Original Article

Prokaryotic Expression of H1N1 Influenza A Virus Haemagglutinin Protein Globular Domain (HA1)

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Abstract

Background: Influenza viruses are a significant cause of morbidity and mortality. The influenza virus pandemics, 1918, 1977, and especially the most recent one, A/H1N1/2009, made evident the need for generating recombinant Influenza H1N1 antigens which are essential to develop both basic and applied research programs. Among influenza virus proteins, haemagglutinin (HA) is a major surface antigen of influenza virus, thus it is highly topical in influenza research and vaccine engineering programs. Alternatively, expression of fragments of the HA (HA1 and HA2) proteins in prokaryotic systems can potentially be the most efficacious strategy for manufacture of large quantities of influenza vaccine in a short period of time.

Materials and Methods: The gene encoding the HA1 protein of the influenza A/Puerto Rico/8/34 was amplified by PCR, then cloned into pTZ57R/T cloning vector. The fidelity of the HA1 open reading frame was confirmed by bidirectional sequencing, then sub-cloned into pET28a prokaryotic expression plasmid, and proteins containing HA1 N-terminally fused to His-Tag were produced in Escherichia coli BL21 through IPTG inducing. The accuracy of the expression was confirmed by running time coursed fraction samples taken before and after the IPTG induction in SDS-PAGE, Western blot analysis were also used for confirmation of the recombinant protein.

Results and Conclusion: The HA1 protein produced here could be considered and evaluated as a protective antigen, which its immunogenicity potential needs to be assessed in animal models along with proper control groups. Moreover, it could be subjected for polyclonal antibody preparation, which, in turn, may be used as an essential material in western blot analyses, as well as in other immunological applications, such as ELISA, immunocytochemistry, immunohistochemistry, and other immunological and serological studies.

Keywords: Influenza A, H1N1, Hemagglutinin, Prokaryotic Expression

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Introduction

Influenza is considered to be one of the most severe threats to human health and animal welfare. In temperate climates, seasonal epidemics occur mainly during winter while in tropical regions, influenza may occur throughout the year, causing outbreaks more irregularly. Influenza occurs globally with an annual attack rate estimated at 5%–10% in adults and 20%–30% in children (1).

All commercial influenza vaccines are produced by propagating the virus in embryonated chicken eggs (2).Their production system has some limitations, such as the need for specific pathogen-free (spf)

embryonated eggs, required time for adaptation to the new circulating virus subtypes, long production time, and limited capacity(3).Further processing is also needed to separate and inactivate viral particles and to purify the haemagglutinin (HA) protein, the primary vaccine antigen. This technology is slow and requires one embryonated egg per vaccine dose (2).

Several alternative strategies have been proposed to produce pandemic and seasonal influenza vaccines, but the expression and purification of a single antigenic protein in bacteria culture may be the simplest and fastest strategy for generating large quantities of new influenza vaccines (4). Moreover, recombinant vaccines produced in bacteria, free of other viral and cellular components, are expected to reduce complications associated with whole virus vaccines such as pyogenic reaction and Guillain-Barre syndrome (5).

Influenza A viruses are single stranded, negative-sense RNA viruses, comprising eight genome segments that are enveloped by a lipid bilayer containing haemagglutinin (HA) and neuraminidase (NA) glycoproteins. Both surface proteins are targeted by human humoral immunity (6), but haemagglutinin is the most abundant glycoprotein on the surface of influenza viruses (7), approximately 25% of total viral protein (8), and functions in viral entry into the host cell (9-11).

HA is synthesized as a precursor polypeptide HA0 in the endoplasmic reticulum, where it is assembled into trimers (3), and this homotrimer has a receptor binding pocket on the globular head of each monomer (12). Then, via the Golgi network, HA trimer is exported to the cell surface where it is cleaved by host proteases into two subunits: HA1 and HA2. The HA1 subunit forms a globular head which contains the receptor binding site, a main target for neutralizing antibodies (3, 13).

Antibodies reactive toward HA have been associated with host resistance and a decrease in disease severity. It has been shown that 60% of antibodies produced during an influenza infection are reactive toward the HA protein (9). Antibodies against HA protein block virus attachment, thereby decreasing the number of cells infected. They can also function to prevent fusion (12). Thus, only neutralizing antibodies directed against the HA glycoprotein are the primary mediators of protection against influenza virus infection (6, 14, 15), and are most effective in vivo (12).

