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Cloning and Expression of Influenza H1N1 NS1 Protein in *Escherichia Coli* BL21

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Background: The influenza virus is globally pathogenic and it is usually associated with zoonotic respiratory diseases. This virus has caused a number of pandemics with high mortality rates. The non-structural protein-1 (NS1) of influenza A viruses is a non-essential virulence factor that has multiple accessory functions during a viral infection. This protein is highly conservative. It has been shown that this protein plays a major role against the immunity responses of host cells.
Objectives: The aim of this study was to produce the recombinant influenza NS1 protein by the use of a bacterial production system, in order to evaluate the immunological and structural features of this protein in future researches.
Materials and Methods: The NS1 gene construct was artificially synthesized; subsequently it was sub-cloned into the pQE30 expression vector. The expression vector was then transformed into the BL21 cells and induced by IPTG. Finally, the expression was evaluated by SDS-PAGE and Western blotting techniques.
Results: The NS1 gene was successfully cloned and transformed into expression cells. As a result, a 23 kDa band was observed both on the SDS-PAGE and nitrocellulose paper after Western blotting.
Conclusions: Based on the results of this study, it could be concluded that the NS1 gene of influenza A H1N1 virus (A/Shiraz/14/2010 strain) could be cloned and the rNS1 protein (recombinant NS1 protein) could be expressed using a bacterial protein translation system. Since this protein is a conservative protein among influenza A viruses, it could be used as a potent vaccine for the prevention of various types of pandemics caused by influenza A.

Keywords: Cloning; Expression; Influenza A Virus; Non-structural Protein

1. Background

Influenza is commonly defined as a type of viral infection caused by the influenza virus. This virus is extremely pathogenic and is typically known as the causative agent of zoonotic respiratory diseases. Influenza A viruses contain single-stranded, negative sense, segmented RNA genomes, consisting of eight segments of viral RNA (vRNA), which encode 11 known proteins. This enveloped virus is a member of the family Orthomyxoviridae and it is classified into subtypes depending upon its surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Depending on the strain, the influenza A virus genome (8 RNA segments) encodes 10 or 11 proteins.

The last segment (segment 8), which is also the shortest one, encodes an mRNA that is alternatively spliced in order to express the nonstructural protein-1 (NS1), and the nuclear export protein (NEP) (1). The NS1 protein is not present in the virion and is only expressed in infected host cells (2). The full-length NS1 is probably a homodimer protein, with both RNA binding and effector domain contributions. NS1 is a multifunctional protein that significantly links the protein-RNA and protein-protein interaction (3, 4). Researchers have demonstrated that the phylogenetic relationship of the NS1A gene isolated from genetically distinct infected cells, verifies that the NS1A protein undertakes evolutionary divergence (5). The viral NS1 protein is generally considered as the common factor by which all influenza A viruses antagonize host immune responses, which is a survival mechanism of the virus (6, 7). There are only three known A subtype influenza viruses affecting human cells (H1N1, H2N2, and H3N2), and both H3N2 and H1N1 are currently circulating among humans (2).

Implication for health policy/practice/research/medical education:
Influenza is a viral respiratory disease of global importance, which makes a specific and efficient worldwide vaccine essential. This study was part of a larger research for the development of an influenza vaccine.

2. Objectives

Influenza NS1 proteins play a key role in viral infections, as well as in various kinds of influenza H1N1 pandemics reported globally. The aim of the present study was to clone and express the NS1 protein of the influenza A H1N1 virus (A/Shiraz/14/2010 strain), in order to provide preventive strategies for the Iranian population in the future.

