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# Diagnostic performance of the canine *Influenza A Virus* subtype H3N8 hemagglutination inhibition assay

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## Abstract

Canine *Influenza A virus* subtype H3N8 (H3N8 CIV) was recognized in 2004 as a novel respiratory pathogen for dogs. To date, infections have been diagnosed in thousands of dogs in 38 U.S. states. Diagnostic techniques such as reverse transcription polymerase chain reaction (RT-PCR) and virus isolation may yield false-negative results if samples are collected after virus shedding has ceased. Therefore, serology is often necessary to confirm diagnosis. The hemagglutination inhibition (HI) assay is the test of choice for serological diagnosis of influenza infections in animals. However, discrepancies exist between diagnostic laboratories and research groups in some of the test parameters for the H3N8 CIV HI assay and the cutoff antibody titer for seropositivity. The objectives of the current study were 1) to assess the diagnostic performance of a H3N8 CIV HI assay using field sera from canine infectious respiratory disease outbreaks and 2) to evaluate the effect of test parameter variations on test performance, including the use of different red blood cell (RBC) species, serum treatment methods, and virus isolates. Based on a receiver operating characteristic analysis using serum microneutralization assay titers as the gold standard, the H3N8 CIV HI assay described in the present study is highly sensitive (99.6%) and

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Sources and manufacturers

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specific (94.6%) when the cutoff antibody titer for seropositivity is 32. Evaluation of parameter variations determined that the sensitivity and specificity of the H3N8 CIV HI assay depend on serum pretreatment with a receptor-destroying enzyme or periodate, use of 0.5% turkey or chicken RBCs, and use of antigenically well-matched H3N8 virus strains.

**Keywords**

Canine influenza virus; diagnosis; H3N8; hemagglutination inhibition; serology; sensitivity; specificity

## Introduction

Canine *Influenza A virus* subtype H3N8 (H3N8 CIV), a respiratory pathogen of dogs,[4] was initially identified in the racing Greyhound population in the United States, although all naïve dogs are susceptible to infection.[4,6,8,9,17] Since the initial report in 2004, H3N8 CIV infections have been documented in thousands of dogs with widespread geographic distribution in 38 U.S. states.[2–4,8,9,13,15,22,24,26,27] To date, there is no evidence of H3N8 CIV circulation in other countries.[5,18,19,23] Similar to other host-adapted mammalian *Influenza A virus* strains, H3N8 CIV is efficiently transmitted between dogs and can cause outbreaks of influenza-like illness (ILI) in communal housing settings such as racing Greyhound kennels, boarding and/or training facilities, and animal shelters.[3,4,8,9,13,15,22,24,36]

Molecular analyses of H3N8 CIV isolates indicate prior transmission of a Florida sublineage (clade 1) H3N8 equine *Influenza A virus* (H3N8 EIV) from horses to dogs, followed by viral adaptation to the new canine host.[4,13,22,24] H3N8 CIV isolates form a monophyletic group that is molecularly and epidemiologically distinct from currently circulating H3N8 EIV in the United States.[24] Signature amino acid changes in the H3 protein, particularly around the receptor-binding pocket, distinguish CIV isolates from their EIV ancestors, and additional changes in endemic isolates indicate the virus continues to adapt to dogs.[4,13,14,22,24]

As for other *Influenza A virus* strains, H3N8 CIV can be diagnosed in clinical specimens by virus isolation or reverse transcription polymerase chain reaction (RT-PCR) using subtype-specific primers.[4,8,9,20,22,24] Peak virus shedding from the upper respiratory tract occurs during the 2- to 4-day incubation period and rapidly declines during the clinical phase to cessation typically by day 7.[4,6,9,17] Reverse transcription polymerase chain reaction is more sensitive than virus isolation[8,9,24]; however, both assays can yield false-negative results if clinical samples are collected outside the viral shedding window.[6,8,9] Therefore, serological assays are recommended for confirmation of H3N8 CIV infections, either as a diagnostic aid for current cases or as an epidemiological tool for surveillance and assessment of disease prevalence.[8,9] Serum antibodies to the CIV hemagglutinin (H3) protein are detectable at 7–10 days after infection and continue to rise to high levels after 14 days.[4,6,17]

The hemagglutination inhibition (HI) assay is considered the test of choice for serological diagnosis of *Influenza A virus* infections in animals.[32,33] In the HI assay, serum antibodies specific for the hemagglutinin protein subtype interfere with virus binding to red blood

cells (RBCs), thereby inhibiting hemagglutination and indicating previous virus infection. A variety of factors can influence the sensitivity and specificity of the HI assay, including the matching of the sialic acid (SA) receptor type on the RBC surface to that preferred by the viral hemagglutinin protein, the presence of nonspecific hemagglutinins and/or nonspecific inhibitors of hemagglutination in serum, and the binding specificity of serum antibodies for the particular hemagglutinin subtype of the test virus.[32,33] Although test conditions may require modification for the detection of novel viruses or viruses that have undergone antigenic drift due to point mutations in the hemagglutinin protein,[21,28] standard HI conditions have been generally established for endemic influenza viruses in avian species, pigs, horses, and human beings.[31–33]

