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Evaluation of a particle concentration fluorescence immunoassay (PCFIA) for the detection of antibodies to pseudorabies virus

by

David Ryg Kinker

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GENERAL INTRODUCTION

Pseudorabies (PR) has been described as economically the most significant viral disease of swine.[31] This dubious honor has been attained in the United States partially as a result of the eradication of hog cholera. The success of the hog cholera control and eradication program, coupled with the availability of diagnostic tools deemed adequate to detect PR infected swine, encouraged swine industry leaders to seek federal and state regulatory assistance in an effort to eradicate pseudorabies from domestic swine in the United States. A national pseudorabies eradication program involving the combined efforts of the swine industry, federal and state regulatory agencies, and state and federal veterinary diagnostic laboratories was officially initiated January 1, 1989.[4] The stated goal of the program is the complete eradication of this disease by 1999. This ambitious undertaking relies almost exclusively on the serological testing of large numbers of swine in order to determine herd and animal infection status and to monitor the effectiveness of eradication efforts.

The advent of the national eradication program has had a clear impact on the demands placed on state veterinary diagnostic laboratories. That impact is most evident in the large swine producing states in the northern midwestern section of the country. Iowa is the leading swine producing state in the country, with over 35,000 herds and 1.6 million breeding swine in 1990.[115] The Veterinary Diagnostic Laboratory at Iowa State University performed nearly 275,000 PRV serology tests in 1990, and the current trend is clearly upward; over 206,000 PRV serology tests were performed in just the first six months of 1991. Nine different serological assays for the detection of

antibodies against pseudorabies virus (PRV) were used at some time during the last year in this laboratory. These serological assays fall into two main categories: screening assays that detect the humoral immune response induced either by vaccination or infection, and differential assays that detect the response to infection, but not to vaccination with a companion gene-deleted vaccine. The screening assays include the standard serum virus neutralization (SVN) test,[41] a commercial latex agglutination test (LAT),[a] and two commercial enzyme-linked immunosorbent assays (ELISAs).[b,c] Four commercial differential PRV ELISAs[d,e,f,g] are in use in this laboratory, and another was discontinued as a result of performance problems.[h] The availability of the differential diagnostic assays as companions to gene-deleted vaccines has caused the percentage of screening tests performed as compared with the total number of tests performed to decrease. However, the actual number of screening tests performed at this laboratory continues to increase due to the dramatic increase in the overall demand for PRV serological testing. Over one half of the total number of PRV tests performed for the first six months of 1991 (nearly 108,000 tests) consisted of screening assays applied

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[a] Pseudorabies Virus Antibody Test Kit - Latex Agglutination, Viral Antigens Inc., Memphis, TE.

[b] HerdChek®: Anti-PRV (S), IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, ME.

[c] DiaSystems® CELISA PRV™, TechAmerica™ Diagnostics, Omaha, Nebraska.

[d] HerdChek®: Anti-PRV-gpX, IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, ME.

[e] HerdChek®: Anti-PRV-gI, IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine.

[f] DiaSystems® CELISA OmniMark™ PRV, TechAmerica™ Diagnostics, Omaha, Nebraska.

[g] ClinEase-PRV®, SmithKline Beecham, Lincoln, Nebraska.

[h] Tolvid® Diagnostic, Agdia, Inc., Elkhart, Indiana.

to sera from nonvaccinated swine. A need has arisen for the development of a screening assay for PRV that can process large numbers of swine sera quickly, and also maintain or improve the high levels of specificity and sensitivity achieved by currently available test procedures.[7,101]

A particle concentration fluorescence immunoassay[45] (PCFIA) has been developed for the detection of antibodies to pseudorabies virus (PRV) in swine sera. The PCFIA is an automated procedure that involves the use of a computer-linked multipurpose instrument, the IDEXX™ Screen Machine.[i] Manual pipetting of the sera and sample diluent is required, but all subsequent steps are performed automatically.

The PCFIA for pseudorabies virus is a competitive immunoassay using polystyrene particles coated with partially purified PRV antigen. The conjugate is a fluorophore-labelled monoclonal antibody to PRV. The amount of particle-bound fluorescence is measured as photon counts by front-surface fluorimetry. The PCFIA allows the automated testing of large numbers of samples in a short period of time. Once samples have been pipetted, a 10 plate batch run (944 samples) is completed in 1 hr 45 min. To maximize throughput, a final batch may be set up to run unattended overnight.

The goal in the development of the PRV PCFIA was to create a screening assay that performed comparably to conventional PRV serology procedures commonly used in veterinary diagnostic laboratories, but was better suited for

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[i]IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine.

the testing of large numbers of sera. This thesis describes a project to evaluate the specificity, sensitivity and practicality of the PCFIA for PRV.

LITERATURE REVIEW

Introduction

Pseudorabies is a disease of significant economic importance in most swine producing areas of the world.[31,34] Those countries without pseudorabies include Australia, which has managed to avoid importation of the disease, and Great Britain, which has essentially eradicated PRV by a control and eradication program based on slaughter with compensation and salvage of marketable pigs for human consumption.[92] Pseudorabies was first described in the scientific literature in 1902 by a Hungarian, Aládar Aujeszky,[5] as a fatal infectious disease in cattle, dogs, and cats, and the disease is commonly referred to as Aujeszky's disease in Europe. In 1934, Shope[103] demonstrated antibodies against pseudorabies in swine from the midwestern United States. However, the presence of PRV infections in cattle in the United States as early as 1813 has been inferred from references in the popular press to a condition called "mad itch".[39]

The overall economic impact of PR to the U. S. swine industry is difficult to calculate. Estimates range from more than 21 million dollars per year (in 1987)[38] to from 30 to 72 million dollars annually.[51] The cost of PR outbreaks has been estimated at approximately 10,000 dollars per year per outbreak.[51] A significant portion of the overall cost of PR to the swine industry may be attributed to the regulatory and diagnostic testing requirements due to its status as a controlled disease in swine.

The United States swine population in 1990 consisted of 276,585 herds with 6,922,100 breeding swine.[115] The national average prevalence of PR in

U.S. swine herds on December 31, 1990 was 2.4%.[4] The disproportionate impact of PR on the states with higher concentrations of swine is reflected in the 37.2% prevalence rate of infected herds in Iowa in 1990.[115]

Pseudorabies virus has a wide host range, including cattle,[47,111] sheep,[111,116] goats,[34] dogs,[28] and cats[28] among the domestic animals, and is nearly always fatal in these species.[34] However, the pig is considered to be the reservoir host for PRV.[34,81] Clinical symptoms in the pig range from unapparent to death of the affected pig.[34] The severity of the disease is usually age-related; the younger the pig, the more severe the symptoms and the higher the mortality rate.[10,21,34,81,125] Pseudorabies clinical signs may include fever, anorexia, vomiting, depression, ataxia, tremors, paralysis, convulsions, and even sudden death.[21,34] Respiratory involvement is generally present, and may be the primary response in older pigs.[21,80] Adult swine exhibit varying degrees of fever, sneezing, coughing, and anorexia, and the mortality rate among this age group may reach 2% in a susceptible herd. However, the greatest risk in infections of adult swine is to the pregnant sow. PRV will cross the placental barrier and infect the embryo or fetus, resulting in embryonic resorption, fetal abortion, mummification, or stillborn or weak infected newborn piglets, depending on the stage of pregnancy at the time of infection and other factors relating to the severity of the virus challenge.[23,34,55,125] Other factors affecting the course and severity of the clinical disease due to infection with PRV include the virulence of the virus strain, the virus dose and route of infection, host species, level of stress, and the immune status of the host.[10,125]

The natural means of transmission of PRV in swine occurs primarily by exposure to infectious upper respiratory secretions via the oral and nasal routes.[21,34,65,88,125] Initial virus replication occurs in the nasopharyngeal region and the upper respiratory tract.[34,123,125] Invasion of the central nervous system and, presumably, centrifugal spread of the virus occurs by the neural pathways, probably via the axoplasm.[34,98,99,123] Viremia occurs, although it is of low titer and intermittent.[34] PRV infects peripheral blood lymphocytes, and can be distributed to all parts of the body in this manner.[34,125]

Pseudorabies virus can persist in a latent state in pigs that have recovered from the disease.[22,97,125] Latency can occur in the presence of maternal antibodies,[71,118] and in pigs vaccinated with either killed or modified live vaccines.[75,102] Schoenbaum et al. reported that, although vaccination did not reduce the rate of occurrence of latent infections, shedding of the virus following reactivation was reduced in vaccinated pigs.[102] In contrast, van Oirschot and Gielkens reported that intranasal vaccination did lessen the ability of virulent virus to cause latent infections, although it did not totally prevent it.[119] The detection of latently infected domestic and feral swine remains a serious impediment to the success of the eradication of PR in the swine population.[51,75] Pigs that maintain a humoral immune response can be detected by a sufficiently sensitive serological assay, but detection of a serologically negative latently infected pig remains a difficult task. A number of methods have been used to detect PRV in latently infected pigs. Reactivation of latent PRV can be stimulated by the use of

immunosuppressants such as dexamethasone[71,75] or prednisolone.[125] Tissue explant and co-cultivation virus isolation techniques have successfully identified the presence of latent virus in various tissues, especially neural tissues such as the trigeminal ganglion.[97,118,125] Molecular DNA hybridization techniques have been shown to be more sensitive than cell culture methods in the detection of pigs latently infected with PRV.[18,35,64,96]

Properties of Pseudorabies Virus

Pseudorabies (Aujeszky's disease) virus has been formally named Suid herpesvirus 1.[61] PRV is a member of the family Herpesviridae, and is further assigned to the subfamily Alphaherpesvirinae, along with herpes simplex virus in man.[12,77] The PRV virion is approximately 180 nm in diameter, and is made of the four typical herpesvirus architectural elements: a central core containing the DNA and bound fibrillar protein; an icosadeltahedral capsid with 162 protein capsomers; a globular tegument surrounding the capsid; and a double or triple lipid envelope containing glycoproteins and lipoproteins.[12,95,125]

The PRV genome consists of a linear, double stranded DNA with a molecular weight of approximately 90×10^6 daltons and with a size of approximately 145 kilobase pairs.[125] The DNA molecule can be visualized as consisting of four functional regions. Starting at the left (5') end of the molecule, a long unique (U_L) segment is followed by an internal repeat (IR) sequence, a short unique (U_S) sequence, and, finally, by the terminal repeat (TR) sequence of base pairs.[13,125]

Seven known glycoproteins are coded for by the PRV genome.[118] Glycoproteins gII,[73] gIII,[91] and gH[120] are encoded by genes in the U_L segment. Glycoproteins gI,[125] gX,[89] gp50,[121] and gp63[72] are encoded by genes located in the U_S segment of the genome. Table 1[118,125] summarizes selected properties of six of the PRV glycoproteins (gH was only recently identified and very little is known about it). All of the glycoproteins except gX are structural components of the viral envelope. In contrast, gX is synthesized in large quantities in infected cells and excreted into the supernatant of infected cell cultures.[11] The function of gX is unknown. Only gII and gp50 are essential to viral replication. The virulence of PRV is multigenically controlled.[118] At least three glycoproteins, gI, gp63, and gIII, may play a role in the release of the virus from infected cells.[118,125] Glycoprotein gII may be important in the process of membrane fusion and penetration of the infected cell.[125] Adsorption of the virus to the target cell may be mediated by gIII and gp50.[125] Glycoproteins gIII and gp50 serve as major immunogens for PRV, with a possible lesser role for gI and gII.[125]

Thymidine kinase (TK) is a nonglycosylated protein encoded by a gene on the U_L segment of the genome. The TK gene is an important virulence factor for PRV that enables the virus to replicate in neural tissue, which is deficient in thymidine kinase.[51] The establishment of a latent infection in neural tissue is believed to be facilitated by the TK enzyme.[112]

Pseudorabies Virus Vaccines

Vaccination of domestic animals for pseudorabies has been practiced for many years; the earliest reference to vaccination found as part of this

Table 1. Properties of the PRV glycoproteins[125,120]

Property	gI	gII	gIII	gX	gp50	gp63
Functions	release	penetration?	release adsorption temp. stability	?	adsorption?	release
MW (mature)	130 kd	155 kd	90 kd	95-99 kd	50-60 kd	63 kd
Gene location	U_S[a]	U_L[b]	U_L	U_S	U_S	U_S
Structural gp	+	+	+	-	+	+
Essential gp	-	+	-	-	+	-
Virulence	+	?	+	-	?	+
Immunogen						
Neutralizing in vivo	±	±	+	-	+	-
Induce CTL[c] in mice	-	?	+	-	?	-
Protective (vaccine)	?	?	+	-	+	?

[a] U_S = short unique genome segment
[b] U_L = long unique genome segment
[c] CTL = cytotoxic T cells

literature review was published in 1936, and was titled "La vaccination des animaux contre la maladie d'Aujeszky est-elle possible?"[90] The same question may be asked today, but rephrased in the context of whether it is possible to eradicate PR from the swine population in the face of the widespread use of vaccination as an integral part of the eradication effort. Currently licensed vaccines in the United States are listed in Table 2, along with information about the source of the vaccine strain, gene deletions, and the availability of companion serological diagnostic tests.

