Comparison of bacteriological, immunological, and serological procedures for use in the diagnosis of Haemophilus pleuropneumoniae

by

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A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
MASTER OF SCIENCE

Department: Veterinary Microbiology and Preventive Medicine
Major: Veterinary Microbiology

Iowa State University
Ames, Iowa

1987

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DEDICATION

Dedicated to the memory of my father in law, Jose, whose untimely death occurred before this work was completed and whose support and unselfish interest made possible my graduate studies.

INTRODUCTION

Diseases associated with the respiratory system of the pig have been the subject of many scientific studies during the past two decades. One bacterial agent which has received considerable attention from hog producers, veterinarians and researchers within recent years is Haemophilus pleuropneumoniae, a primary cause of pneumonia in swine throughout the world.

It was not until the middle to late 1970s that individuals associated with the United States pork industry began to realize the true economic impact of the disease. In Europe the swine industry had recognized H. pleuropneumoniae as a primary cause of pneumonia associated with a marked increase of affected lungs at slaughter and the isolation of new serotypes of the organism. In some sections of Canada and the United States the disease began to reach epizootic proportions in the late 1970s. This trend has continued with a marked increase of laboratory confirmed cases in the last two years. Although the rise in incidence of H. pleuropneumoniae can be attributed mainly to confinement-rearing of swine, the frequent exchange of seed stock between states and countries has certainly contributed to the movement of disease.

The diagnosis of pneumonia in swine caused by Haemophilus pleuropneumoniae has been described as an integration of four basic diagnostic evaluations: clinical, pathological, bacteriological and serological (Nielsen 1985). Diagnostic laboratories play a significant role in the confirmation of pleuropneumonia in swine by the isolation and identification of the causative agent. They are also instrumental in providing accurate antibiotic sensitivity

profiles on the H. pleuropneumoniae strains isolated. A small number of laboratories in the United States provide the service of serotyping isolates and testing serum samples for antibody titers. This information is of great importance to the practicing veterinarian and swine producer as they formulate treatment and prevention programs for the affected herds. Bacteriologists continue to be frustrated by their inability to recover this organism directly from tissue under certain conditions, and they have attempted to circumvent this problem by development of dilution techniques (Little and Harding 1980, Pijoan et al. 1983), selective media (Kume et al. 1985), and rapid detection methods (Mittal et al. 1983c). These techniques have met with varying levels of success.

Another area of concern in the diagnostic laboratory is the interpretation of H. pleuropneumoniae serology results. It is incumbent upon the serologist to assist the field veterinarian and producers in making decisions about purchase of seed stock, movement of hogs from farm to farm for breeding purposes, vaccination programs, and depopulation on the basis of herd serology profiles. This is especially difficult when there is an endemic, chronic infection present with few or no clinical signs other than poor feed efficiency and rate of gain (Riising 1982). Chronically infected animals may serve as inapparent carriers of H. pleuropneumoniae, and constitute a potential threat to susceptible animals in contact with them. Identification of carriers is considered a feature of great epidemiological importance in any effort to control the spread of disease caused by this organism. Serology is considered the appropriate tool to detect these animals but unfortunately little work has been conducted to determine the relationship of positive titers

to the actual presence of the organism (antigen) in the pig lung.

The success of isolating H. pleuropneumoniae from lung tissues depends largely on the stage of infection of the animal at necropsy. Whereas pure populations of H. pleuropneumoniae are readily obtainable from lungs in peracute or acute cases of infection (Hoffman et al. 1985), it is often difficult to isolate the organism from chronic lung lesions. In chronically infected animals, H. pleuropneumoniae is not only present in very low numbers, but it is also frequently overgrown by coexisting or secondary bacteria such as P. multocida (Little 1970, Harrison et al. 1978), beta or alpha hemolytic streptococci and C. pyogenes. This bacterial overgrowth makes restreaking for isolation and serotyping extremely difficult. Another problem encountered in diagnostic facilities which may severely impair the recoverability of H. pleuropneumoniae is the necessity to work with tissues from animals which have been heavily treated with antibiotics. Tissues may also become severely autolyzed while in transit, especially during the warm months of the year. The myriad of bacteria present in these tissues, especially Proteus spp., Bacillus spp. and micrococci, may readily mask a pathogen such as H. pleuropneumoniae. Another limitation of direct culture is that technical staff responsible for processing of tissues may not adequately sample the appropriate areas from those lungs which have minimal lesions or bacteria entrapped in a few necrotic or fibrotic foci. Finally, conventional culture techniques may be quite laborious and time consuming, especially in specific situations where extra efforts must be expended to accomplish the necessary goals. An example of this is the attempt to make an accurate diagnosis in a herd in which a mixed infection of more than a

single H. pleuropneumoniae serotype is suspected. If working from a culture plate, numerous colonies must be individually cloned and serotyped in order to prove that two or more different serotypes are present. This is certainly not a time or cost effective methodology that would be practical to employ in diagnostic laboratories with large case loads.

The first major goal of this study was to evaluate the potential usefulness of two procedures, the co-agglutination and peroxidase anti-peroxidase tests, for the detection of H. pleuropneumoniae in porcine tissue. Co-agglutination is based on the detection of antigen by means of protein A-linked antibodies. This procedure allows the direct detection and simultaneous serotyping of H. pleuropneumoniae from affected tissues. Peroxidase anti-peroxidase (PAP) is an extremely sensitive immuno-histological technique which has been widely applied in biological analyses. The application of these two techniques has been significant in the detection and identification of disease-producing bacteria and other pathogenic agents. This research compares these methods with conventional culture techniques, and evaluates their usefulness in a diagnostic situation.

Among the advantages of the co-agglutination technique is the potential for obtaining a diagnosis of H. pleuropneumoniae as well as serotyping of the organism in less than half an hour. Detection and serotyping may take more than 48 hours for completion when standard culturing methods are employed. This direct immunologic method increases the possibility of detecting chronic carrier animals in which lesions may not be apparent on gross examination and in which the number of bacteria is low. Nonviable H. pleuropneumoniae or cellular remnants of the organism may be the only link to making a confirmed

diagnosis in animals treated heavily with antibiotics or in animals with resolved lesions. Because the test detects capsular antigen, viable bacteria need not be present in order to make a positive identification. For similar reasons the co-agglutination test allows the detection of H. pleuropneumoniae antigen in autolyzed tissue, which is difficult to analyze by standard culture techniques. Another important feature of the technique is the possibility of detecting more than one serotype of the organism infecting lungs simultaneously, a possibility difficult to ascertain by conventional culture.

The PAP test is a relatively new enzymatic technique which has definite advantages over other antibody-conjugated techniques. The amplification factor of the enzyme molecule allows the production of many detectable molecules of color indicating the presence of specific antigen, as compared with the small amount of visible light produced by a fluorescent molecule. Sophisticated equipment, such as that required for fluorescence microscopy, is not necessary to perform the PAP test. The technique is thus more affordable for smaller laboratories. In addition, the PAP procedure may potentially be used in retrospective studies of tissues maintained either as stained slides or in paraffinated blocks.

The second major goal of this study was to determine the relatedness between the presence of H. pleuropneumoniae or specific antigen in the lung and the serologic response of the animal. This was accomplished by comparing the results obtained from tissues and sera from slaughter animals from swine herds with differing clinical and vaccination histories. A comparison was made between the complement fixing antibody titers for H. pleuropneumoniae and results of the standard culture technique,

co-agglutination test and the PAP test.

The justification of this research is based on the expectation that either a co-agglutination test or a PAP test could provide an improved diagnostic method for detection of H. pleuropneumoniae in swine. A more sensitive tool for the detection of H. pleuropneumoniae antigen, in combination with serology testing, could also be useful in slaughter plant surveys if control programs were instituted for SPF herds and other seed stock suppliers.

REVIEW OF LITERATURE

Incidence

Reports of porcine pneumonia caused by Haemophilus pleuropneumoniae first came from California (Olander 1963) and Argentina (Shope 1964, Shope et al. 1964); however, a similar condition was documented somewhat earlier by Pattison et al. (1957) and Matthews and Pattison (1961) in which the etiologic agent was described as a Haemophilus-like organism. The peracute or acute pneumonia described in these reports suggested a high incidence among growing hogs from seven to 12 weeks of age.

Widespread occurrence of the disease has been reported from Europe (Nicolet and Konig 1966, Nielsen and O'Conner 1984, Nicolet 1971, Perrin et al. 1979, Brassine and Dewaele 1976, Gunnarsson 1975), the United Kingdom (Little 1970), Canada (Schiefer et al. 1974) and other countries with confined and intensive production of pigs. The existence of more than one serotype was suspected (Shope 1964) and later defined on the basis of capsule polysaccharides which have been shown to be antigenically specific. In addition to the three serotypes (1, 2, and 3) described initially by Nicolet (1971), serotypes 4 and 5 were reported on the basis of agglutination reactions (Gunnarsson et al. 1977). Later reports were made identifying serotypes 6 and 7 (Nielsen 1982, Rosendal and Boyd 1982). Recently serotypes 8 (Nielsen 1984) and 9 (Nielsen 1985)[1] have been recognized.

The geographical distribution of the nine existing serotypes does not

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[1] Unpublished report, Dr. R. Nielsen, The State Serum Laboratory, Copenhagen, Denmark.

follow any specific pattern or relationship to the spread of the disease (Table 1). Serotype 1 is present in Argentina, Australia, Canada and the United States. Serotype 2 is prevalent in Denmark, Italy, Japan, Sweden, Switzerland and Yugoslavia. Serotype 5 is most commonly found in Belgium, Canada, Holland, Taiwan and the U.S. The rest of the serotypes are widespread throughout the world but occur only infrequently (Nicolet 1985). In the midwestern United States, serotypes 5 and 1 are the most common isolates. In published incidence reports from Illinois and Iowa (Rapp et al. 1985b, Schultz et al. 1983, Schultz et al. 1982) serotypes 5 and 1 were isolated most frequently. Rapp et al. (1985b) reported serotyping results from 141 H. pleuropneumoniae isolates obtained from swine in Iowa and Illinois during the years 1980 to 1982. Fifty-five percent were identified as serotype 5, 34 percent were serotype 1 and seven percent were serotype 7. Hoffman et al. (1985) reported that serotype 1 was the most prevalent serotype among field isolates from Iowa herds from 1983 through May, 1985 (Table 2).

The perceived increase in the incidence of disease attributed to H. pleuropneumoniae infections in the recent past has created some concern among diagnosticians and producers. In a recent report (Hoffman et al. 1985) the number of cases confirmed by culture at the Iowa State University Veterinary Diagnostic Laboratory significantly increased between the years of 1976 to 1984 (Table 3). The prevalence of H. pleuropneumoniae antibody titers in Iowa swine was also found to be quite high, with 68 percent of the herds having at least one animal with a CF titer of 1:8 or greater (Schultz et al. 1982). Similar patterns of increasing prevalence of H. pleuropneumoniae

Table 1. Occurrence of different Haemophilus pleuropneumoniae serotypes among countries (Nicolet 1985, Riising 1982)

Country	Serotype identified
Argentina	1
Australia	1, 7
Belgium	1, 3, 5
Canada	1, 2, 3, 5, 7
Denmark	2, 6, 8
Finland	2, 5
France	1, 3, 7, 9
GRD	2, 5
Holland	1, 2, 3, 4, 5, 6, 9
Hungary	2
Japan	2, 3
Sweden	1, 2, 3, 4
Switzerland	2, 3, 7, 9
Taiwan	2, 5
U.K.	2, 3, 8, 9
U.S.A.	1, 3, 4, 5, 7

Table 2. Serotypes of 175 isolates of Haemophilus pleuropneumoniae identified at the Veterinary Diagnostic Laboratory, Iowa State University (Hoffman et al. 1985)

		SEROTYPE						
YEAR	NUMBER	1	2	3	4	5	7	NT[a]
1983	35	51.4%	0%	0%	0%	42.9%	2.9%	2.9%
1984	96	50.0%	0%	0%	0%	40.6%	7.4%	2.1%
1985 (Jan to May)	44	68.2%	0%	0%	0%	25.0%	6.8%	0%
TOTAL	175							

[a]Nontypeable isolates.

Table 3. Haemophilus pleuropneumoniae isolates at the Veterinary Diagnostic Laboratory, Iowa State University, from 1976 through 1984 (Hoffman et al. 1985)

YEAR	NUMBER OF ISOLATIONS
1976	2
1977	9
1978	7
1979	33
1980	69
1981	85
1982	129
1983	131
1984	304

have been reported for Nebraska, South Dakota, California (Schultz et al. 1982), Illinois (Didier et al. 1984), and Pennsylvania (Harrison et al. 1978).

Taxonomy

Among the facultatively aerobic Gram negative rods, the family Pasteurellaceae includes three closely related genera: Pasteurella, Haemophilus and Actinobacillus (Mannheim 1984). The validity of using growth requirements as the unique criterion for inclusion in one of these genera has been questioned based on DNA sequence homology studies. It has been suggested that the heterogeneity of these three genera should be redefined (Pohl 1981). It has been suggested that H. pleuropneumoniae could be included in the tribe Actinobacilleae on the basis of DNA relatedness with organisms such as Actinobacillus lignieresii, Actinobacillus equuli, Pasteurella ureae, Actinobacillus suis, Actinobacillus capsulatus and the Actinobacillus sp. isolated from the sow vagina (Ross et al. 1972). Currently, however, the genus Haemophilus is still defined on the basis of X and V factors required for growth. The X factor (protoporphyrin IX, protoheme or hemin factor) is required by Haemophilus species that lack intermediate enzymes in the hemin biosynthesis pathway (Biberstein et al. 1963, Kilian 1974) (Figure 1). The V factor, nicotinamide adenine dinucleotide (NAD) or NAD phosphate (NADP), is present in red blood cells but its intracellular location and the presence of NADase in the blood of many species make this factor unavailable for NAD-dependent Haemophilus species that require it for growth (Krumwide and Kutlner 1938). Both X and V factors are present in chocolate agar media in which the NAD has been liberated from

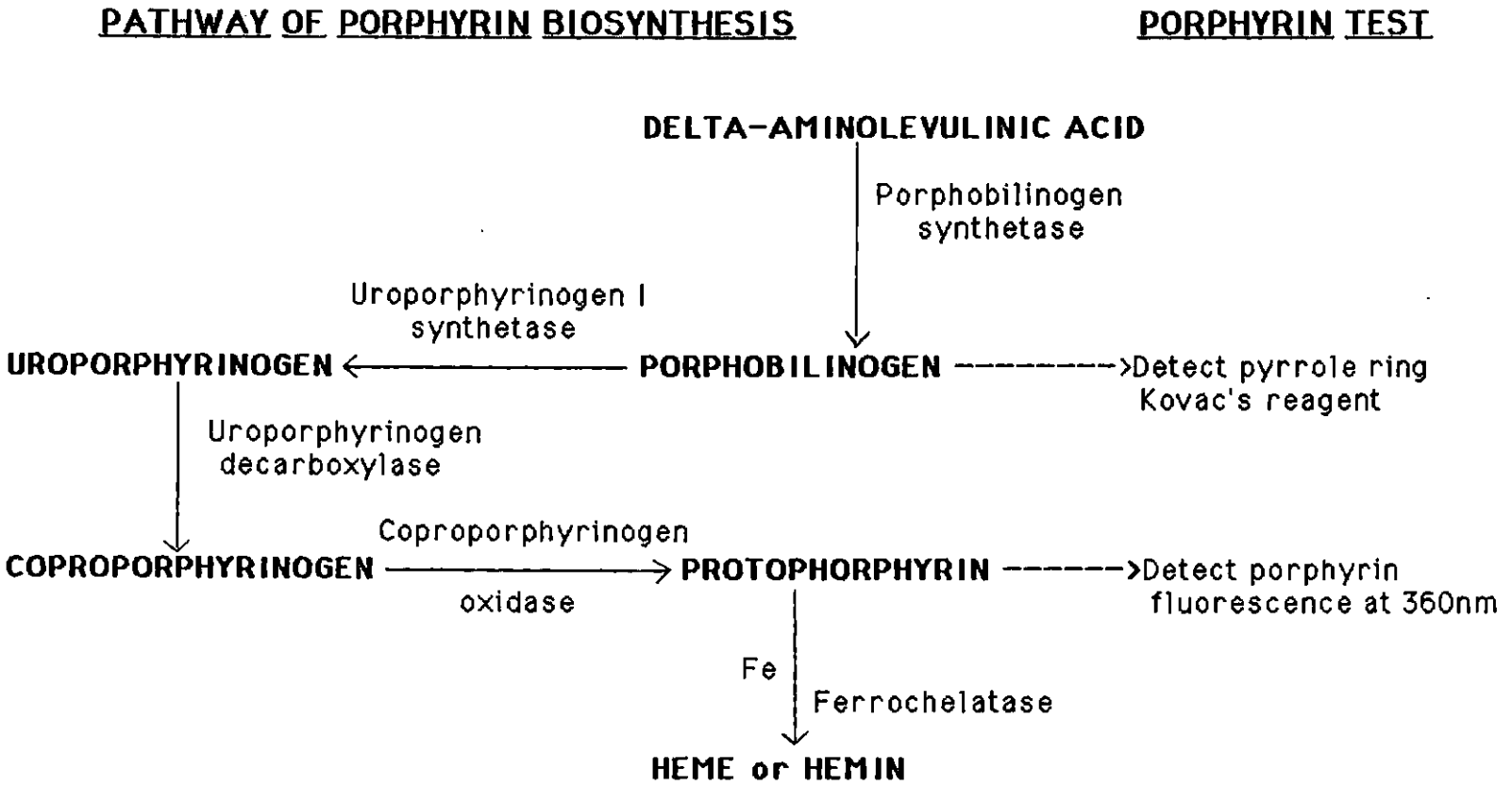


Figure 1. Pathway of porphyrin biosynthesis (Biberstein et al. 1963, M. Kilian 1974)

the cells. More often V factor is supplied by the simultaneous growth of NAD-producing organisms such as Staphylococcus spp. The growth of the Haemophilus colonies in close proximity to the nurse is known as satellitism. This technique is commonly used in diagnostic laboratories for propagation of the organism on blood agar plates by cross-streaking Staphylococcus epidermidis over the previously inoculated area. Biochemical reactions are often utilized to characterize H. pleuropneumoniae. These include urease production, d-amino levulinic acid (ALA) transformation, carbohydrate fermentations, alkaline phosphatase activity and nitrate reduction. The biochemical reactions of the genus are summarized in Table 4.

On blood agar plates, two different types of H. pleuropneumoniae colonies are usually described (Kilian et al. 1978). One colony type is described as a round, hard, "waxy" type that tends to adhere to the platinum loop, and is also differentiated as being "sticky" in nature. This colony is about 1 mm in diameter with a grayish-white color and produces a narrow zone of hemolysis. The other colony type is larger (2 to 3 mm) and flatter, and is soft, smooth and glistening. It produces a wider zone of hemolysis and does not have the adhesive property. The presence of the polysaccharide capsule mentioned above on some young cultures gives rise to the adherent, iridescent, waxy type of colony. This type colony tends to change to the smooth type after a variable number of nonselective passages. A positive CAMP reaction is usual when growth is observed near the staphylococcal nurse colony. Microscopically, the characteristic cell is a Gram negative coccobacillus about 0.2 um wide and variable in length. The appearance of the organism may vary from that of filaments to individual short rods

Table 4. Family Pasteurellaceae: general identification features (Mannheim 1984, Kilian and Frederiksen 1981, Ross et al. 1972)

	H. pleuropneumoniae	H. parasuis	H. "minor taxon"	A. ligneresii	A. equili	A. suis	H. parahaemolyticus	H. somnus	H. equigenitalis	P. multocida	P. haemolytica	P. ureae
Haemolysis	+	−	−	−	−	+	+	−	−	−	−	−
Raffinose	d[a]	−	+	−	+	+	−	−	−	d	+	d
Trehalose	−	−	d	−	+	+	−	+	−	d	−	−
Esculin	−	−	−	−	−	+	−	−	d	−	−	−
NAD required	+	+	+	−	−	−	+	−	−	−	−	−
ALA to porphyrin	+	+	+	+	+	+	+	+	−	+	+	+
L-Arabinose	−	−	−	d	d	+	−	d	−	d	−	−
L-Ramnose	−	−	−	d	+	+	−	−	−	−	−	−
D-Galactose	+	+	+	+	+	+	d	d	−	+	+	−
D-Mannose	+	+	+	+	+	+	−	+	−	+	−	+
Maltose	+	+	+	+	d	−	+	+	−	+	+	+
Galactosidase	d	d	−	+	+	+	d	d	−	d	d	−
CAMP reaction	+	−	−	−	−	−	+	−	−	−	+	−
Inulin	−	+	−	−	−	−	−	−	−	−	−	
Dulcitol	−	−	−	−	−	−	−	−	−	d	d	−
Nitrate red.	+	+	+	+	+	+	+	−	+	+	+	+
Urease	d	−	+	+	+	+	d	−	−	−	−	+

[a]Delayed reaction.

depending on the growth conditions.