In the first reports on H3N8 CIV infection of dogs, an HI assay was utilized for documentation of viral infection.[4,22] The HI antibody titer cutoff for seropositivity was set based on testing sera from uninfected specific pathogen–free (SPF) dogs and SPF dogs experimentally inoculated with an H3N8 CIV isolate.[4,22] As H3N8 CIV has continued to spread through the U.S. canine population, reference and research laboratories have used different versions of the originally reported HI assay for diagnosis or disease surveillance, resulting in discrepancies between laboratories in the HI cutoff antibody titer selected for seropositivity as well as the test parameters used. Unless the diagnostic performance of the different HI assays has been defined, such discrepancies can lead to misdiagnosis of dogs, interlaboratory variability, and unreliable disease surveillance data. Therefore, the first objective of the current study was to assess the diagnostic performance of the HI assay described in the initial reports for H3N8 CIV infections in dogs using a panel of field sera collected during canine infectious respiratory disease outbreaks. The second objective was to evaluate the effect of test parameter variations on the diagnostic performance of the H3N8 CIV HI assay, including the use of different RBC species, canine serum pretreatment methods, and different virus isolates.

## Materials and methods

All studies were approved by the University of Florida Institutional Animal Care and Use Committee.

### Assessing the diagnostic performance of the H3N8 CIV HI assay

**H3N8 CIV HI and serum microneutralization assays.——**The H3N8 CIV HI and serum microneutralization (MN) assays were performed as described in previous reports.[4,22] Sera collected from SPF dogs[4] housed in a barrier research facility and normal ferret sera were used as negative controls for both assays, while polyclonal goat anti-EQ/Miami/63 serum and sera from SPF dogs experimentally infected with the A/canine/Florida/2004 (CN/FL/04) isolate of H3N8 CIV served as positive controls. For the HI assay, serial 2-fold dilutions of serum (25 μl) pretreated with a receptor-destroying enzyme (RDE) were tested with 4 hemagglutinating units (HAU) of the CN/FL/04 isolate per 25 μl and 0.5% turkey RBCs in phosphate buffered saline (PBS; 50 μl). The end-point antibody titer was defined as the reciprocal of the last serum dilution that completely inhibited hemagglutination. For the MN assay, serial 2-fold dilutions of RDE-treated sera were added to the CN/FL/04 isolate

($2 \times 10^3$ $TCID_{50}$ /ml) and Madin–Darby canine kidney (MDCK) cells.[4,25] The end-point antibody titer was defined as the reciprocal of the highest serum dilution that gave 50% neutralization of 100 $TCID_{50}$ of the CN/FL/04 isolate.[4] Based on reactivity of negative and positive control sera, seropositivity of a single sample was defined as a MN antibody titer ≥80.[4,25]

**Serum samples.——**To assess the diagnostic performance of the H3N8 CIV HI assay, HI and MN test results were analyzed for sera collected from 595 dogs. This sample contained 520 racing Greyhounds,[4,22] 35 shelter dogs,[22] and 38 pet dogs involved in respiratory disease outbreaks in the United States as well as 2 SPF dogs experimentally infected with the CN/FL/04 isolate.[4] Paired sera were collected from 212 (36%) dogs. For these dogs, the first samples were collected within 2 weeks of the reported onset of clinical signs or exposure to dogs exhibiting ILI. The second samples were collected from 2 weeks to 3 months after the first sample. Of these dogs, 113 (53%) seroconverted as defined by at least a 4-fold rise in HI or MN antibody titer between the first and second samples, 79 (37%) had positive MN (≥80) titers for both samples, and 20 (10%) had negative MN titers for both samples. The HI and MN values for the second samples of each of the 212 paired sets were used in the correlation and receiver operating characteristic (ROC) analyses described below. For the remaining 383 (64%) dogs, single samples collected from 2 weeks to 3 months after respiratory illness or known exposure to dogs with ILI were included in the analyses. Of these 383 dogs, 313 (82%) were seropositive by MN testing, and 70 (18%) were seronegative.

**Intra-assay and interassay variability of the H3N8 CIV HI assay.——**A subset of 12 serum samples previously tested in both the HI and MN assays were retested in a blinded fashion in the H3N8 CIV HI assay in order to assess intra-assay and interassay variability. This subset included 6 CIV-seronegative samples and 6 CIV-seropositive samples with HI antibody titers ranging from 32 to 1,024. For intra-assay testing, each sample was tested 5 times in the same microtiter plate using the same virus and RBC preparations. For interassay testing, each sample was tested in duplicate on 3 separate days using fresh virus and RBC preparations each day.

**Data analyses.——**Statistical analyses comparing the H3N8 CIV HI and MN test results for the 595 serum samples were conducted with standard software.[a,b] The Spearman rank order correlation test was used to determine the correlation of the HI and MN antibody titers. Receiver operating characteristic analysis was used to determine the sensitivity, specificity, and positive and negative predictive values of the HI assay using the MN assay titers as the gold standard. The H3N8 CIV-seropositive ($n = 502$) samples were coded “1” based on MN titers ≥80, and seronegative ($n = 93$) samples (MN titer <80) were coded “0.” The H3N8 CIV HI antibody titer cutoff for seropositivity was based on the optimum sensitivity and specificity values determined in the ROC analysis. Analytical results with a $P$ value <0.05 were considered significant. The percentage coefficient of variation (%CV) was calculated for the intra-assay and interassay variability of the H3N8 CIV HI assay.