Commercially available vaccines for PR are either modified live virus (MLV) or killed virus preparations. The use of MLV vaccines may be preferable in certain situations. MLV vaccines may stimulate longer lasting antibody production than killed products, and may induce an immune response in vaccinated pigs that mimics natural infection better than killed vaccines.[76] In addition, MLV vaccines may be administered by the intranasal route,[24,76,99,117,119,123] which may be a superior route of vaccination because it induces local immunity at the normal portal of virus entry,[76] and may be superior to intramuscular vaccination in overcoming passive maternal antibody interference with the active immune response to vaccination.[125]

Potential disadvantages to the use of MLV vaccines include the risk of reversion to greater virulence in the host animal,[67,70,81,84] although reversion has not proved to be a problem in actual practice.[25,76] Infectious contaminants are a greater risk in live vaccines; adventitious viruses found in various vaccines have included avian leukosis and other retroviruses, bovine viral diarrhea virus, porcine parvovirus, and cytomegalovirus.[76]

Table 2. Pseudorabies vaccines and companion differential diagnostic tests licensed in the United States

Vaccine	Manufacturer	PRV Strain	Deletion	TK	Differential Test
Bio-Ceutic (MLV)	Boehringer/ Ingelheim	Bartha	gI, gp63	+	HerdChek:Anti-PRV-gpI (IDEXX)
OmniMark (MLV)	TechAmerica (NovaGene, Inc.)	PRV(dlg92dltk) (from BUK)	gIII	-	CELISA OmniMark PRV
Pseudo-Cell (MLV)	Grand Labs	Field	None	+	None
PR-Vac (MLV)	SmithKline Beecham (SKB)	Norden (from BUK)	gI	+	ClinEase (SKB) HerdChek:Anti-PRV-gI
PR-Vac (killed)	SmithKline Beecham	Norden (from BUK)	gI	+	ClinEase (SKB) HerdChek:Anti-PRV-gI
PRV/Marker (MLV)	SyntroVet	from Iowa S-62	X	-	HerdChek:Anti-PRV-gpX (IDEXX)
PRV/Marker Gold (MLV)	SyntroVet	from Iowa S-62	gX, gI	-	HerdChek:Anti-PRV-gpX HerdChek:Anti-PRV-gI
PRV-mune (killed)	Oxford Laboratories	Field	None	+	None
Suvaxyn PRV (killed)	Solvay	Field	None	+	None
Tolvid (MLV)	Upjohn	PRVΔtkΔgX-1 (from Rice)	gX	-	PR gX-Tolvid (Agdia)

Another possible risk of MLV vaccines is the excretion of vaccinal virus to animals in close proximity to the vaccinated pig,[25,67,81] but post-vaccinal viral excretion has been reported not to occur for a number of vaccine strains.[25,44,66] Another potential risk of the use of MLV vaccines is the possibility that a vaccine strain replicating in the host might genetically recombine with a virulent field strain.[40,48] This possibility could have an impact on the current vaccination strategy relied upon for eradication and control programs, as discussed below. This strategy relies on the use of vaccines with known genetic deletions for specific immunogenic glycoproteins. Companion differential serology tests that detect the humoral response to the missing glycoprotein can then differentiate between infection and vaccination responses. Genetic recombination of a gene-deleted vaccine strain with a virulent field strain of PRV could result in either the restoration of the missing gene product in the vaccine strain, or in the deletion of the marker gene in the virulent strain. In the former case, the pig would be detected by the differential assay because antibodies against the diagnostic glycoprotein would be present. In the latter case, the antibody response to a gene-deleted virulent strain would not be detected by the differential test. However, this would be of practical concern only if the gene-deleted virulent strain were to become the sole or predominant strain presented to the immune system, an occurrence that has not been reported in the literature, and would seem an unlikely event. Because gene deletions generally decrease the virulence of the virus, the gene deleted strain would probably not compete well against the nondeleted strain in the host animal.

Killed virus vaccines avoid nearly all of the potential problems associated with the use of MLV vaccines, providing inactivation is complete.[67,70,76] However, killed PRV vaccines have been reported to confer less protection than MLV vaccines,[25,66,67] although equal efficacy has also been reported.[2,25] Killed vaccines must be given parenterally, and, due to the lack of viral replication, there is generally a lower antibody titer, a shorter duration of the immune response, and less local immunity at the sites of viral entry into the pig.[70,76] Two doses of killed PRV vaccines are generally necessary to confer adequate protection, while a single MLV dose is usually considered adequate.[70,76]

Subunit vaccines constitute a third class of vaccines that have been evaluated experimentally, but are not available commercially. Pseudorabies virus subunit vaccines have been prepared from non-ionic detergent extracts containing mostly glycoproteins from the viral envelope.[59,85,86,93] Platt developed a subunit vaccine by purifying a Triton-X-100 crude viral extract with the use of lectin affinity chromatography.[86] A 98 kd antigen found in high concentration in the maintenance media of infected cell cultures, but not found in the lectin purified vaccine preparation, was designated the subunit diagnostic antigen (SUDA).[87] The SUDA was used to create an indirect ELISA that could distinguish between antibody responses due to infection and vaccination.[87] This diagnostic antigen was probably the same as gX,[115] so this test may be considered a precursor of the present day anti-gX differential ELISAs.[20,60]

Many of the early PR vaccines were attenuated live virus vaccines developed by the serial passaging of field strains of virus in cell cultures or in non-porcine hosts.[9,16,105,118,125] This was true for the Bartha vaccine virus strain,[8] which was isolated from a pig, then attenuated by serial passages in porcine kidney cell cultures, followed by adaptation to chicken embryo fibroblasts (CEF). Similarly, serial passages of a field isolate of PRV in the chorioallantoic membrane of embryonated chicken eggs, followed by passages in CEF cultures, led to the creation of the attenuated BUK (Bucharest) virus strain.[127] Other attenuated vaccine strains were derived from the BUK strain, including the Norden vaccine strain[113] and the genetically engineered vaccine strains created by Kit and others.[53,54] The attenuation of the BUK and Bartha-derived "conventional" vaccine strains was due to genetic mutations induced by the serial passaging in the heterologous cell or tissue cultures.[51,118] These mutations are still being elucidated, but share in common deletions in the U_S segment of the PRV genome that code for the gI and gp63 glycoproteins.[51,118,125] These deletions may be complete or only partial. Thus, most vaccine strains derived from both the BUK and Bartha strains do not produce gI, but the BUK clone selected by Kit to produce his genetically engineered vaccine strains happened to be gI positive.[51] Similarly, most Bartha derived strains do not produce any gp63, while most BUK derived strains produce an altered (truncated) gp63.[118,125]

As mentioned previously, the current strategy for the integration of vaccination into a control or eradication program for PR involves the use of deletion mutant vaccine virus strains coupled with companion diagnostic

serology assays that can differentiate between the humoral immune response due to vaccination and infection.[76,118] European countries, especially those countries with high densities of swine populations, such as the Netherlands and Germany, have moved towards adoption of gI-deleted vaccines as the only approved vaccine type.[118] The situation in the United States has been less regulated. As a result, several types of gene deleted vaccines with their companion glycoprotein specific antibody assays are currently commercially available. Table 2 lists the licensed PR vaccines currently available in the U.S. Vaccines that do not express the gI, gX or gIII glycoproteins are available in this country, although the gI and gX deleted vaccines predominate. These vaccines must meet additional minimal standards above and beyond the usual requirements for efficacy and safety. These additional requirements relate to the need for the glycoprotein specific antibody assay to be able to detect infected animals reliably, and differentiate them from vaccinated animals. The deleted glycoprotein used as the differentiating diagnostic antigen must be present and produced at uniform levels by all field strains of PRV, it must induce a detectable antibody response that persists over the time interval for which serologic monitoring is typically done, the deleted antigen must not be necessary for adequate protection against infection, and the antigenic variation of the diagnostic antigen must not be so great as to affect the ability of the diagnostic test to reliably and accurately detect pigs that have been infected with PRV.[68]

Vaccination has been shown to reduce mortality, viral shedding, and the severity of clinical signs in pigs subsequently infected with virulent

PRV.[2,25,27,66] However, vaccination will not prevent infection of pigs or the establishment of latent infections in pigs exposed to virulent strains of PRV.[25,27,75,102] Therefore, vaccination alone will not lead to the eradication of PRV from infected swine herds. It is necessary that infected pigs, including latently infected pigs, be identified and removed from the swine population. This may best be accomplished by the widespread serological surveillance of the swine population, with subsequent culling of those pigs that have been exposed to field strains of PRV.[118]

Serological Assays for Pseudorabies Virus

A wide variety of serology tests have been devised over the years for the detection of the humoral immune response to pseudorabies virus.

The standard serological diagnostic procedure for PRV in recent years has been the serum virus neutralization (SVN) test.[7,14,41] The earliest reference in the literature to the SVN test for PRV was by Glover in 1938.[32] The test is now commonly performed in microwell plastic assay plates, using porcine kidney (PK-15) or other susceptible tissue culture cell lines.[41] Wittman[125] and others report that the sensitivity of the SVN test depends on a number of factors: the type of cell culture, the use of a macro- or microtest, the concentration of cells used, the dose of virus used, whether sera is heat inactivated and, if so, for how long and at what temperature, the length of time and the temperature of the serum-virus incubation period, the presence or absence of complement, the length of time of the final incubation period following the addition of the cells, the type of dilutor used, and so on. Bitsch and Eskildsen[14,15] found that increasing the incubation of the serum-virus

mixture at 37°C from 1 hour to 24 hours dramatically increased the sensitivity of the test. The addition of guinea pig or rabbit complement was found to be necessary to detect neutralizing antibody during the very early (four to eight or nine days postchallenge) immune response.[14,15] Complement was found to be necessary to detect the initial IgM response, as well as that of complement requiring IgG subclasses, which were found to predominate in the early immune response.[14,15] This finding was supported by the work of Rodák et al.,[94] who were unable to demonstrate neutralizing IgM antibodies in an SVN assay lacking complement, even if IgM was present in high titers. Complement independent neutralization was found to be dependent on the appearance of IgG antibodies at about seven or eight days following infection. The standard SVN procedure used in the United States[41] is not optimized for sensitivity, according to the previously mentioned findings. The recommended incubation period of the serum-virus mixture is one hour at 37°C, no complement is added, and the test is read after 48 hours.[41]

A variety of assays have been devised for the detection of the humoral immune response to PRV. These include the complement fixation test (CFT),[30] the microimmunodiffusion test (MIDT),[36] the indirect radioimmunoassay (IRIA),[49] the radial immunodiffusion enzyme assay (RIDEA),[46] the countercurrent immunoelectrophoresis test (CIET),[101] radioimmunoprecipitation (RIP),[121] the indirect hemagglutination (IHA) test,[37] an Elisadisc test,[6] a dot enzyme test,[3] and numerous noncommercial enzyme-linked immunosorbent assays (ELISAs).[17,78,107,109] However, none of these alternate test procedures supplanted the SVN test in veterinary

diagnostic laboratories in the United States on a widespread basis prior to the commercial availability of PRV serology diagnostic test kits in 1986. Requirements for special instrumentation, new technique development, or the lack of standardized test protocols has restricted the use of these procedures primarily to research applications.

The ELISA has been found to offer superior sensitivity as compared to the SVN test, as well as the capability to perform larger numbers of serum assays in a shorter period of time, at relatively low expense.[7,17,29,94] The development of a standardized PRV ELISA procedure[106] at the National Veterinary Services Laboratories (NVSL) set the stage for the current proliferation of commercially available ELISA kits in the United States. However, the NVSL PRV ELISA required that a laboratory perform certain time consuming operations no longer necessary with the commercial kits, such as the coating of microtitration wells with antigen, and the titration of the enzyme conjugate.[106] Consequently, this assay was somewhat tedious to perform, and its use at the Iowa State University Veterinary Diagnostic Laboratory was restricted mainly to the testing of either toxic sera or sera with low SVN titers.

Table 3 lists the commercial PRV serology diagnostic kits currently licensed in the United States, as well as their license dates, manufacturer, and companion marker vaccines. This table contains information gathered from various sources.[50,122]

Commercially available diagnostic kits for the serological detection of antibodies to PRV in swine first appeared in the United States in January,

Table 3. Commercially available pseudorabies serology diagnostic kits (November, 1991)

Kit	License date	Manufacturer/ distributor	Companion vaccines	Diagnostic antigen
Screening Assays:				
DiaSystems® CELISA PRV™	8/89	TechAmerica™ Diagnostics (IDEXX Laboratories)	none	PRV (gII)
HerdChek®:Anti-PRV (S)	1/86	IDEXX Laboratories	none	PRV
HerdChek®:Anti-PRV (V)	1/86	IDEXX Laboratories	none	PRV
Pseudorabies Virus Antibody Test Kit - Latex Agglutination	5/86	Viral Antigens	none	PRV
Differential Assays:				
ClinEase-PRV®	10/89	SmithKline Beecham	PR-Vac (MLV & killed)	gI
DiaSystems® CELISA OmniMark™ PRV	6/90	TechAmerica™ Diagnostics	OmniMark™ PRV	gIII
HerdChek®:Anti-PRV-gpX	8/88	IDEXX Laboratories	SyntroVet PRV/Marker®	gX
			SyntroVet PRV/Marker Gold®	gX
HerdChek®:Anti-PRV-gI	5/90	IDEXX Laboratories	BioCeutic	gI
			SyntroVet PRV/Marker Gold®	gI
			PR-Vac (MLV & killed)	gI
TOLVID® Diagnostic	11/89	Agdia, Inc./The Upjohn Co.	TOLVID®	gX

1986,[122] with the introduction of the IDEXX HerdChek® anti-PRV screening and verification ELISAs.[33,108] A latex bead agglutination test (Pseudorabies Virus Antibody Test Kit - Latex Agglutination) from Viral Antigens Inc. was next licensed in May, 1986.[100,101] These assays detected humoral immune responses both to infection with PRV and to vaccination with either killed or modified live virus (MLV) vaccines, and are classified as screening assays.

