This is to certify that the

thesis entitled

DEVELOPMENT OF AN ELISA FOR THE EVALUATION OF SERUM IMMUNE RESPONSES IN PIGS EXPOSED TO ACTINOBACILLUS PLEUROPNEUMONIAE

presented by

Tesfaye Belay

has been accepted towards fulfillment
of the requirements for

Master's degree in Microbiology

Martha A. Mulks
Major professor

Date Nov. 6, 1989

DEVELOPMENT OF AN ELISA FOR THE EVALUATION OF SERUM IMMUNE RESPONSES IN PIGS EXPOSED TO ACTINOBACILLUS PLEUROPNEUMONIAE

by

Tesfaye Belay

A THESIS

submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1989

ABSTRACT

DEVELOPMENT OF AN ELISA FOR THE EVALUATION OF SERUM IMMUNE RESPONSES IN PIGS EXPOSED TO ACTINOBACILLUS PLEUROPNEUMONIAE

by Tesfaye Belay

The purpose of this thesis research was to analyze the immune responses of pigs to Actinobacillus (Haemophilus) pleuropneumoniae (App), the causative agent of contagious porcine pleuropneumonia. To detect and quantitate antibodies to App, a sensitive and reproducible enzyme-linked immunosorbent assay (ELISA) procedure was developed using App outer membranes as the antigens. The ELISA then was used to measure serum antibodies to App serotypes 1 and 5 in vaccinated, infected, and unexposed pigs.

It was found that 1) the magnitude of the immune responses of animals experimentally infected with either App serotype was dose dependent, and that these animals raised significant ELISA antibody titers against both the challenge serotype and heterologous serotype; 2) animals vaccinated with either an App serotype 5 outer membrane vaccine or a commercial bacterin also raised high antibody responses, which were protective against subsequent challenge with either App serotype 5 or 1; and 3) piglets born of an immune sow had significant serum antibody titers to App at birth, which declined continuously to background levels by 5 to 7 weeks after birth.

DEDICATED TO MY PARENTS

ACKNOWLEDGEMENTS

I wish to express my appreciation to my major advisor, Dr. Martha H Mulks, for her guidance in my research and the writing of the thesis. Thanks are due to Dr. Brad Thacker for his assistance in animal studies and statistical analysis of my data. I also extend my thanks to my other committee members, Drs. Robert Brubaker, Walter Esselman and Ronald Patterson for their advice in my study. I wish to express my appreciation for the assistantship awarded to me from the College of Osteopathic Medicine and World Health Organization that made it possible for me to continue my studies at Michigan State University.

Table of contents

LIST OF TABLES

List of figures Page

Chapter I

Figure

Chapter II

Figure

Chapter III

Figure

INTRODUCTION

Porcine contagious pleuropneumonia is a respiratory disease of swine caused by Actinobacillus pleuropneumoniae (App) (1,2). This disease is economically important in pig raising countries around the world (1,2,3,4). The major economic losses are due to high mortality rate, deteriorated growth rate of infected pigs and increased veterinary fees (4,5). Although several studies have been conducted to develop better methods for the prevention and control of the disease, there are no completely effective vaccines. Various serological tests have been developed for App serotyping (e.g co-agglutination) and/or detection of App antibodies (complement fixation). The cell surface components of App that contribute to induction of protective immunity in pigs have not been completely defined although circulating antibodies to capsule, lipopolysaccharides (LPS), outer membrane proteins (OMPs) and hemolysins have been detected. The purpose of this investigation was to develop a reproducible and sensitive enzyme-linked immunosorbent assay (ELISA) procedure and to utilize this ELISA to analyze the serum immune responses of infected pigs, vaccinated pigs, and passively immune piglets.

LITERATURE REVIEW

Actinobacillus pleuropneumoniae (App), previously known as Haemophilus pleuropneumoniae (Hpp) is a gram negative, nonmotile, nonspore forming, frequently encapsulated pleomorphic rod, with predominantly coccobacillary forms. It grows on enriched culture media, requiring V factor (nicotinamide adenine dinucleotide, NAD) but not X factor (hemin). On sheep blood agar, colonies usually produce a zone of B-hemolysis (6). Recently,the genus name Haemophilus was replaced by Actinobacillus based on data demonstrating that isolates share greater DNA homology, outer membrane protein profiles on gel electrophoresis and morphological and biochemical relatedness with members of the genus Actinobacillus than members of the genus Haemophilus (7,8).

Porcine pleuropneumonia can be either acute fibrino-hemorrhagic or chronic localized necrotizing pneumonia with pleuritis in natural or experimental pig infections (1,2). Although all ages may be affected, pigs about 3 to 5 months of age are the most vulnerable (3,9). The spread of the disease into a new herd is by importation of acutely infected pigs or asymptomatic App carriers or chronically infected pigs (1-3,9). The disease is transmitted via respiratory routes by direct contact from pig to pig (1,2,8). High fever, severe respiratory distress, coughing and anorexia are some of the clinical symptoms of porcine pleuropneumonia (2,8).

Mortality varies greatly (0.4 to 100%) depending on the immune status of the herd. Morbidity is also high (10 to 100%) as measured by a decrease in growth rate and decreased body weight gain of pigs from birth to market or slaughter stage (3-5).

Recovery from clinical disease has been enhanced by treatment with antibiotics (3,9) although antibiotic resistant strains have been isolated (10-11).

Serotyping and antigen characterization of App: At present 12 serotypes have been described based upon capsular antigenic serotype-specificity (12-20). Nontypeable strains have also been reported (5,20). Early studies on serotyping described serotype-specific antigens to be capsule, LPS or both (14-18). More recently, Inzana et al.(21), have demonstrated that the capsular polymer is the serotype-specific antigen for serotype 5. From cross reactions between serotypes, it has been concluded that serotypes of App possess type specific and common species specific antigenic determinants on their cell surface antigens (12,20). Heterogeneity within a single serotype has also been described (12). Tests that have been used to determine the serotype include tube agglutination (13) slide agglutination (13,16 18), coagglutination (19,22), counterimmuno-electrophoresis, immunodiffusion (14,21,23), direct immunofluorescence and indirect fluorescence (13,23,24) and complement fixation (14,25). The heterogeneity within a

species and cross-reactions between serotypes are a problem for both serodiagnosis and vaccination and thus require further development of specific and sensitive tests. The pattern of geographic distributions of App serotypes varies around the world (3,13,26). Serotypes 1, 5 and 7 are common in the United States (13). Serotypes 2,3,4 and 9 are found in Canada while serotype 2 is common in Western European countries (3,12,16,24,26). There are also App isolate reports from Japan, Australia and Taiwan (26).

A Western blot procedure has been used in our laboratory to detect porcine antibody to App outer membrane proteins (OMPs), LPS and capsular polysaccharide antigens. We have identified major OMP antigens with estimated molecular weights of 16-18 kDa, 28-29 kDa, 39-43 kDa, 40-42 kDa, 66 kDa and 97 kDa by comparison with standards of known molecular weights (BioRad) separated by SDS-PAGE and stained with Coomassie blue-silver (27). Similar findings were shown by Rapp et al.(28,29). High molecular weight capsular polysaccharides, rough type LPS (12-14 kDa) in all serotypes and laddered smooth type LPS in serotype 4 and 7, have also been identified. There are differences in the OMP and LPS profiles between serotypes but, in general, little variation is seen among isolates of the same serotype (30).

Pathogenesis: The pathogenesis of porcine pleuropneumonia has not been fully elucidated, particularly the conditions allowing for the proliferation of the organism

in the lower respiratory tract and development of clinical diseases. Many stress situations such as transportation, extreme weather conditions and possibly bacterial and viral respiratory infections may be significant in allowing App to escape the natural defenses of the upper respiratory tract of a pig and invade the lung (3,8). Rapid multiplication of encapsulated bacteria and release of toxic products, including endotoxin, cause alveolar and vascular damage followed by death of alveolar macrophages and neutrophils facilitating the lesion development (31,32).

In the acute clinical form of porcine pleuropnuemonia, the lesions are histologically characterized by hemorrhagic necrosis, exudation of fibrin, infiltration of an unidentified population of degenerated mononuclear cells and thrombosis of pulmonary vessels (2,32-34). In chronic infection, severe clinical disease is not a common phenomenon but pigs show reduced appetite, decreased growth and chronic cough (1,2). Pathological studies have indicated that there is fibrinous pleuritis and necrosis with variable size and distribution in lungs of chronically App infected pigs (1,2).

Virulence factors: Capsule, LPS and hemolysins and/or cytotoxins are the known virulence factors involved in App infection (1,31-45).

Hemolysins: Three types of extracellular hemolysins have been described in App serotypes that are responsible for the necrosis feature of chronic and acute porcine contagious

pleuropnuemonia (34-40,43-45). It has been shown that App serotype 2 broth cultures contain an extracelullar hemolysin known as a heat-stable carbohydrate with hemolytic activity, an anti-phagocytic, and a cytotoxic substance to swine macrophage and neutrophil cells (36-40). In contrast, other studies have suggested that bacteria free-culture supernatants of App serotypes contain heat-labile extracellular protein hemolysins and has been shown that they are cytotoxic to neutrophils and contribute to extensive necrosis seen in pulmonary lesions of diseased pigs (34,35, 43-46). Recently, a 105 kDa Ca^{2+}induced protein extracellular hemolysin and a 27 kDa cohemolysin protein have been identified in several App serotypes (43-45). Furthermore, the 12 App serotypes have been categorized into groups depending on requirement of Ca^{2+} for hemolysin biosynthesis and/or activity (45).

Capsule: It has been shown that capsule is required for colonization of App in the host. Its role is most likely to protect bacterial cells from bactericidal action of complement and antibody thus allowing proliferation during initial phases of infection (21,41,42). Experimental animals intratracheally inoculated with capsular materials did not develop pulmonary lesions. Therefore, it is suggested that capsule is not responsible for lesion development in the lungs of App infected pigs (33, 41,42).

Endotoxin/LPS: There have been several studies to

determine the role of endotoxin in the pathogenesis of App infection (31-35, 41). Rough LPS extracted from App serotype 5, for example, induced lesions of typical acute App infections in pigs; smooth LPS also induced lesions, although to a lesser degree; suggesting involvement of LPS in pathogenesis (41). App appears to produce endotoxin like other gram-negative bacteria and its endotoxin is partially responsible for the massive inflammatory edema, congestion of alveolar capillaries and blood vessels and intravascular fibrinous thrombosis in the lungs of infected pigs (1,3,21,26,33,41).

In most studies of pathogenesis so far carried out, there is no evidence of the involvement of adhesins in attachment to respiratory mucosal cells in App infection. Recently, however, pili-like structures in App, P.multocida and P.haemolytica have been suggested by Carlos Pijoan (International Conference on the Haemophilus, Actinobacillus, Pasteurella group of organisms, Guelph, Canada 1989:18).

Involvement of outer membrane proteins of App in pathogenesis has not been reported but it was recently shown that outer membrane proteins of 76 and 105 kda were expressed in iron-restricted App growth conditions (47). The function of these proteins is speculated as receptors of iron-carrying compounds in App serotypes (47).

Comparative studies in pathogenesis (42,48,49) have suggested that different serotypes of App exhibit differences

in degree of virulence when injected into the lower airway of pigs. This could be due to differences in capsular and lipopolysaccharide chemical composition. The difference in virulence among App of serotypes 1, 5 and 7 has been shown to be low, but serotype 3 is less virulent than 1 (48). The lack of virulence of some serotypes and/or strains is probably due to insufficient capsular material to avoid destruction or removal in the respiratory tract by host defense mechanism (26,41-42,48-49).

The heat-labile protein hemolysins (105 and 27 kDas), the heat-stable carbohydrate hemolysin or cytotoxins, and LPS may have a major role in damaging tissue cells directly or as a result of damage to macrophages and polymorphonuclear cells. Damaged or killed phagocytic cells may release their toxic contents to the environment, causing severe damage to tissues that can lead to intensive lesion development. These, therefore, appear to be necessary in the development of lesions in App infection. The capsule and possibly the outer membrane proteins are probably necessary for the establishment of infection in pigs.

Immunity and control: What constitutes a protective immune response to App infection has not been well defined, although protective immunity can occur after naturally or experimentally induced App infections as well as in vaccinated animals (50-52). Studies have shown that animals which have been infected with App either naturally or by

experimental intranasal or intratracheal inoculations with live organisms, are in general immune to further infections with organisms of the same serotype and are at least partially resistant to challenge with organisms of heterologous serotypes (30,53). In contrast, immunization with formalin-killed organisms usually induces only partial protection against challenge with the homologous serotype, and no significant protection against heterologous serotypes is observed (53). The results of several experiments strongly suggest that the immune response of animals to parenteral vaccination is different from the response seen after infection (30,53,54). The important part of the defense mechanism against App infection could be a local barrier preventing the agent from penetrating the respiratory mucosa. During infection antibodies against deep-seated common antigens of most serotypes may be produced that increase resistance to reinfection. By parenteral vaccination little local antibody response may be elicited or it may stimulate anticapsular antibody production to protect against homologous but not heterologous infection. Evaluation of secretory immune responses in pigs is also underway in our laboratory to understand immunity in resistance to App infection.

The transfer of immunoglobulins across the placenta of a pig to its fetus is not a common process (56). However, it has been shown that antibodies to App are present in sera of

piglets (57, 58). The passive transfer of immunoglobulins in piglets occurs by absorption of colostral immunoglobulins at birth (57). Transfer of humoral antibodies from sows to piglets may protect them against infection of App during the first weeks of age because it was shown that mortality and morbidity rates in colostrum-deprived piglets were almost 100% after four days from birth, but these rates were low among suckled piglets from immune sows (57).

The protective efficacy of anticapsular antibodies has been investigated in pigs and mice by Rosendal et al.(59). After immunization of pigs with two different capsule extracts as vaccines, partial protection and reduced mortality were observed during a subsequent challenge with a homologous serotype of App. It was also demonstrated that passively administered specific monoclonal anticapsular antibodies prevented pigs from death due to induced App infection (60). Complete protection against infection, however, has not been provided by antibody to capsule (21,59,60).

Fenwick, et al.(61) investigated the potential protective effects of immunity against common lipopolysaccharide core antigens of gram negative bacteria by inducing App infections in pigs which had received a vaccine of an Rc mutant of E. coli J5. This Rc mutant E.coli contains a mutation in UDP-galactose epimerase which makes it unable to incorporate exogenous galactose into LPS resulting

in exposure of the LPS core (61-63). There were increased titers to E. coli J5 after vaccination and protection against App re-infection in pigs suggesting the exposure of cross-reacting immunodeterminants of App. In another study, immunogenicity of oligosaccharides of App serotype 5 was improved in experimental animals by conjugation of its LPS to tetanus toxoids (62).

Neutralizing antibodies to hemolysin have been demonstrated in the serum of both pigs and rabbits immunized with concentrated culture supernatant (hemolysin) as well as in the sera of pigs that have survived natural infection with App serotype 1 (35, 40). Protection studies by using hemolysins as vaccine components have been developed in our laboratory. Our data in these vaccination trials showed that bacterin vaccines containing hemolysin were more effective than similar vaccines without hemolysin (Mulks, unpublished data).

Several studies have shown that variations in degree of protection are obtained with different vaccines depending on the quality of vaccine (age of culture for vaccine), route of immunization, type of adjuvant incorporated, dose of vaccine and other factors (50-52,55,59,62-64). It is suggested that vaccination with bacterins prevents the acute form of porcine pleuropneumonia and improves the rate of weight gain, and decreases the magnitude of pleuritis at slaughter of chronically infected pigs (50-52). Other means of controlling

outbreaks of porcine pleuropneumonia include ventilation of facilities in which animals are housed, minimization of temperature changes, avoidance of overcrowding of pigs in barns, avoidance of introduction of sero-positive pigs to a new herd, elimination of infected animals from the herd and antibiotic therapy (3,4,65).

Despite the many reports and studies conducted on App, porcine pleuropneumonia continues to cause economic losses for the pig industry. A more complete understanding of the pathogenesis of App infection, especially the role of specific virulence factors; a more effective, cross-reactive vaccine; and a sensitive and specific serodiagnostic test that permits rapid evaluation of immune status and identification of asymptomatic animals are all needed in order to prevent and control the disease.

CHAPTER 1

DEVELOPMENT OF AN ELISA PROCEDURE

ELISA, a widely used type of serological assay, is beginning to be used for the detection of antibodies in the serum of swine infected or vaccinated with App (21,25,61,66). Several different antigens, including capsular polysaccharide, LPS, and whole cells, have been tried. Our procedure used purified outer membranes of App. To develop a sensitive and reproducible ELISA assay, we tested and standardized several variables to optimize ELISA reactions and minimize background and nonspecific reactions and define titer within the context of our particular ELISA assay. Variables tested included a) selection of solid phase by comparison of different plates b) comparison of coating buffers, coating procedure and optimum antigen concentration for coating. c) antiserum dilutions d) choice of conjugated enzyme-substrate system e) temperature and incubation times.

MATERIALS AND METHODS

Bacterial Strains: App strain No.178, serotype 5, received from V. Rapp, Iowa State University, and strain No.27088, serotype 1, from the American Type Culture Collection, were used throughout the serological investigation.

