RESEARCH REPORT
SWINE HEALTH

Title: **Comparison of Serological Assays in Diagnosing Differing Serotypes of Swine Influenza Virus (SIV) - NPB #03-162**

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Abstract:
Swine influenza virus (SIV) is an economically important pathogen of swine. Recently, new subtypes of the virus have emerged among US swine herds. In addition, apparent changes in the genetic structure of this virus have resulted in strains that often differ antigenically within subtypes. As a result of these changes in the virus, our ability to diagnose SIV infection and thus determine appropriate vaccine strategies has become increasingly difficult in the field. The study reported here compares the ability of a subtype-specific commercial enzyme-linked immunosorbant assay (ELISA), hemagglutination inhibition (HI), and the serum neutralization (SN) assays to detect antibodies elicited by different isolates within multiple subtypes of SIV. Pigs were infected with genetically different isolates of the 3 major circulating subtypes within swine population (H1N1, H1N2, and H3N2). When all pigs within a group collectively reached HI reciprocal titers ≥160 against the homologous virus for that group, serum was collected for use in the study. The serum was assayed and statistical analyses performed to compare the ability of these three different antibody assays to detect antibodies that were produced. Differences were found between these assays in the cross-reactivity among isolates and the ability of the each assay to accurately detect antibodies. These differences will provide important information to diagnostic laboratories, veterinarians and swine producers as they attempt to evaluate serological results based on antibodies produced following infection with SIV.

Introduction:

Over the past several years, swine influenza virus (SIV), a common respiratory pathogen of swine, has been rapidly evolving in the United States. A H1N1 virus, composed of primarily swine genetics, was the primary subtype isolated in the U.S. until 1998, when a H3N2 subtype was isolated in pigs in several states. Since then, viruses of the H3N2 subtype have continued to spread throughout the U.S. swine population (4, 9). Further diversity of the H3N2 viral subtype has been described, and viruses are often classified into one of three clusters (11). This indicates a range of genetic variation among the H3 molecules of circulating viruses in this subtype (1). In 1999, the emergence of an additional subtype, H1N2, was reported in the U.S. Analysis of the H1N2 isolates indicated that they were the result of reassortment between the classical H1N1 and the H3N2 subtype of SIV (8). Recently, a new reassortant H1N1 strain containing portions of the H3N2 virus has also been isolated in the US. Cross-immunity between the different subtypes of influenza A viruses appears to be quite variable, increasing the difficulty in developing effective diagnostic capabilities and vaccination strategies (5, 15, 16).

SIV infection can be diagnosed using methods that detect the virus including: virus isolation, antigen detection by immunoassay, and molecular-based assays such as RT-PCR. Diagnosis of infection by virus isolation can be very difficult as the virus is shed for a very short period of time following infection, often for only 3 to 5 days making timing of sample collection critical (9). Diagnosis of SIV infection can also be determined by measuring antibody production using; the hemagglutination inhibition (HI), ELISA, or serum neutralization (SN) assays (14). Detection of serum antibodies is the most common method used to diagnose infection and to determine the subtype of virus inducing the immune response. Currently, the HI test is the assay most commonly used to detect SIV antibodies, and is based on the ability of the hemagglutinin (HA) protein present on the viral surface to agglutinate red blood cells (RBC). This test has a moderate level of sensitivity depending on whether the test antigen used in the assay is recognized by the antibodies (3). Typically, the diagnosis of an SIV infection by the HI assay requires a four-fold increase in titer in paired acute and convalescent serum samples. Moreover, the HI assay is subject to nonspecific serum inhibitors, and frequent antigenic drift may lead to false negative results (3). A commercial ELISA (IDEXX HerdChek SIV) has been licensed for both the H1N1 (6, 7); and the H3N2 subtypes. However their ability to differentiate antibodies elicited against different isolates has not been well documented. The SN assay, which measures antibodies that are capable of neutralizing the virus, is labor intensive, virus specific, and the correlation to antibodies detected by the other assays is largely unknown (1, 13). Production of neutralizing antibodies is important in clearing the virus from the host as well as preventing infection (12, 18) and therefore a more complete understanding of the relationship between these three assays is of interest.