The first commercial kit designed to distinguish between vaccinated and infected pigs, defined as a differential ELISA, was licensed in August, 1988. The IDEXX HerdChek anti-gX assay[20] is a competitive ELISA (CELISA) designed to be used as a companion differential test for the SyntroVet PRV/Marker® MLV vaccine.[19] A CELISA[33] from TechAmerica™ (DiaSystems® CELISA PRV™) was next licensed in August, 1989. This assay is similar to the HerdChek anti-PRV ELISA and the latex agglutination test (LAT) in that it does not distinguish between infected and vaccinated pigs. In October, 1989, Norden Laboratories (now SmithKline Beecham) released a gI differential ELISA, ClinEase-PRV®, for use with their MLV and killed PRV vaccines, PR-Vac (killed and MLV).[69] This release was followed closely by the licensing, in November, 1989, of Agdia's differential gX CELISA (Tolvid® Diagnostic) for use with the Tolvid® MLV vaccine[60] produced by The Upjohn Company. Two more differential ELISAs have been licensed more recently: TechAmerica™ Diagnostics' DiaSystems® CELISA OmniMark™ PRV kit[52] for use with the OmniMark™ PRV gIII deleted vaccine from NovaGene, Inc.,[54] and IDEXX Laboratory's HerdChek®:Anti-PRV-gI CELISA

kit for use with any of three gI deleted marker vaccines (from SmithKline Beecham, SyntroVet, and Boehringer/Ingelheim).

The PRV serology diagnostic kits listed above are all available commercially, and are licensed by the United States Department of Agriculture (USDA) for use by state-accredited diagnostic laboratories.

Particle Concentration Fluorescence Immunoassay (PCFIA)

The particle concentration fluorescence immunoassay (PCFIA) evaluated here is the first test of this type for the detection of antibodies to PRV. Previous research applications of the PCFIA technique include the detection and quantitation of human immunoglobulins,[1,45,57,58,62,79] murine immunoglobulins[45] and murine antiviral antibodies,[82,83] and the detection of various endogenous proteins.[26,42] The PCFIA technique has also been used to detect the presence of drugs in race horses,[56,63,104,114,126] and commercial tests are available for this purpose. A commercial PCFIA for the detection of antibodies to *Brucella abortus* in cattle[74,107] was licensed[122] in 1987 by the manufacturer that developed the PRV PCFIA.[j]

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[j] IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine.

MATERIALS AND METHODS

Swine Sera

Positive and negative field swine sera

For this portion of the study, 2,262 field serum samples from nonvaccinated swine herds of known status for PRV infection were evaluated by the PCFIA.

Positive field swine sera Sera included in the positive population set of field swine sera (n=619) had previously tested positive by at least two conventional PRV serological assays, including the serum virus neutralization (SVN) test. The SVN test results were considered to be positive at an endpoint dilution of ≥ 1:2. The HerdChek screening ELISA was used to verify the SVN positive result for nearly all samples, but a small number of the sera were verified as positive by the latex agglutination test (LAT) instead. The PRV positive field sera were obtained from nonvaccinated pseudorabies infected herds submitted to the Iowa State University Veterinary Diagnostic Laboratory as part of the Iowa Pseudorabies Disease Program.

Negative field swine sera All sera in the negative population set of field swine sera (n=1643) had been previously tested as negative by the IDEXX HerdChek® screening ELISA[k] for PRV antibodies. The negative set consisted of sera that were obtained from nonvaccinated pseudorabies-free swine herds for which sera had been submitted to the Veterinary Diagnostic Laboratory.

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[k] IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine.

Challenged pig antisera

An additional 241 sera were collected from 35 experimentally infected nonvaccinated pigs ranging from 4 to 11 weeks of age at the initiation of the study. Sera were collected daily from day 4 postchallenge (PC) to day 10 PC, then again at days 14 and 21 PC. The challenge virus strain of PRV was either the Becker strain or the pneumotropic strain VDL 4892.[20] Challenge doses were administered intranasally, and ranged from 10^5 to 10^7 $TCID_{50}$ of virus per pig (Table 4 summarizes this information). These sera were then tested by the experimental PCFIA for PRV, as well as by eight other PRV serological assays currently in use at the Veterinary Diagnostic Laboratory.

NVSL PRV check set

The 1990 PRV ELISA screen and latex agglutination test check set of 30 swine sera was provided by the National Veterinary Services Laboratories (NVSL).[1]

Weak positive and suspect sera

A set of 619 swine sera determined to be either weakly positive or suspect by the HerdChek PRV screening ELISA was selected from field samples submitted to the Veterinary Diagnostic Laboratory. Criteria used for selection were that the sera had tested either weakly positive (S/P ratio of 0.43 to 1.00) or as suspect (S/P ratio of 0.38 to 0.43) on the screening ELISA.

[1] National Veterinary Services Laboratories, Ames, Iowa.

Table 4. Groups of pigs infected intranasally with pseudorabies virus

Sample #	Group ID	Pig ID	Age (weeks)	Strain of PRV	Dose ($TCID_{50}$)
1	802-15	66	8	Becker	4.9×10^6
2		67			
3		68			
4		69			
5		70			
6	804-8	396	4	ISU 4892	3.7×10^7
7		397			
8		398			
9		399			
10		400			
11	804-9	81	10-11	ISU 4892	3.7×10^7
12		82			
13		83			
14		84			
15		85			
16		86			
17		87			
18		88			
19		89			
20		90			
21	91-2	113	4-5	ISU 4892	2.1×10^6
22		114			
23		115			
24		116			
25		117			
26	802-19	71	10-11	Becker	4.1×10^6
27		72			
28		73			
29		74			
30		75			
31	802-19	118	10-11	ISU 4892	5.0×10^5
32		119			
33		120			
34		121			
35		122			

Plugged wells

Swine sera of poor quality can cause the filter at the bottom of the assay plate microwell to become clogged, an occurrence described as a "plugged well". Sera causing plugged wells on initial testing by the PCFIA were characterized according to the criteria of clarity, color, the presence of particulates, and the freshness of the sample. Attempts were also made to treat the offending sera, first by centrifugation (3000 rpm for 15 min) and finally by filtration (0.2 µm).[m]

Serological Assays

PCFIA

PCFIA reagents Reagents for the PRV PCFIA were supplied by IDEXX Laboratories as part of an experimental test kit.[n] The solid phase consisted of 0.6-0.8 µm polystyrene latex particles coated with PRV antigen (Shope strain), diluted in phosphate buffered saline (PBS) with protein stabilizers, and preserved with sodium azide and thimerosal. The conjugate consisted of a monoclonal antibody to the gII glycoprotein of PRV, labelled with phycoerythrin, a high-output fluorophore. Strong positive, weak positive and negative control antisera to PRV were provided pre-diluted in sample diluent. Sample diluent consisted of PBS with protein stabilizers and preservatives. The wash solution consisted of PBS and preservatives.

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[m] Spin-X™ centrifuge filter unit, 0.22 µm cellulose acetate, Costar®, 205 Broadway, Cambridge, MA.

[n] IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine.

PCFIA Assay Plates Specially designed 96 well assay plates similar in appearance to standard microtiter plates were used. A 0.2 µm cellulose acetate filter located at the base of each microwell allowed the rinsing and removal of all well contents not bound to the latex particles by the use of a vacuum applied to the plate by the Screen Machine. The vacuum was applied to a port that communicated with a sump area beneath the membrane filter in each plate (Figure 1).

PCFIA Assay Procedures The PCFIA for antibodies to PRV is a competitive or blocking fluorescence immunoassay. PRV-coated polystyrene particles serve as the solid phase. Diluted test sera are incubated with the coated particles, and antibodies directed against PRV antigens became attached to the solid phase. A conjugate is then added to the test well and allowed to compete with sample antibodies for sites on the antigen. The conjugate used for this evaluation consisted of a monoclonal antibody (MAb) directed against the gII PRV antigen, which is present in all strains of PRV. The MAb was labeled with the fluorophore phycoerythrin. Fewer antigenic sites are available for the conjugate if a serum sample contains anti-PRV gII antibodies. Conversely, a serum sample with no anti-PRV antibodies will not block the antigenic sites, allowing the labeled conjugate to attach to the coated particles. Any unattached conjugate is removed during the filtration and wash steps, resulting in lower photon counts for those wells containing sera from swine exposed to PRV.

The Screen Machine consists of a multi-purpose automated instrument designed to perform the various reagent dispense, incubation, and wash steps,

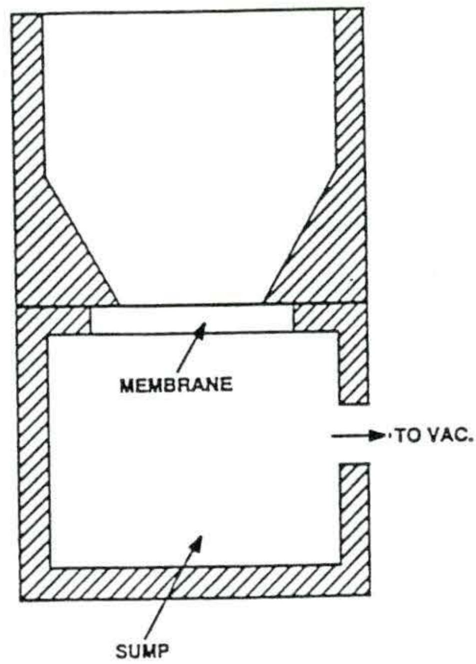

Figure 1. Cross-section of a PCFIA assay plate well, showing the membrane filter, the vacuum port, and the sump

in addition to detecting the fluorescence at specific wavelengths in each well. Certain maintenance and calibration procedures were performed at regular intervals. For this study, the following calibration procedures were performed as part of each assay: a lamp calibration routine, an assay using reference performance verification particles (PVPs), and a reference plate assay. The PVP assay provided information concerning all aspects of instrument performance, including pipetting accuracy, separation (vacuum), and fluorimetry performance. The reference plate assay provided information specifically about the fluorimetry performance.

A partitioned tray was filled with the following test reagents: PRV-coated polystyrene particles, anti-PRV gII:phycoerythrin conjugate, and wash solution. The reagent tray was inserted into the Screen Machine for automated dispensing.

The PCFIA procedure required the addition of 12 negative control samples and two each of strong and weak positive control samples to the first assay plate of each batch run. A batch run consisted of from 1 to 10 plates. Fifty µl of prediluted control sera was added to the prescribed well, and 50 µl of sample diluent was added to each of the remaining wells. A 1:11 dilution of each serum was prepared by adding five µl of each serum sample to the wells containing sample diluent.

The assay plates were then placed in a 10-plate capacity elevator tray in the Screen Machine for automatic dispensing of reagents, incubations, separations, and fluorescence detection. Coated particles (20 µl per well) were pipetted into each well, followed by a 14 minute incubation. Conjugate (20 µl

per well) was then pipetted into each well, followed by a 9 minute incubation. Vacuum was applied for 60 seconds, followed by a wash (50 µl per well). The Screen Machine is capable of reading fluorescence by three channels (channels A, B and C) simultaneously. The emission fluorescence of the bound phycoerythrin was read at 575 nm by channel C of the Screen Machine while a vacuum was applied to the plate (channel A had been reserved for the fluorophore, fluorescein isothiocyanate, which is used in a PCFIA for brucellosis). In addition, a reference particle using Texas red as a fluorophore was read at 620 nm by channel B. The reference particles had been previously mixed with the PRV-coated particles, and served to validate certain assay performance criteria, including proper filtering and pipetting. The test results were calculated as an S/N value, which is the ratio of the test sample signal to the mean of the negative control sera signals. Test results were calculated as a ratio in order to minimize the effect of test to test variation. The signal values used to calculate the S/N value were themselves a ratio of the test and reference channel photon counts for each well. Thus, the S/N formula was defined as:

$$S/N = \frac{\text{Sample channel C counts/sample channel B counts}}{\text{Neg. control channel C counts/Neg. control channel B counts}}$$

<u>Other serological assays</u>

As mentioned previously, 241 swine sera from 35 intranasally infected pigs were tested by the PCFIA in an attempt to assess the ability of the PCFIA to detect the early immune response in infected pigs. These same sera were

also tested by eight other PRV serological assays in order to compare the performance of all of the PRV serological assays currently in use at the Iowa State University Veterinary Diagnostic Laboratory. Test results from the PCFIA were compared with results from each of the other eight assays by the chi-square method, testing the null hypothesis that the PCFIA and the compared test did not differ in the number of sera detected as positive (or suspect) and negative.

The eight assays consisted of the serum virus neutralization (SVN) test, two commercial screening ELISAs (DiaSystems® CELISA PRV™ and HerdChek®:Anti-PRV (S)), four commercial differential ELISAs (ClinEase-PRV®, DiaSystems® CELISA OmniMark™ PRV, HerdChek®:Anti-PRV-gpX, and HerdChek®:Anti-PRVgI), and a commercial latex agglutination test (Pseudorabies Virus Antibody Test Kit-Latex Agglutination). Tables 5 and 6 summarize various features and characteristics of the experimental PCFIA and the seven commercial PRV antibody tests. The SVN test was performed essentially as described in the standard procedure adopted by the American Association of Veterinary Laboratory Diagnosticians (AAVLD) in 1977.[41] All seven of the commercial PRV antibody tests listed above were performed according to the manufacturers' instructions.

