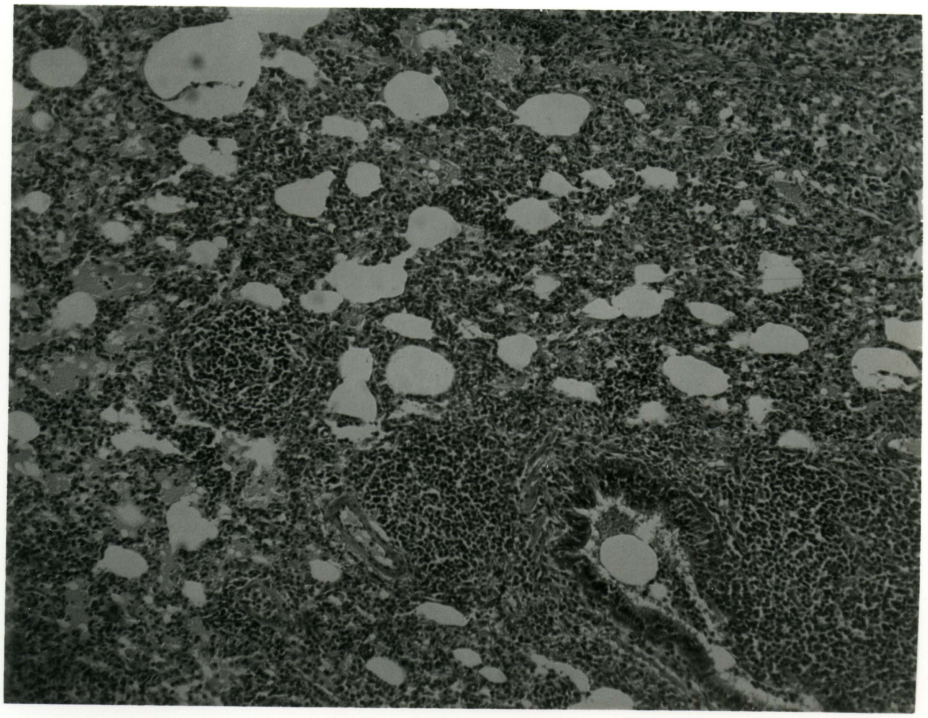
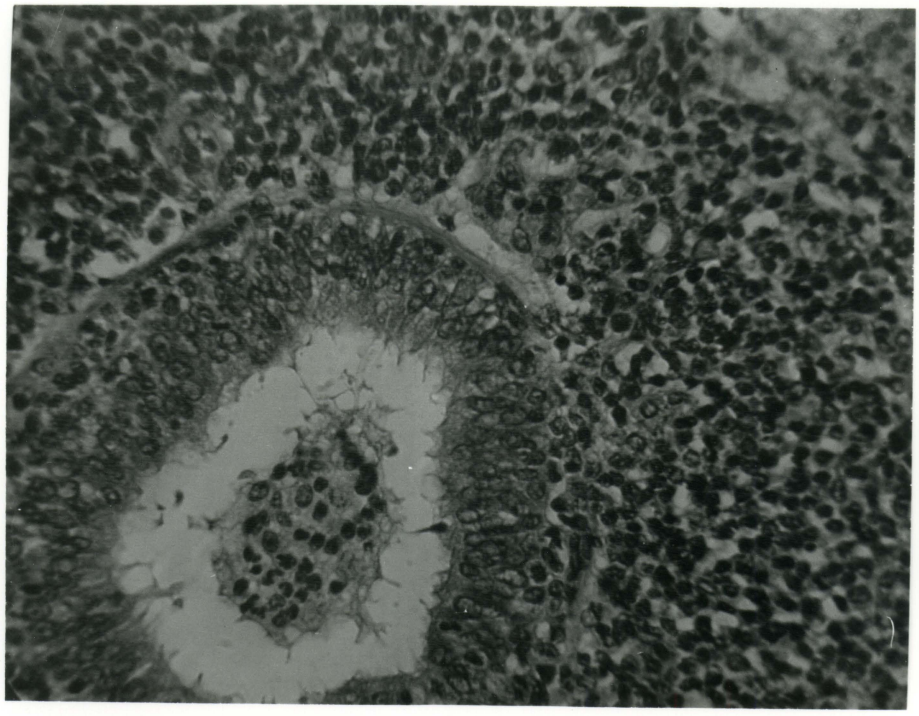


Figure 5. Peribronchiolar lymphoid hyperplasia and bronchiolar exudation of lymphoid cells and neutrophils. Lesion from nonvaccinated control pig inoculated with broth culture of M. hyopneumoniae. Hematoxylin and eosin stain. X430

Figure 6. Perivascular lymphoid hyperplasia. Note migration of lymphoid cells from the lumen of the blood vessel. Lesion from a control pig inoculated with broth culture of M. hyopneumoniae. Hematoxylin and eosin stain. X430



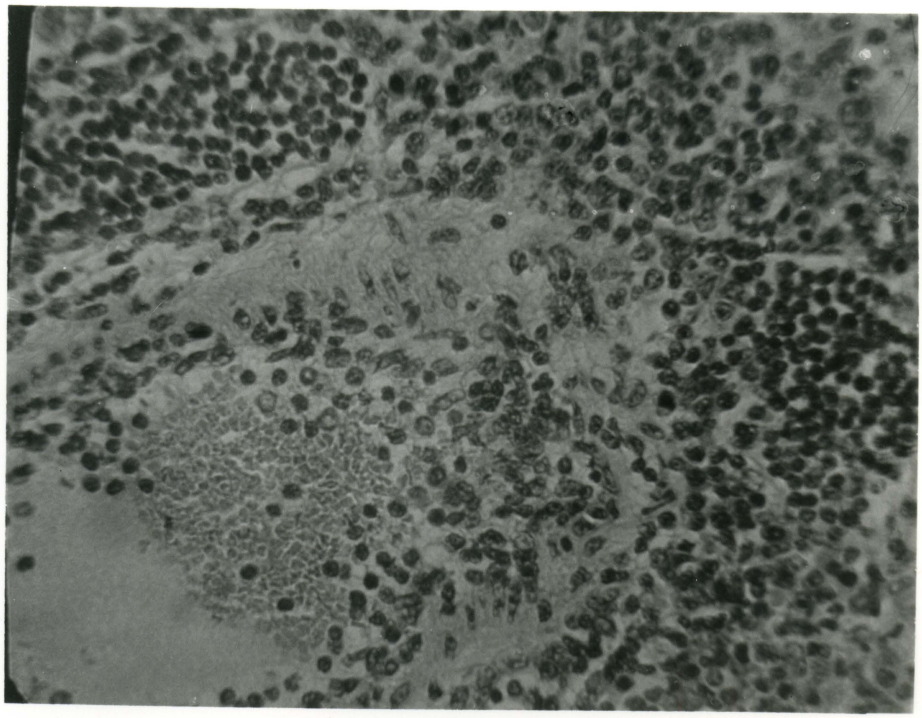
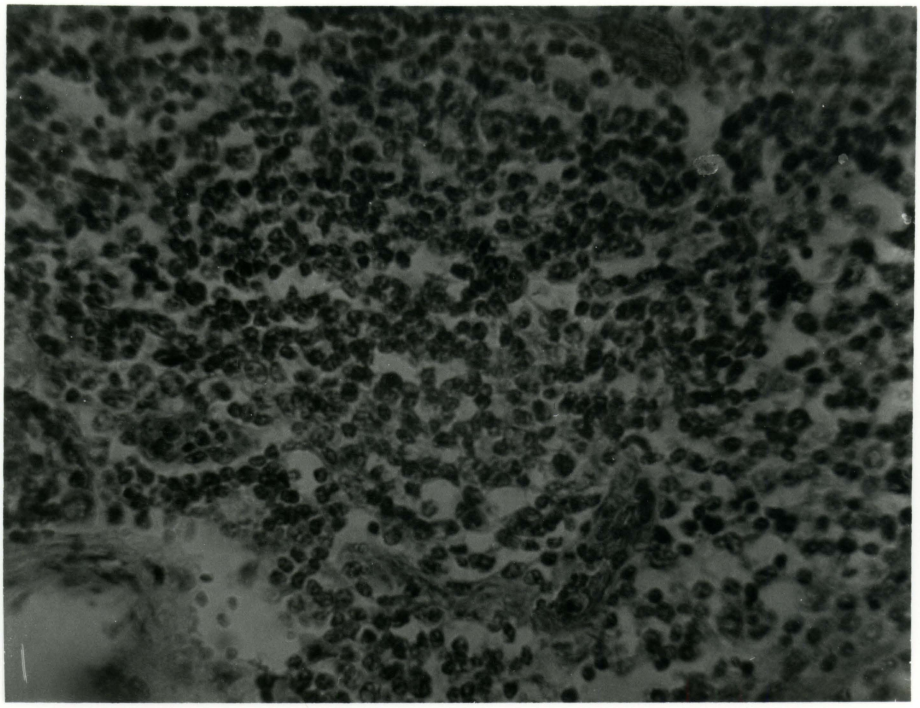