As mentioned above, each HA monomer consists of an HA1 and an HA2 subunit. The HA1 subunit contains the receptor-binding site that mediates viral attachment to the cell membrane, whereas the HA2 subunit contributes to membrane fusion (6, 15, 16). The HA1 domain contains most of the antigenic sites and is more prone to mutations and antigenic drift than other parts of the protein (6, 17). Researches have also shown that HA1 fragment, rather than HA2, is the site of HA and host cell interactions, and the majority of the antibodies are reactive against HA1(6, 8).

That was why we have chosen to work on the globular head of the HA protein, HA1, which has more important role in attachment to the host cell, so in contrast with virus other proteins, blocking it could be happened before the infection of the host cell happens, and against which most human antibodies are made during an influenza infection (9).

One concern may be that complete viral particle may be more immunogenic than recombinant peptides because the former are poly-antigenic and undergo post-transcriptional modifications such as glycosylation. But the researches have shown that glycosylation is not a mandatory requirement for influenza vaccine efficacy (4).

Methods

Viral RNA extraction and Reverse-transcription. Influenza A virus (A/Puerto Rico/8/34 (H1N1)), has been propagated on Madin-Darby Canine Kidney (MDCK) cell line,supplemented with 5% fetal calf serum (FCS), to increase the viral titerto 512 HAU/ml (Haemagglutinin Unite). Total RNA was extracted from 200 µl of the supernatant of infected MDCK cells using Invitrogen PureLink® Viral RNA/DNA Mini Kit (cat# 12280-050).

Amplification of HA1 gene by RT-PCR. To perform RT-PCR, 50 µl of the extracted RNA is first converted into cDNA using M-MuLV reverse Transcriptase (Fermentas cat# EP0451) and the universal primer (uni 12), introduced for amplification of all genomic fragments of all influenza A strains (5’-AGCAAAAGCAGG-3’) (18).

The HA1 gene (978 bp) was specifically amplified by PCR of the cDNA, using High Fidelity (Fermentas,cat#K0191) enzyme and two synthetic oligonucleotide primers (HA1-F-NheI, 5'-GCAGCTAGCGACACAATATGTATAGGTTACC ATG-3'; HA1-R-SalI, 5'-TCAGTCGACTTATCTGGATTGAATGGACGGA ATGTTC-3'). As underlined in the reverse primer, a termination codon has been designed on the reverse primer, because trough the influenza virus infection, the HA gene is being translated intact, and the cleavage between the two fragments (HA1 and HA2) happens afterwards. That is why HA gene itself has the termination codon on the end of the HA2 segment, and not in the end of HA1 fragment. According to our cloning strategy,a restriction enzyme cut site, NheI sequence, and another restriction enzyme cut site, Sall sequence, was introduced in the forward and reverse primers respectively.

Amplification reaction was carried out under the following profile: 3 min at 95°C followed by 31 cycles at 95°C for 30 sec. 58°C for 30 sec, and 72°C for 60 sec, with a final extension step at 72°C for 10 min.

Then the PCR products were analyzed on 1% (w/v) agarose gel electrophoresis.

Cloning and subcloning of HA1. PCR products were cleaned up by QIAquick PCR Purification Kits (QIAGEN cat#2810), and were cloned into pTZ57R/T cloning vector InsTAclone PCR Cloning Kit (Fermentas cat# k1213).

The recombinant vectors were transformed into E.coli DH5ɒ Calcium Chloride competentcells, then the clones were screened by choosing white colonies among blue/white bacteria colonies grew on the culture plate. The insertion of the interested sequence was confirmed by Colony-PCR on the selected white colonies, along with one blue colony as a negative control. Taq DNA Polymerase 2x Master Mix Red (Ampliqon cat#180301) and HA1 specific primers were used in the Colony-PCR. The recombinant vector pTZ57R/T+HA1 was confirmed using restriction enzyme analysis, and subsequently, it was subjected to automatic sequencing bidirectionally using M13 universal primers.

The HA1 gene was excised from the confirmed clones by NheI/BamHI double digestions, and then cloned into prokaryotic expression vector pET28a (Novagen),linearized with the same digestions, in order to create the HA1 expression plasmid pET28a+HA1 (Fig. 1). The fidelity of pET28a+HA1 was confirmed by colony PCR and vector restriction map (Fig. 4).