Epizootiology and Predisposing Factors of H. pleuropneumoniae Infection

The epizootiology of swine pneumonia caused by H. pleuropneumoniae infection is an important factor in the clinical diagnosis of the disease. The relationship between pulmonary disease and stress factors has been well established. Outbreaks of swine pneumonia in the western hemisphere usually involve the introduction of replacement animals from infected herds followed by improper management of the herd relative to ventilation, temperature, humidity or transportation (Roth 1984, Switzer et al. 1981, Curtis et al. 1976, Straw et al. 1986, Desrosiers and Moore 1986). Stress factors which alter the defense mechanisms of the respiratory tract could be considered as predisposing to the appearance of an outbreak in susceptible populations.

The pseudostratified ciliated columnar epithelium of the upper respiratory tract of the pig is normally covered by a mucous blanket. The cilia and the mucus play important roles as primary defense mechanisms, and both can be altered by factors such as chemical irritation from the bedding materials, extreme temperatures and dust in buildings which are poorly ventilated (Switzer et al. 1981). When the beating of the cilia is impaired and the secretion of mucus reduced, aerosolized particles such as bacteria or virus particles can easily enter the lower respiratory tract (Switzer et al. 1981). Three different kinds of cells play major roles in the local cellular resistance at the level of the alveolar wall: type 1 pneumocytes, type 2

pneumocytes, and the alveolar fixed macrophage. The most important cell type is the alveolar macrophage, which functions as a phagocyte and antigen presenting cell.

Epidemiological studies of the disease have stressed the importance of transmission by direct contact (Nielsen and Mandrup 1977, Schultz 1985, Nicolet 1985). The role of aerosolized particles has been shown to be important for the spread of H. pleuropneumoniae respiratory infections. The introduction of carrier boars or replacement gilts plays a major role in carrying the disease into a susceptible herd. Indirect transmission by clothes, boots and other inanimate objects is also possible.

Clinical Signs

Cyanosis, dyspnea, coughing, anorexia and epistaxis are common signs seen as clinical manifestations of H. pleuropneumoniae infection. In addition, sudden death without any apparent clinical signs can also occur, usually 24 to 48 hours after the introduction of infected animals into a susceptible herd (Nielsen and Mandrup 1977). The clinical signs produced by the infectious agent have been attributed to an endotoxic shock (Nordstoga and Fjolstad 1967). The morbidity and mortality will depend on the immune status of the herd, but commonly range from eight percent to 40 percent morbidity and from 0.4 percent to 24 percent mortality (Schultz 1985). In herds with no previous exposure sudden onsets of clinical disease with fatalities may occur in 24 hours or less. In acute outbreaks the clinical signs may include anorexia, dyspnea, high fever and cyanosis of the skin followed by the death of some animals. During the final phase of the peracute stage the animal may

adopt a sitting position, and there may be nonproductive, open-mouthed coughing that may later progress to vomiting. Death often occurs after 24 hours, although some animals will survive and proceed into a subacute or chronic form or stage (Nielsen 1982).

Chronically affected animals frequently may develop secondary pneumonia and pleuritis and appear unthrifty. The pigs may suffer from intermittent coughing and anorexia and, as a consequence, they generally grow poorly and convert feed inefficiently. The epidemiological importance of these animals in spreading the disease and maintaining the organism is one of the primary concerns of producers and veterinary practitioners (Nielsen and Mandrup 1977).

The differential diagnosis of respiratory disease caused by H. pleuropneumoniae should include infections by other Gram negative bacteria such as Bordetella bronchiseptica, Pasteurella spp. and Haemophilus parasuis. Mycoplasmal infections are found preceding, accompanying or following H. pleuropneumoniae infections (Ross 1981). Mycoplasma hyorhinis is described as a common secondary invader of porcine pneumonias (Ross 1981) while Mycoplasma hyopneumoniae is a primary infectious agent. Gram positive bacteria such as Streptococcus spp., Corynebacterium pyogenes and Staphylococcus aureus are often isolated from lungs of pigs chronically infected with H. pleuropneumoniae (Hoffman 1986)[1]. No causal relationships between viral agents and H. pleuropneumoniae pneumonias were described in

[1] Personal communication, Dr. L. J. Hoffman, Bacteriology Section, Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University, Ames, Iowa.

the literature consulted, although adenoviruses have been isolated from clinical cases of pneumonia in which H. pleuropneumoniae was considered the etiological agent (Schiefer et al. 1974).

From a clinical aspect, pneumonias in the pig are hard to differentiate even though the epidemiology, vaccination history and symptoms are generally of value. However, it is necessary to isolate and identify the organism in order to confirm the diagnosis.

Pathology

The gross pathological lesions resulting from H. pleuropneumoniae infections have been well characterized in both the experimental and naturally occurring disease (Olander 1963, Shope 1964, Shope et al. 1964, Nielsen 1970, Mylrea et al. 1974, Little 1971). Several researchers (Mylrea et al. 1974, Sanford and Josephson 1981, Schiefer et al. 1974, Schiefer and Greenfield 1974) have described a correlation between the type of lesion and the duration of infection. The duration of infection also appears to be related to the immune status of the herd. In fatal acute septicemia the gross lesions reflected circulatory microscopic changes associated with endotoxic shock. Accumulation of blood stained fluid in thoracic, pleural and pericardial cavities was the predominant lesion in pigs which died acutely. This fluid was either a serous or serosanguineous effusion from which the etiologic agent was isolated (Schiefer and Greenfield 1974). At this stage the lungs were an enlarged uniform mass of reddish, hemorrhagic tissue in which blood stained edematous fluid filled the alveoli and conducting airways. The first 24 hours of infection were characterized by congestion of the lungs with some

roughening of the pleural membranes. Pigs that survived more than 24 hours developed cellular reactions characterized by the appearance of fibrin deposits and adhesions of the pleural membranes. The involvement of bronchial lymphatics was characterized by enlargement, edema and congestion (Schultz 1981, Sanford and Josephson 1981, Schiefer et al. 1974).

Two to four days after infection the gross pathological lesions were characterized as a necrotizing fibrinohemorrhagic pleuropneumonia with extensive consolidation of the lungs. The formation of sequestered foci of necrotic pulmonic tissue at this stage, as seen in abattoir sampling studies, has been considered an important feature in animals from herds where infection persists; such foci may serve as sources of infective bacteria (Olander 1985). The perpetuation of the infection by pigs with these chronic lesions may also account for the failures in controlling the disease by mass antibiotic medication (Mylrea et al. 1974, Sanford and Josephson 1981).

In clinically recovered animals, the resolution of the affected areas of the lungs will leave consolidated foci of necrotic tissue surrounded by connective tissue. The size of these areas depend on the degree of the involvement during the acute phase. A fibrotic lesion may be accompanied by persistent pleural adhesions in chronically affected animals.

The microscopic and ultrastructural lesions of the affected areas of the lungs in naturally occurring and experimental infections with H. pleuropneumoniae pneumonia have been studied extensively (Perfumo et al. 1983, Nielsen 1970, Bertram 1985, Nordstoga and Fjolstad 1967). The first reaction is the accumulation of platelets along the pulmonary endothelium. It has been speculated that the presence of endotoxin results in thrombi formation,

inflammatory edema and degeneration of endothelial cells (Bertram 1985). The cellular or secondary reaction is characterized by the appearance of macrophages and polymorphonuclear cells which are mainly neutrophils (Bertram 1985) although some earlier studies described the infiltration as being mainly lymphocytic (Mylrea et al. 1974). After the first 24 hours of infection, thickening of the alveolar wall by neutrophils, fibrin, macrophages and clumps of the organism resulted in alveolar collapse and a 30 percent reduction in air space when compared with normal lung tissue (Bertram 1985). The third or fibrotic reaction is characterized by the organization of fibrinous exudates extending the fibrosis through the adjacent pleura (Mylrea et al. 1974). This fibrotic reaction may lead to the formation of persistent necrotic sequestra.

Prevention and Control

Immunity against H. pleuropneumoniae is well documented (Nielsen 1974, 1976, 1979, 1982, 1984). In herds previously exposed to H. pleuropneumoniae, sows passively transfer antibodies to the piglets via the colostrum. These antibody titers, as measured with the CF test, may persist in the piglets for up to 60 days (Nielsen 1974). At approximately eight weeks, pigs should be vaccinated to prevent later infections if the situation dictates it. Experimentation with the various federally licensed vaccines available today demonstrated variable results (Wilson 1980, Rosendal et al. 1981a, Riising 1980, Scholl et al. 1978, Henry 1982, Straw et al. 1985). Autogenous bacterins have been generally more effective than polyvalent vaccines in controlling H. pleuropneumoniae infection in problem herds,

although pleuritis has been reported at slaughter in some vaccinated animals (Riising 1982). Several factors were considered when efficacy was evaluated, such as reduced mortality in vaccinated animals, diminished presence of lesions at slaughter, improvement in the rate of feed conversion and, finally, decreased shedding of the organism. There is still some controversy concerning the use of the correct adjuvant in order to avoid undesirable granulomas (Straw et al. 1985, Higgins et al. 1985). Oil-adjuvanted vaccines generally elicited good antibody titers but also induced an undesirable granulomatous inflammatory response (Henry 1982, Straw et al. 1985). Phosphate and aluminum hydroxide adjuvants produced fewer undesirable tissue reactions, but the immune response elicited was not as good as with oil adjuvants. The most commonly used vaccines are either young whole cell inactivated cultures of the organism or washed suspensions, the former being more acceptable (Henry 1982).

Treatment with broad spectrum antibiotics is desirable in animals displaying severe clinical signs and as a preventive measure when susceptible animals are introduced at any other time in an H. pleuropneumoniae-infected herd (Wilson and Osborne 1985). The route of administration for clinically affected pigs is generally parenteral; the rest of the herd may be treated by water medication (Schultz 1985). The organism is often sensitive in vitro to most of the commonly used antibiotics in swine practice, including penicillin, spectinomycin, ampicillin and gentamicin. Nevertheless, several studies (Schultz and Anderson 1983, Schultz and Ross 1983, Hirsh et al. 1982) indicate substantial changes, some of them plasmid mediated, in the sensitivity of H. pleuropneumoniae isolates to the earlier used antibiotics.

Some of these changes in the sensitivity have been attributed to antibiotic medication added to the feed of postweaning pigs (Hirsh et al. 1982).

In Europe, Nielsen et al. (1976a) proposed serological blood testing by the complement fixation test followed by the removal of the seropositive animals and their litter mates to control the disease. This protocol is probably practical only in a small confinement unit, which is not the predominant type of operation seen in the United States.

It is important to determine the serotype or serotypes of H. pleuropneumoniae present in a particular region or country because recommendations for the preparation and use of serodiagnostic antigens and vaccines are often made on the basis of this information. Since there are some cross-reactions between the different serotypes in the complement fixation test used for diagnosis (Table 5), monospecific antigens should be used when searching for new serotypes in a geographic area.

Economic Significance

The economic impact of H. pleuropneumoniae pneumonia is significant and is manifested in one or more of the following ways: increased mortality, increased time to reach market weight, increased veterinary and drug costs, and increased labor expenses (Friendship et al. 1984, Desrosiers and Martineau 1984). A reduction in growth rate is perhaps of greatest concern, and it is usually associated with chronically infected herds. Some variations are observed in the time required for these animals to reach market weight. It has been estimated that approximately three additional weeks are required to finish hogs from H. pleuropneumoniae infected herds. Accurate

Table 5. Homologous and heterologous H. pleuropneumoniae serotype CF reactions[a]

ANTISERUM SEROTYPE (STRAIN)	ANTIGEN SEROTYPE (STRAIN)										
	1 (4074)	2 (1536)	3 (1421)	4 (M62)	5 (K17)	5 (M1)	5 (200)	5 (B&B)	7 (WF83)	7 (53)	7 (160)
1(4074)	≥128	–	–	–	8	–	–	4	–	–	–
2(1536)	–	32	–	–	–	–	–	–	–	–	–
3(1421)	–	–	16	–	–	–		–	–	–	–
4(M62)	–	–	–	≥128	64	16	–	32	32	128	32
5(K17)	–	–	–	–	64	32	32	32	±	–	8
5(M1)	–	–	–	–	8	16	–	16	–	–	–
5(200)	–	–	–	–	≥128	128	≥128	3	–	–	–
5(B&B)	–	–	–	–	64	32	16	64	–	–	–
7(WF83)	–	–	–	32	64	8	16	32	32	128	64
7(53)	–	–	±	64	≥128	64	64	64	64	≥128	64
7(160)	–	–	–	32	32	8	–	32	32	128	64

[a]Convalescent sera and antigens were prepared at the Veterinary Medicine Research Institute, Iowa State University. The complement fixation titers were determined by Mrs. Theresa Young.

information about real costs of the damage induced by the disease around the world are unavailable, but it is probably in the billions of dollars.

Laboratory Diagnosis

Most veterinary laboratories confirm the diagnosis of H. pleuropneumoniae by conventional isolation and identification of the organism. Serotyping of isolates is performed by direct agglutination, coagglutination or the fluorescent antibody method. Serological diagnosis of H. pleuropneumoniae is basically conducted by the CF test and should be used on a herd basis only. Several other techniques have been developed in an attempt to promptly and accurately detect this disease (Nicolet et al. 1981, Pijoan et al. 1983, and Mittal et al. 1983a).

Isolation and identification

It is generally necessary to attempt culture of H. pleuropneumoniae because of the need for valid antibiotic sensitivity testing. Diagnostic laboratories have approached the recovery and culture of H. pleuropneumoniae from animals by several different techniques. The standard technique employed to isolate H. pleuropneumoniae is by culture at 37° C on 5 percent blood agar supplemented with a streak of Staphylococcus epidermidis to supply nicotinamide adenine dinucleotide (NAD), which is also referred to as V factor. Another culture method involves the use of chocolate agar, in which the ruptured red blood cells liberate the NAD required for growth (Kilian 1974). Kume et al. (1984) used a solid medium called "S agar" to isolate H. pleuropneumoniae. This medium included chicken

meat infusion broth and chicken serum as enrichment factors. Other suitable media are available commercially (e.g., Haemophilus Isolation Medium, Remel Lenexa, Kansas, and Casman Agar, Difco, Detroit). These media contain factors (hemin and NAD) required for some Haemophilus species to grow.

One of the main concerns in chronically infected animals relates to the difficulty of recovering H. pleuropneumoniae because of the overgrowth of other organisms, especially Pasteurella multocida. It has been conjectured that P. multocida may actually inhibit the growth of H. pleuropneumoniae (Little 1970, Harrison et al. 1978). Dilution techniques have been developed to minimize this problem (Little and Harding 1980, Pijoan et al. 1983). The practicability of these techniques for use in diagnostic facilities is limited by cost and the extra labor required. The relative improvement in results obtained by these methods as compared to conventional techniques is minimal, making their use on a daily basis in a diagnostic facility difficult to justify. On the other hand, diagnostic laboratories are confronted regularly with problems inherent in the use of conventional procedures for isolation of H. pleuropneumoniae. The probability of isolating and identifying H. pleuropneumoniae by culture is reduced significantly by a number of factors commonly associated with tissues or animals submitted to veterinary diagnostic laboratories. Tissues are often autolyzed, making recovery of pathogenic bacteria difficult due to overgrowth of contaminants. Culture attempts are often unsuccessful because the necropsied pig was previously treated with antibiotics. Lastly, many lungs are taken from animals in a subacute or chronic stage of infection where few pathogens persist. In these

animals, recovery of H. pleuropneumoniae may be limited by excessive numbers of secondary bacteria.

Another area of concern in laboratories where swine diseases are routinely evaluated is the identification and differentiation of bacteria closely related to Haemophilus pleuropneumoniae, namely Actinobacillus spp., Haemophilus parasuis, Haemophilus taxon "minor group", urease negative Haemophilus and Haemophilus taxon C (Nicolet 1985).

Serotyping

Serotyping of H. pleuropneumoniae has been shown to depend on the presence of capsular antigens (Nicolet 1971), although the nature of the specific antigen in serotypes 6 and 9 is still unknown. Some cross-reactions between serotypes 4 and 7 and between 3, 6 and 8, have been attributed to the presence of more than one antigen; i.e., a specific capsular antigen and a second antigen, probably a lipopolysaccharide, that is responsible for the cross-reactivity (Pijoan 1985).

The success of control and eradication programs for H. pleuropneumoniae requires identification of the H. pleuropneumoniae serotype(s) present in a geographical region. Protection conferred by H. pleuropneumoniae bacterins is known to be serotype specific; therefore, it is important that all of the pathogenic serotypes present in a given region be included in a vaccine regimen (Nielsen 1979).

Besides the nine H. pleuropneumoniae serotypes described, two nontypeable strains have been reported. Also, the occurrence of a "minor taxon" which differs from H. pleuropneumoniae in carbohydrate fermentation

has been reported (O'Reilly et al. 1984). Each of the nine serotypes have been described on the basis of agglutination reactions.

The identification of H. pleuropneumoniae serotypes has been accomplished using a variety of test procedures, including rapid slide agglutination (Mittal et al. 1982, Rapp et al. 1985b), tube agglutination (Gunnarsson et al. 1977), ring precipitation (Mittal et al. 1982), co-agglutination (Mittal et al. 1983b) and indirect fluorescent antibody (Rapp et al. 1985b).

The standard serotyping technique currently in use is the slide agglutination test using serotype-specific hyperimmune serum produced in rabbits. Antiserum is mixed with a saline suspension of the antigen, which has previously been harvested from an 18-hour culture grown on modified Casman agar. The indirect fluorescent antibody test was shown to be less serotype-specific than rapid slide agglutination (Rapp et al. 1985b); the best results were obtained on cultures rather than tissue.

Co-agglutination and peroxidase anti-peroxidase procedures

Two immunological assay procedures that appear to offer significant advantages over other methods for both the identification and serotyping of H. pleuropneumoniae in tissue are a co-agglutination technique and a peroxidase anti-peroxidase (PAP) test. The evaluation of these procedures was a primary purpose of this research; each method is discussed in some detail in the following sections.

