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Serologic and immunologic studies on Mycoplasma hyopneumoniae pneumonia of swine

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SEROLOGIC AND IMMUNOLOGIC STUDIES ON *MYCOPLASMA HYOPNEUMONIAE* PNEUMONIA OF SWINE

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INTRODUCTION

Extensive work has been done on virus pneumonia of pigs (VPP) or swine enzootic pneumonia (SEP) as to its pathology, transmission and significance. However, only after the demonstration that the etiological agent was a mycoplasma did the research on this subject begin to make meaningful progress.

Since the etiological agent is a mycoplasma, the term VPP has been abandoned in favor of the more explicit name, mycoplasma pneumonia of swine or MPS. The agent, which has been named Mycoplasma hyopneumoniae, is a small, cocco-bacillary, filtrable and weakly Gram negative staining organism, capable of growing in cell-free medium.

This present study was focused on the immunologic responses elicited by M. hyopneumoniae in its host, the pig. It includes serologic response of pigs to Mycoplasma hyopneumoniae infections, serologic diagnosis of MPS, and active and passive immunization against the disease.

LITERATURE REVIEW

This literature review is composed of four parts; 1) *Mycoplasma hyopneumoniae* pneumonia of pigs; 2) serologic tests employed for the diagnosis or identification of non-swine mycoplasmal diseases or mycoplasmas; 3) serologic tests employed in the diagnosis or identification of swine mycoplasmal diseases or swine mycoplasmas; 4) mycoplasma vaccines and vaccination procedures currently used for immunization against mycoplasma infections.

Mycoplasma hyopneumonia Pneumonia of Pigs

Mycoplasma hyopneumoniae is the causative agent of a wide spread swine pneumonia sometimes referred to as swine enzootic pneumonia (SEP) or virus pneumonia of pigs (VPP). The causative agent was isolated, characterized, propagated and named by Maré and Switzer (1965). The size of the organism is estimated to be between 110 mu and 225 mu in diameter. Observation of Giemsa stained preparation revealed coccoid to cocco-bacillary bodies with occasional ring forms. Cultures of organism grown in cell-free medium were capable of producing pneumonia in 80 to 100 per cent of the inoculated pigs (Maré, 1965). Goodwin *et al.* (1965) were also able to grow a mycoplasma from their J strain of enzootic pneumonia. The name *M. suipneumoniae* was given to this isolate (Goodwin *et al.* 1967). The cultures of *M. hyopneumoniae* and *M. suipneumoniae* were indistinguishable by the growth inhibition and metabolic inhibition test.

according to the same authors. The specific name of M. hyopneumoniae thus has priority and unless it can be shown to be a nomen dubium, M. suipneumoniae must be regarded as a later homonym (Edward and Freundt, 1969).

Details of history, incidence, pathology, and transmission of mycoplasmal pneumonia have been reviewed by Maré (1965 and 1969) and by Switzer (1969). Briefly, the MPS is spread from one pig to another by direct contact. Young pigs usually contract MPS from their mothers (Young, 1964). The prevalence of MPS has been estimated to vary from 34 to 74 per cent (Young and Underdahl, 1955; Betts 1952; Young and Underdahl, 1960). However, there are several agents that can result in grossly similar lesions (Pattison, 1956; Jericho, 1968). Caution must therefore be used in the evaluation of the incidence of MPS and economic effect on gross pathology alone.

The macroscopic lesions of mycoplasmal pneumonia are found predominantly in the apical, cardiac and intermediate lobes of the lung. It is characterized by clearly demarcated, reddish-blue to light pinkish-grey colored lesions in those lobes. Microscopic lesions consist of alveolar interstitial thickening, giant cell formation, neutrophil infiltration, septal cell proliferation, and peribronchiolar and perivascular lymphoid hyperplasia (Maré 1965).

There were no definitive diagnostic aids until recent development of complement-fixation test (Roberts 1968; Takatori et al. 1968;

Boulanger and L'Ecuyer 1968; and Hodges et al. 1969). There is no effective treatment for MPS.

Serologic Tests Employed for the Diagnosis or Identification of Non-swine Mycoplasmal Diseases or Mycoplasmas

Immunofluorescence

Liu (1957) demonstrated the causative agent of human primary atypical pneumonia (the Eaton agent) in the bronchial epithelium of chicken embryos by using direct immunofluorescence (IMF). The agent was found to multiply exclusively in the cytoplasma of the bronchiolar and air-sac lining epithelium. Marmion and Goodburn (1961) used IMF to stain infected chicken embryo lungs and found that the infectious particles were minute cocco-bacillary bodies resembling mycoplasma. Proof that the fluorescent staining cocco-bacillary bodies were indeed mycoplasma was provided by growth of the agent on cell-free medium and its characterization (Chanock et al. 1962). The name Mycoplasma pneumoniae was given to the agent.

Clark et al. (1963) used IMF in their study of Mycoplasma hominis and found that specific fluorescent staining was a suitable procedure for identification of the mycoplasma. The antigens of this mycoplasma as revealed by IMF were found to remain in the insoluble residue (cell membrane fraction) after exposure of M. hominis to sonic vibration for 30 minutes followed by centrifugation. Immunofluorescence had also been used for the demonstration

of mycoplasma contamination in cell cultures. Carski and Shepard 1961 showed that inoculated mycoplasma randomly distributed over tissue culture monolayer preparations. And later Malizia et al.(1961), also used IMF in their study of tissue culture origin mycoplasma. However, these two groups of investigators presented completely opposite interpretation as to the localization of mycoplasma in tissue culture cells. The former group found the organisms on the cell membrane and in inter-cellular spaces, whereas the latter group found them to be intracytoplasmic.

In spite of the specificity the IMF has, the technique has not enjoyed wide application due to the laborious preparations required. However, Del Giudice et al. (1967) described a direct IMF for rapid identification of mycoplasma colonies growing on agar plates that eliminated subculturing the organism and greatly reduced the time needed for identification. The procedure was shown to be effective for detection of mixtures of mycoplasma serotypes on primary isolation plates. Immunofluorescence has also been used for the identification of M. pneumoniae in direct smears of sputum (Hers and Masurel, 1967), however, the applicability of this technique was limited.

Masiga and Stone (1968) applied IMF to the study of Mycoplasma mycoides. They found it to be a specific identification technique as evaluated by four criteria: 1) fluorescence was not observed with M. agalactiae, M. mycoides var. capri and M. bovigenitalium; 2) fluorescence could be eliminated by adsorption of the antiserum with M. mycoides; 3) fluorescence was not observed in non-inoculated broth smears; and 4) contagious bovine pleuropneumonia immune sera

inhibited the IMF reaction.

Immunofluorescent staining of Eaton agent infected chicken embryo lungs by human convalescent serum was markedly enhanced by heat-labile substances in fresh human serum and guinea pig serum (Liu 1961). However, subsequent findings of Lind (1965) indicated that a diminution of fluorescent staining sometimes occurred when fresh guinea pig serum was added to human convalescent serum and that the enhancement effect was partially dependent upon the source of conjugated antiglobulin employed.

Complement fixation

Both hemolytic and conglutinating complement fixation (CF) tests had been tried by various workers for the diagnosis of contagious bovine pleuropneumonia (CBPP) (Heslop 1921, 1922; Walker 1922). Most of these workers had difficulties with the test. The first satisfactory method was that of Campbell and Turner (1936). This test has been widely used and is still regarded as the best single test for the diagnosis of CBPP.

Chanock et al. (1962a and b) successfully propagated the Eaton agent in a broth medium and identified it as a mycoplasma. They prepared a phenol-treated CF antigen from cultures of the organism and found that although CF was less sensitive than IMF, both tests yielded similar results with paired sera from persons with atypical pneumonia. Taylor-Robinson et al. (1963) used the CF test to study the antigenic relationships of mycoplasmas. The CF test when compared with IMF and indirect hemagglutination test, was found to be

the least sensitive, i. e., antibody titers were the lowest (Taylor-Robinson et al. 1966b). This may account for the fact that the CF test detected 85 per cent of the M. pneumoniae infection that were diagnosed by other serological techniques in their study.

Gamble (1956) noted that killed M. mycoides inoculated into experimental animals would not produce CF antibodies and proposed that CF antibodies were formed in response to metabolic products of living and actively metabolizing organisms. The antigens present in M. pneumoniae that react in the CF test are believed to be lipid in nature since they can be extracted by chloroform or ether (Kenny and Grayston 1965; Sobeslavsky et al. 1966; Marmion et al. 1967; Beckman and Kenny 1968). Satisfactory boiled CF antigen has also been demonstrated by Lemcke (1965) from the CBPP organism. Some loss of CF activity after boiling has been noted. The heat stable CF antigen was located in lipid extracts of concentrated suspensions. The major heat stable CF antigens of M. pulmonis were water soluble and were probably protein or protein complexes (Deeb 1967 a and b).

The M. pneumoniae CF antibodies in sera were present entirely or in part as 19s gamma-globulin (Schmidt et al. 1966) since the antibody activity was destroyed by treatment with mercaptoehhanol and by collection of blood onto filter paper discs. The antibody was capable of passing the placental barrier and was detectable at low levels in the sera of newborn infants (Weiser et al. 1967).

The CF test has had limited use in the study of avian mycoplasmosis. Gianforte et al. (1955) employed the CF test to

establish the identity of several isolates from various species. Lemcke (1964) used it for the differentiation of avian mycoplasma into 17 species and Frey and Hanson (1969) applied it to serotyping, assaying quantity of antigen produced in various media and for detecting mycoplasma antigen in infected tissues.

Agglutination test

Helsop (1922) used both microscopic and macroscopic agglutination tests in the study of M. mycoides and found that both negative and positive sera agglutinated the antigen. He could demonstrate the presence of agglutinins in the serum of a hyper-immunized calf and in the sera of animals naturally infected with CBPP, by the use of a macroscopic agglutination test. He believed that the macroscopic agglutination test was reliable and was simpler than the CF test to use as a mean of detecting the acute disease in live cattle. However, later workers (Yamagiwa et al. 1930; Campbell 1938) obtained disappointing results with the agglutination test especially with sera from chronic cases. Yamagiwa et al. (1930) found strong agglutination reaction frequently occurred with sera from normal cattle.

Klienberger (1938) studied mycoplasma antigens by means of the agglutination test. She classified 16 strains into 7 different serologic types. Norman et al. (1950) found a marked antigenic similarity between five strains of human mycoplasma studied by the agglutination test. They observed that the homologous and heterologous sera agglutinated mycoplasma at the same dilution or differed

in titer by only one or two dilutions.

Priestley (1951) utilized the method of Campbell (1938) to devise a slide flocculation test for the diagnosis of CBPP. The so called wet method of the test consisted of mixing antigen and serum on a slide and waiting for flocculation to occur. The dry method consisted of placing a loopfull of serum on a slide and allowing it to dry in the air. The dried serum was re-dissolved in two loopfulls of concentrated antigen. The test was believed to be a satisfactory method of serodiagnosis and was believed to be more sensitive than CF.

Newing and Field (1953) further simplified the preparation of the tests developed by Campbell and Priestley. They used stained antigen and whole blood from infected cattle for the test. This simplified test was found as satisfactory as the CF test. Agglutinin persisted in the blood of vaccinated animals for some time after CF antibodies had disappeared. Further, it was concluded, on the basis of CF and agglutination test results obtained, that the routine CF test could be replaced by the agglutination test. The latter test being more practical under field condition was less subject to erroneous interpretations (Newing and MacLeod 1955).

An agglutination test antigen for testing chicken sera for M. gallisepticum antibodies was developed by Jungherr et al. (1956) but the antigen was less reliable in detecting agglutinin in turkey sera. Adler and Yamamoto (1956) developed an agglutination test antigen which was adequate for detecting M. gallisepticum antibodies in turkey sera. Dierks et al. (1967) used the agglutination test

to characterize avian mycoplasma. It was found that antigen made from organisms grown in medium enriched with dextrose was less sensitive in slide agglutination tests (Adler and DaMassa 1968).

The agglutination test has also been used to study the antigens of mycoplasma from human and tissue culture sources (Bailey et al. 1961; Tully 1963). Kerr et al. (1964) found that macroscopic agglutination occurred on a glass slide between one to three minutes after mixing one loopfull of serum and two drops of sonicated antigen. They tested acute and convalescent primary atypical pneumonia sera against M. pneumoniae and other mycoplasma. Convalescent sera and reference rabbit M. pneumoniae antiserum agglutinated only the M. pneumoniae antigen while one antiserum of avian origin weakly agglutinated the M. pneumoniae antigen. It appeared that mycoplasma of different origin shared some similar antigen as revealed by agglutination studies.

Latex agglutination

Latex particles are a colloidal suspension of hydrophilic particles. The stability of the suspension is maintained through the presence of a surface active agent (Alfrey 1954). Even so, the latex particles are in a comparatively unstable suspension. The addition of a small amount of proteins (10 ug/ml of latex) will cause the latex particles to flocculate. Large amounts of protein increase the particle stability. This is the typical protective effects of a hydrophilic colloid on a hydrophobic one. As small amounts of protein are adsorbed, the initial surface on the latex particle is neutralized, causing instability. Upon further addition

of protein, the particles acquire a net charged surface due to the adsorbed protein and are thereby stabilized (Singer et al. 1961). The adsorption can be described by a Langmuir adsorption equation and by the mass action law. It can be expressed as:

$$\frac{r}{a} = KN-Kr$$

where r is the amount of protein adsorbed; a is the equilibrium amount of protein remaining in solution; N is the maximum amount that can be adsorbed, and K is a binding constant.

The adsorption process is in two steps. The amount of protein adsorbed in the first step is proportional to the latex particles, and a second step is a second layer of protein coat which contains about half the amount of adsorbed protein present in the first layer. The adsorbed proteins form a random immobile film with the molecules oriented parallel to the particle surface. Further adsorption of the second layer of protein coat thus takes place in the interstices between molecules already coated, but now the molecules are perpendicularly oriented to the particle surface (Oreskes and Singer, 1961).

Latex particles with their surface coated with proteins of various kinds can be agglutinated by specific antiserum (Christian et al. 1958; Carlisle and Saslaw, 1968; Oreskes and Singer, 1961). Mycoplasma cells of human, avian, swine, goat, and tissue culture origins were able to adsorb to the latex particle and thus could be agglutinated by immune sera (Morton 1966). This adsorption was demonstrated by electron microscopy.

In sera from rabbit undergoing immunization with mycoplasam antigen, the presence of anti-mycoplasma antibodies were detected much sooner in the mycoplasma latex agglutination reaction test than in the agar-gel diffusion reaction or the growth inhibition tests (Morton, 1966). The sera of guinea pigs and monkeys reacted in a specific fashion whereas rabbit serum requires pre-treatment to eliminate its non-specific agglutinin. Human sera given such pre-treatment still contain a non-specific agglutinin (Kende 1969). The use of carbohydrate-free mycoplasma antigen as described by Morton and Roberts (cited by Kende 1969) could not remove these non-specific reactions.

Growth inhibition test

Inhibition of the growth of mycoplasma in a liquid medium by immune serum was first demonstrated by Priestley (1952) who used Mycoplasma mycoides and fresh convalescent blood from cattle infected with the organism. Subsequently, immune inhibition of M. hominis was demonstrated by incubating this organism with dilutions of antiserum for various lengths of time, and then subculturing the mixture in nutrient broth or on agar medium (Edward and Fitzgerald 1954). After an appropriate period of growth, the agar medium was examined for mycoplasma colonies. It was found that 1) irreversible inhibition of M. homonis by immune serum required 6 to 48 hours of exposure to the serum , 2) such inhibition occurred more rapidly with higher dilutions of antiserum at 37 C than at room temperature, and 3) inhibition of mycoplasma growth

in broth medium by immune serum did not require a heat-labile accessory factor.

Yamamoto and Adler (1958) incorporated specific antisera into broth and agar media to inhibit the growth of avian mycoplasmas and obtained variable results. However, Edward and Karnarek (1960), Fabricant (1960), Kelton and Van Rockel (1963) and Dierks et al. (1967) have successfully demonstrated the growth inhibiting reactions with hyperimmune rabbit sera when it was incorporated into agar medium. They employed the reaction for serotyping and characterization of mycoplasmas of avian origin. The growth of Mycoplasma mycoides var. mycoides was also inhibited by immune serum from cattle which had recovered from infection with M. mycoides (Domermuth et al. 1967); the inhibitory activity of immune serum was completely removed by adsorption with M. mycoides.

Huijsmans-Evers and Ruys (1956) reported a method of inhibiting human genital mycoplasma on solid medium by application of antisera saturated paper discs. Clyde (1964) perfected the test, and Stanbridge and Hayflick (1967) further modified the test by drying antibody impregnated paper discs at 5 C. When stored at -20 C, these discs had been found to retain inhibiting activity for longer than seven months. Since the discs could be stored for longer period of time, the test had significant advantages over other tests.

It was observed by Edward and Fitzgerald (1954) that a heat-labile accessory factor was not required for the inhibition of mycoplasma growth in broth medium, however, Bailey et al. (1961) found that fresh serum had a potentiating effect for the inhibition

reactions. Riggs et al. (1967) also stated that immunoglobulins G and M inhibited mycoplasma growth and that inhibition was greatly enhanced by a heat-labile component of fresh serum. The same observation on accessory factor effect of fresh serum has been obtained by others (Fernald et al. 1967a; Taylor-Robinson et al. 1966a). Yet, Domermuth and Gourlay (1967) found the presence or absence of complement produced no detectable change in the inhibiting capacity of immune serum.

The substance in antisera responsible for growth inhibition of mycoplasma was found to be associated with the water-soluble globulin fraction. The fraction of the globulin removed from sera by agglutinating antigens also inhibited growth of mycoplasma in a similar manner (Clyde 1964). Cottew (1963) suggested that the growth inhibiting antibody exerted its effect by agglutination. His suggestion was supported by the work of Davies and Hudson (1968) who observed that animals that did not develop any complement fixing antibody titer still developed a persistent weak agglutination reaction, and a concurrent low growth inhibition titer. The demonstration that growth inhibiting antibody was not connected with immunity led them to believe that resistance to contagious bovine pleuropneumonia did not involve circulating antibody but was more probably due to some form of cell mediated immunity (Davies and Hudson 1968).

Growth inhibition test for organisms producing acid

Cell suspensions of Mycoplasma mycoides, the organism of bovine contagious pleuropneumonia have the ability to utilize glucose, fructose, and mannose. Glucose, pyruvate, lactate, and in the presence of catalase, glycerol are oxidized quantitatively to acetate and CO_2. Anaerobically, pyruvate underwent dismutation to acetate, lactate and CO_2. Neither glucose nor glycerol were attacked anaerobically (Rodwell and Rodwell 1953), yet in spite of the inability to breakdown glucose under anaerobic condition, it was concluded that glucose oxidation in M. mycoides proceeded by way of the Embden-Meyerhoff pathway (Rodwell and Rodwell 1954). A strain of Mycoplasms laidlawii was found to have a hexose monophosphate shunt since glucose-6-phosphate dehydrogenase, ribose-5-phosphate isomerase and transketolase were demonstrated (Castrejon-Diaz et al. 1963). Van Dermack and Smith (1964) presented evidence for a citric cycle in mycoplasma. Neimark and Pickett (1960), Gill (1962), and Edward (1950) also observed utilization of glusose by mycoplasma organisms. In the presence of an indicator such as phenol red, the oxidation of glucose to the lactate or acetate would change the color of the broth medium to yellow. The decrease of pH and consequent color change of the indicator were associated with growth of the organisms. The above phenomenon were inhibited by the addition of specific antiserum (Taylor-Robinson et al. 1966 b and c). It was also found that unheated horse serum was necessary for the specific inhibitory effect. Without it the effect was greatly reduced (Taylor-Robinson et al. 1965).

They indicated that a heat-labile accessory factor present in horse or guinea pig serum was important for the demonstration of specific antibody activity.

Growth inhibition test for organisms utilizing arginine

The rapid utilization of arginine by mycoplasma was first described by Smith (1955) who demonstrated that strains of Mycoplasma hominis caused disappearance of citrulline. He attributed this activity to a desimidation pathway in the organisms which yielded a mole of citrulline and a mole of ammonia for each mole of arginine utilized. A degradation pathway for citrulline was also described (Smith 1959):

1. Arginine ⟶ Citrulline + ammonia
2. Citrulline + inorganic phosphate ⟶ ornithine + carbamyl phosphate
3. Carbamyl phosphate + ADP ⟶ ATP + amonia + CO_2

Razin and Cohen (1963); Schimke and Barile (1963) and McCarty et al. (1964) all reported the same ammonia forming mechanism in mycoplasmas. Barile and Schimke (1963), used this mechanism as the basis for a chemical method for detecting contamination of cell cultures by mycoplasma. They utilized the citrulline level in the medium as an indication of contamination. Purcell et al. (1966a) found that addition of specific antiserum to the medium would inhibit the ability of the arginine utilizing organisms to utilize arginine, and thus the alkaline shift produced by the metabolism of the organism was inhibited.