Table 5. Comparison of the PCFIA for pseudorabies virus with six commercial ELISAs and the latex agglutination test

Test name	Test abbreviation	Type of test	Serum dilution	Antigen	Conjugate	Indicator	Wavelength (nm)
PCFIA (IDEXX)	PCFIA	PCFIA	1:11	PRV (gII)	anti-PRV gII: phycoerythrin	N/A[a]	575
HerdChek PRV Ab Screen (IDEXX)	IDEXX Screen	indirect ELISA	1:20	PRV	anti-swine IgG: HRP[b]	ABTS[c]	405-410
DiaSystems PRV Ab CELISA (TechAmerica)	TA Screen	CELISA	none	PRV	anti-PRV gII: HRP	ABTS	405-410
HerdChek PRV gI Ab Test (IDEXX)	IDEXX gI	CELISA	1:2	PRV (gI)	anti-PRV gI: HRP	TMB[d]	650
ClinEase PRV gI Ab Test (SmithKline Beecham)	SKB gI	indirect ELISA	1:5	PRV gI	anti-swine IgG: HRP	ABTS	405-410
HerdChek PRV gpX Ab Test (IDEXX)	IDEXX gX	CELISA	1:2	PRV (gX)	anti-PRV gX: HRP	TMB	650
DiaSystems GIII PRV CELISA (TechAmerica)	TA gIII	CELISA	none	PRV gIII	anti-PRV gIII: HRP	TMB	630
Latex Agglutination PRV Test (Viral Antigens)	LAT	latex agglutination	1:4	PRV	N/A	N/A	N/A

[a] N/A = not applicable.

[b] HRP = horseradish peroxidase.

[c] ABTS = 2,2-azino-di(3-ethylbenzthiazoline sulfonate).

[d] TMB = tetramethylbenzidine.

Table 6. Result calculations and threshold values for the PCFIA and six commercial pseudorabies ELISAs

Test name	Result	Result calculation	Positive threshold	Negative threshold	Suspect range
PRV PCFIA (IDEXX)	S/N	$\frac{\text{Sample count/sample ref count}}{\text{Negative count/negative ref count}}$	≤ 0.90[a]	> 0.90	none
HerdChek® PRV Ab Screen (IDEXX)	S/P[b]	$\frac{\text{Sample OD - Negative OD}^{c}}{\text{Positive OD}^{d}\text{ - Negative OD}}$	> 0.43	< 0.38	0.38-0.43
DiaSystems PRV Ab CELISA (Tech America)	raw OD	none	≤ the greater of: 0.32 x Negative OD, or Positive OD + 0.15	>threshold	none
HerdChek® PRV gI Ab Test (IDEXX)	S/N[e]	$\frac{\text{Sample OD}}{\text{Negative OD}}$	<0.60	>0.70	0.60-0.70
ClinEase PRV gI Ab Test (SmithKline Beecham)	S/C[f]	$\frac{\text{2 x Sample OD - Negative OD}}{\text{Positive OD - Negative OD}}$	>1.0	<0.80	0.80-1.00
HerdChek® PRV gpX Ab Test (IDEXX)	S/N	$\frac{\text{Sample OD}}{\text{Negative OD}}$	<0.60	>0.70	0.60-0.70
DiaSystems GIII PRV Ab CELISA (Tech America)	S/N	$\frac{\text{Sample OD}}{\text{Negative OD}}$	<0.65	>0.75	0.65-0.75

[a] S/N ≤ 0.90 selected as the positive threshold for this evaluation on the basis of results from other studies reported here.

[b] S/P = sample to positive control ratio.

[c] Negative OD = mean negative control optical density.

[d] Positive OD = mean positive control optical density.

[e] S/N = sample to negative control ratio.

[f] S/C = sample to positive control ratio.

RESULTS

PCFIA Serology Results

Positive field swine sera

The S/N values for the 619 swine sera making up the positive population set of field swine sera ranged from 0.09 to 0.98 (Figures 2 and 4), with a mean S/N of 0.36 and a standard deviation of 0.16. Only two positive set samples resulted in S/N values greater than or equal to 0.90. Using a positive S/N threshold of ≤ 0.90, the sensitivity of the PCFIA was 99.7% for this group of known positive field sera.

Negative field swine sera

The S/N values for the 1,643 negative population set of field swine sera ranged from 0.71 to 2.04 (Figures 3 and 4), but were clustered tightly around the mean (mean S/N = 1.09, standard deviation = 0.07). Only two samples resulted in S/N values less than 0.90. Using a positive S/N threshold of ≤ 0.90, the specificity of the PCFIA was 99.9% for this set of known negative field sera.

Challenged pig antisera

A total of 241 sera from 35 pigs experimentally infected with PRV were tested by the PCFIA. If a positive S/N threshold of ≤ 0.90 is used, the PCFIA detected 0% of the sera as positive at 5 days postchallenge (PC), 50% at 6 days PC, 82% at 7 days PC, and 100% at days 8 through 23 PC (Figures 5-11 and Tables 7 and 8).

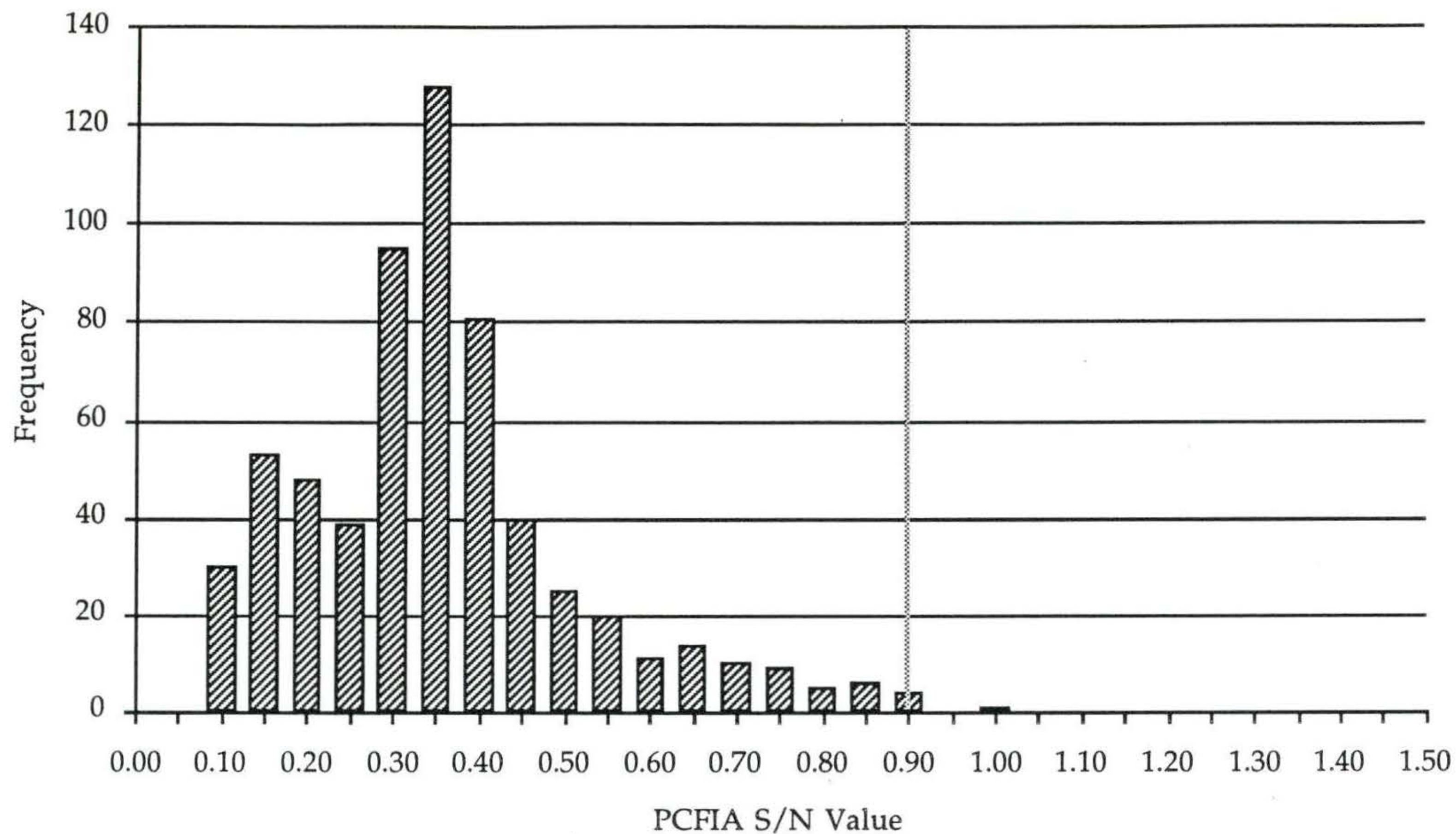

Figure 2. PCFIA S/N frequency distribution for pseudorabies positive swine population (n = 619 sera). Sera were collected from nonvaccinated commercial swine herds. A positive threshold of S/N ≤ 0.90 resulted in a sensitivity of 99.7% for the PCFIA

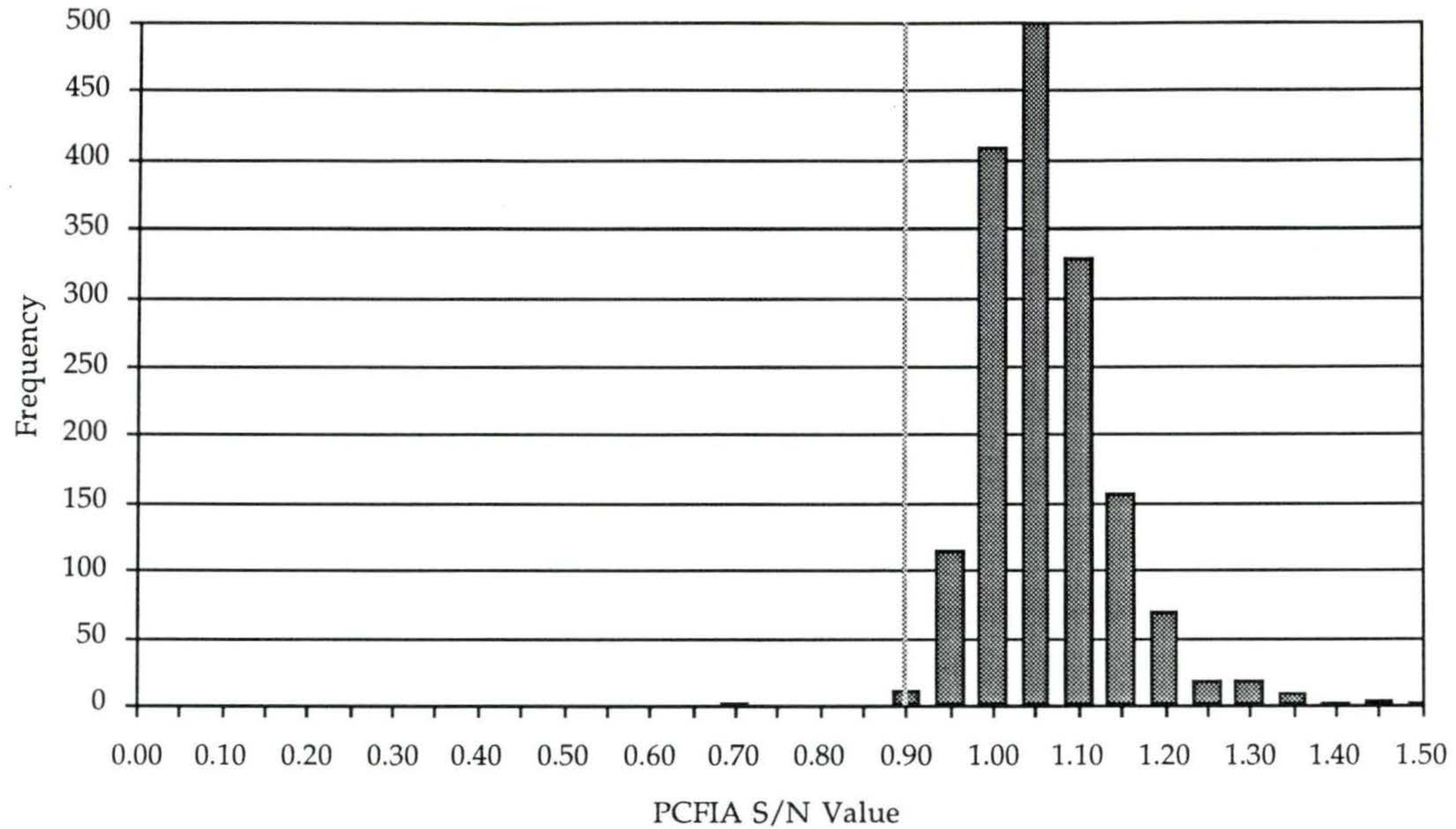

Figure 3. PCFIA S/N frequency distribution for pseudorabies negative swine population (n = 1,643 sera). Sera were collected from nonvaccinated commercial swine herds. A positive threshold of S/N ≤ 0.90 resulted in a specificity of 99.9% for the PCFIA