The co-agglutination technique The co-agglutination technique, as described by Mittal et al. (1983c) for the diagnosis of H. pleuropneumoniae

pneumonia, basically detects H. pleuropneumoniae capsular antigen in the lung tissue by means of serotype-specific antibodies coated by protein A from Staphylococcus strains. Protein A is a major cell wall component of most strains of Staphylococcus aureus. It was first described by Verwey (1940) and later by Jensen (1958), who demonstrated the ability of normal human sera to agglutinate S. aureus by "natural antibodies". Further investigations by Kronvall and Williams (1969) and Forsgren and Sjoquist (1966) with gamma G myeloma globulins demonstrated the "pseudoimmune" characteristic of the reaction (Figure 2). This affinity of protein A for immunoglobulins, notably IgG, stimulated studies of its structural features. The composition of protein A, initially suspected to be polysaccharide in nature (Jensen 1958), was shown later to consist mainly of a polypeptide chain with a molecular weight of 42,000 with little or no carbohydrate present (Bjork 1972, Goding 1978). Four tyrosine residues on its surface are responsible for its biologic activity. These correspond to four segments consisting of more than 50 amino acids, each responsible for Fc-binding sites (Figure 3). Just two of its four Fc-binding sites are expressed in reactions with IgG molecules (Sjoquist et al. 1972) and, although the major immunoglobulin which binds to protein A is IgG, important differences exist among different species (Table 6). The protein nature of the Fc-binding sites on the surface of the reactive staphylococci was demonstrated by its sensitivity to trypsin (Lind and Mansa 1968). The proportion of this protein linked to the peptidoglycan of the staphylococcal cell wall has been estimated at 90 percent, although some is also released during cell growth (Willet 1984, Morse 1980). The exact portion of the immunoglobulin G molecule to which these protein A Fc receptors attach has

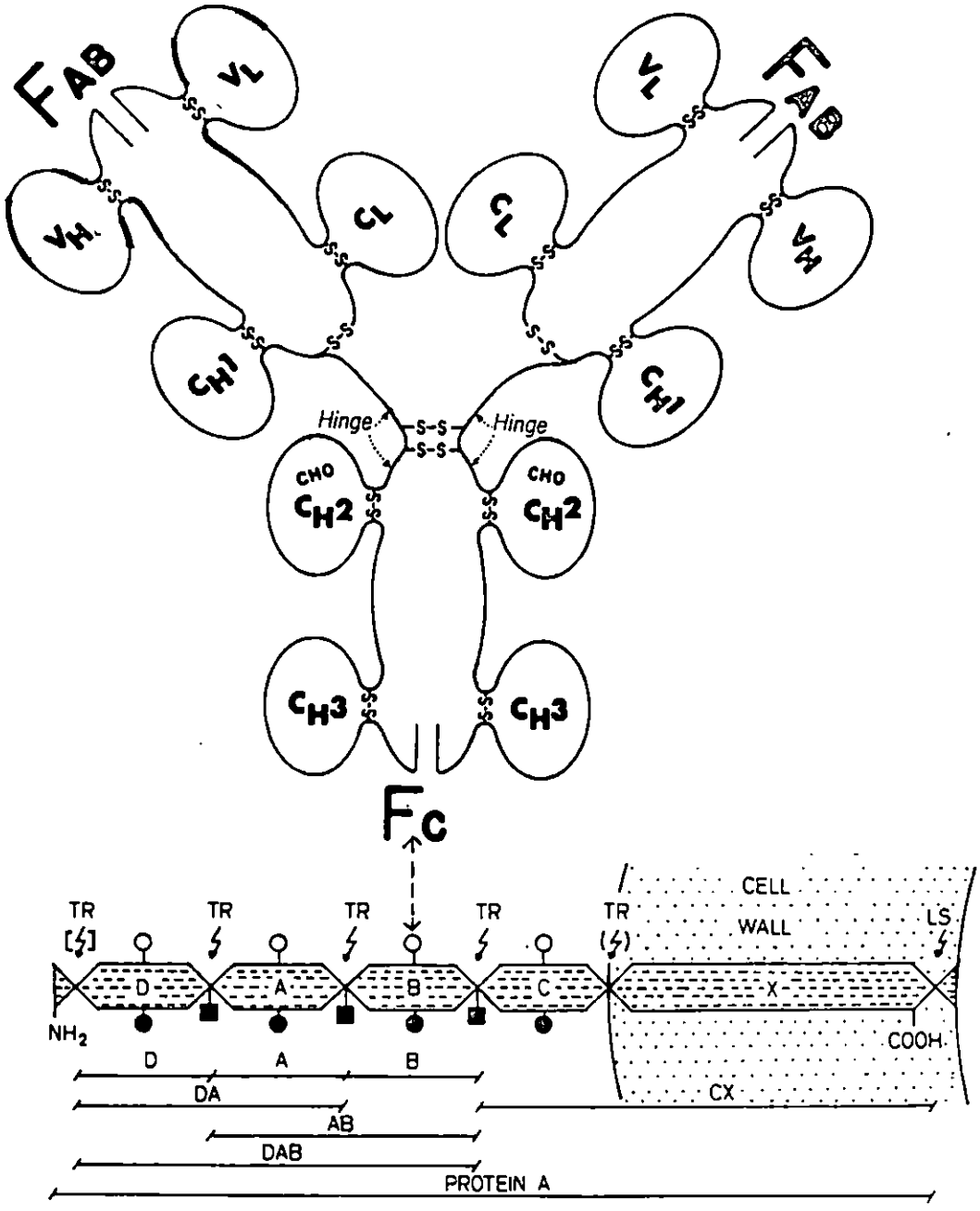


Figure 2. Proposed mechanism for the binding of immunoglobulins to the protein A molecule (Willet 1984)

Fab "antigen-binding fragments" of the immunoglobulin molecule
Fc "crystallizable fragment" of the immunoglobulin molecule
O protein A Fc receptor (four per molecule)

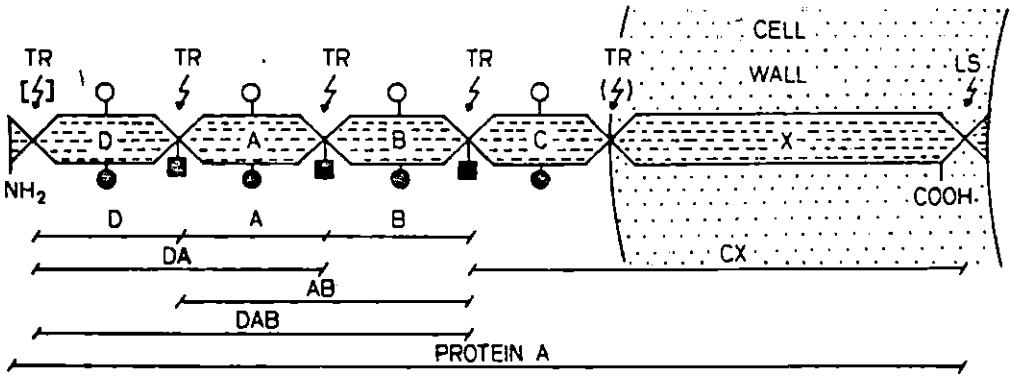


Figure 3. Structure of cell wall protein A from staphylococcal strains (Willet 1984)

TR Trypsin enzymatic cleavage site
O Fc receptors

Table 6. Protein A reactivity of immunoglobulins from various species (Outteridge 1985, Langone 1982b, Goding 1978)

Species	Immunoglobulin subclass bound	%Bound
Horse	IgG	67
	IgM	16
	IgG(T)	20
Dog	IgG	47
	IgM	36
	IgA	40
Pig	IgG	94
	IgM	35
	IgA	23
Sheep	IgG1	2
	IgG2	33
Cow	IgG1	26
	IgG2	53
Rabbit	IgG	93
	IgM	trace
Human	IgG1	95
	IgG2	90-95
	IgG4	90-95
	IgA2	trace

been shown to be localized at the CH2-CH3 interface of the molecule for human beings and guinea pigs (Langone 1982b).

The production of protein A by staphylococcal strains is restricted to S. aureus. Staphylococcus epidermidis was found to lack protein A in a study of 47 strains by Kronvall et al. (1971). In an excellent study by Langone (1982a) 95 percent of S. aureus strains were found to produce protein A. Five percent of these positive strains released the protein into the medium, making it impossible to find any cell bound product. Although a strong positive correlation was found between protein A production and coagulase production and thermostable nuclease activity, 75 percent of Staphylococcus hyicus protein A negative strains were coagulase and thermonuclease positive. The Cowan I strain of S. aureus has been found to produce the most protein A, either as cell bound or as extracellular product.

The co-agglutination test was used in early studies to bind gamma globulins (Forsgren and Sjoquist 1966); differences in binding affinity were shown among subclasses of immunoglobulins (Willet 1984, Kronvall and Williams 1969, Klein 1982). Voller and Bidwell (1986) found enzyme-labeled protein A useful when added instead of the anti-species immunoglobulin conjugate in immunosorbent (ELISA) assays. This technique is currently used for typing streptococci (Christensen et al. 1973, Kronvall 1973), precipitating antigen-antibody complexes, separating cells, and selectively absorbing IgG prior to analysis of other immunoglobulin classes (Langone 1982c). Co-agglutination tests are widely used in the diagnosis of Haemophilus infections in man (Welch and Hensel 1982, Collins and Kelly 1983).

The use of the co-agglutination procedure for the detection of H. pleuropneumoniae antigen in diseased swine lungs was first reported by Mittal et al. (1983c). Protein A from Cowan I strain Staph. aureus was used in combination with specific anti-H. pleuropneumoniae antiserum. Their studies indicated that the method was very effective for detecting H. pleuropneumoniae antigen in lungs that were negative by standard culture procedures.

In summary, the reactive capability and plasticity of this cell wall protein, basically due to its immunoglobulin binding capacity, makes protein A a reagent with a tremendous potential in immunologic assays. As for its specific application in the diagnosis of H. pleuropneumoniae, the co-agglutination assay's great sensitivity in detecting small amounts of capsular antigen is of potentially great value in the identification of chronically affected herds. This capability, combined with the serological testing of swine herds suspected of harboring H. pleuropneumoniae, would facilitate the planning of successful eradication programs.

The peroxidase anti-peroxidase test Enzyme immunoassays have previously been used for the diagnosis of H. pleuropneumoniae; an ELISA has been developed for the detection of antibodies to H. pleuropneumoniae in serum (Nicolet et al. 1981). Several enzyme immunoassays have been developed to detect specific antigens in tissues; the most sensitive reported are peroxidase anti-peroxidase (PAP) procedures (Bourne 1983, Burns 1975) and the avidin-biotin technique (Roth and Marchand 1985). The PAP method, also known as the unlabelled antibody method, was developed to detect antigens in tissues by an immunohistochemical technique that eliminated the

use of artificially conjugated antibodies (Mason et al. 1969, Sternberger et al. 1970). The greater sensitivity of nonconjugated antibodies over the horseradish peroxidase labelled conjugates can be attributed in part to the amplification of the three step method (Boorsma 1983). Initial attempts to intensify the localization of antigen in tissues using the unlabelled antibody method required separate staining steps with rabbit anti-peroxidase and peroxidase (Sternberger 1979, Figure 4). The development of a soluble complex of horseradish peroxidase-anti-horseradish peroxidase (PAP) simplified the procedure by reducing the number of staining steps (Sternberger 1969, 1979, Sternberger and Cuculis 1969, Sternberger et al. 1970). The sequential procedure, as illustrated in Figure 5, involves the following steps: 1) application of rabbit anti-H. pleuropneumoniae antiserum to tissue sections, 2) goat anti-rabbit immunoglobulin added in excess so it will leave one combining site free after reacting with the rabbit antibody, 3) peroxidase anti-peroxidase (rabbit) complex added (reacts as antigen with the free combining sites of anti-IgG), and 4) addition of hydrogen peroxide combined with a suitable chromogen, such as 3-amino-9-ethyl carbazol (AEC).

While working on the diagnosis of syphilis, Sternberger et al. (1970) produced his soluble PAP complex by the addition of antigen in moderate excess to a washed immune precipitate that had been dissolved at a pH of 2.3 at 1o C. After neutralization, reequilibration of peroxidase anti-peroxidase (PO-anti-PO) into soluble complexes of homogeneous composition was accomplished. The soluble PO-anti-PO complexes were separated from PO by precipitation with ammonium sulfate. The complex was shown to have a

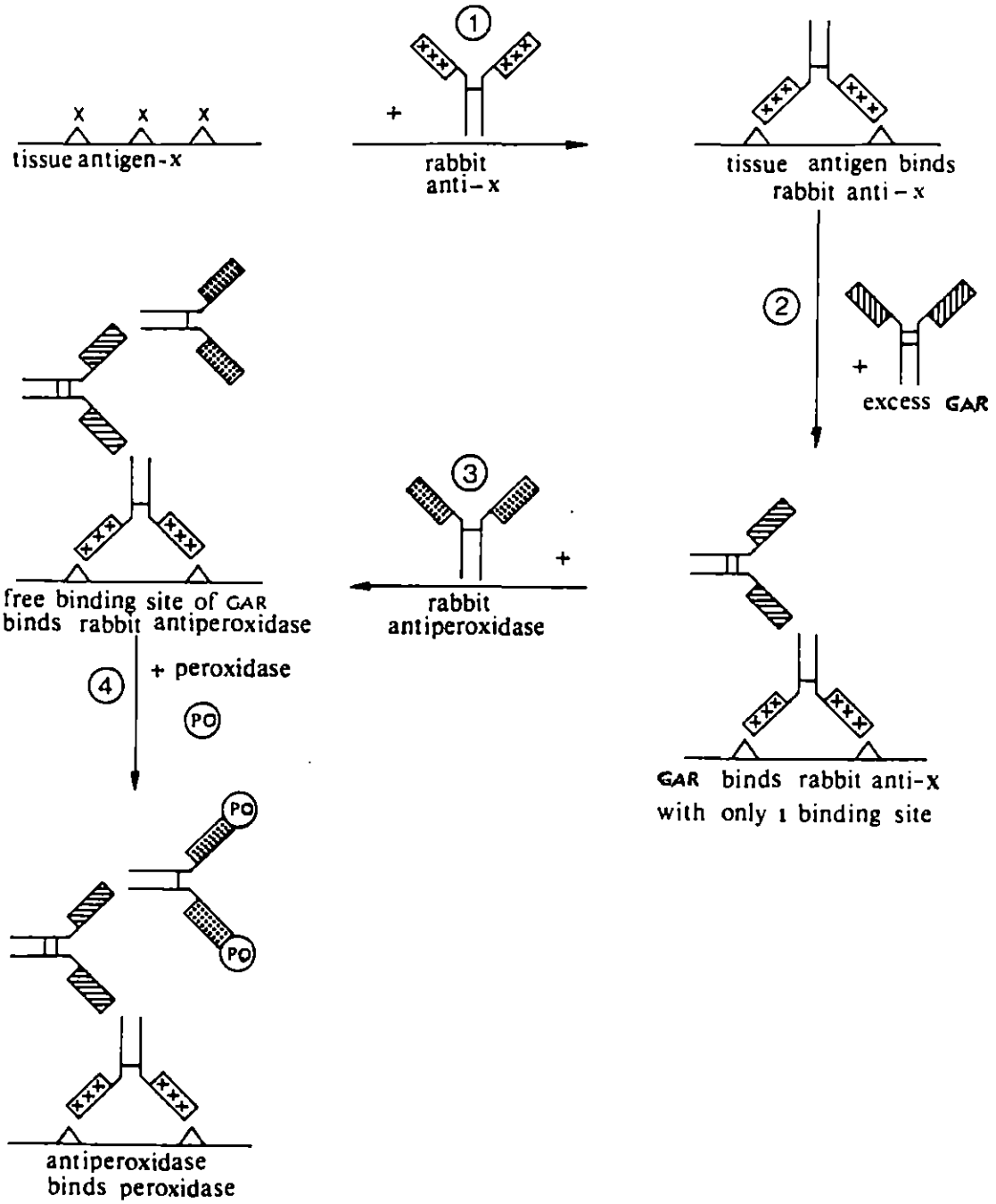


Figure 4. Staining sequence of the original unlabelled antibody enzyme method (Vandesande 1983)

GAR goat anti-rabbit immunoglobulin
PO horseradish peroxidase

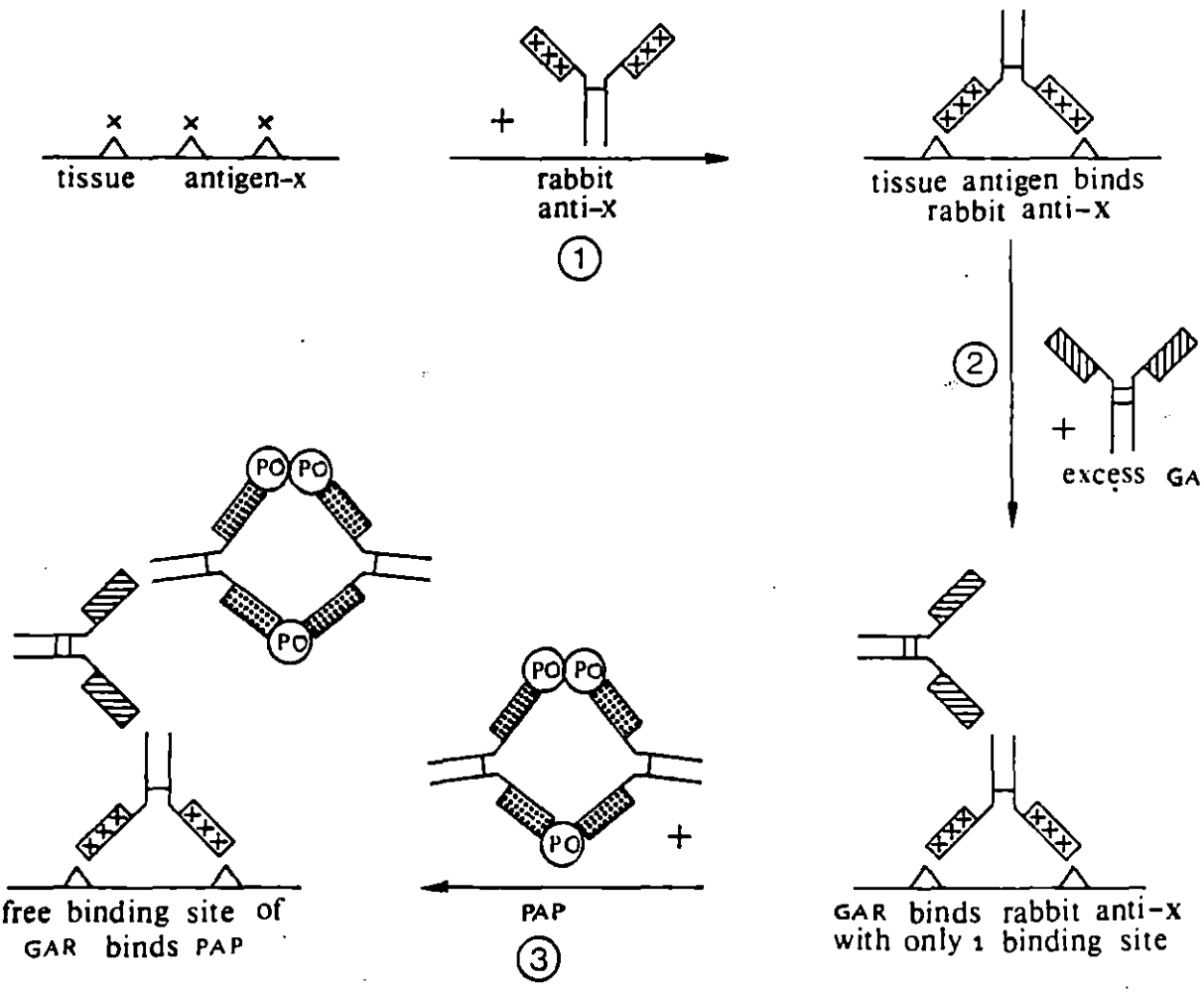


Figure 5. Staining sequence in the PAP method (Vandesande 1983)

GAR goat anti-rabbit immunoglobulin
PO horseradish peroxidase

molecular ratio of PO to anti-PO in PAP of 3:2, which seems to be due to the more stable conformation of this complex (Figure 6) compared with others. Several aspects of the PAP test make it a highly sophisticated technique for detection of antigens. It possesses a greater sensitivity than many other tests because there are no conjugated antibodies. The PAP is up to 100 times more sensitive than immunofluorescence, and 100 thousand times more sensitive than a radioimmunoassay. This is a result of the natural affinity of the antigen (PO) and the specific antibody (anti-PO) to form complexes that remain soluble in solution (Moriarty et al. 1973, Vandesande 1983, Short and Walker 1975). High dilutions of the antiserum used as primary antibody have been shown to detect very small amounts of antigen, and the colored reaction product can be observed with a simple light microscope (DeLellis et al. 1979). Thus, there are no requirements for expensive and sophisticated instrumentation for the evaluation of PAP test results, as there are with other procedures such as immunofluorescence and radioimmunoassays.