All DNA manipulations including double digestion, T4 ligation, and agarose gel electrophoresis were carried out as described by Sambrook and Russell(19).

Expression of HA1 recombinant protein. The pET28a+HA1 transformed into the E.coli BL21 (λDE3) (Novagen) competent cells. After culturing transformed cells on LB-agar plates containing 50μg/ml kanamycin at 37°C overnight, one colony was inoculated in 15ml of LB medium supplemented with 50μg/ml kanamycin, and incubated until reaching to 0.5 to 1 optical density at 600nm.

The cultures were induced by isopropyl-β-D-galactoside (IPTG) at a final concentration of 1mM afterwards. Time course fractionating from cultures started before inducing and continued for 4 hours at 37°C temperature.

SDS-PAGE and Western Blotting. To assess His-tagged proteins, total bacterial cell proteins (TCP) were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) beside of protein ladder (Fermentas/cat#SM0431). Acrylamide gel staining was performed by Coomassie brilliant blue R-250.

For further characterization, the separated proteins on SDS-PAGE were transferred electrically to nitrocellulose membrane and blocked with Tris-buffered saline-Tween (TBST) + 1% Bovin Serum Albumin (BSA) at 4°C overnight, then probed with Anti-His6 monoclonal antibody Peroxidase Conjugate (Roche Cat#04 905 318 001) diluted 1:300 in TBS-T, for 90 min on the shaker at room temperature. After washing with TBS-T, the specific protein bands were visualized using diaminobenzidine (DAB substrate) (Sigma Cat#D5637).

Results

HA1 gene was amplified successfully from the cDNA using designed primers. Figure 2 shows the formation of 999 bps amplicons compared to the DNA

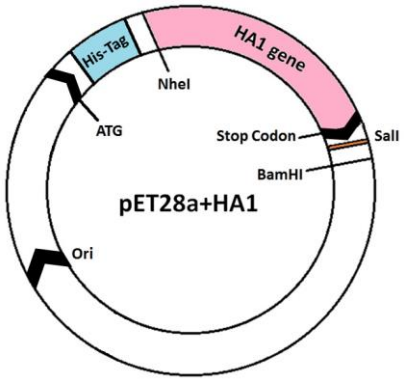

Figure 1. Construction ofpET28a+HA1. Schematic representation of cloned HA1 in pET28a, containing an N-terminal 6 histidine tag.

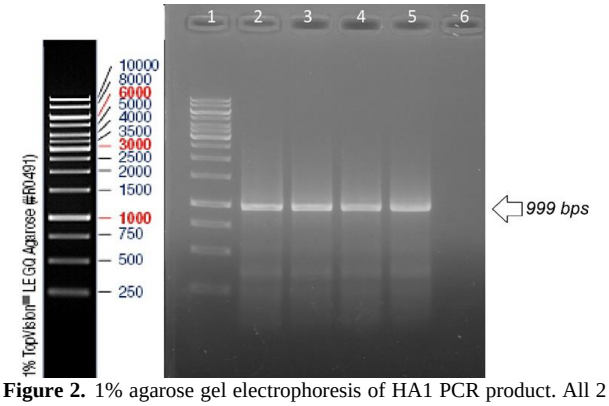

Figure 2. 1% agarose gel electrophoresis of HA1 PCR product. All 2 to 5 numbered lanes are HA1 amplicons, and 6th lane is no template control (NTC). GeneRuler 1kb DNA Ladder (Fermentas Cat#SM0313) has been used.

molecular weight marker, and no signal was observed in the negative control.

The accuracy of the pTZ57R/T+HA1 vector was confirmed through Colony PCR, restriction map analysis, using single digesting with XhoI, KpnI, BamHI and PaeI (SphI), along with double digesting with NheI/BamHI(Fig. 3), and also through DNA sequencing. Results also demonstrated the absence of amino acid substitution when aligned with the counterpart region from influenza A virus strain A/Puerto Rico/8/34.

pTZ57R/T+HA1 Plasmid construct was verified using Colony PCR with HA1 specific primers (Fig. 4), and restriction enzyme digestion (Fig. 5). As demonstrated in Fig.5, restriction enzyme digestion also verified the sub cloning of HA1 into pET28a, resulting in the formation of pET28a+HA1.