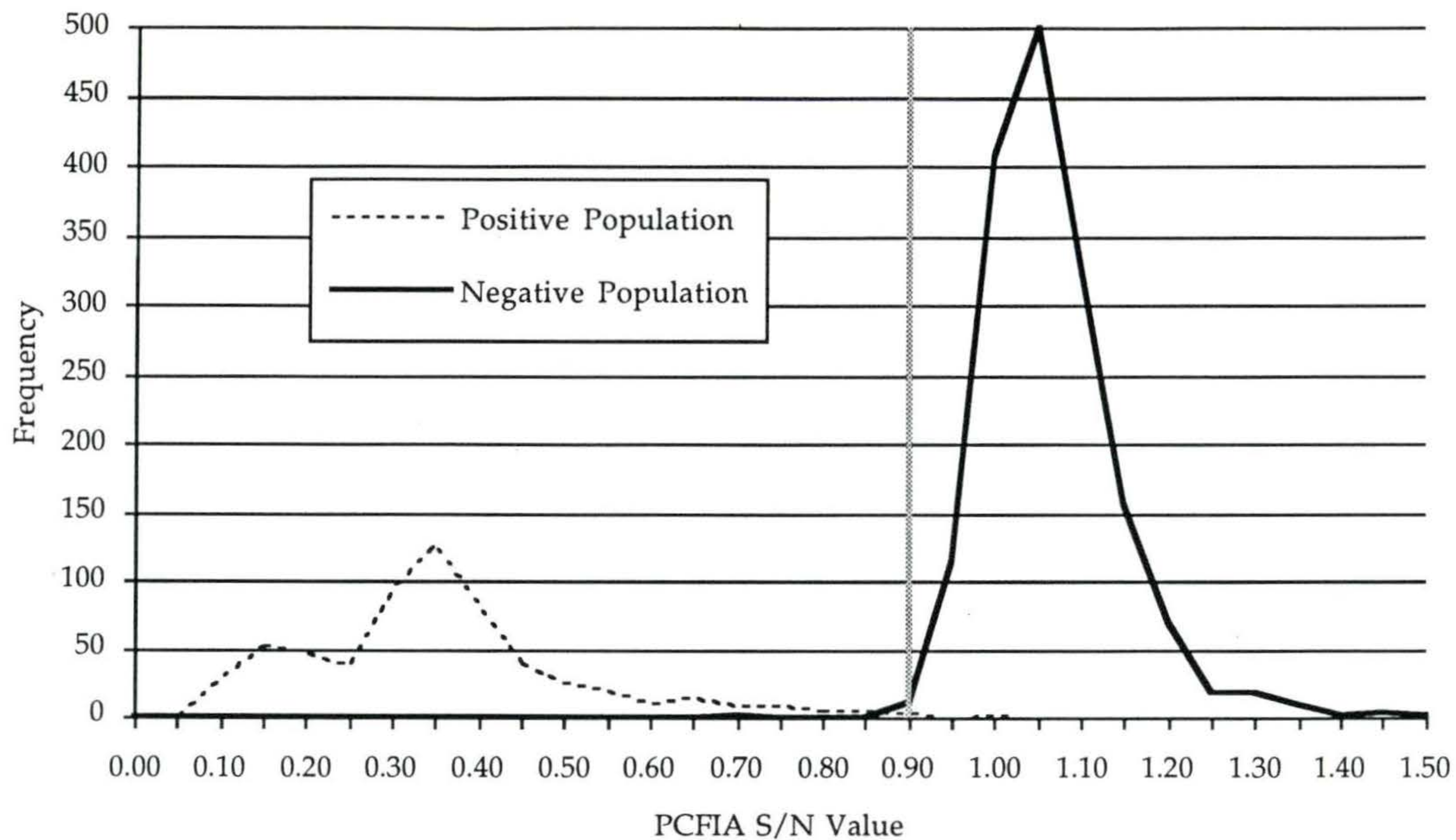

Figure 4. PCFIA S/N frequency distribution for pseudorabies negative (n = 1,643 sera) and pseudorabies positive (n = 619 sera) swine populations. Sera were collected from nonvaccinated commercial swine herds. A positive threshold of $S/N \leq 0.90$ was selected for evaluation of the PCFIA

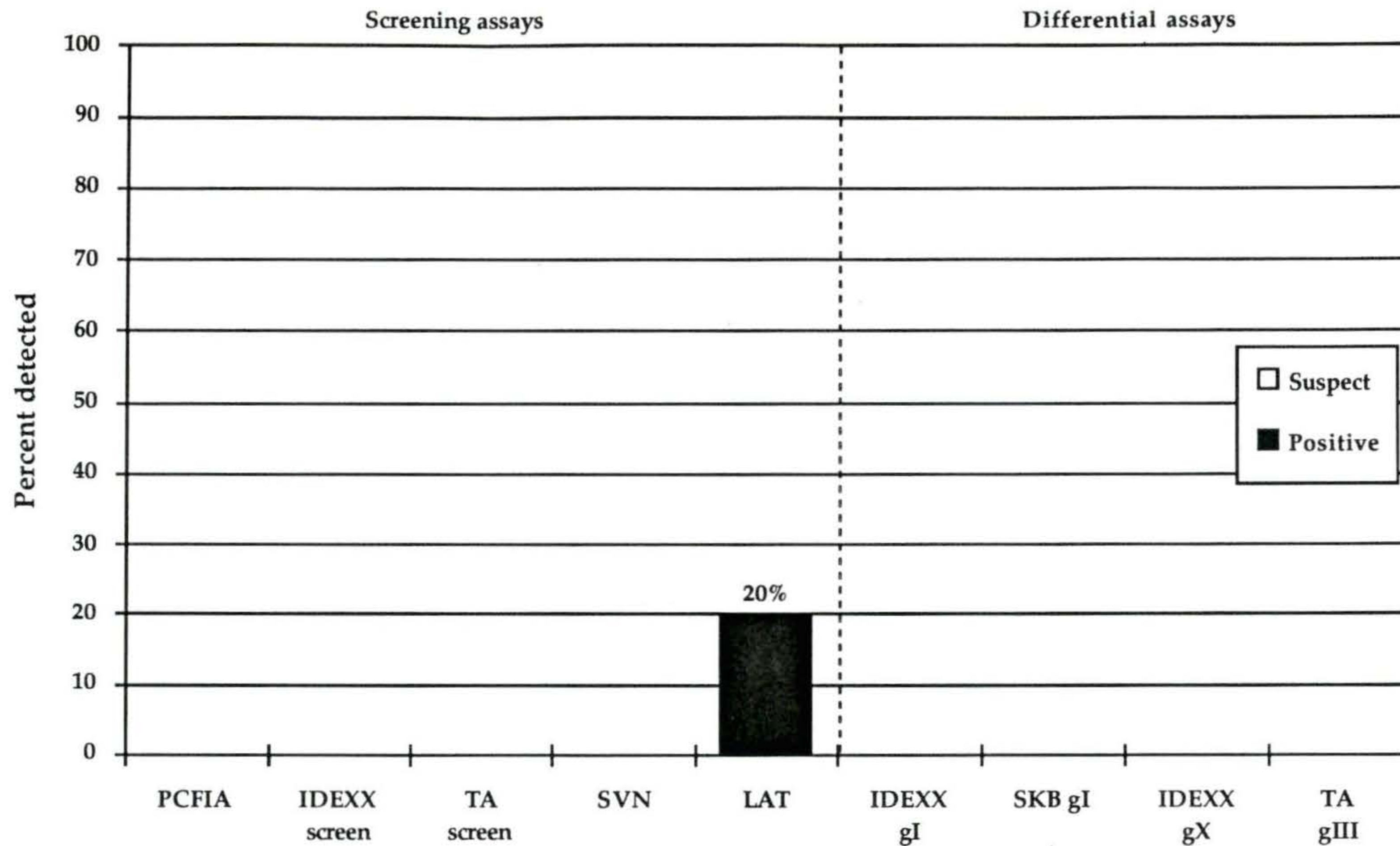

Figure 5. 5 days PC: percent swine serum samples detected as positive or suspect by nine serological assays at five days postchallenge (n = 35)

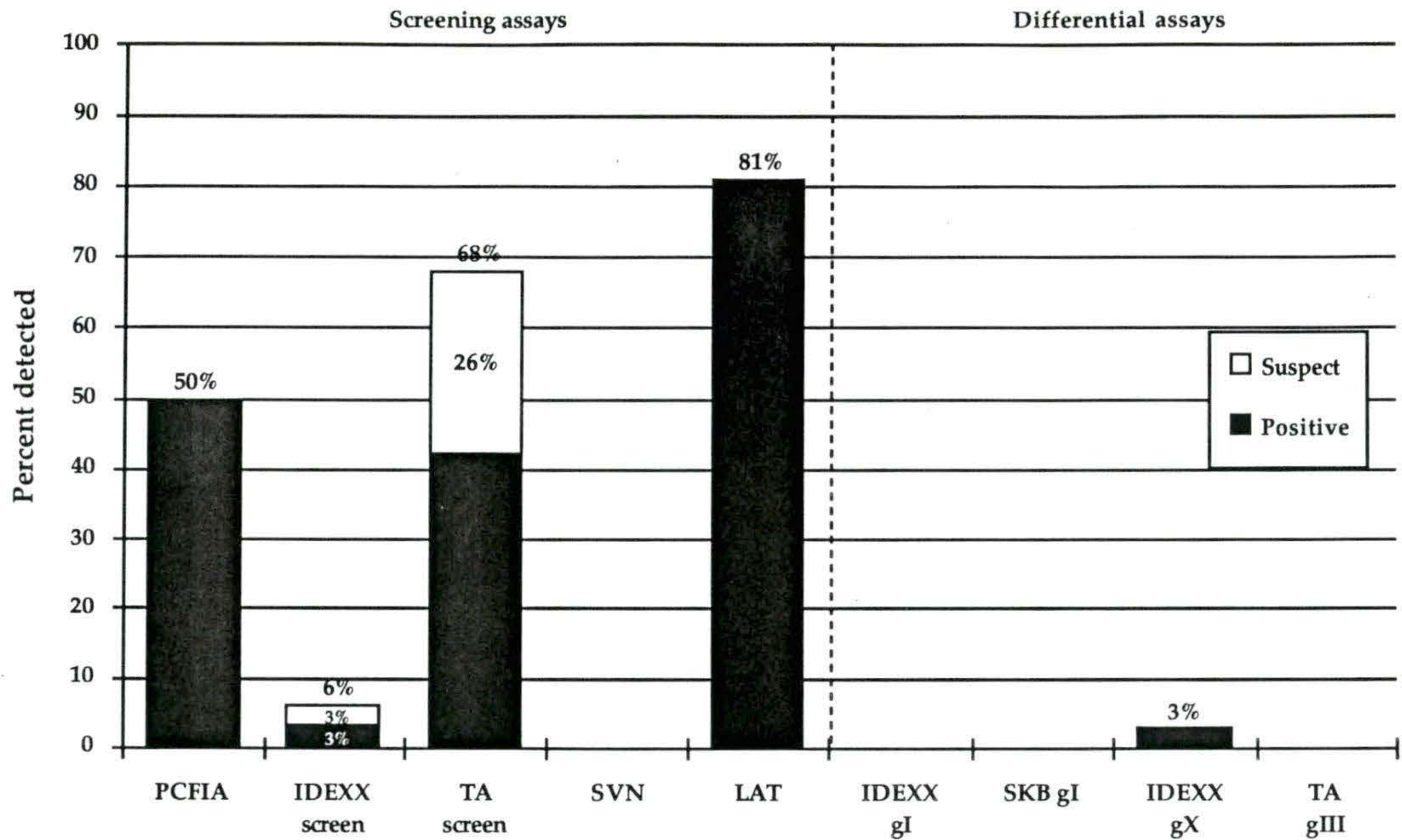

Figure 6. 6 days PC: percent swine serum samples detected as positive or suspect by nine serological assays at six days postchallenge (n = 32)