Certain precautions must be taken in order to avoid problems such as the destruction of the antigen by the use of strong fixing solutions, interference by endogenous peroxidase, and nonspecific staining. Freshly prepared formaldehyde was found to be much better than glutaraldehyde as a fixative for immunohistochemistry (Farr and Nakane 1981). Less cross-linking of proteins afforded by the fresh formaldehyde allows improved penetration of antibodies into the sample, with good retention of antigenic reactivity and adequate morphology. In any case, this problem is of minor importance since the method employs highly diluted antiserum capable of detecting 99 out of 100 antigenic determinants. It is suggested that titrations of the primary

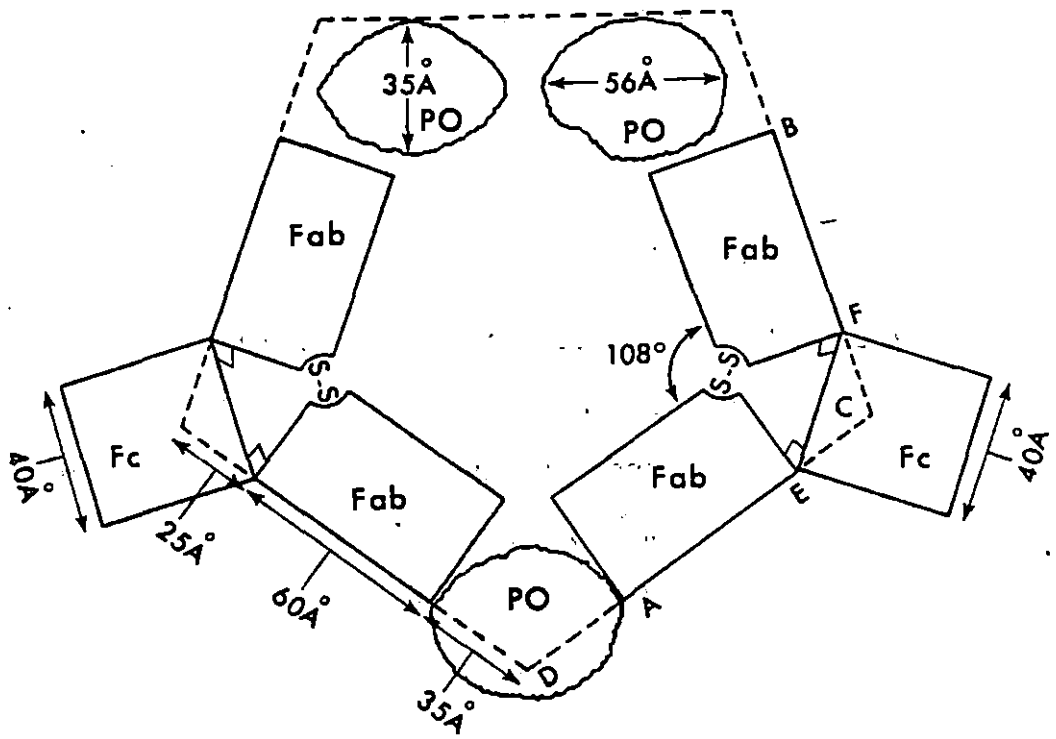


Figure 6. Scale diagram of a molecule of peroxidase anti-peroxidase complex in a ratio 3:2 (Sternberger 1970)

PO horseradish peroxidase

Fab antigen-binding site of antibody

Fc crystallizable fragment of antibody

antibody be started at 1:100, depending on the antibody content of the serum and also on the amount of antigen suspected in the tissue. Dilutions of 1:1000 are commonly suggested for the primary antibody (Sternberger et al. 1970). Endogenous peroxidase normally present in different tissue cells is not destroyed by the paraffin embedding, and should be suppressed or inhibited to avoid visualization resulting in nonspecific background. Among the most commonly used methods recommended to inhibit this activity is the incubation of the tissue section in a 0.5 percent solution of hydrogen peroxide in 0.0125 percent absolute methanol for 30 minutes (Streefkerk 1972, Hrapchak 1980). Alternative methods for inhibiting endogenous peroxidase activity include incubation with acid alcohol or phenylhydrazine (5×10^{-3} M) in PBS for 15-30 minutes (Burns 1982), or either methanol and nitroferricyanide (Streefkerk 1972) or pepsin (Reading 1977) for 20-30 minutes at 37^{0} C.

The catalytic activity of a single molecule of enzyme on several molecules of substrate results in a magnification of the reaction leading to the colored reaction product. This gives the PAP technique a tremendous potential for the detection of antigen in highly contaminated or decomposed tissue, and in tissue from chronically infected animals which may contain only a small quantity of antigen (Yolken 1982).

Several applications of the PAP in diagnostic microbiology and immunology have been documented. These include the demonstration of Mycoplasma suipneumoniae in pig lungs (Bruggmann et al. 1977), Mycoplasma pulmonis from mouse nasopharyngeal and conjunctival swabs (Hill 1978), Chlamydia psittaci from the conjunctiva of a cat (Woodland et al. 1978), Salmonella spp. from various animal tissues (McRill et al. 1984) and

leptospires from broth cultures (Tripathy and Hanson 1974). In an interesting report by Buxton (1978), the binding sites of Clostridium welchii type D epsilon toxin were localized by the use of an antiserum to the epsilon prototoxin believed to bind the same receptor site as the toxin. Horseradish peroxidase-labelled sheep antiserum served as the enzymatic reagent. The immunoperoxidase technique has been used successfully in electron microscopy studies for the detection of Streptococcus mutans in dental plaque (Berthold et al. 1974), and for locating immunoglobulins and the secretory component in human intestinal mucosa (Isobe et al. 1977).

In summary, the PAP technique has several advantages over conventional enzymatic and /or conjugated antibody methods. The preparation is permanent and does not have to be observed in a defined period of time. Examination can be made by light microscopy which makes it affordable for most diagnostic laboratories. Finally, it permits the staining of isolated microorganisms in the infected tissues, and allows the study of the cell-antigen relationships which could reveal important aspects of pathogenesis. There are also commercially prepared reagents available which make it a fast and relatively easy procedure to perform.

Serology

A variety of serological assay techniques have been used as diagnostic or screening tools to detect the presence of serum antibodies directed against H. pleuropneumoniae in swine. The complement fixation test is the standard procedure used in most diagnostic and research facilities. An enzyme-linked immunosorbent assay (ELISA) method has been developed by Nicolet et al.

(1981) and modified by Morrison et al. (1984). An indirect hemagglutination (IHA) test and a 2-mercaptoethanol (2-ME) test were also developed for the serodiagnosis of H. pleuropneumoniae pneumonia (Mittal et al. 1983a, 1984). The potential of these tests as reliable diagnostic tools requires further investigation, especially in terms of specificity and cross-reactivity.

Modifications in the preparation of the H. pleuropneumoniae antigen have been attempted in an effort to to alleviate problems which are inherent in serodiagnostic systems. For example, Gunnarsson (1979b) obtained highly serotype-specific immune-precipitation reactions with antigen prepared by phenol-water extractions of whole cells. This treatment was thought to expose superficial serotype-specific antigens as well as common species-specific antigens by eluting polysaccharides and lipopolysaccharides (Gunnarsson 1979a). However, some cross-reactivity and lower titers were detected among different serotypes when the extraction method was used to prepare antigens for the complement fixation test. Mixed antigen consisting of whole cells from all known serotypes was considered the best screening antigen for routine use due to the presence of capsular materials (Gunnarsson 1979b).

A second important factor affecting the CF test is the type of immunoglobulin fixing the complement (Mittal et al. 1984). IgM is the primary immunoglobulin class involved in seroagglutination reactions and, along with IgG, functions as a complement-fixing antibody in the CF test. IgM is also the immunoglobulin most frequently involved in cross-reactions in serology tests (Tizard 1982). Anti-H. pleuropneumoniae IgG was shown to be more serotype specific than IgM, and could be detected in the first three weeks after

infection (Mittal et al. 1984). An agent like 2-mercaptoethanol (2-ME) will break disulfide bonds on the IgM molecule, thus inactivating the antigen binding sites. Any remaining reactivity is thought to be due to IgG molecules present in the serum. These IgG antibodies are considered to be more serotype-specific than the IgM antibodies. The 2-ME test earlier used in brucellosis eradication programs (Alton et al. 1975) was adapted by Mittal et al. (1984) for the detection of H. pleuropneumoniae serotype-specific antibodies with excellent results, although antibody titers decreased more abruptly than with the CF test. The 2-ME agglutination test was determined to be highly sensitive and may be used simultaneously with the CF test in monitoring herds for H. pleuropneumoniae infection.

A third factor related to the performance of the CF test concerns certain technical difficulties which are due to the procomplementary and/or anticomplementary properties of swine sera. These problems can be alleviated by supplementing the test reagents with freshly collected calf serum or specific pathogen free pig serum. These phenomena and some nonspecific reactions due to the complexity of the CF test led Nicolet et al. (1981) to develop an enzymatic immunoassay for the detection of H. pleuropneumoniae antibodies. He accomplished this by developing an ELISA test procedure using an antigen extracted with ethylenediaminetetraacetic acid (EDTA). The ELISA proved to be more sensitive than the CF test, and nonspecific reactions were not observed. This seemed to be due to the mechanism of extraction of the antigen used in the test, which exposed important surface materials thought to be at the site of insertion of the lipopolysaccharide into the outer membrane. In any case, the special

equipment required and the technical skills needed for the extraction of the antigen by this method made the ELISA a sophisticated device not easily applied in most diagnostic facilities.

At the present time, the CF test is the preferred serological method used in the serodiagnosis of H. pleuropneumoniae infection. There are some contradictory reports concerning cross-reactivity observed in the CF test between H. pleuropneumoniae and other closely related organisms (Bachmann 1972, Rapp et al. 1985a, Rosendal and Mittal 1985, Kume et al. 1984, Pijoan 1985). It should also be emphasized that there are some minimal cross-reactions between different serotypes using the CF test (Table 5).

Lombin et al. (1982) determined that the sensitivity of the CF test was increased by the use of a pooled antigen preparation as opposed to a monospecific antigen.

Serum titers detected by the CF test in naturally infected animals are highly variable. Some infected animals never manifest significant titers and other animals return from a positive to a negative status in a short period of time. Studies of the epidemiology of H. pleuropneumoniae infection suggest the conclusion that it is important to identify carrier animals because of their potential for spreading the disease. Animals with negative or low CF titers may actually be carriers of the organism, and there is a need for developing highly specific and sensitive tests in order to detect these animals (Ross 1985).

The hidden presence of a causal agent may often be the principal factor affecting the prevalence of a disease in a swine herd (Goodwin 1985). Clinical signs such as coughing may not be significant and pathological lesions may be

absent after long periods of time even though the herd is still infected. It is necessary that animals such as these be detected if we wish to achieve and maintain H. pleuropneumoniae-free herds. Complement fixation is currently the test used by most practicing veterinarians for surveying herds and also for making decisions regarding introduction of new seed stock into a herd.

MATERIALS AND METHODS

This project was comprised of three separate experiments. In the first experiment pigs were artificially infected with H. pleuropneumoniae so that lungs and sera could be recovered from known positive animals for the development and standardization of the cultural, immunological and serological techniques designated for use in this project. The second experiment involved assaying lungs from pigs referred to the Veterinary Diagnostic Laboratory, Iowa State University, with naturally occurring pneumonia in an effort to compare co-agglutination and peroxidase anti-peroxidase techniques with conventional culture for detection of H. pleuropneumoniae infection. The third part of the project was a field study in which slaughter hogs were surveyed for presence of H. pleuropneumoniae in the lungs, and these results were compared with serum antibody titers.

Experimental Infection of Pigs

Experimental animals

Twenty 6 to 8 week old crossbred gilts and barrows ranging in weight from 35 to 60 pounds were purchased from the Iowa State University swine teaching herd. There was no history of H. pleuropneumoniae infection in the herd. The pigs were weighed, ear tagged and then assigned randomly to four different pens. They were fed ad libitum with a 14 percent protein creep growing-finishing ration without antibiotics.

Preparation of antigen

Strain K17 of H. pleuropneumoniae serotype 5 was used as the challenge

organism. Cultures were maintained in a modified Mycoplasma broth (Gibco Diagnostics, Appendix A) mixed 1:1 with skim milk and stored at -70^{0} C. Broth cultures prepared by inoculating 10 ml volumes of modified Mycoplasma broth with 0.5 ml of the skim milk stock cultures were incubated at 37^{0} C for six hours and were then used for infecting pigs.

Challenge of animals

Upon arrival all pigs were bled from the anterior vena cava and their sera were evaluated for antibodies to H. pleuropneumoniae by the complement fixation test (Slavic and Switzer 1972). All animals were found to be seronegative for the organism. Following a seven day acclimation period, four pigs in each group were exposed intranasally to 0.5 ml (4×10^{8} CFU/ml) of H. pleuropneumoniae serotype 5. Four pigs (one in each pen) were left uninoculated to determine if natural transfer of infection might occur and induce an antibody response.

A similar challenge was administered to all pigs three days after the first exposure. All pigs were bled from the anterior vena cava 10 days post challenge and the serum samples were examined for CF titers. From this time on, pigs were bled every 7 days and serum titers determined. All pigs were challenged a third time eleven days after the second challenge with 1.0 ml (8.7×10^{7} CFU/ml) of H. pleuropneumoniae serotype 5 culture by the same route as before. Those pigs with titers lower than 1:32 14 days after the second exposure were challenged two more times, 11 and 26 days later. Previously uninoculated animals were also exposed at this time. The first of these challenge doses consisted of 2 ml (1×10^{8} CFU/ml) of serotype 5

administered intranasally, and the final challenge consisted of 0.5 ml (2.6×10^8 CFU/ml) of the culture delivered intratracheally with a three inch needle and syringe. All the pigs were sacrificed by electrocution (on the dates shown in Tables 8a and 8b in the RESULTS section). The animals were euthanatized and necropsied according to a schedule based on their CF titer in an attempt to correlate titer levels with the presence or absence of H. pleuropneumoniae and/or pathognomonic lung lesions. Lungs were evaluated at necropsy and the severity of the observed gross lesions was scored on a scale of zero to three as follows:

0 = No lesions suggestive of H. pleuropneumoniae infection.

1 = Few foci of necrosis and pleural adhesions.

2 = Moderate involvement of the lung with variably sized foci of necrotic tissue sequestered by connective tissue and fibrosis of pleural surfaces.

3 = Extensive involvement of the lung including fibrinous pleuritis and large areas of consolidation with fibrinohemorrhagic and/or fibrinonecrotic lesions.

Bacteriological culture of lungs and tonsils

Lung and tonsillar tissue were removed and cultured for H. pleuropneumoniae. Two 5 percent bovine blood agar plates were used, one plate was incubated aerobically and the other anaerobically. Anaerobic conditions were achieved by a Gas-Pack system (BBL Co., Becton-Dickinson, Cockesville, MD). Lung and tonsil sampling was done by searing the tissue with a heated spatula, incising the tissue with a sterile scalpel and swabbing

the exposed area with a sterile cotton swab. Swabs were used to inoculate the blood plates and streaking followed. A "nurse" culture of Staphylococcus epidermidis was streaked across the inoculated plate. Plates were incubated overnight at 37° C and bacteria were identified using conventional morphologic, cultural and biochemical characteristics as defined by Kilian (1974, 1976, 1985) and Biberstein et al. (1977).

Serotyping

Serotyping of all H. pleuropneumoniae isolates was performed by means of the rapid slide agglutination procedure described previously (Rapp 1985b). H. pleuropneumoniae colonies were transferred to Mycoplasma broth media (Gibco) and incubated overnight. Casman agar (Difco) supplemented with 0.0125 gm/l of NADH and five percent sterile horse serum was inoculated from the Mycoplasma broth using a cotton swab. Following overnight incubation on Casman agar the growth was harvested with 0.15 M sodium chloride solution. This suspension of bacteria was used as the antigen in the rapid slide agglutination test to react against type-specific antiserum. One drop of the antigen was mixed with one drop of serotype-specific antiserum. Positive agglutination (clumping of cells) was generally readable within one minute.

Co-agglutination test

Preparation of antigen The reference numbers of the H. pleuropneumoniae cultures used in this study were as follows: serotype 1 strain reference 4074, serotype 5 strain reference K17, and serotype 7 strain

reference WF83. Cultures were maintained in modified Mycoplasma broth base media (Gibco Diagnostics, Madison, WI.) broth in a ratio of 1:1 with skim milk and stored at -70° C until needed for antigen preparation.

Cultures of serotypes 1, 5 and 7 were thawed and four drops were inoculated into four ml of Mycoplasma broth base media (Gibco Diagnostics, Madison, WI) (Frey et al. 1968, 1973) supplemented with NADH (Sigma Chemical Co., St. Louis, MO) and horse serum, as described by Rapp (1985b) and outlined in Appendix A. The broth cultures were incubated overnight at 37° C. These cultures were then used to seed plates of Casman agar supplemented with two percent NADH and five percent horse serum. After seven hours of incubation at 37° C, the growth was harvested with 0.15 M sodium chloride, checked for purity by inoculating blood agar plates with and without nurse colonies, and inactivated with 0.3 percent formaldehyde solution for 72 hr at 4° C. The suspensions were checked for sterility following inactivation.

Antisera production Twelve White New Zealand rabbits were purchased from Laboratory Animal Resources in the College of Veterinary Medicine at Iowa State University. The rabbits weighed approximately three pounds each and were two months of age at the beginning of the project. The rabbits were housed and identified, and blood samples were collected. Serum samples were tested by the complement fixation test in order to establish the absence of detectable serum antibodies for H. pleuropneumoniae.

The rabbits were divided into three groups of four animals each prior to inoculation with the inactivated H. pleuropneumoniae reference strains 1, 5 and 7. The first two series of inoculations were given subcutaneously two

weeks apart. Each series consisted of four one ml dosages at a concentration of 1×10^9 CFU/ml (one ml per limb). One week after the second subcutaneous infections were administered, a series of three intravenous injections of one ml each containing 4×10^9 CFU/ml were given at two-day intervals. Rabbits were bled by heart puncture 10 days after the last injection, and the undiluted serum was checked for specificity by slide agglutination against known cultures. The slide agglutination test was also used to detect cross-reactivity between H. pleuropneumoniae antisera and closely related organisms such as P. haemolytica , P. multocida, A. suis and H. parasuis. The antisera were divided into three ml aliquots and stored in plastic tubes at -18^0 C.

Preparation of protein A-coated antisera Five ml of staphylococcal protein A (Calbiochem Behring Diagnostics, La Jolla, CA) were allowed to react with 0.5 ml of H. pleuropneumoniae antiserum for 30 minutes at room temperature. The suspension was then washed twice with PBS and resuspended to a concentration of 10 percent (vol/vol) in PBS containing one gram of sodium azide and one gram of bovine serum albumin per liter (Sigma Chemical Co.). The suspension was then stored at 4^0 C.

Tissue processing Approximately one cubic cm of affected tissue was taken from lungs which were obtained from necropsied animals. The piece was ground in a mortar and pestle in the presence of sterile sand and three ml of saline. Tonsils were trimmed with scissors due to the hardness of the tissue before it was ground. After grinding, the suspension was boiled in a water bath for ten minutes and centrifuged at 8000 x g for 30 minutes. One drop of the clear supernatant was reacted with one drop each of types 1,

5 and 7-specific protein A-bound antisera on a plastic blood typing slide (Med-Tek Co., MN). Positive reactions consisting of definite clumping of antigen usually occurred within the first minute of mixing.

Controls Negative serum controls were assayed with nonimmune antisera from rabbits. Negative tissue controls were performed with tissues from a known H. pleuropneumoniae-free herd[1] and from field cases received at the Iowa State University Veterinary Diagnostic Laboratory with no pneumonic lesions. Lungs which were positive for H. pleuropneumoniae by culture were used as positive controls. Cross-reactivity of antisera with closely related bacteria was determined using lungs which were culture positive for P. multocida, S. suis, H. parasuis, A. suis, and B. bronchiseptica.