A labile accessory factor present in unheated guinea pig serum potentiated the inhibitory effect of the antiserum. Further, Purcell et al. (1966b) found that they could also use this test for measurement of antibody to T strain of mycoplasma. It was used for detecting antigenic differences among isolates belonging to the same species. The results were in good agreement with results of nucleic acid homology studies of mycoplasma relationships. It appeared that the serological difference measured by the test reflected basic genetic difference between species (Purcell et al. 1967).

Growth inhibition test utilizing tetrazolium reduction

Tetrazolium salts can be used as indicators of electron transfer. Compounds such as triphenyltetrazolium chloride (TPTC) are converted from a soluble, colorless salt to an insoluble formazan in reduced state. The reduction of TPTC is dependent on enzyme systems, more specifically, the dehydrogenases (Kun and Abood, 1949). The dehydrogenases system is coenzyme I or coenzyme II linked (Mattison et al. 1947). Flavoproteins are also involved in the enzymatic reduction of TPTC (Kun 1951; Brodie and Gots 1951). That mycoplasma had the ability to reduce tetrazolium compounds was first discovered by Somerson and Morton (1953). They noted that broth culture of M. pneumoniae reduced TPTC to a red formazan. In the presence of specific antiserum the ability to reduce TPTC was inhibited (Jensen 1964).

Based on this obervation, Senterfit and Jensen (1966) devised a

method for measuring growth-inhibiting antibody by adding serially diluted antisera to broth cultures and observing the inhibition of TPTC reduction. They concluded that the presence of antibody correlated well with the resistance to disease.

Gel diffusion

Kurotchkin (1937) reported that a substance active in the specific precipitating reaction was present in the serum of animals infected with contagious bovine pleuropneumonia, and that it could also be extracted from the affected lungs. It was suggested that the antigen extracted from cultures of Mycoplasma mycoides was a specific carbohydrate. The high specificity and sensitivity of the substance indicated the possibility of its use for diagnostic purposes, especially for the detection of chronic infections (Kurotchkin 1937). White (1958) observed a strong precipitin reaction when hyperimmune sera were tested with exudate or saline extract of a lung lesion while culture and extracts of the organism gave the same results. Sera from cattle dying of CBPP when tested against immune rabbit sera often gave one or two lines of precipitation demonstrating the presence of circulating antigens probably released from extensive lesions and exudates which contained a high concentration of diffusible antigen (White 1958).

Gel diffusion has been used for the study of the serological relationship among mycoplasmas (Taylor-Robinson et al. 1963; Taylor-Robinson et al. 1964; and Taylor-Robinson et al. 1965). Lemcke (1965)

studied 16 strains of mycoplasma previously determined to be distinct by CF and found that gel diffusion revealed each of the 16 to have a consistent pattern of three to seven precipitin lines with its homologous antiserum. However, cross reactions did occur between some strains, especially with antiserum produced with the aid of an adjuvant. The appearance of cross reactions was attributed to the sharing of certain antigenic components. Taylor-Robinson et al. (1965) found that M. pneumoniae antigen did not react in the gel diffusion test with antisera to the other human mycoplasma nor did M. pneumoniae antisera produce lines with antigens prepared from the other human mycoplasma. In addition, there was no reduction in intensity and number of precipitin lines after absorption of M. pneumoniae antiserum with heterotypic mycoplasma antigen. These findings indicated that M. pneumoniae was antigenically distinct.

Hemagglutination inhibition and hemadsorption inhibition

It was observed by Van Herick and Eaton (1945) that a mycoplasma isolated from hen's egg agglutinated erythrocytes. Subsequently a hemagglutination inhibition test for a naturally occurring mycoplasma infection of poultry air saculitis was described based on the phenomenon (Jungherr et al. 1953) that the addition of antiserum inhibited the hemagglutination reaction. This technique had been widely used in avian mycoplasma work, particularly in the measurement of antibody to M. gallisepticum (Fahey and Crawley, 1954).

Crawley and Fahey (1957), and for the characterization of avian mycoplasmas (Kleckner, 1960; Yoder and Hofstad, 1964; Dierks et al. 1967). Apart from avian mycoplasmas, only a few strains of animal mycoplasma produce a direct hemagglutination and at very low titer (Manchee and Taylor-Robinson, 1968). Of the mycoplasmas of human origin, only M. pneumoniae had agglutinating antibody at low level (Somerson et al. 1967), yet with the addition of horse serum in the hemagglutination system, high level of hemagglutination could be obtained. Hemagglutination appeared to be the mechanism by which horse serum diluent caused high hemagglutination titer of M. pneumoniae since both horse serum and M. pneumoniae had hemagglutination to vervet erythrocytes (John et al. 1966).

The hemadsorption of mammalian erythrocytes to colonies of M. pneumoniae was reported by Del Giudice and Pavia (1964). Mycoplasam from avian and animal sources, i.e., M. gallisepticum, M. agalactiae, M. bovigenitalium, and M. pulmonis could hemadsorb erythrocytes from a wide range of species . Hemadsorption was reduced by growing of colonies on agar and by the addition of specific antiserum to the colonies. Generally the antisera titers obtained by hemadsorption inhibition were low in comparison with those obtained by metabolic inhibition and therefore it was not useful for routine serologic testing (Manchee and Taylor-Robinson, 1968).

The adsorption of M. pneumoniae and M. gallisepticum to red blood cells appeared to depend upon neuraminic acid receptors on the red blood cells, whereas M. orale and M. plumonis seemed to utilize other types of receptors. Pretreatment of red cells with receptor

destroying enzyme, neuraminidase, or influenza B virus, removed the adsorption receptors for M. pneumoniae. Similarly, pretreatment of M. pneumoniae colonies with neuraminic acid containing material prevented adsorption of erythrocytes. The mycoplasma receptor sites were thought to be lipid or lipoprotein in nature (Sobeslavsky et al. 1968), or protein in nature (Manchee and Taylor-Robinson 1968). The adsorption of mycoplasma to neuraminic acid receptors of various cells has been postulated to have a role in virulence of M. pneumoniae since this type of attachment provides an unusual opportunity for peroxide, secreted by the organism, to attack the tissue cell membrane without being rapidly destroyed be catalase or peroxidase presented in extracellular body fluid (Sobeslavsky et al. 1968).

Formalized M. gallisepticum can be used for hemagglutination and in the hemagglutination-inhibition tests. The antigen retained its hemagglutination ability for an extended period (Adler and DaMassa 1967).

Indirect hemagglutination test

Tannic acid agglutination of red blood cells has been known for a long time. Reiner and Fischer (cited by Herz and Kaplan, 1968) described the possible mechanism of the effect. They suggested that tannic acid altered the surface properties of the erythrocytes, changing them from a hydrophilic to a hydrophobic state. Freund (1929) assumed that tannic acid, like homologous antibody, brings about a change in the surface potential of the cells, which in the presence of certain electrolytes

results in their aggregation.

Treatment of erythrocytes with tannic acid causes a reduction in anion permeability (Edelberg, 1952), and an alteration of osmotic resistance (Bohmann, 1944). Exposure of human erythrocytes to tannic acid under conditions commonly employed in indirect hemagglutination tests, resulted in irreversible inactivation of acetyl choline esterase, a membrane enzyme (Herz, 1968). It had been suggested that in low concentration tannic acid traversed the erythrocyte membrane (Bohmann, 1944), yet Herz and Kaplan (1968) have demonstrated that cytoplasmic enzymes were not affected when intact erythrocytes were exposed to tannic acid in conditions which caused irreversible inactivation of acetyl choline esterase (Herz, 1968). This finding does not support the contention that tannic acid is able to traverse the red blood cell membrane, but indicates that this agent only interact with specific segments of the outer surface of the human erythrocyte membrane (Edelberg, 1952).

Treatment of sheep red blood cells with suitable concentration of tannic acid rendered them capable of adsorbing certain protein molecules from solution. Red blood cells which have adsorbed in this way are agglutinated by homologous antiserum (Boyden, 1951). This method of detecting antigen-antibody reaction was further standardized by Stavisky (1954).

Treatment of red blood cells with tannic acid facilitated the attachment of Mycoplasma mycoides. When the antigen-coated red blood cells came in contact with specific antibody, hemagglutination resulted (Anon., 1957).

Dowdle and Robinson (1964) successfully employed the indirect hemagglutination test for the diagnosis of mycoplasma infection and found that the test was immunologically specific and sensitive. The test was modified by Taylor-Robinson et al. (1964) and was used to identify mycoplasma and to establish their relationship with other mycoplasmas. Neimark (1968) and Lind (1968) further simplified the test by fixing antigen-coated tanned erythrocytes with glutarylaldehyde and formalin, respectively. The fixed erythrocytes were equally sensitive and could be stored for six months without losing activity. This finding greatly expanded the applicability of the indirect hemagglutination test to the diagnosis of mycoplasma infections.

Serologic Tests Employed for the Diagnosis or Identification of Swine Mycoplasmal Diseases or Swine Mycoplasmas

There are five established swine mycoplasma species. The serological test applied to each of the species is reviewed.

Mycoplasmas hyorhinis

An indirect hemagglutination test was developed for use with M. hyorhinis by Ross and Switzer (1963). Washed sheep red blood cells modified with antigens from heated M. hyorhinis cell suspensions were agglutinated by hyperimmune rooster, rabbit, or swine antisera. However, sera from naturally or experimentally infected pigs did not consistently produce agglutination. These workers also found serological variations between the various isolates of M. hyorhinis.

Tully (1966) used immunofluorescence to compare two isolates of M. hyorhinis with several other species of mycoplasma and found those isolates were similar to each other yet distinct from other mycoplasm examined. Purcell et al. (1966a) also found that M. hyorhinis was a distinct species but was very similar to several mycoplasma isolated from non-porcine sources.

Mycoplasma granularum

Dinter et al. (1965) showed that M. granularum is antigenically unrelated to other swine strains by both growth inhibition and gel-diffusion studies. Immunofluorescence studies (Tully, 1966) had also revealed distinction between M. granularum and M. hyorhinis. Serological methods for the measurement of response of pigs to infection have not been evaluated.

Mycoplasma hyoarthrinosa

Mycoplasma hyoarthrinosa has been demonstrated in the joint cavities of swine even in the absence of microscopic lesions in adjacent tissues by the use of indirect immunofluorescence (Robinson, et al. 1967a). Robinson et al. (1967b) used an indirect hemagglutination plate test to monitor the serologic response in pigs experimentally infected with M. hyoarthrinosa and found that the serological response varied from undetectable in several pigs to a titer of 1:614 in one pig at nine days post-infection. The serologic response reached its maximum on the ninth day and declined afterward.

Mycoplasma hyogenitalium

It was reported that this organism was serologically distinct from M. hyorhinis or M. granularum (Moore et al. 1966) but the details of how this was accomplished were lacking.

Mycoplasma hyopneumoniae

Using the standard complement fixation (CF) test, Roberts (1968) detected antibodies to M. hyopneumoniae in pig sera. Boulanger and L'Ecuyer (1968) also demonstrated antibodies to the organism at two to three weeks after experimental infection by the use of a modified direct complement fixation test. The antibodies remained detectable for more than 200 days. An indirect CF test was also able to demonstrate antibodies but the titer obtained was lower than with the modified direct test. These workers could differentiate M. hyopneumoniae from M. hyorhinis by the CF test, however, when hyperimmune rabbit sera were tested, only slight antigenic differences were observed and there was enough cross-reactivity to render this source of antibodies impractical for species differentiation. Takatori et al. (1968) also demonstrated CF antibodies in pigs experimentally infected with M. hyopneumoniae. They reported that antibodies first appeared two to three weeks after inoculation. The CF antigen of M. hyopneumoniae was stable to heat and trypsin and was associated with both ether soluble and insoluble fractions of the organisms.

Goodwin et al. (1969) detected CF antibodies in pigs infected with M. hyopneumoniae and found no correlation between either the

extent of the pneumonic lesions or the immune state and the titer obtained. They also successfully employed an indirect hemagglutintion test for the detection of antibodies against M. hyopneumoniae. The mycoplasma antigens were prepared by concentrating organisms to one one-hundredth of the original volume. The appropriate antigen concentration used for the test was determined by block titration. This antigen dilution was then used to sensitize tanned sheep red blood cells. None of the sera from 12 pigs killed at 12 to 22 days postinfection had detectable titers. However, high indirect hemagglutination titers were obtained with sera from pigs infected 16 or more weeks previously.

Mycoplasma Vaccines and Vaccination Procedures Currently Used for Immunization Against Mycoplasma Infections

Contagious bovine pleuropneumonia

Vaccination against contagious bovine pleuropneumonia (CBPP) was based on the use of "culture vaccine" (Walker, 1921-22), which consisted of a strain of the organism attenuated by serial culture for a varying number of generations in 10 per cent bovine serum broth. An initial course of three doses at intervals of three weeks was given, followed by a single yearly dose. Local reactions and levels of immunity following vaccination were variable. Vaccines of this nature was thought to increase the number of "carriers" and render control more difficult (Sheriff and Piercy, 1952). Therefore, further work was

done to adapt a strain of CBPP organism to embryonated hens eggs. Cattle were immunized by the subcutaneous inoculation of ninth egg passage infected chorio-allantoic membranes. The cattle were challenged with virulent pleural exudates taken from CBPP infected cattle. Although there were occasional variations in virulence, the authors claimed that the avianized strain was a safer, more stable and more effective immunizing agent than those in use for the control of CBPP.

The avianized vaccine, in a lyophilized state, was found to be viable after two and half years of storage in vacuo in the frozen state. It was still capable of inducing immunity in cattle, and no appreciable properties had changed (Hyslop, 1955).

Priestley (1955) prepared a vaccine by reconstituting the dried organism in a suitable fluid and immediately mixing with one per cent agar. After the agar had set, animals were inoculated in the tail with 0.3 ml of the vaccine. Twelve out of 18 bulls tested gave positive flocculation reaction after 14 to 18 days. All animals were resistant to challenge with virulent CBPP agent. The authors also found evidence that cattle could be immunized with vaccines stored in the dry state for more than two months. Piercy and Knight (1955-56) propagated the organism in embryonated hens eggs, which were then used as the inoculum for vaccine production. They found vaccination with various lots of vaccines produced varying degrees of protection. They found that after 44 egg passages, the organisms failed to elicit an immune response in cattle and concluded that early generations of adapted strains should be used for vaccine production. Later, Piercy and Knight (1958), in a trial with an attenuated vaccine strain demonstrated

that it conferred a significant degree of protection against challenge. Progressive passage through embryonated hens eggs did not lessen its immunizing ability for at least 18 passages. However, they still recommended the use of early generation egg adapted strains for vaccine production.

The efforts of Piercy and Knight (1958) to increase the immunizing ability of vaccine by various manipulations were not very successful. They also observed the existence of naturally resistant animals which they considered to be a probable explanation of the failure of well attenuated vaccine to immunize the whole of any given cattle population.

Poultry mycoplasma infections

Van Rockel et al. (1953) reported that resistance to challenge was increased in birds recovered from M. gallisepticum infection. Adler (1958) however, was not able to protect turkeys with various killed vaccines or with cultures of this organism propagated in broth. Adler et al. (1960) prepared live and killed vaccines and inoculated chickens by various routes. They then challenged the birds with virulent M. gallisepticum and found that a vaccine prepared by sonic disruption of the organisms did not produce immunity in chickens. Similarly, an aluminum hydroxide-adsorbed formalin-inactivated vaccine did not immunize chicken. Only an attenuated live culture vaccine administered intramuscularly immunized the chickens for about two months. Turkeys did not respond to the vaccine.

Warren et al. (1968) prepared a vaccine by formalin inactivation of M. gallisepticum harvested from broth or from a glass surface. The formalinized vaccine increased resistance of one-day-old chicks to air sac infection by the homologous strain of the organism when it was administered by intranasal or subcutaneous routes.

Human mycoplasma pneumonia

Couch et al. (1964) propagated M. pneumoniae, human primary atypical pneumonia agent, on an agar surface and in tissue culture in attempt to attenuate the agent. They harvested the organism and inoculated 42 antibody-free volunteers via the nasopharynx. Thirty-eight of these volunteers developed serologic evidence of infection and the agent was recovered from 21 of 27 volunteers. Twenty-three of the 42 developed detectable illness after inoculation including eight who developed febrile illness. These workers also found that pneumonia and bullous myringitis occurred in those volunteers inoculated with tissue culture propagated agents but not in those receiving the agar-grown agents. This difference suggested that propagation on a cell-free agar medium resulted in a decrease in the virulence of M. pneumoniae for man. It also indicated that it might be possible to develop suitable attenuated strain of M. pneumoniae for use in immunoprophylaxis.

Jensen et al. (1965) used a chemically defined medium for the production of a Mycoplasma pneumoniae vaccine. The vaccine, after formalin inactivation, was studied in animals and human subjects. It

was found to elicit antibodies in rabbits and monkeys and induced resistance to multiplication of mycoplasma in the lung of vaccinated hamsters. It was well tolerated in preliminary studies with 36 human subjects. Encouraging evidence of antigenicity was obtained when 25 of 30 subjects developed significant rise in mycoplasma growth-inhibiting antibody titers. It was concluded that the vaccine would probably confer some protection against naturally occurring or experimentally produced infection, however, the extent of protection was not determined.

Other formalized vaccines for M. pneumoniae were prepared by Metzgar et al. (1966). These vaccines were aqueous or were incoporated in alum or emulsified peanut oil adjuvant. All of the vaccine preparations were highly antigenic in animals and in man. Alum vaccines were better than aqueous vaccines, and those in adjuvant (peanut oil) appeared to give the best results. Furthermore, no local or systematic reactions of clinical consequence was noted in any person vaccinated. There was evidence that alum vaccine stimulated a high degree of protective antibody in pre-school children and that it protected children and adults against disease.

Weibel et al. (1967) followed up this work and noted that the vaccine given in two doses one month apart, stimulated homologous antibody. As the result of the vaccination, a 28 per cent reduction in mild cases, and a 35 per cent reduction in severe illness was noted. The reduction in severe cases was difficult to evaluate because the number of cases was small.

The studies of Smith et al. (1967) on Metzgar's vaccine showed

that it produced protective and growth inhibiting antibodies in about 50 per cent of the volunteers. One of the individuals that had an antibody level became ill after infection, however, illness occurred in 10 of 13 men in a control group. The protective action of the vaccine was statistically significant ($P < 0.004$). It was also suggested that the vaccine-induced growth inhibiting antibody was associated with significant, but not complete, protection against experimental infections.

In contrast to the protective effect, an anomalous effect was observed in volunteers who did not develop detectable antibody following vaccination. Experimental infection of this group led to more severe illness than that observed in the control group. Therefore, they warned that caution should be exercised when an inactivated vaccine of low potency was evaluated in man. These workers also found that after prolonged passages in artificial medium, M. pneumoniae became less infectious, however, the property of virulence for man could not be completely disassociated from that of infection.

Studies of Mogabgab (1968 a,b) on the protective effects of the inactivated Mycoplasma pneumoniae vaccine prepared by Metzgar et al. (1966) also supported the findings that the vaccines were effective. In military recruits receiving vaccines, three-fourths of the men developed a neutralizing antibody following a single injection of vaccine. The second dose did not increase the response appreciably. The degree of immunity provided by the vaccine was, as evaluated by this worker, adequate.

Swine mycoplasma vaccines

Betts et al. (1955) stated that there was no field evidence to show that a natural attack of enzootic pneumonia produced any appreciable degree of immunity, however, Lannek and Bornfors (1957) showed that a strong immunity developed in experimentally induced pneumonia. Pigs were infected, allowed to recover and then challenged 118 days after the primary infection. All the challenged pigs were free from enzootic pneumonia at slaughter, whereas all the positive controls had pneumonia. Goodwin et al. (1969) also found that pigs recovered from the disease were strongly immune to challenge and no lung lesions developed when inoculated with lung suspensions that produced extensive lesions of enzootic pneumonia in control animals.

