

SWINE HEALTH

Title: Development of an indirect ELISA to diagnose SIV using antibodies against the viral matrix protein - **NPB #03-036**

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Abstract:

The objective of this study was to establish an indirect ELISA that can be used to detect exposure to all swine influenza virus (SIV) subtypes by detecting antibodies against conserved SIV matrix proteins. A second objective was to determine if this assay can also be used to differentiate SIV infection from vaccination. Serum from SIV-vaccinated and/or SIV-experimentally infected pigs were used as antibody positive serum and serum from naive pigs was used for negative controls. Recombinant matrix proteins M1 and M2 proteins (rM1 and rM2) were produced using a baculovirus expression system, an insect cell system that produces high levels of proteins. The rM1 and rM2 proteins were semi-purified by ultracentrifugation and verified using dot blot and western blot assays using monoclonal anti-influenza M1 and M2 antibodies. The presence of antibodies to rM1 and rM2 proteins in serum from pigs vaccinated and/or infected with SIV was detected by immunoblot analysis. Differences in the intensity of the bands detecting antibodies to the M1 and M2 proteins suggest that the level of anti-M1 and M2 antibodies varied depending on the type of SIV exposure. Anti-M2 antibodies were consistently detected in pigs which had been experimentally infected with different SIV subtypes. These results suggest that anti-M2 antibodies can be utilized to detect exposure by the pigs to all SIV subtypes tested to date.

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Introduction:

Swine influenza virus (SIV) is a type A influenza virus of the family *Orthomyxoviridae*. SIV infects the epithelial cells of the respiratory tract and causes acute respiratory disease in swine. SIV plays an important role in the porcine respiratory disease complex (PRDC) that is characterized by slow growth, decreased feed efficiency, anorexia, fever, cough, and dyspnea in finishing pigs (9). Studies have demonstrated that infection with SIV and either porcine reproductive and respiratory syndrome virus (PRRSV) or *Mycoplasma hyopneumoniae* (MHYO), which are commonly isolated from pigs with PRDC, will increase the severity and duration of the respiratory disease (26-28). Over the past few years, SIV has increased in economic importance in the United States. A H1N1 subtype consisting of primarily swine genetics (classical H1N1) was the primary strain of SIV until 1998, when a H3N2 subtype consisting of a combination of swine, avian and human genes emerged in swine herds in several states. Since its emergence, the H3N2 virus has spread throughout the U.S. swine population (15, 21, 29). In 1999, the emergence of an H1N2 subtype was reported in the U.S. Analysis of H1N2 isolates indicate that they are the result of reassortment between the classical H1N1 SIV and the newly emerged H3N2 SIV (2, 3, 14). There is minimal cross-immunity and cross-reactivity between the different subtypes of influenza A viruses. As a result it has been difficult to develop successful vaccination regimens and SIV diagnostic assays that will protect and detect against the various new subtypes and genetically diverse viruses (1).

SIV infection can be detected by various methods including virus isolation, antigen detection by immunoassay, ELISA and molecular-based assays such as RT-PCR. A common problem in diagnosing SIV is the timing of sample collection (25). To diagnose infection by organism identification is difficult as the virus is shed for a very short period of time following infection (7, 20, 26). The hemagglutinin-inhibition (HI) test is the most common serological assay used to detect antibodies and the assay is based on recognition of the SIV hemagglutinin (HA) protein. This test has a moderate level of sensitivity and a high degree of specificity (13). However, to accurately diagnose a recent SIV infection requires a four-fold increase of HI titer in paired serum samples. Moreover, the HI assay is subject to nonspecific serum inhibitors, and frequent antigenic drift may lead to false negative results (10). Antigen-capture type ELISA and RT-PCR detect the virus and exhibit high sensitivity (4, 6, 19, 24). However, the problem of proper timing of sample collection remains due to the brief period of viral shedding. Therefore, a diagnostic test that could detect antibodies to all SIV subtypes has increased flexibility and fewer time constraints in collecting samples would be beneficial.