E. coli strain BL21 (λDE3) harboring pET28a+HA1 was cultured from HA1 protein expressionist's-PAGE of TCP for HA1 protein on 4-12% polyacrylamide gel demonstrated the expected size equal to 38.39 KDa, showed in Fig. 6, in which it is evident that before induction, target protein had not been expressed, but as the IPTG was added to the culture medium, the recombinant protein expression was started and increased during time courses.

HA1 protein sample was further confirmed through Western blotting, conducted by Anti-His6 monoclonal antibody Peroxidase conjugate, and the expected polypeptide band, which wasN-terminally His-tag fused with a molecular weight of about 38.39 kDa (Fig. 7).

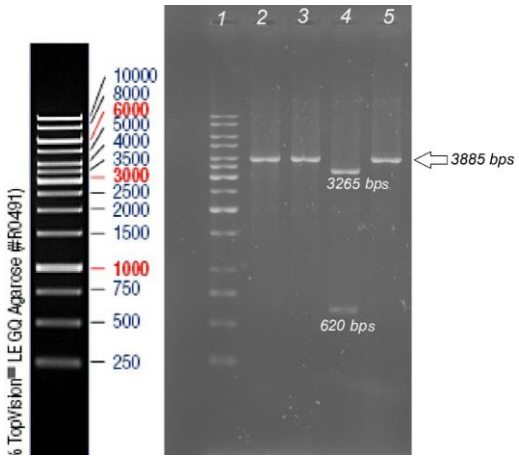

Figure 3. pTZ57R/T+HA1 plasmid restriction enzyme analysis on 1% agarose gel electrophoresis. Lane 2, pTZ57R/T+HA1 single digested by BamHI, 3885 bps; lane 3: pTZ57R/T+HA1 single digested by KpnI, 3885 bps; lane 4, pTZ57R/T+HA1 double digested by PaeI, resulting in two bands, 3270 bps and 615 bps; lane 5, pTZ57R/T+HA1 single digested by XhoI, 3885 bps; GeneRuler 1kb DNA Ladder (Fermentas Cat#SM0313)has been used.

Discussion

The H1N1 influenza subtype is a significant viral agent affecting public health and has been responsible for 3 pandemics and several influenza epidemics globally. As the H1N1 virus has been suggested to become potentially a seasonal influenza agent with annual outbreaks, development of rapid, convenient and specific laboratory diagnosis seems necessary to treat patients efficiently and prevent the virus spreading (6).

The structures of HA antigenic sites vary not only among different subtypes of viruses but also

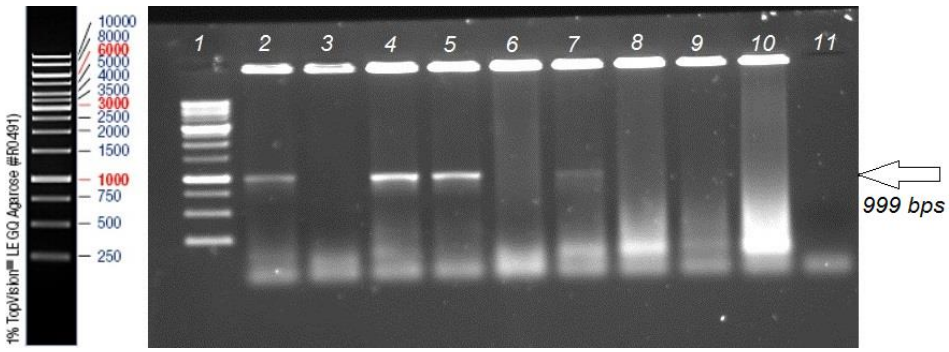

Figure 4. Colony-PCR Results of Cloning the HA1 in pET28a. Lane 2, 4, 5, 7: colonies containing HA1 gene, with the amplified band standing at 999 bps; lane 3, 6, 8, 9, 10: colonies without HA1 gene; lane 11: No Template Control; GeneRuler 1kb DNA Ladder (Fermentas Cat#SM0313) has been used.