[a] XLSTAT, Addinsoft, New York, NY.
[b] SigmaStat, Systat Software, San Jose, CA.

## Effect of test parameter variations on diagnostic performance of the H3N8 CIV HI assay

**Serum samples.——**To determine the impact of test parameter variations on the diagnostic performance of the H3N8 CIV HI assay, subsets of seronegative and seropositive sera previously tested in the HI and MN assays were retested with different RBC species, serum treatment methods, and different H3N8 CIV and EIV isolates. Sera from SPF dogs were used as negative controls.

**RBC sources.——**Commercially available chicken, turkey, goose, horse, dog, sheep, and cow RBCs[c] collected with Alsever solution as the anticoagulant were used in hemagglutination (HA) and HI assays as described below. Red blood cells were stored at 4°C and used within 2 weeks of collection. All RBCs were washed 2 to 3 times with PBS prior to use in the HA and HI assays.

**Serum treatments.——**Sera were either untreated or treated with RDE[d] or periodate[e] followed by heating at 56°C for 30 min to inactivate potential nonspecific inhibitors of HA in canine sera. For RDE treatment, serum samples were incubated with RDE (1 volume of serum to 3 volumes of RDE) for 16 hr at 37°C prior to heat inactivation. This treatment resulted in a final serum dilution of 1:4 for use in the HI assay. Periodate treatment was conducted following a standard World Health Organization (WHO) protocol[32] that included, in a step-wise fashion, the treatment of 1 volume of serum with 2 volumes of trypsin[e] solution (200 mg trypsin in 25 ml of 0.1 M phosphate buffer), inactivation at 56°C for 30 min, incubation with 3 volumes of a 0.011 M metapotassium periodate solution for 15 min at room temperature (23°C), incubation with 3 volumes of a 1% glycerol[e] solution for 15 min at 23°C, and addition of 0.85% saline for a final serum dilution of 1:10 for use in the HI assay.

**Viruses.——**The following H3N8 CIV and EIV isolates were used in the study: CN/FL/04 and A/canine/Jacksonville/2005 (CN/Jax/05)[f]; A/canine/FortCollins/3/2006 (CN/CO/06), A/canine/Weld/2007 (CN/CO/07), and A/canine/ColoradoSprings/2009 (CN/CO/09)[g]; A/canine/Pennsylvania/96978/2009 (CN/PA/09) and A/equine/NewYork/1999 (EQ/NY/99)[h]; H3N8 CN/FL/04 inactivated by binary ethylenimine (BEI) treatment, an alkylating agent that inactivates the virus without altering virus proteins[i]; and A/equine/Kentucky/1991 (EQ/KY/91), A/equine/Kentucky/1995 (EQ/KY/95), and A/equine/Ohio/2003 (EQ/OH/03).[j]

All canine isolates were cultured in MDCK cells using a standard protocol[4] with the described modifications. Briefly, approximately 75% confluent MDCK cells grown in 75-$cm^2$ flasks were inoculated with a 1:750 dilution of virus stock in PBS. The flasks were incubated for 1 hr at 35°C in a humidified atmosphere containing 5% $CO_2$ and then washed with sterile PBS. Dulbecco modified Eagle medium[k] supplemented with 1 μg/ml

[c] Lampire Biological Laboratories, Pipersville, PA.
[d] Denka-Seiken, Tokyo, Japan.
[e] Sigma-Aldrich, St. Louis, MO.
[f] Viruses provided by Dr. R. Donis, Centers for Disease Control and Prevention, Atlanta, GA.
[g] Viruses provided by Dr. G. Landolt, Colorado State University, Fort Collins, CO.
[h] Viruses provided by Dr. E. Dubovi, Cornell University, Animal Health Diagnostic Center, Ithaca, NY.
[i] Viruses provided by Dr. N. Lakshmanan, Intervet Schering Plough Animal Health, Millsboro, DE.
[j] Viruses provided by Dr. T. Chambers, University of Kentucky, Lexington, KY.

of TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone)–treated trypsin,[e] 0.3% final concentration bovine serum albumin (BSA),[k] HEPES (N-2-hydroxyethylpiperazone-N-2-ethanesulfonic acid) buffer,[l] and penicillin-streptomycin[l] was added to the flasks, followed by incubation at 35°C in a humidified atmosphere containing 5% $CO_2$ for 3–5 days. The culture supernatant was harvested upon observation of a cytopathic effect, clarified by centrifugation at 700 rcf for 10 min, and the HA titer determined with the HA assay using 0.5% turkey RBCs in PBS as previously described.[4] The viral culture supernatant was stored at –80°C until future use. All equine virus isolates were cultured in embryonated chicken eggs as previously described.[4] Allantoic fluid aliquots from equine virus cultures were stored at –80°C until use.

**HA assay.——**Prior to evaluating the effect of using different RBC species in the HI assay, the RBC binding preferences of the CN/FL/04 isolate were assessed in HA assays. Serial 2-fold dilutions of MDCK supernatant containing infectious H3N8 CN/FL/04 were incubated with an equal volume (50 µl) of 0.5% chicken, 0.5% turkey, 0.5% goose, 1% horse, 1% dog, 1% sheep, and 1% cow RBCs prepared in PBS or PBS containing 0.5% BSA (PBS/BSA). Incubations were performed in V-bottom microtiter plates for 30 min or 1 hr at 23°C and 4°C. The HAU, defined as the reciprocal of the last virus dilution that completely agglutinated RBCs, were compared for the different RBC species and incubation conditions. The RBC binding preferences were evaluated based on the HAU and known SA receptors for each RBC species. The RBC species with the highest HAU for H3N8 CN/FL/04 were selected for use in subsequent HI assays.

