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Original Research Article

DEVELOPMENT AND EVALUATION OF ANTIGEN CAPTURE ELISA FOR THE DETECTION OF INFLUENZA VIRUS A

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Abstract: The aim of the present study was to develop an Antigen Capture ELISA (AC-ELISA) for the diagnosis of avian influenza virus infections. For this purpose, the nucleoprotein (NP) of the virus was captured by a monoclonal antibody (D'C4) and then detected using a rabbit polyclonal antibody. The developed AC-ELISA did not show cross-reaction with other viral and bacterial pathogens of poultry, while it was able to detect H9 serotype of avian influenza virus as well as H1 and H3 types of human influenza viruses. The sensitivity of this AC-ELISA for the detection of an H9 avian influenza virus strain H9N2 (A/Chicken/Iran/AH-1/06) was 10 times greater than a hemagglutination assay and was comparable with the sensitivity of the RT-PCR method. Furthermore, this method could recognize the influenza virus in tracheal swabs of experimentally infected chickens following 3-5 days post-infection. Based on the obtained results, it can be concluded that the developed AC-ELISA is able to detect H9, H1, and H3 influenza virus serotypes and is sufficiently sensitive and specific for the detection of infections caused by H9 serotype but, its applicability, sensitivity, and specificity for the detection of other serotypes of the virus remain to be determined.

Key words: influenza virus A; nucleoprotein; monoclonal antibody; antigen capture ELISA

Introduction

The genus A influenza viruses in the family *Orthomyxoviridae* cause significant economic losses in the poultry industry (1) as well as diseases in mammals, including horses, pigs, dogs, cows, and human beings (2). In recent years, several epidemics of influenza have appeared in human and animal populations. Due to frequent genetic alterations in the genome of the genus A influenza viruses (3), designing a universal hemagglutinin-(H) and/or neuraminidase- (N) based diagnostic method for these viruses is problematic. However, it has been shown that nucleoprotein (NP) is a highly conserved protein among different strains of genus A influenza viruses (3, 4, 5, 6, 7). Furthermore, the results of Glickman *et al.* (1995) revealed that monoclonal antibodies (Mabs) can bind with high affinity to NP and M (Matrix protein) proteins of influenza virus while hemagglutinin is not an appropriate candidate for virus detection, due to antigenic drifts (8). Therefore, the detection of NP in samples prepared from acute infections can be used as a diagnostic method for all types of the genus A influenza virus (9).

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Regarding the wide spread occurrence of the type H9N2 in Iran since 1998, as well as the probability of the appearance of other pathogenic types, such as H5N1, developing strategies in order to detect and control the infections is necessary (10).

The aim of this study was to design an Antigen Capture ELISA using a NP-specific monoclonal antibody for the diagnosis of infections caused by the genus A influenza viruses.

Material and methods

An NP-specific monoclonal antibody (D'C4 Mab), previously produced by Neisi *et al.* (11) in our laboratory, was used in this study. This Mab has been shown to react with H5, H7, and H9 serotypes of avian influenza viruses as well as H1 and H3 serotypes of human influenza viruses. To obtain an adequate amount of the Mab, D'C4 hybridoma (10^6 cells) was injected intra-peritoneally to three BALB/c mice. The mice have been previously treated with 0.5 ml of incomplete Freund's adjuvant through intra-peritoneal injection one week previously. The ascites fluids produced in these mice were harvested and centrifuged (10 min, 2000 rpm), and the supernatant was stored at -20 °C, as the Mab (1).

For production of the polyclonal antibody against NP, two female rabbits were intramuscularly injected with 100 µg of recombinant NP protein (previously produced in our laboratory by Jaidari *et al.*, (12) emulsified in ISA70 adjuvant at 0, 14, and 28 days. Ten days after the last injection, antibody production was evaluated using indirect ELISA, and the serum of the rabbit with the higher antibody titer was prepared and stored (13).

