A comparative study on the efficacy of semi-purified, viral glycoproteins as antigen in the indirect hemagglutination test for pseudorabies antibody

by

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CHAPTER I. INTRODUCTION

Pseudorabies (PR) or Aujeszky's disease is an economically significant infectious herpesvirus disease that occurs primarily in swine worldwide. Clinically, PR is characterized by respiratory failure in growing and adult swine, central nervous system (CNS) disturbances and high mortality rates in neonatal pigs and reproductive failure in gilts and sows.

The cost of the disease in the United States alone, from deaths, abortions, reproductive failure, and reduced growth rates was estimated at $33.9 million in 1981 (4). This estimate did not include the cost of using vaccines to prevent the infection. A more recent survey of the economic losses due to PR in the state of Iowa indicated that the annual production losses in 1985 was approximately $152.9 to $170.4 million (82). In addition the cost of prevention and control of PR including vaccination and serology was estimated at $31.5 million for a total annual cost of approximately $184 to $201 million (82). As a result of such economic losses, serious efforts are being made in an attempt to control and eventually eradicate the disease from the swine population. The success of any control or eradication program requires sensitive and rapid diagnostic tests. The test should be able to detect early antibody so that infected pigs can be identified quickly and isolated to minimize the spread of the virus.

Presently, the most widely used diagnostic tests for PR antibodies are the serum neutralization (SN) test and the enzyme-linked

immunosorbent assay (ELISA). Although both the SN test and ELISA are considered sensitive and specific, each has some distinct disadvantages. The SN test can only be performed in laboratories equipped with cell culture facilities. The test also requires a minimum of 48 hours to complete and cannot be used to test poor quality serum samples that are frequently toxic to cell cultures. Although the ELISA is a rapid test and can be used to test poor quality serums, it requires labile reagents that are adversely affected by improper storage. The ELISA also requires relatively expensive equipment to be run properly. The recommended equipment cost in 1983 for establishing ELISA capability in a diagnostic laboratory was $27,720 (25). Clearly, the availability of a sensitive and specific test not dependent on expensive equipment and labile reagents would be of great use in diagnostic laboratories especially in countries where animal health funds are limited.

Recent studies by Haffer et al. (21, 22) and Labadie and Toma (32) have demonstrated that the indirect hemagglutination test (IHAT) was both sensitive and specific for detecting pseudorabies virus (PRV) antibody in swine. These investigators also demonstrated that the IHAT was able to detect PRV specific antibodies as early as five days post-infection (p.i.). In addition, the IHAT does not require expensive equipment and the reagents used are not highly labile. In view of these advantages, the IHAT is an ideal test for field laboratory use.

In our laboratory, attempts to duplicate the IHAT described by these investigators were unsuccessful due to extensive nonspecific hemagglutination (NSH) reactions that occured when whole PRV were used as

antigen. The following study was conducted to determine if a more gentle method of processing whole virions as antigen or if the use of any one of four different semi-purified viral glycoprotein preparations would eliminate the problem of NSH and possibly increase the sensitivity of the test.

CHAPTER II. LITERATURE REVIEW

Introduction

Pseudorabies, also known as Aujeszky's Disease, Mad Itch, and Infectious Bulbar Paralysis, was first described by Aujeszky in 1902 who showed that the disease was caused by a filterable agent or more appropriately, a virus (2). Pseudorabies (PR) is most important as an infectious viral disease of swine. The disease is especially important in young pigs which suffer central nervous system (CNS) disorders and high mortality. Other animals in which PR has been reported include cattle, sheep, goats, dogs, cats, captive foxes (19) and raccoons (52); all of which develop fatal infection. Swine are considered to be the natural host of pseudorabies virus (PRV) and can be inapparently infected with the virus.

Pseudorabies is distributed worldwide, particularly in those areas that have an intensive swine industry. In the United States, PR has been a significant problem particularly in the states of Illinois (63), South Dakota (26), Missouri (14), and Iowa (33). Other countries that currently have problems with PR include the USSR (42), Mexico (54), China (58), Japan (43), Taiwan R.O.C. (34), Singapore (18), and most European countries (1, 10, 15, 44, 71). The widespread distribution of PR may have resulted from the importation and exportation of infected pigs by countries that lack efficient quarantine and serological testing procedures.

In the United States, the prevalence of the disease continues to increase and is causing a significant economic impact in the swine industry. Thawley et al. (69) cited data indicating that the prevalence of PR in the USA rose from 3.73 % in 1978 to 8.39 % in 1981. In Iowa, a serum survey for PRV antibodies in 1980 indicated that approximately 10 % of the swine population was serologically positive (48). A subsequent survey conducted in early 1982 showed that 26 % of swine herds were serologically positive for PR in Iowa, while in Illinois and Missouri, the figures were 10 % and 9.5 %, respectively (14). These data clearly indicate that PR is spreading and is rapidly reaching endemic status. In terms of cost of the disease, it has been estimated in Iowa that in 1985, losses from deaths, abortions, reduced conception rates, and reduced growth rates due to PR totalled $152.9 to $170.4 million (82). When prevention and control costs were taken into account, the total cost increased to $184 to $201 million.

The Virus

Pseudorabies virus, also known as Herpesvirus suis I (23), belongs to the family Herpesviridae. Members of this family are characterized by double-stranded DNA surrounded by an icosahedral capsid containing 162 capsomeres. The size of the capsid ranges from 110 to 230 nm in diameter (23). The capsid, in turn, is surrounded by a glycoprotein-rich envelope. There are four major and three minor virus glycoproteins in the membrane (23). Three of the four major virus glycoproteins (125K, 74K, and 58K; gIIa, gIIb, and gIIc, respectively) are covalently linked

by disulfide bridges (23). The fourth major virus glycoprotein (98K; gIII) is not complexed to any other protein (23). The three minor virus glycoproteins (130K , 98K, and 62K; gI, gIV, and gV,, respectively) form a noncovalently linked complex with a 115K nonglycosylated protein (23). Hampl et al. (23) consider that the 98K protein is the most important virus glycoprotein with respect to inducing an immune response in the host. Other investigators (3, 40b) have shown that the antigen composition of the a virulent Bartha "K" and Norden vaccine strain of PRV lack the 130K and 98K protein that are present in virulent strains.

Pseudorabies virus is relatively stable in the environment. Solomkin and Tutushin and Ustenko as cited in Davies and Beran (12b) reported that the virus can survive in the environment for periods ranging from 10 to 49 days. The virus can also survive the variations of pH between 5 and 9 (4) and temperature between 4 C and 37 C (12b). Its tolerance to wide ranges of pH and temperature may facilitate the transmission of the disease by fomites.

The Disease in the Pig

The transmission of PRV in swine normally occurs via the nasal and oral routes. The severity of clinical signs and mortality of infected pigs are inversely proportional to the animals' age. Pigs less than 15 days old are severely affected and often experience 100 % mortality. Common signs of infection include nasal discharge, sneezing, coughing, pneumonia, somnolence, incoordination, trembling, and convulsion.

Occasionally there is paralysis. In adult swine the clinical signs are usually inapparent and mortality is rare. However, certain virulent strains of PRV can kill pigs over 12 weeks old (60).

Pigs can also be infected transplacentally, and on rare occasion, the virus can also infect fetuses by entering through the vagina (19). When in-utero infection occurs during the first 30 days of pregnancy, fetal death and reabsorption may occur. Infection during the middle trimester often results in abortion while stillbirth and mummified fetuses are common if infection occurs during the last trimester of pregnancy. Occasionally pigs are born infected with the virus. Such pigs become prostrate and comatose within one to two hours after the first signs are noticed and death occurs within 12 to 24 hours (19).

Pigs that survive acute and chronic PR infection can be latently infected with the virus. Studies indicated that the virus genome can persists within the cells of the central nervous system for a very long time (56). These pigs become carriers and can serve as a primary source of virus in future outbreaks following reactivation of latent infection due to stress or other undefined conditions (51).

Control of Pseudorabies

Basically, PR can be controlled by: a) prevention of clinical disease by vaccination, and b) elimination of the virus from the individual herds and eventually the entire swine population. The use of vaccines to control or prevent PR infection has been the most common method of control to date. Both modified live virus (MLV) and killed

virus vaccines are commercially available today. Examples of MLV vaccines available include those utilizing the BUK strain which are marketed by Pitman-Moore, Washington Crossing, NJ., and Norden Laboratories, Lincoln, NE. The avirulent Bartha K strain is marketed by Bioceutic Corporation, Kansas City, MO. Examples of killed vaccines are those marketed by Jensen-Salsbury Laboratories, Charles City, IA. and Norden Laboratories, Lincoln, NE. Both MLV and killed vaccines provide good protection. However, MLV vaccines generally induce higher titers in a shorter time (51) which make MLV vaccines more suitable for use during acute outbreaks. The use of either type of vaccine however, will not prevent infection by wild-type virus. Consequently, once a vaccine program is initiated, it usually must be continued at a significant cost to the producer.

Recently, a study known as the Marshall County Pilot Project has been initiated to evaluate the effectiveness of different methods used to eliminate PR from swine herds (33). The PR status of all cooperating herds in Marshall County was determined by testing for the presence of serum neutralizing antibodies. Attempts were then made to eliminate PR from the infected herds by one of three basic methods:

1. Depopulation and repopulation. This method consists of depopulating, cleaning, and disinfecting swine premises which are left vacant for a minimum period of 30 days before repopulation with PRV free pigs. Newly introduced pigs are tested for PRV antibodies 30 days after repopulation.

2. Test and removal. This method involves testing the entire herd every 30 days for six months and removing all positive pigs. Thereafter, 25% of the herd is monitored for PRV antibodies every three months for a year. Those herds that remain seronegative are then designated as PRV free.
3. Controlled vaccination program. Briefly, this method involves separating seropositive pigs from the seronegative pigs. Subsequently, all seronegative pigs are vaccinated. Six months following the last evidence of clinical symptoms, the vaccination program is continued only in the breeding herd. The progeny of the breeding stock are then isolated in a separate facility, and monitored for PRV antibodies. If no PRV antibodies are detected after 60 days, the progeny are used to establish a new breeding herd and the original breeding herd is eliminated.

The PR cleanup procedures used in the Marshall County Pilot Project were very effective. Seventy-five percent of the participating herds employed the controlled vaccination program. Of the 32 originally infected herds, all were reclassified as PRV free as of March 1, 1986 with the exception of two herds. The average time period involved in attaining pseudorabies-free status was 14.9 months.

Diagnostic Tests

An essential requirement for any effective control program is the availability of reliable, inexpensive, and easy to use diagnostic tests

for the detection of PRV antibodies. A good diagnostic test should be sensitive, specific, and able to detect PRV antibody early so as to limit the spread of the virus to other pigs in the herd. The test should also be rapid, easy to use and economical. To date, several PRV diagnostic tests are available. These include the serum neutralization (SN) test, enzyme-linked immunosorbent assay (ELISA), microimmunodiffusion test (MIDT), indirect hemagglutination test (IHAT), indirect fluorescence antibody test (IFAT), latex macroagglutination test (LMAT), complement fixation (CF) test, radial immunodiffusion enzyme assay (RIDEA), countercurrent immunoelectrophoresis (CIE), and the indirect solid-phase microradioimmunoassay (IRIA). The rest of this chapter will describe, evaluate, and compare these tests.

Serum neutralization test

The principle of the SN test is based on the ability of the antiserum to neutralize the infectivity of homologous virus *in vitro*. The SN test has many applications in animal virology. Serum neutralization tests are used for the identification of virus isolates, to determine immune status of the virus hosts, to study antigenic relationships between viral strains, to evaluate the immunogenicity of viral vaccines, and to study the epizootiology of viral diseases (11).

The earliest application of the SN test for PRV was the work done by Horvath in 1958 (27). In this study, the SN test was used to titrate the virus instead of titrating the antibody (constant-serum-varying-virus). Horvath named this assay "the quantitative semi-micro tissue culture

method." This method used live virus as antigen which was originally isolated from the brain of a PRV infected pig. Immune serum was obtained from a guinea pig which was hyperimmunized with inactivated PRV, and subsequently challenged with virulent virus (27). Virus was assayed by adding a constant amount of high titered PRV antiserum to serial dilutions of the virus. The serum-virus mixtures were incubated at room temperature for one hour and then added to a tissue culture system consisting of chicken heart fragments obtained from 12 to 13-day-old embryonated eggs. The virus-tissue culture system was further incubated at 37 C for five days and examined for the presence of virus induced CPE in cell monolayers growing around tissue explants. The 50 % endpoint of the assay was determined and the virus titer was expressed as infective dose (50 %) per 0.1 ml (ID_{50} / 0.1 ml). Horvath (27) concluded that by using tissue cultures, conditions could be created which permit titration of virus far more exact than those obtainable in the most carefully conducted animal experiments.

