Journal of Animal and Veterinary Advances 12 (23): 1651-1656, 2013
ISSN: 1680-5593

Preparation of Monoclonal Antibodies Against HA Protein of H9 Subtype Avian Influenza Virus and Establishment Antigen Capture ELISA

[1]Yang Cui-Jun, [1]Ge Jian, [1]Wu Shu-Qin, [1]Chang Bao, [1]Xu Tong and [2]Wang Jin-Liang
[1]Hebei North University, 075000 Zhangjiakou, China
[2]Binzhou Academy of Shandong,
Institute of Animal Science and Veterinary Medicine, 256600 Binzhou, China

Abstract: To produce Monoclonal Antibodies (McAb) against HA protein of H9 Avian Influenza Virus (AIV) and establish an Antigen Capture ELISA (AC-ELISA). BALB/c mice were immunized with HA recombination protein and 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 was developed for detection of H9 AIV using polyclonal antibody as capture antibody and specific monoclonal antibody as detecting antibody. Hybridoma cell stably secreting anti-HA McAb designated 5F10, the immunoglobulin type of McAb identification shown 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

INTRODUCTION

Avian Influenza Virus (AIV) belongs to the Orthomyxoviridae family, Flu virus, influenza A virus (Pysina and Gorbunova, 1970). This virus is divided into three types which are HPAIV, LPAIV and NPAIV. The subtype H9 which were reported belongs to LPAIV (Homme and Easterday, 1970). In 1994, H9N2 strains were separated by Chen Bolun at first time and now H9N2 were exsited in the country (Chen *et al*., 1994; Wu *et al*., 2010). 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 (Guo *et al*., 1999). In 1999, the events that H9N2 infected human have great significance in the reseach of this virus on public health (Nagarajan *et al*., 2009).

The process of H9 subtype virus infection, the Hemagglutinin (HA), Neuraminidase (NA) and Matrix protein (M2), etc. membrane protein induces immune response to neutralize antibody in the bodies and the Nucleoprotein (NP), Matrix protein (M1) and other internal protein induce cellular immune responses (Carrat and Flahault, 2007). Hemagglutinin (HA) is the surface glycoprotein of AIV and it is the mian main component of membrane spike and it play a key role in the process of virus adsorption and penetrating and it directly involve in the pathogenic process of AIV, so it is the major virulence factor and protective antigen (Okamatsu *et al*., 2008; Guo *et al*., 2003; Shahsavandi *et al*., 2011), the reseach of HA protein has an extremely important scientific significance. In this study, on the basis of prokaryotic expression of recombinant HA protein, the monoclonal and polyclonal antibodies of HA protein were prepared and the method which was antigen capture ELISA was set up to rapid detect this method could rapid diagnosis and differential diagnosis on H9 subtype avian influenza virus.

MATERIALS AND METHODS

Virus, cell and animals: H9 AIV, ILTV, NDV, EDSV, IBV and 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 form Beijing laboratory animal research center.

Reagents: 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 were purchased from life technologies invitrogen.

Corresponding Author: Yang Cui-Jun, Hebei North University, 075000 Zhangjiakou, China

Preparation of antigens: The main antigen region of HA protein in H9 AIV was highly expressed in the pET30a prokaryotic expression system and it had good immunogenicity, purified the purity protein reached up to 95%, the concentration of protein is 0.856 mg mL^{-1} in BCA Method.

Animal immune: The process and method of immune based on reference (Liu, 1994; Ge *et al*., 2009), the famale BALB/c mouses which were 6 weeks were choosed, complete Freund's adjuvant was injected in subcutaneous and the injection volume of complete Freund's adjuvant was 0.1 μg. On the 15 days, incomplete Freund's adjuvant was injected. On the 30 days, incomplete antigen was injected. Then, after 3 days, the spleen of mouse was fused.

The method of ESA screen was establish: The serum of immune mouse regarded as positive contral and the culture supernatant of SP2/0 cell regarded as the negative contral, the H9 AIV which was inactivated regarded as the coating antigen, the best concentration which was the reaction between coating antigen and primary antibody that was determined by square chessboard law titration trial in the positive contral OD_{450nm}≈1.0 in the negative contral OD_{450nm}<0.2, the biggest value of P/N was choosen.