Chemicals and media: Brain Heart Infusion was bought from Difco Laboratories, Detroit, Mi. Bovine serum albumin (BSA), Tween 20, NAD, DTT, lysosyme, sucrose, Trizma base, and Sodium phosphate salts were obtained from Sigma Chemical Company, ST. Louis, MO. Oxalic acid, sodium carbonate, sodium bicarbonate, and sodium chloride were purchased from J. T. Baker Inc. Pillisburg, N.J.

Serum preparation : Blood from experimental pigs for serum preparation was obtained by jugular vein puncture using the vacutainer system. Blood was allowed to clot at room temperature for one hour. The serum was harvested by centrifugation and stored at -20^{o}C until used.

Complement fixation Test: Complement Fixation antibody titers were determined at the Iowa Veternary Diagnostic Laboratory, formalin-killed whole cells of App serotypes of 1, 5 and 7 as antigen was used (67). The maximum complement fixation titer measured is 1:128. Titers less than 1:8 are considered insignificant.

Statistical Analysis: The significance of differences in serum antibody titers between experimental groups were analyzed by Students't-test or Least Significant Difference

(LSD), a multiple comparison of variance with a multicomputer program, Statistix^{R}(NH Analytical Software, St. Paul, Minnesota).

For statistical analysis, reciprocal ELISA titers were transformed as follows: <60 = 0, 60 = 1, 120 = 2, 240 = 3, 480 = 4, 960 = 5, 1920 = 6, 3840 = 7, 7680 = 8.

The actual geometric mean ELISA titers shown in Tables were calculated from the arithmetic mean of transformed titers by the following relationship: Arithmetic mean titer is multiplied by logarithm of 2, then the inverse logarithm of the product is multiplied by 30. The same formula was used for the standard deviation calculation.

Preparation of outer membranes by sucrose gradient.

We used a modified Osborn and Munson method to separate inner and outer membranes of App serotypes (68).

Cultures of both strains, within two subcultures of isolation from pigs, were grown in Brain Heart Infusion Broth (BHI) supplemented with NAD (10ug/per ml) overnight at 37°C in a water bath shaker. Cells were harvested by centrifugation at 13400 X g for 15 minutes at 4°C and resuspended in 40 ml of phosphate buffer (.033M pH 7.00), and centrifuged at 13400 x g for 15 minutes at 4°C. The cell pellets were rapidly resuspended in a 10 ml cold solution of (.75M sucrose - 0.1M Tris-acetate (pH 7.8)-0.2mM DTT), and freshly prepared cold lysosyme (150 ug/ml of cell suspension) was added. After 5 minutes at room temperature, conversion to spheroplast form was completed by slowly adding 2 volumes of 5mM EDTA (pH 7.5) - 0.2mM DTT. Cell suspensions were centrifuged at 16000 X g for 15 minutes, and the pellet was resuspended in 2.5ml of .25M sucrose - .01M Tris-acetate (pH 7.8) 5mM EDTA (pH 7.5) - 0.2mM DTT and sonicated on ice until the suspensions were translucent. The sonicated cell suspensions were centrifuged for one hour at 140,000 X g in a type 70.1 Ti rotor to pellet the membranes. Sucrose solutions of 55%, 45% and 40% by w/w in 5mM EDTA, pH 7.5, were layered in 12ml ultraclear tubes and 10-15 drops (about 0.5ml) of membrane suspension was layered on top of the gradient. Centrifugation was carried out at 140,000 X g for 20-24 hours

in a type SW-41 rotor. The outer membrane bands were collected with a pasteur pipette from the 45-55% sucrose interface. Protein concentration was determined by the Bio Rad protein assay procedure (69). The outer membrane preparations were assayed for quality control by standard immunoblotting against strongly positive pig sera. Preparations were aliquoted and stored at -20^{o}C until used for ELISA plate coating as antigens.

RESULTS

1. Optimization of ELISA: Development of the ELISA procedure was based on the methods described by Engvall and Perlman (70).

a) Solid phase: Microtiter plates from several different sources were tested as solid phase for binding App outer membrane antigens. All of the plates showed reproducible results when assayed against ascertained positive and negative pig sera. The polystyrene microtiter Gibco plate (Gibco Laboratories, Grand Island, N.Y.) was selected for further use because of its excellent binding capacity for antigen and minimum non-specific binding, as well as its availability and low cost.

b) Coating conditions and antigen concentration: Four different buffer solutions were tested as antigen diluents for coating the plates. These were 1) phosphate buffered saline (PBS), (.1M sodium phosphate + .15M NaCl, pH 7.5); 2) carbonate buffer (0.5M sodium carbonate, pH 9.6); 3) Tris-buffered saline (TBS) (.02M Tris-acetate, pH 7.5, plus 0.5M NaCl); and 4) neutral phosphate; (.1M sodium phosphate, pH 7.0). Among the 4 buffers tested, the commonly used carbonate buffer was selected for further use.

Concentrations of outer membrane antigens ranging from 0.01 to 2.00 ug protein per well in carbonate buffer were tested by checkerboard titration for ELISA plate coating. Optimal antigen coating level per well was determined to be 1

ug protein in 100 ul coating buffer per well. Incubation of plates overnight at 37°C was found to be a more consistent method of coating antigens than incubation at 4°C. After coating, plates were washed twice with Tris-buffered saline plus 0.05% Tween 20 (TTBS), then wells were blocked with 3% BSA in TBS for 1 hour at room temperature. Plates were again washed 2 times with TTBS, dried throughly at 37°C for 3 hours, wrapped and stored until used. Coated and blocked plates wrapped with aluminium foil can be stored at -20°C for one month. There was no loss of activity when compared to fresh, one day old and one week old plates. On the other hand, plates coated by incubation at 4° C gave variable optical density (OD) values when assayed after two days or 2 weeks. Outer membrane preparations could be stored for up to six months at -20° C.

c) **Serum dilutions:** Normal saline was used as a serum diluent in our experiments. Positive and negative control sera at 1:25 or 1:60 as an initial dilution, each with a series of eight two-fold dilutions, were examined. With the 1:25 to 1:3200 dilution series, we found inconsistent OD values at the higher dilutions. The use of this serum dilution series also created a problem of rapid substrate color development and high OD values exceeding the maximum limits of the programed ELISA reading at 405. The 1:60 to 17680 two-fold dilution series, however, was found to give consistent results. There was a linear decrease in OD values

with the dilution series, and the threshhold value,(0.333) was obtained within the 8-point dilution series.

d) Enzyme-substrate system: Horseradish peroxidase linked to protein A (Bio-Rad laboratories, Richmond, Ca.) was used as the conjugate. Its potency was tested at dilutions, 1:5000, 1:10,000, 1:20,000 and 1:40,000. At 1:5000 conjugate dilution, intense color development was observed throughout all wells within 5 minutes after substrate addition. At 1:20,000 or 1:40,000, the OD values were low even in extended time of incubation. The decrease in OD values was linear and provided consistent results with the 1:10,000 dilution and this was used in the remaining experiments. Enzyme working strength was stable for about 6 months stored at -20^{o}C.

We attempted preparation of enzyme substrate by dissolving 10mg of ABTS in 10 ml of citric phosphate buffer (pH, 4, .1M citric acid + .2M of sodium phosphate raised to 100 ml of water) plus 166 ul of 30% hydrogen peroxide (70). However, inconsistent OD readings were obtained with this substrate. To eliminate this inconsistency, we chose to use a commercial horseradish peroxidase substrate kit (Bio-Rad, Richmond, Ca.) containing 5-Aminosalicylic,2,2 -azino-di(3-ethylbenzthiazoline-6-sulfonic acid),(ABTS) plus 30% peroxide.

e) Temperature and incubation times: Optimal incubation times for antiserum dilutions and for horseradish peroxidase conjugate were determined to be one hour at room temperature.

Color development was for 15 minutes, also at room temperature. Incubating reaction assays at 30°C gave no significant OD difference from assays performed at room temperature.

2.ELISA Protocol:

The final ELISA protocol was as follows: Wells of polystyrene microtiter Gibco plates were coated with 1 ug outer membrane proteins per well, using 50mM sodium carbonate, pH 9.6 as coating buffer. After incubating the plates at 37°C overnight, wells were washed twice with TTBS, blocked with 3% BSA in TBS for one hour at room temperature, and washed twice with TTBS. Two-fold serial dilutions of serum (1:60; 1:120; 1:240; 1:480; 1:960; 1:1920; 1:3840 and 1:7680) in normal saline were incubated for an hour at room temperature. After washing the wells 3 times with TTBS, Horseradish Peroxidase-tagged protein A diluted 10,000 in 1% BSA in TTBS was added to the wells for one hour incubation at room temperature. Excess conjugate was removed by washing 2 times with TTBS and once with TBS. Chromogenic enzyme substrate, ABTS was added for 15 minutes at room temperature. The reaction was stopped by addition of 2% oxalic acid and the optical density of each well was read at 405nm using a BioTek automated ELISA reader (Bio Tek Instruments Inc, Winooski, Vt).

3.Determination of ELISA antibody titer :

We defined titer as the reciprocal of the lowest serum dilution which yields an optical density greater or equal to

0.333. This threshhold value equals the mean optical density yielded by negative control sera, at a 1:120 dilution, assayed 100 times, ± 3 standard deviations (mean = 0.231, standard deviation = 0.034).

4.Test of reproducibility :

Known positive and negative control pig sera were assayed in 6 replicates for 6 days against serotype 1 and serotype 5 outer membranes. The purpose was to test the reproducibility of the ELISA assay and to establish standard internal controls to be run concurrently in each sample test. Mean optical densities versus reciprocal serum titers are plotted in figures 1 and 2 for serotypes 1 and 5 respectively. The ELISA titration curves for the positive controls were typically S-shaped. Optical densities declined continuously with successive serum dilutions and asymptotically approached zero. There were no significant differences in OD values within replicates of each assay performance on the same day.

Coefficients of variation were calculated to determine the precision of the assay. The values of the coefficients of variation were within the acceptable range, all far less than 20%. Coefficient of variation values tended to be greater at the higher dilutions and decrease at lower serum dilutions. There was good plate-to-plate reproducibility, showing very slight optical density value variations between batches of plates. The endpoint titers, 1920 and 3840 of positive

controls for serotype 5 and serotype 1, respectively, remained consistent during the repeated assaying of the samples. The OD readings of negative serum at the 120 dilution-point remained below the threshhold value, 0.333 during the course of the experiments. We designed a quality control such that if the positive control serum titer varied more than two-fold from the geometric mean titers,(1:3840 against serotype 1 and 1:1920 against serotype 5), ELISA assays were discarded and the tests repeated .

5. Comparison of ELISA and complement fixation (CF) test

The sensitivity of the ELISA procedure was evaluated by assaying preinfection serum samples from pigs from two different sources. ELISA and CF serotiters of non-specific pathogen free (non-SPF) and specific pathogen free (SPF) pigs against outer membrane antigens are shown in Table 1. The ELISA titers of the non-SPF animals were two or more times greater than the titers of SPF animals. The SPF pigs showed essentially the same titer as the internal negative control. There was a significant difference in geometric mean ELISA titers between the 2 sources of animals as evaluated using t-test ($p < 0.05$). The complement fixation test, however, failed (100%) to show any antibody titers in the non-SPF animals. The CF test was unable to discriminate between the SPF and non-SPF samples.

Table 1: Comparison of ELISA and complement fixation (CF) App serotiters of pigs from SPF and non-SPF herds.

		Serotiters[a]		
		ELISA[b]		CF
Groups	No.pigs	Sero. 5	Sero. 1	
Non-SPF[c]	6	381±43 (240-480)	270±50 (120-480)	0
SPF[d]	7	66±39 (60-120)	66±39 (60-120)	0

[a] Titers presented as reciprocal geometric mean ± 1 standard deviation with range in titers in parenthesis.

[b] Antigen = purified outer membrane prepared by sucrose density ultracentrifugation, from either App serotype 1 (27088) or serotype 5 (I-178).

[c] Pigs from a conventional herd that were not exposed to App.

[d] Pigs from an SPF herd that were housed in isolation facilities.

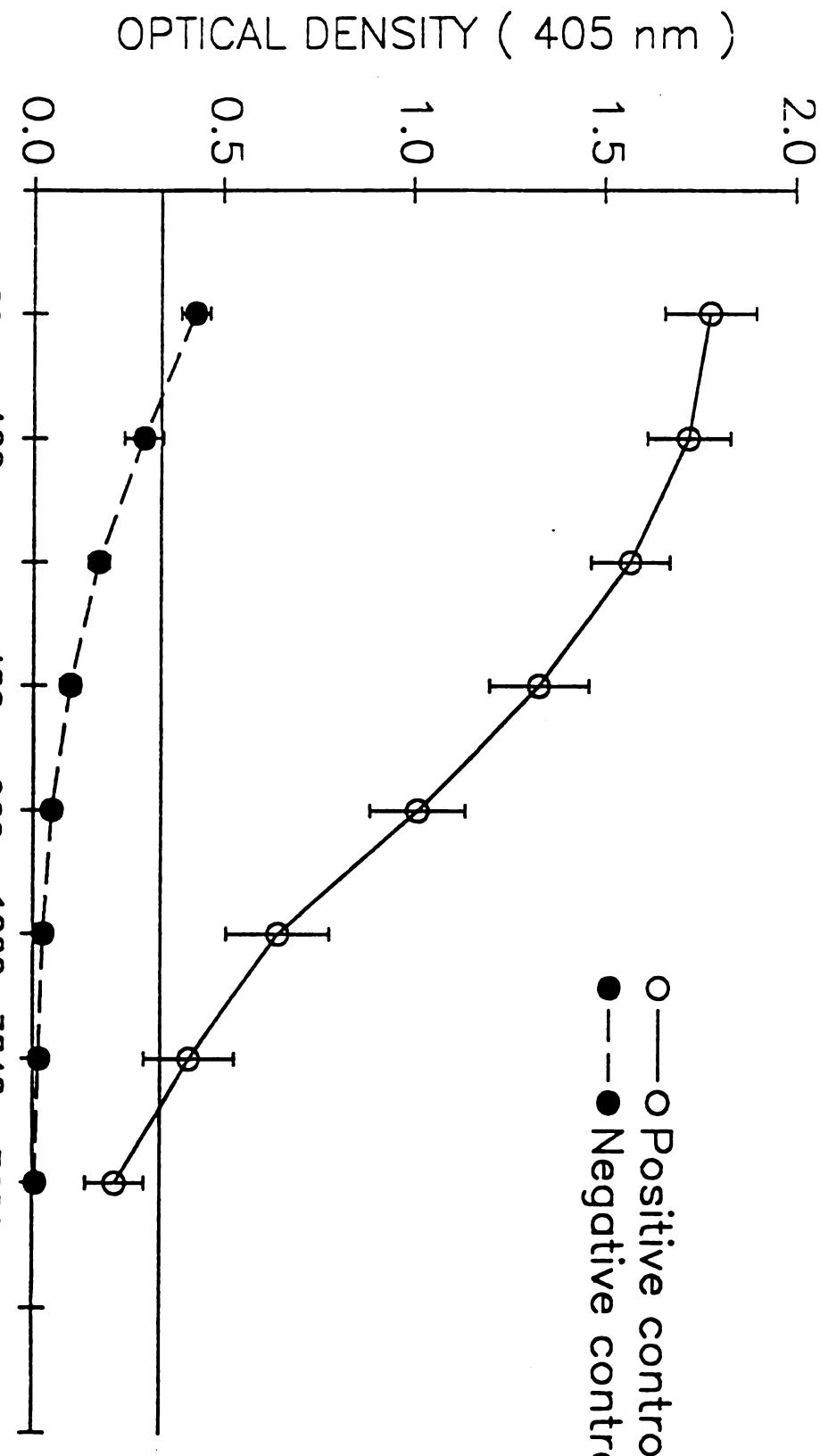

Figure 1: Mean optical densities ± standard deviation versus serum dilutions of positive and negative control sera against serotype 1 outer membrane antigens.