**HI assay.——**Serial 2-fold dilutions of untreated sera (starting dilution of 1:10), periodate-treated sera (starting dilution of 1:10), or sera treated with RDE (typical starting dilution of 1:8; however, 1:10 when compared to periodate-treated and untreated sera) were incubated in V-bottom microtiter plate wells with 4 HAU per 25 µl of infectious H3N8 CIV or EIV isolates or inactivated CN/FL/04 for 30 min at 23°C. The serum/virus mixtures were then incubated at 23°C for 30 min with an equal volume of 0.5% chicken or turkey RBCs in PBS or for 60 min with an equal volume of 1% dog RBCs in PBS/BSA. All samples were tested in duplicate for the determination of end-point antibody titers. For each serum sample, antibody titers generated by HI assays with different parameters were compared to the antibody titers determined by the HI assay performed as described above in the section on diagnostic performance assessment. Because titer differences of 1 dilution (i.e., 2-fold) are recognized as an acceptable margin of error, HI titers that differed by 2 or more dilutions (i.e., 4-fold or more) were considered indicative of altered sensitivity or specificity.

## Interlaboratory HI assay comparison

A panel of 32 canine sera tested by the HI assay at the University of Florida (UF) was also tested in a blinded fashion at the Cornell University Animal Health Diagnostic Center (CU AHDC) and Colorado State University (CSU). The HI assay procedure was similar between laboratories, with the following variations. At UF, sera were pretreated with RDE

[k] Gibco®, Invitrogen Corp., Carlsbad, CA.
[l] Thermo Fisher Scientific Inc., Waltham, MA.

and tested with 0.5% turkey RBCs and BEI-inactivated CN/FL/04. At CU AHDC, sera were treated with periodate and preadsorbed with turkey RBCs prior to HI testing with 0.5% turkey RBCs and a recombinant virus containing CN/FL/04 H3 as the test antigen. At CSU, sera were pretreated with RDE and tested with 0.5% chicken RBCs and A/canine/Boulder/148902/2006.

## Results

### Assessing the diagnostic performance of the H3N8 CIV HI assay

For the 595 serum samples, the HI antibody titers ranged from 4 to 2,048, and the MN antibody titers ranged from 8 to 12,540. The Spearman rank order correlation coefficient comparing the H3N8 CIV HI and MN assay antibody titers was 0.83 ($P < 0.001$). The ROC analysis resulted in an area under the curve of 0.984 (95% confidence interval = 0.975–0.992; $P < 0.001$). The ROC-generated sensitivity, specificity, and positive and negative predictive values are shown in Table 1. Based on a sensitivity of 99.6% and specificity of 94.6%, the optimal cutoff antibody titer for HI seropositivity was 32. At this cutoff, the positive predictive value was 99.0%, and the negative predictive value was 97.8%. There were 7 (1.2%) samples that had discordant HI and MN results: 5 samples were seropositive by HI but not MN, and 2 samples were seropositive by MN but not HI.

The %CV for the H3N8 CIV HI intra-assay variability testing was 4.1%. The %CV for the interassay variability testing was 3.0%. For all replicates, the HI antibody titers correctly classified the dogs as H3N8 CIV infected or uninfected using a cutoff titer of 32.

### Effect of test parameter variations on diagnostic performance of the H3N8 CIV HI assay

**RBC binding preferences of H3N8 CIV.——**To determine the SA binding preference for H3N8 CIV, RBCs from different species were tested in an HA assay. The HAU for H3N8 CN/FL/04 with various RBC species are summarized in Table 2. The HAU were highest with chicken and turkey RBCs, followed by goose and canine RBCs. Incubation temperature and addition of BSA to the viral suspension had little effect on HAU for each RBC species.

**Use of different RBC species in the H3N8 CIV HI assay.——**To examine the effect of RBC source on HI assay performance, RDE-treated sera from 11 H3N8 CIV-infected dogs and 14 uninfected dogs were incubated with chicken, turkey, or canine RBCs. Regardless of the RBC source, all of the samples from uninfected dogs had HI antibody titers of 4, and all of the samples from infected dogs had HI antibody titers ≥32. As shown in Table 3, the HI antibody titers for infected dogs were 2- to 8-fold higher when tested with chicken or turkey RBCs compared to canine RBCs; the geometric mean antibody titer (GMT) was significantly lower ($P < 0.001$) when canine RBCs were used.

An additional 46 serum samples from dogs infected with H3N8 CIV were RDE-treated and tested with 0.5% chicken or turkey RBCs in the H3N8 CIV HI assay. The HI antibody titers ranged from 32 to 1,024. In general, the HI antibody titers between the 2 RBC sources were equivalent; however, 30% (14/46) of the samples had a 2-fold lower titer with chicken RBCs compared to turkey RBCs, resulting in 1 dog being misclassified as seronegative (data not shown).