Figure 7. Lymphoid hyperplasia and alveolar infiltration of lymphoid cells and neutrophils. Lesion from a pig vaccinated with lung-antigen. Pig had no gross lesion. Hematoxylin and eosin stain. X430

Figure 8. Lung from a pig challenged intranasally with M. hyopneumoniae fluid culture and pneumonic lung suspension (Experiment #3). Atelectasis. Note absence of peribronchiolar lymphoid reaction. Hematoxylin and eosin stain. X100



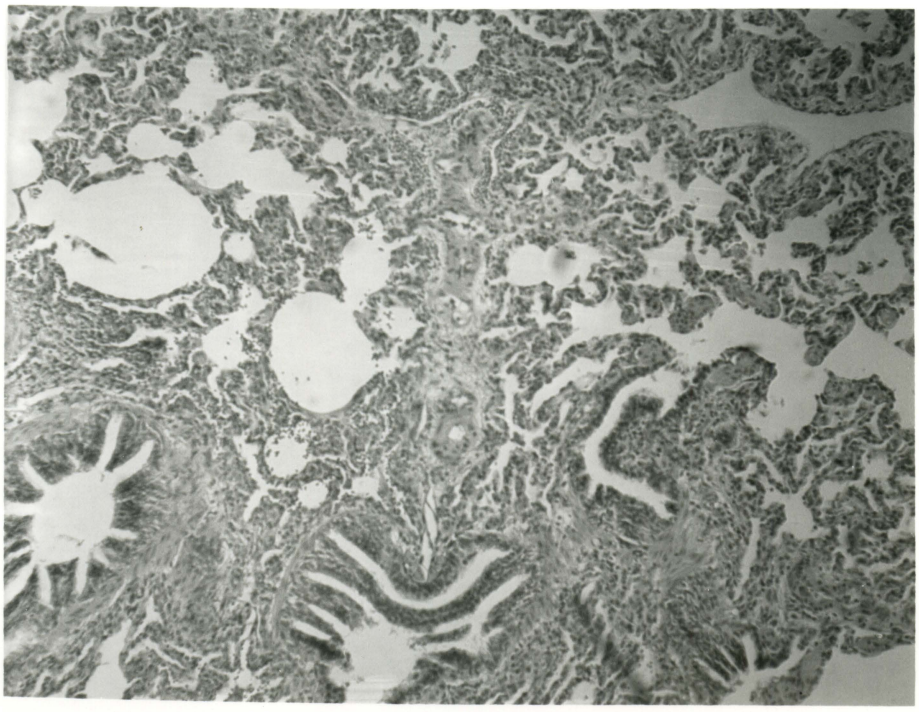


Table 3. CF antibodies[a] against M. hyopneumoniae in pigs vaccinated by the subcutaneous (s/c), footpad, intraperitoneal (i/p), and intratracheal (i/tr) routes

Pig nos.	Weeks of trial: 0 s/c footpad	1	3 s/c footpad	6 challenge	8	11 2nd challenge	14 necropsy
				Vaccinates: Unit #3			
Y6680B	<8	1024	1024	2048	1024	512	>128
H6727B	<8	128	256	1024	512	128	128
Y6770B	<8	32	64	128	128	64	64
Y6760B	<8	256	256	512		256	256
				Vaccinates: Unit #21			
H6723B	<8	512	1024	1024	1024	1024	>128
Y6772B	<8	1024	2048	2048	1024	256	>128
Y6774B	<8	128	1024	1024	1024	512	>128
Y6764G	<8	128	512	512	256	128	-
				Vaccinates: Unit #20[b]			
Y6761B	<8	<8	<8	<8	±8	<8	<8
Y6675B	<8	8	32	16	32	32	32
Y6681B	<8	<8	32	32	32	<8	<8
H6726G	<8	64	64	32	32	16	16
				Nonvaccinates[c]			

[a]CF titers are expressed as the reciprocal of the highest serum dilution in which 70% or more of RBC were intact.

[b]Vaccinates did not receive the footpad inoculation on first day.

[c]All 12 nonvaccinates remained serologically negative after challenge inoculations.

which were not given the footpad injection during the primary immunization, had a titer range of 8 to 64 both at the 2nd and 3rd weeks; the footpad and subcutaneous booster inoculations in the 3rd week did not cause any increase in the titers. One of these vaccinates in unit #20 remained serologically negative after all the immunizations. No CF antibodies titers were detected in the serums of the 12 nonvaccinated control pigs after they were challenged several times with M. hyopneumoniae.

None of the 12 nonvaccinated controls and the 12 vaccinates had any macroscopic pneumonic lesions at necropsy. Except for occasional mild atelectasis (Figure 8), the lungs of all the pigs were microscopically normal.

Experiment IV

The serologic titers of the vaccinates and their response to challenge inoculations are presented in Table 4. Complement-fixing antibodies were not detected in pigs immunized with the heat-inactivated vaccine until 2 weeks after the primary immunization. The 3 vaccinates in isolation unit #7 which received footpad immunization in addition to the subcutaneous injections had much higher antibody titers than any of the 4 vaccinates that received only the subcutaneous inoculations. In the third week post-vaccination the titers of the 3 vaccinates in unit #7 were 512, 1024, and 2048, while the other vaccinates had titers of 128 or 256. These three vaccinates maintained the higher CF antibody titers throughout the duration of the trial. Only one of the 5 nonvaccinated control pigs developed a positive CF titer after the challenge inoculation.

Two of the 5 control pigs had gross lesions typical of mycoplasmal

Table 4. Serologic titers[a] and response to challenge inoculation in pigs immunized with heat-inactivated M. hyopneumoniae vaccine (Experiment IV)

Isolation unit	Pig nos.	Pre-vaccination	Weeks after first vaccination							Gross pneumonia (Estimated) (%)	Microscopic lesion
			1	2	3	4[b]	5	6	7		
#7[c]	Y7114G	<8	<8	128	512	512	128	64	64	0	Negative
	Y7111B	<8	<8	128	1024	512	128	64	64	0	Negative
	Y7131B	<8	<8	512	2048	1024	512	<128	<128	0	Negative
#8	Y7112G	<8	<8	128	128	64	64	16	16	0	Negative
	Y7130B	<8	<8	32	256	256	64	16	16	0	Negative
	Y7132G	<8	<8	128	256	256	256	64	64	0	Negative
	Y7131G	<8	<8	128	256	256	64	32	32	0	Negative
#3	Y7133G	<8	<8	ND[d]	ND[d]	<8	<8	<8	<8	4	Positive
(Nonvaccinates)	Y7115G	<8	<8	ND	ND	<8	<8	<8	<8	0	Mild lymphoid hyper-plasia
	H7160G	<8	<8	ND	ND	<8	<8	<8	<8	0	0
	H7160B	<8	<8	ND	ND	<8	<8	<8	<8	0	0
	H7092	<8	<8	ND	ND	<8	<8	16	32	8	Positive

[a]CF titers are expressed as the reciprocal of the highest serum dilution in which 70% or more of RBC remained intact.

[b]Pigs were challenged intranasally in the 4th week after primary vaccination.

[c]Vaccinates in unit #7 were given footpad inoculation on the 2nd and 3rd week in addition to the subcutaneous injections.

[d]Not done.

pneumonia (4% and 8%, respectively) at necropsy, whereas none of the 7 vaccinates showed any gross lesions. The characteristic histologic lesions in one of the infected pigs (pig #7092) are illustrated in Figures 9 and 10. One of the control pigs without gross lesions had mild peribronchiolar lymphoid infiltration.

Statistical Analysis of Immunization Results

Chi-square was used for analysis of the results obtained in the immunization trials. The results of this analysis are presented in Table 5.

Table 5. Chi-square analysis of the results of immunization trials

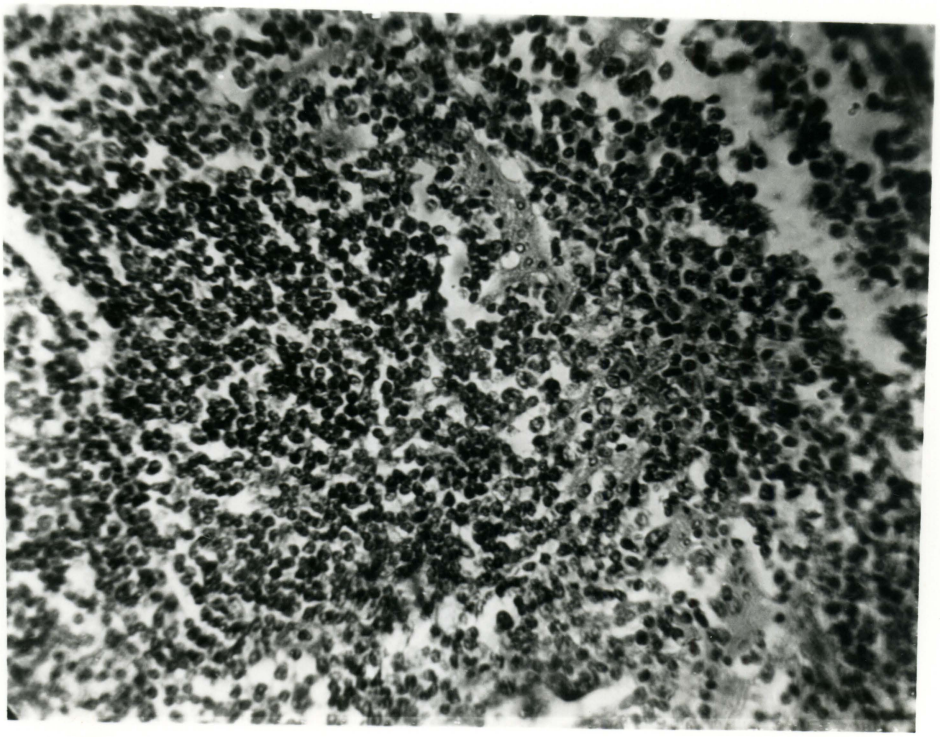
Experiment	Type of vaccine	Vaccinated pigs infected	Control pigs infected	X^2
I	Live vaccine	1/4	3/5	1.102 NS*
II	Heat-inactivated flask-antigen	1/4	3/4	6.95 NS
	Live lung-antigen	3/4	0/4	
IV	Heat-inactivated vaccine	0/7	2/5	5.51**
	Combined results of heat-inactivated vaccine	1/11	5/9	5.10**