In the next step, AC-ELISA was developed as follows: 100 µl of diluted D'C4 ascites fluid (1:100 to 1:320000) in ELISA coating buffer ($NaHCO_3$ 84.01 g/mol, Na_2Co_3 103.99 g/mol, pH 9.6) was added to wells of an ELISA micro titer plate (SPL, Korea) and incubated overnight at 4 °C. Then the plate was washed 3 times with phosphate buffer saline (PBS) containing 0.05% Tween-20 (PBST). The free spaces were blocked using PBST plus 5% of skim milk for 3 hours at 37 °C. After washing, as previously mentioned, 100 µl of different dilutions of the recombinant NP were added to the columns of plate and incubated 2h at 37 °C. Washing was repeated and then a constant dilution of the Mab in PBST plus 2% skim milk was added to wells and incubated at room temperature for one hour. After three rinses, 100 µl of peroxidase conjugated anti-rabbit IgG (Sigma, USA), diluted in PBST plus 2% skim milk, was added to all wells and incubated for 30 minutes at room temperature. Washing was done four times, and then 100 µl of chromogen-substrate solution (Tetra methyl benzidine and H_2O_2 in acetate buffer) was loaded into all wells. The plate remained for 10 minutes in a dark room, and then reaction was stopped by adding 100 µl of 1 M HCl. Finally, the absorbance of wells was measured at 450 nm (4, 13).

To determine the sensitivity and specificity of the designed AC-ELISA, several experiments were performed. The specificity of the assay was evaluated by using different antigens, as follows: H9N2 serotype of avian Influenza virus (A/Chicken/Iran/AH-1/06), H3N2 and H1N1 human influenza viruses and also some avian viral and bacterial pathogens, including Avian Reovirus (vaccine strain, Merial, France), Infectious bronchitis virus (IBV, vaccine strain, Razi Institute, Iran), B1 and Lasota strains of Newcastle disease virus (NDV, vaccine strains, Razi Institute, Iran), Infectious bursal disease virus (IBDV, vaccine strain, Razi Institute, Iran), Infectious laryngotracheitis virus (ILTV, vaccine strain, Razi Institute, Iran), *Pasteurella multocida*, *Escherichia coli* and *Ornithobacterium rhinotracheale* (all clinical isolates), and *Mycoplasma synoviae* and *Mycoplasma gallisepticum* antigens (SOLEIL diagnostics- France).

In terms of sensitivity, the ability of AC-ELISA for the detection of A/Chicken/Iran/AH-1/06 (H9N2) influenza virus was compared to Hemagglutination assay (HA) and RT-PCR. For this purpose, 1:20 to 1:10240 dilutions of the virus were prepared and tested by HA and AC-ELISA, in triplicate. The allantoic fluid (AF) of an embryonated chicken egg was used as a negative control.

Different dilutions of the virus, i.e., 1:100, 1:250, 1:500, 1:1000, 1:2000, 1:4000, 1:6000 and 1:7000 were also prepared for testing by RT-PCR. RNA was extracted using a High pure viral RNA kit (Takapouzist, Iran) and the cDNA was synthesized by using a commercial cDNA synthesis kit (kit, Takara, Kyoto) and H9 gene-specific forward primer (CTY CAC ACA GAR CAC AAT GG) (14). PCR reactions, performed by the H9 gene-specific forward and reverse (GTC ACA CTT

GTT GTT GTR TC) primers (14), contained 2 µl of cDNA, 10 picomols of each primer and 12.5 µl of 2X PCR Master Mix (Amplicon, Denmark), in a final volume of 25 µl. Thermal cycling parameters were as follows: an initial denaturation step (95 °C, 5 min); 35 cycles of denaturation (95 °C, 30 s), annealing (50 °C, 40s) and extension (72 °C, 40 s); and a final elongation step (72 °C, 10 min).