3. Material and Method

3.1. Preparation of the NS1 Gene Construct

The 597 bp nucleotide sequence of the influenza H1N1 NS1 protein (A/Shiraz/14/2010) was obtained from the NCBI (ID No. HQ606471.1). According to the available data, the NS1 gene was considered for codon optimization using the Encorbio website (www.encorbio.com/protocols/codon.htm), and then it was artificially synthesized (Euorofins, China) and inserted into a pCR 2.1 plasmid. Universal M13 primers with the following sequences were used in order to amplify the NS1 sequence using a PCR technique:

M13 F 5´- GTA AAACGACGGCCAGTG -3´

M13R 5´- GGAAACAGCTATGACCATG -3´

The PCR reaction contained 0.1 μg of DNA, 1 × PCR buffer, 1.5 mM MgCl2, 0.1 mM of dNTP, 20 pmol each of forward and reverse primers, and 1 unit of Taq DNA polymerase (Cinnagen, Iran). By adding distilled water, the final volume of the reaction was set to 20 μl. The PCR cycling program was conducted at the following temperatures: denaturation at 94°C for 30 sec, annealing at 49°C for 30 sec, and extension at 72°C for 45 sec. The previously mentioned cycle was repeated 30 times. The reaction was settled at 94°C and 72°C for 5 min before and after PCR cycling, respectively (8). The amplified PCR products were subjected to electrophoresis using 1.5% agarose gel stained with SYBr green and the band was observed under a UV transilluminator.

To subclone the NS1 gene into the expression vector, the NS1/pCR 2.1 plasmid was digested with HindIII and KpnI restriction enzymes. The digestion product was analyzed by electrophoresis on 1.5% agarose gel and the desired band was recovered using a DNA gel extraction kit (Qiagene, USA). The purified fragment was inserted into a digested pQE30 vector and transformed into a Top10 *E*scherichia *coli* strain. Recombinant plasmid was extracted by a plasmid extraction kit (Qiagene, USA). The recombinant plasmid was confirmed with PCR analysis with universal primers and digested by HindIII and KpnI.

3.2. The Expression of the NS1 Protein

The recombinant construct was transformed into *E. coli* BL21 in order to express the previously mentioned protein, and it was cultured on Luria broth agar medium containing 50 $\mu g.ml^{-1}$ of ampicillin. The transformed colony was inoculated in 3 mL x medium (1.2% BACTO TRYPTON, 2.4% yeast extract, 0.04% glycerol, 1% M9 salts) (M9 salts contained: 6.4% $Na_2H_2O_4$-$7H_2O$, 1.5% KH_2PO_4, 0.025% NaCl, and 0.05% NH_4Cl), then incubated at 37°C in a shaker at 200 rpm overnight. After 16 hours, the cultured bacteria was inoculated in a 50 mL flask and incubated at 37°C in a shaker at 200 rpm until the OD reached 0.6 at 600 nm, followed by induction of the cells with 1 mM IPTG for 6 hours at 37°C. The cells were harvested using 4 000 rpm centrifuge for 15 min. The pellet was resuspended in a lysis buffer (glycerol 10%, Tris (pH 8), PMSF 10mM, and 1% Triton X-100) (Merck, Germany). The fraction containing NS1 recombinant protein was analyzed by 12% SDS PAGE electrophoresis (8).

3.3. SDS-PAGE and Western Blot Analysis

Results of the SDS-PAGE were confirmed by a Western blot using a His-tag monoclonal antibody as the primary antibody and anti-mouse HRP conjugated immunoglobulin (Abcam, UK) as the secondary antibody (9).

4. Results

4.1. NS1 Gene Cloning

The synthetic plasmid (pCR2.1) containing the NS1 gene was transformed into the *E. coli* XL1-Blue strain bacteria and cultured on LB medium containing ampicillin. The extracted plasmid (pCR2.1) was uploaded and analyzed by electrophoresis on 0.8% agarose gel. The 597 bp NS1 gene was prepared from the pCR2.1 vector and inserted into HindIII and KpnI restriction sites of the pQE30 expression vector.

The PCR product was subjected to electrophoresis and the 860 bp bands were observed, which confirmed the amplification of the NS1 gene (Figure 1). The PCR product was also digested by restriction enzymes (HindIII and KpnI); and after a 2-hour incubation, a 660 bp band was observed under the UV illuminator. The digested plasmid was sub-cloned into the expression vector (pQE30). The NS1/pQE30 construct is demonstrated in Figure 2.