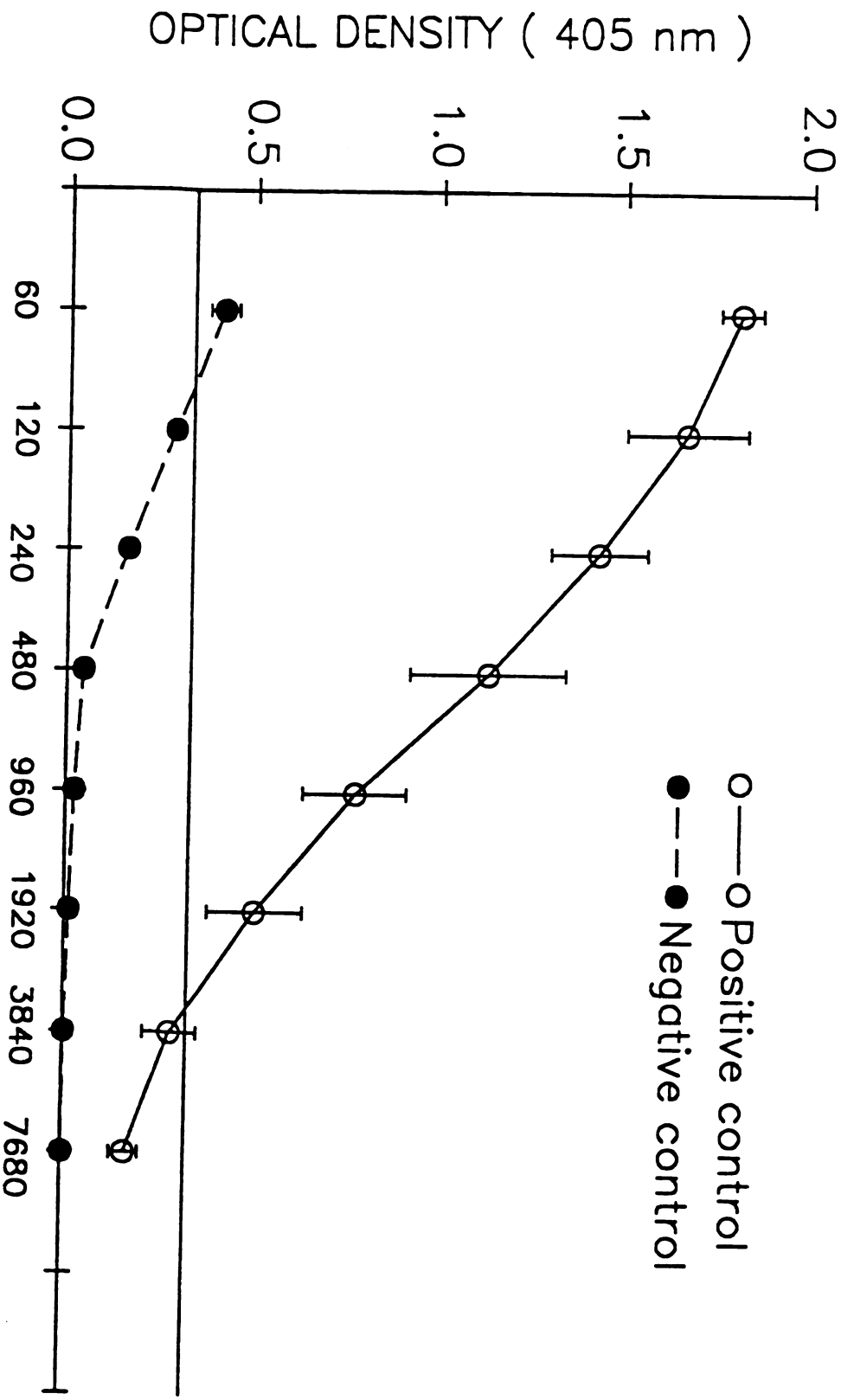

Figure 2: Mean optical densities ± standard deviation versus serum dilutions of positive and negative control sera against serotype 5 outer membrane antigens.

DISCUSSION

We first evaluated the conditions under which the ELISA can best be performed using partially purified outer membrane antigens by designing a series of preliminary experiments. The various parameters affecting sensitivity and specificity of an ELISA for detection of antibodies to App were evaluated and optimized.

Outer membrane antigens separated by sucrose gradient ultracentrifugation bound readily to the walls of polystyrene plates incubated at 37^{o}C compared to incubation at the 4^{o}C. Because of the stability of the preparations, it was possible to store antigen-coated assay plates for one month at -20^{o}C.

Normal saline as serum diluent was found to reduce non-specific binding of serum proteins to the plates. A starting serum dilution of 1:60 and antigen concentration of 1ug protein per well provided the best results of all the dilution series tested indicating that the antigen and antibody concentrations were at optimum level for interaction. The buffers used had the proper pH and ionic strength for antigen-antibody reactions. Tween-20 and bovine serum albumin acted to saturate unbound sites on the plates, thus decreasing nonspecific binding of reactants (70).

Horseradish peroxidase conjugated to staphylococcal protein A worked very efficiently as an indicator in the assay at a dilution of 1:10,000. It was confirmed in previous studies that staphylococcal protein-A has strong reactivity

to swine IgG and even to IgM and IgA immunoglobulin classes (71).

The reliability of the ELISA procedure was demonstrated by the fact that no significant differences in mean OD values were observed during repeated assay of reference serum samples on different days. The coefficients of variation were small for the replicates at each dilution point, ranging from 19% to 4.8% and 24% to 4.6% from the highest to the lowest dilution points for serotype 1 and serotype 5, respectively. The magnitudes and ranges of coefficients of variation for both controls at each dilution point remained almost the same in the repeated assays. This suggests that conditions which can affect the repeatability of ELISA assay were monitored to a significant level in the experiment. However, the relatively large percentages at the highest dilution points of the positive controls, where the OD value is below the threshhold value was also reflected in the negative control serum. This is consistent with the fact that high coefficient values are associated with low OD values, as characteristics of negative sera usually encountered in any immunoassays.

Although slight variations could be observed due to various errors such as pipetting, extended serial dilution, timing, temperature and background of buffers, the reproducibility of the ELISA assay allowed us to use it to evaluate the immune responses of experimental animals against App outer membrane antigens and compare its sensitivity and

specificity to complement fixation. The complement fixation test has been the method of choice for serodiagnosis of porcine pleuropneumonia (53,67), even though there are technical problems due to pro and anticomplementary activity of swine sera and subjective error in the interpretation of hemolysis (25,66).

The results, summarized in Table 1, suggest that ELISA is a reliable discriminator of SPF and non-SPF pigs compared to the CF test. Detectable ELISA titers of the non-SPF pigs against outer membranes of the App serotypes tested could be indicative of past infection with or exposure of the conventional pigs to other gram negative bacteria that have cross-reactive antigens to App. Our Western blot data suggest that, for example, the 16-18 kDa OMP is found in antigenically similar forms in many species of gram- negative bacteria. It is possible to conclude from this that specificity of our ELISA may be decreased if pigs are exposed to other bacteria that have cross-reacting antigens with App serotypes. From the results obtained, it appears that ELISA is more sensitive than the more conventional diagnostic test, complement fixation. In samples where a low level of App antibodies is suspected, the ELISA assay seems to be a better choice to use than the complement fixation test. Similar results in other laboratories showed that depending on the quality of antigen preparation, ELISA was more sensitive than the complement fixation test (25,66).

The outer membrane preparation used in coating ELISA plates contains capsular material, LPS and OMPs. Thus, our ELISA may measure antibodies against a wide range of antigens, including capsule, LPS and outer membrane proteins. Such a range of antibodies have been detected by Western blotting analysis in our laboratory. In contrast, the complement fixation test usually measures only antibodies against capsule, as demonstrated in previous studies (67).

A disadvantage we found in using our ELISA assay is the apparent lack of serotype specificity because all sera we tested reacted with both serotype 1 and serotype 5 antigens. However, the titers were always higher for the particular serotype outer membrane antigen to which the animals had been experimentally exposed. This is discussed in more detail in Chapters II, III, and IV of this thesis.

Another problem of our ELISA procedure was the requirement of extended serial dilutions of a test serum to reach an endpoint titer. As a result, a large quantity of serum samples could not be processed in a relatively short time. The modification of serial dilution is absolutely necessary to reduce reagent costs, increase the number of samples screened and decrease technical time, making the procedure more readily available for research and diagnostic use. However, analysis of the data from these and other experiments suggest that we can develop a standard curve correlating OD at a single serum dilution (for example,

1:480) with endpoint titer, and thus perform accurate measurement of titers with an assay using a single dilution of each test serum.

CHAPTER II

EVALUATION OF IMMUNE RESPONSES OF App VACCINATED PIGS

Currently licensed commercial vaccines are not entirely effective for controlling porcine respiratory pleuropneumonia. Although they reduce the death rate and in some cases the severity of lung lesions in infected pigs, they do not afford complete protection against infection, and are particularly ineffective against App serotypes not included in the vaccine. In addition, there is often granuloma and abscess formation at the site of injection and occasionally there are deaths due to the vaccine alone (55).

There is evidence of strong immunogenicity and broad cross-reactivity of outer membrane proteins among most App serotypes (28,29,47). However, the efficacy of an outer membrane protein vaccine in protection against App infection in pigs has not been evaluated previously. In this study, the serum immune responses of experimental animals to an App outer membrane vaccine were analyzed by ELISA, CF and Western blot tests, and the protective efficacy of this App serotype 5 outer membrane subunit vaccine, as well as a commercial bacterin containing whole killed cells of App serotypes 1, 5 and 7, was evaluated. The purpose of this part of the study was to examine if there is a correlation between ELISA titers and protective efficacy of the App vaccines.

MATERIALS AND METHODS

Experimental design: Twenty four App-free pigs, 6-weeks old and weighing about 12 kilograms each, were used in the study. Ten pigs were vaccinated intramuscularly with 5 mg outer membranes of App strain I-178, serotype 5, plus Freunds' incomplete adjuvant, three times at two week intervals. The vaccine contained outer membrane prepared as described for the ELISA antigen, suspended in saline and mixed 1:1 with the adjuvant. Five pigs were vaccinated with a commercial whole cell-killed bacterin containing App serotypes 1, 5, and 7 twice at three week intervals. Nine pigs were used as nonimmunized controls. Each animal was bled weekly and serum immune response was evaluated by complement fixation, ELISA and Western blotting analysis.

Serum immune responses of two other groups of pigs from separate vaccine trials (6 non-SPF pigs from trial # MSU-86 and 3 SPF pigs from trial # BCHpp3), vaccinated with a commercial bacterin were compared to those of the 5 commercial bacterin vaccinated pigs from this trial (FF4/BC). Protective potency of the commercial vaccine in both SPF vaccination trials was tested by subsequent challenge with a 50% lethal dose of App serotype 1.

Experimental challenge: To examine protection, two weeks after the last vaccination pigs were intratracheally inoculated with either serotype 5, the homologous serotype, or serotype 1, the heterologous serotype, under light

anesthesia. Five of the outer membrane vaccinated and five control pigs were challenged with a 50% lethal dose of App I-178 (5×10^7 CFU in 10 ml of saline) and the rest of the experimental animals were challenged with a 50% lethal dose of App 27088 (5×10^6 CFU). The 5 commercial vaccinates were challenged with a 50% lethal dose of App 27088 (5×10^6 CFU). Clinical signs (rectal temperature, respiration rate, appetite, and depression) were assessed repeatedly after challenge and the severity of pneumonia plus pleuritis were estimated at necropsy.

Clinical signs observed in pigs after experimental challenge were graded as follows: 0 = no clinical signs; 1 = mild, off-feed, depressed, increased respiration rate for 3-4 hours;
2 = mild-moderate signs for 4-12 hours and 0% mortality;
3 = moderate clinical signs for 12-24 hours, 0% mortality;
4 = moderate-severe clinical signs for 24-36 hours, 0-50% mortality; 5 = severe clinical signs for more than 36 hours, severe weight loss, and greater than 50% mortality in other pigs.

Percent pneumonia and pleuritis of all the seven lung lobes were estimated at necropsy by dissecting the lung lobes and estimating the percent damage to each lobe. This data was inserted into a formula that weighs the contribution of each lobe with the following values: left cranial = .04, left middle = .09, left caudal = .25, accessory = .05, right

cranial = .07, right middle=.15 and right caudal= .35.

Animals that died during the course of the experimental challenge were necropsied immediately, survivors were euthanized and necropsied 7 days post infection.

The challenge inocula were prepared as follows: An overnight culture of App on BHI + V agar was used to inoculate 30 ml of BHI + V broth. The broth culture was incubated at 37^{o}C with rapid shaking for 3-4 hours, to mid-to late log phase. Optical density was measured and cfu/ml estimated by comparison to a standard curve. Cells were harvested by centrifugation, washed once and diluted with sterile saline.

RESULTS

Nine of the 10 pigs vaccinated with the outer membrane, the 5 commercial bacterin vaccinated pigs, and the 9 control pigs were evaluated for serum antibodies to App by ELISA and CF 6 weeks post vaccination as shown in Table 2.

Geometric mean ELISA serotiters of outer membrane vaccinated SPF pigs were two-fold higher against serotype 5 App than those of whole cell bacterin immunized SPF pigs. However, both the outer membrane and bacterin vaccinated pigs showed extremely high titers compared to the control pigs. Analysis of variance of ELISA titers using least square differences (LSD) indicates that there was a significant titer difference between the outer membrane vaccinated and commercial bacterin vaccinated pigs ($p < 0.05$).

Geometric mean ELISA serotiters of the outer membrane vaccinated pigs were about three-fold higher against the homologous serotype outer membrane (serotype 5) than titers against the heterologous serotype outer membrane antigens (serotype 1). This was a statistically significant titer difference as compared using the paired t-test. In contrast, the mean ELISA endpoint titers of the commercial bacterin vaccinated SPF pigs against both serotype 1 and 5 were not statistically different from one another.

Complement fixation serotiters six weeks after vaccination also showed a marked increase in titers of vaccinates over the control pigs (Table 2). The geometric

mean complement fixation titer of bacterin immunized pigs was about twice that of outer membrane vaccinates, although analysis of variance showed no significant difference between the vaccinate groups at 6 week post-vaccination. The range of titers in both vaccinate groups was from 1:32 to 1:128.

Table 2: Comparisons of ELISA and complement fixation (CF) App serotiters in pigs six-weeks after vaccination.

		Serotiters[a]		
		ELISA[a]		CF
Group	No.pigs	Sero. 5	Sero. 1	
OM-Vacc[b].	9	6110±41 (3840-7680)	1780±45 (960-3840)	59±6 (32-128)
Bacterin[c]	5	2910±45 (1920-3840)	2205±41 (1920-3840)	97±2 (32-128)
Controls	9	70±41 (60-120)	64±38 (60-120)	0±0

[a] Titers presented as reciprocal geometric mean ± 1 standard deviation, with range in titers in parenthesis.

[b] Purified outer membrane of App serotype 5 (5mg) in Freunds' incomplete adjuvant administered three times at two-week intervals.

[c] Vaccinated twice at three-week intervals with a commercial bacterin containing App serotypes 1, 5 and 7.

Six-week ELISA and CF serotiters of the commercial bacterin vaccinated animals from the 3 vaccination trials were compared (Table 3). Within each trial group, the geometric mean ELISA titers were essentially the same against both serotype 1 and serotype 5 outer membrane antigens. In contrast, the geometric means of ELISA titers of the two SPF groups against both serotypes were significantly greater than those of non-SPF animals. Among the non-SPF group, the two pigs with the lowest ELISA titers before vaccination had the highest titers 6 week post-vaccination.

Table 3: Comparison of serotiters of pigs from three vaccination trials vaccinated with a commercial bacterin[a].

		Serotiters[a]		
		ELISA[a]		CF
Group	No.pigs	Sero. 5	Sero. 1	
FF4/BC2	5 (SPF)	2910±45 (1920-3840)	2205±41 (1920-3840)	97±2 (32-128)
BCHpp3	3 (SPF)	3840±0 (3840)	3840±0 (3840)	128±0 (128)
MSU86	6 (non-SPF)	1358±54 (960-3840)	1210±43 (960-1920)	43±6 (0-64)

[a] Vaccinated twice at three-week intervals with a commercial bacterin containing App serotypes 1,5 and 7.

[b] Titers presented as reciprocal geometric mean ± 1 standard deviation, with range in titers in parenthesis.

Figure 3 illustrates the serum antibody response of App serotype 5 outer membrane vaccinated pigs over 6 weeks post-vaccination as measured by ELISA assay. The serotiters of all pigs ranged from 60 to 120 before vaccination. Cross-reactions were observed between the two serotypes. Serotiters against serotype 5 antigens were greater than those against serotype 1 antigens, even though they were parallel. Homologous titers ranged from 3840 to 7680 and heterologous titers from 960 to 3840 through weeks 3 to 6. Titers of vaccinates showed a marked increase reaching maximum levels at week 3 post vaccination and remaining constant for 6 weeks thereafter, while titers of control pigs showed no increase throughout the six weeks.

The immune responses of the 5 commercial vaccine immunized SPF pigs over time are summarized in Figure 4. The pigs showed equivalent titers to both serotypes throughout all 6 weeks. The ELISA titers against serotype 5 were lower than those of outer membrane vaccinated pigs, but titers against serotype 1 were similar. Significant titer differences against serotype 5 were obtained between outer membrane vaccinated and bacterin vaccinated groups except at week 5 when compared using LSD test. Unlike the outer membrane-vaccinated pigs, commercial vaccine immunized pigs differed significantly in their responses within the group. Two pigs showed a drastic rise in titers by week 2 post-vaccination while three pigs demonstrated gradual rise in

titers reaching peak level (week 5) after booster injection. A slight decline in titers was observed by week 6.

Complement fixation tests on sera from weeks 2, 4 and 6 post vaccination showed that the outer membrane vaccinated pigs had no CF titers on week 2, whereas the bacterin vaccinated pigs had titers ranging from 1:32 to 1:64. All vaccinates showed titers of 1:64 to 1:128 on weeks 4 and 6. In contrast, ELISA titers of outer membrane vaccinated pigs against serotype 5 ranged from 480 to 960 after 1 week post-vaccination, whereas no CF titers were observed. The ELISA titers also ranged from 3840 to 7680 on weeks 4 and 6 after vaccination. Regression analysis has shown a positive correlation between the ELISA and CF tests ($r = .96$).