**Use of different serum treatments in the H3N8 CIV HI assay.——**The HI antibody titers for untreated and RDE-treated sera from 11 H3N8 CIV-infected dogs and 15 uninfected dogs are shown in Table 4. When turkey RBCs were used in the HI assay, all of the dogs were correctly classified as uninfected or infected regardless of whether the sera were untreated or treated with RDE. When chicken RBCs were used in the HI assay, 13 of 15 (87%) of the uninfected dogs were false positive when the serum was not treated with RDE. Regardless of RBC source, most of the infected dogs had titers that were 2- to 4-fold higher in untreated sera. In comparing RDE to periodate treatment for samples from 4 uninfected and 6 infected dogs, the HI antibody titers were equivalent for the uninfected dogs (data not shown). The titers for periodate-treated sera were 2-fold lower than RDE-treated samples from the infected dogs, but there were no false-negative results.

**Use of different virus isolates in the H3N8 CIV HI assay.——**Sera from 14 H3N8 CIV-infected and 14 uninfected dogs were tested in the HI assay with infectious H3N8 CN/FL/04 or virus inactivated with BEI. The HI antibody titers were equivalent or 2-fold lower when inactivated CIV was used (data not shown). The lower titers with the inactivated virus resulted in false-negative results for 3 of 4 samples from infected dogs that had HI titers near the cutoff for seropositivity.

To evaluate consistency in antibody titers when different H3N8 CIV isolates were used in the HI assay, sera from 46 dogs infected with H3N8 CIV between 2004 and 2005 were tested using 5 H3N8 CIV isolates (CN/Jax/05, CN/CO/06, CN/CO/07, CN/CO/09, CN/PA/09) acquired from dogs in different geographic regions from 2005 to 2009 (data not shown). The HI antibody titers for these samples ranged from 32 to 1,024 when tested with the CN/FL/04 isolate. The HI titers with the 5 other isolates were equivalent to or 2-fold lower than the titers with the CN/FL/04 isolate, but there were no false-negative results.

To determine whether H3N8 EIV isolates could be substituted for canine isolates in the HI assay, sera from 46 infected dogs were tested with 4 H3N8 EIV isolates (EQ/KY/91, EQ/KY/95, EQ/NY/99, EQ/OH/03) acquired from horses in different geographic regions from 1991 to 2003 (data not shown). The HI antibody titers to EQ/OH/03 were equivalent to or within 1 dilution of those obtained with the CN/FL/04 isolate, but no dogs were false negative. The HI titers to EQ/NY/99 were 2- to 4-fold lower than those with the CN/FL/04 isolate, resulting in misclassification of 4 infected dogs (8.7%) as uninfected. The HI titers to EQ/KY/95 and EQ/KY/91 were 4- to 8-fold lower than those for the CN/FL/04 isolate, and 8 infected dogs (17.4%) were misclassified as uninfected.

**Interlaboratory H3N8 CIV HI assay comparison.——**A panel of 32 canine sera tested at UF was also evaluated in a blinded fashion by H3N8 CIV HI assays at CU AHDC and CSU (data not shown). The laboratories differed in the use of chicken or turkey RBCs, serum treatment with periodate or RDE, and the source of H3N8 CIV. Of these samples, 20 were identified as seronegative and 9 were identified as seropositive at all 3 laboratories. The HI titers were equivalent or within 1 dilution for all but 1 of the 9 seropositive samples; however, the titer difference did not result in misclassification. For 3 samples, the HI titers were 16 or 32 depending on the laboratory, suggesting that samples with titers near the seropositivity cutoff may be classified as negative or positive by different laboratories.

## Discussion

Using a panel of field sera collected during canine respiratory disease outbreaks, the present study demonstrated a high correlation between H3N8 CIV antibody titers determined in MN and HI assays performed as described in previous reports.[4,22] The ROC analysis results determined that the H3N8 CIV HI assay is highly sensitive and specific when the cutoff antibody titer for seropositivity is 32. The precision of the HI assay was demonstrated by %CVs <5% for intra-assay and interassay replications. Based on the evaluation of test parameter variations, the optimal sensitivity and specificity of the HI assay depended on 1) treatment of serum samples with RDE or periodate, 2) use of 0.5% turkey or chicken RBCs, and 3) use of infectious H3N8 CIV isolates or contemporary H3N8 EIV isolates that are closely related to CIV.

The HI assay is considered the WHO/World Organization for Animal Health (OIE) test of choice for serological diagnosis of influenza A infection in animals.[32,33] The seropositivity cutoff titer of 32 for the H3N8 CIV HI assay described in the current study provides the highest sensitivity (99.6%) and specificity (94.6%). Lower cutoff values increased the number of false-positive findings and decreased specificity. Sensitivity and specificity are fixed characteristics of diagnostic tests, whereas the predictive values of a positive or negative test result are affected by the prevalence of the tested parameter. However, a benefit of ROC analysis is that it allows an estimate of diagnostic accuracy that is independent of prevalence.[11] Therefore, in locations where H3N8 CIV is known to circulate, the HI assay described in the present study is a reliable and sensitive diagnostic test as long as the test antigen is representative of contemporary circulating viruses and sera are collected at proper time points. When a single serum sample is tested, seronegative results may be true negative due to lack of infection or false negative if the sample was collected prior to the appearance of antibodies. Seropositive results are most likely true positive, indicating infection at some time in the past. For large-scale serological surveys to determine H3N8 CIV seroprevalence, especially in populations where virus circulation is unknown, the HI assay is recommended as a sensitive and specific confirmatory test of high-throughput screening assays such as competitive nucleoprotein enzyme-linked immunosorbent assays.[5,10]

Use of RBC species other than the turkey or chicken in the H3N8 CIV HI assay decreased sensitivity. Turkey and chicken RBCs provided comparable results for most samples tested, but nearly one third of the samples had a 2-fold lower titer with chicken RBCs. While this is within the acceptable margin of error (i.e., 1 serum dilution), samples with antibody titers near the seropositivity cutoff may test false negative with chicken RBCs. In this situation, it may be prudent to test another sample collected 2 weeks later before declaring the infection status.

