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Full Length Research Paper

Serologic detection of Avian influenza H5 antibodies using a competitive enzyme-linked immunosorbent assay (ELISA)

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A competitive enzyme-linked immunosorbent assay (cELISA) for avian influenza (AI) H5 specific antibody detection was previously developed. In this study, additional serum samples from infected and vaccinated birds were tested and the results were compared to other serological tests. Using the samples from experimentally infected chickens, the H5 cELISA was shown to be comparable to other serological assays. Using samples from free-living blue-winged teals and vaccinated birds, a correlation was found between the H5 cELISA and the hemagglutination-inhibition (HI) assay. In conclusion, the H5 cELISA will be a useful tool for the serological diagnosis, surveillance of AI H5 infections, and for measuring of protective antibody levels.

Key words: Avian influenza, serologic diagnosis, competitive enzyme-linked immunosorbent assay, protective antibody.

INTRODUCTION

Avian influenza viruses (AIV) are classified as either highly pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI), based on their virulence in domestic poultry. During the past decade, HPAI H5N1 viruses emerged in Asia causing outbreaks in poultry and cross-species transmission to humans (Ellis et al., 2004; Chen et al., 2005; Liu et al., 2005; Gilbert et al., 2006; Kilpatrick et al., 2006). AIV of the H5 subtype is of concern to animal health due to the fact that the H5 subtype is one of two hemagglutinin (HA) subtypes capable of becoming HPAI (Senne et al., 1996; Spackman, 2008). The continued global spread of Eurasian HPAI H5N1 and the increasing fear over the pandemic potential of these viruses demonstrate the need to improve and enhance AI virus detection methodologies. The rapid and early detection of H5 subtype infections is a key to the control of the disease. Antibody detection is widely used for evaluating and confirming prior virus exposure. Competitive enzyme-linked immunosorbent assays (cELISAs) are commonly used for specific antibody detection, due to their sensitivity and simplicity. The significant advantages are: (i) cELISAs are easy to perform and scale up to accommodate the screening of large numbers of sera; (ii) the use of a recombinant antigen negates the need to work with live AI H5 viruses in the Bio-safety laboratory level-3 containment; (iii) cELISAs do not normally require the highly purified antigens needed in indirect ELISAs; (iv) cELISAs are suitable for the detection of antibodies from different species eliminating the need for special reagents.

Several AI nucleoprotein (NP)-based cELISAs have been reported, validated and commercialized (Shafer et al., 1998; Starick et al., 2006; Zhou et al., 1998; Song et al., 2009). However, the NP cELISA is a non-subtype specific approach. Type A influenza seropositive water

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waterfowls (wild and domestic) are commonly found (Abdel-Ghafar et al., 2008), indicating that the birds have encountered an influenza A virus, but no information on the AI virus subtype can be deduced. The H5-specific cELISA can more rapidly identify whether an H5 subtype virus is circulating than other nonsubtype-specfic tests. Prabakaran et al. (2009) described an H5 cELISA using a mAbs which recognized a linear epitope located in the HA1 region of H5 HA. Recently, a similar method using a baculovirus-expressed recombinant H5 and a mAb in a cELISA has been reported (Dlugolenski et al., 2010), but, they found lower sensitivity for chicken sera. In the present study, more serum samples collected from infected and vaccinated birds were tested using a newly developed H5 cELISA. In this cELISA, a recombinant H5 antigen and a monoclonal antibody (mAb) reacted with a conformational epitope located in the H5 HA were used (Yang et al., 2009). The cELISA results were compared to other serological tests for the AI H5 specific antibody detection.

MATERIALS AND METHODS

Recombinant H5 and monoclonal antibody

The recombinant AI H5 antigen and the mAb (#9, F37H5N1-45) used in the cELISA were generated as described previously (Yang et al., 2009).

