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Establishment of antigen capture ELISA detection method for H9 subtype avian influenza virus HA protein

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ABSTRACT

The objective of present research was to establish antigen capture ELISA detection method for H9 subtype avian influenza virus. BALB/c mice were immunized with HA recombination protein, and then the mouse splenic cells were fused with SP2/0 cells, hybridoma cell stably secreting anti-HA McAb was screened by ELISA coated with the AIV antigen, an antigen capture ELISA based on monoclonal antibody was developed for detection of H9 AIV. Hybridoma cell stably secreting anti-HA McAb designated 5F10, the immunoglobulin type was IgG2b type with κ chain. The H9 AIV AC-ELISA showed no cross-reaction with other five avian viruses (ILTV, NDV, EDSV, IBV, and IBDV), the sensitivity eight times higher than HA test, Comparing with RT-PCR, the concordance was 94.8 %, the sensitivity was 97.1 %, and the specificity was 94.4 %. The H9 AIV AC-ELISA has good specificity, sensitivity and repeatability, could be used for diagnosing H9 subtype AIV infections.

Key words: H9 influenza virus; Protein HA; Monoclonal antibodies; Antigen capture ELISA; Diagnosis

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INTRODUCTION

Avian Influenza Virus (AIV) belongs to the Orthomyxoviridae family, Flu virus, influenza A virus [1]. This Virus is divided into three types which are HPAIV, LPAIV and NPAIV. The subtype H9, which belongs to LPAIV, was first reported by Homme PJ in 1970 [2]. In 1994, H9N2 was identified as separate strain by Chen Bolun for first time, and now H9N2 is found to be prevalent in China [3,4].This Virus can cause immune suppression of the host, and it can synergy with other pathogenic microorganisms in poultry incidence and cause significant economic losses to the world poultry industry[5]. In 1999, H9N2 infected human [6] had thus that molecular characterization it is of great significance. In the process of H9 subtype virus infection, the hemagglutinin (HA), neuraminidase (NA) and matrix protein (M2) etc. membrane protein elicit the immune response to neutralize antibody in the bodies, and the nucleoprotein (NP), matrix protein (M1) and other internal protein induce cellular immune responses [7]. Hemagglutinin (HA) is the surface glycoprotein of AIV, and it is the main component of membrane spike, and it plays a key role in the process of virus adsorption and penetration, and is directly involved in the pathogenic process of AIV, so it is a major virulence factor and protective antigen [8, 9, 10] and the the molecular characterization of HA protein is of extreme scientific significance. H9 subtype avian influenza virus detection methods were mainly about antibody and antigen detection method, with the widely used of H9 subtype avian influenza virus vaccine, the antibody detection methods were not suitable for clinical diagnosis. However, virus isolation and identification, hemagglutination test (HA) and other diagnostic methods are low sensitivity and specificity, RT-PCR and other molecular biological methods are sensitive, specific, but require high demand for technical personnel and expensive equipment at operation level. The ELISA diagnosis method is sensitive and specific, simple operation, fast detection, especially suitable for application in basic veterinary department. In this study, recombinant HA protein were expressed in prokaryotic expression system, using the HA protein to prepare monoclonal and polyclonal antibodies, established antigen capture ELISA detection method for rapid detection of H9 subtype avian influenza virus, which provides a simple, specific, sensitive detection method for the rapid diagnosis of H9 subtype avian influenza virus.

EXPERIMENTAL SECTION

1.1 Materials

H9 AIV, ILTV, NDV, EDSV, IBV, IBDV were saved in the laboratory. The cell of SP2/0 and MDCK were saved in the laboratory too. The mouse of BALB/c and ICR were brought from Beijing laboratory animal research center. Complete Freund′s adjuvant, incomplete Freund′s adjuvant and PEG（MW4000）were purchased from Sigma. SBA ClonotypingTM System / HRP antibody sub-class kits, Goat Anti-mouse IgG/HRP, goat anti-rabbit IgG/ HRP and goat anti-mouse IgG/ FITC were purchased from Southern Biotech. Protein A-Sepharose 4B was purchased from Life Technologies Invitrogen.

