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BRIEF COMMUNICATION

Development of an antigen-capture enzyme-linked immunosorbent assay using monoclonal antibodies for detecting H6 avian influenza viruses

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The H6 subtype of avian influenza virus (AIV) infection occurs frequently in wild and domestic birds. AIV antigen detection is preferred for controlling AIV as birds are infected before they produce antibodies. The purpose of this study was to develop an early diagnostic method for AIV detection. Six monoclonal antibodies (mAbs) developed from a field H6N1 AIV strain were tested for their ability to bind to viruses. The two that showed the greatest binding ability to AIVs were used for antigen detection. An antigen-capture enzyme-linked immunosorbent assay (ELISA) to detect H6 AIVs was developed using these mAbs. One mAb was coated onto an ELISA plate as the capture antibody. The other mAb was used as the detector antibody after labeling with horseradish peroxidase. The antigen-capture ELISA detected H6N1 AIVs but not H5 AIVs, human H1N1, H3N2 influenza or other viruses. This antigen-capture ELISA could be used to specifically detect H6N1 AIV.

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Introduction

The avian influenza virus (AIV) is classified into subtypes based on antigenic differences in their surface hemagglutinin (HA) and neuraminidase (NA) glycoproteins.[1] To date, 16 HA subtypes and nine NA subtypes have been recognized.[2,3] Many bird species have been shown to be susceptible to the AIV.[4] Subtype H6 (hereafter called H6) AIV infection occurs frequently in both wild and domestic birds.[5–10]

Two diagnostic approaches, antigen and antibody detection, have been developed to detect AIV infection. Antigen detection is preferred for controlling AIV outbreaks, as birds are infected before they can produce antibodies. The antigen detection methods include virus isolation, nucleic acid detection using reverse transcriptase-polymerase chain reaction (RT-PCR), and antigen-capture enzyme-linked immunosorbent assay (AC-ELISA). Compared with RT-PCR, AC-ELISA has several advantages, such as rapid, automated, objective results that are inexpensive to obtain. Several commercial ELISA kits have been developed based on AIV nucleoprotein detection,[11,12] which is common to all subtypes of AIVs, thus they are unable to differentiate between different subtypes. Chen et al. developed monoclonal antibodies against H6 AIVs.[13] Although antibody detection is a good method for surveillance, it cannot detect early infection before the antibody forms. Thus, to control AIV before it spreads, it is necessary to use a method to detect viruses at an early stage. For this reason, an AC-ELISA specifically for detecting H6 AIV was developed.

Materials and methods

Monoclonal antibodies

The method for the preparation of monoclonal antibodies developed by this laboratory has previously been published.[13] Six monoclonal antibodies (mAbs), CH11-D10, EB2-B3, EB2-E5, EB2-F9, FF9-F5 and FF9-F7, were tested for their binding ability with an H6N1 virus. EB2-B3 and EB2-E5 showed strong binding ability to A/chicken/Taiwan/2838V/00 and so were used for AC-ELISA.

Viruses

The following H6N1 AIV strains were used for virus detection:

- A/chicken/Taiwan/2838N/00;
- A/chicken/Taiwan/2838V/00;
- A/chicken/Miaoli/2896/00;
- A/chicken/Taichung/3072/03;
- A/chicken/Taoyuan/3115/03;
- A/chicken/Taichung/3127/03;
- A/chicken/Yilan/3152/03; and
- A/chicken/Yilan/3153/03.

The H5 AIV strains A/duck/Yulin/04 (H5N2), A/chicken/1209/03 (H5N2), A/chicken/3360/05 (H5N2) and A/duck/Hong Kong/820/80 (H5N3), 1 Newcastle disease virus (2872/00) and 1 infectious bronchitis virus (3071/03) were used to evaluate the specific detection ability of this method. The virus titers were measured in specific pathogen-free chicken eggs as 50% egg infective dose (EID_{50})[14] and adjusted to 10^7 EID_{50} for the following tests. Two human influenza viruses, A/Taiwan/71280(H1N1), A/Taiwan/71328(H3N2), from Chung Gung University were also tested by this AC-ELISA. The viruses were in $>1 \times 10^2$ plaque-forming units in titers and inactivated with 0.1 M 2-bromoethylamine hydrobromide (BEI, Sigma) in 0.2 N NaOH solution. The origins of the viruses used in this study are shown in Table 1.