The central hypothesis of the proposed research is that antibodies elicited against genetically distinct isolates within the same or different subtypes of SIV may react differently in various serological assays, making the diagnosis of SIV infection difficult to interpret for producers and practitioners. In addition, correlation between influenza antibody assays has not been well defined. The reduced ability to accurately detect and classify the specific virus circulating within a herd makes diagnostic interpretation and vaccine selection difficult under field conditions. The study reported here is investigating the level of cross-reactivity between viruses of the same subtype using the three previously described antibody detection assays. These results will enable us to improve diagnostic accuracy as well as enable the industry to determine if new vaccines need to be developed for the control of individual viral isolates

Objectives:

Our long term goal is to increase our understanding about the relationship between the assays commonly used for SIV diagnostics and the influence that test antigens used in these assays have on results. We compared the three commonly used SIV antibody detection assays (ELISA, HI, and SN). Eight SIV isolates were used in this study, and serum from each group has been collected to measure the antibody reactivity pattern using the three assays.

Material and Methods:

Infection of pigs

Thirty-two crossbred 4-week-old pigs seronegative for SIV, porcine reproductive and respiratory syndrome, and *Mycoplasma hyopneumoniae* were obtained from a commercial swine farm. The pigs were identified by ear tags and randomly assigned to groups with stratification of body weight upon arrival. Groups of 4 pigs were housed in separate isolation rooms at Iowa State University. Serum was collected from pigs prior to challenge and used as negative control sera for all the serological assays. Pigs in groups 1-4 were initially challenged intratracheally at approximately 5 weeks of age. Because the initial challenge failed to elicit a sufficient antibody response as measured by HI, pigs were challenged a second time at 7 weeks of age For the first challenge, pigs were inoculated with 5 ml of $1x10^6$ $TCID_{50}$/ml of the assigned virus and for the second challenge; a dose of 5 ml of $1x10^8$ $TCID_{50}$/ml was used. Groups 5-8 were challenged once at 9 weeks of age with a dose of 5 ml of $1x10^8$ $TCID_{50}$/ml of the assigned virus. Serum was collected and aliquoted to assay when sera from all pigs within a group reached HI reciprocal titer of greater then or equal to 160 against the homologous virus.

Viral isolates

Eight viruses within the three subtypes currently recognized to be circulating among swine herds within North America were used in this study and included a classical H1N1 isolate, 2 triple reassortant H1N1 isolates, 2 H1N2 isolates, and 3 isolates of the H3N2 viral subtype. Experimental groups and virus designation used for this study are shown in Table 1. A group name was designated for each experimental group (according to the strain characteristic), which is used throughout this report.

Table 1: Virus Name and Group Designation

Group	Group name	Virus strain	Number of pigs
1	H1N2A	A/Swine/Wisconsin/R33f/01 (H1N2)	4
2	cH1N1	A/Swine/IA/40776/92 (H1N1)	4
3	tH1N1A	A/Swine/H02NJ56371/02 (H1N1)	4
4	H3N2C1	A/Swine/Texas/4199-2/98 (H3N2)	4
5	H1N2B	A/Swine/Indiana/9K035/99 (H1N2)	4
6	tH1N1B	A/Swine/IA/35233/99 (H1N1)	4
7	H3N2C2	A/Swine/Texas/00036/02 (H3N2)	4
8	H3N2C3	A/Swine/Wisconsin/R7c/01 (H3N2)	4

The viruses used in this study were obtained from 4 different sources and are as follows: H1N2A, H1N2B, tH1N1A, and H3N2C3 were provided by Dr. Christopher Olsen, University of Wisconsin-Madison, Madison, Wisconsin. The genotype of H1N2A is as follows: HA, M, NP and NS are classical swine lineages, NA and PB1 are human lineages, and PA and PB2 are avian lineages. The H1N2A and H1N2B were shown not to cross react serologically by Dr. Olsen's laboratory. The H3N2C3 was characterized by Dr. Olsen as a representative of a clade 3 virus as described by Webby et al. (17). The genotype of tH1N1A was reported as follows: HA, NA, M, NP and NS are classical swine lineage, PB1 is human lineage, and PA and PB2 are avian lineage. During the course of the experiment, this virus was determined to actually be a combination of both H1N1 and H3N2 viruses. Isolate tH1N1B was provided by Dr. Bruce Janke, Iowa State University, Ames, Iowa. Strain cH1N1 represents the classical H1N1 strain and H3N2C1 represents a clade 1 H3N2 virus. Both of these viruses were obtained from the National Veterinary Services Laboratory, Ames, Iowa. H3N2C2, represents a clade 2 H3N2 virus, and was provided by Dr. Marie Gramer, University of Minnesota, St. Paul, Minnesota.