1.2 Preparation of antigens

Using RT-PCR amplification main antigen region of H9 subtype avian influenza virus HA protein, then directionally cloning it into pET30a expression vector, transformation of BL21 expression bacterium, which were expressed in inclusion body form with IPTG induction. The protein was purified by nickel ion affinity resin in denaturing conditions, the purity of the purified protein was higher than 95%, the purified protein concentration was 0.856 mg/mL detection by BCA method, and the recombinant protein had good immunogenicity.

1.3 Immunization protocol

The protocol and method of immunization was based on reference [11, 12]. The first immunization was using 0.1μg antigen emulsified with complete Freund's adjuvant after subcutaneous injection of six week old female BALB/c mice. On the 15 day, 0.1μg antigen emulsified with incomplete Freund's adjuvant after abdominal subcutaneous injection. On the day 30, using 0.1μg antigen directly. Then, after three days, the spleen of the mouse was dissected and splenocytes were fused with myeloma cells.

1.4 The ELISA for detection of antigen

The serum of immunized mouse regarded as positive control, and the culture supernatant of SP2/0 cell regarded as the negative control, the H9 AIV which was inactivated regarded as the coating antigen, using checkerboard titration test to determine the optimal concentration of envelope antigen (1:100, 1:500, 1:1000, 1:2000) and antibody （1:50、1:100、1:200、1:400, in the positive control OD450nm≈1.0, in the negative control OD450nm <0.2, the biggest value of P/N was chosen

1.5 The cell was fused

The cell was fused by conventional method [11.13.14], the spleen cells of immune mouse was fused with the SP2/0 cells on logarithmic growth phase by the help of PEG（MW4000）in sterile conditions, peritoneal macrophages of ICR mouse was chosen as the feeder cells, the fused cells were suspended by HAT, then the suspension was spilt in 96-well plates. After five days, half of the HAT was exchanged for HT medium. After 7 ~ 10 d, HT was the medium, regular observation, the medium was changed and testing in a timely manner.

1.6 Screening and cloning of the positive clones

Based on above 1.5 section established indirect ELISA method to screen positive clones, positive cell using limited dilution method (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600) for 3 times subcloning, all of the cell culture supernatant of ELISA test results were positive clones is monoclonal antibody of H9 subtype avian influenza virus HA protein, and the McAb were builded plants and frozen after expanding culture.

1.7 The preparation of ascites

The 0.1mL pristane was injected in the intraperitoneal of health BALB / c mice that were 8 weeks old. After 7~10 days, 0.1mL positive hybridoma cells (about 5X 10^6~10X10^6)was injected in the intraperitoneally. After 7~10 days, the abdomen of the mouse was expanded, the ascites was collected and centrifuged at 12000 r/min and the supernatant fluid which contains the monoclonal antibody was frozen in -20℃.

1.8 The identification of monoclonal antibodies

1.8.1 Routine characterization

Using colchicine for chromosome identification of hybridoma; monoclonal antibody subclass identification according to SBA ClonotypingTM System/HRP subtype identification kit, monoclonal antibody put in microplate, 37℃ after 30min incubation, antibody with HRP markers against mouse types, subtypes were added, 37℃ incubating for 30min later, with TMB chromogenic substrate and terminated with dilute sulfuric acid, to judge the monoclonal antibody subclasses by microplate absorbance detection; monoclonal antibody ascites by 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000, 1:64000, 1:128000, 1:256000, 1:512000, 1:1024000, 1:2048000 fold dilution, respectively detected by established H9 avian influenza virus indirect ELISA method to the determination of antibody titer.