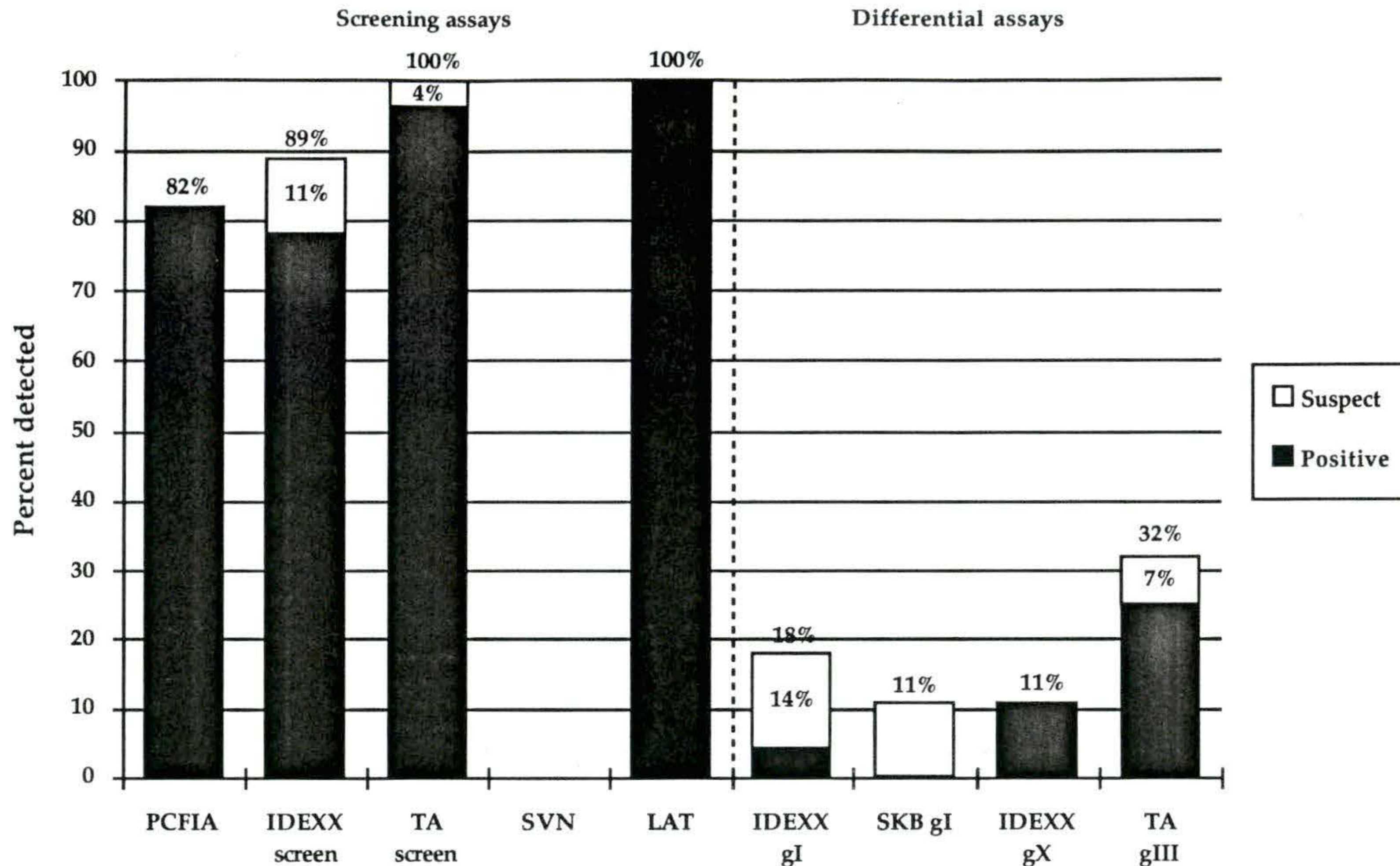

Figure 7. 7 days PC: percent swine serum samples detected as positive or suspect by nine serological assays at seven days postchallenge (n = 28)

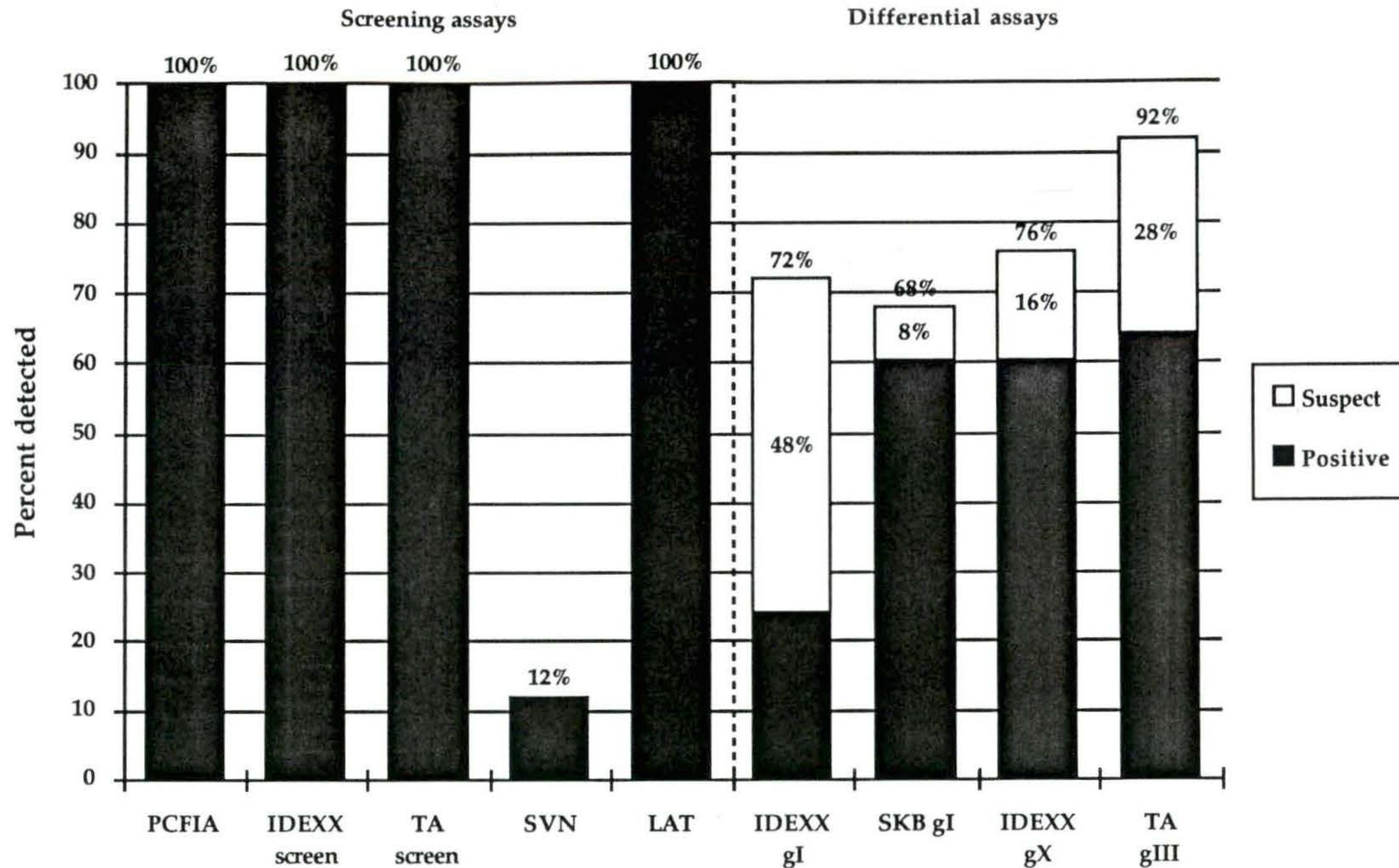

Figure 8. 8 days PC: percent swine serum samples detected as positive or suspect by nine serological assays at eight days postchallenge (n = 25)

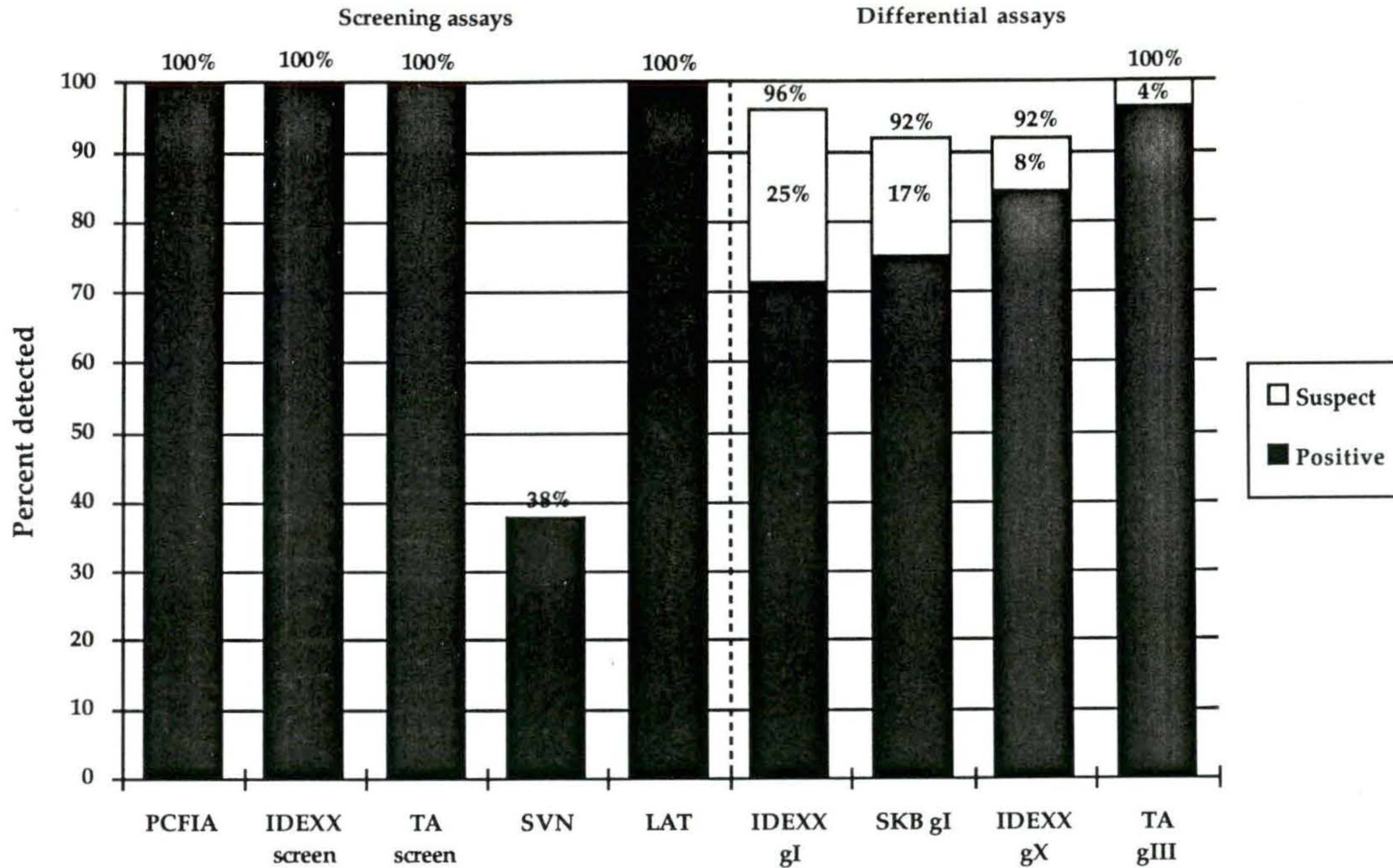

Figure 9. 9 days PC: percent swine serum samples detected as positive or suspect by nine serological assays at nine days postchallenge (n = 24)

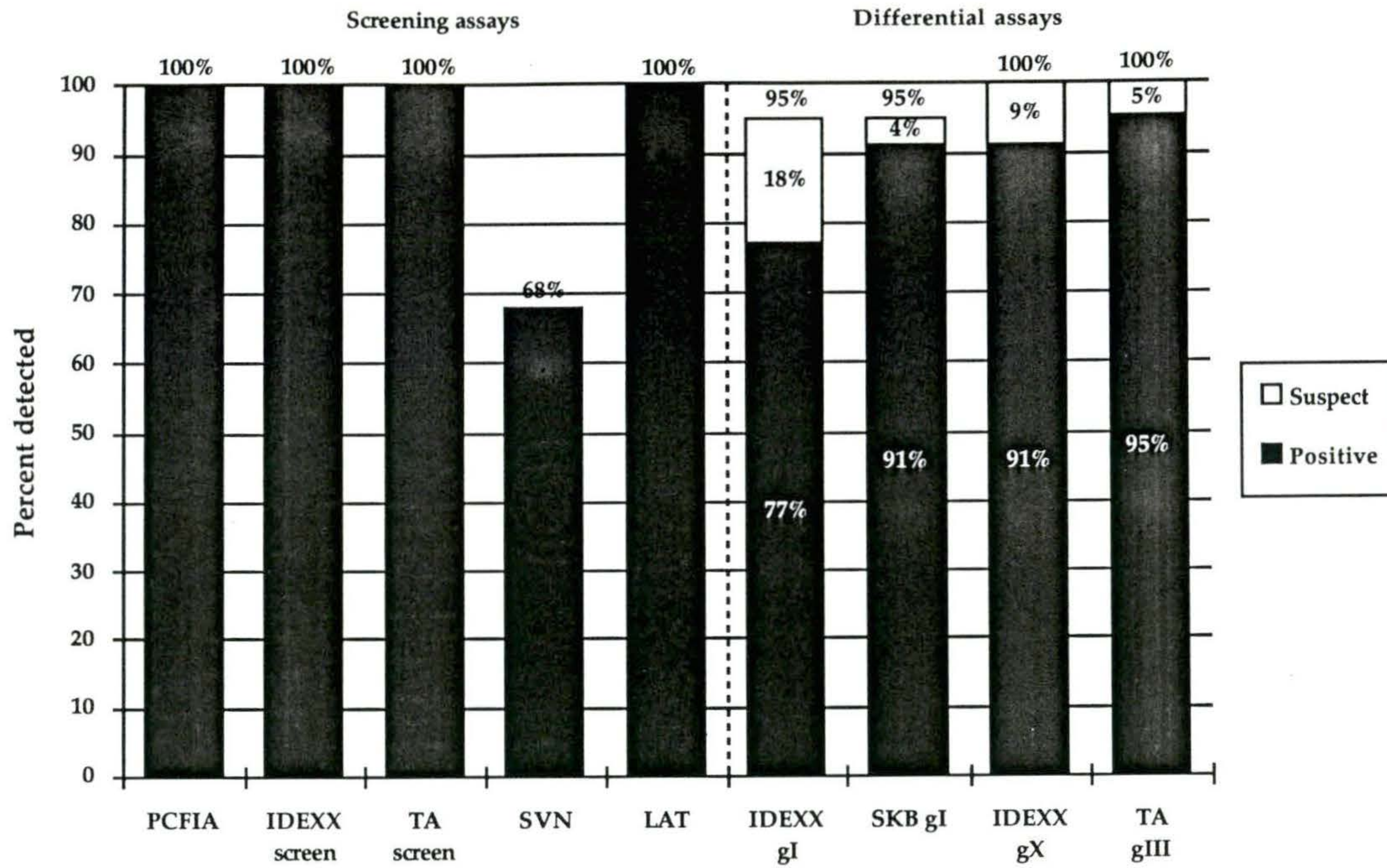

Figure 10. 10 days PC: percent swine serum samples detected as positive or suspect by nine serological assays at 10 days postchallenge (n = 22)