The cell was fused: The cell was fused by conventional method (Liu, 1994; Pan *et al*., 1997; Dai *et al*., 2010), 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 choosen as the feeder cells, the fused cells were suspended by HAT then the suspension was spilt in 96 well plates. After 5 days, half of the HAT was exchanged for HT medium. After 7~10 days, HT was the medium, regular observation, the medium was changed and testing in a timely manner.

Screening and cloning of the positive cells: The positive cloning were screened by indirect ELISA, the positive cell were subcloned beyond 3 times by the method of limited dilution until all cells was positive then the Monoclonal Antibodies (McAb) against HA protein of H9 AIV were produced and the McAb were builded plants and frozen after expanding culture.

The preparation of ascites: The 0.1 mL pristane was injected in the intraperitoneal of health BALB/c mice which were 8 weeks old. After 7~10 days, 5×10^6~10×10^6 positive hybridoma was injected in the intraperitoneal.

After 7~10 days, the abdomen of mouse was expanding obviously, the ascite was taken and centrifuged, the supernatant fluid which included the monoclonal antibodies was gathered and frozen in -20°C.

The identification of monoclonal antibodies

The identification of Hybridoma chromosome: Hybridoma chromosomes were identified by Colchicine Method.

The identification of monoclonal subantibody: The identification of monoclonal subantibody was accorded to the instructions of SBAClonotypingTMSystem/HRP kit.

The determination of antibody titer: The value of monoclonal antibody ascites was identified by the AC-ELISA which was established in H9 AIV.

IFA: H9 VIA which was diluted 100 doubles were inoculated in MDCK cells and were cultivated in 37°C for 48 h and were fixed with cold carbinol. Then, the culture supernatant of hybridomas were regarded as the primary antibody, the goat anti-mouse IgG/FITC the second antibody and the MDCK cells which were not infected with this virus were regarded as the negative control, all results were observed by fluorescence microscopy.

The preparation and Western-blotting analysis of McAb against HA protein: The female of New Zealand White rabbits were immuned by conventional immunization schedules (Yu *et al*., 2008). The purified HA recombinant protein 500 μg (the content of antigen) which was emulsified by equal volume of complete Freund's adjuvant was injected in upon rabbits which was about 3 kg. After 15 days, incomplete Freund's adjuvant was injected. On the 30 days, incomplete antigen was injected. Then, after 7 days, the rabbits were taken blood on the heart, the reactivity between HA protein and polyclonal antiserum was detected by Western-blotting.

The purified of antibody: The rabbit anti-H9 AIV-HA hyperimmune serum IgG was purified throught saturated ammonium sulfate by conventional method. The ascites which included monoclonal antibody were concentrated and purificated by protein A affinity chromatography kits.

The estabilished of AC-ELISA

The process of AC-ELISA: The purified polyclonal antibody which was suitable diluted by carbonate buffer (0.05 mol L^{-1}, pH 9.6) was used to coated ELISA plates and put at 4°C refrigerator throught overnight. The plates was washed 3 times and each time was 3 min. H9 AIV

antibodies or samples which were duilted in perpor were added in the reaction plates and the plates were put at 37°C for a certain period and were washed with the former. The HRP which was suitable diluted was added in the reaction plates and the plates were put at 37°C for a certain period and were washed with the former. The substrate-color reagent of TMB was added in the reaction plates and the plates were put at 37°C for a certain period, the reaction was terminated by 2 mol L^{-1} H_2SO_4. The absorption value (OD_{450nm}) of degradations were detected by microplate reader.

The optimized of AC-ELISA

The best concenration 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, antigen detection and detection antibody were determined by square chessboard law titration trial (Li *et al*., 2011). The positive contral was $OD_{450\,nm}\approx1.0$, the negative contral was $OD_{450\,nm}<0.2$, the biggest value of P/N was choosen.

The choosen of HRP and it's working time: At the same reaction condition, the diffierent dilution times and working time of HRP was determined by square chessboard law titration trial, the positive contral was $OD_{450nm}\approx1.0$, the negative contral was $OD_{450nm}<0.2$, the biggest value of P/N was choosen.