1.8.2 Reaction activity

Using indirect immunofluorescence assay (IFA) method, 1:100 dilution H9 subtype avian influenza virus and inoculated with MDCK cells, cultured at 37 □ for 48 h in medium which containing 2% bovine serum, 4℃ cold methanol fixed 20min, hybridoma cell culture supernatant as antibody, FITC labeled Sheep anti mouse IgG as anti-antibody, uninfected MDCK cells were used as negative control, observed under the fluorescence microscope.

1.9 Preparation of HA protein polyclonal antibody and Western-blot detection

The female of New Zealand White rabbits were immunized by conventional immunization schedules [15]. The purified recombinant HA protein (500 μg antigen content) emulsified in 0.6mL complete Freund's adjuvant neck back subcutaneous injection immune to 3 kg female New Zealand white rabbits; immunity after 15d and recombinant protein 500μg (antigen content) and 0.6ml Freund's incomplete adjuvant emulsified neck back subcutaneous immunization; the 30d recombinant protein (500μg antigen content) directly enhance immune after subcutaneous immunization, heart blood after 7d. The reaction of HA protein with polyclonal antiserum was detected by Western blot method, the recombinant protein by SDS-PAGE electrophoresis, transfer to cellulose nitrate membrane, 8% skim milk closed, adding preparation serum (1:100 dilution) 37℃ for 1h, adding HRP labeled Goat anti rabbit IgG (1:5000 dilution) 37℃ for 1h, color DAB buffer solution.

1.10 Antibody purification

Using saturated ammonium sulfate purified Rabbit anti H9 AIV-HA serum, 10ml serum at 4℃ by adding ammonium sulfate to 45% saturation, action 1h, 12000 r/min centrifugal 30min, abandon the supernatant, precipitation dissolved in 10ml PBS, dialysis 24h, -20 store. Using Invitrogen's Protein A affinity chromatography column kit manual for monoclonal antibody ascites concentration, purification.

1.11 The development of AC-ELISA

1.11.1 Optimization of AC-ELISA reaction conditions

1.11.1.1 The best concentration of coated antibody, antigen detection and detection antibody

The best combination of three factors was determined by orthogonal experimental design. The best concentration of coated antibody(1:100、1:300、1:500、1:700、1:1000 dilution), antigen detection(1:10、1:20、1:40、1:80 dilution)and detection antibody(1:2500、1:5000、1:10000、1:20000、1:4000 dilution) were determined by square chessboard law titration trial [16]. The positive control was OD_{450nm}≈1.0, the negative control was OD_{450nm} <0.2, the biggest value of P/N was chosen.

1.11.1.2 The determination of HRP and it's working time

At the same reaction condition, The different dilution times (1:2500、1:5000、1:10000、1:20000)and working time(30、60、90、120min)of HRP was determined by square chessboard law titration trial, the positive control was OD_{450nm}≈1.0, the negative control was OD_{450nm} <0.2, the biggest value of P/N was chosen.

1.11.1.3 The determination of reaction time of AC-ELISA

At the same reaction condition, the working time of antigen detection was respectively 30 min、60 min、90 min、120 min, the working time of detection antibody was respectively 30 min, 60 min, 90 min and 120 min, the working time of HRP was respectively 30 min, 60 min, 90 min and 120 min, the working time of substrate was respectively 5 min, 10 min, 15 min and 25 min, then ,the best reaction time of antigen detection, detection antibody and substrate were determined. The positive control was OD_{450nm}≈1.0, the negative control was OD_{450nm} <0.2, the biggest value of P/N was chosen.

1.11.2 The standard of positive and negative about AC-ELISA

The negative samples of allantoic fluid and chicken cloaca which were detected both through Hemagglutination test (HA) and RT-PCR were 42 copies. The samples were detected through optimized AC-ELISA, the OD_{450nm} $\overline{X}$ and SD were calculated, the positive control was OD_{450nm} (samples) ≥ $\overline{X}$ +3SD, the negative control was ≤ $\overline{X}$ +2SD, if $\overline{X}$ +2SD<OD_{450nm} (samples) < $\overline{X}$ +3SD of samples need to recheck.

