Effect of age on the susceptibility of pigs to *Mycoplasma hyopneumoniae* pneumonia

by

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CHAPTER I. INTRODUCTION

Mycoplasmal Pneumonia of Swine (MPS) or Enzootic Pneumonia (EPP) is a chronic pneumonia characterized by a nonproductive cough, high morbidity and low mortality. Mycoplasma hyopneumoniae, syn. Mycoplasma suipneumoniae, was shown to be the etiological agent of this respiratory syndrome (Maré and Switzer 1965, and Goodwin et al. 1965).

MPS has been reported in almost all major swine-producing countries and it has been considered the most prevalent and the most important respiratory disease of swine (Switzer and Ross 1975 and Whittlestone 1979).

Economic loss results from a depression in growth rates and reduction in feed/gain ratio. In some infected herds, feed conversion is reduced by as much as 20% (Whittlestone 1973).

The incidence of MPS ranges from 45% to 75% among slaughterhouse pigs (Switzer and Ross 1975 and Whittlestone 1979). The herd incidence and severity of MPS is variable. It depends upon the production system, concentration of pigs, management, ventilation and other environmental factors (Lindquist 1974).

Within clinically affected herds, the incidence of enzootic pneumonia decreases progressively with increasing age of the animals (Pullar 1948). Under experimental

conditions, it appears that inoculation of M. hyopneumoniae induces MPS in swine of all ages but, results obtained in attempts to produce MPS by exposure of susceptible pigs to previously infected ones have been contradictory. In addition, unpublished evidence obtained by Switzer and Ross (1975) indicates that the peak of complement-fixing antibodies among naturally-infected pigs was from 5 to 12 months of age. Furthermore, investigation of naturally-occurring MPS has indicated that the disease is rare in pigs less than 6 weeks of age. These observations may suggest that younger pigs are relatively more resistant to M. hyopneumoniae than older pigs.

MPS is thought to occur as a clinically evident disease for several years within a herd; however, Keller (1976) has suggested that undetected, subclinical, long-standing infections may also exist. These subclinical animals could disseminate the disease within and among herds.

Aerosol and direct contact are the mechanisms by which MPS is thought to be transmitted. On a herd basis, the most important reservoirs of the disease are infected sows which transmit the agent to their suckling offspring. These piglets, when mixed with others from different litters, spread the infection among them (Switzer and Ross 1975). Most efforts for total control of MPS have focused on breaking

down this cycle of transmission. As a result, specific pathogen free (SPF) and minimal disease herds were established. MPS free herds can be established by several mechanisms. Some of them include utilization of stock from others already shown to be free of MPS, cross-suckling (onto MPS-free sows) of piglets removed at birth from their infected mothers, artificial rearing of piglets obtained by surgical techniques, and artificial insemination of MPS-free sows (Whittlestone 1979). In general, the herds established in this way have been monitored for the presence or absence of clinical disease and by the lack of suggestive MPS lung lesions at the abbattoir (Keller 1976). Breakdown of the MPS-free status has been a common problem in most systems. The breakdown is thought to be due to carrier hosts, fomites, airborne infection or clinical expression of long-term subclinical infections (Whittlestone 1979). As mentioned previously, Keller (1976) presented evidence for the existence of a subclinical condition, consequently, a goal to be achieved is the identification of these M. hyopneumoniae carriers.

Minimal disease herds have been established by the utilization of chemotherapeutic regimens and/or by the elimination of infected animals.

The possible existence of age-susceptibility to M. hyopneumoniae could bring a new approach to the maintenance

of the minimal disease herds and to the control of SPF status. More economic and strategic chemotherapeutic measures could be developed if there were an age-susceptibility to M. hyopneumoniae. Furthermore, these susceptible pigs could be utilized in monitoring the SPF-herd status. Vaccination schemes, not yet available, could be brought out in such a way that maximum immunity could be induced in that particular susceptible age.

As a consequence, the main objective of this work was to determine if there is age-susceptibility to MPS. In the present work, pigs of 3 different ages were placed in contact with M. hyopneumoniae-infected pigs. The results obtained by morphological evaluation, detection of the agent and serological response in these different age groups of pigs revealed no differences thus negating the hypothesis of age-susceptibility to M. hyopneumoniae.

CHAPTER II. LITERATURE REVIEW

Literature reviews on Enzootic or Mycoplasmal Pneumonia of swine have been presented by Betts (1953), Maré and Switzer (1965), Huhn (1970), Whittlestone (1973) and Switzer and Ross (1975). As a consequence, the present literature review will concentrate on three aspects: Major steps in the clinicopathological identification of Enzootic or Mycoplasmal Pneumonia in swine and the identification characteristics of Mycoplasma hyopneumoniae, its etiological agent; epidemiology of Mycoplasmal Pneumonia, and; diagnosis of Mycoplasma hyopneumoniae pneumonia.

Mycoplasmal Pneumonia of Swine (MPS)

Shope (1931) demonstrated that swine influenza was caused by the synergistic action of the swine influenza virus and Haemophilus influenza suis. Furthermore, he noted a second pneumonia characterized by low morbidity, absence of prostration and chronicity.

Following Shope's work, Köbe (1932) described a widespread enzootic pneumonia of young pigs in Germany. Glässer (1939) stated that this disease was not confined to piglets but affected older pigs too, although less acutely. These authors regarded their virus as either identical with Shope's

swine influenza virus or at least a less virulent variant of it.

In Australia, Pullar (1948) recognized and described a chronic infectious contagious pneumonia of unknown etiology. In addition, he made a clear cut clinical distinction between his "infectious pneumonia of pigs" and swine influenza. Gulrajani and Beveridge (1951), in attempting to isolate new strains of swine influenza virus from outbreaks of respiratory disease in Ireland, recovered a filterable agent from pneumonic lung. When inoculated into pigs, their agent produced a pneumonia identical to that described by Pullar (1948). The inoculated pigs did not develop antibodies against the swine influenza virus so the authors concluded that they had isolated a new viral agent.

Betts and Campbell (1956), studying the effect of antibiotics and sulfamezathine on the causative agent of a virus pneumonia of pigs (VPP), reported its susceptibility to chlortetracycline and oxytetracycline. This susceptibility was determined by the capability of the drugs to prevent infection in pigs. But these drugs did not influence the course of infections that were established prior to initiation of medication. Discussing their findings, the authors raised the question of whether this agent was really a true virus.

L'Ecuyer (1962) successfully propagated the etiological agent of VPP in primary swine kidney and Hela cell cultures. The agent was detected in these in vitro cultures by its ability to produce typical gross and microscopic lesions in pigs inoculated with second passage, cell culture fluids. After several passages on Hela cells it was observed to lose pathogenicity.

Betts and Whittlestone (1963), using the J strain of VPP, observed a cytopathic effect in plasma clot cultures prepared from lung and nasal mucosa of an infected pig. Fluids from these cultures induced similar cytopathic changes in pig lung monolayer cultures. Early serial passage material in cell cultures was pathogenic for pigs but not after 20 passages. Throughout the experiment, pleomorphic organisms (PO) were observed microscopically in stained touch preparations. The role of these PO was uncertain at that time but, the authors suggested that they were a commensal, synergistic with the primary or the causal agent of the pneumonia. In order to separate the role of the PO, thought to be a PPLO, from that of the virus, Goodwin and Whittlestone (1964) devised a cell-free-medium for isolation of the PO. They succeeded in isolating the PO in boiled pig lung cell cultures and in cell-free medium enriched with serum, lactalbumin and yeast extract. High dilutions

of cell-free cultures induced pneumonic lesions in which pleomorphic organisms were observed. Their work showed that the agent could not be a virus. Although they suggested that the agent probably was a PPLO, they did not prove it.

Maré and Switzer (1965) isolated a small cocco-bacillary organism in a cell-free medium from lesions of experimentally transmitted VPP. The agent was reisolated in cell-free medium and successfully cultivated on agar medium where colonies characteristic of mycoplasmas developed. On the basis of size, morphology, staining characteristics, penicillin resistance and failure to revert to a bacterial form when propagated in absence of bacterial inhibitor, Maré and Switzer named the organism of Mycoplasma hyopneumoniae. This was the first concrete evidence establishing a mycoplasma as the etiological agent of VPP.

Some months later, Goodwin, Pomeroy and Whittlestone (1965) reported the propagation of their "J" strain agent on solid medium. The morphology, growth requirements and antibiotic sensitivity of the PO then described were similar to those described by Maré and Switzer (1965). In addition, such cultures were used to induce typical lesions of VPP in experimental pigs. They proposed the name Mycoplasma suipneumoniae for the agent.

Because the etiologic agent was a mycoplasma, Maré and Switzer (1965) suggested that the disease be called "Pulmonary Mycoplasmosis of Swine" or "Mycoplasmal Pneumonia of Swine" (MPS). Goodwin et al. (1965) retained the name "Enzootic Pneumonia of Pigs" (EPP) for the same condition.

Goodwin et al. (1967), comparing M. suipneumoniae and M. hyopneumoniae by the growth-inhibition and metabolic-inhibition tests, found that they were antigenically indistinguishable. Both strains were shown to be serologically distinct from known mycoplasmas isolated from swine and other animal species. Thus, based on priority, Mycoplasma hyopneumoniae has become the accepted name for the etiological agent of MPS (Subcommittee on the Taxonomy of Mycoplasmatales 1974).

The question has been raised of whether M. hyopneumoniae by itself will cause EPP or whether the synergistic effect of resident flora is necessary. To solve this problem, Hodges et al. (1967) attempted and successfully reproduced the disease by inoculation of gnotobiotic pigs with a pure culture of the agent.

Histopathological changes caused by M. hyopneumoniae are not pathognomonic (Jericho 1977). Other Mycoplasma sp. can induce similar lesions and their role in the naturally occurring disease is disputed among researchers.

Mycoplasma hyorhinis, a polyserositis-producing mycoplasma, was isolated from pneumonic lungs of pigs (Switzer, 1955). Several investigators incriminated this agent as a secondary invader in cases of MPS (L'Ecuyer et al. 1961, L'Ecuyer and Switzer 1963, Switzer 1967 and Goodwin et al. 1967). In contrast, Gois and Kuksa (1974a), using M. hyorhinis inoculated intranasally in gnotobiotic piglets, induced extensive lung lesions in 12 of 20 inoculated piglets as well as polyserositis. Meyling (1971), examining natural cases of pneumonia by the direct fluorescent antibody test, observed that lungs which were negative for M. hyopneumoniae were positive for M. hyorhinis. Furthermore, M. hyorhinis was located on the surface of the bronchial epithelium as had been shown in Mycoplasma hyopneumoniae pneumonia.

M. flocculare (Friis 1972), a culturally and morphologically similar organism to M. hyopneumoniae, was reported by Friis (1974). He found that M. flocculare is not of primary etiological importance in the porcine enzootic pneumonia complex. In addition, Friis (1976), in a study of the incidence and pathogenicity of M. flocculare, concluded that the whole of the respiratory tract is the natural habitat of the organism and that its presence in the Danish SPF system has never resulted in clinical outbreaks of pneumonia.

Mycoplasmal Epidemiology

The factors involved in the epidemiology of a disease are those related to the microorganism, host and environment. In the present review, an effort has been made to characterize these factors throughout the acquired knowledge in different mycoplasmal diseases.

Factors related to the microorganism

Among those factors pertinent to the microorganisms, the most important are: antigenic composition, degree of virulence, specie(s) specificity, tissue tropism, survival of the microorganism away from the host, susceptibility of the microorganism to various drugs and disinfectants, persistence of infection in the host and route of infection. Each of these factors is subject to variability. For example, high level of variability in antigenic type within a species may make control by vaccination difficult. Consequently success in control of a disease requires the best possible understanding of all factors involved in its epidemiology.

Antigenic variability Heterogeneity between strains of the same species of mycoplasma has been well-documented by Kenny (1979). Forshow (1972) found reproducible differences between proteins from different strains of

M. pulmonis extracted with phenolacetic acid and examined by polyacrylamide gel electrophoresis. In other work, Forshow and Fallon (1972) found heterogeneity among 27 strains of M. pulmonis using gel-diffusion and metabolic inhibition techniques. Serological heterogeneity among strains of M. hyorhinis was demonstrated by the indirect hemagglutination test (Ross and Switzer 1963), growth inhibition test (Goiś et al. 1974), metabolic inhibition test (Goiś et al. 1974) and latex agglutination test (Goiś et al. 1974).

Species specificity The host specificity of mycoplasmas is not absolute. At the cellular level it is possible to cross infect tissue culture cells with mycoplasmas isolated from species other than those from which the tissue culture cells were derived.

M. pneumoniae, M. orale and M. hominis of human origin have been shown to infect monkey kidney cells (Chanock et al. 1960), chick embryo cells (Somerson and Cook 1965) and hamster kideny cells (BHK21), (MacPherson and Russel, 1966). M. pulmonis, of rodent origin, has been shown to infect human (Hela) tissue culture cells (Nelson 1960). M. hyorhinis of swine origin has infected human (HEP-2) tissue culture cells. In addition, M. bovigenitalium

of cattle origin infected pig kidney tissue culture cells (Afshar 1967).

Regarding mycoplasma host specificity, isolation studies have pointed to evidence against absolute host specificity. M. arginini has been isolated from cattle (Leach 1970), sheep (Barile et al. 1968 and Leach 1970), goats (Barile et al. 1968), swine (Orning et al. 1978), chamois (Leach 1970) and captive wild cats (Hill 1971). M. agalactiae has both sheep and goats as its host where it causes contagious agalactia (Watson et al. 1968), pleurisy and pneumonia among goats in Australia (Cottew and Lloyd 1965) and granular vulvovaginitis in goats (Singh et al. 1974).

M. hyopneumoniae appears to have only the pig as its host. There has been no report of isolation of M. hyopneumoniae from any other host. Lung suspensions of swine affected with VPP were utilized by Fulton et al. (1953) in several attempts to produce disease in experimental animals. These lung suspension were filtered or treated with antibiotics and, when inoculated into pigs they produced typical lesions of EPP. These lung suspensions failed to induce disease in guinea pigs and mice inoculated intranasally and subcutaneously. They also failed to induce any lesions in eight-day-old chicken embryos. Similarly, a

Rhesus monkey inoculated intranasally with the lung suspension developed no disease. In a similar study, Plowright (1953) found no lesions in young weaned mice inoculated intranasally as well as in unweaned mice inoculated intraperitoneally or intracerebrally. In contrast, Betts (1953) induced pneumonia on two occasions in inoculated ferrets and Goodwin et al. (1968) cultured M. hyopneumoniae in the yolk sac of chicken embryos.

Tissue tropism Mycoplasmas usually infect mammalian hosts by attaching to surface epithelium of the respiratory or genital tract. Certain pathogenic mycoplasmas, such as M. pneumoniae, remain extracellularly but others, capable of systemic invasion, localize in joints, brain, pleura and subcutaneous tissue (Fernald 1979).

In swine, Mycoplasma hyopneumoniae appears to be restricted to the respiratory tract. Goiś and Kuska (1974b) administered M. hyopneumoniae to 8 gnotobiotic piglets via the intranasal route; at necropsy, 28 days after inoculation, 5 of these animals had lung lesions and M. hyopneumoniae was recovered only from the respiratory tract. Attempts to isolate the agent from organs other than the respiratory tract failed. In contrast, strains of M. hyorhinis and M. hyosynoviae, isolated from the respiratory tract, caused mycoplasmemia with colonization and

production of disease in other organs. Switzer (1953) isolated a filterable agent from the nasal mucosa of swine. This agent was identified as a PPLO and was named M. hyorhinis (Switzer 1955). Switzer (1953) inoculated the agent intraperitoneally into pigs and the inoculated animals developed peritonitis, pleuritis and pericarditis. In addition, the author reported that the same agent was isolated from similar lesions in pigs from different herds in Iowa.

M. hyosynoviae (Ross and Karmon 1970), previously named M. granularum (Switzer 1964), causes uncomplicated, nonsuppurative arthritis in 3 to 6 month old (40 kg to 100 kg) and young adult swine (Ross and Duncan, 1970). Goiś et al. (1974b), working with gnotobiotic piglets infected intranasally with M. hyosynoviae, produced slight pneumonia in one pig and the organism was recovered from the tonsils of 5 out of 6 piglets. Furthermore, it was isolated twice from the lung, spleen and nasal mucosa and once from the liver, but not from the joints.

Affinity for host tissues may vary among strains of a certain mycoplasmal species. Varley and Jordan (1978) inoculated three strains of M. gallisepticum (S6 low passage, S6 high passage and A514 high passage) into 18-day-old chick embryos. During the first 28 days following hatching, the virulent S6 strain was recovered more frequent-

ly from a wider variety of tissues in the chicks than were the other strains of the organism. This strain produced more severe clinical disease including nervous signs, respiratory lesions and swollen hocks. Nervous symptoms were observed only with low passage S6 but both S6 strains were isolated from brain and joints, suggesting strain affinity for these sites. All strains were isolated from respiratory tissue, indicating a common affinity for that site. In a similar study with turkey poults, the same authors (Varley and Jordan 1978) found the same affinity of S6 for nervous tissue in that host. Switzer and Ross (1975) discussing factors involved in production of polyserositis-arthritis in swine by M. hyorhinis, pointed out that strains appeared to differ in predilection for specific serous surfaces. In other words, some strains produced lesions on all serous surfaces while others may have a tropism for pericardial or joint surfaces.

Variation in virulence In vitro passage of mycoplasmas generally decreases their virulence. This has been observed with M. arthritidis (Golightly et al. 1970), M. gallisepticum (Power and Jordan 1976) and M. pneumoniae (Couch et al. 1964 and Dajani et al. 1965). Thus, the attenuation of mycoplasmas by in vitro passage is used for development of live vaccines (reviewed by Whittlestone 1976a).

Variability of virulence among different strains of all three species of pathogenic swine mycoplasma appears to be common. Goiš and Kuksa (1974a) found that two strains of M. hyorhinis differed in virulence for gnotobiotic pigs. The two strains (Nell0 and S218) had been subcultivated only two times in fluid medium prior to inoculation into experimental pigs. None of 19 gnotobiotic piglets infected intranasally with M. hyorhinis strain Nell0 at 6 days of age developed clinical disease and there was no evidence of spread of the organism in the body. Mycoplasmas were recovered merely from the upper and lower respiratory tract. In contrast, of 21 piglets infected with strain S218 at 6 days of age, 15 developed clinical disease and 18 showed evidence of the spread of the infection in the body. Ross (1973) compared the capability of two strains (33R and S149) of M. hyosynoviae to produce disease in pigs. Strain 33R was isolated from arthritic as well as from grossly normal joints and lymph nodes of two inoculated pigs. Moreover, infection with this strain resulted in production of complement fixing antibody within 10 days after inoculation. In contrast, cultures of all joints and lymph nodes of pigs inoculated with strain S149 were negative and complement-fixing antibodies was not detectable

within 10 days of infection. Both strains were isolated from nasopharyngeal areas from all inoculated pigs. The severity of lameness and the number of affected joints were higher with strain 33R than strain S149.

Goodwin et al. (1968) compared two strains of M. hyopneumoniae, one after it had been passaged many times in yolk sac of chicken embryos. The higher passage strain induced smaller pneumonic lesions than the lower passage strain although the former contained nearly 100 times more colony-forming units. This fact induced the author to suggest the possibility of attenuation of virulence by passing M. hyopneumoniae in chicken embryos.

Components of virulence The pathogenic potential, long-term resistance to defense mechanisms in the immuno-competent host and, extreme fragility of mycoplasmas suggest that they have unique mechanisms for surviving in their avian and mammalian hosts. These properties may be considered as virulence factors or components of virulence. In a systemic functional approach, these factors of virulence can be divided into four components: attachment, toxin production, induction of detrimental host immune response and capability to circumvent or block immunological attack.

Attachment Some mycoplasmas initiate infection by attaching to ciliated airway epithelial cells with consequent cell damage. M. gallisepticum, M. pneumoniae and M. pulmonis have specialized terminal structures which have been related to gliding movement and or attachment to host cells (Bredt and Radestock 1977).

Maniloff et al. (1965), in an ultrastructural study of M. gallisepticum, described a highly structured bleb and an infrableb region which was composed of a granular material but which had little internal structure. Uppal and Chu (1977), in an electron microscopic study of fowl trachea infected with M. gallisepticum, showed that the blebs were attached to the plasma membrane of epithelial cells. In addition, slight to moderate changes were observed at the cellular level.

Biberfeld and Biberfeld (1970), studying the ultrastructure of M. pneumoniae cultivated in broth on glass and plastic surfaces, described a specialized structure at the thinner end of M. pneumoniae; this structure consisted of a dense rod surrounded by electronlucent cytoplasm and ending with a platelike thickening. Collier and Clyde (1971), studied the interaction between M. pneumoniae and the human respiratory epithelial cell. The host parasite

interaction led to impairment of cellular function which was reflected by disorganization and loss of ciliary motion and an associated sequence of cytopathological changes denoting progressive cell injury. Furthermore, they found that the structure described by Biberfeld and Biberfeld (1970) was serving as a means of cell attachment to host cells.

Subsequently, Collier and Carson (1980), using freeze-fracture preparations of M. pneumoniae, found numerous membrane-associated particles embedded in the inner side of the bimolecular leaflet. Moreover, M. pneumoniae cells subjected to freeze-fracture techniques frequently have areas of the fractured membrane with particle-free zones and/or blebs. These particle-free areas have been observed to be aligned closely to host tracheal epithelium in organ culture.

In a study on the motility of M. pulmonis, Bredt and Rodestock (1977) observed two morphologically distinct forms. One was a round cell with a protruding flexible stalk thickened at its end, and the other form was an elongated cell with a tapered leading end. Both forms showed gliding movement. They suggested that these structures could be important for adherence and motility. On the other

hand, an avirulent strain of M. pneumoniae, with terminal structure, did not attach or cytoadsorb (Collier 1972); this observation suggested that some sort of biochemical mediator was required.

The ability to hemadsorb and hemagglutinate has been associated with virulence and pathogenicity because many pathogenic mycoplasmas such as M. pneumoniae, M. gallisepticum, M. pulmonis and M. agalactiae have these properties. Nonetheless, hemadsorption is not universally essential in all mycoplasmal diseases because, some highly pathogenic mycoplasmas (e.g., Mycoplasma mycoides subsp mycoides) do not hemadsorb (Barile 1979).

Sobeslavksy et al. (1968), utilizing monkey, rat, and chicken tracheal epithelial cells as well as monkey, rat, guinea pig and chicken erythrocytes, found that M. pneumoniae and M. gallisepticum appeared to attach to erythrocytes or tracheal epithelial cells by neuraminic acid receptors on these cells. In contrast, M. orale types 1 and 3 and M. pulmonis seemed to utilize another type(s) of receptor(s). Pretreatment of red cells or tracheal epithelial cells with receptor-destroying enzyme, neuramidase or influenza B virus removed the adsorption receptor for M. pneumoniae. Similar pretreatment of M. pneumoniae colonies with neuraminic acid-containing materials prevented adsorption of erythrocytes and of respiratory tract cells.

Manchee and Taylor-Robinson (1969), using 17 mycoplasma serotypes observed that only four were found to use neuraminic acid receptors; these were M. pneumoniae, M. gallisepticum and M. synoviae and M. AR1. Not all strains of a serotype behaved alike; there were differences among strains of M. gallisepticum in susceptibility of receptors to destruction by neuramidase. This observation suggested to the authors that there was a receptor gradient which might reflect differences in virulence.

Lloyd (1975), in an attempt to clarify the role of sialic acid, suggested that it may be a factor controlling cell behavior: a) by contributing to the structural properties of the cell surface and, b) by protecting or blocking some specific glycopeptides from recognition or adhesion. In other words, sialysation of a cell decreases its adhesion to other cells such as occurs with neoplastic and mitotic cells. Consequently, Barile (1979) suggested that mycoplasma binding to sialic acid may inhibit function of these receptor sites, resulting in cellular dysfunction.

Evidence has been presented that receptors on mycoplasmas which bind to eucaryotic cells are proteins. Hu et al. (1977), using a brief pretreatment of M. pneumoniae with protease, prevented mycoplasma attachment to the sensitive host cell without reducing viability of the microorganism.

Gel electrophoresis analysis of mycoplasma proteins revealed that a major protein was missing after exposure of intact mycoplasmas to protease.

The attachment of swine mycoplasmas to epithelial cells has been demonstrated by immunofluorescence and by electron microscopy. L'Ecuyer and Boulanger (1970), Meyling (1971) and Livingston et al. (1972), using immunofluorescence, found that M. hyopneumoniae cells occurred mainly as a coating of the bronchial and bronchiolar epithelium infected pigs. Meyling (1971) reported that M. hyorhinis, besides its bronchial localization, was found forming foci in the alveolar tissue. Livingston et al. (1972), using transmission electron microscopy (TEM), described a loss of cilia and the presence of M. hyopneumoniae on the bronchial surface. Mebus and Underdahl (1977) examined lungs of gnotobiotic pigs inoculated with M. hyopneumoniae by means of the scanning electron microscope (SEM). They confirmed the bronchial localization of the organism and found the same changes reported by Livingston et al. (1972) in the tracheas of infected animals.

Toxin production As a consequence of mycoplasma-cell host interaction, there are alterations in macromolecular synthesis, ciliostasis and chromosomal aberrations.

Hu et al. (1975) studied alterations in the metabolism of hamster trachea in organ culture after initial attachment of virulent M. pneumoniae organisms to respiratory epithelial cells. Modifications in host cell RNA and protein synthesis preceded decreased uptake of metabolic precursors, ciliostasis and other cytopathology. Furthermore, they found that mediation of tissue injury required metabolic activity, as detected by continued protein synthesis by attached mycoplasmas. Moreover, as a result of mycoplasma endonuclease activity, many mycoplasmas can use RNA and DNA as a source of nucleic acid precursors (Cassel et al. 1978). Fogh and Fogh (1973) observed a reduction in chromosome number, increased chromosome aberrations and the appearance of new chromosome varieties in heteroploid cells after infection with M. fermentans. Cassel et al. (1978) suggested that these changes are likely due to mycoplasma utilization of host cell components and/or release of toxic metabolic wastes.

Gourlay (1965) reported the isolation of a galactan containing lipopolysaccharide (LPS) from the urine of cattle infected with M. mycoides subsp. mycoides. When inoculated in combination with cultures of the live mycoplasma into susceptible or immune animals the galactan

caused a more severe disease. The galactan was weakly pyrogenic in rabbits and nontoxic for cattle, but was lethal for fowl embryos.

Induction of detrimental host immune response

Possible mechanisms for induction of a detrimental host immune response and consequent tissue damage include mycoplasma antibody and complement-mediated cytolysis or other cell damage, autoimmunity, production of immune complexes and production of rheumatoid factors. These mechanisms are especially likely in mycoplasma disease because of the close mycoplasma host cell interaction.

The cytocidal effect occurring when lymphoid cells infected with M. hyorhinis are killed by a combination of antimycoplasma antibody and complement provides evidence that a close association between mycoplasmas and host cells may lead to damage of the "innocent bystander" host cell (Cassel et al. 1978).

The participation of autoimmunity as an inducer of tissue damage in mycoplasma diseases is supported by the fact that antibodies that are reactive with a variety of host tissue are produced in several mycoplasmal diseases. This phenonemon was observed with M. pneumoniae (Maisel et al. 1967), M. mycoides (Shifrine and Gourlay 1965) and

M. gallisepticum (Adler et al. 1973).

Roberts and Little (1970a) obtained evidence of an autoimmune response associated with EPP. They used a lung extract as antigen for the complement fixation test (CF). In tests with 400 paired porcine blood samples, they found heat-labile antibodies directed against both M. hyopneumoniae and lung tissue. Forty serum samples reacted with M. hyopneumoniae antigen and 17 serum samples reacted with the lung extract at a serum dilution of 1/40 or above. Serum samples with high complement fixing antibody titers against M. hyopneumoniae had antibody against lung extract. A positive reaction with the lung extract was accompanied by a positive reaction with M. hyopneumoniae antigen.

Development of autoimmunity may result from altered antigenicity of host tissue, adsorption of host antigen by mycoplasmas and presence of a common antigen between mycoplasmas and host cells.

Razin et al. (1973) showed that A. laidlawii bound large quantities of lysozyme, cytochrome C and bovine serum albumin. In each case, the bound protein retained its immunogenicity. This phenomenon could account for autoimmunity as well as a way for the mycoplasma or its antigens to persist in the host by disguising itself with host antigens (Cassel and Hill 1979).

Mycoplasma arthriditis and M. mycoides are known to share reacting antigens with host tissue. Cahill et al. (1971), using complement fixation, immunofluorescence and agar gel double diffusion, demonstrated an antigenic relationship between rat tissue and M. arthritidis. Antibodies against M. arthritidis conjugated with fluorescein reacted with rat, hamster and mouse skeletal muscle in frozen sections. As a control, unlabelled normal rabbit serum and rabbit anti-M. arthritidis serum were used. They suggested that this heterogenetic antigen(s) may enable the mycoplasmas to become established in the host. Shifrine and Gourlay (1965) described an antigenic similarity between the galactan of M. mycoides subsp. mycoides and a pneumogalactan isolated from normal bovine lung.

The production of immune complexes can be responsible for disseminated intravascular coagulation and glomerulonephritis. Disseminated intravascular coagulation was reported by DeVos et al. (1974) in a fatal case of M. pneumoniae infection. The precipitous fall in C'3 level, simultaneously with the rise of specific and nonspecific antibodies, was used as an argument for the presence of immune complexes. Immune complex glomerulonephritis was reported by Cassel et al. (1978) in mice with chronic M. pulmonis infection.

Rheumatoid factors (anti-gamma-globulin antibodies) have been identified in infections caused by M. synoviae and M. gallisepticum (Roberts and Olesiuk, 1967). Ross (1973) found antiglobulin activity in sera from pigs which had lesions of arthritis caused by M. hyorhinis. Infected pigs which had no lesions had no antiglobulin activity. In addition, agglutinins for globulin-sensitized latex particles were found in synovia from arthritic joints.

Evasion of immune system Mycoplasmas could evade immunological destruction by several mechanisms: organism seclusion within sites devoid of immune mechanisms, release of blocking antigens, phagocyte dysfunction, alteration of lymphocyte responsiveness and antigenic variation (Cassel et al. 1978).

According to Whittlestone (1976a), the intimate relationship of mycoplasmas causing respiratory disease with the surface of the respiratory epithelium and penetration between the cilia protects them against removal by the mucociliary clearance mechanism as well as from phagocytosis. Stanbridge (1971) suggested that host cell cytoplasmatic processes may prevent phagocytosis as well as prevent access of specific antisera or drugs.

The release of soluble antigen and formation of immune complexes may facilitate the survival of infecting

organisms by blocking specific cellular or humoral effector mechanisms (Cassel and Hill 1979). Eng and Frøhulm (1971) described a "not-cell-bound-antigen" of M. pneumoniae occurring in broth cultures of the organism and being readily filtrable through membranes which effectively retain colony-forming units. This antigen was found to react with complement-fixing antibody.

