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Original Research Article

Cloning and bacterial expression of hemagglutinin antigen stem of H9N2 avian influenza virus

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A B S T R A C T

Avian influenza virus (AIV) is one of the main causes of high mortality and economic losses in poultry industry. Therefore, more research in structural identification, efficient and rapid detection methods, control and prevention of the disease are needed. Current study was conducted to clone and express the main region of hemagglutinin (HA) stem of H9N2 AIV, which consists of a part of HA1 region, cleavage site, and a part of HA2, in prokaryotic expression system. Considering the HA nucleotide sequence data of the isolated H9N2 AIVs from Iran, a conserved encoding sequence of the target HA gene of influenza virus strain A/Chicken/Iran/772/1998 (H9N2), was selected, amplified, inserted in pET28-a, and transformed into the BL21 (DE3) E. coli. Gene expression was induced by IPTG and analyzed on SDS-PAGE. Nucleotide sequencing and western blotting were performed to analyze the cloned gene, and the expressed proteins, respectively. Amplified gene was successfully cloned in pET28-a, transformed, and expressed. Sequencing confirmed the cloned gene completion and its correct position. SDS-PAGE and western blotting confirmed the expected expressed protein with an approximate 22 kDa molecular weight. The present study can serve as a fundamental experiment for the expression of the main region of HA stem in H9N2 AIV. The observations suggest that due to the conservation of this region among other H9N2 subtypes, the produced protein can be used as a candidate in developing rapid diagnostic tests and vaccines. Further research to evaluate the immunity of this expressed HA protein in the lab animal models challenged with influenza isolates, is under way. Meanwhile, another study addressing the ability of the respective protein in the detection of specific antibodies against the virus in clinical specimens will be done.

Keywords

Prokaryotic expression; Hemagglutinin; Influenza; H9N2.

Introduction

Avian Influenza virus (AIV) is one of the most dangerous infectious pathogens belonging to the Orthomyxoviridae family and the genus influenza virus. Due to antigenic characteristics of virus core proteins, AIVs are grouped into three

types of A, B and C (Fouchier *et al.*, 2005; Swayne and Suarez, 2000). The viruses have been shown to infect a great variety of both wild and domestic birds, such as turkey, chicken, quail, pheasant, chukar and other minor domestic poultry (Cameron *et al.*, 2000; Liu *et al.*, 2003; Guan *et al.*, 1999). Backyard chickens and wild waterfowl birds such as ducks play an important role in the epidemiology of low pathogenic avian influenza viruses (LPAIVs) and have always been considered as subclinical carriers (Hadipour *et al.*, 2011; van den Berg *et al.*, 2008). In addition, AIVs may cause acute and highly contagious respiratory diseases in human and other mammals. The virus transmission to human may occur occasionally and during close contact with contaminated birds' secretion sources and surfaces. In December 2003, H9N2 virus was isolated from a 5-year-old child with clinical signs in Hong Kong but the source of the infection was unknown (Butt et al., 2005). In the last two decades, the LPAIVs such as H9N2 have been identified as an etiological infectious agent with serious economic loss in poultry industry in Asia, Middle East, Europe and America (Halvorson *et al.*, 1998; Nili and Asasi, 2002, 2003).

Out of twelve recognized proteins of AIVs, hemagglutinin (HA) and neuraminidase (NA) project through viral envelope and are available for interaction with cellular molecules (Hughes *et al.*, 2001, Sriwilaijaroen *et al.*, 2012). HA is encoded by RNA segment 4 (Webster *et al.*, 1992). The cleavage site of HA protein is different in highly pathogenic avian influenza viruses (HPAIVs) and LPAIVs with a pair of di-basic amino acid residues. Consequently, in HPAIVs the structure is cleaved with ubiquitous proteases such as furin and PC6; however, in LPAIVs, the only enzyme which cleaves the HA protein is trypsin-like protease leading to different pathogenicity of them (Senne *et al.*, 1996).