The choosen of reaction time of AC-ELISA: At the same reaction condition, the working time of antigen detection was respectively 30, 60, 90 and 120 min, the working time of detection antibody was respectively 30, 60, 90 and 120 min, the working time of HRP was respectively 30, 60, 90 and 120 min, the working time of substrate was respectively 5, 10, 15 and 25 min then the best reaction time of antigen detection, detection antibody and substrate were determined. The positive contral was $OD_{450nm}\approx1.0$, the negative contral was $OD_{450nm}<0.2$, the biggest value of P/N was choosen.

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

Specific test: The ILTV, NDV, EDSV, IBV and IBDV were 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 other poultry virus.

Sensitive test: The inactivated allantoic fluidthe 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 throught OD_{450nm}, the values were sure.

Repeatability test

The intro-batch repetition test: In the same experimental conditions, select the H9 AIV which include 3 positive samples and 3 negative samples were selected each sample was repeated 6 times and detected by AC-ELISA, the results of detection were gone to statistical analysis.

The inter-batch repetition test: In the same experimental conditions, select the H9 AIV which include 3 positive samples and 3 negative samples were selected, the samples were detected by AC-ELISA, the results of detection were gone to statistical analysis.

Conformity test: The samples which were detected by RT-PCR were positive and then the samples were detected by AC-ELISA. The results of RT-PCR were regarded as the standards, the compliance rates, sensitivity and specificity were calculated between AC-ELISA and RT-PCR.

RESLUTS

The method of indirect ELISA screen was establish: The best concentration of coating antigen was 1:1000 and the best dilution times of primary antigen was 1:100 by square chessboard law titration trial.

The establish of monoclonal antibodies: The H9 AIV HA recombinant protein was immunogen. After fused, the virus were screened by AC-ELISA, a positive clone was successfully obtained and purified for 4 times, a hybridoma cell strain which could secrete monoclonal antibodies were obtained, the cell strain was named 5F10.

The result of monoclonal antibodies identified

The identification of McAb cells strain chromosome: The number of McAb cell strain chromosome was 108. This number is close to the total of the number of spleen cells chromosome and myeloma cells chromosome, the result indicated that this hybridoma cells strain was fused with spleen cells and myeloma cells.

The identification of McAb: The result indicated that the heavy of 5F10 was IgG2b and the light chain of 5F10 was κ chain.

The detection of McAb value: The value of monoclonal antibody ascites which was identified by the AC-ELISA was 1.024×10^6.

The result of IFA: The result showed when the McAb fused with the MDCK cells which were infected by H9 AIV, the green fluorescence light could emit at fluorescence microscopy. When the MDCK cells were not infected by H9 AIV, the green fluorescence light could not see at fluorescence microscopy (Fig. 1).

The production and detection of polyclonal antibody against HA protein: The result of Western-blotting shown in Fig. 2. HA protein could spectic react with polyclonal antiserum, expression bacteria with empty vector could not react with polyclonal antiserum.

The best coating concentration was 1:500, the best dilution was 1:20, the best action time was 1 h at 37°C. The best concentration McAb was 1:10000, the best action time was 1 h at 37°C. The best concentration of HRP was 1:5000, the best action time was 1 h at 37°C. The best colorate time of substrate was 15 min at 37°C.

The standard of AC-ELISA: The OD_{450nm} numerical distribution of 42 copies negative samples shown Fig. 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 were obtained, if OD_{450nm} (sample) $\geq$0.136, the result was positive, if OD_{450nm} (sample) <0.114, the result was negative, if $0.114\leq OD_{450nm}$ (sample) <0.136, the result must detect again.

The result of specific test: From Table 1, the established H9 AIV AC-ELISA had not reaction with other 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
Result	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

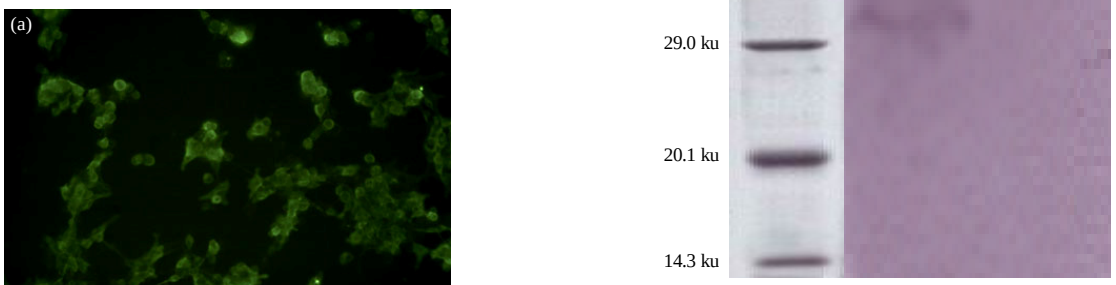

Fig. 2: Western blotting of polyclonal antibody; purified protein reacts with polyclonal antibody; control of pET30a bacteria lysate