Following SIV infection, a timely and effective immune response is induced. The antibody response is rapid, with antibodies appearing as early as 3 days after experimental infection (18). Various structural proteins of the virus particle are known to induce an antibody response. SIV structural proteins include the matrix 1 protein (M1), the major envelope glycoproteins including hemagglutinin (HA), neuraminidase (NA) and a minor surface protein, the matrix 2 protein (M2). While the SIV HA and NA are considered the most immunogenic proteins, amino acid changes may result in subtype shifting and more minor genetic mutations, causes antigenic drift. The genetic changes in the HA proteins is the primary cause of altered binding of neutralizing antibodies to the virus. Changes in the proteins allows the virus to escape binding by antibodies generated to previous strains, thus escaping control by the immune system (5, 8). Alteration in viral proteins can lead to vaccine failure as well as reduced detection and false negative results by the HI test. In contrast, the matrix proteins, including the M1 and M2 proteins, are highly conserved protein antigens that are nearly invariant in all influenza A viruses (12, 22, 23). The M1 protein underlies the viral lipid envelope and provides rigidity to the membrane. The M2 protein is expressed abundantly on the surface of infected cells, but is present in small quantities on the surface of the mature virion (17). A study in pigs revealed that the antibody response to the M2 protein, which was low after a primary influenza virus H3N2 infection, was boosted by a subsequent infection with H1N1, indicating that antibodies to M2 may play a role in immunity to different subtypes of SIV (11). Consequently, detecting the immune response induced by the SIV matrix proteins may be an alternative strategy for diagnosing SIV exposure independent of subtype. ***In this study, we hypothesized that pigs exposed to different SIV subtypes, either by vaccination or infection, will produce anti-matrix antibodies that can be detected using a matrix protein-based ELISA.*** Accordingly, this study should increase our ability to diagnose SIV infection by studying the level of antibody response to the matrix proteins. The level of the anti-matrix

antibodies in pigs infected with SIV will then be compared to pigs vaccinated with SIV bivalent vaccine. Potentially, if there is a difference of the level of antibodies produced between the SIV-infected pigs and SIV-vaccinated pigs it may be utilized to interpret the way of SIV exposure. The level of antibody produced will be studied using an indirect ELISA based on recombinant SIV matrix proteins.

Objectives:

1. Clone and express the SIV matrix proteins utilizing baculovirus expression system
2. Determine the presence and the levels of antibodies induced by the SIV matrix proteins by evaluating serum samples from pigs vaccinated and/or experimentally infected with different SIV subtypes
3. Develop an indirect ELISA to detect exposure to different SIV subtypes

Materials and Methods:

Serum samples: Sera were acquired from 6 groups of pigs summarized in Table 1.

Table 1. Groups of pigs utilized for serum samples

Group	SIV vaccination	SIV infection
1	No	No
2	Yes	H1N1 strain A*
3	Yes	H3N2
4	Yes	No
5	No	H1N1 strain A
6	No	H3N2
7	No	H1N2
8	No	H1N1 strain B**

* strain A = classical H1N1 virus consists of swine genes

** strain B = triple reassortant H1N1 virus consists of swine, avian and human genes

All experimentally infected pigs were infected with at least 5 ml of 10⁶ TCID₅₀/ml of the appropriated SIV strain via intratracheal route. Groups of pigs that were vaccinated were given intramuscularly a commercial bivalent (H1N1/H3N2) vaccine.

Design of Oligonucleotides: The M1 and M2 gene was amplified by RT-PCR using primers specified to create cloning sites to the amplified product. RT-PCR primers were designed on the basis of sequence information obtained from the Influenza Sequence Database at Los Alamos National Laboratories, Los Alamos, N.M. (<http://www.flu.lanl.gov>). To identify conserved sequences in the influenza virus gene segments, entropy plots were created through the Bioedit software package (available through <http://www.mbio.ncsu.edu/RnaseP/info/programs/BIOEDIT/bioedit.html>).