A tissue culture derived M. hyorhinis, a frequent contaminant in swine pneumonia, is prepared as a vaccine for the prevention of swine enzootic pneumonia (Pfizer, Inc. 1968). The vaccine is inactivated by adding formalin. Alumnium hydroxide gel is used as an adjuvant. The manufacturer recommends all gilts, sows and boars be vaccinated. Vaccination is repeated six weeks later. Pregnant gilts and sows should be vaccinated ten to 30 days before each farrowing; boars are vaccinated twice a year.

The vaccination is given subcutaneously and the dosage is 20 ml for each vaccination. No side effects were reported. The vaccine is claimed to be able to free pigs in heavily infected herds from symptoms of pneumonia a few months after vaccination.

MATERIALS AND METHODS

Source of Mycoplasma hyopneumoniae

Mycoplasma hyopneumoniae strain #11 (L'Ecuyer, 1962) was used throughout the course of the study. This strain was isolated from a pig that had gross consolidation of the apical and cardiac lobes of the lung. It was maintained in cell-free medium and was able to reproduce typical pneumonic lesions in experimental pigs 80 to 100 per cent of the time (Maré, 1965). A detailed history of this strain has been documented (Huhn, 1968). A lyophilized culture derived from the 20th passage of this organism in cell-free medium was used as the initial inoculum. The culture was subcultured at three to four day intervals and incubated at 37°C.

Culture Media and Growth of Mycoplasma

Liquid medium

Dulbecco's phosphate buffer (DPB) was used as the basis of a liquid medium for the growth and isolation of Mycoplasma hyopneumoniae. The formulation and preparation of components of DPB medium was as follows:

A. Dulbecco's phosphate buffered saline

Dulbecco's buffered phosphate saline was prepared as described (Dulbecco and Vogt, 1954).

B. Swine serum

Swine serum was processed according to the method of Sachs

(cited by Boulanger, 1954).

1. The pH of the serum was adjusted to between 4.3 to 4.5 with 1 N HCl, being careful not to allow it to drop below pH 4.2.
2. Allow to stand overnight at 4 C.
3. Centrifuge serum at 590 xG for 15 minutes; discard sediment.
4. Supernatant was then clarified through Whatman GF/A glass filter pad and Selas #10, #01, and #02 filters.
5. The pH was adjusted to 7.0 with 1 N NaOH.
6. Store at -20 C until use.

C. Lactalbumin hydrolysate* (Enzymatic)

Lactalbumin hydrolysate was added to the medium at a final concentration of 0.5 per cent.

D. Yeast extract (Hayflick, 1965)

Yeast extract was prepared by the following procedure:

1. Add 250 gm of yeast** to 1 liter of distilled water.
2. Heat to boiling and stir.
3. Filter through two sheets of Whatman #1 filter paper.
4. Adjust the pH to 8.0 with 1 N NaOH.
5. Dispense in 5-8 ml aliquote, and autoclave at 15 pound pressure for 15 minutes. A precipitate may form after autoclaving, avoid using the precipitate.

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*Nutritional Biochemicals Corporation, Cleveland, Ohio.

**Fleischmann type 20-40 yeast, Standard Brands Inc. New York, New York.

6. Store at -20 C until use.

7. The yeast extract was used at a final concentration of 1 per cent.

E. Dextrose

Sufficient dextrose was added to give a final concentration of 0.25 per cent.

F. The DPB medium was prepared by combining the following materials:

Dulbecco's phosphate buffer	790 ml
Lactalbumin hydrolysate	5 gm
Yeast extract	10 ml
Dextrose	2.5 gm
Swine serum	200 ml

1. The pH was adjusted to 7.4 with 1 N NaOH.

2. The medium was clarified through Selas #10, and #01 filters.

3. The medium was sterilized through a Selas #02 or #03 filter.

4. It was then tubed in screw-caped tubes or flasks.

5. The medium was incubated at 37 C for 48 hours and then stored at 4 C until used.

Solid medium

The solid medium was composed of the following materials:

A. Agar*	1 gm
Hank's balanced salt solution** (1X)	20 ml

*Bacto agar, PPLO agar or Noble agar: Difco Laboratories Inc. Detroit, Mich. Oxoid agar: Calab Laboratories, Inc, Chicago Heights, Ill.

**Microbiological Associates, Bethesda, Maryland.

The pH was adjusted to 7.4 and the mixture autoclaved at 15 pound pressure for 15 minutes.

B. Filter sterilized DPB medium 80 ml

After cooling the agar preparation to 50 C and warming the DPB medium to 50 C, A and B were mixed and pipetted in six to seven ml amounts into plastic petri dishes*. The agar congealed at room temperature. The plates were then incubated at 37 C overnight.

Growth of organism in fluid medium for routine maintanance

Each culture tube containing about six ml of DPB medium, was inoculated with 0.6 ml of approximately four-day-old mycoplasma culture. The culture was incubated at 37 C.

Growth of organism in fluid medium for antigen production

Fifty ml of three-day-old culture was inoculated into 500 ml of DPB medium in a screw-caped flask and incubated at 37 C. After five days incubation, the organisms were harvested by centrifugation at 18,000 rpm (35,000G) for 30 minutes in a Beckman model L-2-65 preparative ultracentrifuge. The sediment was washed twice with phosphate buffered saline (PBS) (pH 7.4) and finally resuspended in deionized water. The amount of water added to resuspend the sediment was 1/100 of the original volume of the culture medium.

*Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, California.

Growth of organism on solid medium

One-tenth ml of a three-day-old mycoplasma culture was inoculated onto the surface of the agar. It was incubated at 37 C for two to three hours to obtain initial drying. It was then sealed in a plastic bag to prevent excess evaporation and incubated at 37 C in an inverted position. Daily examination of the surface of the plates with a dissecting microscope was made for 10 days.

Growth of organism on plastic and glass surface

Ten ml of DPB medium was added to a plastic* or glass flask** and one ml of three-day-old M. hyopneumoniae culture was inoculated into it. The flasks were incubated at 37 C. The culture fluid was decanted and fresh medium added every fourth day. Frequent microscopic examination was made to see whether the organism was able to attach and grow on the glass or plastic surface.

Swine Infection Procedures

Source of experimental swine

All pigs used in this work were obtained from the respiratory disease free heard maintained at the Veterinary Medical Research Institute, Iowa State University. This herd is founded on surgically derived stock and has been free of respiratory disease for more than

*Falcon Plastics, Division of B-D Laboratories, Inc., Los Angles, California.

**Corning Glass Works, Corning, New York.

16 years. Most of the pigs used were Yorkshires and Hampshires although a few cross-breds between the two were used. The age of the pigs used in the experiments ranged from 6-14 weeks.

The experimental pigs were housed in strict isolation for the duration of each experiment unless otherwise specified. In all cases the pigs were alloted to treatments by randomized selection of numbers. The pigs were fed a complete 17 per cent protein ration free of antibiotics.

Infection of pigs

Each pig was given five ml of a three-day-old culture of *M. hyopneumoniae* daily for three days. The culture was administered by intranasal instillation with the snout of the pig held in an up-right position as the culture fluid was dripped slowly into the nostril. Pigs were necropsized after observation periods ranging from three weeks to 11 months depending on the nature of the experiment.

Necropsy examination

All pigs were electrocuted and exsanguinated. Blood and lung tissue were collected for various purposes as indicated.

Tissue sections

Lung tissues were collected and fixed in 10 per cent formalin immediately after death. After three days fixation, the tissues

were trimmed, processed and embedded in paraplast* tissue embedding medium. They were sectioned at six micron and mounted on glass slides with an albumin fixative. All sections are stained with Harris's hematoxylin and counter stained with eosin Y (U.S. Armed Force Institute of Pathology, 1960).

Blood

Collected blood was allowed to clot at room temperature, and the serum separated by centrifugation. It was then stored at -20 C until use. None of the serum samples used in this study were heat inactivated.

Mycoplasma isolation

Portions of lung lesions were aseptically removed and ground in the presence of DPB medium. Five-tenth ml of the lung homogenate was inoculated into a tube of DPB medium for the isolation of M. hyopneumoniae. Two hundred I.U/ml of penicillin was added to the medium to retard the growth of contaminants. The inoculated medium was incubated at 37 C and subcultured at two-day-intervals. The isolation attempt was considered to be negative if growth of M. hyopneumoniae could not be demonstrated after 10 transfers.

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*Sherwood Medical Industries, Inc., St. Louis, Mo.

Demonstration of the presence of M. hyopneumoniae

Cultures grown in DPB medium were used for the demonstration of growth of M. hyopneumoniae. Portions of the medium were taken out and centrifuged at approximately 590 xG for 15 minutes. A smear of the sediment was prepared on a glass slide. The slide was then fixed and stained according to the procedures described by Maré (1965), and examined for the presence of Mycoplasma hyopneumoniae.

Serological Tests

Preparation of buffered solutions

Phosphate buffered saline (PBS) The PBS was composed of the following:

A. KH_2PO_4 0.9078 gm in 1,000 ml of 0.85% NaCl

B. Na_2HPO_4 1.4845 gm in 1,000 ml of 0.85% NaCl

The desired pH was achieved by varying the proportions of the two solutions. The pH 7.4 PBS was made of 19 ml of A and 81 ml of B, and the pH 6.4 contains 73 ml of A and 27 ml of B.

Veronal buffered saline (VBS) A VBS described by Hirschfeld (1960) was used for immunoelectrophoresis. It was composed of the following:

	electrode buffer	agar buffer
veronal	13.80 gm	16.60 gm
sodium veronal	87.60 gm	105.10 gm
calcium lactate	3.84 gm	15.36 gm
distilled water to	10 liter	10 liter

The pH of both of the buffers was 8.6.

Preparation of antigens

Mycoplasma hyopneumoniae cultures were concentrated one-hundred fold by centrifugation and then suspended in distilled water. The sedimented organisms were processed in three ways to make three different antigens.

Antigen I The 100x concentrated organisms were alternatively frozen in a solid carbon dioxide-alcohol bath and thawed in 70 C running tap water. After 20 cycles of freezing and thawing, an appropriate amount of NaCl and merthiolate* were added to make a final concentration of 0.85 per cent and 1:10,000 respectively. It was then stored at 4 C. This antigen preparation was designated as Antigen I.

Antigen II Adequate two per cent sodium lauryl sulfate** (SLS) in distilled water was added to 100x the concentrated mycoplasma suspension to make a final concentration of SLS of 0.2 per cent. The cloudy suspension cleared within minutes. The suspension was then dialyzed against 5 M ammonium sulfate (pH 8.0) overnight. The precipitate was pelleted and then dialyzed against pH 7.4 PBS for 48-72 hours to remove ammonium sulfate. Merthiolate was added to a final concentration of 1:10,000, and it was then stored at 4 C. This antigen preparation was designated as Antigen II.

Antigen III One part of solution containing two per cent SLS and two per cent sodium desoxycholate (DOC) was added to nine parts of the 100x concentrated mycoplasma suspension to lyse

*Eli Lily and Co., Indianapolis, Indiana.

**Sigma Chemical Corporation , St. Louis, Missouri.

the organisms. Then a volume of 24 per cent ammonium sulfate equal to that of the lysed organisms was added and the mixture allowed to stand at room temperature for 30 minutes. The flocculating material was pelleted by centrifugation at 2,000 rpm (590 xG) for 15 minutes and resuspended in pH 7.4 PBS. The process of SLS-DOC lysis and ammonium sulfate precipitation was repeated once. The second sediment was reconstituted to the starting volume with PBS, and dialyzed against PBS for 48-72 hours to remove ammonium sulfate. Merthiolate was added to make a final concentration of 1:10,000. The antigen preparation was then stored at 4 C and was designated as Antigen III.

Production of antisera

Experimentally infected pig sera Experimental pigs were inoculated intranasally with Mycoplasma hyopneumoniae and were bled at frequent intervals after inoculations. The sera of the pigs were collected, separated and stored at -20 C.

Hyperimmune pig sera Pigs were inoculated with vaccines G and F intramuscularly. Details of vaccine preparation and vaccination procedures are presented in the section on Active Immunization Trials.

Vaccinated pigs were bled at weekly intervals, and were infected with three doses of three-day-old mycoplasmas culture two weeks after the last vaccination. The pigs were necropsied three weeks later. Blood was collected from the pigs, the sera separated and stored at - 20 C.

Naturally infected pig sera Blood samples from either conventional or specific pathogen free (SPF) herds were obtained through various cooperating veterinarians. Some of the samples were from herds that had a history of pneumonia whereas others were from herds that had no known history of pneumonia. Sera from the blood samples were separated and stored at - 20 C.

Treatment of sera with mercaptoethanol One per cent 2-mercaptoethanol* was prepared in PBS pH 7.4. One tenth ml of 2-mercaptoethanol was added to 0.9 ml of serum and incubated at 37 C for one hour. The treated serum was then dialyzed against PBS pH 7.4 for 24 hours to remove 2-mercaptoethanol, and the dialyzate was tested and compared with untreated serum by means of the indirect hemagglutionation test.

Indirect hemagglutionation test

Swine red blood cells (rbc), collected from the anterior vena cava, were washed three times at 1500 rpm (340 xG) with PBS at 7.4. One ml of the washed and packed cells was then resuspended in 80 ml of 1:40,000 tannic acid** in pH 7.4 PBS and incubated in a 37 C water bath for 15 minutes. The tannic acid treated cells were then sedimented by centrifugation at 2000 rpm (590 xG) and the pellet washed once and finally resuspended in adequate amount of PBS pH 7.4 to give a 10 per cent rbc suspension.

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*Sigma Chemical Co., St. Louis , Missouri

**Mallinkrodt Chemical Works, St. Louis, Missouri

For the indirect hemagglutination test, 0.5 ml of 10 per cent rbc, 0.3 ml of antigen I, II or III, and 2 ml of pH 6.4 PBS were mixed and incubated in a 37 C water bath for 20 minutes. The antigen treated cells were then centrifuged at 1,500 rpm (340 xG) for five minutes and washed once with PBS pH 7.4. The pellet was resuspended in adequate PBS pH 7.4 to make a final concentration of one percent.

The antiserum dilution was carried out in plastic trays* with U-shaped wells. PBS with two per cent gelatin was used as the diluent. To each well was added 0.05 ml of diluent. Five one-hundredth ml of serum was added to the first well in a series. Dilutions were made by means of a diluter** which carried 0.05 ml of fluid from one well to the next thus making a two-fold dilution.

Twenty-five thousandth ml of one per cent antigen treated red blood cells was added to each well containing serially diluted antisera by means of a dropper≠. The plate was then sealed with a plastic sealer, shaken for one minute on a shaking table≠≠, and incubated at 37 C for one hour. The results were read, following incubation, with the aid of a mirror and recorded.

Preservation of antigen coated red blood cells

A. The procedure of Dowdle and Heyward (1968) was used for the preservation of antigen coated rbc. Briefly, adequate antigen

*Limbro Chemical Co. Inc., New Haven, Conn.
**Cooke Engineering Co., Alexandria, Virginia.
≠Cooke Engineering Co., Alexandria, Virginia.
≠≠Arthur H. Thomas Co., Philadelphia, Pa.

treated rbc were added to 1:150 horse serum diluent in 0.85 per cent saline which contained 11 per cent polyvinylpyrolidone, to make a final rbc concentration of five per cent. Two ml of the rbc were then distributed to ampules, quickly frozen in a dry ice-alcohol bath, sealed, and stored at -70 C. Untreated rbc were similarly preserved and served as a control.

B. The procedures of Hubert et al. (1963) were used. Antigen-coated rbc were diluted to 0.05 per cent final concentration with PBS pH 7.4 as the diluent, and an equal amount of a mixture of freshly prepared 10 per cent dextrose and 15 per cent lactose in distilled water was added. Two ml of the rbc-preservative mixture were transferred to an ampule which was then sealed, quickly frozen and stored at -70 C.

Selected ampules were thawed each week. The rbc were pelleted by centrifugation, and resuspended to the original volume with PBS pH 7.4. A total intact rbc count was made to determine the extent of cell lysis under the conditions of preservation.

Agar gel diffusion test

The double diffusion technique of Ouchterlony (1949) was used. The diffusion agars were prepared as follows:

A.	0.85 % NaCl	100 ml
	Noble agar*	1 gm
B.	PBS pH 7.4	100 ml
	Noble agar	1 gm

*Difco Laboratories, Inc., Detroit, Michigan

C.	Veronal buffered saline	100 ml
	Noble agar	1 gm

All preparations were autoclaved at 15 pound pressure for 15 minutes. Seven ml of hot agar were pipetted to plastic petri dish and allowed to congeal at room temperature. The wells were cut with the aid of a gel punch assembly*; the distance between each well was seven mm.

Two tenth ml of preinoculation and 2, 4, 6, 8 and 10 weeks postinoculation sera from experimentally infected pigs were pipetted into surrounding wells and ran against 0.2 ml of Antigen I, II and III, respectively, in the center reservoir. The plates were incubated at 37 C overnight prior to examination.

Immunoelectrophoresis

The procedures of Hirschfeld (1960) were used. The veronal buffered solutions described previously were used for the immuno-cell** and gel. Gel buffer was diluted five times and one per cent Noble agar added. The agar was then autoclaved at 15 pound pressure for 15 minutes and two ml of hot agar was spread on the surface of a clean glass slide and allowed to solidify. The appropriate holes and trough were made with the aid of a cutter≠.

The test samples were added to the wells and the electrophoresis was accomplished in an immuno-cell for 90-100 minutes.

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*GRAFAR Corp., Detroit, Michigan.

**E-C Apparatus Corp., Philadelphia, Pa.

≠Buchler Instruments, Fort Lee, New Jersey.

The agar in the center trough was then peeled off and rabbit anti-porcine sera* added to the trough, and the slide incubated overnight at 37 C in an moist chamber. The slide was dried following incubation, and washed with saline for five to six hours to remove excess protein and dried again. The dried slide was then stained with amido black (2%) followed by destaining in a destainer containing alcohol, water and acetic acid in the ratio 5:5:1. The stained slide could be kept for a long period of time.

Metabolic inhibition test

The metabolic inhibition test of Taylor-Robinson et al.(1966a) was used with slight modification. The test was carried out in acid cleaned, sterile, screw-capped glass tubes. Each tube contained 0.4 ml DPB medium containing one per cent horse serum. Four-tenth ml of four week post-infection sera was added to the first tube and a two-fold dilution of serum made. To each dilution was added 0.4 ml of three-day-old culture and incubated at 37 C. The tubes were examined daily for 10 days.

Stability of antigen

Antigen preparations were stored at 4 C. Aliquots of antigens were taken out at weekly intervals to sensitize swine rbc for the indirect hemagglutination test. A pre-inoculation, a hyperimmune and a 120 day post-infection sera were used to determine the stability of antigens in storage.

*Hyland Division, Travenol Laboratories, Inc., Los Angeles, Calif.

Specificity of the indirect hemagglutination test

Preliminary observations indicated that Antigen II worked best in the IHA test so this antigen was used for the determination of the specificity of this test. Sera from pigs infected with M. hyopneumoniae, M. granularum, M. hyorhinis, and from horses hyperimmunized with Erysipelothrix insidiosa* were tested for their respective reactivity with Antigen II sensitized rbc. Comparison with the CF test (Takatori et al. 1968) was also made.

Duration of passively transferred antibody

Thirty ml of pooled antiserum derived from pigs vaccinated with vaccine F was filter sterilized and injected intraperitoneally into each of three pigs weighing 20 to 30 pounds. The pigs were bled at frequent intervals and the sera of those pigs were tested by means of indirect hemagglutination test for antibodies to Mycoplasma hyopneumoniae. Antigen II was used for the IHA test.

Antiserum derived from pigs vaccinated with vaccine G was similarly treated. The blood samples drawn from the three pigs used for this experiment were also tested for detectable antibodies to M. hyopneumoniae.

Active Immunization Trials

All actively immunized pigs were inoculated intramuscularly with one ml of the appropriate vaccine preparation. A week later

*Professional Biological Co., Denver, Colorado.

they were inoculated with two ml, and another week later with three ml of the same preparation administered by the same route. All the immunized pigs were challenged with five ml of three-day-old M. hyopneumoniae culture each day for three days, two weeks after the last inoculation unless otherwise stated.

Experiment I

The one hundred-fold concentrated suspension of organisms was extracted with three volumes of ether. The mixture was gently shaken a few times and then left at 4 C overnight. The ether soluble fraction was aspirated off and the remaining aqueous phase was dialyzed against distilled water for 18 hours. The dialyzate was then mixed throughly with an equal volume of incomplete Freund adjuvant. Five hundred units per ml of penicillin and 500 ug/ml of streptomycin were added to the vaccine to prevent the growth of contaminants. This vaccine was designated as vaccine A.