1.12 Specific test

0.1mL of ILTV, NDV, EDSV，IBV and IBDV were each carried cross-reactivity tests by the established AC-ELISA, and the negative and positive of H9 AIV controls were established. the test was to make sure if the AC-ELISA has reaction with another poultry virus.

1.13 Sensitive test

The inactivated allantoic fluid the of H9 AIV whose values of HA was 28 were diluted in 1:2、1:4、1:8、1:16、

1:32、1:64、1:128、1:256、1:512、1:1024、1:2048、1:4096，and detected by the established AC-ELISA, and through OD_{450nm}, the values were sure.

1.14 Repeatability test

1.14.1 The intro-batch repetition test

In the same experimental conditions, select the H9 AIV, which include three positive samples and three negative samples, were selected. Each sample was repeated 6 times and detected by AC-ELISA; the results of detection were gone to statistical analysis.

1.14.2 The inter-batch repetition test

In the same experimental conditions, select the H9 AIV, which include three positive samples and three negative samples were selected, the samples were detected by AC-ELISA, the results of detection were gone to statistical analysis.

1.15 Conformity test

Established RT-PCR detection method for H9 subtype avian influenza virus based on reference [17], the samples that were detected by RT-PCR were positive, and then the samples were detected by AC-ELISA. The coincidence of RT-PCR were regarded as the standards, the compliance rates, sensitivity and specificity were calculated between AC-ELISA and RT-PCR. (Coincidence rate = detection results in the same number / total samples, sensitivity = positive samples the same number / total number of positive samples, specificity = negative samples the same total number / negative samples).

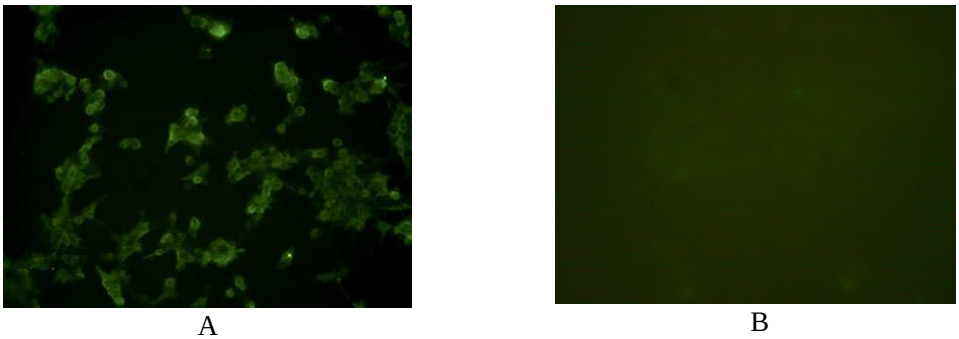
RESULTS

2.1 Establishment of indirect ELISA screening method and hybridoma monoclonal antibodies cell lines

By matrix experiments the optimal coated antigen concentration is 1:1000, antibody optimal dilution for 1:100. Screened by indirect ELISA, successfully obtained one positive clone, four times subcloning and purification, one strains of secreting specific monoclonal antibody hybridoma cell line were obtained, named 5F10.

2.2 Identification results of monoclonal antibody and polyclonal antibodies against HA protein

McAb cell line chromosome number is 108, and the chromosome number approach of myeloma cells with spleen cells, Which indicates the hybridoma cells were spleen cells with myeloma cell fusion cells; McAb subtype identification results show that 5F10 heavy chain is IgG2b, the light chain is κ chain; antibody titer of indirect ELISA for detection of monoclonal antibody ascites as high as $1.024X10^6$; McAb and H9 subtype of AIV infected MDCK cell specific binding, fluorescence microscopy, bright green fluorescence, green fluorescence in uninfected MDCK cells were not (Figure 1). As shown in Figure 2, the recombinant HA protein and polyclonal antisera that reacted specifically.



