Standardization of ELISA for Detection of Avian Influenza Virus Antibodies

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Summary

An indirect enzyme-linked immunosorbent assay (ELISA) was developed for the rapid and efficient large scale screening of antibodies to avian influenza virus (AIV) infection in chicken. Antigen was a whole-purified influenza virus produced from the H9N2 subtype. Optimum dilution for goat anti-chicken conjugate to be used in the ELISA was 1:1000, as determined by signal-to-noise ratio. The antigen concentration was 0.375µg of protein per well, as determined by checkerboard titration. The sensitivity of the ELISA was compared with hemmaglutination inhibition test under field exposure. After testing of 656 field sera, the correlation coefficient for the results of two tests was significant ($r=0.929$, $P<0.001$). Testing 8 standard antisera of various subtypes (H1, H2, H4, H5, H6, H7, H9 and H10) of AIV and AIV antibody positive and negative sera determined specificity of the ELISA. Antisera to all 8-hemmaglutinin subtypes were strongly positive. Both the sensitivity and specificity of the ELISA was compared to the other test. Thus, the ELISA was able to detect specific AIV antibodies and suitable for screening large numbers of samples in diagnostic laboratories.

Key words: avian influenza, ELISA, HI, antibody

Introduction

Avian influenza has emerged as a disease with significant potential to disrupt commercial poultry production often resulting in extensive losses. Type A Orthomyxoviruses that are widely distributed in avian species causes avian influenza. In addition, mammals including seals and domestic swine can be infected and may play a role in the emergence of new strains of virus (Jordan 1990, Beard 1970). The hemagglutinin (H) and neuraminidase (N) are two structurally distinct envelope glycoproteins attached to the lipid bilayer to form the surface of the virion. The antigenic differences of H and N antigens of influenza viruses provide the basis of their classification into subtypes. Serologically there are 15H and 9N subtype

antigens (Fatunmbi *et al* 1989, Snyder *et al* 1985). Serologic surveillance for AIV is an essential part of early detection for prevention and eradication programs. In addition to agar gel precipitin (AGP) and hemmaglutination inhibition (HI) tests, which are commonly used, for detection of antibodies to AIV, various forms of enzyme immunoassays have recently been developed and proved to be more sensitive and specific than AGP and HI. The AGP test detects precipitating antibody produced primarily against the ribonucleoprotein of the virus, and is therefore type specific (Areans *et al* 1990, Snyder *et al* 1985, Meulemans *et al* 1987, Abraham *et al* 1986). The AGP test requires large quantities of reagents and 24-48h for results to be obtained. Furthermore, the AGP test may not be suitable as a universal assay for some other species of birds. Serum samples from waterfowl do not contain good precipitin antibodies (Beard 1970, Lamichhance & Kirkerggard 1997, Zhou *et al* 1997). The HI test detects antibodies produced against the hemmaglutinin surface glycoprotein of the virus, and is therefore subtype specific. The HI test is more sensitive and rapid than the AGP test, but is complicated due to the existence of 15 hemmaglutinin types of AIV. The indirect ELISA has been previously demonstrated to be an effective assay for detection of specific antibody resulting from exposure to many agents that affect poultry (Areans *et al* 1990, Meulemans *et al* 1987). To detect viral type specific antibodies an indirect ELISA using crude, purified and whole viral antigens on the solid-phase to detect viral type specific antibodies, primarily against the ribonucleoprotein (NP) and matrix proteins (MP) carry out. It is a suitable semi-automated test for screening large numbers of sample, making it attractive for use in a surveillance program (Abraham *et al* 1986, Areans *et al* 1990, Beck & Swagne 1997, Fatunmbi *et al* 1989, Snyder *et al* 1985).

During the recent outbreak of avian influenza (H9N2) in different parts of Iran, we were encouraged the development of a rapid and semi-automated ELISA as a surveillance tool for identification of chicken flocks infected with AIV. Thus, the objective of the present study was to prepare a reliable indirect ELISA for detection of AIV antibodies in chicken.