Four pigs were injected intramuscularly with the vaccine. They were challenged with three doses of live mycoplasma culture two weeks after the last vaccination. A group of four pigs served as positive controls and were also challenged with three doses of mycoplasma culture. All pigs were necropsied three to four weeks postexposure.

Experiment II

This vaccine was prepared the same as vaccine A except that 0.3 per cent formaldehyde replaced the antibiotics. The preparation was designated vaccine B.

Four pigs were vaccinated and another four served as positive controls. They were necropsied four weeks post-infection.

Experiment III

Four pigs were infected by intranasal exposure to three doses of M. hyopneumoniae culture. Three weeks later they were immunized with vaccine B. The immunization scheme was the same as described in Experiment I. The pigs were necropsied two weeks after the last inoculation to determine the effect of immunization on elimination of lesions in infected pigs.

Experiment IV

The vaccine used in this experiment was the same as that described in Experiment II. However, the pigs in this trial received only two doses of vaccine at weekly intervals, and the dosage for both vaccinations was two ml each.

Four pigs were vaccinated and another four served as positive controls. They were infected on three consecutive days with three-day-old M. hyopneumoniae culture and necropsied three weeks post-exposure.

Experiment V

The 100-fold concentrated organsims were lysed with two per cent SLS, extracted with ether overnight and then dialyzed against distilled water for 48 hours. An equal amount of incomplete Freund adjuvant

was added to the dialyzate and sufficient formaldehyde was added to make a final concentration of 0.3 per cent. This vaccine was designated as vaccine C.

Eight pigs were used. Four were vaccinated and the other four served as positive controls. They were necropsied four weeks after infections.

Experiment VI

Vaccine C was used in this trial. Four pigs received two doses of two ml each a week apart. They were exposed to M. hyopneumoniae culture two weeks after the last vaccination. Four un-vaccinated control pigs were also exposed at the same time. All pigs were necropsied four weeks postinfection.

Experiment VII

Vaccine C, as well as a modification of it was used in this experiment. This modification consisted of omission of the ether extraction step. The modified preparation was designated as vaccine D.

Fifteen pigs were used in this experiment: three of the pigs received vaccine C, six received vaccine D and the remaining six received no vaccine. A week after the first vaccination, three of the six un-vaccinated pigs were infected with live culture. Two weeks after the last vaccination (four weeks after the infection) three of the six pigs receiving vaccine D were removed and housed with the infected pigs in an attempt to determine whether contact

infection would occur in vaccinated pigs. They were housed together for three months.

The remaining six vaccinated pigs and three un-vaccinated pigs were given an intranasal dose of live culture of M. hyopneumoniae daily for three days. They were necropsied 25 days after the last exposure.

Experiment VIII

Vaccine preparations E and F were used in this trial. The former was prepared from organisms lysed with a final concentration of 10 M SLS at 37 C for two hours. This lysate was mixed with an equal amount of incomplete Freund adjuvant. Vaccine F was prepared by freezing the organism suspension in a dry ice-alcohol bath followed by thawing in 65-70 C running tap water; after twenty cycles of freezing and thawing, an equal amount of incomplete Freund adjuvant was added. Both vaccines had 500 i.u./ml of penicillin and 500 ug/ml of streptomycin added.

Each vaccine was given to four pigs. One of the pigs receiving vaccine E died from a ruptured umbilical hernia and a second pig died of unknown causes during the course of the trial. All surviving pigs were infected with three doses of live Mycoplasma hyopneumoniae culture two weeks after vaccination along with a group of four positive control pigs. They were all necropsied four weeks after infection.

Experiment IX

Vaccine F and G were used. The preparation of vaccine G was as follows:

The concentrated organisms were lysed with two percent SLS at room temperature for 15 minutes followed by dialysis against 1.3 M ammonium sulfate overnight. The precipitate was further dialyzed against distilled water to remove ammonium sulfate. An equal amount of incomplete Freund adjuvant and 500 i.u./ml of streptomycin was added following dialysis.

Eight pigs were given vaccine F and three were given vaccine G. They were infected with live M. hyopneumoniae culture along with four positive control pigs. All pigs were killed four weeks postinfection.

Experiment X

A three-day-old culture of M. hyopneumoniae was treated with formaldehyde at a final concentration of 1:2,000. The preparation remained at room temperature for 24 hours before it was used. The bacterin was designated as vaccine H.

Four pigs were used for this trial. They received, by intranasal instillation, five ml of the bacterin daily for three days. One week after the bacterin administration, they were infected with three daily doses of M. hyopneumoniae culture. Three pigs of about the same age served as positive controls and were infected and necropsied at the same time as those receiving bacterin.

Experiment XI

This experiment was an enlarged version of Experiment VII and IX. Because of the larger number of pigs involved, they were housed in open pens instead of the usual isolation units. Fifty pigs were divided randomly into five pens with 10 pigs in each pen. One pig died from the porcine stress syndrome during transportion from the swine herd to the experimental area. Ten pigs in pen 1 and five of the pigs in pen 3 were vaccinated with vaccine F. The pigs in pen 2 and the remaining five pigs in pen 3 received vaccine G; pigs in pens 4 and 5 served as negative and as positive controls.

Serum samples were taken from each of the pigs before vaccination and two weeks after vaccinations. Five pigs in pen 5 were necropsied prior to infecting pigs with mycoplasma culture. The lungs were all grossly normal. All the remaining pigs were then infected with five ml of three-day-old M. hyopneumoniae culture daily for three days. The pigs in this trial were sacrificed four weeks after infection.

Passive Immunization Trials

Immune sera from appropriate source were sterilized by passing through Selas #10, #01 and #02 filters. All pigs in these trials received intraperitoneal injection of immune sera and were infected with five ml of M. hyopneumoniae culture four, 24, and 48 hours after immunization.

Experiment I

Sera from pigs receiving vaccine A were pooled and filter sterilized. Two pigs weighing about 40 pounds received 50 ml of the serum each and were infected with M. hyopneumoniae culture. Two pigs of about the same weight served as positive controls.

The trial was repeated once with six pigs involved; three were immunized and the other three served as positive controls. All pigs were necropsied 21 days postinfection.

Experiment II

Sera from pigs receiving vaccine F were used. Thirty ml of serum was injected into each 20 to 30 pound pig. Three pigs were passively immunized with the serum and three others served as positive controls. All pigs were exposed to live organisms and were kept in isolation for 24 days before they were necropsied.

Experiment III

The experimental design in this trial was the same as that in Experiment II except that the antisera was derived from pigs receiving vaccine G. Six pigs were used in this trial. Four of them were passively immunized. Of these four, two were challenged with mycoplasma culture along with two positive controls. The two remaining immunized but not challenged pigs were then housed with the infected positive control pigs in order to determine whether contact infection could be established in passively immunized pigs. All pigs were sacrificed three weeks postinfection.

Experiment IV

This trial started concurrently with Experiment XI of the Active Immunization trial so that both trials shared the same positive controls.

Ten pigs of 20-30 pound weight were used. They were inoculated with 30 ml of pooled sera from pigs receiving vaccine G and were infected with mycoplasma culture four, 24 and 48 hours post-immunization. They were sacrified four weeks postexposure.

Statistical analysis of immunization results

Chi-square was used for analysis of results obtained in both active and passive immunization trials.

RESULTS

Growth of M. hyopneumoniae

Liquid medium

The growth of M. hyopneumoniae in DPB could be visualized after two to three days of incubation at 37 C by the development of a yellow color due to acid production. A delicate spiral of growth could be demonstrated by gently shaking the tube.

Solid medium

None of the agar preparations made in this study was capable of supporting the growth of M. hyopneumoniae as no typical "fried egg" colonies were seen. However, pin-head sized colonies with a smooth surface were seen regularly under 30X magnifications. When a block of agar containing these colonies was transferred to DPB medium, characteristic growth of mycoplasma was observed in the broth medium. Giemsa stained preparation of this growth revealed organisms characteristic of M. hyopneumoniae.

Growth on plastic surface

Few Mycoplasma hyopneumoniae were able to attach to the plastic surface, and the attachment did not seem to be firm as it could be shaken off even with gentle motion. Mycoplasma hyopneumoniae is therefore considered unable to grow on plastic surface.

Growth on glass surface

Patches of mycoplasma colonies were present on glass surface after five to seven days incubation and several changes of growth medium . The colonies were irregular in shape and size. The clumps of cells did not spread out and no confluent sheet of cells was observed.

Demonstration of organism by Giemsa stain

Small bluish-purple coccoid organisms, with an approximate size of 200-400 mμ, were present in Giemsa stained preparations. The organisms were generally present in small clusters, but ring forms were occasionally seen especially in older culture. No filamentous or granular form were seen.

Serologic Tests

Indirect hemagglutination test antigens

Sera with a titer of 1:12 or higher were considered positive in the indirect hemagglutination test.

Antigen I This antigen detected antibodies in experimentally infected pigs 42 days after infection. Because this antigen was less sensitive than antigen II it was not used to any extent in this study.

Antigen II The antibody response in experimentally infected pigs was detected 14 days post-infection with this antigen. It reached a peak on the 63rd to 77th day and remained there until

the 193rd day. The titers of antibody started to fall after that, but remained detectable 325 days after infection (Figure 1).

The antibody response in pigs put in contact with those pigs that had been infected two months previously was detected seven to eight weeks post-exposure. It reached the maximum level at the 13th to 19th week post-exposure and remained at that level for more than 28 weeks (Figure 2).

Pigs that were put in contact with those that had just been infected with M. hyopneumoniae culture developed detectable antibodies five to six weeks post-exposure.

The antibodies were detectable in the vaccinated pigs seven days after the first inoculation and reached a peak on the 14th day (seven days after the second inoculation). The antibody titer did not increase further upon administration of the third dose of vaccine.

Antigen III With this antigen, antibodies were detected on the 36th day after inoculation. They reached a peak on the 63rd day, remained there for 106 days, and declined gradually but remained detectable 320 days post-infection. This antigen preparation was more sensitive than Antigen I but was less sensitive than Antigen II and therefore was not used extensively for serological tests.

Indirect hemagglutination test for the detection of experimentally infected pigs

Antigen II was used for this study. Three groups of pig sera were tested. All of the pigs in these three groups were experimentally

Figure 1. Serologic response to experimental infection

Fig. I Serologic Response To Experimental Infection

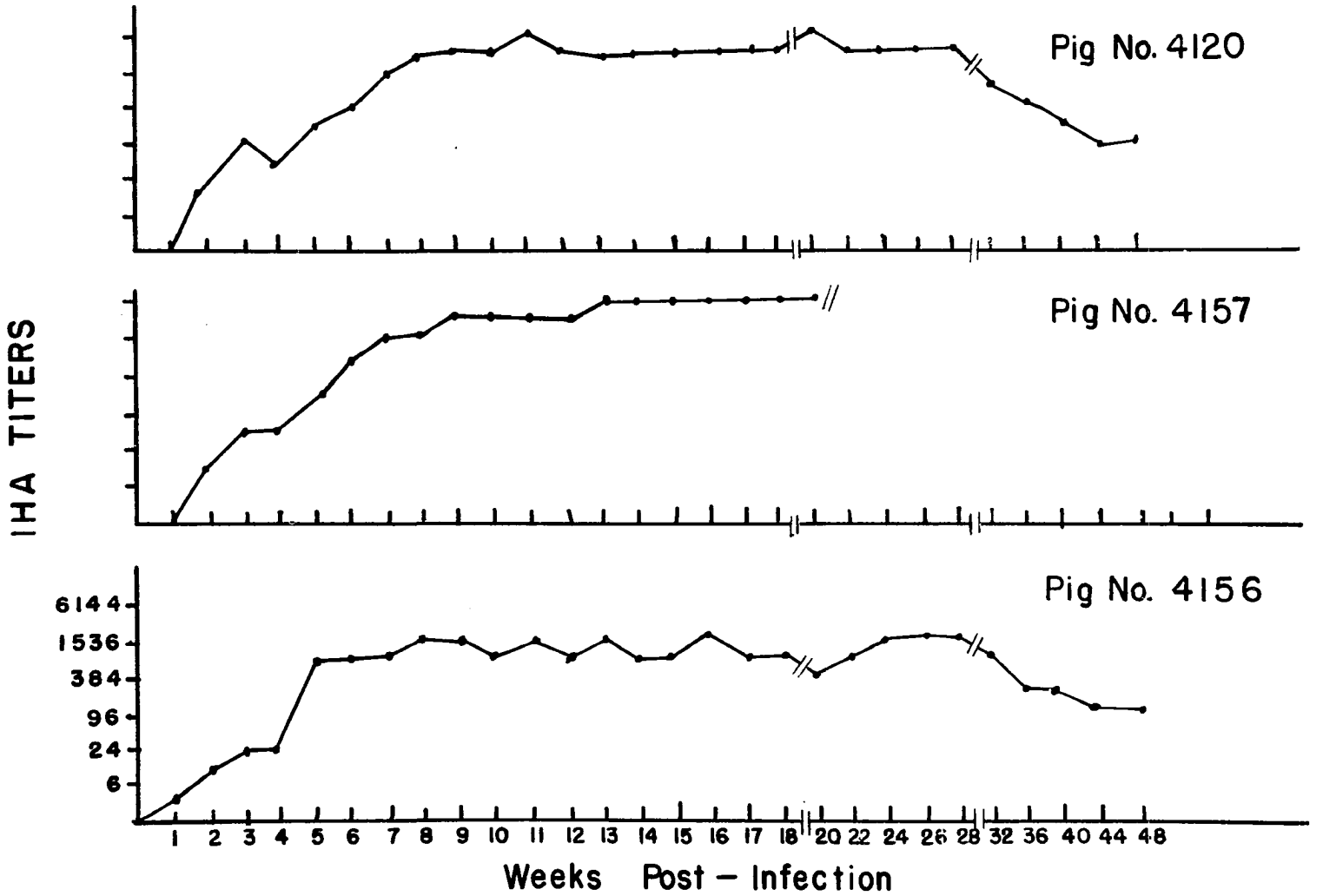
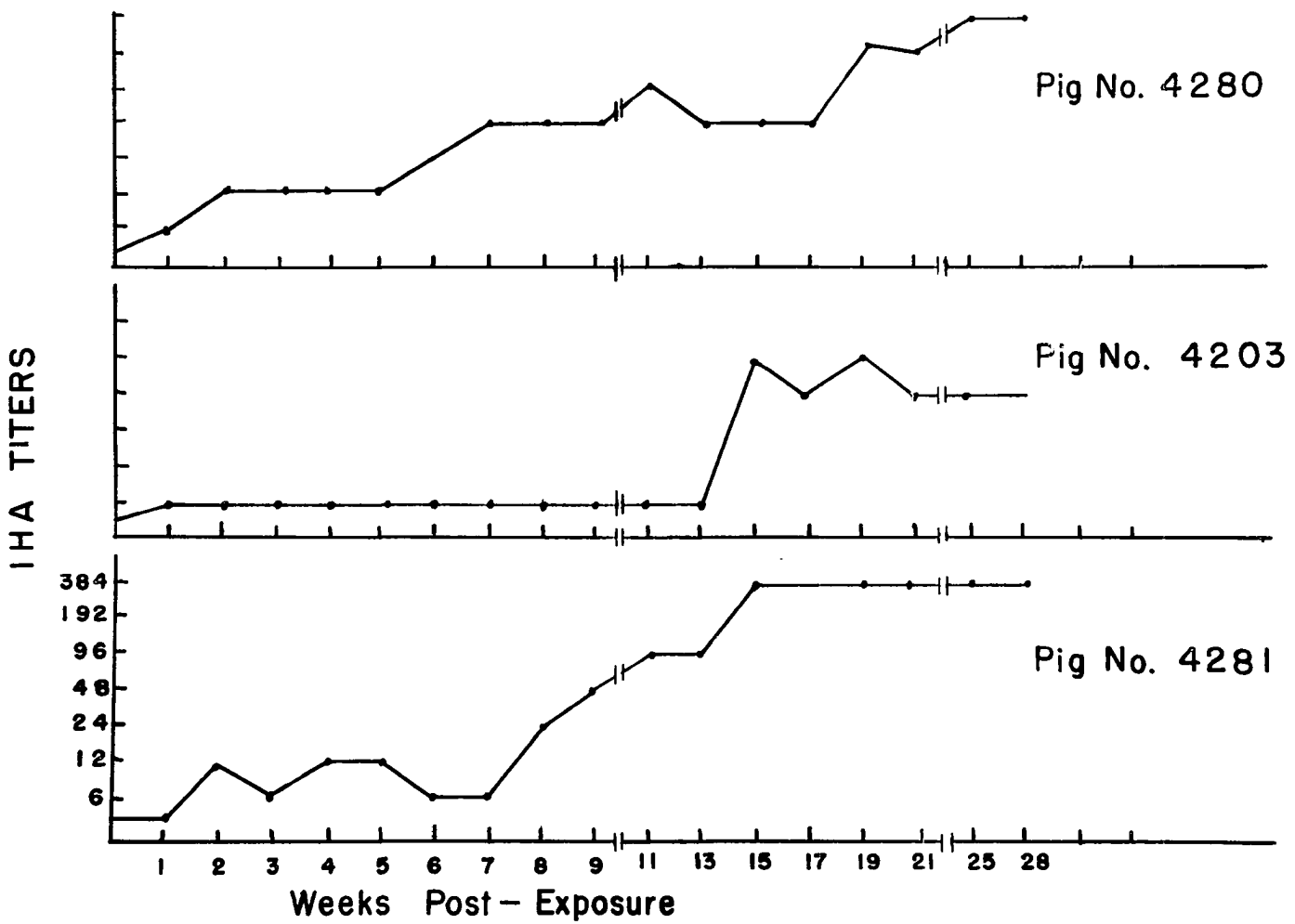


Figure 2. Serologic response to contact infection

Fig. 2 Serologic Response To Contact Infection



infected and necropsied three to four weeks post-inoculation.

Group I contained 24 pigs of which 20 developed lung lesions following experimental inoculation. Nineteen out of the 20 pigs with lesions were serologically positive. One of the pigs with lesion did not have an antibody titer. Among the four lesionless pigs, one was serologically positive while the other three were negative.

There were 20 pigs in Group II. Among them, four were uninfected controls which were all negative for M. hyopneumoniae antibodies when tested by IHA test. All of the inoculated pigs developed lesions, and 15 out of 16 were serologically positive.

Group III was composed of 30 pigs. Among them, 26 pigs developed pneumonic lesions. Twenty-three of those 26 pigs with lesion were serologically positive while the remaining three were negative. Two of the four lesionless pigs were serologically positive, while the other two were negative (Table 1).

Indirect hemagglutination test for the detection of naturally infected pigs

Twenty-two herds of pigs of various origins and locations had been examined for the presence of Mycoplasma hyopneumoniae antibodies. Among them, only one herd was consistently free of antibody. The others had detectable antibodies in one of 10 to 10 of 10 pigs tested. Of the total 433 pig sera examined, 291 had antibody titers (67.2 per cent).

There were slaughter data available on 169 of the pigs tested.

Table 1. Results of IHA tests on sera from experimentally infected pigs

Group	Serologic Results	With Lesion	Without Lesion
I	Positive	19/20*	1/4**
	Negative	1/20	3/4
II	Positive	15/16	0/4***
	Negative	1/16	4/4
III	Positive	23/26	2/4**
	Negative	3/26	2/4

*Number of pigs reacting to serologic test over number of pigs with lesions.

**Number of pigs reacting to serologic test over number of pigs without lesions.

***Un-infected control pigs.

Eighty seven(51.5 per cent)of the pigs had macroscopic pneumonia as against 82 with grossly normal lungs. Antibodies were detected in 78.1 per cent of the pigs with macroscopic lung lesions and in 68.2 per cent of the pigs without macroscopic lesions. The data on six SPF herds are presented in Table 2.

Duration of passively transferred antibodies

Both F and G vaccine derived antibodies reached maximum level six hours after they were passively transferred. The former remained at that level for about eight days then started to fall whereas the latter remained for 30 to 40 days. The vaccine F derived antibodies decreased to an insignificant level 18 to 25 days

Table 2. Mycoplasma hyopneumoniae antibodies in Iowa SPF herds as compared with lung lesions

Sample no.	Herd I		Herd II		Herd III		Herd IV		Herd V		Herd VI	
	IHA	Lesion	IHA	Lesion	IHA	Lesion	IHA	Lesion	IHA	Lesion	IHA	Lesion
1	256	NA*	32	--**	2	--	4	S	3	--	256	+
2	256	+≠	32	s≠≠	2	--	4	--	6	--	128	--
3	256	--	4	NA	32	--	256	--	3	--	256	--
4	256	--	8	--	8	NA	4	--	3	--	256	NA
5	256	--	2	S	32	--	32	--	3	--	128	--
6	64	--	2	NA	32	+	8	S	48	--	64	S
7	128	+	16	NA	8	+	4	+	12	--	16	--
8	128	--	2	--	16	--	16	+	3	--	64	--
9	128	--	16	--	16	--	64	--	3	--	64	--
10	64	--	2	--	16	--	8	--	6	--	64	--

*Not available.