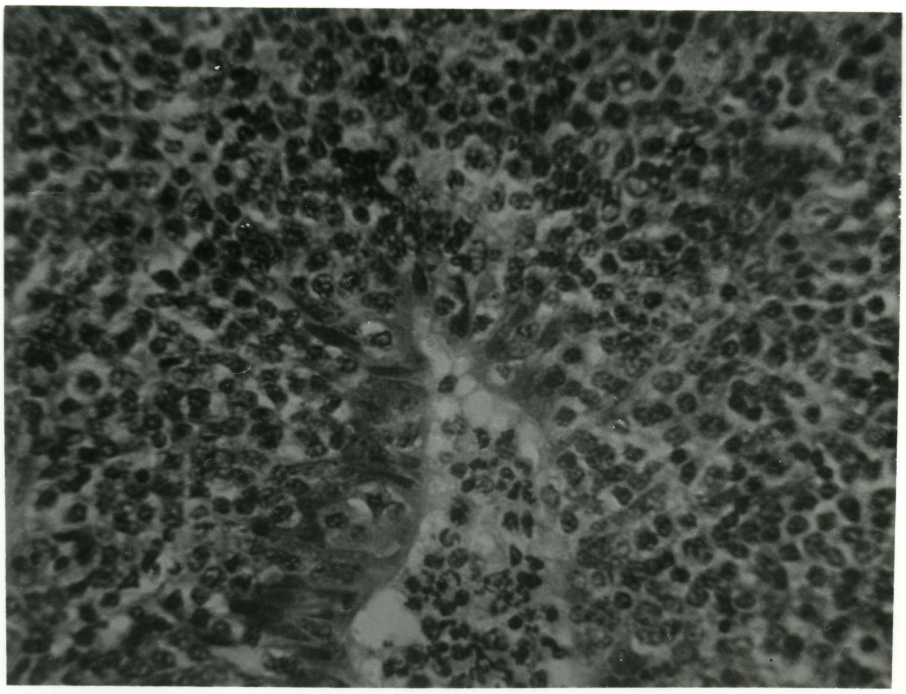
*NS = not significant at $p \leq .05$ level.

**Significant at $p \leq .05$ level.

Figure 9. Lymphoid hyperplasia and alveolar infiltration of lymphoid cells and neutrophils. Tissue from nonvaccinated control pig inoculated with M. hyopneumoniae culture. Hematoxylin and eosin stain. X430

Figure 10. Peribronchiolar lymphoid hyperplasia and bronchiolar exudation of lymphoid cells and neutrophils. Tissue from nonvaccinated control pig inoculated with M. hyopneumoniae culture. Hematoxylin and eosin stain. X430





Influence of Passage Level on the Virulence of *M. hyopneumoniae* and the Effect of Age of Pigs on Susceptibility to Experimental Infection

No CF antibodies against *M. hyopneumoniae* were detected in the serums of the 3 young pigs and the 3 old pigs exposed intranasally with high passage culture of *M. hyopneumoniae*. Similarly, none of the 6 pigs (3 young and 3 old) inoculated with the low passage culture of the mycoplasma responded serologically.

No gross lesions of mycoplasma pneumonia were observed in the lungs of any of the 12 pigs at necropsy. Histologic examination of the lungs was not carried out.

Effect of Swine Feed on Development of Lesions from Challenge Inoculation with *M. hyopneumoniae*

Experiment I

Sensitivity tests of VMRI feed filtrate Wide zones of growth inhibition of *M. hyopneumoniae* were consistently observed in plates containing the discs dipped in the VMRI feed filtrate. Under the 100X magnification of the compound light microscope, inhibition zones were observed to extend 0.5 to 1mm from the discs. Both the high passage and the low passage broth cultures of *M. hyopneumoniae* were significantly inhibited by the feed filtrate. No inhibition zones were observed around control discs not dipped in the feed filtrate.

M. hyopneumoniae colonies did not develop on the plates inoculated with a mixture of the feed filtrate and the mycoplasma culture except for a few scattered colonies with distorted morphology which were observed in

two such plates. Growth inhibition occurred with both the high passage and low passage cultures. Abundant typical colonies were observed in control plates inoculated with the mycoplasma culture only.

Comparison of VMRI Contract feed and Swine Nutrition feed The results obtained in the comparisons of the two feed filtrates are summarized in Table 6. Wide zones of inhibition (up to 1mm) were observed around discs soaked in the VMRI feed filtrate, whereas very narrow inhibition zones (about 0.1mm) were seen around some of the discs dipped in the Swine Nutrition feed filtrate. No growth inhibition of M. hyopneumoniae occurred around control blank discs.

Typical M. hyopneumoniae colonies were observed in plates inoculated with a mixture of Swine Nutrition feed filtrate and the mycoplasma culture. No M. hyopneumoniae colonies were seen in plates inoculated with a mixture of the VMRI feed filtrate and the broth culture.

In the tube-dilution test, no growth of M. hyopneumoniae occurred in the 1:2 to 1:128 dilutions of the VMRI feed filtrate. Beyond the 1:128 dilution of the filtrate growth of the mycoplasma occurred. With the Swine Nutrition feed filtrate, inhibition of M. hyopneumoniae growth occurred only in the 1:2 and 1:4 dilutions.

Sensitivity tests of individual components of swine feed No growth inhibition of M. hyopneumoniae was observed in the disc sensitivity tests of filtrates prepared from the following Swine Nutrition feed components: ground corn, soybean meal, vitamin premix, dicalcium phosphate, calcium carbonate, and iodized salt. However, narrow zones of inhibition (0.05 to 0.1mm) were observed around discs dipped in the filtrate prepared from the

Table 6. Inhibition of growth of M. hyopneumoniae by swine feed filtrate: comparison of VMRI feed and feed from Swine Nutrition Farm

a. Disc Sensitivity Tests:

VMRI feed filtrate undiluted	Swine Nutrition feed filtrate undiluted
Wide zone of growth inhibition (0.2-0.5mm)[a]	Very narrow zone of inhibition (0.05mm)

b. Feed filtrate mixed with equal volume of M. hyopneumoniae broth culture:

VMRI feed	Swine Nutrition feed
No growth, i.e., no colonies formed	Growth, i.e., typical colonies formed

c. Tube dilution test:

VMRI feed		Swine Nutrition feed	
1:2	No growth	1:2	No growth
1:4	" "	1:4	" "
1:8	" "	1:8	Growth
1:16	" "	1:16	"
1:32	" "	1:32	"
1:64	" "	1:64	"
1:128	" "	1:128	"
1:256	Growth	1:256	"
1:512	"	1:512	"
1:1024	"	1:1024	"

[a]Estimation of size of inhibition zone as seen with the 100X magnification of compound light microscope.

trace mineral premix. In the tube-dilution test, inhibition of mycoplasma occurred in the 1:2 and 1:4 dilutions of the trace minerals premix filtrate.

Antibiotic assay

The result of the antibiotic assay of the VMRI swine feed indicated that the feed sample was negative for tylocine and tetracycline.[1] The American Cyanamid Company gave the following report on the antibiotic assay of a sample of the VMRI contract feed: "The sample appeared to contain a small amount of crystalline chlortetracycline when examined microscopically. Upon assay, the sample was found to contain less than 1:3gm of chlortetracycline activity per ton of feed."[2] Eli Lilly Company reported that the feed sample was negative for tylocine.[3]

Experiment II

All 4 pigs fed the Swine Nutrition feed and 3 of the 4 pigs on the VMRI contract feed responded serologically to the intranasal inoculation of mycoplasmal pneumonic lung suspension. Complement-fixing antibodies against M. hyopneumoniae were detected in the 7 pigs two weeks after the intranasal exposure.

At necropsy, all 4 pigs receiving the Swine Nutrition feed had extensive gross lesions of mycoplasmal pneumonia. One of these pigs had gross pneumonia score of 15%, another had a score of 15% and two had 20% of the lung pneumonic. The lesions were characterized microscopically by

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[1]Dr. M. Jurgens, Animal Sci. Dept., ISU, Ames, Ia. Personal communications. 1972.