Materials & Methods

Virus strain. The avian influenza virus was isolated in Department of Poultry Diseases, Razi Vaccine & Serum Research Institute during the recent outbreak of avian influenza in Iran. The isolate was confirmed by CVL (Central Veterinary Laboratory Weybridge, UK) and coded as A/chicken/ Iran/ 259/ 1998 (H9N2). This

strain was propagated in specific poathogen free (SPF) (Lohman, Germany) emberyonated chicken eggs and used as antigen.

Preparation of the ELISA antigen. Avian influenza virus strain A /chicken /Iran /259 /1998 (H9N2) was propagated in 10-day-old SPF emberyonated chicken eggs. After 48h incubation at 37C, the infectious allantoic fluids were harvested and pooled. Freshly allantoic fluids were clarified of particulate host debris by centrifugation at 8000*g* for 20 min at 4C. Supernatants were freezed and thawed three times. Virus in the clarified fluid was precipitated at 4C with 8% (w/v) polyethylene glycol 6000 (PEG-6000). This solution was kept at 4C for 2h with gentle stirring. The mixture was then centrifuged at 10000*g* for 1h at 4C. The obtained pellet was suspended in appropriate volume of TEN buffer (0.01M Tris HCl [pH 7.4], 0.1M NaCl, 0.001M EDTA) and dialyzed against TEN buffers. It homogenized with the vortex shaker and later by sonication (1 min at a setting of 1 amp). Further purified by sucrose-gradient centrifugation (30-60%) at 280000*g* for 4h at 4C (Heyward *et al* 1977). The viral band was harvested and dialyzed against TEN buffer and its protein concentration was determined using the method of Lowry (1951). In order to disrupt virus particles and release internal antigens sodium dodecylsulphate (SDS) was added to a final 0.5% concentration incubated at 37C for 30 min. It was finally divided into aliquots, frozen at -70C and stored until use.

Conjugate titration. Conjugate (Sigma, Saint Louis, USA) was titerated, using a checkerboard titration method with 250 hemagglutination units of AIV belonging to subtype H9N2 (0.75μg of protein per well of coating buffer: 0.05M carbonate bicarbonate, pH 9.6). 100 microliters of this solution was used to coat wells of a micro-ELISA plate (Griener Labortechnik, Germany). Known AIV antibody positive and negative chicken sera were used. Wells in rows 1 to 6 were coated with increasing dilution of positive serum. Negative serum was added to wells in rows 7 to 12. The last row (H) was used as a conjugate control. The conjugate was diluted in wash buffer (PBS, pH 7.4, containing 0.05% Tween20) and used at a two-fold dilutions beginning from 1:400 dilution, rows A to G were coated with increasing dilution of conjugate. Absorbencies were read at 405nm on an ELISA reader. The optical density (OD) reading of AIV antibody positive and negative sera were evaluated at a single serum dilution, using the signal-to noise (S/N) ratio. The S/N ration was the ratio of observed OD reading of AIV antibody positive serum to the observed OD reading of AIV antibody negative serum, at the same dilution. The S/N ratio was evaluated for different conjugate dilutions.

Antigen titration. Antigen was titerated, using a checkerboard titration in which each row of wells from A to H was coated with different concentration 0.01-1.5μg per well. To the coated wells, known positive and negative sera were added. Predetermined optimal conjugate was used. Results were read as described above.