Peroxidase anti-peroxidase test

Lung and tonsillar tissues were fixed in neutral buffered formalin (Fisher Scientific, Itasca, IL) for 24 hours. Dehydration and clearing procedures were conducted according to Scheenan and Hrapchak (1980, p. 59), and paraffin blocks were made. Sections (4 mm) were cut and maintained at 60° C for one hour in a drying oven, and then deparaffinized in Hemo-de (Fisher Scientific, Itasca, IL) for 20 minutes. They were dehydrated by 10 consecutive dips in 100 percent alcohol, followed by the same process in 95 percent alcohol. Slides were then immersed in PBS for five minutes. Incubation procedures were conducted in a 37° C humidity

[1] Animal Resource Station, Iowa State University.

chamber on an electric warming plate (American Scientific Products, Minneapolis, MN) with the exception of the primary antibody, which was incubated for four hours at room temperature. Slides were laid in a water moistened paper towel bed, and two drops of three percent hydrogen peroxide (Immunon, Lipshaw, Detroit, MI) were added to inhibit endogenous tissue peroxidase activity (Bourne 1983). After a five minute incubation period, the slides were washed with PBS. Two drops of protein blocking agent (PBA Universal, Lipshaw, Detroit, MI) were then added. This step was followed by incubation of the slides for five minutes and another PBS wash. Pooled H. pleuropneumoniae rabbit antisera diluted 1:1000 was generously added over the circled piece of tissue and allowed to react for four hours at room temperature. Additional priming antibody was added if drying of the slide occurred. Following another wash with PBS, two drops of donkey anti-rabbit or linking antibody (Lipshaw, Detroit, MI) were added and the slides were incubated at 37° C for five minutes followed by another gentle wash with PBS. Finally, two drops of the PAP complex (Lipshaw, Detroit, MI) were applied to the tissue section, and was incubated for five minutes at 37° C. Following another rinse with PBS, two drops of the chromogen, prepared by adding five ml of Millipore-filtered and deionized water, two drops of sodium acetate concentrate, two drops of three percent hydrogen peroxide and two drops of 3-amino-9-ethyl carbazol (AEC, Lipshaw, Detroit, MI), were applied to the slide. There was a final incubation of 10 minutes after which slides were washed and placed in PBS. A three minute counterstaining step with Mayer's hematoxylin (hematoxylin 1.0 g, distilled water one liter, sodium iodate 0.2 g, ammonium or potassium alum 50 g, citric acid 1.0 g and chloral

hydrate 50 g) followed. After washing in tap water, the slides were immersed in ammonia water (25 drops of concentrated ammonium hydroxide in 500 ml of tap water) and finally washed for a minute in tap water. The preparations were mounted with aqueous mounting medium (Lerner Lab, New Haven, CT) and covered with coverslips (Fisher Scientific, Itasca, IL) (refer to Appendix C for details of procedure).

Controls Controls were performed each time slides were prepared in order to measure variables in the test.

Nonimmune sera was substituted for the primary antibody in order to serve as negative serum controls.

Specimens from known H. pleuropneumoniae-free herds and field cases known to be negative for H. pleuropneumoniae by culture and co-agglutination were used as negative tissue antigen controls.

Known infected tissues were included as positive tissue controls.

Tissues known to be infected with other bacterial agents, including B. bronchiseptica, P. multocida, Salmonella spp. and H. parasuis, were run in order to check for possible cross-reactions.

Interpretation Slides were examined with a light microscope. Positive slides were identified by the reddish-brown AEC stain surrounding areas of tissue damage in which clumps of clearly stained bacteria could be observed. Slides were interpreted to be negative when no stained organisms or cell fragments were observed against the blue background produced by the counterstain.

Complement fixation test

The complement fixation test was conducted according to the Laboratory Branch complement fixation (LBCF) method (U.S. Department of Health, Education, and Welfare, 1965, 1975) with modifications by Slavic and Switzer (1972) and personnel in the serology section of the Veterinary Diagnostic Laboratory, Iowa State University.

Antigen preparation Reference strains of serotypes 1, 5 and 7 (0.3 ml) were inoculated into Mycoplasma broth base (American Scientific Products, Minneapolis, MN) in screw cap tubes and incubated for 24 hours at 37° C. Additional tubes were inoculated (0.3 ml) and incubated overnight (12-18 hr). To each tube, 0.8 ml of NADH solution, prepared with 10 mg of NADH/ml of PBS, was added and mixed. Modified Casman agar was inoculated with 0.5 ml of antigen in 100 mm diameter Petri dishes and incubated for six hours at 37° C. The growth was harvested with 2.5 ml of PBS and centrifuged at 13,000 x g for 20 minutes at 4° C. The supernatant was discarded and one more wash was done with PBS. Antigen was diluted in PBS at a ratio of 2.5 ml per plate and inactivated with merthiolate (thimerosal) at a ratio of 1:2000. Antigen was stored at 4° C in two ml aliquots.

Preparation of sheep red blood cells Blood was collected from sheep and mixed with equal volumes of modified Alsever's solution (Dextrose 20.5 g, sodium citrate 8.0 g, citric acid 0.55 g, sodium chloride 4.2 g, distilled water q.s. to 1000 ml). This mixture was stored for at least one week but no longer than four weeks before using. Sheep red blood cells were washed twice in barbital buffer and diluted in PBS to a four percent cell suspension. A 2.8 percent solution of the cells was prepared using the

cyanmethemoglobin method.

Complement Guinea pig lyophilized complement (Gibco, Grand Island, NY) was restored with normal swine serum from six to eight week old pigs in order to account for the procomplementary effect of swine serum in the complement titration and to restore the complement fixing activity of the heat-inactivated serum samples (Ross 1980). The normal swine serum was added to each vial of lyophilized guinea pig complement in place of the supplied diluent.

Hemolysin and sensitized sheep red blood cells Commercially available (Difco, Detroit, MI) rabbit anti-sheep red blood cell antiserum preserved with 50 percent glycerin was used. Titrations were performed for every new lot of hemolysin, and for each collection of sheep red blood cells. A hemolysin dilution of 1:2000 was used for the duration of this project. An equal volume of the optimally diluted hemolysin was mixed with the 2.8 percent sheep red blood cells and incubated for 10-15 minutes in a 37^{0} C water bath. These sensitized sheep red blood cells were cooled to room temperature before being used in the CF test.

Test procedure Serum samples were inactivated at 56^{0} C for 30 minutes. Controls included positive antiserum, normal porcine serum, serum anticomplementary (AC), and reagent controls (antigen, complement, hemolysin and diluent). A flow sheet describing the CF test procedure may be found in Appendix D. The criteria used in the final evaluation were as follows: positive = 0-30 percent hemolysis, suspect = 35-65 percent hemolysis, negative = 70-100 percent hemolysis. Sera were considered anticomplementary (AC) when the wells containing no H. pleuropneumoniae

antigen (containing serum dilutions of 1:1 and 1:2) were less than 70 percent hemolyzed.

Analysis of Lungs from Pigs with Naturally Occurring Pneumonia

Ninety-six sets of porcine lungs presented to the Veterinary Diagnostic Laboratory, Iowa State University, were analyzed in this portion of the study. The lungs were obtained either at necropsy in the laboratory or were mailed to the laboratory by field veterinarians. All of the animals involved had experienced a recent respiratory disease syndrome, and in each case H. pleuropneumoniae was strongly implicated by history, clinical signs, and gross pathological lesions. Lungs were examined visually for lesions and then scored from 0-3 as described earlier. Each set of lungs was tested by three diagnostic techniques: direct culture, the co-agglutination procedure and the PAP technique. Specimens were assayed by co-agglutination using specific antisera against serotypes 1, 5 and 7, the three most commonly isolated serotypes in Iowa swine. The PAP technique was conducted using pooled antisera against serotypes 1, 5 and 7.

Survey of Lungs and Serum Samples Collected at Packing Plants

Four hundred twenty-three market weight pigs from 15 different Iowa herds were checked for the presence of H. pleuropneumoniae in lung tissue by culture, co-agglutination and PAP and the presence of serum antibodies to the organism by the CF test. A concerted effort was made to select swine herds with varying histories of H. pleuropneumoniae disease and immune status.

The herds were assigned to one of three groups. Group A consisted of a total of 55 animals from two nonvaccinated herds with no history of H. pleuropneumoniae infection. Group B consisted of 118 animals from six vaccinated herds with no outbreaks of the disease in the recent past. Group C consisted of 250 animals from seven nonvaccinated herds that had experienced recent outbreaks of respiratory disease. A large percentage of this sample group included pigs which had been treated and had subsequently recovered from respiratory disease. The number of pigs examined from each herd was variable depending on the number of animals submitted to the plant on a given date. Samples were collected at the Oscar Mayer plant in Perry, Iowa, and at the Farmland plant in Iowa Falls, Iowa. Basically, the operational configuration of the two plants was quite similar (Figure 7), the main difference being the number of animals processed per hour. Approximately 920 animals per hour were processed at the Oscar Mayer plant, while approximately 700 per hour were processed at the Farmland plant. Pigs were identified by ear tags and skin tattoos while in the holding area in order to correlate the blood with the carcasses as they progressed through the kill floor. Blood samples were collected in Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) immediately following heart sticking of the hog. They were kept at room temperature for three hours and then refrigerated overnight. The following morning the sera were harvested by centrifugation and inactivated at 56° C for 30 minutes.

Lungs were obtained after identification of the carcasses, which moved parallel to the viscera trays. The lungs were identified by cards, bagged and brought to the laboratory, where gross lesions were scored on a scale of 0-3

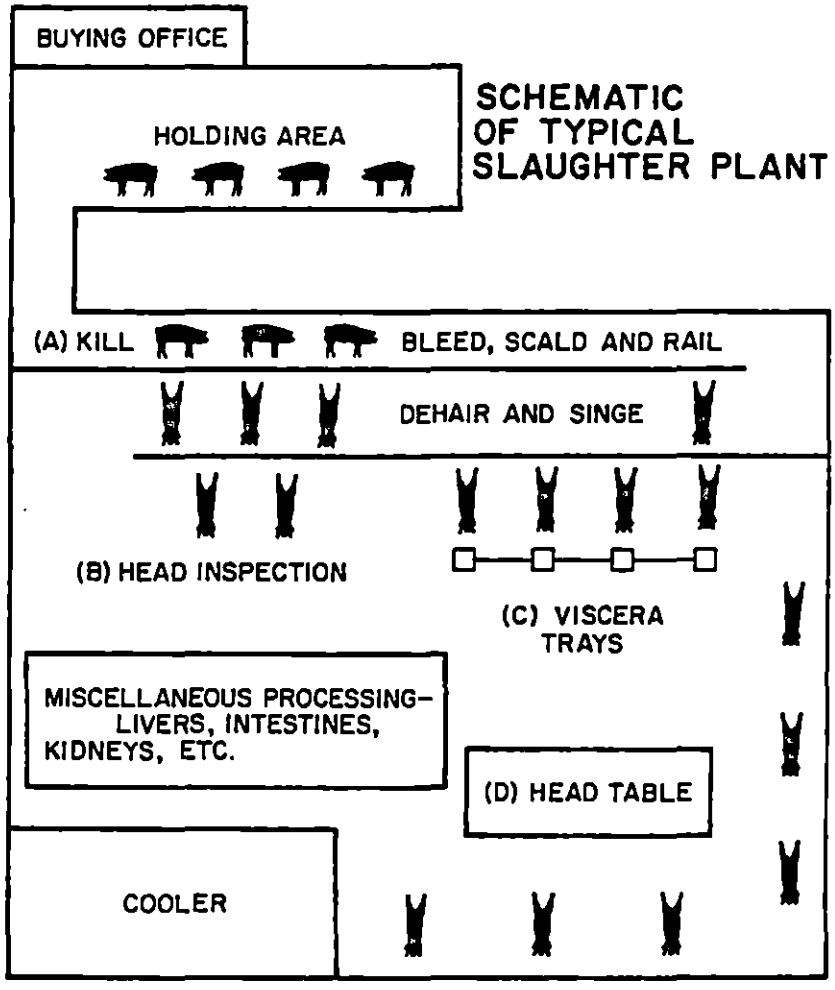


Figure 7. Schematic of a typical packing plant (Straw et al. 1986), and sites in the packing plants where sample collection procedures were performed

Holding area	pig identification by ear tag and tattoo
Site A	blood collection (identification by ear tag)
Site C	lung tissue collection (identification by carcass tattoo)

as previously discussed. All lungs were subjected to standard culture techniques and co-agglutination. All samples from Group C were tested by PAP. As a result of the finding that tissues from specific herds in Groups A and B were uniformly negative by standard culture and co-agglutination and had no observable lung lesions, only 20 percent of the animals from those herds were randomly selected for assay by PAP.

Statistical Methods

The chi-square test was used to determine whether the number of positives detected by co-agglutination, PAP, standard culture and complement fixation were significantly different from each other. Comparisons with CF results were made using two different positive threshold titer levels (1:4 and 1:8). The criteria used to determine the statistical significance of the differences are listed in Table 7.

Table 7. Criteria for statistical significance of differences in number of positives detected by co-agglutination, PAP, standard culture and complement fixation

P-value	(Probability that the observed chi-square statistic is greater than the chi-square value for significance levels listed and one degree of freedom)[a]	Degree of significance
	$P > 0.1$	Not significant
$0.10 > P > 0.05$		Slightly significant
$0.05 > P > 0.01$		Significant
$0.01 > P$		Highly significant

[a]Zone of the tabulated chi-square distribution for 1 degree of freedom (Snedecor and Cochran 1980).

RESULTS

Experimental Challenge Study

Eight of the sixteen pigs intranasally exposed to H. pleuropneumoniae serotype 5 on three different occasions during a two-week period developed detectable serum antibodies to the pathogen within 14 days after the third exposure. These pigs were not further challenged. Complement fixation titers for this group of pigs are shown in Table 8a. One pig died approximately 12 hr after the first challenge due to acute H. pleuropneumoniae infection (this animal was erroneously exposed to a higher volume of H. pleuropneumoniae culture than the others).

A fourth intranasal challenge was administered to the seven pigs which remained seronegative 14 days after the third exposure. The four unchallenged penmates which remained seronegative during the first challenge paradigm were also intranasally exposed at that time. Nine of these animals received a later intratracheal installation of H. pleuropneumoniae in an attempt to enhance the level of serum antibodies. Interestingly, four of the nine intratracheally exposed pigs died within 24 hours postchallenge, and three of these four pigs that died acutely were seronegative at the time of intratracheal delivery. Serology results for all pigs which were challenged differently from those in the initial paradigm can be found in Table 8b.

Differences in clinical signs were observed in all seroreactors. Several pigs were lethargic and anorexic with definite signs of respiratory distress while others manifested no overt signs of disease. Most animals had varying

Table 8a. Progression of complement fixation titers in experimentally challenged pigs: Group A – Pigs seropositive 14 days after the third intranasal challenge dose

Pig#	Dates bled												Date of death[a]
	6/29	7/15		7/22	7/30	8/5	8/12	8/19	8/26	9/2	9/9	9/16	9/23	
68	Neg													7/3[b]
65	Neg	**[c]	≥128	*	≥128	16	32							8/6
57	Neg	**	Neg	*	Neg	16	16	16						8/6
64	Neg	**	64	*	≥128	64	16	64	32	32				8/28
69	Neg	**	4	*	Neg	32	64	≥128	64	32	Neg	Neg		9/14
59	Neg	**	64	*	≥128	≥128	≥128	≥128	≥128	64	64	64	≥128	9/18
54	Neg	**	8	*	16	16	16	32	16	32	Neg	Neg	Neg	9/23
66	Neg	**	≥128	*	≥128	64	8	≥128	≥128	≥128	≥128	64	32	≥128	9/25
62	Neg	**	≥128	*	≥128	32	16	64	≥128	64	64	64	32	≥128	9/25

[a]Pigs were euthanatized on the date shown (except for pig #68).

[b]Pig #68 died after a single intranasal inoculation.

[c]Pigs were inoculated intranasally three times: 7/2, 7/5 and 7/16 (4×10^9 PFU per dose).

Table 8b. Progression of complement fixation titers in experimentally challenged pigs: Group B – Pigs seronegative 14 days after the third intranasal challenge

Pig	Dates bled													Date of death
	6/29	7/15	7/22	7/30	8/5	8/12	8/19	8/26	9/2	9/9	9/16	9/23		
60[a]	Neg	Neg	Neg	Neg *[b]	Neg	Neg	#[c]							8/16[d]
67[a]	Neg	Neg	16	Neg *	Neg	Neg	#							8/16[d]
52[a]	Neg	4	Neg	Neg *	Neg	Neg	#	Neg	≥128	≥128	≥128	64		9/18[e]
70[a]	Neg	Neg	4	Neg *	Neg	64	#	64	32	32	32	16	64	9/25[e]
53	Neg **	Neg *	Neg	Neg *	Neg	Neg	#							8/16[d]
58	Neg **	Neg *	Neg	Neg *	Neg	32	#							8/16[d]
51	Neg **	Neg *	Neg	Neg *	Neg	Neg	#	Neg	Neg	Neg	Neg			9/12[e]
71	Neg **	Neg *	Neg	Neg *	16	16	#	8	≥128	≥128	≥128	32		9/25[e]
55	Neg **	Neg *	Neg	Neg *	Neg	64	#	32	64	64	64	64	≥128	9/25[e]
61	Neg **	Neg *	4	Neg *	32	Neg		Neg	16	Neg	Neg	Neg		9/23[e]
56	Neg **	Neg *	Neg	Neg *	32	64		64	64	32	Neg	32	32	9/25[e]

[a] Contact control pigs–not inoculated prior to 7/31.

[b] Pigs received one (7/31) or four (7/2, 7/5, 7/16 and 7/31) intranasal inoculations.

[c] Intratracheal inoculation (given on 8/15).

[d] Died acutely following intratracheal inoculation.

[e] Euthanatized.

degrees of temperature elevation during the course of the experiment. One of the major objectives of this preliminary study was achieved when 14 of the 20 animals infected developed demonstrable CF titers without becoming acutely ill.

Gross pathological lesions as well as culture, co-agglutination, PAP and CF results for all laboratory challenged animals are presented in Tables 9a and 9b. Lung lesions typical of various stages of H. pleuropneumoniae infection were present in all but one pig involved in this experiment.

Haemophilus pleuropneumoniae was detected by culture, co-agglutination and PAP in three of the five animals which died acutely during this pilot study. Lung lesions in these animals were definitely compatible with acute pleuropneumonic infection. The fourth animal had typical lesions and positive culture results but co-agglutination and PAP tests were not conducted because the lung tissue was inadvertently discarded. The fifth pig to die acutely had normal appearing lungs and negative culture and co-agglutination tests, but had a positive PAP test and a CF titer of 1:32. This was the only animal of those which died from infection to display a positive titer for H. pleuropneumoniae.

Fourteen of the 15 animals which were euthanatized developed complement fixing antibodies at some point during the experimentation. Eleven of these animals were positive in a range between 1:16 to $\geq$ 1:128 at the time of necropsy. In six of these seropositive animals, H. pleuropneumoniae was detected by culture and at least one of the immunological techniques. Of the remaining five animals all were negative for H. pleuropneumoniae on culture but three were positive by PAP and one was positive by co-

agglutination and PAP. The last animal was negative on all three tests.

Three pigs were euthanatized and necropsied after CF titers had dissipated; two of the three were positive only by the PAP test and the other was negative by all three tests. The single animal which was seronegative throughout the testing period had no gross lesions of H. pleuropneumoniae infection and was negative on all three tests.

The co-agglutination test detected type-specific H. pleuropneumoniae antigen. This was confirmed by serotyping (using standard slide agglutination procedures) isolates of H. pleuropneumoniae which had been recovered from test pigs using conventional culture techniques. No cross-reactions were observed and no agglutination was detected in the negative controls.

The PAP technique appeared to be more sensitive than the co-agglutination test in this group of animals; of the total of 19 pigs tested, 15 were positive by PAP while only nine were positive by co-agglutination (Table 10). Clumps of bacteria outlined by the reddish-brown AEC stain (Figures 8 and 9) were seen within the alveoli or associated with small airways. No specific pattern of bacterial localization was observed. These clumps were usually associated with histological lesions easily visualized in parallel sections of lung tissue stained with hematoxylin and eosin. In order to substantiate specificity of this system, smears made from closely related organisms such as A. suis, Haemophilus "minor taxon", H. parasuis and urease negative Haemophilus spp. were assayed by the PAP test. There appeared to be no cross-reactivity problems with the pooled H. pleuropneumoniae antisera. Positive and negative controls were used to validate PAP results throughout the study.

