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Development of a monoclonal antibody-based antigen capture enzyme-linked immunosorbent assay for detection of H7N9 subtype avian influenza virus

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Abstract

In order to establish a rapid detection method for H7N9 avian influenza virus (AIV), monoclonal antibodies (mAbs) against hemagglutinin (HA) of H7N9 were developed to establish an antigen capture enzyme-linked immunosorbent assay (AC-ELISA). AC-ELISA achieved high specificity and sensitivity, with a detection limit of 3.9 ng/ml for H7N9 HA protein (A/Zhejiang/DTID-ZJU01/2013), and 2^{-2} HA unit/100 μl for live H7N9 AIV. The inter- and intra-assay coefficient of variation was less than 10%. Compared with conventional virus isolation detection, the sensitivity and specificity were 94.96% and 88.24%, respectively. AC-ELISA proved to be a rapid and practical technique for detection of H7N9 AIV.

KEY WORDS Avian influenza virus, H7N9, Virus detection, Antigen capture enzyme-linked immunosorbent assay (AC-ELISA), Monoclonal antibody, Assay sensitivity

1 INTRODUCTION

Sporadic human infection with H7 avian influenza virus (AIV) was first detected in 1959[1]. The first instance of H7 AIVs causing severe outcomes was in 2003 in he Netherlands, when 89 cases of highly pathogenic H7N7 AIV were detected, accompanied with evidence of limited human-to-human transmission[2]. In early spring in 2013, the novel H7N9 AIV first infected humans in China[3].

Subsequently, there were five epidemics caused by H7N9 AIV in China, and in the fifth epidemic H7N9 isolates evolved into two new genetic lineages; the Yangtze River Delta and the Pearl River Delta clades[4]. As of July 2019, there were 1568 laboratory-confirmed cases of human infection and 613 deaths, with a mortality rate of ~40%[5]. Current treatment strategies for H7N9 are supportive care and early application of antiviral drugs (within 48h after symptom onset)[5]. Therefore, a rapid, specific and sensitive detection method for detection of H7N9 is urgently required.

During the early stages of the H7N9 outbreak, the first three respiratory specimens from infected patients were analysed by virus isolation, subtyping and pathotyping, which are crucial for further virological studies, particularly during an initial outbreak[3]. Additionally, real-time reverse transcription polymerase chain reaction (RRT-PCR) was also applied to detect H7N9 AIVs with high sensitivity and specificity. However, the above methods are time-consuming, and require biosafety level 3 laboratory access and expensive equipment, making them difficult to implement widely in primary hospitals[6]. Therefore, it is important to establish a simple and convenient H7N9 rapid diagnosis method. To this end, an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) was established based on two specific monoclonal antibodies (mAbs) for detecting viral antigen, resulting in a convenient method for testing many samples simultaneously[7]. Additionally, AC-ELISA can amplify the response signal

through enzyme catalysis, resulting in high sensitivity and specificity[8]. Therefore, an AC-ELISA was developed in the present study to detect H7N9 subtype AIVs rapidly and specifically.

2 MATERIALS AND METHODS

2.1 Cell lines, reagents and viruses

Mouse myeloma SP2/0 cells were grown in Dulbecco's modified Eagles medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics solution consisting of 10,000 units/ml penicillin and 10,000 μg/ml streptomycin (Pen Strep; Gibco). Hybridomas were maintained in DMEM supplemented with 10% FBS, 1% Pen Strep, and 1% hypoxanthine thymidine (HT; Invitrogen)[8].

AIVs (H1-H7, H9-H11), influenza B virus (Yamagata), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV) and avian paramyxovirus (APMV) used in this study (Table S1) were isolated from lung, tracheal and cloacal of poultries (including chickens, ducks and geese) and stored at -80°C in our laboratory. H7N9 A/Zhejiang/DTID-ZJU01/2013(ZJU01) low pathogenic (LP) and A/Guangdong/HP001/2017(HP001) high pathogenic (HP) viruses were isolated from throat swabs of patients in Zhejiang Province and Guangdong, respectively[9]. All viruses were propagated in 9-day-old specific-pathogen-free embryonated

chicken eggs and titrated using hemagglutinin (HA) from 1% chicken red blood cells (RBCs) as described previously[10].

2.2 Development of mAbs

The method for the preparation of mAbs developed in our laboratory has been described previously[9]. Briefly, 6-week-old male BALB/c mice (n = 5) were immunised with split vaccines of H7N9 in novel cytokine adjuvant (Biodragon-immunotech, Beijing, China) twice intraperitoneally at 3-week intervals. Splenocytes were fused with Sp2/0 myeloma cells and hybridomas were screened and selected by ELISA using purified H7 HA protein. Ascitic fluids from positive hybridomas were generated in mice and purified using protein G columns. The isotypes of mAbs were determined using a Mouse Monoclonal Antibody Isotyping Kit (Bio-Rad) according to the manufacturer's instructions. The binding affinity of mAbs was evaluated by indirect ELISA as described previously[7]. The specificity was detected by immunofluorescence assay (IFA) as described previously[11]. The control mAb (1E7) against syphilis and PBS were used as negative controls. The animal experiment was approved by the Institutional Animal Care Use Committee (IACUC) of Zhejiang University (No. 2017-015).

