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Adaptation and limitations of established hemagglutination inhibition assays for the detection of porcine anti–swine influenza virus H1N2 antibodies

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Abstract. Hemagglutination inhibition (HI) has been a reliable method for determining porcine antibody levels to the well-characterized swine influenza virus (SIV) H1N1 and H3N2 subtypes. However, the recent emergence of the novel H1N2 serotype of SIV and the persistence of 2 other serotypes (H1N1 and H3N2) in the United States swine population represents a significant challenge to diagnostics. Both standardized and modified HI protocols were used in a blinded study to examine a collection of 50 control sera representing a total of 12 swine that were experimentally inoculated with one of the 3 SIV subtypes. Using these control sera data, a statistical basis for analysis was established in an attempt to classify 30 field sera with known case histories or seroprevalance into SIV serotype categories. By this approach 57% of the field sera could be classified into specific categories. The remaining samples that could not be classified reliably were most likely composed of heterogeneous anti-SIV antibody populations. These results indicate that although serological differentiation might be possible in a controlled environment, applications of these methods to field samples are currently problematic. Approaches other than HI will be required to fulfill the current need for SIV diagnostics and surveillance when specific serotype identification is required.

From its first clinical identification in 1918 until the 1970s, swine influenza virus (SIV) has been an endemic orthomyxovirus of remarkable antigenic stability in that it has circulated almost exclusively as 1 subtype, H1N1 (classical SIV; cH1N1), in the United States. However, in the mid-1970s genetically and antigenically novel swine influenza A viruses of the H3N2 subtype were detected.[3] Serological studies performed in the late 1970s showed that although 21–25% of the swine in the United States had antibodies to the H1N1 virus, a much lower percentage (1–2%) of swine had anti–SIV H3N2 antibodies.[3] By the mid-1990s the prevalence of anti–SIV H3N2 antibodies in the United States swine population increased.[7] Interestingly, it was during this period that a third SIV subtype, H1N2, emerged first in 1994 in Europe and then in 1999 in a United States (Indiana) swine herd.[4] The predominant genotype of SIV H3N2 since 1997 was determined to be a triple reassortant among human (hemagglutinin [HA], neuraminidase, and polymerase PB1), avian (polymerases PA and PB2), and classical (matrix protein, nonstructural protein, and nucleoprotein) swine influenza A viruses.[6,8] The genome of the H1N2 subtype was determined to contain the HA gene from the 1997–1998 cH1N1 lineage, whereas the remaining gene repertoire was from the H3N2 virus.[4,5] The rise in prevalence of SIV H3N2 appeared to enable genetic reassortment (antigenic shift) between cH1N1 and H3N2 viruses, resulting in the emergence of the new subtype. However, of equal importance for the HA and neuraminidase epitopes is the phenomenon of antigenic drift arising from genetic point mutations. Such antigenic variation may accumulate rapidly within influenza viruses and may result in altered phenotypic properties within serotypes.

A traditional method to quantitate antiviral antibodies against a number of different viruses, including influenza A viruses, is hemagglutination inhibition (HI). Standardized protocols have been established for the detection of porcine antibodies to SIV H1N1 and SIV H3N2.[2] These HI protocols exploit the fact that the 2 subtypes have dissimilar red blood cell (RBC) preferences for their hemagglutination activity. In addition, because of the inherent antigenic differences between the H1 and H3 hemagglutinins, specific antibody responses to these 2 proteins are easily distinguished. Such biological characterizations of the H1N2 (North American) subtype have not been reported in the literature.

The serological identification of the specific serotype causing an outbreak of swine influenza is of great importance for diagnostic applications and for the de-

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termination of immunization strategies. It was therefore attempted to optimize the hemagglutination parameters for detection of the SIV H1N2 serotype. In addition, it was examined whether porcine anti–H1N2 virus antibodies could be distinguished from those to the other 2 SIV serotypes, using established SIV HI protocols. The swine influenza virus H1N2 has a definitive RBC preference that is distinguishable from that of cH1N1 in that, unlike its cH1N1 counterpart, it does not react readily with chicken RBCs. Swine antibody responses to these 2 subtypes may be discernable with the existing SIV HI protocols if the chicken RBC difference can be exploited. However, if both H3N2 and H1N1 viruses are cocirculating in the same population with H1N2, serological determination of each serotype by current HI methodology is not feasible.