Ability of mAbs to bind to H6N1 AIV

Indirect ELISA was used to evaluate the binding affinity of the mAbs to the AIV strain A/chicken/Tw/2838N/00. A microtiter plate was coated with 60, 120, and 240 ng of the virus per well and kept at 4°C overnight. After washing with 340 μL/well of phosphate-buffered saline containing 0.1% Tween 80 (to make phosphate buffered saline with Tween 80 or PBST, pH 7.4), the plate was incubated with 100 μL/well of blocking buffer at 37°C for 30 minutes. After being washed three times with PBST, the plate was incubated with 25 ng and 200 ng/100 μL/well of mAbs at 37°C for 30 minutes. After washing five times, 100 μL/well of goat anti-mouse IgG labeled with horseradish peroxidase (HRP, 1:2500 dilution) was added and incubated at 37°C for 30 minutes. The plate was then washed three times and 100 μL/well of activated 3,3′,5,5′-tetramethylbenzidine dihydrochloride solution (TMB, SureBlue, and microwell peroxidase substrate, Kirkegaard and Perry Lab, Gaithersburg, MD) was added. The reaction was stopped after 10 minutes in the dark by adding 100 μL of TMB stop solution to each well. The optical density (OD) at 450 nm was read with an ELISA reader (EL312e Bio-Kinetics reader, Bio-Tek Instruments, Winooski, VT).

Tracheal samples

Forty-nine tracheal samples consisting of 20 market-age Taiwan Country chickens (10—15 weeks old), 26 adult

Table 1 Influenza viruses used in this study

Virus strain	Subtype	GenBank accession no.
A/chicken/Taiwan/2838N/00	H6N1	EF681870
A/chicken/Taiwan/2838V/00	H6N1	EF681878
A/chicken/Miaoli/2896/00	H6N1	—
A/chicken/Taichung/3072/03	H6N1	—
A/chicken/Taoyuan/3115/03	H6N1	—
A/chicken/Taichung/3127/03	H6N1	—
A/chicken/Yilan/3152/03	H6N1	—
A/chicken/Yilan/3153/03	H6N1	—
A/duck/Yunlin/3233/04	H5N2	—
A/chicken/Taiwan/1209/03	H5N2	AY573917
A/chicken/3360/05	H5N2	—
A/duck/Hong Kong/820/80	H5N3	—
A/Taiwan/71280	H1N1	—
A/Taiwan/71382	H3N2	—

Indicates no GenBank accession number.

broiler breeders, and three broilers that tested negative for avian influenza virus by RT-PCR and virus isolation were used for the cutoff value calculation. The mucosa was scraped from each of the tracheal samples and homogenized with 1 mL of tryptose phosphate. The homogenate was centrifuged at 4500 rpm for 15 minutes at 4°C and then filtered through a 0.45 μm membrane.

AC-ELISA

The mAb EB2-B3, which showed strong virus-binding ability, was diluted to 1 ng, 10 ng, 100 ng and 1000 ng/100 μL in sodium carbonate buffer. This solution was then used to coat a microplate and incubated at 4°C overnight. After washing with PBST once, the plates were incubated with 100 μL/well of blocking buffer for 30 minutes at 37°C. After washing three times, the plates were incubated with 100 μL/well of AIVs or tracheal samples for 30 minutes at 37°C. After being washed five times, 100 μL/well of 1:800 dilution of EB2-E5-mAb-HRP (as detector antibodies) were added to the plate which was kept at 37°C for 30 minutes. Then, after washing five times, 100 μL/well of the TMB solution was added to the plate and then the reaction was stopped after 10 minutes in the dark by adding 100 μL of TMB stop solution. The OD at 450 nm was read with an ELISA reader.