2.3 Establishment of AC-ELISA

AC-ELISA (Figure 1A) was established as described previously[7]. Briefly, mAb at a concentration of 2 mg/ml was labelled with horseradish peroxidase (HRP; Innoreagents) according to the manufacturer's instructions. The captured mAb was diluted to 0.4 μg/ml in coating buffer (pH 7.2) and 100 μl was used to coat each well of a microtitre plate. The plate was incubated at 4°C overnight. After washing five times with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST), the plate was blocked with 5% bovine serum albumin (BSA) at room temperature for 2 h. After rinsing five times, 100 μl of sample was added and incubated at room temperature for 2 h. After five more washes, 100 μl of HRP detection mAb (1:500 dilution) was added and incubated at room temperature for 1h. For colour development, 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB; KPL) was added and the reaction was stopped by addition of 100 μl of 0.1 M sulfuric acid. The optical density (OD) value was measured at a wavelength of 450 nm using an ELISA reader (Bio-Rad).

2.4 Determination of cutoff value

To determine the AC-ELISA cutoff value, 30 H7-free clinical samples (cloacal swabs and throat swabs) verified by virus isolation were collected from live poultry markets in Zhejiang province. All samples were eluted in 1 ml of PBS containing 0.5% BSA, penicillin G and streptomycin sulphate. Sample mixtures

were vortexed and centrifuged at 3000 rpm for 30 min, and the supernatant was used for detection by developed AC-ELISA[10]. The mean and standard deviation (SD) of the OD values of the 30 samples were calculated, and the cutoff value was calculated as the mean plus three SD. Samples with OD value greater than or equal to the cutoff value are considered positive, while samples less than the cutoff value are negative.

2.5 Specificity, sensitivity and reproducibility of AC-ELISA

Specificity was evaluated using different subtype AIVs (Table S1) and other viruses, including influenza B virus, NDV, IBDV, IBV and APMV[6]. All viruses were tittered and diluted to 2^5 HAU/100 μl, then detected by AC-ELISA.

Sensitivity was determined using a serial dilution of purified H7-HA protein from A/Zhejiang/DTID-ZJU01/2013(ZJU01) and a standard curve for quantifying H7-HA protein as described previously[8]. We also used serial dilutions of H7 AIVs, including H7N3 (A/duck/Zhejiang/DK16/2013, DK16), H7N7 (A/chicken/Jiangxi/C25 /2014, C25), LP-H7N9 (A/Zhejiang/DTID-ZJU01/2013, ZJU01) and HP-H7N9 (A/Guangdong /HP001/2017, HP001). The detection limit of AC-ELISA was determined based on the highest dilution detected above the cutoff value.

Reproducibility was determined by intra- and inter-assays and the coefficient of variation (CV) was calculated[12]. Intra-assay variation was assessed using

AC-ELISA with the same batch, and all samples were repeatedly detected three times in three parallel wells. Inter-assay variation was evaluated using AC-ELISA with different batches, and all samples were detected in three parallel wells.

2.6 Determination of the ability to detect clinical samples

A total of 156 cloacal swabs and throat swabs were collected from live poultry markets in Zhejiang to evaluate the ability of AC-ELISA to detect field samples[10, 13]. Field samples were also detected by virus isolation, the current gold standard for virus detection[14]. All samples were treated as described above prior to detection.

3 RESULTS

3.1 Selection of mAbs for AC-ELISA

Five mAbs (1H10, 2D1, 2F5, 2D5 and 2G2) were finally developed by hybridoma technology, and their subtypes and binding abilities were determined. In affinity tests, two mAbs displayed strong binding to H7N9-HA. The specificity of mAbs (2D5 and 2F5) were detected by IFA and the results were described in Figure S1. The results showed that mAbs 2D5 and 2F5 were specific for H7 AIVs and showed no cross-reaction with other viruses. In the isotype analysis, 2D5 and 2F5 were found to belong to IgG1 and IgG2a, respectively. Thus, these two mAbs were selected for detection and capture antibodies, respectively.

3.2 Determining the cutoff value for AC-ELISA

Thirty clinical samples confirmed negative for H7 subtype AIVs by virus isolation were used to determine the cutoff value for AC-ELISA. The cutoff value was calculated as the mean + three SD[6]. The cutoff for AC-ELISA was determined to be 0.062 (mean = 0.038, SD = 0.008).

3.3 Specificity, sensitivity and reproducibility of AC-ELISA

The specificity, sensitivity and reproducibility of AC-ELISA were determined as described above. A total of 23 viruses were detected by AC-ELISA, including four H7N9, two H7-AIVs and 17 non-H7 viruses. There was no cross-reactivity detected when AC-ELISA was applied to detect non-H7N9 viruses (Table S1 and Figure 1B). Additionally, a phylogenetic analysis of the tested H7 AIVs in the background of the whole diversity of H7 AIVs was constructed using molecular evolutionary genetics analysis (MEGA) software version 6.0 (Figure S2). And it suggested that the AC-ELISA was specific for H7N9 AIVs.