Molecular cloning and expression of viral genes: The BaculoDirect™ Baculovirus Expression Systems (Invitrogen, Carlsbad, California) was used for expression of the matrix proteins. Viral RNA was isolated from MDCK cells infected with the SIV strain A/swine/IA/40776/92 (H1N1). The integrity of the base sequence in the plasmid was verified by sequence analysis. Recombinant baculovirus carrying the matrix genes was constructed according to the manufacturer’s instructions. To express the proteins, recombinant baculovirus carrying the matrix genes were inoculated onto 60% monolayers of SF21, insect cells (Invitrogen, Carlsbad, California) cultured in Excel 405 (JRH Bioscience, Lenexa, Kansas) with antibiotic-antimycotic (Gibco BRL, Gaithersburg, Maryland). The baculovirus was allowed to absorb onto the cells for 1 hour at room temperature. Subsequently, the cells were cultured at 27⁰C for 72 hours.

Antigen preparation: Positive and negative antigens for ELISA were partially purified from SF21 insect cells infected with recombinant baculovirus carrying the matrix genes or SHAM inoculated, respectively. At 72 hours post infection, SF21 insect cells in 75-cm² flask were frozen at -20⁰C. Cells were thawed and clarified by

centrifugation at 10,000 X g for 10 min at 4⁰C. The protein concentration was determined and the supernatant containing either matrix (M Ag) or SF21 cell protein (Negative Ag) antigens were dispensed in aliquots and kept at -70⁰C.

ELISA: To coat plates for the indirect ELISA, the optimum concentration of antigen was determined by checkerboard titration. Modified ELISA methods, utilizing the recombinant matrix proteins were performed as described previously (21). Both positive (M Ag) and negative antigens were diluted in carbonate-bicarbonate coating buffer, pH 9.5 (22). Immunolon 2HB polystyrene microtiter plates (Dynex Technologies Inc., Chantilly, Virginia) were coated with positive and negative antigen by adding 100 µl of diluted M Ag or no Ag into wells. The coated plates were incubated at 4⁰C for 36-40 h and then stored at -20⁰C.

The microtiter plates were thawed and incubated at room temperature, washed 3 times with 30 second incubations using PBST wash buffer (0.1 M PBS, 0.1% Tween 20, pH 7.2). Sera were diluted to an appropriate dilution and incubated in triplicates with M Ag and negative antigens. Conditions were varied and adjusted as necessary. A peroxidase-labeled anti-swine immunoglobulin G was used as the conjugated antibody, and 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABST) was subsequently used as a substrate. The optical density (OD) was measured at 405 nm using a microtiter plate reader.

Results:

Objective 1: Clone and express the SIV matrix protein

1.1 Design of oligonucleotide (primers)

Complete sequences of the matrix gene from 4 isolates, A/swine/Iowa/15/30 (H1N1), A/swine/Wisconsin/1/61 (H1N1), A/swine/Iowa/17672/88 (H1N1) and A/swine/Minnesota/593/99 (H3N2) were retrieved from Genbank. The accession numbers of each isolates are M33045, M63519, M63522 and AF251430 respectively. A multiple sequence alignment program was utilized to align the sequences. The conserved regions were predicted and the protein translation-starting site of M1 and M2 proteins were located. Two pairs of primers (M1 and M2) were designed based on the conserved regions to amplify both matrix gene 1 and 2. The size of M1 gene is 756 base pairs (bp) and M2 gene is 291 bp (Figure 1). Each amplified product was designed to contain a specified sequence to recombine with the baculovirus entry vector.

1.2 Construction of a baculovirus entry vector and verification

Both the M1 and M2 genes were cloned into a pENTR Directional TOPO vector. Each recombinant entry vector (M1 and M2) was transformed into TOP 10 *E.coli* cells. Individual colonies were selected and analyzed by PCR to confirm the presence of the M1 and M2 cDNA in the pENTR Directional TOPO vector (Figure 2). In addition, the selected clones have been sequenced to verify the correct orientation of the matrix cDNA.