Considering phagocytosis, data available indicate that mononuclear and polymorphonuclear phagocytosis occur only in the presence of specific antibody (Fernald, 1979). In contrast, Simberkoff and Elsbach (1971), found that following incubation of M. hominis or M. arthritidis for 2 hours with rabbit peritoneal exudate granulocytes or leukocytes from human peripheral blood, there was no killing of mycoplasmas in the presence or absence of type-specific antiserum. Furthermore, killing of E. coli in the same system, was diminished when the leukocytes were preincubated with mycoplasmas.

Mycoplasmas can be regarded as nonspecific B-cell or T-cell mitogens and/or as T-lymphocyte suppressors. Barile and Leventhal (1968) found that depletion of arginine from medium was responsible for inhibition of lymphocyte transformation induced by phytohaemaglutinin (PTH). Simberkoff et al. (1969) demonstrated that extracts of five arginine-utilizing mycoplasmas inhibited PHA-induced lymphocyte

mitosis while extracts of five glucose-utilizing mycoplasmas did not. In addition, they report that arginine deiminase inhibited antigen- and PHA-stimulated lymphocyte transformation. These observations suggested to the authors that arginine is an essential amino acid for the lymphocyte. On the other hand, Roberts (1972), utilizing seven species of mycoplasmas isolated from pigs, found that M. hyorhinis strains which are dextrose-utilizing and arginine nonutilizing, inhibited PHA stimulation to a similar degree to that of the arginine utilizing species (M. iners, M. hyosynoviae and M. gallinarum). M. hyopneumoniae and M. granularum inhibited the stimulatory effect of PHA to a lesser extent than the arginine-utilizing species. By analogy, he speculated that depletion of an essential ingredient required by the lymphocytes was responsible for the phenomenon.

Biberfeld and Gronowicz (1976) showed that M. pneumoniae is a polyclonal activator for mouse B lymphocytes and that it has a mitogenic effect on guinea pig spleen cells in vitro. They suggested that this stimulation of B cells could account for the early production of nonspecific IgM antibody in natural infections. In a later paper, Biberfeld (1977) reported that M. pneumoniae can induce non-antigen-specific antibody production in human B lymphocytes

without stimulation of DNA synthesis in these cells. Naot and Ginsburg (1978), working with lymph node cell suspensions from athymic nude mice described the same results with different strains of M. neurolyticum. Naot et al. (1979) demonstrated that M. pulmonis was mitogenic for rat B and T lymphocytes. This mitogenic activity was more effective on B cell than on T cells. They suggested that nonspecific activation of B lymphocytes by invading mycoplasmas is an essential part of the host's defense mechanism and that B and T lymphocyte stimulation plays a role in the lesion produced. Mitogenic activity was thought to be associated with a membrane protein.

Aldridge et al. (1977) showed that strains of M. arthritidis and M. hominis were capable of inducing normal CBA mouse lymphocytes to produce a cytotoxic response against ^{51}Cr-labeled allogenic and syngeneic target cells. This effect was considered a possible mechanism for in vivo cell damage and inflammation. Eckner et al. (1974) found that 4 to 5 week-old BALB/c mice showed less thymic cellularity than noninoculated controls following inoculation with extracts of M. arthritidis. T-dependent areas of thymus and lymph nodes were depleted too. Moreover, suppression of in vitro responses to PHA and concanavalin A was observed but the antibody response to sheep erythrocytes was preserved. This suggested that selective suppression on

a T-cell subpopulation had occurred.

Biberfeld and Sterner (1976) have demonstrated that anergy to tuberculin skin reaction occurs in patients with M. pneumoniae pneumonia. Adegboye (1978a) employed the lymphocyte transformation test and the delayed-hypersensitivity skin test to monitor cell-mediated immunity (CMI) of pigs inoculated intranasally with M. hyopneumoniae. CMI was demonstrated during the chronic stages of MPS or after the pigs had recovered. The author evaluated the lymphocyte transformation response in pigs reinoculated intranasally or inoculated intradermally with M. hyopneumoniae antigen. A transient decline of lymphocyte response was interpreted as a possible evidence of immunosuppression. Considering that immunosuppression could be reflected as depletion of the paracortex in the local lymph nodes, Adegboye (1978b) studied the immunological response of bronchial lymph node of M. hyopneumoniae inoculated pigs. He detected a depletion of lymphoid cells in the paracortical region of lymph nodes in 2 of 23 pigs.

The production of large amounts of IgG_1 in M. pulmonis-infected mice was considered an anomalous lymphocyte response to the infection (Cassel and Hill 1979). Their reasoning was that IgG_1 is both noncomplement-fixing and nonopsonizing antibody so that it could act as a "blocking antibody", thus protecting the organism from host defense,

Another way by which mycoplasmas can evade the immune

mechanism is by variation of their antigenic make up. Antigenic drift during M. pulmonis infection was suggested by Ogata et al. (1967), who found 58 different serotypes of M. pulmonis within only five rat colonies. In this case, immunological evasion could theoretically be provided by selection of antigenically altered mutants.

Disguising of mycoplasmas with host antigen may be another mechanism by which the organism may persist in the host (Cassel and Hill 1979). This phenomenon could be a consequence of mycoplasma and host cell fusion and/or an exchange of antigen (Cassel et al. 1978). In fact, Razin et al. (1973) demonstrated the capability of mycoplasmas for binding of exogenous proteins and Wise et al. (1978) reported that M. hyorhinis selectively acquired alloantigen from murine T lymphoblastoid cell surfaces. In addition, Razin et al. (1980) observed M. pneumoniae and red blood cell interaction by SEM microscopy.

Survival of the organism away from the host It appears that the capability, among mycoplasma species, of surviving away from the host is quite variable.

Orning et al. (1978) recovered M. arginini from all samples examined from a swine waste disposal system; this indicated a great capability of the cited species for surviving, at least, in that environment. Windsor and Massiga

(1977) found that M. mycoides survived 72 hours in placenta and for longer periods in infected urine and hay. In contrast, Goodwin (1972a) was unable to induce mycoplasmal pneumonia or isolate the mycoplasma from pigs put in rooms which had housed coughing, infected pigs 5 to 47 minutes prior to repopulation.

Infectivity of M. hyopneumoniae for susceptible swine in contaminated pig lungs is retained at -30°C for 20 months, 4°C for 4 days, 20°C for 1 day, 37°C for 4 hours and 42.5°C for 2 hours (Whittlestone, 1973). A similar pattern of survival was observed in attempts to isolate the agent from pneumonic pig lungs kept at different temperatures (Whittlestone, 1973).

Being a respiratory disease, M. hyopneumoniae pneumonia is transmitted mainly by the airborne route; therefore, the size of particles expelled by pigs and the effect of climatic factors on these particles plays an important role in dissemination of the disease.

According to Hatch (1961), retention of inhaled particles in the respiratory tract is similar in man and animals. Total retention is essentially 100 percent for inhaled particles 10μ in diameter and larger. Retention remains high down to 5μ and then drops off reaching a minimum of 20 to 30 percent at 0.25 to 0.5μ below which retention

increases again, returning to 60 percent or better for submicroscopic particles (< 0.1μ).

The highest probability for deposition of inhaled particles in the respiratory spaces of the lung occurs within the size range of 1 to 2μ (gravity settlement) and in the submicroscopic region below 0.2μ (precipitation by diffusion). Above 1 to 2μ, penetration to and deposition in the lobules falls off with increasing size simply because most larger particles are trapped in upper respiratory tracts. Above 10μ, the probability for penetration to the lobules is essentially zero. Below 1 to 2μ lobular deposition falls off because the efficiency of removal by gravity settlement within the lobules themselves decreases. At 0.25 to 0.5μ, the combination of precipitation by gravity and diffusion are at a minimum so that these are the size of lowest probability for deposition of inhaled particles. The probability of lobular deposition increases below 0.25μ because the force of precipitation by diffusion increases as particle size become smaller.

Kundsin (1968) investigating the size of viable airborne particles, found that mycoplasma aerosols had median diameters of $2.1 \mp 0.5\mu$.

Mycoplasmas in airborne particles are subject to change in relative humidity and irradiation damage. Wright

et al. (1968), studying the effect of relative humidity on the survival of airborne M. pneumoniae, found that low and high humidity were best for survival of M. pneumoniae in 2 mμ atomized particles. The most lethal relative humidity was 60 to 80 percent at which level fewer than 1 percent of the organisms survived over a 4 hour observation period. Hatch et al. (1970), looking for the effect of abrupt change in relative humidity, observed that a change in the relative humidity of more than 18 percent in either direction from a lethal mild range humidity decreased the rate of biological decay. In addition, double humidity shifts (dry to middle range level and to high humidity range) were very detrimental with very few survivors after 8 minutes.

Airborne M. pneumoniae was exposed to near ultraviolet (3000-4000 A) light, daylight, fluorescent light or total darkness at 25, 50 and 95 percent of relative humidity (Wright and Bailey 1969). Without light, survival of the organism was good at both high and low relative humidity with poorest survival at 50 percent of relative humidity. Ultraviolet light decreased survival of airborne organisms at all levels of relative humidity.

Susceptibility of mycoplasma to various drugs Mycoplasmas are insensitive to penicillins and have variable sensitivity to tetracyclines, chloramphenicol and streptomycin (Clyde 1979). Ogata et al. (1971) compared the susceptibility of 15 strains of mycoplasmas isolated from animals including M. hyopneumoniae, M. hyorhinis and M. granularum from swine to 22 commonly used antibiotics and 9 nitrofurans. They found that there were no marked differences in susceptibility to these drugs with the exception of erythromycin and oleandomycin. Actinomycin D and mitomycin C were the most active of all agents. Tylosin, botromycin, spiramycin and tetracycline followed them in activity. Kusagamosin, polymyxin B and colistin were noninhibitory. These results did not differ greatly from those obtained by Newhnam and Chu (1965). In a comparison of susceptibility of 9 strains of M. hyopneumoniae and 1 strain of M. hyorhinis to 51 antimicrobial agents Williams (1979) found that all M. hyopneumoniae strains were resistant to penicillins and peptides. They were susceptible to sulfonamides and tetracyclines. In other antibiotic classes, there was variability in susceptibility. All strains of M. hyopneumoniae were susceptible to 33 of the 51 agents. M. hyorhinis was susceptible to 19 of the 33 agents that M. hyopneumoniae was susceptible to. All strains of

M. hyopneumoniae differed from M. hyorhinis in that they were susceptible to cephaloglycin and nitrofurazone. Drews et al. (1975) found that the minimal inhibitory concentration of tiamulin for M. hyopneumoniae was 0.031 micrograms per milliliter.

Transmission of mycoplasma diseases Mycoplasma diseases can be transmitted by direct and indirect contact; however, direct contact appears to be much more important than indirect methods.

The spread of M. gallisepticum occurs almost entirely by direct means, through the egg or, by infective airborne droplets from the respiratory system of carriers or clinically affected fowl (Yoder 1978). Jordan (1979) suggested that the relatively short survival time of M. gallisepticum away from the host greatly reduces fomite transmission although it can occur.

The transmission of M. mycoides subsp. mycoides has always been assumed to be by direct contact between infected and healthy cattle, but Windsor and Massiga (1977) were able to infect three out of six cattle by feeding them hay heavily infected with M. mycoydes subsp. mycoides. In addition, some infected cattle may excrete up to 10^6 M. mycoides organisms per milliliter in their urine as a consequence of kidney lesions. The organism also may be shed

by way of infected placentae.

Cassel and Hill (1979), reviewing epidemiology of M. pulmonis, observed that newborn of affected rat dams acquired the organism by way of aerosol during the first few weeks of life. They may also acquire the organism in utero. Once rats and mice have acquired the organism, a slowly progressive respiratory disease begins and it can persist for life assuring presence of a "carrier state".

Current evidence indicates that transmission of M. hyopneumoniae is by direct contact with respiratory tract secretions from infected swine and by aerosols. Indirect proof includes the findings that: a) Mycoplasma hyopneumoniae was isolated only from the respiratory tract and attempts to isolate the agent from other organs have failed (Goiś and Kuksa 1974b), b) the organism was isolated from the nasal cavities of diseased pigs (Goodwin 1972b), c) mycoplasmas are viable in airborne particles fulfilling the condition of airborne disease (Kundsin, 1965) and d) transmission among swine penmates has been observed (Farrington, 1976 and Etheridge et al. 1979).

Transmission of mycoplasmal pneumonia of swine by lungworms (Metastrongylus spp.) was investigated by Preston and Switzer (1976). They found that pigs fed lungworm-larvae-infected earthworms, collected from a herd of swine infected with M. hyopneumoniae resulted in lungworm

infection in the SPF recipient pigs, but failed to produce pneumonia or complement-fixing antibody against M. hyopneumoniae.

Whittlestone (1979), considering the high number of breakdowns to MPS in minimal disease herds, suggested the possibilities of transmission by carrier hosts, transfer by fomites, long-distance airborne infection or clinical expression of a long-term, subclinical infection.

In a survey of boar semen, Schulman and Estola (1974), isolated M. hyopneumoniae once and M. hyorhinis 3 times from 101 samples of semen. Confirmation of this finding has not appeared.

Keller (1976), analyzing three herds that had breakdowns in SPF status for M. hyopneumoniae, concluded that M. hyopneumoniae infections may remain subclinical in SPF herds for months or years.

Host factors involved in the epidemiology of mycoplasma diseases

The host factors involved in the epidemiology of mycoplasmal diseases are: genetic constitution, age, sex and status of immunity.

Genetic constitution In spite of the paucity of data about genetic constitution, it is an important factor in susceptibility of the host to mycoplasma diseases.

Taylor-Robinson and McCormack (1979) reviewed factors involved in human genitourinary infection caused by Mycoplasmataceae. They found several reports indicating that isolation of genital mycoplasmas is more frequent in black than white men. Furthermore, the colonization rates among nonpregnant black women are higher. In pregnant women, the same pattern was observed with M. hominis and ureaplasmas. The authors pointed out that the extent to which these differences are due to race or sexual activity was not very well defined. However, in the same report, McCormack cited unpublished findings that susceptibility of black women to colonization with M. hominis and ureaplasmas was higher than white women when the sexual experience factor was controlled.

Hannan (1971), in a study of the arthritogenic properties of Sabin's type C murine mycoplasma (M. pulmonis), found that the incidence of arthritis was dependent on the strain of mouse used. The organism was injected intravenously into 25 female mice from each of five different strains.

Barden et al. (1973) compared the susceptibility of

Piney Woods miniature swine and Yorkshire swine to M. hyorhinis infection. The course of arthritis induced following intraperitoneal inoculation was similar in the two groups of pigs during the first month after infection. After that, the Minis improved while the Yorkshires developed chronic arthritis. The acute synovitis seen in the two breeds was similar, but was more intense in the Yorkshire pigs. The Minis did not develop bone or cartilage destruction but the Yorkshires did. On the other hand, Ross (1973) found that Hampshire swine more rapidly developed clinical lameness after infection with M. hyosynoviae than did Yorkshire swine. In addition, some of the Yorkshires developed mild lesions of arthritis that did not result in detectable clinical lameness.

Age susceptibility Age susceptibility appears to be a fact among some mycoplasmal diseases. In man, there is a high incidence of M. pneumoniae infection in university and military settings. At the University of North Carolina, Tulane University and the University of Wisconsin, approximately half of pneumonia cases could be associated with M. pneumoniae infections (Clyde 1979). Foy et al. (1979), monitoring penumonias due to M. pneumoniae in a large medical care group in Seattle, Washington, between 1963 and 1975, found that age-specific attack rates for M. pneumoniae

pneumonia among children aged five to nine years (about six per 1,000) were about twice the rates for younger children and four-times those of adults. In infants younger than six months of age, M. pneumoniae disease was rare. In addition, they found that a higher proportion of infections among children aged five to nine years than among adolescents aged 15-19 years resulted in pneumonia. On the other hand, Foy et al. (1966) studying M. pneumoniae infection in families, reported that the grade of infection among children less than 5 years of age was as high as 5 to 14 year old children. These data suggested that a lower proportion of infections leads to pneumonia in children less than five years of age. Noah (1974) reported epidemiological data for M. pneumoniae infections in the United Kingdom from 1967 to 1973. The highest attack rates were in children aged 5-9 years old. Adults over 65 years appeared to be affected uncommonly.

In rats, the effect of age on susceptibility to M. pulmonis is not fully understood; however, Cassel and Hill (1979) indicated that older rats succumb more readily. They suggested that the immune system of older rats had decreased competence.

Among swine mycoplasmas, M. hyorhinis and M. hyosynoviae appear to exhibit a clear cut age susceptibility.

Carter (1954) isolated a PPLO organism from swine in 3 herds suffering from clinical signs of Glasser's disease. This PPLO was similar to M. hyorhinis isolated by Switzer (1953 and 1955). In these three herds, the Glasser's disease type condition occurred in swine from one to four months of age. Goiś and Kuksa (1974a), using gnotobiotic pigs, showed that susceptibility to M. hyorhinis decreased with age. Of 21 piglets infected with strain S218 at 6 days of age, 15 developed clinical disease and 18 showed systemic invasion. In contrast, none of 10 piglets infected with the same strain at 8 weeks of age developed clinical disease. Schulman et al. (1970), isolated M. hyorhinis more frequently from pigs over 2 weeks of age. In their study, the organism was isolated more frequently from pneumonic than from a normal lung.

In regard to M. hyosynoviae, Ross and Duncan (1970) found that pigs up to 6 weeks of age have a low incidence of infection even though their dams may be infected; M. hyosynoviae arthritis is found in swine as small as 30 kg but usually occurs after they weigh 40 to 100 kg (3 to 6 months old). Similar observations were made by Roberts et al. (1972) in an outbreak of M. hyosynoviae arthritis in Britain. Pullar (1948) pointed out that all ages of swine were affected by "infectious pneumonia" but that the

incidence of the disease decreased with age. His observations were confirmed by MacPherson and Shanks (1955) who found 39 of 670 (5.8%) sows with lesions of pneumonia while 554 of 1000 (55.4%) bacon pigs had lesions of pneumonia. Holmgren (1974a), studying the immunological and epidemiological status of two herds with enzootic pneumonia, found acute pneumonia in 2 to 5-week-old pigs and acute or chronic pneumonia in 10 to 12-week-old pigs. No macroscopic signs of pneumonia could be shown in pigs 4 to 5-months-old in either of the herds. Willeberg et al. (1978), in a clinico-epidemiological analysis of respiratory disease in a cohort of bacon pigs, found a peak incidence of enzootic pneumonia at 16 to 19 weeks of age.

Huhn (1971) induced pneumonia in 4-hour-old pigs by intranasal inoculation of pneumonic lung suspension containing M. hyopneumoniae. This finding led him to conclude that pigs may be considered susceptible to EPP at birth. Goodwin (1972b) was unable to induce pneumonia in six 7-week-old pigs kept in contact with M. hyopneumoniae-infected pigs. The agent was not recovered from lungs and nasal mucosae of contact pigs, but, four of six inoculated pigs had lesions and the microorganism was reisolated from all 6 of these pigs. Lam and Switzer (1972) failed to reproduce MPS by contact in three 7-8-week-old pigs in contact with three pigs which had been infected experimentally

two months previously. In that study, 2 of the 3 inoculated pigs had pneumonia when necropsied. In another experiment, they put 3 normal pigs (6 weeks old) in contact with a group of 3 pigs a few hours after inoculation. Both groups developed pneumonia. Ross (personal communication) was unable to induce MPS in ten 25-day-old pigs in contact with 4 pigs that had been infected 3 weeks previously with M. hyopneumoniae. Etheridge et al. (1979) placed 45 pigs aged 6 to 9 weeks on contact with M. hyopneumoniae-inoculated pigs and when killed 28 to 74 days later, 35 of the contact pigs had gross lesions of EPP. M. hyopneumoniae was isolated from 33 of the 45 pigs. Nine of the 12 pigs with no gross lesions had microscopic lesions.

Sex There is a paucity of studies dealing with the relationship of sex to susceptibility of the host to mycoplasmal disease. Foy et al. (1979) found M. pneumoniae disease more frequent in females than males in the 30-39 year age group (1.8 versus 1.2 per 1000). This observation suggested to the authors that exposure of women to their children overwhelmed their immunity. In an epidemiological study, Willeberg et al. (1978) found no evidence of a differential between sexes in susceptibility to MPS.

Immunological status It seems that the reaction of the host to mycoplasmal infection is very peculiar. The same agent can induce different responses in different hosts. Antisera to M. pulmonis protect mice against pneumonia following intravenous (Taylor and Taylor-Robinson 1976) or intranasal (Cassel et al. 1973) exposure to that organism. Classical cell-mediated immunity does not appear to play a major role against M. pulmonis infection in mice since immunity cannot be transferred passively with immune cells (Taylor and Taylor-Robinson 1976). In contrast, passive transfer of immunity using spleen cells, but not serum, from immunized rats protected them against M. pulmonis pneumonia (cited in Cassel and Hill 1979).

Swine appear to develop strong immunity following natural infection and the humoral response appears to play a role in protecting the animal against M. hyopneumoniae pneumonia. Lannek and Bornfors (1957) found that pigs recovered from EPP were strongly immune to challenge by inoculation with pneumonic lung suspension. The status of the disease was based on X-ray monitoring of challenged pigs. Goodwin et al. (1965), using a lung suspension containing M. hyopneumoniae, confirmed Lannek and Bornfors findings. Goodwin (1965), using old sows in an EPP eradication program, reported that piglets from older sows did not develop clinical signs or lesions of pneumonia. However, when gilts

from these litters had grown and farrowed for the first time, their litters were affected with the disease. This phenomenon needs to be explained. Lam and Switzer (1971a), utilizing sera from M. hyopneumoniae and Freund's adjuvant-immunized-pigs reduced the incidence and the grade of pneumonia in those pigs which received the convalescent sera intra-peritoneally.

Several serological tests are used to detect antibodies in serum from M. hyopneumoniae-infected pigs; but, there is no correlation known between resistance and titer. This fact was established for complement-fixing, metabolism-inhibiting (Goodwin et al. 1969a, 1969b) and indirect hemagglutinating antibody (Goodwin et al. 1969a and Lam and Switzer 1971b).

Antibodies to M. hyopneumoniae have been detected in tracheobronchial secretions; but, the role of these local antibodies in resistance to infection is not known. Holmgren (1974b) found indirect hemagglutinating antibodies (IHA) in tracheobronchial secretions of 14 pigs 2 weeks post-inoculation; immunochemical characterization of this early antibody response in one pig showed that IHA-antibodies were high molecular weight IgA, probably secretory IgA. Durisić et al. (1975) detected secretory antibody in colostrum of sows inoculated with M. hyopneumoniae antigen and adjuvant.

As in other mycoplasmal diseases, M. hyopneumoniae induces a cell-mediated reaction. However, the role of this cell-mediated immunity in protection has not been determined.

Roberts (1973) detected cell-mediated immunity to M. hyopneumoniae in pigs by means of transformation of sensitized lymphocytes by specific antigen, inhibition of macrophage migration and the production of an intradermal, delayed type, hypersensitivity reaction. Lymphocyte transformation was observed during 5 to 6 weeks post inoculation; the increase of isotope uptake was 1.5- to 3-fold compared with that of the controls. The intradermal tests were carried out seven weeks post inoculation. Adegboye (1978a), in an attempt to demonstrate a cell-mediated immune response during M. hyopneumoniae infection in pigs, found significant transformation of lymphocytes 15 weeks after inoculation. This effect was demonstrated up to 44 weeks postinoculation, the longest time examined. Skin hypersensitivity was demonstrated in all tests performed between 20 and 46 weeks after inoculation. In this study, differing from that of Roberts (1973), the index of lymphocyte stimulation was considered significant if it was equal to or above 3.0. In this study, pigs were given repeated doses of pathogenic M. hyopneumoniae intranasally. The two pigs which gave the strongest skin reaction 34 weeks after primary inoculation were those which had no pneumonia at slaughter. This fact and the late cell-

mediated immune response suggested to the author that intradermal and lymphocyte transformation tests may correlate with resistance to MPS.

Intercurrent infection in mycoplasmal diseases Intercurrent infection can result in no effect, synergistic effect, or an ameliorative influence on the course of the disease.

Katzen et al. (1969), in two controlled experiments, showed that M. gallisepticum inoculated into birds suffering from Marek's disease decreased mortality due to the latter disease to 50 percent compared to greater than 90 percent mortality in the uninoculated controls. Jordan (1979), reviewing M. mealagridis infection in turkeys, stated that intercurrent infections with other avian pathogens seem to play a relatively minor role in disease caused by that agent, but that synergism may exist between M. meleagridis and E. coli in poults and with M. synoviae in sinusitis in turkeys.

A synergistic effect of M. hyopneumoniae in swine pneumonia has been observed with Pasteurella multocida (Smith et al., 1973), Ascaris suum (Underdahl and Kelley 1957). Metastrongylus elongatus (Mackenzie 1963) and swine adenovirus (Kasza et al. 1969).

Effect of ecological factors in the development of mycoplasmal pneumonia of swine

In a systemic approach, ecological factors can be divided into climatic effects and the role of production system and husbandry. Climatic factors including humidity, volume of air per pig, ventilation, noxious gases, and dust in the air play an important role in determining prevalence and severity of MPS. Whittlestone (1976b), reviewing the literature on the seasonal influence on pneumonia occurring in fattening pigs, pointed out that winter was the worst time for clinical and pathological occurrence of MPS. In contrast, Willeberg et al. (1978) did not find any seasonal influence on the rate of clinical respiratory disease, deaths due to respiratory disease and lesions found at meat inspection in a Danish herd examined over a four year period.

Eernstman (1963) showed that high temperature and high humidity reduced EPP. Furthermore, he found that high humidity and low temperature were detrimental to the health of the respiratory system of pigs. Jericho (1968) reported that lowering relative humidity to 50 percent was associated with coughing and respiratory distress due to a drying of the mucous blanket covering the respiratory tree. Gordon (1963a) confirmed that high humidity and temperature reduced the incidence of EPP and found that the number of bacterial colonies was lowest in the air of those houses with the

highest absolute humidity (Gordon 1963b).

Lindquist (1974), used farm records and lesions at slaughterhouses to investigate the influence of housing and environment on the health status of fattening pigs. He pointed out that pigs from houses with an air volume of 3 m per pig have a lower incidence of pneumonia than those from houses with volume of less than 3 m per pig. This effect was confirmed by Bäckström and Bremer (1978).

Bäckström and Bremer (1978) studied the relationship between disease incidence in fatteners registered at slaughter and environmental factors in herds. They found that ventilation (subjectively graded) was best in herds with the lowest incidence of pneumonia and pleurisy. In a controlled experiment, Jericho et al. (1975) found that lungs from pigs fattened in a force-ventilated building showed a higher incidence of lung consolidation than did pigs from a more open naturally ventilated environment during both summer and winter. Aalund et al. (1976) found that ventilation (shaft and fan) or no ventilation (no shaft) was associated with increased risk of respiratory disease as compared to spontaneous, slow air replacement through a shaft. Ammonia and dust have been implicated in the pathogenesis of EPP (Jericho, 1968). The author suggested that these exogenous factors interfere with the muco-ciliary apparatus. The capacity of the muco-ciliary apparatus as an air purifier is

related to the mucous surface velocity. Mucous surface velocity is dependent upon viscosity of the mucous layer, frequency of ciliary beat and secretory function of the globlet cells (Carson et al. 1966). Ammonia was incriminated as a factor reducing the mucous surface velocity (Carson et al. 1966). In the field situation, chronic coughing, without evidence of pneumonia, and reduced growth rates have been reported in swine housed in barns with high odor levels of ammonia (Andersen 1970). Furthermore, Kovacs et al. (1967) detected an increase in both the incidence and severity of pneumonia in swine housed in barns with high ammonia and dust concentrations. Doing and Willoughby (1971) studied the effect of ammonia and organic dust on swine under controlled conditions. Pigs exposed 2 to 6 weeks to 100 ppm of ammonia had an increase of 50 to 100 percent in the thickness of the tracheal epithelium with a concomitant decrease in the number of tracheal globlet cell. Similar lesions were detected in the nasal epithelium of the same pigs.

Husbandry factors which have been suggested to play a role in the prevalence or intensity of EPP are: herd size, type of production system, repopulation of fattening units, density, manure management and hygiene.

Lindquist (1974) found that pigs from buildings containing less than 500 animals tended to have a lower incidence

of pneumonia than was associated with more than 500 pigs per building. Bäckström and Bremer (1978) reported that those farms which produced higher numbers of fatteners had a higher incidence of pneumonia than others producing lower numbers of fatteners. Aalund et al. (1976) found a twenty-fold increase in risk of lung lesions for herds producing more than 500 hogs in the three year period from 1969 to 1971 in Denmark.

In regard to type of production system, production strictly in batches results in a lower incidence of pneumonia than when continuous production (Lindquist 1974 and Bäckström and Bremer 1978) or continuous production in batches (Lindquist 1974) are used.

Bäckström and Bremer (1978) found that herds classified in a low-pneumonia-incidence group stocked their growing and finishing units with their own feeder pigs while in herds in the high-incidence group, piglets were purchased from other sources, especially at a market. This is in agreement with findings of Aalund et al. (1976).

The density of pigs per unit obviously is related to volume of air per pig and stress. Lindquist (1974) found that pigs from houses with a total area equal or greater than 0.7 m^2 per pig had a lower incidence of pneumonia than those from houses with a total area less than 0.7 m^2 per

pigs.

Manure management may also influence severity of pneumonia, an effect undoubtedly related to the production of gas. Lindquist (1974) observed that pigs from houses with solid manure handling had a tendency to a lower incidence of pneumonia than those from houses with liquid manure handling.

Diagnosis of M. hyopneumoniae Pneumonia

Detection of the organism or antigen

Isolation of M. hyopneumoniae The isolation of M. hyopneumoniae from lungs is achieved by broth culture followed by serological identification. According to Whittlestone (1979), three problems limit and have to be overcome in attempting isolation of the microorganism: a) M. hyopneumoniae is nutritionally fastidious, b) other more rapidly growing mycoplasmas, especially M. hyorhinis, are present in swine lungs, and c) some strains, even under ideal conditions, are difficult to isolate.

To overcome these problems, several media have been developed and one of the most significant advances in this area was the medium developed by Friis (1975). Other recently developed media were those of Goodwin (1976), Etheridge et al. (1979) and Chenglee et al. (1980).

Whittlestone (1979), reviewing the ways of overcoming

the presence of M. hyorhinis in mixed infections pointed out four methods; a) utilizing M. hyorhinis antiserum in the media, b) incorporating specific inhibitors in the media, c) increasing the concentration of serum, favoring M. hyopneumoniae, rather than M. hyorhinis, and d) making five to seven passes at weekly intervals, after which M. hyopneumoniae but not M. hyorhinis continues to multiply.

A method of isolation used widely is that described by Friis (1975). A 10% suspension of lung tissue is made in broth and ten-fold dilutions are made up to 10^{-8} in broth culture. Cultures are incubated at 37°C. Cultures showing an acid shift from red (pH 7.4) to yellow (pH 6.8) are subcultivated to broth or agar.