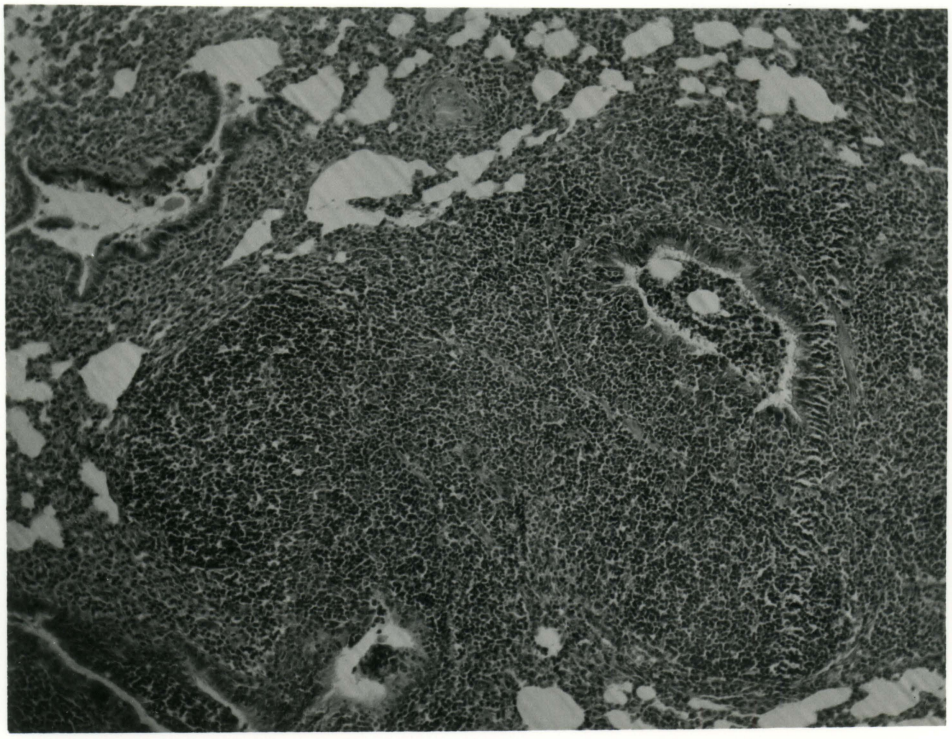
[2]Part of a personal letter to Dr. W. P. Switzer from Dr. Robert G. Eggert, American Cyanamid Company, New Jersey.

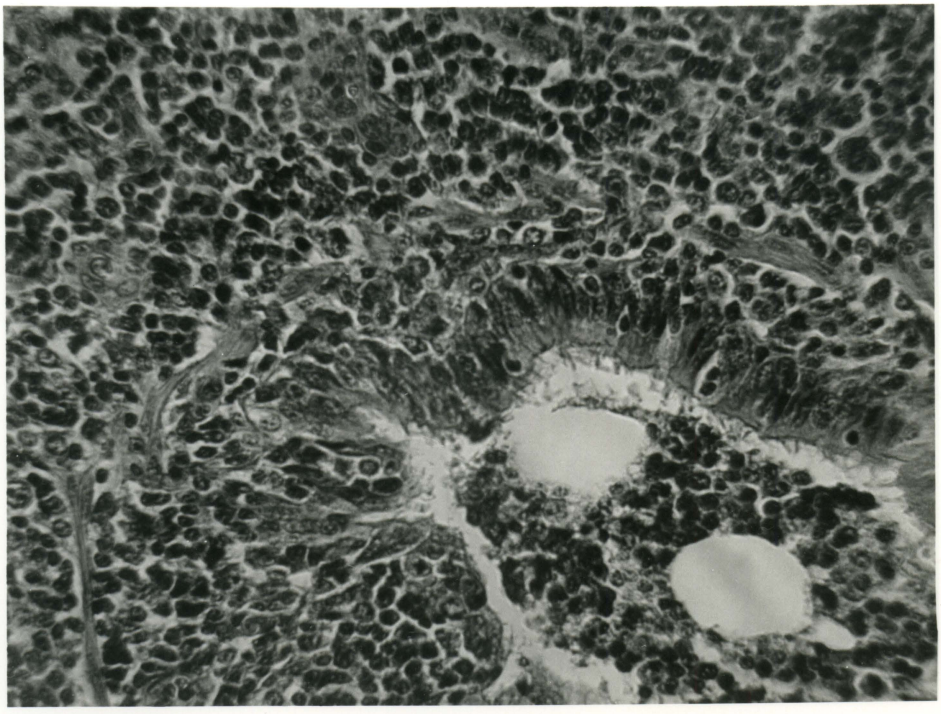
[3]Paul Gorham, Eli Lilly Company, Greenfield, Indiana. Personal communication.

severe peribronchiolar and perivascular lymphoid hyperplasia, alveolar interstitial thickening, septal cell proliferation and neutrophil infiltration (Figures 11 and 12). Two of the 4 pigs fed the VMRI contract feed had gross lung lesions; the pneumonic scores were 3% and 4%, respectively. One of serologically positive pigs showed no gross lesions, but microscopically the lung had mild lymphoid cell infiltration.

Figure 11. Severe peribronchiolar and perivascular lymphoid hyperplasia, alveolar interstitial thickening, septal cell proliferation and bronchiolar exudation of lymphoid cells and neutrophils. Note hyperplastic nodular formation. Lesions from a pig inoculated with lung suspension containing M. hyopneumoniae. Hematoxylin and eosin stain. X100

Figure 12. Peribronchiolar lymphoid hyperplasia and bronchiolar exudation of lymphoid cells and neutrophils. Lesion from a pig inoculated with lung suspension containing M. hyopneumoniae. Hematoxylin and eosin stain. X430





DISCUSSION

Recent research by independent groups of investigators has established that the etiologic agent of chronic pneumonia variously called "Infections Pneumonia of Pigs", "Swine Enzootic Pneumonia", and "Virus Pneumonia of Pigs" is a mycoplasma. The causative mycoplasma has been characterized and named M. hyopneumoniae (Maré and Switzer, 1965). The preferred name of this disease is mycoplasmal pneumonia of swine.

Mycoplasmal pneumonia of swine probably has worldwide distribution, as indicated by the reports cited in the literature review. The chronic pneumonia has been described as probably the world's most economically important single disease of swine (Switzer, 1970) because of its widespread geographical distribution and the enormous economic loss it causes the world's swine industry. In England and United States, the annual economic loss due to swine mycoplasmal pneumonia has been estimated in hundreds of millions of dollars (Betts, 1956; Young, 1956; Goodwin, 1963; Switzer, 1970). This great economic loss is caused, not by any significant mortality rate, but by the chronicity of the disease, high herd morbidity, loss of vigor, stunted growth and decreased feed efficiency produced in affected animals.

On account of the highly infectious nature of mycoplasmal pneumonia of swine, much of the efforts in its control have been directed at measures which break the cycle of infection. However, such measures as isolation and controlled rearing of the breeding stock or depopulation of infected herd and repopulation with surgically derived specific pathogen-free (SPF) pigs have proved cumbersome, expensive and not totally reliable. Similarly,

chemotherapy and chemoprohylaxis are at present ineffective in the control of the disease. While chlortetracycline may prevent the development of lesions in pigs infected with M. hyopneumoniae, it does not eliminate the organism from the lungs nor does it prevent transmission of the organism to in-contact pigs (Huhn, 1969). These problems and the increasing economic effects of the disease in the swine industry have created the need for investigations of immunization as an alternative or complementary control measure. There are indications that some immunity is induced in swine which have recovered from natural or experimental infection of mycoplasmal pneumonia (Lannek and Bornfors, 1957; Goodwin et al., 1969a). These suggest encouraging prospects for vaccination as a feasible immunoprophylactic measure.

The objective of the present study was to evaluate the ability of M. hyopneumoniae vaccines to promote recovery in infected pigs by enhancing the elimination of lesions, and to provide protective immunity in susceptible swine. Such an immunization study required the production of adequate quantities of the mycoplasm antigen and the application of a serodiagnostic technique that is relatively simple, economical, reliable, and specific.

Successful cultivation of M. hyopneumoniae in cell-free medium made feasible the production of sufficient amounts of the antigen. Gentle shaking of the culture flasks during incubation was found to be a critical factor. Stationary incubation of the flasks resulted in low yield of packed cells. Shaking the flasks at a speed greater than 10 to 15 oscillations per minute caused an increased precipitation of medium proteins. It is not certain why shaking the culture flasks resulted in

higher antigen yield. It has been suggested that shaking caused an increased aeration of the culture and hence an increased growth of the mycoplasma (Slavik, 1971).

A modified microtitration complement fixation test (Slavik and Switzer, 1972) was employed in determining the serologic response of swine to experimental infection with M. hyopneumoniae and to parenteral immunization with vaccines. The CF test was modified by heating the test serums at 56°C for 30 minutes in order to destroy the natural hemolysins to sheep RBC which are present in swine serum. The sensitivity of the test was also increased by reconstituting the commercial lyophilized guinea-pig complement with unheated serum from young normal pigs. This modification provided "enhancement" of the guinea-pig complement before its titer was determined; it also restored the complement-binding activity of the heat-inactivated test serums. It is believed that the normal swine serum supplied C'3 component to the guinea-pig complement, which has C'3 as the limiting factor (Bankowski et al., 1953; Slavik, 1971).