The use of tissue culture by Horvath (27) as a virus indicator system for the SN test instead of live animal models provided a basis for other researchers to develop an even simpler in vitro SN test procedure. Although the techniques for the SN test vary greatly, the constant virus-varying serum technique is currently the most common method used for titrating antibodies in serum. An example of this method was described by Lodetti et al. (35). These investigators performed a microculture plaque reduction assay in trays consisting of glass wells each 1.5 cm in diameter and one cm deep with metal covers. Essentially, serum-virus

mixtures were incubated with cells in suspension for five hours at 37 C. During this period, cells adhered to the surface of the wells. After incubation, maintenance medium containing 3 % methylcellulose and 10 % fetal calf serum was added to each well without removing the growth medium. The trays were then incubated at 37 C in a humidified atmosphere containing five percent CO_2 for three days and the 50 % endpoint was determined.

This method was an improvement over the method used by Horvath (27) because it was easier to use and endpoints were easier to establish. Lodetti et al. (35) also found their method to be more sensitive. Generally, they found that the microculture plaque reduction test consistently detected titers one to two $\log_2$ dilution higher than the conventional tube test.

Wirahadiredja and Rondhuis (80) developed an even simpler SN method which made it possible to screen large numbers of serum samples in a relatively short period of time. The test was performed in flat bottomed microtiter plates. Serum samples were inactivated at 56 C for 30 minutes and serially diluted in two-fold steps. Twenty five ul of diluted serum and PRV suspension containing 100 $TCID_{50}$'s were mixed and incubated at room temperature for 30 minutes. Subsequently, $10^{4.1}$ viable cells in growth medium were added to the wells containing the mixture. The preparation was incubated at 37 C for seven days and the 50 % endpoint was determined. The SN titer was then expressed as the highest serum dilution which completely inhibited the development of cytopathic effect (CPE) on monolayers. These investigators also stressed the importance of

having a cytotoxicity control, negative and positive serum controls, and a cell control in each microtiter plate.

It was suggested by both Wirahadiredja and Rondhuis (80) and Lodetti et al. (35) that the differences in the incubation periods of the virus-serum mixture prior to inoculation onto the tissue culture could influence the sensitivity of the test. Such a relationship was demonstrated by Bitsch and Eskildsen (8) who found that the sensitivity of the SN test could be increased by four $\log_2$ dilutions when serum-virus mixtures were incubated at 37 C for 24 hours instead of one hour. However, Bitsch and Eskildsen (8) also found that prolonged incubation produced some false positive reactions. In one experiment, they found that two of 24 serum samples from PRV free pigs reacted positively when incubated with virus at 37 C for 24 hours. The same two serums were negative when a one hour incubation period was used. These workers concluded that although nonspecific reactions can occur when using prolonged incubation periods for detecting PRV antibodies, such reaction are rare at dilutions of 1:4 and 1:8. These workers also concluded that the increased sensitivity shown by prolonged incubation proved to be useful for the early detection of PRV antibodies and that the occasional false positive reactions obtained would be acceptable (4).

In addition to the length of the serum-virus incubation period, other factors can also influence the results of the SN test. Crandell et al. (11) proposed that six such factors should be closely controlled. These factors include: virus growth conditions in the assay host, the virus growth curve, sensitivity of the virus assay system, the kinetics

of neutralization, virus stability, and virus strain differences.

Hill et al. (24) took the preceding variables into consideration and standardized the microtiter SN test that is currently being used by official diagnostic laboratories today. The standardized test uses the Iowa strain of PRV having a titer between 100 to 500 $TCID_{50}$, the MDBK cell line, and known strong positive, weak positive,and negative serum controls. A control on serum toxicity was also included.

Despite its usefulness, the SN test does have some limitations. Detectable neutralizing antibody does not appear in the serum of infected pigs until about seven days after infection (11). Because of this time delay in the occurrence of detectable neutralizing antibody, early infection may go undetected. Serum toxicity is also an occasional problem which interferes with the SN test. Crandell et al. (11) suggested that serum toxicity could be greatly reduced by using vacutainers (Becton-Dickinson, Rutherford, NJ) to collect the blood. Serum toxicity can also be avoided by using higher dilutions of serum samples for testing, but to do so reduces the sensitivity of the test.

Crossreactivity is another factor which can also interfere with the test. Crandell et al. (11) reported that reciprocal crossreactions can occur between PRV and infectious bovine rhinotracheitis (IBR) virus and their respective antibodies. This report is of concern because IBR virus has been isolated from swine and IBR virus antibody has been demonstrated in swine serum (11).

Although the test is widely accepted, there has been some controversy as to what constitutes a minimum positive SN titer. Some

workers consider a titer of 1:2 to be positive while others consider 1:4 to be the minimum positive titer (11, 80). Crandell et al. (11) suggested that serum samples testing SN positive at 1:2 should be further tested by the indirect fluorescence antibody test.

Enzyme-linked immunosorbent assay

The first use of the ELISA for the detection of antigen specific immunoglobulins was made by Engvall and Perlmann in 1971 (16). Since then, the ELISA has had a significant impact on the field of diagnostic medicine. Figure 1 briefly summarizes the methodology used for the detection of antibody by the ELISA.

Several groups of investigators have reported on the use of the ELISA for the detection of PRV antibody (9, 13, 16, 25, 60, 61, 71, 73, 74, 77). Snyder and Stewart (60) discussed a procedure which utilized antigen consisting of PRV infected porcine kidney (PK) 15 or swine testicle (ST) cells which were grown, infected, and subsequently acetone fixed in 96 flat bottom well microtiter plates. The control antigen consisted of fixed virus free cells. These investigators used peroxidase labelled rabbit anti-porcine immunoglobulin as conjugate and hydrogen peroxide as the enzyme substrate. Both alpha-aminosalicylic acid-hydrochloride (5-ASA) and 2, 2'-azino-di-3-ethyl-benzthiozoline (ABTS) were compared as colorimetric indicators. Both indicators were satisfactory but 5-ASA was found to be more stable. Test results were

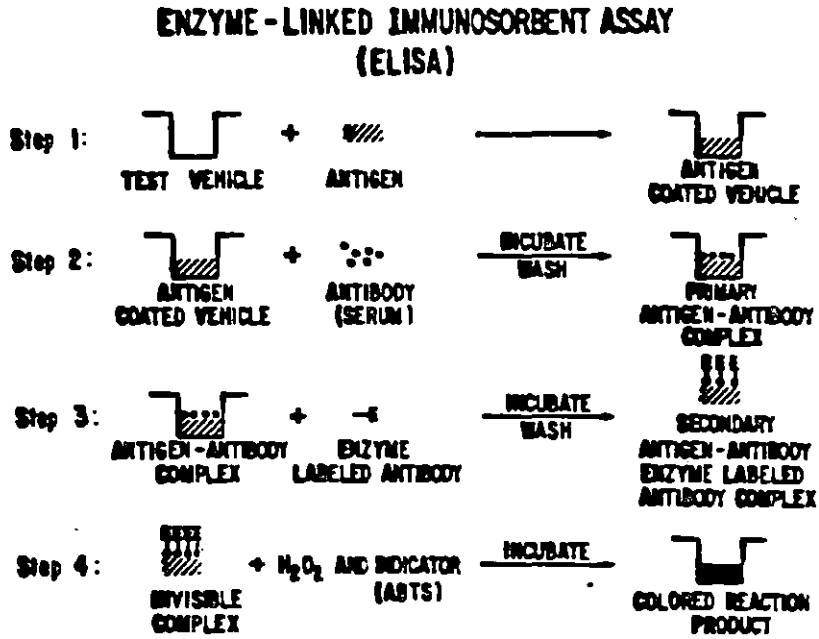

Figure 1. A four-step outline of the ELISA (from National Veterinary Services Laboratories, Ames, IA)

interpreted by calculating the optical density (OD) ratio obtained by reacting test serum with positive antigen and control antigen. Ratios of 1.5 or greater were considered to be positive. These workers also found that specific reactions could be enhanced by incorporating guinea pig complement with the conjugate. Their results also showed a 98.3 % correlation between the ELISA and the SN test and that the ELISA detected 80 % more positive serum samples than the SN test. Snyder and Stewart (60) however reported that the ELISA did not yield reliable quantitative results. This unreliability was attributed to the wide range of variability of test readings obtained from single serum samples. For example, the coefficient of variation ranged from 7.3 to 15 %. Snyder and Stewart (60) speculated that the variation may have been due to the variation of antigen concentration from well to well and/or to the quantities of reagents used. Variation was less with PRV infected ST cells than with PRV infected PK-15 cells.

Snyder and Stewart (60) subsequently modified their test by utilizing Triton X-100 solubilized PRV infected Vero-Maru cells as antigen. The change in antigen provided more reproducible results. These investigators also recommended that positive reactions should be represented by OD ratios of at least 1.5 as calculated by the formula:

$$\text{S/N ratio} = \frac{OD_{(PRV)}}{OD_{(NHC)}}$$

where S/N ratio is the specific reaction represented by the OD produced

by reacting test serums with PRV antigen, i.e., $OD_{(PRV)}$ divided by the nonspecific reaction represented by the OD produced by reacting test serum with normal host cell antigens, i.e., $OD_{(NHC)}$. Today, the National Veterinary Services Laboratories, Ames, IA recommend that the S/N ratio should be increased to 1.8. They also recommend that the $OD_{(PRV)}$ of the serum should be greater than the mean of the negative control minus the mean of the positive control divided by two, i.e., (NC - WPC / 2).

Independently, Briaire et al. (9) described a procedure that used partially purified PRV as antigen. The virus was clarified by low speed centrifugation and partially purified by high speed centrifugation for one hour at 30,000 rpm in a Beckman R 30 rotor (9). The pellet was resuspended in its original volume in PBS. The antigen was coated overnight at 4 C onto polystyrene cuvettes or flat bottom microtiter plates. No control antigen was used.

The specificity of an individual serum reaction was determined by calculating the ratio of extinction coefficients, (E) at 546 nm derived from reacting test serum and a known negative serum in the ELISA. This value, designated as P/N was determined from the formula:

$$P/N = \frac{E_{(sample)} - E_{(blank)}}{E_{(negative)} - E_{(blank)}}$$

where $E_{(sample)}$, $E_{(negative)}$, and $E_{(blank)}$ represent the $E_{546\ nm}$ of the serum tested, the mean $E_{546\ nm}$ of 30 negative reference serums and the $E_{546\ nm}$ of a saline control, respectively (9). The extinction

coefficient, was calculated by the formula:

$$E_{546\ nm} = \frac{A}{L}$$

where A is the absorbance or OD and L is the path length in cm, i.e., the width of the cuvette used in the ELISA. A sample was regarded as positive if the P/N value was equal to or greater than 2.0 (9). It was observed that there were no differences in results between the ELISA performed in cuvettes or in microtiter plates (9).

Briaire et al. also demonstrated that the ELISA was more sensitive than the SN test. For example, 64 serum samples obtained from vaccinated and PRV infected pigs that were SN negative when tested by the conventional SN test scored positive by the ELISA (9). When these same serums were tested by a modified SN test using a 24 hours preincubation period instead of one hour, 13 serums were positive (9). The SN titers were also directly proportional to the $\log_{10}$ of the P/N value (9).

Although there is a positive correlation between the ELISA procedure suggested by Briaire et al. (9) and the SN test, the lack of control antigen has been criticized by Todd et al. (71). Todd et al. argued that the virus antigen absorbance values of negative serum samples employed in the interpretation of the results by Briaire et al. (9), varied between 0.144 and 0.467. He further indicated that the antigen absorbance values of serums from two negative herds were also significantly different. He suggested that the differences in absorbance values produced by reacting

test serums with the positive virus antigen and negative host cell antigen would be a more reliable parameter for interpreting ELISA results (71).

In an attempt to determine the point between positive and negative responses of serum samples tested, Todd et al. (71) analyzed 129 negative serum samples. Statistical analysis of these 129 negative serum samples showed that 99.98 % gave differences in absorbance values below 0.15 (71). Consequently an OD difference of 0.15 was selected by Todd et al. as the point above which ELISA responses were recorded as positive.

Todd et al. also found that the ELISA was more sensitive than the SN test. They reported that 29 SN negative serum samples were positive when tested by ELISA. It was suggested that the failure of the SN test to detect antibody in such serum samples could be due to the measurement of different antibody by both tests (71). They speculated that at some point after infection, non-neutralizing antibodies to other viral antigen may still be present at levels detectable by the ELISA (71).

In France, the ELISA for the detection of PR antibody was also independently described by workers at the Contagious Disease Laboratory of the National Veterinary School at Alfort (74). Conjugate consisted of enzyme-labelled protein A (73). Antigen consisted of Triton X-100 solubilized PRV infected cells which had been clarified with KCl sucrose (0.25 M) for 10 minutes at 700 X g. Control antigen was prepared in exactly the same way from noninoculated cells. The results were expressed as the OD of the serum in the presence of viral antigen less the OD of the same serum in the presence of cell control antigen.

An index was then calculated by multiplying this difference by 10. Serum samples with indexes lower than one were considered negative. Serum samples with indexes between one and two were retested; and serum samples with indexes greater than two were considered positive (73). The French workers found good correlation between the results produced by ELISA and the SN test (73). However, they commented that the sensitivity of the ELISA varied between laboratories (73).