Materials and methods

Viruses. The SIV cH1N1 subtype, A/SW/IL/1976/31, was propagated in 9-day-old embryonated hen eggs. The H3N2 serotype, A/SW/TX/4199/98, was propagated in Madin Darby canine kidney cells according to a previously published protocol.[2] Both viruses had been obtained from the National Veterinary Services Laboratory (NVSL; Ames, IA) for routine SIV HI testing in the University of Illinois Veterinary Diagnostic Laboratory (UI VDL; Urbana, IL). A 2001 Illinois isolate that was serotyped (NVSL) and genotyped (A/Swine/Illinois/100084/01; H1N2 IL)[4] as a purely H1N2 strain was also used in this study and propagated using the SIV H3N2 protocol. Viruses were used as obtained without further purification. To obtain acceptable hemagglutination titers, the H1N2 virus was concentrated from clarified infected cell lysates by ultracentrifugation (113K $\times$ *g* for 1 hr at 4 C). The pelleted virus was then suspended at 1/100 of the original volume in minimum essential media[a] containing penicillin[a] (1 U/ml), streptomycin[a] (2 μg/ml), amphotericin B[a] (2.5 ng/ml), and gentamicin[a] (50 μg/ml) and then stored at 4 C until use.

Serum samples. Porcine serum samples were obtained from 20 different diagnostic field cases that had been submitted to the UI VDL. Sample selection was based on characteristically high–anti-H1N1, high–anti-H3N2, high–anti-H1N1 and -H3N2, or undetectable anti–H1N1 and –H3N2 SIV antibody levels. Additional serum samples were obtained from convalescent pigs in a herd where the H1N2 IL virus was isolated. Because these animals had not been vaccinated against SIV, there was a high probability that only anti–H1N2 virus antibodies would be present in their sera. Likewise, 30 more samples were obtained from an unvaccinated North Carolina swineherd approximately 7 wk after an H1N2 virus infection. In addition, 50 anti-SIV control sera were obtained from NVSL: 13 samples from pigs inoculated with SIV cH1N1 (12 of these samples were from 4 different pigs for which each sample had been 2-fold serially diluted), 24 sera representing 5 animals that had been infected with SIV H3N2 and then sequentially bled during a 16-day period, and 13 samples obtained from 2 pigs that had been inoculated with SIV H1N2 IL and then bled sequentially during a 38-day period.

Hemagglutination. Hemagglutination assays were performed as described previously.[2] To optimize the hemagglutination conditions for the H1N2 virus, guinea pig,[b] chicken,[c] and tom turkey[b] RBCs were examined. Chicken and tom turkey erythrocytes are components of the standard hemagglutination protocols for the H1N1 and H3N2 viruses, respectively. Guinea pig RBCs were examined for their potential use because such cells are known to agglutinate many different types of viruses. V- and U-bottomed 96-well plates[d] were compared for their resolution properties. The hemagglutination protocols were performed at both room temperature and 4 C, and their results were compared to determine the optimum temperature for SIV H1N2.

Hemagglutination inhibition. The standardized SIV HI protocols for detection of anti-H1N1 and -H3N2 antibodies were used as described previously.[2] Both standardized assays (H1N1 and H3N2) were then modified by replacing the challenge virus with H1N2 IL and the indicator RBCs with guinea pig or tom turkey species. Thus, 6 different HI assays were used to analyze the control sera from NVSL as well as the selected field samples. These assays were designated as T11V11BC, T11V12BT, T11V12BG, T32V32BT, T32V12BT, and T32V12BG. The first 3 characters, T11 or T32, indicate the respective H1N1 or H3N2 HI protocol used; the second 3 characters represent the challenge virus used in the assay: V11 (H1N1), V12 (H1N2), or V32 (H3N2); the last 2 characters represent the indicator RBC used: BC (chicken), BT (tom turkey), or BG (guinea pig). All HI assays were performed at room temperature using V-bottomed (for T11 protocols) or U-bottomed (for T32 protocols) 96-well plates. The anti–SIV cH1N1 control sera were examined at undiluted as well as 2-fold serial dilutions (1:2 to 1:8) for validation purposes. The 30 sera from the North Carolina outbreak were diluted 1:2 to have sufficient sample volumes to complete all necessary protocols. All other sera were used as collected.