The pattern of increase in antibody titer over time in sera of the 6 non-SPF pigs immunized with the commercial vaccine is shown in Figure 5. The purpose of including these animals in this study was to compare serological immune responses of non-SPF and SPF animals. The titers of these pigs before vaccination were greater than those of SPF pigs, but post-vaccination titers were lower than those reached by SPF pigs. Almost the same titers were found against both serotype antigens. More variation of titers was shown within this group than those of SPF pigs. Individual titers ranged from 480 to 3840. Sera samples from two pigs (33.3%) with low titers (120 or 240) before vaccination dramatically increased titers to 3840, whereas four pigs (66.7%) initially with

relatively high titers (480) increased slightly up to 960 after subsequent vaccination. Titers rose progressively and reached maximum level at the fourth week post vaccination and a slight fall in titers was seen in eight weeks post vaccination.

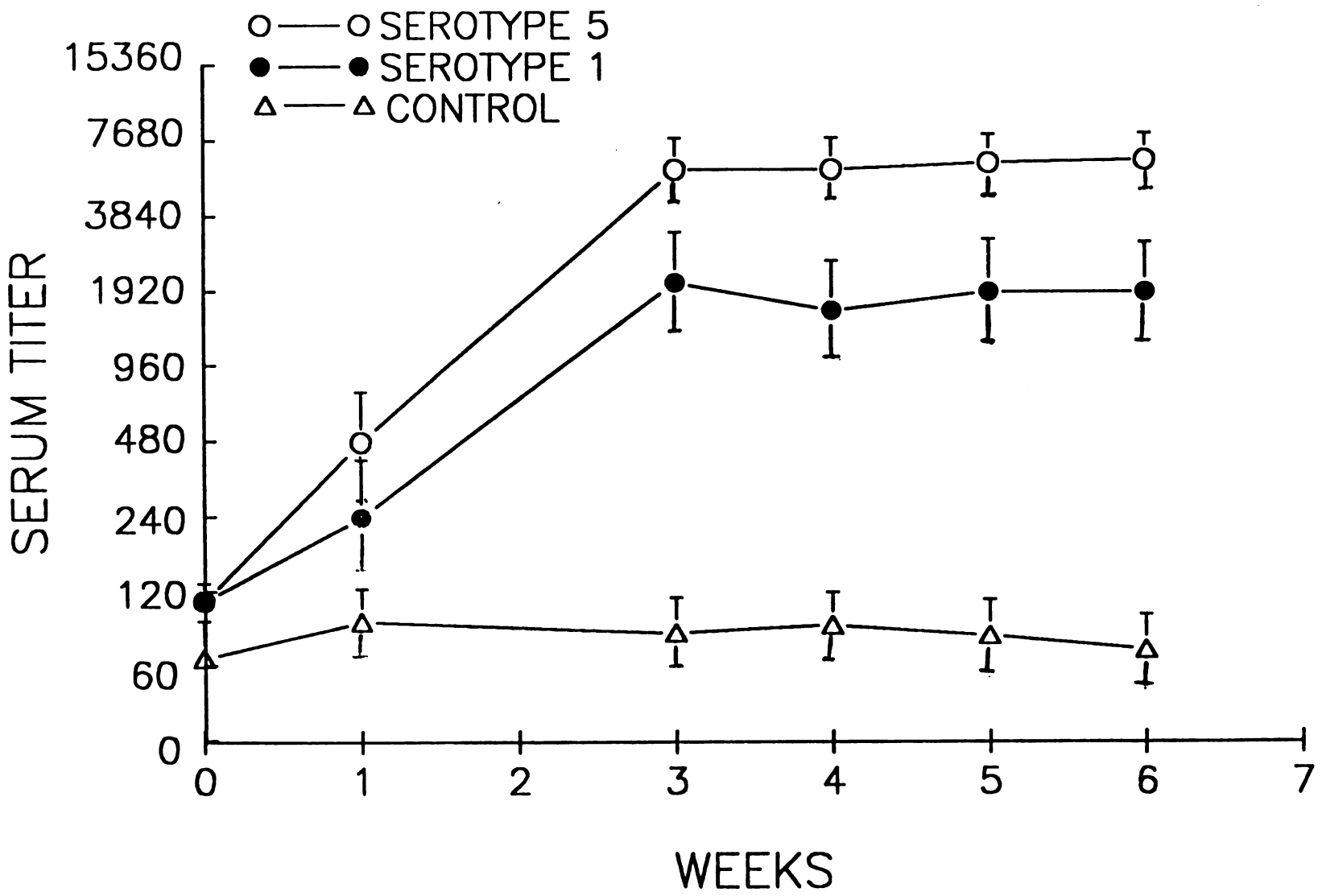

Figure 3: Serum immune responses of App serotype 5 outer membrane subunit vaccinated SPF pigs against serotype 1 and serotype 5 outer membrane antigens.

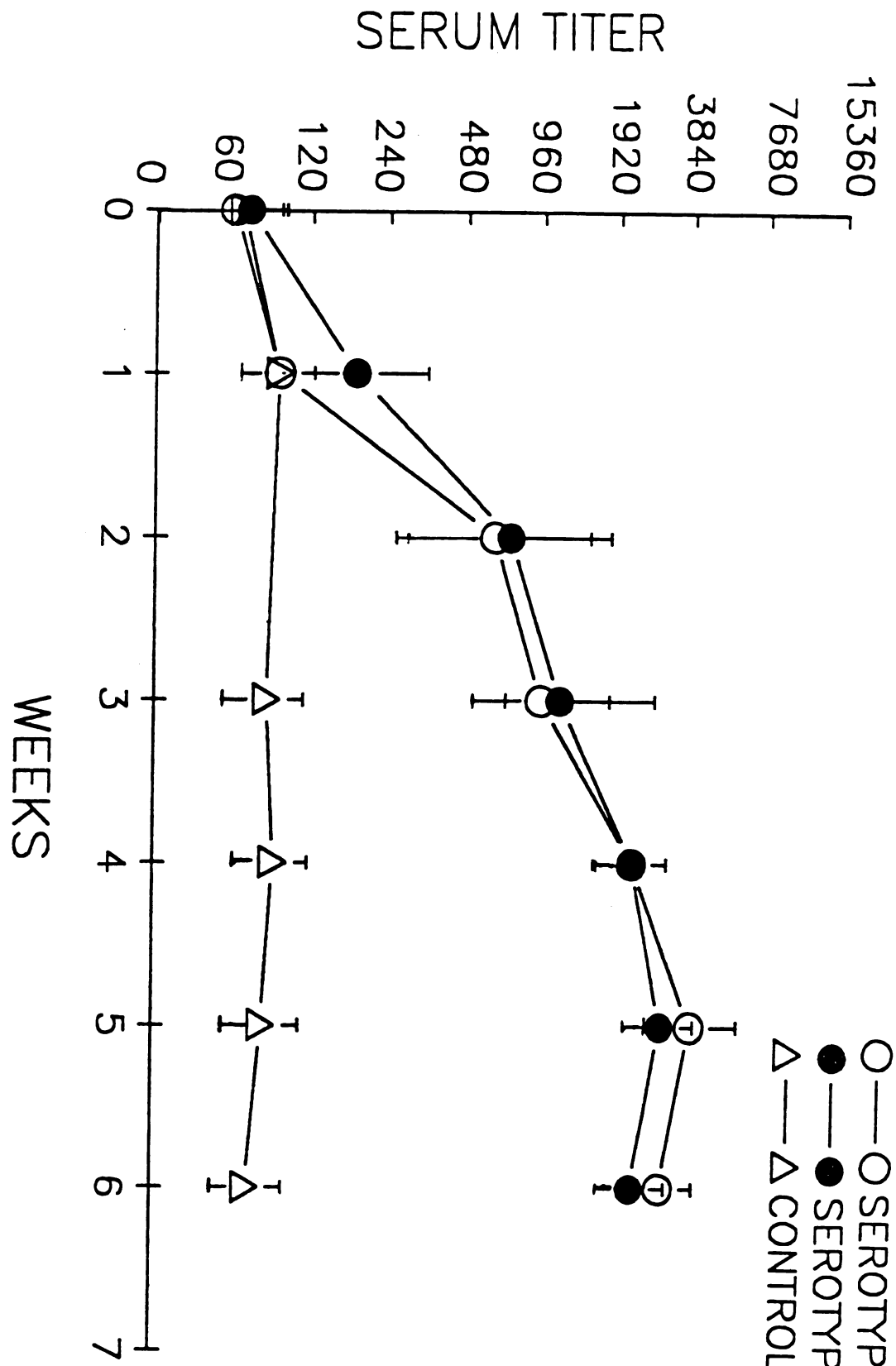

Figure 4: Serum immune responses of commercial vaccine immunized SPF pigs against serotype 1 and serotype 5 outer membrane antigens.

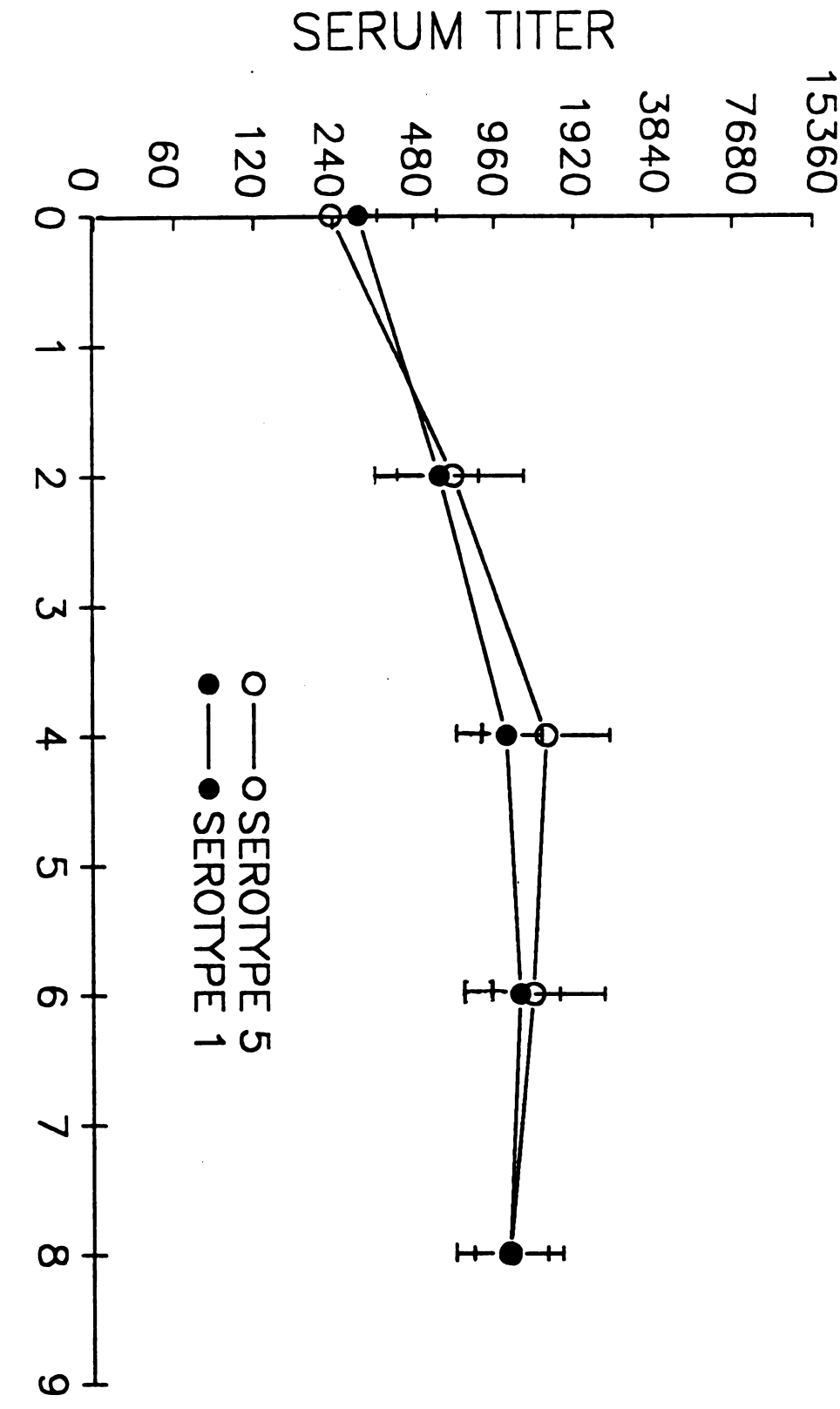

Figure 5: Serum immune responses of commercial vaccine immunized non-SPF pigs against serotype 1 and serotype 5 outer membrane antigens.

Comparison of ELISA serotiters and percent pneumonia after challenge is shown in Table 4. The App serotype 5 outer membrane (OM) vaccine afforded complete protection against mortality and significant protection against morbidity in this trial. Despite challenge with 50% lethal doses of either serotype 1 or serotype 5, none of the OM vaccinated animals died. Clinical signs ranged from mild to moderate, with more severe signs in the animals challenged with serotype 1 than in those challenged with seroytpe 5. The mean percent pneumonia in the OM vaccinated animals was 6.4±10. However, the average percent pneumonia was 0.95±1.36 in serotypes 5 challenged pigs as compared to 11.78±10.7 in serotype 1 challenged pigs.

Analysis of variance using LSD indicates that there was a significant difference in lung score between the homologous and heterologous serotype challenged pigs.

ELISA titers of these pigs at challenge ranged from 3840 to 7680 against serotype 5 and 960 to 3840 against serotype 1. There was no direct correlation between titer at challenge and severity of clinical signs or pneumonia.

In contrast, 50% of the controls died within sixty hours after challenge, and all showed moderate to severe clinical signs such as high temperature, respiratory distress, anorexia and depression. None of the control pigs had detectable titers to App serotypes at challenge. The surviving control pigs had a mean percent pneumonia of 48±28

when necropsied 7 days after challenge, which is about 8 times that of outer membrane vaccinated pigs. Lung scores and clinical signs in the control pigs were similar with both challenge serotypes.

Seven of the 8 whole cell bacterin vaccinated SPF pigs from the 2 vaccination trials challenged with serotype 1 had mean percent pneumonia of 24.4±33 at necropsy (Table 5). The mean percent pneumonia of the bacterin vaccinated pigs was about half of the controls. The mean percent pneumonia of trial BCHpp3 animals was 34.7±43 and those of FF4/BC was 11.4±20. Significant variation in lung lesions or clinical signs was observed among the vaccinates, but there was no correlation between ELISA titer at challenge and severity of infection.

Table 4: ELISA serotiters and protection against experimental challenge in pigs immunized with purified outer membranes of App serotype 5 in Freund's incomplete adjuvant.

Pig#	Group	ELISA titer at challenge[a] Sero.5	Sero.1	Challenge serotype[b]	Percent pneumonia	Clinical score
10	OM-Vacc.	3840	960	5	0.0	1
11		3840	1920	5	0.0	3
12		7680	960	5	0.0	1
13		7680	1920	5	3.5	2
4		7680	3840	5	1.25	3
14		ND	ND	1	4.75	2
3		7680	3840	1	25.85	3
7		7680	1920	1	23.6	4
8		7680	1920	1	4.7	2
9		7680	1920	1	0.0	2
1	Control	60	60	5	72.2	5
2		120	120	5	3.7	5
5		60	60	5	19.4	4
6		60	60	5	60.2	5
15		60	60	5	59.0	5
23		60	60	1	44.0	4
35		60	60	1	86.8	5
50		60	60	1	18.5	3
55		60	60	1	63.3	5

[a] Titers reported as reciprocal, with serotype 5 and 1 antigens used in assays.

[b] Challenge dose = 50% lethal dose of serotype 5 or of serotype 1; administered by percutaneous intratracheal injection.

ND = not done

Table 5: ELISA serotiters and protection after experimental challenge in pigs vaccinated with a commercial whole cell bacterin containing App serotypes 1, 5 and 7.

Pig#	Group	ELISA titer at challenge Sero.1	Challenge serotype[a]	Percent pneumonia	Clinical score
24	BCHpp3	3840	1	16.35	3
25	BCHpp3	3840	1	84.0	5
33	BCHpp3	3840	1	3.60	1
27	FF4/BC	1920	1	2.5	1
30	FF4/BC	1920	1	1.63	1
42	FF4/BC	3840	1	41.50	2
44	FF4/BC	1920	1	M	M
51	FF4/BC	1920	1	0	1

[a] 5×10^6 CFU of serotype 1 administered by percutaneous intratracheal injection.

M = missing

DISCUSSION

The results indicate that following immunization with adjuvanted App serotype 5 outer membrane or whole cell bacterins, all pigs developed significant antibody titer compared to those pigs given saline solution (Table 2). Vaccination with outer membrane antigens induced higher antibody responses than did vaccination with bacterins as reflected by higher ELISA titers in pigs of the same group.

The apparent higher relative ELISA serotiters of outer membrane vaccinated pigs over the whole cell bacterin immunized pigs may be due to : a) use of the same antigen preparation for coating ELISA plates and vaccine preparation, b) greater stimulation of immune system of the outer membrane vaccinated pigs boosted three times compared to bacterin immunized pigs boosted twice, c) use of partially purified cell wall components that may contribute to production of antibodies against capsule, LPS and outer membrane proteins, d) use of Freunds' incomplete adjuvant in outer membrane subunit vaccine, which may more stimulate the animals immune system more than the adjuvant used in the bacterin, or e) variation of vaccine doses used in injection.