**Negative gross lung lesion.

≠positive lung lesion.

≠≠Scarring in the lung.

after they were transferred as against 30 to 50 days for the vaccine G derived antibodies. The duration of passively transferred F and G antibodies are presented in Figures 3 and 4, respectively.

Specificity of Indirect hemagglutination test

There was no serological reaction when Antigen II was tested against antisera to M. granularum or M. hyorhinis. Furthermore, there was no observable reaction when the latter two organisms made into IHA antigens with the procedures were used to make Antigen II and were tested against M. hyopneumoniae sera.

Serological reaction of Mycoplasma hyopneumoniae Antigen II with Erysipelothrix insidiosa antisera was observed at low level (Figure 5).

Excellent correlation was obtained between indirect hemagglutination and previously reported complement-fixation results. The results of the comparison are presented in Table 3. The complement-fixation data were obtained from Takatori et al. (1968).

Treatment of pig sera with 2-mercaptoethanol

The titer of sera from experimentally infected and from hyper-immunized pigs, after treatment with 2-mercaptoethanol, generally decreased by two-fold. Most of the serologically positive sera from SPF pigs were completely devoid of antibody titer after treatment. A few decreased in titer by only four-fold.

Figure 3. Fate of passively transferred Ab-F-ab

Fig. 3 Fate of Passively Transferred Ab — F-ab

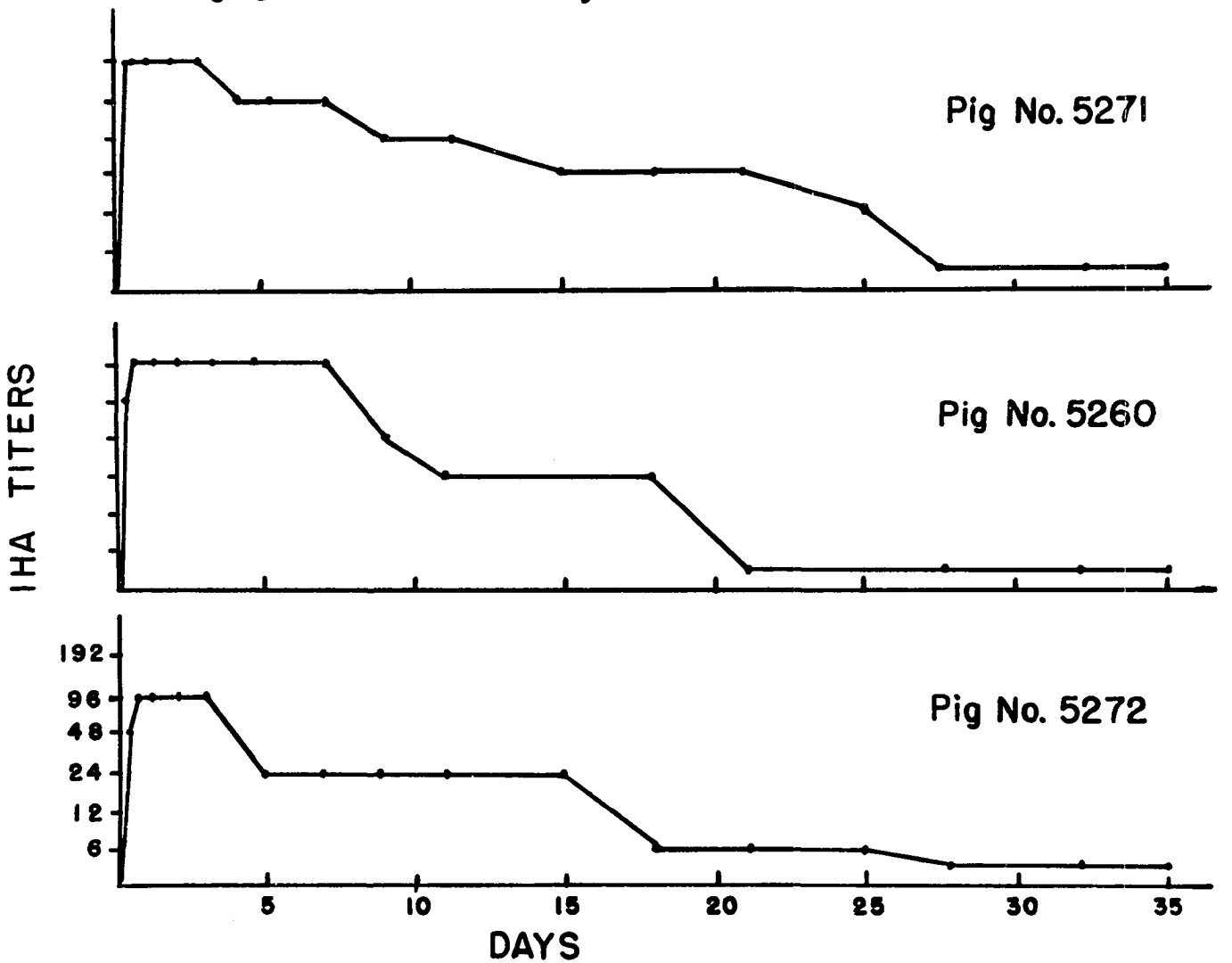
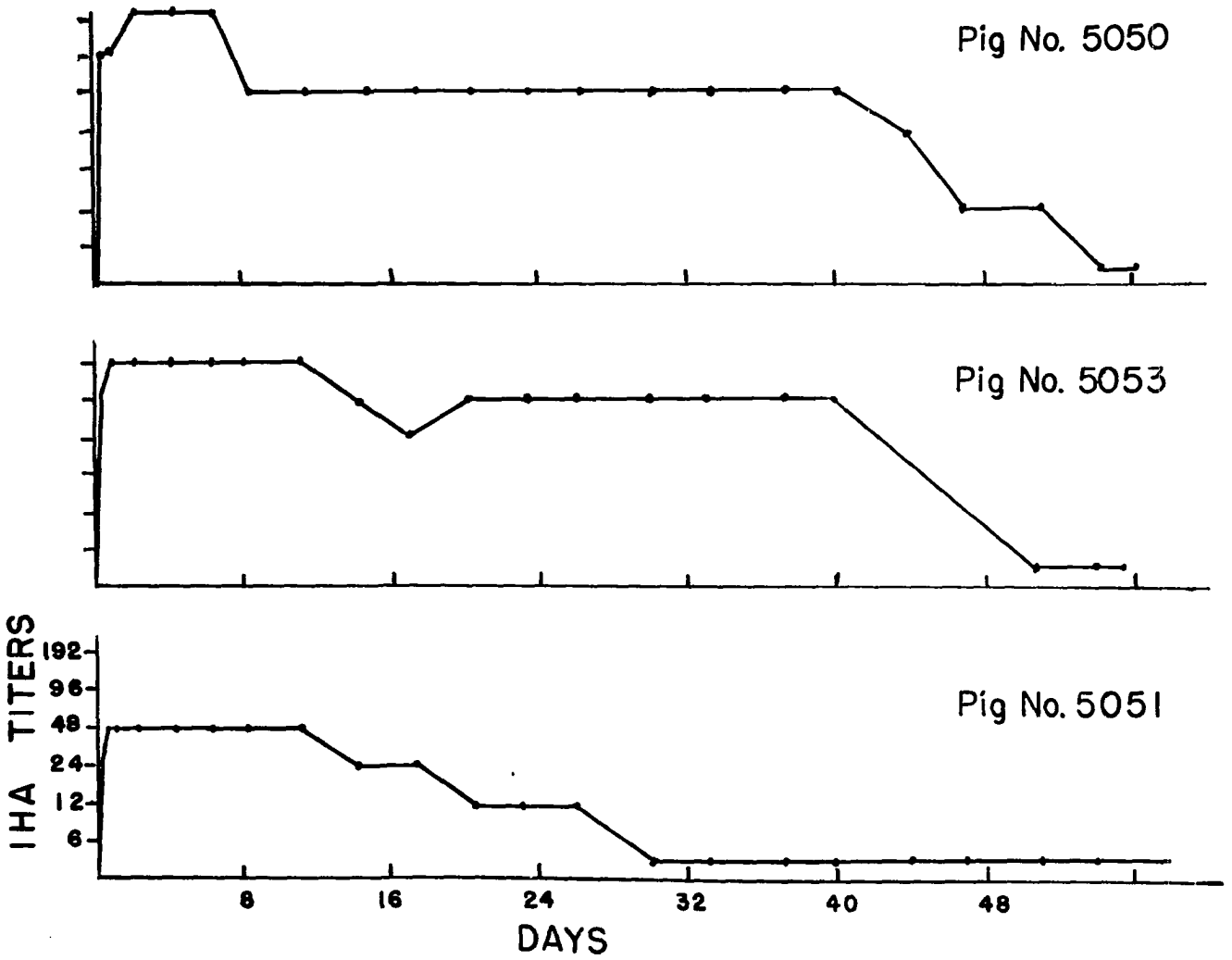


Figure 4. Fate of passively transferred Ab-G-ab

Fig. 4 Fate of Passively Transferred Ab — G - ab



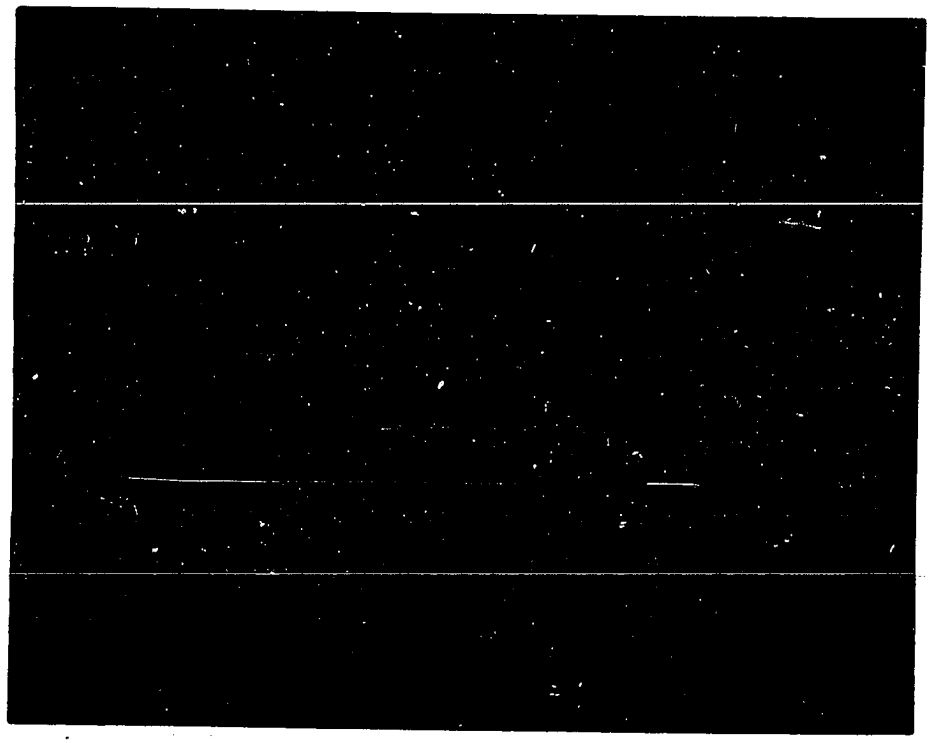


Figure 5. Indirect hemagglutination patterns

From top row to bottom row

Row 1: preinoculation serum
Row 2: 120 day post-inoculation serum
Row 3: hyperimmune pig serum
Row 4: Erysipelas antiserum rabbit origin
Row 5: Erysipelas antiserum horse origin
Row 6: diluent control

Two-fold dilution starting from 1:3 utilizing Antigen II.

Table 3. Comparison between antibody titers in naturally infected pig sera as measured by the CF* and IHA tests

Sample No.	Herd A CF	Herd A IHA	Herd B CF	Herd B IHA	Herd C CF	Herd C IHA
1	32	192	2	2	64	256
2	128	96	2	2	16	256
3	32	192	2	2	4	256
4	256	384	2	2	32	256
5	64	384	2	2	4	256
6	128	96	2	2	256	256
7	64	192	2	2	128	256
8	64	192	2	2	16	32
9	32	64	2	2	64	32
10	32	NS**	2	2	64	64
11	--	--	2	2	---	---
12	--	--	2	2	---	---

*CF results from Takatori et al. 1968.

**No sample remaining.

Preservation of red blood cells

Method A All cells, antigen treated and control cells, were lysed when they were thawed several minutes after freezing.

Method B Ninty-five per cent of the cells were lysed when thawed one week after freezing; 100 per cent lysis was observed in those thawed the second week.

Since neither of the preservation methods was satisfactory for swine red blood cells, attempts to preserve antigen coated cells were abandoned.

Agar-gel diffusion

Antigen I was found to be better than the other two antigen preparations for this purpose since it gave more intense precipitin

lines. It was therefore used for the test.

Sera from pigs hyperimmunized with vaccines A, F and G each gave two distinct and one faint bands in gel diffusion. Pre-inoculation sera did not react with Antigen I in the gel diffusion system. The precipitin antibodies started to appear seven days after the first vaccination. The intensity of the precipitin lines increased as the days after immunization increased. There was no precipitation reaction when experimentally infected and naturally infected pig sera were used.

Immunoelectrophoresis

No precipitation reaction was observed when infected lung homogenate was used as the antigen. A faint precipitin line was found in the IgG region when Antigen I was pipetted into the center trough and run against sera from F and G vaccine inoculated pigs after the serum had already undergone electrophoresis. There was no reaction between antigen I and pre-infection and 12 week post-infection sera.

Metabolic inhibition test

No metabolic inhibition of Mycoplasma hyopneumoniae by infected pigs sera or hyperimmune sera was observed in the system utilized.

Isolation of Mycoplasma hyopneumoniae

Isolation of M. hyopneumoniae from experimentally infected pigs was achieved consistently. None of the negative control pig lungs yielded M. hyopneumoniae. None of the attempts to isolate mycoplasma

from lesionless lungs of pigs that had been vaccinated and then infected were successful. Organisms were recovered from pigs that had been immunized and infected but developed lesions.

Stability of antigen

Antigen preparation was stable at 4 C for more than two months.

Animal Infection

Inoculation of pigs

Pigs inoculated intranasally with M. hyponeumoniae evidenced some mild coughing two weeks after infection. No other clinical signs were observed. On necropsy, there were no gross lesions except in the respiratory tract. The trachea contained mucous that was occasionally blood tinged. The lungs of the infected pigs had purple to gray colored, well demarcated lesions in the apical, cardiac and sometimes in the intermediate lobes.

Negative control pigs receiving no inoculations all had grossly normal lungs.

Microscopic lesions

Experimentally infected pigs The lung tissue was edematous and hemorrhagic. The hemorrhage is believed to be due to electrocution. Extensive lymphoid infiltration and/or hyperplasia were present around the bronchioles and blood vessels. Moderate numbers of lymphoid cells, neutrophils, and eosinophils were present in the interstitial

region. The lumen of some of the bronchioles was slightly constricted due to the hyperplasia of lymphoid cells and sometimes contained septal cells, neutrophils, and cell debris. Fibrosis of the interstitial area was observed in a few cases (Figure 6).

Immunized pigs with no lesions A few lymphoid cells were observed in the peribronchiolar regions; hemorrhage was noticeable. The lungs were slightly emphysematous (Figure 7). No other lesions were noticeable.

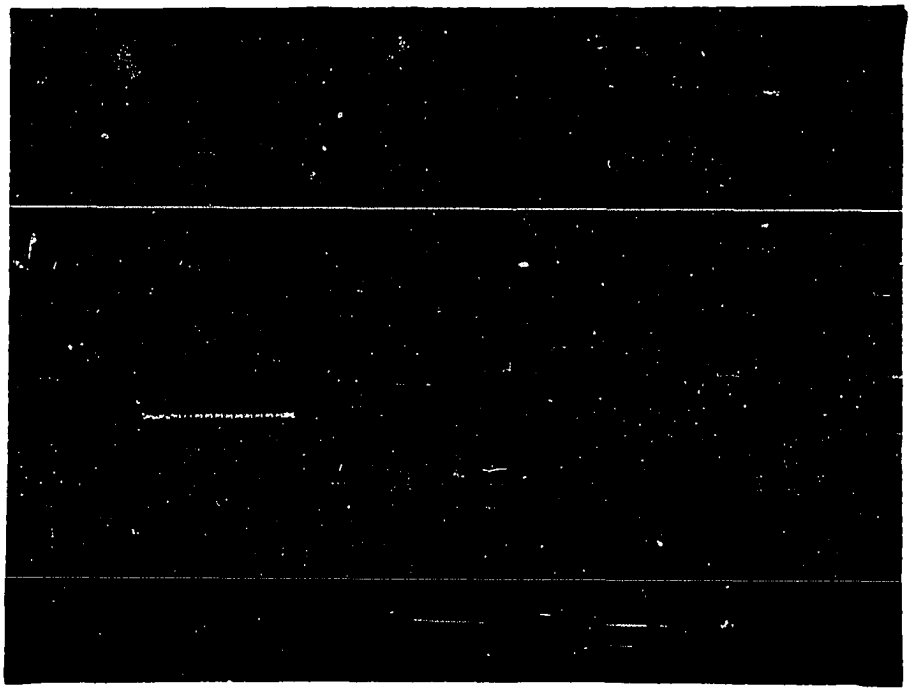
Immunized pigs with lesions Massive lymphoid infiltration was present in the peribronchiolar areas and resulted in the stenosis or complete blockage of air passages. Areas of necrosis were also seen. Extensive fibrosis was present in the interstitial and perilobular regions. Alveoli were collapsed.

The pathological lesions of pigs passively immunized then infected were similar to those in actively immunized then infected pigs.

Formalinized bacterin immunized pigs All four pigs inoculated with vaccine H, had more extensive gross lesions than the infected controls. Affected lobules of the lungs were edematous and had a more severe lymphoid and neutrophilic infiltration in both the perivascular and peribronchiolar areas. Secretory glands in the bronchi were significantly increased. The lumen of the bronchi were filled with lymphocytes, neutrophils, septal cells and cell debris. There was an increased amount of fibrous tissue in the interlobular septum. (Figure 8).