Due to the potency of H9N2 virus to cause the next pandemic in human, finding effective methods to control and prevent the virus infection is important (Maines *et al.*, 2008). Vaccination has been shown to be one of the most effective tools to control and prevent the influenza infection and reduce its complications. In doing so, both conventional inactivated vaccines and recombinant vector vaccines can be used (Capua and Alexander, 2006). The current egg-based technology for producing inactivated influenza vaccine was created in the 1950s (Osterholm, 2005). Encouraging recombinant vaccine systems based on the expression of viral proteins have been pursued in the world.

The objective of the present study was cloning and expression of HA gene of H9N2 influenza A virus in prokaryotic expression system.

Materials and Methods

Influenza Viruses

Influenza Virus strain A/Chicken/Iran/772/1998 (H9N2), was kindly provided by *Razi* Vaccine & Serum Research *Institute*, HesaraK, Karaj, Iran. It was grown in the allantoic cavities of 9-day, embryonated chickens' eggs at 35˚C for 3 days. The infectious allantoic fluid was aliquoted and evaluated for EID50 with Reed and Muench method and stored at -70˚C Reed and Muench, 1938).

RNA Isolation

The viral genomic RNA was extracted

from 100-200 µl of allantoic fluid using the guanidium thiocyanate-phenol-chloroform method (RNX^{TM}-plus isolation, Cinnagen, Iran) according to the manufacturer's instructions. One ml of RNX solution was added to the allantoic fluid and the samples were incubated at room temperature for 5 minutes. Then, 0.2 ml chloroform was added; the contents were mixed, and the mixture was centrifuged at 12,000 g (4°C) for 15 minutes. The aqueous phase was recovered and transferred to the fresh tube. An equal volume of isopropanol was added and after storing for 15 minutes on ice, the samples were centrifuged at 12,000 g (4°C) for 15 minutes. Afterwards, the supernatant was removed and the RNA pellet was washed with 1 ml of 75% cold ethanol by several inverting and subsequently centrifugation for 8 minutes at 7,500 g (at 4°C). The RNA pellet was then dried at room temperature for 10-15 minutes and resuspended in 50-60 µl of nuclease free water, and stored at -70°C.

Primers and cDNA Synthesis

Oligonucleotide primers (Sec4F and Sec4R2) were designed according to the 20 available hemaglutinin nucleotide sequence data of H9N2 influenza viruses, which were isolated from Iran previously, from the GenBank database of the National Center for Biotechnology Information (NCBI). The sequences were analyzed by Lasergene sequence analysis software package (DNAStar, Madison, WI, USA) and the primer sets were designed based on the common segment which carried NheI and HindIII restriction cut sites with respect to the cleavage site of the molecule. The extracted RNA was used for synthesis of the first strand cDNA using M-MuLV reverse transcriptase (Fermentase, Lithuania). Eight µl of RNA was mixed with 2 µl of 20 µM reverse primer and 2 µl water and was incubated at 65-70°C for 10 min and then, on ice for 5 min. Later, 4 µl of the reaction buffer, 40 U of the RiboLock^{TM} RNase Inhibitor and 2 µl of 10 mM dNTPs mix, were added to the initial solution. After incubation at 37°C for 5 min, 200 U of RevertAid^{TM} M-MuLV Reverse Transcriptase was added to the reaction tube which was then incubated for 1 h at 42°C. Finally, cDNA was stored at -20°C until performing the PCR.

Polymerase Chain Reaction (PCR)

The single strand cDNA was used as the template for double strand DNA synthesis. PCR was carried out using pfu DNA polymerase (Fermentas, Lithuania) in a 25 µl reaction containing one unit of enzyme, 8 mM MgSO4, 200 pmol of each dNTPs and 400 nM of each of the primers Sec4F and Sec4R2. The reaction mixture was heated to 94°C for 3 min and then, incubated for 35 cycles of 94°C for 50 sec, 61°C for 50 sec and 72°C for 1 min, and 5 min at 72°C for an additional extension. The PCR products were analyzed on 1% agarose gel.