Immunization with the serotype 5 OM vaccine raised antibody titers against both serotype 5 and serotype 1. However, homologous titers (against serotype 5) were always higher than heterologous titers (against serotype 1), probably due to the immune response to serotype-specific

antigenic determinants in serotype 5, such as capsular polysaccharide. The cross-reactivity was verified in our laboratory by Western blot analysis. The OM vaccinated animals produced antibodies against cross-reactive outer membrane proteins with estimated molecular weights of 16-18, 29, 38-42, and 55 kDs and against lipopolysaccharides of other App serotypes (72). However, antibody to capsular polysaccharide was specific for serotype 5. This indicates that there is a high level of antigenic similarity of lipopolysaccharides and outer membrane proteins between the serotypes while capsule is serotype-specific antigen.

The data on the time-course of antibody responses indicated that the outer membrane vaccinated animals produced antibodies rapidly as reflected by significant titers during week 1 post-vaccination. The rise in antibody level was relatively slow and variable among the 5 bacterin vaccinates compared to the outer membrane vaccinated animals. There was no significant decline, especially in homologous titers of the outer membrane vaccinated pigs. Western blot analysis has demonstrated that antibodies raised against outer membrane proteins are persistent at high levels for at least 6 weeks. The slight decreasing trend observed in ELISA titers towards the end of the 6 week study is possibly due to elimination of short lived antibodies, for example, to LPS, as indicated by Western blot analysis in our lab.

Bacterin immunized groups showed virtually identical

antibody responses over the 6-week period for both serotype 1 and serotype 5, possibly because the vaccine was composed of equal amounts of antigens of the respective serotypes. Bacterins mainly raise antibodies against capsular polysaccharides (67) or lipopolysaccharides (61) and as a result complement fixation showed higher titers in sera of whole cell bacterin immunized SPF pigs. The quantity of capsular material is relatively low in outer membrane preparation compared to the bacterin content. The reason for variation in the serological responses observed among the bacterin immunized pigs is not clear, but could be due to several factors, including variation in the immune status of each animal at the start of each experiment. The same reasoning may hold true regarding the variation observed between the bacterin vaccinated SPF pigs from the 2 vaccination trials. In addition, these trials were conducted using diffrent production lots of vaccine and the experimental animals were from 2 different herds.

The non-SPF pigs showed lower ELISA and complement fixation titers than the SPF pigs 6 week post-vaccination (Table 3). The data suggests that low levels of cross-reacting antibodies found in many non-SPF pigs block the ability to respond maximally to App vaccines. This conclusion is supported by our study demonstrating that passive immunity blocks response of piglets to vaccination (30).

The analysis of the protective efficacy of the vaccines shows that immunization of pigs with either OM vaccine or commercial whole cell bacterin completely prevented mortality when the vaccinates were challenged with a 50% lethal dose of either the homologous or the heterologous App serotype. On the other hand, nonvaccinated pigs infected with the same doses had 50% mortality of the animals. Vaccination, however, did not prevent development of lesions in the vaccinates.

Analysis of variance using LSD has revealed that the controls and vaccinates had significantly different percent pneumonia and severity of clinical signs. Furthermore, the homologous serotype challenged pigs and the heterologous serotype challenged pigs showed a significant difference in percent pneumonia ($p < 0.05$).

The findings indicate that protection of pigs from mortality or reduced lung lesion development depended upon the high magnitude of the serum titers after vaccination compared to the controls. However, some pigs with maximum titers showed significant lung lesions while others with relatively lower titers had insignificant lung scores. A negative correlation between percent pneumonia and ELISA titers, ($r = -0.57$) or complement fixation titers ($r = -0.61$) was found by simple regression analysis. There was also a negative correlation between ELISA titers and clinical signs. Considering the survival rate, there was a correlation

between the ELISA titers and protection efficacy of the vaccines. In general, a titer of 1920 or higher affords significant protection compared to controls. In comparison, data from other laboratories suggests that a complement fixation titer of 1:32 is indicative of a protective level of antibody.

This investigation supports previous studies that demonstrated vaccination with App bacterins (49-52) or R mutant of E. coli J5 (61-63) or capsule (59) confer partial protection against App challenge (49-52,59-61,63). In this investigation, however, the clinical signs and extent of lung lesions noticed in vaccinates due to challenge were less severe compared to those observed in previous studies (49-52).

The homologous serotype challenged App serotype 5 outer membrane subunit vaccinates showed the least clinical signs and insignificant lung lesions. This may be due to protection provided by serotype-specific capsular polysaccharide antibodies. The reason heterologous serotype challenge caused relatively high percent pneumonia and increased severity of clinical signs could be due to the antigenically distinct capsules of serotype 1 and of serotype 5. The serotype-specific anticapsular antibodies might not have an effect on the encapsulated heterologous serotype. Thus, the bacteria was able to establish itself in the lung. On the other hand, cross-reactive antibodies to lipopolysaccharides or outer

membrane proteins might have a role in recognizing cross-reactive antigenic determinants of the challenge heterologous serotype. As a result, less severe clinical signs and reduced lung lesions were manifested. Similar findings demonstrated that experimentally induced immunity to serotype 2 was protective against challenges with serotypes 1, 4 and 5 (52).

The mechanism of protection against App infection afforded by humoral immunity in the vaccinates could be due to the involvement of the antibodies in opsonization, or in increased complement-mediated bacteriolysis, or directly in agglutination of the infectious agent (60,61). Cellular immunity induced by vaccination also has a major role in protection from App infection, even though it is a lower response than cellular immunity induced by infection (73,74).

In summary, the results of this study are: Pigs vaccinated with adjuvanted outer membrane antigens or the commercial bacterin vaccinated pigs raised antibodies against serotypes 1 and 5 surface antigens. Using the sensitive and reproducible ELISA procedure, it was possible to quantitate levels of serological responses. Outer membrane vaccinated pigs mounted higher ELISA antibody titer than the bacterin immunized pigs. The antibodies produced in all vaccinates persisted for at least 6 weeks with slight decreases. The ELISA procedure was able to detect low levels of antibodies 1 week after vaccination not detectable by complement fixation.

ELISA and CF tests showed a correlation in detection of antibodies against App outer membrane antigens. All vaccinates were completely prevented from mortality, whereas 50% of the nonvaccinated pigs died due to a challenge with a 50% lethal dose of serotype 5 or serotype 1. Outer membrane vaccinated pigs developed less severe lung lesions than did bacterin immunized pigs indicating that the outer membrane antigen subunit vaccine has a better protective efficacy than the commercial bacterin. There was also cross-protection of pigs against heterologous challenge suggesting that antibodies to cross-reactive outer membrane antigens, such as outer membrane proteins and lipopolysaccharide, can provide significant protection against App infection.

CHAPTER III

EVALUATION OF IMMUNE RESPONSES OF APP INFECTED PIGS

The following study was initiated to analyze the serum immune responses of pigs surviving experimental infection with different dosages of a virulent strain of App serotype 5 or serotype 1. Relationships between the inoculated dosages, and the mortality and morbidity rates of serotype 5 infected pigs was also examined.

MATERIALS AND METHODS

Experimental design. Late log phase cultures of App serotype 5 (reference strain I-178) or App serotype 1 (ATCC 27088) grown in BHI + NAD broth were used for infection. Cells were harvested by low speed centrifugation, washed with sterile saline, and diluted in saline to obtain the desired colony forming units (cfu)/ml.

a) In one experiment, App free pigs (20-25 kg) were inoculated intratracheally under light anesthesia with either high (6×10^7 cfu) or medium (2×10^7 cfu) or low (1×10^6 cfu) dosages of App I-178 or saline. We have previously determined that the 50% lethal dose equals 5×10^7 cfu for App I-178 in this experimental model. Six pigs were used in each group. Blood samples were taken at 0, 8, 16 hours for hematological analysis. Clinical signs (rectal temperature, respiration rate, appetite, depression, and dyspnea) were assessed repeatedly after infection. The severity of clinical

signs was graded as described in chapter II. The severity of pneumonia was also estimated at necropsy as described in chapter II. Surviving pigs were bled weekly for six weeks and serum immune responses were evaluated by complement fixation, ELISA and Western blot.

b) In a second experiment, nine pigs were inoculated intratracheally under light anesthesia with either a lethal (1×10^9cfu) or high (1×10^8cfu) or medium (1×10^7cfu) dose of App 27088. We have previously determined that the 50% lethal dose equals 1×10^7 for App 27088 in this experimental model. Three pigs were used in each treatment group. Animals were monitored as described above. Among the nine pigs, 5 pigs (3 medium and 2 high dose animals) survived the infection. They were bled weekly for 6 weeks and serum immune responses were evaluated by ELISA and complement fixation.

RESULTS

The outcome of intratracheal infection of SPF pigs with various dosages of App strain I- 178, serotype 5, is summarized in Table 6. Mortality in the high, medium, and low dose groups and saline inoculated controls was 50%, 16.6%, 0%, and 0% respectively. Increased respiratory rates, reduced appetite, depression and other clinical symptoms were more pronounced in the high dose group animals than in the medium dose group, and both high and medium dose animals had more severe clinical signs and lung lesions than the low dose animals. Total leukocyte counts increased almost two-fold in all infected pigs after eight hours of infection and remained elevated, with no significant change seen between the 8^{th} hour and 16^{th} hour samples. In the controls, no difference in leukocyte counts was observed during the initial 16 hours post infection. Immature neutrophil counts markedly increased in the high and medium dose groups by 8 hours, and further increased by 16 hours after infection in the high dose group but decreased in the medium dose group. Immature neutrophils also increased in the low dose group, but neither the rate of increase nor the maximum value was as high as the high and medium dose infected groups.

Gross post mortem findings showed that all the pigs that died within 24 hours had an acute hemorrhagic fibrinous pneumonia with lesions identical to those seen in naturally App infected pigs. Pigs necropsied 9 days after infection

showed a chronic-active type of lesion. Pigs necropsied at 42 days post infection had a chronic fibrosing bronchopneumonia. A significant difference in the extent and severity of lung lesions was observed between the groups (Table 6). The average pleuritis score of the high dose group was double that of the medium dose group and twenty times higher than that of the low dose group. The average pneumonia score of the high group was also three times larger than that of the medium dose group and seven times that of the low dose group. All the control animals had 0% pneumonia and 0% pleuritis.

Figure 6 shows the pattern of antibody rise in the treatment groups after infection. ELISA titers against serotype 5 antigen increased rapidly, reaching the maximum levels by week 2 and falling slowly for the remaining 4 weeks, especially in the high and medium dose groups. The mean serotiters versus serotype 5 (homologous titers) of the high and medium dose animals were similar throughout the 6 weeks study, and were about 4 fold higher than mean titers of the low dose group at corresponding time points. Analysis of variance using LSD shows no statistically significant difference in homologous serotiters between the high and the medium dose groups; however, both were statistically different from the low dose and control animals. The low dose and the control animals were also statistically different in titer from each other.

Heterologous titers (versus serotype 1 antigens) were

lower than homologous titers in all infected groups, as illustrated in Figure 7. Mean heterologous titers of the high dose group were statistically greater than those of medium group throughout the study, in contrast to titers against serotype 5, where both treatment groups demonstrated similar responses. Indeed, mean heterologous titers were statistically different for the high, medium, low, and control groups at most time points,with higher heterologous titers observed with the corresponding larger bacterial doses.

Geometric mean complement fixation serotiters of all the treatment groups are shown in Table 7. All pigs had no detectable CF titer before experimentally induced infection. The high dose and 2 pigs of the medium dose groups had significant CF titers by week 2 post infection, with the former showing a statistically higher mean titer than the latter, because one pig among the medium dose group had no CF titer at all. The CF titers of the high dose group animals were one fold higher than the titers of the medium dose animals. As a result, there was a positive correlation between the ELISA titers and complement fixation titers only in the high dose group. The titers of the high dose animals remained elevated at 4 and 6 weeks after infection. All the low dose group animals and the controls, however, showed insignificant titers (<1:8) throughout the study.

Table 6: Clinical and hematological outcomes of experimental infection of SPF pigs with various dosages of App serotype 5[a].

Parameters	Inoculating dose			
	High	Medium	Low	Control
Mortality	3/6	1/6	0/6	0/6
%Pleuritis	53±32	24±20	2.2±4	0
%Pneumonia	53±24	18±17	7.2±16	0
Clinical score	4	3	0	0
Total Leukocytes[b] after infection.				
0 hrs	20.2	22.9	29.3	24.5
8 hrs	38.3	46.9	39.8	24.0
16 hrs	41.9	44.0	43.3	22.9
Immature Neutrophils[b] after infection.				
0 hrs	78.0	181.8	54.5	55.8
8 hrs	4514.2	4386.8	169.2	263.7
16 hrs	5608.8	2450.3	919.7	ND

[a] Bacteria were inoculated by percutaneous intratracheal injection. Inoculating doses were: high = 6×10^7 cfu, medium = 2×10^7 cfu, low = 1×10^6 cfu and control = 10 ml of saline.

[b] Counts x 1000/ mm^3

ND = not done

Table 7: Complement fixation serotiters six weeks after experimental infection of SPF pigs with various dosages of App serotype 5.

Weeks after infec.	Serotiters[a] by inoculating dose[b]			
	High	Medium	Low	Control
0	0	0	0	0
2	64±0	21.3±18	1±2	0
4	48±22	26.6±33	1±2	0
6	80±67	26.6±33	4±8	0

[a] Titers reported as geometric mean ± 1 standard deviation.

[b] High = 6×10^7 cfu, medium = 2×10^7 cfu, low = 1×10^6 cfu, and control = 10 ml of saline.

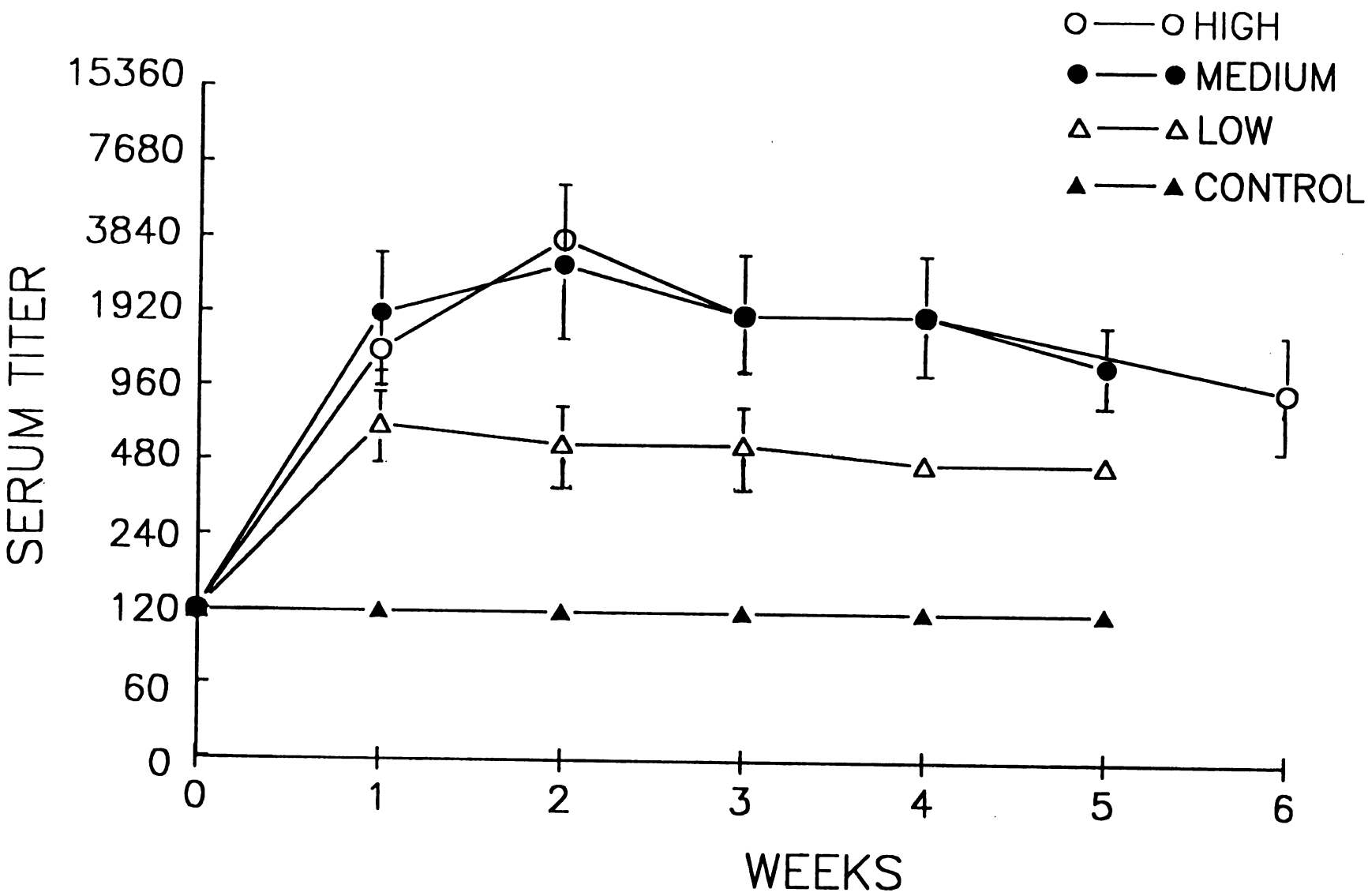

Figure 6: Serum immune responses of various doses of serotype 5 infected pigs to serotype 5 outer membrane antigens.