The recombinant protein was expressed and detected using different techniques including SDS-PAGE and Western blotting; in both methods, the expression of the 23 kDa protein was confirmed and compared with the control group.

4.2. SDS-PAGE Analysis

The expression of recombinant NS1 protein was detected and compared with control forms of the bacteria by SDS PAGE analysis (Figure 3).

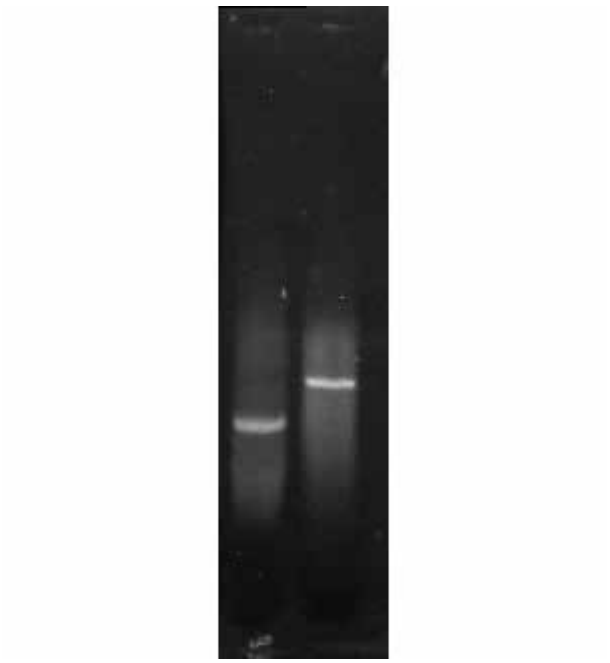
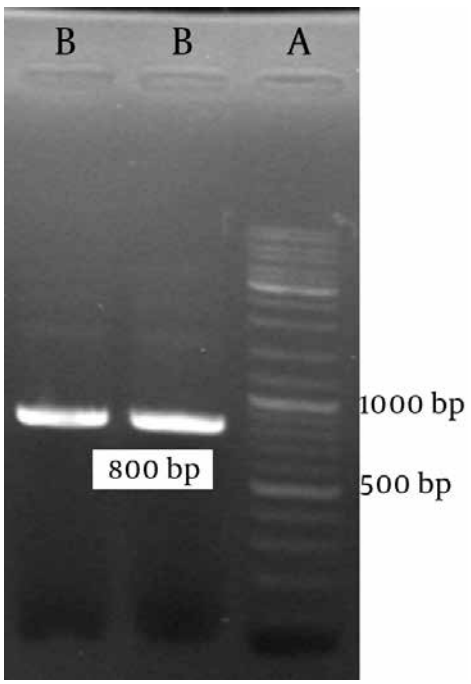


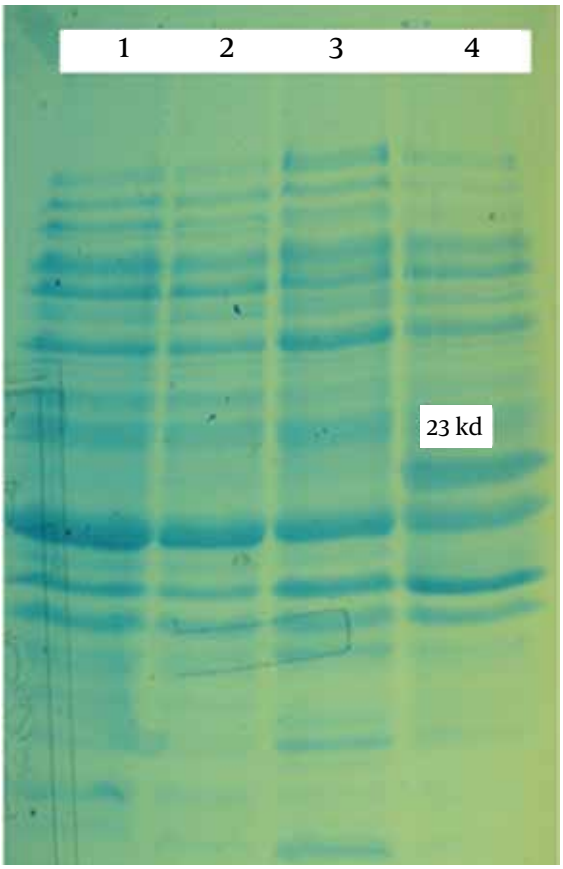
Figure 1. A) Digested NS1PCR product by HindIII and KpnI restriction enzymes, B) The 800bp universal PCR product of cloned NS1 into pCR1.2 before digestion