Statistics. Mean HI titers of sera subjected to different treatment–virus–RBC combinations were compared using Student's *t*-tests for paired data. Because multiple tests were run simultaneously, the probability of type 1 error was elevated across the analysis. Therefore, a Bonferroni correction was applied such that results were considered statistically significant only when $P < 0.05/n$, where n was the number of tests run.[1] Descriptive statistics were generated, and *t*-tests performed using commercially available computer programs.[e,f]

Differences in $\log_2$ titers (T11V11BC–T11V12BT and T32V32BT–T32V12BT) were tabulated for all control sera. Means and standard deviations were calculated for the distributions of these differences. Normal distributions were then generated with means and standard deviations identical to the empirical distributions. Ninety-five percent confidence intervals were calculated from these normal distributions as the symmetrical range within which 95% of the values of the distribution were contained. These analyses were performed using a computer program.[g]

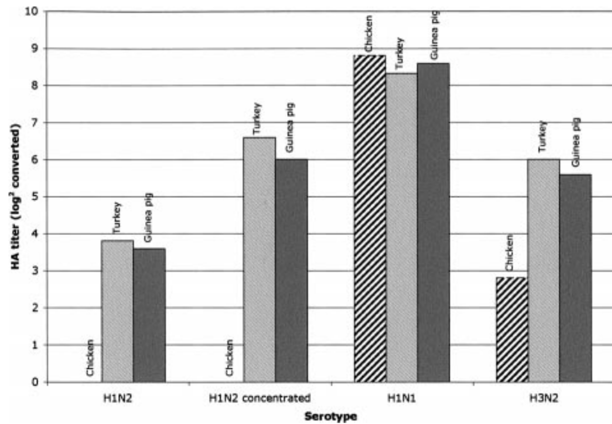


Figure 1. Hemagglutination of different blood types by the 3 serotypes of SIV. Hemagglutinin titers ($\log_2$ converted) of H1N1, H1N2, and H3N2 SIV were determined at room temperature using RBCs originating from chicken, guinea pig, or tom turkey. Results for both concentrated and unconcentrated H1N2 viral samples are included in the graph.