1.3 Production of the virus and expression of the recombinant proteins

An LR recombination reaction was performed. The M1 and M2 gene was transferred from the entry clone to the BaculoDirect™ Linear DNA to generate the recombinant baculovirus. The recombinant baculovirus was transfect into SF9 insect cells and the selected with ganciclovir. The selected virus was further propagated and infected into SF21 insect cells to produce the rM1 and rM2 proteins.

Dot blot analysis was performed to verify the recombinant proteins. The proteins collected from the cell lysates and culture supernates were blotted onto a nitrocellulose membrane and were stained with monoclonal anti-influenza M1 and monoclonal anti-influenza M2 antibodies separately (Figure 3). A 10% denatured polyacrylamide gel electrophoresis (PAGE) was run to confirm the size of the proteins. The proteins rM1 and rM2 migrated to the expected level of approximately 27 and 15 kDa respectively.

Objective 2: Determine the presence and the levels of antibodies induced by the SIV matrix proteins by evaluating serum samples from pigs vaccinated and/or experimentally infected with different SIV subtypes

Immunoblot analysis was used to confirm the presence of M1 and M2 antibodies in the pig serum. The rM1 and rM2 proteins were separated using 10% PAGE electrophoresis followed by transfer to a nitrocellulose membrane. Non-reducing conditions were used to retain the native structure of the recombinant proteins. The membrane was incubated with serum collected from pigs experimentally infected and/or vaccinated with SIV as described above. Negative serum and a monoclonal anti-influenza M2 antibody that recognizes the M2 native structure were included as negative and positive controls respectively. The results of the rM2 immunoblot analysis (Figure 4) demonstrated that pigs infected with different SIV subtypes produced detectable levels of anti-M2 antibodies. Differing intensity of the bands suggests that the amount of anti-M2 antibodies produced may vary according to frequency of exposure and type of exposure to SIV (vaccinated or infection). As pigs that were vaccinated and infected (lane 7) had a higher intensity of the bands compared to pigs that were either infected (lane 2) or vaccinated (lane 6) alone. The results of the study reported here suggests that the M1 proteins play an important role in inducing CMI response to SIV-heterosubtypic infection (16).

Objective 3: Develop an indirect ELISA to detect SIV

Serum samples. Known positive serum samples ($n = 30$) used in this study were collected from pigs experimentally exposed to SIV by infection ($n = 22$) or vaccination ($n = 8$). Known negative serum samples ($n = 28$) were collected through sequential bleeding from control pigs in the study. The SIV-exposed and SIV-unexposed status were confirmed by the hemagglutination-inhibition (HI) test which detects the HI antibody titers against matched specific SIV isolates. A summary of the serology results is shown in table 2.

Results. As shown in table 3, the levels of M1 and M2 IgG antibodies specific to SIV were significantly greater in the pigs that were exposed to SIV, either by infection or vaccination compared to the unexposed serum samples. With the current assays, detection of serum antibodies requires the homologous SIV isolate. However, in this study we were able to detect M1 and M2 SIV specific antibodies in all the SIV infected serum samples which were collected from pigs experimentally infected with heterologous SIV isolates (table 1). Interestingly, the result demonstrated that the level of the matrix SIV specific antibodies from the vaccinated pigs were significantly higher than the pigs that were SIV infected. However, more serum samples are required to validate the findings and to define the specificity and the sensitivity of the test.

Discussion:

As discussed in the introduction, SIV has emerged as a problem pathogen to the US swine industry. With the emergence of new subtypes and antigen different isolates, diagnostic detection has become problematic. The purpose of this grant was to develop a diagnostic assay that would detect SIV antibodies independent of subtype. We have successfully developed antigens to the matrix proteins (M1 and M2) which are highly conserved and thus less variable than the proteins currently used. Based on the research described here, we have determined that the M2 protein appears to be immunogenic and antibodies to the protein are detectable in the serum of pigs experimentally inoculated with SIV isolates of different subtype and genetic makeup.

There is potential for this ELISA assay to be used to determine if the pigs have been exposed to any SIV isolate. We are still investigating whether the ELISA as developed will be able to differentiate between antibodies developed through vaccination or infection. The information described here will provide a tool for producers and veterinarians to use in determining the health status of a herd as well as whether the respiratory disease found in a herd is due to SIV or other pathogens.