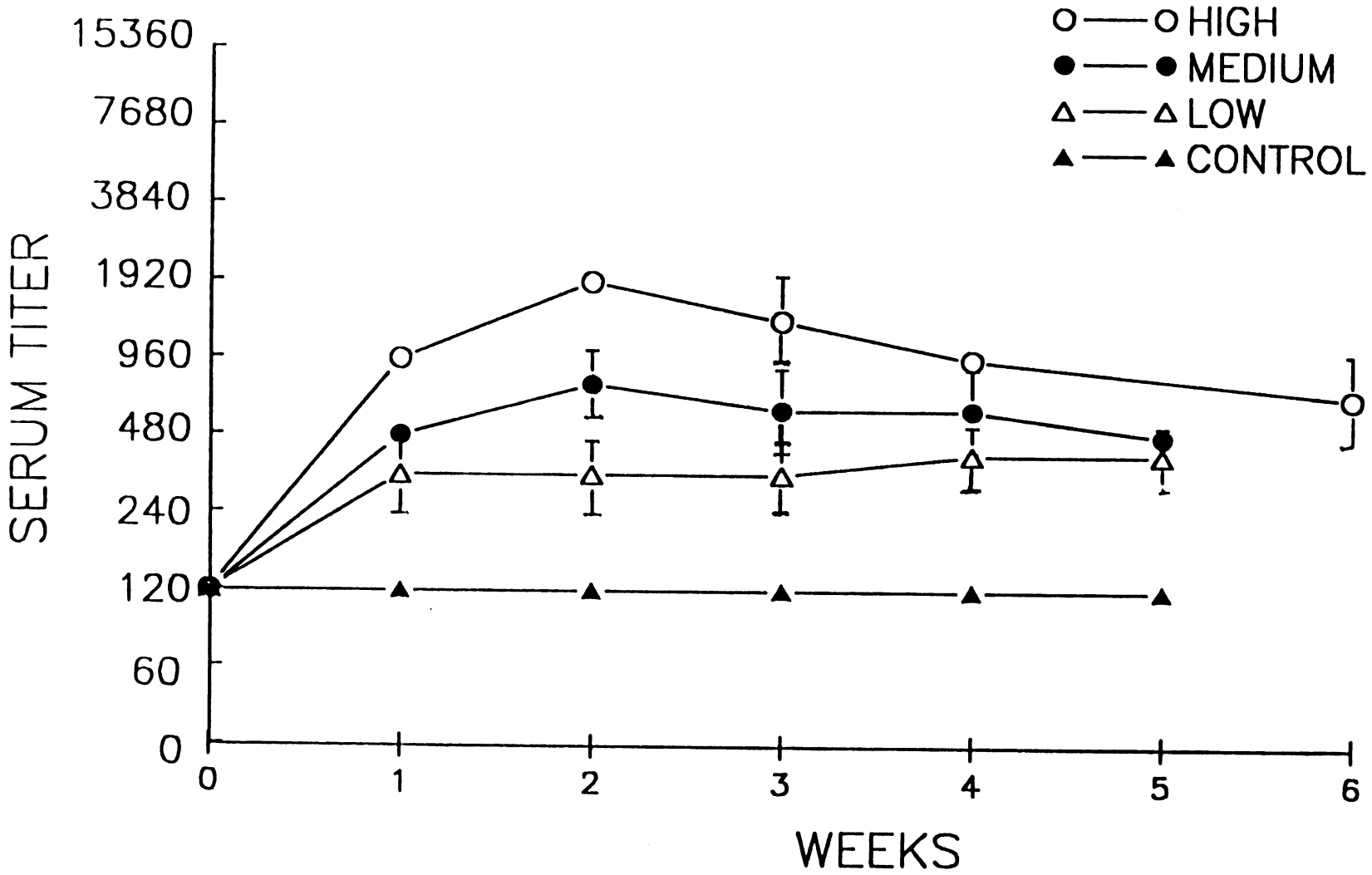

Figure 7: Serum immune responses of various doses of serotype 5 infected pigs to serotype 1 outer membrane antigens.

ELISA and complement fixation serotiters of pigs infected with App serotype 1, 6 week post-infection, are shown in Table 8. All the survivors of serotype 1 infection showed significant serotiters against both serotype 1 and 5 antigens. The geometric mean ELISA titer against serotype 1 of the high dose animals was slightly higher than the mean of the medium dose group. Both groups showed the same mean ELISA titers against serotype 5. In both groups, homologous ELISA titers were at least double the heterologous titers. The geometric mean complement fixation titer of the high dose group animals was also higher than the mean of the medium group animals(Table 8).

Serum immune response of one pig among the medium dose App serotype 1 infected animals is illustrated in Figure 8. ELISA titers against both serotype 1 and 5 antigens increased progressively reaching the maximum levels by week 4 after infection and then declining slightly. Homologous titers (versus serotype 1 antigens) were greater than heterologous titers (versus serotype 5 antigens) throughout the 6 weeks of the study.

Serum immune response of one of the high dose App serotype 1 infected pigs is also shown in Figure 9. The homologous ELISA titers of the high dose animal were greater than that of medium dose animal. However, the heterologous titers were not statistically different between the high and the medium dose animals. The same pattern of antibody titer

rises or declines were noticed throughout the 6 weeks in both pigs.

Table 8: ELISA and CF serotiters six weeks after experimental infection of pigs with various dosages of App serotype 1.

		Serotiters[a]		
		ELISA[a]		CF
Dose[b]	No.pigs	Sero. 1	Sero. 5	
Medium	3	1920±0	960±0	51±8
High	2	2715±49	960±0	91±16

[a] Geometric mean ± 1 standard deviation. Serum samples were assayed in triplicates and repeated 3 times on different days.

[b] Medium = 7×10^6 cfu; High = 7×10^7 cfu.

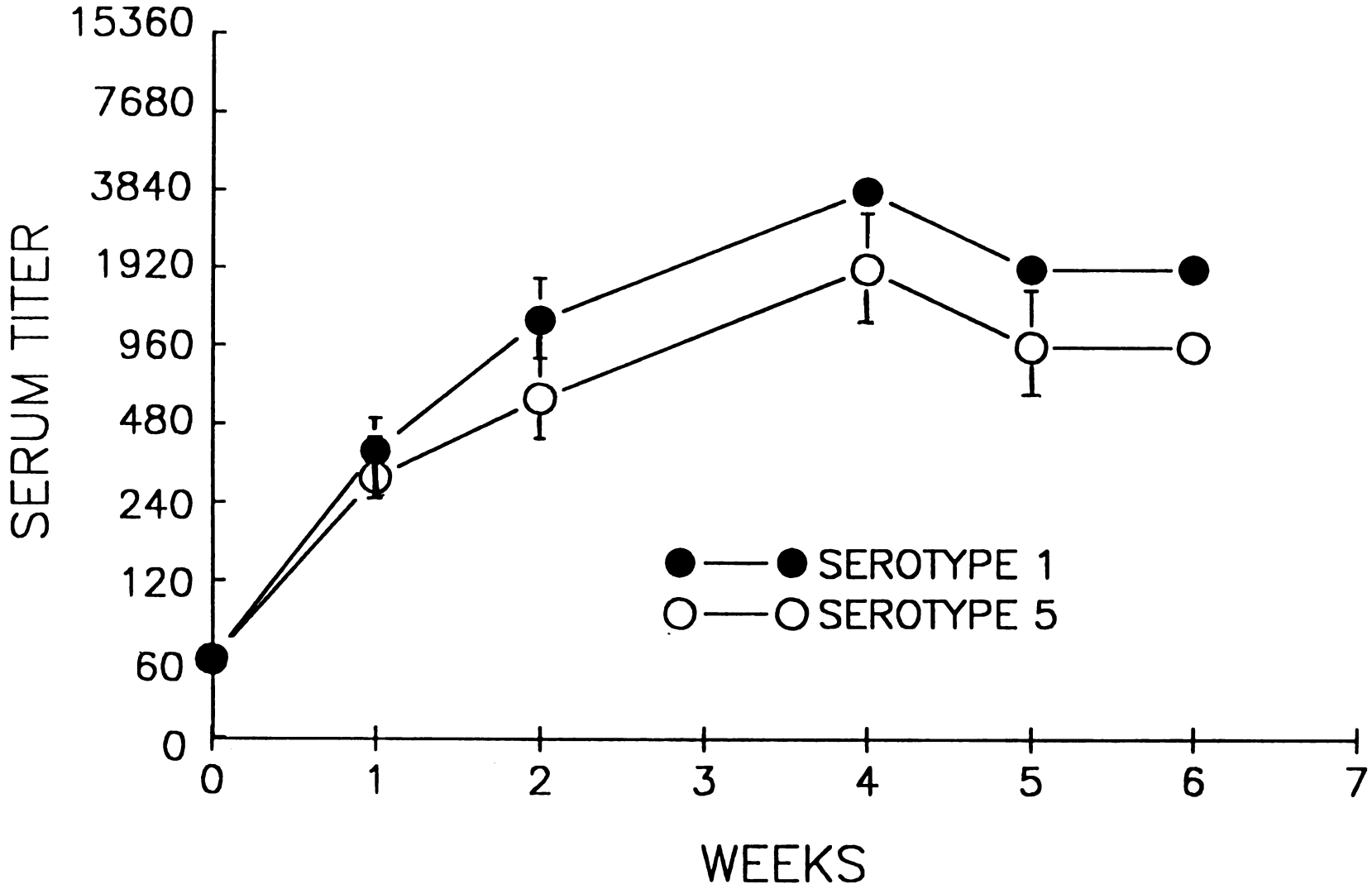

Figure 8: Serum immune responses of an App serotype 1 infected pig against serotype 1 and serotype 5 outer membrane antigens. Samples assayed in triplicates and repeated 3 times on different days.

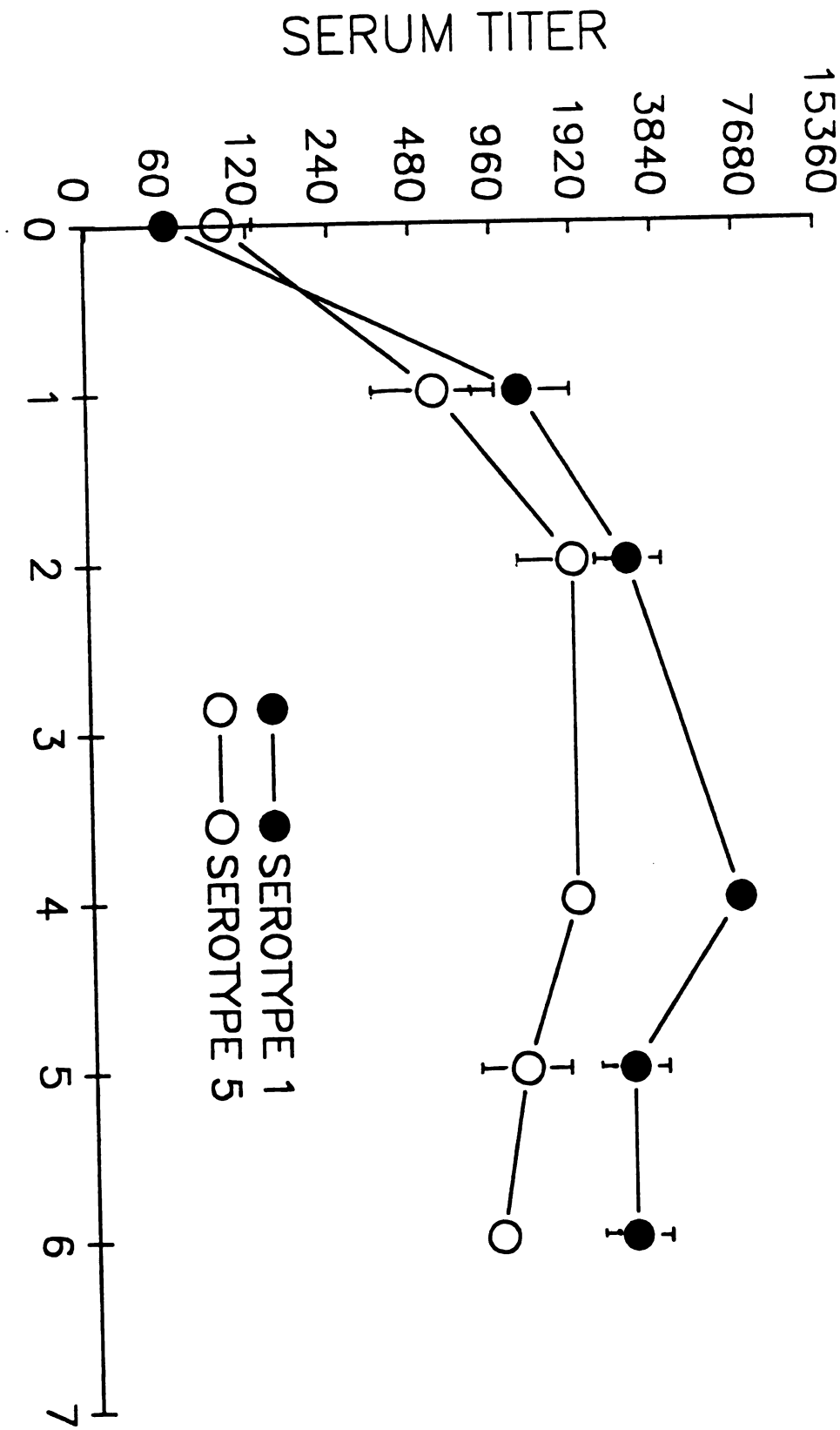

Figure 9: Serum immune responses of an App serotype 1 infected pig against serotype 1 and serotype 5 outer membrane antigens. Serum samples assayed in triplicates and repeated 3 times on different days.

DISCUSSION

The results indicate that there was a direct correlation between the dosage used in these experimental infections and the severity of the induced disease. This direct relationship was also reflected by the increases in leukocyte and neutrophil counts in the high dose group animals compared to those of the medium or low dose animals. Mortality rates, severity of clinical signs, and extent of lung lesions, increased with each increase in dose.

There could be wide variation in results of experimentally induced App infections depending on differences in virulence of App strains, route of inoculation, dose and preparation of inocula, and immune status of experimental animals. Despite these factors, the findings of the present study were consistent with other findings obtained in previous works (32,34-35,48). For example, development of the acute forms of lung lesions in the high dose group animals were consistent with previous results observed in pigs infected with App serotype 5 (32). Also, Rosendal et al. (48) reported that a 10^8 cfu of App serotype 1 lead to acute lethal porcine pleuropneumonia, whereas 10^7 to 10^4 of the same culture developed the chronic nonlethal disease in pigs.

The roles of different virulence factors in the pathogenesis of App were not investigated in this study, but other workers have shown that App endotoxin (34,61,63), heat-

labile extracellular hemolysins(34,35), and heat-stable, extracellular hemolysin (36-42) all contribute to the development of lung lesions. Endotoxin-macrophage-interaction and macrophage-generated chemotactic factors to neutrophils are believed to be responsible for the increases in leukocyte and neutrophil counts (34).

The serological responses of the animals showed a good correlation to the inoculating doses. The higher ELISA serotiters observed in the high and medium dose groups compared to the low dose App serotype 5 infected pigs suggest that retention and presentation of greater amounts of App surface antigens to the immune systems of the animals might have taken place resulting in production of a large quantity of antibodies. High quantities of antibodies reflected by high ELISA titers were verified by Western blotting analysis. Both the high and medium dose groups had strong antibody responses to the 16-18, 29, 38-42, 55, and 94 kD outer membrane proteins, the LPS, and to capsular polysaccharide of serotype 5. The low dose pigs, however, only responded to the 16-18 and 29 kD proteins and weakly to LPS and serotype 5 capsule.

The immune responses of the three treatment groups were similar in the pattern of increases and decreases of antibody titers over time, the main difference being in quantity of antibodies, which were dependent on inoculation doses. The maximal levels of antibody responses exhibited at week 2 by

ELISA were verified by Western blot which showed responses to LPS and capsule were strongest at week 2.

Like the antibody responses detected in outer membrane vaccinated animals discussed in chapter II, serum antibodies to outer membrane antigens appeared within one week post-infection, increased for 2-4 weeks, and persisted with a slight decrease for at least 6 weeks. The decline in antibody levels was relatively high compared to those of vaccinates. However, the vaccinates received two booster injections that could have stimulated a longer lasting secondary immune response.

The results of App serotype 1 infection study, although performed with a very limited number of animals, indicated a dose-dependent relationship supporting the more complete study of pigs infected with serotype 5. There was a direct correlation between the concentration of culture inoculated and the serological responses of the surviving animals. Mortality rates and severity of clinical signs also correlated with inoculating doses in this experiment.