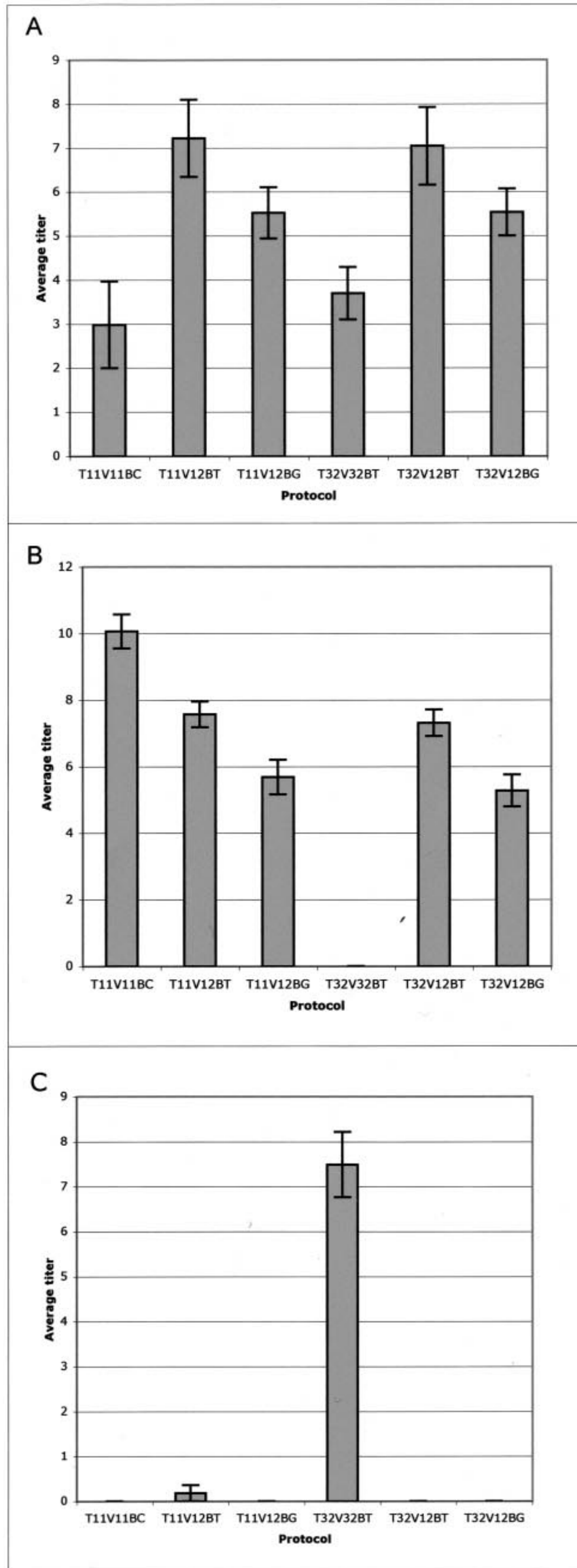
Results

Hemagglutination optimization. Optimal hemagglutination characteristics were established for the H1N2 IL virus using different RBCs, incubation temperatures, and plate types. Repeated analysis showed that neither plate type nor incubation temperature significantly altered the observed viral titer (data not shown). In contrast to the cH1N1 serotype, the H1N2 virus was able to hemagglutinate only the tom turkey and guinea pig RBCs and failed to react with the chicken RBCs (Fig. 1). To test the possibility that the H1N2 IL virus titer was below that capable of hemagglutinating chicken RBCs, the virus was concentrated by ultracentrifugation and then titered using all 3 species of RBCs. As before, the H1N2 IL virus could not measurably hemagglutinate chicken RBCs and maintained a slight preference for tom turkey over guinea pig RBCs (Fig. 1).

Hemagglutination inhibition. To resolve the ability of the standardized and modified HI assays to differentiate antibody responses to each SIV serotype, control sera titer values obtained from each assay using treatment (T11 vs. T32) and blood types (BT vs. BG) as criteria for evaluation were compared. Use of the modified protocols (T11V12BT, T11V12BG, T32V12BT, and T32V32BG) resulted in anti-H1N2 titers being greater than those obtained by examination with the standard H1N1 protocol (T11V11BC). Titers from the T32V32BT protocol were not statistically different from those from the T11V11BC protocol; however, both T11V11BT and T32V12BT had significant differences from the standard T11V11BC ($P = \ll 0.0001$ for both T11V12BT and T32V12BT). Because of this difference the effect of the serum treatment was evaluated in relation to titer (T11V12 vs. T32V12). Interestingly, a slightly higher titer was observed (an approximately 2-fold difference but not statistically significant) when the H1N1 serum treatment (T11V12 assays) was used (Fig. 2A). Likewise, a significantly higher antibody titer was obtained when using tom turkey instead of guinea pig RBCs for both treatments: T11V12BT > T11V12BG ($P = 0.0002927$, 2 tailed) T32V12BT > T32V12BG ($P = 0.006213$, 2 tailed) (Fig. 2A). Anti–cH1N1 virus control sera had significant titers in all assays, which used either H1N1 or H1N2 as the challenge viruses, and had the highest titers when the traditional assay for H1N1 was used (T11V11BC); this was due to cross-reactivity between the anti–H1N1 virus antibodies and the H1N2 virus (Fig. 2B). As expected, only anti–H3N2 virus control sera yielded significant titers in its traditional assay (T32V32BT) because of the lack of recognition of the anti–H1 virus antibodies with the challenge virus, H3N2 (Fig. 2C).

The control sera for both H1N2 and H3N2 viruses were obtained sequentially from experimentally infected pigs. The anti–H3N2 virus control serum was analyzed using the T32V32BT protocol, whereas the T11V12BT protocol was used to examine anti–H1N2 virus antibody responses. The 12 serially diluted anti–H1N1 virus control sera were used to validate the experimenter's technique (data not shown). As expected, a 2-fold increase in the dilution factor resulted in the observed titer being reduced by half. The 30 anti–H1N2 virus field sera obtained from North Carolina were examined in the same manner as the anti–H1N2 control sera. Cross-reactivity of the antibodies was not observed. However, the resultant titers for these samples were low in the modified protocols (T11V12BT and T32V12BT) and negative in the standardized H1N1 assay (T11V11BC), and, consequently, the sera were not subjected to statistical analysis.