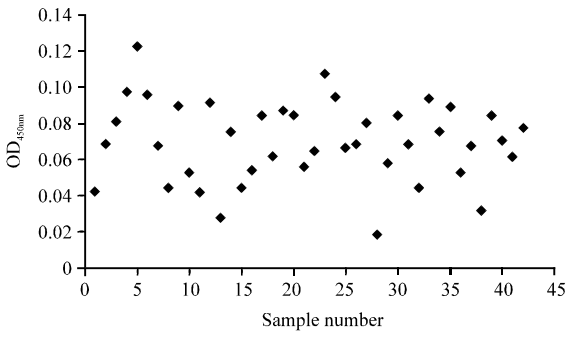

Fig. 3: Distribution of OD_{450nm} of negative samples

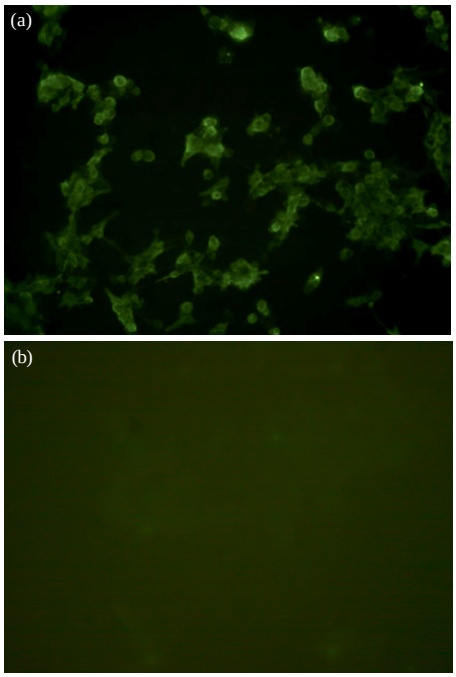

Fig. 1: The IFA result of McAb reacted with the MDCK. a) McAb reacted with MDCK which infected H9 AIV; b) negtive control

Table 2: Sensitivity of HA and AC-ELISA

Result	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

Table 3: Intro-batch repetition test

Testing sample	Number of replications (OD_{450nm}) 1	2	3	4	5	6	$\bar{X}$	SD	CV
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}) 1	2	3	4	$\bar{X}$	SD	CV
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

SD = Standard Deviation; CV = Coefficient of Variation (%); $\bar{X}$ = Average

Table 5: Comparison between AC-ELISA and RT-PCR

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

The result of sensitive test: Form Table 2, the inactivated allantoic fluidthe 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 was positive, the sensitiveness of AC-ELISA was 8 doubles to the HA test.

The result of repeatability test: The CV value of intro-batch repetition test was <5% (Table 3), the CV value of inter-batch repetition test was <10% (Table 4), all this indicated that AC-ELISA had good repeatability.

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 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).

DISCUSSION

In the world, to monitor the avian influenza is mainly used serological methods such as AGP, HI, ELISA, the method of antibody detection to disease epidemics and control has obviously lag what can not meet the current needs of the poultry industry. Especially, the vaccines of H9 subtype avian influenza virus were extensively immune, the antibody methods detection can not meet the basic requirements of clinical detection. At present, the etiological diagnosis of avian influenza virus is mainly isolation, identification and molecular biology methods. The isolation and identification of virus is accurate, effective and straight forward but this method which is time-consuming and requires a longer time is not able to meet the needs of defending and treating the acute and highly infectious diseases and those are not used in grassroots units. The method of ELISA has the advantages of sensitivity and specificity, simple operation, rapidly detection and high-throughput advantages, etc. ELISA is especially apply to primary veterinary sector and a large area of animal disease investigation.