Cloning

The DNA fragment was initially cloned in intermediated cloning vector pTZ57R/T (Fermentase, Lithuania). The amplicons were extracted and purified from agarose gel using the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) and subsequently cloned into the T added EcoRV blunt site of pTZ57R plasmid, using T4 DNA ligase (Fermentas, Lithuania) at 16°C for overnight. The recombinant plasmid (pTZ57R/HA) was transformed into the XL1-blue E. coli,

competent cells. Transformants were grown on TSA plate with 100 µg/ml of ampicillin. Some colonies were subjected to PCR using M13 universal primers and two gene fragment-carrying colonies were chosen for plasmid isolation and sequencing. The purified intermediated recombinant plasmid, pTZ57R/HA and also expression vector, pET28-a (Novagen, San Diego, USA) were cut with restriction enzymes NheI (Fermentase, Lithuania) and HindIII (Takara, Japan) and were electrophoresed on 1% agarose gel to confirm the correct size. The released fragment (HA gene) and cut pET28-a were extracted from gel and ligated using T4 DNA ligase at a 1:3 ratio of vector at 16°C for overnight. The ligation product (pET28-a/HA) was transformed into competent BL21 (DE3) E. coli and transformed cells were selected on TSA plates containing 25 µg/ml kanamycin. The selected clones were further analyzed by PCR using specific and also universal T7 primers and finally, recombinant plasmid of two positive clones was sequenced by a commercial facility using T7 promoter and terminator primers. The aminoacid sequences distance analysis between the produced protein (2811) and the other 20 available hemaglutinin nucleotide sequence data of H9N2 influenza viruses isolated was performed by Lasergene sequence analysis software package (DNAStar, Madison, WI, USA).

Expression and SDS-PAGE analysis

Five ml of LB medium with 25 µg/ml kanamycin was inoculated with a few fresh colonies of recombinant clone and incubated at 37˚C with shaking. When the culture OD_{600} reached 0.6, protein expression was induced with a final concentration of 1mM of isopropyl β-*D*-1-thiogalactopyranoside (IPTG), and then, incubated at 37˚C for an additional 16 hrs. The overnight culture was centrifuged at 5000g for 15 min at 4˚C and the supernatant was discarded. To prepare cell lysate for SDS-PAGE, the pelleted cells were washed three times with cold PBS, resuspended in 100 µl of cell lysis buffer (0.225 M Tris-HCl pH 6.8, 20 % glycerol, 2 % SDS, 0.1 M DTT and 0.02 % Bromophenol blue) and stored at -20˚C. The protein profile of antigens was examined by SDS-PAGE using a 4 % stacking and a 12 % separating acrylamide gels in vertical electrophoresis apparatus.

Western-blotting assay

To confirm that the expressed protein was as expected, western-blotting assay was done. First, the respective influenza virus with determined titer based on hemagglutination test (HA) was injected into a goat 5 times for 5 continuous weeks and the sera were separated each time. To understand the presence and the highest titer of secreted antibodies against the injected virus, agar gel immunodiffusion (AGID) and hemagglutination inhibition (HI) were performed on the sera and the highest titer was used for western-blotting. Having done the SDS-PAGE assay, as mentioned above, the gel was immersed in a transfer buffer, and the proteins were transferred onto the nitrocellulose membrane. The membrane was incubated overnight with a blocking solution (4% nonfat dried milk), and subsequently incubated with the goat positive sera for 1 h at 37°C. Then, the membrane was incubated for 1 h with horse-radish peroxidase (HRP)-conjugated horse anti-goat IgG (Vector Laboratories, USA) according to the manufacture's protocol. Finally, 3,3- Diaminobenzidine tetrahydrochloride (DAB, Sigma, Germany) was used to visualize the band on the paper.