Competitive ELISA

Briefly, microtiter plates (Nunc-Immunoplate Maxisorp, Roskilde, Denmark) were coated with 100 µl/well (10µg/ml) of recombinant H5 in carbonate buffer (pH 9.6) overnight at 4°C. After washing, equal volumes (50 µl) of diluted test sera (1:5) and hybridoma culture supernatants (1:500) were added to the plates and incubated at 37ºC for 1 h with agitation. Then HRP-conjugated anti-mouse IgG (1:3000, Jackson Immuno-Research Laboratories, West Grove, PA) was added and incubated at 37ºC for 1 h with subsequent washing. TMB substrate (Sigma-Aldrich, St Lucia, MO), was added and colour development was stopped after 15 min with 50 µl/well of 2.0 M sulphuric acid. The OD was determined using an automated plate reader (Photometer Multiskan Reader, Labsystems, Foster, VA). Results were expressed as a percentage of inhibition and derived using the following formula: Percentage of inhibition (PI) = [(negative reference serum OD–test sample OD)/ (negative reference serum OD–positive reference serum OD)] × 100%. A cut-off value of 40% was established based on the negative sera tested.

The positive control serum used in the cELISA was from a Canada goose vaccinated with H5N2-A/mallard/British Columbia/373/2005 (Pasick et al., 2007). The negative control chicken serum was obtained from Sigma-Aldrich, St Lucia, MO (Cat. No. C5405).

Negative sera

To determine diagnostic specificity of the cELISA, 161 true negative sera (158 chicken and 3 duck) were collected by the Chinese Animal Health and Epidemiology Center, Shandong, China. An additional 174 negative control sera were collected from domestic poultry submissions by different laboratories in Canada. All negative sera were classified as such based on the Hemaggutination-Inhibition (HI) assay results.

Sera from experimentally infected chickens

Ten chickens were infected with 10^6 50% egg infectious doses (EID50) of A/Ty/CA/35621/1984-H5N3. Sera from infected chickens were collected at 0, 7, 14, 21 and 28 days post infection (dpi).

Sera from wild waterfowl

The 50 serum samples were collected from free-living blue-winged teals (Anas discors) in Alberta, Saskatchewan, and Manitoba (Environment Canada, under a Canadian wildlife Service Permit 10-MB/SK/AB/ON/NS/PE-S008).

Sera from vaccinated birds

179 chickens, 12 ducks and 13 geese were immunized with H5 vaccines at lease three times. The sera were collected by the Chinese Animal Health and Epidemiology Center, Shandong, China. The other 180 chickens were vaccinated with DNA vaccines (pCAG-H5), containing a H5 gene of A/Hanoi/30408/2005.

Hemaggutination-Inhibition assay

The HI assay was performed using the standard procedure. All serum samples were examined using 4 HA units of A/duck/British Columbia/26-6/2005-H5N2 or A/chicken/Vietnam/14/2005-H5N1 as test antigen. HI titers were regarded as positive when a serum dilution ≥ 1/16 (2^4) inhibited the agglutination of chicken erythrocytes with 4 HA units of test antigen (World Organisation for Animal Health, 2008).

RESULTS AND DISCUSSION

The negative serum samples (n=335), as determined by the HI assay, were examined using the H5 cELISA. A cut-off value of 40% inhibition was established which provided a clear distinction between positive and negative sera. All 335 negative samples tested are within this range (<40% inhibition). To determine immune response kinetics, sera from the ten experimentally infected chickens were tested using the NP cELISA (Zhou et al., 1998), the agar-gel immunodiffusion (AGID) using the antigen obtained from SPAFAS (Charles River SPAFAS, North Franklin, CT, USA) (World Organisation for Animal Health, 2008), the IDEXX indirect ELISA kit for influenza antibody detection (IDEXX Laboratories Inc., Maine, USA), the HI assay and the H5 cELISA (Yang et al., 2009). In the HI assay, serum samples were examined using the homologous antigen. The H5 cELISA results were compared with those obtained from three other diagnostic tests (Table 1). Three of the eight chickens tested showed positive antibody responses (37.5%) at 7 dpi in the H5 cELISA. The NP cELISA

Table 1. Comparison of the H5 cELISA to other serological assays for experimentally infected chickens.