Lay Interpretation:

Respiratory disease caused by swine influenza virus (SIV) has become a serious health and economic problem to the U.S. swine industry. Prior to 1998, SIV in the U.S. swine population consisted of a single subtype of the virus, a H1N1 virus comprised of swine genetics. In 1998, a new subtype of the virus emerged, a H3N2 virus that was made up of genetics from avian and human influenza lineage in addition to the swine lineage of the original virus. The emergence of this virus has resulted in viruses that differ genetically. As a

result, diagnostics and disease control have become more problematic to the swine industry. The goal of the research reported here was to develop a diagnostic assay that could be used to detect SIV, independent of subtype or genetic makeup. We used proteins in the virus that show little variation to develop this assay. Currently, we have demonstrated that using these proteins, we can detect antibodies in the serum of pigs infected with SIV isolates that differ by subtype and genetic make up. The assay developed will provide information on whether a herd is infected. We hope the assay will assist in determining SIV infection as well as vaccination status.

Figure 1. RT-PCR product of M1 and M2. RNA was isolated from strain A/swine/IA/40776/92 (H1N1) grown in MDCK cells, followed by RT-PCR analysis and agarose gel electrophoresis. Lanes 1 and 3, negative control; lane 2, M1; lane 4, M2; lane 5, positive control; lane M, 100 bp molecular weight marker.

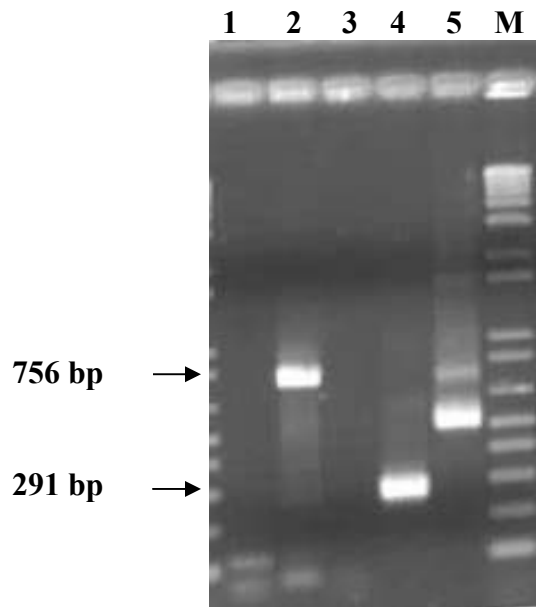


Figure 2. PCR analysis of baculovirus entry clones. Lanes 1 to 5, clones from M1 recombinant entry vector; lanes 6 to 15, clones from M2 recombinant entry vector; lane M, 100 bp molecular weight marker. PCR products from lane 1, 3, 4 and 6 to 8 were further analyzed by DNA sequencing.