A B

Fig.1 Identification of the McAb against H9 subtype AIV in MDCK by IFA

A: McAb with H9 subtype AIV infected MDCK; B: Negtive control

2.3 AC-ELISA conditions to determine the test results

2.3.1The determined of the best reaction condition

The best coating concentration of polyclonal antibody was 1:500, the best dilution was 1:20, the best action time was 1h at 37℃. The best concentration McAb was 1:10000, the best action time was 1h at 37℃. The best concentration of HRP was 1:5000, the best action time was 1h at 37℃. The best coloration time of the substrate was 15min at 37℃.

2.3.2 The standard of AC-ELISA

The OD_{450nm} numerical distribution of 42 copies negative samples saw figure 3. The value of OD_{450nm} was mainly distributed between 0.04 and 0.10, the samples occupied 88.1% in all samples. The reasonable distribution of data can regard as the basis of making decision criteria.

The $\bar{x}$ and SD of OD_{450nm} was respectively 0.070 and 0.022. According to the principles of statistics, the standard of AC-ELISA was obtained, if OD_{450nm}（sample）≥0.136, the result was positive, if OD_{450nm}（sample）＜0.114, the result was negative, if 0.114≤OD_{450nm}（sample）＜0.136, the result must detect again.

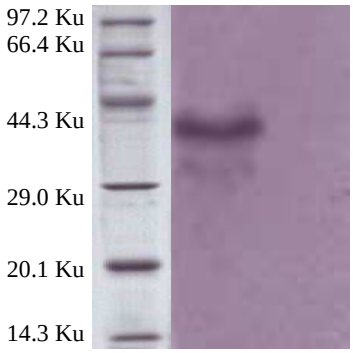


Fig.2 Western blotting of polyclonal antibody
1. Purified protein react with polyclonal antibody
2. Control of pET30a bacteria lysate

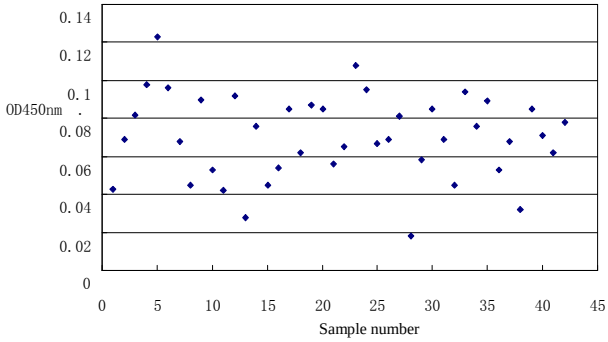


Fig.3 Distribution of OD_{450nm} of negative samples

2.5 The established of AC-ELISA

2.4 The result of specific test

From table 1, the data could see, the established H9 AIV AC-ELISA had not reaction with another poultry virus that indicated that this method had highly specific to H9AIV.

Table 1 Result of cross-reaction

	Other viruses of poultry					Positive control	Negative control
	ILTV	NDV	EDSV	IBV	IBDV	H9 AIV +	H9 AIV -
OD_{450nm}	0.058	0.041	0.038	0.052	0.036	1.082	0.054

2.5 The result of sensitive test

From table 2, the data could see, The inactivated allantoic fluid the of H9 AIV whose values of HA was 2^8 were diluted in 1:2048, the value of OD_{450nm} was 0.142, the samples were positive, the sensitiveness of AC-ELISA was 8 doubles to the HA test.

Table 2 Sensitivity of HA and AC-ELISA

	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
HA	+	+	+	+	+	+	+	+	-	-	-	-
ELISA OD_{450nm}	2.155	1.865	1.464	1.135	0.935	0.761	0.536	0.449	0.342	0.256	0.142	0.088

2.6 The result of repeatability test

The C.V value of intro-batch repetition test was less than 5 % (see table 3), The C.V value of inter-batch repetition test was less than 10 % (see table 4), all this indicated that AC-ELISA had good repeatability.