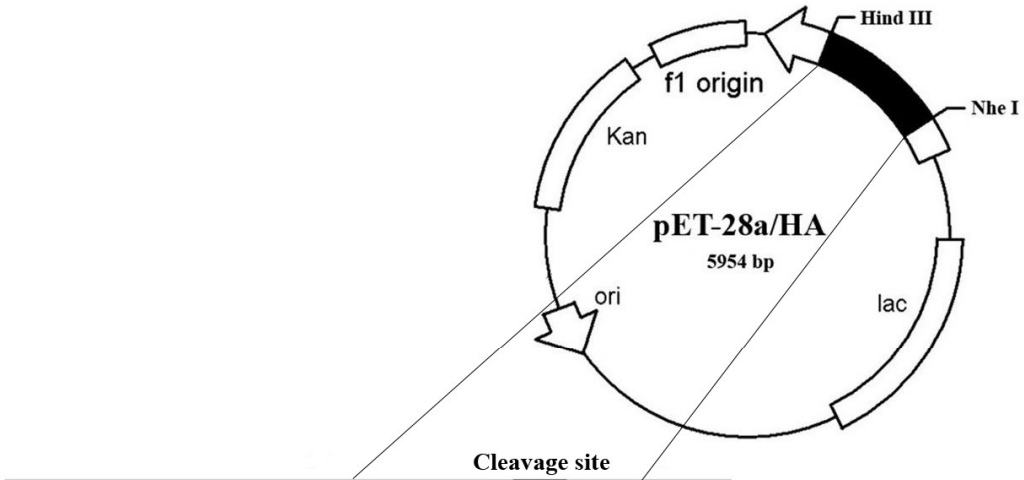
Results and Discussion

The EID_{50}/ml of the growth virus in the infectious allantoic fluid was 10^9. Sec4F and Sec4R2 which carried NheI and HindIII restriction cut sites, respectively, were designed to amplify a 585 bp (195 aa) fragment beside the cleavage site of HA gene (including a part of HA1, cleavage site, and a part of HA2), and this fragment was named as 2811. The HA gene was amplified by RT-PCR, identified by 1% agarose gel electrophoresis, and both recombinant pTZ57R/ HI and, the expression vector, pET28-a, were successfully digested by NheI and HindIII restriction enzymes. Then, the released insert was well purified and cloned into the digested pET28-a vector and sequencing confirmed the cloned gene completion (Figure 1). The results of SDS-PAGE after induction confirmed the production of expected recombinant protein with approximate 22 kDa molecular weight in total lysate of E. coli BL21 (DE3). AGID test demonstrated that the titers of antibodies against the virus increased after the 2nd week and the HI results showed that the highest titer was in the 5th week with the HI quantity of 1:512. The western-blotting also confirmed the expected protein. The aminoacid sequences distance analysis between the produced protein (2811) and the other 20 available hemaglutinin nucleotide sequence data of H9N2 influenza viruses isolated from Iran has been shown in Figure 2.

Nowadays, genetic recombination technology has been developed rapidly in the world, and both prokaryotic and eukaryotic expression systems are applied extensively. Despite the limitations of bacterial expression systems including inability to perform the post-translational modifications, they are used frequently due to their rapid growth rate, capacity for continuous fermentation, and relatively low cost (Belshe *et al.*, 1988; Haag and Ostermeier, 2009). Since 1986, pET expression vectors, as a powerful and highly selective system, have been widely used for quick production of a large quantity of proteins (Moffatt and Studier, 1986). This system is under the control of strong bacteriophage T7 transcription. In the present study, the target sequence was cloned into pET28-a vector, which bears an N-terminal His•Tag®/ thrombin/ T7•Tag® structure as well as an optional C-terminal His•Tag sequence. Moreover, E. coli BL21 (DE3) strain which is used for the transformation step of the recombinant vector in this study contains T7 polymerase upon IPTG induction (Haag and Ostermeier, 2009; Studier, 2005).