Table 9a. Standard culture, co-agglutination, peroxidase anti-peroxidase and complement fixation results from pigs challenged with H. pleuropneumoniae: Group A - Pigs seropositive 14 days after the third intranasal inoculation

Pig#	Lung lesions (gross)	Culture[a] (lungs)	Culture (tonsil)	Co-ag	PAP	CF titer (at death)
68[b]	acute diffuse lesions	high HPP	NT[c]	pos	pos	NT
65	diffuse adhesions	mod HPP	low beta Strep	pos	pos	32
57	small hemorrhag. lesions & adhesion	mod HPP	few contam.	neg	pos	16
64	acute lesions diffuse	mod HPP	NT	pos	pos	32
69	fibrinous pleuropneumonia	sterile	low Kleb sp high beta Strep	neg	pos	neg
59	severely affected	high HPP	low E coli, low	pos	neg	≥128
54	resolved, few adhes., abscess	high P mult	high beta Strep low Kleb sp	neg	pos	neg
66	few hemorrhagic foci mod alpha Strep	high HPP & low Staph	high alpha Strep	pos	pos	≥128
62	resolved, few adhesions	no growth	high Micrococcus	neg	pos	≥128

[a] Refer to Appendix E for abbreviations of names of bacteria.

[c] Not tested.

[b] Pig #68 died after one intranasal (IN) inoculation. All other pigs received 3 IN inoculations.

Table 9b. Standard culture, co-agglutination, peroxidase anti-peroxidase and complement fixation results from pigs challenged with H. pleuropneumoniae: Group B – Pigs seronegative 14 days after the third intranasal inoculation

Pig#	Lung lesions (gross)	Culture[a] (lungs)	Culture (tonsil)	Co-ag	PAP	CF titer (at death)
60[b]	acute diffuse	high HPP	few alpha Strep	pos	pos	neg
67[b]	acute diffuse	high HPP	high beta Strep	pos	pos	neg
52[b]	scattered lesions few adhesions	high P mult	low Kleb, mod. beta Strep	neg	pos	64
70[b]	resolved, few adhesions, abscess	high HPP high alpha Strep	high Staph sp high E coli	pos	pos	64
53[c]	diffuse areas consolidation	high HPP	low beta Strep	NT	NT	NT
58[c]	normal	no growth	low contaminant	neg	pos	32
51[c]	resolved, chronic adhesions	sterile	mod. Kleb, mod. E coli, high beta Strep	neg	neg	neg
71[c]	resolved, few adhes., abscess	no growth	high B strep, low Kleb sp	neg	pos	≥128
55[c]	adhes., congestion accessory lobe	no growth	high beta Strep	pos	pos	64
61[d]	resolved, chronic adhesions	low alpha Strep	high beta Strep	neg	neg	neg
56[d]	resolved, chronic lesions	no growth	mod hem E coli	neg	neg	32

[a] Refer to Appendix E for list of bacterial abbreviations.

[b] Contact control pigs–received one intranasal and one intratracheal inoculation.

[c] Pigs received four intranasal and one intratracheal inoculations.

[d] Pigs received four intranasal inoculations.

Table 10. Experiment 1. Comparison of standard culture, co-agglutination and peroxidase anti-peroxidase techniques for the detection of H. pleuropneumoniae in experimentally challenged pigs

	POSITIVE			NEGATIVE			
	A	B	TOTAL	A	B	TOTAL	
Standard Culture	5	5	10 (50%)	3	7	10	20
Co-agglutination	4	5	9 (47%)	4	6	10	19[a]
PAP	7	8	15 (79%)	1	3	4	19[a]

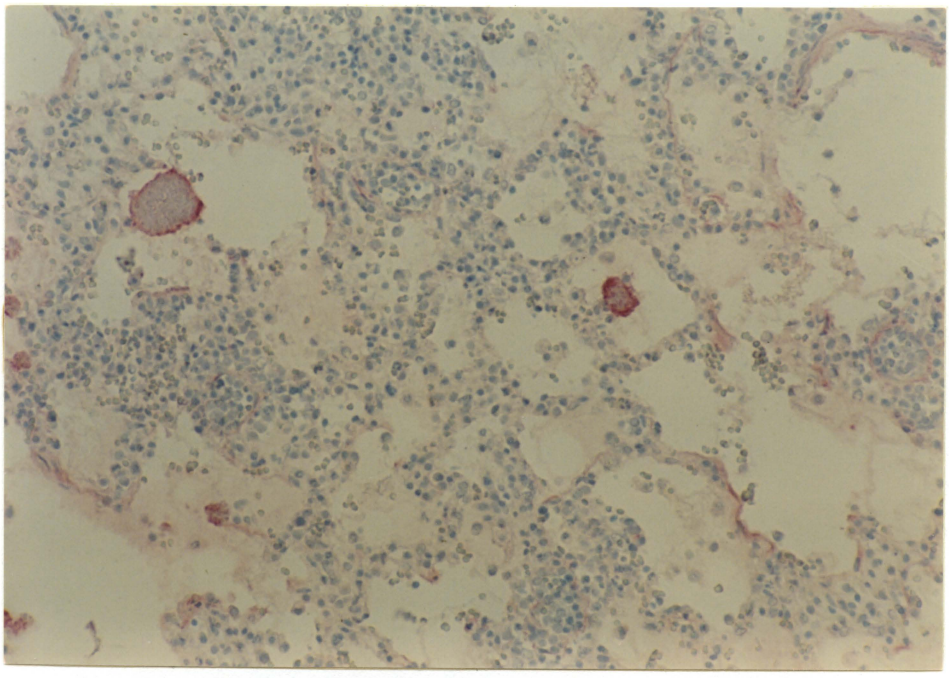
GROUP A - Seropositive after three intranasal inoculations

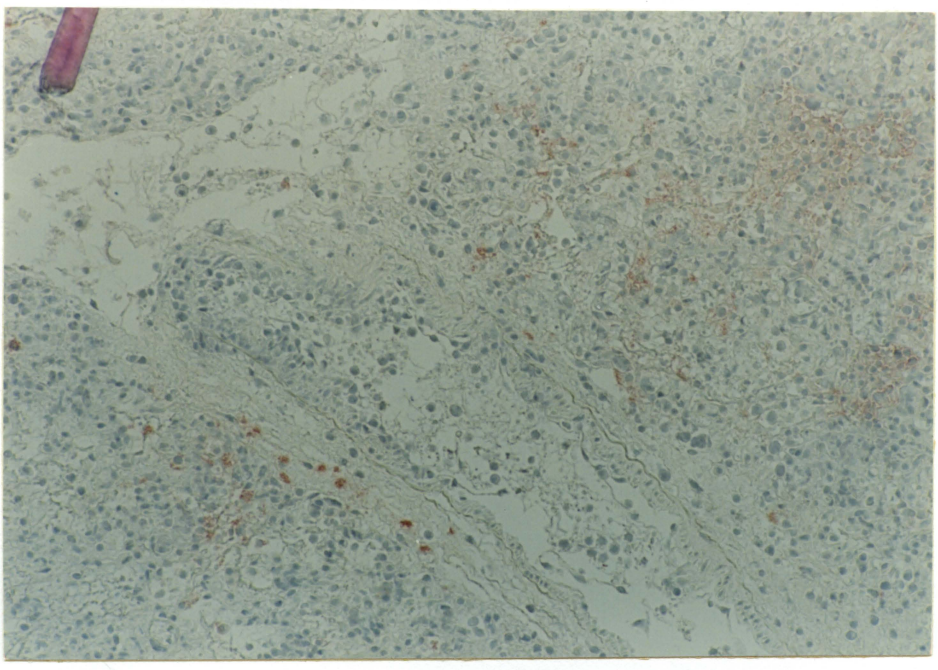
GROUP B - Seronegative after three intranasal inoculations

[a]Procedure was not performed on all pigs in the challenge study.

Figure 8. Haemophilus pleuropneumoniae staining by means of peroxidase anti-peroxidase technique. Granules are deposits of reddish-brown AEC-stained material (arrows). x 325.

Figure 9. Haemophilus pleuropneumoniae staining by means of peroxidase anti-peroxidase technique. Reddish-brown clumps of granular material stained with AEC (arrows). Alveoli are filled with filled with fibrin and cellular debris. x 325.





Haemophilus pleuropneumoniae was not demonstrable in tonsillar tissue removed from experimentally challenged pigs by standard culture, co-agglutination or peroxidase anti-peroxidase techniques. Grinding both tonsils resulted in negative co-agglutination results, while the same test was usually positive within 15 seconds when lung tissue from the same animal was examined. All tonsils assayed for the presence of H. pleuropneumoniae by the PAP test were negative. No histological tonsillar lesions were observed. Tonsil evaluations proposed for the other portions of this study were excluded on the basis of these negative results.

Naturally Occurring Pneumonia Survey

Lesion descriptions, standard culture results, and the results from the co-agglutination and PAP procedures are presented in Appendix E for each of the 96 animals included in the naturally occurring pneumonia survey. Haemophilus pleuropneumoniae was recovered from 44 out of the 96 lungs using standard culture techniques (Table 11). These same lungs were consistently positive by one or both of the immunological techniques. Of the 52 lungs negative by culture for H. pleuropneumoniae, 13 lungs were found to be positive for the organism by PAP, while co-agglutination detected nine positives. This represents a 13 percent and a nine percent increase for the PAP and the co-agglutination tests respectively over the conventional culture method in confirming the presence of H. pleuropneumoniae in infected lung tissue. Statistically, the number of positives detected by the co-agglutination and PAP tests were each significantly different ($P < .01$) than the number detected by standard culture. The co-agglutination and PAP results were not

Table 11. Experiment 2. Comparison of standard culture, co-agglutination and peroxidase anti-peroxidase techniques in the diagnosis of H. pleuropneumoniae infection from 96 cases obtained at the Veterinary Diagnostic Laboratory, Iowa State University

	POSITIVE	NEGATIVE	TOTAL
Standard Culture	44 (46%)	52 (54%)	96
Co-agglutination	53 (55%)	43 (45%)	96
PAP	57 (59%)	39 (41%)	96

significantly different from each other.

Serology results were unobtainable from the pigs evaluated in this portion of the study because the majority had been necropsied by a veterinarian, who then forwarded the tissues to the diagnostic laboratory. The remaining animals were delivered to the laboratory by the owners; however, most of these pigs had succumbed to infection prior to delivery.

Packing Plant Survey

The results of standard culture, co-agglutination, PAP and complement fixation tests used to assay 425 lungs and serum samples collected at two different slaughter plants are shown in Table 12. The individualized data including gross pathological lesions from 15 herds are presented in Appendix F. The majority of these lungs had no significant lesions. When present, lesions were commonly found in the dorsal portions of the caudal lobes as poorly defined granular areas with fibrinous pleuritis over the area of lesion. In two nonvaccinated herds with no history of the disease, the difference in standard culture results and co-agglutination and/or PAP were highly significant ($P < .001$). Out of 57 animals, there were none positive by conventional culture, while seven were positive by the co-agglutination test and 12 by PAP. There were 15 animals with H. pleuropneumoniae CF antibody titers in the range 1:8 - 1:32.

Animals from six herds vaccinated for H. pleuropneumoniae were evaluated at slaughter. There had been no recent significant respiratory infections in any of these herds. One out of 118 animals was positive by standard culture as compared to nine by co-agglutination and 15 by PAP

(Table 12). The difference in positive results between culture and co-agglutination or PAP was highly significant ($P < .001$). Twenty eight animals had CF titers to H. pleuropneumoniae (1:4 to $\geq$ 1:128).

Samples were collected from seven herds which had experienced varying degrees of respiratory problems in the recent past. On the basis of previous slaughter checks or positive serology it was concluded that each herd had experienced H. pleuropneumoniae infection, but a confirmed laboratory diagnosis had never been made. A total of 250 animals from these seven herds were tested. Thirty nine lungs were positive for H. pleuropneumoniae by culture, 182 positive by co-agglutination and 129 positive by PAP. The number of positives detected by each of the two immunologic techniques was significantly different ($P < .001$) from those detected by culture. There was no significant difference between co-agglutination and PAP test results.

The PAP and co-agglutination methods detected a greater number of H. pleuropneumoniae-positive animals than the complement fixation test when CF titers of 1:8 and higher were considered positive, but not when CF titers of 1:4 and higher were considered positive. The standard culture method detected significantly fewer positives than the complement fixation test ($P < .001$) regardless of whether CF titers of 1:4 or 1:8 were used as the positive threshold.

Table 12. Experiment 3. Comparison of results obtained by standard culture, co-agglutination, PAP and complement fixation in the diagnosis of H. pleuropneumoniae infection from packing plant survey of animals

	POSITIVES				NEGATIVES				TOTAL
	A	B	C	TOT	A	B	C	TOT	
Standard culture	0	1	39	40	57	117	211	385	425
Co-agglutination	7	9	182	198	50	109	68	227	425
PAP	12	15	129	156	45	103	121	269	425
CF[a]	15	28	172	215	42	90	78	210	425
CF[b]	15	26	113	154	42	92	137	271	425

GROUP A - Herds with no history of H. pleuropneumoniae pneumonia (non-vaccinated).

GROUP B - Vaccinated herds with no outbreaks in the recent past.

GROUP C - Non-vaccinated herds with recent outbreaks of the disease.

[a] 1:4 titer considered positive.

[b] 1:8 titer considered positive.

DISCUSSION

Techniques based on immunologic principles for the efficient detection or identification of bacterial agents have become popular in recent years. In this study two such techniques, co-agglutination and peroxidase anti-peroxidase (PAP), were compared to the standard culture method for detection of Haemophilus pleuropneumoniae. Results from preliminary pilot studies developed to standardize the techniques indicated that the co-agglutination and PAP tests were more sensitive than conventional culture. Several individual animals with low or negative complement fixation (CF) antibody titers were found to be positive for H. pleuropneumoniae by co-agglutination or PAP tests. These pigs were assumed to be potential carriers of the organism.

Analysis of test results from 96 cases of naturally occurring pneumonia in swine provided additional evidence that both the co-agglutination and the PAP techniques were highly sensitive and sufficiently specific for use as alternative or supplemental tests to standard culture procedures for the detection of H. pleuropneumoniae infection. This information was corroborated by data obtained from the packing plant study in which lungs of pigs from 15 different herds were evaluated by each of the three techniques. It was shown that animals which had experienced a previous episode of respiratory disease due to H. pleuropneumoniae could be identified much more readily with either the co-agglutination or PAP test than with conventional culture methods. Whether or not a positive coagglutination or PAP test correlates closely with the presence of viable organisms is unknown

at this time since both tests will detect viable and non-viable bacteria as well as cell fragments.

Co-agglutination test results were highly specific, accurate and rapid. Despite the inconvenience of grinding tissue, the test was straightforward and provided the advantage of combining detection of H. pleuropneumoniae with serotyping in a single procedure. Another advantage of co-agglutination over the standard culture procedure was detection of antigen in highly decomposed and contaminated tissue. Sampling larger pieces of tissue may also result in an increased probability of organism detection. Use of co-agglutination precludes antibiotic sensitivity testing because there is no recovery of colonies on laboratory media. This constitutes a disadvantage for daily diagnostic work in which the veterinary practitioner is expected to initiate treatment promptly with the appropriate antimicrobial. On the other hand, veterinarians often initiate treatment with drugs which are well established in their efficacy against pleuropneumonia prior to obtaining a confirmed diagnosis from the diagnostic laboratory. Laboratory results are often necessary only to confirm the suspected infection. The co-agglutination technique may serve a useful diagnostic function in cases in which a rapid determination of the serotype present in a herd is required.

The PAP technique, although highly sensitive and specific for the detection of antigen in formalin-fixed tissues, was found to be much too time consuming for a daily diagnostic routine. Moreover, the reading of the PAP slides was definitely subjective. Previous experience in the interpretation of results from chronically infected lungs was found to be beneficial when observing the slides. Variables such as primary antibody dilutions and the

presence of endogenous peroxidase in phagocytic and red blood cells could confuse the diagnostician. Some knowledge about the pathogenesis of the disease is also required in order to search for the bacteria in tissue sites where it is most likely to be found. The technique was expected to be extremely sensitive based on previous comparisons with other immunologic procedures (Sternberger et al. 1970). However, in this study the co-agglutination test proved as sensitive as the PAP procedure, perhaps due to the methodology of lung sampling. A single three to five mm thick slice of tissue was cut from the suspected infected area for the PAP test, while for the co-agglutination test a total sample size of approximately one cubic cm of tissue was taken from three or four different sites.

Results of samples collected at packing plants indicated that co-agglutination and PAP techniques were effective in detecting H. pleuro-pneumoniae in lungs of animals which displayed no overt signs of respiratory disease at slaughter. One part of the study involved 250 pigs from seven non-vaccinated herds. Each herd had experienced at least one episode of respiratory disease during the finishing period. Although previous slaughter surveys and serologic testing had suggested the presence of H. pleuro-pneumoniae, no definitive diagnosis had been made at a diagnostic facility. There were 198 out of 250 pigs which tested positive for H. pleuropneumoniae by co-agglutination, PAP or both, but only 39 of the animals were positive by direct culture of lungs. All of those positive by culture were also positive by one or both of the immunological procedures. These data clearly support a confirmation of H. pleuropneumoniae infection in each of the herds tested.

Lungs were also obtained at slaughter from 118 pigs originating from six

different farms where vaccination programs for H. pleuropneumoniae had been introduced within the past few years following known outbreaks of the disease. Haemophilus pleuropneumoniae was recovered by culture from only one animal while 18 animals were positive by co-agglutination, PAP or both tests. Even though positive animals were detected by these sensitive methods, the low number suggests that immunoprophylaxis probably reduced the rate and severity of infection in these herds.

Specimens from pigs in two unvaccinated herds with no history of H. pleuropneumoniae infection were also collected. There were 14 of 58 animals in this group which had lungs positive for H. pleuropneumoniae by co-agglutination and/or PAP. The organism was not recovered from any of the lungs by conventional methods. These results support the premise that H. pleuropneumoniae invades many Iowa herds without causing overt clinical disease (Schultz et al. 1982).

The complement fixation test is the serological procedure most commonly employed in diagnostic laboratories to monitor animal exposure to H. pleuropneumoniae. The results obtained with this test, especially when interpreted on a herd basis, accurately reflect the presence or absence of H. pleuropneumoniae infection. Moreover, the complement fixation test is particularly useful for detecting chronically infected herds where clinical signs are subtle and intermittent. Diagnosticians are frequently asked to interpret the significance of low serum antibody titers to H. pleuropneumoniae in swine, and to predict the potential of these animals as carriers of the organism. There has been little research conducted to determine the relationship between seropositive test results and the actual presence of the

organism (or antigen) in the lung of the host.

The CF titers of pigs challenged several times with H. pleuropneumoniae in the pilot study were highly variable. This inconsistency in immunological response to H. pleuropneumoniae has been previously noted (Nicolet et al. 1971, Schultz et al. 1982). It is significant to note that 12 challenged pigs which were seropositive (at a serum dilution of 1:8) had lungs which were positive for H. pleuropneumoniae by culture of lungs or by co-agglutination or PAP. Of the four animals which had no CF titers at necropsy, two were positive by PAP and two were negative by all tests. The PAP detected two animals which failed to develop measurable antibody titers. Tonsils from experimentally infected pigs were consistently negative for H. pleuropneumoniae by culture, co-agglutination and PAP, which is contradictory to data published by Bachmann (1972) suggesting that CF positive titers correlated with H. pleuropneumoniae infection in tonsillar tissue.