Heating the mycoplasma antigen at 52°C to 54°C for 30 minutes before its titer was determined yielded an antigen with a high CF activity and a low level of anti-complementary activity. Slavik (1971) has suggested that heat-treatment "cleaned" the mycoplasma by removing metabolic wastes and medium constituents coating the organism and thereby revealed more specific antigenic determinants on the cell membrane.

The specificity of the modified CF test had been evaluated by testing M. hyopneumoniae, M. hyorhinis, and M. hyosynoviae antigens against the heterologous and homologous antiserums. No cross-reactions were demonstrated among these agents of swine mycoplasmal infections. However,

nonspecific reactions with other swine pneumonia agents had not been adequately evaluated. Evaluation of the accuracy of the modified CF test indicated that it is capable of detecting 85% or more of pigs with gross lesions of mycoplasmal pneumonia (Switzer, 1972). In the present study, all the serologic diagnoses were supplemented with gross and microscopic examination of the lungs and, in some cases, by the isolation of the mycoplasma.

In the first immunization experiment, antibodies against M. hyopneumoniae were detectable in all the pigs throughout the course of the trial. At necropsy, however, one of the pigs naturally infected and then immunized and 2 controls (naturally infected but not immunized) had no gross lesions of mycoplasmal pneumonia and no scars in the lungs. The most likely explanation is that these infected pigs had completely recovered from the disease but still had detectable circulating antibodies. The infected and then immunized pigs maintained a higher average antibody titer than the infected nonvaccinates (Figure 2). Since only 1 of 4 infected vaccinates had gross lesions, whereas there were 3 of 5 infected non-vaccinates with lesions, it appeared that vaccination may have influenced recovery. However, statistical analysis of the results indicated that vaccination had no significant effect in curing the infected animals. Thus, while parenteral immunization of pigs already infected with M. hyopneumoniae caused an elevation of complement-fixing antibodies, it did not significantly enhance the elimination or resolution of established lesions. There was no correlation between the level of CF antibody and the estimated percentage of gross pneumonia. Although pig #6100 had a CF titer of 2048 in the 5th week, it was the only infected vaccinate that had gross

pneumonic lesions at necropsy. Similarly, pig #6130 which maintained the highest CF titer among the infected nonvaccinates had the highest estimated pneumonic score (6%). Lam (1970) has reported that intramuscular immunization of pigs already infected with M. hyopneumoniae resulted in more extensive microscopic lesions. More severe lymphoid hyperplasia, alveolar lymphoid infiltration, and hyperplastic nodular formations were observed in the lungs of infected vaccinates than were seen in lungs of infected control animals. Microscopic examinations of the lungs were not made in this trial so the histopathologic effects of subcutaneous vaccination of the infected pigs was not evaluated.

In the second immunization trial, two types of vaccines were evaluated for their ability to provide protective immunity against M. hyopneumoniae infection. The lung-antigen consisted of a 10% concentration of frozen mycoplasmal pneumonic lung suspension. The second vaccine was prepared from a 10% suspension of mycoplasma harvested from flask cultures; the flask-antigen was heat-inactivated and contained incomplete Freund's adjuvant. No CF antibodies against M. hyopneumoniae were detected in the serums of pigs vaccinated with the lung-antigen. The lung had been obtained originally from a pig that had gross and microscopic lesions typical of mycoplasmal pneumonia. Vials of the same batch of the frozen pneumonic lung had been used in previous experiments to induce mycoplasmal pneumonia. All these suggested that the pneumonic lung contained viable mycoplasma. The reason for the poor antigenicity of the lung material is, therefore, not clear and whether some type of antibodies not detectable by the CF test were induced is not known. The lung-antigen failed to protect the immunized pigs because 3 of the 4 vaccinates had lesions typical of

mycoplasmal pneumonia.

Among the pigs vaccinated with the heat-inactivated, adjuvant-containing flask-antigen, CF antibodies were not detected until 2 weeks after the primary inoculation. Since blood samples were collected at 7-day intervals, it is probable that antibodies could have been detected sometime between the first and second weeks post-vaccination. At the time of challenge inoculation, the CF titers of the flask-antigen vaccinates ranged from 128 to 512. Only one of these 4 vaccinates had gross lesions at necropsy; however, chi-square analysis of the data showed that the subcutaneous immunizations with the flask-antigen did not significantly protect the pigs.

CF antibodies were detected in 3 of 4 positive control challenged intranasally with M. hyopneumoniae; all three zero-positive pigs had pneumonic lungs at necropsy. The remaining positive control was serologically negative and had no pneumonic lesions. Similarly, all 4 negative controls remained serologically negative and all were pneumonia-free at necropsy.

The third immunization trial was designed to evaluate the cumulative effect of M. hyopneumoniae vaccines administered by different routes in eliciting resistance to challenge infection. Each vaccinate was immunized by the subcutaneous, footpad, peritoneal, and the intratracheal routes. The subcutaneous and footpad vaccinations were given to induce circulating serum antibodies; the intraperitoneal vaccination given after the administration of mineral oil was designed to "prime" the immune--responsive peritoneal macrophages which are actively functional in cell-mediated immunity; and the intranasal immunization with inactivated M. hyopneumoniae

antigen was made with the hope of eliciting some local immunity (humoral or cellular). The reasoning was that if the mycoplasmal antigen was actually capable of producing significant protection, then the synergistic or cumulative action of humoral antibodies and local or cellular immunity elicited by the different routes of vaccination should effect such a protection. If protection was achieved, then it would be possible by a process of selective elimination in subsequent trials, to find which vaccine and route of administration are best capable of inducing resistance to mycoplasmal pneumonia.

Vaccinates which received both the subcutaneous and footpad injections on the first day of immunization maintained much higher antibody titer than did those vaccinates that received only the subcutaneous inoculations.

It was impossible, however, to interpret the results of this trial and to evaluate the protective effect of the vaccines because none of the 12 nonvaccinated control pigs could be infected with challenge inoculation of M. hyopneumoniae. It was not evident why the pigs could not be infected when challenged with both the broth culture of the organism and the pneumonic lung suspension. But, as a result of it, other experiments were performed to evaluate the virulence and pathogenicity of the test organism and to determine the effect of age of the pigs on susceptibility to infection.

In the fourth immunization trial the pigs were vaccinated with heat-inactivated, adjuvant-containing 10% suspension of the harvested mycoplasma antigen. As was observed in the third experiment, vaccinates which received footpad inoculations in addition to the subcutaneous inoculations developed significantly higher levels of CF antibodies than did those

vaccinates that received only the subcutaneous vaccination. It is not certain why footpad inoculation, given either during the primary immunization or as a booster injection, caused the production of higher levels of circulating antibodies. One explanation is that the footpad contains a rich layer of adipose tissue. The antigen is entrapped in the fat depot and is retained for a longer period of time. The pressure created when an animal steps on its footpads provides a "pumping action" which releases only a small amount of the antigen at a time. The prolonged retention of the antigen and its constant but gradual release to antibody-forming sites may result in a more prolonged production of antibody and a higher level of circulating antibody. When adjuvant-containing antigen is administered subcutaneously, a granuloma is formed at the inoculation site. It is probable that antigen is retained much longer in the footpad than in the granuloma, thereby causing antibody formation over a more extended period of time.

Two of the 5 nonvaccinated controls had gross pneumonic lesions and one other control pig had a mild lymphoid reaction when the lung was examined microscopically. All 7 vaccinates were protected from the challenge infection. The protection conferred by the heat-inactivated M. hyopneumoniae vaccine was statistically significant. All 3 vaccinates which received both subcutaneous and footpad inoculations as well as all 4 vaccinates given only the subcutaneous injections were protected. It is not known, therefore, if footpad inoculation, which results in higher CF antibody level, also conferred greater protection. Further investigation of the protective value of vaccines administered by the footpad route should be carried out.

When the results of immunization Experiments #2 and #4 are pooled, it is noted that only 1 of 11 pigs vaccinated with the antigen harvested from flask cultures had pneumonic lesions at necropsy. On the other hand, 5 of 9 nonvaccinates developed lesions typical of mycoplasmal pneumonia. Statistical analysis of these data confirmed that the heat-inactivated, adjuvant-containing vaccine administered by subcutaneous or footpad route conferred significant protection. At the time of challenge inoculations, all the vaccinates that were protected had CF titers ranging from 256 to 1024. Only pig #6421 (Experiment #2) with a CF titer of 512 had pneumonic lungs. These findings suggested there is correlation between vaccine-induced circulating antibody titers and protective immunity, or that serum antibodies are capable of inducing resistance to challenge infection. Goodwin et al. (1969b) have reported that intramuscular vaccination with formalin-inactivated mycoplasma antigen provided little or no protective immunity to mycoplasmal pneumonia, and that any immunity produced did not correlate with CF serum titers. It is probable that the contrasting results reflect differences in the mode of inactivation of the mycoplasma antigen and the route of immunization. It is likely that heat-treatment is more effective than formalinization in removing nonspecific medium constituents from the antigen surface and in revealing the more specific antigenic determinants on the cell membrane. Lam and Switzer (1971b) have shown that formalinized M. hyopneumoniae vaccine inoculated intranasally was ineffective in inducing protective immunity. These workers reported that when the M. hyopneumoniae antigen was extracted with ether or was lysed with 2% sodium lauryl sulfate (SLS) and then injected intramuscularly with adjuvant, significant protection was conferred in vaccinated animals.