A variety of nonspecific hemagglutinins and inhibitors of HA have been identified in animal and human sera, and limited studies with few samples have shown that canine serum may have low levels of nonspecific hemagglutinins for avian RBCs and nonspecific inhibitors of HA for some influenza viruses.[1,30] The presence of nonspecific agglutinins to avian RBCs was not specifically addressed in the current study; however, based on the reported comparison testing between the 3 laboratories, HI antibody titers were comparable (i.e.,

equivalent or within 1 serum dilution) whether or not sera were preadsorbed with turkey RBCs. This suggests that nonspecific agglutinins do not appear to be a significant concern with canine sera. The present study did demonstrate, however, that serum treatment is required to inactivate nonspecific inhibitors of agglutination in canine serum. Nonspecific inhibitors of HA were demonstrated by higher antibody titers in untreated sera from infected dogs compared to titers obtained after RDE or periodate treatment and heat inactivation. Nonspecific inhibitors of HA were particularly evident when untreated sera were tested with chicken RBCs rather than turkey RBCs: chicken RBCs resulted in false-positive reactions for nearly all of the truly uninfected dogs tested. Therefore, to maintain high specificity, canine sera should be treated with RDE or periodate and heat inactivation prior to testing in the H3N8 CIV HI assay.

Although use of inactivated H3N8 CIV in the HI assay would be advantageous to reduce biosafety risks to laboratory personnel and generation of biohazardous waste, BEI-inactivated virus reduced the sensitivity of the HI assay when testing sera from infected dogs with antibody titers near the cutoff for seropositivity. In addition, RBC streaming was at times more difficult to interpret than with infectious viruses. Binary ethylenimine treatment is supposed to preserve viral proteins; however, it is not known if this alkylating treatment induces hemagglutinin protein folding or cross-linking that could reduce exposure to antigenic epitopes. Further analysis of viral inactivation methods, such as use of β-propiolactone, is warranted before inactivated H3N8 CIV could be reliably substituted for an infectious virus in the HI assay.

The use of multiple H3N8 CIV isolates recovered from dogs in 2005 to 2009 did not alter the sensitivity and specificity of the HI assay. Molecular and serological analyses indicate that significant antigenic change has not occurred in H3N8 CIV isolates to date.[2,22,24] If antigenic drift does occur in the future, then use of earlier isolates in HI testing of sera from dogs exposed to drifted viruses will compromise the sensitivity of the assay, leading to misdiagnosis of truly infected dogs. This underscores the value of continual molecular monitoring of new isolates to maintain diagnostic test accuracy.

Many diagnostic laboratories have H3N8 EIV isolates in storage for serological diagnosis of influenza-infected horses, and some have attempted to use these isolates for diagnosis of H3N8 CIV infection. Phylogenetic analyses of H3N8 CIV and EIV isolates have shown that the canine viruses are most closely related to contemporary H3N8 EIV isolates of the Florida sublineage (clade 1) recovered from horses since 2000.[4,13,22,24] This genetic relatedness likely explains why use of a 2003 clade 1 Florida sublineage EIV isolate (EQ/OH/03) in the CIV HI assay did not affect sensitivity and specificity, but use of earlier American lineage H3N8 EIV isolates resulted in decreased sensitivity and misdiagnosis of truly infected dogs. While avian, swine, and human H3 isolates were not evaluated in the present study, it is assumed that their use in the HI assay would not identify dogs infected with H3N8 CIV based on the genetic disparity between the H3 proteins.[4] A previous report demonstrated that HI antibody titers for dogs truly infected with H3N8 CIV were ˂16 when tested with human H3N2 viruses.[4]

The binding preferences of H3N8 CIV were assessed in HA assays with a variety of avian and mammalian RBCs. This is important not only to selection of the best RBCs for use in the HI assay to maintain diagnostic accuracy, but the agglutination patterns for RBCs from different species can also provide insight into viral specificities for SA receptors.[16] The current study demonstrated that H3N8 CIV preferred avian and canine RBCs, which primarily have SAα2,3-Gal and SAα2,6-Gal receptors with *N*-acetylneuraminic acid (Neu5Ac) as the SA species.[12,16,21,35] The greater agglutination of chicken or turkey RBCs compared to canine RBCs may be due to a relative altered density of these SA receptors on canine RBCs. There was less viral agglutination of horse and ruminant RBCs, which primarily express SAα2,3-Gal receptors with *N*-glycolylneuraminic acid (Neu5Gc) as the SA species.[16] H3N8 EIV binds both Neu5Acα2,3-Gal and Neu5Gcα2,3-Gal receptors, but Neu5Gcα2,3-Gal receptors are required for viral entry and subsequent replication in the equine epithelial cell.[29] The RBC agglutination patterns of H3N8 CIV suggest that the canine H3 protein prefers Neu5Acα2,3-Gal receptors over Neu5Gcα2,3-Gal receptors. This supports the findings of a 2010 report showing reduced binding of H3N8 CIV to Neu5Gcα2,3-Gal receptor analogs.[34] Although more sophisticated methods such as glycan microarrays have been developed for assessing viral SA receptor preferences, results from the current study also suggest that the altered SA preference of CIV compared to EIV may be an important adaptation of the horse virus to the dog. This adaptation may be associated with the predictable amino acid substitutions near the H3 receptor-binding pocket that have become the molecular signature for H3N8 CIV.[4,22,24]