It was observed that when titers (as determined by the different assays) of the same antiserum were compared (i.e., titer of serum x in T11V11BC − titer of serum x in T11V12BT), similar variations were found within the antiserum sample categories (anti–H1N1, –H1N2, and –H3N2). Because such comparisons resulted in titer distinctions that were serotype specific, it was believed that the prevailing anti-SIV antibodies in the serum samples could be categorized by using the different assays (T11V11BC, T11V12BT, T32V32BT, and T32V12BT) and then subjecting the resultant titers to certain comparisons (titer in T11V11BC − titer in T11V12BT and titer in T32V32BT − titer in T32V12BT). Depending on the differences between the assay titers, the unknown sample could be categorized into a serotype category by using normal distributions that were established by using the control antisera in the assays. These antisera



data were then grouped to establish normal distributions representative of the difference in antibody titers for each subtype (Fig. 3). After creation of the normal distributions, the HI data from both the control and the field sera were overlaid onto the appropriate distribution to determine whether the field sera could be grouped by subtype. Figure 3A, 3B indicates that the overlap among the distributions was too great to enable sera to be categorized reliably by an individual comparison. However, using both comparisons in conjunction with each other was a reliable means of identification for the control sera. Thus, the anti–SIV H1N1 and –SIV H1N2 sera could be differentiated from the anti–SIV H3N2 sera (Fig. 3A) using the comparisons of T11V11BC–T11V12BT, whereas the anti–SIV H1N1 sera were differentiated from the H1N2 antiviral sera using T32V32BT–T32V12BT (Fig. 3B). The field samples were then subjected to this statistical analysis to identify the serotype category (established by the control sera) to which they belonged. Both the field and the control titer differences were compared, and then the normal distribution was overlaid onto the titer differences to determine the possible relationship between the field and the control sera (Figs. 4, 5). By this approach approximately 57% of the field sera could be classified into specified categories. Furthermore, samples that were categorized as having exclusively anti–SIV H1N2 antibodies were properly identified 84% of the time. However, some field samples were composed of heterogeneous anti-SIV antibody populations, as is evident from a shift in the titer comparisons, and, therefore, could not be reliably classified.

Discussion

By comparing results from the different HI protocols, it was possible to determine the identity of the viral serotypes of the control serum samples. Normal distributions and 95% confidence intervals demonstrated that control sera sorted into mutually exclusive and readily distinguishable groups, reflective of the serotypes of the original infecting virus. Use of the 2 established SIV HI protocols for detection of antibodies to the H1N1 and H3N2 serotypes enabled identification and differentiation of their respective control sera. In this regard the anti–SIV H1N1 and –SIV H1N2 sera could be differentiated from anti–SIV H3N2 sera (Fig. 3A) by comparing the titers from

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Figure 2. Effect of HI protocol on determination of anti-SIV antibody titers. Using the indicated protocols (T11V11BC, T11V12BT, T11V12BG, T32V32BT, T32V12BT, and T32V12BG), the average HI titers ($\log_2$ converted) obtained for SIV subtype **A**, H1N2, **B**, H1N1, and **C**, H3N2.