Identification is achieved by means of the metabolic inhibition test (Taylor-Robinson et al. 1966), epi-immunofluorescence (DelGuidice et al. 1967) growth precipitation test (Gois and Kuksa 1975) and growth inhibition test (Clyde 1964).

Immunofluorescence test The application of the immunofluorescence technique to the study of mycoplasmas was first reported by Liu (1957). He described the bronchial localization of the etiological agent of atypical pneumonia of man in experimentally infected chick embryos.

L'Ecuyer and Boulanger (1970), Meyling (1971) and

Livingston et al. (1972), using the direct fluorescent antibody technique (DFA), described the bronchial and bronchiolar location of M. hyopneumoniae in pneumonic swine lungs. In addition, Meyling (1971) observed that M. hyorhinis could often be demonstrated with distribution and localization similar but not identical to that of M. hyopneumoniae. In slaughter house material, he found a good correlation between isolation of M. hyopneuomiae and DFA. Similar results were obtained by Holmgren (1974a). Amanfu et al. (1980), evaluating the sequential detection of M. hyopneumoniae antigen in lungs of infected pigs by DFA, observed maximum fluorescence at 4 to 6 weeks post-inoculation with persistence up to 12 weeks, the longest time studied. Goiś et al. (1975), used the indirect immunofluorescence technique (IFA) to assess the presence of M. hyopneumoniae in pigs from an infected herd at an abattoir. They found that, of 39 positive lungs, 10 were detected by culture and IFA, 11 were positive only by IFA and 18 were positive only by culture. No explanation was offered for this discrepancy.

Enzyme-linked immunoperoxidase technique The enzyme-linked immunoperoxidase technique was used by Bruggmann et al. 1977a) to demonstrate M. hyopneumoniae antigen in frozen lung sections and in bronchial smears. In bronchial

smears the organism was seen as reddish brown pleomorphic spots, in the form of colonies, randomly distributed, or at the surface of scraped epithelial cells. In frozen tissue sections, they bordered the bronchial epithelium.

Direct examination of Giemsa-stained pneumonic lung touch preparations for organisms with the morphology of M. hyopneumoniae Pleomorphic organisms (PO) which could be detected regularly in touch preparations from the lungs of pigs infected experimentally with several strains of the causative agent of enzootic pneumonia were described by Whittlestone (1958). These PO organisms were identified as Mycoplasma hyopneumoniae (Goodwin et al. 1965). Goodwin and Whittlestone (1967) used this method as a preliminary diagnostic method within the EPP control scheme in England. In spite of the fact that this method is nonspecific, Giger et al. (1977) found that it correlated well with results obtained by immunofluorescence, histopathology and a immuno-peroxidase technique.

The method has the advantage that dried touch preparations are very stable and results can be available within a few hours. In contrast, interpretation is subjective and false positives may occur due to M. flocculare infections (Whittlestone 1979).

Detection of antibody response to M. hyopneumoniae

Complement fixation (CF) test Using unheated serum, Roberts (1968) detected CF antibodies against M. hyopneumoniae in pigs inoculated with either pig lung suspension or broth cultures, whereas control sera were negative. Boulanger and L'Ecuyer (1968), utilizing a modified direct-CF test, found a good correlation between presence of typical lung lesions in experimentally infected pigs and CF antibodies in serum. Moreover, detection of antibodies was possible as early as 14 days after intratracheal exposure and for at least 267 days thereafter. No cross reactions were observed between M. hyorhinis and M. hyopneumoniae. Slavik and Switzer (1972), using complement provided by desiccated guinea pig serum, reconstituted in serum from young pneumonia-free pigs and an antigen heated at 52°C to 54°C for 15 minutes, overcame the problem of poor complement binding and procomplementary activity of the antigen. In this way, it was possible to detect antibodies to M. hyopneumoniae in test sera at dilutions as low as 1:8. The CF test may yield some false positive, or false negative reactions or cross reactions. Hodges and Betts (1969) found that sera from 2 pigs infected with A. granularum and 1 of 16 pigs infected with M. hyorhinis did produce low titer cross reactions. Wood et al. (1976) compared sera from SPF and non-SPF swine in Illinois. They found that of 458 serum samples from 8 specific pathogen free

(SPF) swine herds, 9.4% of sera were positive while of 128 serum samples from 8 non-SPF herds, 51.7% of sera were positive. They suggested that either the test was detecting nonspecific reactions or the SPF system was ineffective in maintaining their M. hyopneumoniae-free status. In an evaluation of diagnostic procedures for detecting MPS, McKean et al. (1979) found a high correlation between CF results and gross and macroscopic lesions in butcher-weight swine. But, they found a greater number of swine with microscopic lesions than with CF titers. This fact suggested to them that CF titers declined before resolution of microscopic lesions occurred.

Indirect hemagglutination (IHA) test Using the IHA test, Goodwin et al. (1969a) found that pigs killed 12-22 days after infection had titers less than 1:5, but high titers were obtained 16-60 weeks after infection. The latter pigs had very low CF titers at that time. This suggested that the IHA test could be more suitable for long-standing infections.

Lam and Switzer (1971b) developed an IHA test for detection of antibodies to M. hyopneumoniae. They used tanned swine erythrocytes and treated their antigen with sodium dodecyl sulfate followed by ammonium sulfate precipitation. In evaluating this test, they detected antibodies

in 57 of 62 (92%) experimentally inoculated pigs that had pneumonia and in 3 of 8 pigs that failed to develop lesions. In naturally occurring infections, antibodies were detected in 68 of 87 (78.1%) pigs having macroscopic lesions and in 56 of 82 (68.2%) pigs not having lesions. The resolution of pneumonic lesions with persistence of the titer and the nonpathognomonic status of the lesions were cited as reasons for the lack of correlation between IHA antibodies and lung lesions. Holmgren (1974c), using IHA with formalinized, tanned swine erythrocytes, detected antibodies against M. hyopneumoniae in 87 out of 98 (89%) sera from slaughter pigs believed to be free from the disease. No cross reactions were observed with M. hyorhinis. Holmgren (1974a) monitored the status of M. hyopneumoniae infection in two herds with a high prevalence of EPP by using the DFA test to detect antigen in pneumonic lung tissue and the IHA test to detect antibodies in affected pigs. He found a good correlation between these two techniques in 10-to 12-week-old pigs but in 2-to 5-week-old pigs no IHA titers were observed even though all pneumonic lungs were DFA positive. All 2-to 5-week-old pigs had acute pneumonia while IHA negative 10-to 12-week-old pigs had an acute pneumonia too. IHA positive 10-to 12-week-old pigs tended to have chronic lesions.

Enzyme-linked immunoassay (ELISA) Bruggmann et al. (1977b) developed the ELISA test for the detection of M. hyopneumoniae antibodies. Positive sera were observed at 2 weeks post infection and all sera tested between 3 and 50 weeks after inoculation were positive. They described the following advantages for this test: all classes of immunoglobulins can be detected, the sensitivity is identical to radioimmunoassay and results can be measured quantitatively.

Cross reactions between M. hyopneumoniae and M. hyorhinis have been observed (Bruggmann 1978). In order to eliminate this cross reaction, Bruggmann (1978), using antigen fractionation techniques, found the specific antigen of M. hyopneumoniae among 3 to 4 proteins in the molecular range between 28,000 and 175,000 daltons. Armstrong et al. (1978), using Bruggmann et al. antigen (1977b), in an evaluation of the ELISA test, found that 33 sera from SPF pigs devoid of gross MPS lesions were ELISA negative and 32 of 33 were CF negative. From 18 MPS suspect pigs, all were CF antibody positive while 13 of 18 were ELISA positive. In testing ELISA specificity, using M. hyorhinis, M. hyopneumoniae and M. hyosynoviae antiserum against homologous and heterologous antigen, they observed that heterologous antiserum had titers greater or equal to 4000 against homologous antigens. They concluded that the ELISA was a promising method for diagnosing MPS but, the specificity of the M. hyopneumoniae antigen

needed to be increased before the procedure could be used under field conditions.

Indirect immunofluorescence (IFA) test Meyling (1972) detected IFA antibodies in infected pigs at 3 weeks and maximum fluorescence occurred at 6 to 8 weeks after inoculation. In a slaughterhouse survey, he found that 67% of 265 sera diluted 1:10 had IFA antibodies. He also found that 369 of 375 sera from pneumonia-free SPF swine were negative for IFA. The 6 positive sera which gave trace reactions were reevaluated and considered negative.

Indirect immunoperoxidase (IIP) test Bruggmann et al. (1976) used the IIP test to detect M. hyopneumoniae antibodies in an infected herd and in an SPF herd. No antibodies were detected in the SPF herd but in the infected herd a positive correlation of 97.7% was obtained between presence of antibodies and evidence of the disease.

Tube agglutination test Fujikura et al. (1970) demonstrated antibodies against M. hyopneumoniae by using the tube agglutination test. All pigs inoculated with a culture of the organism or with pneumonic lung suspensions developed agglutination titers. No cross reaction was observed between M. hyopneumoniae and M. hyorhinis with the heterologous immune serum. In pigs less than 3 months of

age, agglutinating antibody was demonstrated at a higher frequency than was CF antibody.

Roberts and Little (1970b), utilizing a stained antigen plate agglutination test, were unable to detect a serological response in pigs to two strains of M. hyopneumoniae.

Pijoan and Boughton (1974) described a tube agglutination test. They studied several variables in the reaction and concluded that temperature variation affected the results strongly. The test worked well for hyperimmune sera but field sera from pigs with experimental M. hyopneumoniae infection did not agglutinate even when they possessed CF titers. They suggested that the test detected only macroglobulins.

Metabolic inhibition (MI) test Taylor-Robinson et al. (1966) found the MI technique relatively specific and sensitive in detecting human mycoplasma antibody. The test, with M. hyopneumoniae infected pigs, has not been very encouraging. Takatori (1970) was unable to detect any MI activity in sera from experimentally infected pigs and Goodwin et al. (1969a) found nonspecific inhibitory substances in the sera of some pigs both before and after infection.

CHAPTER III. MATERIALS AND METHODS

Experimental Animals

All experimental pigs were obtained from the respiratory-disease-free herd at Iowa State University. The original stock was obtained by caesarean section. All these animals and the subsequent generations of pigs have been maintained under strict isolation for 2 years. All pigs were either crossbred Yorkshire and Hampshire or purebred Yorkshire breeding. They were fed a 16% protein grower ration with no added antibiotics. Each pig was ear-notched for identification.

Experimental Design

Experiment A

Respiratory-disease-free pigs were inoculated with *M. hyopneumoniae* in order to provide a source for natural transmission of the agent among three different age groups of animals. These inoculated pigs were designated seeders. Ten 6-week-old pigs were inoculated for this purpose; nine were used as seeders in the experiment and one was designated to be necropsied before starting the contact period to ascertain that the disease had been established. Six of the seeders were inoculated intratracheally and three (490, 492 and 495) were inoculated intranasally because of their small

size which did not permit intratracheal inoculation. The inoculum, consisting of pneumonic lung suspension containing M. hyopneumoniae strain 11, was given 18 and 21 days prior to use of the seeders for contact-transmission of the agent.

Eighteen respiratory-disease-free pigs derived from 5 litters but of different ages were used. They were separated into three groups of 6 animals; the first group consisted of 3- to 4-week-old pigs, the second group were 6- to 7-week-old pigs and a third group were 12- to 13-week-old pigs. The three age groups of pigs were kept in separate isolation units with identical environmental conditions. The unit size was 6 m^2. Each group was exposed by contact to 3 seeders. Every day, the 3 groups of seeders were moved, in a systematic way, from one age group to another in order to provide a uniform opportunity for contact. The 3 age groups of pigs were exposed to seeders for 27 days. At the end of this period the nine seeders were necropsied. The 3 groups of contact-exposed pigs were kept in their units until 41 to 43 days after the beginning of contact when they were necropsied. During this period they were observed clinically. Necropsies were carried out on two alternate days. On each necropsy day, half of each age group of pigs were killed. Two control pigs for each age group were kept in a separate isolation unit and approximately at the same time, they were necropsied and subjected to the same microbiological and

pathological examinations as the exposed pigs. Nasal secretions were collected from each pig before inoculation or before placing it in the isolation unit. These collections were used for microbiological examination. Blood samples were collected from each pig prior to inoculation or prior to placing it in a unit. Blood collections were continued at weekly intervals until each pig was euthanized.

Experiment B

Nine 8- to 9-week-old pigs were inoculated for use as seeders; six were used in the experiment and three were necropsied before starting the contact period to ascertain that the disease had been established. These pigs were inoculated intranasally. Inoculum, consisting of pneumonic lung suspension containing M. hyopneumoniae strain 11, was given 21, 20 and 19 days prior to use of the seeders for contact-transmission of the agent.

Twelve respiratory-disease-free pigs derived from 3 litters of different ages were used. They were separated into two groups of six animals. Each group was formed with three 3-week-old pigs and with three 11- to 12-week-old pigs. Both groups were kept in separate isolation units with identical environmental conditions. The unit size was 6.38 m^2. Each group was exposed by contact to three seeders. The two groups were kept with the seeders for 20 days. At the

end of this period the seeders were necropsied and each pig of both groups was moved to an individual Smidley hog house with a wood slat porch. The house area was 4 m^2 and the porch was 3.30 m^2. Each Smidley hog house was provided with an automatic waterer and self-feeder. The accumulated manure below the hog house was removed only at the end of the experiment. Hog houses were 4.5 m distant from each other and they were disposed in two parallel rows of 11 and 9 hog units. The distance between the rows was 10.5 m. The experimental pigs were distributed randomly among the houses in an alternate way. Respiratory-disease-free pigs were placed in the intercalate houses in order to detect possible indirect contact. These latter pigs were designated sentinel pigs; three were 6 to 7-weeks-old and three were 14- to 15-weeks-old. Twenty-nine to 30 days after they had been placed in individual hog house units, the two groups of contact-exposed pigs were necropsied, respectively. The sentinel pigs were necropsied 60 days after they had been placed among the other pigs. Two control pigs for the 3-weeks-old contact-exposed pigs and four control pigs for the other age group were kept in isolation and at the appropriate time, they were necropsied and subjected to the same microbiological and pathological examination.

Nasal secretions were collected from the seeders before inoculation and before being placed in contact with the 3-

week-old and 12-week-old groups of pigs. Nasal secretions were collected from both groups of pigs prior to their contact with seeders and before putting them in the Smidley hog houses. Similarly, nasal swabs were taken from sentinel pigs before they were put in their hog houses. Blood samples were collected and processed as described in Experiment A except for sentinel pigs. Blood was collected from the sentinel pigs before locating them in the Smidley hog houses and at necropsy.

Inoculation of Pigs with Pneumonic Lung Inoculum

Pneumonic lung inoculum (PLI)

The pneumonic lung inoculum (PLI) used was prepared from four pigs inoculated with a serial-passaged culture of M. hyopneumoniae strain 11 (Maré and Switzer 1965). The pigs were killed 38 days post inoculation at which time their lungs were collected aseptically in preweighed 500 ml. sterile beakers. The lungs were homogenized by using mortars and pestles and by using sterile alumina.[1] Homogenates with a final concentration of 10% were prepared in Friis (1975) broth medium containing no antibiotics. The 10% PLI was centrifuged in 40 ml glass tubes at 800 rpm for 5 minutes at 6-8°C. This procedure removed alumina and large pieces of lungs. In order to check purity, the pooled PLI

[1]Walker-Anderson Company, Chicago, Illinois.

was examined by bacteriological and mycoplasmal culture techniques as described on pages 72-75. No bacteria or mycoplasmas were isolated from the PLI used except M. hyopneumoniae. In order to estimate the titer of M. hyopneumoniae in the PLI, ten-fold dilutions of PLI were made in Friis broth medium. These dilutions were incubated at 37°C and, as described on page 75, they were observed for color change for 21 days. The color change from red to yellow, was indicative of M. hyopneumoniae growth. The highest dilution of PLI which changed color was considered the final titer of M. hyopneumoniae in color-changing units (CCU). The PLI was stored in 26 or 31 ml volumes at -70°C.

On the day of inoculation, a 24 hr low-passage (17th) culture of M. hyopneumoniae strain 11 grown in Friis broth medium (1975) was added to thawed PLI in the ratio of 1:4. This final inoculum was kept in an ice bath until inoculation. As described previously, the final inoculum was checked for purity and the CCU was determined. In Experiment A, the CCU in the inoculum used to inoculate seeders on the first day was 10^7 and on the second day inoculum was found to contain 10^6 CCU. In Experiment B, the CCU of the inoculum used for seeders was found to be 10^7, 10^8, and 10^7 CCU, respectively, on days 1, 2 and 3 of inoculation.

Intratracheal inoculation

The intratracheal inoculation of seeders was performed according to Bentley and Farrington (1980). Briefly, the pigs were held in a vertical position and a rubber catheter was passed through the mouth into the trachea. The mouth was kept open by means of a porcine mouth speculum inserted between the upper and lower jaws. Passage of air through the catheter coincident with respiratory movements was considered indicative of intratracheal localization. Using a 20 ml syringe attached to a Luer-Lock adapter on the endotracheal tube, 5 ml of inoculum and 15 ml of air were inoculated quickly into the lungs. An additional 20 ml of air was inoculated to assure that the tube had been voided of inoculum.

Intranasal inoculation

Pigs were held with their head up and when the animal made a strong inspiratory movement by partial obstruction of the other nostril, inoculum was given through a syringe. Half of the inoculum was deposited in each nostril.

Isolation and Identification of Bacteria from the Nose, Trachea and Lung

Tracheal and nasal swabs were collected from all pigs at necropsy time. They were collected according to methods noted on page 79.

Nasal secretions from live animals were collected in a manner similar to that of Goiś et al. (Goiś, M., Barnes, H. J., and Ross, R. F. Production of atrophic rhinitis with Pasteurella multocida in pigs naturally infected with Haemophilus parasuis. In preparation. Veterinary Medical Research Institute, Ames, Iowa). Calcium alginate swabs on flexible aluminum shafts[1] were inserted into the nasal cavities of the pigs. Swabs were then placed into vials containing 0.2 ml of beef heart infusion broth (BHI) with 5% of swine serum and 2 units of bacitracin. Following 30 minutes at room temperature, the bacitracin treated swabs were processed for bacteria and mycoplasma isolation. Lung homogenate and Friis broth utilized to wash the left cardiac lobe as described on page 75 and both tracheal and nasal swabs were streaked on 5% horse blood agar and MacConkey agar[2] with 1% dextrose.[2] A Staphylococcus epidermidis culture was streaked diametrically on each blood agar plate as a nurse colony for Haemophilus sp. isolation. Plates were incubated aerobically at 37°C. They were examined

[1]Calgiswab, Inolex, Glenwood, Illinois.

[2]Difco Laboratories, Detroit, Michigan.

after 24 hr and 48 hr incubation for Pasteurella multocida, Bordetella bronchiseptica and Haemophilus sp. Colonies on blood agar characteristic of P. multocida were picked after 24 hr of incubation and subcultured to blood agar in order to check purity. After that, such cultures were passaged into tryptose phosphate broth[1] and incubated for 24 hr. Nonfermenting colonies growing on MacConkey agar after 48 hours were picked and inoculated on MacConkey agar to check purity. These cultures were passaged to tryptose phosphate broth and incubated for 24 hours. Suspect broth cultures of P. multocida and B. bronchispetica were Gram-stained and subjected to biochemical tests. Carbohydrate utilization was determined in phenol red broth base containing 0.5% of the following carbohydrates; dextrose, lactose,[1] and mannitol.[1] Urea agar,[1] Simmons citrate agar,[1] and sulfide-indole-motility (SIM) agar[1] were utilized for additional information for identification (Carter 1975).

Suspect Haemophilus sp. colonies growing close to S. epidermidis were picked and streaked on a blood agar plate with a nurse colony in order to check purity and their dependence on the nurse culture. Blood agar and BHI agar with and without nurse colony were utilized too. The Minitek system[2] (Back and Oberhofer 1978) and Micro-ID system[3]

[1]Difco Laboratories, Detroit, Michigan.
[2]BBL Microbiological Systems, Cockeysville, Maryland.
[3]General Diagnostics, Morris Plains, New Jersey.

(Edberg et al. 1980) were used for biochemical characterization of the Haemophilus sp.

The Minitek system is a micromethod which utilizes reagent-impregnated discs acting as substrates for various enzymatic reactions. As prescribed by the manufacturer, a suspension of the test organism was prepared in Neisseria inoculum broth[1], and using the Minitek pipettes, 0.05 ml of suspension inoculum was delivered to each of 12 wells in a microtray. Each well except one, identified ONPG was promptly overlaid with 3 drops of sterile mineral oil, after which the inoculated trays were placed in the Minitek humidor[1] and incubated overnight at 37°C. Reactions of the individual tests were recorded. The indole test was performed by adding two drops of Kovac's reagent to the ONPG disk. The trays were reincubated for an additional 24 hours after which the test for nitrate reduction was performed by adding 1 drop of each nitrate reagent to the oil overlaying the dextrose-nitrate disk. A wooden applicator stick was used to promote contact between the reagents. The micro-ID system consists of 15 biochemical reactions on paper impregnated disks, each in its own compartment in a hard plastic tray. Each compartment was inoculated with

[1]BBL Microbiological Systems, Cockeysville, Maryland.

0.2 ml of bacterial suspension. After 4 hours of incubation in air at 37°C, 2 drops of 20% KOH were added to the Voges-Proskauer well. The Micro ID was tilted to wet the reagent discs in the first five compartments. In both the Minitek and Micro ID systems the positive or negative reactions were recorded as indicated by the manufacterer.

Isolation and Identification of Mycoplasmas from the Nose, Trachea and Lung

M. hyopneumoniae was isolated from lungs using the procedure described by Friis (1975).

In Experiment A, approximately 1 gm of lung was homogenized by using mortar, pestle, sterile alumina and 5 ml of Friis broth medium without antibiotics. This lung homogenate was utilized for bacteria and mycoplasma isolation.

Kaklamanis et al. (1969) have shown that extract of tissue kills mycoplasmas. As a consequence, in Experiment B, two procedures were used for mycoplasma isolation.

The right cardiac lobe was homogenized as described previously. The left cardiac lobe was sampled by means of a washing technique. The entire left cardiac lobe or a portion of it was collected at necropsy and put into a sterile Petri dish. In the laboratory, the most distal portion of the cardiac lobe was cut in order to expose the

terminal bronchi. Friis broth (5 ml), without antibiotics, was delivered through the main proximal bronchus with a sterile disposable 5 cc syringe fitted with a 22 x 1" sterile disposable needle. The Friis broth medium which passed through the bronchial system was collected into the sterile Petri dish. From the Petri dish, the Friis broth medium was aspirated into the same syringe and put back into its original tube. From this Friis broth medium used to wash the left cardiac lobe and from the lung homogenate serial ten-fold dilutions were made to 10^{-7} in 1.8 amounts of Friis broth with antibiotics contained in rubber stoppered tubes. The Friis broth medium contained 25% SPF pig serum. Cultures were incubated at 37°C in a roller drum. Growth of M. hyopneumoniae was evidenced by a shift from red (pH 7.4) to yellow (pH 6.8) of the phenol red. The occurrence of a color shift indicated the time for subcultivation which was done in Friis broth or in agar medium. The passage on agar was done after 3 to 6 passages in broth. A drop of Friis broth was inoculated on Friis solid agar medium and the plates were incubated at 37°C in a humid atmosphere with 5 to 10% CO_2.

The tracheal and nasal swabs were first streaked on blood agar and MacConkey agar plates, then they were swirled and expressed into 5 ml of BHI with 20% of turkey serum (BHI-TS) (Ross and Switzer 1963). This medium

contained 2,000 IU penicillin per ml and 1:4,000 thallium acetate. Five ml tubes of BHI-TS were inoculated with 0.5 ml of lung homogenate or with 0.5 ml of Friis used to wash the left cardiac lobe. These BHI-TS broth tubes were examined daily for opalescence, tetrazolium reduction, as indicated by a color change to pink, pellicle formation, and a deposit that swirled when the tubes were agitated. A drop of culture medium showing any of the above changes was placed on BHI-TS agar plates, streaked for isolation and incubated at 37°C. First passage BHI-TS broths were subcultivated routinely after 72 hours regardless of whether the above changes were detected or not. Friis broth and primary and secondary cultures of BHI-TS were discarded if no color change or turbidity occurred within 21 days of incubation.

The identification of M. hyopneumoniae was achieved by using the direct epi-immunofluorescence test as described by Møller (1979) but using fixed colonies according to Amanfu (1980). Plates of solid agar medium with colonies suggestive of M. hyopneumoniae were kept in a refrigerator at 4°C for 48 hours. Stainless steel or plastic rings were pressed down into the agar encircling one or several suspect colonies. The rings containing agar with mycoplasmal colonies were removed gently from the plate with a spatula and placed in holes in acrylic carrier slides. The rings were built

with one end bevelled and the other with a collar which prevents the agar plug from falling out. The colonies were then fixed for 60 minutes with absolute methanol dropped into the ring over the colonies. The methanol, as well as other reagents, was retained by the collar of the ring. Methanol was removed by suction with a Pasteur pipet. Colonies were covered with PBS (pH 7.4) for 20 minutes at room temperature. The PBS was removed and the colonies were covered with a drop of the appropriate dilution of fluorescein-conjugated antiserum. Slides containing the colonies were incubated in Petri dishes at room temperature for 30 minutes. The colonies were then washed two times with PBS (pH 7.4), counterstained with chelated azo dye counterstain for 2 minutes (Potgieter and Ross, 1972) and washed two times with chilled distilled water. The slides with rings containing the stained colonies were examined under vertical U.V. illumination[1] from a 12 volt tungsten filament and a blue excitatory filter M. hyopneumoniae (strain J), M. hyorhinis (strain 7), and M. flocculare (strain Ms42) colonies were used as known positive controls. Conjugated M. hyorhinis antiserum was used as heterologous control.

[1]American Optical Company, Scientific Instruments Division, Buffalo, New York.

Blood Sample Collection

Blood samples were collected from the cranial vena cava at time intervals described previously. After clotting at room temperature, the serum was separated and centrifuged at 1,700 rpm for 10 minutes in order to remove blood cells. Serum was frozen and kept at -20°C. The same procedure was utilized with blood collected at necropsy.

Necropsy Technique

At the appropriate time, according to the experimental design, the pigs were electrocuted and exsanguinated by cutting the right brachial blood vessels.

In pigs from Experiment A, the right thoracic cavity was opened aseptically by reflecting the right thoracic wall dorsally. Approximately 2 gm of pneumonic lung tissue were collected aseptically for microbiological culture examination. When no gross lesions were visible, the same amount of lung was collected from the most distal portion of the right cardiac lobe.

In pigs from Experiment B, the right thoracic cavity was opened as was described for Experiment A and the same amount of pneumonic tissue was collected but with priority for the right cardiac lobe. Independent of lesions, 2 gm of the right cardiac lobe were collected. If the right

cardiac lobe did not show lesions, the sample was restricted to the most distal portion of it. In addition, the left thoracic wall was opened aseptically and, independent of lesions, 50 to 100% of the left cardiac lobe was collected aseptically for microbiological culture examination.

The lungs were then removed from the thoracic cavity. The trachea was opened aseptically near the thoracic inlet and secretions were collected by inserting a sterile cotton-tipped swab.

Any lung hepatization resembling that described by Pullar (1948) was considered as a typical lesion and recorded in the following way: 1 = no gross lesions; 2 = up to 25% of the affected lobe; 3 = 25 to 50% of the affected lobe; 4 = 50 to 75% of the affected lobe and 5 = 75 to 100% of affected lobe. The total lesion score of each pig was calculated. Bronchial and mediastinal lymph nodes were observed for edema, congestion and enlargement. In the next step, the head was separated from the body at the atlanto-occipital articulation and the snout was cut transversally at the level of the first upper premolar tooth with a band saw. Turbinate atrophy was scored on a scale of 0 to 5; 1 = mild atrophy of ventral scroll of ventral turbinate; 2 = moderate to severe atrophy of ventral scroll of ventral turbinate; 3 = mild atrophy of dorsal scroll of ventral turbinate; 4 = moderate to severe atrophy of dorsal scroll

of ventral turbinate; 5 = severe atrophy of dorsal and ventral scroll and ventral turbinate and 0 = no atrophy of dorsal and ventral scrolls of ventral turbinates. The head was cut sagittally on the midline. Both nasal cavities were exposed by aseptically removing the septum nasi. Nasal secretions were collected from the nasal mucosa of the proximal portion of the nasal cavities by using sterile cotton-tipped swabs.

Immunofluorescence Technique

Preparation of tissue for immunofluorescence tests

The procedure used to prepare tissue for the immunofluorescence test was that described by Amanfu et al. (1980). Small pieces of lung (1x1x0.5) were usually taken at the junction of grossly normal tissue and diseased tissue to include bronchus and several small bronchioles. Samples were embedded in OCT[1] fluid medium contained in small flat-bottom plastic wells.[2] These were frozen on dry ice, wrapped in aluminum foil and stored at -70°C. Six slides were made from each lung specimen by cutting the tissue 4 µm thick with a cryo-cut microtome.[3] After that, specimens were

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

[2]Linbro Scientific Inc., Hamden, Connecticut.

[3]American Optical Corp., Scientific Inst. Div., Buffalo, New York.

fixed for 10 minutes in absolute methanol. The slides were air-dried and stored at -20 C or below until stained.

Direct immunofluorescence (DFA) test

The direct immunofluorescence (DFA) test used in the present work was that described by Amanfu et al. (1980).

Conjugated porcine IgG antibody against M. hyopneumoniae was used to detect M. hyopneumoniae antigen and conjugated rabbit IgG antibody against M. hyorhinis was used to detect M. hyorhinis antigen in cryostat lung sections. The fluorescein isothiocyanate (FITC) conjugated M. hyopneumoniae antibody and M. hyorhinis antibody were prepared by Theresa Young and Barbara Zimmermann at the Veterinary Medical Research Institute. Briefly, globulins from hyperimmune sera against M. hyopneumoniae strain 11 and M. hyorhinis strain 7 produced respectively in swine and rabbit were precipitated from the serum with saturated ammonium sulfate solution and the IgG fraction from globulins was separated in Sephadex G200.[1] The IgG was conjugated to fluorescein isothiocyanate[2] in a ratio of 1:90. Free fluorescein dye was removed from conjugated IgG by gel filtration with Sephadex G25.[1] The titration and specificity of each

[1]Pharmacia Fine Chemicals Inc., Piscataway, New Jersey.

[2]BBL, Cockeysville, Maryland.

conjugate were achieved by using two-fold dilutions of conjugates to stain lung sections from pigs infected with M. hyopneumoniae or M. hyorhinis.