ELISA test. The enzyme immunoassay was performed in rigid polystyrene plates with 96 flat-bottomed wells. Wells were coated by passive adsorption, using 0.1ml purified antigen (0.375μg protein/well) diluted in carbonate bicarbonate buffer, pH 9.6 and incubated overnight at 4C. Fluid was dumped from antigen coated target plates, and the plates were tapped dry. A blocking solution (0.3ml/well) containing 1% bovine serum albumin and 0.05% Tween20 prepared in PBS was added and incubated for 90 min at 20C. Plates were then dumped, and all wells washed three times for 3 min with washing buffer. Serum was diluted 1:100 in dilution buffer (PBS containing 0.05% Tween20 and 0.25% bovine serum albumin) and 100ul of serum was incubated on the plate for 30 min. The plate was washed five times with washing solution. 100 microliters of HRP-labeled goat anti-chicken IgG (H+L) conjugate was incubated for 30 min at room temperature. The plate was washed again as described above. 100 microliters of ABTS (2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, Boehringer mannheim, Germany) substrate was incubated in the wells for 15 min. The substrate reaction was stopped using 1% stop solution (2.5M H_2SO_4). Optical density (OD) values are obtained at 405nm using a 96-well ELISA reader (Anthos 2001, anthos Labtech Instruments, Salzburg, Austria).

Antisera. A panel of antisera against various subtype of AIV (H1, H2, H4, H5, H6, H7, H9 and H10) was obtained from CVL, AIV antibody positive and negative sera was obtained from KPL (Kirkegaard and Perry Laboratories, OR Outside, USA) and assayed by ELISA.

HI tests. The HI test was a standard beta test (Beard *et al* 1989), using 4 hemagglutinin units, the first dilution beginning 1:10. The antigen used for the HI tests was prepared from β-propiolactone inactivated (0.01%) allantoic fluid harvested from chicken embryos incubated with the avian influenza strain A/ chicken /Iran /259/1998 (H9N2). A total of 656 sera from different poultry flocks were selected from various areas of Iran. All sera were tested by HI and ELISA.

Results

Conjugate and antigen titration. A peak of S/N ratio of 8 conjugate dilution was observed at 1:100 chicken serum dilution for the 1:800 conjugate dilution (Fig. 1).

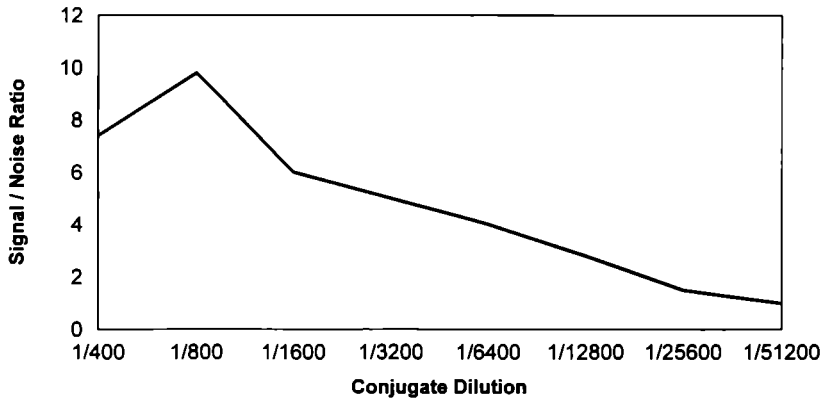

Figure 1. *Signal to noise ratio to determine optimal conjugate dilution*

The high absorbency value obtained in control wells at conjugate dilution of 1:400 was due to nonspecific attachment of conjugate to the antigen solid phase. A concentration dilution of 1:1600 had low absorbency values with control, but did not have a significant difference between values of the known AIV-positive and AIV-negative sera. The S/N ratio is the best method for detecting optimal conjugate dilution. The 1:1000 dilution was chosen because results of a subsequent experiment indicated low adsorption of the solid phase and a satisfactory difference between AIV-positive and negative sera. When a 1:1000 conjugate dilution was used, an optimum antigen concentration of 0.375µg of protein/well (128HU/well) was determined (Fig. 2).

Determination of positive/negative cut-off. After several control serum tested by ELISA, the mean of sample-to-positive (SP) ratio value plus two standard deviation (SD) of the controls serum was 0.278 [0.155+(2×0.0615)]. The cut-off for chicken sera was therefore set at 0.278 (Fig. 3).

ELISA assay. Antisera prepared to reference influenza virus strains representing the 8-hemmaglutinin subtypes were strongly positive and results were shown in table1. Evaluation of field exposure by ELISA and HI show comparative HI and ELISA results of 656 field sera collected from flocks which suspected to infectious with AIV H9N2 subtype (Table 2).