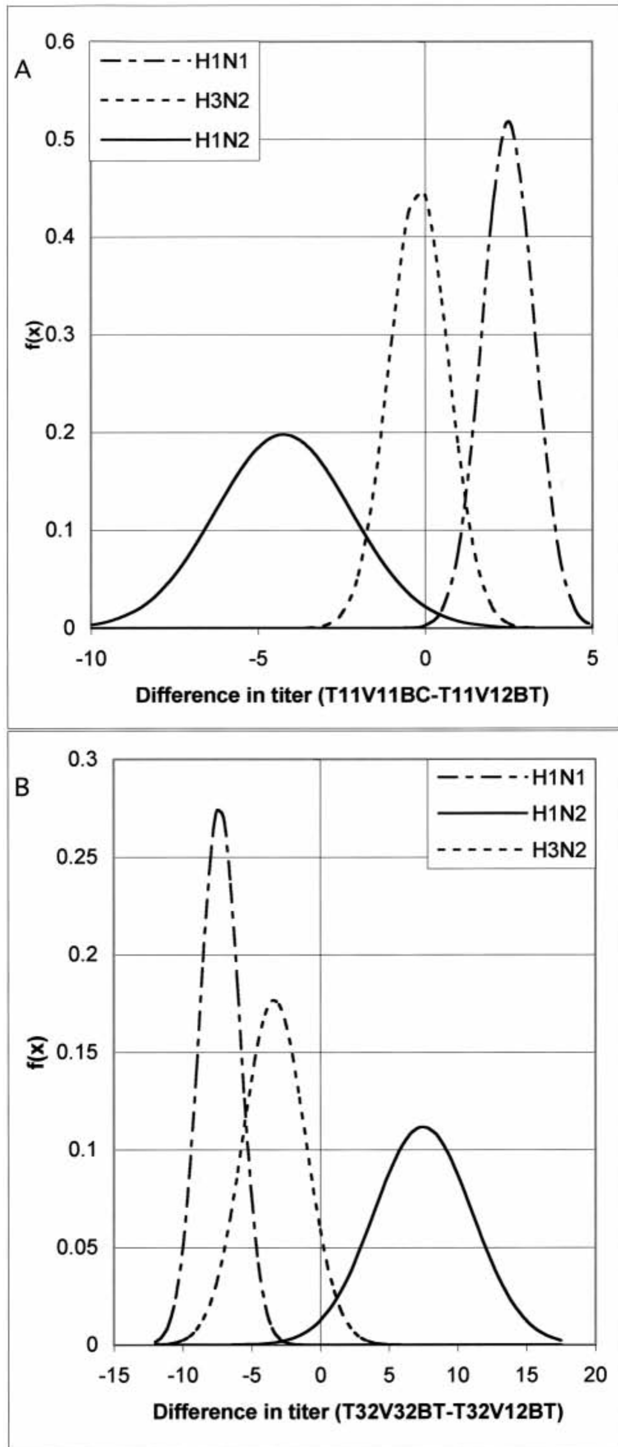
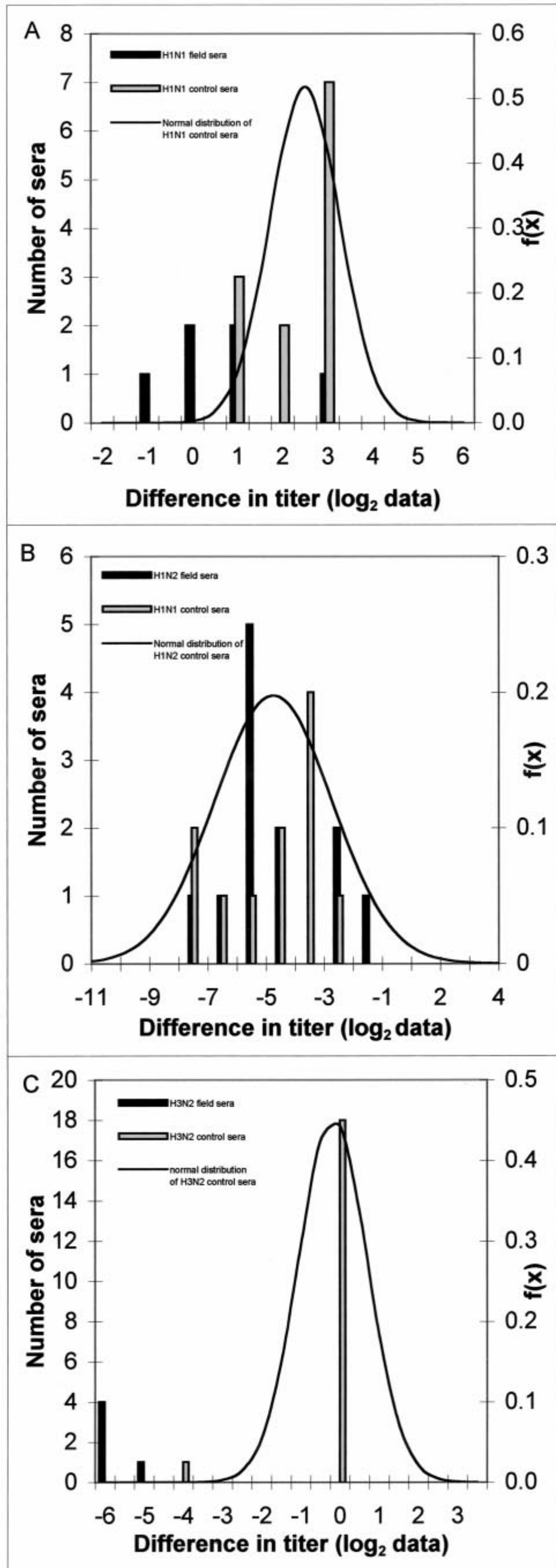


Figure 3. Normal distributions *(f(x))*, with means and variances identical to the means and variances of the empirical distributions of the anti-H1N1, -H3N2, and -H1N2 control sera. The titer comparisons are representative of **A**, T11V11BC − T11V12BT, and **B**, T32V32BT–T32V12BT. Titers were $\log_2$ transformed before the comparison.