For staining, sections of lung on slides were placed in a rectangular dish containing moist filter paper. The sections were covered with a drop of the appropriate dilution of conjugate. Dishes were then sealed with a microtiter plate sealer[2] and incubated for 30 minutes at 37°C. Excess unbound conjugated IgG was removed by two sequential rinsings with PBS pH 7.4 for 5 minutes in a glass jar on a stirrer. The sections were then counterstained with azo-dye (Potgieter and Ross. 1972) for 30 seconds and washed twice in distilled water for two minutes. Stained sections were carefully blotted dry, mounted with phosphate buffered glycerin pH 7.4 and examined with incident ultraviolet light. A binocular Leitz Ortholux microscope[2] equipped with a dark field condenser and an Osram HBO 200 mercury vapor lamp was used. A blue excitatory filter (BG12) and a yellow barrier filter (K530) were used. Sections were generally examined at 100X.

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[1]Pharmacia Fine Chemicals Inc., Piscataway, New Jersey.

[2]BBL, Cockeysville, Maryland.

The intensity and location of fluorescence in bronchi and bronchioli were scored as follows: 1 = scattered granular green yellow fluorescent particles lining the bronchi with no fluorescence in bronchioli; 2 = thin coating of yellow green particles lining the bronchi with no bronchiolar fluorescence; 3 = thin coating of yellow green particles lining the bronchi but with green yellow fluorescent granules lining the bronchioli; 4 = continuous thin coating of yellow green fluorescent particles lining bronchi and bronchioli.

Lung sections of specific pathogen free (SPF) pigs were utilized for controls. The optimal working dilution of each conjugate was the dilution at which minimum nonspecific fluorescence and maximum bronchial and bronchiolar specific fluorescence was seen. In addition, the specifity of conjugates was determined by using two-fold dilutions of conjugate in the epi-immunofluorescence test as described on page 75. This test was performed with colonies from several porcine mycoplasmas isolated from swine and from other species. The cross reactivity of the swine M. hyopneumoniae antiserum with M. hyorhinis antigen was removed by adsorption for 24 hours at 4°C with deoxycholate extract of M. hyorhinis organisms.

Indirect immunofluorescence

All pulmonary lobes examined by DFA were examined also by IFA in order to determine the correlation between the two techniques. Fluorescein conjugated goat IgG antibodies against rabbit IgG was used to detect rabbit IgG antibodies against M. hyopneumoniae and M. hyorhinis in cryostat lung sections following treatment with the rabbit antisera.

A commercial goat anti rabbit IgG conjugated with fluorescein isothiocyanate (FITC) with an F:P ratio of 2.5 mg:gm was used. Antisera against M. hyopneumoniae and M. hyorhinis were prepared by Barbara Zimmermann at VMRI. M. hyorhinis antiserum was prepared by several inoculations of rabbits with whole formalized cells of M. hyorhinis strain 7. Sera from 4 hyperimmunized rabbits (281, 282, 283 and 284) were pooled and used in this work. M. hyopneumoniae antiserum was prepared by several inoculations of sodium deoxycolate extract (Ross and Karmon, 1970) of M. hyopneumoniae strain 11 in rabbits. Serum from one rabbit (253) was utilized in this work.

Titration and specificity of fluorescein-conjugated anti rabbit IgG and M. hyopneumoniae and M. hyorhinis antisera was achieved by checkerboard titration using one homologous, one heterologous and a normal system. The checkerboard

[1]Coppel Laboratories, Cochranville, Penn.

titration was carried out on lung sections from pigs experimentally infected with M. hyopneumoniae or M. hyorhinis and on lung sections from normal SPF pigs. Two-fold dilutions from 1:2 to 1:1280 were used. The homologous system included M. hyopneumoniae and M. hyorhinis diseased lung sections stained, respectively, with anti M. hyopneumoniae and M. hyorhinis antisera. The heterologous system consisted of M. hyopneumoniae and M. hyorhinis diseased lung sections stained, respectively, with M. hyorhinis and M. hyopneumoniae antisera and normal rabbit serum. The normal system consisted of SPF normal lung sections stained with both antisera and normal swine serum. The end point was taken as that point where minimum nonspecific fluorescence and maximum bronchial and bronchiolar specific fluorescence were seen. In addition, the specificity of conjugate and antisera was determined in the indirect epi-immunofluorescence test as described by Møller (1979) but using colonies on agar fixed with methanol according to Amanfu et al. (1980). The mycoplasma colonies utilized as antigen were: M. hyorhinis (strain 7), M. hyosynoviae (strain S16), M. flocculare (strain Ms42) and M. hyopneumoniae (strains 11 and J). The cross reactivity of rabbit antiserum against M. hyorhinis with M. hyopneumoniae was removed by adsorption for 24 hours at 4°C with deoxycholate extract of M. hyopneumoniae organisms (Ross and Karmon 1970). M. hyopneumoniae antiserum was subjected to

swine liver powder sorption (Cherry et al., 1960) in order to eliminate a diffuse green nonspecific background fluorescence.

The classical IFA test was run at 37°C according to Cherry et al. (1960) but with washing and timing according to Rosendal and Black (1972). Cryostat lung sections on slides, setting in a rectangular dish containing moist filter paper, were covered with a drop of the appropriate dilution of antiserum. Dishes were sealed with a microtiter plate sealer[1] and incubated for 30 minutes at 37°C. Excess unbound antiserum was removed by two sequential rinsings in PBS (pH 7.4) for 10 minutes in a glass jar on a magnetic stirrer. The sections were blotted dry and arranged again in the rectangular dish where they were covered with a drop of conjugated antibody against rabbit IgG. The dishes were then sealed and incubated for 30 minutes at 37°C. Excess fixed conjugate was removed by two sequential rinsings in PBS (pH 7.4) for 10 minutes and one rinsing in distilled water. Some slides were counterstained with azo-dye (Potgieter and Ross 1972) for 30 seconds and washed twice in distilled water. The washed sections were blotted dry, mounted in phosphate buffered glycerin (pH 7.4) and examined with incident ultraviolet light as described previously for

[1]Cook Engineering, Alexandria, Virginia.

DFA. The intensity and location of fluorescence in bronchi and bronchioles were scored as for the DFA test.

Histological Techniques

Preparation of tissue for histological examination

Pieces (1x2x0.5) of pneumonic lung from lobes with lesions as well as similar pieces from all normal lobes were collected according to Figure 1.

Lung samples as well as a section of trachea were put in 10% buffered formalin for 48 hours and processed according to standard paraffin technique and stained with hematoxylin and eosin as indicated in "Histologic and Special Staining Techniques" (Armed Forces Institute of Pathology 1968).

Evaluation of microscope lesions

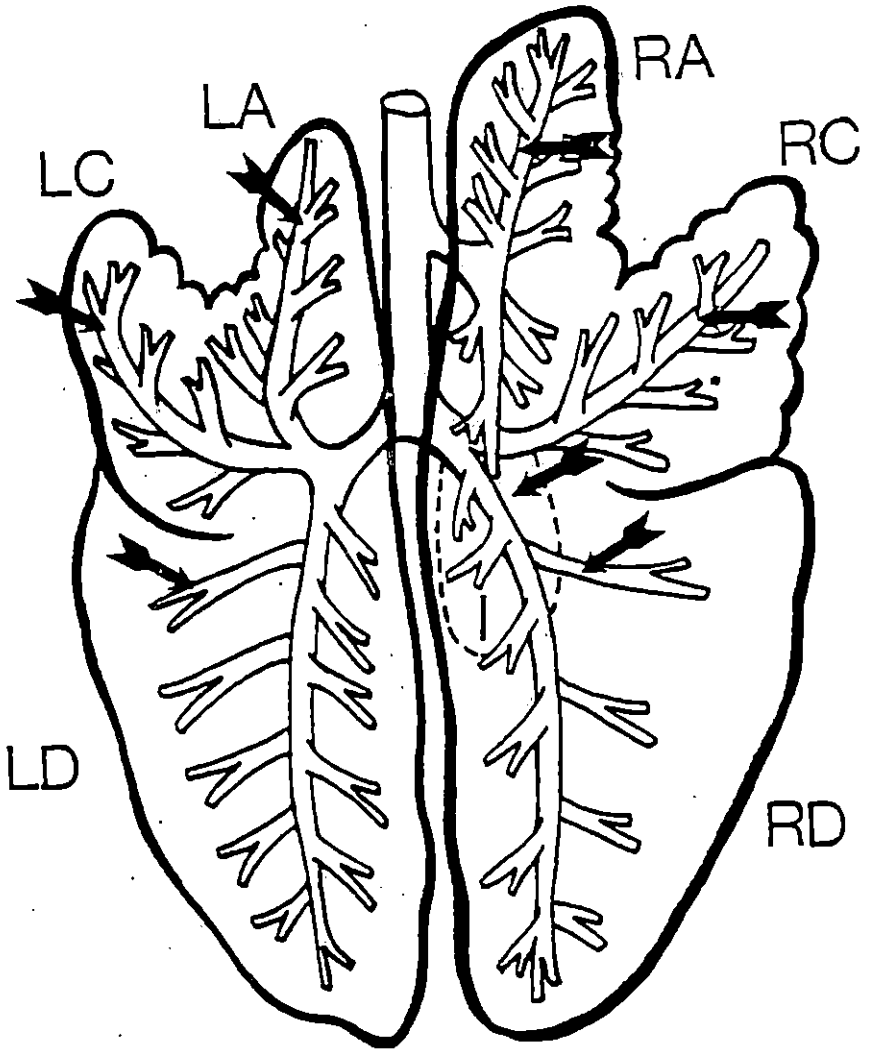
The lung sections were examined with a light microscope for the stage of the disease and its severity.

The stages of the disease were divided into acute (early), acute (late), subchronic and chronic (Histopathologic classification of Mycoplasmal pneumoniae of swine, Barnes, M. J. 1977, Veterinary Medical Research Institute, Iowa State University). The intensity of the mycoplasmal lesion was determined by dividing the total number of bronchi plus bronchioli present in the lung section by the number of affected bronchi plus bronchioli. Affected bronchi

Figure 1. Schematic drawing of pig lung showing sample collection sites for microscopic lesions examination (Adapted from Neurand, K., Wissdorf, H. and Mensow, C. 1970. Beitrag zur Bronchialverzneigung beim Schwein. Berl. Munch. Tierartzl. Wach. 23:467-470)

RA = Right apical lobe
RC = Right cardiac lobe
RD = Right diaphragmatic lobe
LA = Left apical lobe
LC = Left cardiac lobe
LD = Left diaphragmatic lobe
I = Intermediate lobe

(Arrows indicate bronchi serving lobes and sites for collection of samples for microscopic lesions examination)


RA
LA
RC
LC
LD
RD

or bronchioli was defined as those with peribronchial and peribronchiolar lymphoid hyperplasia which extended into the adjacent interstitium and through the muscular layer into the submucosa or by marked peribronchial and peribronchiolar lymphoid hyperplasia. The severity of the lesions were classified from 0 to 4, according to the coefficient defined above. Lung sections with scores equal to zero were classified as belonging to class 0. Lung sections with scores equal or above 10, between 5 to 9.9, between 2 to 4.9 and between 1 to 1.9 were classified into 1, 2, 3, and 4 classes, respectively.

Complement Fixation (CF) Test

Complement fixing antibodies to M. hyopneumoniae and M. hyorhinis were detected by using a direct microtitration system where the reaction volume was 0.125 ml (Slavik and Switzer 1972). Lyophilized guinea pig complement was reconstituted with normal unheated swine serum from 6 to 8-week-old respiratory disease-free pigs. Five 50% hemolytic units of complement were used and the optimal concentration of antigen was determined by block titration. The antibody titer was expressed as the reciprocal of the highest serum dilution which gave approximately 70% or more of haemolysis.

Enzyme-linked Immunosorbent Assay (ELISA) Test

The enzyme linked-immunosorbent assay (ELISA) test (Bruggmann et al. 1977b) adapted to the microtiter system[1] was utilized in this study. Microtiter wells[2] were coated with a sodium dodecyl sulfate extract of M. hyopneumoniae strain J. Plates were washed with buffer and serial dilutions of test sera from 1:10 to 1:1280 were made in the microtiter wells. The microtiter plates were kept at room temperature for 5 hours and then they were washed with buffer. A peroxidase-conjugated IgG fraction of rabbit antiserum against swine IgG (heavy and light chain)[3] was added in the appropriate dilution to each well and the microtiter plates were incubated overnight. The plates were washed with buffer and the enzyme substrate, 5-aminosalicylic acid, and hydrogen peroxide were added. The enzymatic reaction was stopped by adding sodium hydroxide (Molar solution) 15 minutes after the beginning of the reaction. The color change in the fluid was assumed to be equivalent to the amount of conjugate fixed. The amount of conjugate fixed was therefore equivalent to the amount of antibody bound to antigen. The degree of color change was measured by

[1]Adapted and run by Theresa Young, Research Associate Veterinary Medical Research Institute, Iowa State University, Ames, Iowa.

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

[3]Cappel Laboratories, Inc., Cochranville, Pennsylvania.

using a colorimeter capable of measuring results directly in the wells of the microtiter plates (Clem and Yolken 1977). Wells containing antigen, substrate, and sodium hydroxide were used to adjust the machine to zero. Approximately 9 negative sera were used each time that the test was run. Normally, negative control sera were those collected from the experimental pigs before their utilization in the experiments.

A preliminary analysis revealed that results obtained varied significantly from one day to the next. In addition, sera from some negative control pigs caused more intense color change than others.

Dilution of serum appeared to reduce this background. In fact, the variation in color change with negative sera decreased with dilution. The influence of age of pigs on background or nonspecific color change was evaluated by using preexposure and postexposure sera from 6 sentinel pigs. Three pigs were 6 to 7 weeks old and 3 pigs were 14 to 15 weeks old prior to use as sentinels. Following use as sentinels the pigs were euthanized when they were 14 to 15 and 22 to 23 weeks old, respectively. Lesions of MPS and M. hyopneumoniae were not detected in these pigs. The preexposure and the necropsy sera were evaluated with the ELISA test on the same day. Means of optical density for corresponding dilutions of the preexposure and necropsy sera

did not differ ($p > 0.05$).

In order to overcome the day-to-day variation, two criteria were utilized to classify pigs as positive or negative with the ELISA test. In the first criterion, any optical density reading at a given dilution above 3 standard deviations from the means of the negative control sera for that dilution was considered positive (Chao et al. 1979). This procedure of classification will be called the three standard deviation criterion.

The second criterion utilized discriminant analysis. By this criterion, an unknown serum was classified as positive or negative according to the discriminant functions. Discriminant functions were defined by utilizing ELISA test results obtained with 34 sera from contact-exposed pigs; 14 sera were defined as positive and 20 as negative. A serum collected at necropsy, was classified as positive if it was from a pig with microscopic lesions of MPS and M. hyopneumoniae as determined by DFA or isolation procedures. In addition, the animal from which the sera was collected must have been CF-positive at least one time during the period of observation. Negative sera were those collected from experimental pigs before their utilization in the experiment (preexposure serum).

Dilutions 1:40 and 1:80 were utilized to build the

discriminant functions. At these dilutions, the background of serum appeared to be less important. In fact, the variance at these dilutions of negative controls was approximately half that at 1:10 dilution.

In order to overcome the time to time variation, the mean of negative controls for each day was subtracted from all positive and negative sera. As a consequence, the means of negative sera were adjusted to zero and the means of positive sera became the difference between the mean of positive and negative serum before adjustment.

Two squared distance functions were determined according to Snedecor and Cochran (1967), one for the positive and another for the negative serum.

$$D^2_-(X) = 1{,}247.578(\underline{X}_{40})^2 - 2{,}552.91(\underline{X}_{80}\underline{X}_{40}) + 1{,}865.763(\underline{X}_{80})^2 - 13.456$$

$$D^2_+(X) = 393.12(X_{+40})^2 - 732.98(X_{+80}\ X_{+40}) + 430.463(X_{+80})^2 - 10.46$$

If $D^2_-(X) < D^2_+(X)$, Unknown sera will be classified as negative

If $D^2_+(X) < D^2_-(X)$, Unknown sera will be classified as positive

D^2 = Squared distance function

X = Unknown sera

$\underline{X}_{40}$ = Optical density reading at 1:40 dilution of sera to be classified (X) minus the optical density reading mean of negative controls at 1:40 dilution

$\underline{X}_{80}$ = Optical density reading at 1:80 dilution of sera to be classified (X) minus the optical density reading mean of negative controls at 1:80 dilution

X_{+40} = Optical density reading at 1:40 dilution of sera to be classified (X) minus 0.284 (constant)

X_{+80} = Optical density reading at 1:80 dilution of sera to be classified (X) minus 0.203 (constant)

This procedure for classifying pigs positive or not to ELISA test will be called discriminant analysis criterion.

Statistical Analysis

In both experiments, data were tested for significance by using the chi-square test. In addition, contingency tables were utilized for testing the independency or association among attributes observed.

CHAPTER IV. RESULTS

Experiment A

Clinical signs

Seeders Coughing was observed in 1 pig 8 days after inoculation and the rest of the pigs 11 to 15 days after inoculation. Coughing was noticed more frequently after forced exercise.

Pigs exposed by contact to seeders Only 6 of the 18 pigs from all three age groups were observed to cough; one from the 3-week-old group, two from the 6-week-old group and three from the 12-week-old group. The pigs were observed to cough first from 31 to 34 days after contact-exposure had begun. Coughing was observed only when the pigs were forced to exercise.

Necropsy findings

Lung hepatization with uniform color was observed in all groups of pigs. The colors observed were from pale gray to pink and to red (Figure 2). These lesions, although not pathognomonic, were considered characteristic of *M. hyopneumoniae* pneumonia.

The number of pigs with gross lesions of pneumonia among the principal experimental groups is summarized in Table 1. There was no difference in the proportion of animals in the 3 age groups with gross lung lesions ($P > 0.05$).

Figure 2. Gross lesion of MPS in lung of a pig exposed by contact to M. hyopneumoniae

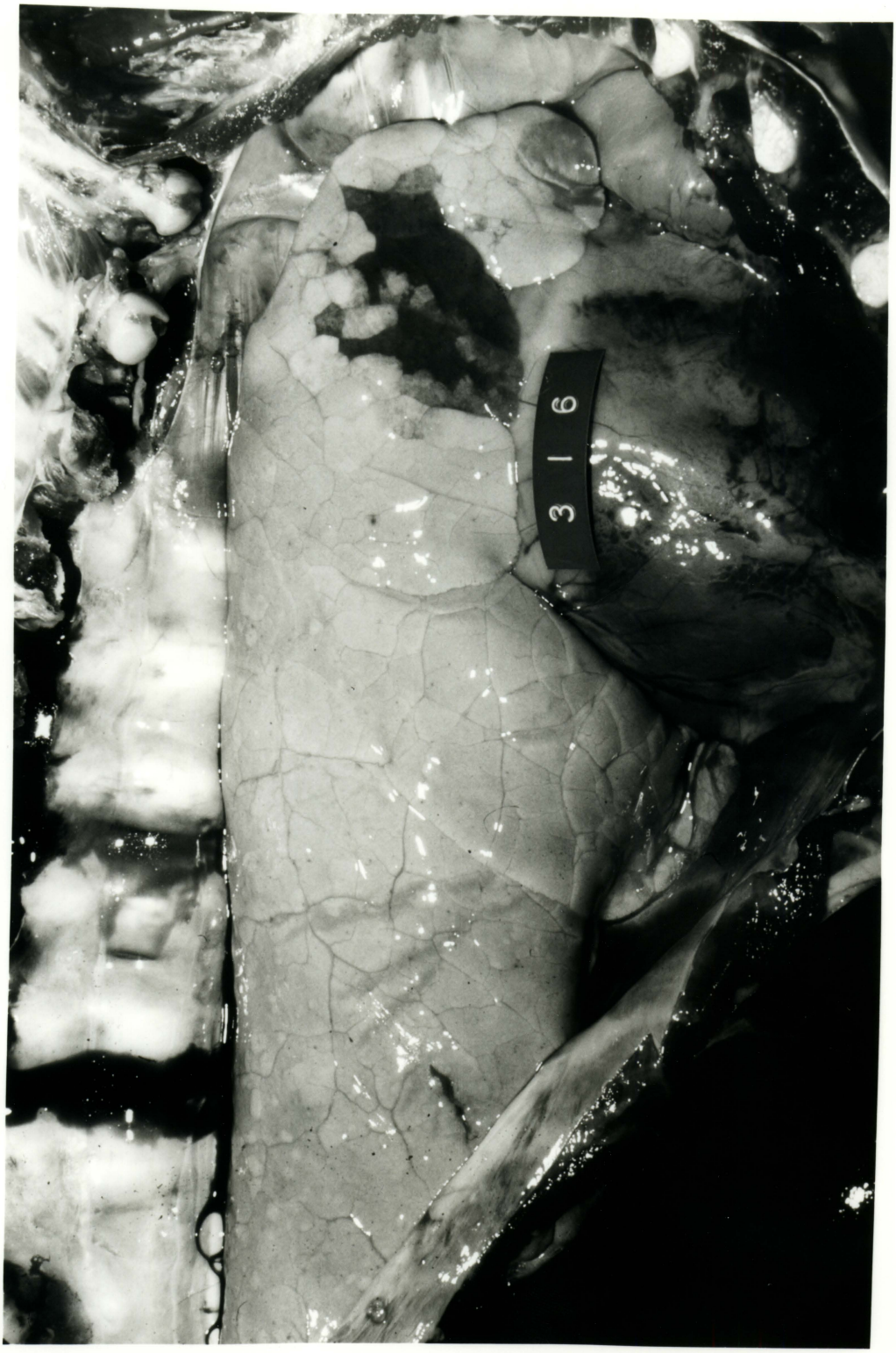

3 1 6

Table 1. Severity of pneumonia in three age groups of contact-exposed pigs: Number of pigs with gross lesions of pneumonia

Experimental groups	Total number of pigs	Number of pigs with pneumonia	Number of pigs without pneumonia	Chi-square
3[a]	6	3	3	1.50*
6	6	4	2	
12	6	5	1	

[a]Weeks of age.

*$P > 0.05$.

The severity of pneumonia in the three age groups of pigs was evaluated by the number of lobes with lesions (Table 2). For purposes of analysis, four categories of pigs with an increasing number of MPS-affected lobes were established. No differences were detected in the distribution of pigs in these categories ($P > 0.05$).

The severity of pneumonia was scored according to the approximate percentage of each lobe affected. The sum of all lobular scores from a pig constituted its gross lesion pneumonia severity score. Gross severity scores are given in Table 3. For purposes of analysis, three categories of pigs with increasing severity of gross pneumonia scores were determined. Pigs with no gross lung lesions were scored 7. (Each normal lobe received a score of

Table 2. Severity of pneumonia in three age groups of contact-exposed pigs: Number of lobes with gross lesions of pneumonia

Experimental groups	Total number of pigs	Number of pigs in pooled categories of number of lobes with pneumonia				Chi-square
		0 Lobes	1-2 Lobes	3-4 Lobes	5-7 Lobes	
3[a]	6	3	2	1	0	3.99*
6	6	2	2	0	2	
12	6	1	2	1	2	

[a]Weeks of age.

*P > 0.05.

Table 3. Severity of pneumonia in three age groups of contact-exposed pigs: Gross lesion severity score

Experimental groups	Total number of pigs	Number of pigs in categories of gross lesion severity score			Chi-square
		7	8-11	12-15	
3[a]	6	3	2	1	2.00*
6	6	2	2	2	
12	6	1	2	3	

[a]Weeks of age.

*P > 0.05.

1, hence a completely normal pig received a score of 7. The most affected ones were classified in the category 12-15 while the least-affected ones were classified in the 8-11 category. No differences were detected in gross lesion severity scores among the three age groups of pigs ($P > 0.05$).

All seeder pigs, except one, had gross lesions characteristic of MPS (Table 4).

Gross pneumonia appeared more frequently in the right apical and right cardiac lobes (Table 5) but, analysis indicated these differences were not significant ($P > 0.05$).

Edema of bronchial lymph nodes was observed in two seeder pigs and in one each of the 6- and 12-week-old pigs, respectively.

Atrophy of nasal turbinate was found in all groups of pigs (Tables 4 and 6). There were more affected pigs among younger than among older pigs ($P < 0.05$). In general, mild to moderate atrophy of the ventral scroll of the ventral turbinate was observed in all groups.

No macroscopic lesions were found in control pigs.

Histopathology

Microscopic lesions suggestive of MPS in lungs of contact-exposed pigs were classified into four stages: acute (early), acute (late), subacute and chronic.

Table 4. Necropsy findings in M. hyopneumonia-inoculated pigs used as seeders in Experiment A

Pig number	Pneumonia severity score	Enlargement of bronchial lymph nodes	Turbinate atrophy score
486	13[a]	1[b]	0/0[c]
487	10	1	0/0
488	15	3	0/0
489	11	1	0/0
490	7	1	0/0
491	13	3	1/0
492	15	1	0/0
493	9	1	0/0
494	14	1	0/1

[a]Each normal lobe received a score of 1, hence a completely normal pig received a score of 7. A total score above 7 indicates that lesions were observed. Higher scores indicated more lung tissue was involved.

[b]1 = No gross enlargement of bronchial lymph nodes,

3 = Moderate enlargement of bronchial lymph nodes.

[c]0 = No atrophy of the ventral turbinate was observed,

1 = Mild atrophy of ventral scroll of the ventral turbinate.

Table 5. Distribution of lesions of pneumonia among the lobes in lungs of seeder and contact-exposed pigs

Experimental animals	Number of pigs	Total number of lobes	Number of each lobe with lesions of pneumonia							Chi-square
			LA[a]	LC[b]	LD[c]	RA[d]	RC[e]	RD[f]	I[g]	
Seeders	9	63	3	6	5	8	8	4	6	9.37*
Pigs exposed by contact	18	126	6	7	4	6	7	6	3	3.518*

[a]Left apical lobe.

[b]Left cardiac lobe.

[c]Left diaphragmatic lobe.

[d]Right apical lobe.

[e]Right cardiac lobe.

[f]Right diaphragmatic lobe.

[g]Intermediate lobe.

*$P > 0.05$.

Table 6. Atrophy of nasal turbinates in three age groups of pigs exposed by contact to M. hyopneumoniae

Experimental groups	Total number of pigs	Pigs with turbinate atrophy	Pigs without turbinate atrophy	Chi-square
3[a]	6	6	0	7.200*
6	6	5	1	
12	6	2	4	

[a]Weeks of age.

*$P < 0.05$.

The histopathologic lesions found in the acute (early) stages of MPS (Figure 3B) were characterized mainly by perivascular cuffing of lymphocytes and peribronchial and peribronchiolar lymphoid tissue hyperplasia. Frequently, lymphoid proliferation extended into the adjacent interstitium and through the muscular layer into the submucosa compromising the lumina of the airways. Very few cells and little exudate were observed in bronchi, bronchioli and alveoli. Alveolar walls were normal or thickened.

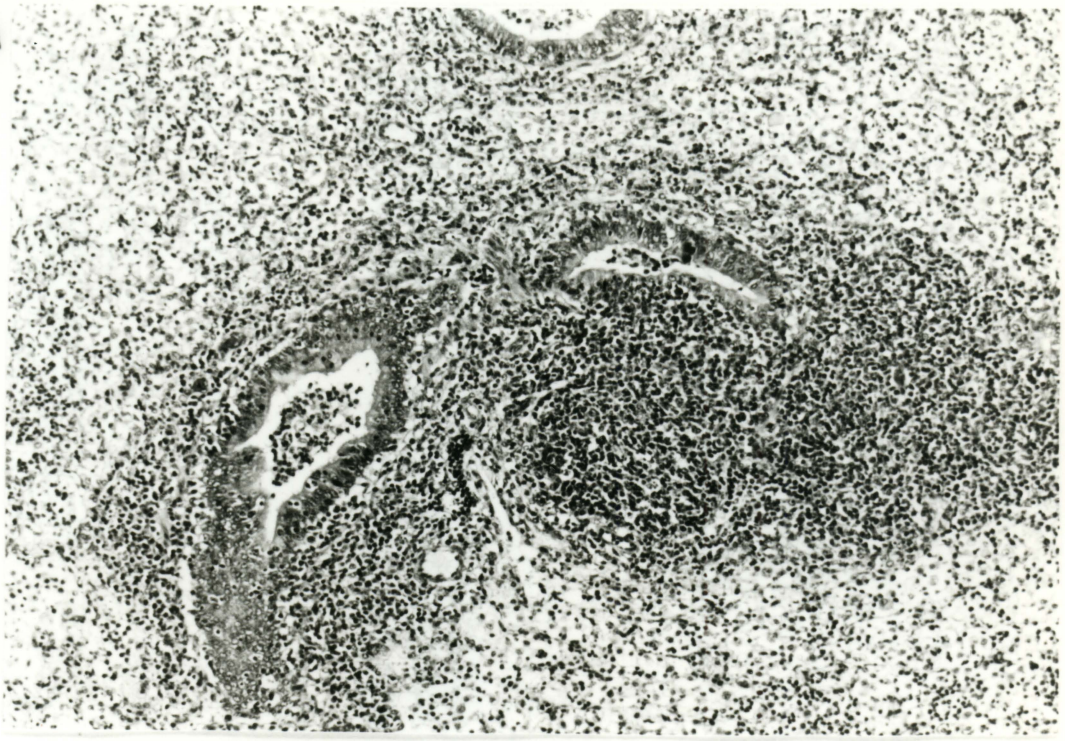
In the acute (late) stage (Figure 3A), vascular changes were similar to the early stage but with greater lymphocytic cuffing. Lymphoid tissue was increased with formation of germinal centers. This hyperplastic lymphoid tissue

Figure 3. Sections of lungs of contact-exposed pigs

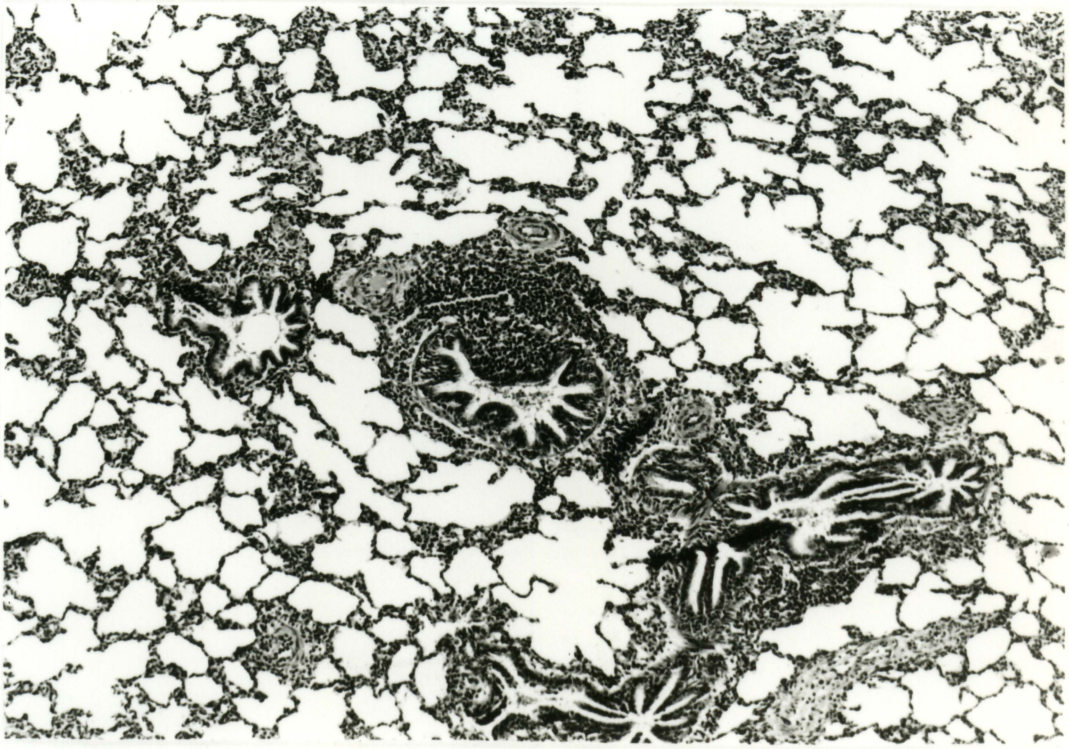
A - Acute (late) state of MPS. Notice peri-bronchiolar cuffing of lymphocytes and presence of cellular infiltrate in alveoli and bronchioli (H&E) (100 x magnification)

B - Acute (early) stage of MPS. Notice lymphoid proliferation extended through the muscular layer into the submucosa and the absence of cellular infiltrates in alveoli and bronchioli (H&E) (100 x magnification)

A



B



produced a moderate to marked compression of the lumina of airways. Type II alveolar cells were rounded and sloughed into alveoli, often filling the lumina. Minimal numbers of neutrophils (PMN) and other mononuclear cells were located in alveoli, bronchi and bronchioli. Edema, and less frequently, fibrin was observed inside alveoli. Thickening of alveolar walls and patchy loss of cilia in bronchi and bronchioles were observed.