They also found that passive transfer of serums from the hyperimmunized pigs conferred protection to the passively immunized pigs. These findings suggested that the protective immunity was mediated by circulating serum antibodies. The probability of such an immune mechanism is supported by the results obtained in the present study. These preliminary findings indicate that, as with contagious bovine pleuropneumonia and chronic respiratory disease of poultry, mycoplasmal pneumonia of swine may be controlled by parenteral immunization with vaccines.

The failure to infect any of the 12 control pigs (Experiment #3) with both low passage and high passage cultures of M. hyopneumoniae prompted the speculation that the virulence of the test organism had been significantly reduced or totally lost under laboratory conditions. The fact that the experimental pigs were 6 to 8 months old at the time of challenge exposure and that none of the pigs could be infected with pneumonic lung suspension raised another possibility. Namely that the pigs had become too old to be infected. It was considered probable that pigs which are 6 months or older become more resistant to infection. An experiment was carried out to test the virulence of the broth cultures and the effect of age on susceptibility to mycoplasma infection.

None of the young pigs (4 weeks old) and the old pigs (8 months and older) was infected with the low passage culture; similarly, the high passage failed to infect any of the young and old pigs inoculated intranasally. These findings suggested that the age of the pigs could not account for their failure to develop pneumonia. This view is supported by the successful experimental infection of old pigs in a subsequent immunization trial (Experiment #4).

The laboratory-maintained culture of M. hyopneumoniae had maintained growth initiating titer of 10^{-7} or more. It seemed likely that the mycoplasma was still capable of producing disease in the natural host system, although it is probable that growth initiating titer is not an appropriate measure of virulence or pathogenicity. The broth culture had been used in previous experiments to elicit typical mycoplasmal pneumonia lesions. More significantly, when the experimental pigs were fed the Swine Nutrition feed instead of the VMRI contract feed, the broth culture was used successfully to infect the pigs (Experiment #4). These findings indicate that some factor other than the virulence of the test organism was responsible for the failure to infect the pigs. Since only 2 of the 5 control pigs were infected with the broth culture, the possibility remains that the pathogenicity of the mycoplasma had been altered somewhat by laboratory propagation.

Sensitivity tests of the VMRI feed supplied on the most recent contract bid revealed that the feed was inhibitory to M. hyopneumoniae. The growth of the mycoplasma was consistently inhibited by the feed filtrate both in the solid medium and in fluid medium. A comparison of the VMRI swine feed and the feed supplied by the Iowa State University Swine Nutrition Farm showed that in dilutions up to 1:128 the VMRI feed filtrate inhibited the growth of M. hyopneumoniae. On the other hand, inhibition of the organism occurred only in the 1:2 and 1:4 dilutions of the Swine Nutrition feed filtrate. There was a significant difference in the inhibition caused by the two feed filtrates.

Sensitivity tests of the individual components of the Swine Nutrition feed were performed and only the Trace minerals premix component was

inhibitory to M. hyopneumoniae. The Trace minerals premix inhibited the organism at the same dilution level as the complete Swine Nutrition mix; it is probable, therefore, that the Trace mineral component accounted for the slight inhibition produced by the Swine Nutrition feed. Since the VMRI feed and Swine Nutrition feed contained essentially the same amount of trace minerals at almost exact concentrations, the trace minerals in the VMRI feed could not account for the high degree of inhibition produced by the VMRI feed.

In vivo comparison of the effects of the two feeds was done. All 4 pigs which were fed the Swine Nutrition feed developed extensive lesions of mycoplasmal pneumonia following intranasal instillation of pneumonic lung suspension. The estimated pneumonic scores were 15% to 20%. In contrast, only 2 of 4 pigs which were fed the VMRI feed developed lesions and the pneumonic scores were 3% and 4%. The results obtained from these experiments indicated that the VMRI feed contained some water-soluble substance which had an inhibitory effect on M. hyopneumoniae. The nature of the inhibiting substance is not known and it is not certain if the material is a feed additive or a naturally occurring inhibitor. Antibiotic assays of the feed showed only an insignificant level of the antibiotic chlortetracycline which is known to inhibit the growth of M. hyopneumoniae. It is possible, though unlikely, that the inhibitor is some other type of feed additive antibiotic. The contract for the VMRI ration specifies that under no conditions is such to be included in the feed. It has been suggested[1] that the inhibitor is a naturally produced by-product of contaminant mold.

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[1]Switzer, W. P. Veterinary Medical Research Institute, Personal communication.

The source of such a mold could be commercially supplied corn or soybean meal which had become moldy before it was incorporated into the swine feed.

It seems premature to speculate on the significance or nonsignificance of the inhibiting substance. Further investigations are suggested to verify the inhibition activity and to determine the nature of the inhibitor. It is quite possible that inhibition reported here is of no real significance, and that analysis of future feed supplies may not reveal any more activity. If mycoplasmal inhibition is consistently observed, as the results obtained in the present study indicate, it will be important to attempt an identification and characterization of the inhibitor. The inhibitor may have potential in the control of swine mycoplasmal pneumonia either as a feed additive or a medication. In any case, the results experienced in the current study point out the desirability of pretesting any feed supply used in future pig transmission trials before the trial is undertaken.

SUMMARY

Heat-inactivated Mycoplasma hyopneumoniae vaccine mixed with incomplete Freund's adjuvant was evaluated for its ability to prevent mycoplasmal pneumonia of swine. The protective effect of a live vaccine prepared from mycoplasma pneumonic lung suspension was also evaluated. Efficacy of the vaccines was tested by intranasal challenge inoculation of immunized pigs with either M. hyopneumoniae broth culture or pneumonic lung suspension. The serologic response of the pigs to immunization and the challenge infection was determined by a modified microtitration complement-fixation (CF) test.

The heat-inactivated vaccine injected by the subcutaneous and footpad routes protected 10 of 11 pigs from pneumonia. All vaccinated animals developed high CF antibody titers. Five of 9 nonvaccinated control pigs were infected by the challenge exposure. The pneumonic-lung vaccine failed to elicit serologic response or to induce resistance to mycoplasmal pneumonia.

The ability of a live vaccine to produce recovery of pigs already infected with mycoplasmal pneumonia was evaluated. This vaccine did not significantly promote recovery, despite the elevation of CF antibodies beyond the level elicited by natural infection.

The results suggested that, while CF circulating antibodies induced by vaccination were associated with protective immunity, they were unable to enhance the elimination of established lesions.

An attempt was made to evaluate the cumulative effect of M. hyopneumoniae vaccines administered by the subcutaneous, footpad,

intraperitoneal, and intratracheal routes to produce resistance to infection. The results of the trial could not be interpreted because of the failure to infect any of the control pigs by intranasal challenge inoculations of broth cultures and pneumonic lung suspension. The virulence of the mycoplasma was then tested and the effect of age of the experimental pigs on susceptibility was also studied.

Filtrates prepared from the supply of Veterinary Medical Research Institute (VMRI) swine feed used in this trial were tested in vitro and were found to cause consistent inhibition of growth of M. hyopneumoniae. The feed was compared with feed supplied by the Iowa State University Swine Nutrition Farm. All 4 pigs receiving the Swine Nutrition feed developed extensive mycoplasmal lesions, following intranasal inoculation of pneumonic lung suspension; pneumonia lesions were estimated to be 15% to 20% of total lung. Only 2 of 4 pigs receiving the VMRI feed developed pneumonic lesions estimated to be 3% and 4% of the total lung. This points out the need to pretest feed used in mycoplasmal pneumonia of swine transmission trials.

REFERENCES

Adler, H. E. 1970. Avian Mycoplasmal Diseases. In Sharp, T. J., ed. The role of Mycoplasmas and L-Forms of bacteria in diseases. P. 240-261. Charles C. Thomas Publisher, Springfield, Ill.

Adler, H. E. and Lamas Da Silva, J. M. 1970. Immunization against M. gallisepticum. Avian Dis. 14: 763-769.

Adler, H. E., McMartin, D., and Shifrine, M. 1960. Immunization against mycoplasma infections of poultry. Am. J. Vet. Res. 21: 482-485.

Bankowski, R. A., Wichmann, R., and Kummer, M. 1953. A complement-fixation test for identification and differentiation of immunological types of the virus of vesicular exanthema of swine. Am. J. Vet. Res. 14: 145-149.

Benneth, S. C. J. 1932. Contagious bovine pleuropneumonia: control by culture vaccines. J. Comp. Path. Therap. 54: 257-264.

Betts, A. O. 1952. Respiratory diseases of pigs. V. Some clinical and epidemiological aspects of virus pneumonia of pigs. Vet. Rec. 64: 283-288.