Figure 2. Confirmation of NS1/pQE30 by Vector Universal Primers



A) 100 bp DNA ladder, B) 800bp PCR product of the cloned fragment by plasmid universal primers

Figure 3. Comparison Between Protein Expressions at Different Times After Induction



Lane 1: lysate of bacterial cell content pQE30, Lane 2: lysate of host cell (control), Lane 3: lysate of bacterial cell content NS1/pQE30 before induction, Lane 4: lysate of expressed recombinant bacteria 5 hours after induction by IPTG.

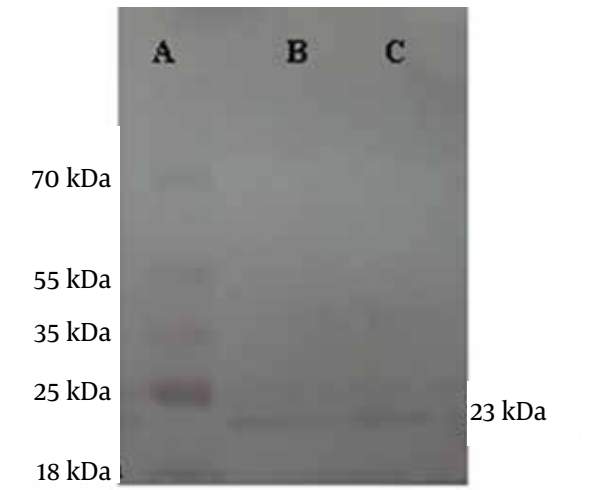


Figure 4. Western Blot Analysis of Expressed Protein with His-tag Monoclonal Antibody

4.3. Subsection style

The expressed 23kd protein was confirmed by Western blotting. The brown band for positive expression against the pre-stained protein marker (Fermentas, Lithuania) appeared 5 hours after induction (Figure 4).

5. Discussion

Influenza is a viral respiratory disease of global importance; it is caused by the influenza virus, which is an RNA virus with segmented genome and antigenic diversity. Influenza is responsible for about 20 000 deaths and 114 000 hospitalizations annually (2). The NS1 protein binds to the double-stranded RNA and forms dimers *in vivo*, and performs several important functions for the replication of the virus in its host (1). Influenza viruses lead to seasonal epidemics, which are due to mutations in the viral surface glycoproteins. The viral NS1 protein is widely regarded as the common factor by which all influenza A viruses antagonize host immune responses. The available data indicate that the main function of NS1 is to antagonize IFN-α/β mediated antiviral responses (10). The influenza A virus NS1 protein has a unique regulative property in mammalians, with which it can undergo a series of reassortment events under harsh pressure (11). Thus, many researchers have conducted extensive researches on the influenza A virus in order to find an effective vaccine according to the NS1 properties and features. Metreveli carried out a comparative study on the NS1 gene extracted from H7N7, H4N6, H12N5 and H6N8 subtypes (2, 11).