Due to the growing interest in the ELISA for pseudorabies diagnosis, a commercial ELISA test (Enzygnost Aujeszky^{TM}) has been developed by Research Laboratories, Behringwerke AG, D3550 Marburg, West Germany and subsequently licensed by the Federal Health Office in West Germany. This test was evaluated by Dopatka et al. (13) who reported a 97.6 % agreement between the ELISA and SN test after testing 1478 different pig serums in four different laboratories.

Other applications of the ELISA as a diagnostic tool are also available. For example, the ELISA can also be used to detect antibodies in body fluids other than serum. Dopatka et al. (13) found that liver and kidney fluids were good sources of antibody. Williams et al. (77) were also able to detect antibodies to PRV in porcine colostrum with the ELISA.

In summary, the ELISA appears to be an ideal test for the serological diagnosis of PR. Its sensitivity was found to be comparable to that of the SN test. Four groups of investigators (13, 71, 74, 77) demonstrated that the test was able to detect PRV specific antibody as early as seven days post infection and three groups (9, 41, 77) concluded

that the ELISA was more sensitive than the SN test. Stewart et al. (65) however suggested that the ELISA was less sensitive than the SN test but more sensitive than the MIDT. The ELISA has also been reported to be more sensitive than the IFAT (9). The ELISA can be automated to screen large numbers of serum samples and only requires three to four hours to produce results. In addition, the ELISA can be used to test serums that are cytotoxic to cell cultures and as such cannot be tested by the SN test.

Despite the advantage of the ELISA, the assay does have some significant disadvantages. The initial cost of equipment is high. The recommended equipment cost in 1983 for establishing ELISA capability in a diagnostic laboratory was $27,720 (25). Secondly, nonspecificity can be a problem. Snyder et al. (62) suggested two probable source of such nonspecificity: a) substrate conversion due to bacterial growth in serum samples, and b) cross reaction between vaccine and ELISA antigen. In support of the first possibility, Snyder et al. (62) cited that 44 % of the bacterial species isolated from contaminated serum produced substrate conversion ranging from weak to strong in an alkaline phosphatase system. However, only 17 % of these bacterial isolates caused a positive ELISA reaction in the horseradish peroxidase system. In support of the second possibility, Snyder et al. (62) showed that serum from three pigs which had been inoculated with calf serum reacted positively to ELISA antigen that was produced in cells grown in medium supplemented with calf serum.

Microimmunodiffusion test

In 1948, Ouchterlony (45) developed an *in vitro* method for testing the toxin producing capacity of diphtheria bacteria. This technique was used for identifying antigen specific precipitating antibodies or precipitins in agar gel. It was based on the principle that the reaction between the antigen and specific precipitin will result in the production of distinct lines or bands of precipitate in gel. This technique has subsequently been used to detect antibodies to many types of herpesvirus infections such as herpes simplex (72), herpes varicella-zoster (68), and PRV (20, 28, 40, 59). The technique is commonly referred to as the agar gel immunodiffusion test (AGIDT).

Smith and Stewart (59) conducted one of the first studies to evaluate the AGIDT as a diagnostic test for PRV antibody in swine and compared the AGIDT to the SN test using a double blind protocol. The AGIDT used by Smith and Stewart was a modification of the method described by Pearson et al. (47) who used the test to detect the presence of antibodies to equine infectious anemia virus. The test was run in one percent agarose gel prepared in 0.1 M borate-buffered saline, pH 8.5. Antigen consisted of heat inactivated PRV (Shope strain) which was propagated in embryonic pig kidney cells. The results of their study revealed 100 % correlation between the AGIDT and SN test if the SN titer of serums was $\geq$ 1:32. The correlation dropped to 86 % when serums having an SN titer of 1:16 were tested.

Gutekunst et al. (20) modified the AGIDT by developing the microimmunodiffusion test (MIDT). These workers reported that optimum

results were obtained using 0.69 % agarose in 0.05 M Tris buffer (pH 7.2) containing 0.025 % sodium azide but no NaCl. They reported that the presence of NaCl resulted in loss of definition of the precipitin lines. They also noted that an increase or decrease of pH from the optimum 7.2 also caused loss of definition of the precipitin lines. Antigen for the MIDT was prepared as described by Smith and Stewart (59) with the exception that virus was concentrated by precipitation with ammonium sulphate (42.5 % w/v) and then resuspended and dialyzed against distilled water. The viral antigen preparation was then concentrated to approximately one one-hundredth of the original volume with polyethylene glycol (m.w. 20,000). The test was run on standard slides containing 2.25 ml of agarose with wells measuring 2.5 mm in diameter and spaced 2.5 mm apart. Individual wells were filled with 20 ul of serum or antigen. Reactions were allowed to develop for 48 hours at 25 C. Results of this study revealed that the MIDT was less sensitive than the SN test. Ninety-four percent of serums with SN titers of 1:8 were positive by the MIDT and only 64 % of serums with SN titers of 1:4 were positive.

In an attempt to further improve the sensitivity of the MIDT, Medveczky and Tuboly (40a) evaluated an antigen prepared by solubilizing Bartha's K strain in an aqueous solution consisting of Triton X-100, sodium deoxycholate, and beta-mercaptoethanol. Test reactions were allowed to develop at 37 C for 48 hours. Test serums were regarded as positive if at least one independent precipitation line or highly visible arc appeared in the system containing the reference serum sample. The

latter phenomenon facilitated evaluation particularly of serums with low SN titers.

Medveczky and Tuboly (40a) found the sensitivity of their MIDT to be higher than that reported by Gutekunst et al. (20). One-hundred percent and 85 to 90 % serums with SN titer of 1:4 and 1:2, respectively tested positive in the MIDT. These investigators attributed the increased sensitivity to the solubilization of the antigen by detergents. They speculated that the solubilization procedure resulted in the separation of virus proteins, including those responsible for precipitation, from viral lipoproteins and that the beta-mercaptoethanol facilitated the diffusion of antigen in the gel by disrupting disulphide bridges resulting in a higher concentration of viral proteins participating in the reaction. Medveczky and Tuboly also found the test to be highly specific. Negative pig serums did not react falsely nor did the PRV positive serum samples react nonspecifically with PK or calf testicle cell antigens.

Further evaluation of the sensitivity and specificity of the MIDT for PRV antibodies was done by Johnson et al. (28). These investigators followed the standard MIDT protocol suggested by Gutekunst et al. (20) and evaluated sensitivity and specificity by using the following statistically valid formulas:

$$\text{Sensitivity} = \frac{(M+)\ (SN+)}{(M+)\ (SN+) + (M-)\ (SN+)} \times 100\ \%$$

$$\text{Specificity} = \frac{(M -)\ (SN -)}{(M -)\ (SN -) + (M +)\ (SN -)} \times 100\ \%$$

Where (M +) is the number of MIDT positive serums; (M -) is the number of MIDT negative serums; (SN +) is the number of SN positive serums; and (SN -) is the number of SN negative serums.

Johnson et al. (28) found the sensitivity to be 100 % when testing serums with SN titers of 1:4 or higher that were collected from naturally infected pigs. However, the sensitivity of the MIDT was found to be only 34.8 % when used to detect maternal antibody, and only 26.04 % when used to detect antibodies in sows vaccinated with attenuated PRV vaccine (Norden Lab., Lincoln, NE) (28). Johnson et al. attributed this lower sensitivity to smaller amounts of precipitating antibody in the serum of vaccinated pigs or piglets with maternal antibodies.

Based on the above reports, it appears that the MIDT is almost as sensitivity as the SN test for detecting antibodies in naturally infected pigs. In addition, the MIDT has several advantages over the SN test. First, it is a very simple test to perform and requires less expertise to conduct. Second, the results of the test can be obtained rapidly. The test can be read after 48 hours of incubation while the preliminary results of the test can be obtained in as little as 24 hours. Third, the MIDT is comparatively less expensive when compared to the SN test. Smith and Stewart (59) estimated the cost of the AGIDT test per serum sample to be $ 0.68 compared to $ 3.00 to $ 5.00 for the SN test. The cost is even less for the MIDT which requires smaller volumes of test reagents and

agar. Fourth, the MIDT can be used to assay cytotoxic, hemolyzed, or contaminated serum samples. Smith and Stewart (59) indicated that two severely hemolyzed serum samples were positive by the AGIDT but were toxic to embryonic PK cells used in the SN test. Gutekunst et al. (20) found that 13 serum samples were positive out of 71 samples which were too toxic, contaminated, or markedly hemolyzed to be evaluated in the SN test. Medveczky and Tuboly (40a) indicated that four out of six serum samples which were cyototoxic in the SN test were MIDT positive.

The disadvantages of MIDT include: 1) difficulty in accurately determining antibody endpoints making it difficult to detect changes in antibody levels due to vaccination or infection, and 2) lack of sensitivity for detecting precipitation antibodies in both vaccinated or passively immunized pigs.

Attempts have been made by Medveczky and Tuboly (40a) and Gutekunst et al. (20) to standardize the MIDT for diagnostic laboratory use. Both groups proposed that standard reference serums with SN titers of 1:2 and 1:4 should be tested with each lot of serum. Gutekunst et al. further proposed that such reference serums should be collected only from naturally infected or nasally infected pigs to minimize the possibility of false reactions.

Indirect hemagglutination test

The indirect hemagglutination test, also known as the passive hemagglutination test, has been used widely for the diagnosis of many virus infections including those caused by herpesviruses (6, 21, 32, 64,

76, 81). One of the most extensive studies on the IHAT was done by Bernstein and Stewart (6) who adapted the test for detecting antibodies against cytomegalovirus (CMV). Antigen consisted of whole virions suspended in PBS. Test serums were initially diluted 1:10 in PBS containing one percent heat inactivated, normal rabbit serum which was previously absorbed with a 50 % suspension of sheep red blood cells (RBCs). The diluted serums were then heat inactivated at 56 C for 30 minutes and absorbed at 4 C for 30 minutes with 0.1 ml of a 50 % suspension of washed sheep RBCs to remove heterophilic antibodies. Serums were then diluted two-fold in microtiter plates and allowed to react with antigen coated sheep RBCs at room temperature for two to four hours and refrigerated until read. Wells were read as positive when the cells were completely and uniformly agglutinated or when a large circle of partially agglutinated cells coated the bottom of the wells. Small rings of unagglutinated cells and buttons were read as plus-minus and negative reaction, respectively.

Bernstein and Stewart (6) compared the sensitivity of the IHAT to that of the complement fixation (CF) test and found that the IHAT generally produced serum titers from five to 10 times higher than the CF test. They further reported that the test was highly sensitive and reproducible and detected both IgM and IgG (6). Bernstein and Stewart (6) also showed that the IHAT was highly specific by indirect hemagglutination inhibition test. The addition of homologous antigen to CMV serum samples consistently produced inhibition while the use of heterologous antigen from herpes varicella-zoster virus produced no

inhibition. Specificity was also demonstrated by the absence of positive IHAT reactions when serums with high antibody level to related herpesvirus were tested (6).

Bernstein and Stewart (6) also noted that the use of sheep RBCs which were aged for at least two weeks generally gave sharper endpoints than fresh RBCs. They also suggested that the best settling pattern for endpoints determination was produced when the diluent contained one percent rabbit serum (6). In the absence of rabbit serum the RBCs often failed to form compact buttons and endpoint were very difficult to read.

Two studies on the use of the IHAT for detecting PRV antibody in swine have been made by Haffer et al. (21) and Labadie and Toma (32). Both groups of investigators used tanned sheep RBCs sensitized with whole virions. They reported that IHAT results correlated 100 % with SN test results of serums with SN titers ranging from 1:2 to 1:256. Haffer et al. also demonstrated that PRV antibody could be detected in swine as early as five days p.i., two days before the appearance of neutralizing antibody. They attributed the early detection of antibody to the ability of the IHAT to detect IgM. These workers also demonstrated that the IHAT was suitable for detecting antibody in cytotoxic serums. Labadie and Toma also demonstrated that the test was highly specific. No crossreactivity was observed when serums with antibody titers to IBR virus, porcine parvovirus (PPV), and transmissible gastroenteritis (TGE) virus, were tested.

Indirect fluorescence antibody test

The indirect fluorescence antibody test for detecting PRV antibody in swine was evaluated by Wirahadiredja and Rondhuis (80). These investigators used antigen that consisted of a mixture containing equal quantities of PRV infected and noninfected baby hamster kidney cells. One tenth ml of this mixture was placed into test wells of epoxy-coated microprint slides. The slides were air-dried for 24 hours at room temperature and stored at -25 C until used. Sheep anti-pig IgG labelled with fluorescein isothiocyanate (FITC) was used as conjugate. These investigators reported that frequent false positive reactions occurred when serums from PRV free pigs were tested. They also reported that the test lacked sensitivity. For example, of 39 serums with PRV SN titers of 1:4, only 27 (69.2 %) were identified as positive by the IFAT. However, when serums with SN titers of 1:16 and 1:32 were tested by the IFAT, 95.7 % and 100 %, respectively were positive.