Betts, A. O. 1953. Pneumonic diseases of the pig caused by viruses: A review of the literature. Brit. Vet. J. 109: 99-106.

Betts, A. O. 1956. VPP Virus Pig Pneumonia. Jen-Sal. Jour. 39: 2.

Betts, A. O. and Whittlestone, P. 1963. Enzootic or virus pneumonia of pigs: production of pneumonia with tissue culture fluids. Res. Vet. Sci. 4: 471-479.

Betts, A. O., Whittlestone, P., and Beveridge, W. I. B. 1955. Investigations on the control of virus pneumonia of pigs in the field. Vet. Rec. 67: 685-689.

Börnfors, S. and Lannek, N. 1958. Studies on the immunity of swine enzootic pneumonia. Nord. Vet. Med. 10: 426-430.

Boulanger, P. 1969. Diagnosis of porcine enzootic pneumonia by serologic methods. Proceedings of the N. American symposium on swine respiratory diseases. P. 46-57.

Boulanger, P. and L'Ecuyer, C. 1968. Enzootic pneumonia of pigs: complement-fixation test for the detection of mycoplasma antibodies in the serum of immunized rabbits and infected swine. Canad. J. Comp. Med. 32: 547-554.

Boulanger, P. and L'Ecuyer, C. 1970. Complement-fixation tests for diagnosis of porcine enzootic pneumonia. Vet. Rec. 86: 448.

Carnaghan, R. B. A. 1962. Immunity in infectious synovitis of chickens. J. Comp. Path. 72: 433.

Chanock, R. M. 1970. Control of acute mycoplasmal and viral respiratory tract disease. Science 169: 248-256.

Clyde, W. A. 1971. Immunopathology of experimental M. pneumoniae disease. Infect. & Immunity 4: 757-763.

Dafaalla, E. N. 1956. A preliminary investigation into the adjuvant action of some substances on dried contagious bovine pleuropneumonia organisms. Vet. Rec. 68: 393-395.

Davies, G. 1969. The persistence of M. mycoides in the host after vaccination with T_1 broth vaccine. Res. Vet. Sci. 10: 225-231.

Denny, F. W., Wallace, C. A., and Glezen, P. W. 1971. M. pneumoniae disease: clinical spectrum, pathophysiology, epidemiology, and control. J. Infect. Dis. 123: 74-92.

Domermuth, C. H. 1962. Vaccination of chickens with M. gallisepticum. Avian Dis. 6: 412-419.

Dzu et al. 1971. Zur ätiologie und diagnose der mykoplasmose der schweine. I. Experimentelle infektionen mit mykoplasmen und plasteurellen. Mn. Vet. Med. 26: 169-181.

Estola, T. and Schulman, A. 1966. Isolation of swine enzootic pneumonia agent from pigs with enzootic pneumonia in Finland. Acta Path. Microbiol. Scand. 66: 113-123.

Fernald, G. W. 1969. Immunological aspects of experimental M. pneumoniae infection. J. Infect. Dis. 119: 255-266.

Fernald, G. W. 1972. In vitro response of human lymphocytes to M. pneumoniae. Infect. & Immunity 5: 552-558.

Fernald, G. W. and Clyde, W. A. 1970. Protective effects of vaccines in experimental M. pneumoniae disease. Infect. & Immunity 1: 559-565.

Friis, N. F. 1969. Mycoplasma suipneumoniae isolated in Denmark. Acta Vet. Scand. 10: 295-297.

Friis, N. F. 1971a. Mycoplasmas cultivated from the respiratory tract of Danish pigs. Acta Vet. Scand. 12: 69-79.

Friis, N. F. 1971b. Mycoplasma hyorhinis as a causative agent in pneumonia of pigs. Acta Vet. Scand. 12: 116-119.

Fujikura, T., Namioka, S., and Shibota, S. 1970. Tube agglutination test of M. hyopneumoniae infection in swine. Nat. Inst. Anim. Hlth. Quart. (Tokyo) 10: 42-43.

Gilbert, F. R., Davies, G., Read, W. C. S., and Turner, G. R. T. 1970. The efficacy of T_1 strain broth vaccine against contagious bovine pleuropneumonia: In-contact trials carried out 6 and 12 months after primary vaccination. Vet. Rec. 86: 29-33.

Glässer. 1939. Zum schweingrippeproblem. Deutche tierärztliche Wochenschrift 47: 209-212.

Gois, M., Cerny, M., and Veznikova, D. 1970. Relationship between metabolic-inhibition antibodies and colonial growth of M. hyorhinis and other porcine mycoplasmas in the presence of rabbit and pig sera. Res. Vet. Sci. 2: 161.

Gois, M., Valicek, L., and Savadina, M. 1968. Mycoplasma hyorhinis: a causative agent of pig pneumonia. Zentralblatt fur Vet. Med. 15B: 230-240.

Goodwin, R. F. W. 1963. The economic effect of enzootic pneumonia in a large herd of pigs. Brit. Vet. Jour. 119: 298-306.

Goodwin, R. F. W., and Hodgson, R. G. 1970. The passive hemagglutination test for the detection of M. suipneumoniae and the possible diagnosis of enzootic pneumonia of pigs. J. Hyg. (Cambridge) 68: 327-336.

Goodwin, R. F. W., and Hurrell, J. M. W. 1970. Further observations on the problem of isolating M. suipneumoniae from cases. J. Hyg. (Cambridge) 68: 313-325.

Goodwin, R. F. W. and Whittlestone, P. 1963. Production of enzootic pneumonia in pigs with agent grown in tissue culture from natural disease. Brit. J. Experimental Path. 44: 291-299.

Goodwin, R. F. W. and Whittlestone, P. 1964. Production of enzootic pneumonia in pigs with microorganism grown in medium free of living cells. Vet. Rec. 76: 611-613.

Goodwin, R. F. W., Hodgson, R. G., Whittlestone, P., and Woodhams, R. L. 1969a. Immunity in experimentally induced enzootic pneumonia of pigs. J. Hyg. (Cambridge) 67: 193-208.

Goodwin, R. F. W., Hodgson, R. G., Whittlestone, P., and Woodhams, R. L. 1969b. Some experiments relating to artificial immunity in enzootic pneumonia of pigs. J. Hyg. (Cambridge) 67: 465-476.

Goodwin, R. F. W., Pomeroy, A. P., and Whittlestone, P. 1965. Production of enzootic pneumonia in pigs with mycoplasma. Vet. Rec. 77: 1247-1249.

Goodwin, R. F. W., Pomeroy, A. P., and Whittlestone, P. 1967. Characterization of M. suipneumoniae, a mycoplasma causing enzootic pneumonia. J. Hyg. 65: 85-96.

Goodwin, R. F. W., Pomeroy, A. P., and Whittlestone, P. 1968. Attempts to recover M. suipneumoniae from experimental and natural cases of enzootic pneumonia. J. Hyg. 66: 595-603.

Gulrajani, T. S. and Beveridge, W. I. B. 1951. Studies on respiratory diseases of pigs. IV. Transmission of infectious pneumonia and its differentiation from swine influenza. J. Comp. Path. Therap. 61: 118-139.

Hodges, R. T., Betts, A. O., and Jennings, A. R. 1969. Production of pneumonia in gnotobiotic pigs with pure cultures of M. hyopneumoniae. Vet. Rec. 84: 268-273.

Hudson, J. R. 1965. Contagious bovine pleuropneumonia: the immunization value of the attenuated KH_3J. Austr. Vet. J. 41: 43-49.

Hudson, J. R. and Turner, A. W. 1963. Contagious bovine pleuropneumonia: A comparison of the efficacy of two types of vaccine. Austr. Vet. J. 39: 373-385.

Huhn, R. G. 1970. Enzootic pneumonia of pigs: a review of the literature. Vet. Bull. 40: 249-257.

Hyslop, N. St. G. 1956. Duration of immunity in cattle vaccinated with egg-adapted contagious bovine pleuropneumonia vaccine. Brit. Vet. J. 112: 519-522.

Jensen, K. E., Senterfit, L. B., Chanock, R. M., Smith, C. B. and Purcell, R. H. 1965. An inactivated M. pneumoniae vaccine. J. Amer. Med. Assoc. 194: 248-252.

Karrer, H., Meyes, K. F., and Eddie, B. 1950. The complement-fixation inhibition test and its application to the diagnosis of ornithosis in chickens and in ducks. I. Principles and technique of the test. J. Infect. Dis. 87: 3-23.

Karst, O. and Mitchell, S. 1972. Intranasal vaccination of cattle with an attenuated Gladysdale strain of M. mycoides van mycoides. J. Comp. Path. 82: 171-178.

Kenny, G. E. 1971. Immunogenicity of M. pneumoniae. Infect. & Immunity 3: 510-515.

Köbe, K. 1932. Ueber Benfunde von influenza-ahnlichen Bakterien biem schwein. Berliner und Münchener tierärztliche Wochensehrift 48: 209-210.

Laboratory Branch of the Communicable Disease Center. 1965. Standardized diagnostic complement-fixation method and adaptation to micro-test. United States Dept. of Health, Education, and Welfare. Public Health monograph 74.

Lam, K. M. 1970. Serologic and immunologic studies on M. hyopneumoniae pneumonia of swine. Unpublished Ph.D. thesis, Library, Iowa State University, Ames, Iowa.