DPI	NP cELISA		H5 cELISA		IDEXX ELISA		AGID		HI*	
	P/T**	Positive (%)	P/T**	Positive (%)	P/T**	Positive (%)	P/T**	Positive (%)	P/T**	Positive (%)
0	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
7	6/8	75	3/8	37.5	4/8	50	4/8	50	0/8	0
14	9/10	90	9/10	90	9/10	90	8/10	80	6/7	85.7
21	8/9	88.9	8/9	88.9	8/9	88.9	8/9	88.9	5/5	100
30	8/9	88.9	8/9	88.9	8/9	88.9	8/9	88.9	5/5	100

*A/Ty/CA35621/1984-H5N3 was used as the antigen, P/T**: number of sera positive/number of sera tested.

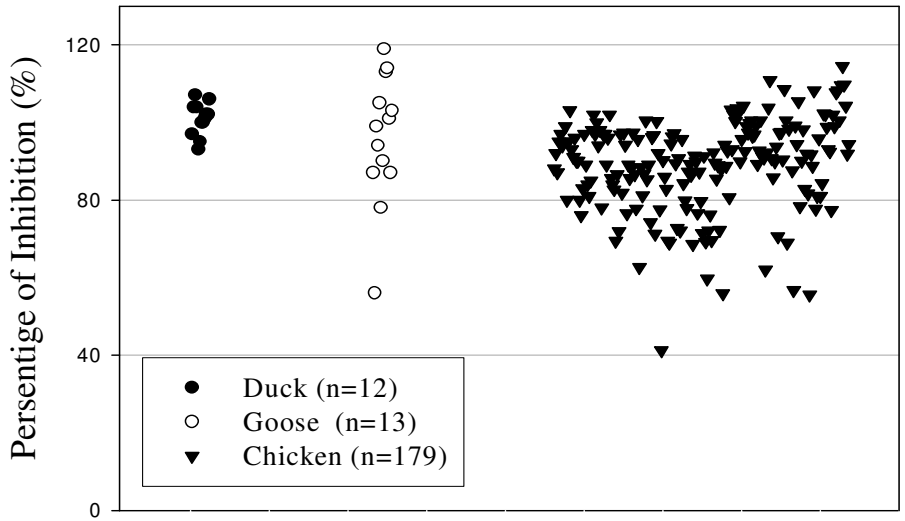

Figure 1. The H5 cELISA results for the serum samples from vaccinated ducks, geese and chickens. Recombinant baculovirus H5 was coated onto microtiter plates. Equal volumes (50 µl) of test sera (1:5) from vaccinated birds and mAb #9 (1:1000) were added to the plates and allowed to compete at 37℃ for 1 h with agitation. Then HRP conjugated anti-mouse IgG was added and incubated at 37℃ for 1 h with subsequent washing. The OPD was added and colour development was stopped after 15 min with 50µl/well of 1.0 M sulphuric acid. The OD was determined at 490 nm on an automated plate reader. Results were expressed as a percentage of inhibition and derived by the following formula: Percentage of inhibition (PI) = [(negative reference serum OD–test sample OD)/ (negative reference serum OD–positive reference serum OD)] × 100%. The cut-off value was established at 40% of inhibition base on the negative sera tested.

detected a positive reaction in six out of the eight chickens (75%) at the same time point. Seroconversion rates in the early stage of infection as determined by H5 cELISA and HI assay were lower than those detected by the NP-cELISA (75%), the IDEXX indirect ELISA (50%) and the AGID (50%). Yewdell et al. (1985) and Prokudina et al. (1991) demonstrated that kinetics of the HA accumulation are different from those of cell surface and extracellular NP accumulation. They indicated that NP protein is expressed on the surface of virus infected cells before HA. As a result, the antibody against NP protein appears earlier than HA explaining why seroconversion, as determined for NP specific antibodies, is detected earlier than the H5 specific antibodies as determined by the H5 cELISA and the HI assay. After 14 dpi, all five tests - NP cELISA, H5 cELISA, IDEXX indirect ELISA, AGID and HI assay produced identical results. Our results are consistent with the report published by Katz et al. (1999) which indicated that the kinetics of the antibody response in H5N1-infected persons was usually detected 14 days after symptom onset. Using a microneutralization assay, antibodies against H5N1 virus were also detected 14 days after the beginning of symptoms in humans.