In the subacute stage (Figure 4), alveoli were free of cells and inflammatory exudate. Lymphoid follicles persisted and distorted bronchi and bronchioles. Alveolar walls were often markedly thickened.

The chronic stage of M. hyopneumoniae pneumonia was not observed.

Free erythrocytes were sometimes present in alveolar spaces. This may have resulted from electrocution. Clumping and loss of tracheal cilia was noticed in all stages of the disease but no inflammatory cells were observed in the lamina propria of trachea.

Table 7 summarizes the occurrence of different stages of MPS among the experimental pigs. Almost all MPS-affected pigs (85%) had acute (late) stage lesions. Different stages of MPS were observed in one pig. This may indicate that spread of infection from one lobe to

Figure 4. Section of lung of contact-exposed pig. Subacute stage of MPS. Notice persistence of lymphoid follicles, absence of cellular infiltrate in alveoli, bronchus and bronchiolus and thickening of alveolar walls (H&E) (100 x magnification)

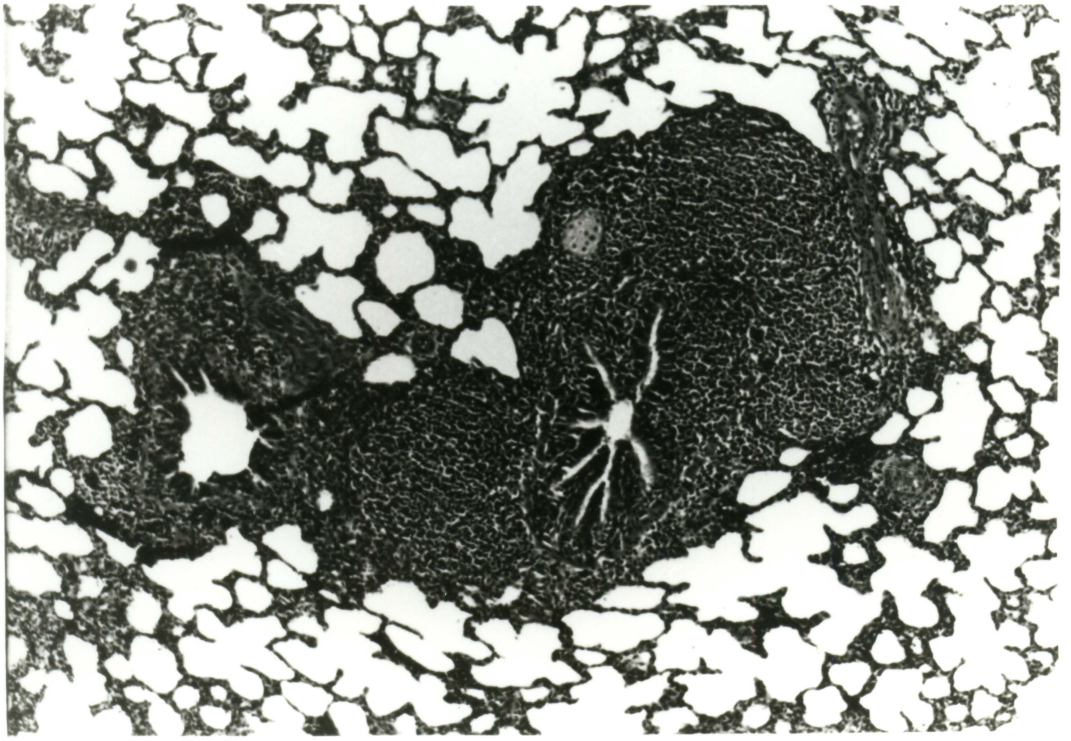


Table 7. Severity of pneumonia in three age groups of contact-exposed pigs: Histopathologic classification of MPS lesions in lungs

Experimental groups	Total number of pigs	Number of pigs in histopathologic stages of MPS categories			
		AE[a]	AL[b]	AE+AL	SA[c]
Seeders	9	0	6	0	3
3[d]	6	2	3	0	0
6	6	0	2	1	0
12	6	0	5	0	0

[a]Acute (early) stage.

[b]Acute (late) stage.

[c]Subacute stage.

[d]Weeks of age.

another or between pigs had occurred. Acute (early) lesions occurred only in 2 pigs in the 3-week-old group. Neither pig had any gross lesions. This may indicate that infection occurred later during the experiment.

The effect of age on the number of lobes with microscopic lesions of MPS is summarized in Table 8. For purposes of analysis, contact-exposed pigs were classified into four categories on the basis of number of affected lobes. Those pigs which did not show any lung lesions were classified in category 0. Those pigs which had 5 to 7 lobes

Table 8. Severity of pneumonia in three age groups of contact-exposed pigs: Number of pigs with microscopic lesions of MPS

Experimental groups	Total number of pigs	Number of pigs in pooled categories of number of pneumonic lobes				Chi-square
		0	1-2	3-4	5-7	
3[a]	6	3	2	1	0	5.644*
6	6	3	1	0	2	
12	6	1	1	2	2	

[a]Weeks of age.

*$P > 0.05$.

with microscopic lesions were classified in category 5-7. There were no differences in the distribution of pigs in these cateogories ($P > 0.05$).

The severity of microscopic lesions was evaluated according to the number of bronchi and bronchioli with characteristic lesions of MPS in a section from each lobe. The sum of all lobular microscopic MPS scores from a pig constituted its microscopic MPS severity score. Microscopic MPS severity scores, for analysis purposes, were placed into 4 categories (Table 9). Analysis of the distribution of pigs among these categories revealed no differences in severity of microscopic lesions among the

Table 9. Severity of pneumonia in three age groups of contact-exposed pigs: Histopathologic MPS severity scores

Experimental groups	Total number of pigs	Numberof pigs in categories of microscopic MPS severity scores				Chi-square
		0	1-8	9-16	17-24	
3[a]	6	1	4	1	0	7.78*
6	6	3	1	0	2	
12	6	1	2	2	1	

[a]Weeks of age.

*P > 0.05.

three age groups of pigs (P > 0.05).

Increased PMN were observed in bronchi, bronchioli and alveoli in some cases of MPS. This was observed in 2 pigs each from both the 3- and 6-week-old groups and in 1 from the 12-week-old group. All these pigs, except the 12-week-old ones, had B. bronchiseptica in their nasal cavities or tracheas and one had it in the lung.

Purulent bronchopneumonia and purulent broncho-bronchiolitis were observed, respectively, in two pigs from the 12-week-old group of pigs.

B. bronchiseptica was isolated from nose, trachea and lung of the pig with purulent bronchopneumonia.

A multifocal granulomatous pneumonia was observed in one

or more lobes of lungs from 2 control pigs, 1 seeder pig and 1 pig belonging to the 3-week-old group. Foreign bodies, suggestive of plant material were observed within macrophages and giant cells (Figure 5).

Alveolar wall thickening was a common feature among all groups of pigs, including controls. Cells involved in the alveolar thickening were those which normally comprise lung tissue. In these areas, folding of bronchial and bronchiolar mucosa suggested some degree of atelectasis (Figure 6).

Isolation of bacteria and mycoplasmas from lungs, tracheas and nasal cavities of pigs

Bacteria and mycoplasmas isolated from lungs, tracheas and nasal cavities are summarized in Tables 10 and 11.

Bordetella bronchiseptica was the only pathogenic bacteria isolated. It was isolated more frequently from the tracheas and nasal cavities of pigs but, it was detected 3 times in lungs. B. bronchiseptica was isolated from all experimental groups but it appeared to occur more frequently among 3- and 6-week-old pigs. Pigs apparently became infected with B. bronchiseptica during the experiment because, preexposure nasal samples from all pigs were culture-negative for the organism. The source of infection was not determined.

M. hyopneumoniae was the only mycoplasma isolated. Its occurrence among the three age groups of pigs appeared not to

Figure 5. Histopathological section of lung of a control pig (Experiment A). Multifocal granulomatous pneumonia (notice multinucleated giant cells in the pneumonic focus) (H&E stain) (200x magnification).

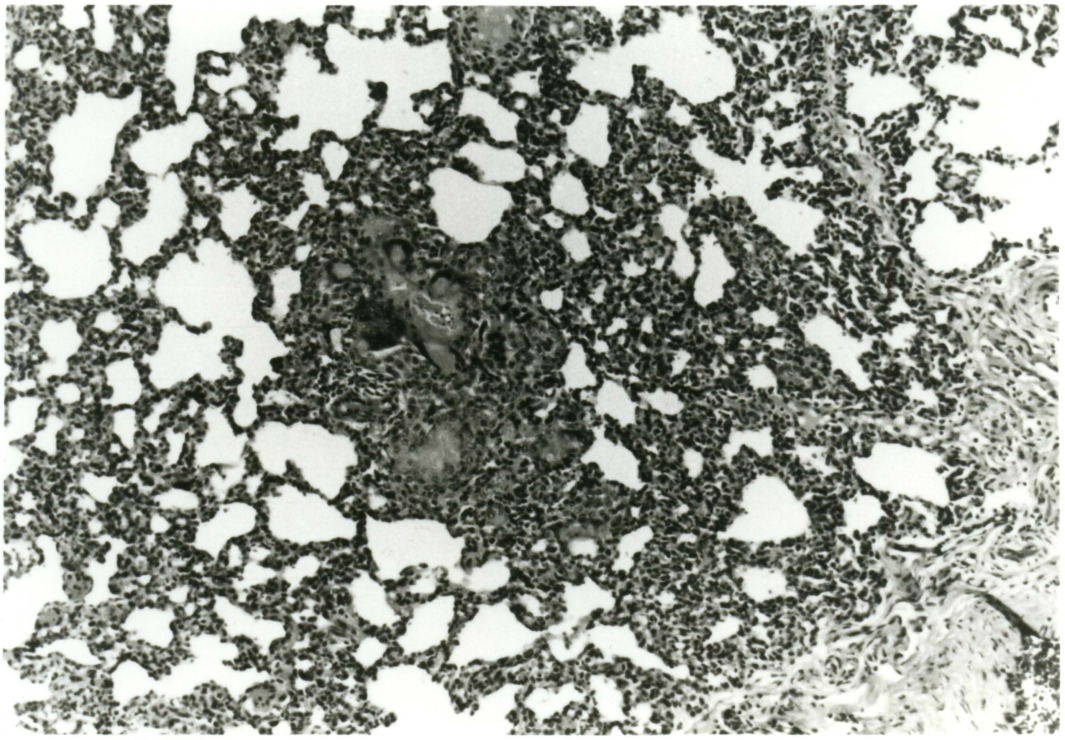


Figure 6. Sections of lungs of control pigs

A - Normal lung (H&E stain) (100 x magnification)

B - Normal lung showing some degree of atelectasis (notice the folding of bronchial mucosa and the thickening of the alveolar walls) (H&E stain) (100 x magnification)

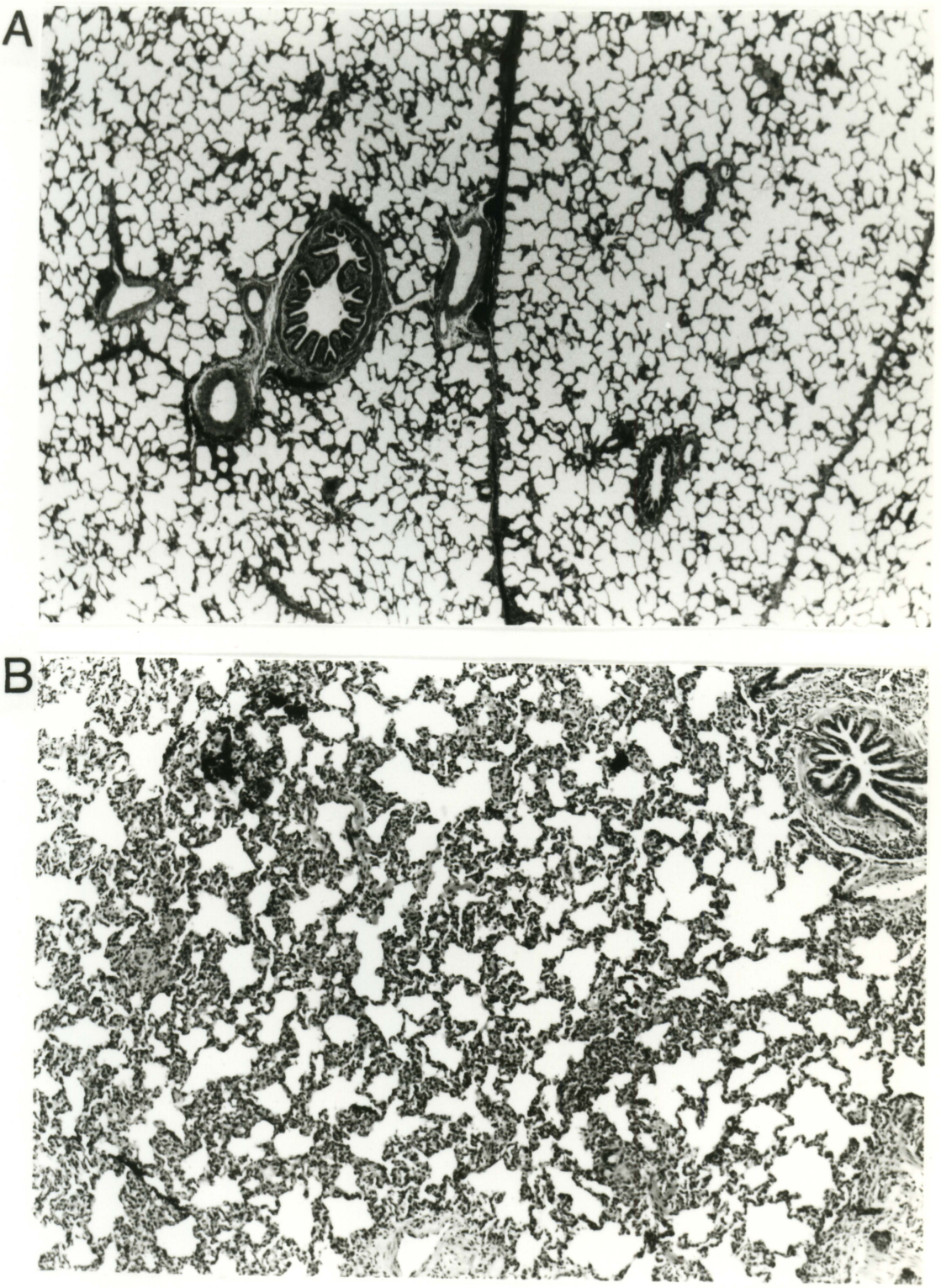

A
B

Table 10. Summary of Bordetella bronchiseptica isolation in three age groups of M. hyopneumoniae contact-exposed pigs

Experimental groups	Total number of pigs	Number of pigs with B. bronchiseptica		
		Lung	Trachea	Nasal cavities
Seeders	9	1	1	4
3[a]	6	0	5	5
6	6	1	3	5
12	6	1	2	1

[a]Weeks of age.

be different ($P > 0.05$). Isolation of M. hyopneumoniae was accomplished only in pigs that had macroscopic lesions of MPS. From 85% of those pigs having macroscopic MPS lesions, M. hyopneumoniae was isolated.

M. hyopneumoniae was isolated from all seeders but not from any of the control pigs.

Effect of atrophic rhinitis lesions and presence of B. bronchiseptica on the severity of MPS

The influence of turbinate atrophy and presence of B. bronchiseptica in the respiratory tract on the severity of MPS was determined by comparing the number of lobes with pneumonic lesions and the gross lesion pneumonia score

Table 11. Summary of M. hyopneumoniae isolation in three age groups of contact-exposed pigs

Experimental groups	Total number of animals	Geometric means of CCU[a] titers of M. hyopneumoniae	Number of pigs culture-positive for M. hyopneumoniae	Number of pigs culture-negative for M. hyopneumoniae	Chi-square
Seeders	9	171	9	0	
3[b]	6	30	3	3	2.270*
6	6	390	3	3	
12	6	80	4	2	

[a]CCU - color change units.

[b]Weeks of age.

*P > 0.05.

between two groups of contact-exposed pigs formed by the presence or absence of the considered factors. Analysis revealed that B. bronchiseptica and turbinate atrophy had no influence on the occurrence and severity of gross lesions in lungs of contact-exposed pigs (Tables 12 and 13; $P > 0.05$).

Immunofluorescence examination of cryostat sections of lungs

Three procedures were utilized to detect M. hyopneumoniae antigen in frozen sections of lung; DFA and IFA with azo-dye counterstain and IFA test without counterstain.

Mycoplasmal antigen was detected on the surface of bronchi and bronchioli by means of all the procedures as a granular or thin coating of bright-yellow fluorescent particles.

A diffuse green nonspecific background fluorescence was observed in lung sections with the IFA test without counterstain (Figure 7A). This nonspecific fluorescence was eliminated almost completely by adsorption of M. hyopneumoniae antiserum with swine liver powder and by the utilization of azo-dye counterstain (Figure 7B).

Tables 14 and 15 summarize the results of the immuno-fluorescence tests obtained with the three age groups of pigs. There were no differences in the occurrence of M. hyopneumoniae antigen and the intensity of fluorescence in

Table 12. Influence of nasal infection with B. bronchiseptica and turbinate atrophy in M. hyopneumoniae contact-exposed pigs on the number of lobes with gross lesions of pneumonia

Factor considered	Total number of pigs	Number of pigs in pooled categories of number of lobes with pneumonia				Chi-square
		0	1-2	3-4	5-7	
Presence of B. bronchiseptica	13	4	5	1	3	1.23*
Absence of B. bronchiseptica	5	2	1	1	1	
Presence of turbinate atrophy	13	4	5	2	2	1.53*
Absence of turbinate atrophy	5	1	1	1	2	

*P > 0.05.

Table 13. Influence of nasal infection with B. bronchiseptica and turbinate atrophy in contact-exposed pigs on the severity of gross lesions of pneumonia

Factor considered	Total number of pigs	Number of pigs in pooled categories of gross lesion scores			Chi-square
		7	8-12	12-15	
Presence of B. bronchiseptica	13	4	5	4	0.548*
Absence of B. bronchiseptica	5	2	1	2	
Presence of turbinate atrophy	13	4	5	4	0.548*
Absence of turbinate atrophy	5	2	1	2	

*P > 0.05.

Figure 7. Cryostat lung sections of contact-exposed pig stained by IFA

A - Cryostat section without counterstain (notice the thin coating of M. hyopneumoniae antigen on bronchial epithelial surface (80 x magnification)

B - Similar section with counterstain. Notice the red background provided by the counterstain (80 x magnification)

A

B

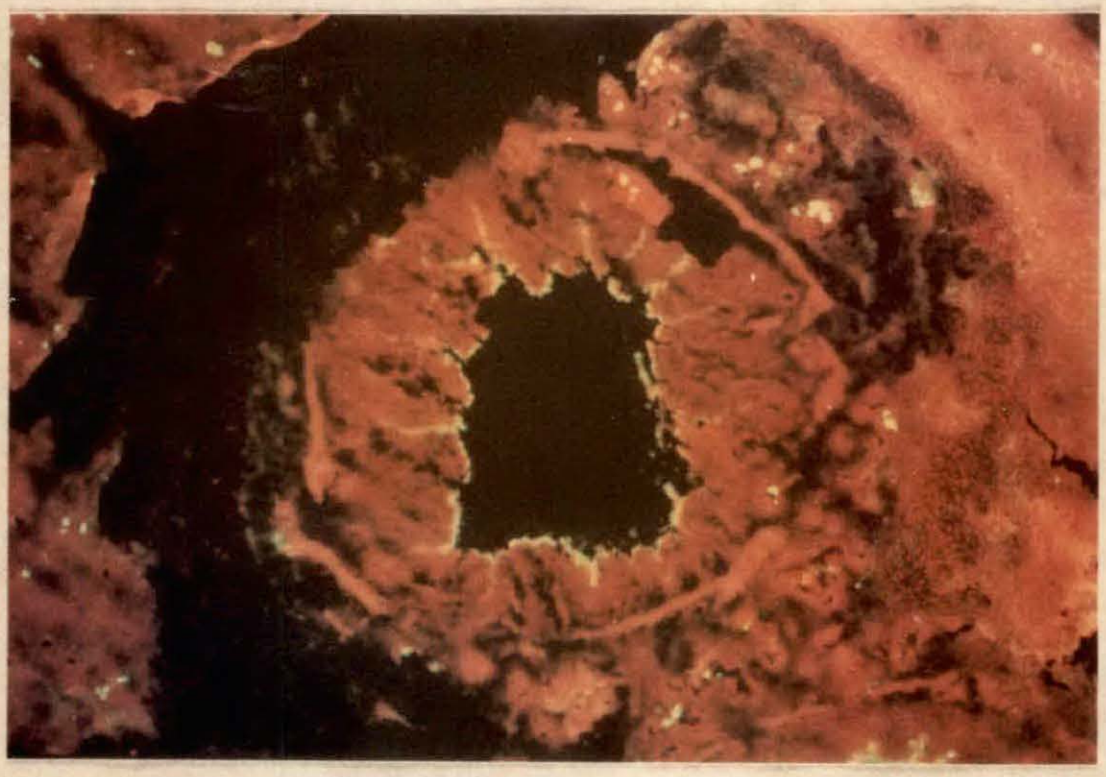


Table 14. Number of contact-exposed pigs immunofluorescence-positive for M. hyopneumoniae in three age groups

Immunofluorescence procedure	Total number of pigs in each group	Number of pigs positive in experimental groups			Chi-square
		3[a]	6[b]	12[c]	
DFA	6	3	4	5	0.830*
IFA without counterstain	6	4	4	5	0.320*
IFA with counterstain	6	3	4	5	0.830*

[a]Three weeks of age.

[b]Six weeks of age.

[c]Twelve weeks of age.

*$P > 0.05$.

cryostat lung sections from the three age groups of pigs ($P > 0.05$).

Except for one lobe from a seeder pig, specific fluorescence was seen only in bronchi and bronchioli of lungs that had gross lesions indicative of MPS. No specific fluorescence was observed in control pigs.

Tables 16 and 17 present a comparison of the immunofluorescence techniques used. Analysis indicated that DFA, IFA with counterstain, and IFA without counterstain were equally sensitive for detection of mycoplasmal antigen.

Table 15. Immunofluorescence (DFA) intensity scores for lung from three age groups of contact exposed pigs

Experimental groups	Total number of pigs	Number of pigs in graded categories of fluorescence		Chi-square
		1-2[a]	3-4	
3[b]	6	3	0	5.63*
6	6	1	3	
12	6	1	4	

[a]1 = Scattered granular green yellow fluorescent particles lining the bronchi with no fluorescence in bronchioli.
2 = Thin coating of yellow green particles lining the bronchi with no bronchiolar fluorescence.
3 = Thin coating of yellow green particles lining the bronchi but with green yellow fluorescent granules lining the bronchioli.
4 = Continuous thin coating of yellow green fluorescent particles lining bronchi and bronchioli.

[b]Weeks of age.

*P > 0.05.

Table 16. Comparison of DFA and IFA procedures for detection of M. hyopneumoniae antigen in lungs of contact-exposed pigs

Total number of contact-exposed pigs	Number of lobes examined	Number of lobes DFA positive	Number of lobes IFA positive (without counterstain)	Number of lobes IFA positive (with counterstain)	Chi-square
18	31	14	15	14	0.709*

*P > 0.05.

Table 17. Comparison of intensity of fluorescence obtained in lungs of contact-exposed pigs with the DFA and IFA procedures

Immunofluorescence procedure	Total number of pigs	Total number of FA-positive lobes	Number of lobes in graded categories of fluorescence				Chi-square
			1[a]	2	3	4	
DFA with counterstain	18	15	2	3	9	1	8.316*
IFA without counterstain	18	15	3	4	4	4	
IFA with counterstain	18	14	1	7	5	1	

[a]1 = Scattered granular green yellow fluorescent particles lining the bronchi with no fluorescence in bronchioli.
2 = Thin coating of yellow green particles lining the bronchi with no bronchiolar fluorescence.
3 = Thin coating of yellow green particles lining the bronchi but with green yellow fluorescent granules lining the bronchioli.
4 = Continuous thin coating of yellow green fluorescent particles lining bronchi and bronchioli.

*$P > 0.05$.

Furthermore, no significant differences were observed among the three techniques in the intensity of immunofluorescence obtained ($P > 0.05$).

The counterstain facilitated interpretation of the immunofluorescence tests virtually eliminating the non-specific green background and providing a marked contrast between the red color from the counterstain and the bright-yellow-green color of the specific fluorescence.

Complement fixation (CF) test

Complement-fixing (CF) antibodies against M. hyopneumoniae were first detected in sera from seeder pigs (Table 18) two weeks after inoculation.

These antibodies were detected in all seeders from the second to the seventh week after inoculation when they were euthanized. The highest CF antibody titers were obtained from 3 to 7 weeks postinoculation (Figure 8).

Table 19 summarizes CF antibody responses to M. hyopneumoniae in the three age groups of contact-exposed pigs. CF antibodies were detected first at three weeks after beginning of contact with seeder pigs. The maximum number of CF-positive contact-exposed pigs occurred at 4 weeks after contact had begun. Appearance and disappearance of CF titer as a function of time was observed in almost all contact pigs.

Table 18. Complement-fixing antibody titers in sera from M. hyopneumoniae-inoculated pigs (seeders)

Pig number	Weeks after inoculation							
	0	1	2	3	4	5	6	7
486	<4[a]	<4	64	128[b]	64	128	128	128
487	<4	<4	64	128	64	64	64	32
488	<4	<4	64	128	128	128	128	64
489	<4	<4	32	128	128	128	128	64
490	<4	<4	16	64	128	128	128	128
491	<4	<4	32	64	128	128	128	64
492	<4	<4	32	128	128	128	128	128
493	<4	<4	64	128	32	32	64	128
494	<4	<4	32	64	128	64	128	128

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

[b]Highest dilution tested.

Table 19. Titers of complement-fixing antibody M. hyopneumoniae of three age groups of contact-exposed pigs

Pig number	Experimental groups	Weeks after exposure to seeders						
		0	1	2	3	4	5	6
111	3[a]	<4[b]	<4	<4	32	64	32	<4
112		<4	<4	<4	<4	<4	<4	8
113		<4	<4	<4	32	32	<4	<4
114		<4	<4	<4	<4	<4	<4	<4
115		<4	<4	<4	<4	<4	<4	<4
116		<4	<4	<4	32	<4	<4	<4

[a]Weeks of age.

[b]Titers are expressed as reciprocals of highest dilution of sera in which 70% or more of complement was fixed.

Table 19 (Continued)

Pig number	Experimental groups	Weeks after exposure to seeders 0	1	2	3	4	5	6
310	6	<4	<4	<4	<4	16	<4	4
311		<4	<4	<4	<4	<4	<4	<4
312		<4	<4	<4	16	16	<4	16
314		<4	<4	<4	<4	<4	<4	<4
315		<4	<4	<4	<4	<4	<4	<4
316		<4	<4	<4	<4	<4	<4	<4
576	12	<4	<4	<4	<4	<4	<4	<4
580		<4	<4	<4	<4	<4	<4	16
582		<4	<4	<4	<4	32	<4	16
583		<4	<4	<4	32	16	<4	<4
584		<4	<4	<4	<4	<4	<4	<4
585		<4	<4	<4	32	32	<4	<4

Analysis revealed no differences in the proportion of pigs in three age groups that developed CF antibodies to M. hyopneumoniae (Table 20). The highest CF antibody titers among the contact-exposed pigs were detected from 3 to 4 weeks after contact had begun (Figure 9). Contact-exposed pigs had a titer approximately 1:30 that of seeders (Figures 8 and 9).

Table 21 compares the relationship between detection of CF antibody to M. hyopneumoniae at different intervals after contact with isolation of M. hyopneumoniae from lungs of pigs at necropsy. No significant relationship was detected at 5 and 6 weeks after contact began. M. hyopneumoniae occurrence was significantly higher than CF antibodies in sera ($P < 0.05$).