Finally, the potential of the developed AC-ELISA for the diagnosis of avian influenza virus in clinical specimens was assessed by testing tracheal swabs collected from experimentally infected chickens. Five seronegative chickens were inoculated intra-ocularly and intranasally by 0.2 ml of 1:10 dilution of A/Chicken/Iran/A H-1/06 (H9N2) influenza virus and tracheal swabs were collected from 1 to 7 days after inoculation. The swabs were vortexed in 1 ml PBS and the released secretions were stored at -70 °C. Serum samples were also collected at 7th days and presence of antibody against the virus was investigated by Hemagglutination inhibition (HI) assay (13, 15). Tracheal swab samples were also prepared from 39 seronegative healthy chickens, as negative controls and for calculation of the cut-off point. The recombinant NP (with a concentration of 0.075 µg/ml) was applied as a positive control in this experiment. All samples were double tested in AC-ELISA and the mean absorbance of each swab was recorded. The optical density ratio of each swab sample to positive control (SP index) was calculated and the cut-off point was determined using the following formula:

Cut-off = mean SP values of seronegative healthy chickens + 3 SD

For comparison, 35 swabs collected during seven days from five experimentally infected chickens were also tested by RT-PCR, as described.

Results

In the checkerboard optimization of AC-ELISA, the optimal dilutions of the NP-specific monoclonal antibody, rabbit polyclonal antibody, and peroxidase conjugated anti-rabbit antibody were 1:5000, 1:1000 and 1:3000, respectively. Figure 1 shows the results of assessment of different antigens in AC-ELISA. As can be found, the absorbance of H1N1, H3N2, and H9N2 serotypes of influenza virus was approximately equal to the positive control (NP protein) while those of the other tested pathogens were equal to the negative control. These results confirm the high specificity of the designed AC-ELISA and the absence of false positive results (Figure 1).

The results of sensitivity assays have been presented in Table 1. As it suggests, the developed AC-ELISA can detect H9N2 influenza virus up to 0.1 HA unit. However, the sensitivity of the AC-ELISA was less than RT-PCR. The results of RT-PCR reaction revealed that an expected 488 bp Amplicon (a DNA segment of 488 bp) was produced in 1:100 to 1:6000 dilutions of the H9N2 virus in the allantoic fluid.

Optical densities of tracheal swabs from 39 uninfected healthy chickens in AC-ELISA were equal to PBS as the negative control (0.16), while the optical density was significantly higher for experimentally infected chickens, in some cases depending on the day of sampling, even greater than the OD value of the positive control (NP protein). Based on the calculated SP indices, the cut-off point of the designated AC-ELISA was 0.123. The SP indices of all samples prepared from infected chickens, 3 to 5 days after inoculation, were located above the cut-off line, i.e., were positive. Furthermore, some SP indices related to swabs collected between 2 and 6 days after experimental infection were also positive (not shown). To compare the AC-ELISA and RT-PCR, 35 tracheal swab samples from five experimentally infected chickens were also tested by RT-PCR. Following RT-PCR, it was confirmed that it was able to detect the H9 gene in swab samples collected from 2 to 6 days after infection and the most detections were 3 and 4 days after infection. OD values from experimentally infected chickens and the results of RT-PCR are shown in Table 2. At 7 days post-infection, all five experimentally infected chickens were positive in HI test, with the titers varying from 1:64 to 1:256.

Discussion

Rapid diagnosis of avian influenza plays a major role in the control of its epidemics. This subject is more prominent regarding the disease caused by highly pathogenic strains. Therefore, diagnosis using methods such as virus isolation, nucleic acid detection, and antigen detection is superior to serological methods (1, 9). Virus isolation is a time-consuming method and commonly has