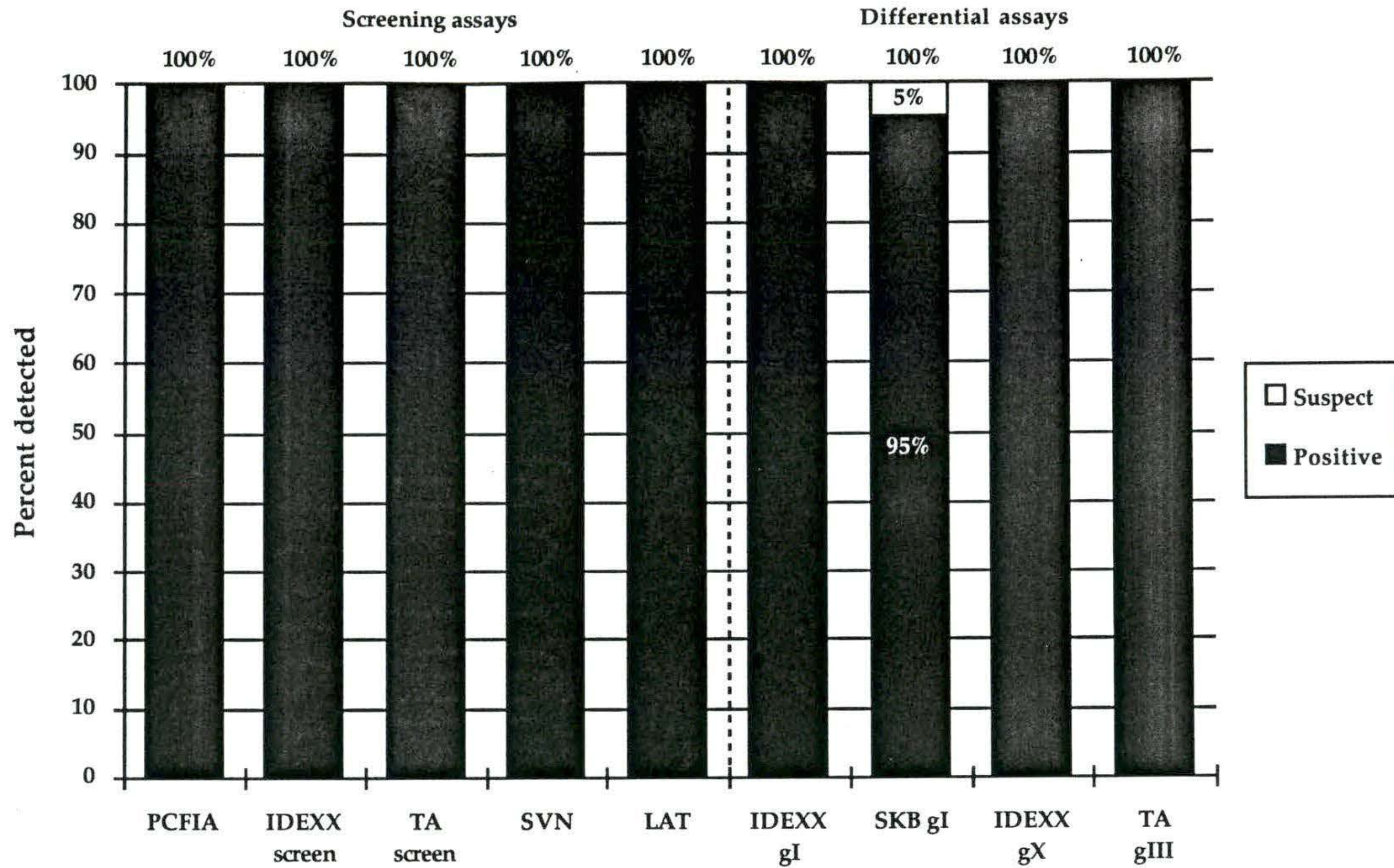

Figure 11. 14 days PC: percent swine serum samples detected as positive or suspect by nine serological assays at 14 days postchallenge (n = 21)

Table 7. Comparison of results from nine PRV serological tests in the detection of the early immune response in pigs 5 to 7 days after experimental infection with pseudorabies virus

Test	Days PC	No. sera	No. positive/suspect	No. negative	% positive	P[a]
PCFIA	5	35	0	35	0.0	
IDEXX screen	5	35	0	35	0.0	n.s.[b]
TA screen	5	35	0	35	0.0	n.s.
SVN	5	33	0	35	0.0	n.s.
LAT	5	35	7	28	20.0	< 0.01
IDEXX gI	5	35	0	35	0.0	n.s.
SKB gI	5	35	0	35	0.0	n.s.
IDEXX gX	5	35	0	35	0.0	n.s.
TA gIII	5	35	0	35	0.0	n.s.
PCFIA	6	32	16	16	50.0	
IDEXX screen	6	32	2	30	6.3	< 0.001
TA screen	6	31	13	18	41.9	n.s.
SVN	6	32	0	32	0.0	< 0.001
LAT	6	32	26	6	81.3	< 0.01
IDEXX gI	6	32	0	32	0.0	< 0.001
SKB gI	6	32	0	32	0.0	< 0.001
IDEXX gX	6	32	1	31	3.1	< 0.001
TA gIII	6	32	0	32	0.0	< 0.001
PCFIA	7	28	23	5	82.0	
IDEXX screen	7	28	25	3	89.3	n.s.
TA screen	7	23	22	1	95.7	n.s.
SVN	7	28	0	28	0.0	< 0.001
LAT	7	28	28	0	100.0	< 0.05
IDEXX gI	7	28	5	23	17.9	< 0.001
SKB gI	7	28	3	25	10.7	< 0.001
IDEXX gX	7	28	3	25	10.7	< 0.001
TA gIII	7	28	9	19	32.1	< 0.001

[a] P = the probability that the results from the PCFIA and the compared test do not differ, as calculated by the chi-square test.

[b] n.s. = no statistical difference between the PCFIA and the compared test.

Table 8. Comparison of results from nine PRV serological tests in the detection of the early immune response in pigs 8 to 10 days after experimental infection with pseudorabies virus

Test	Days PC	No. sera	No. positive/suspect	No. negative	% positive	P[a]
PCFIA	8	25	25	0	100.0	
IDEXX screen	8	25	25	0	100.0	n.s.[b]
TA screen	8	25	25	0	100.0	n.s.
SVN	8	25	3	22	12.0	< 0.001
LAT	8	25	25	0	100.0	n.s.
IDEXX gI	8	25	18	7	72.0	< 0.005
SKB gI	8	25	17	8	68.0	< 0.01
IDEXX gX	8	25	19	6	76.0	< 0.01
TA gIII	8	25	23	2	92.0	n.s.
PCFIA	9	24	24	0	100.0	
IDEXX screen	9	24	24	0	100.0	n.s.
TA screen	9	24	24	0	100.0	n.s.
SVN	9	24	9	15	37.5	< 0.001
LAT	9	24	24	0	100.0	n.s.
IDEXX gI	9	24	23	1	95.8	n.s.
SKB gI	9	24	22	2	91.7	n.s.
IDEXX gX	9	24	22	2	91.7	n.s.
TA gIII	9	24	24	0	100.0	n.s.
PCFIA	10	22	22	0	100.0	
IDEXX screen	10	22	22	0	100.0	n.s.
TA screen	10	22	22	0	100.0	n.s.
SVN	10	22	15	7	68.2	< 0.005
LAT	10	22	22	0	100.0	n.s.
IDEXX gI	10	22	21	1	95.5	n.s.
SKB gI	10	22	21	1	95.5	n.s.
IDEXX gX	10	22	22	0	100.0	n.s.
TA gIII	10	22	22	0	100.0	n.s.

[a] P = the probability that the results from the PCFIA and the compared test do not differ, as calculated by the chi-square test.

[b] n.s. = no statistical difference between the PCFIA and the compared test.

Results for the eight other PRV antibody tests are presented alongside the PCFIA data in Figures 5-11 and in Tables 7 and 8. The latex agglutination test appeared to be more sensitive than the PCFIA in detecting the early immune response to infection with PRV. Results of the PCFIA were significantly different from those of the LAT on days 5 and 6 postchallenge ($P < 0.01$), and on day 7 postchallenge ($P < 0.05$). The PCFIA appeared to be more sensitive than the SVN test for this set of swine sera. Results of the PCFIA were significantly different than those of the SVN test on days 6 through 9 postchallenge ($P < 0.001$), and on day 10 postchallenge ($P < 0.005$). The PCFIA results did not differ significantly on any test date with the TechAmerica screening ELISA. The PCFIA results differed significantly from those of the IDEXX screening ELISA only for day 6 postchallenge ($P < 0.001$).

The PCFIA appeared to be more sensitive than all four differential ELISAs for days 6 and 7 postchallenge ($P < 0.001$). On day 8 postchallenge, the PCFIA results differed significantly from those of the IDEXX gI ($P < 0.005$), the SmithKline Beecham gI ($P < 0.01$), and the IDEXX gX tests ($P < 0.01$), but not from those of the TechAmerica gIII ELISA. By days 9 and 10 postchallenge, no significant differences were found between test results from the PCFIA and the differential ELISAs.

Figure 12 and Table 9 summarize the respective performance of each test regarding four key parameters: the time (number of days postchallenge) required to detect at least one, 95%, and 100% of the sera as either positive or suspect, and the time from initial detection to ≥ 95% detection. Suspect

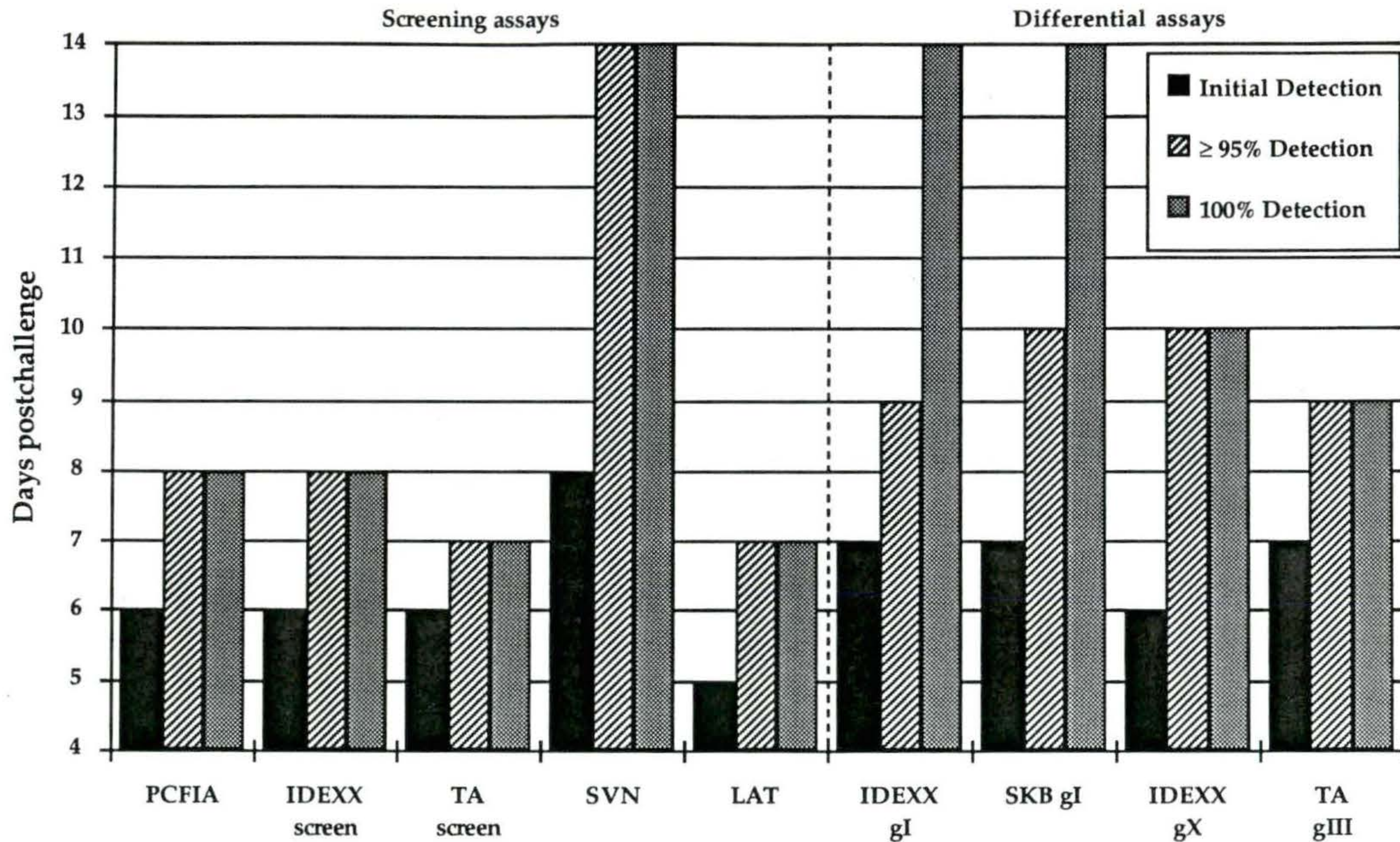

Figure 12. Comparison of the time (number of days postchallenge) required for nine serological assays to detect at least one, 95%, and 100% of the postchallenge swine serum samples as either positive or suspect for antibodies against pseudorabies virus (n=35 at day 4 postchallenge, n=35 at day 5, n=32 at day 6, n=28 at day 7, n=25 at day 8, n=24 at day 9, n=22 at day 10, and n=21 at day 14)

Table 9. Comparison of results from nine PRV serological tests in the detection of the early immune response in pigs to intranasal infection with pseudorabies virus

Test name	Initial PRV detection	≥ 95% PRV detection	100% PRV detection	Time from initial to ≥ 95% detection
PRV PCFIA (IDEXX)	6 days PC	8 days PC	8 days PC	2 days
HerdChek PRV Ab Screen (IDEXX)	6 days PC	8 days PC	8 days PC	2 days
DiaSystems PRV Ab CELISA (TechAmerica)	6 days PC	7 days PC	7 days PC	1 day
Serum virus neutralization	8 days PC	14 days PC	14 days PC	6 days
Latex agglutination PRV Test (Viral Antigens)	5 days PC	7 days PC	7 days PC	2 days
HerdChek PRV gI Ab Test (IDEXX)	7 days PC	9 days PC	14 days PC	2 days
ClinEase PRV gI Ab Test (SmithKline Beecham)	7 days PC	10 days PC	14 days PC	3 days
HerdChek PRV gpX Ab Test (IDEXX)	6 days PC	10 days PC	10 days PC	4 days
DiaSystems GIII PRV CELISA (TechAmerica)	7 days PC	9 days PC	9 days PC	2 days

results were pooled with positive results using the rationale that either result is cause for further action in our laboratory, whether it be retesting the serum, rebleeding the pig, or simply reporting the result as it stands.