Latex macroagglutination test

The latex macroagglutination test was first described by Bercks and Querfurth (5) who reported that antigen coated latex beads usually ranging in diameter from 0.8 to 0.9 um could be specifically agglutinated with antiserum. Antigen is usually adsorbed to the beads by either direct adsorption or by first coating the beads with antigen specific immunoglobulin and subsequently allowing antigen to interact with the globulin coated beads. Antigen can also be attached more uniformly to beads by utilizing a carbodiimide reaction between carboxylated latex

beads and oxidized antigen (53). Two groups of investigators have evaluated the LMAT for detecting PRV antibody (39, 79). The more extensive study conducted by McGregor et al. (39) involved 200 serums with SN titers ranging from <20 to >5000. These investigators reported that LMAT results correlated 100 % with the SN test results. They also reported that the LMAT detected PRV antibody in all serums at day 8 p.i. compared to only two of five pigs when tested by SN test. In a more limited study Wilson and Schipper (79) reported an 88 % agreement between the LMAT and SN test results.

Complement fixation test

The complement fixation (CF) test has been used for the serological diagnosis of bacterial, fungal, chlamydial, mycoplasmal, rickettsial, and viral diseases (75). Mayer has discussed the theoretical principles of this test in great detail (37). The CF test is based on the ability of complement to bind to antigen-antibody complexes. The sensitivity and specificity of the test is dependent upon optimizing the quantity of both antigen and complement in the test system. A positive test reaction is indicated by the presence of free complement which is detected by the lysis of sheep RBCs sensitized with rabbit anti-sheep immunoglobulin.

The CF test has not been frequently used with pig serum because of procomplementary activity associated with pig serums. Eskildsen (17) however demonstrated that it was possible to modify the CF test and use it to detect PRV specific antibody in swine serum. This modification consisted of treating swine serums with 2-mercaptoethanol in TRIS buffer,

pH 7.2 at 56 C for 20 minutes followed by freeze-thawing at -20 C and 37 C, respectively and clarification by low speed centrifugation. Although the preceding treatment removed the procomplementary activity, it also had the undesirable effect of inactivating specific IgM activity.

In a subsequent study, Bitsch and Eskildsen (8) used the modified CF test and found a high degree of agreement with the SN test. They reported that CF titers were detected earlier than the SN titer and were also up to one $\log_2$ dilution higher. These investigators also reported that occasionally test serums reacted with control antigen suggesting that the test may lack a degree of specificity.

The ability of the CF test to detect PRV specific antibody earlier than the SN test suggests that it can be used as an alternative diagnostic test in an eradication program where early detection is important. However, due to the laborious procedure for removing procomplementary activity and lack of specifity, the CF test may not be suitable to screen large numbers of serums.

Radial immunodiffusion enzyme assay

In 1984, Joo et al. (29) described the radial immunodiffusion enzyme assay (RIDEA) for the detection of PRV antibodies. This technique combines the principle of radial immunodiffusion and ELISA. Basically, a Triton X-100 disrupted subunit fraction of PRV is coated onto polystyrene petri dishes and overlayed with one percent agar in PBS, pH 7.2. Circular wells three mm in diameter are cut in the agar and filled with 0.015 ml of test serum. After overnight incubation, the gel is

peeled off, and the petri dish is washed with PBS, flooded with peroxidase conjugated rabbit anti-pig IgG, and allowed to incubate at 25 C for one hour. The petri dish is washed a second time and overlaid with agar containing enzyme substrate and color indicator. The presence of antigen-antibody reactions is indicated by the appearance of a dark purple circular zone which is permitted to develop over a 15 hour period.

The antibody titer of a serum is directly propotional to the diameter of the colored circular zones. It was found that diameters of 7, 8, 9, 10, 11, and 12 mm were equivalent to SN titers of 1:2 to 1:4,1:2 to 1:8, 1:4 to 1:16, 1:8 to 1:32, 1:16 to 1:32, and 1:32 to 1:64, respectively. Diameters of six mm or less were considered to represent a negative reaction since most known negative serums tested, produced diameters of six mm or less.

Joo et al. (29) reported that the sensitivity and specificity of RIDEA were comparable to SN test results. The test was further evaluated by Thawley et al. (70) who found that the sensitivity and specificity of the test was similar to that reported by Joo et al. with the exception that maternally transferred antibodies were only detected in 76.1 % of 283 on piglets that tested SN positive.

In summary, the RIDEA compares favorably with the SN test with respect to sensitivity and specificity. It is relatively simple to run and can be used in the field. Its major disadvantages are the cost of test reagents, and a difficulty in precisely determining endpoint titers. The diameter of the colored circular zones only correspond to a range in SN titers and not a definite endpoint. The inability of the test to be

adapted to a microsystem may prevent it from being used to screen and to test large numbers of serums.

Indirect solid-phase microradioimmunoassay

In 1978 Kelling et al. (30) developed the indirect solid-phase microradioimmunoassay (IRIA) for the detection of PRV antibodies utilizing antigen prepared according to the method described by Gutekunst et al. (20). The test was run by incorporating PRV antigen and control cell antigen in separate wells of microtiter plates. The plates were air-dried overnight at room temperature and fixed with absolute methanol for 20 minutes. Two-fold dilutions of the serum samples were made and added to the wells containing the viral and control antigens. After a 90 minute incubation period, the wells were washed and treated with ^{125}I-labelled rabbit anti-pig IgG for one hour at 37 C. The conjugate was aspirated from each well and the plates were air-dried. Plates were secured in a metal template and a three mm diameter section was punched out from the bottom of each well and placed in gamma counting vials for counting. The binding ratio for individual serum samples was calculated by dividing the average counts per minutes (cpm) of wells containing PRV antigen by the average cpm of wells containing control cell antigen (24). A serum was considered positive if the binding ratio was greater than or equal to two.

Kelling et al. (30) found that the results from the IRIA correlated closely with the SN test and MIDT. Moreover, they reported that the IRIA titers were two to 32 times greater than the corresponding SN titers for

10 of 12 serums indicating that the IRIA was more sensitive than the SN test. The authors, however, did not address the specificity of the test.

Besides being more sensitive than the SN test, the IRIA also offers other advantages. First, test results can be quantitated. Secondly, the test can be used to detect antibodies in cytotoxic serums and in serums that have been contaminated with bacteria. Thirdly, test results can be obtained in approximately three hours, as compared with 48 hours required for the SN test and MIDT.

However, one important disadvantage of the IRIA is its use of ^{125}Iodine which requires special handling and safety precautions. The IRIA also requires the use of expensive scintillation equipment which may not be available to many laboratories.

Countercurrent immunoelectrophoresis

Countercurrent immunoelectrophoresis is based on the principle of electrophoresing antigen and antibody in opposing directions in agarose gel which results in the formation of a distinct immunoprecipitate when optimum concentrations of antigen and antibody interact. The test was adapted for the detection of PRV antibody by Papp-Vid and Dulac (46). Antigen consisted of whole inactivated virions that were precipitated from lysate of infected cells and suspended in veronal buffer, pH 8.2. Lysate from noninfected cells was processed in the same manner and used as the control antigen. Individual wells were spaced four mm apart in agarose gel prepared in veronal buffer, pH 8.2. Wells were filled with 10 ul of antigen on the cathode side and 10 ul of serum on the anode

side. The antigen and antibody were electrophoresed at 10 volts/cm for 60 minutes. Distinct immunoprecipitate lines consistently formed at approximately 1.2 mm from the well containing antibody and was readily visible by examination in indirect light. Williams et al. (78) demonstrated that the visualization of the immunoprecipitation line could be enhanced by staining with commassie brilliant blue R-250 or treating gels with a mixture of 2 % zinc chloride and 2 % sodium phosphotungstate in distilled water.

The sensitivity of CIE compared favorably with both the SN test and MIDT. According to Papp-Vid and Dulac, all serums with SN titers of 1:3 or greater also tested positive by CIE. However, CIE could not detect PRV antibody in serum samples with an SN titer of 1:2. They also pointed out that circulating antibodies were detected by the CIE as early as seven days p.i. In contrast, Williams et al. (78) showed that there was 100 % agreement between the SN test and CIE, but only when serums with SN titers of 1:32 or greater were tested which suggested that the CIE is less sensitive than the SN test. Papp-Vid and Dulac (46) also reported that the CIE was highly specific. No false positive reactions were observed when PRV antibody negative serums or serums obtained from pigs infected with porcine CMV were tested.

The CIE is a particularly useful test because results can be obtained in as little time as one hour. The test is also relatively simple to perform, does not require expensive equipment, and can be used to economically screen large numbers of serums for PRV antibody. The test has the added advantage of being able to test serums that are

cytotoxic. However, one disadvantage of CIE is that, like the MIDT, it is not a reliable quantitative test.

Concluding Remarks

The SN test, ELISA, IHAT, and LMAT are serological tests that are comparable in terms of sensitivity and specificity. All four tests are suitable for large scale screening of swine serums. However, more tests can be run in a single day by the ELISA and the SN test than by either the IHAT and LMAT. Both the ELISA, IHAT, and LMAT can produce results in one day compared to two days that are required by the SN test. These three tests also have the added advantage of being able to detect antibody in cytotoxic serums which cannot be tested by the SN test. The IHAT and LMAT are superior to the SN test and the ELISA in their ability to detect antibody earlier. However, antibody could be detected earlier by the ELISA if anti-pig IgM were included in the enzyme conjugate. The IRIA is also a highly sensitive test but its requirement for ^{125}I makes it less desirable as a routine laboratory test. The RIDEA is also as sensitive as the SN test. However, this test requires at least two days for results and uses relatively large quantities of materials and reagents. It probably will not be used for large scale PR testing. The MIDT, IFAT and the CF test are not as sensitive as the SN test, ELISA, IHAT, or LMAT. Each of these tests is also more laborious to perform and as such are not practical for large scale PR testing.

CHAPTER III. MATERIALS AND METHODS

Virus, Cells, Media, Buffers and Reagents

Pseudorabies virus strains BE and K were used for antigen production. Pseudorabies virus strain BE was used as challenge virus in the pig studies. Porcine kidney (PK) 1a cells and bovine lung (BL) cells were obtained from the Veterinary Medical Research Institute, Iowa State University, Ames, IA. Growth medium (GM) consisted of Eagle's minimum essential medium (MEM) with Earle's salts (Gibco, Madison, WI) supplemented with 10 % fetal calf serum. Antibiotics were incorporated in the medium at the rate of five ug/ml gentocin, or 100 ug/ml streptomycin, and 100 IU/ml penicillin G. Fungizone was added to all medium at the rate of 1.65 ug/ml. Maintenance medium (MM) was GM supplemented with two percent fetal calf serum.

Antigen coating buffer (ACB) for the ELISA consisted of 0.02 M carbonate/bicarbonate buffer, pH 9.6 (Table 1). Wash solution (WS) was 0.01 M phosphate buffered saline (PBS), pH 7.2 containing 0.05 % Tween 20 (Table 2). Serum/conjugate diluent (SCD) and substrate diluent (SD) were 0.05 M Tris/saline buffer, pH 7.4 (Table 3) and 0.05 M citric acid solution, pH 4.0, respectively. The color indicating system was 2,2' Azino-di-(3-ethylbenzthiozoline-6-sulfonic acid (ABTS). Substrate was a 1/50 dilution of a 50 % solution of hydrogen peroxide. The conjugate used was horseradish peroxidase labelled goat anti-porcine IgG (National Veterinary Services Laboratory (NVSL), Ames, IA).

The serum diluent and washing buffer for the modified IFAT was 0.01 M PBS, pH 7.4 (Table 2). Mouse anti-pig IgM and IgG monoclonal antibodies were obtained from Dr. P. S. Paul, Veterinary Medical Research Institute, Iowa State University, Ames, IA.

Buffers and reagents used in the IHAT included 0.15 M PBS, pH 6.7 and pH 7.2 (Table 4) and 1 % (w/v) reagent grade tannic acid (Mallinckrodt Chemical Works, St. Louis, MO). Alsever's solution (Table 5) was used for the collection of sheep RBCs.

Antigen extraction buffer (EB) consisted of 0.025 M tris/tricine (TT) buffer pH 8.6, (Table 6) (Bio-Rad Laboratories, Richmond, CA) containing 1 % (v/v) Triton X-100.

Table 1. Preparation of 0.02 M carbonate/bicarbonate buffer, pH 9.6

Reagents	Amounts
Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Sterile deionized water	q.s. to 1 liter

Adjust pH to 9.6 using 5M NaOH and store at 4 C in a sealed container no longer than 1 week.

Table 2. Preparation of 0.01 M phosphate buffered saline

Reagents	Amounts	
	pH 7.2	pH 7.4
Na_2HPO_4	1.19 g	1.15 g
NaH_2PO_4	0.22 g	-
$NaH_2PO_4.H_2O$	-	0.28 g
NaCl	8.50 g	8.50 g
Tween 20	0.50 ml	-
Sterile deionized water	q.s. to 1000 ml	

Adjust pH with 1 N HCl or 1 N NaOH.

Table 3. Preparation of 0.05 M Tris/saline buffer, pH 7.4

Reagents	Amounts
NaCl	8.70 g
EDTA	0.37 g
Tris base	6.05 g
Bovine serum albumin (BSA, Fraction V)	10.00 g
Tween 20	0.50 g
Sterile deionized water	q.s. to 1000 ml

Adjust pH with 1 N HCl or NaOH.