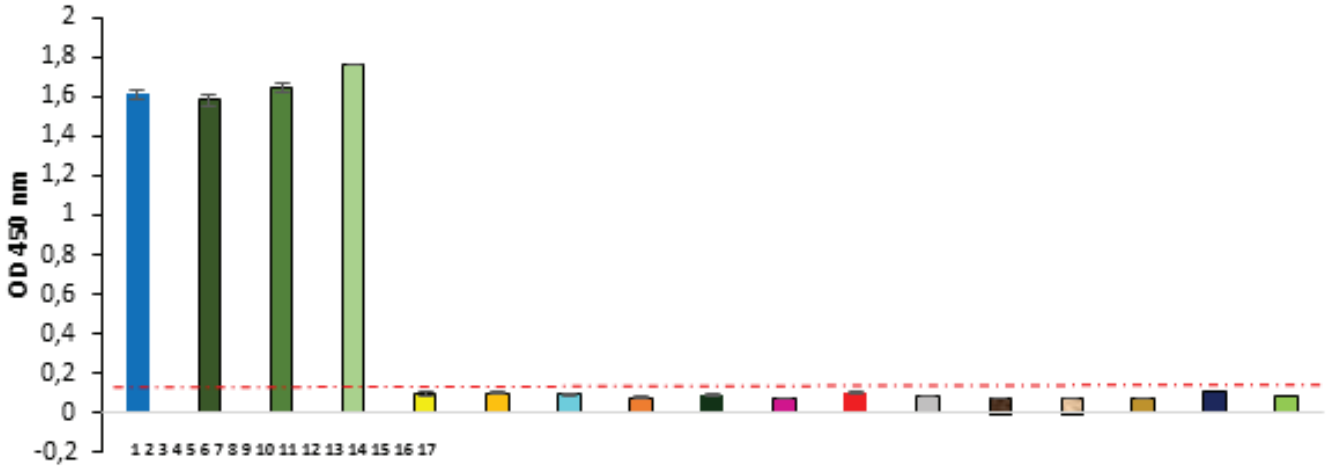

Figure 1: Results of cross-reaction of AC-ELISA with different viral and bacterial antigens.

1. Recombinant NP (0.075 µg/ml), 2. H9N2 1:160 dilution, 3. H1N1 1:30 dilution, 4. H3N2 1:30 dilution, 5. AF 1:30 dilution, 6. PBS 7. NDV B1 strains, 1:50 dilution, 8. (NDV Lasota strains, 1:50 dilution, 9. IBV, 1:50 dilution, 10. IBDV, 1:50 dilution, 11. ILTV, 1:50 dilution, 12. Avian Reovirus 1:50 dilution, 13. *Mycoplasma synoviae* 1:50 dilution, 14. *Mycoplasma gallisepticum* 1:50 dilution, 15. *Ornithobacterium rhinotracheale* (ORT) 1:50 dilution, 16. *Escherichia coli* 1:50 dilution, 17. *Pasteurella multocida* 1:50 dilution

Table 1: The sensitivity of AC-ELISA for H9N2 detection compare to HA titration and RT-PCR

dilution of virus	1:100	1:250	1:500	1:1000	1:2000	1:4000	1:5000	1:6000	1:7000
HA	+	+	+	–	–	–	–	–	–
AC-ELISA	+	+	+	+	+	+	+	–	–
RT-PCR	+	+	+	+	+	+	+	+	–

Table 2: Results (OD values) of AC-ELISA assay and RT-PCR on samples prepared from experimentally infected chickens

day	0	1	2	3	4	5	6	7
Inf. chick 1	0.112	0.117	0.408	2.33	2.46	1.01	0.17	0.165
Inf. chick 2	0.111	0.098	0.113	1.351	2.328	1.815	0.448	0.138
Inf. chick 3	0.127	0.136	0.205	0.782	2.445	2.426	0.182	0.174
Inf. chick 4	0.125	0.147	0.316	1.125	2.484	0.845	0.43	0.254
Inf. chick 5	0.11	0.113	0.57	2.241	1.928	0.741	0.229	0.125
RT-PCR	–	–	+	+	+	+	+	–

low sensitivity. RT-PCR is used for nucleic acid detection but despite its suitable specificity and sensitivity, in comparison with other diagnostic methods it is expensive, needs an expert operator, and is affected by contaminations, which leads to false positive results (1).