RT-PCR

RNA from tracheal homogenates was prepared using TRIzol (Life Technologies, Frederick, MD, USA). The presence of H6 AIV was detected by RT-PCR. Briefly, copy DNA was synthesized, followed by amplification of the *HA* gene by RT-PCR using a H6 HA gene-specific primer.[15] RT-PCR was initiated by reverse transcription at 42°C for 45 minutes, denatured at 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 50°C for 40 seconds, and 72°C for 40 seconds. The amplified products were further elongated at 72°C for 10 minutes.

Determination of cutoff value

Forty-nine tracheal samples that were determined by RT-PCR to be negative for AIV were used to calculate the cutoff value of the AC-ELISA. The cutoff value was calculated as the mean plus two standard deviations.

Evaluation of AC-ELISA

Twofold serial dilutions of eight H6N1 AIV strains in allantoic fluid, quantified by EID_{50}, were used as the antigens in the AC-ELISA. The detection limit of the AC-ELISA was determined according to the highest dilution that showed up above the cutoff value. In addition, two H5N2 AIVs—the 1 Newcastle disease virus and 1 infectious bronchitis virus—were used to test the specific binding of this AC-ELISA. For comparison, besides tracheal homogenates, allantoic fluid from three specific pathogen-free eggs was also used as the negative control.

Results

The ability of mAbs to bind to H6N1 virus

All of the mAbs tested except CH11-D10 showed an ability to bind to A/chicken/Taiwan/2838V/00. EB2-B3 and EB2-E5 showed stronger binding ability than the other monoclonal antibodies. The OD values of the former two antibodies reached about 3.00. EB2-B3 and EB2-E5 were therefore selected for the following AC-ELISA.

Determination of the cutoff value

After searching for an optimized condition by using different dilutions of both mAbs, mAb EB2-B3 at 100 ng/well and mAb EB2-E5-HRP at a dilution of 800× were used for the following ELISA. The mean OD and standard deviation of the 49 negative chicken tracheal samples were 0.07 and 0.02, respectively. Thus, the cutoff value for this AC-ELISA was 0.11 (the mean plus two standard deviations).

Detection limit different H6 AIVs on AC-ELISA

The OD values of the H6N1 viruses ranged from about 2.0 to >2.5 at different original virus concentrations (Fig. 1) and that of the tracheas and specific pathogen-free egg allantoic fluid of negative controls was <0.11. The detection limits of this AC-ELISA for A/chicken/Taiwan/2838N/00, A/chicken/Taiwan/2838V/00, A/chicken/Miaoli/2896/00, A/chicken/Taichung/3072/03, A/chicken/Taoyuan/3115/03, A/chicken/Taichung/3127/03, A/chicken/Yilan/3152/03 and A/chicken/Yilan/3153/03 were 2.0×10^6, 2.1×10^6, 3.3×10^7, 2.8×10^6, 1.1×10^7, 2.2×10^6, 1.3×10^6 and 1.3×10^5 EID_{50}/mL, respectively. The detection limits were