Table 4. Preparation of 0.15 M phosphate buffered saline, pH 6.7 and 7.2

Reagents	Amounts
Solution A	
KH_2PO_4	101.70 g
Sterile deionized water	q.s. to 1 liter
Solution B	
Na_2HPO_4	105.95 g
Sterile deionized water	q.s. to 1 liter
Solution C	
NaCl	42.50 g
Sterile deionized water	q.s. to 1 liter
To make 1 liter of 0.15M PBS pH 6.7 (5X), add	
Solution A	339.00 ml
Solution B	161.00 ml
Solution C	500.00 ml
To make 1 liter of 0.15M PBS pH 7.2 (5X), add	
Solution A	380.00 ml
Solution B	120.00 ml
Solution C	500.00 ml
Dilute to 1X and adjust pH using 1N NaOH.	

Table 5. Preparation of Alsever's solution

Reagents	Amounts
Dextrose	20.5 g
Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	8.0 g
Citric acid ($C_6H_8O_7$)	0.6 g
Sodium chloride	4.2 g
Sterile deionized water	q.s. to 1 liter

Sterilize by filtration (0.22 um).

Adjust pH to 6.1 $\pm$ 0.1 with 1 N HCl or 1 N NaOH.

Table 6. Preparation of 0.025 M tris/tricine buffer, pH 8.4

Reagents	Amounts
TRIS-base	9.8 g
Tricine	4.3 g
Calcium Lactate	0.1 g
Sodium azide	0.2 g
Sterile deionized water	q.s. to 1 liter

Adjust pH with 1 N HCl and 1 N NaOH.

Antigen Production

Preparation of whole pseudorabies virus antigen

Porcine kidney (PK) 1a cells were propagated in GM at 37 C in 890 cm^2 plastic roller bottles. Confluent cell monolayers were infected with the BE strain of PRV at a multiplicity of infection of 10. After one hour, virus inoculum was removed and replaced with MM. Virus infected cells were harvested using sterile glass beads when 90 to 100 % of the cells showed cytopathic effects (CPE). Harvested cells were freeze-thawed three times to disrupt the cells and the virus suspension was clarified twice by centrifugation at 800 X g for 30 minutes.

The virus suspension was inactivated with beta-propiolactone (BPL) by the following protocol. A 10 % dilution of stock BPL (Sigma Chemical Company, St. Louis, MO) was prepared in cold (4 C) sterile deionized water. Diluted BPL was slowly added to cold constantly agitated virus suspension at the rate of one ml BPL per 50 ml of suspension. The pH of the suspension was adjusted to approximately 7.0 with 1 N NaOH. The temperature of the suspension was then raised to 37 C and maintained at this level for 72 hours. During this time period the preparation was continuously agitated and the pH monitored and adjusted as necessary to avoid acidity. Bacterial contamination was prevented by adding 0.5 ml of a one percent thimerasol solution to each 100 ml of suspension before BPL treatment.

Inactivated virions were removed from the suspension by two methods of ultracentrifugation: a) centrifugation at 50 K X g for 30 minutes, and b) centrifugation at 100 K X g for two hours through a 36 % (w/v)

sucrose solution prepared in 0.15 M PBS, pH 6.7 onto a 72 % (w/v) sucrose cushion. Pelleted virus from the first method was resuspended in 0.15 M PBS to one tenth of the original volume. Virus that accumulated on top of the sucrose cushion in the second method was harvested and dialyzed against 0.15 M PBS to remove the sucrose. Hereafter, these two virus preparations are referred as cushioned (CUS) antigen and pelleted (PEL) antigen.

Preparation of lectin-purified, PRV glycoprotein antigen

Lectin purified antigen was prepared by the method described by Platt (49). In brief, PK-1a cells were propagated at 37 C in 890 cm^2 plastic roller bottles. The cells were inoculated with either the BE or Bartha K strain of PRV. Cells were removed from the surface of roller bottles with the aid of glass beads when 100 % of the monolayer showed CPE. The cells were separated from the medium by centrifugation at 1.9 K X g for 15 minutes and the packed cell volume was determined. The cells were then washed twice in serum-free medium and resuspended in EB at the rate of 1.5 ml buffer per one ml of packed cell volume. The cell preparation was sonically disrupted with a Bronson sonifier, model 350 equipped with a microtip. The output was set at two and the duty cycle at 50 %. Power was supplied discontinuously for 20 pulses. Occasionally, an additional ten pulses were required for complete cell disruption. During this procedure the cell suspension was kept in an ice bath. Following sonication, an additional 1.5 ml of extraction buffer per one ml of original cell volume was added. The preparation was then

gently agitated for 24 to 48 hours at 4 C and then centrifuged at 100 K X g for 90 minutes. The clear middle layer of the centrifuged material constituting crude viral antigen (CVA) was harvested and stored at 4 C until used.

Pseudorabies virus glycoproteins were removed from the CVA by lectin affinity column chromatography utilizing either Lens culinaris agglutinin (LCA) or Ricinus communis agglutinin (RCA) which was covalently immobilized on agarose beads (E-Y Laboratories, San Mateo, CA). Prior to use, the lectin preparation was equilibrated with 10 volumes of EB followed by three volumes of EB containing 2.5 % (w/v) mannose. The column was then washed with 10 volumes of mannose free EB, loaded to capacity with CVA and stored overnight at 4 C. Unabsorbed antigen was removed from the column by washing with 10 volumes of EB followed by five volumes of TT buffer without detergent. The specifically adsorbed antigen was eluted from LCA agarose or RCA agarose columns with three volumes of EB containing 2.5 % (w/v) mannose and galactose, respectively. The eluted antigens, hereafter referred to as LCA or RCA antigen, were concentrated to approximately 15 % of the original volume of CVA by ultrafiltration utilizing a Diaflo PM 30 membrane (Amicon, Danvers, MA) which has a molecular weight limit of approximately 30,000. The concentrated antigen preparations were equilibrated by dialysis against 0.15 M PBS, pH 6.7.

Microtiter Serum Neutralization Test

The procedure for the microtiter serum neutralization test was done as described by Hill et al. (24). In brief, serum samples were inactivated at 56 C for 30 minutes. Duplicate, serial two-fold dilutions of serums were made with 0.05 ml volumes of Earle's balanced salt solution in standard 96-well, flat bottom microtiter plates. An equal volume (0.05 ml) of the virus suspension containing 300 $TCID_{50}$ were added to each well containing the diluted serum. The plates were agitated for two minutes and subsequently incubated at 25 C for at least one hour. The plates were agitated for two minutes and subsequently incubated at 25 C for at least one hour. Five hundreth ml of cells suspension containing approximately 20,000 MDBK cells were added to each well. The plates were then incubated at 37 C for 48 hours in a 5 % CO_2 atmosphere. Serum neutralization titers were defined as the highest dilution (log_2) of the serum that gave complete neutralization in both wells. Titers were then expressed as the geometric mean of a minimum of two separate tests. All tests were run with appropriate negative and positive serum controls and cell controls. The virus antigen concentration was confirmed for each test by back titration.

Enzyme-Linked Immunosorbent Assay

The ELISA was performed according to the procedure outlined by Snyder and Erickson (61). Control and test serums were diluted 1:20 in 0.05 M Tris buffer, pH 7.4. The diluted serums were added in duplicate at the rate of 100 ul to individual antigen coated and control wells of

96-well plates (Immunlon 1, Dynatech Laboratories, Alexandria, VA) and allowed to incubate at 25 C for 30 minutes on an orbital shaker set at 60 cycles per minutes. Samples were removed from the wells and the plates were washed three times with 0.01 M PBS, pH 7.2. A three minute soak period was used between each washing. An optimal dilution of conjugate was then added to the test system and allowed to react for 30 minutes at 25 C on an orbital shaker. Plates were again washed as above, treated with 50 ul per well of enzyme substrate containing ABTS and incubated at 25 C for 20 minutes on an orbital shaker. The enzyme substrate reaction was stopped by adding 50 ul per well of a 1:400 aqueous dilution of 48 % reagent grade, hydrofluoric acid. The optical density (OD) of the resulting color reaction was read with the Dynatech MR 580 Microelisa Auto Reader using a test filter wavelength of 405 nm and a reference filter wavelength of 450 nm. Mean absorbance ratios were then calculated by dividing the mean absorbance values produced by reacting test serum with PRV antigen, i.e., signal (S) and normal host cell antigen, i.e., noise (N). Serums were considered positive for PRV antibody if the S/N ratio was $\geq$ 1.8 and the OD of a test serum reacted with test antigen exceeded a control midpoint value. The control midpoint value was the mean of the sums of the OD produced by separately reacting both negative serum and weak positive serum with test antigen. Individual ELISA titers represented the reciprocal of the last serum dilution to produce a positive ELISA reaction (13).

The test and control antigen used in the ELISA was supplied by NVSL, Ames, IA and consisted of detergent solubilized whole PRV infected and

virus free Vero-Maru cells, respectively. Microtiter plates were coated with antigen by adding 100 ul/well of antigen preparation in ACB. The plates were incubated for three hours at 37 C and then at 4 C until used. The antigen was left on the plates during storage. Plates were washed three times with WS prior to use. There was no soak period between the washings.

The optimum antigen concentration was determined by first selecting the concentration that yielded OD values of 0.15 $\pm$ 0.025 and $\geq$ 1.00 when reacted with negative and strong positive serums, i.e., SN titer $\geq$ 1:64, respectively. Subsequently, normal host cell antigen was diluted to a concentration that yielded the same OD $\pm$ 20 % when reacted with negative serums as was produced when negative serum was reacted with test antigen.

The optimum dilution of conjugate was determined by selecting the highest dilution of conjugate that produced the greatest OD when weak and strong positive control serums were reacted with positive antigen and the lowest OD when these serums were reacted with normal host cell antigen.

Modified Indirect Fluorescence Antibody Test

Antigen for the modified indirect fluorescence antibody test (IFAT) consisted of acetone fixed, PRV infected BL cells grown in four chambered Lab Tek slides (American Scientific Products, Minneapolis, MN). The titer of the virus inoculum used to prepare the slides was predetermined so that approximately 20 % of the cell monolayer exhibited CPE after a 36 to 48 hours incubation period. Occasionally, a single chamber of cells was left uninoculated to serve as a control for nonspecific

fluorescence. Fixed slides were air-dried, packaged in air-tight plastic bags containing anhydrous calcium chloride as desiccant and stored at -70 C until used.

The IFAT was executed by adding 50 ul of individual test serums to virus infected cells, taking extreme care to avoid cross contamination with different serums. The antigen/serum preparations were incubated in a humidified chamber for 30 minutes at 37 C and then washed three times in gently agitating PBS, pH 7.4. Each wash cycle lasted 10 minutes. Subsequently, the antigen preparations were stained and washed as described above first with either mouse anti-swine IgM (IFAT-M) or IgG (IFAT-G) monoclonal antibody and secondly with goat anti-mouse Fc conjugate. The preparations were then examined by ultraviolet microscopy using a UV-microscope equipped with epifluorescence 10X oculars and 40X lens. A 450-490 nm exciter filter and 520 nm barrier filter were routinely used.

Positive reactions were determined by comparing each test reaction to that produced by negative and weak positive control serums. A reaction was considered positive if it fluoresced greater than the fluorescence produced by control negative serum and equal to or greater than that induced by weakly positive control serum. Titers were expressed as the reciprocal of the last dilution of serum to produce an obviously positive reaction. Individual tests were repeated at least twice.

Indirect Hemagglutination Test

Antigen preparation

Normal sheep RBCs were collected aseptically in an equal volume of Alsever's solution. The cells were centrifuged at 800 X g for 10 minutes and the buffy coat was removed. The packed cells were washed three times in 0.15 M PBS, pH 7.2, resuspended in the same buffer, and adjusted to a 5 % concentration. A 2.5 % (v/v) L-gluteraldehyde solution in 0.15 M PBS, pH 7.2 was then slowly added at a rate of one ml fixative for every 10 ml of cell suspension. Fixation of RBCs was allowed to proceed for one hour at 25 C. The fixed cells were collected by centrifugation, washed three times in 0.15 M PBS, pH 7.2 and resuspended in the same buffer to a final concentration of 2.5 % (v/v) for sensitization with antigen. A 50 % cell suspension was also prepared and used for the absorption of serum samples.