In contrast, antigen detection methods, especially AC-ELISA, have advantages such as rapid analysis of a large number of samples, desirable specificity and sensitivity, and lack of contamination risk in comparison to RT-PCR (4, 9).

Several Antigen Capture ELISA have been developed for the diagnosis of influenza viruses. Chomel *et al.* designed an immunocapture ELISA with 97% specificity and considerable sensitivity (15). In another study, the same researchers

subjected the nasal swabs of patients infected with H1N1 to immunocapture ELISA. The results revealed that this method can detect NP antigen in the nasopharyngeal swabs of infected humans even seven days after sampling (16). Ji *et al.* (13), using monoclonal and polyclonal antibodies specific for NP of H3N8, developed an AC-ELISA. Their results indicated that this AC-ELISA has sufficient sensitivity and specificity for the diagnosis of H3N8 and H7N1 strains of the equine influenza virus. Based on the report of Ji et al. (13), their designed AC-ELISA was able to find influenza viruses in nasal swabs prepared from experimentally infected horses from the 3rd to the 7th days after infection.

The investigations of Davison *et al.* (17), Gerentes *et al.* (18), Cattoli *et al.* (19), Wang *et al.* (20), He *et al.* (21), Velumani *et al.* (22), and Ho *et al.* (23) are examples of studies on developing AC-ELISA for the detection of all or specific serotypes of avian influenza viruses. Using two monoclonal antibodies, EB2-B3 and EB2-B5, specific for H6, Chen *et al.* (4) developed an AC-ELISA that was able to only detect H6 serotypes. He *et al.* (24) designed a dual function ELISA for the simultaneous detection of H7 and its specific antibody. This immunoassay had high sensitivity and 100% specificity for the diagnosis of H7 antigen and its specific antibody (24). Quan-wen et al. (1) produced recombinant H9 and its monoclonal antibody and then designed an AC-ELISA, which had 99.4% correlation, 97.1% sensitivity, and 94.4% specificity, in comparison with RT-PCR.

Due to the widespread prevalence of the H9N2 serotype of influenza virus in Iran and the risk of epidemics with H5 and H7 highly pathogenic serotypes, in the present study, an AC-ELISA based on NP and a NP-specific monoclonal antibody that can react to several serotypes of avian and human influenza viruses was designed. Following the optimization of principal variables, the sensitivity and specificity of this test for the detection of clinical infections caused by avian influenza virus were assessed. The developed AC-ELISA did not react with any other viral or bacterial antigens tested in this study and, therefore, its specificity was 100%.

In the study of Quan-wen *et al.* (1), the specificity of developed AC-ELISA, investigated using Egg drop syndrome virus, Infectious laryngotracheitis virus, Infectious bursal disease virus, Infectious bronchitis virus, and Newcastle disease virus, was estimated to be 94.4%.

The detection limit of the influenza virus with our developed AC-ELISA was as 0.1 HA unit. For comparison, the sensitivity of AC-ELISA in the study of Ho *et al.* (23) was estimated as 1-2 HA. Quan-wen *et al.* (1) also showed that the sensitivity of their developed AC-ELISA was limited to 8 HA. Therefore, it appears that the ELISA developed in our study is more sensitive than the hemagglutination assay was.

To compare it with the RT-PCR method, we also tested tracheal swabs collected from experimentally infected chickens by RT-PCR. Using RT-PCR, the virus was again detected most frequently between the 2nd and 6th days post-infection. Similar results have been found by Noroozian and Vasfi Marandi (25) that using RT-PCR detected H9 virus from 3 to 7 days after experimental infection.

Velumani et al. (22) also developed an AC-ELISA for H7N1 diagnosis and detected this virus in tracheal swab samples of experimentally infected chickens at the 3rd to 7th days after infection. They also used RT-PCR simultaneously and found that this method can detect the cDNA of the virus at the 3rd to 7th days after infection.

However, in the study of Chen *et al.* (4), the sensitivity of AC-ELISA for the H9 virus was 10^2 times less than that of RT-PCR.