All eight viruses used as test antigens in the HI and SN assays were propagated in Madin-Darby Canine Kidney (MDCK) cells. Following growth of each virus in cell culture, clarification was carried out by centrifugation. The final titer for each virus was derived through determination of the hemagglutinin (HA) units of each strain, using a HA test, utilizing 0.5% turkey RBC's. Virus titer was also measured through type A influenza specific protein detection by immunocytochemistry staining of the virus infected MDCK cells and the calculation of tissue culture infective dose ($TCID_{50}$/ml) was based on the Reed-Muench method (10). Multiple aliquots of the stock virus were stored at -80^{0}C prior to use in the study.

Assays

Serum was tested using three swine influenza antibody assays: HI, SN, and commercial H1N1 and H3N2 ELISA assays (IDEXX Laboratories, Westbrook, ME). The SN and HI assays were performed using viruses and antiserum of each of the subtypes against the same HA subtypes (H1's compared to H1's and H3's compared to H3's). ELISA assays were performed using antiserum from all pigs in groups challenged with all 3 subtypes of virus to determine cross reactivity between isolates and subtypes and how these results correlate with data obtained from the HI and SN assays. The ELISA was performed according to the manufacturer's instructions

The HI assay was performed following the standard protocol used by the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL). A comparison of HI serological cross-reactivity was made between all groups challenged with viruses of the H1N1 and H1N2 subtypes (groups 1, 2, 3, 5, and 6). Similarly, a comparison of serological HI cross-reactivity was made between all groups challenged with viruses of the H3N2 subtype (groups 4, 7, and 8). All HI tests used 8 HA units of turkey RBC's. Cross-reactivity data is shown in tables 2 and 3.

The SN assay was performed using a MDCK cell monolayer, and a constant amount of virus diluted from stock to a standard SN working titer of 100 $TCID_{50}$/0.1ml and performing serum dilutions. SIV infected cells in the SN assay were detected by indirect immunocytochemistry, utilizing a monoclonal antibody that detects type A influenza virus nucleoproteins, followed by a labeled rabbit anti-swine secondary antibody.

Titers for the HI and SN assays are reported as the geometric mean group titer based on the reciprocal of the last antibody dilution that completely inhibited hemagglutination or infection respectively. ELISA data is reported as S/P ratios according to the manufacturer's standard protocol.

Statistical analysis

HI and SN assay data is reported as the reciprocal of mean titers against each of the test antigens used in these assays (Tables 2-5). Groups infected with viruses of the H3N2 subtype (Groups 4, 7, and 8) were compared, and a separate similar comparison of groups infected with viruses of the H1N1 and H1N2 subtypes (Groups 1, 2, 3, 5, and 6) was made. Variance between serum groups was analyzed using a one-way ANOVA, Statistix 8 (Analytical Software Tallahassee, FL 2003), with the serum group being used as the categorical variable and the serum titer against each of the individual test antigens being used as the dependent variable. Statistical differences between each group's mean titer against each test antigen was determined by least significant difference based on each group's mean titer. The differences between groups reported, based on test antigen used, were significant with a p-value < 0.05.

ELISA data is reported as mean S/P ratios using each of the two ELISA assays (Table 6 and 7). A comparison was made between both ELISA assays using serum from groups challenged with each of the viruses of the H3N2 subtype. A similar comparison was made between ELISA assays using serum from groups challenged with viruses of the H1N1 and H1N2 viral subtype. ELISA S/P ratios were analyzed using a one-way ANOVA with the serum group as the categorical variable and the ELISA S/P ratio as the dependent variable. Differences between each group were determined by least significant difference based on mean S/P ratios using each ELISA.