Tanning and sensitization of sheep RBCs with antigen were done according to the method described by Yeager (81) with modifications. The optimum concentration of tannic acid and antigen was determined by cross titration. Tanning was accomplished by mixing an equal volume of a 2.5 % RBC suspension with an equal volume of tannic acid diluted in 0.15 M PBS, pH 7.2 to 1:5,000, 1:10,000, 1:20,000, and 1:40,000. The RBCs were allowed to react at 37 C with the different dilutions of tannic acid for 15 minutes, washed three times in 0.15 M PBS, pH 7.2, and resuspended in 0.15 M PBS, pH 6.7 to a 2.5 % suspension. The tanned RBCs were divided into aliquotes and mixed with equal volumes of specific dilutions of individual antigens prepared in 0.15 M PBS, pH 6.7. The cell-antigen

mixtures were reacted at 37 C with intermittent gentle agitation every 10 minutes for one hour. The antigen coated or sensitized cells were collected by centrifugation, washed three times in 0.15 M PBS, pH 7.2 and resuspended to a 0.5 % suspension in 0.15 M PBS, pH 7.2 containing 0.5 % gelatin (w/v) (Difco Laboratories, Detroit, MI) and 0.1 % Tween 20 (v/v) (Sigma Chemical Co., St. Louis, MO). Control cells were prepared in an identical manner except that no antigen was used.

The optimal conditions for tanning and sensitization of RBCs were then determined by using each antigen preparation in the IHAT as described below to titer a known positive and a known negative serum. The optimal antigen dilution was that which produced the highest titer for the positive serum and the least NSH when reacted with the negative serum.

Execution of the indirect hemagglutination test

Prior to testing, individual serums were heat inactivated for 30 minutes at 56 C. Non-specific agglutinins were adsorbed by adding 0.1 ml of a 50 % suspension of fixed sheep RBCs to one ml of test serum and incubating at 25 C for 30 minutes. The adsorbed serums were clarified by low speed centrifugation. Two sets of serial two-fold dilutions of the serums were made in 0.025 ml volumes of 0.15 M PBS, pH 7.2 containing 0.5 % (w/v) gelatin and 0.1 % (v/v) Tween 20 in 96-well, V-bottom microtiter plates (Dynatech, Alexandria, VA). Subsequently, 0.025 ml of sensitized RBCs was added to individual wells. The microtiter plates were then gently agitated to disperse the cells, allowed to incubate at

room temperature for one to two hours and read. Agglutination reactions were graded negative, 1+, 2+, 3+, and 4+ as illustrated in Figure 2. Only grades of 3+ and 4+ were considered to be a positive indication of antibody presence. Titers of individual serums were determined at least twice. Controls consisting of known high and weak positive serums, negative serums, and diluent were run against sensitized and nonsensitized cells each day the test was run.

Production of Pseudorabies Virus Antibody in Experimental Pigs

Three groups of pigs were nasally infected with PRV. Gruops 1 and 3 consisted of ten and seven secondary SPF pigs and were infected with 10^3 PFU of PRV contained in two ml of MM. Group 2 consisted of two minimal disease pigs that were similarly infected with 10^5 PFU of PRV. Serums were similarly collected from Group 3 pigs through day 7 p.i., the last day of survival. The serums from each group were separately pooled by day, aliquoted and stored at -70 C until used. Serums of individual pigs were also aliquoted and similarly stored.

Collection of Pseudorabies Antibody Positive Field Serums

Field serums with PRV SN titers ranging from 1:2 to 1:8 were obtained from the Veterinary Diagnostic Laboratory at Iowa State University, Ames, IA. Out of 240 serums tested, 30, 30, and 32 serums had titers of 1:2, 1:4, and 1:8, respectively. Care was taken to select only those serums that tested positive at the same titer in two consecutive tests. The PRV antibody status of each serum was confirmed

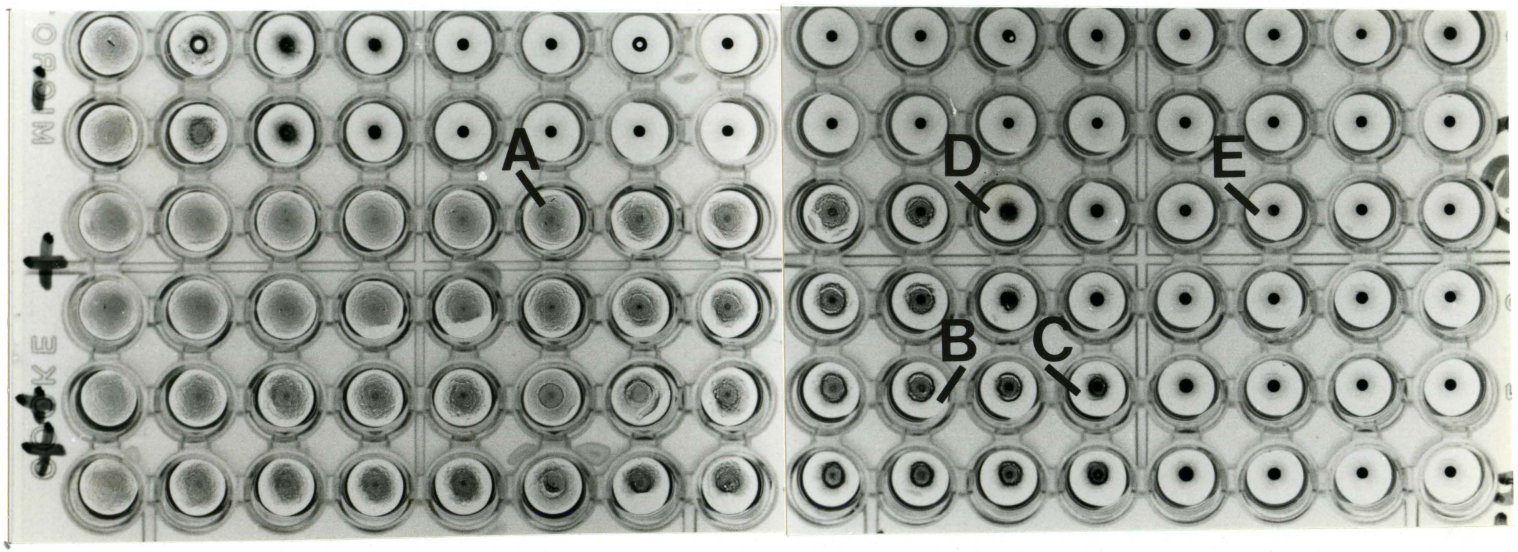

Figure 2. Typical IHAT reactions. A, B, C, D, and E represent the degree of hemagglutination of 4+, 3+, 2+, 1+, and negative (button)

by the ELISA, IFAT-M, and IFAT-G. Serums with PRV specific IgM were considered to represent early infections. Serums with no PRV specific IgM were considered to represent long standing infections.

Evaluation of Specific Antigens in the IHAT

Initially, whole virion preparations and solubilized viral glycoproteins extracted by LCA affinity chromatography from cells infected with the BE strain of PRV were compared as IHAT antigen using pooled serums collected from four surviving pigs of Group 2. The suitability of each preparation as IHAT antigen was determined by comparing: a) the antibody titers of pooled serum by day, and b) the degree of NSH at the lower serum dilutions. Results of these initial comparisons indicated that the viral glycoprotein preparation was superior to whole virions as IHAT antigen. Consequently, further evaluations were made only with viral glycoproteins of the BE and K strains of PRV which were extracted from detergent solubilized, infected cells by LCA or RCA affinity chromatography.

The sensitivity of each glycoprotein preparation was assessed by comparing the specific IHAT antibody response of surviving pigs of Group 1. Results from these comparisons revealed that there were no significant differences between the glycoprotein preparations. Consequently, further evaluation of the IHAT was made only with strain BE glycoproteins that were extracted from solubilized , virus infected cells by LCA affinity chromatography. This evaluation was made by comparing: a) the days that antibody was first detected by the IHAT, SN test, ELISA,

IFAT-M, and IFAT-G, and b) the persistence of the antibody titers. The sensitivity of the IHAT was further compared to the SN test by comparing IHAT titers of field serums with SN titers of 1:2, 1:4, and 1:8.

The specificity of the IHAT was evaluated by testing 30 PRV antibody negative field serums. Eleven PRV negative field serums with transmissible gastroenteritis (TGE) virus titers ranging from 4.3 to 6.7 (log_2), and 12 PRV negative serums with a porcine parvovirus titers ranging from 9.7 to 12.7 (log_2). In addition, three serums from pigs experimentally infected with infectious bovine rhinotracheitis (IBR) virus and 10 bovine field serums with geometric mean titers ranging from 3.7 to 6.3 and 5.3 to 7.3 (log_2) were also tested for crossreactivity in the IHAT.

The suitability of the IHAT for testing cytotoxic serums was evaluated with 30 cytotoxic field serums consisting of 14 PRV positive and 16 PRV negative serum as determined by the ELISA.

Statistical Analysis

Differences between geometric mean titers were analysed by Student's t-test utilizing pooled variance of the day analyzed. The treatments were defined as either different antigen preparations or different serological tests.

CHAPTER IV. RESULTS

Establishment of IHAT Parameters

Diluent content

The data summarized in Table 7 demonstrate the necessity of incorporating both gelatin and Tween 20 in diluent to prevent NSH of antigen-sensitized RBCs. Spontaneous hemagglutination occurred at all dilutions of normal serum tested in the absence of gelatin or Tween 20. Nonspecific hemagglutination however was essentially eliminated when a combination of 0.1 % (v/v) Tween 20 and 0.5 % (w/v) gelatin were present in the diluent. The use of either Tween 20 or gelatin alone at concentration up to 0.1 % and 0.5 % respectively lowered the amount of nonspecificity but not to the same degree that was observed when both Tween 20 and gelatin were used together.

Pre-test absorption of serums

The effect of absorbing heterophilic antibody from swine serum with nonsensitized RBCs is summarized in Table 8. Three of six negative serums caused NSH of sensitized RBCs at dilutions > 1:2 prior to absorption. This NSH was reduced by absorption but not eliminated. All serums continued to cause NSH at the 1:2 dilution. Attempts to eliminate NSH at the 1:2 dilution by treating serums with kaolin prior to RBC absorption were not successful. Consequently, the minimum dilution of serum to be tested by the IHAT was set at 1:4.

Table 7. The effect of gelatin and Tween 20 on nonspecific hemagglutination (NSH) of LCA sensitized sheep RBCs

Negative serum number		Nonspecific Hemagglutination titers					
	% Gelatin	none	0.05 %	0.05 %	0.50 %	0.50 %	none
	% Tween 20	none	none	0.10 %	none	0.10 %	0.10 %
1		SA[a]	6[b]	4	2	1	4
2		SA	5	4	2	1	3
3		SA	5	3	2	1	3

[a]SA = Spontaneous agglutination at all serum dilutions tested.

[b]Reciprocal of the last dilution ($\log_2$) of normal porcine serum at which NSH occurred.

Table 8. The effect of absorption with sheep RBCs on the removal of nonspecific hemagglutination (NSH) due to heterophilic antibodies

Serum number	Reciprocal of last dilution (log_2) at which NSH occurred	
	Unabsorbed	Absorbed
1	2	1
2	1	1
3	3	1
4	1	1
5	3	1
6	1	1

Sensitization of RBCs with antigen

The optimum concentrations of tannic acid and antigen preparation to be used to sensitize L-gluteraldehyde fixed sheep RBCs was determined by cross titration. Results of these titrations using lectin derived viral glycoprotein, cushioned virions, and pelleted virions are summarized in Tables 9, 10, and 11 respectively. The optimum concentration of tannic acid and antigen was considered to be that combination which yielded the lowest NSH and the greatest difference between specific and nonspecific hemagglutination. When the results of two different antigen/tannic acid combinations were similar, the combination requiring the lowest amount of antigen was considered optimal. Using these criteria, the optimum

Table 9. Optimization of the IHAT using LCA glycoprotein antigen

Stock antigen dilution (log_2)	Geometric mean IHAT titers (log_2)[a] of positive / negative serum controls				
	Tannic acid dilutions				
	1:5,000	1:10,000	1:20,000	1:40,000	none
3	11.5 / 7.0	12.0 / 5.0	11.0 / 2.0	5.5 / 3.5	2.0 / 2.0
4	9.0 / 2.5	10.5 / 2.0	8.5 / <2	4.0 / 2.0	2.0 / <2
5	5.5 / <2	9.0 / <2	6.5 / <2	3.0 / 2.0	<2 / <2
6	3.0 / 2.0	3.5 / <2	2.5 / <2	2.0 / 2.5	<2 / 2.0

[a]Titers are expressed as the reciprocal of serum dilutions (log_2). Geometric means represent 2 replicate tests of positive and negative serum controls.

Table 10. Optimization of the IHAT using cushioned whole pseudorabies virions

Stock antigen dilution (log_2)	Geometric mean IHAT titers (log_2)[a] of positive / negative serum controls				
	Tannic acid dilutions				
	1:5,000	1:10,000	1:20,000	1:40,000	none
3	9.5 / 7.0	9.0 / 6.5	8.0 / 7.0	5.5 / 4.5	2.5 / 2.0
4	9.0 / 6.0	8.5 / 3.5	7.5 / 3.0	5.0 / 4.0	2.5 / <2
5	4.0 / 3.5	4.5 / 3.0	4.5 / 4.0	2.5 / 4.0	2.5 / 4.0
6	2.5 / <2	<2 / 2.5	<2 / 2.5	2.0 / 3.0	3.0 / 3.5

[a]Titers are expressed as the reciprocal of serum dilutions (log_2). Geometric means represent 2 replicate tests of positive and negative serum controls.