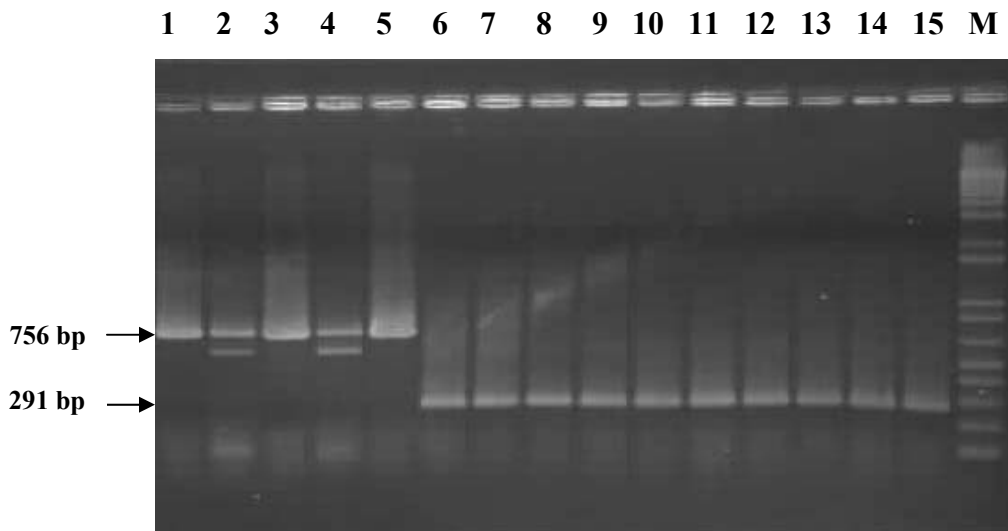


Figure 3. Dot blot analysis of unpurified rM1 and rM2 proteins. Lane 1, incubated with monoclonal anti-influenza M1 antibody; lane 2, incubated with monoclonal anti-influenza M2 antibody.

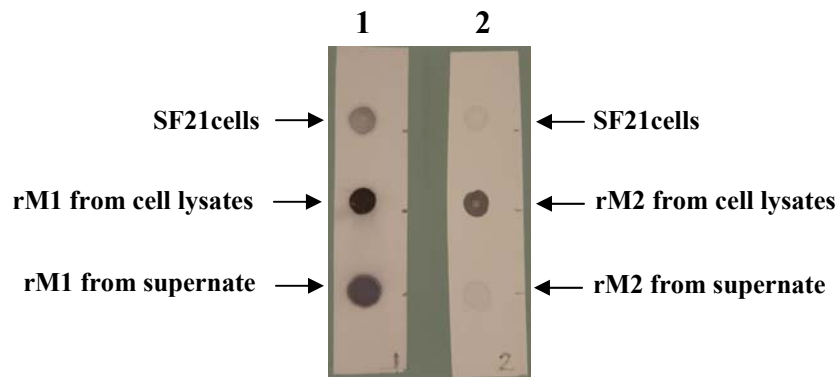


Figure 4. Western blot analyses to detect antibodies to M2 proteins from pig serum. Lane 1, incubated with monoclonal-influenza M1 antibody; lane 2, incubated with pig serum infected with H3N2 SIV; lane 3, incubated with pig serum infected with H1N1 SIV; lane 4 and 5, incubated with pig serum unexposed to SIV antigen; lane 6, incubated with pig serum SIV vaccinated; lane 7, incubated with pig serum SIV vaccinated and infected with H1N1 SIV; lane M, molecular weight marker.

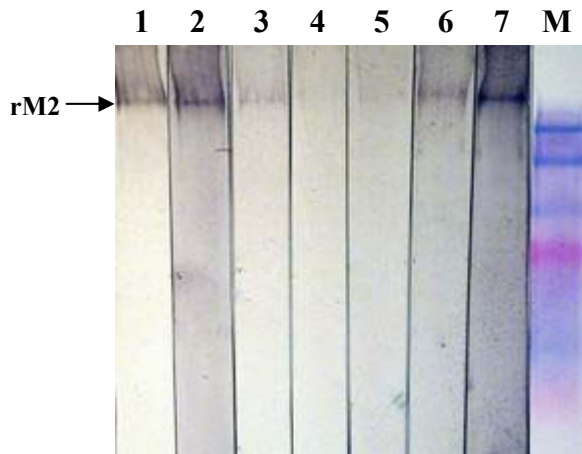


Table 2. Status of pig serum samples detected by hemagglutinin-inhibition (HI) test against matched specific SIV isolate

Test	Vaccinated	SIV Infected	Unexposed	Total
HI (+)	8	22	0	30
HI (-)	0	0	28	28
Total	8	22	28	58

Table 3. Mean O.D ± standard error detected by M1-ELISA and M2-ELISA

Status	M1-ELISA (p<0.0001)	M2-ELISA (p<0.0001)
Vaccinated	0.49 ± 0.03 ^c	0.71 ± 0.05 ^c
Infected	0.32 ± 0.02 ^b	0.53 ± 0.03 ^b
Unexposed	0.15 ± 0.02 ^a	0.23 ± 0.03 ^a

^{a, b, c} Means with different superscripts within a column are statistically different

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