Infection with either serotype 5 or serotype 1 induced not only dose dependent titers against the infecting serotype but also strong cross-reactive responses against the heterologous serotype. The ELISA affords good quantitation of antibody titers against a mixture of cell surface antigens, including outer membrane proteins, LPS and capsule, but does not as yet allow for dissect the immune response to determine

titers against specific antigens. However, western blot analysis of sera from serotype 5 infected animals indicates that serum antibodies to outer membrane proteins appeared within 1 week post-infection persisted at a high level for at least 6 weeks, and cross-reacted with outer membrane proteins from serotypes 1-7. Responses to LPS were maximal at 2 weeks post-infection and cross-reacted with LPS from serotypes 2,3,4 and 5. Responses to capsular polysaccharide were maximal at 2-4 weeks post infection and were serotype specific. Similar results were seen with sera from serotype 1 infected pigs.

A major difference between ELISA and CF tests was observed, where the serological responses of the low dose App serotype 5 infected animals were not detected by CF test. The main reason could be due to low production of anticapsular antibodies in the animals so that CF test is not sensitive enough to demonstrate titers against particulate pooled antigens of three serotypes. ELISA and complement fixation tests positively correlated in detection of antibodies to App in the App serotype 1 infected pigs. Both tests showed difference in antibody responses between the groups. This observation is in agreement with only the high dose of serotype 5 infected pigs.

In summary, the experimental model of App infection produced clinical signs of porcine pleuropneumonia similar to those that occur in natural infection, in a dose dependent

fashion. Experimental infection with serotype 5 or serotype 1 elicited immune responses to a varity of outer membrane antigens. Antibody responses were maximal 2 to 4 weeks after infection and persisted with slight decrease for at least 6 weeks. Although, there was a significant correlation between the dose and the level of the ELISA antibody titers, there was no significant difference in homologous titers between high and medium dose of serotype 5 infected pigs.

Cross-reactivity between both serotypes was observed as reflected by significant heterologous titers and as verified by Western blot analysis. Antibodies were cross-reactive to outer membrane proteins and LPS. Anticapsular antibodies were serotype specific.

The ELISA test, unlike complement fixation, was sensitive enough to detect the serological immune responses of all pigs infected with various doses of App. ELISA and CF tests positively correlated in detecting antibodies against App outer membrane antigens only in high dose serotype 5 infected pigs and in sera of high and medium dose App serotype 1 infected pigs.

CHAPTER IV

EVALUATION OF PASSIVE IMMUNITY IN PIGLETS OF A SEROTYPE 5 INFECTED SOW

Colostral transfer of immunity to App in piglets and the correlation between complement fixation titers and resistance to infection was demonstrated by Nielsen (57). Passively acquired antibodies protect pigs against infections during the early part of their lives, but interfere with the development of active immunity following vaccination (72). Poor response of piglets to vaccination has been a major problem in the programs to control porcine pleuropneumonia (57,72). The duration of passive antibodies to App in piglets needs to be evaluated in order to determine the optimal time for vaccination for the development of active immunity.

MATERIALS AND METHODS

Experimental design: Nine piglets farrowed from a gilt that had been experimentally infected at 3 months of age, and reinfected at 5 months, with App serotype 5 were followed in the study. The gilt's complement fixation titer at farrowing was 1:64. The piglets were allowed to suckle and were bled at one week intervals for eleven weeks. Their serum immune responses were evaluated by complement fixation, ELISA and Western blotting analysis.

RESULTS

Geometric mean ELISA and CF serotiters of piglets are shown in Figure 10. Eight out of the nine piglets had an ELISA endpoint titer of 960 and the other pig had 480 against serotype 5 one week after birth. Mean ELISA titers of the nine piglets dropped steadily, to a low of 120-240 at 5-7 weeks. After 8 weeks, however, 7 out of the 9 piglets had increasing ELISA titers and all the piglets showed increases in ELISA titers 11 weeks after birth.

Detectable ELISA titers against serotype 1 were demonstrated in all but 1 of the piglets one week after birth. ELISA titers against serotype 1 also decreased steadily, to a low of 60 at 4-5 weeks. A rise in titer against serotype 1 was also observed in weeks 8-11 of the study, although titers were low compared to the homologous titers (Figure 10).

Comparison of geometric mean ELISA serotiters of piglets against serotype 5 using t-test showed that they were significantly different at week 1 from those of week 5 or week 11 of age ($p < 0.05$). There was also a statistically significant difference between the means of ELISA titers of weeks 5 and 11.

Eight of the 9 piglets had a CF titer of 1:128 and the other piglet had 1:64 one week after birth. A steady decrease in titers was also shown by complement fixation test. Most of the piglets had no significant complement fixation titers

after the third week from birth. Complement fixation and ELISA tests correlated in demonstrating detectable titers only during the few weeks. However, the complement fixation test did not detect any rise in titers after 8 weeks in contrast to the ELISA test (Table 9).

Table 9: Comparison of ELISA and complement fixation serum titers of piglets farrowed from an immune sow.

Week	ELISA serotiter[a]	CF serotiter[b]
1	960±42	118.5±21
3	285±49	25±4
5	202±43	13.7±7.2
8	320±53	3.2±3
11	411±55	0±0

[a] Geometric mean ± 1 standard deviation of nine piglets assayed against serotype 5 outer membrane antigens.

[b] Geometric mean ± 1 standard deviation of nine piglets tested against pooled antigens of serotypes 1, 5, and 7.

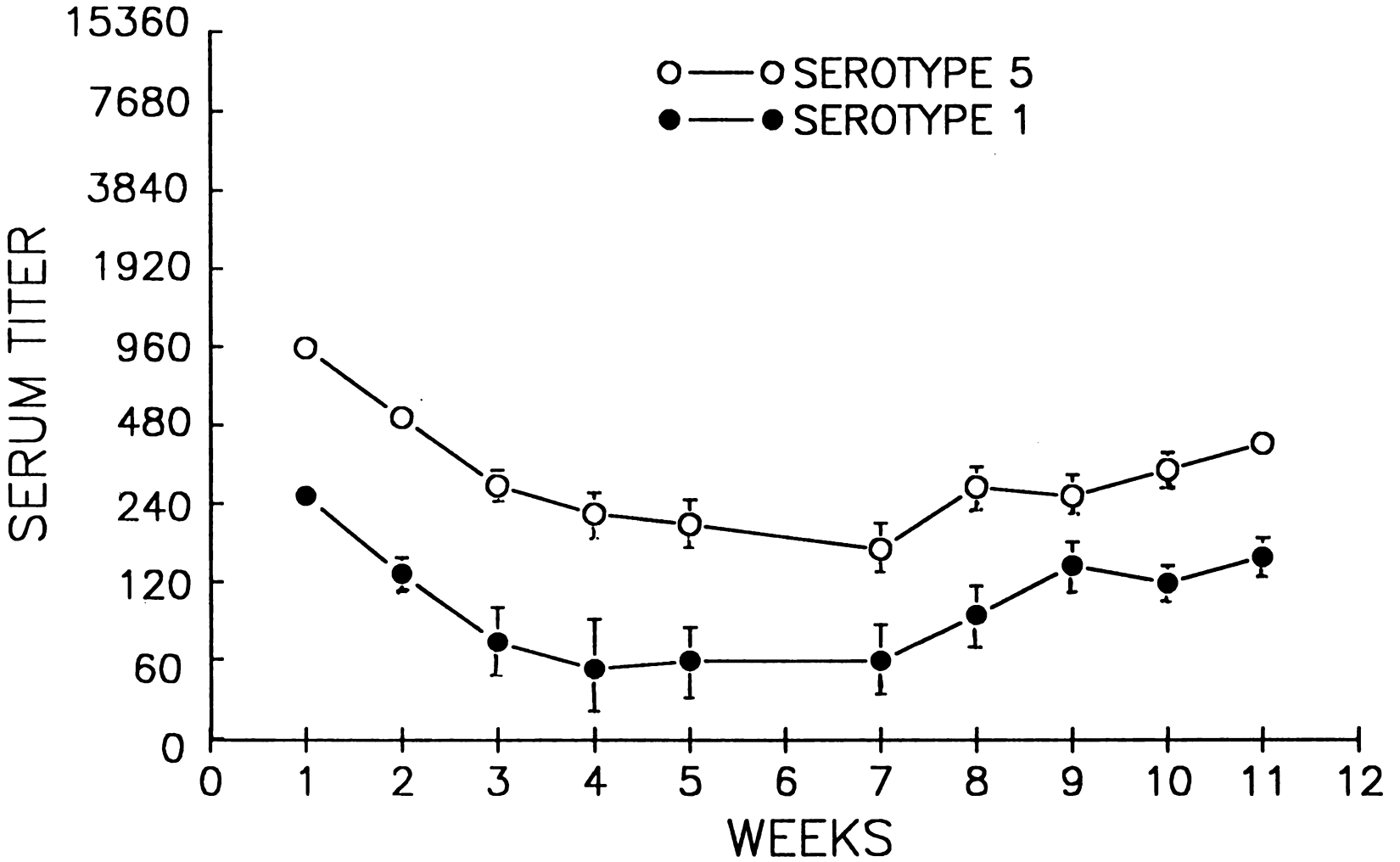

Figure 10: Passive immunity of piglets against serotype 1 and serotype 5 outer membrane antigens.

DISCUSSION

The results indicate that there is significant passive transfer of antibody to App in colostrum, and that anti-App titers decrease steadily, with a half-life of about 7 to 10 days. The half-life was consistent with results from other studies; it is generally expected that passively acquired immunoglobulins decay continuously, with lgG and lgM half-lives about 15 and 5 days, respectively (75).

Both ELISA and CF tests showed the same trend of decline in titers although, the ELISA test was more sensitive and able to show significant titers in some of the piglets between weeks 4 to 7. The present results agree with the findings reported by Nielsen (57), who demonstrated that CF titers in piglets from immune sows declined within 6 weeks from birth.

In this study, the rate of antibody decay varied slightly among the piglets, although all piglets had the same initial ELISA titers. The slight variations could be due to differences in many factors among piglets, such as increase in blood volume, biological degradation, lost through mucosal surface of the piglets and subjective errors in ELISA assaying procedure(76). The main reasons for the steady decline of the passively transferred antibodies could be because of antigen-antibody reaction and clearance of circulating antibodies of the animals, as suggested also by others (75).

One of the main findings in this study was the rise in ELISA titers in all the piglets by eight weeks after birth. As the piglets grew in the isolation facilities, it is possible that the piglets were exposed to other gram negative bacteria that may share common cell wall components with App. Therefore, cross-reactive antibodies could develop that can react to App outer membranes after the decay of the specific antibodies of App transferred through colostrum feeding. For example, the rise in titers in the piglets could be due, in part, to antibody to a 16-18 kD outer membrane protein contained as a common cross-reactive antigen in many gram negative bacteria (42). In our laboratory, we have found by Western blot analysis that post pigs, including uninfected controls from both SPF and non-SPF herds, have detectable serum antibodies against the 16-18 KD cross-reactive outer membrane protein.

ELISA serotiters were supported by Western blot analysis that showed the presence of declining amounts of antibodies until 4 or 5 weeks with a gradual increase in the weeks 8-11 of the study. The reactivity was initially to outer membrane proteins, LPS and capsule of serotype 5; however, antibodies to capsule and LPS disappeared by 4-6 weeks, and antibodies during weeks 7-11 were mainly against the 16-18 kDa protein as well as 1 or 2 other major outer membrane proteins.

Complement fixation, on the other hand, did not detect significant antibody levels after 19 days from birth indicating that complement fixation is not as sensitive a

test as either Western blot or ELISA. ELISA detected low levels of colostrum-derived antibody in piglets that were negative by CF, and also detected antibodies in response to exposure of the piglets to cross-reactive bacteria not detected by CF test.

It has been demonstrated by Nielsen (57), that passively transferred antibodies in piglets are protective against App infection during the first few weeks of life. Colostrum-fed piglets from immune sows were protected from development of acute App disease after challenge, whereas those from non-immune sows developed severe clinical signs after infection. On the other hand, colostral antibodies interfere with the development of active immunity in piglets and other newborn animals (72, 75, 76). It was demonstrated by Mulks, M. H. and Brad Thacker (72) that passive immunity was able to interfere development of active immunity with vaccination in pigs. In their studies, piglets vaccinated at the age of 11 weeks and above, had detectable CF titers, whereas those vaccinated at the age of 5 and 8 weeks had no CF titers. These results were verified by Western blotting analysis. Therefore, determining duration and levels of colostral antibodies in the present study may have contributions to the prevention and control of porcine pleuropneumonia because it can be used in planning strategies for pigs to immunize against App infection.

In summary, it was observed that passively transferred antibodies of piglets decayed steadily with a half-life of 7

to 10 days. Although they had the same initial titers, a slight variation in the rate of decay of antibodies in the piglets was observed. Some of the piglets had no significant ELISA titers between 5 and 7 weeks after birth. Almost all the piglets had negative CF titers after 3 weeks from birth. Significant ELISA serotiters were observed against serotype 1 outer membrane antigens during week 1 after birth.

The rise in titers against both serotype antigens after week 8 from birth, probably due to contamination by other organisms, was not detected by complement fixation. ELISA and Western blot tests were more sensitive than complement fixation test in the detection of low levels of antibodies in the piglets.

CONCLUSION

The ELISA procedure developed using App outer membranes as the antigen is a sensitive and reproducible method to measrure serum antibody levels against Actinobacillus pleuropneumoniae. As discussed in chapter 1, Coefficients of variation calculated from the OD values were less than 20% during repeated assaying of reference positive control sera for both serotype 1 and serotype 5. In addition, the endpoint ELISA titers of the positive control sera remained the same during the repeated assaying on different days. The sensitivity of ELISA is demonstrated by its ability to indicate measurable titers in the non-SPF pigs that were not detected by the CF test (Table 1).

Specificity of the ELISA may be decreased if pigs are exposed to other bacteria that possess antigens that crossreact with App surface antigens. we have consistently found that animals from non-SPF herds have higher background titers against App, as measured by our ELISA, than animals from SPF herds. Western blot analysis indicates that a 16-18 kDa outer membrane protein, found in many gram-negative bacteria besides App, may be a major crossreactive antigen contributing to these background titers. This reduced specificity may in some cases be an advantage. We have found that pigs with no detectable CF titer and a low level but measurable ELISA titer, such as non-SPF pigs and passively immune piglets, do not respond well to vaccination against

App and may not develop protective immunity against App infection. The ability to detect low levels of crossreactive antibodies may make it possible to alter vaccination protocols to provide improved protection in these animals.

The correlation of ELISA and CF tests in detection of antibody titers against App outer membrane antigens is summarized in Table 10. Regression analysis indicated that CF titers and ELISA titers were positively correlated. Simple correlation analysis yielded correlation coefficients of approximately 0.8 regardles of the infection or vaccination status of the pig. ELISA titers of positive pigs were distributed over a range similar to the CF titers. However, the ELISA was more sensitive than CF in detecting low serum antibody levels. Many samples that were negative by CF test had significant ELISA titers; in most cases, these samples were from non-SPF or passively immune pigs. In general, it can be concluded that the CF and ELISA tests positively correlated in the detection of antibody levels to App, but the ELISA assay was more sensitive than the CF test. The results of the study collectively suggest that ELISA is a dependable test for evaluating the immune responses in vaccinated pigs, infected pigs and piglets. It can be used to follow the immune responses of experimental animals with time, and to evaluate the immune responses in vaccinated pigs, infected pigs and piglets. Currently, evaluation of the protective efficacy of vaccines requires sacrifice of

experimental animals after challenge for pathological examination, which is not cost effective. Our data suggest that ELISA may be useful in the evaluation of vaccines, at least in the initial stages of development. In addition, use of ELISA to determine levels and duration of colostral antibodies in piglets may help to solve the problem of passive antibody interference with the development of active immunity by vaccination (72).

Preliminary data indicates that endpoint titers can be accurately determined by performing the ELISA at a single serum dilution, measuring the optical density, and comparing with a standard curve. OD values at three dilution points (240, 480, 960) were analyzed and found to correlate well with endpoint titers. For example, the means of ODs were not significant from one another for all the vaccinates of this study at the 240 dilution point. However, the OM vaccinated and the commercial bacterin immnized pigs showed a significant difference in OD readings at 480 and 960 dilution points ($p < 0.05$). A similar manner of OD differences was supported by statistical analysis that there were significant differences between the high and/or medium mean absorvance values and the low dose of App serotype 5 infected pigs at dilution points, 480 and 960 ($p < 0.05$).

Development of this ELISA procedure into a commercially feasible serodiagnostic test rather than a research tool will require several modifications to improve both the cost-

effectiveness and the sensitivity. Use of a single serum dilution, even in triplicate, rather than the 8-point dilution series, will increase the number of samples that can be run on a single ELISA plate, and thus decrease the cost per sample, by a factor of 3 or 4. It will be necessary to evaluate a large number of samples from both infected and vaccinated animals to develop an accurate standard curve for this procedure, two different methods are under examination. One would be to utilize purified antigens, such as individual outer membrane proteins, that are unique to App and are also found in antigenically similar forms in all App serotypes. A second possibility would be to use a monoclonal anitody against a common App antigen in a competitive ELISA procedure. Both of these methods are currently under investigation.