Table 11. Optimization of the IHAT using pelleted whole pseudorabies virions

Stock antigen dilution (log_2)	Geometric mean IHAT titers (log_2)[a] of positive / negative serum controls				
	Tannic acid dilutions				
	1:5,000	1:10,000	1:20,000	1:40,000	none
3	9.0 / 7.0	9.5 / 7.5	7.0 / 5.5	6.5 / 6.5	3.0 / 2.0
4	5.5 / 3.5	9.0 / 3.0	6.0 / 4.5	7.5 / 5.5	3.5 / 2.0
5	2.5 / 2.0	4.0 / <2	4.5 / 2.0	4.0 / 2.0	<2 / 2.0
6	<2 / 2.0	<2 / <2	2.0 / 2.5	2.5 / 2.0	<2 / <2

[a]Titers are expressed as the reciprocal of serum dilutions (log_2). Geometric means represent 3 replicate tests of positive and negative serom controls.

tannic acid concentration for all three antigen preparations was found to be 1:10,000. Higher and lower concentrations of tannic acid resulted in increased nonspecificity with a concurrent loss of sensitivity.

It was also very important that the dilution of tannic acid be made just prior to the tanning procedure. If this precaution were not taken, the diluted tannic acid deteriorated rapidly even at 4 C and was not effective.

Evaluation of IHAT Antigens

The effect of LCA, whole pelleted virions and cushioned virions on the sensitivity of the IHAT was evaluated by: a) comparing the time p.i. that the test using each antigen first detected PRV antibody and b) comparing the differences in the IHAT titers of test serums. Titers were expressed as the reciprocal of the highest dilution ($\log_2$) that produced hemagglutination. Mean IHAT titers of test serums were adjusted by substracting the reciprocal of the highest dilution ($\log_2$) of normal serum that produced NSH, i.e., background due to extensive NSH produced by negative serum when both pelleted and cushioned whole virion antigens were used in the IHAT. The background was one for LCA antigen and four for both pelleted and cushioned antigens. The results of these comparisons are summarized in Table 12.

In addition to minimizing NSH, the use of LCA antigen also made the IHAT more sensitive. Antibody was first detected at day 6 p.i. when LCA antigen was used in the IHAT. In contrast, antibody was first

Table 12. The effect of different antigen preparations on the sensitivity of the IHAT

Days post-infection	Geometric mean titers ± S.E (log_2)[a]		
	Antigen preparations[b]		
	LCA	PEL	CUS
0	neg[c]	neg	neg
2	neg	neg	neg
3	neg	neg	neg
4	neg	neg	neg
5	neg	neg	neg
6	1.7 (0.2)	neg	neg
7	4.5 (0.3)	2.5 (0.3)	2.7 (0.3)
10	12.0 (0.3)	7.3 (0.2)	6.7 (0.2)
14	11.5 (0.3)	7.0 (0.3)	6.2 (0.2)
21	9.7 (0.2)	5.0 (0.3)	4.2 (0.2)

[a]Individual titers represent the reciprocal of the last dilution (log_2) of test serum producing hemagglutination less background in 3 test replicates performed on pooled serums of 4 surviving pigs. Background is the reciprocal of highest dilution of negative control serum producing hemagglutination. Background for LCA, PEL, and CUS = 1, 4, and 4, respectively.

[b]LCA is PRV glycoprotein extracted from infected cells with *Lens culinaris* agglutinin; PEL and CUS represent pelleted and cushioned whole PRV virions respectively.

[c]neg = negative.

detected at day 7 p.i. when cushioned and pelleted whole virions were used as antigens. Furthermore, the mean titers detected with LCA antigens were 2.0 to 5.3 $\log_2$ dilutions higher than those detected with whole virion antigens ($p < 0.05$). No statistically significant differences were observed between the titers of serums which were tested in the IHAT with cushioned and pelleted whole virions.

Experiments comparing lectin affinity purified antigens derived from cells infected with the virulent BE strain of PRV and the avirulent K strain of PRV are summarized in Tables 13, 14, and 15. No significant differences were observed between IHAT titers when either LCA or RCA antigens derived from cells infected with strain BE were used as IHAT antigen (Table 13). However, RCA antigen produced titers that were slightly higher between days 7 and 14 p.i. than the titers produced with LCA antigen. Similarly, the strain of PRV from which IHAT antigen was derived did not affect the sensitivity of the test. No significant differences or trends were observed between IHAT titers when antigen were compared that were derived from cells infected with PRV strain BE or K and extracted with either LCA (Table 14) or RCA (Table 15).

Comparison of the IHAT to the SN Test, ELISA and Modified IFAT

The sensitivity of the IHAT using LCA antigen from cells infected with the BE strain of PRV was compared to the SN test, ELISA, and IFAT using serums that were collected sequentially from three separate groups of pigs that survived nasal challenge with PRV. The results of these

Table 13. Comparison of IHAT sensitivity using glycoprotein antigen extracted with LCA or RCA agglutinins from cells infected with strain BE of PRV

Days post-infection	Geometric mean titers ± S.E. (Log_2)[a]	
	Antigen[b]	
	LCA	RCA
0	neg[c]	neg
2	neg	neg
3	neg	neg
4	neg	neg
5	neg	neg
6	neg	neg
7	3.7 (0.3)	4.0 (0.6)
10	10.0 (0.6)	11.0 (0.6)
14	10.3 (0.3)	11.2 (0.9)
21	10.6 (0.3)	10.3 (0.3)

[a]Titers are expressed as the reciprocal of serum dilutions (log_2). Geometric mean values represent 3 test replicates performed on pooled serums from 4 surviving pigs.

[b]LCA represents Lens culinaris agglutinin derived PRV glycoprotein; RCA represents Ricinus communis derived PRV glycoprotein.

[c]neg = negative.

Table 14. Comparison of IHAT sensitivity using Lens culinaris agglutinin extracted viral glycoprotein from cells singularly infected with strain BE and K of PRV

Days post-infection	Geometric mean titer ± S.E. (log_2)[a]	
	Antigen[b]	
	Strain BE	Strain K
0	neg[c]	neg
2	neg	neg
3	neg	neg
4	neg	neg
5	neg	neg
6	neg	neg
7	3.3 (0.3)	3.7 (0.3)
10	11.0 (0.6)	10.7 (0.3)
14	10.3 (0.3)	10.3 (0.3)
21	10.0 (0.6)	10.0 (0.0)

[a]Titers are expressed as the reciprocal of serum dilutions (log_2). Geometric mean values were calculated from 3 replicates performed on pooled serums from 4 surviving pigs.

[b]Antigen extracted with LCA from cells infected with PRV strain BE and K.

[c]neg = negative.

Table 15. Comparison of IHAT sensitivity using Ricinus communis agglutinin extracted viral glycoprotein from cells singularly infected with strains BE and K of PRV

Days post-infection	Geometric mean titer ± S.E. (log_2)[a]	
	Antigen[b]	
	Strain BE	Strain K
0	neg[c]	neg
2	neg	neg
3	neg	neg
4	neg	neg
5	neg	neg
6	neg	neg
7	3.7 (0.3)	3.3 (0.3)
10	11.7 (0.3)	11.3 (0.7)
14	11.3 (0.9)	11.7 (0.3)
21	10.7 (0.3)	11.0 (0.0)

[a]Titers are expressed as the reciprocal of serums dilutions (log_2). Geometric mean values were calculated from 3 replicates performed on pooled serum from 4 surviving pigs.

[b]Antigen extracted with RCA from cells infected with strain BE and K of PRV.

[c]neg = negative.

comparison are summarized in Tables 16, 17, and 18 and demonstrate that the IHAT is more sensitive in detecting PRV specific antibodies than either the SN test, ELISA or IFAT-G. For example, PRV antibody was first detected by the IHAT in Group 1 (Table 16) by day 6 p.i. In contrast, no PRV antibody was detected by the SN test, ELISA, or the IFAT-G until day 10 p.i. Similarly, antibody was first detected by the IHAT in Groups 2 and 3 (Tables 17 and 18) on days 5 and 6 p.i. respectively. In contrast, antibody was first detected by the SN test, ELISA, and IFAT-G in Groups 2 and 3 on day 7 p.i., two and one days respectively after antibody was first detected by the IHAT. Antibody was first detected by the IHAT and IFAT-M on day 5 in Group 2. However, IFAT-M did not detect antibody in Groups 1 and 3 until after day 7 p.i. The IFAT-M detected antibody in Group 3 pigs by day 7 p.i. before neutralizing antibodies were detected, i.e., two days after antibody was detected by the IHAT. Antibody titers as determined by the IHAT, SN test, ELISA, IFAT-M, and IFAT-G reached a maximum titers between days 10 and 14. These titers varied only slightly through day 21 in Group 1. Similarly, all titers remained essentially unchanged through day 56 in Group 2 with the exception of those titers measured by IFAT-M. Antibody was last detected by IFAT-M at day 21 p.i.

Suitability of the IHAT for Field Serums

The IHAT was used to test 92 field serums with PRV SN titers ranging from 1:2 to 1:8. The results of this comparison are summarized in Table 19. Forty-nine of these serums had PRV specific IgM and represented early virus infection. The remaining 43 serums, seven of which with

Table 16. The development and persistence of PRV antibody titers in 4 pigs surviving challenge with PRV as determined by the IHAT, SN test, ELISA, and modified IFAT.

Days post-infection	Geometric mean titer ± S.E.[a] (log_2)				
				Modified IFAT	
	IHAT	SN test	ELISA	anti-pig IgM	anti-pig IgG
0	neg[b]	neg	neg	neg	neg
2	neg	neg	neg	neg	neg
3	neg	neg	neg	neg	neg
4	neg	neg	neg	neg	neg
5	neg	neg	neg	neg	neg
6	1.7 (0.2)	neg	neg	neg	neg
7	4.5 (0.3)	neg	4.0 (0.3)	neg	neg
10	12.0 (0.3)	1.7 (0.3)	5.6 (0.3)	7.6 (0.3)	5.6 (0.3)
14	11.5 (0.3)	3.3 (0.3)	6.9 (0.3)	6.0 (0.3)	6.6 (0.3)
21	9.7 (0.2)	4.3 (0.3)	7.6 (0.3)	5.0 (0.3)	8.0 (0.3)

[a]Titers are expressed as the reciprocal of serum dilutions ($\log_2$). Geometric means represent 3 replicate tests of pooled serums from 4 surviving pigs.

[b]neg = negative.

Table 17. The development and persistence of PRV antibody titer in 2 pigs surviving challenge with PRV as determined by the IHAT, SN test, ELISA, and modified IFAT

Days post-infection	Geometric mean titers ± S.E. (log_2)[a]				
				Modified IFAT	
	IHAT	SN test	ELISA	anti-IgM	anti-IgG
0	neg[b]	neg	neg	neg	neg
1	neg	neg	neg	neg	neg
2	neg	neg	neg	neg	neg
3	neg	neg	neg	neg	neg
4	neg	neg	neg	neg	neg
5	1.3 (0.7)	neg	neg	3.9 (0.3)	neg
6	3.5 (0.3)	neg	neg	4.6 (0.3)	neg
7	7.2 (0.2)	1.3 (0.3)	4.0 (0.3)	6.0 (0.3)	3.6 (0.3)
8	8.5 (0.3)	2.0 (0.3)	5.0 (0.3)	6.6 (0.3)	4.0 (0.3)
9	10.0 (0.0)	3.0 (0.3)	5.3 (0.3)	7.6 (0.3)	6.0 (0.3)
10	12.0 (0.3)	3.3 (0.3)	6.0 (0.3)	8.6 (0.3)	6.3 (0.3)

14	10.0 (0.6)	4.7 (0.3)	9.0 (0.3)	8.0 (0.3)	8.6 (0.3)
21	10.7 (0.2)	5.0 (0.6)	9.6 (0.3)	4.6 (0.3)	9.3 (0.3)
28	10.0 (0.6)	4.7 (0.3)	11.0 (0.3)	neg	9.0 (0.3)
42	10.5 (0.0)	5.3 (0.3)	10.6 (0.3)	neg	9.0 (0.3)
56	9.7 (0.2)	6.7 (0.3)	9.6 (0.3)	neg	9.7 (0.2)

[a]Titers are expressed as the reciprocal of serum dilutions ($\log_2$). Geometric means represent 3 replicate tests of pooled serums from 2 surviving pigs.

[b]neg = negative.

Table 18. The development and persistence of PRV antibody titers in 7 pigs challenged with PRV as determined by the IHAT, SN test, ELISA, and modified IFAT

Days post-infection	Geometric mean titer ± S.E.[a] (log_2)				
				Modified IFAT	
	IHAT	SN test	ELISA	anti-pig IgM	anti-pig IgG
0	neg	neg	neg	neg	neg
2	neg	neg	neg	neg	neg
3	neg	neg	neg	neg	neg
4	neg	neg	neg	neg	neg
5	1.4 (1.1)	neg	neg	neg	neg
6	3.9 (1.2)	neg	neg	neg	neg
7	5.0 (0.7)	0.9 (0.5)	neg	3.9 (0.5)	neg

[a]Titers are expressed as the reciprocal of serum dilutions (log_2). Geometric means represent 3 replicate tests of 7 individual pigs.