HA protein is one of the main membrane antigens of influenza A virus, also recognized as a major target molecule for host immune system during the viral infection (Sriwilaijaroen *et al.*, 2012). HA monomer, consisting of two linked subunits; HA1 (~324 amino acids) and HA2 (~222 amino acids, (Webster *et al.*, 1992), totally weighs about 60 kDa in the unglycosylated form (Sriwilaijaroen *et al.*, 2012). This surface glycoprotein is expressed as a trimer and plays important roles both in the virus attachment and the fusion between the viral envelope with the host cell endosomal membrane (Sriwilaijaroen *et al.*, 2012). During the viral fusion step, cleavage of HA protein by cellular enzymes into HA1 and HA2 is

Figure.1 Plasmid map of pET-28a/HA and the amino acid sequence of 2811 fragment



RSNGNLIAPWYGHVLSGGSHGRILKTDLNSGNCVVQCQTEKGGLNSTLPFHNI
SKYAFGNCPKYVRVKSLKLAVGLRNVPARSSRGLFGAIAGFIEGGWPGLVAGW
YGFQHSNDQGVGMAADRDSTQKAIDKITTKVNNIVDKMNKQYEIIDHEFSEVE
TRLNMINNKIDDQIQDVWAYNAELLVLLENQKTLDE

Figure.2 Sequence distance analysis between the amino acid sequence of hemagglutinin stem of influenza virus strain A/Chicken/Iran/772/1998 (H9N2) and the other 20 hemagglutinin nucleotide sequence data of H9N2 influenza viruses isolated from Iran. The Genbank accession numbers of the isolates are available on the right

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
1		99.5	99.5	99.5	99.5	99.5	100.0	98.5	98.5	96.9	96.9	96.9	96.4	98.5	97.9	95.9	96.4	96.4	98.5	95.9	96.9	1	2811(Produced protein)
2	0.5		100.0	99.0	99.0	99.0	99.5	97.9	99.0	96.4	96.4	97.4	96.9	97.9	98.5	95.4	96.9	96.9	99.0	96.4	96.4	2	GQ497117
3	0.5	0.0		99.0	99.0	99.0	99.5	97.9	99.0	96.4	96.4	97.4	96.9	97.9	98.5	95.4	96.9	96.9	99.0	96.4	96.4	3	GQ497118
4	0.5	1.0	1.0		100.0	99.0	99.5	99.0	99.0	97.4	97.4	96.4	95.9	99.0	98.5	96.4	95.9	95.9	99.0	95.4	97.4	4	GQ497119
5	0.5	1.0	1.0	0.0		99.0	99.5	99.0	99.0	97.4	97.4	96.4	95.9	99.0	98.5	96.4	95.9	95.9	99.0	95.4	97.4	5	GQ497120
6	0.5	1.0	1.0	1.0	1.0		99.5	99.0	97.9	96.4	96.4	96.4	95.9	97.9	97.4	95.4	95.9	95.9	97.9	95.4	96.4	6	GQ497121
7	0.0	0.5	0.5	0.5	0.5	0.5		98.5	98.5	96.9	96.9	96.9	96.4	98.5	97.9	95.9	96.4	96.4	98.5	95.9	96.9	7	GQ497122
8	1.6	2.1	2.1	1.0	1.0	1.0	1.6		97.9	96.4	96.4	95.4	94.9	97.9	97.4	95.4	94.9	94.9	97.9	94.4	96.4	8	GQ497123
9	1.6	1.0	1.0	1.0	1.0	2.1	1.6	2.1		97.4	97.4	96.4	95.9	97.9	99.5	96.4	95.9	96.9	100.0	96.4	97.4	9	GQ497124
10	3.1	3.7	3.7	2.6	2.6	3.7	3.1	3.7	2.6		100.0	93.8	93.3	96.4	96.9	97.9	93.3	94.4	97.4	93.8	100.0	10	GQ497125
11	3.1	3.7	3.7	2.6	2.6	3.7	3.1	3.7	2.6	0.0		93.8	93.3	96.4	96.9	97.9	93.3	94.4	97.4	93.8	100.0	11	GQ497126
12	3.1	2.6	2.6	3.7	3.7	3.7	3.1	4.8	3.7	6.4	6.4		99.5	95.4	96.4	93.3	99.0	99.5	96.4	99.0	93.8	12	GQ497128
13	3.7	3.1	3.1	4.2	4.2	4.2	3.7	5.3	4.2	7.0	7.0	0.5		94.9	95.9	92.8	98.5	99.0	95.9	98.5	93.3	13	GQ497129
14	1.6	2.1	2.1	1.0	1.0	2.1	1.6	2.1	2.1	3.7	3.7	4.8	5.3		97.4	95.4	94.9	94.9	97.9	94.4	96.4	14	GQ497130
15	2.1	1.6	1.6	1.6	1.6	2.6	2.1	2.6	0.5	3.1	3.1	3.7	4.2	2.6		96.9	96.4	96.9	99.5	96.4	96.9	15	GQ497131
16	4.2	4.8	4.8	3.7	3.7	4.8	4.2	4.8	3.7	2.1	2.1	7.0	7.6	4.8	3.1		93.3	93.8	96.4	93.3	97.9	16	GQ497132
17	3.7	3.1	3.1	4.2	4.2	4.2	3.7	5.3	4.2	7.0	7.0	1.0	1.6	5.3	3.7	7.0		98.5	95.9	97.9	93.3	17	GQ497133
18	3.7	3.1	3.1	4.2	4.2	4.2	3.7	5.3	3.1	5.9	5.9	0.5	1.0	5.3	3.1	6.4	1.6		96.9	99.5	94.4	18	GQ497134
19	1.6	1.0	1.0	1.0	1.0	2.1	1.6	2.1	0.0	2.6	2.6	3.7	4.2	2.1	0.5	3.7	4.2	3.1		96.4	97.4	19	GQ497135
20	4.2	3.7	3.7	4.8	4.8	4.8	4.2	5.9	3.7	6.4	6.4	1.0	1.6	5.9	3.7	7.0	2.1	0.5	3.7		93.8	20	GQ497136
21	3.1	3.7	3.7	2.6	2.6	3.7	3.1	3.7	2.6	0.0	0.0	6.4	7.0	3.7	3.1	2.1	7.0	5.9	2.6	6.4		21	GQ497127
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		