Figure 8

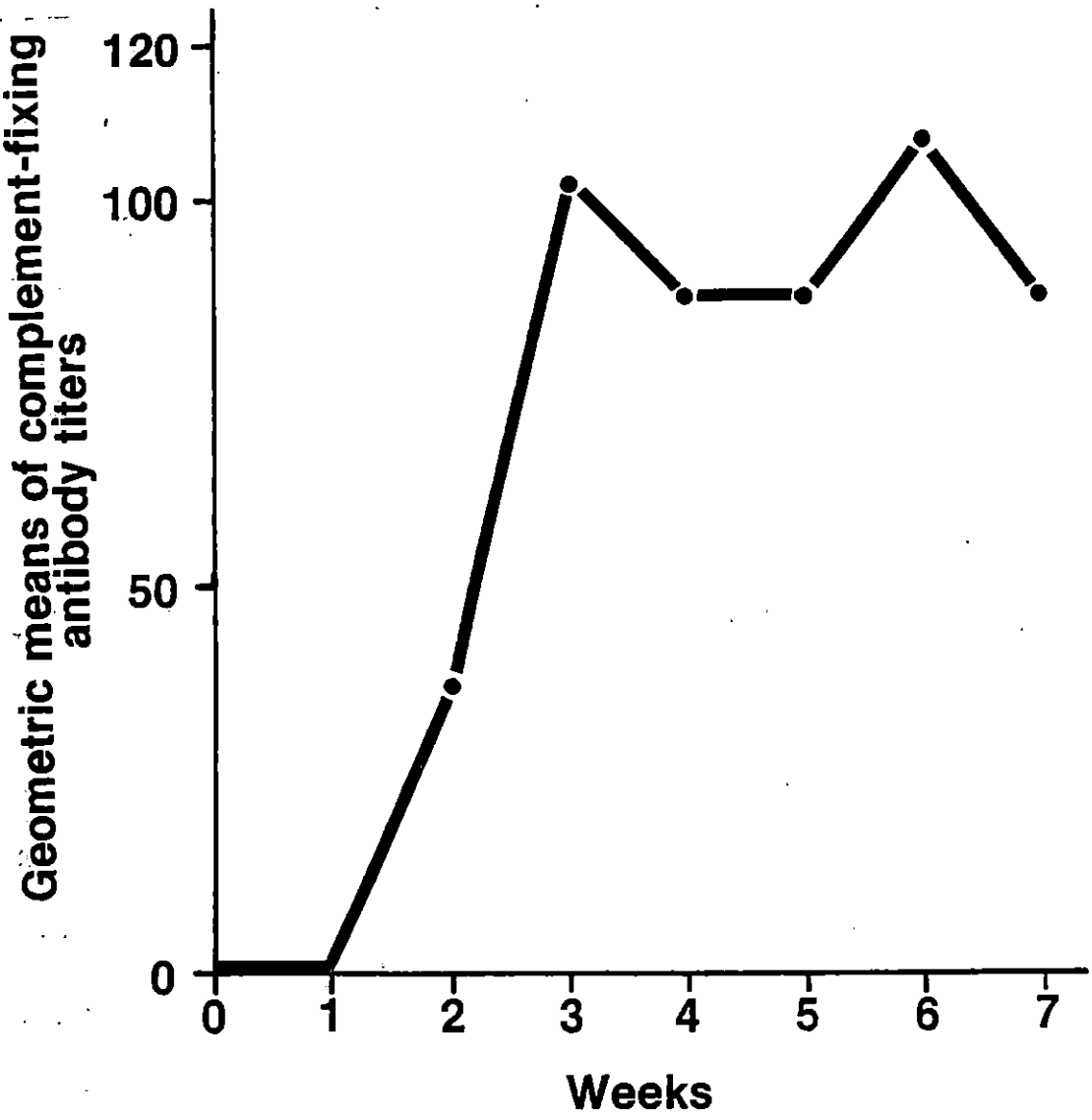


Figure 8. Relationship between titer of complement-fixing antibody and time interval after inoculation with M. hyopneumoniae

Table 20. Complement-fixing M. hyopneumoniae-antibody response of three age groups of contact-exposed pigs

Experimental groups	Total number of pigs	Number of pigs CF positive	Number of pigs CF negative	Chi-square
3[a]	6	4[b]	2	1.802*
6	6	2	4	
12	6	4	2	

[a]Weeks of age.

[b]Pigs which had detectable CF antibodies at least 1 time.

*P > 0.05.

Table 21. Relationship between detection of complement-fixing (CF) antibodies at different intervals after contact-exposure and the presence of M. hyopneumoniae in lung tissue at necropsy

Number of pigs	Weeks after contact began	Number of M. hyopneumoniae positive pigs[a] at necropsy	Number of CF-positive pigs	Chi-square
18	3	12	6	2.777*
18	4	12	7	1.777*
18	5	12	1	11.250**
18	6	12	5	4.012**

[a]DFA-positive or M. hyopneumoniae isolated at necropsy 6 weeks after exposure began.

*P > 0.05.

**P < 0.05.

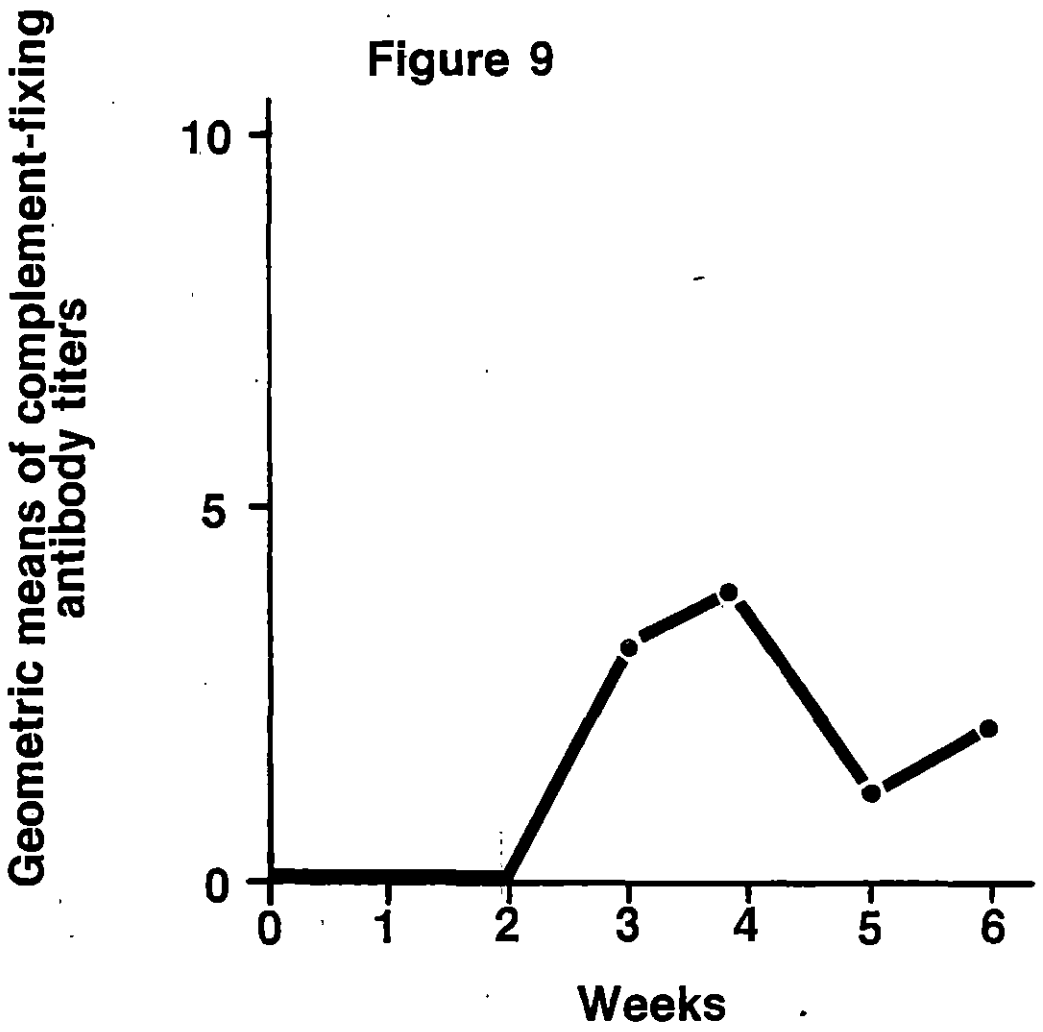


Figure 9. Relationship between complement-fixing M. hyopneumoniae-antibody titers and time interval in contact-exposed pigs after contact began

Enzyme-linked immunosorbent antibody assay (ELISA) test

ELISA antibodies against M. hyopneumoniae were detected one week post inoculation in sera of 1 seeder pig (Tables 22 and 23), using the three standard deviation criterion and of 7 pigs using the discriminant analysis criterion.

The discriminant analysis criterion classified all seeders as positive from 2 weeks after inoculation until they were euthanized (Table 23). Using the three standard deviation criterion, 7 of 9 were positive at 3 weeks PI and 9 of 9 positive at 4 weeks PI (Table 22). ELISA titers appeared to be highest at 5 to 6 weeks after inoculation (Figure 10).

Tables 24 and 25 summarize the ELISA antibody response to M. hyopneumoniae in contact-exposed pigs. According to the three standard deviation criterion (Table 24), ELISA antibody was present in 3 of the 8 pigs one week after beginning of the contact period. Using the same criterion, the maximum number of ELISA-positive pigs (10 of 18) occurred at five weeks after exposure to seeders. The highest ELISA titer among these pigs was observed at the 4th week after exposure to seeders (Figure 10). Contact-exposed pigs had ELISA titer approximately 1:20 that of seeders (Figures 10 and 11).

According to the discriminant analysis criterion (Table 25), ELISA antibody-positive animals (4 of 18) occurred

Table 22. ELISA titers in sera from M. hyopneumoniae-inoculated pigs (seeders) using three standard deviation criterion

Pig number	Weeks after inoculation							
	0	1	2	3	4	5	6	7
486	<40[a]	<40	80	160	320	640	640	640
487	<40	<40	<40	320	640	320	320	80
488	<40	320	320	640	640	640	1280[b]	1280
489	<40	<40	160	160	160	1280	640	320
490	<40	<40	<40	<40	320	640	1280	320
491	<40	<40	<40	320	320	640	640	640
492	<40	<40	<40	<40	160	320	320	320
493	<40	<40	160	320	320	640	320	160
494	<40	<40	<40	320	320	320	320	640

[a]Titers are expressed as reciprocals of the highest dilution of sera by the three standard deviation criterion.

[b]Highest dilution tested.

Table 23. ELISA results obtained with sera of M. hyopneumonia-inoculated pigs (seeders) using the discriminant analysis criterion

Pig number	Weeks after inoculation							
	0	1	2	3	4	5	6	7
486	+	+	+	+	+	+	+	+
487	-	+	+	+	+	+	+	+
488	-	+	+	+	+	+	+	+
489	-	+	+	+	+	+	+	+
490	-	-	+	+	+	+	+	+
491	-	+	+	+	+	+	+	+
492	-	-	+	+	+	+	+	+
493	-	+	+	+	+	+	+	+
494	-	+	+	+	+	+	+	+

Figure 10

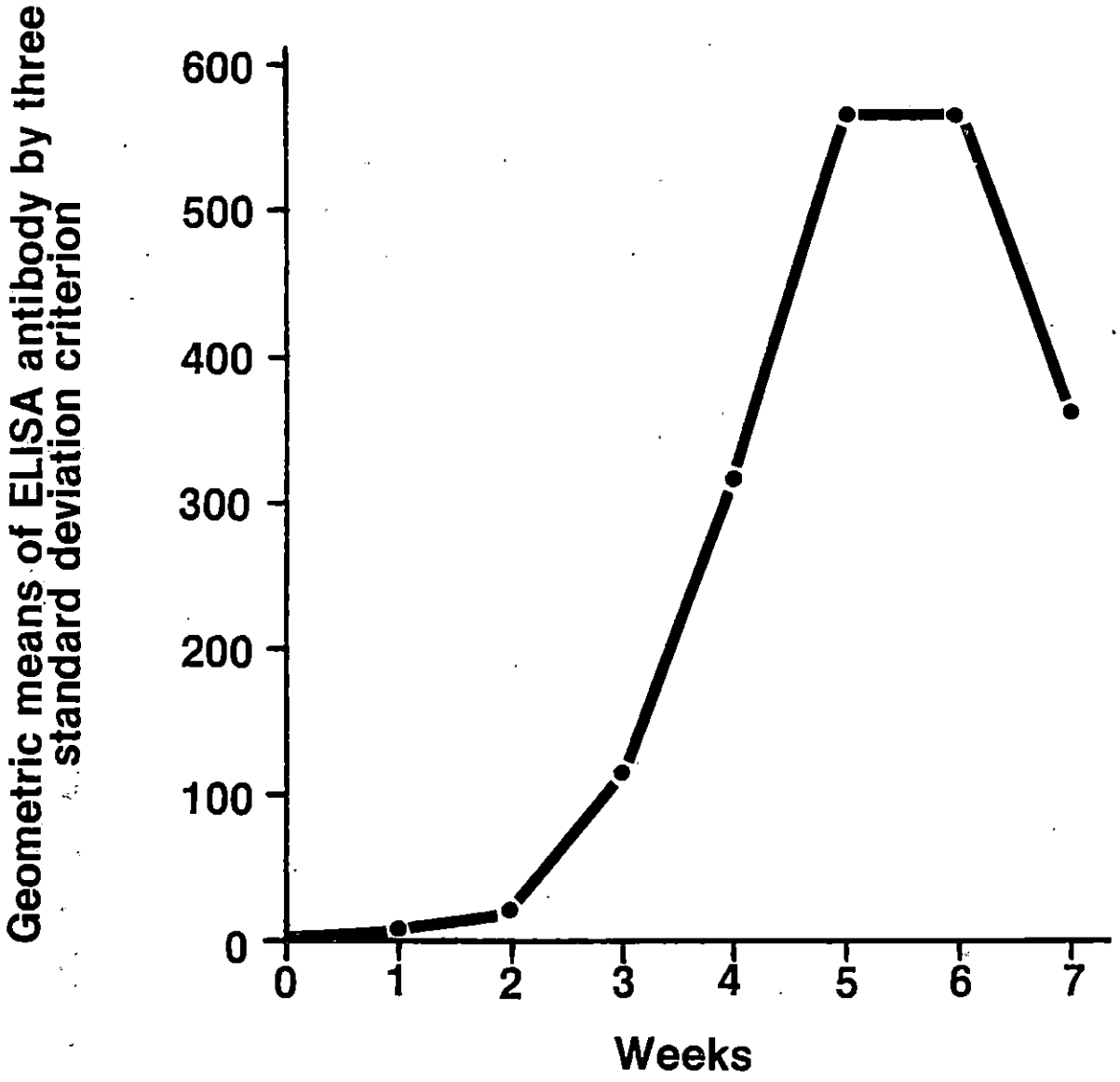


Figure 10. Relationship between ELISA titer and time interval after inoculation with M. hyopneumoniae

Table 24. ELISA titers against M. hyopneumoniae in three age groups of contact-exposed pigs using the three standard deviation criterion

Pig number	Experimental groups	0	1	2	3	4	5	6
111	3[a]	<40[b]	<40	40	80	80	160	80
112		<40	<40	<40	<40	<40	40	80
113		<40	<40	<40	<40	<40	<40	ND
114		<40	<40	<40	<40	40	<40	40
115		<40	<40	<40	<40	<40	<40	ND
116		<40	<40	<40	<40	<40	<40	ND
310	6	<40	<40	<40	40	<40	40	80
311		<40	<40	320	<40	80	<40	<40
312		<40	<40	<40	<40	<40	40	40
314		<40	<40	640	160	640	160	ND
315		<40	160	<40	40	160	<40	ND
316		<40	160	160	160	640	160	ND
576	12	<40	<40	160	160	160	40	ND
580		<40	<40	<40	<40	<40	160	ND
582		<40	<40	<40	<40	40	40	40
583		<40	<40	<40	<40	<40	<40	<40
584		<40	<40	<40	<40	<40	<40	ND
585		<40	160	160	160	160	160	ND

[a]Weeks of age.

[b]Titers are expressed as reciprocals of highest dilution of sera positive by the three standard deviation criterion.

Table 25. ELISA titers against M. hyopneumoniae detected in three age groups of contact-exposed pigs using the discriminant analysis criterion

Pig number	Experimental group	Weeks after exposure to seeders 0	1	2	3	4	5	6
111	3[a]	-	-	+	+	+	+	+
112		-	-	-	+	+	+	+
113		-	-	-	+	+	+	ND
114		-	-	-	+	+	-	+
115		-	-	-	-	-	+	ND
116		-	-	-	+	+	+	ND

[a]Weeks of age.

Table 25 (Continued)

Pig number	Experimental groups	Weeks after exposure to seeders 0	1	2	3	4	5	6
310	6	-	-	+	+	+	+	+
311		+	-	+	+	+	-	+
312		-	-	-	+	+	+	+
314		-	-	-	-	+	-	ND
315		-	-	-	+	-	-	ND
316		-	-	-	+	+	-	ND
576	12	-	-	-	+	+	+	ND
580		-	-	-	-	-	+	ND
582		-	-	-	-	+	+	+
583		-	-	-	-	-	-	+
584		-	-	-	-	-	+	ND
585		-	-	+	+	+	+	ND

first at the second week after beginning of contact. The maximum number of ELISA-positive pigs (13 of 18) observed among the contact-exposed pigs was at the 4th week.

A comparison of proportions of ELISA-positive pigs among the three age groups of pigs is presented in Table 26. Analysis indicated there were no differences among the age groups in development of ELISA-positive status ($P > 0.05$). In fact, discriminant analysis criterion indicated that all pigs seroconverted.

Table 27 compares the occurrence of ELISA antibodies and detection of M. hyopneumoniae in lung tissue. From 3 to 6 weeks after the beginning of contact-exposure,

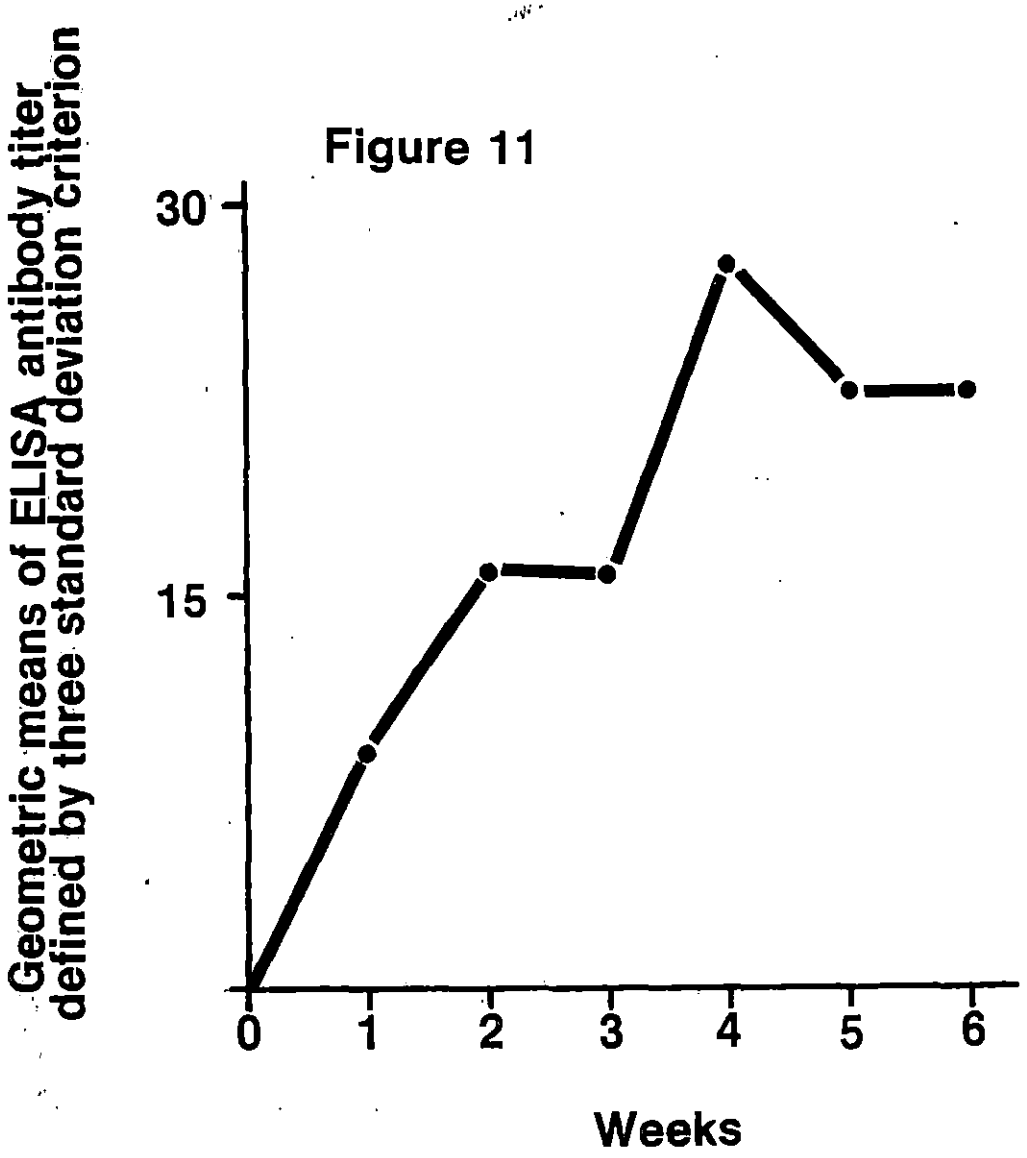


Figure 11. Relationship between ELISA M. hyopneumoniae-antibody titers and time interval in contact-exposed pigs

Table 26. ELISA against M. hyopneumoniae in three age groups of contact-exposed pigs

Experimental groups	Total number pigs	Number of ELISA[a] positive pigs	Chi-square	Number of ELISA[b] positive pigs	Chi-square
3[c]	6	3[d]	3.868*	6	0.0*
6	6	6		6	
12	6	4		6	

[a]Positive pigs defined by three standard deviation criterion.

[b]Positive pigs defined by discriminant analysis.

[c]Weeks of age.

[d]Pigs which had an ELISA titer at least once during the observation period.

*P > 0.05.

Table 27. Relationship between detection of ELISA antibodies at different intervals after contact-exposure and the presence of M. hyopneumoniae in lung tissue at necropsy

Total number of pigs	Weeks after contact began	Number of M. hyopneumoniae positive pigs[a] at necropsy	Number of ELISA[b] positive pigs	Chi-square	Number of ELISA[c] positive pigs	Chi-square
18	3	12	7	1.783*	12	0.0*
18	4	12	9	0.457*	13	0.0*
18	5	12	10	0.116*	12	0.0*
8	6	6	6	0.0*	8	0.571*

[a]DFA-positive or M. hyopneumoniae isolated at necropsy 6 weeks after exposure began.

[b]ELISA-positive pigs defined by three standard deviation criterion.

[c]ELISA-positive pigs defined by the discriminant analysis criterion.

*$P > 0.05$.

there were no differences between these two factors ($P > 0.05$).

Experiment B

Clinical signs

Coughing was first observed from 11 to 20 days after inoculation. Increased coughing was noted after forced exercise.

Contact-exposed and sentinel pigs were never observed coughing.

Necropsy findings

Macroscopic lesions characteristic of MPS, as described in Experiment A, were observed in all groups of pigs.

Numbers of pigs with gross lesions of pneumonia are presented in Table 28. No differences in the proportion of pigs with gross lesions were detected in the 3-week-old and 11-week-old groups of pigs ($P > 0.05$).

The severity of pneumonia in the two age groups of pigs was evaluated by categorizing the number of lobes with pneumonia (Table 29). Analysis of the distribution of pigs in these categories revealed no differences among the two age groups of pigs ($P > 0.05$).

Table 30 summarizes the gross lesion severity scores of the two age groups of pigs as defined in Experiment A

Table 28. Severity of pneumonia in two age groups of contact-exposed pigs: Number of pigs with gross lesions of pneumonia

Experimental groups	Number of pigs	Number of pigs with pneumonia	Number of pigs without pneumonia	Chi-square
3[a]	6	4	2	2.400*
11	6	6	0	

[a]Weeks of age.

*$P > 0.05$.

Table 29. Severity of pneumonia in two age groups of contact-exposed pigs: Number of lobes with gross lesions of pneumonia

Experimental groups	Total number of pigs	Number of pigs in pooled categories of number of lobes with pneumonia				Chi-square
		0 lobes	1-2 lobes	3-4 lobes	5-7 lobes	
3[a]	6	2	1	0	3	2.57*
11	6	0	2	1	3	

[a]Weeks of age.

*$P > 0.05$.

Table 30. Severity of pneumonia in two age groups of contact-exposed pigs: Gross lesion severity score

Experimental groups	Total number of pigs	Number of pigs in categories of gross lesion severity score			Chi-square
		7	8-11	12-16	
3	6	2	1	3	2.46*
11	6	0	2	4	

[a]Weeks of age.

*P > 0.05.

(page 97). No differences were detected among the two age groups of pigs ($P > 0.05$).

Necropsy findings for seeder pigs used in Experiment B are presented in Table 31. All lung lesions were typical of MPS.

Gross lesions of pneumonia were observed in all lobes of all experimental groups. No significant differences were found in the occurrence of MPS lesions among the different lobes (Table 32).

Edema of bronchial lymph nodes was observed in two seeder pigs, in two pigs from the 3-week-old group and in 3 from the 11-week-old group.

Mild turbinate atrophy was observed in 3 of the 12 contact-exposed pigs.

No macroscopic lesions were found in control and in 5 of the 6 sentinel pigs. One sentinel pig had macroscopic lesions suggestive of MPS but histological examination revealed only a moderate degree of atelectasis.

Histopathology

Histopathological lesions characteristic of acute (early), acute (late) and subacute stages of MPS were found and summarized in Table 33. Acute late stage MPS was the most common stage among the contact-exposed pigs. Acute early lesions were observed in 3 pigs from the 3-week-age

Table 31. Necropsy findings in M. hyopneumoniae-inoculated pigs used as seeders in Experiment B

Pig number	Pneumonia severity score	Enlargement of bronchial lymph nodes	Turbinate atrophy score
62	12[a]	1[b]	0/0[c]
63	14	2	1/1
64	13	1	1/1
65	10	1	0/0
66	7	1	0/0
69	13	2	0/0

[a]Each normal lobe received a score of 1, hence a completely normal pig received a score of 7. A total score above 7 indicates that lesions were observed. Higher scores indicated more lung tissue was involved.

[b]1 = No gross enlargement of bronchial lymph nodes.
2 = Moderate enlargement of bronchial lymph nodes.

[c]0 = No atrophy of the ventral turbinate was observed.
1 = Mild atrophy of ventral scroll of the ventral turbinate.

Table 32. Distribution of lesions of pneumonia among the lobes in lungs of seeder and contact-exposed pigs

Experimental animals	Number of pigs	Total number of lobes	Number of each lobe with lesions of pneumonia							Chi-square
			LA[a]	LC[b]	LD[c]	RA[d]	RC[e]	RD[f]	I[g]	
Seeders	6	42	2	3	3	3	4	4	5	3.86*
Pigs exposed by contact	12	84	7	3	7	6	4	7	5	5.235*

[a]Left apical lobe.

[b]Left cardiac lobe.

[c]Left diaphragmatic lobe.

[d]Right apical lobe.

[e]Right cardiac lobe.

[f]Right diaphragmatic lobe.

[g]Intermediate lobe.

*$P > 0.05$.

Table 33. Severity of pneumonia in three age groups of contact-exposed pigs: Histopathologic classification of MPS lesions in lungs

Experimental groups	Total number of pigs	Number of pigs within histopathologic stages of MPS categories					
		AE[a]	AL[b]	AE+AL	SA[c]	AL+SA	AE+AL+SA
Seeders	6	0	1	0	1	3	1
3[d]	6	3	1	2	0	0	0
11	6	0	2	3	0	0	1

[a]Acute (early) stage.

[b]Acute (late) stage.

[c]Subacute stage.

[d]Weeks of age.

group. Only one of these 3 pigs had macroscopic lesions of MPS. Furthermore, from 9 pigs having acute early stage-MPS, only 4 lobes of 12 showing this stage of MPS had gross lesions of pneumonia. Different stages of MPS occurred in 6 pigs.

The effect of age on the number of lobes with microscopic MPS lesions and on the severity of microscopic lesions are presented in Tables 34 and 35. Evaluation of the distribution of pigs among categories of number of lobes affected by MPS and among graded categories of severity of microscopic lesions revealed no differences between the two age groups of pigs ($P > 0.05$).

In one control pig, very mild acute, early stage lesions of MPS were observed but, macroscopic lesions, M. hyopneumoniae isolation, a positive FA test, and presence of CF and ELISA antibodies were not observed.

Clumping and loss of tracheal cilia was noticed in all observed stages of the disease but, no inflammatory cells were observed in the lamina propria of tracheas.

Focal purulent pneumonia was the only histopathological lesion found besides MPS pneumonia. It was found in a sentinel pig. Free erythrocytes, as in Experiment A, were sometimes observed in alveolar spaces. Alveolar wall thickening, as in Experiment A, was a common feature among

Table 34. Severity of pneumonia in two age groups of contact-exposed pigs: Number of pigs with microscopic lesions of MPS

Experimental groups	Total number of pigs	Number of pigs in pooled categories of number of pneumonia lobes			Chi-square
		1-3	4-5	6-7	
3[a]	6	3	0	3	2.14*
11	6	1	1	4	

[a]Weeks of age.

*P > 0.05.

Table 35. Severity of pneumonia in three age groups of contact-exposed pigs: Histopathologic MPS severity scores

Experimental groups	Total number of pigs	Number of pigs in categories of microscopic MPS severity scores			Chi-square
		1-8	9-16	17-25	
3[a]	6	3	1	2	1.520*
11	6	1	2	3	

[a]Weeks of age.

*P > 0.05.

all groups of pigs.

Isolation of bacteria and mycoplasma from lungs, tracheas and nasal cavities of pigs

Haemophilus parasuis was isolated from preinoculation nasal secretions from one of nine pigs selected to be used as seeders. This H. parasuis-positive pig was eliminated. Six of the remaining 8 pigs were selected to be seeders. Bacteriological examination of these 6 pigs, before contact began, and at necropsy day, revealed no Haemophilus sp., B. bronchiseptica or P. multocida. In addition, these agents were not isolated from nasal cavities of the two age groups of pigs before the beginning of the contact period. Similarly negative results were obtained when samples from nasal cavities, tracheas and lungs of these pigs were cultured at necropsy.

M. hyopneumoniae was the only mycoplasma isolated from lungs (Table 36). Its occurrence among both age groups of contact-exposed pigs appeared not to be different ($P > 0.05$).

M. hyopneumoniae was isolated from all seeders but samples from control and sentinel pigs were negative for this organism as well as M. hyorhinis.

Table 37 summarizes the results of M. hyopneumoniae isolation, DFA and pathological findings from the right and left cardiac lobes of 6 seeder pigs and 12 contact-

Table 36. Summary of M. hyopneumoniae isolation in two age groups of contact-exposed pigs

Experimental groups	Total number of animals	Number of pigs culture-positive for M. hyopneumoniae	Number of pigs culture-negative for M. hyopneumoniae	Chi-square
Seeders	6	6	0	
3[a]	6	3	3	1.777*
11	6	6	0	

[a]Weeks of age.

*P > 0.05.

Table 37. Summary of M. hyopneumoniae isolation, DFA and gross and microscopic findings with the right and left cardiac lobes of two age groups of contact-exposed pigs

Pig numbers	Group of pigs	Left cardiac lobe				Right cardiac lobe			
		ISO[a]	DFA[b]	MACRO[c]	MICRO[d]	ISO	DFA	MACRO	MICRO
62	Seeders	+	+	+	+	+	-	-	+
63		+	-	-	+	+	-	+	+
64		+	+	+	+	+	-	+	+
65		-	-	-	-	+	+	+	+
66		-	-	-	+	+	+	-	+
69		+	+	+	+	+	+	+	+
226	3[e]	-	-	-	-	-	-	-	+
227		+	+	+	+	+	+	+	+
228		+	+	+	+	+	+	+	+
229		-	-	-	+	-	-	-	-
230		-	-	+	+	-	-	-	-
680		+	+	+	+	+	+	+	+
233	11	+	+	+	+	+	+	+	+
511		+	+	+	+	+	-	-	+
513		+	+	+	+	+	+	+	+
515		+	+	+	+	+	+	+	+
516		-	-	-	-	+	-	+	+
517		+	+	+	+	+	-	-	-

[a]Isolation of M. hyopneumoniae.