To assess the H5 cELISA's ability to detect H5 specific antibodies in field samples, 50 sera collected from wild waterfowl were tested and compared with results of the HI assay (antigen: A/Dk/BC26-6/05-H5N2). Five of the 50 serum samples showed positive results by both H5 cELISA and HI assay indicating that these five birds have been infected with AI H5 viruses previously. Another 5 samples were negative for H5 antibodies by HI assay, but produced percentage inhibition values in the positive range with the H5 cELISA (>40%). The negative results obtained from the HI assay could be due to the use of a non-homologous antigen on the field infecting virus.

The HI antibody test is a good method for measuring the amount of protective antibody produced in an immune response, which is important for evaluating levels of protection in response to vaccination (Suarez and Schultz-Cherry, 2000; de Jong et al., 2003). In comparison, antibodies against NP are not neutralizing and therefore do not prevent infection, and are thus not a good measure of protection (Suarez and Schultz-Cherry, 2000; Qiao et al., 2003). To evaluate the correspondence between the HI assay and the H5 cELISA in sera from vaccinated birds, the correlation between the percentage of inhibition and HI titer was examined. The sera from the vaccinated birds (n=204) were collected and then tested by the H5 cELISA and the HI assay where the homologous strain to the vaccine was used as the test antigen. Both the H5 cELISA and the HI assay identified all 204 sera from vaccinated birds as positive (Figure 1). The observation that some sera demonstrated greater than 100% inhibition may be due to the fact that the positive reference serum used in the test was unable to fully inhibit binding of the mAb. It is important to note that a new cut off value would need to be selected if other laboratories do not use the same reference serum in the H5 cELISA.

The sera from the chickens vaccinated with DNA vaccines (pCAG-H5) were tested using the H5 cELISA and the HI assay. The correlations of coefficient (R^2) of determination values of 0.56 (P= 0.013) were established between these two assays (Figure 2). This demonstrates the potential of the H5 cELISA to evaluate seroprotection in vaccinated animals. Perez-Ramirez et al. (2010) found that a NP-cELISA test performed better on duck samples than on samples from other species. In contrast, the H5 cELISA performed equally well for chicken, duck and geese samples. The non-species specificity is a major advantage of the H5 cELISA. As only 12 duck and 13 geese samples were examined in this study, more sera will need to be tested in order to obtain full validation for the H5 cELISA.

The mAb (F37H5N1-45, #9) which competes with H5 infected sera for binding to recombinant H5 antigen is crucial to the high specificity requirement for H5 cELISA development. The mAb #9 did not show HI activity indicating that this antibody might not be in direct competition with the binding site of hemagglutinating antibodies. It is possible that the binding site for this mAb is located on HA1, but is not associated with the ligand binding site on sialic acid receptors presented on the surface of red blood cells. A similar observation has been reported (Yang et al., 2010). The mAb #9 used for assay development failed to recognize 4 of 13 H5 viruses as reported previously (Yang et al., 2009), presumably due to differences in the binding sites of these isolates. It is possible that the H5 cELISA would fail to determine antibodies in sera from animals infected with these viruses. It is also significant that two of the viruses not recognized by this mAb are recent isolates. This suggests limitations in the use of a 1966 virus strain for assay development, because of continuing antigenic evolution of the virus. Moreover, H5 viruses will continue to evolve, necessitating on-going validation of the assay to confirm its ability to detect antibodies against current H5 viruses. Another limitation of this assay might be that a single mAb is unable to compete with other H5 epitopes against which the polyclonal response would generally be generated. Therefore the assay will work only if the H5 strains that test animals were exposed to a shared epitope.