Sensitivity was simultaneously evaluated by quantifying H7-HA protein and live viruses. As shown in Figure 1C, the detection limit for H7-HA protein was 3.9 ng/ml, based on the standard curve. When detecting serially diluted H7N9 virus (Figure 1D), AC-ELISA could detect as little as 2^{-2} HAU/100 μl for ZJU01, 2^{-1} HAU/100 μl for HP001, and no positive reaction was observed for DK16 (H7N3) or C25 (H7N7).

The reproducibility of AC-ELISA was determined by intra- and inter-assays, and the results revealed that all coefficients of variation were <10% (Table S2 and Table S3), suggesting this method is highly reproducible.

3.4 Clinical applications of AC-ELISA

Using 156 clinical samples, AC-ELISA found that 14.10% (22/156) of samples were positive for H7N9 AIVs. All samples were further analysed by virus isolation, and the results showed that 10.90% (17/156) of samples were positive (Table 1). Thus, compared with virus isolation, the sensitivity and specificity of AC-ELISA was 94.96% and 88.24%, respectively.

4 DISCUSSION

During the annual surveillance of AIVs in poultry, H7 AIVs have been found to circulate in many countries, including Vietnam, Cambodia, The Netherlands and China[1, 15, 16]. H7N9 AIV first infected humans in 2013, and has caused five epidemics in China. The first epidemic lasted 6 months, and the following four occurred annually during September and October from 2013 to 2017[4]. During the fifth wave, the emergence of highly pathogenic avian influenza (HPAI) H7N9 viruses raised wide global concern[5]. Since 2017, H7N9 infection has decreased due to the application of an ambivalent H5N1/H7N9 vaccination program in poultry in China[1]. However, sporadic infections caused by HPAI H7N9 still occur, and the virus has gained the ability to be transmitted between ferrets, raising

concerns that it will cause a sixth pandemic[17]. Therefore, a rapid antigen detection method is necessary to monitor and control H7 AIVs.

In recent years, AC-ELISA has been applied widely to detect various AIVs including H5, H6, H7, H9N2 and H10N8[6, 8, 14, 18, 19]. A sandwich ELISA was developed with a detection limit of 0.45 ng/ml for the H7N9-HA protein derived from A/Anhui/1/2013 (H7N9), and 1 and 2 HAU/50μl for live H7N9 AIVs[20]. An additional sandwich ELISA was developed with a lowest detection limit of 10 ng/ml for recombinant H7 protein and 0.5^{-2} HAU/50μl for live H7 AIVs, respectively[21]. However, the above ELISA assays are not only sensitive for H7N9, but also other H7 subtype AIVs. In the present study, a specific AC-ELISA for detecting H7N9 AIVs was developed based on two murine mAbs recognising H7-HA. The detection limit was 3.9 ng/ml for the H7-HA protein and 2^{-2} to 2^{-1} HAU/100 μl for live H7N9 AIVs, including the Yangtze River Delta and the Pearl River Delta lineages. Furthermore, the AC-ELISA developed in this study showed no cross-reactivity with other H7 AIVs or other viruses, and achieved a comparable or superior performance for detecting H7N9 AIV. Compared with virus isolation, our AC-ELISA displayed comparable sensitivity and specificity for the detection clinical samples, with a shorter detection time and simpler operation.

In conclusion, an AC-ELISA for rapid, sensitive and specific identification of H7N9 subtype AIVs was established that may play an important role in the detection and prevention of H7N9 influenza viruses.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTION

FY and HW conceived the study and drafted the manuscript; YX, FL and HW designed and performed the study protocol; FY, HP, NW and HW made the analysis and interpretation of the database. All authors read and approved the final manuscript.

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Table 1 Detection sensitivity of the H7N9 strip using clinical specimens

		Virus isolation		**Total**
		positive	negative	
AC-ELISA	positive	15	7	22
	negative	2	132	134
	total	17	139	156

Note: AC-ELISA and virus isolation methods are highly correlated (p <0.001).

Figure legends

Figure 1 (A) The Antigen capture enzyme-linked immunosorbent assay (AC-ELISA) procedure. (B) AC-ELISA specificity. All viruses were tested at a fixed concentration of 2^5 HAU/100 μl. NC, negative control (PBS); PC, positive control (H7-HA protein). (C) Standard curve for AC-ELISA using purified H7-HA protein from A/Zhejiang/DTID-ZJU01/2013(H7N9). There is a high dose-reaction relationship between the optical density (OD) value and the logarithmic concentration of H7-HA protein, and the detection limit is 3.9 ng/ml. (D) Detection limit for ZJU01(A/Zhejiang/DTID-ZJU01/2013) and HP001(A/Guangdong/HP001/2017). The cutoff value is denoted by the dotted line. AC-ELISA, antigen-capture enzyme-linked immunosorbent assay.