Figure 6. Peribronchiolar lymphoid hyperplasia observed in experimentally infected pig. X100 H and E stain

Figure 7. Microscopic lesion of an immunized then challenged pig with no gross lesion. Moderate cellular response in peribronchiolar region. The lung was slightly emphysematous. X100 H and E stain



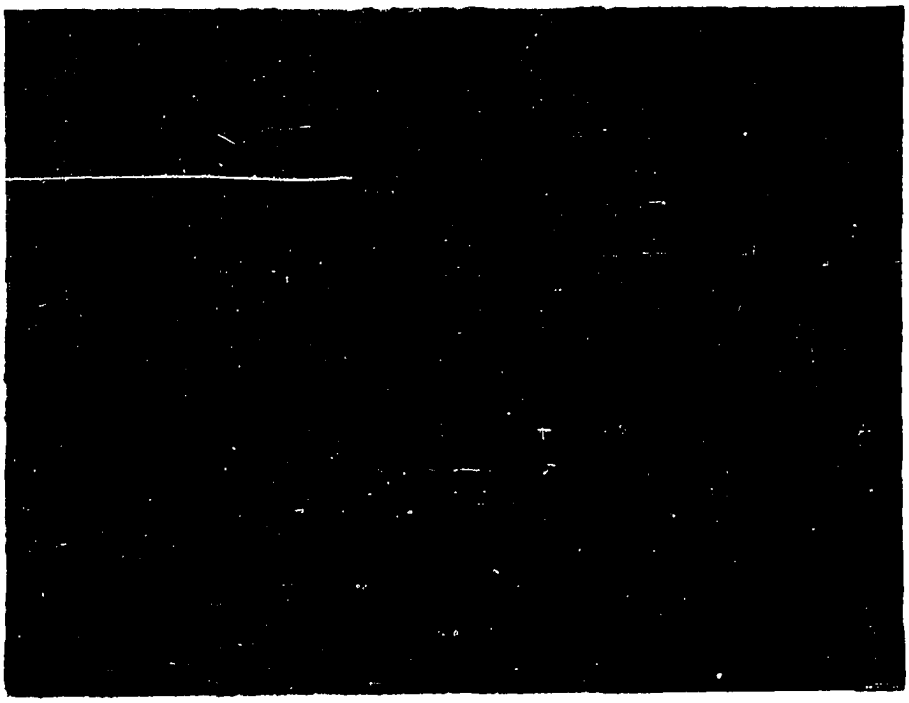
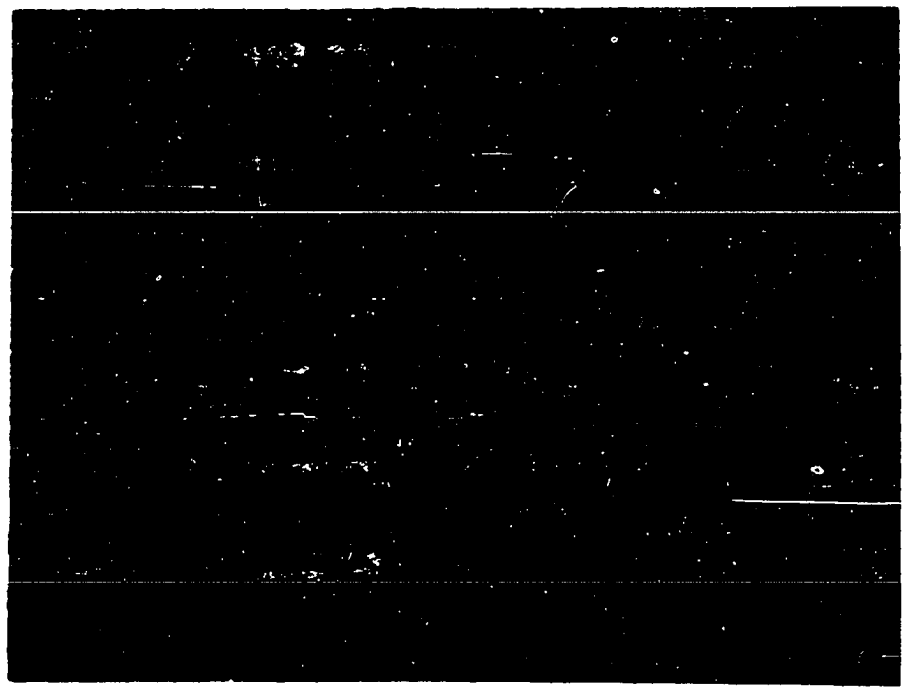


Figure 8. Formalinized bacterin administered intranasally (vaccine H). The pig had extensive gross lesions. Massive lymphoid infiltration, and septal cell proliferation. X100 H and E stain

Figure 9. Tissue from pig that was infected then inoculated with vaccine B. Nodular proliferation of lymphoid cells is apparent. Mitotic figures are present in many areas. X100 H and E stain



Infected then immunized pigs Distinct hyperplastic lymphoid nodules were present (Figure 9) throughout the lobules. In some instances, the lymphoid cells invaded into the bronchi. Mitotic figures were frequently observed in the lymphoid cells. Interstitial areas were edematous and contained hemorrhage.

Active Immunization

Experiment I

None of the four vaccinated pigs had gross lesions whereas four out of four positive control pigs developed lesions of mycoplasmal pneumonia.

Experiment II

Three of four vaccinated pigs were grossly normal while the remaining one had pneumonia. All four positive control pigs had extensive pneumonia.

Experiment III

One of the three inoculated then immunized pigs developed lesions. One of the remaining two had scars in the lung while the other animal had no pneumonic lesions.

Experiment IV

Two of the four vaccinated pigs had no lesions while three out of four positive controls had pneumonia.

Experiment V

Four out of four vaccinated pigs developed lesions as did the four positive controls.

Experiment VI

One out of four vaccinated pigs was protected from pneumonia. Four out of four positive controls were infected.

Experiment VII

Three out of three pigs receiving vaccine C and three out of three pigs receiving vaccine D developed pneumonia. Three out of three positive control pigs also had pneumonia. None of the three pigs that received vaccine D and were then put in close contact with infected pigs had pneumonic lesions.

Experiment VIII

Two of the pigs that received vaccine E died during the course of immunization. One of the two surviving vaccinated pigs developed pneumonic lesions upon challenge; the remaining pig's lung was grossly normal.

Three out of four pigs that received vaccine F had lungs that were grossly normal, whereas four out of four control pigs were infected.

Experiment IX

Six out of eight pigs immunized with vaccine F, and two out of three pigs immunized with vaccine G were protected from mycoplasma pneumonia. All of the four positive control pigs were infected.

Experiment X

None of the four pigs receiving formalinized bacterin intranasally were protected from infection. On the contrary, these four pigs had more extensive lesions than the four positive control pigs.

Experiment XI

Five negative control pigs were necropsied prior to challenge of the remainder and all five had grossly normal lungs. Ten out of 14 pigs receiving vaccine F and 13 out of 15 pigs receiving vaccine G were protected from mycoplasmal pneumonia. Eleven out of 15 positive controls developed pneumonia following challenge.

The results of active immunization trials were summarized in Tables 4 and 6.

Passive Immunization

Experiment I

Antiserum collected from Experiment I (vaccine A) described in the Active Immunization results was passively transferred to pigs. It protected two out of five pigs from mycoplasmal pneumonia.

Table 4. Active immunization of swine against Mycoplasma hyopneumoniae

Expt. no.	Vaccine used	Vaccinated pigs infected*	Control pigs infected*
I	A	0/4	4/4
II	B	1/4	4/4
IV	B	2/4	3/4
V	C	4/4	4/4
VI	C	3/4	4/4
VII	C	3/3	
	D	3/3	3/3
	D**	0/3	
VIII	E	1/2***	4/4
	F	1/4	
IX	F	2/8	
	G	1/3	4/4
X	H	4/4	4/4
XI	F	4/14	
	C	2/15	11/15

*Number of pigs that had pneumonia over the number of pigs in trial.

**Contact challenge exposure.

***Two of the pigs died during the experiment.

All five non-immunized positive controls became infected.

Experiment II

One of three pigs receiving antisera from F vaccine immunized pigs was protected from mycoplasmal pneumonia. Both positive control pigs developed disease.

Experiment III

Two out of two positive control pigs became infected whereas all four pigs receiving antisera from G vaccine immunized pigs were protected from mycoplasmal pneumonia.

Experiment IV

Nine out of 10 immunized pigs were protected from mycoplasmal pneumonia while 11 out of 15 pigs became infected. The protected pigs received antisera from the same source as was used in Experiment III.

The results of the passive immunization trials are summarized in Tables 5 and 6.

Table 5. Results of passive immunization trials

Expt. no.	Passive immunized pigs infected*	Control pigs infected*
I	3/5	5/5
II	2/3	2/2
III	0/2	2/2
	0/2**	
IV	1/10	11/15

*Number of pigs that had pneumonia over number of pigs in trial.

**Contact challenge exposure.

Table 6. Statistical analysis of results of immunization trials

Active Immunization		Passive Immunization	
Vaccine	χ^2 Value	Antibody derived from vaccine	χ^2 Value
A	8.0**		
B	4.2**	A	1.2
C	1.05	F	0.83
D	0.0	G	16.3**
E	2.4		
F	15.8**		
G	14.3**		

**Significant at 0.025 per cent level.

DISCUSSION

The indirect hemagglutination test described by Dowdle and Robinson (1964) was first utilized in the present study for the attempted detection of antibodies against M. hyopneumoniae without much success. This method detected a low level of antibodies in serum from an infected pig. This serum from an infected pig hemolyzed the sheep rbc utilized for the IHA test. Since the hemolysis impaired the indirect hemagglutination reaction, and since the sensitivity of the test procedure was low, it was decided to use swine rbc as the carrier and to modify the preparation of antigens. Goodwin et al. (1969) reported that an IHA test with intact M. hyopneumoniae utilizing sheep rbc as carrier could yield IHA antibody titers of higher than 1:40,000. In the early stage of the present study, the intact mycoplasma attached to sheep rbc had been used as the antigen for the IHA test, however, it was abandoned as no indirect hemagglutination reaction was observed.

The indirect hemagglutination test developed for the detection of antibodies against M. hyopneumoniae was capable of detecting antibodies 14 to 21 days after exposure. The antibodies persisted for more than 320 days in swine. Goodwin et al. (1969) with their method, found no detectable antibody for three to four weeks post-infection, yet obtained high antibody titers in pigs infected four months previously.

Antibody response in pigs exposed by contact infection showed variation. Pigs in contact with infected pigs on the day of infection

produced antibody earlier than those that were in contact with pigs that had been infected two months previously. These previously infected pigs had antibody titers of 1:192 or higher. The efficiency of transmission of this disease was probably decreased by the development of an adequate antibody titers as no lesions were found in pigs that were put in contact with them. It is possible that antibody produced in the pigs was in some way capable of decreasing the virulence or quantity of mycoplasma transmitted.

It was found that in hyperimmunized pigs, two inoculations produced the maximum antibody titer and the third inoculation did not further increase the antibody titer. This finding is in agreement with the observation made by Goodwin et al. (1969) who found that in immune pigs, further challenge would not increase IHA or CF antibody titers. All of the parenterally vaccinated pigs in this present study, had antibody titers of 1:3,072 or higher yet only 48 out of 75 (64 per cent) pigs were protected from pneumonia. It is possible that the antibody measured by indirect hemagglutination is not completely associated with protection. It is also probable that the challenge dose of organisms was much greater than any likely to be encountered in the usual swine production system.

When the antibody elicited by vaccine G were passively transferred to healthy pigs, it protected the pigs from pneumonia 13 out of 14 times. The possible explanation may be that the vaccine immunized pigs were exposed two weeks after receiving the last dose of vaccine and there was not time enough for protection to attain an

optimal level. The protective antibody may require a longer time to reach a significant level, and therefore, when the antisera was obtained five weeks after immunization or three weeks after challenge, it passively conferred immunity. If this is correct, it should be possible to challenge pigs four to five weeks after immunization and obtain better protection than was obtained in the present trials. Further studies are necessary to answer this question.

Since the passively transferred antibody conferred immunity in pigs, it is probable that the protective mechanism is a circulating antibody instead of local immunity. The finding that circulating antibody is the protective mechanism in swine pneumonia is different from the observation made on contagious bovine pleuropneumonia. Lloyd (1967) reported that immunity could not be transferred by using antisera obtained from cattle. But Smith (1967) by using mice as a model, was able to demonstrate the suppression of bacteriemia by passively transferred antibody from recovered mice. Davies and Hudson (1968) demonstrated that growth inhibiting antibody in cattle was not associated with immunity and concluded that resistance to CBPP did not involve circulating antibody but was probably due to some form of cell mediated immunity. Since Smith was using mice as the experimental animal, and the other workers were using cattle, the conflicting results could be due to the differences in animal models.

Antibodies derived from pigs receiving vaccine G, when passively transferred, remained at a detectable level in pigs for 51 days as compared with 31 days for passively transferred antibodies derived

from pigs vaccinated with F vaccine. The F vaccine stimulated antibody protected fewer pigs from pneumonia than did the G vaccine stimulated antibody. This indicates that it is some property of antibody other than IHA titer alone that is responsible for protection. Both F and G vaccines gave about the same percentage of protection in actively immunized pigs, yet only antibody from pigs receiving G vaccine conferred a significant degree of protection when it was passively transferred.

The duration of protection in those passively immunized pigs and the ability of the protective antibody to pass via colostrum to confer protection to new born pigs was not determined.

Goodwin et al. (1969) observed a non-specific antibody titer in one of their experimental pigs. They did not indicate the origin of the antibody. In the course of the present study, sera from two litter-mates were found to have antibody titers before infection. The antibody, however, was removed by 2-mercaptoethanol treatment, and was therefore believed to be a 19s antibody. Treatment by 2-mercaptoethanol decrease antibody titers of sera from infected and hyperimmunized pigs by two-fold. This indicated that the antibody in sera from infected and hyperimmunized pigs was not extremely sensitive to 2-mercaptoethanol and that it probably was a 7s immunoglobulin. The finding that mycoplasmal pneumonia antibodies produced in pigs is a 7s immunoglobulin is in agreement with the findings of Lynn (1967) who found antibody produced in rabbits to S-2 strain of swine mycoplasma was associated with 7s immunoglobulin.

Schmidt et al. (1966) reported that in human atypical pneumonia,

the CF antibody was entirely or in the major part 19s immunoglobulin based on various methods of separation and characterization including 2-mercaptoethanol treatment. However, Fernald et al. (1967 a and b) noted that 2-mercaptoethanol treatment only partially reduced CF titers against M. pneumoniae in early convalescent sera but had no effect on later sera. They suggested that a transition to 2-mercaptoethanol-resistant antibody or 7s immunoglobulin had occurred. The human sera used by the former workers were from patients showing significant increase in CF antibody suggesting convalescent. The latter workers were using hamster sera. The conflicting results between these two groups of workers were not resolved. Investigation of the possible occurrence of the sequential antibody transition observed by Fernald, is needed in M. hyopneumoniae pneumonia.

Fernald et al. (1967 a and b) also noted that IHA antibody of human and rabbit origin to M. pneumoniae was destroyed by 2-mercaptoethanol and suggested that it was a 19s immunoglobulin. This finding is different from our observation in swine which indicated the IHA antibody is a 7s immunoglobulin.

The limited amount of comparative work between CF and IHA tests revealed an excellent correlation. Hodges and Betts (1969), using the CF test, reported that in infected herds, antibodies were detected in 81 per cent of those pigs with macroscopic lesions of pneumonia and in 69 per cent of pigs without macroscopic lesions. They also found antibodies to M. hyopneumoniae in 61 per cent of bacon pigs with macroscopic pneumonia and 12 per cent of animals without macroscopic lesion. The present study showed that 87 out of 169 pigs with

lung data available had macroscopic lesions of pneumonia (51.5 per cent). Antibodies were detected in 78.1 per cent of the pigs with macroscopic lesions and in 68.2 per cent of the pigs without macroscopic lesions. The present results and those reported by Hodges and Betts (1969) indicated that there is some correlation between pneumonic lesions and antibody titers. This data also shows that a high per centage of the lesionless pigs had antibody titers to M. hyopneumoniae. This poses a severe problem in evaluation of lesionless swine as false positive reactors or as lesionless carriers.

The lack of absolute correlation between antibody titer and gross pneumonic lesions in field samples could be due to various reasons. First of all, the serologic tests, i. e., IHA or CF test, might not be reliable; second, there may be organisms other than mycoplasma that are capable of producing the same gross pneumonic lesions; and third, there may be other organisms that share antigens with M. hyopneumonia.

Good correlation between CF and IHA tests, no cross-reaction among M. hyopneumoniae, M. granularum, and M. hyorhinis as detected by CF and IHA tests, and no cross-reactions between swine influenza and Mycoplasma hyopneumoniae (Takatori et al. 1968) indicate the specificity of the serologic tests utilized for the detection of antibodies against M. hyopneumoniae. In addition, the evidence that the IHA could detect antibody in 95 per cent of experimentally infected pigs supported the notion that the IHA test is specific for the detection of antibodies against M. hyopneumoniae in pigs.

Jericho (1968) warned that some features of pneumonia were

common to more than one pneumonic conditions and could not be used as the basis for definition of a specific pneumonia. Proliferation of intraplumonary lymphoid tissue and alveolar septal cells probably constitute this type of lesions and should not be unduly relied upon for the recognition of swine mycoplasmal pneumonia. The author listed similar histopathological lesions caused by M. hyopneumonie (Maré and Switzer, 1965), human adenovirus (Jennings and Betts 1962), animal enterovirus (Meyer et al. 1966) and influenza virus (Nayak et al. 1965), and cautioned against using one specific gross or pathologic lesion for a definitive diagnosis of a pneumonic condition. Since many organisms can cause lesions resembling mycoplasmal pneumonia, it may explain some of the lack of correlation between the gross pneumonia and the IHA serologic results.

The finding that erysipelas antiserum cross reacted with M. hyopneumoniae may also account for the lack of correlation. Antigen II coated swine rbc had reacted with Erysipelothrix insidiosa antiserum made by hyperimmunizing rabbits or horses. Post-infection serum from M. hyopneumoniae infected pig also reacted with E. insidiosa coated swine rbc used as the IHA antigen. The finding poses a problem in the application of the IHA test to field swine.

The antigen preparations used for the IHA test could be stored at 4 C for up to two months without losing any antigenicity, however, the swine rbc preparation required frequent laborious washings and tanning procedures. Therefore, attempts were made to preserve the antigen-coated rbc by freezing. Neither of the two methods of preservation attempted was successful. The excessive hemolysis of swine

rbc may be due to the unusual fragility of swine red blood cells.

Swine rbc were used because of the presence of a natural hemolysin in swine sera to sheep rbc. The hemolysin was heat stable and was difficult to absorb from swine sera. In addition, the hemolysin was present in minimal amount in young pig, and increased in potency as the pig aged. Since most field samples tested were from pigs five months of age or older, the hemolysin titers were high. This hemolytic effect masked the antigen-antibody reaction. The swine rbc was not as good an antigen carrier as sheep rbc which generally gave one to two-fold higher titers in the test. This disadvantage was offset by the detection of low levels of antibody activity by the swine rbc antigen carrier system.

Lysis of organisms by SLS was very effective. Cloudy suspensions of mycoplasma were cleared in a few minutes. Most of the SLS was removed in the first change of buffer solution. Engleman and Morowitz (1968) reported that after 36 hours of dialysis against three changes of buffer solution, the dialyzate contained only 1.7 per cent of the initial amount of SLS as determined by radioactivity counts.

The aggregated dialyzate was then forcefully resuspended and treated with merthiolate. The original purpose of adding the merthiolate was to prevent contaminants from growing (Rodwell et al. 1967) while the antigen was stored at 4 C, but later it was found that the merthiolate addition was essential for the demonstration of antigen-antibody activity in the IHA test. Fresh antigen devoid of merthiolate failed to react with antiserum whereas the same antigen preparation with merthiolate added at a 1:10,000 final concentration

was capable of detecting antibody. Also, the addition of merthiolate helped prevent the re-aggregation and precipitation of the antigen during storage at 4 C. The role of merthiolate in binding antigen to the carrier rbc was an unexpected finding that deserves further exploration.

Lannek and Bornfors (1957) showed that a strong immunity developed in pigs experimentally infected with enzootic pneumonia even though Betts et al. (1955) found no evidence of any appreciable degree of immunity in pigs that were naturally infected by mycoplasmal pneumonia. Lannek and Bornfors (1957) infected a group of pigs and allowed them to recover and then challenged them four months after primary infection. They found all the re-infected pigs were free from enzootic pneumonia, whereas all the positive controls had pneumonia. The authors did not demonstrate neutralizing antibody in those re-infected pigs. Goodwin et al. (1969) found that pigs recovered from the disease were strongly resistant to challenge. The pigs developed virtually no lung lesions when infected with virulent inoculum that produced extensive lesions of enzootic pneumonia in control pigs.

The results of these workers are very difficult to interpret because the conditions necessary to insure the recovery of pigs from mycoplasmal pneumonia were not specified. In the present study two out of three swine still had pneumonia 11 months after infection.

The commercial SEP vaccine* produced and marketed for a short time in Sweden is the only known vaccine utilized for the prevention

*Chas. Pfizer, Inc., Sweden

of mycoplasmal pneumonia in swine. The vaccine contains formalinized M. hyorhinis the causative agent of a polyserositis and arthritis in swine. This mycoplasma is a frequent secondary invader of swine pneumonia (L'Ecuyer, 1962). According to available information, 10,000 doses of the SEP vaccine (Pfizer, Inc., 1968) were used from 1962 to 1968 in several herds of pigs. Some previously severely infected herds, through a systematic vaccination program, were able to bring the pneumonia under control. Since M. hyorhinis is serologically distinct from M. hyopneumoniae (Switzer, 1969), the established etiological agent of mycoplasmal pneumonia or swine enzootic pneumonia, the use of a M. hyorhinis vaccine for the prevention of mycoplasmal pneumonia is difficult to understand.

The vaccine preparations used in the present study contained M. hyponeumoniae. Vaccine H contained formalinized organisms. The rest of the preparations were made from lysed organisms or components of M. hyopneumoniae. The preliminary results obtained in the present study by the use of M. hyopneumoniae for immunization against this disease indicates considerable potential for this procedure to aid in the control of this particular swine pneumonia. Both F and G vaccines used in the present study protected about 80 per cent of the immunized pigs from pneumonia.