[b]Direct immunofluorescent test.

[c]Macroscopic MPS lesion.

[d]Microscopic MPS lesion.

[e]Weeks of age.

exposed pigs.

Isolation of M. hyopneumoniae was accomplished mainly from pigs having macroscopic or microscopic lesions of MPS. It was isolated from a right cardiac lobe with no lesions of MPS. The pig from which this right cardiac lobe was cultured had 4 lobes with MPS lesions.

Table 38 summarizes the results of the comparison between isolation of M. hyopneumoniae, direct immunofluorescence, MPS-macroscopic and MPS-microscopic lesions. The procedures did not differ in ability to detect diseased pigs. Isolation procedures used for the two lobes differed. Right cardiac lobe was homogenized while the left cardiac lobe was sampled by mean of a washing technique. Considering the above results, it appears that the two techniques are comparable for isolation of M. hyopneumoniae from lung tissue.

Immunofluorescence examination of cryostat sections of lungs

Cryostat lung sections were examined by means of the same immunofluorescence test described in Experiment A.

Tables 39 and 40 summarize results of immunofluorescence tests with lung from the two age groups of pigs. Occurrence of M. hyopneumoniae antigen and intensity of specific fluorescence in cryostat lung sections did not differ among the two age groups of pigs ($P > 0.05$).

Table 38. Comparison between M. hyopneumoniae isolation, DFA, MPS macroscopic lesions and MPS microscopic lesions from two age groups of contact-exposed pigs

Lobe of lung	Number of lobes evaluated	Number of lobes culture-positive for M. hyopneumoniae	Number of lobes DFA-positive	Number of lobes with macroscopic lesions	Number of lobes with microscopic lesions	Chi-square
Left cardiac	12	7	6	7	10	3.2*
Right cardiac	12	9	6	7	10	2.2*

*P > 0.05.

Table 39. Number of contact-exposed pigs immunofluorescence-positive for M. hyopneumoniae in two age groups

Immuno-fluorescence procedure	Total number of pigs in each group	Number of pigs positive in experimental groups		Chi-square
		3[a]	11[b]	
DFA	6	3	5	1.5*
IFA without counterstain	6	4	5	0.0*
IFA with counterstain	6	4	5	0.0*

[a]Three weeks of age.

[b]Eleven weeks of age.

*P > 0.05.

Table 40. Immunofluorescence (DFA) intensity scores for lung from two age groups of contact-exposed pigs

Experimental groups	Total number of pigs	Number of pigs in graded categories of fluorescence		Chi-square
		1-2[a]	3-4	
3[b]	6	0	3	0.076*
11	6	1	4	

[a]1 = Scattered granular green yellow fluorescent particles lining the bronchi with no fluorescence in bronchioli.
2 = Thin coating of yellow green particles lining the bronchi with no bronchiolar fluorescence.
3 = Thin coating of yellow green fluorescent particles lining bronchi and bronchioli.
4 = Continuous thin coating of yellow green fluorescent particles lining bronchi and bronchioli.

[b]Weeks of age.

*P > 0.05.

Specific fluorescence with the DFA test was seen only in bronchi and bronchioli of lobes which had gross lesions of MPS (Table 37). In one case, an IFA-positive test was obtained with lung from a pig with microscopic lesions of MPS but without gross lesions. The pig was CF, ELISA and culture-negative for M. hyopneumoniae.

Tables 41 and 42 summarize the results of comparisons of the immunofluorescence techniques. No differences were detected among DFA, IFA with counterstain or IFA without counterstain in detecting mycoplasmal antigen. Furthermore, no differences were observed in intensity among the techniques ($P > 0.05$).

Complement fixation (CF) test

Complement-fixing (CF) antibodies against M. hyopneumoniae were detected first in sera from seeder pigs at 3 weeks PI (Table 43). In contrast to Experiment A, CF antibodies were detected consistently in all seeders only at 6 and 7 weeks after inoculation. In addition, the highest titer was obtained with a sample collected 6 weeks after inoculation (Figure 12).

Table 44 summarizes CF antibody responses to M. hyopneumoniae in the two age groups of pigs exposed by contact. CF antibodies were first detected 5 weeks after the beginning of contact. At this time, 3 of 12 pigs had

Table 41. Comparison of DFA and IFA procedures for detection of M. hyopneumoniae antigen in lungs of contact-exposed pigs

Total number of contact-exposed pigs	Number of lobes examined	Number of lobes DFA positive	Number of lobes IFA positive (without counterstain)	Number of lobes IFA positive (with counterstain)	Chi-square
12	25	15	16	16	0.041*

*P > 0.05.

Table 42. Comparison of intensity of fluorescence obtained in lung of contact-exposed pigs with the DFA and IFA procedures

Immunofluorescence procedures	Total number of pigs	Total number of FA-positive lobes	Number of lobes in graded categories of fluorescence				Chi-square
			1[a]	2	3	4	
DFA with counterstain	12	15	2	0	9	4	7.494*
IFA without counterstain	12	16	0	4	8	4	
IFA with counterstain	12	16	2	5	6	3	

[a]1 = Scattered granular green yellow fluorescent particles lining the bronchi with no fluorescence in bronchioli.
2 = Thin coating of yellow green particles lining the bronchi with no bronchiolar fluorescence.
3 = Thin coating of yellow green particles lining the bronchi but with green yellow fluorescent granules lining the bronchioli.
4 = Continuous thin coating of yellow green fluorescent particles lining bronchi and bronchioli.

*$P > 0.05$.

Table 43. Complement-fixing antibody titers in sera from M. hyopneumoniae-inoculated pigs (seeders)

Pig number	Weeks after inoculation							
	0	1	2	3	4	5	6	7
62	<4[a]	<4	32	32	32	32	32	16
63	<4	<4	32	64	64	64	64	64
64	<4	<4	64	32	32	64	128[b]	64
65	<4	<4	<4	<4	8	<4	16	8
66	<4	<4	<4	16	8	<4	8	8
69	<4	<4	32	32	16	64	64	64

[a]Titers expressed as reciprocals of highest dilution of sera in which 70% or more of complement was fixed.

[b]Highest dilution tested.

Table 44. Complement-fixing antibody titers to M. hyopneumoniae in three age groups of contact-exposed pigs

Pig number	Experimental groups	Weeks after exposure to seeders							
		0	1	2	3	4	5	6	7
226	3[a]	<4[b]	<4	<4	<4	<4	<4	<4	<4
227		<4	<4	<4	<4	<4	<4	32	32
228		<4	<4	<4	<4	<4	<4	16	32
229		<4	<4	<4	<4	<4	<4	<4	<4
230		<4	<4	<4	<4	<4	<4	<4	<4
684		ND	<4	<4	<4	<4	<4	16	16
233	11	<4	<4	<4	<4	<4	<4	8	16
511		<4	<4	<4	<4	<4	32	32	32
513		<4	<4	<4	<4	<4	4	16	16
515		<4	<4	<4	<4	<4	<4	32	64
516		<4	<4	<4	<4	<4	<4	<4	<4
517		<4	<4	<4	<4	<4	4	16	<4

[a]Weeks of age.

[b]Titers are expressed as reciprocals of highest dilution of sera in which 70% or more of complement was fixed.

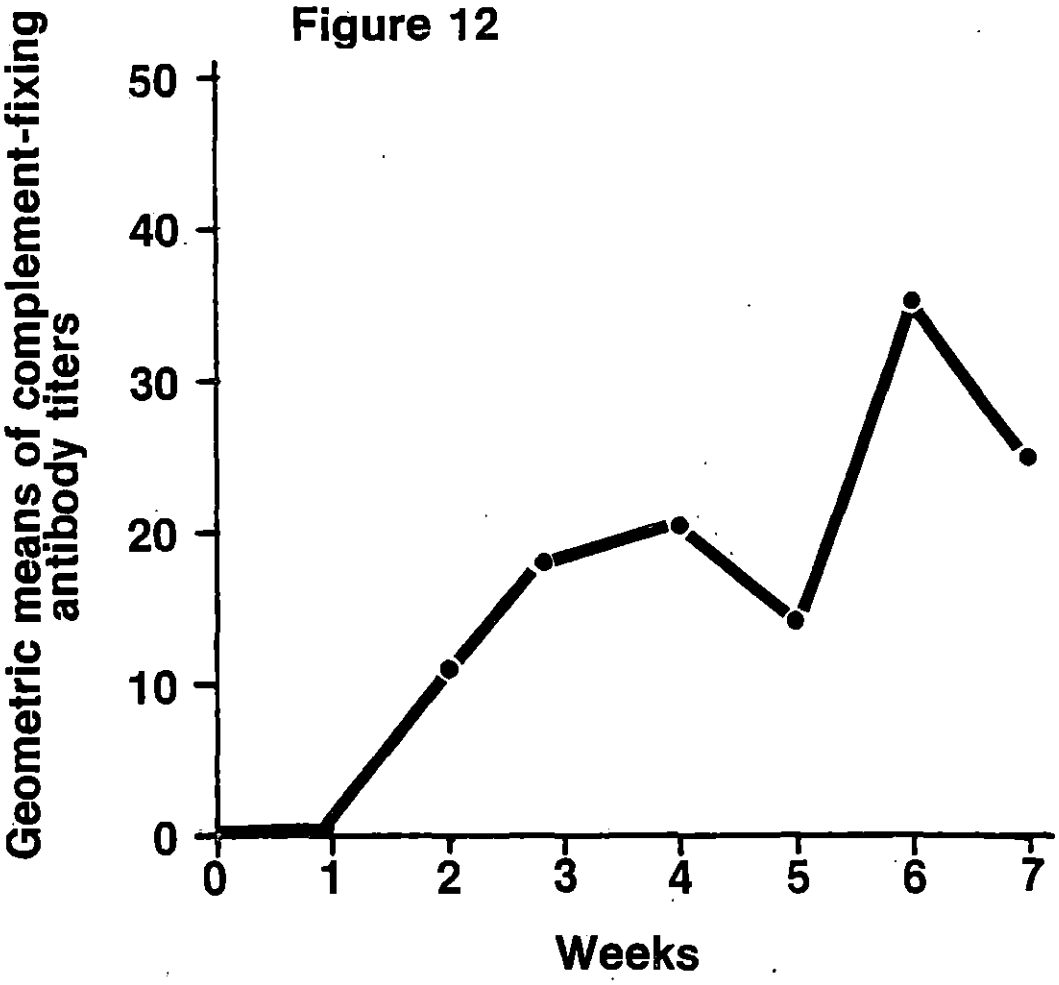


Figure 12. Relationship between titer of complement-fixing antibody and time interval after inoculation with M. hyopneumoniae

seroconverted. The maximum number of CF-positive, contact-exposed pigs observed was at 6 weeks after contact had begun. At this time, 8 of the 12 (66.6%) pigs were positive.

No difference was detected in the proportion of pigs developing CF antibodies in the two age groups ($P > 0.05$, Table 45).

The highest CF titer among the two age groups of pigs was observed 6 weeks after contact had begun (Figure 13). Comparing the highest geometric mean (GM), CF titer from seeders and contact-exposed pigs (Figures 12 and 13), contact-exposed pigs had a GM approximately 1:7 that of seeders. In contrast to Experiment A, appearance and disappearance of a CF titer was observed in only one pig.

Table 46 summarizes the comparison between the occurrence of CF antibodies at various intervals and detection of M. hyopneumoniae in lung tissue at necropsy. From 3 to 5 weeks after beginning of the contact period, there was a significantly higher number of pigs with M. hyopneumoniae in their lungs than CF titers in sera ($P < 0.01$). On the other hand, no difference between the occurrence of these two factors was observed at 6 and 7 weeks after contact had begun ($P > 0.05$).

Table 45. Complement-fixing antibody titers to two age groups of contact-exposed pigs

Experimental groups	Total number of pigs	Number of pigs CF positive	Number of pigs CF negative	Chi-square
3[a]	6	3[b]	3	0.375*
11	6	5	1	

[a]Weeks of age.

[b]Pigs which had detectable CF antibodies at least 1 time.

*P > 0.05.

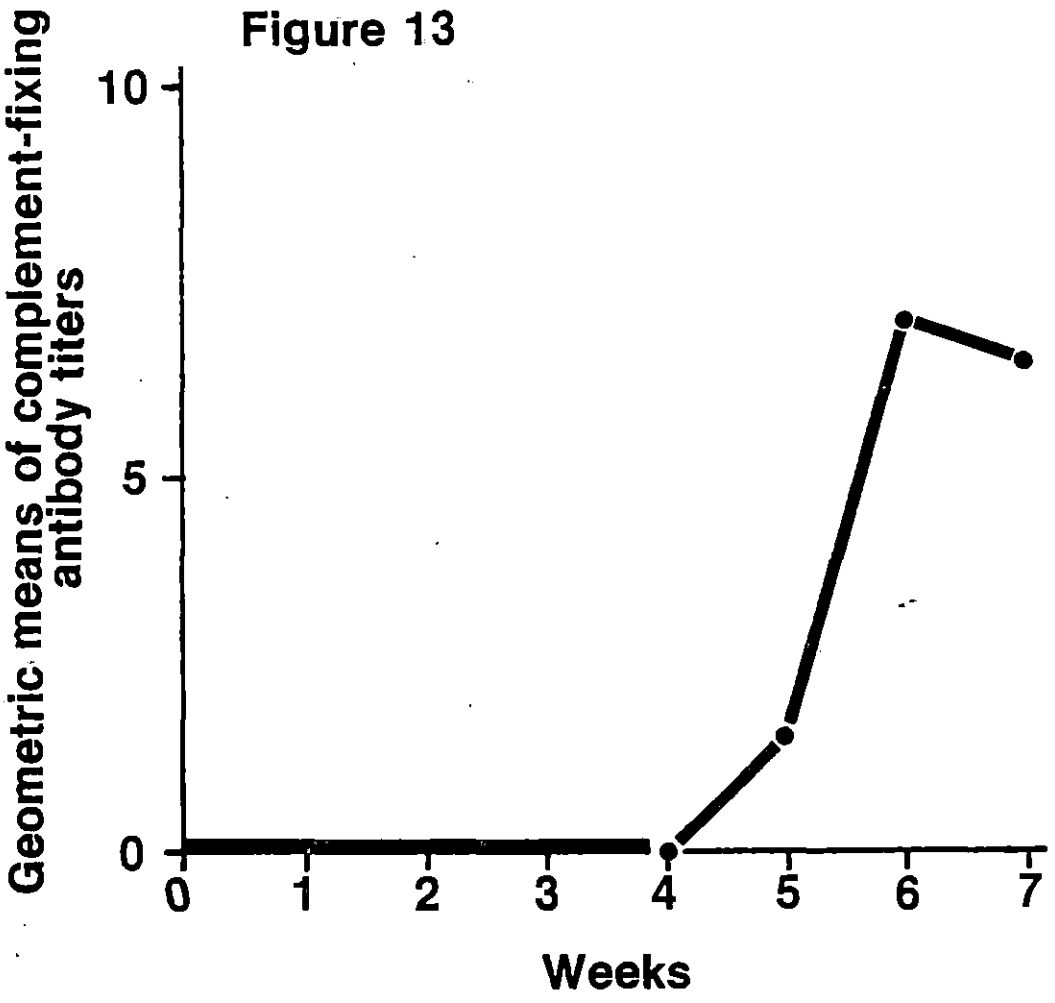


Figure 13. Relationship between complement-fixing antibody titers and time interval in contact-exposed pigs

Table 46. Relationship between detection of complement-fixing (CF) antibodies at different intervals after contact-exposure and the presence of M. hyopneumoniae in lung tissue at necropsy

Number of pigs	Weeks after contact began	Number of M. hyopneumoniae positive pigs[a] at necropsy	Number of CF-positive pigs	Chi-square
12	3	9	0	11.37**
12	4	9	0	11.37**
12	5	9	3	4.16*
12	6	9	8	0.20*
12	7	9	7	0.375*

[a]DFA-positive or M. hyopneumoniae isolated at necropsy 6 weeks after exposure began.

*P > 0.05.

**P < 0.01.

Enzyme-linked immunosorbent antibody assay (ELISA) test

First detection of ELISA antibodies against M. hyopneumoniae in sera from seeders was at 1 week after inoculation (Tables 47 and 48). According to the three standard deviation criterion, only one pig had antibody at this time while the discriminant analysis criterion classified 2 pigs as positive.

The discriminant analysis criterion classified all seeders as positive from the 4th week after inoculation until they were euthanized (Table 48). In contrast, the three standard deviation criterion identified all pigs as positive only at seven weeks after inoculation.

The highest ELISA titer was observed at 7 weeks after inoculation (Figure 14). Comparison revealed little difference between the highest GM of ELISA titer from seeders and from contact-exposed pig (Figure 14 and 15). In addition, the temporal development of antibody in the two groups appeared similar (Figures 14 and 15).

Tables 49 and 50 present the ELISA antibody responses of contact-exposed pigs. Both criteria, 3 standard deviation and discriminant analysis, detected the first ELISA-positive pig at the second week and the maximum number of pigs with positive titers at 6 and 7 weeks after the beginning of contact.

Table 47. ELISA antibody titers in sera from M. hyopneumoniae-inoculated pigs (seeders) using three standard deviation criterion

Pig number	Weeks after inoculation							
	0	1	2	3	4	5	6	7
62	<40[a]	<40	<40	<40	<40	<40	<40	40
63	<40	<40	<40	<40	<40	<40	80	160
64	<40	80	160	40	80	640	320	640
65	<40	<40	<40	<40	<40	80	80	160
66	<40	<40	<40	<40	<40	80	<40	80
69	<40	<40	<40	<40	<40	80	160	160

[a]Titers are expressed as reciprocal of the highest dilutions of sera by the three standard deviation criterion.

Table 48. ELISA results obtained with sera of M. hyopneumoniae-inoculated pigs (seeders) using the discriminant analysis criterion

Pig number	Weeks after inoculation							
	0	1	2	3	4	5	6	7
62	-	-	+	+	+	+	+	+
63	-	-	+	-	+	+	+	+
64	-	+	+	+	+	+	+	+
65	-	+	+	+	+	+	+	+
66	-	-	-	-	+	+	+	+
69	-	-	-	-	+	+	+	+

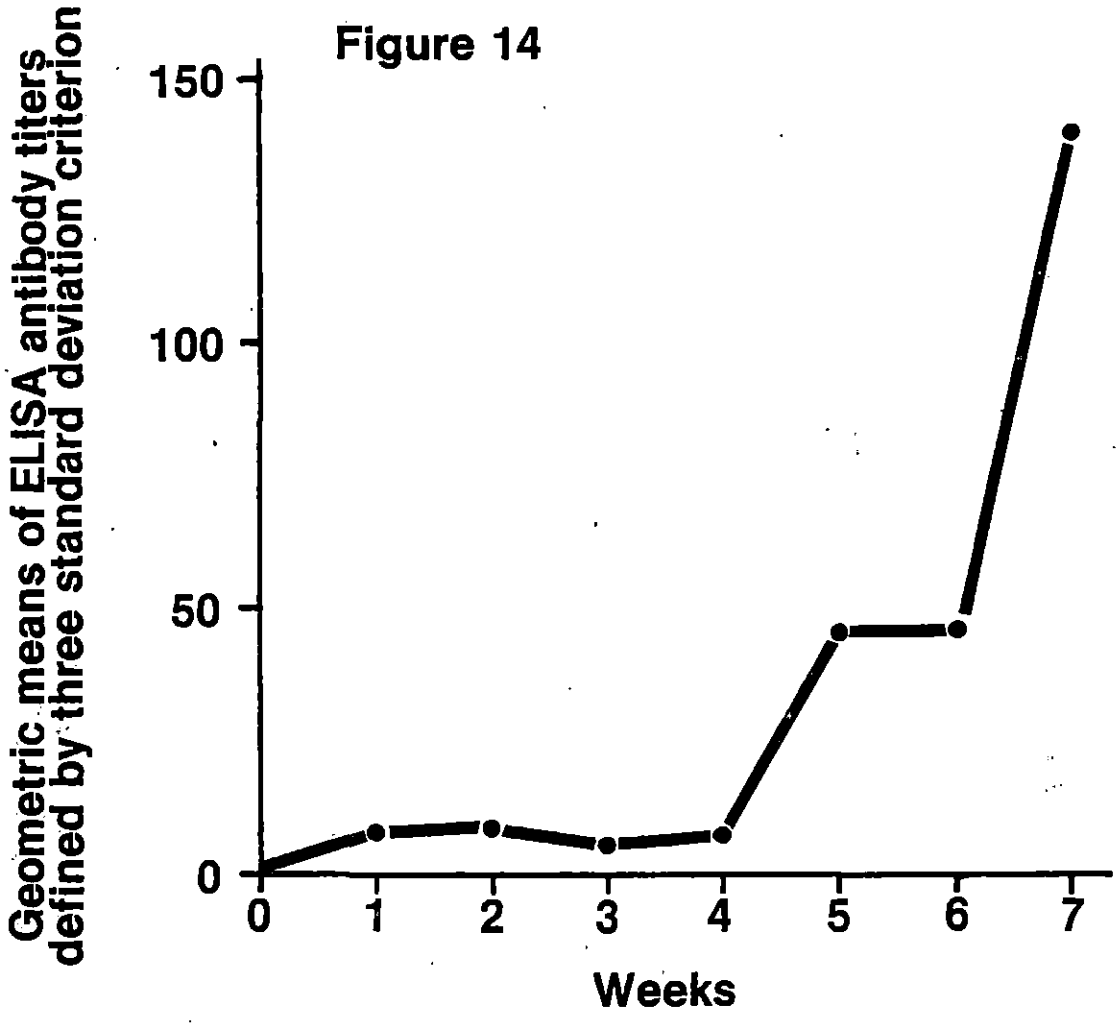


Figure 14. Relationship between titer of ELISA and time interval after inoculation with M. hyopneumoniae

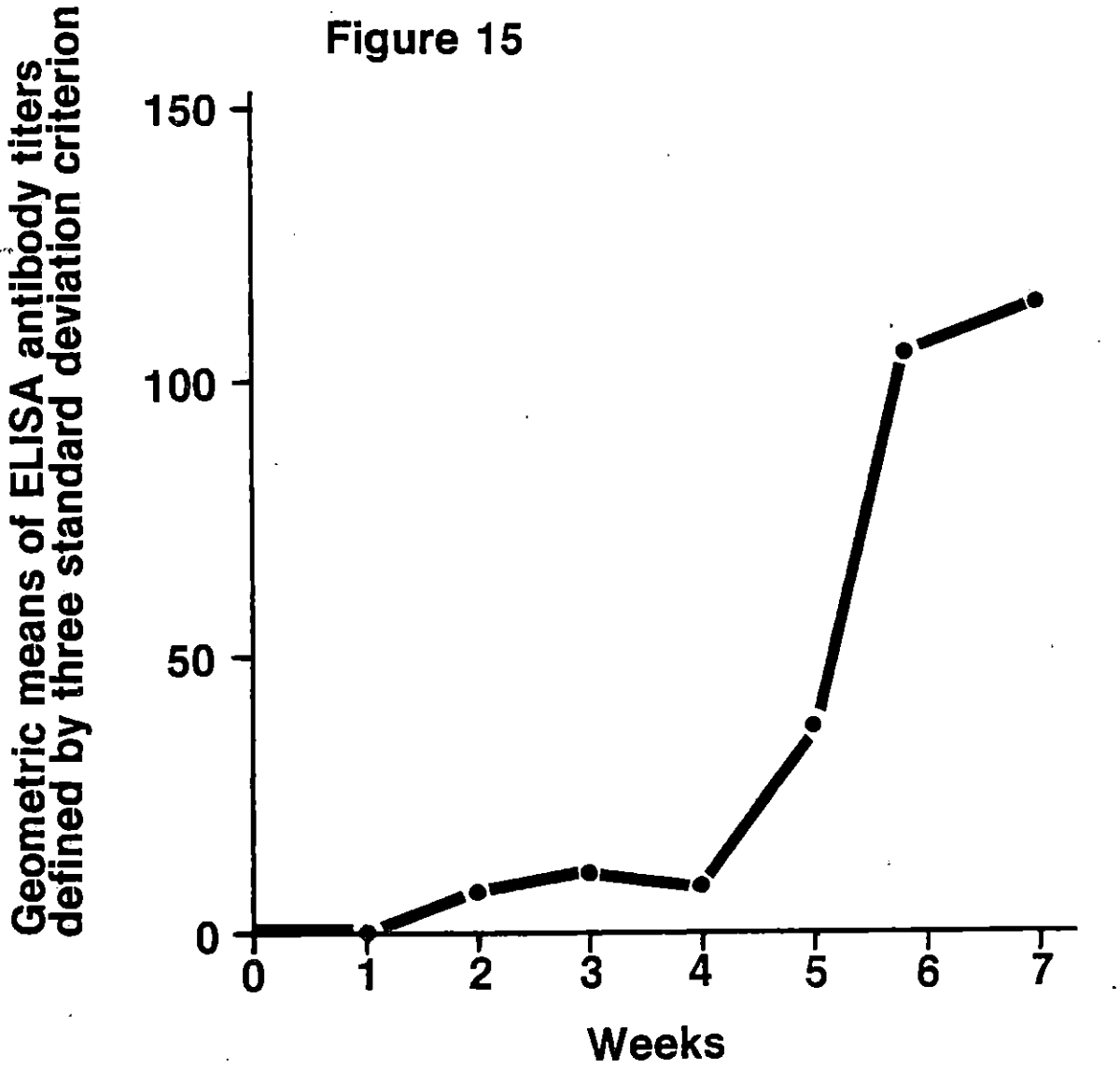


Figure 15. Relationship between ELISA titers against M. hyopneumoniae and the timer interval in contact-exposed pigs

Table 49. ELISA titers against M. hyopneumoniae in two age groups of contact-exposed pigs using three standard deviation criterion

Pig number	Experimental groups	Weeks after exposure to seeders							
		0	1	2	3	4	5	6	7
226	3[a]	<40[b]	<40	<40	<40	<40	<40	320	320
227		<40	<40	<40	<40	<40	320	640	640
228		<40	<40	160	160	160	320	640	640
229		<40	<40	<40	<40	<40	<40	<40[c]	<40
230		<40	<40	<40	<40	<40	40	1280	1280
684		<40	<40	<40	80	<40	80	160	160
233	11	<40	<40	<40	<40	<40	40	1280	1280
511		<40	<40	<40	<40	<40	<40	<40	<40
513		<40	<40	<40	80	160	320	320	320
515		<40	<40	<40	<40	<40	80	80	160
516		<40	<40	<40	<40	<40	<40	<40	<40
517		<40	<40	<40	<40	<40	<40	40	40

[a]Weeks of age.

[b]Titers are expressed as reciprocals of the highest dilution of sera positive by the three standard deviation criteria.

[c]Highest dilution tested.

Table 50. ELISA against M. hyopneumoniae detected in two age groups of contact-exposed pigs using the discriminant analysis criterion

Pig number	Experimental groups	0	1	2	3	4	5	6	7
226	3[a]	-	-	-	-	-	+	+	+
227		-	-	-	-	-	-	+	+
228		-	-	+	+	+	+	+	+
229		-	-	-	-	-	-	-	-
230		-	-	-	-	-	+	+	+
684		-	-	-	+	-	+	+	+
233	11	-	-	-	-	-	+	-	+
511		-	-	-	+	+	+	+	+
513		-	-	-	+	+	+	+	+
515		-	-	-	-	-	-	+	+
516		-	-	-	-	-	-	-	-
517		-	-	-	-	-	+	+	+

[a]Weeks of age.

No difference was detected in ELISA-positive pigs among the two age groups of pigs (Table 51, $P > 0.05$).

Detection of ELISA antibodies at various intervals after contact-exposure and detection of M. hyopneumoniae in lung tissue at necropsy are compared in Table 52. There were no differences between the two factors from 5 to 7 weeks after the beginning of contact exposure ($P > 0.05$).

Comparison of ELISA and CF Tests for Detection of Early M. hyopneumoniae Infection

In order to compare the ELISA and CF test results, data from Experiments A and B were pooled. This was done in an attempt to reduce the probability of error. ELISA-positive pigs were defined by two criteria; three standard deviations and discriminant analysis. Comparison of the two criteria using pooled data from contact-exposed pigs revealed no differences (Table 53, $P > 0.05$). However, more pigs were positive by the discriminant analysis criterion than by use of 3 standard deviations. In addition, results obtained with the discriminant analysis criterion at intervals from 3 to 7 weeks after contact-exposure began concurred with detection of M. hyopneumoniae at necropsy. Concurrence was not obtained with the 3 standard deviation criterion until 5 weeks after contact-exposure began (Table 54, $P > 0.05$.

Table 55 presents a comparison of the CF and ELISA

Table 51. ELISA against M. hyopneumoniae in two age groups of contact-exposed pigs

Experimental groups	Number of pigs	ELISA-positive pigs[a]	Chi-square	ELISA-positive pigs[b]	Chi-square
3[c]	6	5[d]	0.444* NS	5	0.00*
11	6	4		5	

[a]Positive pigs defined by the three standard deviation criterion.

[b]Positive pigs defined by the discriminant analysis criterion.

[c]Weeks of age.

[d]Pigs which had an ELISA titer at least once during the observation period.

*P > 0.05.

Table 52. Relationship between detection of ELISA antibodies at different intervals after contact-exposure and the presence of M. hyopneumoniae in lung tissue at necropsy

Total number of pigs	Weeks after contact began	Number of M. hyopneumoniae positive pigs[a] at necropsy	Number of ELISA positive[b] pigs	Chi-square	Number of ELISA positive[c] pigs	Chi-square
12	3	9	3	4.16*	4	2.685*
12	4	9	2	6.04*	3	4.16*
12	5	9	7	0.187**	8	0.0**
12	6	9	9	0.0**	9	0.0**
12	7	9	9	0.0**	10	0.0**

[a]DFA-positive or M. hyopneumoniae isolated at necropsy 6 weeks after exposure began.

[b]ELISA-positive pigs defined by three standard deviation criterion.

[c]ELISA-positive pigs defined by the discriminant analysis criterion.

*$P < 0.05$.

**$P > 0.05$.

Table 53. Comparison between three standard deviation and discriminant analysis criteria for defining ELISA test-positive pigs (Experiments A and B pooled)

Total number of pigs observed	Weeks after contact began	ELISA-positive[a] pigs	ELISA-positive[b] pigs	Chi-square
30	1	3	0	0.138*
30	2	7	5	0.104*
30	3	10	16	2.40*
30	4	11	16	1.07*
30	5	17	20	1.01*
20	6	15	17	0.156*
12	7	9	10	0.00*

[a]ELISA-positive pigs defined by three standard deviation criterion.