Results

Results from the HI assays evaluating antibodies to the H1 viruses used in this study found differences in the reactivity observed by antibodies induced by the different viruses against the various antigens used in the study. Antibodies in sera from challenge groups 1 (H1N2A) and 3 (tH1N1A) showed a strong two-way cross-reactivity against both the homologous antigens and the opposite test antigen, while showing moderate reactivity to the other 3 antigens used in the assays. Antibodies in serum from group 3 reacted strongly against the antigens of groups 1 and 5 (tH1N1B). Analyses of serum antibodies collected from challenge groups 5 (H1N2B) and 6 (tH1N1B) show that each group had the strongest serological reactivity with the homologous virus. Interestingly, these groups showed fairly low antibody reactivity with the other four test antigens used for the HI assay.

Antibodies to the viruses of the H3N2 viral subtypes found that serum from challenge group 4 (H3N2C1) reacted strongly with the homologous antigen and the group 8 (H3N2C3) antigen with a weak reactivity with group 7 (H3N2C2). Serum from challenge groups 7 and 8 showed a similar pattern of having the strongest reactivity with the homologous virus for that group and weak reactivity with the other two test antigens.

All negative control serum tested HI negative throughout the study against all viral subtypes used.

SN antibody responses are described in tables 4 and 5. The SN antibody responses tended to be similar to the HI results with most of the antibodies having the highest geometric mean titer to the homologous virus. Overall, the serum reactivity of the SN antibodies to the H1N1 and H1N2 viruses appeared higher than the HI levels. A number of exceptions occurred where SN antibody levels deviated from the trend shown by the HI assay. One such exception was observed with group 1 (H1N2A) which had higher SN antibody titers to the tH1N1A and H1N2B viruses than the homologous virus. As expected, group 2 induced the highest SN antibody titers to the homologous antigen and had similar levels of SN antibodies to the H1N2B virus. As with the HI levels, groups 5 and 6 had the lowest SN titers, having the strongest reaction with the homologous viruses and lower responses to the other viruses used in the study.

SN antibody responses to the H3 viruses were more consistent in their reactivity patterns with those observed in the HI assays. The H3 viruses demonstrated the highest reactivity to the homologous virus and as with the HI responses, group 8 had low SN antibody responses to the H3N2C1 and H3N2C2 viruses.

The results of the ELISA assays are summarized in Table 6. We assayed all serum using both assays. The H1N1 assay determined that groups 1, 2, and 3 were positive for H1N1 antibodies, using a positive cut point >0.4 according to the manufacturers directions. Neither groups 5 or 6 were determined to be positive for H1 antibodies, although both viruses were previously confirmed to be H1N2 and H1N1. The H3N2 assay determined all three of the known H3N2 positive samples as positive with a value of >0.4. In addition, H3N2 ELISA results showed that group 3 was also positive and that interestingly, group 1, which was a H1N2 was within the suspect range.

Discussion

This study provides important data on the serum cross-reactivity patterns of 8 different isolates of SIV. SIV remains a significant problem to the swine industry. Historically, SIV-induced disease in pigs was controlled through sow

vaccination strategies to increase the levels of maternal antibodies in the pigs. These passive antibodies protected the pigs from disease through the nursery stage until the grow-finish stage at which time the virus had less impact on the overall health and production parameters of the pigs. Since the emergence of the H3N2 virus in the late 1990's and the subsequent emergence of genetically diverse viruses, this picture has changed, with nursery pigs in herds of vaccinated sows having significant disease due to SIV. The purpose of this study was to begin assessing the ability of the various assays to detect and quantify antibodies to SIV as well as investigating the cross reactivity of the antibodies to the various viruses and their antigens. To date, we have only assessed cross reactivity by subtype using the HI assay, but we may assess the reactive pattern between the viruses of different subtypes in the future.