Conclusion

The H5 cELISA in combination with a recombinant antigen and a mAb offers a promising approach for rapid, safe and convenient H5 specific antibody detection. Both the recombinant H5 antigen and the mAb can be easily standardized, an important characteristic for diagnostic test quality control. This cELISA appears to be useful for general screening purposes and efficient for estimating

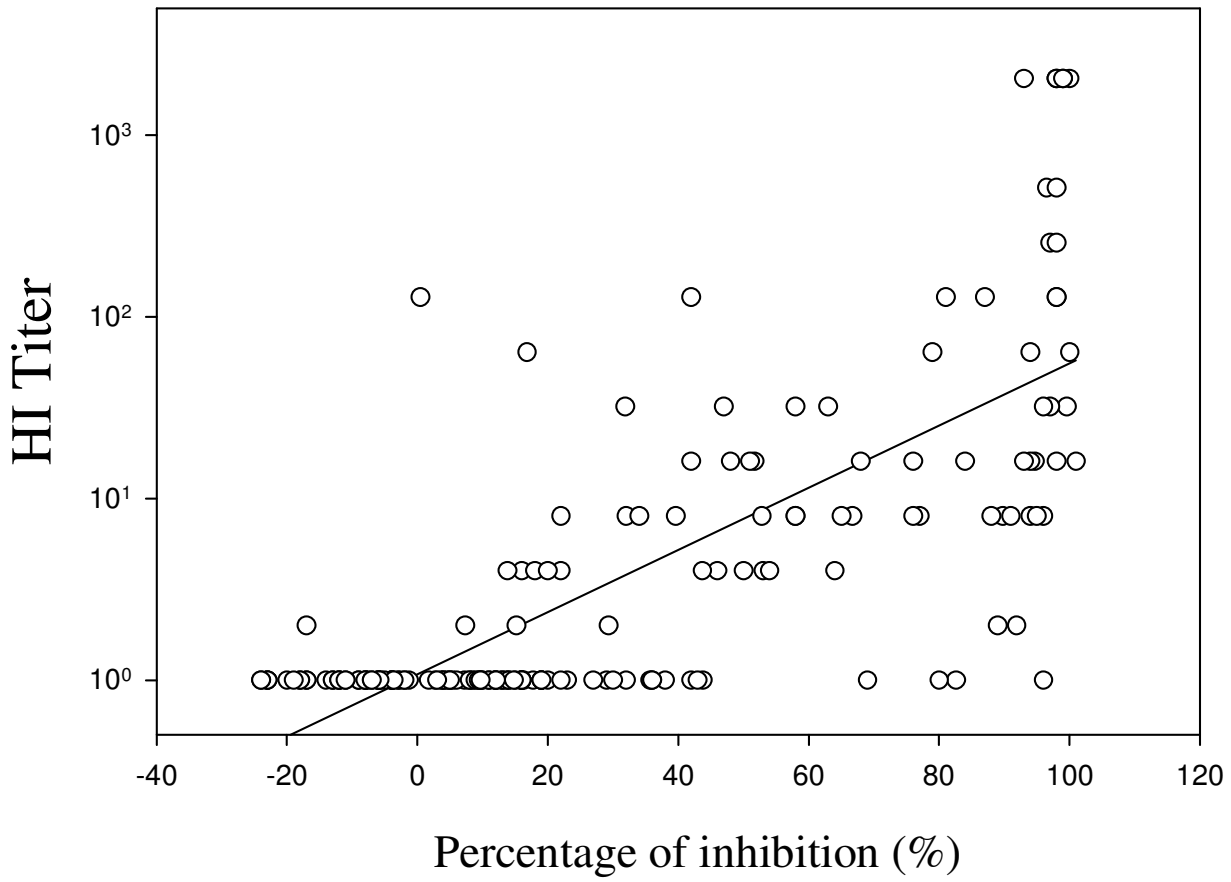

Figure 2. Correlations between the H5 cELISA and the HI assay for sera from vaccinated birds. Chickens (n=180) were vaccinated with a DNA vaccine (pCAG-H5). Correlations between the HI titer and the H5 cELISA were determined (R^2=0.56, P=0.013).

levels of protective antibody. The development of rapid and accurate serological techniques will provide laboratories with quick and definitive diagnoses to facilitate surveillance and disease control efforts against AI H5 subtype.

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