The data obtained from lungs and sera of slaughtered animals suggest a direct relationship between positive CF titers and presence of H. pleuropneumoniae in the lung. Two hundred fifteen of 425 animals evaluated had positive CF titers at slaughter. All but 17 of these animals were positive by culture, co-agglutination or PAP. The co-agglutination and PAP tests were much more sensitive than culture, detecting 198 and 156 positives respectively as compared to 40 by culture. Some of the animals with the highest CF titers were positive by culture, suggesting that there may have been an active infection in these animals at the time of slaughter. Most of the animals with titers of 1:4 or 1:8 were negative by culture, suggesting that more sensitive methods may be necessary to detect antigen or bacteria in

animals which are chronically infected or recovered. Most of the pigs from vaccinated herds were seronegative at slaughter, but there were a few with residual vaccinal titers. Some of the seropositive animals in this group were presumably infected because antigen was detected by either co-agglutination or PAP tests. In nonvaccinated herds there was a high percentage of animals with CF titers for H. pleuropneumoniae. Most of these animals were in the chronic stage of disease or had completely recovered because lesions were minimal and titers were low (1:4-1:16). Detectability of organisms by co-agglutination and PAP corresponded well with the presence of antibody titers.

A significant aspect of this research is the knowledge which has been gained relative to interpretation of CF titers. Nicolet et al. (1971) considered CF titers of 1:10 or higher to be indicative of previous exposure in nonvaccinated herds. Schultz et al. (1982) reported that a titer of 1:20 using Nicolet's methodology corresponded to a titer of 1:4 in the CF microtiter method currently in use at the Veterinary Diagnostic Laboratory, Iowa State University. Nielsen (1982) stated that animals with CF titers as low as 1:4 are potential carriers of the organism. Titers of 1:4 are considered suspect and titers of 1:8 or greater are interpreted as positive at the Iowa State University Veterinary Diagnostic Laboratory. In the present study, H. pleuropneumoniae in some form was shown to be present in lung tissue from market weight pigs with titers as low as 1:4. The selection of these animals for introduction into breeding herds based on the fact that the CF titer was not in the range normally considered to be positive might constitute a real hazard. These low-titered animals may be inapparent carriers that could become active shedders of H. pleuropneumoniae (Nielsen 1982).

If seronegative, a herd can generally be considered free from infection. However, the CF test should be interpreted on a herd basis since there are important differences in the immunological response of animals in the same herd with respect to the production and persistence of complement-fixing antibodies, as was demonstrated in the preliminary challenge experiment in the current study.

In conclusion, this study suggests that CF titers of 1:4 should be considered positive since immunological detection of antigen was consistent with the presence of CF titers at this level. Animals with low titers should be considered potential carriers.

SUMMARY

This research project compared two immunological techniques, co-agglutination and peroxidase anti-peroxidase (PAP), with the standard culture method in the diagnosis of H. pleuropneumoniae infection in swine. In addition, CF titers were compared with results of culture, co-agglutination and PAP tests for 423 market weight pigs. This study was designed to determine the relationship between positive serological results and the presence of H. pleuropneumoniae in lung tissue.

An assay for the antibody responses of swine intranasally infected with H. pleuropneumoniae was performed; complement fixing antibodies (measured weekly) were compared with the the presence of H. pleuropneumoniae in lung tissue as determined by standard culture, co-agglutination and PAP procedures. The complement fixation test detected fewer animals carrying capsular antigen than either the co-agglutination procedure or the peroxidase anti-peroxidase technique.

The co-agglutination and PAP procedures were also shown to be more sensitive than standard blood agar culture for the detection of H. pleuropneumoniae in naturally infected lung tissues from pigs submitted to the Iowa State University Veterinary Diagnostic Laboratory.

Information obtained from investigations of cases of naturally occurring swine pneumonia confirm that the co-agglutination test is a reliable screening test in laboratory diagnosis when a rapid identification and serotyping of suspected H. pleuropneumoniae-infected tissues is required in order to initiate or alter vaccination programs. The PAP test, although highly

sensitive, was considered to have more potential for use on a research basis than in a daily diagnostic capacity.

Four hundred twenty five market weight pigs were studied at slaughter from three pre-selected herd categories in order to obtain information on the occurrence of H. pleuropneumoniae infection in swine herds in Iowa. Animals from vaccinated herds were shown to have fewer or no lesions at slaughter compared with nonvaccinated herds. Complement fixing antibodies were absent in the majority of animals, and H. pleuropneumoniae was detected in a low number of animals. Animals from nonvaccinated herds believed to be free of the organism were shown to carry the bacterium in their lungs. The results correlated with antibody titers of 1:4 or greater on the complement fixation test. Infected, nonvaccinated herds were detected by complement fixation, co-agglutination and peroxidase anti-peroxidase tests but only rarely by standard culture techniques in these market weight pigs.

No important differences were found between complement fixation, co-agglutination and peroxidase anti-peroxidase procedures when a 1:4 CF titer was considered positive. Both the co-agglutination and the PAP tests detected a greater number of positive animals than the complement fixation test when a 1:8 CF titer was considered positive.

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ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Lorraine J. Hoffman for her sincerely enthusiastic support and encouragement during my training and study at the Iowa State University Veterinary Diagnostic Laboratory.

I wish to extend a special thank you to Dr. Vaughn A. Seaton, head of the Veterinary Diagnostic Laboratory, whose acute concern for me and his unselfish interest during some discouraging moments have given me the impetus to persevere.

Sincere thanks are due to the bacteriology personnel at the lab, Louise Henderson and Jay Morgan, as well as Carl Bessman in the serology section, for their help during this project.

The cooperation of Dave Cavanaugh and Dick Lindahl, members of the histology section, is also appreciated.

I am deeply indebted to the head buyers, Dick Palan (Oscar Mayer Co.) and Roger Neering (Farmland Co.), for their invaluable assistance in the collection of samples at their respective packing plants.

Special acknowledgments are extended to Dr. Jorge Garcia Pravia, head of the Microbiology Department at the City Hall Laboratories in Montevideo, Uruguay, for keeping my position and salary during this research endeavor.

Deepest appreciation is extended to Dr. David R. Kinker, my office partner, my confidant, my friend, for his unparalleled unselfish assistance. Without him, the preparation of this manuscript would not have been possible.

Finally, I am profoundly grateful to my wife Lydia who never failed to provide me with the true gifts of life, those of mind, spirit and heart, and to our children Natalia and Nicolas for their understanding during my course of study and work.

This project was supported by USDA Formula Funds project number 410-23-96-00-2319.

APPENDIX A: MYCOPLASMA BROTH BASE (FREY)

Mycoplasma broth base (Gibco Diagnostics, Madison, WI.; Frey et al. 1968) may be used for the cultivation of H. pleuropneumoniae with the addition of NAD and serum.

FORMULA:

	grams/liter
Peptone 140 (Pancreatic digest of casein)	7.5
Peptone 110 (Papaic digest of soy protein)	2.5
Yeast extract	5.0
Sodium Chloride	5.0
Potassium Chloride	0.4
Magnesium Sulfate	0.2
Sodium Phosphate, Dibasic	1.6
Potassium Phosphate, Monobasic	0.1

pH 7.7 at 25° C

MEDIUM PREPARATION:

Dissolve 22.5 g of base in 1000 ml distilled water. Sterilize by autoclaving for 15 min at 250° F. Cool to 50° C before adding other components.

For Haemophilus pleuropneumoniae isolation purposes the following mixture should be added while the media is at 50° C. Add 10 ml of 2 percent NAD or NADH (Sigma Chemical Company, St. Louis, MO) to 100 ml inactivated horse serum and filter sterilize (e.g., 120-0020 Nalgene 0.20 micron filter). Dispense in 5 ml aliquots into 16 x 125 mm sterile tubes (plastic or glass). Store less than 2 weeks.

APPENDIX B: CASMAN AGAR BASE

Casman agar base (Gibco Diagnostics, Madison, WI.) is used in the cultivation of fastidious microorganisms.

FORMULA:

	gram/liter
Peptone 180 (Animal tissue casein polypeptone)	10.00
Peptone 220 (Yeast casein polypeptone)	10.00
Beef Extract	3.00
Niacinamide	0.05
p-Aminobenzoic acid	0.05
Dextrose	0.50
Corn starch	1.00
Sodium chloride	5.00
Agar	13.50

pH 7.3 at 25° C

MEDIUM PREPARATION:

Suspend 43 gm of medium in 1000 ml of demineralized water. Heat to boiling with agitation to dissolve completely. Sterilize by autoclaving for 15 min at 121° C. For H. pleuropneumoniae isolation purposes the following mixture should be added while the media is at 50° C: to 50 ml of horse serum add 10 ml of 0.1 percent NADH (0.0125 gm NADH, Sigma Chemical, in 10 ml deionized water, filtered with a 0.20 mm filter). Dispense 12-15 ml of media per 100 x 15 mm sterile Petri plate.

APPENDIX C: PEROXIDASE ANTI-PEROXIDASE PROCEDURE FOR HISTOPATHOLOGICAL SECTIONS

Fixation: Twenty four hours in 10 percent normal buffered formalin.

Embedding: Cut paraffin sections at 4 µm.

Solutions: (From Lipshaw, Detroit, MI - titered and prediluted)

PBS pH 7.6
3 percent hydrogen peroxide
Blocking reagent (donkey or universal serum)
Primary antibody (rabbit anti-HPP serotype-specific)
Linking reagent (donkey anti-rabbit)
Labeling reagent (peroxidase anti-peroxidase complex)
Substrate solution: 3-amino 9-ethyl carbazol (AEC)
Mayer's hematoxylin
Ammonia water
Aqueous mounting media

Procedure

1. Dry sections in 60° C oven for 1 hour.
2. Deparaffinize sections for 20 minutes in Hemo-De.
3. 10 dips in 2 changes of 100 percent alcohol.
4. 10 dips in 2 changes of 95 percent alcohol.
5. PBS buffer solution for 5 min.
6. Cover section with 3 drops hydrogen peroxide for 5 min.[1]
7. Wash well with PBS.
8. Cover section with 3 drops blocking reagent for 5 min.
9. Wash well with PBS.
10. Cover section with 3 drops primary antibody for 4 hours at room temperature.
11. Wash well with PBS.
12. Cover section with 3 drops of linking (secondary) antibody for 5 min.
13. Wash well with PBS.
14. Cover section with 3 drops of labeling reagent (PAP) for 5 min.

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[1] Incubation times are for humidity chamber with 37° C warming plate except for primary antibody.

15. Wash well with PBS.
16. Prepare substrate solution (AEC) and cover section with 3 drops for 10 min.
17. Wash well with PBS.
18. Counterstain with Mayer's hematoxylin for 3 min.
19. Wash in tap water, blue in ammonia water and wash in tap water for 1 min.
20. Coverslip with aqueous mounting medium.

APPENDIX D: COMPLEMENT FIXATION TEST - MICRO METHOD

1. Label U-bottom microtiter plates (inactivate serum for 30 min at 56° C).
2. 0.025 ml VBD buffer dropped into all wells except first row.
3. 0.05 ml of serum dropped into first row.
4. Dilute by two-fold dilutions using hand diluter.
5. Add 0.025 ml buffer to first and second rows to balance volume, since no antigen will be added to these rows.
6. Refrigerate at least one hour.
7. Add 0.025 ml titered antigen in all wells except first and second rows.
8. Add titered complement (0.05 ml) to all wells.
9. Cover plates and shake.
10. Refrigerate at 4° C overnight.
11. Sensitize sheep blood cells and drop 0.025 ml into all wells.
12. Cover and shake plates.
13. Incubate at 37° C for 30 min. Centrifuge 1 min.
14. Read according to percentage of hemolysis:

Positive	0-30%
Suspect	35-65%
Negative	70-100%

APPENDIX E: LESION DESCRIPTION AND COMPARISON OF STANDARD CULTURE, CO-AGGLUTINATION AND PEROXIDASE ANTI-PEROXIDASE TECHNIQUES IN THE DIAGNOSIS OF H. PLEUROPNEUMONIAE INFECTION IN LUNG TISSUE SUBMITTED TO THE VETERINARY DIAGNOSTIC LABORATORY, IOWA STATE UNIVERSITY

Lesion score:

0 = no lesions suggestive of H. pleuropneumoniae infection
1 = few foci of necrosis and pleural adhesions
2 = moderate involvement of the lung with variably sized foci of necrotic tissue sequestered by connective tissue and fibrosis of pleural surfaces
3 = extensive involvement of the lung including fibrinous pleuritis and large areas of consolidation with fibrinohemorrhagic and/or fibrinonecrotic lesions

Abbreviations for bacteria:

HPP	Haemophilus pleuropneumoniae
P mult	Pasteurella multocida
Strep	Streptococcus spp.
Staph	Staphylococcus spp.
C pyo	Corynebacterium pyogenes
B bronch	Bordetella bronchisepticum
E coli	Escherichia coli
Kleb	Klebsiella spp.

Case Number	Lesion Score	Standard Culture Results	Co-agglutination Results	PAP Results
20245	2	HPP 5	type 5	positive
20453	1	HPP	type 5	positive
22989	1	P mult	type 1	positive
23088	1	HPP	type 1	positive
23075	2	HPP 5	type 5	positive
23292	2	HPP 5	type 5	positive
22033	0	no growth	type 1	positive
22996	1	HPP	type 1	positive
21132	1	HPP 1	type 1	positive

Case Number	Lesion Score	Standard Culture Results	Co-agglutination Results	PAP Results
21426	2	HPP 1	type 1	negative
21544	2	HPP 5	type 5	positive
21632	1	no growth	type 1	positive
22174	1	no growth	type 1	positive
21988	0	no growth	negative	positive
22335	1	no growth	type 5	positive
22356	1	HPP[1]	type 7	positive
22230	0	no growth	negative	negative
22292	1	HPP[1]	type 1	positive
22267	1	HPP	type 1	positive
06	0	alpha & beta Strep	negative	negative
07	0	P mult beta Strep	negative	negative
08	0	beta Strep Staph	negative	negative
09	0	P mult	negative	negative
10	0	beta Strep	negative	negative
29180	2	HPP	type 1	positive
29104	1	HPP	type 5	positive
29084	1	HPP	type 5	positive
29280	1	no growth	negative	negative
29114B	2	no growth	negative	positive
29445	2	HPP	type 7	positive
0082	1	no growth	type 1	negative
29448	3	contaminated	type 5	positive
29444	1	HPP	type 1	negative

[1] H. pleuropneumoniae was isolated from lungs, but isolation and serotyping were unsuccessful due to excessive overgrowth of other bacteria.

Case Number	Lesion Score	Standard Culture Results	Co-agglutination Results	PAP Results
00143	3	no growth	negative	negative
290	3	HPP	type 1	positive
529	2	HPP	type 5	positive
408	1	HPP	type 5	positive
487	2	HPP	type 5	positive
671	3	Strep suis C pyo	type 5	positive
661	0	P mult	negative	negative
1131	1	HPP 5	type 5	positive
1052C	2	HPP	type 5	positive
1290	3	HPP 5	type 5	positive
1289	3	HPP 5	type 5	positive
1624	1	HPP	type 5	negative
1614	2	smooth E coli	negative	negative
1509	1	P mult	negative	negative
1766	0	B bronch Strep equis	negative	negative
1803	0	C pyo	negative	negative
1762	2	HPP	type 1	negative
1750B	3	H parasuis HPP[1]	type 5	positive
1801	3	HPP	type 5	negative
1764	2	HPP 1	type 1	positive
2225	1	Strep suis C pyo	negative	negative
2203	1	HPP Salmonella	type 5	positive
2210	0	Strep suis	negative	negative
2202	2	H parasuis	negative	negative

Case Number	Lesion Score	Standard Culture Results	Co-agglutination Results	PAP Results
2297	2	HPP P mult B bronch	type 5	positive
2355	3	HPP 1	type 1	positive
01	0	no growth	negative	negative
02	0	alpha Strep	negative	positive
03	0	P mult	negative	negative
04	0	no growth	negative	negative
05	0	no growth	negative	negative
2415	3	HPP 1	type 1	positive
2568	2	HPP[1] P mult	type 1	negative
2651	2	HPP	type 5	positive
2769	1	HPP Strep suis	type 5	positive
2765	0	Strep suis	negative	negative
2850	0	smooth E coli	negative	negative
3000	0	P mult	negative	negative
2886	3	C pyo	type 5	positive
3233	2	sterile	negative	negative
3162	0	sterile	negative	negative
3476	1	Salmonella	negative	negative
3520	1	HPP 5	type 5	positive
3491	2	HPP 5	type 5	positive
3564	2	P mult	negative	negative
3893	1	HPP	type 1	positive
3947	3	HPP	type 1	negative
3944	3	HPP	type 1	positive
4033	0	contaminated	negative	negative
4040	0	smooth E coli	negative	negative

Case Number	Lesion Score	Standard Culture Results	Co-agglutination Results	PAP Results
4048	3	P mult HPP[1]	type 1	positive
4062	2	HPP 1	type 1	positive
4167	2	HPP	negative	positive
4234	1	P mult	negative	negative
4320A	3	P mult moderate Proteus	type 1	positive
4320B	3	HPP	type 1	positive
4320C	3	HPP 1	type 1	negative
4320D	3	HPP	type 1	positive
4320E	3	no growth	negative	negative
2209	0	C pyo	negative	negative
2222	0	P mult	negative	negative
2222B	0	P mult	negative	negative
1265	2	HPP 1	type 1	positive

APPENDIX F: COMPARISON OF STANDARD CULTURE, CO-AGGLUTINATION, PEROXIDASE ANTI-PEROXIDASE AND COMPLEMENT FIXATION TECHNIQUES IN THE DIAGNOSIS OF H. PLEUROPNEUMONIAE PNEUMONIA IN MARKET PIGS

Explanations of lesion scores and abbreviations for bacteria may be found at the beginning of Appendix A.

Group A: Herds with no history of H. pleuropneumoniae pneumonia (non-vaccinated).

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
511	0	mod P mult	neg	no test	neg
512	0	mod hem E coli	neg	neg	neg
513	0	mod E coli	neg	no test	no test
514	0	no growth	neg	neg	neg
516	0	high beta Strep	neg	no test	neg
517	0	few contam	neg	neg	neg
518	0	mod E coli	neg	pos	1:16
520	0	mod hem E coli	neg	no test	neg
521	0	few beta Strep	neg	no test	neg
522	0	no growth	neg	no test	neg
523	0	high beta Strep	neg	no test	neg
524	0	no growth	neg	neg	neg
526	0	high Staph spp	neg	no test	neg
527	0	no growth	neg	no test	neg
528	0	high beta Strep	neg	no test	neg
529	0	no growth	neg	no test	neg
530	1	high P mult	neg	neg	neg
531	0	no growth	neg	neg	1:8
532	0	mod P mult	neg	pos	1:32
533	0	low P mult	neg	pos	1:16
534	0	mod hem E coli	pos 5	pos	1:32

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
535	0	few contam	neg	neg	neg
536	0	high beta Strep and P mult	neg	no test	neg
537	0	high P mult	pos 5	pos	1:32
538	0	mod P mult	neg	no test	neg
539	0	high P mult	neg	pos	1:16
540	0	high P mult	neg	no test	neg
541	0	nod hem E coli	neg	no test	neg
542	0	low Staph spp and beta Strep	neg	neg	neg
543	0	few contam	neg	no test	neg
544	0	few contam	neg	neg	neg
545	0	couple P mult	neg	no test	neg
546	1	high P mult	neg	pos	1:8
547	0	high hem E coli	neg	neg	neg
548	0	mod P mult	neg	no test	neg
549	0	high P mult mod beta Strep	neg	pos	1:32
550	0	high hem E coli	neg	no test	neg
641	0	high P mult	neg	neg	neg
642	0	high P mult	neg	no test	neg
643	0	high P mult	neg	no test	neg
644	0	mod P mult mod beta Strep	neg	no test	neg
645	0	mod alpha Strep	neg	no test	neg
646	0	mod P mult	neg	neg	neg
647	0	no tested	neg	no test	neg
648	0	high P mult	neg	neg	neg
649	0	mod contam	neg	neg	neg
650	0	mod P mult	neg	no test	no test
651	0	high b Strep high P mult	neg	neg	neg

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
652	0	few contam	pos 5	pos	1:8
653	0	mod hem E coli	pos 5	pos	1:8
654	0	mod b Strep	pos 5	pos	1:8
655	0	high b Strep	neg	neg	neg
656	0	high b Strep	neg	no test	neg
657	0	mod P mult	neg	pos	1:8
658	0	high P mult	pos 5	neg	1:8
659	1	mod P mult low b Strep	pos 5	pos	1:16
660	0	high P mult	neg	no test	neg

Group B: Vaccinated herds with no outbreaks in the recent past.