The results of this study demonstrate that there is a significant variation in the reactivity of the various viruses against each other determined by different assays. This was true of both the H1 and the H3 viruses. During the course of this investigation, it was determined that virus 3, which was originally thought to be a H1N1 made up of swine, human and avian genetics was actually a combination of both H1N1 and H3N2 viruses. Thus, we detected the H1N1 antibodies when we did the HI assays using the various H1 antigens, but the sera were also positive for the H3N2 antibodies by the ELISA. We were able to confirm that the isolate consisted of both subtypes of viruses using a multiplex RT-PCR assay (2).

It is known that SN antibodies are important in controlling viral infections. Further statistical analysis is ongoing to provide more information on the correlation between the results of the HI and SN assays. However, by observation, it is apparent that the correlation is not directly linear. The ability of the different viruses to agglutinate RBC's may have an effect on the HI serological assay as shown in the results from sera from groups 5 and 6 where reactivity of SN titers was relatively higher than the HI titers. This implies that both viruses, the H1N2B (group 5) and tH1N1B (group 6) have less capability to agglutinate turkey RBC and utilizing these 2 viruses in the HI assay may lower the sensitivity of the test. This study did not assess cross protection between isolates and the significance of the SN antibody levels is unknown. Further studies need to be performed to determine the level of SN antibodies and their correlation to HI antibody levels required for protection against disease. However, SN assays are difficult, expensive and time consuming and are not practical for routine diagnostic use. Nevertheless, the findings of this study provide preliminary information to assist in evaluating the serological results induced by exposure to the various viruses.

The commercial ELISA assays were easy to perform and did detect antibodies to all 3 H3N2 viruses. The sensitivity and accuracy in detecting antibodies to the H1 viruses was more questionable, with the assays not detecting antibodies to viruses in groups 5 and 6. Although both of these viruses appear to be less immunogenic as evidenced by the low HI titers induced and showed less cross reactivity against other H1 antigens, they did show a high reactivity to the homologous virus by SN test.

This study demonstrated that genetically different viruses used as antigens in the various diagnostic laboratories may impact the success of diagnosing SIV infection based on antibody production. These results suggest that care must be taken when using one serological assay to diagnose SIV infection and the use of multiple assays may further increase the accuracy of diagnosis. Unfortunately, as the number of assays performed increases the cost increases as well and this is problematic to swine veterinarians and producers. Further studies will need to be performed continuously as new genetically diverse viruses emerge in the field. However, the results of this study provides important information about the cross reactivity patterns and diagnostics required for accurate determination of SIV infection using a number of genetically diverse SIV isolates. The information provided in this study and the serum will be made available to diagnostic labs and researchers to use in developing and evaluating the prevalence and cross-reactivity between SIV viruses.

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 viruses of the H1N1 subtype 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 new isolates that differ both genetically and antigenically from the original virus as well as showing continuous evolution to new antigenic types. As a result, diagnostics and disease control have become more problematic to the swine industry. The goal of the research reported here was to evaluate the ability of the different serological assays to detect SIV viruses that differ in genetic makeup both by subtype and within subtype. This study demonstrated that different viruses of both the H1 and H3 hemagglutinin subtypes have different cross-reactivity based on the virus used as the test antigen in the HI assay. The results of measuring SN antibodies, thought to be important in disease control, showed similar patterns as with the HI assay. However, the levels of SN antibodies were not tightly correlated with the HI results and further complex statistical analysis is ongoing to determine this relationship. The commercial ELISA assay

detected antibodies to all viruses that were of the H3 subtype, but was less consistent in detecting antibodies to the H1 subtype. Antibodies to two of the H1 viruses, a H1N1 and a H1N2, were not detected by the ELISA, raising a concern about the sensitivity of the test as both of these viruses showed low levels of antibodies measured by both the HI and SN assays. The sera created in this study will be available to researchers and diagnostic laboratories to use in developing and evaluating the prevalence and cross-reactivity between SIV viruses. It is hoped that the information obtained here will aid producers and veterinarians in accurately diagnosing SIV infection within swine herds and facilitate vaccine development and selection to further enhance disease control.