The enhanced sensitivity of AC-ELISA in the present study can be related to the high affinity and avidity of the monoclonal and polyclonal antibodies, leading to an efficient antigen detection of the assay.

In the clinical assay, the introduced AC-ELISA was able to detect the influenza virus in swab samples prepared 3 to 5 days following experimental infection. Some experimentally infected chickens also had positive results at the 2nd to 6th days of experimental infection. The most significant reactions were obtained from samples collected four days after experimental infection. These results are in agreement with the report of Chomel *et al.* (15). They were able to detect a remarkable amount of NP antigen from the 2nd to 5th days, after infection and nearly in all cases, the most significant NP antigen detection was observed at the 4th day of infection. Lack of antigen detection in birds beyond the 5th to 6th days post infection can be due to immune responses which reduce the virus shedding. Because, as demonstrated, the HI titer of chickens was detected at the 7th day

post-infection, and this could be a reason for this finding.

While in the studies of Ji *et al.* (13) and Quan-wen *et al.* (1) the OD index was applied for determining positive or negative swab samples, in the present study the SP index was used. SP is a more accurate index for evaluating the positivity of clinical samples, because by applying this index the effects of possible variations on the outcomes of clinical samples, and positive control (NP antigen) will be eliminated.

With respect to the fact that the clinical experiment was based on the antigen detection in tracheal swabs, several tracheal swabs from uninfected chickens were also collected to determine the cut-off point. Chen *et al.* (4) and Chiu *et al.* (9) have used the mean OD values of negative controls plus 2 SD in order to calculate the cut-off point of their experiments. In the present study, the cut-off point was at first considered as the mean SP value obtained from the uninfected population plus 2 SD but it was consequently revealed that this cut-off point, despite increasing the sensitivity, will decrease specificity. Therefore, the cut-off point was set based on the mean SP value obtained from the uninfected population plus 3 SD.

In conclusion, the designed AC-ELISA has the potential for the detection of (at a minimum) the H9 Avian influenza virus and human H1 and H3 viruses. Further studies are necessary to verify the applicability of the assay for the detection of other influenza virus serotypes.

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RAZVOJ IN PREIZKUS NOVE METODE ELISA Z UJETJEM PROTITELES ZA ODKRIVANJE VIRUSA INFLUENCE A

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Povzetek: Namen raziskave je bil razvoj nove metode elisa z ujetjem protiteles za lažje diagnosticiranje okužbe z virusom ptičje influence. Nukleoprotein (NP) virusa smo najprej vezali z monoklonskimi protitelesi D'C4 in nato kompleks nukleoproteina in monoklonskega protitelesa označili s kunčjimi poliklonskimi protitelesi. Ta metoda ni kazala nobene navzkrižne (napačne) reakcije z drugimi virusi ali bakterijami ptičjega porekla, z njo pa smo lahko zaznali prisotnost virusa ptičje influence serotipa H9, pa tudi viruse človeške influence seroptipov H1 in H3. Občutljivost te metode za določanje linije H9N2 (A/Chicken/Iran/AH-1/106) virusa H9 je bila desetkrat višja v primerjavi z metodo hemaglutinacije in je bila primerljiva z občutljivostjo metode RT PCR. Z njo smo virus lahko določili v sapničnih izpirkih že 3 do 5 dni po okužbi poskusno okuženih piščancev. Iz pridobljenih rezultatov sklepamo, da nanovo razvita metoda elisa z ujetjem protiteles lahko zazna serotype H9, H1 in H3 virusa influence pri piščancih in ljudeh. Dokazali smo, da je metoda zanesljiva in zelo občutljiva za zaznavanje okužb s serotipom H9, uporabnost te nove metode za zaznavanje okužb z drugimi serotipi virusov influence pa bomo morali še dokazati.

Ključne besede: influenca virus A; nulleoprotein; monoklonska protitelesa; elisa z ujetjem protiteles