[b]ELISA-positive pigs defined by the discriminant analysis criterion.

*$P > 0.05$.

Table 54. Relationship between detection of ELISA antibodies at different intervals after contact-exposure and the presence of M. hyopneumoniae in lung tissue at necropsy (Experiments A and B pooled)

Total number of pigs	Weeks after contact began	Number of M. hyopneumoniae positive pigs[a] at necropsy	ELISA[b]	Chi-square	ELISA[c]	Chi-square
30	3	21	10	6.67*	16	1.128**
30	4	21	11	5.42*	16	1.128**
30	5	21	17	0.645**	20	0.07**
20	6	15	15	0.0**	17	0.156**
12	7	9	9	0.0**	10	0.25**

[a]DFA-positive or M. hyopneumoniae isolated at necropsy 6 weeks after exposure began.

[b]ELISA-positive pigs defined by three standard deviation criterion.

[c]ELISA-positive pigs defined by the discriminant analysis criterion.

*$P < 0.05$.

**$P > 0.05$.

Table 55. Comparison of CF and ELISA tests for detection of M. hyopneumoniae antibodies in sera from contact-exposed pigs (Experiments A and B pooled)

Number of pigs	Weeks after contact	Number of CF-positive pigs	Number of ELISA-positive[a] pigs	Chi-square
30	3	6	16	5.811*
30	4	7	16	4.512*
30	5	4	20	15.625*
20	6	13	17	1.2**
12	7	7	10	0.946**

[a]ELISA-positive pigs defined by the discriminant analysis criterion.

*$P < 0.05$ level.

**$P > 0.05$.

tests for detection of M. hyopneumoniae antibodies in contact-exposed pigs. The CF test and ELISA test differed significantly in their ability to detect antibodies in the early stages of infection ($P < 0.05$). The ELISA test appeared to be more sensitive from 3 to 5 weeks after contact began. At 6 and 7 weeks after contact began, the longest period observed, the tests did not differ ($P > 0.05$).

The relationship between presence of M. hyopneumoniae in lung tissue at necropsy with CF antibody in sera (pooled from Experiments A and B) of contact-exposed pigs at weekly intervals after exposure is analyzed in Table 56. This analysis revealed that the proportions of both factors were similar only at 6 and 7 weeks after exposure to seeders had begun ($P > 0.05$).

Table 56. Relationship between detection of CF antibodies at different intervals after contact-exposure and the presence of M. hyopneumoniae in lung tissue at necropsy (Experiments A and B pooled)

Total number of pigs	Weeks after contact begun	Number of M. hyopneumoniae positive pigs[a] at necropsy	Number of CF-positive pigs	Chi-square
30	3	21	6	13.19*
30	4	21	7	11.31*
30	5	21	4	17.55*
30	6	21	13	3.32**
12	7	9	7	0.375**

[a]DFA-positive or M. hyopneumoniae isolated at necropsy 6 weeks after exposure began.

*$P < 0.05$.

**$P > 0.05$.

CHAPTER V. DISCUSSION

Age-susceptibility of pigs to M. hyopneumoniae was the hypothesis tested in this study. Evaluation of the occurrence and intensity of MPS in the different age groups of pigs did not support this hypothesis. During the course of this work, two additional contributions have resulted: 1) a new criterion was devised for discrimination of positive and negative pigs by means of the ELISA test, and 2) the ELISA test was found to be more efficient than the CF test for detection of early infection with M. hyopneumoniae.

Our failure to demonstrate age-susceptibility to MPS is in agreement with results obtained by Pullar (1948) and Holmgren (1974a).

In doing this study, some problems were encountered. First of all, the seeder pigs and susceptible pigs needed to be in contact with one another for a certain length of time. The minimum time required for contact transmission was not known, so detection of the CF antibody response was used. Appearance of CF titers gave an indication that natural transmission of the disease had occurred. During the time of exposure to seeders the susceptible pigs became older. As a consequence, susceptibility may have changed during the period of exposure. In Experiment A, contact-exposed pigs were kept together until the end of the

experiment. This may have facilitated cross infection among them. There is no way to determine the possible effect of this factor on the results. Some considerations may be valid. Pigs in Experiment A were separated in units by age. Consequently, after removal of seeders, the number of mycoplasmas in the air depended upon the amount of mycoplasmas expelled by the contact-exposed pigs. This amount would be related to the degree of infection in each age group and to the degree of susceptibility of the pigs. Thus, cross infection may not interfere in the interpretation of results. Another point to be considered is that the time from exposure to seeders to the day of necropsy may not have been long enough to permit establishment of infection and development of disease and elimination of the agent with the establishment of a secondary infection in other pigs.

Our failure to detect an influence of age on the occurrence and severity of lesions may have been due to the fact that the number of experimental animals was not great enough.

The gross and microscopic lesions observed in both experiments did not differ from those seen in naturally-affected (Pullar 1948) or in experimentally-inoculated (Maré and Switzer 1965, Goodwin et al. 1965 and Livingston et al. 1972) pigs. The lobular distribution of gross lesions appeared not to be different in lungs of seeders

or contact-exposed pigs. These findings are in disagreement with Pullar (1948) and Betts (1952) who reported that enzootic pneumonia was more frequent in the apical and cardiac lobes. Livingston et al. (1972), in an experimental study of MPS, found that gross pneumonia occurred more frequently in the right cardiac and apical lobes of the lungs. They hypothesized that the lobular distribution and frequency among different lobes was related to mechanical barriers produced by the walls of the trachea and bronchi. Tortuous routes to less affected lobes provided more opportunity for removal of mycoplasmas from the tidal air by the mucociliary clearance mechanism. Our failure to detect a difference in this study may have been due to the fact that the number of pigs was too small. In the first experiment, a trend was observed for greater frequency of MPS lesions in the right cardiac lobe but in the second experiment this was not observed.

Turbinate atrophy was found in all groups of pigs from Experiment A. Its occurrence was associated with the presence of B. bronchiseptica in their nasal cavities. Furthermore, younger pigs were more affected than older ones. These findings are in agreement with Switzer and Farrington (1975) who reported that B. bronchiseptica is capable of producing turbinate atrophy mainly in young pigs, that is, pigs

infected at 4 weeks of age or less. M. hyopneumoniae has been reported to act synergistically with other organisms in the production of pneumonia (Smith et al. 1973, Underdahl and Kelley 1957 and Kaska et al. 1969). Analysis revealed no evidence of synergism between M. hyopneumoniae and B. bronchiseptica in this study. B. bronchiseptica pneumonia appears to occur mainly in baby pigs (Duncan 1965). This author was unable to induce pneumonia in 4-week-old pigs inoculated with pure cultures of B. bronchiseptica. On the other hand, he was able to induce pneumonia in 5-day-old pigs reared in isolation from the dams. In pigs raised with the dams, pneumonia was induced only in 2 of 20 3-day-old piglets. Age of piglets, strain of B. bronchiseptica, and stressing factors were incriminated as predisposing factors for turbinate atrophy. Considering that, in Experiment A, the youngest pig was 3 weeks old at the beginning of the experiment and that the infection by B. bronchiseptica occurred at this age or later; susceptibility to B. bronchiseptica pneumonia did not appear to be a problem. This may explain the lack of association between B. bronchiseptica infection and macroscopic lesions of MPS.

In Experiment B, 3 pigs had mild atrophy of nasal turbinates. No reasons were found for this finding since the pigs were SPF and B. bronchiseptica, Haemophilus sp. and P. multocida were not isolated from them. Usually,

turbinate evaluation is done by examination of a cross section of the nasal cavity made at the level of the first and second premolar teeth. A misinterpretation of the normal turbinate structure might have occurred. This may occur because of distortion produced by the saw in the turbinate while cross cutting the nasal cavity or by sectioning the nasal cavity cranial to the second premolar tooth. At this point, differences in development of the scroll of the turbinate may lead to an erroneous diagnosis of turbinate atrophy (Switzer and Farrington 1975). In addition, genetic variability must be considered. Taking into account these findings, it appears that examination of turbinates cross-sectioned at various levels could provide a better evaluation.

In Experiment A, 85% of contact-exposed pigs with lesions of MPS had lesions of the acute (late) stage of the disease. According to H. J. Barnes ("Histopathologic classification of mycoplasma pneumonia of swine", VMRI, Iowa State University, 1977), this stage is expected to occur from 3 to 6 weeks after inoculation. As a result, the bulk of infection in Experiment A was supposed to occur between 1 and 3 weeks after contact-exposure. This hypothesis is supported by the fact that ELISA antibodies were detected 2 weeks after exposure and the strong proportional association between ELISA-positive status at 3 weeks after contact-

exposure and M. hyopneumoniae-positive status at necropsy. In Experiment B, only 67% of pigs had lesions of acute (late) stage MPS. In this experiment ELISA antibodies appeared first at the second week after exposure began but the association between M. hyopneumoniae-positive status at necropsy and ELISA-positive status was observed only at 5 weeks after contact-exposure began. Furthermore, occurrence of acute (early) stage disease was higher in Experiment B than in A. These observations may indicate that pigs from Experiment A were infected earlier than those from Experiment B. This possible difference may relate to the fact that seeders from Experiment A were inoculated by the intratracheal route; thus, liberating more infective material in the environment than those inoculated by the intranasal route in Experiment B. MPS was slightly more severe in seeders from Experiment A than in those from Experiment B.

In Experiment B, pigs were separated and housed individually after 20 days of contact. As a consequence, those pigs which developed MPS must have been infected at this time. The last pigs to become infected during the time of exposure would have the earliest stage MPS lesions at necropsy. The earliest stage MPS lesions observed may be determined by an infection which occurred close to the end of the contact-exposure period, 4 weeks before necropsy. This is supported by the failure to detect evidence of aerosol transmission of

M. hyopneumoniae to sentinel pigs interspersed in houses between the contact-exposed pigs. The earliest stage observed was acute (early). This stage occurred in three pigs from the 3-week-old group. According to H. J. Barnes ("Histopathologic classification of mycoplasmal pneumonia of swine", VMRI, Iowa State University, 1977), this stage occurs between 1 and 3 weeks after infection. Observation of this stage four or perhaps more weeks after exposure suggests that this acute (early) stage may occur 3 weeks after infection or development of MPS disease is slower in younger pigs.

Acute (early) stage microscopic lesions were not associated with gross lesions of MPS. Cryostat lung sections of seven lobes from seven contact-exposed pigs having acute early stage MPS were examined by immunofluorescence techniques. Two of them were IFA positive. This observation supports the characterization of the early (acute) stage of the disease (H. J. Barnes, "Histopathologic classification of mycoplasmal pneumonia of swine", VMRI, Iowa State University, 1977).

The bronchopneumonia and bronchobronchiolitis observed in Experiment A as well as the increase of PMN in lesions characteristic of MPS were associated with the presence of B. bronchiseptica. These lesions probably were the manifestation of an early infection because late stages of

B. bronchiseptica infection are characterized by sheets of connective tissue (Duncan 1965) in lung parenchyma. This late condition was not observed in this study.

The effect of B. bronchiseptica on microscopic MPS scores was felt to be minimal because the scores were based on the number of bronchi and bronchioli with characteristic lesions of MPS. In this case, the presence of exudate was not considered.

The occurrence of a mild, acute, early stage MPS lesion in a control pig (pig number 90) without any evidence of M. hyopneumoniae infection is not conclusive because this type of reaction is considered to be nonspecific (Jericho 1977).

Multifocal granulomatous pneumonia occurred in Experiment A. This probably was caused by the aspiration of small pieces of food. Whittlestone (1957) described this lesion in 57 of 323 cases of pneumonia. Similar observations were made by Amanfu (1980).

Some degree of atelectasis was found in both experiments. Atelectasis may result either from incomplete expansion of lungs at birth or from collapse of a previously normal lung. The pneumothorax occurring at necropsy with subsequent focal collapse of lung is the probable explanation for cases found in both experiments. The systematic

collection of samples from normal macroscopic lobes for histopathology from the border of lobes may coincide with those parts of the lung which are subjected to a higher degree of atelectasis when the thoracic cavity is opened. This may explain the relatively high occurrence of atelectasis.

In both experiments, the isolation of M. hyopneumoniae among different age groups of pigs did not differ significantly. These findings suggested there are no differences among pigs of different ages in susceptibility to establishment and growth of M. hyopneumoniae in lungs.

Isolation of M. hyopneumoniae and immunofluorescence results were associated with detection of microscopic as well as macroscopic lesions at necropsy. McKean et al. (1979), in an evaluation of diagnostic procedures for detection of MPS in naturally infected pigs, found that isolation of M. hyopneumoniae was not associated with microscopic lesions of MPS. In their study, Eagle's medium was utilized for isolation of M. hyopneumoniae. In this regard, Friis medium appears to be better (Farrington and Switzer 1976 and Friis 1975). In addition, McKean et al. (1979) pointed out that attempts to isolate M. hyopneumoniae from lung samples obtained at slaughter often were impaired by bacterial overgrowth. Amanfu et al. (1980) in a sequential

study of the presence of M. hyopneumoniae antigen in cryostat sections of pigs found that fluorescence was observed to be more intense at 4 to 6 weeks PI with a tendency to decrease in intensity at 8 to 12 weeks PI. This suggested a decrease in number of M. hyopneumoniae cells in the more advanced stages of the disease. The nonspecificity of morphological changes induced by M. hyopneumoniae (Jericho 1977) and the decrease in amount of the organisms in the late stage of infection point out the weakness of using a single test for diagnosis. Epidemiologic investigations obtained with a battery of tests may be the best approach.

The isolation of H. parasuis from one pig from Experiment B was not repeated in subsequent examinations. In addition, extensive examination was done among pigs from the SPF herd which provided the experimental animals. No haemophili were recovered. Possibly the organism was an inadvertant, laboratory-obtained contaminant.

The good association between isolation of M. hyopneumoniae from homogenized lung and from bronchial washings with DFA, MPS macroscopic and microscopic lesions suggests that both isolation techniques may have similar efficiency. A direct comparison using the same lobe was not possible because the utilization of one isolation technique removed a

certain number of organisms; thus, interfering with the efficiency of another. This finding contrasts with those of Kaklamanis et al. (1969) who reported that extracts of tissue in lung homogenates kill mycoplasmas. Failure to detect this difference in our study, if it exists, may be due to the small number of observations. Another point to be considered is that the time between necropsy and culture procedures was so short that a deleterious effect of tissue homogenates was not detected. The washing technique has the advantage of eliminating the problem of unequal distribution in serial dilutions of inoculum caused by pieces of incompletely ground lung; thus, more accurate estimates of number of organisms can be obtained.

Distribution and intensity of staining of M. hyopneumoniae antigen was similar by both the direct and indirect immunofluorescence tests. The antigen appeared as a granular or in a yellow green-layer on the surface of the bronchial and the bronchiolar epithelium. Similar localization of M. hyopneumoniae was described by L'Ecuyer and Boulanger (1970), Meyling (1971), Livingston et al. (1972), Giger et al. (1977) and Amanfu et al. (1980). The lack of immunofluorescence in alveolar walls or spaces may have been due to the requirement for a special environment by M. hyopneumoniae. Such conditions appear to be provided by the ciliated epithelial lining of airways. Mebus and Underdahl (1977), using SEM of

trachea and bronchi from gnotobiotic pigs inoculated with M. hyopneumoniae, were unable to detect mycoplasmas in sections of small bronchioles almost devoid of ciliated cells. In addition, macrophages may play a role in the clearance of mycoplasmas from alveoli (Livingston et al. 1972).

In both experiments, analysis of the results obtained with immunofluorescence tests revealed that the occurrence of M. hyopneumoniae antigen and intensity of fluorescence were similar in all groups of pigs. Intensity of fluorescence is related to the amount of M. hyopneumoniae in lungs (Amanfu et al. 1980). Thus, the close similarity between the age groups examined in this study suggest that the severity of infection with M. hyopneumoniae did not differ between the age groups. These results are in agreement with those obtained by isolation of the agent. As a result, no difference in susceptibility to establishment of M. hyopneumoniae infection was detected.

As one could expect, no differences were observed in the efficiency of DFA, IFA without counterstain, and IFA with counterstain for detection of M. hyopneumoniae antigen in lung sections. In addition, no significant differences were observed in intensity of fluorescence. The IFA appears to be more suitable for less equipped laboratories because the fluorescein marked globulin can be bought

commercially. In addition, the antiserum can be used at higher dilutions than with the direct procedure.

Results obtained by means of FA tests corresponded closely to those obtained by culture procedures and macroscopic and microscopic evaluation for MPS lesions. These results are in agreement with those obtained by L'Ecuyer and Boulanger (1970), Livingston et al. (1972), Giger et al. (1977) and Amanfu et al. (1980). In spite of this fact, a few lobes that were culture-positive were negative by FA while in one case the reverse occurred. Similar findings were observed by Livingston et al. (1972), Goiś et al. (1975) and Amanfu et al. (1980). It may be that a lower number of M. hyopneumoniae can be detected by culture procedures than by FA tests. In addition, examination of different microbiological samples, although from the same lobe may have occurred. Consequently, use of several techniques appears to be the best approach for diagnostic purposes. For example, lesions of pneumonia could be examined by FA, then if negative the specimens could be examined by culture technique.

The azo-dye counterstain reduced nonspecific fluorescence and provided a better contrast with no evidence of reduced sensitivity. The necessity of using a counterstain could in part depend on the potency of antiserum used. Potent antiserum can be used at higher dilutions so that nonspecific fluorescence is of less significance.

Seeder pigs from Experiment A developed CF antibodies at 2 weeks PI. This result is in agreement with Boulanger and L'Ecuyer (1968), Takatori et al. (1968), Hodges and Betts (1969) and Slavik and Switzer (1972). All pigs seroconverted by 3 weeks after inoculation and the CF-titers remained approximately steady until necropsy day. Seeders from Experiment B developed CF-antibodies by 3 weeks PI but all of the pigs did not seroconvert until 6 weeks PI. In addition, higher titers were observed in seeders from Experiment A. These discrepancies observed between the two groups of seeders are thought to be due to different methods used for inoculation. In the intratracheal method natural barriers from the nose to the trachea are by-passed. As a consequence, all inoculum is deposited into lungs. When intranasal inoculation is used, only atomized particles may reach the lungs while the rest of the inoculum is swallowed or expelled by sneezing or coughing. CF and ELISA tests detected an earlier and stronger serological response in pigs inoculated by the intratracheal route than that in pigs inoculated by the intranasal route or infected by contact-exposure. This stronger serological response may be a reflection of the amount of antigen contacting immunologically responsive cells under the three types of exposure.

In Experiment A, the development of CF antibodies in

contact-exposed pigs occurred earlier than in Experiment B, 3 and 5 weeks respectively. Slavik and Switzer (1972) and Amanfu (1980) detected CF-positive pigs in contact-exposed pigs at 5 and 4 weeks after the beginning of exposure, respectively. The peak incidence of seroconversion of contact-exposed pigs was 4 weeks in Experiment A and 6 weeks in Experiment B. These observations indicated that pigs in Experiment A were infected earlier than those from Experiment B. These differences may have resulted from the different routes of inoculation used for infection of seeders.

Appearance and disappearance of CF antibodies was observed in almost all contact-exposed pigs in Experiment A and in one in Experiment B. This observation was similar to those obtained by Slavik and Switzer (1972) and Amanfu (1980). Several mechanisms can be postulated to explain this phenomenon. The low CF titers observed suggests that these titers were near the lower limit detectable by the test. Thus, the inherent variability of the test could be responsible for the discontinuous appearance of CF antibodies.

The early primary response to most immunogens is characterized by a predominance of IgM antibody; the IgG class of antibody appears somewhat later. In addition, IgM antibody production is usually transient, and within two weeks after initiation of the immune response IgG predominates (Herscowitz

(1978). IgM antibody is a more effective complement-fixing antibody than IgG. Consequently, the shift from IgM to the IgG stage may lead to less efficiency on the part of the CF test. Furthermore, low affinity antibodies are usually produced in the earliest stage of the infection (Herscowitz 1978). Low affinity antibodies are less effective in complement-dependent damage of biological membranes (Six et al. 1973). Mycoplasma pulmonis-infected mice produce large amounts of IgG_1 (Cassel et al. 1974) which is both non-complement-fixing and nonopsonizing. Similar events may occur to some degree with M. hyopneumoniae thus, reducing the efficiency of the CF-test. One of the above described factors or a combination of them, may have caused the variability observed.

Seeders from both experiments had ELISA antibody at 1 week PI. In Experiment A, all pigs seroconvected at 2 weeks after inoculation while in Experiment B, only at 4 weeks PI. These data differ from those obtained by Bruggmann et al. (1977b) and Armstrong et al. (1980). Bruggmann et al. (1977b) detected ELISA-positive pigs only at 4 weeks PI when all 3 observed pigs seroconverted. Several factors may contribute to these discrepancies; different routes of inoculation, strain of M. hyopneumoniae, dose,

and different ELISA tests or modified tests were utilized. In addition, different criteria were used to define positive and negative pigs. Consequently, comparison of results obtained with the ELISA tests used in different laboratories is difficult.

In Experiment A, the appearance of ELISA-positive pigs in contact-exposed pigs occurred earlier than in Experiment B, 1 to 2 and 2 weeks after exposure, respectively. In addition, the maximum occurrence of seroconversion was observed at 4 to 5 weeks and 6 to 7 weeks post-exposure, respectively. These observations are in agreement with the CF test results, thus supporting the hypothesis that infection occurred earlier in Experiment A. Armstrong et al. (1980) reported different findings in 5 pigs exposed by contact. He detected no antibodies with the ELISA test until 52 days after contact exposure. The discrepancy in these results has been discussed previously.

The undulating appearance of CF titers observed with contact-exposed pigs did not occur with the ELISA test. The more consistent results observed with the ELISA test probably relates to the fact that it detects all immunoglobulins independently of their class and biological features (Bruggmann et al. 1977b). The CF

test is antibody class and subclass dependent.

Preliminary analysis of ELISA test results revealed that two factors were impeding interpretation of results: day by day variation of the background color and pig to pig variation due to apparently nonimmunological reactions. A good criterion for interpretation of results must take these variables into account. Chao et al. (1979) used a statistical approach to overcome these problems. He defined a positive serum as that serum whose optical density reading was higher than 3 standard deviations above the mean of the control readings. Using this criterion, he considered positive those readings which had less than a 1:100 probability of belonging to the normal distribution of the negative readings. The discriminatory function criterion used in this study, besides taking into account the variability of negative readings, considered also the variability of positive results thus establishing, based on probability, the best boundaries between the positive and negative populations.

The discriminant analysis criterion appeared to be more efficient than the three standard deviations criterion because antibody could be detected earlier and it was more strongly associated with detection of M. hyopneumoniae.

Two misclassifications were observed with the discriminant analysis criterion. This may have been due to an error in doing the technique or to a characteristic of the serum. A few misclassifications were observed with the three standard deviation criterion. In this case, discontinuous results were observed in the serial dilution of serum. Evaluation of both criteria with sera from swine with naturally-occurring MPS should be done so that their usefulness can be further determined.

In order to compare the relative efficiency of ELISA and CF tests, a retrospective analysis was done between the presence of M. hyopneumoniae at necropsy and the presence of serologically-positive pigs for both tests from 3 weeks after beginning of contact-exposure until necropsy. This was possible because, according to experimental design, pigs from Experiment B must have been infected at 3 weeks after beginning of contact exposure. In Experiment A, the appearance of older lesions of MPS and the earlier serological response than in Experiment B suggested infection developed earlier. Thus, it was assumed that pigs from this experiment were already infected at 3 weeks after contact-exposure had begun.

In Experiment A, M. hyopneumoniae-positive status at necropsy was associated retrospectively with CF-positive status only at 3 and 4 weeks after contact exposure but not at 5 to 6 weeks. This paradoxical observation appears to be a consequence of the undulatory appearance of the CF titers.

Analysis of individualized experiments and of pooled data from both experiments indicated that M. hyopneumoniae-positive status at necropsy was associated retrospectively better, earlier and more consistently with ELISA results than those of the CF-test. In addition, ELISA and CF test results were associated only at 6 and 7 weeks after contact had begun. These observations indicate that the ELISA test is more efficient in detecting early infection than the CF test. These results are in disagreement with Armstrong et al. (1980) who compared ability of the ELISA, IHA, IFA and CF tests to detect antibodies against M. hyopneumoniae in contact-exposed pigs. The CF test was the most sensitive while the ELISA test the least one. In their work, Armstrong et al. (1980) suggested that use of chromatographically purified IgG explained the late response detected with ELISA. This explanation contrasts with Bruggmann et al. (1976) and Bruggmann et al. (1977b). These authors indicated that ELISA test detected all immunoglobulin classes because only the Fc part of IgG is class specific, but not the antigenic

determinants of the Fab pieces. The latter observation is in agreement with Herscowitz (1978). In the present study, peroxidase conjugated IgG antiswine IgG (heavy and light chain) was used; thus, detecting all classes of immunoglobulins. This characteristic of the ELISA test associated with its sensitivity to detect minimal amounts of antibody, less than 1 ng/ml (Engvall and Perlmann 1972), may explain its better efficiency. Another possibility to be considered is that the ELISA test may be detecting antibodies of low affinity. Engvall and Perlmann (1972) suggested this possibility after they had compared the ELISA and Farr techniques for detection of an early antibody response against the dinitrophenyl group (DNP) in rabbits.

Cross reactions between M. hyopneumoniae antigen and hyperimmune serum against M. flocculare were observed with the ELISA test. Cross reactions among swine mycoplasmas by this test have been reported by Bruggmann (1978), Armstrong et al. (1978) and Nicolet et al. (1980). This appears to be the most important problem with the ELISA test which must be overcome.

The occurrence at necropsy of a few M. hyopneumoniae-positive pigs with no ELISA antibody indicates that caution must be taken in interpreting negative results. Schuller and Swoboda (1980) compared histopathology, CF, FA and ELISA tests for diagnosing M. hyopneumoniae infection. They did not

find a good association between the tests employed and stressed the importance of running more than one test to obtain a definitive diagnosis of MPS.

Summary

The effect of age on the susceptibility of pigs to M. hyopneumoniae was evaluated in this study. Two experiments were carried out. In both experiments, exposure of experimental pigs was provided by contact with M. hyopneumoniae-inoculated pigs. Exposure time was 27 and 20 days, respectively, in Experiments A and B. Pigs were 3, 6 and 12 weeks of age at the start of Experiment A and 3 and 11 weeks of age in Experiment B.

The three age groups of pigs from Experiment A were housed by age in three units until necropsy 6 weeks after exposure began. Pigs from Experiment B were kept isolated from each other in Smidley hog houses until 7 weeks after exposure began when they were euthanized. Sentinel pigs were housed in houses between those of contact-exposed pigs in order to detect aerosol infection.

No differences were observed between age groups of pigs in occurrence and severity of MPS lesions or in detection of M. hyopneumoniae in lung tissue. These observations suggest that pigs from 3 to 12 weeks in age are equally susceptible to MPS.

A good association was found between isolation of M. hyopneumoniae, immunofluorescence test results and presence of macroscopic and microscopic lesions of MPS. In spite of this fact, the lack of complete agreement between these factors indicates that for definitive diagnosis a battery of tests is needed.

Direct and indirect immunofluorescence tests were equally efficient for detection of antigen in cryostat lung sections. Indirect immunofluorescence appeared to be more appropriate for less-equipped laboratories because FITC-labelled antiglobulin serum can be bought commercially and the specific antiserum can be used in higher dilutions than it is in the direct procedure. Utilization of an azo dye counterstain reduced nonspecific fluorescence and provided a good color contrast without interfering with sensitivity of the test. CF antibody titers were detected first in inoculated pigs from 2 to 3 weeks PI and in contact-exposed pigs at 3 to 5 weeks after contact began. Undulatory appearance of CF antibody occurred in contact-exposed pigs. Possible mechanisms for this event were discussed.

ELISA antibody titers were detected first in inoculated pigs at 1 week PI and in contact-exposed pigs at 1 to 2 weeks after contact began. ELISA antibodies were associated better, earlier and more consistently with M. hyopneumoniae-positive

status at necropsy than those detected with the CF-test.

Two criteria devised for detection of ELISA positive and negative pigs were compared: three standard deviations and discriminant functions criteria. Discriminant analysis criterion appeared to be more efficient than three standard deviations because it was better related to the M. hyopneumoniae-positive status.

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APPENDIX

Enzyme-linked Immunosorbent Assay (ELISA) Test

The enzyme-linked immunosorbent assay (ELISA) test (Bruggmann et al. 1977b) was adapted to the microtiter system by Theresa Young.

Antigen preparation

The M. hyopneumoniae antigen was prepared from strain J according to Bruggmann et al. (1977). Several concentrations of antigen were tested; 1 µg/ml, 2.5 µg/ml, 5 µg/ml and 10 µg/ml. The concentration of 5 g/ml gave the best contrast between positive and negative sera.

Peroxidase conjugated IgG fraction of rabbit anti swine IgG

A peroxidase conjugated IgG fraction of rabbit anti-serum against swine IgG (heavy and light chain)[1] was diluted 1:100, 1:1,000 and 1:2,000 in 0.05M tris-HCl (pH 7.4), 0.15M sodium chloride, 1 mM EDTA (TBSE), plus 0.1% bovine serum albumin and 0.05% of Tween 20. A dilution of 1:1,000 gave the best results with positive and negative sera.

Substrate preparation

Just before use, 80 mg of 5- aminosalicylic acid was dissolved in 100 ml hot (60°C) 0.02 M sodium phosphate, pH 6.0. This solution was filtered and 0.5 ml of a 1

[1]Cappel Laboratories, Inc., Cochranville, Pennsylvania.

per cent hydrogen peroxide solution was added.

ELISA test

The test was performed essentially as described by Bruggman et al. (1977b), but with proportional reduction of the reagents. Disposable microtiter plates[1] were incubated with 100 µl/well of antigen diluted to 5 µg/ml for three hours at 37°C. Just before testing, the microtiter wells were washed three times with 0.15 M sodium chloride containing 0.05 per cent Tween 20. The coated microtiter plates were incubated 5 hours at room temperature with dilutions of pig serum prepared in TBSE, containing 0.1 per cent bovine serum albumin, 0.02 per cent sodium azide and 0.05 per cent Tween 20. Then, the microtiter wells were washed as described above and 100 µl of rabbit anti-swine IgG peroxidase conjugate, diluted 1:1,000, was added to each well. The plates were sealed and incubated overnight at room temperature and then washed as described above; 100 µl of a substrate solution in 0.02 sodium phosphate, pH 6.0 was added to the wells. After an incubation time of 15 minutes the enzyme reaction was stopped by adding 20 µl of 1 N sodium hydroxide. The reaction product had brown color. The degree of color change was measured by using a colorimeter capable of measuring results directly

[1]Cook Engineering Company, Alexandria, Virginia.

in the wells of the microtiter plates (Clem and Yolken 1977).

The interpretation of results was achieved by using 3 standard deviation and discriminatory function criteria as described on page 92.