The correlation coefficient for the results of ELISA and HI was significant ($r=0.929$, $P<0.001$). The sensitivity, specificity and correlation rate were 94.4%, %87.8 and %93, respectively.

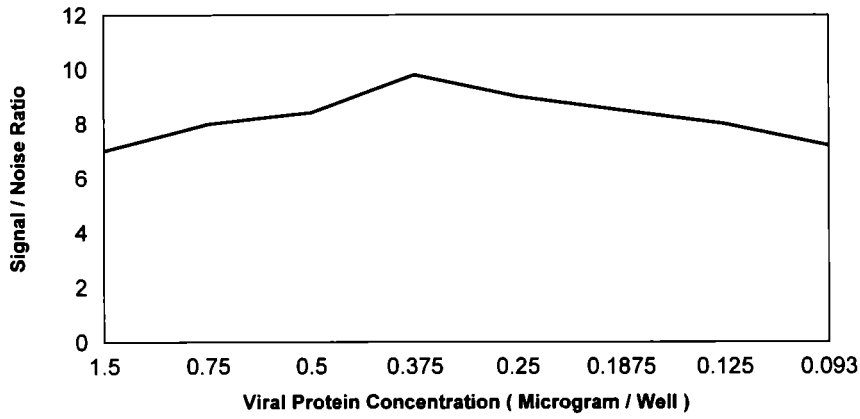

Figure 2. *Signal to noise ratio to determine optimal viral protein cocentration*

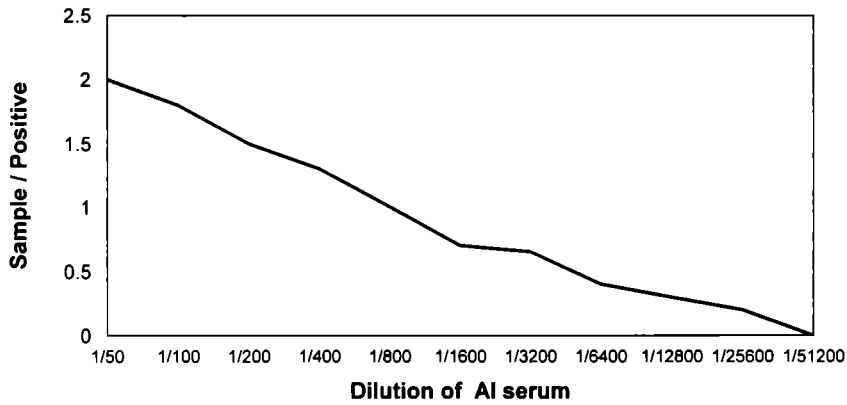

Figure 3. *Dilution curve for avian influenza antiserum*

Table 1. *Influenza ELISA values of standard antisera to viruses from 8-hemmaglutinin subtypes*

Virus antiserum	H 1	H 2	H 4	H 5	H 6	H 7	H 9	H 10	Cont.(-)	Cont.(+)
Average SP Value(1,2)	2.488	2.458	1.119	1.255	1.178	0.958	0.744	1.875	0	1
AIV ELISA titer (3)	5779	5744	1856	2190	2043	1480	1026	3950	0	1800
AIV ELISA SP range(4)	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Positive

1. Values are arethematic mean of 8 replicate samples.

2. Sample SP value = $\frac{\text{OD of sample - average OD of normal control}}{\text{average OD of positive control - average OD of normal control}}$

3. Log. 10 titre = (1.464 * Log. 10 SP)+ 3.197

4. SP ranges : Negative <0.278, Positive >.278

Table 2. *The results of 656 field sera tested for AIV antibody using the ELISA and HI*