Table 2: HI Serum reactivity among viruses of H1N1 and H1N2 subtypes

				Test Ag		
Group	Serum Group	H1N2A	cH1N1	tH1N1A	H1N2B	tH1N1B
1	H1N2A	1575.8[a*]	260.0[a]	519.8[b]	260.0[a]	260.0[b]
2	cH1N1	196.9[b]	394.0[a]	160.0[c]	260.0[a]	260.0[b]
3	tH1N1A	1039.7[a]	394.0[a]	1575.9[a]	320.0[a]	519.8[a]
5	H1N2B	34.8[c]	34.8[b]	28.3[d]	226.3[a]	34.8[d]
6	tH1N1B	34.8[c]	69.6[b]	34.8[d]	49.2[b]	139.3[c]
	Negative	<10	<10	<10	<10	<10

* Data reported as reciprocal of geometric mean titers
[a,b,c] Values with superscripts within each column are significantly different (P<.05)

Table 3: HI Serum reactivity among viruses of H3N2 subtype

			Test Ag	
Group	Serum Group	H3N2C1	H3N2C2	H3N2C3
4	H3N2C1	787.9[a*]	40.0[b]	787.9[a]
7	H3N2C2	20.0[b]	1280.0[a]	69.6[b]
8	H3N2C3	17.4[b]	34.8[b]	452.5[a]
	Negative	<10	<10	<10

* Data reported as reciprocal of geometric mean titers
[a,b,c] Values with superscripts within each column are significantly different (P<.05)

Table 4: SN Serum reactivity among viruses of H1N1 and H1N2 subtypes

				Test Ag		
Group	Serum Group	H1N2A	cH1N1	tH1N1A	H1N2B	tH1N1B
1	H1N2A	452.5[a,b]	393.9[b,c]	452.5[a]	640.0[a,b]	640.0[a]
2	cH1N1	485.0[a,b]	1280.0[a]	640.0[a]	1280.0[a]	787.9[a]
3	tH1N1A	787.9[a]	787.9[a,b]	1280.0[a]	787.9[a,b]	320.0[a,b]
5	H1N2B	139.2[c]	139.2[d]	80.0[b]	1114.3[a]	160.0[b]
6	tH1N1B	160.0[b,c]	278.5[c,d]	452.5[a]	278.5[b]	640.0[a]
	Negative	<10	<10	<10	<10	<10

* Data reported as reciprocal of geometric mean titers
[a,b,c,d] Values with superscripts within each column are significantly different (P<.05)

Table 5: SN Serum reactivity among viruses of H3N2 subtype

			Test Ag	
Group	Serum Group	H3N2C1	H3N2C2	H3N2C3
4	H3N2C1	1575.8[a]	160.0[b]	905.1[a]
7	H3N2C2	196.9[b]	1575.8[a]	196.9[c]
8	H3N2C3	98.5[c]	113.1[b]	452.5[b]
	Negative	<10	<10	<10

* Data reported as reciprocal of geometric mean titers
[a,b,c] Values with superscripts within each column are significantly different (P<.05)

Table 6: IDEXX ELISA Mean S/P Ratios among H1N1 and H1N2 Subtype

Group	Serum Group	H1N1 ELISA	H3N2 ELISA
1	H1N2A	0.590[a]	0.349[a,b]
2	cH1N1	0.795[a]	0.189[b]
3	tH1N1A	0.603[a]	0.674[a]
5	H1N2B	0.128[b]	0.152[b]
6	tH1N1B	0.170[b]	0.111[b]
	Negative	- 0.03	-0.016

* Data reported as group mean S/P ratio with a > 0.4 positive cut-off value
[a,b] Values with superscripts within each column are significantly different (P<.05)

Table 7: IDEXX ELISA Mean S/P Ratios Among H3N2 Subtype

Group	Serum Group	H1N1 ELISA	H3N2 ELISA
4	H3N2C1	0.037[a]	1.767[a]
7	H3N2C2	0.024[a,b]	0.952[b]
8	H3N2C3	-0.004[b]	0.404[c]
	Negative	- 0.03	-0.016

* Data reported as group mean S/P ratio with a > 0.4 positive cut-off value
[a,b,c] Values with superscripts within each column are significantly different (P<.05)

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