Table 3 Intro-batch repetition test

Testing sample	Number of replications（OD_{450nm}）						Average	Standard deviation	Coefficient of variation (%)
	1	2	3	4	5	6	$\bar{x}$	SD	C.V
1	1.119	1.131	0.996	1.055	1.121	1.086	1.085	0.0517	4.77
2	0.522	0.583	0.556	0.561	0.552	0.523	0.550	0.0235	4.27
3	0.206	0.187	0.195	0.196	0.189	0.191	0.194	0.0068	3.51
4	0.106	0.112	0.104	0.098	0.108	0.107	0.106	0.0046	4.34
5	0.093	0.087	0.084	0.085	0.081	0.088	0.086	0.0041	4.77
6	0.061	0.056	0.058	0.054	0.055	0.059	0.057	0.0026	4.56

Table 4 Inter-batch repetition test

Testing sample	Different time（OD_{450nm}）				Average	Standard deviation	Coefficient of variation (%)
	1	2	3	4	$\bar{X}$	SD	C.V
1	1.102	1.123	0.938	1.063	1.057	0.0828	7.83
2	0.502	0.578	0.542	0.563	0.546	0.0330	6.04
3	0.213	0.176	0.186	0.195	0.193	0.0157	8.13
4	0.112	0.098	0.095	0.107	0.103	0.0079	7.67
5	0.077	0.091	0.085	0.087	0.085	0.0059	6.94
6	0.066	0.059	0.062	0.055	0.061	0.0047	7.70

2.7 The result of conformity test

The positive results of RT-PCR were 35, the positive results of AC- ELISA were 34, and the negative of the result of AC- ELISA was 1.

The negative results of RT-PCR were 195, the negative results of AC- ELISA were 184, and the positive results of AC- ELISA were 11. AC-ELISA relative to the RT-PCR, the conformity was 94.8%, the sensibility was 97.1%, and the specificity was 94.4% (table 5).

Table 5 Comparison between AC- ELISA and RT-PCR

RT-PCR		AC- ELISA		Coincidence rate	Sensibility	Specificity
Positive	Negative	Positive	Negative			
35	----	34	1	94.8%	97.1%	----
----	195	11	184		----	94.4%

DISCUSSION

By using double antibody sandwich method to establish antigen capture ELISA method have very strict requirements of antibody specificity, and obtain specific antibodies requires high purity of immune antigen. Because of the H9 subtype avian influenza virus antigen preparation process is complex, lack of standardization, not easy to purify, and there is a risk of the spread of the virus, so get the artificial antigen specific H9 subtype avian influenza viral protein by using gene engineering technology has gradual importance. Because the HA protein is AIV specific, protective antigen, the antibody level of HA protein produced with whole virus antigen antibody level were almost consistency. For the expression of HA protein, it is important to choose the expression system, the Escherichia coli expression system has the advantages of low cost, convenient, simple operation, cultivating a large quantity, and pET30a prokaryotic expression vector expression of the N-terminal His tag which can be purified by affinity chromatography, the purity of nickel ions, can guarantee the maximum possible recombinant protein the purification process, simple, easy to mass production. In view of this, this study uses the pET30a prokaryotic expression vector in BL21 (DE3) to obtain the recombinant protein with high purity, the recombinant proteins were prepared highly specific monoclonal antibodies and polyclonal antibody. This study will be creative polyclonal and monoclonal antibodies were used as the capture antibody and detection antibody, polyclonal antisera because as capture antibody, has a wide range of capture sample, and the detection of antibody against H9 was used AIV HA protein specific monoclonal antibody, has an extremely high subtype specific, so the research establishment antigen capture ELISA method has the repeatability, sensitivity and specificity of good, and has the advantages of rapid, simple, economical, safe, high throughput, this method still needs further optimization and improvement of this method, firstly, the need for sample detection amount, accurately to determine the positive detection rate and false positive rate; secondly, the method can also be compared with the commercial ELISA kit or other ELISA detection method, in order to make the method more perfect, promotion and a wider range of applications.

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