T11V11BC with those from T11V12BT. Although the control sera containing anti–SIV H1N2 IL antibodies were readily identified, they were indistinguishable from anti–SIV H1N1 antibodies when using the original H1N1 HI protocol (T11V11BC). However, anti–SIV H1N1 sera could be differentiated from H1N2 antiviral sera by comparing T32V32BT with T32V12BT (Fig. 3B). Even though H1N2 control sera had observable antiviral titers in traditional H1N1 protocol (T11V11BC), these titers were significantly lower than those titers observed in the modified protocols (T11V12BT and T32V12BT). In contrast, when the anti–SIV cH1N1 control sera were examined, the relationship was reversed. This anomaly was anticipated because of the presence of the antigenically varied HA of the 2 viral H1 subtypes. The cH1N1 virus used in the test protocols was isolated in 1976, whereas the HA genotype of the H1N2 viruses is more closely related to cH1N1 isolates obtained in 1997–1998.[5] Such phenotypic changes were manifested by the hemagglutination properties: SIV cH1N1 (1976) reacted more readily with chicken than with guinea pig or tom turkey RBCs, whereas the H1N2 virus demonstrated a preference for tom turkey RBCs and failed to detectably hemagglutinate chicken RBCs (Fig. 1). Overall these results demonstrated that the traditional H1N1 protocol is currently optimal for identifying anti–H1N1 virus antibodies, whereas the use of the modified protocol (T11V12BT) in this study is more proficient for detection of anti–H1N2 virus antibodies.

Based on the case history, each field sample was assigned to one of 4 specific categories (anti–SIV H1N1, –SIV H3N2, or –SIV H1N2, or mixed infection). Subsequently, field sera HI data were examined by the statistical approach in an attempt to identify the different serotypes involved in the disease episodes (Figs. 4, 5). In some cases it was apparent that antibodies to one viral subtype were only a proportion of the anti-SIV antibodies present in the sample. Therefore, the sera that contained significant amounts of anti-SIV antibody to more than one subtype could not be reliably classified. Consequently, the presence of multiple anti–SIV subtype antibodies was a limiting factor for dependable classification of swine sera using this method of analysis. The data from the remaining 30 samples obtained from North Carolina that contained only anti–SIV H1N2 antibodies were not analyzed statistically because of either low or negative HI titers. Although these samples were obtained during the convalescent stage (approximately 7 weeks after SIV H1N2 was isolated from the animals), it is possible that the antibody response to the H1N2 virus did not reach a sufficient level or persist long enough to produce a detectable cross-reaction with the cH1N1 virus.



The results of this study demonstrate that anti–SIV H1N2 IL antibodies can be identified using current diagnostic HI methods partly because of the HA in the H1N2 subtype being derived from the cH1N1 lineage.[4,5] Although the HA of the H1N2 IL subtype has evolved significantly through the process of antigenic shift, as evidenced by the loss of its preference for chicken RBCs, there still remains cross-reactivity of anti-H1N1 antibodies with the H1N2 virus. Consequently, anti–H1N2 virus antibodies also may have a high degree of cross-reactivity with the cH1N1 virus, and as a result, it is possible to identify such antibodies with the use of the established H1N1 protocol (Fig. 2). However, this also infers that the HI assay, regardless of the challenge virus used, cannot reliably differentiate between anti–SIV cH1N1 and –SIV H1N2 antibodies. In this regard, when the anti-SIV antibody levels in the North Carolina sera were determined using a reassortant H1N1 virus (North Carolina origin; G. Erickson, personal communication), the HI titers were similar to those found in this study using the H1N2 IL rather than the cH1N1 virus. The reason for these comparable titers is most likely that the HA-coding sequence of this reassortant H1N1 virus has a high degree of sequence homology with the reassortant H1N2 but not with the cH1N1 HA gene region (R. Webby, personal communication).

In this study it was determined that, if both SIV subtypes H3N2 and H1N1 are cocirculating in the same population with the H1N2 virus, accurate serological determination of each subtype is not feasible by current HI methodology even if coupled with statistical analysis. However, because of cross-reactivity between anti-H1N1 and -H1N2 antibodies, outbreaks of SIV still can be detected even if the specific serotype causing the outbreak (H1N1 or H1N2) cannot be elucidated. Therefore, approaches other than HI will be required to meet the needs of SIV diagnostic and surveillance applications when specific serotype identification is required. This may include enzyme-linked immunosorbent assay technology that uses selective SIV HAs and neuraminidases as test antigens reflective of the known circulating viral subtypes as well as potential reassortants such as H3N1. In addition, such an approach would enable more definitive evaluation of