NVSL PRV check set

Evaluation of the PCFIA using the 1990 PRV check test for ELISA screen and latex agglutination tests, provided by the National Veterinary Services Laboratories, resulted in a range of S/N values from 0.22 to 1.26, with no overlapping of sample values classified as positive or negative by the NVSL. All positive check test sera S/N values were less than or equal to 0.90. All negative check test sera S/N values were greater than 1.02. Using a positive S/N threshold of $\leq$ 0.90, all check set samples were correctly identified by the PCFIA.

Weak positive and suspect sera

A total of 619 field sera from unvaccinated swine were tested by the PCFIA after previously being identified as weakly positive or suspect by the HerdChek Screening ELISA for PRV.[o] Samples determined to be weakly positive included 376 sera with S/P values from 0.43 to 1.00. The suspect ("retest") group of samples included 279 sera with S/P values from 0.38 to 0.43. All 619 swine sera were also tested by the latex agglutination test (LAT) for PRV.[p] The results indicate a close match between the LAT and PCFIA when 0.90 is used as the positive threshold for the PCFIA (Table 10). The LAT

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[o] IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine.
[p] Viral Antigens Inc., Memphis, Tennessee.

and PCFIA agreed on 92% of the sample results while the PCFIA results matched those from the ELISA for only 2% of the samples. A total of 571 sera determined to be weakly positive or suspect by the ELISA were negative by both the LAT and the PCFIA.

Table 10. Summary of PRV PCFIA and latex agglutination test (LAT) serology results for 619 swine sera determined to be either weakly positive (S/P from 0.43 to 1.00) or suspect (S/P from 0.37 to 0.43) on the IDEXX HerdChek screening ELISA

	PCFIA negative	PCFIA positive	Total
LAT negative	571	12	583
LAT positive	10	26	36
Total	581	38	619

Plugged wells

The incidence of plugged wells on the PCFIA ranged from 1-2% for the routine testing of fresh samples, to more than 10% for sera of poor quality. A total of 216 sera causing plugged wells were identified and described, and 145 of these were treated by centrifugation. This treatment resulted in 81 (66%) of the sera no longer causing plugged wells, while 64 sera (44%) continued to cause plugged wells. Filtration of 28 of the 64 sera that continued to cause plugged wells resulted in only 2 plugged wells (7%).

Of the 216 sera causing plugged wells initially, only six (2.8%) were described as "normal" in appearance according to the criteria of color, clarity, presence of particulates, and viscosity (Table 11). The majority of samples causing plugged wells were identified as abnormal in appearance: 79.2% by abnormal color, 81.9% by abnormal clarity, 88.9% by the presence of particulates, and 39.8% by abnormal viscosity.

Table 11. Visual description of 216 swine sera causing plugged wells on the PCFIA for PRV

	Color		Clarity		Particulates		Viscosity	
	No.	%	No.	%	No.	%	No.	%
Normal	45[a]	20.8%	39	18%	24	11.1%	130	60.2%
Abnormal	171[b]	79.2%	177[c]	81.9%	192[d]	88.9%	86[e]	39.8%

[a] Slightly hemolyzed (red or brown) sera were classed with normal colored sera.

[b] Abnormal color descriptions ranged from red to dark brown or green.

[c] Abnormal clarity descriptions ranged from cloudy to opaque.

[d] Precipitates (white or otherwise) were the most frequently described particulates.

[e] Abnormal viscosity descriptions ranged from slightly thick to clotted blood.

SUMMARY AND DISCUSSION

This study evaluated a screening PCFIA for antibodies to pseudorabies virus by the testing of a wide variety of sera, including those from swine herds of known PRV exposure and vaccination status, and experimentally infected pigs. The true status of each field serum was inferred by the results from conventional serology tests used in the Iowa State University Veterinary Diagnostic Laboratory, including the serum virus neutralization test, a screening ELISA, and a latex agglutination test. Results of this testing indicated that the PCFIA for PRV reliably detected the presence of antibodies to PRV in swine sera at a sensitivity and specificity comparable or superior to other commonly used serological assays, including the ELISA, the serum virus neutralization test and the latex agglutination test.

This study was intended to provide information that would facilitate the setting of threshold S/N values for the interpretation of test results. The optimal threshold for a screening assay would theoretically maximize test sensitivity while maintaining an acceptable level of test specificity. Thus, the threshold should identify nearly all true positive pigs (minimize false negatives), but should minimize the number of true negative pigs incorrectly identified as exposed to PRV (minimize false positives). The tight grouping of the negative population around the mean observed here allowed the establishment of a positive threshold at a level high enough to ensure adequate sensitivity of the test for screening purposes.

A positive threshold set at $S/N \leq 0.90$ resulted in extremely high levels (>99%) of both sensitivity and specificity for the positive and negative pools of

field sera. However, these groups of field sera were deliberately chosen as well defined groups that might be expected to lead to such results. The positive group of field sera had all tested positive previously by the SVN test with a serum dilution of 1:2 or greater. We consider the SVN test procedure performed in our laboratory to be a screening assay of low to moderate sensitivity, but high specificity. Therefore, sera testing positive on the SVN test would be expected to be detected as positive by an assay that was designed to perform comparably to the more sensitive ELISA screening assay. Similarly, the negative group of field sera had all previously tested negative by the extremely sensitive screening ELISA, and therefore probably did not contain many borderline sera that might cause problems on the PCFIA. Nonetheless, the PCFIA performed well with these groups of field sera, and did not produce any unexpected results.

The sensitivity of the test in detecting the early immune response in experimentally infected pigs was quite good in comparison with the eight other assays these samples were tested by. Figure 12 and Tables 7, 8 and 9 provide a summary of the ability of the nine PRV antibody tests to detect the early antibody response to experimental infection of young pigs. The statistical significance of the results from the PCFIA as compared with each of the other eight assays is indicated in Tables 7 and 8, as determined by the chi-spuare test.

The PCFIA initially detected at least one serum sample as positive at 6 days postchallenge (PC). The time to initial detection of at least one serum

sample as positive or suspect for the other eight tests ranged from 5 days PC for the latex agglutination test to 8 days PC for the SVN test.

The PCFIA detected 100% of the challenged swine sera as positive by day 8 PC. The other eight tests required from 7 days PC (for the Tech America screening ELISA and the latex agglutination test) to 11-14 days PC (for the SVN test, the HerdChek gI ELISA, and the ClinEase gI ELISA) to detect 100% of the sera as positive or suspect.

Generally, the screening assays, including the PCFIA, initially detected positive sera earlier than the differential assays, and also detected the majority of sera as positive more quickly as well. The exception to this generalization was the SVN test, which performed poorly with this group of swine sera. The SVN test did not detect any positive sera until 8 days PC, and did not detect 100% of the sera as positive until 14 days PC. This latter value may be misleading, because sera were not collected between day 10 and day 14 PC, so that the majority of sera may actually have been detected by the SVN somewhat earlier than is indicated here. Also, as was discussed in the literature review, the procedure followed in our laboratory for the SVN test would not be expected to perform well in this comparison, because it cannot detect the early IgM response in the absence of supplemental complement. For similar reasons, the superior performance of the LAT at detecting the early immune response is not surprising, because IgM is known to be highly efficient in agglutination reactions (reportedly about 750 times as efficient as IgG).[110] These results also confirmed an earlier report concerning the high

sensitivity of the LAT in comparison with the SVN and ELISA screen tests, especially for the early immune response.[101]

The specificity of the PCFIA was measured, in part, by the large negative field sera group, but also by the testing of the group of 619 field sera that had tested as weakly positive or suspect by the screening ELISA. The majority of this group of sera (583 of the 619 sera tested) were confirmed by neither the LAT nor the SVN test in our laboratory, and we consider them to be false positive results. The close correlation (92%) between the PCFIA and the LAT results for this type of sample may be a significant feature of the PCFIA for laboratories experiencing a high rate of unconfirmed retests of screening ELISA results.

Plugged microwells are a hazard unique to the PCFIA technique. The process by which the unbound reagents and sera are removed from the reactant wells involves filtration under a vacuum through a filter at the bottom of the wells. Sera containing particulate or other insoluble material, such as lipid films or bacteria, can plug the filter and prevent the removal of unbound reagents, and also prevent concentration of the coated particles at the surface of the filter. These occurrences are termed plugged wells, and constitute an invalid test. The incidence of plugged wells has not proven to be of great importance in the testing of bovine sera by the brucellosis PCFIA,[¶] but swine sera is frequently of lower quality, and often contains a lipid layer on the surface. An evaluation of the incidence of plugged wells using the PRV PCFIA was considered an important aspect in the determination of the

¶ Personal communication from Peggy Jo Fague, IDEXX Laboratories, One IDEXX Drive, Westbrook, Maine.

practicality of the assay for routine screening in a veterinary diagnostic laboratory. Our experience with the occurrence of plugged wells strongly indicates that the PCFIA requires good quality sera to be practical in a real world setting. However, this may prove to be more of an aid than a hindrance to a desired result of valid test results. Poor quality sera may produce inaccurate results in assay procedures that do not call attention to conditions such as heavy bacterial contamination, or the presence of particulate material. The PCFIA requires that the test serum be reasonably free of such contaminants, and provides a validation procedure to ensure that such is the case. A plugged well rate of 1-2% was routine in this study for sera that was less than one week old and in what we consider to be "normal" condition (not cloudy, greatly discolored, containing particulate matter, or highly viscous in nature). This is a rate of retesting that would appear to be reasonable, considering the speed with which an assay can be completed. Samples causing plugged wells could be retested by another procedure, or a replacement sample of higher quality could be requested for retesting by PCFIA. Preselection of abnormal appearing sera, and removal for testing by another procedure, might be a practical method of lowering the rate of plugged well occurrence, since about 80-90% of the sera causing plugged wells were abnormal in appearance. Data presented here indicate this rate could be decreased even further if routine centrifugation was made part of the sample preparation routine. Centrifugation and the use of kaolin to adsorb

contaminants have been used successfully to decrease plugged well rates by laboratories using the PCFIA for brucellosis.[r]

The specific S/N values obtained from performing the PCFIA may be expected to vary depending on alterations made to reagents, controls and assay procedures. Simply substituting a negative control with a higher photon count will shift all S/N values down a corresponding degree. The results reported here reflect the particular configuration of test procedures, reagents and negative control sera provided by the manufacturer in the experimental kits under evaluation.

The PCFIA enabled the rapid testing of larger numbers of sera than would be practical using alternative methods. Estimates of the number of sera that could be tested by the PCFIA are presented in Table 12.[107] A single technician is estimated to be able to test up to 2,880 sera per day, or up to 748,800 sera per year. Commercial availability of a PCFIA for PRV would greatly facilitate the rapid testing of the large numbers of swine sera that has been mandated by the national pseudorabies eradication program.

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[r] Personal communication from Peggy Jo Fague, IDEXX Laboratories, One IDEXX Drive, Westbrook, Maine.

Table 12. Estimate of the instrument throughput and technician time for the PCFIA for antibody to pseudorabies virus[107]

Instruments	Technicians	Batch runs per day[a]	Samples per day	Tech. time per sample[b]	Samples per year
1	1	3	2,880	10 sec	748,800
1	1-1.5	4	3,840	11 sec	998,400
1	2	5	4,800	12 sec	1,248,000
2	2	7	6,720	9 sec	1,747,200
2	3	10	9,600	9 sec	2,496,000

[a] Instrument completes 10 plate batch run (960 samples) in 1 hr 45 min

[b] Based on an 8 hr day and includes sample preparation time

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