Table 10: Comparison of ELISA titers against App serotype 5 or serotype 1 and complement fixation titers in exposed pigs.[a,b,c]

	ELISA Endpoint titer							
CF titer	60	120	240	480	960	1920	3840	7680
< 4	18	19	6	25	6	2	0	1
4	0	0	6	3	0	0	0	0
8	0	3	0	0	0	0	0	0
16	0	0	2	1	0	0	1	0
32	0	1	0	3	4	4	0	2
64	0	0	0	0	0	5	5	6
128	0	0	0	0	0	15	10	8

[a] Data presented as the number of pigs with each CF-ELISA titer combination.

[b] Titers listed as reciprocals.

[c] Serum samples were obtained from pigs after exposure to App serotype 5 or serotype 1 either by experimental infection, vaccination with a commercial bacterin, or vaccination with an adjuvanted serotype 5 OM preparation, and from piglets of an immune sow.

BIBLIOGRAPHY

1. Olander, H. J. 1963. A septicemic disease of swine and its causative agent,Haemophilus parahemolyticus. Ph. D. Thesis. University of California, Davis, Calif.

2. Shope, R. E. 1964. Porcine contagious pleuropneumonia. 1. Experimental transmission, etiology and pathology. J. Exp. Med. 119: 357-368.

3. Sanford, S. E. and G. K. A. Josephson. 1981. Porcine Haemophilus pleuropneumonia Epizootic in Southern Ontario: Clinical, Microbiological, Pathologicaland some Epidemiological Findings. Can. J. Comp. Med. 45: 2-7.

4. Hunneman, W.A. 1986. Incidence, economic effects and control of Haemophilus pleuropneumoniae infections in pig. Vet. Quarterly. 8(1): 83-87.

5. Schultz, R. A., R. F. Ross, A. Gunnarsson, R. Nielsen. 1983.Serotyping of 50 different isolates of Haemophilus pleuropneumoniae from swine pneumonia in Iowa and surrounding states. Vet. Med. Small Ani. Clin. 1451-1453.

6. Kilian, M., J. Nicolet and E. L.Biberstein 1978. Biochemical and serological characterization of Haemophilus pleuropneumoniae (Matthew and Pattison) Shope 1964 and proposal of a neotype strain. Int. J. Syst. Bact. 28: 20-26.

7. Macinnes, J. J.and S. Rosendal. 1987. Analysis of major antigens of Haemophilus (Actinobacillus) pleuropneumoniae and related organisms. Infec. Immun. 55:1626-1634.

8. Pohl, S. H. U. Bertschinger, W. Freederiksen and W. Mannheism. 1983. Transfer of H. pleuropneumoniae and the Pasteurella haemolytica-like organism causing porcine necrotic pleuropneumonia to the genus Actinobacillus (Actinobacillus pleuropneumoniae Comb. nov.) on the basis of phenotypic and Deoxyribonucleic Acid relatedness. Int. J. Syst. Bacteriol. 33: 510-514.

9. Sebunya, T. N. K., J. R. Saunders and A. D. Osborne. 1982. Characteristics of Haemophilus pleuropneumoniae isolates and some epidemiological findings on porcine Haemophilus pleuropneumonia in Saskatchewan. Can. Vet.J. 23: 224-228.

10. Vaillancourt, J. P.et.al. 1988. Changes in susceptibility of Actinobacillus pleuropneumoniae to antimicrobial agents in Quebec (81-86). JAVMA. 193: 470-473.

11. Gilbride, K. A., S. Rosendal and J. L. Bruton. 1989. Plasmid mediated antimicrobial resistance in Ontario isolates of Actinobacillus (Haemophilus) pleuropneumoniae.Can J. Vet. Res. 53: 38-42.

12. Nielsen, R. 1986. Serology of Haemophilus (Actinobacillus) pleuropneumoniae serotype 5 strains: Establishment of subtypes A and B. Acta. Vet. Scand. 27: 49-58.

13. Rapp, V. J., R. F. Ross, and B. Zunmermann, E. 1985. Serotyping of Haemophilus pleuropneumoniae by rapid slide agglutination and indirect fluorescent antibody tests in swine. Am. J. Vet. Res. 46(1). 185-192.

14. Gunnarsson, A. 1979. Serologic studies on porcine strains of Haemophilus parahaemolyticus. Extraction of type-specific antigens. Am. J. Vet. Res. 40: 469-472.

15. Rosendal, S. and D. A. Boyd. 1982. Haemophilus pleuropneumoniae serotyping. J. Clin. Microbiol. 16: 840-843.

16. Kamp, E. M., Johan, K. Popma and Leo A. M. G. Van Leengoed.1987. Serotyping of Haemophilus pleuropneumniae in the Netherlands with emphasis on heterogeneity within serotype 1 and (proposed) serotype 9. Vet. Microbiol. 13: 249-257.

17. Neilsen,R. 1986. Serological characterization of Haemophilus (Actinobacillus) pleuropneumniae strains and proposal of a new serotype 12. Acta. Vet. Scand. 27: 453-455.

18. Mittal, K. R., R. Higgins and S. Lariviere. 1982. Evaluation of slide agglutination and Ring precipitation tests for capsule serotyping of Haemophilus pleuropneumniae. J. Clin. Microbiol. 15: 1019-1023.

19. Mittal, K. R., R. Higgins and S. Lariviere. 1983. Identification and serotyping of Haemophilus pleuropneumniae by coagulation test. J. Clin. Microbiol. 18: 1351-

20. Mittal, K.R., R. Higgines and S. Lariviere. 1987. An evaluation of agglutination and coagulation techniques for serotypig of Haemophilus pleuropneumoniae isolates. Am. J. Vet. Res. 48(2): 219-226.

21. Inzana, T. J. and B. Mathison. 1987. Serotype specificity and immunogenecity for capsular polymer of Haemophilus pleuropneumoniae serotype 5. Infec. Immun. 55(7): 1580-1587.

22. Hunter, D. and J. Livingstone. 1986. Detection of Haemophilus pleuropneumniae antigens using the coagulation test. Vet. Rec. 118: 129.

23. Piffer, I. A., G. R. Carter, A. A. F. Botovchenko. 1986. Identification of serotypes of Haemophilus pleuropneumniae by Counterimmunoelectrophoresis. Vet. Rec. 118: 292-294.

24. Rosendal, S. L. Lombin and J. Demoor. 1981. Serotyping and detection of Haemophilus pleuropneumniae by indirect fluorescent antibody technique. Can. J. Comp. Med. 45: 271-274.

25. Nicolet, J., Ph. Paroz, M. Krawinkler and A. Baumgarter. 1981. An Enzyme-Linked Immunosorbent assay, using an EDTA-extracted antigen for the serology of Haemophilus pleuropneumoniae. Am. J. Vet. Res. 42(12): 2139-2142.

26. Sebunya, T. N. K and J. R. Saunders. 1983.Haemophilus pleuropneumniae infection in swine: A Review. JAVMA. 182: 1331-1337.

27. Gorg, A.,W. Postel, J. Weser, H.W. Schivargain, and W.M. Boesken. 1985. Horizontal SDS electrophoresis in ultrathin pore-gradient gels for the analysis of urinary proteins. Science Tools (Sweden) 3: 5-9.

28. Rapp, V. J., and Richard F. Ross. 1986. Antibody response of swine to Outer membrane components of Haemophilus pleuropneumoniae during infection. Infect. Immun. 54(3): 751-760.

29. Rapp, V.J. 1986. Outer membrane protein profiles of Haemophilus pleuropneumoniae. Infect. Immun. 52(1): 414-420.

30. Mulks, M.H., D.Y. Hunter-Simon, J.W. Sprague and B.J. Thacker. 1986. Proc. IPVS. 1986: 266.

31. Bertram, T. A. 1986. Intravascular macrophages in Lungs of pigs infected with Haemophilus pleuropneumniae. Vet. Pathol. 23: 681-691.

32. Liggett, A.D., and L.R. Harrison. 1987. Sequential study of lesion development in experimental Haemophilus pleuropneumonia. Res. Vet. Sci. 42: 204-221.

33. Liggett, A. D., L. R. Harrison and R. L. Farrell. 1986. Acute inflammatory effects of intratracheally instilled E. coli endotoxin and sonicated suspension of Haemophilus pleuropneumniae in swine. Can. J. Vet. Res. 50: 526-531.

34. Udeze, F.A., K.S. Latimer and S. Kadis. 1987. Role of Haemophilus pleuropneumoniae Lipopolysaccharide endotoxin in the pathogenesis of porcine Haemophilus pleuropneumonia. J. Vet. Res. 48(5): 768-773.

35. Rosendal, S., J. Devenish, J.I. Macinnes, J.H. Lumsden, S. Watson and H. Xun. 1988. Evaluation of heat-sensitive, neutrophil-toxic, and hemolytic activity of Haemophilus (Actinobacillus) pleuropneumoniae. Am. J. Vet. Res. 49(7): 1053-1058.

36. Bendixen, P. H., P. E. Shewen, S. Rosendal and Bruce N.W. 1981. Toxicity of Haemophilus pleuropneumoniae for porcine lung macrophages, peripheral blood monocytes and testicular cells. Infect. Immun. 33: 673-676.

37. Kume, K., T. Nakai and A. Sawata. 1986. Interaction between Heat-stable Hemolytic Substance from Haemophilus pleuropneumoniae and porcine microphages in in vitro. Infec. Immun. 51(2): 563-570.

38. Pijoan, Carlos. 1986. Effect of Pasturella Multocida and Haemophilus pleuropneumniae toxins on swine alveolar macrophages. Vet. Immunol. and Immunopathol. 13: 141-149.

39. Nakai, T, A. Sawata and K. Kume. 1983. Characterization of the hemolysin produced by Haemophilus pleuropneumniae. Am. J. Vet. Res. 44: 344-347.

40. Nakai, T., A. Sawata and K. Kume. 1984. Pathogenecity of Haemophilus pleuropneumoniae for laboratory animals and possible role of its hemolysin for production of pleuropneumonia. Jpn. Vet. Sci. 46: 851-858.

41. Fenwick, B. W., B. I. Osburn, and H. J. Olander 1986. Isolation and biological characterization of two lipopoly-saccharides and a capsular-enriched polysaccharide preparation from Haemophilus pleuropneumoniae. Am. J. Vet.Res. 47(7). 1433-1441.

42. Jensen, A. E. and T. A. Bertram. 1986. Morphological and Biological Comparison of Virulent and Avirulent isolates of Haemophilus pleuropneumoniae serotype 5. Infect. Immun. 51(2): 419-424.

43.Frey, J and J. Nicolet. 1988. Regulation of hemolysin expression in Actinobacillus pleuropnuemoniae serotype 1 by Ca ion. Infec. Immun. 56: 2570-2575.

44.Frey, J. , J. Perrin., and J. Nicolet. 1989. Cloning of a Cohemolysin,the CAMPfactorofActinobacillus pleuropneumoniae. Infec. Immun. 57: 2050-2056.

45. Frey, J. , J. Perrin. and J. Nicolet. 1988. Biochemical and genetic characterization of Actinobacillus pleuropnuemoniae hemolysin. Abst. Conf. Res. Workers. Ani. Dis. 68 16: 1987.

46.Van Leengoed, L. A. , E. M. Kump., and J. M. A. Pol. 1989. Toxicity of Actinobacillus pleuropnuemoniae to porcine lung macrophages. Veterinary Microbiology, 19 337-349.

47. Deneer, H. R. and A. A Potter. 1989. Effect of Iron restriction on the outer membrane proteins of Actinobacillus (Haemophilus) pleuropneumoniae. Infec. Immun. 57: 798-804.

48. Rosendal, S., D.A. Boyd, and K.A. Gilbride. 1985. Comparative virulence of porcine Haemophilus bacteria. Can.J. Comp. Med. 49: 68-74.

49. Brandreth, S.R. and I.M. Smith. 1987. Comparative virulence of some English strains of Haemophilus pleuropneumoniae serotype 2 and 3 in the pig. Res. Vet. Sci. 42: 187-193.

50. Neilsen, R. 1976. Pleuropneumonia of swine caused by Haemophilus parahaemolyticus. Studies on the protection obtained by vaccination. Nord. Vet. Med. 28: 337-348.

51. Rosendal, S. D., S. Carpenter, W. R. Mitchell et al 1981.Vaccination against pleuropneumonia in pigs caused by Haemophilus pleuropneumniae. Can. V. J. 22: 34-35.

52. Nielsen, R. 1979. Haemophilus parahaemolyticus serotypes pathogenecity and cross-immunity. Nord. Vet. Med. 31: 407-413.

53. Nielsen, R.1984. Haemophilus pleuropneumoniae serotypes: Cross protection experiments: Nord. Vet. Med.36:221-234

54. Reynolds, H. Y. 1974. Pulmonary host defences in rabbits after immunization with Pseudomonus antigens: The interactionof bacteria,antibodies,macrophages and lymphocyte. J. Infect. Dis. 130: 134-142.

55. Straw, B. E., N. J. Machachlam, W. T. Corbett, P. B. Carter andH.M.Schey.1985.Comparisonoftissue reactions produced byHaemophilus pleuropneumoniae vaccines madewith six different adjuvants in swine. Can.J.Comp. Med. 49: 149-151.

56. Sterzl, J.,J, Rejnek and J. Travnicek. 1966. Impermeability of pig placenta to antibodies. folia Micribiolo. 11: 7-10.

57. Neilsen, R. 1975. Colostral transfer of immunity to Haemophilus parahaemolyticus in pigs. Nord. Vet. Med. 27:319-328.

58. Chaniago, T. D. et.al. 1978. Immunoglobulins in the blood of foetal pigs. Aust. Vet. J. 54: 30-33.

59. Rosendal, S., O.P. Miniats and P. Sinclair. 1986. Protective Efficacy of Capsule extracts of Haemophilus pleuropneumoniae in pigs and mice. Vet. Microbiol. 12: 229-240.

60. Koruvo, A., L. Lindberg, J. Tuomi, J. Schroder. 1988. Use of monoclonal antibodies to serotype-specific antigens of Haemophilus pleuropneumoniae serotype 2 in passive immunization. AMJ Vet. Res. 49: 2072-2075.

61. Fenwick, B.W., J.S. Cullor, B.I. Osburn, and H.J. Olander. 1986. Mechanisms involved in protection provided by immunization against core lipopolysaccharide of E. coli J_5 from lethal Haemophilus pleuropneumoniae infections in swine. Infect. Immun. 53(2): 298-304.

62. Fenwick, B.W. and B.I. Osburn. 1986. Vaccine potential of Haemophilus pleuropneumoniae oligosaccharide-tetanus toxoid conjugates. Infect. Immun. 54(2): 583-586.

63. Fenwick, B.W., B.I. Osburn, J.S. Cullor, S.C. Henry and H.I. Olander. 1986. Mortality in Swine herds ende mically infected with Haemophilus pleuropneumoniae: Effect of immunization with cross-reacting lipopolysaccharide core antigens of E.coli. Am. J. Vet. Res. 47(9): 1888-1891.

64. Kume, K. and T. Nakai. 1988. Efficacy of a bivalent vaccine containing sero 2 and 5 strains of Haemophilus pleuropneumoniae in pigs or in guinea pigs. Jpn. J. Vet. Sci. 50: 237-241.

65. Schultz, R. A. 1985. Compendium on swine haemophilus pleuropneumonia, distributed on the annual meeting of the American Association of Swine Practitioners March 24-26, 1985.

66. Morrison R.B. et. al. 1984. An Enzyme-linked assay, Using heat treated antigen for the detection of Haemophilus pleuropneumoniae antibodies.International pig veterinary society proceeding # 8 .

67. Schultz, R., T. Young and R. Ross. 1982. Prevalence of antibodies to Haemophilus pleuropneumoniae in Iowa swine. Am. J. Vet. Res. 184: 1848-1851.

68. Osborn, M. J. and R. Munson 1974. Separation of inner (cytoplasmic) and outer membranes of gram-negative bacteria. Meth. Enzymol. 31: 642-653.

69. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

70. Voller, A. and D. Bidwell. 1986. Enzyme-Linked Immunosorbent Assay.In N.R. Rose et al (eds).Manual of clinical laboratory immunology, 3rd edition. American Society for Microbiology, Washington, D.C., 99-109.

71. Goudswaard, J., J. A van der Donk, A.Noordzij, R. H.Van Dam and J. P. Vaerman. 1978. Protein A reactivity of various mammalian immunolobulins. Scand. J. Immunol. 8: 21-28.

72. Mulks,M. H.and Brad. Thacker. Abst. Conf. Res. Workers Ani. Dis. 68: 16,1987.

73. Lazary, S., E. Scholl and E. Rivera. 1977. Immune Response to Haemophilus parahaemolyticus(Hp) infection in the pig. I. In vitro test to detect sensitized cells in the blood of infected and vaccinated animals. Vet.

Microbiol. 3: 143-154.

74. Fenwick, B.A. and B. Osburn. 1986. Immune responses of polysaccharides of Haemophilus pleuropneumoniae in convalesent and immunized pigs. Infec. Immun. 54: 575-582.

75. Tyler, W. J. et. al. 1989. Preferential decay of passively acquired immunoglobulins recognizing shared gram-negative core antigens in neonatal swine. Am J. Vet. Res. 50: 480-482.

76. Paul, P. S., W.L. Mengeling, E. C. Pirtle. 1982. Duration and biological half-life of passively acquired colostral antibodies to porcine parvovirus. Am. J. Vet. Res. 43: 1376-1379.