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
601	0	no growth	neg	neg	neg
602	0	few contam	neg	neg	neg
603	0	low alpha Strep	neg	pos	1:8
604	0	mod alpha Strep	neg	neg	neg
605	0	low hem E coli	neg	neg	neg
606	0	couple contam	neg	neg	neg
607	0	mod hem E coli	neg	neg	1:4
608	0	few alpha Strep	neg	neg	neg
609	0	few alpha Strep	neg	neg	neg
610	0	mod contam	neg	neg	neg
611	0	high beta Strep	neg	neg	neg
612	0	few alpha Strep	neg	pos	1:8
613	0	high beta Strep	neg	neg	neg
614	0	few beta Strep	neg	neg	neg
615	0	mod Gram -	neg	neg	neg
616	0	mod a & b Strep	neg	neg	no test
617	0	mod beta Strep	neg	neg	neg
618	0	no growth	neg	neg	neg
619	0	mod beta Strep	neg	neg	neg
620	1	high beta Strep	pos 5	pos	1:16
621	0	high P mult	neg	neg	neg
622	0	high alpha Strep	neg	neg	neg
623	0	mod P mult	pos 5	neg	neg
624	0	high beta Strep	neg	neg	neg
625	0	high P mult	neg	neg	neg
626	0	few beta Strep	neg	neg	neg
627	0	few hem E coli	neg	neg	neg
628	0	mod Staph spp	neg	neg	neg

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
629	0	few contam	neg	neg	neg
630	0	mod contam	neg	neg	neg
631	0	mod contam	pos 5	neg	neg
632	0	mod P mult	neg	neg	neg
633	0	mod Pseudom	neg	neg	neg
634	0	few contam	neg	neg	neg
635	0	mod Staph spp	neg	neg	neg
636	0	few contam	neg	neg	neg
637	0	few contam	neg	neg	neg
638	0	no growth	neg	neg	neg
639	0	high Staph spp	neg	neg	neg
640	0	high alpha Strep	neg	neg	neg
661	0	high P mult	neg	neg	neg
662	0	high P mult low beta Strep	neg	neg	neg
663	0	mod P mult mod C pyo	neg	neg	neg
664	0	high P mult	neg	neg	neg
665	0	high beta Strep	neg	neg	neg
666	0	high beta Strep	neg	neg	neg
667	0	low beta Strep	neg	neg	neg
668	0	no growth	neg	neg	neg
669	0	mod hem E coli	neg	neg	neg
670	0	high P mult	neg	neg	neg
671	0	mod Pseudom	neg	neg	neg
672	0	mod E coli	neg	neg	neg
673		mod P mult high beta Strep	neg	neg	neg
674	0	mod alpha Strep	neg	neg	neg
675	0	high beta Strep	neg	neg	neg
676	0	mod P mult	neg	neg	neg

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
677	0	mod P mult	neg	neg	neg
678	0	mod P mult	neg	neg	neg
679	0	mod P mult high beta Strep	neg	neg	neg
680	0	low contam	neg	neg	neg
971	0	low contam	neg	neg	neg
972	0	no growth	neg	neg	neg
977	0	low contam	neg	neg	neg
979	0	high P mult	neg	neg	neg
981	0	high P mult	neg	neg	neg
987	0	mod P mult	neg	neg	neg
988	0	no growth	neg	neg	neg
989	0	high P mult	neg	neg	neg
990	0	high P mult	neg	neg	neg
994	0	mod alpha Strep	neg	neg	neg
996	0	no growth	neg	neg	neg
997	0	no growth	neg	neg	neg
998	0	low P mult	neg	neg	neg
1000	0	low alpha Strep	neg	neg	neg
901	0	no growth	neg	pos	≥1:128
902	0	low contam	neg	neg	neg
903	0	mod contam	neg	neg	1:16
904	0	low beta Strep	neg	pos	1:32
905	0	no growth	neg	neg	no test
906	0	high alpha Strep high C pyo	neg	pos	1:32
907	0	few contam	neg	neg	1:32
908	0	high P mult	neg	neg	no test
909	0	low contam	neg	pos	1:64
910	0	low contam	neg	neg	1:8
911	0	mod alpha Strep	neg	neg	AC

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
912	0	low contam	pos 5	neg	1:16
913	0	mod P mult	neg	neg	no test
914	0	high P mult	neg	neg	no test
915	0	low contam	neg	neg	1:16
916	0	low bacilli	neg	neg	1:16
917	1	high contam low HPP[1]	pos 5	pos	1:16
918	1	mod alpha Strep	pos 5	pos	1:32
919	0	no growth	neg	pos	1:16
920	0	no growth	neg	neg	1:16
921	0	no growth	neg	neg	neg
922	0	high P mult	neg	neg	neg
923	0	low P mult	neg	neg	neg
924	0	no growth	neg	neg	neg
925	0	high Staph	neg	pos	1:16
926	0	mod P mult mod alpha Strep low beta Strep	neg	neg	AC
927	0	no growth	neg	neg	neg
928	0	mod P mult	neg	pos	1:8
929	0	high P mult	neg	neg	neg
930	0	low alpha Strep	neg	neg	neg
931	0	low alpha Strep	neg	neg	neg
932	0	few contam	neg	neg	no test
933	0	mod P mult	neg	neg	neg
934	0	mod alpha Strep mod E coli	neg	neg	1:4
935	0	no growth	neg	neg	neg

[1] H. pleuropneumoniae was isolated from lungs, but isolation and serotyping were unsuccessful due to excessive overgrowth of other bacteria.

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
936	0	mod P mult	neg	neg	neg
937	0	mod beta Strep	neg	neg	1:8
938	0	high P mult	pos 5	neg	1:16
939	0	low contam	pos 5	neg	1:16
940	0	high P mult low Proteus	neg	neg	neg
941	0	no growth	neg	neg	AC
942	0	no growth	neg	neg	neg
943	0	high P mult	neg	pos	1:8
944	1	no growth	neg	pos	1:16
945	0	high alpha Strep	neg	neg	neg
946	0	no growth	neg	neg	neg
947	0	few contam	neg	neg	neg
948	0	mod alpha Strep	pos 5	neg	1:8
949	0	no growth	neg	neg	neg
950	1	high alpha Strep	neg	pos	1:32

Group C: Nonvaccinated herds with recent outbreaks of the disease.

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
301	1	high P mult	pos 5&7	pos	1:8
302	1	high Bacilli	pos 5	pos	1:4
303	0	high a & b Strep	pos 5	neg	1:4
304	0	low P mult	neg	neg	1:16
305	1	high Bacilli	pos 5&7	pos	1:16
306	1	high E coli	pos 5	pos	1:16
307	0	few P mult high Staph spp.	pos 5&7	neg	neg
308	0	high a Strep	pos 5&7	pos	1:4
309	0	mod P mult	pos 5	neg	neg
310	0	high P mult	neg	neg	neg
311	0	high alpha Strep high P mult	pos 5	neg	neg
312	0	mod alpha Strep	pos 5	neg	1:4
313	0	few contam	pos 5&7	pos	1:4
314	0	high P mult	pos 5&7	pos	1:4
315	1	mod contam	pos 5	pos	1:16
316	0	high E coli	pos 5&7	neg	1:16
317	0	high P mult	neg	neg	neg
318	0	few beta Strep	pos 5&7	neg	1:4
319	0	high P mult	pos 5	neg	suspect
320	1	mod P mult	pos 5&7	pos	1:16
321	1	high bacilli	pos 5&7	pos	1:4
322	1	mod contam	pos 7	pos	1:32
323	1	high bacilli	pos 5	pos	1:4
324	0	high P mult	pos 5&7	pos	1:4
325	0	high P mult low bacilli	pos 5	pos	1:32
326	0	high P mult	pos 5	neg	neg

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
327	0	few contam	pos 5	pos	1:4
328	1	few P mult	neg	neg	neg
329	1	high P mult	pos 5	pos	1:32
330	0	high contam	pos 5&7	pos	1:4
701	0	high beta Strep	neg	neg	neg
702	1	no growth	pos 5	pos	1:8
703	0	mod P mult	neg	neg	neg
704	0	high P mult	neg	neg	neg
705	0	high P mult few beta Strep	neg	neg	neg
706	0	mod beta Strep	neg	neg	neg
707	0	mod P mult	neg	neg	neg
708	1	mod alpha Strep	pos 5	neg	1:8
709	0	high P mult	neg	pos	1:8
710	0	high beta Strep	neg	neg	neg
712	1	high P mult high beta Strep	pos 5	neg	1:16
713	1	high contam low HPP[1]	pos 5	pos	1:4
714	1	mod beta Strep low HPP[1]	pos 5	pos	1:4
715	1	high contam mod HPP[1]	pos 5	pos	1:4
716	1	high alpha Strep	neg	pos	1:32
717	0	high bacilli	neg	neg	neg
718	1	no growth	pos 5	pos	1:8
719	1	high contam low HPP[1]	pos 5	pos	1:4
720	0	high P mult	neg	neg	neg
721	0	mod contam	neg	neg	neg
722	0	high P mult	neg	pos	1:32

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
723	0	high alpha Strep	neg	pos	neg
724	1	high beta Strep	pos 5	pos	1:4
725	1	mod P mult	pos 5	pos	1:8
357	0	low P mult	pos 5	neg	1:4
358	1	low HPP[1]	pos 5	pos	1:4
359	0	low P mult alpha Strep	pos 5	pos	1:4
360	1	low P mult alpha Strep	pos 5&7	pos	1:32
361	0	high P mult	pos 5	neg	neg
362	1	mod P mult	pos 5&7	pos	1:32
363	0	mod P mult beta Strep	pos 5&7	neg	suspect
364	1	high hem E coli	pos 5	pos	1:32
365	0	no growth	pos 5	pos	suspect
366	0	few contam	neg	pos	neg
367	1	high P mult	pos 7	pos	1:64
368	1	high P mult low HPP[1]	pos 5&7	neg	1:4
369	0	high P mult	pos 5	pos	1:4
370	1	high P mult high hem E coli	pos 5	neg	1:4
371	1	high P mult	pos 5&7	neg	neg
372	0	few contam	pos 5&7	pos	suspect
373	0	mod P mult	pos 5&7	neg	suspect
374	1	high P mult	pos 5	pos	1:32
375	0	mod P mult mod alpha Strep	pos 5&7	pos	1:4
376	0	high P mult	neg	neg	1:16
377	0	high hem E coli	pos 5&7	pos	suspect
378	0	mod P mult	pos 5	pos	1:4
379	0	high P mult	pos 5	neg	1:4

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
380	1	high P mult	pos 5&7	neg	1:4
381	0	high bacilli	pos 5	neg	suspect
382	1	high P mult	pos 5&7	pos	1:4
383	0	low P mult	pos 5&7	pos	suspect
384	1	low alpha Strep	pos 5	No test	1:32
385	0	high P mult	pos 5&7	pos	1:4
386	0	high P mult	neg	neg	neg
387	0	no growth	pos 5	pos	1:32
388	1	high P mult	pos 5&7	neg	1:8
389	2	mod P mult mod HPP[1]	pos 5&7	pos	≥1:128
390	1	high P mult	pos 5	neg	1:4
391	0	high P mult	pos 5	pos	1:4
392	0	mod Gram neg	pos 5	pos	suspect
393	1	high bacilli	pos 5&7	pos	1:16
394	1	high P mult	pos 5&7	neg	1:16
395	1	low P mult low alpha Strep	pos 5&7	pos	1:4
396	0	mod P mult	pos 5&7	neg	1:4
397	0	few contam	pos 5&7	pos	suspect
398	1	high P mult high contam	pos 5&7	pos	1:16
399	1	low P mult low HPP[1]	pos 5	pos	1:8
400	0	low P mult	pos 5&7	pos	1:4
726	0	few contam	neg	neg	neg
727	0	high P mult	neg	neg	neg
728	1	mod P mult	pos 5	pos	1:4
729	1	high beta Strep	pos 5	pos	1:4
730	1	no growth	pos 5	pos	1:8
731	0	few P mult	neg	neg	neg

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
733	0	no growth	neg	pos	no test
735	1	no growth	pos 5	pos	1:8
736	1	mod P mult	pos 5	neg	neg
737	1	low P mult	pos 5	pos	1:8
738	0	no growth	neg	neg	neg
739	0	high P mult	pos 5	pos	≥ 1:128
740	0	mod P mult	neg	neg	1:8
741	1	high P mult	pos 5	pos	1:8
742	0	mod P mult	neg	pos	neg
743	0	high beta Strep	neg	neg	1:16
745	0	no growth	neg	neg	neg
746	0	mod P mult low beta Strep	neg	neg	neg
747	1	high P mult few beta Strep	pos 5	pos	1:4
331	1	high alpha Strep	pos 5&7	pos	1:4
332	1	mod alpha Strep	pos 5&7	pos	1:4
333	1	few contam low HPP[1]	pos 5&7	pos	1:4
334	2	high bacilli mod HPP	pos 5&7	pos	1:32
335	0	mod P mult	pos 5	neg	neg
336	2	high beta Strep	pos 5	pos	1:32
337	1	mod P mult	neg	pos	1:4
338	0	high contam low HPP[1]	pos 5&7	pos	1:4
339	0	few contam	pos 5	neg	neg
340	?	high contam high HPP	pos 5&7	pos	1:64
341	0	high P mult	pos 5&7	pos	1:4
342	0	mod P mult low HPP[1]	pos 5&7	pos	1:4

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
343	0	mod contam low HPP	pos 5&7	pos	1:4
344	2	high contam low HPP	pos 5	pos	1:32
345	0	high P mult	pos 5	pos	1:4
346	0	high P mult	pos 5	pos	1:16
347	2	few beta Strep	pos 5&7	pos	1:32
348	3	high alpha Strep high HPP	pos 5	pos	≥1:128
349	2	no growth	pos 5	neg	1:64
350	0	mod contam	pos 5&7	pos	1:8
351	0	high P mult	pos 5	neg	1:4
352	0	mod P mult	pos 5	neg	1:4
353	0	mod contam	pos 5	neg	1:4
354	0	high P mult	pos 5	pos	1:4
355	0	mod contam high HPP	pos 5&7	pos	1:4
356	2	high Staph high HPP	pos 5&7	pos	1:16
801	1	few alpha Strep	pos 5	neg	1:64
802	1	high P mult	pos 5	neg	1:64
803	2	high alpha Strep low HPP	pos5	neg	1:4
804	1	few contam	pos 5	neg	1:4
805	1	mod P mult	pos 5	pos	1:16
806	1	mod alpha Strep	pos 5	neg	1:16
807	1	mod P mult low HPP	pos 5	pos	neg
808	1	mod alpha Strep	pos 5	pos	1:32
809	1	few P mult	pos 5	pos	1:4
810	0	mod P mult	neg	pos	no test

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
811	2	mod contam low HPP	pos 5	pos	1:64
812	1	few alpha Strep	pos 5	pos	1:64
813	0	high hem E coli	pos 5	neg	1:4
814	2	mod HPP	pos 5	neg	1:64
815	1	high alpha Strep	pos 5	pos	1:64
816	1	mod contam low HPP	pos 5	pos	1:32
817	1	mod alpha Strep	pos 5	pos	1:16
818	2	low HPP low hem E coli	pos 5	neg	1:4
819	0	mod beta Strep	neg	pos	1:4
820	1	few contam	pos 5	neg	1:4
821	2	mod HPP mod P mult	neg	neg	1:4
822	1	mod contam low HPP[1]	pos 5	pos	1:16
823	3	mod HPP mod hem E coli	pos 5	pos	1:16
824	2	high hem E coli low HPP	pos 5	pos	1:32
845	0	high P mult	pos 5&7	pos	1:16
846	1	high P mult	pos 5&7	pos	1:64
847	1	mod P mult	pos 5&7	pos	neg
848	0	mod P mult	pos 5	pos	neg
849	1	high P mult mod HPP	pos 5&7	pos	neg
850	0	high beta Strep	pos 5&7	neg	neg
851	1	mod P mult	pos 7	neg	1:32
852	1	mod P mult	pos 5&7	neg	1:32
853	1	high P mult	pos 5&7	neg	1:16
854	1	high contam	pos 5	neg	1:16

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
855	0	high P mult	neg	neg	1:8
856	0	mod P mult	neg	neg	neg
857	0	high P mult	pos 5	pos	1:8
858	2	mod P mult	pos 5&7	neg	≥1:128
859	1	high P mult high beta Strep	neg	pos	neg
860	1	high contam	pos 7	neg	neg
861	1	high P mult mod HPP	pos 5	pos	neg
862	0	high P mult	pos 5&7	pos	1:64
863	0	low contam	pos 5	pos	1:16
864	0	mod P mult	neg	neg	neg
865	1	high beta Strep	pos 5	pos	1:32
866	1	high P mult mod HPP[1]	pos 5	pos	1:32
868	0	high P mult high alpha Strep	pos 5	neg	neg
869	0	mod P mult	pos 7	neg	1:8
870	0	mod beta Strep	neg	neg	neg
871	0	high P mult	neg	pos	neg
872	0	high P mult	neg	pos	neg
551	1	no growth	pos 5	pos	1:64
552	0	no growth	pos 5	neg	1:16
553	1	mod hem E coli	pos 5	pos	1:16
554	1	no growth	pos 5	neg	1:16
555	0	no growth	neg	neg	neg
556	2	high P mult	pos 5	pos	1:64
557	1	high P mult	neg	pos	1:32
558	1	high P mult	neg	neg	neg
559	1	no growth	neg	pos	1:32
560	0	few contam	neg	neg	1:8

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
561	1	no growth	pos 5	pos	1:64
562	2	no growth	pos 5	pos	1:64
563	1	high P mult	neg	neg	1:8
564	0	no growth	neg	neg	1:8
565	1	mod P mult	pos 5	neg	1:32
567	0	mod P mult	neg	neg	neg
568	0	no growth	neg	neg	neg
569	0	few contam	neg	neg	1:8
570	1	mod contam	neg	neg	1:8
571	1	mod contam low HPP[1]	pos 5	neg	1:32
572	0	mod Gram -	neg	neg	neg
573	0	high P mult	neg	neg	neg
574	1	mod P mult	neg	pos	1:32
575	0	low E coli	neg	neg	neg
576	2	mod P mult	pos 5	pos	1:64
577	1	mod hem E coli	pos 5	pos	1:32
578	0	few contam	neg	neg	neg
581	0	mod contam	neg	neg	neg
582	1	mod alpha Strep	neg	neg	neg
584	1	mod P mult	neg	neg	no test
585	2	no growth	pos 5	pos	1:64
586	0	few hem E coli	neg	neg	no test
587	2	high P mult	neg	pos	1:64
588	0	mod P mult	neg	neg	neg
589	2	mod contam low HPP[1]	pos 5	pos	1:32
590	2	high P mult	neg	pos	1:8
825	1	high contam	pos 5&7	pos	1:8
826	1	mod alpha Strep low HPP[1]	pos 5&7	pos	1:8

Ear tag	Lesion Score	Standard Culture	Co-agglut.	PAP	CF
827	2	high beta Strep low HPP	pos 5	pos	1:64
828	1	high HPP	pos 5	neg	1:16
829	1	high P mult	pos 5&7	pos	1:32
830	1	mod HPP, mod P mult	pos 5&7	neg	1:32
831	0	high P mult	neg	neg	no test
832	1	high beta Strep mod P mult	neg	pos	1:32
833	2	high beta Strep mod HPP	pos 5&7	pos	1:64
834	0	high P mult low HPP[1]	pos 5&7	pos	neg
835	1	high beta Strep low HPP	pos 5&7	pos	neg
836	1	high P mult	pos 5&7	neg	1:64
837	0	high P mult low HPP[1]	pos 5&7	neg	neg
838	1	high beta Strep mod P mult	pos 5	pos	neg
839	0	high P mult	neg	pos	neg
840	1	high P mult low beta Strep	pos 5&7	pos	1:32
841	1	high P mult mod hem E coli	pos 5&7	pos	1:32
842	1	mod P mult mod HPP	pos 5	pos	1:32
843	1	high Staph spp	neg	pos	1:64
844	2	high P mult mod Proteus spp	pos 7	pos	1:64