It has been observed that, mutant influenza A viruses that are unable to express NS1 only display high pathogenicity in mice lacking antiviral mediators, for instance STAT1 or the dsRNA-activated protein kinase (PKR) (6). The available data shows that the main function of NS1 in current *in vivo* models is to antagonize IFN-a/b mediated antiviral responses (10). Moreover, the NS1 protein also has multifunctional activities which help the virus to replicate in the host cells. These sorts of functions include: sequential regulation of viral RNA biosynthesis, regulation of viral genome splicing, increases in the viral translation process, and suppression of host immune responses such as the apoptotic pathways and activation of phosphoinosidine Kinase-3 (PIK-3) (10). A number of studies have adopted different transformation methods. Regardless of the method used in transformation, the bacterial strain, *E. coli*, is commonly used as an expression host for a range of therapeutic proteins (9). In the present study we tried to express NS1 recombinant protein of influenza A H1N1 virus (A/Shiraz/14/2010 strain) in order to provide preventive strategies for the Iranian population. However, Ward et al. used the yeast Saccharomyces cerevisiae, as a host for this goal (12). Recent studies have shown that the NS1 protein was expressed in both soluble and insoluble fractions. Typically, the expressed NS1 fusion protein is detected at 26 kDa (13, 14); in the present study a 23 kDa band was observed which is in agreement with previous studies. Based on the results of this study, it could be concluded that the NS1 gene of the influenza A H1N1 virus (A/Shiraz/14/2010 strain), could be cloned and the rNS1 protein (recombinant NS1 protein) could be expressed using the bacterial protein translation system. Since this protein is a conservative protein among influenza A viruses, it can be used as a potent vaccine for the prevention of various types of pandemics caused by influenza A.

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Authors Contributions

All authors participated equally in the manuscript preparation.

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References

1. Geiss GK, Salvatore M, Tumpey TM, Carter VS, Wang X, Basler CF, et al. Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. *Proc Natl Acad Sci U S A.* 2002;**99**(16):10736–41.
2. Metreveli G. Expression of influenza virus nonstructural protein 1 (NS1). Sweden, Uppsala: MSc; 2007.
3. Xia S, Monzingo AF, Robertus JD. Structure of NS1A effector domain from the influenza A/Udorn/72 virus. *Acta Crystallogr D Biol Crystallogr.* 2009;**65**(Pt 1):11–7.
4. Qiu Y, Krug RM. The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). *J Virol.* 1994;**68**(4):2425–32.
5. Zohari S, Gyarmati P, Thoren P, Czifra G, Brojer C, Belak S, et al. Genetic characterization of the NS gene indicates co-circulation of two sub-lineages of highly pathogenic avian influenza virus of H5N1 subtype in Northern Europe in 2006. *Virus Genes.* 2008;**36**(1):117–25.
6. Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, et al. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology.* 1998;**252**(2):324–30.
7. Kochs Georg, García-Sastre Adolfo, Martínez-Sobrido Luis. Multiple Anti-Interferon Actions of the Influenza A Virus NS1 Protein. *J Virol.* 2007;**81**(13):7011–7021.
8. Kazemi B, Seyed N, Bandehpour M, Sharifnia Z, Pakzad P. Cloning, expression and purification of truncated Chlamydia trachomatis outer membrane protein 2 (omp2) and its application in an ELISA assay. *Iran J Immunol.* 2008;**5**(3):148–55.
9. Bandehpour M, Khodabandeh M, Mosaffa N, Sharifnia Z, Ghazanfari T, Kazemi B. An efficient procedure for purification of recombinant human beta heat shock protein 90. *Daru.*

2010;**18**(1):64–8.
10. Hale BG, Randall RE, Ortin J, Jackson D. The multifunctional NS1 protein of influenza A viruses. *J Gen Virol.* 2008;**89**(Pt 10):2359–76.
11. Lih Lih O. Cloning and Overexpression of Influenza A H1N1 NS1 Protein In Escherichia coli. Malaysia: Universiti Teknologi; 2010.
12. Ward AC, Azad AA, Macreadie IG. Expression and characterisation of the influenza A virus non-structural protein NS1 in yeast. *Arch Virol.* 1994;**138**(3-4):299–314.
13. Manasatienkij W, Lekcharoensuk P, Upragarin N. Expression and Purification of NS1 Protein of Highly Pathogenic Avian Influenza Virus H5N1 in Escherichia coli. *Kasetsart J.* 2008;**42**(3):485.
14. Ma F, Li Y, Zheng L. Prokaryotic expression and purification of NS1 protein of human influenza A virus subtype H1N1. Chinese J Biol. 2009;22(3):221-225.