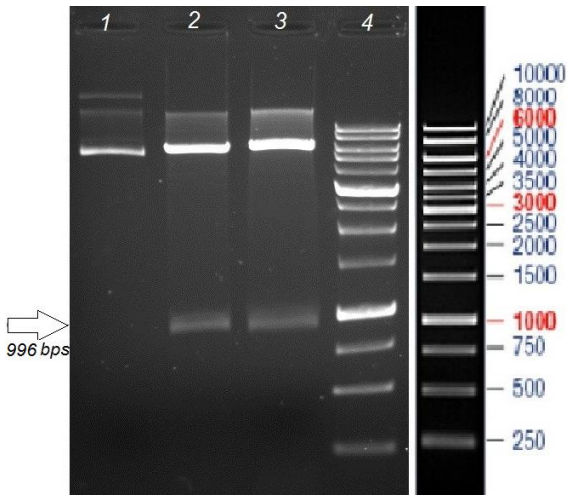

Figure 5. Restriction enzyme analysis ofpET28a+HA1 on 1% agarose gel electrophoresis. Lane 1, Undigested pET28a+HA1 plasmid; lane 2, 3: XhoI double digested pET28a+HA1 plasmids; GeneRuler 1kb DNA Ladder (Fermentas Cat#SM0313) has been used.

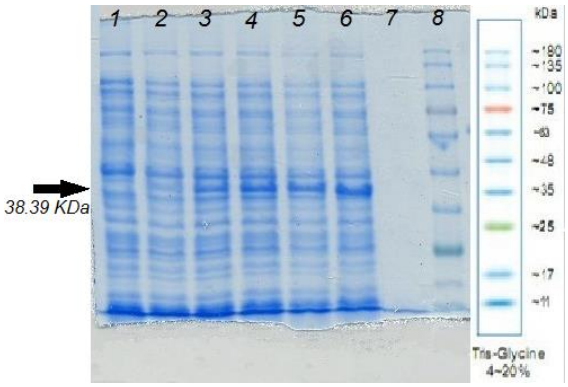

Figure 6.12% acrylamide gel electrophoresis of TCP collected fractions through specific time courses before and after induction. Lane 1, before IPTG induction; lane 2 to 6, collected samples 30 min, 1h, 2h, 3h and 4h after IPTG induction, respectively; lane 7, Prestained Protein Ladder (SinaClon Cat#PR901641).

within the same subtype. The continuous antigenic drifts and occasional antigenic shifts that arise from this continuous evolutionary variation enable human influenza viruses to escape the human immune system. (20) That is why, HA1 part of this membrane protein containing its major, constantly changing epitopes, represents a potential candidate for the discriminative diagnosis of H1N1 infection; and demonstrates the need for these priming studies in order to help complementing our knowledge about biochemical, immunological and serological aspects of influenza HA1 protein.

Recombinant proteins are the most common source of the diagnostic reagents.(6)While each of mammalian, insect, and bacterial expression systems has its advantages (21), the bacterial expression system is one of the most universally used (6). This system has been employed widely due to multiple factors, including its relatively inexpensive cost, ease of manipulation and rapid growth rate (22).

The reason that we chose pTZ57R/T as our cloning vector was that Taq DNA polymerase enzymes add a single 3'-A overhang at the both ends of the PCR product. The structure of these PCR products favors direct cloning into a linearized cloning vector with single 3'-ddT overhangs. Such overhangs at the vector cloning site not only facilitate cloning, but also prevent the recircularization of the vector. As a result, more than 90% of recombinant clones contain the vector with an insert (23). Recombinant clones were then selected based on blue/white screening.

Then, considering the type of the target protein, our cloning method, ongoing application of the expressed protein, and its further extraction and purification, we choose pET28a as our expression vector.

The pET system is one of the powerful expression systems for recombinant proteins in E. coli