Lam, K. M. and Switzer, W. P. 1971a. Mycoplasmal pneumonia of swine: development of indirect hemagglutination test. Amer. J. Vet. Res. 32: 1731-1736.

Lam, K. M. and Switzer, W. P. 1971b. Mycoplasmal pneumonia of swine: active and passive immunization. Amer. J. Vet. Res. 32: 1737-1741.

Lam, K. M. and Switzer, W. P. 1972. Mycoplasmal pneumonia of swine: Serologic responses in pigs. Amer. J. Vet. Res. 33: 1329-1332.

Lannek, N. and Börnfors, S. 1957. Immunity to enzootic pneumonia of pigs following recovery from diseases. Nordisk. Vet. Med. 9: 91-98.

L'Ecuyer, C. L. 1962. Propagation of the primary agent of virus pneumonia of pigs and determination of the microbiology of naturally occurring cases. Unpublished Ph.D. thesis, Library, Iowa State University, Ames, Iowa.

L'Ecuyer, C. L. 1969. Enzootic pneumonia of pigs: propagation of a causative mycoplasma in cell cultures and in artificial medium. Canad. J. Comp. Med. 33: 10-19.

L'Ecuyer, C. and Boulanger, P. 1970. Enzootic pneumonia of pigs: identification of causative mycoplasma in infected pigs and in cell cultures by immunofluorescent staining. Canad. J. Comp. Med. 34: 38-46.

L'Ecuyer, C. L. and Switzer, W. P. 1963. Virus pneumonia of pigs: attempts at propagation of causative agent in cell cultures and chicken embryo. Canad. J. Comp. Med. & Vet. Sci. 27: 91-99.

L'Ecuyer, C., Switzer, W. P., and Roberts, E. D. 1961. Microbiologic survey of pneumonia and normal swine lungs. Am. J. Vet. Res. 22: 1020-1025.

Lindley, E. P. 1965. Experiments with attenuated culture vaccine against contagious bovine pleuropneumonia. Brit. Vet. J. 121: 471-478.

Lipman, R. P., Clyde, W. A., and Denny, F. W. 1969. Characteristics of virulent, attenuated and avirulent M. pneumoniae strains. J. Bacteriology 100: 1037-1043.

Lloyd, L. C. 1967. An attempt to transfer immunity to M. mycoides infection with serum. Bull. Epizoot. Dis. Africa 15: 11-17.

Maré, C. J. 1965. Characterization of causative agent of virus pneumonia of pigs. Unpublished Ph.D. thesis, Library, Iowa State University, Ames, Iowa.

Maré, C. J. 1969. Mycoplasmal pneumonia of swine. Proceedings of the North American symposium on swine respiratory diseases. P. 35-45.

Maré, C. J. and Switzer, W. P. 1965. New species: Mycoplasma hyopneumoniae, a causative agent of virus pneumonia of pigs. Vet. Med. 60: 841.

Maré, C. J. and Switzer, W. P. 1966. Virus pneumonia of pigs. Propagation and characterization of a causative agent. Am. J. Vet. Res. 27: 1687-1693.

McMartin, D. A. and Adler, H. E. 1961. An immunological phenomenon in chickens following infection with M. gallisepticum. J. Comp. Path. 71: 311.

Meyling, A. 1971. M. suipneumoniae and M. hyorhinis demonstrated in pneumonic pig lungs by fluorescent antibody technique. Acta Vet. Scand. 12: 137-141.

Mogabgab, W. J. 1968. Protective effects of inactive M. pneumoniae vaccine in military personnel. Amer. Rev. Resp. Diseases 97: 359-365.

Moore, R. W., Livingstone, C. W. and Redmond, H. E. 1965. Cultural and serological techniques for the differential diagnosis of mycoplasma in swine. Proceedings: 69th annual meeting of the United States Livestock Sanitary Association.

Olson, N. O., Adler, H. E., Da Massa, A. J. and Corstvet, R. E. 1964. The effect of intranasal exposure to M. synoviae and infectious bronchitis on development of lesions and agglutinins. Avian Dis. 8: 623.

Pattison, I. H. 1956. A histological study of a transmissible pneumonia of pigs characterized by extensive lymphoid hyperplasia. Vet. Rec. 68: 490-496.

Pfizer, Chas. Inc. 1968. Circular: SEP vaccine.

Piercy, S. W. and Knight, G. J. 1958. Studies with avianized strains of the organisms of contagious bovine pleuropneumonia. V. Experiments with avianized vaccines at various levels of attenuation. Brit. Vet. J. 114: 245-253.

Poland, J., Endinton, N., Gois, M. and Betts, A. O. 1971. The production of pneumonia with or without pleurisy in gnotobiotic pigs with pure cultures of strain TR32 M. hyorhinis. J. Hyg. (Cambridge) 69: 145-154.

Priestley, F. W. 1955a. Immunization against contagious bovine pleuropneumonia, with special reference to the use of a dried vaccine. J. Comp. Path. Therap. 65: 168-182.

Priestley, F. W. 1955b. Further observations on immunity to contagious bovine pleuropneumonia with special reference to adjuvants. Vet. Rec. 67: 729-733.

Pullar, E. M. 1948. Infectious pneumonia of pigs: I. General description, differential diagnosis, and epidemiology. Austr. Vet. J. 24: 320-330.

Purchase, H. S. 1939. Vaccination against contagious bovine pleuro-pneumonia with "culture virus". Vet. Rec. 51: 31-47; 67-75.

Report of the Panel of the Colloquium on Selected Infectious Diseases of Swine: Report of the Committee on Nomenclature. JAVMA. 159(12): 1799.

Roberts, D. H. 1968. Serological diagnosis of M. hyopneumoniae infection in pigs. Vet. Rec. 82: 362-363.

Roberts, D. H. and Little, L. W. A. 1970a. Serological studies in pigs with M. hyopneumoniae. J. Comp. Path. 80: 211-220.

Roberts, D. H. and Little, L. W. A. 1970b. An auto-immune response associated with porcine enzootic pneumonia. Vet. Rec. 86: 328.

Shope, R. E. 1931. Swine influenza. J. Exp. Med. 56: 349-359.

Slavik, M. F. 1971. Development of microtitration complement-fixation test for diagnosis of Mycoplasmal pneumonia of swine. Unpublished M.S. thesis, Library, Iowa State University, Ames, Iowa.

Slavik, M. F. and Switzer, W. P. 1972. Development of microtitration complement-fixation test for diagnosis of Mycoplasmal pneumonia of swine. Iowa State Journal of Research 47: 117-128.

Smith, C. B., Friedwald, W. T. and Chanock, R. M. 1967. Inactivated M. pneumoniae vaccine: evaluation in volunteers. J.A.M.A. 199: 353-358.

Steinberg, P., Horswood, R., Brunner, H. and Chanock, R. M. 1971. Temperature-sensitive mutants of M. pneumoniae. II. Response of hamsters. J. Infect. Dis. 124: 179-187.

Switzer, W. P. 1955. Studies on infectious atrophic rhinitis. IV. Characterization of a pleuropneumonia-like organism isolated from nasal cavities of swine. Am. J. Vet. Res. 16: 540.

Switzer, W. P. 1967. Swine mycoplasma. J.A.V.M.A. 151: 1656-1661.

Switzer, W. P. 1970. Mycoplasmosis and Mycoplasmal pneumonia. In Dunne, H. W., ed. Diseases of Swine. P. 672-692. Iowa State University, Ames, Iowa.

Switzer, W. P. 1972. Mycoplasmal pneumonia of swine. J.A.V.M.A. 160: 651-653.

Takatori, I. 1969. Demonstration of complement-fixation antibody against M. hyopneumoniae in Japan. Nat. Inst. Anim. Hlth. Quart. (Tokyo) 9: 183-184.

Takatori, I. 1970. Etiology and serological diagnosis of swine enzootic pneumonia. Japan Agric. Res. Quart. 5: 39-45.

Takatori, I., Huhn, R. G. and Switzer, W. P. 1968. Demonstration of CF antibody against M. hyopneumoniae in the sera of pigs infected with swine enzootic pneumonia. Nat. Inst. Anim. Hlth. Quart. 8: 195-203.

Vlaovic, M. S. and Bigland, C. H. 1971. The attempted immunization of turkey hens with viable M. meleagridis. Canad. J. Comp. Med. 35: 338-341.

Walker, J. C. 1921-1922. Bull. Nos. 1 & 2, Dept. of Agricl, Kenya.

Wallis, A. and Thompson, G. 1969. The evaluation of a complement-fixation test for the diagnosis of porcine enzootic pneumonia. Vet. Rec. 85: 573-577.

Warren, J., Senterfit, L. B., and Sieirs, F. 1968. Inactivated culture vaccine against M. gellisepticum infection in chickens. Am. J. Vet. Res. 29: 1659-1664.

Wichmann, R. W., Bankowski, R. A. and DeMassa, A. J. 1960. The cultivation and modification of the avian infections synovitis agent in tissue culture. Avian Dis. 4: 152.

Young, G. A. 1956. Is VPP a new swine disease? Norden News 30: 6.

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