Samples tested	91	22	27	75	119	102	85	74	30	16	15
SP value	0.155	0.187	0.215	0.366	0.513	0.683	0.814	1.03	1.596	1.937	2.755
SD of SP value	0.093	0.077	0.089	0.149	0.209	0.278	0.344	0.435	0.774	0.939	1.335
AIV ELISA titer	102	137	167	362	593	902	1165	1652	3193	4145	6940
SD of ELISA titer	68	54	44	154	282	357	776	979	1249	2316	4730
HI titer (H9N2)	0	1	2	3	4	5	6	7	8	9	10

Sample/Positive ranges: Negative <0.165, Suspect=0.164 - 0.277, Positive>0.278

Discussion

In this study, we have developed a reliable AIV ELISA for the rapid and efficient large scale screening of AIV type A antibody in chicken. Various procedures and materials have been used in the indirect ELISA. The choice of antigen, anti-IgG conjugate, enzyme, and solid phase differs with the needs of a particular investigation.

An effective screening test for influenza virus infection should detect antibodies to any subtype, while at the same time retaining the sensitivity of the subtype specific HI test which detects seroconversion at 4 to 7 days after infection. ELISA has obvious advantages as a screening test, particularly where large numbers of sera are to be tested and the experiments described here demonstrate the sensitivity and specificity of the test for detection of influenza A virus antibodies. Snyder *et al* (1984) described an ELISA standardized for use in chicken and demonstrated its sensitivity in detecting seroconversion following infection of birds with influenza virus. Lamichhance and Kirkegaard (1997) compared serological methods for detection of antibodies to avian influenza virus in chicken sera. They demonstrated that the AIV ELISA was able to detect specific AIV antibodies as early as one week postinfection and its sensitivity and specificity of the ELISA was comparable to the AGP and HI tests.

Using an indirect ELISA Fatunmbi *et al* (1989) identified a broad-spectrum viral antigen for the detection of avian influenza virus specific antibodies. They demonstrated that H9N2 antigen is the best single antigen to use in the ELISA to screen for avian influenza virus antibodies and it detected antibodies against six viruses subtypes (H1, H4, H5, H6, H7, and H9) as early as 4 days postinfection. A whole-purified H9N2 antigen used in this study was able to detect 8 standard antisera of various subtype of avian influenza virus. One possible explanation for the early detection of antibodies is that disrupted viruses used as ELISA antigens would lead to the exposure of internal virus proteins (MP and NP) which are able to react with appropriate antibodies in the test sera. Both the MP and NP are common to all type A virus strains, and antibodies to these antigens as well as H and N antigens will be induced following influenza virus infections. Thus, larger amounts of antibodies would be detected, leading to an early detection of AIV infections.

Lambre and kasturi (1979) reported that influenza antigen at a protein concentration of 10μg/ml gave a low absorbency and a high sensitivity for the assay. Abraham and Visanadan (1986) reported similar finding. In this study, we have determined that antigen at a protein concentration of 3.75μg/ml gave a high

sensitivity. High antigen concentration resulted in an increase in control values and a decrease in the sensitivity of the assay. Antigen concentration <3μg of protein/ml had a low absorbency value, but the sensitivity of the test was also markedly diminished. However, optimal conjugate concentrations must be determined for each system and lot of conjugate. We have determined the optimum dilution of 1:1000 goat anti-chicken conjugate. The high conjugate concentration of 1:400 resulted in an increase in control absorbency values due to nonspecific attachment of conjugate to the antigen solid phase. Conjugate concentration of 1:1600 had low absorbency values with control, but did not have a significant difference between values of the known AIV-positive and AIV-negative sera. In this study, bovine serum albumin (BSA) has been added to antigen-coated wells with PBS/Tween or test serum to reduce nonspecific binding. This has been due to a cross-reaction between the BSA and R-T globulin.

In conclusion, the AIV ELISA that standardized in this study is a specific and sensitive assay for detection of AIV antibody in chicken sera. Sensitivity data reveal that the AIV ELISA has comparable sensitivity to the HI test. The ELISA is a rapid automated and suitable test for screening large numbers of samples and can routinely used in many poultry diseases diagnostic laboratories.

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