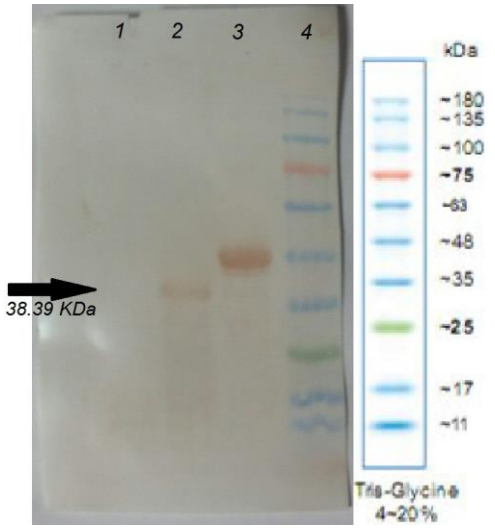

Figure 7. Detection of the recombinant HA1 protein by Western blot analysis using Anti-His6 monoclonal antibody Peroxidase Conjugate. Lane 1, TCP sample of transformed cells before IPTG induction; lane 2, TCP 4h after IPTG induction, showing expected 38.39 kDa HA1+His-tag protein size; lane 3, A known His-tagged protein sample weighed 48 kDa, used as positive control for WB procedure; lane 4, Prestained Protein Ladder (SinaClon Cat#PR901641).

prokaryotic cell. Its expression system is restrictedly under the control of bacteriophage T7 lac promoter, which would be induced by IPTG, for providing T7 RNA polymerase in host cell, in order to start the recombinant protein expression (24).

Problematically, inclusion bodies of the membrane proteins, such as the HA protein may be often produced with incorrect folding using the bacterial system and require denaturation and renaturation before further use. These processes may complicate or inhibit recombinant protein production. As a result, the use of low culture temperature, co-expression of molecular chaperones and proper host strain of *E. coli* has been suggested to promote the soluble expression of recombinant proteins. Hence, to further increase the recombinant protein solubility, the *E. coli* BL21 (DE3) has been chosen as the host strain, because it has previously demonstrated to be appropriate (25).

The results showed that the designed primers have properly amplified the desired gene, and according to the sequencing results, the gene has properly been cloned in pTZ57R/T cloning vector. The recombinant expression vector restriction map also showed the authenticity of sub-cloning the desired gene, and an increasing pattern in target protein expression was observed in SDS-PAGE analysis through a time course at the expected protein weight, and the genuineness of the result recombinant protein was confirmed by Western Blotting results, showing the prosperity in achieving the prokaryotic expression of our desired protein. Our final expressed protein contains His. Tag which can help purifying it more easily for further investigations.

In spite of the problems faced in expressing a viral native gene in a prokaryotic system, mostly caused by codon usage, we importantly could have expressed HA1 gene in E.Coli. Studies show that, despite not being glycosylated in this host, this protein can be refolded to its immunogenic structure, and also induce immune responses (4). But since lack of glycosylation, most of the recombinant HA proteins expressed by E. coli could not agglutinate human RBCs (6), and then the product of this expression method could not still be coated on ELISA plates in order to replace current conventional hemagglutination inhibition (HI) assays (26). In the meanwhile, the recombinant HA1 protein produced in this study can be used for specific polyclonal antibody production in order to be utilized in immunological tests like Western blotting and Eliza. This protein would possibly be very useful for H1N1 virus infection surveys; and also after being purified from LPS and passing animal tests, it can be used for the screening of individuals for vaccination .

As a result, it can also be argued that modifying the incubation temperature and time, and also the inducer concentration is suggested to prevent inclusion bodies. After having done all the required in silico conformational and epitope studies, fusing the HA1 protein to a molecular adjuvant (like C-terminal HSP70 and Flic) can be proposed as well, in order to increase its immunogenicity. It can also be suggested to fuse the HA1 protein to nanoparticles in other to use it in vivo as a nano-composite, and investigate it in further animal trial studies.

Since the Influenza virus is a respiratory tract infection agent, antibodies induced by vaccine that restrict replication throughout the upper and lower respiratory tract are desired. So it might be more logical to direct its vaccinology studies toward producing mucosal antibodies in order to achieve faster and more effective prevention of its infection, because mucosal antibodies are evidently more

effective than systemic antibodies in restricting replication of influenza virus in upper respiratory tract (12).

Conclusion

In the current study, we investigated the probability of expressing the HA1 globular domain instead of the intact gene, so in future studies, after doing all the required in silico studies, the probability of expressing the most important epitopes of HA can be investigated, in order to reduce the expenses of large-scale vaccine production. Also, this study can potentially be a primary step toward modern recombinant influenza vaccine production inside Iran, as the vaccine is the most effective way to prevent infection, especially because of not being able to prevent this infection transmission when occurring in a society.

Conflicts of Interest

None

Acknowledgment

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