In conclusion, the HI assay described in the present study is a highly sensitive and specific assay for serological diagnosis of H3N8 CIV infection in dogs. Misdiagnosis of H3N8 CIV-infected dogs will be minimized by using 0.5% turkey or chicken RBCs, serum treated with RDE or periodate and heat inactivation, infectious H3N8 CIV isolates, and a seropositivity cutoff titer of 32. The precision of the HI assay was demonstrated by %CVs ˂5% for intra-assay and interassay replications. The best diagnostic approach for a recent active infection is to perform the HI assay on paired serum samples, with the first sample collected during the first week of illness or known exposure followed by another sample 2 to 4 weeks later, to document seroconversion as defined by at least a 4-fold increase in titer over time. Moving forward, it will be important to continue monitoring for antigenic drift in the CIV H3 protein to ensure that the H3N8 CIV isolate used in the HI assay is closely related to that in circulation. In addition, accurate knowledge of vaccination history is now imperative to accurately interpret H3N8 CIV HI test results because the assay cannot differentiate antibodies induced by an infection from vaccination with the inactivated whole H3N8 CIV vaccine.[7] This limits the use of the HI assay in future epidemiological studies of H3N8 CIV prevalence in the United States if vaccination status is unknown.

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**Table 1.**

Receiver operating characteristic analysis of antibody titers determined for 595 canine sera in the canine *Influenza A virus* subtype H3N8 hemagglutination inhibition (HI) assay using the microneutralization antibody titers as the gold standard.*

| HI antibody titer | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | True positive | True negative | False positive | False negative |
|---|---|---|---|---|---|---|---|---|
| 4 | 100.0 (99.1–100.0) | 0.0 (0.0–4.9) | 84.4 | — | 502 | 0 | 93 | 0 |
| 16† | 99.6 (98.4–100.0) | 92.5 (84.9–96.5) | 98.6 | 97.7 | 500 | 86 | 7 | 2 |
| 32 | 99.6 (98.4–100.0) | 94.6 (87.6–97.9) | 99.0 | 97.8 | 500 | 88 | 5 | 2 |
| 64 | 97.6 (95.8–98.7) | 95.7 (89.0–98.6) | 99.2 | 88.1 | 490 | 89 | 4 | 12 |
| 128 | 91.2 (88.4–93.4) | 95.7 (89.0–98.6) | 99.1 | 66.9 | 458 | 89 | 4 | 44 |
| 256 | 67.1 (62.9–71.1) | 98.9 (93.5–100.0) | 99.7 | 35.8 | 337 | 92 | 1 | 165 |
| 512 | 39.4 (35.3–43.8) | 98.9 (93.5–100.0) | 99.5 | 23.2 | 198 | 92 | 1 | 304 |
| 1,024 | 18.3 (15.2–22.0) | 100.0 (95.1–100.0) | 100.0 | 18.5 | 92 | 93 | 0 | 410 |
| 2,048 | 0.6 (0.1–1.8) | 100.0 (95.1–100.0) | 100.0 | 15.7 | 3 | 93 | 0 | 499 |

*Numbers in parentheses are 95% confidence intervals. HI antibody titer = reciprocal of the last serum dilution that inhibited red blood cell agglutination by canine *Influenza A virus* subtype H3N8; PPV = positive predictive value; NPV = negative predictive value; — = not calculated.

†No sera had an HI antibody titer of 8; therefore, it is not included in the analysis.

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**Table 2.**

Canine *Influenza A virus* subtype H3N8 (H3N8 CIV) agglutination of avian and mammalian red blood cells (RBCs) under different incubation conditions.

| | | H3N8 CIV HAU‡ | | | |
|---|---|---|---|---|---|
| | | 23°C × 30 min | | 4°C × 60 min | |
| RBC source* | RBC SA† | PBS | PBS/BSA | PBS | PBS/BSA |
| Chicken | 2,3/2,6 Neu5Ac | 32 | 16 | 32 | 16 |
| Turkey | 2,3/2,6 Neu5Ac | 16 | 16 | 16 | 16 |
| Goose | 2,3/2,6 Neu5Ac | 8 | 8 | 8 | 8 |
| Dog | 2,3/2,6 Neu5Ac | 8 | 16 | 16 | 16 |
| Horse | 2,3 Neu5Gc | 2 | – | 2 | – |
| Sheep | 2,3 Neu5Gc | 2 | 1 | 2 | 2 |
| Cow | 2,3 Neu5Gc | – | – | 1 | 1 |

*Species source of RBCs used in the hemagglutination assays. Avian RBC suspensions were 0.5%, and mammalian RBC suspensions were 1.0% in either phosphate buffered saline (PBS) or PBS/bovine serum albumin (BSA).