necessary, which ultimately leads to infectivity of the virus (White *et al.*, 1982). Previous studies indicated that HA1, containing the HA globular head, is the most immune-dominant region of the HA molecule because of its exposure on the envelope of the virus (Clementi *et al.*, 2012). However, its high nucleotide sequence variations between different influenza A subtypes, gives rise to difficulty with producing influenza universal vaccines as well as genus molecular diagnostic assays. On the other hand, the HA2 region is more conserved between different HAs, but with less immunogenicity, when presented to the host immune system (Clementi *et al.*, 2012). Therefore, its application for developing diagnostic tests may be more possible. In the present study, a part of stem region of HA of H9N2 influenza virus was expressed. This produced protein consists of a part of HA1 region, cleavage site, and a part of HA2 region between 254 to 448 aminoacids. Sequence distance analysis contains small differences between the produced protein and the other available HA nucleotide sequence data of H9N2 influenza viruses isolated from Iran. Therefore, it can be concluded that this produced protein can be used as a candidate in developing rapid diagnostic tests for H9N2 influenza viruses in the region.

In conclusion, the target HA gene was successfully cloned in pET28-a vector, transformed to E. coli BL21 (DE3) strain and expressed. The present study can serve as a fundamental experiment for the expression of the main region of HA stem in H9N2 AIV. The observations suggest that the produced protein can be used as a candidate for developing rapid diagnostic tests and vaccines for the respective pathogen. Further research to evaluate the immunity of this expressed HA protein in the lab animal models challenged with influenza isolates, is under way. Meanwhile, another study addressing the ability of the respective protein in the detection of specific antibodies against the virus in clinical specimens will be done.

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