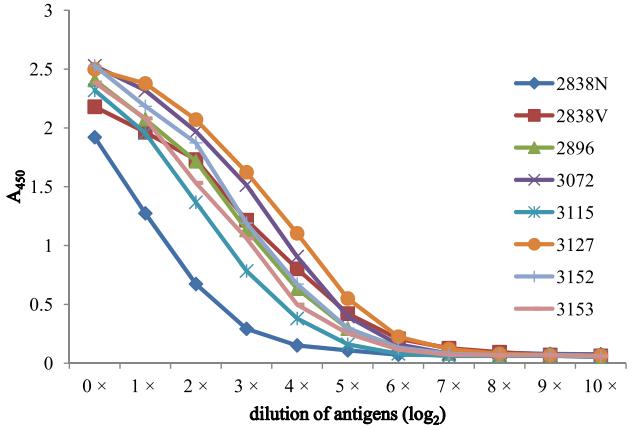

Figure 1. The detection limit of AC-ELISA to different concentrations. Different profiles represent different AIVs. EB2-B3 and EB2-E5-mAb-HRP were used as the coating and the detecting antibodies. 2838N: A/chicken/Taiwan/2838N/00 (H6N1), 2838V: A/chicken/Taiwan/2838V/00 (H6N1), 2896: A/chicken/Miaoli/2896/00 (H6N1), 3072: A/chicken/Taichung/3072/03 (H6N1), 3115: A/chicken/Taoyuan/3115/03 (H6N1), 3127: A/chicken/Taichung/3127/03, 3152: A/chicken/Yilan/3152/03 (H6N1), and 3153: A/chicken/Yilan/3153/03 (H6N1).

different for different viruses. The test was approximately 250 times ($3.3 \times 10^7/1.3 \times 10^5$) more sensitive for A/chicken/Miaoli/3153/03 than for A/chicken/Miaoli/2896/00. With regard to RT-PCR, the detection limit for A/chicken/Taiwan/2838V/00 was 2.6×10^4 EID_{50}. Thus, this AC-ELISA was approximately 100 times less sensitive than RT-PCR for this virus.

Specific AC-ELISA detection of other viruses

The OD values of other H5 AIVs tested—1 Newcastle disease virus, 1 infectious bronchitis virus and two human influenza viruses (H1N1 and H3N2)—were <0.11. No cross-reactivity was observed with these viruses.

Discussion

Subtype H6 AIV infection frequently occurs in both wild and domestic birds in Asia, America and Africa.[5,8–10,16] Although H6 is a subtype with low pathogenicity, outbreaks of H6 AIV in domestic birds have had a serious impact on the poultry industry in California.[8] In addition, recent reports indicate that the H6N1 virus A/teal/HK/W312/97 shared six of eight gene fragments with the H5N1 subtype virus, A/Hong Kong/156/97 (H5N1).[5,16,17] H5N1 caused an outbreak among chickens, with sporadic human cases and deaths in Hong Kong in 1997.[18,19]

No methods of H6 AIV detection are currently available. Thus, to detect H6 AIV at an early stage is important in order to control this disease before it spreads. Although RT-PCR has been successfully applied to the detection of AIV infection on a poultry farm, an antigen detection assay provides an alternative for the direct detection of AIV. Many farms, like those in Taiwan, own their own ELISA reader but not a thermocycler for RT-PCR. This AC-ELISA could be readily adapted for use in such farms.

AIV-negative tracheas were used to determine the cutoff value for interpretation rather than chicken embryonating eggs because the tracheas will be used for detection in the field. Since this AC-ELISA is a screening test, two standard deviations were selected instead of three to define a positive result in order to decrease false-negatives and increase the detection limit and sensitivity.[20] The detection limit of a detection method is dependent on the cutoff value selected as defining a positive result.

The sensitivities of the present AC-ELISA were different for different H6N1 AIVs, ranging from 10^5 to 10^7 EID_{50}/mL. The reason for this is unknown. The amino acid differences in HA sequences among different AIVs were compared, but no correlation was found between amino acid identities and detection limits (data not shown). The differences in the detection limits for different AIVs found when using this method need further study. Another limitation of this method was that it was less sensitive than a rapid test for H5 AIVs.[21] In addition, H6N1 AIVs in other countries could not be tested using this method because importing AIVs from other countries to Taiwan is prohibited.

The possibility of conformational changes in the target antigens exists in clinical samples due to various environmental conditions, so the recognition of conformational and linear HA antigens is important for successful detection. The ability of the mAbs to bind to AIVs was tested for this purpose in the present study. The results showed that the AC-ELISA could catch different strains of H6 AIVs, which is useful in the field.

With a decrease in sensitivity, the testing of tracheal samples tends to yield some false-negative results,[20] which allows some infections to remain undetected. For the treatment of poultry, however, the present AC-ELISA could be used on a flock basis because if one chicken gets an infection, an outbreak will occur.[10] This AC-ELISA should be used on a flock basis because almost all chickens are infected in an outbreak.[9,22]

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