Smith (1969) reported that a large, single dose of inactivated CBPP vaccine given intravenously conferred virtually complete protection against a wide range of challenge in mice, while subcutaneous vaccination failed to produce even slight immunity. Vaccine preparations A through G in the present study were all administered

intramuscularly with incomplete Freund adjuvant. Vaccine H (formalinized bacterin) was administered intranasally and failed to protect pigs from infection, in fact, the vaccinated pigs developed more extensive lesions than the infected control pigs. Since vaccine H was not administered intramuscularly, it is not possible to compare this product with other vaccines. Additional study is required to determine the most effective antigen preparation and administration for the control of the disease.

The failure of vaccines F and G to give complete protection may be due to inadequate dosage or to inadequate immunogenic material present in the vaccines. The yield of M. hyopneumoniae, with present technique, is still meager, and it is hoped that a better growth medium will eventually become available for the more abundant growth of the organism so that it can be more readily studied and utilized in antigen-antibody reactions and in vaccine production.

There were small local abscesses formed as the result of administration of F vaccine. The abscess formation may have been due to contamination of the vaccine since only blood agar plates were used for sterility check. The abscess may also have resulted from the action of the adjuvant. Further effort can probably eliminate this problem.

Cattle vaccinated with the Ti strain of broth vaccine against CBPP were found to have persistent mycoplasma in the body. It was suggested that the survival of viable organisms in the body was correlated with a positive complement fixation test (Davies, 1969). Fernald (1969) was able to isolate Mycoplasma pneumoniae from

immunized and infected hamster lung tissues. He could infect and produce pneumonia in all control animals and in all animals vaccinated parenterally. In previously infected and recovered animals, however, complete protection from pneumonia was observed. The frequency of mycoplasma isolation was significantly less (55 per cent) than in the non-immunized, infected controls. Thus a previous respiratory infection with M. pneumoniae produced partial resistance to re-infection and complete protection against development of pneumonia.

In the present study, attempts to isolate M. hyponeumoniae from lesionless lungs of pigs that had been vaccinated and then challenged were unsuccessful. Organisms were isolated from pigs that had been immunized and infected but developed lesions. This evidence indicates that the immunized, lesionless pigs harbor no recoverable organisms and should not constitute a reservoir of infection.

SUMMARY

An indirect hemagglutination (IHA) test for the detection of Mycoplasma hyopneumoniae infection has been developed. Pigs infected with the agent developed antibodies two to three weeks post-infection. The antibody titer reached its peak on the 63rd day of infection, and remained at that level for 130 days. The level of antibody started to fall off gradually after that, but remained detectable for at least 320 days post-infection.

Healthy pigs in contact with pigs that had just been infected with live culture of M. hyopneumoniae developed antibody titer in five to six weeks. When susceptible pigs were in contact with pigs that had been infected two months previously, the susceptible pigs developed antibody in seven to eight weeks but failed to develop lung lesions. This indicates that adequate neutralizing antibody to retard the transmission of the disease was produced after a period of infection.

Four hundred thirty three swine serum samples from various field herds were tested for antibodies against M. hyopneumoniae. Two hundred ninety one of them were serologically positive (67.2 per cent). There were slaughter data available on 169 of those pigs. Eighty seven (51.5 per cent) of the pigs had lungs that were pneumonic as against 82 that had grossly normal lungs. Antibodies were detected in 78.1 per cent of the pigs with macroscopic lung lesions and in 69 per cent of the pigs without lesions.

Eight vaccine preparations were evaluated in this study for their ability to prevent pneumonia. Vaccines F and G were both found to protect about 80 per cent of pigs from pneumonia. Antibody levels were found to differ by only a two-fold dilution in all of the vaccinated pigs, yet some were protected from pneumonia while others were not. Therefore, it appears that IHA antibody level in vaccinated pigs may not be completely associated with protection.

Serum from pigs receiving vaccine F, when passively transferred, protected one out of three pigs from pneumonia; serum from pigs receiving vaccine G conferred immunity against pneumonia to 11 out of 12 pigs.

No Mycoplasma hyopneumoniae was recovered from the lungs of pigs that were protected from pneumonia by either active or passive immunization.

The sera from experimentally infected and from vaccinated pigs were partially resistant to 2-mercaptoethanol treatment. The antibody were therefore thought to be a 7s immunoglobulin.

LITERATURE CITED

Adler, H. E. 1958. A PPLO slide agglutination test for the detection of infectious sinusitis of turkeys. Poultry Science 37:1116-1123.

Adler, H. E., and DaMassa, A. J. 1967. Use of formalinized Mycoplasma gallisepticum antigens and chicken erythrocytes in hemagglutination and hemagglutination-inhibition studies. Applied Microbiology 15:245-248.

Adler, H. E., and DaMassa, A. J. 1968. Effect of dextrose in medium for the preparation of Mycoplasma gallisepticum plate antigens. Applied Microbiology 16:558-562.

Adler, H. E., McMartin, D., and Shifrine, M. 1960. Immunization against mycoplasma infections of poultry. American Journal of Veterinary Research 21:482-485.

Adler, H. E., and Yamamoto, R. 1956. Preparation of a new pleuropneumonia-like organism antigen for the diagnosis of chronic respiratory disease by the agglutination test. American Journal of Veterinary Research 17:290-293.

Alfrey, J., Bradford, E. B., and Vanderhoff, J. W. 1954. Optical properties of uniform particle size latexes. American Optical Society Journal 44:603-609.

Anonymous. 1957. Annual Report, Department of Veterinary Science, Kenya, for 1956. Government Printer, Nairobi, Kenya.

Bailey, J. S., Clark, H. W., Felts, W. R. , Fowler, R. C., and Brown, T. M. 1961. Antigenic properties of pleuropneumonia-like organisms from tissue cell cultures and the human genital area. Journal of Bacteriology 82:542-547.

Bailey, J. S., Clark, H. W., Felts, W. R., and Brown, T. M. 1963. Growth inhibitory properties of mycoplasma antibody. Journal of Bacteriology 86:147-150.

Barile, M. F., Malizia, W. F., and Riggs, D. B. 1962. Incidence and detection of pleuropneumonia-like organisms in cell cultures by fluorescent antibody and cultural procedures. Journal of Bacteriology 84:130-136.

Barile, M. F., and Schimke, R. T. 1963. A rapid chemical method for detecting PPLO contamination in tissue cell cultures. Experimental Biology and Medicine Society Proceedings 114:676-679.

Beckman, B. L., and Kenny, G. E. 1968. Immunochemical analysis of serologically active lipids of Mycoplasma pneumoniae. Journal of Bacteriology 96:1171-1180.

Betts, A. O. 1952. Respiratory disease of pigs. V. Some clinical and epidemiological aspects of virus pneumonia of pigs. Veterinary Record 64:283-288.

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

Bohlmann, A. 1944. Uber die mirkung von tannin auf einzelzellen. Naunyn-Schmiedebergs Archiv Fur Pharmakologie und Experimentelle, Pathologie. 203:317.

Boulanger, P. 1954. A preliminary note on a method of inactivating a substance or substances in swine serum that interfere with the detection of antibodies by the complement-fixation test. Canadian Journal of Comparative Medicine 18:423-425.

Boulanger, P., and L'Ecuyer, C. 1968. Enzootic pneumonia of pigs: Complement-fixation tests for the detection of mycoplasma antibodies in the serum of immunized rabbits and infected swine. Canadian Journal of Comparative Medicine 32:547-554.

Boyden, S. V. 1951. The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by anti-protein sera. Journal of Experimental Medicine 93:107-120.

Brodie, A. E., and Gots, J. S. 1951. Effects of an isolated dehydrogenase enzyme and flavorprotein on the reduction of triphenyltetrazolium chloride. Science 114:40-41.

Campbell, A. D. 1938. Contagious bovine pleuropneumonia: A report on the use of new antigen for the complement-fixation tests. Council on Science and Industry Research Journal (Australia) 11:112-118.

Campbell, A. D., and Turner, A. W. 1936. Studies on contagious pleuropneumonia of cattle: II. Council on Science and Industrial Research Bulletin 97:11-52.

Carlisle, H. N., and Saslaw, S. 1958. A histoplasma-latex agglutination test. Journal of Laboratory and Clinical Medicine 51:793-801.

Carski, T. R., and Shepard, C. C. 1961. Pleuropneumonia-like (mycoplasma) infections of tissue culture. Journal of Bacteriology 81:626-635.

Castrejon-Diaz, J., Fisher, T. N., and Fisher, E., Jr. 1963. Glucose metabolism of two strains of Mycoplasma laidlawii. Journal of Bacteriology 86:627-636.

Chanock, R. M., Hayflick, L., and Barile, M. F. 1962a. Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. National Academy of Sciences Proceedings 48:41-49.

Chanock, R. M., James, W. D., Fox, H. H., Turner, H. C., Mufson, M. A., and Hayflick, L. 1962b. Growth of Eaton PPLO in broth and preparation of complement fixing antigen. Experimental Biology and Medicine Society Proceedings 110:884-889.

Christian, C. L., Mendez-Bryan, R., and Larson, D. L. 1958. Latex agglutination test for disseminated Lupus erythromatous. Experimental Biology and Medicine Society Proceedings 98:820-823.

Clark, H. W., Bailey, J. S., Fowler, R. C., and Brown, T. McP. 1963. Identification of mycoplasmataceae by the fluorescent antibody method. Journal of Bacteriology 85:111-118.

Clyde, W. A., Jr. 1963. Studies on Eaton agent in tissue culture. In: Conference on new respiratory disease viruses, 1962. American Review of Respiratory Diseases 88:212-217.

Clyde, W. A., Jr. 1964. Mycoplasma species identification based upon growth inhibition by specific antisera. Journal of Immunology 92:958-965.

Cordero, L., Florey, C.du V., and Horstman, D. M. 1968. Persistence of antibodies to Mycoplasma pneumoniae following naturally acquired infections. American Journal of Epidemiology 88:428-435.

Cottew, G. S. 1960. Indirect hemagglutination and hemagglutination inhibition with Mycoplasma mycoides. Australian Veterinary Journal 36:54-56.

Cottew, G. S. 1963. Inhibition of growth of Mycoplasma mycoides by bovine serum. Research in Veterinary Science 4:459-470.

Couch, R. T., Cate, R., and Chanock, R. M. 1964. Infection with artificially propagated Eaton agent (Mycoplasma pneumoniae). American Medical Association Journal 187:442-447.

Crawley, J. F., and Fahey, J. E. 1957. The use of the hemagglutination inhibition test for the control of PPLO infection in poultry. American Veterinary Medical Association Journal 130:187-190.

Davies, G. 1969. The persistence of Mycoplasma mycoides in the host after vaccination with Ti broth vaccine. Research in Veterinary Science 10:225-231.

Davies, G., and Hudson, J. R. 1968. The relationship between growth-inhibition and immunity in contagious bovine pleuropneumonia. Veterinary Record 83:256-258.

Deeb, B. J., and Kenny, G. E. 1967a. Characterization of Mycoplasma pulmonis variants isolated from rabbits. I. Identification and properties of isolates. Journal of Bacteriology 93:1416-1424.

Deeb, B. J., and Kenny, G. E. 1967b. Characterization of Mycoplasma pulmonis variants isolated from rabbits. II. Basis for differentiation of antigenic subtypes. Journal of Bacteriology 93:1425-1429.

Del Giudice, R. A., and Pavia, R. 1964. Hemadsorption by Mycoplasma pneumoniae and its inhibition with sera from patients with atypical pneumonia. Bacteriological Proceedings p. 71.

Del Giudice, R. A., Robillard, N. F., and Carski, T. R. 1967. Immunofluorescence identification of mycoplasma on agar by use of incident illumination. Journal of Bacteriology 93:1205-1209.

Dierks, R. E., Newman, J. A., and Pomeroy, B. S. 1967. Characterization of avian mycoplasma. New York Academy of Sciences Annals 143:170-189.

Dinter, Z., Danielson, D., and Bakos, K. 1965. Differentiation of porcine mycoplasma strains. Journal of General Microbiology 41:77-84.

Domermuth, C. H., and Gourlay, R. N. 1967. A solid medium test for measuring growth inhibition and neutralization of Mycoplasma mycoides by immune bovine serum. Journal of General Microbiology 47:289-294.

Dowdle, W. R., and Robinson, R. Q. 1964. An indirect hemagglutination test for diagnosis of Mycoplasma pneumoniae infections. Society for Experimental Biology and Medicine Proceedings 116:947-950.

Dowdle, W. R., and Heyward, J. T. 1968. Preservation of sensitized red blood cells for serologic diagnosis of Mycoplasma pneumoniae infections. American Journal of Clinical Pathology 49:132-134.

Dulbecco, R. and Vogt, M. 1954. Plaque formation and isolation of pure lines of poliomyelitis virus. Journal of Experimental Medicine 99:167-182.

Edelberg, R. 1953. The action of tannic acid on model system formed of constituents of the erythrocyte membrane. Journal of Cellular and Comparative Physiology 41:37-50.

Edward, D. G. ff. 1950. An investigation of pleuropneumonia-like organisms isolated from the bovine genital tract. Journal of General Microbiology 4:4-14.

Edward, D. G. ff., and Fitzgerald, W. A. 1954. Inhibition of growth of pleuropneumonia-like organism by antibody. Journal of Pathology and Bacteriology 68:23-30.

Edward, D. G. ff., and Freundt, E. A. 1969. Classification of the mycoplasmatales. In Hayflick, L., ed. The Mycoplasmatales and the L-phase of Bacteria. Chapter 5. Pp. 147-200. Appleton-Century-Croft, New York, New York.

Edward, D. G. ff., and Kanarek, A. D. 1960. Organisms of pleuropneumonia group of avian origin: Their classification into species. New York Academy of Sciences Annals 79:696-702.

Eng, J. 1967. Studies on the complement fixation test with Mycoplasma pneumoniae antigen. I. Production of antigen. Pathologica et Microbiologica Scandinavica Acta 71:79-88.

Engleman, D. M., and Morowitz, H. J. 1968. Characterization of the plasma membrane of Mycoplasma laidlawii. III. The formation and aggregation of small lipoprotein structure derived from sodium dodecyl sulfate-solubilized membrane components. Biochemica et Biophysica Acta 150:376-384.

Fabricant, J. 1960. Serological studies of avian pleuropneumonia-like organism (PPLO) with Edward's technique. Avian Diseases 4:505-514.

Fahey, J. E., and Crawley, J. F. 1954. Studies on chronic respiratory disease of chickens. IV. A hemagglutination inhibition diagnostic test. Canadian Journal of Comparative Medicine 18:264-272.

Fernald, G. W. 1969. Immunologic aspects of experimental Mycoplasma pneumoniae infection. Journal of Infectious Diseases 119:255-266.

Fernald, G. W., Clyde, W. A., Jr., and Denny, F. W. 1967a. Factors influencing growth inhibition of Mycoplasma pneumoniae by immune sera. Society for Experimental Biology and Medicine Proceedings 126:161-166.

Fernald, G. W., Clyde, W. A., Jr., and Denny, F. W. 1967b. Nature of the immune response to Mycoplasma pneumoniae. Journal of Immunology 98:1028-1038.

Freund, J. 1929. Phagocytosis of red blood cells treated by tannin. Society for Experimental Biology and Medicine Proceedings 26:876-878.

Frey, M. L., and Hanson, R. P. 1969. A complement fixation test for the study of avian mycoplasma. Avian Diseases 13:185-197.

Gamble, R. M. 1956. Studies on contagious pleuropneumonia with special reference to complement fixation test. British Veterinary Journal 112:3-30.

Gianforte, E. M., Jungherr, E. L., and Jacobs, R. E. 1955. A serologic analysis of seven strains of pleuropneumonia-like organisms from air sac infection in poultry. Poultry Science 34:662-669.

Gill, J. W. 1962. Culture and metabolism of Mycoplasma gallisepticum. Journal of Bacteriology 83:213-218.

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

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

Goodwin, R. F. W., Pomeroy, A. P., and Whittlestone, P. 1967. Characterization of Mycoplasma suipneumoniae. A mycoplasma causing enzootic pneumonia of pig. Journal of Hygiene (Cambridge) 65:85-96.

Hayflick, L. 1965. Tissue cultures and Mycoplasmas. Texas Reports on Biology and Medicine 23:285-303.

Helsop, G. G. 1921. Research into the serological diagnosis of contagious pleuropneumonia. Royal Society, Victoria, Proceedings 33:160-211.

Helsop, G. G. 1922. Further research into the serological diagnosis of contagious pleuropneumonia. Royal Society, Victoria, Proceedings 34:180-195.

Hers, J. F. Ph., and Masurel, M. 1967. Infection with Mycoplasma pneumoniae in civilian in the Netherlands. New York Academy of Sciences Annals 143:447-460.

Herz, F. 1968. On the effects of tannic acid on erythrocyte membrane acetylcholinesterase. Society for Experimental Biology and Medicine Proceedings 127:1240-1245.

Herz, F., and Kaplan, E. 1968. Effects of tannic acid on erythrocyte enzymes. Nature (London) 217:1258-1259.

Hirschfeld, J. 1960. Immunoelectrophoresis--Procedure and application to the study of group-specific variations in sera. Scientific Tools 7:18-25.

Hodges, R. T., and Betts, A. O. 1969 Complement-fixation tests in the diagnosis of enzootic pneumonia of pigs. I. Experimental studies. Veterinary Record 85:452-455.

Hodges, R. T., and Betts, A. O. 1969. Complement-fixation tests in the diagnosis of enzootic pneumonia of pigs. II. Field studies. Veterinary Record 85:455-458.

Hubert, E. G., Kalmanson, G. M., and Guze, L. B. 1963. Preservation of antigen-coated sheep erythrocytes by freezing for use in indirect hemagglutination procedure. Journal of Bacteriology 86:569-572.

Huhn, R. G. 1968. Chemoprophylaxis, chemotherapy, and economic effect of swine enzootic pneumonia. Unpublished Ph.D. Thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.

Huijsmans-Evers, A. G. M., and Ruys, A. C. 1956. Micro-organisms of the pleuropneumonia group (Family of Mycoplasmataceae) in man. Antonie van Leeuwenhoek 22:371-376.

Hyslop, N. St. G., 1955. The viability at low temperatures of a dried egg-adapted (Kenya vaccine) strain of Asterococcus mycoides. Veterinary Record 67:411-412.

Jennings, A. R., and Betts, A. O. 1962. Human adenovirus in pigs. New York Academy of Sciences Annals 101:485-492.

Jensen, K. E. 1964. Antibodies to Mycoplasma pneumoniae measured by inhibition of tetrazolium reduction. Bacteriological Proceedings Pp. 70-71.

Jensen, K. E., Senterfit, L. B., Chanock, R. M., Smith, C. B., and Purcell, R. H. 1965. An inactivated Mycoplasma pneumoniae vaccine. American Medical Association Journal 194:134-138.

Jericho, K. W. F. 1968. Pathogenisis of pneumonia in pigs. Veterinary Record 82:507-520.

John, T. J., Stahl, M., and Fulginiti, V. A. 1966. Cumulative hemagglutination by Mycoplasma pneumoniae and other agglutinins. Journal of Bacteriology 92:1002-1004.

Jones, D. M., and Sequeira, P. J. L. 1966. The distribution of complement fixing antibody and growth inhibiting antibody to Mycoplasma hominis. Journal of Hygiene (Cambridge) 64:441-449.

Jungherr, E. R., Luginbuhl, R. E., and Jacobs, R. E. 1953. Pathology and serology of air sac infection. American Veterinary Medical Association Proceedings 90th Annual Meeting, Pp. 303-312.

Jungherr, E. L, Luginbuhl, R. E., Tourtellote, M., and Burr, W. E. 1956. Significance of serological testing for chronic respiratory disease. American Veterinary Medical Association Proceedings 92nd Annual Meeting, Pp. 315-322.

Kelton, W. H., and Van Rockel, H. 1963. Serological studies of mycoplasma (PPLO) of avian origin. Avian Diseases 7:272-286.

Kende, M. 1969. Antibody response of animals to Mycoplasma pneumoniae measured by latex agglutination. Applied Microbiology 17:275-279.

Kenny, G. E. 1967. Heat-lability and organic solvent-solubility of mycoplasma antigens. New York Academy of Sciences Annals 143:676-681.

Kenny, G. E., and Grayston, J. T. 1965. Eaton pleuropneumonia-like organism (Mycoplasma pneumoniae) complement fixing antigen: Extraction with organic solvents. Journal of Immunology 95:19-25.

Kerr, K. M., Mascoli, C. C., Olson, C., and Campbell, A. 1964. Rapid specific agglutination of Eaton agent (Mycoplasma pneumoniae) Journal of Bacteriology 87:478-479.