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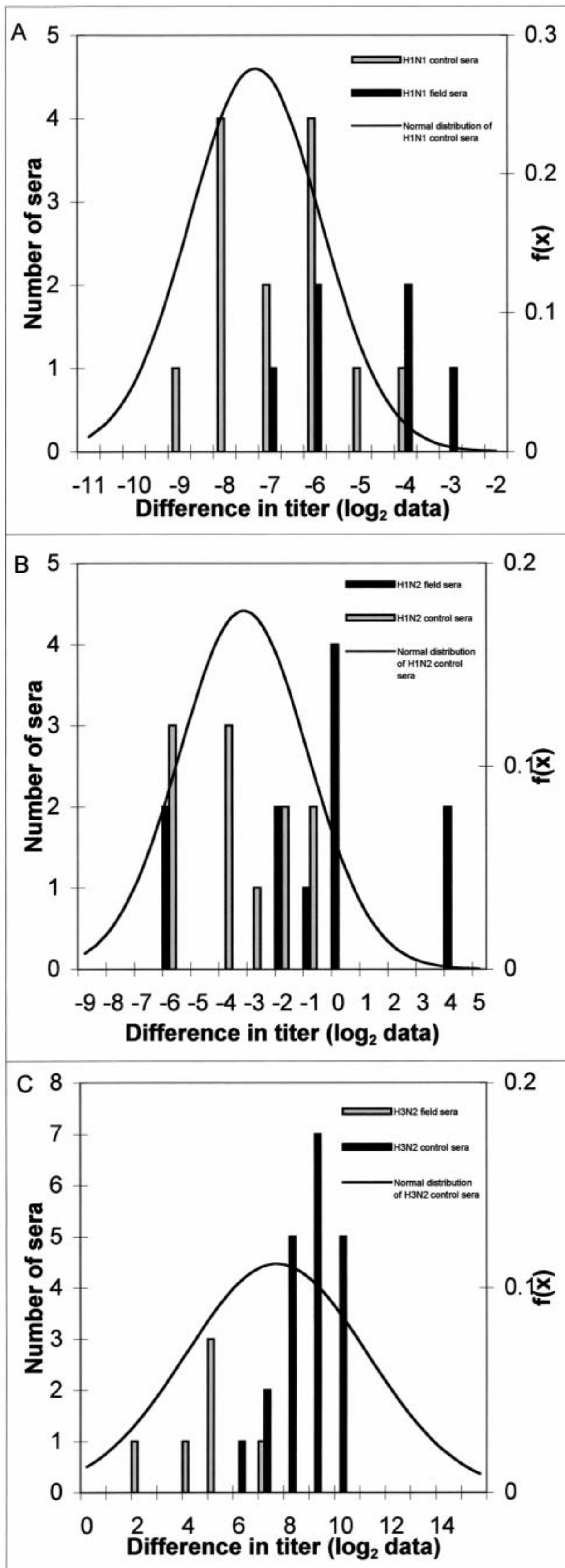
Figure 4. Swine influenza virus HI titer comparisons of T11V11BC–T11V12BT protocols. The log_2-transformed titer data of subtype-specific control sera (NVSL control samples) and field sera (classified by case history obtained from the UI VDL) of the same subtype are shown. The normal distribution obtained from Fig. 3A is applied to each graph according to the subtype that is being examined: **A**, anti–H1N1 virus sera, **B**, anti–H1N2 virus sera, and **C**, anti–H3N2 virus sera.



immunization strategies and the potential for cross-protection afforded by the various viral subtypes.

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Sources and manufacturers

a. Sigma-Aldrich, St. Louis, MO.
b. Lampire Biological Laboratories, Inc., Pipersville, PA.
c. Poultry farm, University of Illinois, Urbana, IL.
d. Fisher Scientific, Hanover Park, IL.
e. KaleidaGraph™, v. 3.51, Synergy Software, Inc., Essex Junction, VT.
f. SAS v. 8.0, SAS Institute, Inc., Cary, NC.
g. Microsoft Excel 98, Microsoft Corporation, Redmond, WA.

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Figure 5. Swine influenza virus HI titer comparisons of T32V32BT–T32V12BT protocols. The log_2-transformed titer data of subtype-specific control sera (NVSL control samples) and field sera (classified by case history obtained from the UI VDL) of the same subtype are shown. The normal distribution obtained from Fig. 3B is applied to each graph according to the subtype that is being examined: **A**, anti–H1N1 virus sera, **B**, anti–H1N2 virus sera, and **C**, anti–H3N2 virus sera.