CONCLUSION

In this study, the rabbit hyperimmune polyclonal antiserum which obtained from high purity H9 AIV HA protein immuned rabbit was regarded as the capture antibody, the specific monoclonal antibodies of against H9 AIV HA protein was regarded as the detection antibody. The sensitivity of AC-ELISA was 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, the laboratory was expected to assemble this into kits, this method could be used for diagnosing H9 subtype AIV infections.

ACKNOWLEDGEMENT

This study was supported by the Natural Science Foundation of Hebei Province (C2009001028).

REFERENCES

Carrat, F. and A. Flahault, 2007. Influenza vaccine: The challenge of antigenic drift. Vaccine, 25: 6852-6862.

Chen, B.L., Z.H. Zhang and W.B. Chen, 1994. Isolation and preliminary serological identification of avian influenza virus-type A from chickens. Chinese J. Vet. Med., 22: 3-5.

Dai, X.G., Q.Y. Teng, H.B. Huang, Z.X. Wang and S.M. Zhang *et al*., 2010. Preparation and characterization of monoclonal antibodies against M1 protein of H9N2 avian influenza virus. Chin. J. Anim. Infect. Dis., 18: 8-12.

Ge, Y., G.H. Deng, X.P. Li and H.L. Chen, 2009. Expression of PB1-1 gene of influenza virus and preparation of a monoclonal antibody against PB1-1 protein. J. Preventive Vet. Med., 31: 726-729.

Guo, X., M. Liao and C. Xin, 2003. Sequence of HA gene of avian influenza A/Chicken/Guangdong/SS/1994 (H9N2) virus. Avian Dis., 47: 1118-1121.

Guo, Y., J. Li and X. Cheng, 1999. Discovery of men infected by avian influenza A (H9N2) virus. Chinese J. Exp. Clin. Virol., 13: 105-108.

Homme, P.J. and B.C. Easterday, 1970. Avian influenza virus infections. IV. Response of pheasants, ducks and geese to influenza a/Turkey/Wisconsin/1966 virus. Avian Dis., 14: 285-290.

Li, J., J.L. Wang, L.C. Zu, J.W. Xie and Z.Q. Shen, 2011. Development and application of PPA-ELISA for detecting antibodies to classical swine fever virus using recombinant E2 antigen. Chin. J. Vet. Sci., 31: 1390-1394.

Liu, X.F., 1994. The Application of Monoclonal Antibody in Agriculture. Anhui Science and Technology Publishing House, Hefei, China.

Nagarajan, S., K. Rajukumar, C. Tosh, V. Ramaswamy and K. Purohit *et al*., 2009. Isolation and pathotyping of H9N2 avian influenza viruses in Indian poultry. Vet. Microbiol., 133: 154-163.

Okamatsu, M., Y. Sakoda, N. Kishida, N. Isoda and H. Kida, 2008. Antigenic structure of the hemagglutinin of H9N2 influenza viruses. Arch. Virol., 153: 2189-2195.

Pan, S., J. Huang, J. Zhou and J. Wu, 1997. Establishment of hybridoma technique and study on fusion conditions of hybridoma cells. Bull. Hunan Med. Univ., 22: 179-182.

Pysina, T.V. and A.S. Gorbunova, 1970. Relation between the pathogenicity of avian influenza viruses for laboratory animals and infection of avifauna. Vopr. Virusol., 15: 298-301.

Shahsavandi, S., A.H. Salmanian, S.A. Ghorashi, S. Masoudi, F. Fotouhi and M.M. Ebrahimi, 2011. Specific subtyping of influenza A virus using a recombinant hemagglutinin protein expressed in baculovirus. Mol. Biol. Rep., 38: 3293-3298.

Wu, Z.Q., J. Ji, K.J. Zuo, Q.M. Xie and H.M. Li *et al*., 2010. Cloning and phylogenetic analysis of hemagglutinin gene of H9N2 subtype avian influenza virus from different isolates in China during 2002 to 2009. Poult. Sci., 89: 1136-1143.

Yu, R.Q., C.F. Zhang, X.W. Zhou and Y.J. Wang, 2008. High level expression of NS1 protein in Escherichia coli and preparation of its polyclonal antibody. Chin. J. Vet. Sci., 28: 921-925.