Kerr, K. M., and Olson, N. O. 1967. Pathology in chickens experimentally inoculated or contact-infected with Mycoplasma gallisepticum. Avian Diseases 11:559-578.

Kleckner, A. L. 1960. Serotypes of avian PPLOs. American Journal of Veterinary Research 21:274-280.

Klienberger, E. 1938. Pleuropneumonia-like organisms of diversed provenance: Some results of an enquiry into methods of differentiation. Journal of Hygiene (Cambridge) 38:458-476.

Kun, E. 1951. Mechansim of enzymatic reduction of triphenyltetrazolium chloride. Society for Experimental Biology and Medicine Proceedings 78:195-197.

Kun, E., and Abood, L. G. 1949. Colorimetric estimation of succinic dehydrogenase by triphenyltetrazolium chloride. Science 109:144-146.

Kurotchkin, T. J. 1937. Specific carbohydrate from Astero-coccus mycoides for serologic tests of bovine pleuropneumonia. Society for Experimental Biology and Medicine Proceedings 37:21-22.

Lannek, N., and Bornfors, S. 1957. Immunity to enzootic pneumonia in pigs following recovery from the disease. Nordisk Veterinaermedicine 9:91-98.

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

Lemcke, R. M. 1964. The serological differentiation of mycoplasma strains from various sources. Journal of Hygiene (Cambridge) 62:199-219.

Lemcke, R. M. 1965. A serological comparison of various species of mycoplasma by agar-gel double diffusion technique. Journal of General Microbiology 38:91-100.

Lind, K. 1965. Role of fresh human or guinea pig serum in the indirect fluorescent antibody test for Mycoplasma pneumoniae antibodies in human sera. Acta Pathologica et Microbiologica Scandinavica 63:639-640.

Lind, K. 1968. An indirect hemagglutination test for serum antibodies against Mycoplasma pneumoniae using formalinized, tanned sheep erythrocytes. Acta Pathologica et Microbiologica Scandinavica 73:459-472.

Liu, C. 1957. Studies on primary atypical pneumonia. I. Localization, isolation and cultivation of a virus in chick embryos. Journal of Experimental Medicine 106:455-467.

Liu, C. 1961. Studies on primary atypical pneumonia. III. A factor in normal serum which enhance the reaction between PAP virus and convalescent serum. Journal of Experimental Medicine 113:111-123.

Lloyd, L. C. 1967. Evidence to the FAO Expert Panel on contagious bovine pleuropneumonia, 1967.

Lynn, R. J. 1967. The immune response of rabbits to various strains of mycoplasma. New York Academy of Sciences Annals 143:654-663.

Malizia, W. F., Barile, M. F., and Riggs, D. B. 1961. Immunofluorescence of pleuropneumonia-like organisms isolated from tissue cell cultures. Nature (London) 191:190-191.

Manchee, R. J., and Taylor-Robinson, D. 1968. Haemadsorption and haemagglutination of mycoplasmas. Journal of General Microbiology 50:465-478.

Manchee, R. J., and Taylor-Robinson, D. 1969. Studies on the nature of receptors involved in attachment of tissue culture cells to mycoplasma. British Journal of Experimental Pathology 50:66-75.

Maré, C. J. 1965. Characterization of a causative agent of virus pneumonia of pigs (VPP). Unpublished Ph.D. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.

Maré, J. A. 1969. Mycoplasmal pneumonia of swine. In proceedings of the North American symposium on swine respiratory diseases Pp. 35-45.

Maré, C. J., and Switzer, W. P. 1965. New species: Mycoplasma hyopneumoniae. A causative agent of virus pig pneumonia. Veterinary Medicine 60:841-846.

Marmion, B. P., and Goodburn, G. M. 1961. Effect of an organic gold salt on Eaton's primary atypical pneumonia agent and other observations. Nature (London)189:247-248.

Marmion, B. P., Plackett, P., and Lemcke, R. M. 1967. Immunochemical analysis of Mycoplasma pneumoniae. I. Methods of extraction and reaction of fractions from M. pneumoniae and from M. mycoides with homologous antisera and with antisera against Streptococcus MG. Australian Journal of Experimental Biology and Medical Science 45:163-187.

Masiga, W. N., and Stone, S. S. 1968. Application of a fluorescent-antibody technique for the detection of Mycoplasma mycoides antigen and antibody. Journal of Bacteriology 96:1867-1869.

Mattison, A. M., Jensen, C. O., and Dutcher, R. A. 1947. Triphenyltetrazolium chloride as a dye for vital tissues. Science 106:294-295.

McCarty, K. S., Woodson, B., Amstey, M., and Brown, O. 1964. Arginine as a precursor of pyrimidine in strain L-929 fibroblasts infected with pleuropneumonia-like organisms. Journal of Biological Chemistry 239:544-549.

Metzgar, D. P., Woodhour, A. F., Vella, P. P., Weibel, R. E., Stokes, J., Jr., Drakes, M. E., Tytell, A. A., and Hillman, M. R. 1966. Respiratory virus vaccines. II. Mycoplasma pneumonia (Eaton agent) vaccines. American Review of Respiratory Diseases 94:1-9.

Meyer, R. C., Woods, G. T., and Simmon, J. 1966. Pneumonitis in an enterovirus infection in swine. Journal of Comparative Pathology 76:397-405.

Mogabgab, W. J. 1968a. Mycoplasma pneumoniae and adenovirus respiratory illness in military and university personnel, 1959-1966. American Journal of Respiratory Diseases 97:345-358.

Mogabgab, W. J. 1968b. Protective effects of inactive Mycoplasma pneumoniae vaccine in military personnel, 1964-1966. American Journal of Respiratory Diseases 97:359-365.

Moore, R. W., Redmond, H. E., and Livingston, C. W., Jr. 1966. Mycoplasma as the etiology of metritis-mastitis syndrome in sows. Veterinary Medicine 61:883-887.

Morton, H. E. 1966. Mycoplasma-latex agglutination reaction. Journal of Bacteriology 92:1196-1205.

Nayak, D. P., Twiehaus, M. J., Kelley, G. W., and Underdahl, N. R. 1965. Immunocytologic and histopathologic development of experimental swine influenza infection in pigs. American Journal of Veterinary Research 26:1271-1283.

Neimark, H. C. 1968. Glutaraldehyde-fixed erythrocytes for indirect hemagglutination with mycoplasma. Nature (London) 219:293-294.

Neimark, H. C., and Pickett, M. J. 1960. Products of glucose metabolism by pleuropneumonia-like organisms. New York Academy of Sciences Annals 79:531-537.

Newing, C. R. 1955. An improved method for the preparation of an antigen for the rapid slide test for contagious bovine pleuropneumonia. British Veterinary Journal 111:378-384.

Newing, C. R., and Field, A. C. 1953. A preliminary report on a rapid field test for contagious bovine pleuropneumonia. British Veterinary Journal 109:397-404.

Newing, C. R., and MacLeod, A. K. 1955-56. Immunity and serodiagnostic methods. East African Veterinary Research Organization Annual Report, 1955-56. Pp 79-89. Government Printer, Nairobi, Kenya.

Norman, M. C., Saslaw, S., and Kuhn, L. R. 1950. Antigenic similarity of five human strains of pleuropneumonia-like organisms. Society for Experimental Biology and Medicine Proceedings 75:718-720.

Oreskes, I., and Singer, J. M. 1961. The mechanism of particulate carrier reactions. I. Adsorption of human r-globulin to polystyrene latex particles. Journal of Immunology 86:338-344.

Ouchterlony, O. 1949. Antigen-antibody reactions in gel. Pathologica et Microbiologica Scandinavica Acta 26:507-515.

Pattison, J. H. 1956. A histological study of a transmissible pneumonia of pigs characterized by extensive lymphoid hyperplasia. Veterinary Record 68:490-494.

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

Piercy, S. E., and Knight, G. J. 1955-56. Contagious bovine pleuropneumonia. East African Veterinary Research Organization Annual Report, 1955-56:67-69.

Piercy, S. E., and Knight, G. J. 1956. Studies with avianized strains of the organism of contagious bovine pleuropneumonia. Veterinary Record 68:367-373.

Piercy, S. E., and Knight, G. J. 1958. Studies with avianized strains of the organism of contagious bovine pleuropneumonia. V. Experiments with avianized vaccines at various levels of attenuation. British Veterinary Journal 114:245-253.

Priestley, F. W. 1951. A slide flocculation test for the diagnosis of contagious bovine pleuropneumonia. Veterinary Record 63:427-429.

Priestley, F. W. 1952. Observations of immunity to contagious bovine pleuropneumonia with special reference to the bactericidal action of blood. British Veterinary Journal 108:153-161.

Priestley, F. W. 1955. Immunization against contagious bovine pleuropneumonia with special reference to the use of a dried vaccine. Journal of Comparative Pathology 65:169-182.

Priestley, F. W. 1957. Further observations on the bactericidal action of cattle blood on the contagious bovine pleuropneumonia organism. British Veterinary Journal 113:464-469.

Purcell, R. H., Somerson, N. L., Fox, H., Wong, D., Turner, H. C., and Chanock, R. M. 1966a. Identification of acid-inducing agent and related mycoplasma as Mycoplasma hyorhinis. National Cancer Institute Journal 37:251-253.

Purcell, R. H., Taylor-Robinson, D., Wong, D., and Chanock, R. M. 1966b. A color test for the measurement of antibody to the non-acid-forming human mycoplasma species. American Journal of Epidemiology 84:51-66.

Purcell, R. H., Taylor-Robinson, D., Wong, D, and Chanock, R. M. 1966c. Color test for the measurement of antibody to T-strain mycoplasmas. Journal of Bacteriology 92:6-12.

Purcell, R. H., Wong, D., Chanock, R. M., Taylor-Robinson, D., Canchola, J., and Valdesuso, J. 1967. Significance of antibody to mycoplasma as measured by metabolic-inhibition techniques. New York Academy of Sciences Annals 143:664-675.

Razin, S., and Cohen, A. 1963. Nutritional requirements and metabolism of Mycoplasma laidlawii. Journal of General Microbiology 30:141-154.

Razin, S., Morowitz, H. J., and Terry, T. M. 1965. Membrane subunits of Mycoplasma laidlawii and their assembly to membrane-like structure. National Academy of Sciences Proceedings 54:219-225.

Riggs, S., Sharp, J. T., and Carpenter, R. R. 1967. Factors involved in growth-inhibition of mycoplasma by immune serum. New York Academy of Sciences Annals 143:784-793.

Roberts, D. H. 1968. Serological diagnosis of Mycoplasma hyopneumoniae infection in pigs. Veterinary Record 82:362-363.

Robinson, F. R., Moore, R. W., and Redmond, H. E. 1967a. Immunofluorescence studies of Mycoplasma hyoarthrinosa infection in swine. American Journal of Veterinary Research 28:141-147.

Robinson, F. R., Moore, R. W., and Redmond, H. E. 1967b. Serologic response of swine to a mycoplasma: Indirect-hemagglutination plate test. American Journal of Veterinary Research 28:149-152.

Rodwell, A. W., Razin, S., Rottem, S., and Argaman, M. 1967. Association of protein and lipid in Mycoplasma laidlawii membranes disaggregated by detergents. Archives of Biochemistry and Biophysics 122-621-628.

Rodwell, A. W., and Rodwell, E. S. 1953. Pathway for glucose oxidation in Asterococcus mycoides. Nature (London) 172:254-255.

Rodwell, A. W., and Rodwell, E. S. 1954. The breakdown of carbohydrate by Asterococcus mycoides. The organism of bovine pleuropneumonia. Australian Journal of Biological Science 7:18-30.

Ross, R. F., and Switzer, W. P. 1963. Comparison of isolates of Mycoplasma hyorhinis by indirect hemagglutination. American Journal of Veterinary Research 24:622-627.

Schimke, R. T., and Barile, M. F. 1963. Arginine breakdown in mammalian cell culture contaminated with pleuropneumonia-like organism (PPLO). Experimental Cell Research 30:593-596.

Schmidt, N. J., Lennette, E. H., Dennis, J., and Gee, P. S. 1966. On the nature of complement-fixing antibodies to Mycoplasma pneumoniae. Journal of Immunology 97:95-99.

Senterfit, L. B., and Jensen, K. E. 1966. Antimetabolic antibodies to Mycoplasma pneumoniae measured by tetrazolium reduction inhibition. Society for Experimental Biology and Medicine Proceedings 122:786-790.

Sheriff, D., and Piercy, S. E. 1952. Experiments with an avianized strain of the organism of contagious bovine pleuropneumonia. Veterinary Record 42:615-621.

Singer, J. M., Alterman, G., Oreskes, I., and Plotz, C. M. 1961. The mechanism of particle carrier systems. III. The stabilizing effect of serum proteins. American Journal of Medicine 30:772-778.

Smith, C. B., Chanock, R. M., Friedewald, W. T., and Alford, R. H. 1967. Mycoplasma pneumoniae infections in volunteers. New York Academy of Sciences Annals 143:471-483.

Smith, G. R. 1967. Mycoplasma mycoides var. mycoides: Production of bacteriaemia and demonstration of passive immunity in mice. Journal of Comparative Pathology 77:203-209.

Smith, G. R. 1969. Effect of route of vaccination on the immune response of mice to a single dose of heat-killed Mycoplasmas mycoides var. mycoides. Journal of Comparative Pathology 79:255-260.

Smith, P. F. 1955. Amino acid metabolism of pleuropneumonia-like organisms. I. General catabolism. Journal of Bacteriology 70:552-556.

Smith P. F. 1959. Amino acid metabolism of PPLO. New York Academy of Sciences Annals 79:543-550.

Sobeslavsky, O., Prescott, B., and Chanock, R. M. 1968. Adsorption of Mycoplasma pneumoniae to neuraminic acid receptors of various cells and possible role in virulence. Journal of Bacteriology 96:695-705.

Sobeslavsky, O., Prescott, B., James, W. D., and Chanock, R. M. 1966. Isolation and characterization of fractions of Mycoplasma pneumoniae. II. Antigenicity and Immunogenicity. Journal of Bacteriology 91:2126-2138.

Somerson, N. L., James, W. D., Walls, B. E., and Chanock, R. M. 1967. Growth of Mycoplasma pneumoniae on glass surface. New York Academy of Sciences Annals 143:384-389.

Somerson, N. L., and Morton, H. E. 1953. Reduction of tetrazolium salts by pleuropneumonia-like organisms. Journal of Bacteriology 65:245-251.

Stanbridge, E., and Hayflick, L. 1967. Growth inhibition test for identification of mycoplasma species utilizing dried antiserum-impregnated paper discs. Journal of Bacteriology 93:1392-1396.

Stavisky, A. B. 1954. Micromethods for the study of protein and antibodies. I. Procedures and general applications of hemagglutination and hemagglutination inhibition reactions with tannic acid and protein-treated red blood cells. Journal of Immunology 72:360-367.

Switzer, W. P. 1967. Swine mycoplasma. American Veterinary Medical Association Journal 151:1656-1661.

Switzer, W. P. 1969. Swine mycoplasmas. In Hayflick, L. ed. The Mycoplasmatales and the L-phase of Bacteria. Pp 607-619. Appleton-Century-Crofts, New York, New York.

Takatori, I., Huhn, R. G., and Switzer, W. P. 1968. Demonstration of the complement-fixation antibody against Mycoplasma hyopneumoniae in the sera of pigs infected with swine enzootic pneumonia. National Institute of Animal Health Quarterly 8:195-203.

Taylor-Robinson, D., Canchola, J., Fox, H., and Chanock, R. M. 1964. A newly identified oral mycoplasma (M. orale) and its relationship to other human mycoplasmas. American Journal of Hygiene 80:135-148.

Taylor-Robinson, D., Purcell, R. H., Wong, D. C., and Chanock, R. M. 1966a. A color test for the measurement of antibody to certain mycoplasma species based upon inhibition of acid production. Journal of Hygiene (Cambridge) 64:91-104.

Taylor-Robinson, D., Shirai, A., Sobeslavsky, O., and Chanock, R. M. 1966b. Serologic response to Mycoplasma pneumoniae infection. II. Significance of antibody measured by different techniques. American Journal of Epidemiology 84:301-313.

Taylor Robinson, D., Sobeslavsky, O., and Chanock, R. M. 1965. Relationship of Mycoplasma pneumoniae to other human mycoplasma species studied by gel diffusion. Journal of Bacteriology 90:1432-1437.

Taylor-Robinson, D., Sobeslavsky, O., Jensen K. E., Senterfit, L. B., and Chanock, R. M. 1966c. Serologic response to Mycoplasma pneumoniae infection. I. Evaluation of immunofluorescence, complement-fixation, indirect hemagglutination, and tetrazolium reduction inhibition tests for the diagnosis of infection. American Journal of Epidemiology 83:287-298.

Taylor-Robinson, D., Somerson, N. L,, Turner, H. C., and Chanock, R. M. 1963. Serological relationships among human mycoplasma as shown by complement-fixation and gel-diffusion. Journal of Bacteriology 85:1261-1273.

Terry, T. M., Engleman, D. M., and Morowitz, H. J. 1967. Characterization of the plasma membrane of Mycoplasma laidlawii. II. Modes of aggregation of solubilized membrane components. Biochimica et Biophysica Acta 135:391-405.

Tully, J. G. 1963. Erythrocyte-modifying capacity and serological reactivity of cell components of human mycoplasma (PPLO) strains. Society for Experimental Biology and Medicine Proceedings 114:704-709.

Tully, J. G. 1966. Mycoplasma granularum of swine origin as a tissue culture contaminant. Society for Experimental Biology and Medicine Proceedings 122:565-568.

U.S. Armed Forces Institute of Pathology, 1960. Manual of histologic and special staining technics. 2nd ed., New York, McGraw-Hill Book Company, Inc.

Walker, J. 1921-22. Annual Reports, Department of Agriculture, Kenya, 1919-1920.

Walker, J. 1923. The application of conglutination reaction to the serum diagnosis of bovine pleuropneumonia (Lung sickness). South African Journal of Science 20:406.

Warren, J., Senterfit, L. B., and Sieiro, F. 1968. Inactivated culture vaccine against M. gallisepticum infection in chickens. American Journal of Veterinary Research 29:1659-1664.

Weibel, R. E., Stokes, J., Jr., Mascoli, C. C., Leagus, M. B., Woodhour, A. F., Tytell, A. A., Vella, P. P. and Hilleman, M. R. 1967. Respiratory virus vaccines. VII. Field evaluation of respiratory syncytial, parainfluenza 1, 2, 3, and Mycoplasma pneumoniae vaccines, 1965-1966. American Review of Respiratory Diseases 96:724-739.

Weiser, O. L., Higaki, H. H., and Nolte, L. B. 1967. Mycoplasma pneumoniae complement-fixing antibody titers at birth. American Journal of Clinical Pathology 47:641-642.

White, G. 1958. Agar double diffusion precipitation reaction applied to the study of Asterococcus mycoides. Nature (London) 181:278-279.

Van Dermarck, P. J., and Smith, P. F. 1964. Evidence for a carboxylic acid cycle in Mycoplasma hominis. Journal of Bacteriology 88:1602-1607.

Van Herick, W., and Eaton, M. D. 1945. An unidentified pleuropneumonia-like organism isolated during passages in chick embryos. Journal of Bacteriology 50:47-55.

Van Rockel, H., Clarke, M. K., Bullis, K. L., Olesiuk, O. M., and Sperling, F. G. 1953. Infectious bronchitis. American Journal of Veterinary Research 12:140-146.

Yamagiwa, S., Itabashi, K., and Ito, S. 1930. Studies on contagious pleuropneumonia in cattle. I. On the practical value of complement-fixation test, applying the virus culture as antigen in the diagnosis of contagious bovine pleuropneumonia. Japanese Society of Veterinary Science Journal 9:42-69.

Yamamoto, R., and Adler, H. E. 1958. Characterization of pleuropneumonia-like organisms of avian origin. II. Cultural, biochemical, morphological and further serological studies. Journal of Infectious Diseases 102:243-250.

Yoder, H. W. Jr., and Hofstad, M. S. 1964. Characterization of avian mycoplasma. Avian Diseases 8:481-512.

Young, G. A.. 1964. Virus pneumonia of pigs. In Dunne, H. W. ed. The Diseases of Swine. Pp. 127-135. Iowa State University Press, Ames, Iowa.

Young, G. A., and Underdahl, N. R. 1955. An evaluation of influenza in midwestern swine. American Journal of Veterinary Research 16:545-552.

Young, G. A., and Underdahl, N. R. 1960. Certification of swine herds as virus pneumonia-free. American Veterinary Medical Association Journal 137:186-189.

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