†Predominant sialic acid (SA) linkages (2,3 and/or 2,6) and species (Neu5Ac = *N*-acetylneuraminic acid; Neu5Gc = *N*-glycolylneuraminic acid) on RBCs.[12,16,21,35]

‡Hemagglutinating unit (HAU) = reciprocal of the last dilution of H3N8 CIV that agglutinated RBCs. Negative indicates that no RBC agglutination occurred by undiluted H3N8 CIV suspension. – = negative.

**Table 3.**

Hemagglutination inhibition (HI) antibody titers for sera from 11 dogs infected with canine *Influenza A virus* subtype H3N8 (H3N8 CIV) tested with 0.5% chicken red blood cells (chRBC), 0.5% turkey RBC (tRBC), or 1% canine RBC (cnRBC).[*]

| | H3N8 CIV HI antibody titer | | |
|---|---|---|---|
| **Sample ID** | **chRBC** | **tRBC** | **cnRBC** |
| 1 | 256 | 64 | 32 |
| 2 | 256 | 128 | 64 |
| 3 | 128 | 128 | 64 |
| 4 | 256 | 256 | 64 |
| 5 | 256 | 512 | 64 |
| 6 | 128 | 128 | 64 |
| 7 | 256 | 256 | 128 |
| 8 | 256 | 512 | 64 |
| 9 | 256 | 512 | 128 |
| 10 | 256 | 512 | 128 |
| 11 | 512 | 512 | 64 |
| GMT | 240 | 256 | 73[†] |

[*]Sera were pretreated with receptor-destroying enzyme (RDE) and serial dilutions incubated with H3N8 CN/FL/04 isolate prior to the addition of RBC suspensions. All serum samples were tested in duplicate. HI antibody titer = reciprocal of the last serum dilution that inhibited RBC agglutination by H3N8 CIV. The cutoff titer for seropositivity in the H3N8 CIV HI assay is 32. Geometric mean antibody titer (GMT) is for 11 dogs.

[†]The GMT for cnRBC is significantly different from chRBC and tRBC ($P < 0.001$).

**Table 4.**

Hemagglutination inhibition (HI) antibody titers for untreated or receptor-destroying enzyme (RDE)–treated sera from 11 dogs infected with canine *Influenza A virus* subtype H3N8 (H3N8 CIV) and 15 uninfected dogs.*

| | | H3N8 CIV HI antibody titer | | | |
|---|---|---|---|---|---|
| | | Turkey RBC | | Chicken RBC | |
| Sample ID | CIV status | Untreated | RDE-treated | Untreated | RDE-treated |
| 1 | Uninfected | 4 | 4 | 32 | 4 |
| 2 | Uninfected | 4 | 4 | 32 | 4 |
| 3 | Uninfected | 4 | 4 | 64 | 4 |
| 4 | Uninfected | 4 | 4 | 32 | 4 |
| 5 | Uninfected | 4 | 4 | 32 | 4 |
| 6 | Uninfected | 4 | 4 | 32 | 4 |
| 7 | Uninfected | 4 | 4 | 32 | 4 |
| 8 | Uninfected | 4 | 4 | 64 | 4 |
| 9 | Uninfected | 4 | 4 | 32 | 4 |
| 10 | Uninfected | 4 | 4 | 4 | 4 |
| 11 | Uninfected | 4 | 4 | 32 | 4 |
| 12 | Uninfected | 4 | 4 | 64 | 4 |
| 13 | Uninfected | 4 | 4 | 32 | 4 |
| 14 | Uninfected | 4 | 4 | 32 | 4 |
| 15 | Uninfected | 4 | 4 | 4 | 4 |
| 16 | Infected | 512 | 64 | 256 | 256 |
| 17 | Infected | 512 | 128 | 256 | 256 |
| 18 | Infected | 512 | 128 | 256 | 128 |
| 19 | Infected | 256 | 128 | 128 | 128 |
| 20 | Infected | 512 | 256 | 512 | 256 |
| 21 | Infected | 512 | 256 | 512 | 256 |
| 22 | Infected | 512 | 512 | 256 | 256 |
| 23 | Infected | 1,024 | 512 | 1,024 | 256 |
| 24 | Infected | 512 | 512 | 512 | 256 |
| 25 | Infected | 1,024 | 512 | 1,024 | 256 |

| | | H3N8 CIV HI antibody titer | | | |
|---|---|---|---|---|---|
| | | Turkey RBC | | Chicken RBC | |
| Sample ID | CIV status | Untreated | RDE-treated | Untreated | RDE-treated |
| 26 | Infected | 512 | 512 | 512 | 256 |

*Serial dilutions of the untreated or RDE-treated sera were incubated with H3N8 CN/FL/04 isolate prior to the addition of 0.5% turkey or chicken red blood cell (RBC) suspensions. All serum samples were tested in duplicate. H3N8 CIV infection status was verified in both the HI and serum microneutralization assays. HI antibody titer = reciprocal of the last serum dilution that inhibited RBC agglutination by H3N8 CIV. The cutoff titer for seropositivity in the H3N8 CIV HI assay is 32. Sera were either untreated or treated with RDE prior to incubation with the virus.