[b]neg = negative.

Table 19. Summary of IHAT results of field serums

Field serums					
Number tested	SN titer[a]	Presence of IgM[b]	# IHAT positive[c]	Geometric mean IHAT titer ± SE (range)	Percent agreement[d]
30	1	Y	23	7.1 ± 0.7 (5.7 to 8.3)	100 %
	1	N	7	6.8 ± 0.2 (5.3 to 8.2)	100 %
30	2	Y	16	8.9 ± 0.8 (8.3 to 9.8)	100 %
	2	N	14	8.8 ± 0.5 (7.7 to 9.3)	100 %
32	3	Y	10	10.3 ± 0.4 (9.0 to 11.2)	100 %
	3	N	22	10.9 ± 0.3 (10.2 to 11.7)	100 %

[a]Serum neutralization titer represents the $\log_2$ of the reciprocal of highest neutralizing dilution.

[b]Y = present; N = not present.

[c]Nonspecific hemagglutination occurred at the 1:8 dilution in 2 IgM-containing serums with SN titers of 1 ($\log_2$) and in 1 IgM-free serum with an SN titer of 2 ($\log_2$).

[d]Percent agreement = percentage of serums tested that were IHAT positive.

SN titers of 1:2, lacked IgM and represented long standing infection. All serums reacted positive at dilutions of 1:8 and higher. Two serums from recently infected pigs and one serum representing an established infection with a 1:4 titer produced NSH at the 1:8 dilution. These three serums represented 3.3 % of the total tested. There were no differences between the IHAT titers of serums with identical SN titers from pigs with early and established PRV infections.

Specificity of the IHAT

Specificity experiments are summarized in Table 20. Thirty PRV antibody negative serums as determined by the SN test, ELISA, and modified IFAT were tested by the IHAT and found negative at the 1:4 serum dilution. Similarly, 11, 12, and 3 PRV antibody negative serums with titers to TGE virus, porcine parvovirus, and IBR virus respectively also produced negative reactions in the IHAT at serum dilutions of 1:4. In addition, 10 bovine serums with IBR virus antibody titers ranging from 5.3 to 7.3 also tested negative in the IHAT.

The Effect of Cytotoxic Serums on the IHAT

Sixteen PRV antibody negative and 14 PRV antibody positive cytotoxic serums were identified by the ELISA and modified IFAT. Antibody to PRV was detected by the IHAT at the 1:4 dilution in all positive serums. No false positive reactions occurred among 15 of 16 PRV antibody negative serums which were tested at the same dilution. A single PRV antibody negative serum produced NSH at the 1:4 but not at the 1:8 dilution.

Table 20. Specificity of the pseudorabies IHAT

Antibody[a]	# tested	Geometric mean titer[b] ± S.E. (range)	# IHAT (PR) positive
normal	30	-	0
IBR[c] (por.)	3	5.3 ± 0.7 (4.7 to 6.3)	0
IBR[d] (bov.)	10	6.3 ± 0.3 (5.3 to 7.3)	0
PPV	12	10.8 ± 0.2 (9.7 to 12.7)	0
TGE	11	5.8 ± 0.3 (4.3 to 6.7)	0

[a]Normal represents absence of antibody to infectious bovine rhinotracheitis (IBR) virus, porcine parvovirus (PPV), transmissible gastroenteritis (TGE) virus, and pseudorabies virus (PRV).

[b]Titers are expressed as the reciprocal of the highest dilution ($\log_2$) showing positive results. Titers for IBR and TGE viruses were determined by the SN test. Titers for PPV was determined by the hemagglutination inhibition test.

[c]IBR (por) represents serums collected from pigs nasally infected with IBR virus.

[d]IBR (bov) represents serums collected from cattle naturally infected with IBR virus.

CHAPTER V. DISCUSSION

Comparison of LCA Derived Viral Glycoproteins and Whole Virions as IHAT Antigens

In earlier studies by Haffer et al. (21, 22) and Labadie and Toma (32), the IHAT was used successfully to detect PRV specific antibody. In both of these studies, the investigators used whole pelleted virions and reported the test to be highly sensitive and specific. In this laboratory however, frequent problems with NSH have occurred when whole pelleted virus was used as antigen. As a result, the lowest dilution at which the IHAT could be read using pelleted whole virions was 1:16 which did not afford the sensitivity necessary to detect low levels of antibody. Consequently, the preceding study was conducted to determine if NSH could be avoided and sensitivity increased by using a gentler method of obtaining whole virion antigen and by using lectin-purified PRV glycoproteins as antigen in the IHAT.

Results of this study conclusively demonstrated that the use of lectin-purified viral glycoproteins in the IHAT virtually eliminated nonspecific activity. The highest dilution at which NSH occurred when negative serums were reacted with both pelleted and cushioned whole virion antigens was 1:16 in contrast to 1:2 when these serums were reacted with the viral glycoproteins (Table 12). Furthermore, the use of viral glycoproteins markedly enhanced the sensitivity of the IHAT. Pseudorabies specific antibody was first detected at day 6 p.i with viral glycoproteins compared to day 7 p.i. when both pelleted and cushioned

whole virion antigens were used. Mean titers detected with viral glycoproteins were generally 2.0 to 5.3 $\log_2$ dilutions higher than titers obtained by whole virion antigens ($p < 0.05$). The superiority of the viral glycoprotein as IHAT antigen may be due to its more purified nature. The use of a purified antigen may provide better antigen presentation on the surface of sheep RBCs and thus facilitate antigen-antibody interaction.

No significant difference in IHAT sensitivity was observed when either LCA or RCA antigen was used in the test. However, the mean IHAT titers observed with RCA antigens were consistently higher than the titers obtained with LCA antigen on three of four days that antibody was detected (Table 12). This very slight difference may have been due to chance alone or the different antigen composition of LCA and RCA antigens as reported by Platt (51) who demonstrated by crossed immunoelectrophoresis that LCA and RCA antigens each possessed a unique antigen in addition to two antigen complexes that were shared. In order to determine which of these two possibilities is correct, further replications should be made with LCA and RCA antigens from additional lots of crude viral antigen.

Similarly, no significant difference in IHAT sensitivity was detected when antigens derived from cells infected with the virulent BE strain or the vaccine K strain were evaluated (Tables 13 and 14). Consequently, the missing glycoprotein of the K strain as reported by Ben-Porat et al. (3) and Mettenleiter et al. (40b) does not appear to have a major influence on the sensitivity of the IHAT.

The IHAT utilizing viral glycoprotein also proved to be highly specific. No false positive reactions were observed among 30 PRV negative field serums. Similarly no false positives were observed when 11, 12, and 3 PRV antibody-free serums with titers to TGE virus, porcine parvovirus, and IBR virus were tested by the IHAT. Ten bovine serums with IBR virus antibody titers ranging from 1:32 to 1:256 also tested negative in the IHAT for PRV antibody. The lack of crossreactivity between PRV and IBR virus antibody is important because both viruses are herpesviruses and IBR virus has been isolated from pigs (11).

Comparison of IHAT Sensitivity to the SN Test, ELISA and Modified IFAT

The IHAT with viral glycoprotein antigen and the IFAT-M were found to be more sensitive than either the SN test, ELISA or IFAT-G in detecting early antibody. In studies involving laboratory infected pigs, the IHAT and IFAT-M detected antibody a minimum of two days earlier than the SN test, ELISA and IFAT-G. The sensitivity of the IHAT was further illustrated when field specimens were assayed for PRV antibody. Currently, there is considerable controversy over what constitutes a positive SN test. In most laboratories, 1:4 is considered to be the minimum positive SN titer. Consequently, pigs can be misdiagnosed especially in the very early stages of a PRV infection. In the present study, the IHAT readily detected PRV antibody in 100 % of 49 serums collected from recently infected pigs (Table 19), 23 of which had an SN titer of 1:2. The IHAT titers of these serums ranged from 5.7 to 11.2. The IHAT also detected titers ranging from 5.3 to 8.2 ($\log_2$) among seven

late serums with SN titer of 1:2. It is anticipated that the IHAT will also detect PRV antibody in serums with SN titers less than 1:2 since IHAT titers were 3.6 to 10.3 dilutions ($\log_2$) higher than the SN titers as illustrated in Tables 16 and 17.

Despite the high degree of sensitivity of the IHAT, 3 of 92 (3.3 %) serums produced NSH at dilutions of 1:8 and lower. Consequently under field condition, approximately three percent of serums may be expected to produce NSH and will have to be screened at dilutions higher than 1:8 which may result in a false negative reaction and loss of sensitivity. Alternatively, such serums could routinely be tested by the IFAT or ELISA.

The preceding observations agree with those of Haffer et al. (21, 22) and Labadie and Toma (32) who reported that the IHAT was able to detect PRV specific antibody at least two days before the SN test. Haffer et al. (21, 22) concluded that early detection was due to the presence of virus specific IgM because treatment of early serums with beta-mercaptoethanol eliminated PRV specific antibody activity. The presence of PRV specific IgM as detected by modified IFAT in serums on the same day that the IHAT first detected antibody in three groups of laboratory infected pigs, together with the absence of SN activity in these serums strongly supports the conclusion of the above workers (Tables 16, 17, and 18).

Establishment of IHAT Parameters

The composition of diluent proved to be the most important consideration with respect to NSH. The combination of both Tween 20 and gelatin in the diluent virtually eliminated NSH of RBCs sensitized with viral glycoprotein and reduced NSH of RBCs sensitized with whole virions by two $\log_2$ dilutions. The use of both Tween 20 and gelatin also reduced the incubation time before the test could be read. In the present study, tests were read after an incubation period of 45 to 60 minutes in contrast to two hours as reported by other investigators (21, 22, 32).

The present study also confirmed the finding by Yeager (81) that sheep RBCs fixed with L-gluteraldehyde can be stored for a period of at least one month without a significant loss in their ability to be sensitized with antigen. She also stated that L-gluteraldehyde treated RBCs can be stored at -70 C or liquid nitrogen without cryoprotectants for a period of up to six months.

General Comments

The IHAT was found to be comparable or superior in sensitivity to the SN test, ELISA and modified IFAT for the detection of PRV antibody. Its principle advantage is that it does not require expensive equipment as do by the SN test, ELISA and modified IFAT. The test is also easy to perform and as such would not require the services of highly trained technicians. The test can also be used to detect antibodies in hemolyzed and cytotoxic serums. These characteristics of the IHAT make it an ideal test especially for those parts of the world where funds for training

laboratory personnel and operating diagnostic laboratories for animal diseases are limited.

SUMMARY

Whole pseudorabies virions and semipurified viral glycoprotein antigens were evaluated for sensitivity and specificity in the indirect hemagglutination test (IHAT) using serums collected from four laboratory infected pigs. Whole virions were collected from disrupted MDBK cells by ultracentrifugation with and without the presence of a sucrose cushion. Viral glycoprotein was extracted by lectin affinity chromatography with Lens culinaris or Ricinus communis agglutinins from detergent solubilized cells infected with either the virulent BE strain or the avirulent K strain of PRV. The glycoprotein antigens were found to be superior to whole virion antigen preparations. Nonspecific hemagglutination (NSH) was absent at final serum dilutions of 1:4 when viral glycoprotein was used as antigen. The use of this antigen also permitted the detection of antibody in laboratory infected pigs as early as five days post infection (p.i.). In contrast, NSH consistently occurred at final serum dilutions as high as 1:16 when whole virions were used as IHAT antigen. Furthermore, the presence of antibody was not detected until seven days p.i. Antibody titers to PRV as detected by viral glycoprotein antigen also ranged from 2.0 to 5.3 times higher than those detected with whole virion antigens during the first 21 days p.i ($p < 0.05$). No differences in IHAT sensitivity and specificity were observed when the different viral glycoprotein preparations were evaluated indicating that antigen differences between strains do not influence the sensitivity of the IHAT.

Comparison of the IHAT using viral glycoprotein antigen also proved to be superior in sensitivity to the SN test, ELISA and IFAT-G. Antibody titers were first detected as early as five days p.i. among 13 laboratory infected pigs. In contrast, the earliest detection by the SN test, ELISA, and IFAT-G was seven days p.i. Detection of antibody with the IFAT utilizing anti-swine IgM conjugate occurred on day 5 p.i. indicating that early detection by the IHAT was due to its ability to detect PRV specific IgM. The same degree of sensitivity was also observed when the IHAT was compared to the SN test for detecting PRV specific antibody in field serums. Thirty serums with SN titers of 1:2 produced IHAT titers ranging from 5.3 to 8.3 ($\log_2$).

The IHAT was also shown to be highly specific. No false positive reactions occurred when 30 PRV negative, 11 TGE virus positive, and 12 parvovirus positive serums were tested in the IHAT. Three IBR virus positive serums from IBR infected pigs and 10 IBR virus positive bovine serums also tested negative in the IHAT. A possible disadvantage of the IHAT is the occassional NSH that may occur when screening field serums for PRV antibody. Three of 92 (3.3 %) field serums produced NSH at dilutions as high as 1:8.

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